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Contents

VOLUME 4 · NUMBER 1

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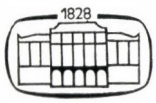

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Farewell to Ludwig Heilmeyer (1899-1969)

I have the sad task to say farewell to Professor L. Heilmeyer on behalf of the Hungarian Haematological Society as well as in my own name. My task is rendered difficult by the fact that I had the privilege to call myself his friend. We had become acquainted as young assistants of Weil's department in Jena, in 1929. Then already Heilmeyer was an enthusiastic researcher, who believed in purposeful scientific work and this was what gave the essence of his life. His main interest then was haematology. He soon realized that a purely morphological trend was not to ensure the best results. Shortly he became aware of the importance of biochemical research in haematology but did not overlook the significance of functional morphology beside the biochemical approach and this was one of his great merits, and at the same time the key for his having been able to take stand in every question of general and clinical haematology.

His first work of importance was the elaboration of a method for determining iron in blood, a procedure which then allowed to clarify many problems of iron deficiency, and the sideropenic and sideroachrestic anaemias. He was among the first to start laboratory investigations into porphyrin synthesis. In addition to the pathogenesis of porphyria erythropoietica he succeeded in elucidating a number of questions of the secondary disorders of porphyrin metabolism. His research involved megaloblastic anaemia, haemolytic diseases, the normal and pathological functions of the spleen. He described several bone marrow types in panmyelopathy. There was in fact no field in haematology in which he was not interested.

His creative activity was not exhausted by analysis, but with the clinician's logic he laid great stress upon synthesis. This aspect was always characteristic of his books, papers and lectures.

Heilmeyer was not only an internationally known experimental haematologist but also an outstanding clinician. At his department one had the opportunity to become acquainted with its excellent spirit and organization, the close coordi-

nation of the scientific, diagnostic and therapeutic activities, with much basic research work and clinical investigations in well-equipped laboratories, as well as with a great number of enthusiastic disciples, who realized Heilmeyer's initiatives and ideas. In this department the analysis of every problem started indeed from, and returned to, the patient's bed.

Heilmeyer was a scientist with exceptional gifts whose opinion was original and clear, and who could always draw the attention to some new, unexpected point of view. He passed away in the strength of his creative power, and haematology has lost a great personality. Our sorrow is lessened by the conviction that the memory of a great personality is kept alive by the results of his creative life.

I. BARTA

Haematologia 4 (1), pp. 5—14 (1970)

Effect of Diazooxonorleucine and N-ethyl Maleimide on the Incorporation of Nicotinic Acid and Nicotinamide into the Pyridine Nucleotides of Human Erythrocytes in vitro*

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Diazooxonorleucine inhibited the incorporation of radioactive nicotinic acid, but not of nicotinamide, into the pyridine nucleotides of human erythrocytes. Inhibition was reflected in an increased accumulation of nicotinic acid adenine dinucleotide, the immediate precursor of NAD. Inhibition of incorporation of nicotinic acid by N-ethyl maleimide and, to a lesser degree, incorporation of nicotinamide paralleled the inhibition of glycolysis produced by this compound. These observations were consistent with the known pathways for the biosynthesis of pyridine nucleotides in mammalian tissues. They also indicated that nicotinamide deamidase activity, if present at all, was extremely limited in human erythrocytes.

The synthesis of NAD by human erythrocytes, as well as by other mammalian tissues, appears to depend upon the presence of the nicotinic acid pathway demonstrated by Preiss and Handler [1, 2]. This pathway requires nicotinic acid, glutamine or ammonia, ATP and phosphoribosylpyrophosphate for the net synthesis of NAD. NAD kinase, the only known enzyme for the formation of NADP from NAD, also requires ATP [2, 3]. Utilization of nicotinamide, when present in physiological concentrations, is not considered to be significant for the synthesis of NAD by human erythrocytes [4, 5]. In other tissues, however, it has been demonstrated that nicotinamide can be utilized for the formation of NAD after it is deamidated by nicotinamide deamidase to nicotinic acid [6—8]. Previous studies have shown that incubation of normal human erythrocytes with radiocarbon labeled nicotinic acid results in the net synthesis of NAD and the incorporation of the radioactive label into NAD and NADP [9]. Incubation with radioactive nicotinamide results in the incorporation of the labeled precursor into NAD and NADP to a much more limited extent than occurs with nicotinic

* These studies were supported by U.S. Public Health Service grant AM-13698. Dr. Jaffé is a Career Scientist of the Health Research Council of the City of New York, award I-169.

** Abbreviations: AMP: adenosine 5'-phosphate; ATP: adenosine 5'-triphosphate; NMN: nicotinamide mononucleotide; NAD: nicotinamide-adenine dinucleotide; NADP: nicotinamide-adenine dinucleotide phosphate; NADase: nicotinamide-adenine dinucleotide nucleotidase; GSH: glutathione (reduced); DON: 6-diazo-5-oxo-L-norleucine; NEM: N-ethyl maleimide.

acid and no net synthesis of NAD is observed. This incorporation can be attributed to an exchange of the nicotinamide moiety mediated by the activity of NAD (?NADP) nucleotidases (NADase) [2, 10].

The studies described here were conducted to obtain information about the presence of nicotinamide deamidase activity in human erythrocytes and about the relative importance of glycolytic activity in the biosynthesis of the pyridine nucleotides. Diazooxonorleucine (6-diazo-5-oxo-L-norleucine or DON), a specific inhibitor of reactions involving glutamine [11], and N-ethyl maleimide (NEM) which forms a stable complex with GSH and other sulfhydryl groups [12] were employed to alter the metabolic activity of the erythrocytes.

Materials and Methods

Whole blood, anticoagulated with heparin, was obtained from healthy adults, and suspensions of washed erythrocytes were prepared as described previously [9]. Reticulocyte-rich suspensions were prepared from blood drawn from a patient (III-26) [13] with a hereditary non-spherocytic hemolytic disorder associated with an altered phospholipid composition of the erythrocytes; the reticulocyte count was 10 %. Flasks for incubation contained 11 ml of washed erythrocytes, 3.3 μmoles and 15 μc of radioactive nicotinic acid or nicotinamide, 1390 μmoles of glucose, 220 μmoles of glutamine, 550 μmoles of inorganic phosphate as an isotonic sodium phosphate buffer solution, pH 7.4, 15 mg of penicillin and 15 mg of streptomycin in a total volume of 20 ml. After dilution with unlabeled compound, the specific activity of the nicotinic acid-7-^{14}C and nicotinamide-7-^{14}C was 1.86 to 2.20×10^6 cpm per μmole.[1] The final concentration of nicotinic acid or nicotinamide was 0.165mM. In the experiment with a 100-fold higher concentration of nicotinamide (16.5mM), the specific activity was reduced to 1.86×10^4 cpm per μmole with unlabeled nicotinamide. When required, DON[2] and NEM[3] were dissolved in the incubation medium just prior to the addition of the washed erythrocytes. The flasks were incubated for 4 hours, unless otherwise indicated, in air at 37° C in a Dubnoff metabolic shaker-incubator. The procedures employed for the isolation of nucleotides from cold trichloroacetic acid extracts of washed erythrocytes, for the identification of the pyridine nucleotides, and for the determination of radioactivity were described in detail previously [14].

After incubation with nicotinic acid-7-^{14}C, a highly labeled material with the spectral characteristics of an adenine-containing compound was eluted from the Dowex 1-X2 resin about 400 ml after NAD and about 200 ml before NADP

[1] Radiocarbon labeled compounds were obtained from New England Nuclear Corporation, Boston, Massachusetts. The purity of the compounds was verified by paper chromatography in two solvent systems.

[2] Kindly provided by Dr. Ralph K. Barclay, Sloan-Kettering Institute for Cancer Research, Rye, New York.

[3] Obtained from Schwarz Bioresearch, Inc., Mount Vernon, New York.

were recovered. This compound was identified as nicotinic acid adenine dinucleotide (deamido-NAD) on the basis of the following properties: 1. relative position in the column chromatographic profile [1, 15], 2. ultraviolet absorption spectrum [1], 3. failure to show increased light absorption at 340 mμ after incubation with ethanol and crystalline yeast alcohol dehydrogenase [6], and 4. identification of radioactive nicotinic acid and unlabeled AMP after alkaline hydrolysis and paper chromatography [1, 14]. The failure to demonstrate nicotinic acid mononucleotide or nicotinamide mononucleotide (NMN) in these experiments probably resulted from the fact that mononucleotides are not readily precipitated from cold trichloroacetic acid extracts by —20 °C acetone [6, 16]. Those fractions which were expected to contain deamido-NAD were collected in all experiments, regardless of the labeled precursor. These fractions were evaporated to dryness, redissolved in water, subjected to paper chromatography, and counted for radioactivity [14]. Deamido-NAD could not be detected in extracts of normal human erythrocytes which had not been incubated with nicotinic acid. All of the radioactive deamido-NAD, therefore, was presumed to have been formed during the period of incubation. The amount synthesized was calculated from the total number of counts recovered from the deamido-NAD fractions obtained on column chromatography divided by the specific activity of the deamido-NAD eluted from the paper chromatogram. A molar extinction coefficient of 15.5 at 260 mμ was used to calculate the concentration of deamido-NAD collected from the paper chromatograms [1]. The amount synthesized per ml of erythrocytes was calculated from the packed erythrocyte volumes of the incubation suspensions which were determined with microhematocrits. Recovery by column chromatography of NAD added to extracts of erythrocytes was about 90 % and recovery of added NADP was about 75 %. The values reported here, however, were not corrected.

Lactate [17] and pyruvate [18] assays were performed on clear filtrates obtained after addition of one vol of each erythrocyte suspension to 6 vol of cold 2 % perchloric acid before and after incubation. Lactate and pyruvate production per ml of erythrocytes were calculated. The concentrations of GSH before and after incubation were determined with 5,5′-dithiobis-(2-nitrobenzoic acid) [19].

Results

The data obtained in these experiments are summarized in Table 1. Extensive incorporation of radioactive nicotinic acid into the NAD of mature erythrocytes was observed after incubation for 4 hours. The radioactivity of the NADP in the absence of inhibitors was about 5 % of that of the NAD. Three to 7 mμmoles of deamido-NAD per ml of erythrocytes accumulated during the 4 hours. Incubation for 16 hours resulted in about a two-fold increase in the labeling of NAD and a four-fold increase in the labeling of NADP over that observed after 4 hours, but did not result in increased accumulation of deamido-NAD.

In contrast to the findings with the immature erythrocytes of rabbits [14], the extent of incorporation of nicotinic acid into NAD in a cell suspension with

Table 1

Effect of metabolic inhibitors on the incorporation of radioactive nicotinic acid and nicotinpyruvate, and concentration

Expt. No.	Inhibitor	Specific activity			Inhibition	
		NAD	NADP	Deamido-NAD	NAD	NADP
	μmoles/ml cells	cpm/μmole × 10^{-3}			%	%
Nicotinic acid-7-^{14}C						
I	Control	814	52	1880		
	Control	831	49	2030		
II*	Control	795	153	2060		
III	Control	818	45	1713		
	DON 1.4	113	10	1860	86	78
IV	Control	905	46	2000		
	DON 1.6	148	11	2019	84	76
V**	Control	1536	187	2368		
	DON 1.6	184	37	2100	88	80
VI	Control	906	12	1800		
	NEM 3.2	783	14	1850	14	0
VII	Control	774	45	1860		
	NEM 9.3	154	6	1950	80	87
Nicotinamide-7-^{14}C						
I	Control	155	9			
II*	Control	329	11			
III	Control	101	6			
	DON 1.4	110	8		0	0
IV	Control	140	8			
	DON 1.6	135	8		3	0
V**	Control	637	53			
	DON 1.6	600	49		6	7
VI	Control	175	7			
	NEM 3.2	165	5		6	29
VII	Control	128	11			
	NEM 9.3	47	1		63	91
VIII***	Control	7.2	1.3			
	DON 1.5	7.1	1.3		1	0

Studies with the same experiment numbers were performed with erythrocytes from the same phlebotomy.

* Erythrocyte suspension from blood with 10% reticulocytes.

** Incubated at 37°C for 16 hours.

*** Incubated at 37°C for 23 hours with 330 μmoles of nicotinamide in 20 ml of incubation suspension.

10% reticulocytes was not increased above that observed with mature cells. Incorporation into NADP, however, was increased by about 4 times and 9 mμmoles of deamido-NAD per ml of cells were formed. Many more extremely immature

amide into pyridine nucleotides, synthesis of deamido-NAD, production of lactate and of GSH in human erythrocytes

Deamido-NAD	Lactate formed	Pyruvate formed	Lactate + pyruvate	Inhibition	GSH Initial	GSH Final	Decrease from initial
mμmoles/ml cells	μmoles/ml cells			%	μmoles/ml cells		%
4.1	14.6	0.1	14.7		3.0	2.9	3
3.5	14.7	0.1	14.8		3.0	2.9	3
9.3	30.1	0.1	30.2		3.3	3.3	0
7.1	13.5	0.2	13.7		2.8	2.6	7
31.7	13.9	0.1	14.0	0	2.9	2.7	7
4.7	12.8	0.1	12.9		2.9	2.9	0
29.0	12.9	0.1	13.0	0	2.9	2.8	3
3.8	43.5	1.6	45.1		2.8	2.7	4
22.8	43.1	1.5	44.6	1	2.8	2.7	4
4.2	14.8	0.2	15.0		3.0	3.0	0
5.8	14.2	0.2	14.4	4	0.9	0.8	76
2.7	14.7	0	14.7		3.0	2.9	3
5.4	2.4	0.2	2.6	82	0.2	0.3	90
< 0.01	14.5	0.1	14.6		3.0	2.9	3
< 0.02	30.3	0.1	30.4		3.3	3.3	0
< 0.02	13.8	0.2	14.0		2.8	2.6	7
< 0.01	13.4	0.1	13.5	4	2.9	2.7	4
< 0.02	12.9	0.1	13.0		2.9	2.8	3
< 0.02	12.9	0.2	13.1	0	3.0	2.9	0
< 0.02	43.0	0.7	43.7		2.8	3.0	0
< 0.01	42.8	1.2	44.0	0	2.8	2.9	0
< 0.01	13.4	0.2	13.6		3.0	3.0	0
< 0.01	13.7	0.2	13.9	0	0.4	0.3	90
< 0.01	14.2	0.4	14.6		3.0	2.9	3
< 0.01	2.1	0.6	2.7	82	0.2	0.3	91
< 0.01	57.1	1.3	58.4		2.7	2.7	0
< 0.01	56.9	1.4	58.3	0	2.9	2.9	0

reticulocytes, by morphologic criteria, were obtained from rabbits with acetylphenylhydrazine-induced reticulocytosis than were seen in the blood of the patient with a compensated hereditary hemolytic disorder.

When 1.4 to 1.6 μmoles of DON per ml of erythrocytes were included in the incubation medium, the incorporation of nicotinic acid into NAD was inhibited by 86 % and incorporation into NADP was decreased by 78 %. The accumulation of deamido-NAD, however, was increased by about six-fold. The presence of

DON did not inhibit glycolysis, as reflected in the production of lactate and pyruvate, and did not decrease the concentration of GSH significantly.

Incubation with a concentration of NEM approximately equimolar to the intracellular concentration of GSH resulted in a 76 % decrease in the concentration of GSH, a minimal (4 %) inhibition of glycolysis, and a 14 % inhibition of incorporation of nicotinic acid into NAD. Incorporation into NADP was not inhibited and the accumulation of deamido-NAD was increased by 38 %. When a three-fold excess of NEM over GSH was employed, incorporation into NAD and NADP, as well as glycolysis, were inhibited to about the same extent (80 to 90 %). Accumulation of deamido-NAD, however, was not impaired.

When an amount of nicotinamide-7-^{14}C equimolar to the amount of nicotinic acid was used as the radioactive precursor, incorporation into NAD was about 17 % of that observed with nicotinic acid, and the labeling of NADP was about 20 %. After incubation for 16 hours, labeling of NAD was increased five-fold and that of NADP six-fold over the values observed after 4 hours. Incorporation of nicotinamide into the NAD of a reticulocyte-rich suspension was increased about two-fold over that obtained with mature erythrocytes, while incorporation into NADP was not altered significantly.

The presence of DON in the concentrations which inhibited the incorporation of nicotinic acid did not significantly inhibit incorporation of nicotinamide. Deamido-NAD accumulation could not be demonstrated in any of these experiments, even after prolonged incubation with or without DON. The quantities listed in the table reflect the recovery of a total of 150 to 300 counts per minute and were, therefore, of questionable significance. Even when nicotinamide was added in a concentration 100 times greater than that used in the other experiments and incubation was continued for 23 hours (Expt. VIII), DON did not inhibit incorporation and deamido-NAD was not detected. In this latter experiment, about three times as much NAD was recovered from the column chromatograms as was recovered when erythrocytes were incubated with the lower concentration of nicotinamide or without added nicotinamide for 4 or 16 hours. Thus, net synthesis of NAD from nicotinamide appeared to have occurred under the conditions of this experiment.

NEM, at a concentration equal to that of intracellular GSH, resulted in no impairment of glycolysis and minimal inhibition of incorporation of nicotinamide into NAD and NADP. A three-fold excess of NEM over GSH, however, produced marked inhibition of glycolysis and of incorporation into the pyridine nucleotides.

Discussion

The observations described here are consistent with the known pathways for the biosynthesis of pyridine nucleotides in mammalian tissues [2]. The final step in NAD synthesis involves amidation of deamido-NAD through the activ-

ity of NAD synthetase in the presence of glutamine (or ammonia) and ATP. The level of activity of this enzyme is considered to be rate-limiting in the protozoan, *Astasia longa*, [20], but not in *Escherichia coli* [21]. The greater inhibition of incorporation of nicotinic acid into NAD than of the formation of deamido-NAD when glycolysis is inhibited by NEM in the present study may indicate either that NAD synthetase is susceptible to sulfhydryl inhibition or that this enzymatic activity is also rate-limiting in human erythrocytes. The greater increase in the formation of deamido-NAD than in the labeling of NAD in a reticulocyte-rich suspension is consistent with the latter concept. Immature erythrocytes (reticulocytes) have an increased glycolytic rate and an increased content of ATP [22] and, therefore, might be expected to demonstrate increased capacities for the synthesis of pyridine nucleotides.

NAD kinase activity also is dependent upon ATP. The extent of labeling of NADP is increased significantly after incubation of human reticulocytes, rather than mature cells, with labeled nicotinic acid. The level of activity of NAD kinase, therefore, does not appear to be rate-limiting under these conditions.

The effect of DON on the incorporation of nicotinic acid into NAD and NADP is supporting evidence for the role of glutamine in the synthesis of NAD. Inhibition of incorporation by DON is reflected in an increased accumulation of deamido-NAD, the immediate precursor of NAD. Glutamine has been demonstrated to be the preferred nitrogen donor for NAD synthesis in yeast [1], while ammonia is the preferred substrate in *Escherichia coli* [23]. Incorporation of nicotinic acid into NAD and NADP, and net synthesis of NAD by human erythrocytes are decreased by at least 50 % when glutamine is omitted from the incubation medium [5, 9]. Thus, glutamine probably is the preferred nitrogen donor in human erythrocytes.

Inhibition of glycolysis in human erythrocytes by NEM when present in a concentration greater than the intracellular concentration of GSH has been demonstrated by Jacob and Jandl [24]. NEM probably impairs metabolic activity by inhibiting sulfhydryl-containing enzymes. The inhibition of incorporation of nicotinic acid which is observed when NEM is present in a concentration equal to or greater than the concentration of GSH probably reflects the comparable inhibition of glycolysis and, therefore, of the generation of ATP. The failure of deamido-NAD accumulation to decrease is difficult to explain, but may indicate preferential utilization of available ATP in this earlier biosynthetic reaction which may not be subject to sulfhydryl inhibition.

Incorporation of radioactive nicotinamide into NAD and NADP can be explained by the exchange reaction which is catalyzed by NADase [2, 10]. The more extensive labeling of NAD than of NADP may reflect the greater affinity of NADase for NAD than for NADP [25]. Hemolysates of reticulocytes appear to possess only slightly greater NADase activity than do lysates of mature erythrocytes [25, 26]. The enhanced incorporation of nicotinamide into the pyridine nucleotides of a reticulocyte-rich suspension, however, may be due to increased activity of this enzyme system in intact immature erythrocytes. The minimal in-

hibition of incorporation of nicotinamide which results from incubation of human erythrocytes with low concentrations of NEM and the significant inhibition which occurs with high concentrations may be evidence that the NADase-mediated exchange reaction is sensitive to sulfhydryl inhibition. In rat erythrocytes, however, the uptake of both nicotinamide and nicotinic acid is inhibited by fluoride, iodoacetate or arsenate [27]. Thus, it is unclear if glycolytic activity is essential for the incorporation of nicotinamide into pyridine nucleotides.

Nicotinamide is a better precursor than nicotinic acid for the synthesis of NAD by mammalian liver *in vivo* [2, 6, 28]. This apparent paradox has been resolved by the finding that rat and rabbit liver contains nicotinamide deamidase activity [7, 8]. In this tissue, therefore, nicotinamide can be converted to nicotinic acid which is then utilized for the synthesis of NAD via the nicotinic acid pathway. Administration of DON plus nicotinamide to mice completely prevents the increased hepatic synthesis of NAD induced in the livers of control animals injected with nicotinamide alone [15]. In the livers of animals treated with DON, deamido-NAD accumulates in amounts approximately equal to the quantities of NAD synthesized in the absence of DON. In human erythrocytes *in vitro*, DON fails to inhibit incorporation of nicotinamide into NAD and NADP, and the formation of significant amounts of deamido-NAD is not observed, even after prolonged incubation. Nicotinamide deamidase activity, therefore, is either absent from mature human erythrocytes or its level of activity is so low that it cannot be detected with the methods employed in this investigation.

Net synthesis of NAD is demonstrable after prolonged incubation of human erythrocytes with high concentrations of nicotinamide [5]. Even under these conditions and in the presence of DON, incorporation is not inhibited and deamido-NAD accumulation is not observed. Synthesis of NAD may result from the activity of NAD pyrophosphorylase which can form NAD from NMN and ATP [2]. NMN is readily synthesized by human erythrocytes from nicotinamide [29], but the level of activity of NAD pyrophosphorylase in these cells is considered to be too low to account for significant synthesis of NAD [29, 30]. Deamidation of NMN, reported to take place in mouse liver [31], might be involved, but accumulation of deamido-NAD would be expected to occur when NAD synthetase activity is inhibited by DON. Thus, the synthesis of NAD in human erythrocytes upon incubation with high concentrations of nicotinamide remains unexplained.

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Haematologia 4 (1), pp. 15—20 (1970)

Erythropoietin Inhibitor in Plasma from Patients with Chronic Renal Failure

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In 20 of 23 patients with chronic renal failure no measurable plasma and urinary erythropoietin activity could be detected in spite of the presence of severe anaemia. In addition, plasma from 11 out of 13 anaemic uraemic subjects induced a significant inhibition of ESF, while normal human plasma was enhancing the erythropoietic effects of ESF. The inhibitor was still present after *in vitro* dialysis and was partially inactivated by heating. It was also shown that the inhibitor could be acting during the stage of stem-cell differentiation, but not during the later stages of erythroid cell development.

The anaemia of renal failure is associated with an increased destruction of red blood cells (RBC) and an impaired erythropoietic response to anaemia. The pathogenesis of the erythroid failure has been postulated to be due to diminished erythropoietin (ESF) production [1, 2] and/or the toxic effects of the metabolic products of uraemia on the erythroid cells [3]. However, the mechanism of the decreased ESF production is not fully understood.

Recently, Fisher et al. [4] suggested the presence of an ESF inhibitor in the normal rat kidney homogenate; an increase in this inhibitor may play an important role in the mechanism of the anaemia of chronic renal failure.

In the present study, the plasma level of the ESF inhibitor in anaemic uraemic patients has been compared with that of normal human subjects. Furthermore, it has been attempted to clarify the mechanism of ESF inhibition by the uraemic plasma.

Materials and Methods

Twenty-three patients with azotaemia due to chronic renal failure were studied. In 15 of these patients, chronic haemodialysis was done twice a week in our hospital. All patients had BUN levels of more than 57 mg per 100 ml.

Plasma and urinary erythropoietin assays were performed in hypertransfused polycythaemic mice as described by DeGowin et al. [5].

The inhibitory effect of anaemic uraemic plasma on human urinary erythropoietin was tested in polycythaemic mice as follows. Five ml of untreated uraemic

plasma were mixed with 1.0 unit of erythropoietin in 0.2 ml of saline. The mixture was incubated at room temperature for 40 minutes. One ml (0.2 unit ESF/ml) of the incubated mixture was given subcutaneously to each of 5 polycythaemic assay mice.

The effect of uraemic plasma (case M. S.) on erythropoiesis was estimated as follows. Five groups of 5 polycythaemic mice each received 0.2 units of ESF in 0.5 ml of saline subcutaneously. Each group was given subcutaneously 1.0 ml of uraemic plasma 6 hours before, simultaneously with, and 12, 24, and 36 hours after a single injection of ESF. Forty-eight hours later 0.5 μci of $^{59}FeCl_3$ was injected intraperitoneally and the incorporation of ^{59}Fe into erythrocytes was measured another 48 hours later.

The erythropoietin used in these studies was extracted from the urine of an anaemic patient with bone marrow erythroid hypoplasia according to the method of Lowy and Keighley [6]. The potency of this urinary erythropoietin was standardized by comparison with International Standard B Erythropoietin obtained from the Bureau of Standards, National Institute of Medical Research, London, England.

Results

Erythropoietin levels in uraemic patients

Results are shown in Figure 1. In 20 of 23 anaemic uraemic patients there was no significant plasma erythropoietin titre as compared to that of the saline

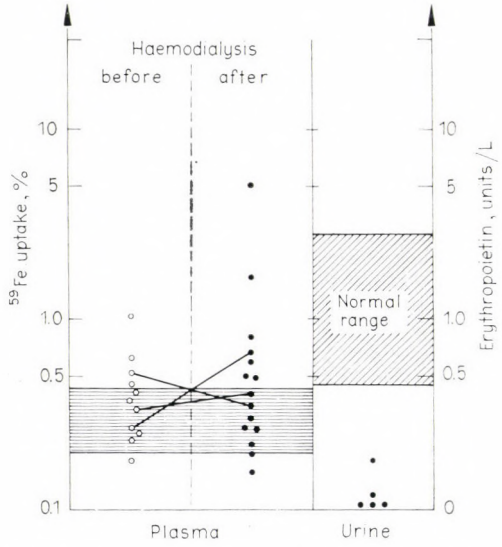

Fig. 1. Plasma and urinary erythropoietin levels in anaemic uraemic patients
(—: Same subjects)
≡: Saline control

controls (Fig. 1, left). Slight or no erythropoietic activity was demonstrable in the urine of 5 uraemic patients, concentrated by the method of Adamson et al. [7], while activity was present in normal concentrated urines (Fig. 1, right).

ESF inhibitor

The effect of uraemic plasma incubated with ESF was compared in polycythaemic mice with that of 5 normal human subjects. As seen in Table 1, the

Table 1

Effects of anaemic uraemic and of normal plasma on the erythropoietic activity of ESF in polycythaemic mice

Treatment	Haemat. per cent.	BUN mg per 100 ml	Dosage (per mouse)		% ^{59}Fe incorporation in RBC of polycythaemic mice*
			ESF units	Plasma ml	
Saline	—	—	—	—	0.31 ± 0.12
ESF	—	—	0.2	—	3.95 ± 0.58
		Normal plasma			
1	39	9.0	0.2	+ 1.0	8.9 ± 0.2
2	45	12.5	0.2	+ 1.0	4.8 ± 0.5
3	42		0.2	+ 1.0	6.9 ± 2.5
4	40	14.0	0.2	+ 1.0	9.1 ± 1.7
5	46		0.2	+ 1.0	6.3 ± 0.6
		Uraemic plasma			
Y. K.	28	100.5	0.2	+ 1.0	6.7 ± 3.5
M. S.	20	66.9	0.2	+ 1.0	0.8 ± 0.2
H. O.	30	65.0	0.2	+ 0.5	2.7 ± 1.2
H. A.	32	60.6	0.2	+ 1.0	1.8 ± 0.4
		During haemodialysis			
K. S.	22	57.0	0.2	+ 1.0	0.9 ± 0.2
M. T.	22	61.5	0.2	+ 1.0	2.2 ± 0.9
K. O.	20	119.7	0.2	+ 1.0	1.4 ± 0.8
Ta. Y.	29	105.5	0.2	+ 1.0	0.8 ± 0.4
Tu. Y.	27	61.6	0.2	+ 1.0	3.6 ± 1.1
H. M.	23	79.0	0.2	+ 1.0	2.8 ± 1.7
A. K.**	24	63.2	0.2	+ 1.0	8.4 ± 2.6
S. T.	19	66.0	0.2	+ 1.0	3.3 ± 0.4
K. I.	23	72.2	0.2	+ 1.0	1.6 ± 0.6

* Each group receiving plasma contained 3—5 mice ± standard error of mean
** ESF activity present in plasma

erythropoietic effect of ESF was markedly accentuated when injected with normal human plasma. On the other hand, the plasma from 11 of the 13 uraemic patients showed a significant inhibitory effect of ESF on ^{59}Fe incorporation in polycythaemic mice.

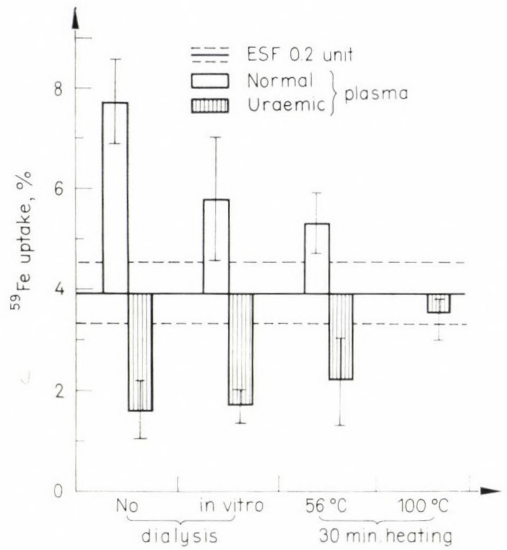

Fig. 2. Inhibitory effect of uraemic plasma

As demonstrated in Figure 2, the inhibitory effect persisted after dialysis of uraemic plasma; it was partially inactivated by heating at 56°C for 30 minutes, while completely inactivated by boiling.

Inhibition of erythropoietin by uraemic plasma

The effect of uraemic plasma (case, M. S.) on erythropoiesis is shown in Figure 3. Iron incorporation in these groups was $1.2 \pm 0.6\,\%$; $0.8 \pm 0.2\,\%$; $0.9 \pm 0.4\,\%$; $1.8 \pm 0.8\,\%$; and $3.4 + 0.5\,\%$, respectively.

Those groups which had received uraemic plasma either prior to or immediately following the injection of ESF, showed a marked depression of exogenous erythropoietic activity, but when the uraemic plasma had been given 24 to 36 hours after the ESF, no inhibition was observed.

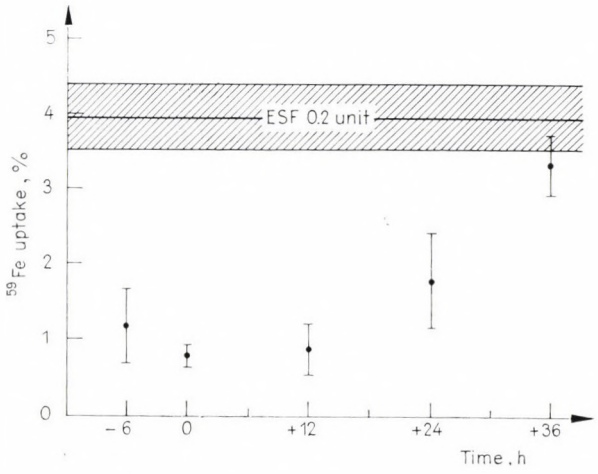

Fig. 3. Interval between injections of erythropoietin and uraemic plasma (Subject: M.S.)

Discussion

The kidney is believed to be the major site of erythropoietin production or activation, ever since in 1957 the renal dependency of circulating ESF in the anephric rat has been demonstrated by Jacobson et al. [8] and the role of ESF in inducing increased erythropoiesis by differentiation of stem cells is now generally accepted. A deficiency of the hormone has been thought to play an important role in the anaemia of uraemic patients [1, 2, 9]. In the present study, no measurable plasma and urinary erythropoietin titres were detected in spite of the presence of severe anaemia in 20 of 23 patients with chronic renal failure (Fig. 1). However, the mechanism by which this decrease of ESF production occurs, has not been clarified.

Recently, Fisher et al. [4, 10] have suggested the presence of an ESF inhibitor in normal rat and uraemic human kidney homogenates and the occurrence of an erythropoiesis inhibiting factor in the plasma of polycythaemic animals has been reported by other investigators [11, 12]. In this sense, the increase of ESF inhibitor might play an important role in the mechanism of the anaemia of chronic renal failure. The present studies demonstrated that the plasma from 11 out of 13 anaemic uraemic subjects induced a significant inhibition of ESF, while normal human plasma enhanced the erythropoietic effect of ESF (Table 1). Although the improvement in ferrokinetics observed in anaemic uraemic patients following dialysis [3, 13, 14] may be related to the removal or inactivation of an ESF inhibitor, the inhibitor found in uraemic plasma withstood dialysis and was not completely inactivated by heating (Fig. 2). Thus, the ESF inhibitor might be bound to some non-dialyzable protein.

In order to clarify the mechanism of ESF inhibition by uraemic plasma, polycythaemic mice were divided into groups and treated with 0.2 unit of ESF on a

single occasion. The uraemic plasma was injected 6 hours before, simultaneously with, and 12, 24 and 36 hours after ESF administration (Fig. 3). The result indicated that inhibition occurred only when the uraemic plasma had been given from 6 hours before to 12 hours after the ESF injection. This suggested that uraemic plasma could be acting during the stage of stem cell differentiation and erythropoietin utilization, and that it was not acting on the later differentiation of erythroid precursors. However, it is not known whether the inhibitor interacts with ESF directly, such as an antigen-antibody combination, or impairs the production of erythropoietin.

From these studies it is assumed that the anaemia of chronic renal failure is due, at least in part, to erythroid failure resulting from a lack of available erythropoietin. This deficiency in ESF activity is probably due to an increase in ESF inhibitor.

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A New Method of Preparing Nitrogen Samples from Haem for Mass Spectrometric Isotope Analysis

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The life span of a select population of the red cells formed in the bone marrow after thermal injury was studied by means of the ^{15}N stable isotope tracer technique. The adequacy of this method depends on the suitable preparation of nitrogen samples from haem for isotope analysis. A new procedure has been worked out for this purpose utilizing oxidation by cupric oxide in high vacuum at a temperature of about 600 °C. Being much less time consuming and at the same time reasonably accurate, the procedure is superior to the methods hitherto used.

Determination of the concentration of the heavy nitrogen isotope ín the circulating haem after administering glycine labelled with ^{15}N makes it possible to estimate the rate of haemoglobin production and the life span of the red cells. The curve obtained can be divided into three main portions, *viz.* a rapid rise in ^{15}N labelling over the first few days followed by a plateau and finally a descending portion corresponding with the isotope's disappearance from the blood haem. This last phase indicates the destruction and elimination of the red cells the labelling of which had presumably occurred in the bone marrow during the administration of the labelled glycine [7].

The method is based on the principle of building the label into the erythrocytes during their development, consequently in the labelling of a select population.

The investigation involves two separate procedures. First, the whole nitrogen content of the haem is liberated in elementary, i.e. gaseous, form. This is then followed by isotopic analysis by mass spectrometry.

Mass spectrometric isotope analysis has developed into a routine practice and if its different sources of error [1] are minimized it ensures an accuracy of $\pm$ 0.01% or less. Accuracy and speed of the method are considerably influenced and in many cases severely restricted by the procedure of preparing the sample in a suitable form for introduction into, and ionization in, the mass spectrometer ion source.

In our case the procedure means the liberation of the nitrogen of a complex and stable compound, of the haem, in elementary gaseous form ensuring the preservation of the nitrogen isotope ratio of the compound and the elimination of impurities adversely affecting the reliability of the mass spectrometric analysis.

In a similar investigation James et al. [2] used the two step procedure described by Rittenberg [3], consisting of conversion of organic nitrogen to ammonia

by the Kjeldahl method and the subsequent oxidation of the ammonia to nitrogen by hypobromite. Due to its complexity the method requires, in addition to an intricate though conventional equipment, a most careful handling to avoid the many possible errors. Its main disadvantage is, however, the demand in working time: 18 hours per sample. In laboratories not restricted in staff, working time and equipment, the Kjeldahl digestion presents no special difficulty. A number of laboratories do not, however, dispose of these prerequisites. A trial has therefore been made with a modified Dumas procedure used in the Institute for Organic Chemistry of Budapest University for the determination of the nitrogen content of organic macromolecules. Though this is a one-step method, new difficulties arose due to the necessity of collecting the nitrogen over a potassium hydroxide solution. It was even more of a drawback that small peaks appeared at the mass numbers M = 26, 27 and 31, indicating the presence of some organic impurities which seemed to contribute to the isotopic peaks M = 29 and 30, as well, undesirably affecting thereby the isotope ratio measurements. Finally, we have decided to make an attempt at the extension of Rolle's method [4] to compounds like haem. This method is based on the oxidation of organic materials by the use of CuO/Cu in evacuated and heated ampoules. As a result of a series of experiments a procedure satisfactorily fufilling the main requirements has been worked out.

Oxidation is carried out in a supremax glass ampoule attached directly to the sample inlet system of the mass spectrometer, by the high vacuum pump of which it can be evacuated. Protection against contamination with air nitrogen is provided by the special design of the ampoule's stop-cock (Fig. 1) and the use of sufficiently heat resistant teflon grease for lubrication.

The isolation of haem from the blood was performed according to Shemin and Rittenberg [5]. After recrystallization according to Fischer [6], 5—7 mg of the haem samples were mixed with 500 mg CuO; of this, 200 mg were in powder form and 300 mg in pieces of small diameter oxidized copper wire. The mixture was filled to the bottom of the reaction ampoule and then layers of 120 mg pure copper in wire form, 150 mg of CaO powder and a new layer of CuO (100 mg powder and 200 mg wire) were added. The uppermost layer was made up of CaO powder to which particles of CuO were mixed. The column thus prepared was covered by a small plug of loose quartz wool to prevent throw-up at the beginning of evacuation.

Before the reaction was started the ampoule was thoroughly evacuated to about 10^{-5} torr. Degassing was made easier by inserting some quartz wool inside the column, at the layer boundaries. After evacuation the ampoule was placed in a cylindrical electric oven approximately up to the sign indicated in Fig. 1, and heating was started. In the middle of the oven the temperature was raised to 620 °C. The stopcock was shielded from direct heat flow by an asbestos bandage and was cooled by a small ventilator. Stopcock and joint were thereby easily kept at room temperature during the whole process. An accidental warming up of stopcock and joint may lead to a leaking in of air. Contamination with air is

controlled by the oxygen and argon peaks, i.e. by the mass spectrometer itself. Every measurement is to be controlled in this way. In the course of our determinations, however, we have never observed an error due to air-leakage.

Overall heating time was 100 minutes to allow oxidation of the organic material. The copious excess of the oxidizing agent and its arrangement in two layers ensured against incomplete oxidation. The peaks at the mass numbers

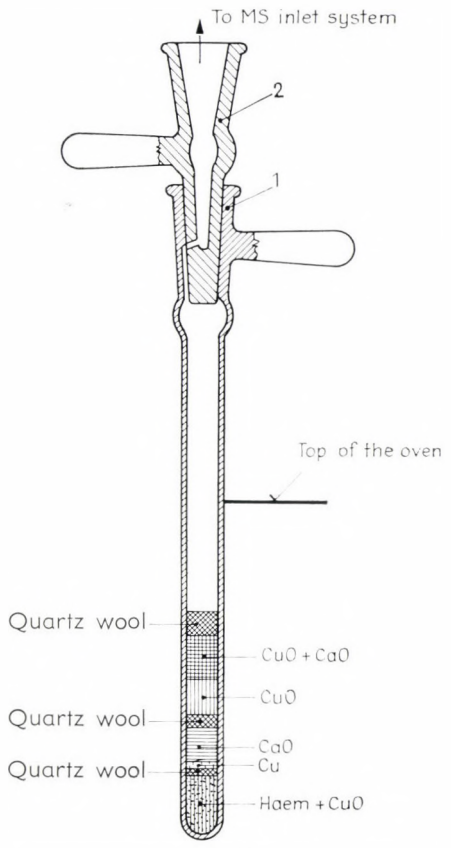

Fig. 1

M = 26, 27 and 31 were thereby made negligible. Platinized quartz wool catalyzer added to the reaction mixture at the beginning of the experiments proved later to be unnecessary.

The CaO layers had the purpose of removing the CO_2 formed from the gas phase. The eventual CO_2 rests were made completely to disappear by cooling by liquid air contained in a Dewar flask replacing the oven after heating, when the ampoule had cooled down to room temperature. Liquid air cooling removed

the water vapour as well. Finally, eventual traces of free oxygen were bound by the pure copper present in the mixture.

The Rittenberg method requires merely the freezing out of water and dry ice-acetone can accordingly be used. In our procedure, the freezing out by liquid air is even more convenient, because liquid air — being used for mercury traps of the diffusion pumps of the mass spectrometer — is at disposal.

The evacuation preceding the reaction is relatively time-consuming. The essential point, however, is that the total duration of the whole procedure is still much shorter than that of the previous methods. Furthermore, no separate evacuating system is needed, as degassing is performed by the pumps of the mass spectrometer.

As the sample nitrogen was also cooled to liquid air temperature and, therefore, a considerable fraction of it became absorbed, the extent of isotope fractionation by the process had to be checked. This was done by the use of tank nitrogen both as sample and standard, the sample nitrogen having been cooled down in the same reaction ampoule filled with the same column. Even if liquid nitrogen had been used for cooling, the difference in ^{15}N contents between the "sample" and the "standard" was insignificant, hardly exceeding one tenth of the normal biological deviation in the ^{15}N percentage, as indicated by the data of Table 1.

Table 1

Experiment No.	Decrease in the atomic percentage of ^{15}N
1	0.00048
2	0.00058
3	0.00044
4	0.00059
5	0.00090
average	0.00060

To determine the overall accuracy of the new method of preparing nitrogen samples from haem and the subsequent mass spectrometric isotope analysis, several measurements of the ^{15}N percentage (atomic) of the same haem sample were carried out at different dates. The results obtained are shown in Table 2. As a standard, tank nitrogen was used, and the sample-standard differences showed the normal biological deviation in ^{15}N content. If the maximum of the deviations of the data in Table 2, i.e. 0.00048, is considered the measure of the possible error, this was less than 0.1% of the normal ^{15}N content, i.e. 0.366%.

In the first series of experiments, aimed at the investigation of the anaemia after thermal injury, the measured ^{15}N contents yielded atom percent excess values between 0.04 and 0.32, i.e. values of two orders of magnitude greater than the cautiously estimated possible error. In the experiments a VARIAN (ATLAS) MAT—M86 type mass spectrometer was used.

Table 2

Experiment No.	Difference in ^{15}N atomic percentage: sample — standard
1	0.00427
2	0.00414
3	0.00462
4	0.00445
average	0.00435

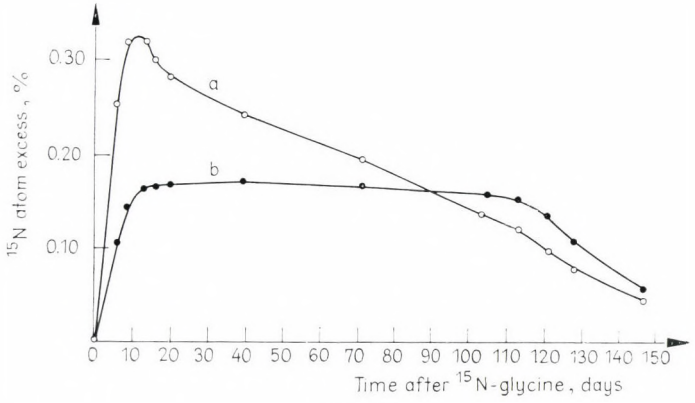

Fig. 2

Table 3

Date	Days after administration of ^{15}N-glycine	^{15}N atom per cent excess in haem	
		burned patient	control
5.15	0	0.0045	0.004
5.21	6	0.256	0.103
5.24	9	0.318	0.145
5.28	13	0.320	0.163
5.31	16	0.295	0.162
6.4	20	0.282	0.166
6.29	45	0.241	0.178
7.26	72	0.194	0.167
8.27	104	0.135	0.159
9.6	114	0.120	0.155
9.13	121	0.094	0.136
9.20	128	0.079	0.109
10.9	147	0.042	0.060

The method's high accuracy might be of use in the interpretation of the fine details of curves showing the variation in time of the ^{15}N content of the blood of the patients. As an example (Table 3) such a curve is reproduced in Fig. 2 (curve a). This experiment was started on the 29th day after the injury. For contrast, the corresponding curve for a control patient is presented (curve b). These experiments are still in progress and will be reported later.

We are indebted to Professor V. Bruckner for permission to carry out the first experiments in his institute, and to Mr. O. Nagy for valuable help in constructing the reaction ampoule.

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The Uptake of Radioactivity of Thymidine, Uridine, Formate, Glycine and Lysine into Cultures of Blood of Normal Human Subjects

Relationships with Mycoplasma Infection

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Experiments have been undertaken to study whether the uptake of radioactive substances into haemocultures of normal human subjects could be related to latent and diffuse mycoplasma infection. Whole blood or washed erythrocytes were incubated in PPLO "Difco" broth, with or without antibiotics, in the presence of 6-^{3}H or 2-^{14}C thymidine, G-^{3}H or 2-^{14}C uridine, ^{14}C Na formate, 2-^{3}H glycine, or G-^{14}C l-lysine. At intervals of several hours or days the centrifuged sediments of the blood cultures were examined for radioactivity in the fractions extractable with warm $HClO_4$ before and after elimination of the acid-soluble fraction, the purines, the nucleic acids, or the protein fraction. In addition, chromatographic fractionation of extracts and autoradiographic examination of smears of incubated erythrocytes were carried out. The findings were qualitatively similar, but quantitatively considerably different. Uptake of thymidine and uridine into the acid-soluble fraction and into the nucleic acids, uptake from formate and glycine into the purine bases and into the nucleic acids, uptake of lysine into the protein fraction were observed. Radioactive or non-radioactive thymidine and uridine, within certain concentration limits, stimulated the uptake of the same nucleosides and of formate. The completed uptake of a certain amount of thymidine radioactivity appeared to exert an inhibitory action on the subsequent uptake. Further, uptake was stimulated under good conditions of oxygenation. In agreement with previous findings it has been concluded that the results may be attributed to a turnover of nucleic acids related to a diffuse and silent infection by mycoplasma or L-forms of schizomycetes and to the multiplication of these micro-organisms in the blood cultures.

Frequent mention has been made, both in past and recent literature, of the finding of bacteria, mycoplasma and virus forms in human subjects in association with neoplastic diseases. Bibliographic data and up-to-date discussion of this subject can be found in the works of Diller et al. [1] and Dmochowski et al. [2]. Pease [3] has considered the possibility that conditions of autoimmunity and malignant degeneration occur as a consequence of an altered tolerance to the L-form of Listeria, which is of very wide distribution, and is found in a variety of morphological forms in the blood of animals of many species as well as of a large proportion of clinically apparently healthy human subjects. According to this author, some common mycoplasmas may play an analogous role. The reader will know that the relations between mycoplasma and the L-forms of bacteria are still a subject of much debate.

We have examined 150 persons of both sexes and all ages, young, adult and aged, who were regarded as clinically healthy, and who were selected at random from the neighbourhood of the Institute.

On the basis of this study we have already described the results of laboratory cultures obtained from plasma in PPLO media. The cultures produced proliferating particles (from 100 mμ to 1 μ in size). In liquid medium these were free in suspension, or were embedded in floccules of filamentous material. After the addition of Ringer's solution, these particles further proliferated while adhering to the fibrin reticulum. On solid medium the particles gave rise to microcolonies with the characteristic aspect of mycoplasma colonies, embedded in the surface of agar [4]. The same particulate material, free in the medium or connected with structural elements, was obtained from cultures of whole blood or of washed erythrocytes. By phase contrast microscopy, or staining with May–Grünwald–Giemsa or Feulgen, or after acridine orange staining and U. V. examination of the entire material, or following incubation with DNase or RNase, structures were easily demonstrated in the larger bodies and the presence of nucleic acids could be recognized.

These particles were clearly distinguishable from other particulate material present in the haemocultures (Heinz bodies), and the observations led us to consider that they were part of the developmental cycle of a micro-organism [5]. Chromatographic [6] and spectrophotometric [7] analysis of the products obtained by extraction from the haemocultures, demonstrated a turnover of purine and pyrimidinic bases. Electron microscopical observations of ultrathin sections of the incubated erythrocytes showed intracellular and extracellular bodies which resembled stages in the growth-cycle of mycoplasmas [8].

All these results were regarded as indicative of the presence in the blood stream of healthy persons of bacterial L-forms or mycoplasmas whose development and multiplication could be detected by means of cultural and biochemical methods. However, as these methods are not easily applicable to a quantitative evaluation of the biochemical processes in culture, the experiments to be described here have been carried out to verify the interpretation of our earlier findings.

Methods

Radioactive material

We used the following products supplied by the Amersham Radio-chemical Centre

6-^{3}H thymidine,	5000 mC/mM;	2-^{14}C uridine,	58 mC/mM;
G-^{3}H uridine,	2730 mC/mM;	2-^{3}H glycine,	923 mC/mM;
G-^{3}H uridine,	760 mC/mM;	^{14}C Na formate,	13.4 mC/mM;
2-^{14}C thymidine,	58 mC/mM;	G^{14}C 1-lysine,	75 mC/mM;

(G-^{3}H uridine of 760 mC/mM specific radioactivity, was used in a single experiment described, as will be pointed out later.)

The radioactive substances were diluted in 0.9 % NaCl, to a specific activity of 0.1 mC/ml, and added to the suspensions in broth to be used for incubation at final concentration of 1 μC/ml; there was no dilution with non-radioactive substances, except in certain cases reported below under Results.

Preparation of the suspensions for incubation

Blood samples were obtained by venipuncture from more than 100 persons, under the conditions described previously, taking the standard precautions against microbial contamination [l.c. 4, 5, 6, 7, 8]. The blood was immediately citrated.

To PPLO Difco broth, with or without antibiotics (potassium penicillin G 100 I. U./ml and streptomycin 0.1 mg per ml), we added whole blood in a proportion of 1 : 10, or alternatively we added erythrocytes collected at 1200 r. p. m. and washed twice with 0.9 % NaCl or erythrocytes washed and resuspended in their own plasma, from which the leucocytes and thrombocytes had been removed by centrifugation at 5000 r. p. m. for 30 minutes.

Careful examination of the washed erythrocyte preparations showed that they contained no or a negligible number of leucocytes (in stained smears less than one in several fields at a magnification of 500 ×).

For each experiment, one suspension in broth was prepared in a vessel kept in an ice bath; after addition of the solution containing the substances under examination (with the exception of the cases mentioned below in which these substances were added after various intervals), the suspension was rapidly transferred to the smaller vessels used for incubation.

Incubation and sample taking

Incubation was done at 37 °C, with exceptions which will be discussed below. The vessels used were bacteriological test tubes, or test tubes with 2.5 cm in diameter, or Erlenmeyer flasks with a base diameter of 5 cm; these vessels held 10 to 30 ml of the suspensions. During incubation, the preparations were either left untouched or were stirred from time to time, or were stirred continuously.

In this manner, the degree of oxygenation was changed in order to determine the optimal conditions.

The preparations to be used for determinations at zero time were tested as soon as the cold specimens had been distributed; from the others, samples were taken at various time intervals during the incubation, as will be described below for each individual case.

Collection of the incubation products

At zero time and, subsequently at successive periods the suspensions were centrifuged at 7000 g for 10 to 20 minutes; in some cases, which will be mentioned

individually, there was a fractionated collection of the material sedimenting at 1200 rpm and at 7000 g. The sediments were washed twice in 0.9 % NaCl, containing non-radioactive forms of the radioactive substances of which the uptake was studied at concentrations at least 1000 times greater than those originally used.

Where the material was intended to be washed with cold $HClO_4$ to eliminate the acid-soluble fractions, or where it was intended for extraction of the nucleic acids, EDTA was added to the suspensions to a final concentration of 0.1 *M* at time zero and at intervals thereafter. Washing of the sediments with non-radioactive substances was carried out in such cases in the presence of 0.01 *M* EDTA.

Determination of radioactivity uptake

Direct extraction with warm $HClO_4$. After further washing with alcohol and ether, the material was dried and then extracted with 2—3 ml $HClO_4$ (0.5 *N*) for 40 minutes at 80 °C. After cooling and centrifugation, the radioactivity was determined in the supernatant.

Extraction with warm $HClO_4$ after removal of the acid-soluble fractions. Before washing with alcohol and ether, the material was washed twice with $HClO_4$ (0.2 *N*) at 0 °C; subsequently, it was extracted with $HClO_4$ (0.5 *N*) at 80° C.

Extraction and precipitation of the purines. Material obtained by incubation of 5 ml of erythrocytes was dried by washing with alcohol and ether, and then extracted with 10 ml $HClO_4$ (0.5 *N*) for 40 minutes at 80 °C; after centrifugation and removal of the supernatant, the extraction was repeated. The combined extracts were lyophilized and hydrolysed with $HClO_4$ (11 *N*) for one hour in a boiling water bath. After dilution with 10 volumes of water containing 275 μg adenine and 275 μg guanine, 1.4 ml 25 % NH_3 and 2 ml 10 % $AgNO_3$ solutions were added. After 24 hours in the refrigerator at 4° C, the precipitate was washed 3 times with 5 ml water, dried, and again dissolved with 2 ml HCl (0.1 *N*). Part of the solution was used for determination of the radioactivity and part for quantitative determination by spectrophotometry.

Extraction and precipitation of RNA. We used the method of Laskov et al. [9], partially modified to fit the particular material studied here. The sediment corresponding to the incubation of 5 ml erythrocytes was resuspended in 10 ml EDTA (10^{-4} *M*) at pH 8; 500 μg inert RNA (Calbiochem) and 10 ml of 90 % phenol were added. After stirring for 60 minutes at 20° C, the aqueous fraction was removed and the phenolic phase was re-extracted with 8 ml EDTA (10^{-4}*M*) at pH 8. The combined aqueous fractions were centrifuged at 10,000 g and the RNA was precipitated from the supernatant with two volumes of 95 % alcohol containing 2 % potassium acetate. After 24 hours in the refrigerator, the precipitate was separated by centrifugation, washed with alcohol and ether, dried and redissolved in 3 ml of $HClO_4$ (0.5 *N*) for 45 minutes at 80 °C. One part

was then used for the determination of radioactivity, and one part for spectrophotometric estimation.

Extraction and precipitation of DNA. Sediment obtained by incubation of 5 ml of erythrocytes was suspended in 10 ml of 10 % NaCl containing 0.01 *M* EDTA, and extracted, under stirring for 5 hours at room temperature; after centrifugation, 500 μg DNA (Calbiochem) was added in a 10 % NaCl solution. After stratification with equal volumes of absolute ethanol, the DNA was removed in the form of threads that clung to the glass stirring rod. After washing, carried out by transferring the glass rod five to ten times to a 2 : 1 alcohol-water bath, the material was dissolved in 0.5 *N* $HClO_4$ at 80 °C for 45 minutes. The solutions were used for determination of radioactivity and for spectrophotometry.

Protein fraction. In the case of incubation with G-^{14}C 1-lysine, after washing with alcohol and ether, we determined the radioactivity present in the extract which had been obtained with warm 0.5 *N* $HClO_4$, and also in the residue left after two successive extractions with warm $HClO_4$ and thereafter hydrolysed with 12 *N* HCl in a closed tube at 105 °C.

Electrophoretic method. For the determination of the degree of purity of the solutions of 6-^{3}H thymidine and G-^{3}H uridine we used 50 μl of the solution containing 100 μC per ml. The material was placed in the centre of a 3 cm wide strip of Whatman's 3 MM paper 3 cm wide. Citrate buffer 0.02 M at pH 3.5; application of 1000 V for 20 minutes. The strips were then dried and cut transversely into 1 cm wide bands for radioactivity determination.

Chromatographic methods. The extracts obtained with 0.5 N $HClO_4$ at 80 °C from incubation products, and the same extracts lyophilized and thereafter hydrolysed with 11 *N* $HClO_4$ for one hour in a boiling water bath, were studied by unidimensional descending chromatography, using 4.5 cm strips of Whatman's paper No 1. For the studies carried out with G-^{14}C 1-lysine, the hydrolysate of the protein fraction was chromatographed in 12 *N* HCl. Control chromatography was carried out with extracts to which known radioactive or non-radioactive substances had been added. The localization of the latter was determined by fluorescence examination, or with ninhydrin, or by exposure to iodine vapour, depending on the nature of the case.

The localization of the radioactive substances was carried out on transverse 1 cm strips cut from the chromatographs.

The solvents used for the development of the chromatographs are given in the descriptions of the individual experiments.

Thin-layer chromatography. Eastman Kodak fluorescent silica gel strips of 4.5 cm; flow stage: water. The strips were cut into transverse 1 cm bands for radioactivity determination. The localization of the substances studied was determined by fluorescence examination and with ninhydrin.

Determination of radioactivity. A three-channel liquid scintillation spectrometer model 725 Nuclear Chicago was used.

The solutions in $HClO_4$ or in HCl, were neutralized, were added, in amounts of 2—2.5 ml to 15 ml of solutions composed as follows: dioxan 1.1;

PPO, 7 g; POPOP, 0.3 g, naphthalene 10 g. After reading, the quenching was determined by adding solutions of known radioactivity. The strips of 4.5×1 cm or 3×1 cm that were cut in succession from chromatographic and electrophoretic strips, were immersed for the determination into a solution composed of: toluol, l.1; PPO, 4 g; POPOP, 0.05 g. The values in counts per minute were referred to the products of incubation of 1 ml of erythrocytes. Results of the determination of chromatographic and electrophoretic material are given in DPM.

Spectrophotometric determinations. Newly synthesized purine, RNA and DNA were, as mentioned above, extracted and precipitated in the presence of non-radioactive carriers added in considerable excess. By means of spectrophotometric (Beckmann DU) determinations, carried out on a part of the same solutions which were used for radioactivity determinations, we assessed the degree of purity of the preparations and obtained the quantitative data from which the total incorporated radioactivity could be calculated.

Autoradiographic method. Smears from suspensions in the process of incubation were determined by the standard method of Messier and Leblond [10] on Kodak NTB 3 emulsion.

Material

The results to be reported were obtained in blood samples taken from more than 100 persons who were regarded as clinically normal; these were of both sexes and of various ages, with different occupations, and all of them lived in the region where our Institute is located.

Results

The variability among the samples was great; in view of the impossibility of presenting all the data, and of the difficulty of adopting an entirely objective criterion in the selection of the results, it was decided to report in detail those which appeared to be representative of the effects observed.

A. *Preliminary tests*

1. *Absence of contamination in the radioactive thymidine and uridine solution.* Since it has been shown [11] that radioactive thymidine solutions may contain contaminating substances with affinity to protein molecules, and that the presence of such contamination may lead to errors in determining the uptake values, the 6-^{3}H thymidine and G-^{3}H uridine preparations were analysed electrophoretically. As can be seen in Fig. 1, no autodecomposition products were found. As regards 2-^{14}C thymidine and 2-^{14}C uridine, the uptake of nucleosides as such was demonstrated chromatographically: the experimental findings are reported below.

2. *Importance of the conditions of oxygenation.* Comparative tests, using incubation by different methods and with equal volumes of the same erythrocyte suspensions demonstrated that aeration was enhancing the uptake of the radioactive substances in question. If the preparation was not stirred, activity uptake was

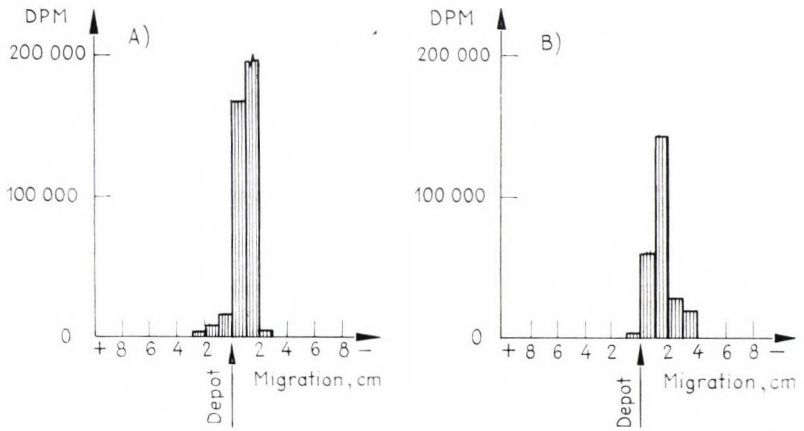

Fig. 1. Electrophoretic analysis of 6-^{3}H thymidine (A) and G-^{3}H uridine (B) preparations. Whatman's 3 MM paper; citrate buffer 0.02 M, pH 3.5; 1000 V for 20 minutes

facilitated if the surface exposed to air was large. The data listed below, for example, show the findings obtained in the 72nd hour of incubation of erythrocyte suspensions in vessels of different shapes, in the presence of 6-^{3}H thymidine:

surface (sq.cm)/volume (cu.cm) = 0.15 c.p.m. incorp. 630

surface (sq.cm)/volume (cu.cm) = 0.60 c.p.m. incorp. 1344

Accordingly, the findings to be reported refer to experiments carried out by methods which provided the most efficient oxygenation.

The possible significance of the preliminary findings presented above will be considered later.

B. *Direct extraction with warm* $HClO_4$

1. *Uptake of 6-^{3}H thymidine and G-^{3}H uridine.* Figure 2A shows the results for thymidine uptake by whole blood; the purpose was to determine the eventual differences brought about by the presence or absence of antibiotics. The incubation temperature was 30 °C: in this condition the uptake was found to increase until the 72nd hour. The presence of antibiotics appeared not to exert a significant influence on uptake. The difference between the two curves in Fig. 2A falls into the limits of variability of the data obtained from experiments carried out in duplicate in a single specimen of blood.

The curves in Fig. 2B show the uptake by washed erythrocytes of thymidine, uridine and of the two nucleosides together. Uridine uptake increased progressively whereas the incorporated quantity of thymidine reached a peak and subsequently decreased. This finding, confirmed by the results of subsequent experiments, is analogous to what had been observed in the uptake of thymidine and uridine by cultures of *E. coli* [12]. The curve of uptake in the presence of both thymidine and uridine practically represented the sum of the values obtained for the two nucleosides separately.

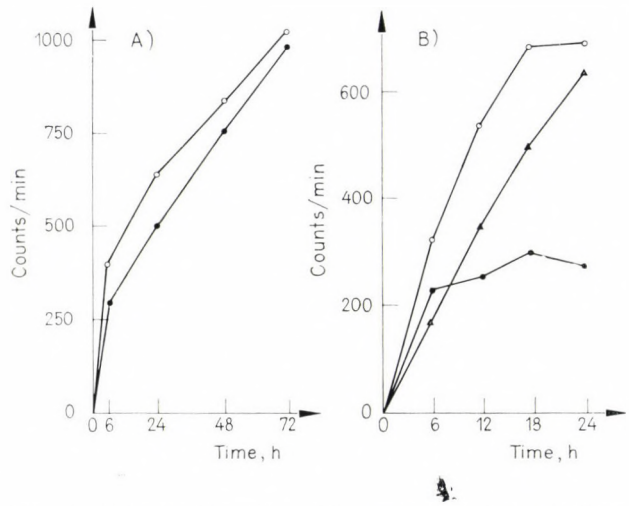

Fig. 2. A. Uptake of 6-^{3}H thymidine. Preparations of whole blood in the presence (●—●) and the absence (o—o) of antibiotics. Incubation at 30 °C. Extraction made directly with warm $HClO_4$. B. Uptake of 6-^{3}H thymidine (●—●), G-^{3}H uridine (▲—▲) and of the two nucleosides together (o—o). Washed erythrocytes; incubation temperature; 37 °C. Direct extraction with warm $HClO_4$

In assessing the quantitative ratios of 6-^{3}H thymidine and G-^{3}H uridine uptake in these and the subsequent experiments, it must be taken into account that the activity of uridine (with the exception of one case quoted separately below) was 45.4 % less than that of thymidine.

Figure 3 shows an autoradiogram of a smear of an erythrocyte suspension incubated in the presence of both thymidine and uridine; we can clearly observe the uptake of radioactivity into a component of erythrocytic nature which appears to be increased in volume and near the point of lysis.

In Fig. 4A, thymidine and uridine uptake by washed erythrocytes is shown up to the 72nd hour.

Although the samples had been taken at long time intervals, the peak times did not coincide; the initially incorporated radioactivity of thymidine was retransferred to the medium at an early stage.

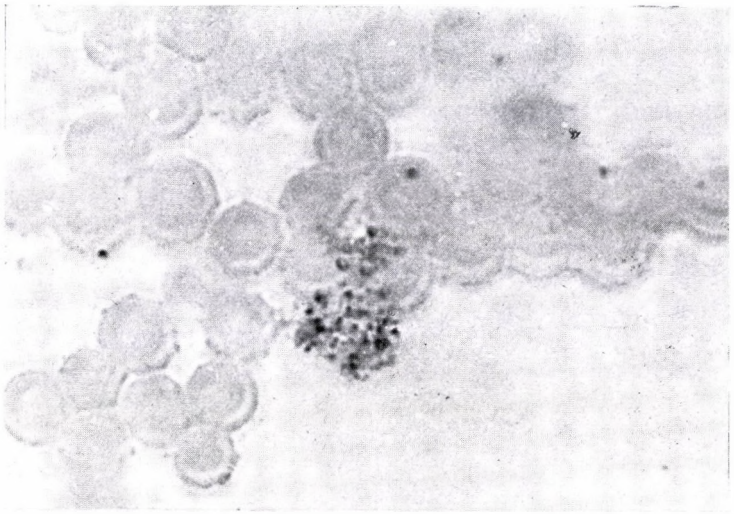

Fig. 3. Autoradiogram. Smear of erythrocytes after 24 hours' incubation in the presence of thymidine and uridine

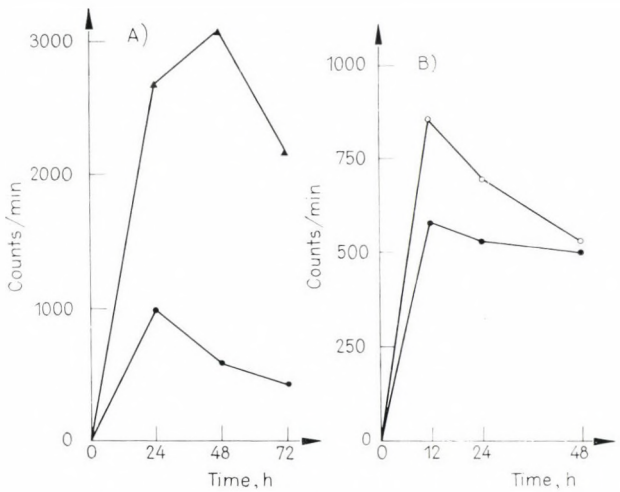

Fig. 4. A. Uptake of 6-^{3}H thymidine (●—●) and G-^{3}H uridine (▲—▲). Washed erythrocytes. Direct extraction with warm $HClO_4$. B. Uptake of G-^{3}H uridine. Washed erythrocytes (●—●), and washed erythrocytes resuspended in their own plasma centrifuged at 5000 r.p.m. (o—o). Direct extraction with warm $HClO_4$

Figure 4B shows a comparative experiment with washed erythrocytes, and with erythrocytes washed and then again suspended in their own plasma, centrifuged at 5000 r.p.m. In the latter case, uridine uptake was considerably greater:

the experimental conditions excluded the possibility of leucocytes and thrombocytes to play a part. The response of this particular blood sample differed from that seen in the majority of cases in that the decrease of uridine activity uptake, which was not followed by a subsequent increase, could already be observed between the 12th and the 24th hour.

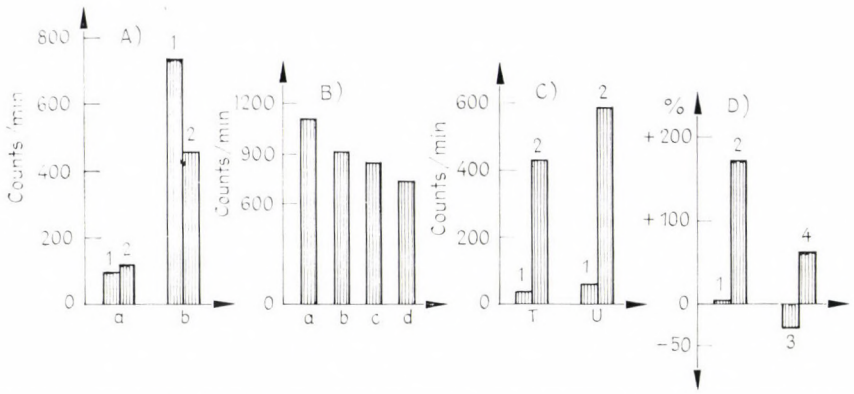

Fig. 5. A. Uptake of G-^{3}H uridine. Direct extraction with warm $HClO_4$. Two cases: a and b. Whole blood incubated in broth without radioactive material for 4 days, then centrifuged at 1200 r.p.m.; the supernatant was again centrifuged at 7000 g; the sediments 1 and 2, respectively, were resuspended in broth containing G-^{3}H uridine: 2 hours' incubation. B. Uptake of 6-^{3}H thymidine. Direct extraction with warm $HClO_4$. Washed erythrocytes. Series of suspensions kept for 2 hours in the presence of radioactive material, beginning immediately (a), or after 24 hours (b), 48 hours (c), or 72 hours (d), of incubation. C. Uptake of 6-^{3}H thymidine (T) and G-^{3}H uridine (U) in concentrations of 0.2 μC per ml (1) or 1 μC per ml (2). Whole blood, 15 hours' incubation. Direct extraction with warm $HClO_4$. D. Uptake of 6-^{3}H thymidine in the presence of non-radioactive thymidine at concentrations 500 times higher (1) and 15000 times higher (3); and of G-^{3}H uridine in the presence of non-radioactive uridine at concentrations 500 times higher (2) and 15000 times higher (4). Washed erythrocytes, 16 hours of incubation. Direct extraction with warm $HClO_4$. Results are given in percentage of the variation from the controls incubated with the radioactive substance alone

The results of another type of experiment, carried out to compare blood samples from two different persons are shown in Fig. 5A. Whole blood was incubated in broth for 4 days, in the absence of radioactive material. Then the suspensions were centrifuged at 1200 r.p.m.; the supernatant, which in one case was fairly clear, and in the other contained much particulate material and was coloured by haemolysis, was centrifuged again at 7000 g. The centrifugation sediments were then added to broth containing G-^{3}H uridine and once more incubated for 2 hours. Uptake was small by the suspensions made from the 1200 r.p.m. and 7000 g centrifugation sediments of the first sample (which showed no haemolysis and slight turbidity), whereas the two resuspended sediments of the second case showed a considerable uptake.

Thymidine uptake was then studied in a series of erythrocyte suspensions from the same subject, which had been exposed for 2 hours to radioactive substances immediately after their preparation, and subsequently at intervals of 24 hours during incubation. As can be seen in Fig. 5B, uptake decreased with time.

Figure 5C shows the findings obtained at incubation of whole blood for a period of 15 hours in the presence of 0.2 μC per ml or 1 μC per ml of thymidine or uridine, corresponding, respectively, for thymidine to 39.3 and 196.5 μ μM per liter, and for uridine to 72 and 360 μ μM per liter. As can be seen, the ratios of the values for activity uptake in the presence of the greater and the lesser concentrations, were 9.5 for thymidine and 9.1 for uridine, much higher than the value for the ratio between the available amounts. If we keep in mind that, in either case, only a small part of the available activity was taken up, we may postulate that the uptake was stimulated, at least within the time limits in question, by the presence in the culture medium of higher concentrations of both tritiated nucleosides.

Next, experiments were carried out with erythrocytes incubated in the presence of 6-^{3}H thymidine and G-^{3}H uridine, with addition of the same nucleosides in their non-radioactive forms at different concentrations. Uptake values in the 16th hour are listed in Fig. 5D, expressed as percentage variations as compared to controls incubated in the presence of the radioactive forms. An increase in the concentration of non-radioactive uridine from 0.80 mM/l to 5.4 mM/l (from 500 to 15 000 times the concentration of the G-^{3}H uridine) led to a considerable increase in uptake; if we consider the ratio of activity dilution, the higher concentration was found to represent a definitely stronger stimulus. With thymidine and the lower concentration of non-radioactive substance added (500 times the radioactive substance; 0.098 mM/l) there was a modest increase in activity uptake; if the concentration of the non-radioactive substance had been increased 15000 times (2.957 mM/l), activity uptake was definitely impaired, in contrast to the case with uridine.

The findings for thymidine may be regarded as due to interactions of several different factors which affect the experimental conditions to a degree which could not be assessed individually, *viz.* a stimulatory effect due to an increase in the concentrations of the nucleoside, the consequence of the dilution of activity; the possible inhibitory action exerted by the activity of incorporated 6-^{3}H thymidine, which inhibition was not, or was not completely, eliminated by dilution with non-radioactive nucleoside (compare with Fig. 2B and see 12).

2. *Uptake of 2-^{14}C thymidine and 2-^{14}C uridine.* The results of these experiments were in all respects analogous to those obtained with the corresponding tritiated nucleosides. The curves in Fig. 6A which refer to the incubation of erythrocytes, show the rising uridine uptake: the fall seen between the 12th and the 24th hour is the consequence of an interruption in the stirring of the preparations, and confirms observations reported earlier; thymidine uptake reached its peak before the uptake of uridine, and subsequently decreased rapidly.

Figure 6A shows the course of 2-^{14}C thymidine uptake in the presence of non-radioactive thymidine at a 50fold concentration [0.845 mM/1]. There was a rapid initial increase of uptake, followed by a slow progressive increase: there was no peak. This finding can be interpreted by analogy to the experiments using 6-^{3}H thymidine in the presence of non-radioactive thymidine: an initial stimulatory action due to the higher concentration of the nucleoside; and a consequence of

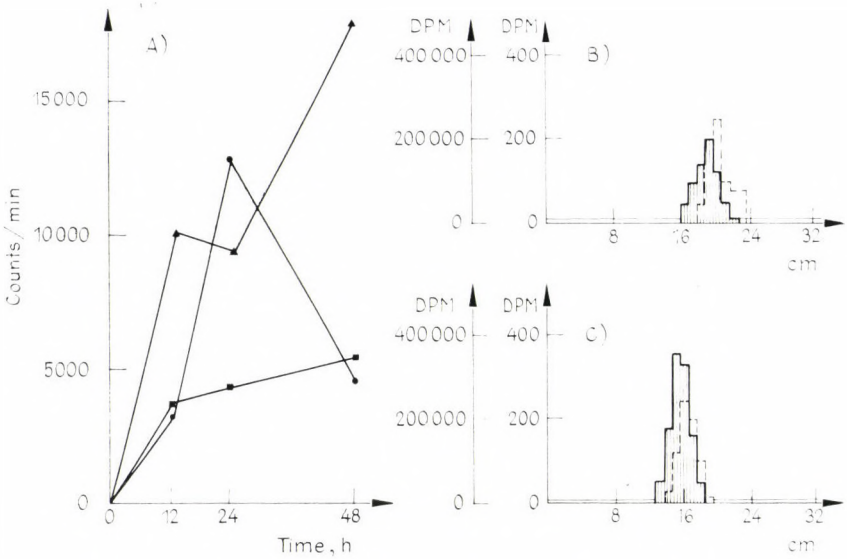

Fig. 6. A. Uptake of 2-^{14}C thymidine (●—●), 2-^{14}C uridine (▲—▲) and 2-^{14}C thymidine + non-radioactive thymidine at 50fold concentration (■—■). Direct extraction with warm $HClO_4$. B and C. Radiochromatography. Running phase: isopropanol, 170 parts; HCl (S. G. I, 19), 41 parts; water added to 250 parts. The extracts in $HClO_4$ (0.5 N) at 80 °C, obtained after 48 hours from the products of incubation reported in Fig. 6A (B in presence of 2-^{14}C thymidine, C in presence of 2-^{14}C uridine) were lyophilized and hydrolysed with $HClO_4$ (11 N); chromatographed as such (————,values on the ordinate at the right) or after addition of the hydrolysates of the nucleosides used in the experiment (— — —, values on the ordinate on the left)

the dilution of activity, which dilution, although leading to a decrease of the number of c.p.m., may thereafter exert a positive action by eliminating the inhibition resulting from the uptake of activity itself.

The extracts in $HClO_4$ (0.5 N) at 80 °C, lyophilized and hydrolysed with $HClO_4$ (11 N) were chromatographed, with and without addition of the products of hydrolysis of the same nucleosides used in the experiment. Running phase: isopropanol, 170 parts; HCl (S. G. 1.19), 41 parts; and water, added to total 250 parts. The results obtained (Fig. 6B and C) showed that thymine and uracil

were incorporated as such, and that the radioactive material used did not contain any impurities measurable by this method.

3. *Uptake of radioactivity from* ^{14}C *Na formate.* Uptake by erythrocyte suspensions showed a rapidly progressive increase (Fig. 7A).

Uptake was estimated also in the presence of non-radioactive thymidine and uridine at different concentrations. The findings reported in Fig. 7B expressed as percentage of the increase during 40 hours of incubation with ^{14}C Na formate showed that in the presence of 1.8 to 5.4 mM/1 thymidine there was a significant

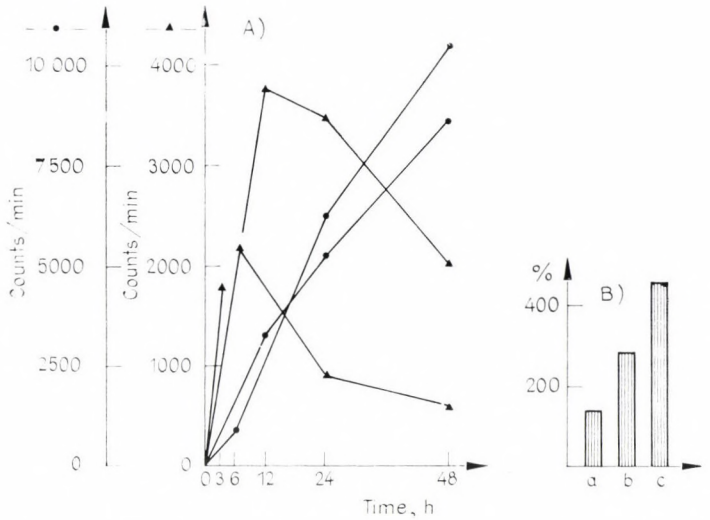

Fig. 7.A. Uptake of radioactivity from ^{14}C Na formate (●—●, left ordinate) and from 2-^{3}H glycine (▲—▲, right ordinate). Washed erythrocytes. Direct extraction with warm $HClO_4$. B. Uptake of radioactivity from ^{14}C Na formate in the presence of non-radioactive thymidine at concentrations from 1.8 to 5.4 mM/1 (a), and of non-radioactive uridine at concentrations from 10 (b) to 30 (c) mM/1. Values expressed as percentage of the difference in uptake, in the presence of ^{14}C Na formate alone. Washed erythrocytes. 40th hour of incubation. Direct extraction with warm $HClO_4$

stimulatory action to be observed which, however, did not change within the concentration limits of the experiment. As the concentration of uridine increased up to 30 mM/1, the uptake of ^{14}C Na formate showed a progressive rise. These findings were in good agreement with previous ones concerning uridine and thymidine activity uptake at varying concentrations of the same radioactive or non-radioactive nucleosides.

The products obtained after incubation with ^{14}C Na formate were examined by paper chromatography in n-butanol—water (86 : 14). The determinations of radioactivity and the localization tests carried out with non-radioactive substances revealed an uptake into purine bases (adenine and guanine), which could not be

identified separately by radiochromatography as they were not sufficiently resolved (Fig. 8A).

4. *Uptake of radioactivity from 2-^3H glycine.* Uptake by erythrocyte suspensions displayed a rapid initial rise followed by a rapid fall (Fig. 7A). The incubation products were subjected to paper chromatography in an isopropanol—HCl (S. G., 1. 19)—water (170 : 41 : 250) system and to thin layer chromatography with silica gel. The radioactivity could be demonstrated in both the adenine and the guanine (Fig. 8B); the thin layer chromatogram of the 6 hour incubation prod-

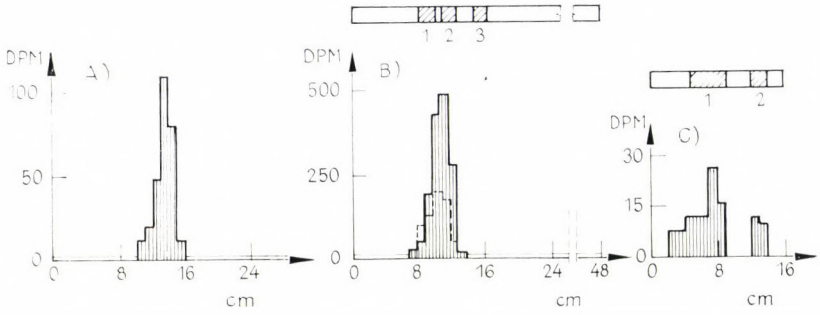

Fig. 8. A. Radiochromatography. Running phase: n-butanol—water (86 : 14). Uptake of radioactivity from ^{14}C Na formate. Washed erythrocytes. 24th hour of incubation. Direct extraction with warm $HClO_4$, lyophilization of the extract and subsequent hydrolysis with $HClO_4$ (11 N). B. Radiochromatography. Running phase: isopropanol, 170 parts; HCl (S. G. 1.19), 41 parts; water, added to 250 parts. Uptake of radioactivity from 2-^3H glycine. Washed erythrocytes. Direct extraction with warm $HClO_4$, lyophilization of the extract and subsequent hydrolysis with $HClO_4$ (11 N), 6th (– – – –) and 12th (————) hour of incubation. Above the radiochromatographic curve, a chromatograph is shown (reading in U.V. and with ninhydrin) prepared with the hydrolysate of the incubation product at the 12th hour with addition of non-radioactive guanine (1), adenine (2), or glycine (3). C. Thin-layer radiochromatography (silica gel). Running phase: water. Uptake of radioactivity from 2-^3H glycine. Washed erythrocytes. 7th hour of incubation. Direct extraction with warm $HClO_4$, lyophilization of the extract, and subsequent hydrolysis with $HClO_4$ (11 N). Above the radiochromatographic trace, a strip prepared with the hydrolysate of the incubation product, with the addition of non-radioactive adenine and guanine (1) and non-radioactive glycine (2); reading in U.V. light and with ninhydrin

ucts also showed 2-^3H glycine, distinctly separated from the adenine and guanine which together formed a single elongated spot with a pronounced maximum (Fig. 8C).

C. *Extraction with warm $HClO_4$ preceded by removal of the acid-soluble fractions*

1. *Uptake of 6-^3H thymidine and G-^3H uridine.* We used uridine with a specific radioactivity of 760 mC/mM. The tests were carried out with erythrocytes. Fig. 9A shows the findings obtained after elimination of the acid-soluble fraction,

and, for comparison, the findings obtained after direct extraction with warm 0.5 N $HClO_4$ of parallel incubation products. A considerable portion of the incorporated activity was soluble in cold acid, and the decrease after cold extraction was greater with the samples incubated with uridine than with those incubated with thymidine. Similar findings were obtained in other, analogous experiments and even though we have to take into account the more rapid turnover of RNA as compared with that of DNA, we must consider the possibility of a partial solubilization and extraction of the RNA in the course of cold washing.

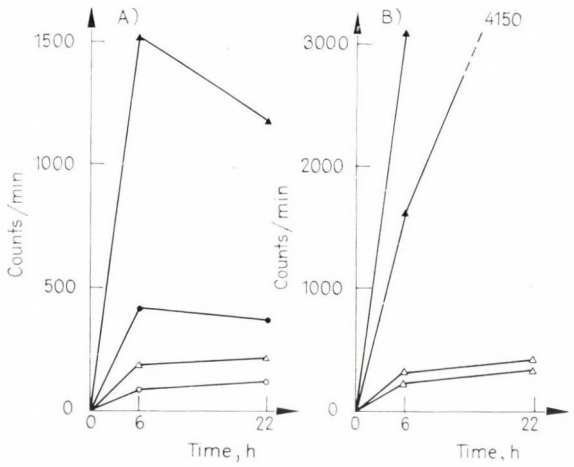

Fig. 9. A. Uptake of 6-^{3}H thymidine and G-^{3}H uridine. Washed erythrocytes. Direct extraction with warm $HClO_4$ (thymidine: ●—●; uridine: ▲—▲), and with warm $HClO_4$ after elimination of the acid soluble fraction (thymidine: o—o; uridine: △—△). B. Uptake of radioactivity from 2-^{3}H glycine. Washed erythrocytes. Direct extraction with warm $HClO_4$ (▲—▲), or with warm $HClO_4$ after elimination of the acid-soluble fraction (△—△)

These findings mean that only a fraction of the incorporated nucleosides is localized in the nucleic acids. If we consider also the time intervals at which the determinations were carried out, we may relate this finding to the development of lytic processes, which have been demonstrated in earlier experiments; it has in fact been shown that with the passing of time, there occurs a loss of the total radioactivity previously incorporated from thymidine and uridine.

2. *Uptake of radioactivity from 2-^{3}H glycine.* Simultaneously incubated erythrocyte preparations were used. The findings obtained in the products of extraction with and without preliminary washing in cold acid were compared. As the curves in Fig. 9B show, a large part of the initially incorporated activity which subsequently returned into the medium (cf. Fig. 7A) was localized in the acid-soluble fraction. The size of the radioactive fraction resistant to cold extraction and soluble in 0.5 N $HClO_4$ at 80 °C, and which accordingly may be considered

to belong to the nucleic acids, was found to change with time in good agreement with the course observed in similar experiments done with thymidine and uridine (cf. Fig. 9A).

D. *Extraction of the purines and of the nucleic acids. Uptake of radioactivity from 2-^{3}H glycine, 6-^{3}H and 2-^{14}C thymidine, 2-^{14}C uridine*

The data presented in Table 1 refer to the radioactivity determined in purified material, extracted after incubation of 5 ml erythrocytes in 25 ml broth for 22 hours.

Table 1

Radioactivity in purines and nucleic acids extracted from 5 ml erythrocytes after 22 hours of incubation
10 cases selected at random

Cases	Uptake of	Incorporation in	C. p. m.
1	2-^{3}H glycine	purines	1350
2	2-^{3}H glycine	purines	2706
3	2-^{3}H glycine	purines	2409
4	6-^{3}H thymidine	DNA	513
5	6-^{3}H thymidine	DNA	113
6	6-^{3}H thymidine	DNA	254
7	6-^{3}H thymidine	DNA	176
8	6-^{3}H thymidine	DNA	907
9	2-^{14}C thymidine	DNA	8200
10	2-^{14}C uridine	RNA	934

E. *Protein fraction*

Incorporation of G-^{14}C l-lysine. Activity uptake into the fraction of whole blood extractible with $HClO_4$ at 80 °C, without previous washing in cold acid, was rapid. Uptake into the protein fraction, which increased with time, was less marked (Fig. 10A).

Figure 11 shows an autoradiogram of a smear prepared in the 72nd hour of incubation; the activity is incorporated into elements of erythrocyte nature, whose changes of volume and transparency reveal structural alterations (phase contrast photograph).

We subjected to paper chromatography in a tertiary butanol–formic acid–water, 70 : 15 : 15 system the hydrolysate of the protein fraction obtained at 72 hours; the same hydrolysate added to the same solution of G-^{14}C l-lysine as had been used in the experiments; and the solution of G-^{14}C l-lysine by itself. It was found that the solution used in the experiment contained not only the amino acid,

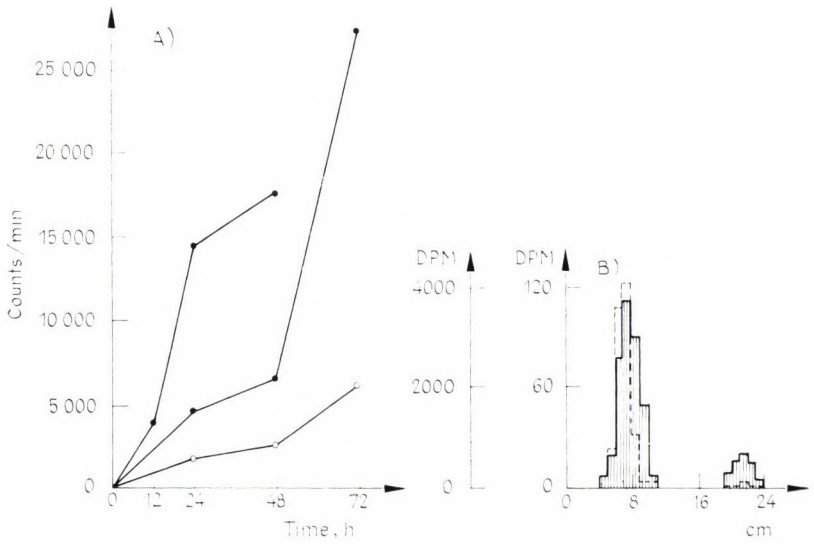

Fig. 10. A. Uptake of radioactivity from G-^{14}C 1-lysine. Whole blood. Product of direct extraction with warm $HClO_4$ (●—●), and hydrolysate of the protein fraction (o—o). B. Radiochromatography. Running phase: tertiary butanol–formic acid–water (70 : 15 : 15). Uptake of radioactivity from G-^{14}C 1-lysine. Whole blood. Hydrolysate of the protein fraction (———, right ordinate), and hydrolysate of the protein fraction with addition of G-^{14}C 1-lysine (— — —, left ordinate)

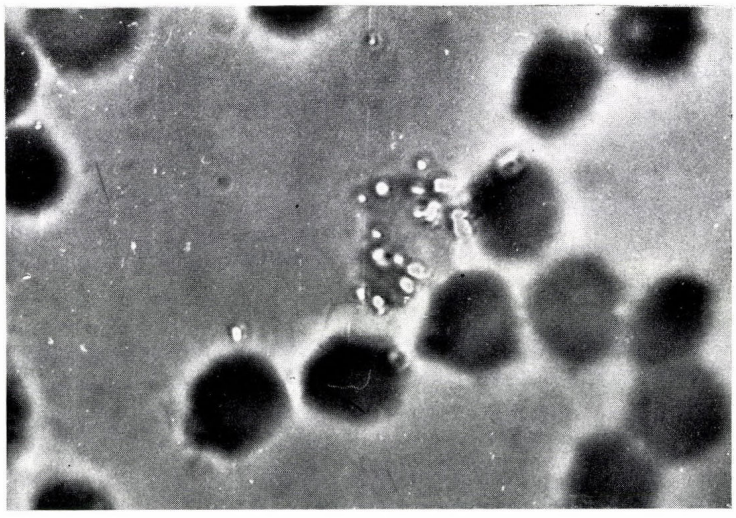

Fig. 11. Autoradiogram of a smear prepared after 72-hour incubation in the presence of G-^{14}C 1-lysine. Phase contrast microscopy

but another substance with an Rf. of 0.67, the radioactivity of which amounted to 1.75 % of the radioactivity of the lysine. In the hydrolysate of the incubation product added to the lysine solution, the activity of the impurity amounted to 2.41 %; in the hydrolysate of the incubation product alone, the activity of the impurity, still with an Rf. of 0.67, amounted to 17.05 % of the radioactivity incorporated as lysine (Fig. 10B). The chemical nature of the impurity was not deter-

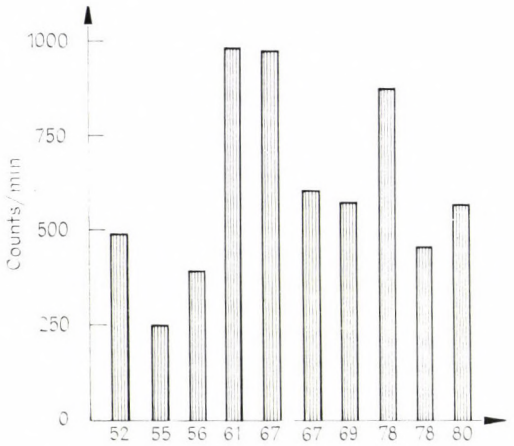

Fig. 12. Uptake of 6-^{3}H thymidine by blood samples from 10 clinically normal male su bject selected at random from among a group of adults and elderly persons. Ages noted below the values of incorporated radioactivity. Washed erythrocytes, 24 hours of incubation. Direct extraction with warm $HClO_4$

mined: the fact that it was present in a very small amount in the solution of G-^{14}C-l-lysine, and was much more concentrated in the incubation product suggested that it was either a substance introduced as such, and which was relatively more easily incorporated, or it was a substance that was directly and more rapidly derived from the lysine under the influence of the incubation, in the presence of blood cells.

Discussion

Tests with cultures of blood from clinically healthy subjects consistently demonstrated a moderate, but widely varying uptake of radioactive thymidine and uridine. The maximum order of magnitude of the uptake observed in these investigations was found to be 5.5×10^{-3} μ μM and 10×10^{-3} μ μM, respectively, for thymidine and uridine, with part of the radioactivity localized in the nucleic acids. To demonstrate the variability in uptake, Fig. 12 shows the results of determinations carried out for purposes of comparison, using washed erythrocytes

taken from 11 persons selected at random: these samples were incubated for 24 hours in the presence of 6-^{3}H thymidine.

Within the concentration limits reported, the uptake was stimulated by the presence of both radioactive and non-radioactive nucleosides.

The radioactivity of formate or glycine was incorporated into the purine bases, also within wide limits of variability. With glycine, in particular, part of the activity was located in the fraction which was not soluble in cold acid but could be extracted with warm $HClO_4$, in other words, in the nucleic acids.

In the experiments with lysine, uptake into an acid-soluble fraction and into the protein fraction was observed.

The determinations were carried out in the course of the incubation, at various but always fairly long time-intervals. Accordingly, the course of the curves represents only approximately the course of the uptake, but the findings obtained in different experimental conditions have, on the whole, been reproducible and in good agreement. The possibility that the phenomena observed are to be attributed to processes of adsorption or to the formation of complexes of various nature could be excluded. Such a conclusion could be drawn from a number of findings such as the relationship between the temperature of incubation, the degree of oxygenation of the blood culture, and the development of the uptake; and the summation of the values for radioactive uptake from thymidine and uridine in the case of incubation with both nucleosides present in excess in the medium; the variations in the degree of uptake in relation to the presence, and to the concentration variations in the solution of the non-radioactive or radioactive forms of nucleosides; the observed uptake into the purines of the radioactivity of formate or glycine, and into the nucleic acids of the radioactivity of the pyrimidine nucleosides.

The relations between the thymidine and uridine uptakes, their changes with the passage of time, the clearly evident inhibition exerted by the radioactivity of the thymidine incorporated on the progress of the incorporation (analogous to what can be observed in tissue cultures and broth cultures of schizomycetes) which inhibitory action with the passage of time is added to the initial stimulatory action exerted by the nucleoside: all these data support the hypothesis of the existence of metabolic activities concerning the nucleic acids in the examined blood cultures.

The phenomena described were observed not only with whole blood, but also with washed erythrocyte suspensions. Although in the former case the possibility that leucocytes and thrombocytes played a part cannot be excluded, it has nevertheless been shown that, in the phenomena considered, a special role has been played by the erythrocytes and/or the particulate material accompanying the erythrocytes in suspension. As stated under Methods, the leucocyte content of the washed erythrocyte preparation was so low as to exclude their significant contribution to the observed activities. This view was confirmed by the autoradiogram and by the uptake of the particulate material that remained free in the suspension after the lysis of the blood cells.

With reference to the uptake of lysine into the proteins, we may postulate a synthesis of haemoglobin by reticulocytes, or of proteins by other cells (thrombocytes, [13]), although such a hypothesis was not supported by the prolonged period of uptake, nor by the considerable amount of radioactivity incorporated in relation to the number of reticulocytes present, nor by the autoradiographic findings.

The uptake of nucleosides into nucleic acids should not be regarded as due to any metabolic activity of erythrocytes or of reticulocytes. Nor does it appear that the more marked uptake of thymidine and uridine into the acid-soluble fraction was due to some metabolic activity connected with the synthesis of the corresponding nucleotides which, so far as we know, are not significant and do not appear in the cycle of activity of the circulating elements of the red series.

Further, according to the literature [14], the activity of these circulating elements cannot be held responsible for the uptake of radioactivity from glycine and formate into purine bases, because no *de novo* synthesis of the bases of the purine nucleotides takes place in erythrocytes.

In view of the modest degree of the uptake phenomena observed, it may be assumed that some of them may have escaped attention or has been regarded as negligible by the earlier investigators concerned with the metabolic activities of erythrocytes; and that they may be considered part of the normal biochemical processes of these cells.

On the other hand, it must be remembered that whereas our findings have always been consistent from the qualitative point of view, quantitatively they varied widely, from one case to another, and this finding is not easily explained as part of a normal situation. The autoradiographic findings, in addition, have shown that the radioactivity is localized in a variable number of cells, that were morphologically altered, or were in a more or less advanced stage of lysis.

Furthermore, if the phenomena observed belonged wholly or in part to the normal metabolic activities of the blood cells, it ought in all probability to be possible to determine them in much shorter periods of time than was the case in the present investigation. The intervals of several hours or days, needed for the demonstration of the reported findings, are instead indicative of phenomena that take place outside the organism, as in the case of multiplication of microbial forms in blood cultures.

The experiments were performed in blood samples carefully collected and handled in order to avoid microbial contamination. The results appeared to be unaffected by the presence of antibiotics at a concentration which still allowing multiplication of some L-forms and mycoplasmas, inhibit most of the possible contaminating bacteria. Finally, no bacterial growth was ever noticed in the specimens even in the absence of antibiotics.

The above-mentioned studies by other authors together with our own investigations, have yielded morphological, histochemical, and biochemical findings that revealed the behaviour of blood cultures at time intervals of the order of those considered here; the findings may be regarded as indicative of the existence of diffuse latent infections in large groups of the population by mycoplasmas or

schizomycetes in their L-phase. In support of these findings, it seems logical to assume that the present results are indicative of the turnover of nucleic acids associated with the multiplication and evolution of similar microbial forms in cultures.

Further investigations of a comparative nature will have to be carried out in large groups of persons, subdivided according to age, sex, social state and habitat, and in health and disease. The purpose of these investigations will be to determine the correlations, if any, between the particular individual and the intensity of the biosynthetic activity of the type considered here, so as to relate to possible modifications of that state of diffuse infection to which it would appear that the activities in question must be attributed.

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Hyperleukocytosis before the Beginning of Treatment in Acute Leukaemia in Children and its Subsequent Repetition at the Onset of Relapses

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The degree of leukocytosis at the beginning of 144 relapses of acute leukaemia, determined by examination of the bone marrow, was studied. In 30 children with leukocytosis 10,000/mm^3 or less before beginning of treatment, 65 relapses were observed, including 4 with leukocytosis above 10,000/mm^3. In the second group of 28 children (leukocytosis over 10,000/mm^3 before treatment), 79 relapses were observed, including 22 with leukocytosis over 10,000/mm^3. In the second group, compared with the first, blastic hyperleukocytosis at the beginning of relapses was significantly more frequent.

An increased number of white blood cells in acute leukaemia before the beginning of treatment is a classic symptom of unfavourable immediate prognosis [4, 10, 13, 15, 16, 18, 20]. It has been observed that acute leukaemia cases characterized by extensive dissemination of leukaemic cells in the peripheral blood manifesting in hyperleukocytosis exhibit a number of peculiarities in the course of the disease. One of them consists in a greater frequency of blastic hyperleukocytosis at the beginning of relapses [1]. We have studied the frequency of hyperleukocytosis at the beginning of relapses and its prognostic implications on a larger number of patients.

Material and Methods

The material of the analysis consisted of children suffering from acute leukaemia in the years 1962—1968. Therapy of the first phase and relapses of leukaemia have changed in the meanwhile. Since 1963, special attention has been given to the initial symptoms of acute leukaemia indicating unfavourable direct prognosis, especially to blastic hyperleukocytosis. In those cases more intensive therapy was applied in the first phase of the disease, consisting of larger doses of prednisone and replacement of 6-mercaptopurine by methotrexate injected intravenously in some cases. Certain patients were treated with combinations of 3, or even 4 drugs. Toward the end of 1964, in addition to pharmacologic therapy, low-protein purine-free diet was introduced in the treatment of all patients during exacerbations and remissions [7]. Changes in the therapy of relapses of acute

lymphoblastic leukaemia included introduction of vincristine (1965), cytosine arabinoside (1967), and rubidomycin (1967); the two last-mentioned drugs, however, were never used in the beginning of the disease.

At the same time, the management of remissions changed little and was the same in cases with immediately poor or better prognosis. In the years 1962—1964, patients in remissions were treated with 6-mercaptopurine in doses of 2.5 mg/kg/24 hr, or 2.5 mg/kg/48 hr. Since 1965, they have been treated by alternate administration of 6-mercaptopurine in doses of 2.5 mg/kg/24 hr and methotrexate in doses of 0.5 mg/kg twice weekly, orally, or less often intramuscularly. The drugs were exchanged periodically every 4—6 weeks. Importance was attached to control during remissions. Clinical examinations, laboratory findings as well as blood and bone marrow studies were always performed by the physician who had previously treated the child in the clinical ward. The examinations were repeated every 14 days, or less often every 3 weeks. Examination of the peripheral blood and the finding of a few parablasts (usually less than 10 per 250 white blood cells) were found to be decisive for the diagnosis of relapse. In spite of the bone marrow being loaded with parablasts at this time, the child usually exhibits no symptoms of the disease, and thrombocytopenia and anaemia are absent [2, 8].

Less often, relapse was indicated by neutropenia or by quickly developing blastic hyperleukocytosis. Further relapses were detected also by routine bone marrow punctures. Cases in which the myelogram contained less than 50 % parablasts were not included in the study on blastic hyperleukocytosis. However, this did not pertain to cases in which bone marrow parablastosis below 50 % was accompanied by the presence of parablasts in the peripheral blood on repeated examinations. This previously described discordance [3], was exceptional in relapses. Only cases of acute lymphoblastic leukaemia were included in the study. Diagnosis of the type of leukaemia was established mainly on the basis of morphological criteria by a group of physicians constantly employed in the haematologic ward. Patients treated with prednisone before leukocyte counts were made were excluded from the analysis. Only cases were selected in which the first complete remission was attained, and which were observed and treated during the remission and during at least one relapse.

Assuming the upper limit of leukocytosis at 10,000/mm^3 before beginning of treatment, 58 cases of acute lymphoblastic leukaemia were divided into two groups:

I. 30 cases with white blood cell counts $<$ 10,000/mm^3 before beginning of treatment;

II. 28 cases with a leukocytosis of $>$ 10,000/mm^3 before beginning of treatment.

Leukocytosis over 10,000/mm^3 in relapses, confirmed by the myelogram, was always associated with the presence of numerous leukaemic cells in the peripheral blood, that is, with blastic hyperleukocytosis. The distribution of the cases in the two groups was similar in different years. The distribution of the cases according to age and sex is shown in Table 1. In 58 children 151 relapses were observed.

Table 1

Age at diagnosis	> 1–4	> 4–7	> 7–10	> 10
Male	11	12	11	3
Female	5	8	5	3
Total	16	20	16	6

Table 2

WBC per mm^3 at the beginning of relapse	Number of relapses treated	Number and percentage of the bone marrow remissions*
$\leq$ 10,000	118	87 (73.81 %)
> 10,000	33	15 (45.45 %)

* Bone marrow remissions depended on the presence of 10 % or less parablasts and blasts in the myelogram.

The percentage of remissions was much smaller in relapses with initial white blood cell counts higher than 10,000/mm^3 (Table 2). Hyperleukocytosis at the beginning of relapse was observed 33 times in 58 children which had 151 relapses. In 7 cases the hyperleukocytosis was preceded by a discontinuation of administration of the antimetabolite by the children's parents for 1—3 months during remission. These relapses were omitted from the analysis. The frequency of relapses with hyperleukocytosis in the two groups has been compared in Table 3. The frequency of

Table 3

WBC per mm^3 at the beginning of the disease	WBC per mm^3 at the beginning of relapses		
	$\leq$ 10.000	> 10.000	
$\leq$ 10.000	61	4	65
> 10.000	57	22	79
Total	118	26	144

$p < 0.001$

relapses with hyperleukocytosis in relation to leukocytosis at the beginning of the disease was compared by means of the χ^2 test. Hyperleukocytosis was significantly more frequent in cases with hyperleukocytosis at the beginning of the acute

phase, compared with patients who had 10,000 white blood cells or less per mm^3 before beginning the treatment.

In the group of 30 children with initial leukocytosis less than $10,000/mm^3$, the mean duration of the first remission was 8.7 months (mean 6.7 months), and the mean survival time of the children 19.6 months (15.3); four children are still under treatment.

In the group of 28 children with initial leukocytosis over $10,000/mm^3$, the mean duration of the first remission was 6.7 months (mean 4.7), and mean survival 16.6 months (mean 11). Two children are still under treatment. The differences were more pronounced in 6 children of the last group with leukocytosis over $100,000/mm^3$ at the beginning of treatment. The mean duration of the first remission in these cases was 3.8 months (median 4), and mean time of survival 9.8 months (median 9.5).

In previous studies it was found that cases with blastic hyperleukocytosis at the beginning of illness, compared with a control group, were characterized by more frequent appearance of meningeal leukaemia [1,9] and tumour of the kidneys resulting in worse immediate prognosis [1].

Cases of acute leukaemia with accumulation of prognostically unfavourable symptoms at the beginning of illness (hyperleukocytosis, brief history, marked hepatosplenomegaly, etc.) showed different further course of the disease. Combined therapy with 3 or 4 drugs during the first acute phase of the disease does not ensure long survival of the children in good condition during remission. 6-mercaptopurine and methotrexate in the remissions fulfil its purpose insufficiently and should be replaced by more intensive therapy [5, 6, 11, 12, 14, 19]. A lot of modern clinical studies justify the rightness of such a management. Many of them are based on previous experimental works [17]. Treatment of acute leukaemia in children has to be individually adjusted.

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Beitrag zu Skelettmanifestationen von Leukosen, Lymphogranulomatosen und Retothelsarkomen

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Es werden Skelettveränderungen bei chronischen leukämischen Myelosen, unreifzelligen Leukosen, chronischen Lymphadenosen, Lymphogranulomatosen und lymphoretikulären Sarkomen anhand eines klinischen Krankengutes beschrieben. Die Art und Häufigkeit von Knochenherden in den Autopsiebefunden eines pathologischen Institutes von an bösartigen hämatologischen Erkrankungen Verstorbenen werden mit den klinischen Ergebnissen verglichen. Es fanden sich herdförmige Osteolysen, umschriebene oder diffuse Osteosklerosen, Osteoporosen und ganz vereinzelt Periostreaktionen. Prozeßbedingte ossäre Veränderungen waren im autoptischen Material häufiger als durch klinische und röntgenologische Untersuchungen nachzuweisen. Aus der Erkennung von Skelettherden bei den untersuchten Krankheitsgruppen lassen sich u. U. spezielle prognostische und therapeutische Folgerungen ableiten.

Bei langjähriger Betreuung eines hämatologischen Krankengutes konnten an einzelnen dieser Patienten Skelettveränderungen beobachtet werden, die im klinischen Verlauf als Selten- oder Besonderheit zu betrachten sind. — In unserem Leukämiematerial aus den Jahren 1956 bis 1965 stehen die *chronischen leukämischen Myelosen* mit 49 auswertbaren Krankheitsverläufen an erster Stelle. Hinzu kommen noch Erhebungen aus der Durchsicht von Autopsiebefunden der Jahrgänge 1960 bis 1964.* Die Zahl der myeloischen Leukosen dieser Studie beträgt 48; d. h. insgesamt auswertbar waren demnach 97 chronische myeloische Leukämien.

Trotz starker Proliferation des myeloischen Gewebes werden bei dieser häufigen Leukämieform nur selten Skelettmanifestationen klinisch und röntgenologisch intra vitam diagnostiziert. Jaffé und Schneider [15, 25] u.a. betonen, daß ossäre Veränderungen erst in relativ späten Krankheitsstadien auftreten. Diese Seltenheit ist damit zu erklären, daß das Erwachsenenskelett über einen größeren Reserveraum für eine expansive Ausbreitung von neoplastischem hämatopoetischem Gewebe verfügt. Bei längeren Verläufen finden sich nach Naumann [20] generalisierte *Osteoporosen*, worauf schon Apitz [2] im älteren Schrifttum hinwies. Heilmeyer [11] dagegen vermißt bei der Mehrzahl der chronischen myeloi-

* Herrn Professor Dr. Coutelle, dem Direktor des Pathologischen Institutes der Martin-Luther-Universität Halle, danken wir für die freundliche Erlaubnis zur Verwertung der Sektionsprotokolle

schen Leukosen diesen Befund. Die Unterscheidbarkeit gegenüber Altersosteoporosen ist mitunter schwierig, wobei rasche Progredienz eventuell für eine leukämische Genese verwertbar ist. — Neben der Osteoporose kommen fleckförmige *Osteolysen*, besonders der proximalen Enden von Röhrenknochen, z. T. kombiniert mit periostalen Belägen vor. Alder [1] beschreibt zirkumskripte Knochenzerstörungen im terminalen Myeloblastenschub. Da bei einzelnen Verläufen *osteosklerotische* Veränderungen angetroffen werden, bleibt stets die Osteomyelosklerose differentialdiagnostisch abzugrenzen, soweit im Einzelfall eine Trennung überhaupt möglich ist. Zur Problematik der therapiebedingten Knochenmarksfibrosen und -sklerosen bei myeloischer Leukämie haben kürzlich Hunstein und Mitarbeiter [12] Stellung genommen. Boidin, Bousser und Delzant [3] beschrieben am Schädeldach und anderen Stellen des Skelettsystems Areale von elfenbeinartiger Sklerose neben hochgradiger Entkalkung. Im übrigen finden sich im Schrifttum zur Skelettbeteilung bei myeloischer Leukämie folgende Angaben: Craver und Copeland [6]: 1mal unter 83 Beobachtungen; Uehlinger [29]: 3 von 67 Patienten: Schneider [25]: 1mal unter 44 chronischen Myelosen.

Bei der Analyse des eigenen Krankengutes von 49 chronischen myeloischen Leukosen konnten wir die nachstehend kurz skizzierten 3 interessanten Skelettveränderungen beobachten.

1. 57jähriger, am 26. 9. 56 erstmalig in unserer Klinik wegen chronischer myeloischer Leukämie aufgenommener Mann mit klassischer Symptomatologie. — Sternalmark und Blutbild: Vorherrschen von Myelo- und Promyelozyten. Initiale Leukozytenzahl: 193 000/mm^3. — Nach einjähriger zytostatischer Behandlung mit Myleran im November 1957 Hüftschmerz links. Röntgenbefund vom Dezember 1957: ausgedehnter osteolytischer unregelmäßig begrenzter Bezirk im Bereich der Beckenschaufel links (Abb. 1). Gezielte Biopsie des röntgenologisch erfaßten Prozesses: dichte Verbände von granulopoetischen Vorstufen. Exitus am 7. 5. 58.

2. 46jährige Patientin. Am 13. 5. 1957 Feststellung einer myeloischen Leukämie mit Leukozytose von 164000/mm^3 und starker Linksverschiebung, jedoch kein terminaler Myeloblastenschub. Krankheitsbeginn vermutlich im Herbst 1956 mit starker Gewichtsabnahme und zunehmender Schwäche. Myleranbehandlung. Röntgenbefund vom 15. 8. 1958: osteoklastischer Herd im Gebiet des Trochanter minor links z. T. auf den Oberschenkelschaft übergehend. Das Gebiet ist etwa kirschgroß (Abb. 2). Exitus am 16. 9. 1958.

3. 1904 geborener Patient. Krankheitsbeginn 1964 mit Schwäche, Inappetenz und Milzvergrößerung. Stationäre Vorbehandlung in einer auswärtigen Klinik mit Bluttransfusionen und Milzbestrahlung. — Aufnahme in die II. Medizinische Universitätsklinik Halle am 16. 3. 1967. Leukozytenzahl bei der ersten Untersuchung 661 000/mm^3, dabei deutliche Linksverschiebung mit Myelozyten- und Promyelozytenvermehrung. Sternalpunktion und Beckenkammbiopsie: granulopoetische Hyperplasie, kein Anhalt für Osteomyelosklerose bzw. -fibrose. — Röntgenbefund vom 11. 4. 1967: am Übergang zwischen mittlerem und unterem Drittel der Diaphysen beider Femura Nachweis einer rechtsseitig aus-

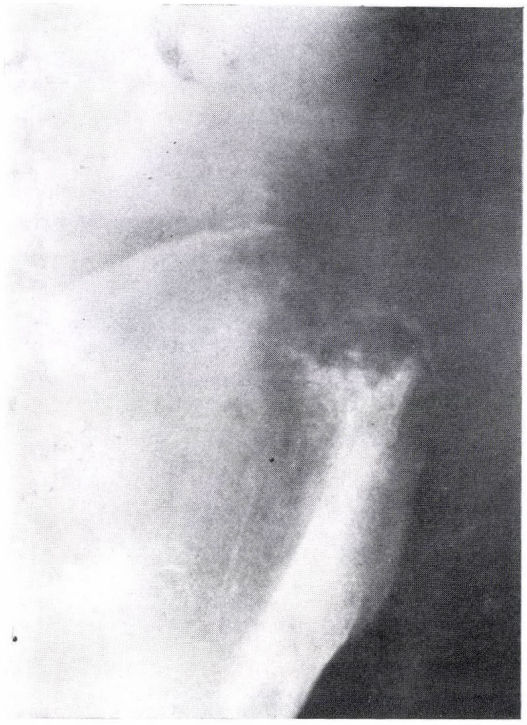

Abb. 1. Großer, unregelmäßig begrenzter osteolytischer Destruktionsherd des Os ileum bei chronischer myeloischer Leukämie

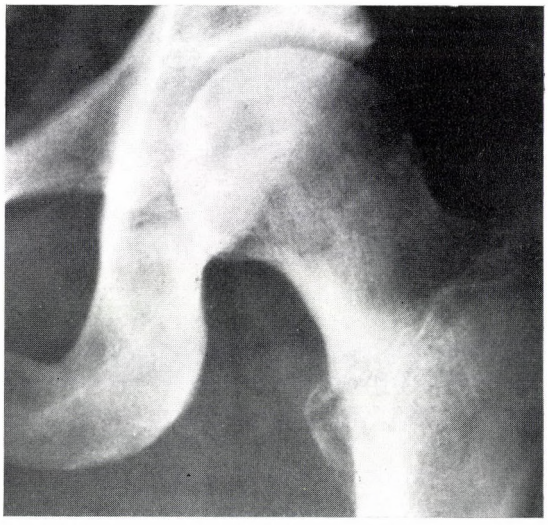

Abb. 2. Osteolytische Umwandlung des Trochanter minor bei chronischer myeloischer Leukämie

geprägten, linksseitig nur angedeuteten Periostauflagerung. Sonst regelrechte Knochenstrukturen (Abb. 3 Periostose der Femurdiaphyse links). — Klinisch sowie anamnestisch kein Hinweis für Phlebopathie, keine Symptome einer Angioorganopathie. Exitus am 25. 9. 1967. Sektion nicht ausgeführt.

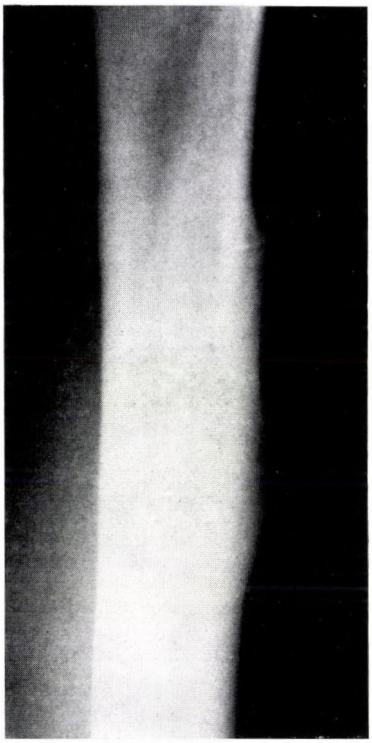

Abb. 3. Ausgeprägte Periostauflagerung der Femurdiaphyse li. bei chronischer myeloischer Leukämie (spezifische Genese nicht bewiesen, vgl. Text)

Die Zuordnung des beiderseits festgestellten periostalen Prozesses im Bereich der Femurdiaphyse im Rahmen des leukämischen Geschehens bleibt ohne autoptische Bestätigung problematisch. In diesem Zusammenhang sei jedoch auf die Feststellung Uehlingers [29] verwiesen, daß bei Leukämien periostale Knochenneubildung eventuell als unspezifische Reaktion in Erscheinung treten kann.

Die Auswertung des Sektionsmaterials zeigte bei 5 myeloischen Leukämien Myelosklerosen mit Einengung der Markräume und bei 3 Patienten osteoporotische Veränderungen sowie an einer Leiche fortgeschrittene Destruktionen mehrerer Rippen.

Für lymphatische Leukosen im Erwachsenenalter liegen die Prozentangaben über eine Skelettbeteiligung etwas höher als bei myeloischer Leukämie, d. h. zwischen 2.7—7 % [6, 25]. Auch hier nimmt die Häufigkeit mit der Progredienz

des Leidens zu, wobei Osteoporosen an erster Stelle stehen. Sie sind nach Jaffé [15] jedoch nicht sehr ausgeprägt. Der Einfluß einer protrahierten Prednisonmedikation kann jedoch die Osteoporose verstärken. Sehr selten wurden osteosklerotische Prozesse gesehen [29], ein Verhalten, das durch die spezielle Altersdisposition der chronischen Lymphadenose zu erklären ist [10]. Auch periostale Prozesse wurden selten beobachtet [27]. Apitz [2] beschrieb multiple Knochendefekte der Schädelkalotte. Erweiterte und mit Tumormassen ausgefüllte Haverssche Kanäle fand Jaffé, Moseley [15, 18] sah bei Demineralisation der Wirbelsäule Wirbelkompressionen, bevorzugt in den unteren Wirbelsäulenabschnitten. Es kommen auch lytische Prozesse im Femurbereich vor, die zu Spontanfrakturen führen können [15, 24]. Eine schlechte Heilungstendenz kennzeichnet solche Frakturen [29].

Differentialdiagnostisch ist bemerkenswert, daß osteolytische Knochenkarzinosen meist auf das Stammskelett beschränkt sind. Neben der Karzinose müssen folgende Prozesse abgegrenzt werden: Tuberkulose, Osteomyelitis, chronische Arthritis, Myelom, Hyperparathyreoidismus und Altersosteoporose [10]. Wie bei chronischer myeloischer Leukämie widersteht die Tela ossea lange Zeit dem expansiven Wachstum. Zu beachten sind in Zukunft bei längere Zeit mit Prednison behandelten Lymphadenosen durch die Steroidtherapie bedingte aseptische Knochennekrosen, denen wahrscheinlich intraossäre obliterierende Arteriopathien zugrunde liegen [16].

Während unter unseren klinischen Beobachtungen von 42 Patienten lediglich bei einem 60jährigen Mann nach 9jährigem Krankheitsverlauf Wirbeldestruktionen wegen lokalisierter Schmerzen vermutet und später autoptisch bestätigt wurden, ergab die Auswertung von 40 autoptischen Befunden 9mal Skelettveränderungen, die sich wie folgt darstellten: 2mal Osteosklerose bei grobknotigen Infiltrationen, 2mal auf das Grundleiden bezogene Osteoporosen, eine osteosklerotische Herdbildung des Schädels und mehrerer Wirbelkörper sowie 4mal Spongiosadestruktionen bei herdförmiger grobknotiger Infiltration. In einem Sektionsprotokoll wurde eine begleitende Osteomyelofibrose vermerkt.

Akute oder unreifzellige Leukosen dominieren im Kindesalter. Hier werden Skelettveränderungen wesentlich häufiger diagnostiziert. Die Prozentangaben liegen zwischen 50—70 % [25]. Die Skelettbeschwerden bei kindlichen Leukosen werden häufig initial als rheumatischer oder osteomyelitischer Prozeß fehlgedeutet. Besonders häufig werden Querlinien oder bandförmige, 2—5 mm breite Aufhellungen juxtaepiphysär beobachtet. Weiterhin werden folgende Veränderungen beschrieben: diffuse Osteoporose, mottenfraßähnliche Osteolyse, endostale Osteosklerosen und periostale Osteophytenbildung. Diese Prozesse können sich rasch, d. h. innerhalb von Monatsfrist nach Krankheitsbeginn entwickeln. Nach ihrem Manifestwerden rechnet man mit einer Krankheitsdauer von etwa 6 Monaten [29]. Fibro- und osteoklastische Reaktionen faßt Pantlen [21, 22] als sogenannte »mesenchymale Kohärenzreaktion« auf. Danach kommt die hämatopoetische Neoplasie möglicherweise als Ursache für die Fehldifferenzierung auch anderer mesenchymaler Elemente in Betracht. Therapeutisch induzierte Remissionen

können sich u. U. restituierend auf die ossären Begleitprozesse auswirken. Diese Feststellung hat in Hinblick auf die höhere Remissionsrate kindlicher Leukosen unter der modernen Intensivtherapie Bedeutung. Hansen [8] sah nach Anwendung eiweißanaboler Steroide 4—8 Wochen nach Therapiebeginn Zunahme der Knochenstrukturdichte. Als interessante Beobachtung teilten kürzlich Schwarz, Hoffmeister und Loewe [26] einen Pseudohyperparathyreodismus im Verlaufe einer akuten Leukose bei einer 69jährigen Patientin mit. — Bei 28 eigenen klinischen Beobachtungen von unreifzelliger Leukose konnten wir keine Knochenherde feststellen. — Die Auswertung des Autopsiematerials von 5 Jahrgängen mit 78 Parablastenleukosen ergab bei 2 Beobachtungen Destruktionsherde der Schädelkalotte, ferner eine Destruktion mehrerer Rippen mit Herdabmessung bis zu Kleinapfelgröße sowie zweimal eine tumorförmige Wucherung mit Auflösung der Spongiosastruktur an mehreren Stellen des Skelettsystems. Hierzu ist zu bemerken, daß nur ein Drittel der Patienten unter 20 Jahre alt war. Busse [4] fand bei akuten Hämoblastosen im Erwachsenenalter in 7.5 % Knochenbeteiligung.

Tabelle 1

Lymphogranulomatose Anzahl und Lokalisation der Knochenherde bei 33 Patienten mit Skelettbeteiligung

Wirbelsäule:	HWS	19
	BWS	27
	LWS	28
Rippen		2
Brustbein		17
Beckenschaufel		4
Kreuz-Steißbein		—
Schädel		2
Schulterblatt		—
Schlüsselbein		—
Oberschenkel		6
Oberarm		—
Distale Extrem.		—

Ossäre Prozesse sind bei *Lymphogranulomatose* von besonderer Bedeutung, da nach Heilmeyer [11] das Skelettsystem der zweithäufigste Sitz extraglandulärer Herde ist. Die Häufigkeitsangaben stehen in Beziehung zur Intensität der Suche [17]. Nach Musshoff [19] fanden sich in 31.2 % der autoptischen Befunde von Lymphogranulomatosen ossäre Herde, während der klinische Nachweis nur bei 10.6 % gelang. Jaffé [15] gibt für klinisch erfaßbare Skelettherde 10—20 % an, denen nach Schrifttumsangaben 34—78 % pathologisch-anatomisch nachgewiesene Knochenmanifestationen gegenüberstehen. Stadium II und III sind am häufigsten befallen. Knochenveränderungen können zu jedem Zeitpunkt der Erkrankung auftreten, sind jedoch als Frühsymptome selten. Die Annahme einer

primären Knochenlymphogranulomatose wird von den meisten Autoren abgelehnt. Nach Moseley [18] können Knochenveränderungen dem übrigen Organbefall um Jahre vorausgehen. Die Herde können entweder durch arrosives Wachstum benachbarter Lymphome oder Weichteilprozesse oder hämatogen entstehen. Über die Häufigkeit nach topographischer Reihenfolge stimmen sämtliche Autoren dahingehend überein, daß Wirbelprozesse an erster Stelle stehen. Dann folgen Becken, Rippen, Skapula oder Klavikula. Nach manchen Angaben kommt auch Femurbefall relativ häufig vor [5, 7]. — Die Auswertung von 51 autoptischen Befunden zeigte nach eigenen Untersuchungen folgendes Bild der Lokalisation von Knochenherden: (s. Tabelle 1).

Grundsätzlich gibt es keinen für Morbus Hodgkin spezifisch pathognomonischen Röntgenbefund. Trotz ausgedehnter disseminierter Knochenherde fehlt häufig ein Röntgennachweis. Erst wenn Kompakta oder Spongiosa ergriffen

Tabelle 2

Retothelsarkom. Anzahl und Lokalisation der Knochenherde bei 41 Patienten mit Skelettbeteiligung

Wirbelsäule:	HWS	20
	BWS	28
	LWS	27
Rippen		2
Brustbein		20
Beckenschaufel		7
Kreuz-Steißbein		4
Schädel		8
Schulterblatt		1
Schlüsselbein		—
Oberschenkel		18
Oberarm		1
Distale Extrem.		1

sind, ist dieser positiv [9]. Osteolysen mit reaktivem sklerotischem Randsaum sind relativ häufig anzutreffen [30]. Die osteolytischen Herde sind nicht so scharf abgegrenzt wie beim Myelom. An der Wirbelsäule wurden Kompressionsfrakturen beobachtet bzw. als seltener Befund eine elfenbeinartige Verdichtung [13]. Senkungsabszesse kommen im Gegensatz zur Tuberkulose nicht vor. Die Zwischenwirbelscheibe bleibt intakt. Gibbusbildung besteht nur selten.

Differentialdiagnostisch sind folgende Krankheitsprozesse mit Knochenbeteiligung abzugrenzen: Myelom, Tuberkulose, Tumormetastasen, Ewingsarkom, Knochenretikulosarkom, eosinophiles Granulom, osteogenes Sarkom, Morbus Gaucher. Die Beckenkammbiopsie kann im Einzelfall eine diagnostische Klärung bringen. Ausgedehnte hämatogene Herde verursachen z. T. eine Panzytopenie [28]. Beziehungen zwischen Schmerzempfindlichkeit und röntgenologi-

schem Skelettbefund sind inkonstant. Nach Musshoff [19] ist die Prognose bei Skelettbefall nicht besonders ungünstig. Die Knochenherde sprechen gut auf Strahlentherapie an und sind durch Zytostatika weniger beeinflußbar. Bei der neuerdings bekanntgewordenen günstigen therapeutischen Beeinflußbarkeit der Stadien I und II sollte der Kliniker neben anderer intensiver Herdsuche an der frühzeitigen Erfassung von Knochenherden besonders interessiert sein. — Bei 44 Hodgkinpatienten unserer Klinik fanden wir einmal eine spezifische Infiltration des Sternums, bei einem anderen Patienten einen osteoplastisch veränderten 7. Brustwirbelkörper, osteoklastische Herde im Lendenwirbelbereich bei einer weiteren Patientin und bei einer jungen Frau einen Rippenherd mit Auftreibung und zystischer Auflockerung der Knochenstruktur (Abb. 4).

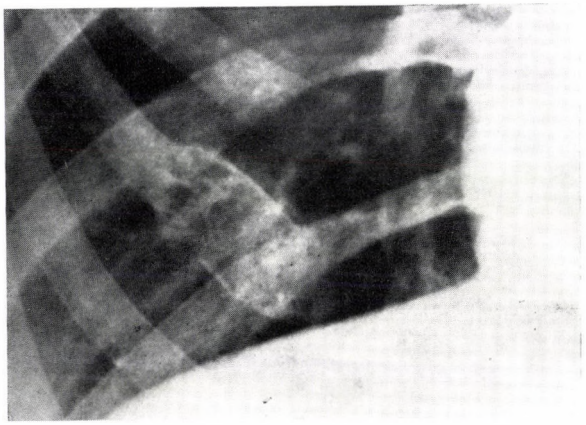

Abb. 4. Knochenauftreibung und Osteolyse mit zystischer Umwandlung des ventralen Anteils der 5. Rippe re. durch lymphogranulomatöse Herdbildung

Das *Retikulumsarkom* (Retothelsarkom, lymphoretikuläres Sarkom) kann im Gegensatz zur Lymphogranulomatose als primäre Knochengeschwulst beginnen, worauf zuerst Parker und Jackson 1939 [23] hingewiesen haben. Die Diagnose ist bioptisch zu sichern. Ivins und Dahlin [14] fanden bei 117 malignen Lymphomen des Knochens 14 Retikulumzellsarkome und 85 gemischt retikulumzellig-lymphosarkomatöse Tumoren. Schneider [25] teilt bei 153 Retikulumzellsarkomen 14mal Skelettbefall mit, davon 4 primär im Knochen lokalisierte Formen. Nach Heilmeyer [11] kommen bei dieser Neoplasie zahlreiche und häufige Metastasenbildungen im Skelett vor, wobei Verwechslungen mit der nacktkernigen Form der Lymphogranulomatose möglich sind.

Differentialdiagnostisch ist besonders an das früh metastasierende Ewingsarkom zu denken, welches häufiger bei Patienten vor dem 20. Lebensjahr auftritt. Die Röntgenbefunde sind uncharakteristisch. Es finden sich fleckige, meist nicht scharf begrenzte Aufhellungen oder spindelförmige Osteolysen. Die langen Röhrenknochen sind bevorzugter Sitz. Spontanfrakturen sind nicht häufig. Periostale Knochenreaktionen können vorkommen. Die Knochenzerstörung ver-

läuft ohne reaktive Neubildung. Multiple Rippenzerstörungen können einen Pancoasttumor vortäuschen. Verwechslungsmöglichkeiten mit einer Reihe anderer ossärer Prozesse sind gegeben (Lymphogranulomatose, Plasmozytom, Leukose u. a.).

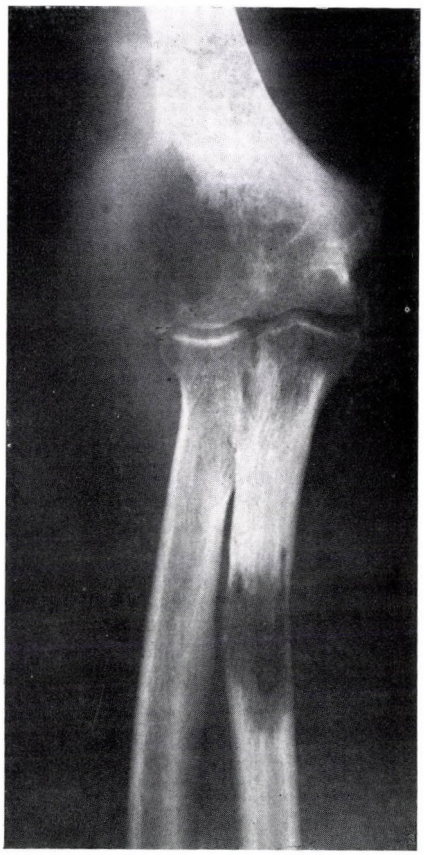

Abb. 5. Ausgedehnte osteolytische Veränderungen des Humerus und der Ulna bei Retothelsarkom

81 ausgewertete autoptische Befunde von Patienten mit Retothelsarkom ergaben folgende Lokalisation von Knochenherden:

Bei 30 in unserer Klinik behandelten Patienten mit Retothelsarkom konnten wir nur einmal röntgenologisch eindrucksvoll einen Knochenbefall nachweisen (s. Abb. 5). Der klinische Nachweis von ossären Defekten gelingt demnach im Vergleich zu den Autopsiebefunden relativ selten. Als Ursache für diesen Unterschied ist anzunehmen, daß die lymphoreticulären Sarkome erst verhältnismäßig spät zu einer Zerstörung der Spongiosastruktur oder der Kompakta des Knochens führen.

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Contribution to the Skeletal Manifestations of Leukoses, Lymphogranulomatoses and Retothelsarcomas

Skeletal manifestations in clinical patients suffering from chronic myeloid leukaemias, leukoses of immature cells, chronic lymphadenoses, lymphogranulomatoses and lymphoreticular sarcomas are described. The characteristics and frequency of osteal islands in the postmortem findings of a pathological institute on patients who died of malignant haematological diseases are compared with the clinical results. Osteolytic islands, circumscript or diffuse osteoscleroses, osteoporoses and quite casually periosteal reactions occurred. Osteal manifestations dependent on the process could be detected more frequently in the autopsy material than in the clinical and roentgenological examinations. In certain cases of the examined disease groups special prognostic and therapeutic conclusions can be drawn from the recognized skeletal manifestations.

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The Management of Polycythaemia vera with Dibromomannitol

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Sixteen patients with polycythaemia vera have been treated with Dibromomannitol (DBM) over a 30-month period. Treatment has been cyclic. Dosage regimen included daily doses from 200 mg to 300 mg for 8 to 15 days. Fifty-one cycles are adequate for evaluation: 27 with a total dose of less than 3000 mg and 24 with a total dose of more than 3000 mg. The control of the disease has been achieved in all the sixteen patients, a single cycle every 4 to 6 months being necessary for such a purpose. Leukopenia and thrombocytopenia occurred in 16% and 18% respectively of overall treatments. A clear dose-response and dose-toxicity rate is shown. The use of DBM in chemotherapeutic control of polycythaemia vera, both for induction and maintenance therapy, is finally discussed.

There are three modalities of therapy now available for the control of polycythaemia vera: phlebotomy, radioactive phosphorus (^{32}P) and chemotherapeutic agents. Patients treated with ^{32}P are likely to live longer than patients treated only with phlebotomies [1, 5, 11—14, 20], but the occurrence of haematologic complications is higher [8, 9, 14, 15]. It is, at present, difficult to determine the quantitative relationship between haematologic complications and ^{32}P treatment [8, 9, 4, 20]. Leukaemia seems to be a delayed complication of polycythaemia and it is likely to be correlated with the total dose of ^{32}P received [8, 9, 12, 15]. The search for chemotherapy in polycythaemia vera arises both from the need to have another effective therapy and to avoid haematologic complications. There are, at present, no published data available on survival time with chemotherapy alone, but good experience has been gained up to date with Busulfan [3, 7, 19]. More recently some other drugs have been studied successfully, as chlorambucil [3], cyclophosphamide [3], melphalan [6] and a piperazine derivative [10].

In a previous paper [18] we have reported preliminary data about the treatment of polycythaemia vera with Dibromomannitol (DBM), an anti-tumor agent mostly effective against chronic myeloid leukaemia [2, 17]. As the first results of this trial were positive, we have continued to use DBM in every patient with polycythaemia vera, in order to better evaluate the effectiveness and the modalities of the action of the drug and to standardize a regimen treatment.

The data pertaining to a thirty-month study and sixteen patients will be reported.

Materials and Methods

Sixteen patients with polycythaemia vera have been studied. Diagnosis was established by direct measurement of the red cell mass with radioisotopic method. Secondary erythrocytosis was excluded by arterial oxygen saturation, pulmonary and cardiac function studies, and other appropriate X-ray and laboratory studies.

Eleven patients were males and five females aged from 27 to 82 years. Nine patients had received radioactive phosphorus prior to treatment, one patient had been treated with Busulfan, three patients had had phlebotomies as the only treatment and the remaining three patients were untreated.

The drug was administered per os, cyclically. Dosage regimen of every cycle included daily doses from 200 mg to 300 mg for 8 to 15 days (total dose ranging from 1400 mg to 3750 mg). Eleven cycles were conducted with a double treatment, i.e. a second identical treatment was repeated 10 to 15 days after the first, unless leukopenia or thrombocytopenia occurred. In such cycles the total average dose was 4700 mg, ranging between 3000 mg and 6125 mg. Dosage schedule included daily doses from 200 mg to 350 mg.

In this study of sixteen patients on chemotherapy treatment, there were 51 cycles with data adequate for evaluation: these cycles can be subdivided into a group of 27 with total dose of less than 3000 mg and a group of 24 with a total dose of more than 3000 mg.

During the whole period of study the patients did not receive any other drug nor were submitted to phlebotomies.

These patients underwent periodical controls by physical examination and appropriate laboratory studies, including routine standard haematological examinations (with direct platelet counts), bone marrow biopsy, SGOT, SGPT, bilirubin level, serum proteins, plasma cortisol level, and gastric function (HCl, pepsin and intrinsic factor secretion).

Responses were evaluated by considering the following data: haematocrit, platelet level, spleen size and subjective complaints.

Results

Haematocrit

Fifty courses of chemotherapy are available for evaluation of haematocrit response. The results are summarized in Table 1. A reduction of haematocrit more than 20 % of the initial value was obtained in 15 treatments (30 %), a reduction between 11 % and 20 % in 16 treatments (32 %) and a reduction of less than 10 % in the remaining 19 treatments (38 %). A significant haematocrit response, accordingly (more than 10 % of the initial value) has been obtained in 31 treatments out of 50 (62 %). In assessing the results as a function of the total dosage administered in every treatment, a clear dose-response rate is shown. With a total dose less than 3000 mg (27 treatments) the haematocrit

Table 1

Haematocrit decrease %	Overall treatments 50 treatments		Total dose ≥ 3000 mg 23 treatments		Total dose < 3000 mg 27 treatments	
0 – 5	20	38%	0	9%	37	63%
6 – 10	18		9		26	
11 – 20	32	62%	36	91%	26	37%
≥ 21	30		52		11	

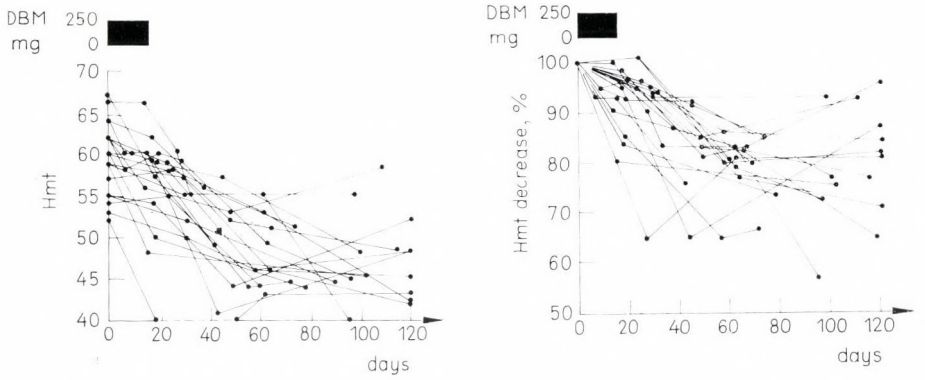

Fig. 1. Patterns of haematocrit decrease. Treatments with total dose of more than 3000 mg. Average total dose 4000 mg. Average dosage schedule 250 mg/day. *Left:* haematocrit decrease in absolute values. *Right:* haematocrit decrease in per cent of the initial values

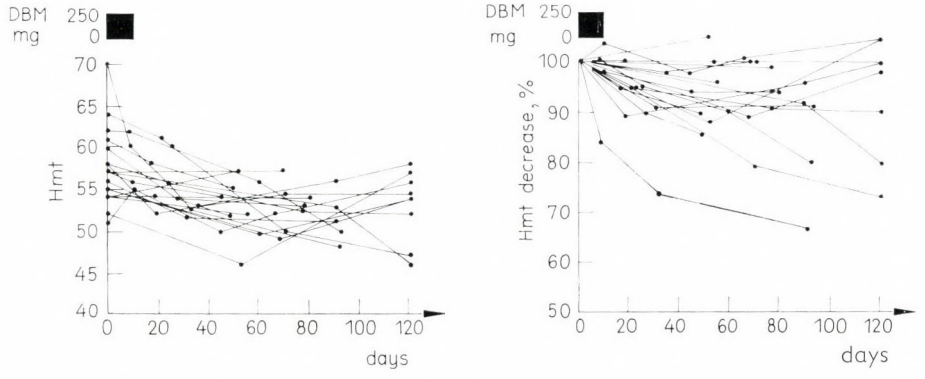

Fig. 2. Patterns of haematocrit decrease. Treatments with total dose of less than 3000 mg. Average total dose 2500 mg. Average dosage schedule 250 mg/day. *Left:* haematocrit decrease in absolute values. *Right:* haematocrit decrease in per cent of the initial values

reduction was significant in 10 treatments (37 %), while with a total dose of more than 3000 mg, such a response was obtained in 21 treatments out of 23 (91 %). The decrease of the haematocrit when a total dose of more than 3000 mg is given, is generally slow and delayed, minimum levels being reached only after 50 to 60 days from the beginning of the treatment (Fig. 1). Patterns of haematocrit variations with a total dose of less than 3000 mg are nearly the same, although the haematocrit does not fall to the same level (Fig. 2).

Thrombocytosis and splenomegaly

Data on DBM effectiveness on thrombocytosis (platelets more than 500,000/mm^3) were available in 12 cycles. Thrombocytosis was corrected in all cycles but two (83 %).

Splenomegaly was present in seven patients. In all cases the spleen was no longer palpable after chemotherapy.

Subjective symptoms

The data on the effectiveness of DBM treatment on subjective complaints concerned with polycythaemia vera (itching, headache, dizziness, sonitus, visual troubles, neurovascular disturbancies of upper and lower extremities) were available in 30 cases. In twenty-one cases (70 %) all subjective complaints disappeared completely, in two cases (7 %) the complaints were reduced and in seven (23 %) there was no significant response.

Haematologic toxicity and side effects

A significant decrease of neutrophilic polymorphonucleocytes (PMN) has been observed in almost all cases. A PMN decrease below 1500/mm^3 has been recorded in 8 treatments overall (16 %), of which seven had been conducted with a total dose of more than 3000 mg and one with a total dose of less than 3000 mg (Table 2).

Table 2

	Overall treatments 51 treatments %	Total dose $\geq$ 3000 mg 24 treatments %	Total dose $<$ 3000 mg 27 treatments %
P. M. N. $< 1500/mm^3$	16	29	3
Platelets $< 100{,}000/mm^3$	18	25	11

Platelet depression was nearly the same. Platelet levels below 100,000/mm^3 were recorded in 9 cases (18 %), of which 6 comprised a total dose of more than 3000 mg and 3 a total dose of less than 3000 mg (Table 2). The platelet level decreased below 50,000/mm^3 in only one case.

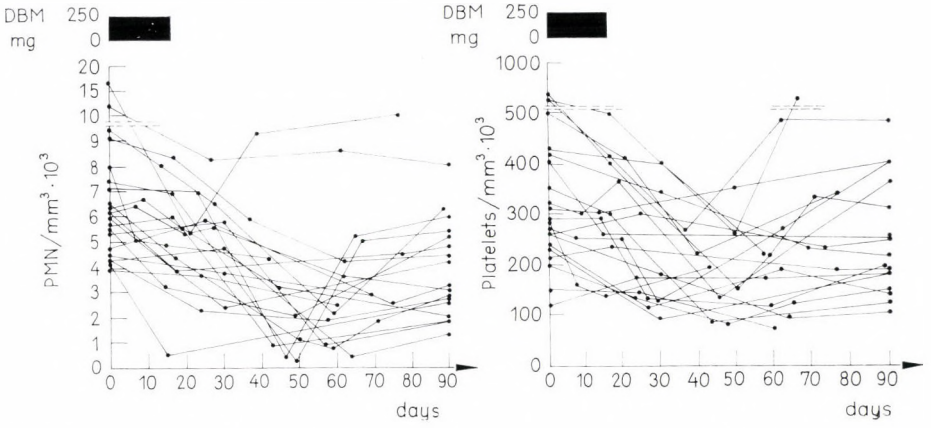

Fig. 3. Patterns of PMN and platelet decrease. Treatments with total dose of more than 3000 mg. Average total dose 4000 mg. Average dosage schedule 250 mg/day. PMN and platelet decrease is early, but lowest values are reached more often between the 40th and the 60th day

Patterns of PMN and platelet reduction are shown in Fig. 3. The decrease is generally rapid and therefore can be evaluated within 20 days from the beginning of the treatment. Sometimes, however, the decrease does not stop and a lower value may be reached after several days, usually between the 40th and the 60th day from the beginning of the treatment.

Extra-haematologic side effects were not observed at all. No change was observed in laboratory studies performed.

Discussion

The haematocrit value is the most simple and valid parameter for a current evaluation of the course of polycythaemia vera. The control of the disease is achieved when haematocrit values are maintained within normal ranges. Such a result has been obtained in all the sixteen patients treated with DBM. A striking quantitative relation exists between the total dose given (dosage schedule including 200 to 300 mg/day) and the response. We assume that a significant haematocrit decrease means a decrease of more than 10 % of the initial value: significant decreases have been obtained in 91 % of the treatments with total dose of more than 3000 mg and in 37 % of the treatments with total dose of less than 3000 mg. The latter treatments (total dose of less than 3000 mg) have nearly always been em-

ployed when the initial values of haematocrit were not very high, with the ultimate purpose of preventing a continuing rise in haematocrit rather than obtaining a significant reduction. First-type treatments (total dose of more than 3000 mg) may well be defined as induction treatments, while second-type treatments fulfil the criteria of a cyclic maintenance therapy.

Haematocrit decrease is nearly always slow and minimum levels occur after 50 to 60 days. In a smaller group of patients, on the contrary, reduction was rapid

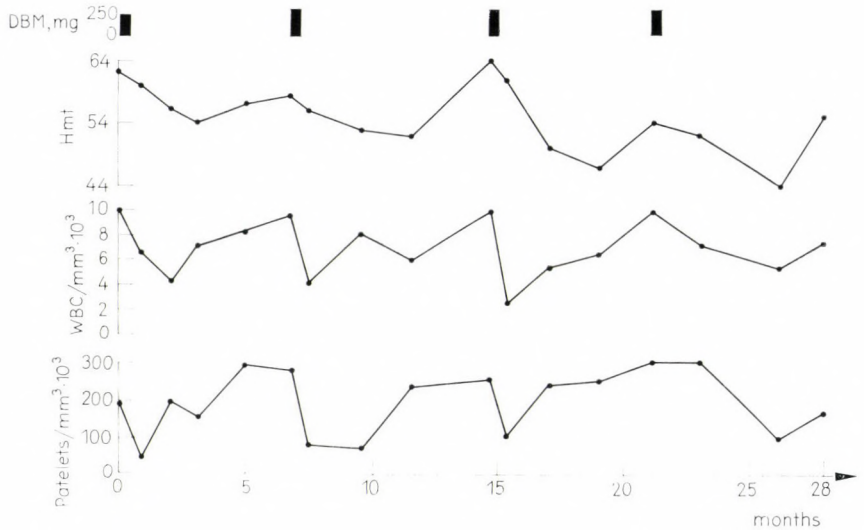

Fig. 4. Cyclic chemotherapy with DBM in a polycythaemic patient over a 28-month period

(Figs 1 and 2). Patterns of haematocrit reduction may be well explained, considering that DBM target cells are mitotable precursors and that these cells must provide the renewal of a cell population whose mean survival time is about 120 days. In cases with faster haematocrit decrease we may assume that the erythrocytes have had a shorter life span and therefore a shorter renewal time. However, reconstitution of erythroid population will occur in four to six month-time, because, as it is shown by the rise of haematocrit to levels above normal, 100 to 200 days (average 156 days) are necessary for relapse. In practice, a single cycle every 4 months is the maximum request to maintain red cell mass within normal range (Fig. 4). More recently we set up another type of regimen treatment to test the effectiveness of two closely successive courses of chemotherapy with an interval ranging between 10 and 20 days (Fig. 5). The aim of this regimen treatment was to give a second course of DBM in a critical moment for bone marrow proliferating pools, that is to say when their size has been already reduced by the first course and a greater proportion of cells have entered the active or proliferating phase. This type of treatment (250 mg/day for 12 days, 15 days interval, then again

250 mg/day for 10 days) has proved to be highly effective. However leukopenia and thrombocytopenia occurred more frequently and recovered more slowly.

The effectiveness of chemotherapy with DBM on thrombocytosis, splenomegaly and subjective symptoms is nearly absolute. In a few cases some subjective complaints were not modified but they were most probably related to a generalized vascular disease rather than to polycythaemia. Haematologic toxicity in this larger series of patients has been shown similar to that seen in our preliminary report

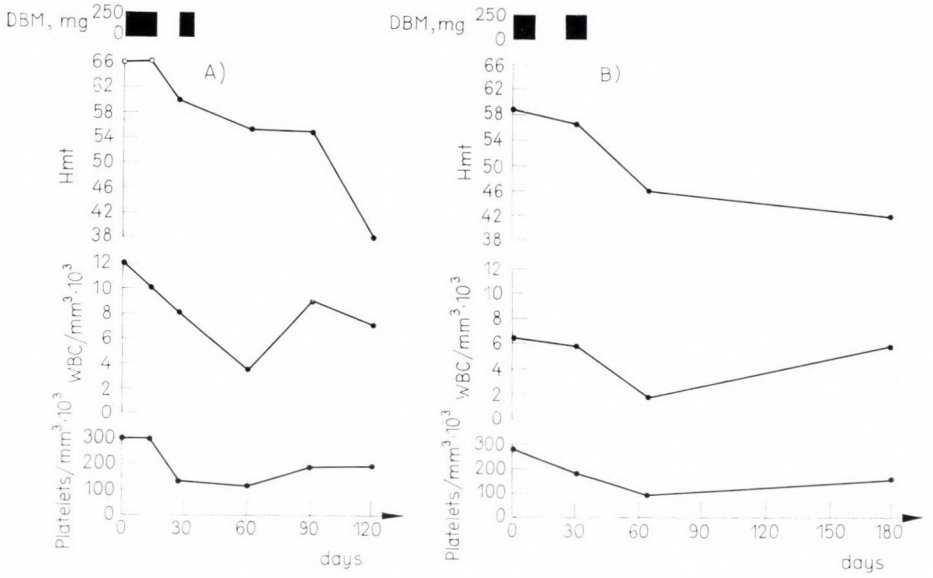

Fig. 5. Results of double treatments in two separate patients with polycythaemia vera. Patient A had an haematocrit decrease from 66 to 38 on 120th day. In Patient B both leukopenia and thrombocytopenia occurred

[18]. This haematologic toxicity does not differ significantly from that reported in other series of polycythaemic patients treated with different drugs [3, 6, 7, 10, 19]. Prolonged cyclic treatment has never produced bone marrow hypoplasia with chronic cytopenia nor was there further evidence of bone marrow aplasia [16]. Polycythaemic patients were never given a daily dose of DBM higher than 350 mg, because from our data on chronic myeloid leukaemia [17] it was clear that higher doses would produce excessive myelosuppression.

Other side effects or gastrointestinal intolerance were not recorded, over a 30-month period.

Chemotherapy management of polycythaemia vera is quite different from other malignancies, because the aim for therapy is not *to cure* but *to control* the disease. Such a control would aim at reaching the longest survival with the minimum risk and discomfort for the patients. Dibromomannitol seems to fulfil

the last of these basic requirements. This 30 months' study has shown that the response rate is similar to that produced by other chemotherapeutic agents, acute haematologic toxicity is slow, chronic cytopenia is not produced and there are no side effects. Finally no resistance to the drug occurred. DBM is effective both for induction and maintenance therapy and thus it appears to occupy a definite place in the chemotherapeutic control of polycythaemia vera.

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Inhibition of Blood Platelet Aggregation by Serum IgM Macroglobulin

Preliminary Report

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The time of platelet aggregation induced by ADP in platelet rich plasma of a patient with IgM macroglobulinaemia was found to be longer and the aggregates smaller than normal. The patient's platelet poor plasma caused a similar change in the reaction of the platelet rich plasma of a healthy subject. Aggregation time was prolonged by isolated IgM macroglobulin as well.

A pathological protein belonging to the class of macroglobulins is encountered in numerous diseases with abnormal proliferation of plasmatic and lymphoid cells. This so-called paraproteinaemia of the gamma fraction according to Waldenström [9] is classified as gammopathy and according to Heremens [3] as a disturbance of the gamma system.

According to clinical observations, the presence of increased amounts of pathological macroglobulins in blood leads to disturbances in the haemostatic processes [4]. It seemed therefore of interest to study the effect of isolated IgM macroglobulins on blood aggregation. Aggregation of blood platelets was followed according to the method of Niewiarowski and Thomas [6]. It is seen in Table 1 that in the platelet rich plasma of a patient with IgM macroglobulinaemia platelet aggregation induced by ADP was considerably prolonged and that the

Table 1

Blood platelet aggregation in platelet rich plasma of a patient with IgM macroglobulinaemia

Test	Aggregation time, sec. induced by ADP (0.5 μg/ml)
PRP control	37
PRP patient	118
PRP control + PPP control	40
PRP control + PPP patient	87

PRP = platelet rich plasma
PPP = platelet poor plasma

platelet aggregates were smaller than in normal subjects. Incubation for five minutes of platelet rich plasma obtained from a healthy subject with an equal volume of platelet poor plasma of the patient, caused a delay in the formation of platelet aggregates induced by ADP.

A purified IgM preparation was obtained from human serum by precipitation of the low-density lipoproteins with dextran sulphate [2] and by subsequent filtration in Sephadex G-200 [5]. IgM macroglobulin was identified by means of immunoelectrophoresis.

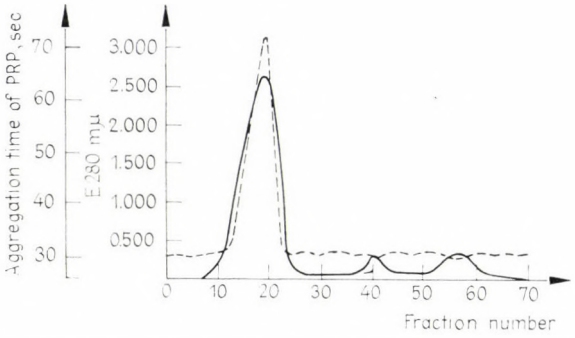

Fig. 1. Sephadex G-200 chromatography of supernatant obtained after precipitation of blood serum with dextran sulphate. ——— absorption at 280 mμ; – – – – aggregation time, sec.

As it is seen in Fig. 1, separation of the serum on Sephadex G-200 column yielded 3 protein fractions, the first one containing IgM macroglobulin at concentration not higher than 0.5 mg/ml. To 0.3 ml platelet rich plasma, 0.2 ml of the fraction obtained from the column and after incubation at 37 °C for 5 minutes 0.1 ml ADP (0.5 μg/ml) was added. The protein fraction containing IgM macroglobulin was found to prolong the time of aggregation as compared to that isolated from normal serum.

It is supposed that adsorption on the platelet surface of a pathological protein with different physical and chemical properties, such as IgM macroglobulin, may cause a change of the electrostatic charge of the platelet surface and thus lead to disturbances in the haemostatic function of the platelets. Pachter et al. [7] and Braunsteiner et al. [1] already suggested that macroglobulins may coat blood platelets, changing in this way their surface and making them less susceptible to aggregating factors. The fact concerning adsorption of macroglobulins on the surface of blood platelets was mentioned by Rosenberg and Dintenfass [8]. These authors have observed disturbances in platelet aggregation in individual cases of Waldenström's macroglobulinaemia.

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Ein weiterer Fall von Anti-N im Serum einer Person der Blutgruppe MN

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(Eingegangen am 28. Juli 1969)

Es wird ein weiterer (4.) Fall von Anti-N bei einer Person der Blutgruppe MN berichtet. Die MN-Antigene und der Anti-N-Antikörper der 82-jährigen deutschen Frau verhalten sich in allen Tests regelrecht. Der Antikörper gehört offenbar zur IgM-Klasse und ist als sogenannter natürlicher Antikörper anzusehen.

Im Jahre 1961 berichteten Metaxas-Bühler und Mitarbeiter [6] über einen gesunden Blutspender, der an seinen Erythrozyten die Blutfaktoren MN und in seinem Serum einen Antikörper der Spezifität Anti-**N** trug. Fünf Jahre später veröffentlichten Greenwalt und Mitarbeiter [4] einen ähnlichen Fall, bei dem die MN-Antigene und der Anti-**N**-Antikörper wie im Schweizer Beispiel der Norm entsprachen. In einem Nachsatz dieser Arbeit sowie in der 4. Auflage von Mollisons bekanntem Buch [7] wird schließlich über einen dritten derartigen Befund berichtet, der von R. Gibb in Bellingham, Washington erhoben und von E. Giblett in Seattle untersucht wurde. Nachstehend schildern wir unsere Untersuchungen zu einer weiteren Beobachtung dieser Art.

Die Probandin E. H. war 82 Jahre alt (1887 geboren), als ihr Blut zur Blutgruppenbestimmung zu uns gelangte. Sie war wegen einer Schlafmittelvergiftung ins Krankenhaus gekommen, zeigte sonst jedoch keine akuten Krankheitssymptome. Sie hat niemals Bluttransfusionen oder -injektionen erhalten. Zwei Geburten 1913 und 1914, keine Aborte. Beide Kinder und drei von vier Geschwistern sind bereits verstorben. Die noch lebende Schwester war für uns nicht erreichbar. Wir konnten lediglich die Blutproben dreier Enkel der Probandin mituntersuchen. Da wenige Tage nach unserer ersten Blutabnahme der Patientin ein zweiter Suizidversuch gelang, stand uns nur eine begrenzte Menge Blut zur Verfügung.

Die Erythrozyten E. H.

Die Blutkörperchen von E. H. hatten folgende Formel: B, MNSS, P_1, CcD.Ee, Lu(a—b+), kk, Le(a+b—), Fy(a+b—), Jk(a+). Mit den Testseren Anti-**s**, — **C**^{w}, —**K**, —**Kp**^{a}, —**Mg**, —**Ny**^{a}, —**Bu**^{a} und — **Wr**^{a} reagierten die Erythrozyten nicht. Der direkte Antihumanglobulintest war mit 5 verschiedenen

Coombs-Seren negativ; je eines dieser Seren reagierte spezifisch mit IgG- bzw. IgM-Antikörpern.

Die Erythrozyten wurden mit 5 Anti-**M**- und 9 Anti-**N**-Seren vom Kaninchen getestet. Humane Anti-M- und -N-Seren sowie MN-spezifische Phytagglutinine standen uns leider nicht zur Verfügung. Der Ansatz erfolgte im Röhrchen: je 0.1 ml 2%ige Erythrozytensuspension in 0.9%iger Kochsalzlösung und Serum in der optimalen Gebrauchsverdünnung wurden eine Stunde bei 22 °C stehen gelassen und dann makroskopisch abgelesen. Die Reaktionen unterschieden sich in der Stärke (in allen Fällen + + oder + + +) nicht von 20 MN-Kontrollen. Mikroskopische Kontrollen zeigten stets regelrechte Agglutinationsbilder.

Titrationen der frischen Blutprobe E. H. wurden mit 5 Anti-**N**- und 4 Anti-**M**-Seren vorgenommen. Gleichfalls frisch entnommene Blutproben der Gruppen M, MN und N dienten als Kontrollen, wobei jedes Kontrollpanel aus 5 Einzelbluten bestand. Die durchschnittlichen Score-Werte dieser Titrationen [3] gibt Tabelle 1 wieder.

Tabelle 1

Reaktionen der Erythrozyten E. H. mit Anti-M- und Anti-N-Seren im Vergleich zu Kontrollbluten (durchschnittliche Score-Werte)

Erythrozyten:	E. H.	MN	M	N
Anti-M (4 Seren)	29.3	32.6	41.8	–
Anti-N (5 Seren)	23.0	22.2	–	29.7

Auch in Absorptionsexperimenten verhielten sich die Erythrozyten E. H. ähnlich wie ein Kontrollpanel aus 3 MN-Blutproben. Die verwendeten 4 Anti-**M**- und 4 Anti-**N**-Seren ließen sich durch gleiche Mengen des MN-Kontrollblutes und der E. H.-Blutkörperchen bis zur Reaktionslosigkeit mit homologen Testerythrozyten M oder N absorbieren. Es gelang jedoch nicht, von den absorbierenden Erythrozyten den Antikörper zu eluieren (Landsteiner-Miller-Technik nach 3), eine Beobachtung, die auch Greenwalt und Mitarbeiter [4] machten.

Die Blute der drei Enkel der Probandin und das ihrer Mutter (Schwiegertochter zu E. H.) gehörten zur Gruppe N. Sie reagierten in zahlreichen Ansätzen ebenso wie andere N-Blute.

Das Serum E. H.

Bereits bei der AB0-Bestimmung fiel das Serum auf, da es auch die B-Testerythrozyten (die zur Gruppe MN gehörten) agglutinierte. Im Antikörpersuchtest agglutinierte das Serum im Kochsalzmilieu bei Raumtemperatur die Testzellen mit 2+ Reaktion, bei 4 °C mit 3+ Reaktion. Papainbehandelte Erythrozyten

reagierten negativ. Im Differenzierungsansatz mit 10 Testbluten ergab sich dann der Verdacht auf einen Antikörper der Spezifität Anti-N, der bei Anwendung weiterer 25 N-, 87 MN- und 49 M-Blutproben der Blutgruppen B und 0 bestätigt wurde. Unterschiede in der Reaktion mit B- und 0-Bluten gleicher MN-Struktur wurden nicht gefunden, dagegen ein deutliches Dosisphänomen zwischen MN- und N-Blut.

Titrationen des Serums E. H. gegen mehrere Testerythrozytensuspensionen in physiologischer Kochsalzlösung wurden bei 4°, 12°, 22° und 37 °C ausgeführt. Die stärksten Reaktionen und höchsten Titerwerte zeigten die Ansätze bei 4° und 12 °C; bei 22 °C waren die Werte deutlich geringer und bei 37 °C reagierten N-Blutkörperchen nur noch mit 1 + Reaktion, MN-Zellen nur vereinzelt mit (+) Reaktion (Tabelle 2). Gegen M-Zellen konnten auch mikroskopisch keine Agglutinationen gesehen werden. Die eigenen Erythrozyten von E. H. wurden durch das Serum deutlich schwächer agglutiniert als andere MN-Erythrozyten: Score-Werte bei 4 °C 3, bei 12 °C 8, bei 22 °C 5 und bei 37 °C 0.

Tabelle 2

Durchschnittliche Titer- und Score-Werte des Serums E. H.

Testblut-körperchen	4 °C		12 °C		22 °C		37 °C	
	Titer	Score	Titer	Score	Titer	Score	Titer	Score
N (4)	8	35	4	29	2	16	1	5
MN (6)	8	28	4	18	2	13	<1	3

Als Titer gilt der reziproke Wert der Serumverdünnung, die noch mindestens eine 1 + Reaktion ergibt. Die Zahlen in Klammern geben die Zahl verwendeter Testerythrozyten an.

Bei Aufschwemmung der Testzellen in AB-Serum waren die Reaktionen wesentlich schwächer. Im Einstufen- und Zweistufen-Papaintest konnte der Antikörper nicht demonstriert werden. Im Thermostabilitätstest reagierte das Serum nach Erwärmung auf 70 °C für 10 min hitzeempfindlich. Es reagierte danach nicht mehr mit 0 N- und B N-Erythrozyten.

Eine verbindlichere Aussage zur Natur des vorliegenden Antikörpers lassen die Ergebnisse der Antiglobulintechnik (Tabelle 3) und der Behandlung des Serums mit 2-Merkaptoäthanol zu. Bei den Untersuchungen der Tabelle 3 war das Serum dreieinhalb Monate bei —30 °C gelagert worden. Verdünnungen des Serums E. H. wurden mit gleichen Teilen einer 20%igen Kochsalzsuspension von 0 N-Erythrozyten 2 Stunden bei 4 °C sensibilisiert und nach viermaligem Waschen mit den verschiedenen Antiglobulinseren bei Raumtemperatur auf der Platte angesetzt. Die AHG-Seren I und II waren sogenannte Breitspektrum-Coombs-Seren eigener Produktion.

Tabelle 3

Anti-Humanglobulin-Reaktionen des Serums E. H. gegen 0 N-Erythrozyten

E. H.-Serum 1:	2	4	8	16	32	64
AHG-Serum I	++	++	++	+	–	–
AHG-Serum II	++	++	+	+	–	–
Anti-IgG	+	+	–	–	–	–
Anti-IgM	++	++	+	+	+	–
NaCl 0.9%	++	+	+	–	–	–

Nach Behandlung des Serums E. H. mit 2-Merkaptoäthanol (Technik nach 7, S. 730) war die agglutinierende Eigenschaft gegen 0 MN- und 0 N-Zellen nicht mehr nachweisbar. Beide Befunde (Antiglobulintechnik und 2-ME-Behandlung) sprechen dafür, daß es sich um einen IgM-Antikörper handelt.

Schließlich seien die Ergebnisse unserer Absorptions- und Elutionsversuche mitgeteilt. In wiederholten Ansätzen zeigte sich stets das gleiche Verhalten: das Serum E. H. ließ sich mit 0 N-Erythrozyten »leer« absorbieren, d. h. es zeigte nach Absorption keine Aktivität mit N-tragenden Blutkörperchen. Die verwendeten Testzellen ergaben vor der Elution einen positiven Agglutinationstest; nach der Hitzeelution konnten jedoch auch mit dem direkten Coombs-Test keine Antikörper mehr nachgewiesen werden. Dennoch gelang es nicht, mit irgendeiner Technik den Antikörper im Eluat wiederzufinden.

Diskussion

Die Untersuchungen am Blut der Probandin E. H. ergaben normale Befunde hinsichtlich der MN-Struktur der Erythrozyten. Dennoch enthält das Serum einen Antikörper der Spezifität Anti-**N**. Da der Antikörper nur schwach mit den eigenen MN-Blutkörperchen reagierte und eine Sensibilisierung in vivo ebensowenig nachweisbar war, ist es unwahrscheinlich, daß hier ein Autoantikörper vorliegt. Die Probandin war — unter Berücksichtigung ihres Alters — gesund und bot keinerlei Zeichen für Autoimmunisierungsvorgänge. Insoweit gleicht unser Fall den beiden früher berichteten [4, 6], während im Bericht von Giblett (nach [4] und [7]) von einem »Autoagglutinin« gesprochen wird. Berichte über derartige Abweichungen vom Ehrlichschen Gesetz des »horror autotoxicus« sind selten. In der Blutgruppenserologie sind nur wenige Fälle spezifischer Isoantikörper gegen autologe Erythrozytenantigene bekannt [1, 2, 4, 5, 6, 7, 9, 10, 11].

Ein isologer Immunisierungsreiz ist nach eingehender Anamneseerhebung nicht nachzuweisen. Die Probandin hat nie Bluttransfusionen bekommen, die letzte Schwangerschaft war vor 55 Jahren. Eine so lange Persistenz eines Immunantikörpers ist mehr als unwahrscheinlich. Es bleibt nur die Annahme eines sogenannten spontanen oder natürlichen Antikörpers, ohne daß bisher eine Erklä-

rung für das Entstehen gegeben werden kann. Für eine zur Bildung der normalen Isoantikörper des AB0-Systems analoge Vermutung, die Produktion spontaner Anti-**N** (und -**M**)-Antikörper könne durch Antigene der intestinalen Darmflora stimuliert werden (sei also letzten Endes heterologen Ursprungs), fehlen bisher experimentelle Beweise [8]. Für die Annahme eines natürlichen Antikörpers sprechen auch die serologischen Charakteristika des Serums E. H.: Wirksamkeit in Kochsalzaufschwemmung, Temperaturoptimum bei tiefen Temperaturen, Thermolabilität bei 70 °C und Vorliegen eines IgM-Antikörpers.

Abweichend von den früher berichteten Beispielen — insbesondere vom Serum A. P. von Metaxas-Bühler und Mitarbeiter [6] und vom Serum Mark V. von Greenwalt und Mitarbeiter [4] — ist das Verhalten in der indirekten Antiglobulintechnik, in der unser Serum positiv reagierte. Zu beachten ist, daß wir die Antikörperbindung bei 4 °C, den Ansatz im Coombs-Test bei 22 °C vornahmen. In den höheren Serumverdünnungen ließen sich dabei auch Reaktionsunterschiede mit Antiglobulinseren gegen verschiedene Immunglobulinklassen demonstrieren.

*

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A Further Case of Anti-N in the Serum of a Person of MN Blood Group

A further (fourth) case of anti-N is reported in a person of the MN blood group. The MN antigens and anti-N antibody of the 82-year-old German woman behaved regularly in all tests. The antibody obviously belongs to the IgM-class and is to be regarded as a so-called natural antibody.

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B-Like Blood Factor of Fur Seals and Sea Lions

Comparative Study with D-Galactose Inhibition, Human Group B Erythrocytes and B-Active Human, Baboon and Rhesus Monkey Saliva[1]

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Fur-seal and sea-lion, but not harbor-seal erythrocytes were shown to possess B-like blood factors. Human anti-**B** serum was shown to possess: 1. a D-galactose-*inhibitable* fraction which cross reacted with both human group B and B-like seal red cells, and 2. a D-galactose-*non*inhibitable fraction, sensitive to 2-mercaptoethanol treatment, which reacted only with human group B red cells. The heteroagglutination of both human group B and B-like seal red cells by catfish anti-**B** heteroagglutinins was specifically inhibited with D-galactose, thus suggesting that immunodeterminant D-galactosyl residues of otherwise dissimilar surface membrane receptor structures provided the basis for the B-like cross reactivity. Variability in the comparative reactivity of human group B and B-like seal red cells with immune plasmas produced by heteroimmunizing catfish with B-active human, baboon and rhesus monkey saliva further illustrated the heterogeneity of: 1. immune responsiveness in the lower vertebrates, and 2. the "blood-group-active" saliva substances of different species of primates.

A B-like erythrocyte antigen of the northern fur seal *(Callorhinus ursinus)* has been reported by Fujino and Cushing [1]. B-like blood factors of other sea-adapted mammals, namely, the California sea lion *(Zalophus californianus)* and the fin-whale, have also been noted by Cushing in his comprehensive review of the blood groups of marine animals [2].

The sea-adapted carnivores in the suborder Pinnepedia (seals, sea lions and walruses) are of special scientific interest because of the unique absence of lactose in their milk [3, 4], as well as their concomitant hypersensitivity to ordinary lactose-containing milk and milk products [5, Nigrelli et al., unpublished observations]. That the absence of a specific galactose-associated disaccharide (lactose) from the milk of the Pinnepedia is not related *per se* to an inability to metabolize simple D-galactose, however, has been indicated by the studies of Mathai et al. [6]. Not only were normal mammalian levels of galactokinase and galactose-1-phosphate uridyl transferase activity found in liver tissue and the blood of both infant and sub-adult sea lions, but these investigators were also able to demonstrate the ability of sea lions to metabolize D-galactose ingested as a simple sugar.

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The reported blood-group-B activity of seals and sea lions, therefore, is especially of interest, because terminal D-galactosyl residues are now recognized as the immunodeterminant oligosaccharide structures responsible for conferring B-like activity to a variety of substances ubiquitously distributed throughout both the plant and animal kingdoms [7—9].

This paper reports: 1. the comparative serological behavior of human group B erythrocytes and of fur-seal and sea-lion erythrocytes tested in parallel with variously absorbed or inhibited agglutinating reagents, and 2. the extent to which agglutination-inhibition tests with simple D-galactose and other B-active soluble substances suggest actual structural analogies between the oligosaccharides presumably associated with B-like fur-seal and sea-lion agglutinogens, on the one hand, and the oligosaccharides now known to be associated with both the soluble and cellular expression of the human A—B—O blood group system [10, 11].

Materials and Methods

Reagent materials. Unless otherwise specified, sterile saline (0.9 % w/v) was the erythrocyte (henceforth "red cell") suspension medium and diluent used in all of the testing procedures. Human red cells were washed at least once with saline and made up as 2 % suspensions from freshly processed typing cells of commercial origin. Thrice-washed 2% test suspensions of fur-seal *(Callorhinus ursinus)*, sea-lion *(Zalophus californianus)* and harbor-seal *(Phoca vitulina)* red cells were similarly made up from blood samples freshly obtained by flipper puncture from young adult specimens maintained at the New York Aquarium, Brooklyn, New York. The sheep red cells available were not specifically typed for their R group status, but appeared to be A-like (i.e., R positive) only with respect to their intermediate agglutinability with anti-$\mathbf{A}_1$ lectin *(Dolichos biflorus)*.

Hemagglutinating reagents of human or plant origin were randomly selected from regular commercial stocks. The plasmas from brown bullhead catfish, *Ictalurus nebulosus* (LeSueur) immunized with human group B secretor saliva, were available from previously reported experiments [12, 13], and the plasmas from white catfish *(I. catus*, Linnaeus) immunized with B-active baboon and rhesus monkey saliva were available from current immunization experiments (Chuba et al., manuscript in preparation) — the only modification in procedure with the white catfish being the restriction of the use of adjuvant (Freund's complete) to only the first of 4 equally spaced immunizing injections given (intra-abdominally and in the pectoral lymph sinuses) over a 3 week period. Except for hemolytic activity which could not be demonstrated beyond the dilution endpoint of natural complement sufficiency, nonimmune catfish plasma specimens were virtually unreactive with all of the red cells tested.

The nonhuman primate materials were available through a collaboration with the Laboratory of Experimental Medicine and Surgery in Primates (LEM-

SIP) of New York University Medical Center located at Sterling Forest, New York.

Standardized red cell stromata for absorption studies were made up in advance by lysing carefully selected, thrice-washed red cells of the types desired with several volumes of distilled water acidified to pH 4.0 with acetic acid [14]. The stromata from the lysed red cells were then washed by alternately packing (2000g for 10 min) and resuspending the stromata (20 % v/v) in several successive changes of regular distilled water. Immediately following the formation of macroscopic floccules, the stromata were washed several more times in successive changes of saline. The washed stromata were finally stored at 4 °C as 35 % suspensions in saline to which sodium azide (0.1 % final concentration) was added as a preservative. Prior to their use as absorption reagents, the preserved stromata were washed at least once in several volumes of saline. The use of red cell stromata, in the place of intact red cells, for the absorption studies on unheated serum or plasma circumvented the problem of interfering hemolysis without hazarding the unwitting destruction of poikilothermic (e.g., amphibian or teleost) antibody activities now known to be sensitive [15, 16, Chuba et al., unpublished observations) to the 56 °C heat-inactivation treatment routinely employed to decomplement hemolytic mammalian sera. Moreover, in other studies, the 56 °C heat elution of thermostable antibodies from red cell stromata could be extended beyond the usual 10 min treatment limit for intact red cells without posing the problem of viscous, discolored eluates.

The simple sugar compounds (a generous gift from the laboratory of the late Dr. Bertram Gessner, New York University School of Medicine) were made up as 10 % stock solutions in Bacto Hemagglutination Buffer (Difco, Detroit). Other testing materials are described in the footnotes of the tables in which they are mentioned.

Testing procedures. The serological tests were performed by the senior author in the Immunohematology Special Service Laboratory of Bellevue Hospital Center, New York City, by methods previously described [12]. The simple sugar agglutination-inhibition tests were performed in the same manner as the inhibition tests with saliva and other "blood-group-active" soluble substances: namely, by incubating one volume each of appropriately diluted hemagglutinating reagent and inhibitor solution for 20 min at room temperature prior to the introduction of one volume of indicator red cell suspension. Hemagglutination titers are expressed as the maximum saline dilution of serum or plasma at which macroscopic agglutination was still detectable following 20 min incubation at room temperature and a light spin.

Results

Routine screening with regular blood-grouping reagents indicated that the fur-seal, sea-lion and harbor-seal red cells were not agglutinable with anti-$\mathbf{A}_1$ lectin *(Dolichos biflorus,)* anti-**H** lectin *(Ulex europaeus)*, or by those human

Table 1

Human group B, fur-seal and sea-lion red cells tested with variously absorbed or inhibited human anti-**B** typing serum 3292

Absorption-inhibition status of serum	Human anti-**B** typing serum 3292 tested with:								
	Human group B red cells			Fur-seal red cells			Sea-lion red cells		
	Serum diluted:			Serum diluted:			Serum diluted:		
	1 : 8	1 : 16	1 : 32	1 : 2	1 : 4	1 : 8	1 : 2	1 : 4	1 : 8
a. Unabsbd. and uninhib.	+++[1]	++	+	+++	++	tr	+++	++	tr
Absorbed with:									
b. Human grp B red cells	0	0	0	tr	0	0	tr	0	0
c. Seal red cell stromata	+++	++	+	0	0	0	0	0	0
d. Sheep red cells	+++	++	+	tr	0	0	+	0	0
e. Human grp A_1 red cells	+++	++	+	+++	++	tr	+++	++	tr
f. Human grp O red cells	+++	++	+	+++	++	tr	+++	++	tr
Inhibited with:									
g. Equine grp B substance[2]	0			0			0		
h. Frog gastric mucus[3]	0			0			tr		
i. Human grp B substance[4]	0			tr			+		
j. D-galactose (100 mg/ml)	+++			0			0		
k. Porcine grp A sub.[2]	+++			++			++		
l. Human grp A substance[4]	+++			++			+++		

[1] Agglutination reactions are graded from "tr" (trace of macroscopic agglutination) to "++++" (single large clump of strongly agglutinated red cells); *e.g.*, "+++" equals large clump of agglutinated red cells with some smaller clumps. "0" equals no reaction.

[2] Commercial blood group substance preparations (Pfizer Diagnostics, N. Y.).

[3] Boiled gastric mucus from bull frog *(Rana catesbiana)*; mucus previously determined in agglutination inhibition tests to have specific B-like, H-like and Forssman-like activity (Chuba et al., unpublished observations).

[4] Boiled human secretor saliva with specified blood group activity.

anti-**A** sera which did not display "heterophilic" agglutinating activity with sheep red cells. Irrespective of the presence or absence of concomitant agglutinating activity for harbor-seal or sheep red cells, however, all of the human anti-**B** sera tested agglutinated fur-seal and sea-lion red cells in reactions which could be significantly weakened following absorption with human group B, but not human group A or group O red cells (e.g., Table 1, b, e, f).

Table 1 shows the comparative reactivity of human group B, fur-seal and sea-lion red cells tested with an anti-**B** serum (3292) which did not display heterophilic agglutinating activity with either harbor-seal or sheep red cells. As shown in the table, the fur-seal and sea-lion red cells reacted in a virtually identical "B-like" manner in all of the tests and will be collectively referred to henceforth simply as "seal red cells". The unexpected reduction in reactivity with seal red cells following absorption of serum 3292 with seemingly unreactive sheep red cells (Table 1, d), will be discussed later in the paper.

The failure of seal red cell stromata demonstrably to reduce the *homologous* anti-**human-B** activity of serum 3292 (Table 1, c) clearly demonstrates a one-sided serological relationship between human group B and seal red cells. Absorption of serum 3292 with human group B red cells and seal red cell stromata, in fact, revealed 2 fractions of anti-**B** activity: 1. a fraction strongly reactive *only* with human group B red cells, and 2. a fraction less strongly reactive with both human group B and seal red cells. The one-sided cross relationship of the cellular B-active materials is also strikingly paralleled in the agglutination-inhibition tests with the B-active soluble substances: e.g., the failure of D-galactose to inhibit the *homologous* anti-**human-B** reactivity of serum 3292 with human group B red cells (Table 1,j), compared to the complete D-galactose inhibition of the *heterologous* reactivity of serum 3292 with seal red cells (Table 1,j and Table 2).

Interestingly, the phylogenetically diverse soluble substances, equine group B substance and bull frog *(Rana catesbiana)* gastric mucus, qualitatively at least, were just as effective as human group B secretor saliva for inhibiting the isoagglutination of human group B red cells by serum 3292 (Table 1, g, h, i). As in the case of the homologous absorption of serum 3292 with human group B red cells, however, homologous inhibition of serum 3292 with human group B secretor saliva significantly reduced, but did not completely abolish the heterologous reactivity of serum 3292 with seal red cells (Table 1, i). On the other hand, heterologous inhibition with equine group B substance completely abrogated both the homologous reactivity of serum 3292 with human group B red cells and the heterologous reactivity of serum 3292 with seal red cells (Table 1, g).

Table 2 shows significant inhibition only by D-galactose, among 5 sugars tested, in the case of: 1. the heteroagglutination of seal red cells by serum 3292, as well as 2. the heteroagglutination of seal *and* human group B red cells by immune catfish plasma 095. As in Table 1, however, the isoagglutination of human group B red cells by serum 3292 was not inhibited with D-galactose.

Table 3 shows the pre- and post-absorption titers of human group B and seal red cells with: (a) human-group-B-saliva-immunized brown bullhead catfish

Table 2

Human group B and B-like seal red cells tested with human anti-**B** serum 3292 and group-B-saliva-immunized catfish plasma 095 following agglutination-inhibition with variously diluted solutions of D-galactose and other sugars

Immune serum or plasma	Simple sugars[1] tested for inhibition	Eight agglutinating units[2] of serum tested with Human grp B red cells Serum mixed 1 : 2 with 10% sugar sol. dil. 1:					Saline
		1	2	4	8	16	
3292 Human anti-**B** unabsbd.	D-galactose	+++					+++
	L-fucose	++					+++
	D-mannose	+++					+++
	NAc-D-gal	+++					+++
	NAc-D-glu	++					+++
095 Catfish, absorbed O red cell stromata	D-galactose	0	tr	+	++	+++	+++
	L-fucose	+++					+++
	D-mannose	+++					+++
	L-glucose	++					+++
	NAc-D-glu	++					+++

Immune serum or plasma	Simple sugars tested for inhibition	Eight agglutinating units of serum tested with Seal red cells Serum mixed 1 : 2 with 10% sugar sol. dil. 1:					Saline
		1	2	4	8	16	
3292 Human anti-**B** unabsbd.	D-galactose	0	0	tr	+	++	+++
	L-fucose	+++					+++
	D-mannose	+++					+++
	NAc-D-gal	++					+++
	NAc-D-glu	+++					+++
095 Catfish, absorbed O red cell stromata	D-galactose	0	0	tr	+	++	+++
	L-fucose	++					+++
	D-mannose	+++					+++
	L-glucose	++					+++
	NAc-D-glu	+++					+++

[1] "NAc-D-gal" equals N-acetyl-D-galactosamine; "NAc-D-glu" equals N-acetyl-D-glucosamine.

[2] "Eight agglutinating units" are equivalent to the saline dilution at which macroscopic agglutination of the red cells specified would persist through 3 more doubling dilutions.

plasmas 076 and 095, (b) B-active-rhesus-monkey-saliva-immunized white catfish plasmas 205, 237 and 242, and (c) B-active-baboon-saliva-immunized white catfish plasmas 219, 225 and 243. Although all of the immune plasma titers were lessened to some extent following absorption with human group O red cell stromata, plasmas 076 and 095 remained strongly reactive with both human group B and seal red cells (Table 3, a). That this residual agglutinating activity was B specific (limited materials precluded further absorption studies on plasma 076 and 095) was indicated by previous findings [12, 13] and the D-galactose-inhibition tests in Table 2.

The immunization of 3 white catfish with B-active rhesus monkey saliva did not evoke appreciable agglutinating activity for seal red cells (Table 3, b) and only the plasma of catfish 205 displayed a fraction of anti-human-B activity (titer of 1 : 32) following absorption of the immune plasma with human group O red cell stromata (Table 3, b).

The immunization with B-active baboon saliva, in contrast, produced quite variable results (Table 3, c). Immune plasma 219 displayed strong agglutinating activity with both human group B and seal red cells in reactions which could be lessened from a titer of 1 : 256 to 1 : 4 or 1 : 8 by absorbing the immune plasma with human group O red cell stromata (Table 3, c). The unabsorbed immune plasma of catfish 225, however, agglutinated seal red cells to a much higher titer (1 : 256) than human group B red cells (titer of 1 : 16) and did not display reactivity with human group B red cells following absorption with human group O red cell stromata (Table 3, c). The unabsorbed plasma of the third catfish immunized with baboon group B saliva (243, Table 3, c) displayed agglutinating activity with both human group B (titer of 1 : 128) and seal red cells (titer of 1 : 64). Following absorption with human group O red cell stromata, plasma 243 still displayed a fraction of activity with human group B red cells (titer of 1 : 64). This anti-**human-B** activity, as expected, could be completely removed by absorbing raw plasma 243 with human group B red cell stromata (Table 3, c). Absorption with either human group O *or* human group B red cell stromata, on the other hand, significantly lessened, but did not completely abolish the reactivity of plasma 243 with seal red cells (Table 3, c).

Table 4 shows the inhibitability of the baboon-group-B-saliva-immunized white catfish plasma with various B-active soluble materials following the absorption of the plasmas with human group O red cell stromata. As shown in Table 4, *only* the baboon saliva inhibited the agglutination of seal red cells, but the more avid-appearing reactivity of plasma 219 and 243 with human group B red cells was completely inhibited by both human and baboon B-active saliva as well as by both D-galactose and N-acetyl-D-galactosamine. Red cells from group B baboons, as expected [17], were unreactive with the 3 baboon-group-B-saliva-

Table 3

Saline agglutination titers of human group B and seal red cells with immune plasmas from catfish variously injected with B-active human, baboon and rhesus monkey saliva

Catfish species and immunizing material	Immune catfish plasma	Plasma unabsorbed		Plasma absorbed with red cell stromata from human			
		tested with:		grp O cells[1] tested with:		grp B cells tested with:	
		human grp B cells	seal red cells	human grp B cells	seal red cells	human grp B cells	seal red cells
a. Brown bullhead, immunized human grp B saliva	076	512[2]	128	256	32	N. D.[3]	N. D.
	095	512	512	64	128	N. D.	N. D.
b. White catfish, immunized rhesus grp B saliva	205	512	<4	32	N. D.	0	N. D.
	237	1024	<4	0	N. D.	N. D.	N. D.
	242	128	<4	0	N. D.	N. D.	N. D.
c. White catfish, immunized baboon grp B saliva	219	256	256	4	8	0	4
	225	16	256	0	16	0	16
	243	128	64	64	4	0	4

[1] None of the raw immune plasmas, however strongly reactive with human group O red cells, displayed residual agglutinating activity with human group O red cells following absorption with human group O red cell stromata.

[2] Titers are expressed as the reciprocal of the maximum saline dilution of the plasma at which macroscopic agglutination was still detectable after 20 min incubation at room temperature and a light spin.

[3] "N. D." equals not done.

immunized catfish plasma following their absorption with human group O red cell stromata. Prior to absorption (not shown in Table 4), the raw immune plasmas weakly hemolyzed, but did not agglutinate the baboon red cells.

Discussion

The present findings are reminiscent of earlier investigations by Friedenreich and With [18], and later by Owen [19], in which absorption studies with red cells of various species of animals indicated that the anti-**B** activity of human anti-**B** serum, far from being serologically homogeneous, actually represents a spectrum of B-related specificities [20].

Table 4

Baboon-group-B-saliva-immunized white catfish plasmas absorbed with human group O red cell stromata and tested with human group B and seal red cells following inhibition with the soluble substances specified below

Immune catfish plasma	Red cell suspensions tested	Inhibited 1 : 2 with				
		saline (control)	human grp B saliva	baboon grp B saliva	D-galactose	N-acetyl-D-galactosamine
219	Human grp B	++	0	0	0	0
	Seal	+	+	0	+	+
	Grp B baboon	0				
225	Human grp B	0				
	Seal	+±	+±	0	+±	+±
	Grp B baboon	0				
243	Human grp B	++±	0	0	0	0
	Seal	+	+	0	+	+
	Grp B baboon	0				

In this study, both human group B and the B-like seal red cells were similarly reactive with D-galactose-*inhibitable* anti-**B** heteroagglutinins evoked by heteroimmunizing catfish with human group B secretor saliva (Table 2). The seal red cells, however, although heteroagglutinable with D-galactose-*inhibitable* human anti-B isoagglutinins, were not in turn reactive with (e.g., did not absorb) D-galactose-*non*inhibitable human anti-**B** isoagglutinins present in the same immune serum (Table 1, c).

These findings thus suggest that "immunodeterminant" D-galactosyl residues provided the basis for the B-like cross reactivity between otherwise dissimilar surface membrane receptor structures respectively present on both human group B red cells and seal red cells. The apparent inability of the D-galactose-*non*inhibitable anti-**human-B** isoagglutinins to cross react with seal red cells could in turn be attributed to the steric interference of the dissimilar portions of the B-like seal agglutinogen molecules. In the case of antibodies specific for carbohydrate determinants, inheritable differences in the carbon linkages between even single analogous pairs of immunodeterminant glycosyl residues could presumably produce sufficient steric variation to prevent certain antibodies from cross reacting with otherwise seemingly closely related antigens. By the same token, antigen-antibody interaction based on complementarity encompassing *only* the terminal D-galactosyl *portions* of B-active receptor structures would presumably be: 1. more readily inhibitable with sufficiently high concentrations of "haptenic" D-galactose molecules, and 2. more likely to display a broad spectrum of cross reactivity with heterogeneous receptor structures possessing terminal D-galactosyl residues (e.g., the interaction of seal and sheep red cells with D-galactose-inhibitable anti-**B** isoagglutinins present in human serum 3292 and the interaction of seal

and human group B red cells with D-galactose-inhibitable catfish anti-**B** hetero-agglutinins).

The foregoing interpretations of the serological findings are compatible both with earlier diagrammatic speculations by Wiener and Karowe [21] and current immunochemical concepts, recently reviewed by Kabat [22, p. 82], regarding variability in the size of the combining sites of antibodies presumably evoked by similar, or closely related antigens.

That the D-galactose-inhibitable and D-galactose-*non*inhibitable human anti-**B** isoagglutinins in this study also represented, perhaps merely by chance, different classes of immunoglobulins is indicated by the supplementary observation (not shown in any of the tables) that treatment of serum 3292 with 2-mercapto-ethanol [23] did not lessen the agglutination titer of 1 : 8 with seal red cells, but did lower the titer with human group B red cells from 1 : 32 to 1 : 8 — thus suggesting that the D-galactose-*non*inhibitable isoagglutinins were 19 S, IgM immunoglobulins.

The unexpected disappearance of most of the anti-**seal-B** activity, following the absorption of serum 3292 with seemingly nonreactive sheep red cells (Table 1, d), could possibly be attributed to the ability of the terminal D-galactosyl residues of some of the sheep oligosaccharides, now believed to be mosaically associated with the Forssman activity of sheep red cells [14], selectively to act as "nonagglutinating" receptors for the D-galactose-inhibitable human anti-**B** agglutinins.

The demonstration of both anti-human and anti-seal hemagglutinins in the immune plasmas of 2 of the 3 white catfish heteroimmunized with baboon group B saliva, compared with the virtual absence of demonstrable agglutinins for seal red cells in the immune plasmas of the 3 white catfish heteroimmunized with B-active rhesus monkey saliva (Table 3), suggests subtle variations in the chemical structure of the soluble "blood-group-active" saliva substances of different species of Old World monkeys. Interesting also is the observation that baboon group B saliva evoked catfish heteroagglutinins which, following absorption with human group O red cell stromata, could be demonstrated only by virtue of their chance cross reactivity with seal red cells (Table 3), and could, in turn, only be inhibited with baboon group B saliva (Table 4). On the other hand, the residual reactivity with human group B red cells of 2 of the 3 baboon-group-B-saliva-immunized catfish plasmas, following their absorption with human group O red cell stromata, could be inhibited with all of the B-active inhibitors tested, as well as with N-acetyl-D-galactosamine (Table 4). The above observations do not, of course, exclude the possibility that the boiled baboon group B saliva used to inject the catfish may also have contained: 1. fortuitously present antigenic material of bacterial or viral origin, or 2. unrecognized baboon-specific saliva antigens which, like the A-B-O-related soluble substances, are not expressed on baboon red cells by agglutinogens of corresponding specificity.

This investigation has thus further illustrated the usefulness of phylogenetically diverse serological interactions, especially when combined with simple sugar

agglutination-inhibition tests, for elucidating fundamental questions regarding: 1. serological cross reactivity involving carbohydrate determinants, and 2. the ubiquitous distribution of blood group A-B-O and Forssman-like activities in nature.

*

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Multiple Myeloma without Paraprotein

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The case history of a patient with multiple myeloma is presented. The diagnosis was based on the typical bone marrow picture and bone deformities. However, paraprotein could not be detected in the blood or urine by either paper electrophoresis or immunoelectrophoresis.

Since the use of electrophoresis has become widespread, the simplest and most reliable way to diagnose multiple myeloma is the detection of the so-called M-component in the serum. However, in about 10 to 25 % of cases there is no characteristic protein peak in the paper electrophoretogram of the serum. Fortunately, Bence-Jones protein can almost invariably be demonstrated in the urine of these patients and thus multiple myeloma can be verified in the overwhelming majority of cases by combined serum and urine electrophoresis. In a very small number of cases, however, paraprotein cannot be found even by the most thorough examination, though the diagnosis of multipe myeloma can be established beyond doubt from the bone marrow picture and bone deformities. Osserman et al. [11] reported 3 such cases, whereas Di Guglielmo [4], Coltman [3] and Forssman [6] reported one each. Löffler et al. [8] described 7, Michot [9] 2 somewhat similar cases.

Description of our case

A. T., 56, female. The case history comprises a strumectomy in 1965 because of Riedel goiter. She has known of her cholelithiasis for ten years but it caused her no complaints. Pains in the bones have occurred since the summer of 1967. It was noticed on X-ray examination that several ribs had broken without any apparent trauma. During November and December 1967 she received treatment because of ischias syndrome l. d. The patient was admitted to our clinic to reveal the cause of diffuse osteoporosis, spontaneous fractures, and circumscript bone defects.

When admitted to the clinic the patient was in very poor condition, she could hardly move. On physical examination the liver was soft and palpable three finger-breadths below the right arch of the ribs, and the edge was rounded off. No other physical alterations could be detected.

More important laboratory results on admission: blood pressure, 110/70 Hgmm; urine, sp.wt., 1016, no pathological changes, no protein or Bence-Jones protein. ESR: 45 mm in 1 hour. Wassermann reaction negative. Peripheral blood count: erythrocytes 3.2 M; Hb, 8.8 %; leucocytes, 4,800; platelets, 140,000. Differential count: polymorphonuclei, 60; eosinophils, 4; monocytes, 7; lymphocytes, 28; plasma cells, 1 %. Bone marrow picture from the sternum: high amounts (28%) of plasmocellular reticulum cells including many young forms and cells with vacuola in the plasma and with two nuclei; erythropoiesis greatly reduced, granulopoiesis preserved. The shape of megakaryocytes is normal, their number appears to be somewhat lower. Colloid tests: all negative. Serum cholesterol: 286 mg %. Serum creatine: 1.47 mg %. Non-protein nitrogen: 41.0 mg %. Serum calcium, 14.0 mg %; serum phosphorus, 3.7 mg %; serum alkaline phosphatase, 20 King-Armstrong units; acid phosphatase, 2.2 King-Armstrong units; serum total protein, 5.6 g %. Paper electrophoresis: albumin, 57.0; alpha-1, 8.1; alpha-2, 12.9; beta, 12.5; gamma, 9.5 %.

X-ray picture on admission: two-direction photographs of the upper and lower half of the dorsal spinal column, of the lumbar spinal column, anteroposterior photographs of the pelvis and of the lower ribs. Results: the 8th vertebra in the dorsal backbone is compressed. The hind part of the body of the vertebra and the arch are more porous and coarser in structure than the normal spongiosa. The upper lamina shows an uneven slightly impressed contour. Vertebras D—4, D—7, and to a small extent D—3, also seem to be compressed. It is noteworthy that the spinal processes are partly or wholly absent on vertebras 5 to 7. Several unevenly contoured rib fractures are present: at two places on the right 3rd rib, and on the right 7th and left 10th ribs. Bone destruction with uneven margin can be seen also on the left 11th and 12th ribs. On the lumbar backbone vertebras 1, 2, 4 and 5 are also partly compressed. Parts suspected of bone destruction can be seen in the region of the left pubic and ischiatic bones. A fresh fracture can be observed on the ramus superior of the left os pubis. Progressed porosis can be seen in the vertebras and in the pelvic bones. On the right side, in a parasacral position, over the linea terminalis there is a loose area poor in structure. On the left side, over the upper contour of the acetabulum, several round lytic areas of cherry-stone size can be discerned.

Immunoelectrophoretic studies

For immunoelectrophoresis antihuman horse serum Batch 249 (Institute for Serobacteriological Production and Research Human, Budapest) was used. Sevac anti IgG, IgA and IgM were employed as monovalent immune sera. Anti IgD was kindly supplied by Dr. D. Rowe. Horizontal starch-gel electrophoresis was carried out according to the method of Poulik. Paraproteins could not be detected by electrophoresis either from serum or from urine. The marked decrease in the level of immune globulins was conspicuous. By quantitative immunodiffusion technique the IgG level proved to be 7.0 mg per ml (normal value 10.0 mg per ml), whereas IgM and IgA levels were greatly diminished.

Alterations characteristic of myeloma could not be detected by starch-gel electrophoresis, but the decrease in antibody titer here also could be observed.

Prednisolon treatment was commenced. On the administration of a daily dose of 30 mg, the serum calcium level rapidly returned to normal (from 14 to 12.5, 10.4 then 10.0 mg %). The anaemia of the patient was controlled at the beginning by transfusions, then we achieved by large doses of Retandrol (750 mg per week) that no more transfusions were necessary. (Altogether 6,000 mg Retandrol were

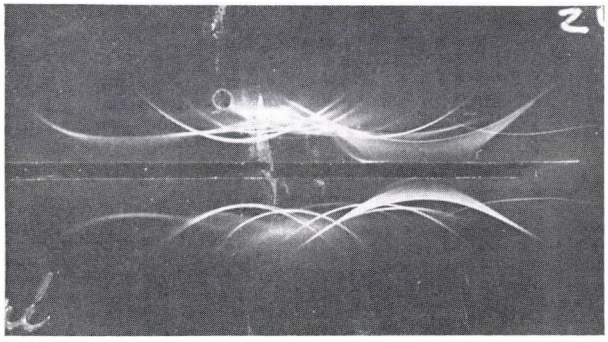

Fig. 1. Immunoelectrophoretic pattern of the serum of A. T., 56, female patient. IgG level moderately, IgA and IgM levels markedly reduced

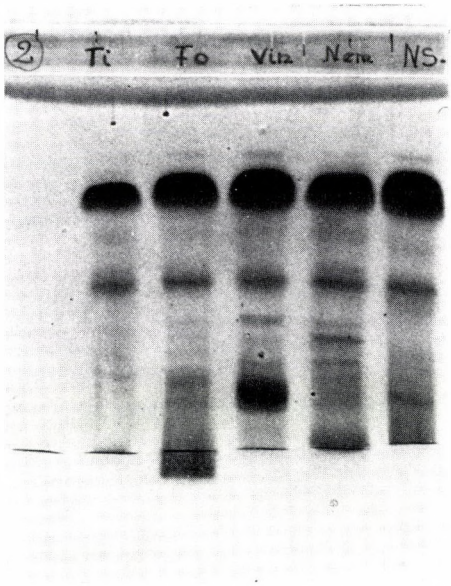

Fig. 2. Starch-gel electrophoresis of different sera. Ti = our patient; Fo, Vin, and Néra = patients with established myeloma; NS = normal control. The partial antibody deficiency of our patient is evident

given.) In three courses, each lasting for nine days, altogether 150 mg Alkeran (Melphalen) were administered.

The patient was discharged from the clinic on April 26, 1968. At this time she could walk with crutches and gained 3 kg weight. More important laboratory results on discharge: ESR, 5 mm in 1 hour; erythrocytes, 3.9 M; Hb, 12.9 g% white blood count, 3,800 (after the cytostatic treatment the latter fell temporarily to 2,000); platelets, 200,000. In the qualitative blood picture no significant changes occurred. Sternal puncture normal. The number of plasma cells fell to 4 %,

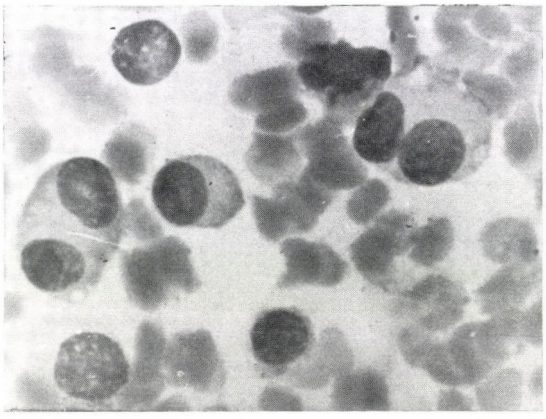

Fig. 3. Bone marrow picture from the sternum. An extensive accumulation of characteristic plasma cells can be observed

young forms were present in very slightly increased numbers. Nonprotein nitrogen: 31 mg %. Serum calcium, 9.8 mg %; total protein, 5.0 g %. Paper electrophoresis: as before. Serum alkaline phosphatase, 13.0 King-Armstrong units. No essential changes were observed on X-ray examination. As for further treatment at home, 10 mg per day of Prednisolon and 1 ampoule of Retabolil every two weeks were prescribed.

Control examinations were performed on September 25, 1968. The patient was then free from complaints and was able to walk without aid. More important laboratory results: ESR, 15 mm in 1 hour; erythrocytes, 3.8 M; Hb, 12.0 g %; white blood count, 3,600; platelets, 160,000. Differential count: polymorphonuclei, 59; eosinophils, 10; monocytes, 1; lymphocytes, 30 %. Serum calcium, 9.9 mg %. Total protein, 6.3 g %. Paper electrophoresis: the same as before. On the anteroposterior X-ray photograph of the pelvis osteoporosis appeared to be less pronounced. The above-described lytic area in the caudal part of the right sacroiliacal joint became somewhat smaller, its margin turned sclerotic. A minimal sclerosis could be detected also on the margins of the lytic areas found on the symphysis and on the ramus inferior of os pubic. Good callus formation was observed at the old pathological fracture on the ramus superior of the left pubic bone.

Discussion

According to our present knowledge the synthesis of immune globulins takes place in the plasma cells. Plasma cell myelomas are those autonomous neoplasias which originate from cells that produce immune globulins also under normal conditions [10], and the neoplastic growth leads to the formation of a large amount of protein, i.e. paraprotein. Physicochemically and immunochemically this protein corresponds to the normal gammaglobulin, but lacks any kind of functional (antibody) specificity. Other authors [15] do not agree with this statement, and regard the protein produced by myeloma cells as specific antibodies.

All normal immune globulin molecules consist of 4 polypeptide chains: 2 light and 2 heavy ones. The light chains are uniform in the different immune globulins (K and L types) and are the carriers of common properties. The heavy chains are different: so far we know alpha, gamma, mμ, and delta types.Depending on which of these polypeptide chains are comprised in the immune globulin molecule, we refer to them as IgA, IgG, IgM, and IgD type immune globulins. If only the light chains associate, Bence-Jones protein is formed, whereas if the heavy chains associate, the heavy chain protein of Franklin is produced.

In 1961 Ossermann pointed out that for the synthesis of one kind of protein one plasma cell clone was responsible and suggested the collective name "monoclonal gammopathy" for diseases like plasmocytoma leading to the synthesis of paraprotein. Immunofluorescent studies also indicate that the normal mature plasma cell is capable of synthesizing only one kind of heavy and light chains.

In the case described above we observed the accumulation of immature young, but morphologically uniform plasma cells in the bone marrow. In addition to diffuse osteoporosis, on several bones there were lytic areas characteristic of myeloma and signs of pathological fractures on several ribs were also detected. However, paraprotein could not be demonstrated in either the serum or urine by paper and immunoelectrophoresis. Starch-gel electrophoresis, a method highly esteemed by many authors, also failed to indicate myeloma.

Myeloma globulin and Bence-Jones protein, which give homogeneous peaks on electrophoresis in some instances, yield 2 to 5 narrow bands on starch-gel electrophoresis. It has been shown by chromatographic and sedimentation experiments that this heterogeneity is not due to differences in the molecular weight, and neither do the antigenic properties of the fractions differ. It is generally assumed that the appearance of several bands is the result of various conformational states within the molecule, or of small differences in the primary structure.

According to our present knowledge, multiple myeloma without paraprotein is always associated with reduced immune globulin (gamma globulin) levels. In our case the total protein concentration of the serum was 5.6 g % and, as in Forssmann and Coltman's case, the diminution of fraction IgA was most pronounced. On administration of Melphalan, Prednisolon, and androgens the serum-protein concentration of the patient increased (6.3 g %), her blood picture improved, and the number of bone marrow plasma cells returned to normal.

As to the lack of paraproteinaemia, in our case two different explanations may be afforded: 1. paraproteins were formed but could not be detected by our methods; 2. the severely damaged plasma cells were no longer capable of synthesizing immune globulin. According to Fahey [5] the following alternatives exist for the pathomechanism of gammopathies: 1. the plasma cells synthesize pathological protein; 2. normal protein is formed but in excessive amount; 3. a great number of plasma cells produce normal protein in normal amounts.

In our opinion, the patient belongs to Group 3.

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Electron Microscopic Investigation of the Developing Forms of Megakaryocytes

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The submicroscopic properties of the developing forms of megakaryocytes in mouse spleen have been investigated. The criteria of the classification of the individual developing forms, the formation of the DMS, the supposed double role of the Golgi apparatus, the changes in the chromatin substance of the cell nucleus and its possible functional background are discussed.

Light microscopic investigations revealed four stages in the classification of the developing forms of megakaryocytes [4]. The main criteria of classification are: nucleus/splasma quotient, changes in the basophilia of the cytoplasm and appearance of specific granules in the megakaryocyte. The development of megakaryocytes from the megakaryoblast stage to the platelet-producing megakaryocyte is not a fractionated but a continuous process. Due to the continuous and very rapid (72 hours) development many transitory forms can be found, which — based on light microscopy — cannot be classified with security in any of the stages. The resolving power of the light microscope does not allow the exact identification of the cytological changes indicating the appearance of a qualitatively new characteristic, for example that of an organelle. Thus, only advanced changes can be evaluated in the light microscope and this makes the electron microscopic study of the development of megakaryocytes reasonable. In this work we report on submicroscopic changes and differentiation processes allowing a more exact classification of the developing forms of megakaryocytes.

Material and Method

In our investigations the spleen of male mice weighing 20—25 g were used. The organs were fixed in 4.5 % glutaraldehyde buffered with Millonig [18] buffer (pH 7.2). After a short washing in buffer post-fixation was carried out in 1 % osmium tetroxide for 1 hour. Following an alcoholic dehydration the materials were embedded in araldite. Sections were prepared by a Reichert OMU—2 type ultramicrotome. The sections were contrasted by uranylacetate [27] and lead citrate [24], then studied by JEM 6C electron microscope.

Results

The early developing forms of mouse spleen megakaryocytes are assembled in groups.

The megakaryoblasts are 20—25 μ in diameter and contain eccentric oval or bean-shaped nucleus. Part of the chromatin substance is concentrated under the nuclear membrane in the form of smaller and bigger granules or is associated with the nucleoli (Fig. 1).

A high amount of free ribosomes is characteristic of the cytoplasm (Fig. 2). In the ribosome field filling up the whole cytoplasm there are a few disseminated mitochondria. In this stage the proliferating mitochondria arranged in groups as well as the appearance of the tubules of the rough surfaced endoplasmic reticulum indicate the differentiation of the cell. The mitochondria are assembled in groups, as a rule at the concave side of the bean-shaped nucleus, in the biggest homogeneous cytoplasmic field (Fig. 1). Further on the cisterns of the ergastoplasm intrude also among the mitochondria (Fig. 3). The Golgi apparatus is located usually in the surroundings of the cytocentrum (Fig. 3).

Depending on the stage of differentiation of the cytoplasm, the cells with segmented nucleus and several centrioles can be regarded as transitory forms between the megakaryoblast and the megakaryocyte stage I. The centrioles are oriented in all directions of the space, therefore, in a single field they can be seen in cross longitudinal or oblique sections (Fig. 4). In these rare developing forms quadrilamellar membranes also occur [14] (Fig. 4).

At the end of the megakaryoblast stage apparently empty vesicules of about 0.15—0.2 μ size demarcated by a membrane appear in the cytoplasm (Fig. 4). They persist even in the thrombocytogenic megakaryocyte. Nothing is known about their role. They do not seem to be in connection with the cell membrane or with the endoplasmic reticulum of smooth surface appearing later.

The non-ergastoplasmic persisting membrane system characterized by smooth surface appears first in megakaryocyte stage I. This was named by Marsh et al. [16] as demarcation membrane system (DMS). It occurs at several places in the cytoplasm, but mainly in the perinuclear areas (Fig. 5). The disseminated foci are made up by the Golgi apparatus and the smooth surfaced endoplasmic reticulum. In the area of these foci the above-mentioned apparently empty vesicules can also be distinguished.

The mitochondria of the cell can be found around these differentiation foci (Fig. 5). The foci are surrounded by large ribosome fields, as long as the majority of the free ribosomes do not disappear from the area of the foci. Very unfrequently mitosis can be observed even in cells showing such degree of differentiation (Fig. 6). Simultaneously with the described cytoplasmic process the segmentation of the nucleus begins, which is shown by the presence of 4—5 apparently separated parts of the nucleus in the cytoplasm. The chromatin substance of these nuclear parts is most evenly distributed; no heterochromatic regions can be seen.

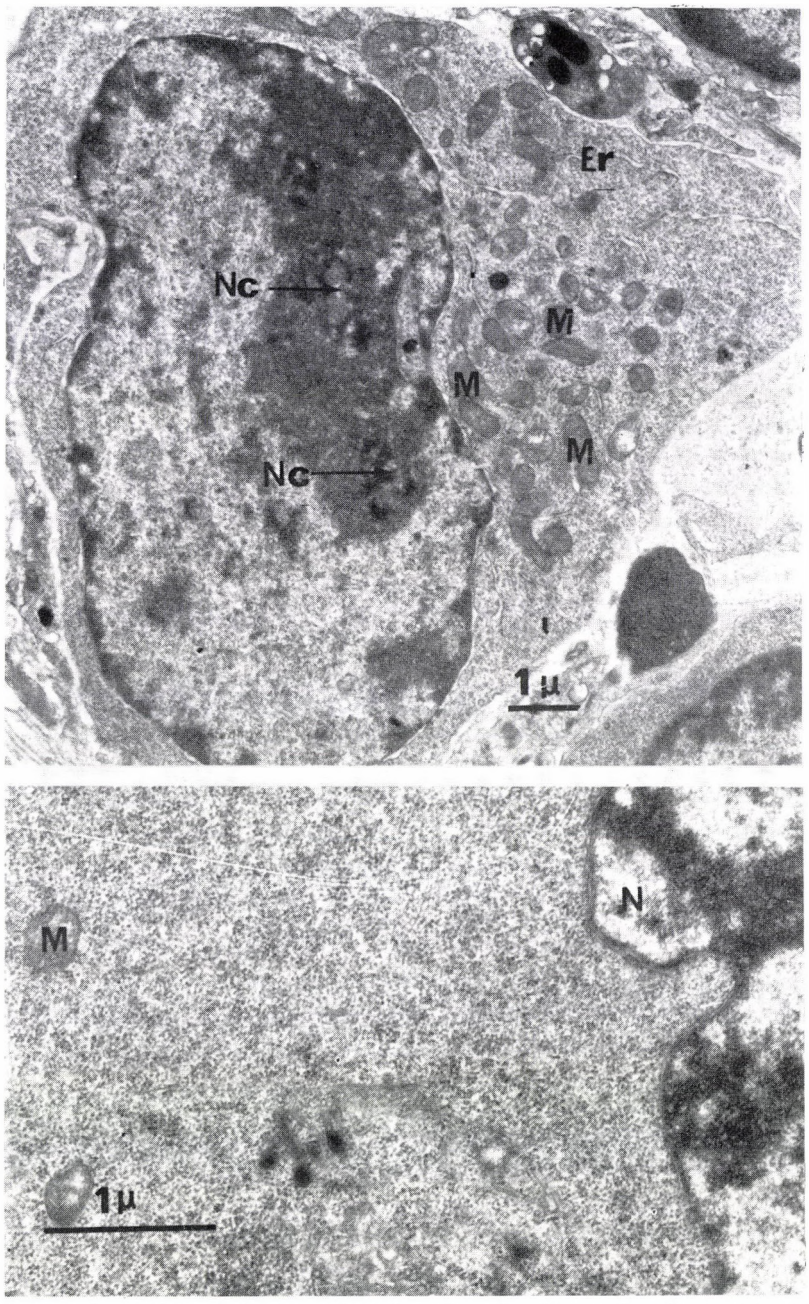

Fig. 1. Megakaryoblast. In its bean-shaped nucleus the chromatin substance is associated with the nucleoli(Nc). At one side of the nucleus the mitochondria(M) are located in groups. In their neighbourhood there are a few ergastoplasmic (Er) cisterns

Fig. 2. Detail of a megakaryoblast. The cytoplasm of the cell is densely filled up by the free ribosomes. Mitochondrium (M), nucleus (N)

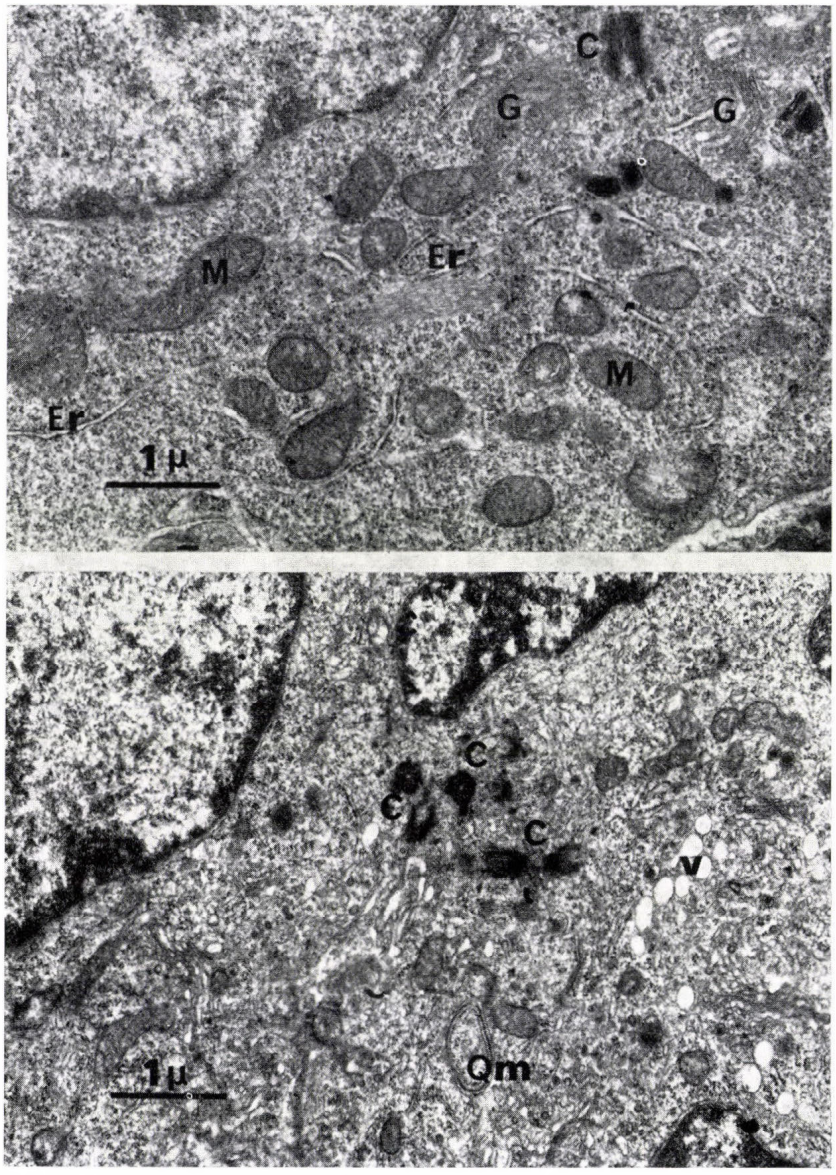

Fig. 3. Detail of a megakaryoblast. The Golgi apparatus (G) of the cell is located around the cytocentrum (C). The accumulated ergastoplasmic(Er) cisterns are among the mitochondria (M)

Fig. 4. Detail from a transitory cell between megakaryoblast and megakaryocyte I. The sections of different planes of several centrioles (C) can be seen in the cell. The apparently empty vesicules of 0.15—0.2 μ in size are demarcated by membranes. The formation consisting of quadrilamellar membranes (Q_m) deserves attention

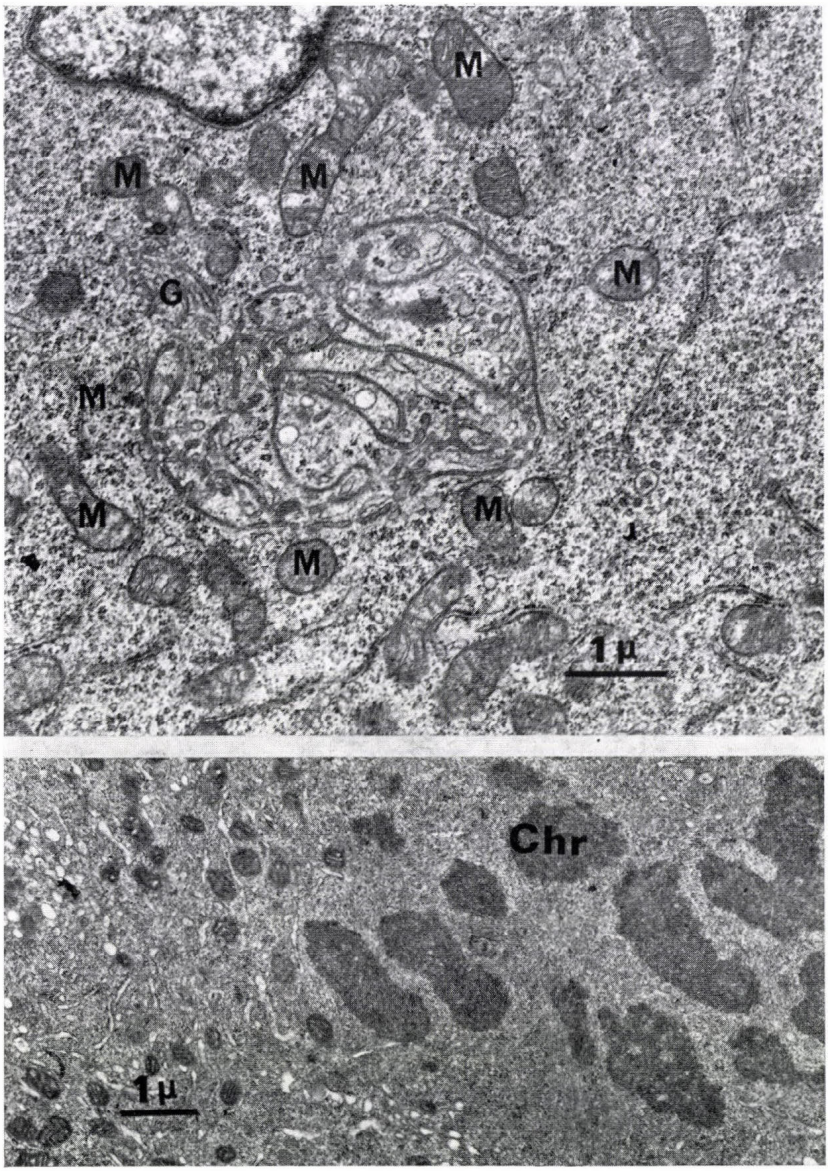

Fig. 5. Detail of megakaryocyte 1. Perinuclearly in the fields consisting of free ribosomes foci of membranes of smooth surface appear. In the area of the foci the Golgi (G) apparatus can be detected. The mitochondria (M) are arranged around the focus

Fig. 6. A cell in the stage of megakaryocyte I in mitosis. Chromosomes (Chr)

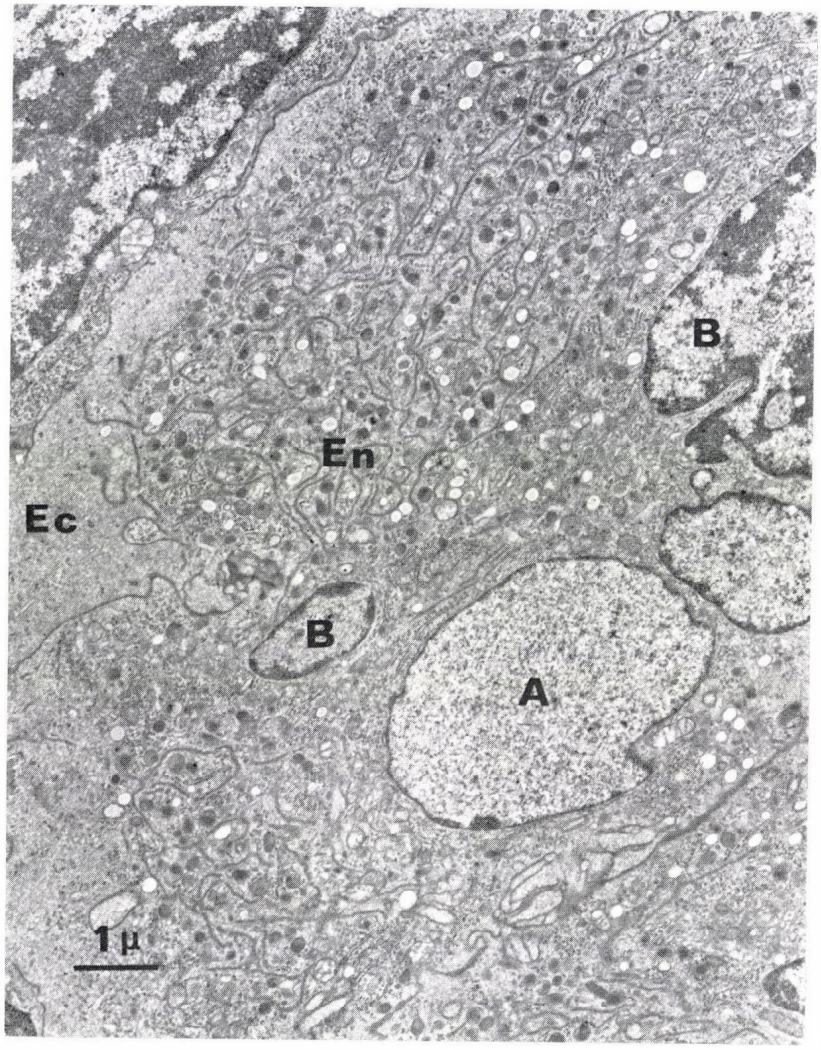

Fig. 7. Detail of megakaryocyte II. The membranes of smooth surface fills up the whole endoplasm (En) of the cell. The specific granules of the cell are located within the membrane-bordered areas. Only hyaloplasm and a few free ribosomes can be found in the ectoplasm of the cell (Ec). It is noteworthy that the individual nuclear parts in the cell show different chromatin distribution (A : nucleus detail with evenly distributed chromatin, B: nucleus detail with granulated chromatin structure)

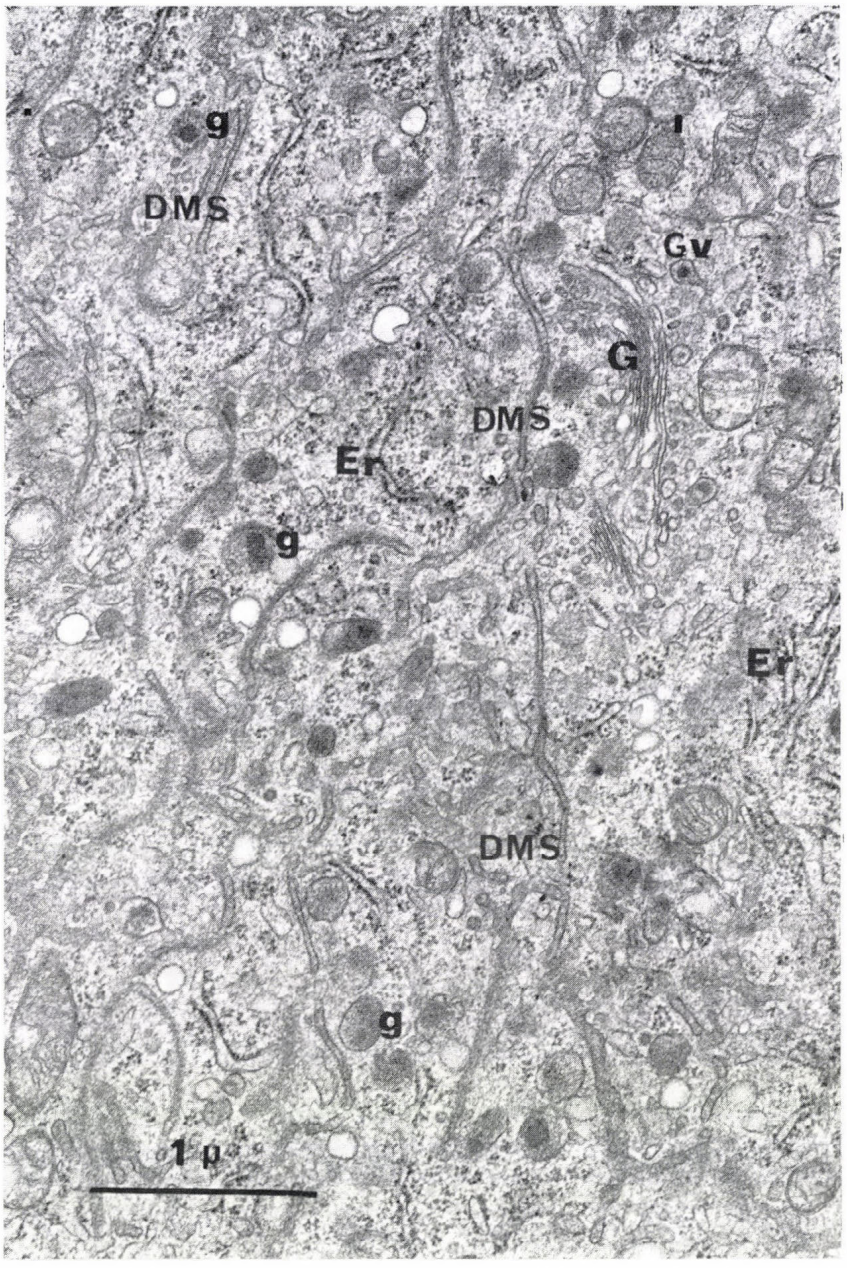

Fig. 8. Detail of megakaryocyte II. The DMS inside contains a substance of medium electron-density. On the convex side of the Golgi apparatus in the detached Golgi vesicules (Gv) the formation of the compact electron dense core can be observed, while on its concave side there is no core formation in the detached vesicules. In the cytoplasm there are disseminated mature granules (g) and a few ergastoplasmic (Er) cisterns as well as a great number of polyribosomes in the neighbourhood of the letter

In megakaryocyte stage II the foci of differentiation in the cytoplasm increase, conflux and fill up the whole cytoplasm with the exception of the marginal ectoplasmatic part (Fig. 7). Parallel with this procedure the number of free ribosomes decreases: they persist only on the surface of the short ergastoplasmic cisterns and in the neighbourhood of these cisterns as polyribosomes (Fig. 8).

We could not observe the connection of the DMS filling up the whole endoplasm with the cell membrane, as we have not seen cell membrane invagination through the ectoplasm in this stage. The cavity of the DMS cisterns is filled by a medium dense, finely granulated material (Fig. 8). In the cisterns of the Golgi apparatus, as well as in the Golgi vesicules at the concave part of the Golgi area in many cases a substance similar in density to that in the cisterns of DMS can be seen (Fig. 8).

The specific granules of the megakaryocyte appear at first in the Golgi zone in the megakaryocyte stage II. In the Golgi vesicules detached at the convex side of the apparatus (Fig. 8) an area of medium density can be seen, in the middle of which there is a core of high electron density. These formations differ from the structure of the mature granules only in their size, former being about twice as great (0.2—0.3 μ).

At the end of the megakaryocyte stage II the ectoplasm is transformed into a thin rim, while the endoplasm is perfectly filled up by the DMS.

Another noteworthy phenomenon occurs in the cells of the megakaryocyte stage II. The parts of nuclei or segments of the single cells differ in chromatin structure. There are parts of nuclei within one cell, the chromatin substance of which is concentrated in the form of bigger or smaller granules, while in other parts of the nuclei it is distributed evenly.

The final stage of the light microscopic differentiation is the mature thrombocytogenic megakaryocyte studied by several authors for light microscopic [10, 17, 19] and submicroscopic [5, 8, 12, 15, 21, 28, 29] characteristics, as well as for functional relation [20].

Discussion

The most characteristic functional feature of the megakaryoblast is its very rapid ribosome production which may be regarded as a preparation for the morphological differentiation taking place in the following stages. The high amount of free ribosomes — mainly in the form of polysomes — appearing in the cytoplasm is conspicuous in this stage. The well-known light microscopic reflection of this stage is an intensive basophilia of the cell. The association of the chromatin substance with the nucleoli can be evaluated as the formation of ribosomes in the nucleus. After the production of a high number of free ribosomes, the nucleus/plasma quotient is gradually shifted light microscopically in favour of the cytoplasm. In the meantime the electron microscopic pictures show the appearance of ergastoplasmic cisterns in the vicinity of the mitochondria flocked together.

The assembly of the two kinds of cytoplasm components may probably be taken for the incipient sign of the foci of differentiation, which leads to the electron microscopical megakaryocyte stage I.

The appearance and accumulation of the foci of differentiation are characteristic of megakaryocyte stage I. These can be observed first in the perinuclear zone of the cytoplasm and show the presence of Golgi apparatus and elements regarded as smooth surfaced endoplasmic reticulum. The foci are surrounded at their borderline to the ribosome fields with mitochondria, which are far more numerous in this stage than in the megakaryoblasts. The ergastoplasmic cisterns are located mainly among the mitochondria or in their direct vicinity. The close topography of the ergastoplasm (polysomes and ergastoplasmic cisterns) and the mitochondria refer to an intensive protein synthesis. Since in this stage no specific formation can be observed in the electron microscopic pictures, it seems probable that the produced protein is used mainly for the synthesis of the smooth surfaced membranes resulting in an increase of the size of the foci of differentiation. The accumulation and increase of foci may occur presumably very rapidly, as in the megakaryocyte population only a very few cells containing such disseminated foci can be found.

The stage in which the foci of differentiation spread over the whole cytoplasm — with the exception of the ectoplasmic part — when they conflux and the specific granule formation begins may be regarded as electron microscopic megakaryocyte stage II.

The proliferated DMS fills up the whole endoplasm of the cell and produces in this manner a compartmental structure. In the inside of the DMS a medium electron dense substance can be found, which is identical with the compound binding ruthenium red on the surface of platelets according to Behnke's [2] investigation. Behnke's [3] examinations with markers (lanthanum nitrate and horse-radish peroxidase) have doubtlessly proven this membrane system being in connection with the megakaryocyte cell membrane. According to our observations the foci of differentiation appear at first beside the nucleus and already in the megakaryocyte stage I they contain a medium dense substance. No invagination was observed in this early stage on the cell membrane; accordingly, there is little probability that the DMS membranes are in direct contact with the cell membrane. It seems more probable that the membranes belong to the membrane system of the endoplasmic reticulum and get in contact with the cell membrane only later, secondarily in the megakaryocyte stage II. The fusion of the two kinds of membrane (thin and thick) may occur according to DeDuve [6] only after the transformation of the thin membrane, an essential step of which is the appearance of a carbohydrate-containing external coat characteristic of the cell surface. Though DeDuve locates the transformation in general in the Golgi apparatus, it is easily possible that the endoplasmic reticulum of the megakaryocyte in itself is able to get transformed into a membrane suitable for the fusion with the cell membrane. This would mean at the same time that the endoplasmic reticulum in the megakaryocytes has been specialized for the production of PAS positive [11]

mucopolysaccharide [22, 23] characteristic of the external coat and for the production of the platelet membrane.

The increase of the cisterns of DMS occurs according to Schultz [26] by the conflux of the vesicules. Following a combined fixation with glutaraldehyde and osmium tetroxide we were unable to observe the presence of vesicule rows and their conflux into cisterns. Therefore, like in Röhlich's experiments with photoreceptors [25], it seems more probable that the newly formed and therefore presumably still labile membrane disintegrates into vesicule rows on osmium treatment. Thus the presence of vesicule rows draws the attention not to the way of the production membranes but to their lability. In our case the presence of vesicule rows may be regarded as "logical artifacts".

The role of the Golgi apparatus in the production of the substance in the inside of the DMS cisterns is not yet elucidated. In some cases we observed vesicules in the concavities of the Golgi apparatus; these contained a substance of similar density as DMS and failed to show the presence of dark nuclei characteristic of megakaryocyte granules. It is possible that these Golgi vesicules transport certain material then they coalesce with the cisterns of the DMS.

In the production of the megakaryocyte granules earlier Jones [13], then Zamboni [29] attributed an important role to the Golgi apparatus. According to our observations a concentration of the substance and a nucleus formation similar to those in the mature granules of megakaryocytes can be seen in the Golgi vesicules detached at the convex side of the Golgi cisterns. Thus, it is conceivable that the Golgi apparatus plays a double role in the life of the megakaryocytes: 1. it transports a substance to the DMS in the vesicules detached at its concave side; 2. the characteristic granules of the megakaryocytes develop from the vesicules detached at its convex side. A similar Golgi function has already been observed by Bainton and Farquhar [1] in the production of the specific and azurophil granules of granulocytes.

Parallel with the development of megakaryocytes the chromatin structure of the cell nucleus is changed. In the megakaryoblast the chromatin is concentrated partly below the nuclear membrane, but mainly around the nucleoli. In the megakaryocyte stage II the chromatin substance of the cell concentrates again and appears under the nuclear membrane as smaller or bigger heterochromatic regions. This chromatin concentration is even more definite in the thrombocytogenic megakaryocyte. No sure explanation can be given for the changes of the chromatin stage of the nucleus, but supposedly it may be connected with the functional activity of the nucleus. Electron microscopic autoradiography of the nucleic acid precursor incorporation in the different stages of development of megakaryocytes may provide further data in this line. It would be most interesting, if these investigations were coordinated with the periods of the intensive ribosome production and of the formation of DMS cisterns and specific granules in the developing forms. It is worth mentioning that the chromatin structures of the individual nuclear parts in the megakaryocyte stage II, differ from each other strongly. Light microscopic autoradiography experiments [7] with ^{3}H-thymidine

indicating the existence of megakaryocytes containing nuclear parts differing in radioactivity are in good concordance with this phenomenon.

The cytoplasm of the cells is separated into ecto- and endoplasm in the megakaryocyte stage II. The ectoplasm is an about 1 μ thick cytoplasm layer inside the cell membrane in which only hyaloplasm and eventually a few free ribosomes can be found. The cell organelles, the megakaryocyte granules and the DMS are located in the area of the endoplasm. Parallel with the maturing of the cell the ectoplasm becomes gradually thinner, but it is retained as a few tenth micron thick layer even in the thrombocytogenic megakaryocyte. Therefore, we can presume that the platelet demarcation occurs not directly on the cell surface — on the ectoplasm — but in the area of the endoplasm. In the light of conception we may attribute an important role to the ectoplasmic part in the liberation of platelets. It is presumable that the ectoplasm, like an envelope, keeps the partially demarcated platelets together. The injury of this ectoplasmic rim due to a yet unknown factor may lead to a "blowing up" of the megakaryocyte.

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Book Review

Blood Replacement. By U. F. Gruber, Springer-Verlag, Berlin—Heidelberg—New York, 1969. With 20 figures. XII, 284 pages. Cloth, DM 24,—; US $6.00.

"Blood replacement presents problems in most fields of medicine . . . The importance of immediate and complete replacement of lost fluid is generally recognized and documented in the treatment of all forms of hypovolaemic shock. In practice, however, the implementation of this principle often presents difficulties . . . Recently there has been increasing doubt concerning the opinion that administration of blood exclusively in all stages of haemorrhagic shock and in all circumstances represents the best therapy . . . Thus there is a real need for so-called blood substitutes . . . What, how much and when? These are the questions raised in the therapy of blood loss and in the treatment of the various stages of haemorrhagic shock . . . This book attempts to answer these questions on the basis of related literature and original investigations" writes the author in the introduction.

Gruber succeeded fully in attaining his aims and presents a good survey of the problem. The literary data are well supplemented by the experience of his own experimental work and clinical practice of long years.

The physico-chemical, biological and pharmacological characteristics of the different blood and plasma preparations as well as of the plasma substituents with or without colloids, their sphere of indications and therapeutical efficacity are compared and discussed in detail.

The monograph consists of four parts. The first deals with the haemodynamical and metabolic changes developing in the course of haemorrhagic shock, as well as with therapeutic considerations. The second part discusses the methods of volume substitution. The dangers and disadvantages of blood transfusion are presented, and the therapeutic results are evaluated. Volume substitution by plasma includes the effects and therapeutic results attainable by fresh plasma, stored pooled plasma, dried plasma, pasteurized plasma-protein and albumin. The physicochemical characteristics of the artificial colloid solutions (dextran, gelatin, PVP, hydroxyethyl-starch preparations, Alginon, Levan), the compatibility of some preparations with other drugs, the metabolism of the dextran and gelatin, their effects on viscosity, sedimentation rate, blood grouping, resistance to infections, blood coagulation, laboratory findings, etc. are discussed in detail; histological and immunological investigations are reported on, and finally the therapeutic results are evaluated. The part dealing with colloid-free solutions includes electrolyte and non-electrolyte solutions. At the end of each point a summary is given and conclusions are drawn. In Part III, results are discussed and practical conclusions are presented. Part IV is a concise summing up of the whole subject in no more than two pages.

The ample list of references includes 1854 titles. In the Appendix, books and reviews on shock, monographs and reviews on blood-transfusion and plasma-expanders, monographs on blood volume determination, its value and its technical problems are given in different groups.

The book was translated into English by L. Oxtoby and R. F. Armstrong; the preface was written by M. Allgöwer, professor of surgery at Basel University.

This excellent work is warmly recommended to our readers.

I. Bernát

Abstracts

Influence of glycolysis on NADH content in human erythrocytes. A. Omachi, C. B. Scott, T. E. Parry (Department of Physiology, University of Illinois, College of Medicine, Chicago, Ill.) *Am. J. Physiol. 216*, 527 (1969)

NADH levels were determined following incubation of intact erythrocytes in balanced saline media at pH 7.4 and 37 °C. The absence of glucose in the medium resulted in a lower NADH content. This change was prevented by the presence of 5 mM fluoride or 10 mM lactate. These results are consistent with Whittam's observation that pyruvate accumulates in red cells incubated in substrate-free media. In the presence of 10 mM glucose, a lower NADH was observed if the medium also contained 10 mM pyruvate or 0.5 mM iodoacetate or if the pH was reduced to 7.0. No difference was seen when the temperature was lowered to 22 °C. These findings are in accord with classical views of glycolysis obtained from studies with cell-free systems and indicate that these views apply also to the intact red cell.

I. Árky

Low binding of 2,3-diphosphoglycerate to haemoglobin F. A contribution to the knowledge of the binding site and an explanation for the high oxygen affinity of foetal blood. C. H. de Verdier, L. Garby (Department of Clinical Chemistry and Clinical Physiology, University Hospital, Uppsala) *Scand. J. clin. Lab. Invest. 23*, 149 (1969)

The binding of 2,3-diphosphoglycerate to human haemoglobin A and F was determined using an ultracentrifugation technique. The binding of the phosphocompound was much lower to haemoglobin F than to haemoglobin A, both in the oxygenated and deoxygenated states. It is concluded that this difference may explain, at least partly, the higher oxygen affinity of foetal blood. The result supports previously advanced arguments that binding of 2,3-diphosphoglycerate to haemoglobin A takes place at βH21 His.

I. Árky

Properties of an adenyl cyclase partially purified from frog erythrocytes. O. M. Rosen, S. M. Rosen (Departments of Medicine and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York) *Arch. Biochem. Biophys. 131*, 449 (1969)

A particulate adenyl cyclase has been purified 150–200fold from the erythrocytes of adult Rana Pipiens. The enzyme catalyzed the synthesis of cyclic 3′,5′-AM^{32}P from α-labelled AT^{32}P in the presence of NaF, Mg^{2+} or Mn^{2+} and a sulfhydryl compound. Catecholamines stimulated enzymic activity in the absence of fluoride to the extent of 50–75% of that demonstrable in the presence of fluoride. The ability of different catecholamines to stimulate enzymic activity paralleled their potency as β-adrenergic compounds. Sensitivity to stimulation by catecholamines was a labile function preserved only by storage of the enzyme in liquid N_2. The purified adenyl cyclase fraction did not contain cyclic 3′,5′-nucleotide phosphodiesterase activity, exhibited optimal activity at pH 8.0, and was inhibited by Zn^{2+} or Ca^{2+}. dATP was the only nucleoside tri-

phosphate other than ATP which was able to serve as a substrate for the formation of a cyclic 3′,5′-nucleotide. Phase microscopy, density gradient centrifugation, enrichment for ATPase activity, and the content of sialic acid and lipid phosphorus all suggested that the particulate enzyme was a fragment of the cell membrane.

G. Gárdos

Effect of reduced glutathione on rat erythrocytes: Production of PNH-like features. N. H. Baum, C. E. Mengel, S. P. Balcerzak (Department of Medicine, Division of Hematology and Oncology, The Ohio State University Hospitals, Columbus, Ohio) *J. Lab. clin. Med. 73*, 277 (1969)

Incubation of rat red cells with GSH resulted in lysis of these cells in serum at a pH optimum of 8.4. Lysis was prevented by heat inactivation of serum and accentuated by addition of thrombin. In addition, these cells also lysed in low ionic strength sucrose solution and had a low acetylcholinesterase activity and increased lytic sensitivity to H_2O_2. They also had a marked shortening of in vivo survival. These findings suggested that the rat erythrocytes had acquired certain in vitro and in vivo lytic features similar to those of human PNH.

I. Árky

Evaluation of stored blood viability by methylene blue reduction. M. D. Sass, C. J. Caruso, D. R. Axelrod (Veterans Administration Hospital, Brooklyn, N. Y.) *J. Lab. clin. Med. 73*, 744 (1969)

Acceptability of stored blood for transfusion is evaluated in a new and simple manner by measuring the uptake of methylene blue by red cells. This measure reflects changes in metabolic activity associated with storage and has a high correlation with viability. Its use may permit recognition of blood suitable for transfusion beyond the storage limit of 21 days.

Ilma Szász

Role of red cell 2,3-diphosphoglycerate in the adaptation of man to altitude. J. W. Eaton, G. J. Brewer, R. F. Grover (Departments of Human Genetics, Anthropology, and Medicine, University of Michigan, Ann Arbor, Mich. and the Cardiovascular-Pulmonary Laboratory, Department of Medicine, University of Colorado Medical Center, Denver, Co.) *J. Lab. clin. Med. 73*, 603 (1969)

The levels of red cell 2,3-diphosphoglycerate (DPG) were measured in a large number of normal men and women residing at high altitudes (10,200 to 11,300 feet). Red cell DPG concentration was also measured in other high altitude residents with excessive polycythemia. Results from both these groups were compared with those from a large sample of Caucasians of both sexes living at a lower altitude (900 feet). It was found that DPG levels were significantly higher in both sexes at high altitude, probably accounting for the frequently reported shift to the right in the oxygen dissociation curve at altitude. In addition, individuals with excessive polycythemia were found to have abnormally high levels of red cell DPG. It is suggested that these high red cell DPG levels are a possible contributory factor in the development of excessive polycythemia at high altitude. The negative correlation between whole blood hemoglobin concentrations and red cell DPG levels is also present in this sample from high altitude.

G. Gárdos

The M-antigen in HK and LK sheep red cell membranes. P. K. Lauf, D. C. Tosteson (Department of Physiology and Pharmacology, Duke University, Durham, N. C.) *J. Membrane Biol. 1*, 177 (1969)

Red cells of all high-potassium-type (HK) sheep and of more than one half of all low-potassium-type (LK) sheep contained the M-antigen and were haemolyzed by isoimmune anti-M antiserum in presence of a guinea pig serum complement. It was characteristic for the haemolysis of HK red cells by the M-antiserum that all HK cells were ultimately haemolyzed at suboptimal antibody concentrations, provided the time of incubation at 37 °C was sufficiently long.

Thus, the M-antigen appears to be expressed on all red cells of an individual HK sheep. The M-antibody was absorbed by HK red cells and their membranes with a high affinity, whereas M-negative LK red cells and their membranes did not bind the antibody. The ratio of the number of antibody units absorbed per cell or membrane to the number of antibody units required for lysis approached unity. The amount of antibody absorbed per membrane was unaffected by ouabain in the presence of ATP, Mg^{++}, Na^+, and K^+. The M-antigen activity depends on the integrity of the red cell membrane and was not detectable after lyophilization of HK membranes or in the membrane protein solubilized by n-butanol. The major M-antibody activity was found among the high molecular weight plasma proteins and may be attributed to the β_2 M globulins. Heterogeneity within the antibody fraction cannot be excluded since some haemolytic activity was detected in a chromatographic fraction containing predominantly γ-globulin. The relationship between the M-antigen and the $Na^+ - K^+$ transport system in sheep red cell membranes is discussed.

G. Gárdos

Structure of membranes: IV. Antigen activity of sheep erythrocyte ghosts after phospholipid fractionated hydrolysis. G. Nanni, U. M. Marinari, I. Baldini, M. Ferro, A. Casu (Istituto di Patologia generale, Università, Genova, Italia), *Italian J. Biochem. 18*, 123 (1969)

The role of native proteins and phospholipids and their probable arrangement in the membrane structure of sheep erythrocytes have been studied by immunological way. By means of haemolysis and haemagglutination tests, production of different kinds of antibodies against sheep erythrocytes, erythrocyte membranes and against stromata depleted of phospholipids by fractionated enzymatic hydrolysis was followed. From the data obtained it would appear that the removal of sphingomyelin from phospholipid-protein architecture of sheep erythrocyte membrane results in a significant decrease in the antigen-antibody reaction at the level of intact sheep erythrocyte. In relation to previous observations, results are discussed regarding the position of phospholipids in the structure of sheep erythrocyte membrane.

Ilma Szász

Deformation of red blood cells in capillaries. R. Skalak, P. I. Branemark (Department of Civil Engineering and Engineering Mechanics, Columbia University, New York; Laboratory of Experimental Biology, Department of Anatomy, University of Gothenburg, Gothenburg, Sweden) *Science 164*, 717 (1969)

The shapes of red blood cells in capillary blood vessels are reinterpreted from observations of human red cells. The parachute or umbrella shape often observed is not an axisymmetric shape as formerly assumed, but is the basic biconcave disk shape of the red cell. The distortion during flow consists of the blunting of the rear end of the cell and the thickening of the front end. The flattened rear portion of the cell becomes squeezed free of cell contents (primarily haemoglobin), which are contained under such circumstances in the bulging front end of the cell.

Ilma Szász

Determination of the life span of blood and bone marrow cells. O. N. Piksanov (Inst. med. radiol. AMH USSR, Obninsk, USSR) *Probl. Gemat. 14*, 4, 46 (1969).

The author presents a formula for the determination of the developmental stage of the formed blood cells. Calculations are mathematical and proceed from the partial formula of these elements and the sum total of their life span.

J. Dávid

Ocena krwinkogubnej czynności śledziony w oparciu o badania z użyciem promieniotwórczego izotopu chromu Cr^{51} (Evaluation of the haemolytic function of the spleen based on investigations with ^{51}Cr). M. Gembicki, S. Dzieciuchowicz) (II Klinka Chorób Wewnętrznych AM, Poznań, Poland). *Pol. Tyg. lek. 24*, 221 (1969).

In 10 patients including 2 patients with splenomegaly but without haemolytic anaemia, 6 patients with various types of haemo-

lytic anaemia, and 2 normal subjects, the survival time of ^{51}Cr-labelled erythrocytes, the spleen-liver index and the sequestration of erythrocytes in spleen and liver were determined. A high spleen—liver index was found in patients with splenomegaly and in most patients with haemolytic anaemia. On the other hand, increased sequestration of erythrocytes in the spleen was observed in only 5 patients with haemolytic anaemia. The authors believe that the spleen—liver index and erythrocyte sequestration in these organs should be determined simultaneously. Increased haemolysis occurring mainly in the spleen is evidenced by a high spleen—liver index and an increased sequestration of erythrocytes in the spleen unassociated with similarly increased sequestration in the liver.

S. Maj

Bdania nad kinetyka ^{59}Fe *i erytrokinetyka* ^{51}Cr *w czerwienicy* (Studies on ^{59}Fe kinetics and ^{51}Cr erythrokinetics in polycythaemia). L. Konopka, K. Rechowicz, S. Pawelski (Instytut Hematologii, Warsaw, Poland). *Pol. Arch. Med. wewnęt. 42*, 39 (1969).

Simultaneous studies of ^{59}Fe kinetics and ^{51}Cr erythrokinetics were made in 11 cases of polycythaemia vera in various stages of the disease and in four cases of secondary polycythaemia associated with kidney disease and ovarian tumour. A considerable enhancement of effective erythropoiesis was found in all cases independent of the duration of the disease, evidenced by an increased haemoglobin synthesis per 24 hours, and by an increased volume of the circulating erythrocytic mass. The said parameters were, however, less distinctly elevated in five cases with iron deficiency. In the three cases of polycythaemia vera with a long duration of the disease, the volume of the circulating erythrocytic mass remained high despite the coexisting shortening of the erythrocyte life span. The identical results obtained in polycythaemia vera and secondary polycythaemias seem to speak against the reliability of isotope methods in the differentiation between the two conditions.

S. Maj

Trudnosci diagnostyczne w zaotrzewenowych wylewach krwi do miesnia biodrowo-ledzwiowego u chorych na hemofilie (Diagnostic difficulties in retroperitoneal haemorrhage into the iliopsoas muscle in patients with haemophilia). F. Loth, K. Mars-Gawlikowska (Oddzia) (Urazowo-Ortopedyczny Szpitala Chirurgii Urazowej Dzieciecej, Warsaw, Poland). *Pol. Tyg. lek. 24*, 306 (1969).

Haemorrhage into the iliopsoas muscle of haemophiliacs presents considerable diagnostic difficulties. Such haemorrhages on the right especially resemble peritonitis or acute appendicitis. An important sign differentiating an acute abdomen from the iliopsoas haemorrhage is the flexion of the hip joint with adduction and lateral rotation of the thigh. Diagnostic errors may be the cause of unnecessary laparotomy with possible tragic consequences in haemophiliacs.

S. Maj

Wyniki leczenia busulfanem (myleranem) czerwienicy prawdziwej (Results of busulphan (Myleran) treatment of erythraemia vera). I. Urasinski, M. Mysik (II Klinika Chorób Wewnętrznych, Kraków, Poland) *Pol. Arch. Med. wewnęt. 42*, 661 (1969).

Immediate and long-term results with busulphan (Myleran) treatment of 36 cases of erythraemia vera are presented. A long-term remission was obtained in 34 patients whereas in two cases the disease was resistant to the treatment. The mean duration of the first remission after the first course of busulphan administration was 39 months. The duration of the subsequent remissions after repeated courses of treatment became shorter and shorter. No clinical complications due to the treatment were seen. An experience of ten years allows the conclusion that treatment of erythraemia vera with busulphan is not inferior to ^{32}P treatment.

S. Maj

Coenzyme forms and total content of B_{12} *compounds in acute leukosis.* N. V. Myasishcheva, G. D. Levina, Yu. I. Lorie, M. O. Raushenbach (Gematol. klin i labor. sist. zabolevan. krovi, Inst. exp. klin. onkol., Moscow, USSR) *Probl. Gemat. 14. 4*, 20 (1969).

The authors studied the vitamin B_{12} content and the composition of its active forms in the serum of patients with acute and chronic leukaemia. Chronic myeloleukaemia was characterized by increased, chronic lymphoid leukaemia by normal vitamin content. The two groups of patients differed from one another and from healthy persons in the distribution of B_{12} coenzymes. The vitamin content in one group of patients suffering from acute leukaemia was normal or reduced, while in another group increased values were observed. The latter were prognostically unfavourable. A relationship was shown between vitamin B_{12} content and the clinicomorphological variant of acute leukaemia. A number of cases exhibited a correlation between vitamin content and the course of leukaemia, as well as between vitamin content and the absolute blast cell count in blood and bone marrow during the disease. Elevation of vitamin B_{12} concentration in acute leukaemia is an unfavourable sign. The ratio of cobamides in acute leukaemia is not uniform. A resistance to combined cytostatic therapy was noted in patients with a prevalence of methyl or oxycobalamine in their blood serum.

J. Dávid

Rybonukleazy leukocytów. Doniesienie I i II (Leucocytic ribonucleases. Part I and II). J. Sznajd, J. Naskalski (III Klinika Chorób Wewnętrznych AM, Kraków, Poland). *Pol. Arch. Med. wewnęt. 42*, 187 and 197 (1969).

The nucleolytic activity of leucocytes was found to depend mostly on a thermostable ribonuclease with a 6.8—7.1 optimum pH. Extracts of normal leucocytes and leucocytes from patients with chronic granulocytic leukaemia did not differ essentially and quantitatively in nucleolytic activity. Three fractions of leucocytic ribonuclease were separated by our own method. All the isolated fractions digested yeast RNA. The decomposition products of one fraction consisted of several nucleotide spots of slight mobility. The products of decomposition due to the activity of the two remaining fractions were similar, consisting of numerous nucleotide spots of high mobility and a small spot of medullar nucleotides.

S. Maj

Transformacja blastyczna limfocytów in vitro pod wplywem fitohemaglutyniny w ziarnicy zlosliwej (Blastic transformation of lymphocytes in vitro by phytohaemagglutinin in lymphogranulomatosis). A. Płuzanska (II Klinika Charób Wewnętrznych, Łódz, Poland). *Pol. Arch. Med. wewnęt. 42*, 655 (1969).

In three-day cultures of white blood cells from patients with lymphogranulomatosis the per cent of lymphocytes undergoing blastic transformation by phytohaemagglutinin depended on the patient's clinical condition. Blastic transformation was much more frequent in the period of remission than in the periods of progression.

S. Maj

Differentiation of lymphocytes of laboratory animals by the number of RNA-containing granules. A. P. Gindin, N. M. Ogienko (Patomorfol. labor. otd. ran. infekc. Inst. epidemiol. i mikrobiolog. im N. F. Gamalei, Moscow, USSR) *Probl. Gemat. 14, 4*, 44 (1969).

Variable numbers of RNA-containing granules differing in shape and size were found in the cytoplasm of lymphocytes and monocytes in the blood of rabbits, guinea pigs, Wistar rats and Cricetus auratus W. According to the number of such granules, the lymphocytes were divided into three groups: cells densely covered with granules were classified into the first group, the second comprised cells with a lesser number of granules and in the third the cells contained only individual granules.

J. Dávid

Isolation and identification of microorganisms from the blood and bone marrow of patients suffering from acute leukosis. T. V. Golosova, V. A. Martynova, G. V. Osechenskaya (Bakteriol. labor. i. genatol. klin. Central. Inst. Gematol. i. Pereliv. Krovi, Moscow) *Probl. Gemat. 14, 4,* 25 (1969).

From the blood, bone marrow, leukocytes and serum of patients suffering from acute leukaemia, the authors isolated cultures of microorganisms consisting of small coccoid or coccobacillary corpuscles forming short and long chains. The isolated microbes exerted weak enzymic activity: they did not ferment sugars, did not form indole and hydrogen sulphide, rarely caused haemolysis or reduced methylene blue. In media used for passage of mycoplasma, the microbes acquired an appearance similar to mycoplasma structures.

J. Dávid

Badania nad zawartoscia kwasu foliowego w surowicy krwi kobiet z nowotworem narzadu rodnego (Serum folic acid level in women with genital neoplasm). T. Pertynski, B. Krawczyk, E. Małafiej, M. Draminski (Oddział Polozniczo-Ginekologiczny Szpitala MSW, Łódz, Poland). *Pol. Tyg. lek. 24,* 161 (1969).

A low serum folic acid level was found in 42 women with genital neoplasm. No correlation was observed between the size of the malignancy and the serum folic acid level.

S. Maj

The effect of prophylactic vaccination on haemopoiesis in mice. T. I. Sergeva, F. I. Zharova (Labor. sistem. zaboleran. krovi, Inst. eksperimental. i klin. onkol., Moscow, USSR) *Probl. Gemat. 14, 4,* 41 (1969)

In $CC_{57}Br$ and $C_{57}BI$ mice, changes in haemopoietic function were observed after prophylactic smallpox vaccination. For three and more weeks the vaccinated animals displayed an enhanced proliferation of undifferentiated haemopoietic elements in the bone marrow and in other haemopoietic organs; there was also an intensified production of the lymphoid series with a depression of neutrophil formation and maturation.

J. Dávid

Fibrynolityczne leczenie zatorów tetniczych (Fibrinolytic treatment of arterial embolism). T. Dorobisz, Z. Jaworski, B. Sampławska, K. Skóra, Z. Szydłowski (I Klinika Chirurgiczna AM, Wrocław, Poland). *Pol. Tyg. lek. 24,* 579 (1969).

In arterial embolism surgical intervention is carried out as a rule. However, in 8 cases with contraindications for this method of treatment or with embolisation of small arteries, the authors used Streptase (Behringwerke). Early results were good in all the cases, while late results were good in 5 cases with involvement of small and middle-sized arteries, and poor in 3 cases of abdominal aorta-embolism. Fibrinolytic treatment is considered a valuable supplementary method to be used after surgical treatment of arterial embolism.

S. Maj

Krazacy antykoagulant u chorych z hemofilia A (Circulating anticoagulant in patients with type A haemophilia). L. Uszynski, S. Łopaciuk (Instytut Hematologii, Warsaw, Poland) *Pol. Arch. Med. wewnęt. 42,* 119 (1969).

A circulating anti-AHG anticoagulant was discovered in three haemophilic patients treated previously with plasma transfusions. The inhibitor was found in the gamma-globulin fraction and was relatively thermostable; it could not be absorbed on aluminium hydroxide and reacted on incubation with factor VIII in a time and temperature dependent way. The anticoagulant was variably related to plasma AHG of various species. Precipitation as well as immunoelectrophoretic tests were negative.

S. Maj

From the International Literature of Haematology

Acta Haematologica (Basel) **41** (1969) No. 5

Peripheral nerve function in pernicious anemia before and after treatment. *Lockner, D., Reizenstein, P., Wennberg, A., Widén, L.* (Dept. of Intern. Med., Karolinska Sjukhuset, Stockholm, p. 257

Effect of heparin on platelet adhesiveness. *Zbinden, G., Tomlin, S.* (Dept. of Med. Univ. of Cambridge, Cambridge), p. 264

Zur Darstellung der Transformationsfähigkeit von Lymphozytenkulturen bei verschiedenen Erkrankungen mittels Feulgen-Zytophotometrie. *Ambs, E., Chesebro, J., Lagerlöf, B.* (Univ.-Kinderklin., 87 Würzburg), p. 276

An evaluation of nuclear arylsulfatase activity in acute leukemias. *Smutka, Paulette, Brunning, R. D.* (Hematol. Lab., Univ. of Minnesota, Minneapolis, Minn.), p. 290

Cytochemical demonstration of the co-enzyme ubiquinone in normal human blood and bone marrow cells. *Puera, B., Nir, E., Efrati, P.* (Kaplan Hosp., Rehovoth, Israel), p. 296

The comparison of the coagulation factors in arterial and venous blood. *Özsoylu, S.* (Dept. of Pediat, Hacettepe Univ. School of Med., Ankara), p. 303

A case of IgAK-myelomatosis with two urinary Bence-Jones proteins (BJK and BJL) and multiple chromosomal abnormalities. *Dammacco, F., Trizio, D., Bonomo, L.* (Clinica Med., Università di Bari, 70124 Bari), p. 309

Blood (New York) **34** (1969) No. 3

Bone marrow reactions to trauma. Stimulation of erythropoietic marrow by mechanical disruption, fracture or endosteal curettage. *Van Dyke, D., Harris, N.* (Univ. of California, Lawrence Radiat. Lab., Berkeley, Calif.), p. 257

The quantitative distribution of the erythron and the RE cell in the bone marrow organ of man. *Nelp, W. B., Bower, Rae Ellen* (Univ. of Washington, School of Med., Seattle, Wash.), p. 276

Anabolic androgenic steroids in the treatment of acquired aplastic anemia. *Sanchez-Medal, L., Gomez-Leal, A., Duarte, Z., Guadalupe Rico, M.* (Inst. of Nat. Nutr. Dept. of Hemat., Mexico City), p. 283

Clinical investigations in the treatment of meningeal leukemia: radiation therapy regimes vs. conventional intrathecal methotrexate. *Sullivan, Margaret P., Vietti, Teresa J., Fernbach, D. J., Griffith, K. M., B. Haddy, Theresa, Lorraine Watkins, W.* (Univ. of Texas, M. D. Anderson Hosp. and Tumor Inst., Houston, Texas), p. 301

The eosinophil response to toxoids and its inhibition by antitoxin. *Speirs, R. S., Turner, M. X.* (City Univ. of New York, Downstate Med. Center, Brooklyn, N. Y.), p. 320

The morphogenesis of Gaucher cells investigated by electron microscopy. *Pennelli, N., Scaravilli, F., Zacchello, F.* (Univ. of Padova, Pediat. Clinic, Inst. of Path. Anat., Padova), p. 331

Protein synthesis in human leukocytes and lymphocytes: 1. Effect of steroids and sterols. *Werthamer, S., Hicks, C., Amaral, L.* (Methodist Hosp., Brooklyn, N. Y.), p. 348

Characteristics of iron dextran utilization in man. *Henderson, P. A., Hillman, R. S.*

(Univ. of New Mexico, Dept. of Gynecol. and Obstet., Albuquerque, N. M.), p. 357

Diisopropylfluorophosphate is not a specific label for the red cell membrane. *Sears, D. A., Weed, R. I.* (Univ. of Texas Med. School, San Antonio, Texas), p. 376

Studies on the control of hemoglobin synthesis: the *in vitro* stimulating effect of a 5 β-H steroid metabolite on heme formation in human bone marrow cells. *Necheles, T. F., Rai, U. S.* (New England Med. Center Hosp., Boston, Mass.), p. 380

Brief report: Hemoglobin-coated charcoal radioassay for serum vitamin B_{12}. A simple modification to improve intrinsic factor reliability. *Hillman, R. S., Oakes, M., Finholt, C.* (Univ. of Washington School of Med., Seattle, Wash.), p. 385

Blut (München) **19** (1969) No. 7

Der Einfluß von Erythropoietin und somatotropem Hormon auf die Retikulozyten-Reifungszeit in vitro. *Engelbrecht, H. M., Remmele, W.* (Pathol. Inst. der Städt. Klin., 62 Wiesbaden), p. 385

Die medikamentöse Beeinflussung experimenteller Knochenmarksfibrosen nach wiederholter Fremdeiweiß-Applikation durch Antiphlogistica und Prednisolon-Acetat. *Cohrs, U., Hauswaldt, Ch., Hunstein, W.* (Med. Univ.-Klinik, 34 Göttingen), p. 394

Protein synthesis in human peripheral blood leukocytes. *Scott, R. B., Abdul-Fadl, Y.* (Dept. of Med., Med. Coll. of Virginia, Richmond, Va.), p. 405

Monoklonales abnormes Karyogramm: 45, XX, 2D−, (17−18). 2C+, Gp+ in den Tumorzellen eines Retothelsarkoms. *Ganner, Elisabeth* (Med. Univ.-Klinik, A−6020 Innsbruck), p. 416

Studies with heterologous antilymphocytic serum as a therapy for chronic lymphocytic leukemia. *Tsirimbas, A. D., Pfisterer, H., Hornung, B., Thierfelder, S., Michlmayr, G., Stich, W.* (I. Dept. of Med., Univ. of Munich, 8 Munich 15), p. 420

Der Bürstenschädel. Eine Übersicht über Vorkommen, Röntgenologie, Anatomie, Verlauf und Bedeutung dieses Symptoms. V. Veränderungen des Skeletts bei schweren Bluterkrankungen. *Reimann, F., Gökmen, E.* (Inst. f. med. Forsch., I. Med. Fakultät der Univ., Istanbul), p. 423

British Journal of Haematology (Oxford) **17** (1969) No. 4

Annotation: The misused reticulocyte. *Hillman, R. S., Finch, C. A.* (Univ. of Washington School of Med., Seattle, Wash.), p. 313

"Non-tropical idiopathic splenomegaly" ("primary hypersplenism"): a review of ten cases and their relationship to malignant lymphomas. *Dacie, J. V., Brain, M. C., Harrison, C. V., Lewis, S. M., Worlledge, Sheila M.* (Depts of Haematol. and Morbid Anat., Royal Postgrad. Med. School, London W. 12), p. 317

The role of puberty in red-cell production in hereditary haemolytic anaemias. *Shahidi, N. T., Clatanoff, D. V.* (Dept. of Pediat. and Med., Univ. of Wisconsin, Madison, Wis.), p. 335

Haemolysis and erythropoiesis. II. Reticulocytosis and rate of haemoglobin rise in haemolytic and deficiency anaemias. *Sánchez-Medal, L., Pizzuto, J., Rodríguez-Moyado, H., Espósito, L.* (Dept. of Haemat., Inst. Nac. Nutr., Mexico City, Mexico), p. 343

Diurnal variations of the serum iron in normal subjects and in patients with haemochromatosis. *Sinniah, R., Doggart, J. R., Neill, D. W.* (Dept. of Path., Queen's Univ. of Belfast, Belfast, N. I.), p. 351

Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets. *Cohen, P., Derksen, A.* (Biochem. Lab., The Univ. of Utrecht, Utrecht), p. 359

Wiskott-Aldrich syndrome: qualitative platelet defects and short platelet survival. *Gröttum, K. A., Hovig, T., Holmsen, H., Foss Abrahamsen, A., Jeremic, M., Seip, M.* (Haemat. Sect. of Med. Dept. A, Oslo Univ. Inst. of Path. Anat., Oslo), p. 373

Effect of ethanol on liver α-aminolaevulinate synthetase activity and urinary porphyrin excretion in symptomatic porphyria. *Shanley, B. C., Zail, S. S., Joubert, S. M.*

(Dept. of Chem. Path., Univ. of Natal, Durban, S. A.), p. 389

Antibody activity against hog intrinsic factor in pernicious anaemia. *Ungar, Berta* (Dept. of Clin. Path., Royal Melbourne Hosp., Victoria, Australia), p. 397

British Journal of Haematology (Oxford) **17** (1969) No. 5

Annotation: Antilymphocytic serum. *Gunn, A.* (Div. of Exp. Biol., Nat. Inst. for Med. Res., London), p. 421

The effect of Arvin on experimental pulmonary embolism in the rabbit. *Olsen, E. G. J., Pitney, W. R.* (Depts of Pathol. and Haematol., Roy. Postgrad. Med. School, London), p. 425

Fibrinolysis at rest and after exercise in hepatic cirrhosis. *Das, P. C., Cash, J. D.* (South-East Scotland Reg. Blood Transf. Res. Lab., Royal Infirmary, Edinburgh 3), p. 431

The mechanism of enhanced streptokinase-induced clot lysis following *in vitro* factor-XIII inactivation. *Henderson, K. W., Nussbaum, M.* (Dept. of Med., New Jersey Coll. of Med. and Dentistry, Newark, N. J.), p. 445

The original haemorrhagic fever: yellow fever. *Dennis, L. H., Reisberg, B. E., Crosbie, J., Crozier, D., Conrad, M. E.* (Georgetown Tumor Service, D. C. Gen. Hosp., Washington, D. C.), p. 455

Electron microscopy of fibrin-like precipitate formed during the paracoagulation reaction between soluble fibrin monomer complexes and protamine sulphate. *Horn, R. G., Hawiger, J., Collins, R. D.* (Dept. of Path., Vanderbilt Univ. School of Med., Nashville, Tenn.), p. 463

Effects of dietary methionine and vitamin B_{12} deficiency on folate metabolism. *Vitale, J. J., Hegsted, D. M.* (Depts of Preventive Med. and Path., Tufts Univ. School of Med., Boston, Mass.), p. 467

Erythropoiesis, red-cell creatine and plasma aldolase activity in anaemia in the rabbit and man. *Griffiths, W. J., Lothian, Elizabeth J.* (Dept. of Haemat., Louis Jenner Lab., St. Thomas's Hosp. and Med. School, London), p. 477

Haemoglobin synthesis in β-thalassaemia. *Modell, C. B., Latter, A., Steadman, J. H., Huehns, E. R.* (Dept. of Chem. Path., Univ. Coll. Hosp. Med. School, London), p. 485

Immunochemical quantitation of human transferrin in pregnancy and during the administration of oral contraceptives. *Jacobi, J. M., Powell, L. W., Gaffney, T. J.* (Dept. of Med., Univ. of Queensland, Brisbane, Australia), p. 503

Folia Haematologica (Leipzig) **92** (1969) No. 1

Veränderungen im Bereich der biochemischen Parameter während Streptokinase-Infusion. *Amery, A., Vermylen, J., Verstraete, M.* (Klin. Inw. Geneesk., Acad. Ziekenhuis, Leuven), p. 7

Fibrinogenspaltprodukte und ihre Komplexe mit Fibrinmonomeren. *Kowalski, E.* (Inst. f. Kernforsch., Abt. f. Radiobiol. u. Gesundheitsw., Warschau), p. 20

Endogene und exogene Fibrinolyse. *Perlick, E., Wilfert, K.* (Med. Univ.-Klinik, 701 Leipzig), p. 28

Dosierung und Dauer der Streptokinasebehandlung beim Herzinfarkt. *Weller, P., Hanke, P., Kujat, G., Frank, I.* (II. Med. Univ.-Klinik, 40 Halle/Saale), p. 33

Zur Behandlung des Herzinfarktes unter besonderer Berücksichtigung fibrinolytischer Substanzen. *Heinrich, H.-G.* (II. Med. Klinik, Städt. Oskar-Ziethen-Krankenhauses, 113 Berlin), p. 38

Über die fibrinolytische Aktivität menschlicher Gewebe. *Perlewitz, J., Markwardt, F.* (Pharmakol. Inst. der Med. Akad., 50 Erfurt), p. 46

In-vitro-Untersuchungen mit Urokinase. *Fischer, M., Pilgerstorfer, H. W.* (I. Med. Univ.-Klinik, A—1090 Wien), p. 51

Hyperfibrinolyse als Ursache von sogenannten "Spontanblutungen". *Perlick, E.* (Med. Univ.-Klinik, 701 Leipzig) p. 57

Antifibrinolytika und deren therapeutische Anwendung. *Fischer, M.* (I. Med. Univ.-Klinik, A—1090 Wien), p. 64

Vergleichende klinisch-pharmakologische Untersuchungen synthetischer Antifibrinolytika. *Markwardt, F., Klöcking, H.-P.,*

Landmann, H. (Pharmakol. Inst. der Med. Akad., 50 Erfurt), p. 78

Tierexperimentelles Verfahren zur Testung von Fibrinolytika und Antifibrinolytika. *Klöcking, H.-P., Markwardt, F.* (Pharmakol. Inst. der Med. Akad., 50 Erfurt), p. 84

Über den Kombinationseffekt von PAMBA und Contrykal. *Richter, M., Markwardt, F.* (Pharmakol. Inst. der Med. Akad., 50 Erfurt), p. 90

Klinische Untersuchungen mit Derivaten der *p*-Aminomethylbenzoesäure. *Vogel, G., Klöcking, H.-P.* (Med. Klinik der Med. Akad., 50 Erfurt), p. 93

Revue Française de Transfusion (Paris) **12** (1969) No. 2

Aspects nouveaux des variations des antigènes de groupes sanguins. *Salmon, Ch., Mannoni, P., Séger, Jeanine* (Centre Départ. de Transf. Sanguine, 75-Paris 12), p. 207

Détermination automatique des groupes sanguins. Réalisation d'un appareil expérimental. *Matte, Cl.* (Centre National de Transf. Sanguine, 75-Paris 15), p. 213

Un nouvel exemple de génotype rhésus cD-/cD-. *Delmas-Marsalet, Y., Goudemand, M. Tippett, Patricia* (Centre Rég. de Transf. Sanguine, 59-Lille), p. 233

Le complexe génique DIV(C)-. *Salmon, Ch., Gerbal, A., Liberge, G. Sy, B., Tippett, P., Sanger, R.* (Centre Départ. de Transf. Sanguine, 75-Paris 12), p. 239

Iso-immunisation fœto-maternelle anti-S: maladie hémolytique du nouveau-né chez les deux descendants. *Brizard, C. P., La Selve, A., Le Petit, J. C.* (Centre Départ. de Transf. Sanguine, Saint-Etienne), p. 249

Évolution de l'activité lysozymique du plasma dans le sang conservé. *Saint-Blancard, J., Le Baron, J.-L., Monteux, G., J., Forlot, P., Jollès, P.* (Centre de Transf.-Réanim. de l'Armée "Jean Julliard", 92-Clamart), p. 259

Mobilité électrophorétique sur acétate de cellulose des lipoprotéines de très faible densité isolées à partir du sérum lipémique. *Burstein, M., Gouzi, M.* (Centre Nat. Transf. Sanguine, 75-Paris 15), p. 271

Phénotype Am avec expression secondaire de l'antigène A dans les hématies. *Serim, N.* (Centre de Transf. de l'Hôp. Numune Haydarpasa, Istambul), p. 277

Note sur la survie des microfilaires en sang conservé. *Kremer, M.* (Inst. de Parasitol., 67-Strasbourg), p. 281

Scandinavian Journal of Haematology (Copenhagen) **6** (1969) No. 4

The activator time. A method for control of activator activity during thrombolytic therapy. *Blix, S.* (Ullevål Hosp., Dept. of Kroghstotten, Oslo), p. 221

Some effects of pH and temperature upon normal human red cell adenosine triphosphate. *Scott, G. L., Oates, M., Grimes, A. J.* (St. Thomas Hosp., Jenner Lab., London), p. 227

Effects of atropine on platelet structure and function. *White, J. G.* (Mayo Bldg., Dept. of Pediat., Minneapolis, Minn.), p. 236

An evaluation of aryl sulphatase activity in leukaemic cells. *Boysen, G.* (Osterbrogade 54, Copenhagen), p. 246

Hereditary thrombocytopenia with excessively prolonged bleeding time. *Stavem, P., Jeremic, M., Hjort, P. F., Wislöff, F., Vogt, E., Öyen, R., Abrahamsen, A. F., Sövde, A.* (Rikshosp., Sect. Haemat., Oslo), p. 250

Glycogen in human peripheral blood leukocytes. III. Effect of female hormones. *Abdul-Fadl, Y., Scott, R. B.* (Med. Coll. of Virginia, Dept. of Med., Richmond, Va.) p. 262

Serum iron as differential diagnostic aid in acute leukaemia in children. *Moe, P. J.* (Barneklin Haukeland Hosp., Bergen, Norway), p. 270

Neutrophil segmentation and radial segmentation. Effects of cooling and demecolcine on the lobulation on neutrophil nuclei. *Norberg, B.* (Lasarettet, Dept. of Intern. Med., Lund 5), p. 274

A heterogeneity of the HD-receptor, demonstrable by HD-cold antibodies: HD_1/HD_2. Immunochemical aspects. *Roelcke, D., Uhlenbruck, G., Bauer, K.* (Univ. of Heidelberg, Inst. of Immunol., Heidelberg), p. 280

Metformin and fibrinolysis. *Heikinheimo, R.* (Hamenpuisto 1, Tampere, Finland), p. 288

Permanent fixation of lymphoid cells exposed

to dye exclusion test. *Claesson, M. H., Hoyer, P. E., Ropke, C.* (Inst. of Med. Anat., Copenhagen), p. 291

Scandinavian Journal of Haematology (Copenhagen) **6** (1969) No. 5

The relationship between haem and globin synthesis by erythroid precursors in refractory normoblastic anaemia. *Kramer, S., Viljoen, E., Becker, D., Zail, S. S., Metz, J.* (S. Afr. Inst. Med. Res., Johannesburg, S. A.), p. 293

Ultrastructure of the specific granules of human neutrophil granulocytes. Studies in fatal granulomatous disease and toxic granulation. *Egeberg, J. C., Bro-Rasmussen, F., Andersen, V.* (Anat. Dept. B., Univ. of Copenhagen, DK—2200 Copenhagen N), p. 303

Cytoplasmic microtubules and radial-segmented nuclei (Rieder cells). Effects of vinblastine, sulfhydryl reagents, heparin and caffeine. *Norberg, B.* (Dept. of Intern. Med. A, S—220 05 Lund 5), p. 312

Mechanisms of leukocyte production and release. X. Eosinophil proliferation in rats of different ages. *Alexander, P., Jr., Monette, F. C., LoBue, J., Gordon, A. S., Chan, P.-C.* (Dept. of Biol., New York Univ., New York, N. Y.), p. 319

Haemolysis induced by subcellular fractions of rabbit spleen and liver. *Hurwic, M.* (Dept. of Radiobiol. and Hlth. Prot., Inst. of Nuclear Res., Warsaw), p. 327

Major surgery in a patient with thrombasthenia and rapid removal of normal platelets. *Barkve, H., Neset, G.* (Haemat. avdeling, Rikshosp., Oslo 1), p. 338

Variation in the individual subject of some parameters in blood coagulation and fibrinolysis. A longitudinal study in young, healthy women. *Ygge, J.* (Med. kliniken II, Sahlgrenska sjukhuset, S—413 45 Göteborg), p. 343

Impaired phagocytosis by peripheral blood granulocytes in systemic lupus erythematosus. *Brandt, L., Hedberg, H.* (Dept. of Intern. Med., Lasarettet, Lund), p. 348

Mathematical correlation between ADP-induced platelet aggregation and subsequent disaggregation. A parameter for the velocity of disaggregation. *Holdrinet, A., Ewals, M., Reichert, W. J., Haanen, C.* (Dept. of Intern. Med., Univ., Hosp., St. Radboud, Nijmegen, The Netherlands), p. 354

Scandinavian Journal of Haematology (Copenhagen) **Suppl. No. 7** (1969)

Subcellular particles of human platelets. *Day, H. J., Holmsen, H., Hovig, T.* (Temple Univ., School of Med., Dept. of Med., Philadelphia, Pa), p. 1—35

Scandinavian Journal of Haematology (Copenhagen) **Suppl. No. 8** (1969)

The blood platelet release reaction. *Holmsen, H., Day, H. J., Stormorken, H.* (Rikshosp., Univ. Inst. of Thrombosis Res., Oslo), p. 1—26

Scandinavian Journal of Haematology (Copenhagen) **Suppl. No. 9** (1969)

The release reaction of secretion. *Stormorken, H.* (Rikshosp., Univ. Inst. of Thrombosis Res., Oslo), p. 1—24

Scandinavian Journal of Haematology (Copenhagen) **Suppl. No. 10** (1969)

Serum protein binding of vitamin B_{12}. *Beal, R. W., Gizis, E., Meyer, L. M.* (Long Island Jewish Hosp., Div. of Hemat., Jamaica, N. Y.), p. 1—39

Transfusion (Philadelphia) **9** (1969) No. 4

Blood substitutes in the Soviet Union. A review. *Kiel, F. W.* (Office of the Asst. Chief of Staff for Intelligence, Dept. of the Army, Washington, D. C.), p. 169

Oxygen equilibria and biochemical changes of whole blood stored in different preservation media. *Huisman, T. H. J., Boyd, E. M., Kitchens, J., Mayson, S., Shepeard, W. L.* (Med. Coll. of Georgia, Augusta, Ga.), p. 180

Effects of adenine on clotting factors in fresh blood, stored blood, and stored fresh frozen plasma. *Mooreside, D. E., Graybeal, F. Q., Jr., Langdell, R. D.* (Dept. of Pathol., Univ. of North Carolina School of Med., Chapel Hill, N. C.), p. 191

Formation of inosine triphosphate and of ^{14}C-labeled 2,6-diaminopurine ribonucleoside di- and triphosphates in stored human erythrocytes. *Blair, D. G. R., Dommasch, M.* (Dept. of Cancer Res., Nat. Cancer Inst. of Canada, Univ. of Saskatchewan, Saskatoon, Saskatchewan, Canada), p. 198

Coexistence of MN erythrocytes and apparent anti-M antibody. *Schmidt, A. P., Taswell, H. F.* (Mayo Graduate School of Med., Univ. of Minnesota, Rochester, Minn.), p. 203

Generation and survival of angiotensin in nonfrigerated plasma. *Vandongen, R., Gordon, R. D.* (Dept. of Path., Univ. of Adelaide, Adelaide, S. Australia), p. 205

Effect of anticoagulants on calcium and magnesium content of human platelets. *Aledort, L. M., Hirsch, C., Udkow, M., Puszkin, E.* (Mount Sinai School of Med., New York, N. Y.), p. 212

Information needs for hospital blood bank inventory control. *Jennings, J. B.* (Operations Res. Center, Massachusetts Inst. of Technol., Cambridge, Mass.), p. 214

Hemolytic disease of the newborn due to anti-Js^{b}. *Wake, E. J., Issitt, P. D., Reihart, J. K., Feldman, R., Luhby, A. L.* (Blood Bank, Metropolitan Hosp., Dept. of Clin. Pathol., Pediat. and Pathol., New York Med. Coll., New York, N. Y.), p. 217

Vox Sanguinis (Basel) **17** (1969) No. 2

HL—A antigens and genes. I. A study of unrelated Norwegians. *Thorsby, E.* (Blood Bank and Dept. of Immunohaemat., Ullevål Hosp., Oslo), p. 81

HL—A antigens and genes. II. Studies of the antigens MH(= HL—A5) of the 4 sub-locus. *Thorsby, E.* (Blood Bank and Dept. of Immunohaemat., Ullevål Hosp., Oslo) p. 93

HL—A antigens and genes. III. Production of HL—A typing antisera of desired specificity. *Thorsby, E., Kissmeyer-Nielsen, F.* (Blood Bank and Dept. of Immunohaemat., Ullevål Hosp., Oslo), p. 102

Complement-fixing platelet iso-antibodies. III. Quantitation of the HL—A2 antigen on platelets. *Svejgaard, A.* (Blood Bank and Blood-Grouping Lab., Århus Kommunehosp., 8000 Århus), p. 112

$E^{ed_{8}hj}$(=E^{9}), a new allele in the E blood-group system of the pig. *Dinklage, H., Schahmirzadi, H., Hradeczký, J., Hojný, J.* (Inst. f. Tierzucht u. Haustiergenet., Univ. Göttingen, Göttingen), p. 129

The enhancement of *Ulex europaeus* anti-H activity by human serum. *Voak, D., Lodge, T. W., Reed, J. V.* (Reg. Blood Transf. Centre, Sheffield S10, England), p. 134

Occurrence and assay of alpha-N-acetylgalactosaminyl transferase in the gastric mucosa of humans belonging to blood-group A. *Tuppy, H., Schenkel-Brunner, H.* (Inst. f. Biochemie, Univ. Wien, A—1090 Wien), p. 139

Heterogeneity of the human haptoglobin 1—1 molecule by electrophoresis of serum in starch gels at pH 4.9. *Fagerhol, M. K., Jacobsen, J. H.* (Nat. Blood Group Ref. Lab., Nat. Inst. of Publ. Hlth., Oslo), p. 143

Antibody/latex erythrocyte label. A new method for the detection and counting of minor red cell populations. *Fimmel, P. J.* (Dept. of Haemat., Royal Perth Hosp., Perth, Australia), p. 146

The rapid papainisation of red cells for the detection of Rh antibodies. *Izatt, M. M., Blue, A. M., Muir, W., Sheridan, R.M., Morrison, C. J.* (Glasgow and West of Scotland Reg. Blood Transf. Service, Law Hosp., Carluke, Lanarkshire, Scotland), p. 157

Vox Sanguinis (Basel) **17** (1969) No. 3

Treatment of shock with dextrans and gelatins. Effects and side effects. *Lundsgaard-Hansen, P.* (Dept. of Exp. Surg., Inselhosp., 3008 Bern), p. 161

Automated detection of Gm factors of human γ G globulin. *Litwin, S. D., Camp, F. R., Jr.* (Dept. of Med., Cornell Univ. Medical School, New York, N. Y. 10021), p. 194

Separation and differential enzymic inactivation of the F and V isoantigens of the

bovine erythrocyte. *Hatheway, C. L., Weseli, D. F., Ludwick, T. M., Hines, H. C.* (Div. of Biol. Products, Michigan Dept. of Publ. Hlth., Lansing, Mich.), p. 204

The effect of bacterial T-activating enzymes on the red cell Sp_1 antigen. *Marsh, W. L., Nichols, M. E.* (Serol. and Genet. Lab., New York Blood Center, New York, N. Y. 10021), p. 217

The inheritance of the Kell blood groups in a Caucasian population sample. *Lewis, M., Kaita, H., Chown, B.* (Rh Lab., Winnipeg 3, Man., Canada), p. 221

Complement-fixing platelet iso-antibodies. IV. A comparison of human, guinea pig and rabbit complement. *Svejgaard, A* (Blood Bank and Blood-Grouping Lab., Århus Kommunehosp., 8000 Århus), p. 224

Further observations on human anti-LDL antibodies: serological behaviour, frequency and mode of formation. *Bütler, R., Brunner, E.* (Central Lab. of Blood Transf. Service, Swiss Red Cross, 3000 Bern), p. 230

Serum albumin variants in New Guinea indigenes. *Weitkamp, L. R., Shreffler, D. C., Saave, J. J.* (Dept. of Human Genet., Univ. of Michigan Med. School. Ann. Arbor, Mich.), p. 237

News Item

SYMPOSIUM ON VIRUS HEPATITIS ANTIGENS AND ANTIBODIES

organized by
the International Society of Blood Transfusion
(in conjunction with: the International Society of Haematology)
MUNICH, Thursday, August 6, 1970

Speakers (having accepted):

I — *Morning session* (9—11 a.m.)
10—12 minutes presentations

Chairmen: A. J. ZUCKERMAN (Great Britain)
R. W. McCOLLUM (USA)
B. S. Blumberg (USA) — T. J. Greenwalt (USA) — R. W. McCollum (USA) — K. Okachi (Japan) — A. M. Prince (USA) — J. P. Soulier (France) — F. Wewalka (Austria) — R. Wright (Great Britain) A. J. Zuckerman (Great Britain)

II — *Afternoon session* (3—6 p.m.)
6—8 minutes — short presentations

Chairmen: B. S. BLUMBERG (USA)
J. P. SOULIER (France)
L. F. Barker (USA) — H. Berthold (West Germany) — F. Deinhardt (USA) — D. J. Gocke (USA) — G. A. LoGrippo (USA) — T. London (USA) — F. O. MacCallum (Great Britain) — R. H. Purcell (USA) — C. Rittner (West Germany) — P. Taylor (Great Britain)

and *general discussion* between all the speakers.

All applications for this symposium should be sent to Professor Ag. J. P. SOULIER — Secretary General of the International Society of Blood Transfusion — 6, rue Alexandre-Cabanel, Paris 15^{e} (France) — and a copy should be forwarded to Professor Dr. Theodor M. FLIEDNER — XIII. International Congress of Haematology, 8 München 12, Postfach 200.

The participants may either pay the inscription fee of the Haematology Congress (which will give also the right to attend this symposium) or pay a one-day fee for this special day (Tageskarte).

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An International Symposium on Regulation of Erythropoiesis and Haemoglobin Synthesis will be held from August 10th to 13th, 1970, in Prague, Czechoslovakia. Those who require further details or who wish to present papers containing original contributions should write to: Dr. Jan Neuwirt, Institute of Pathological Physiology, U. Nemocnice 5, Prague 2, Czechoslovakia.

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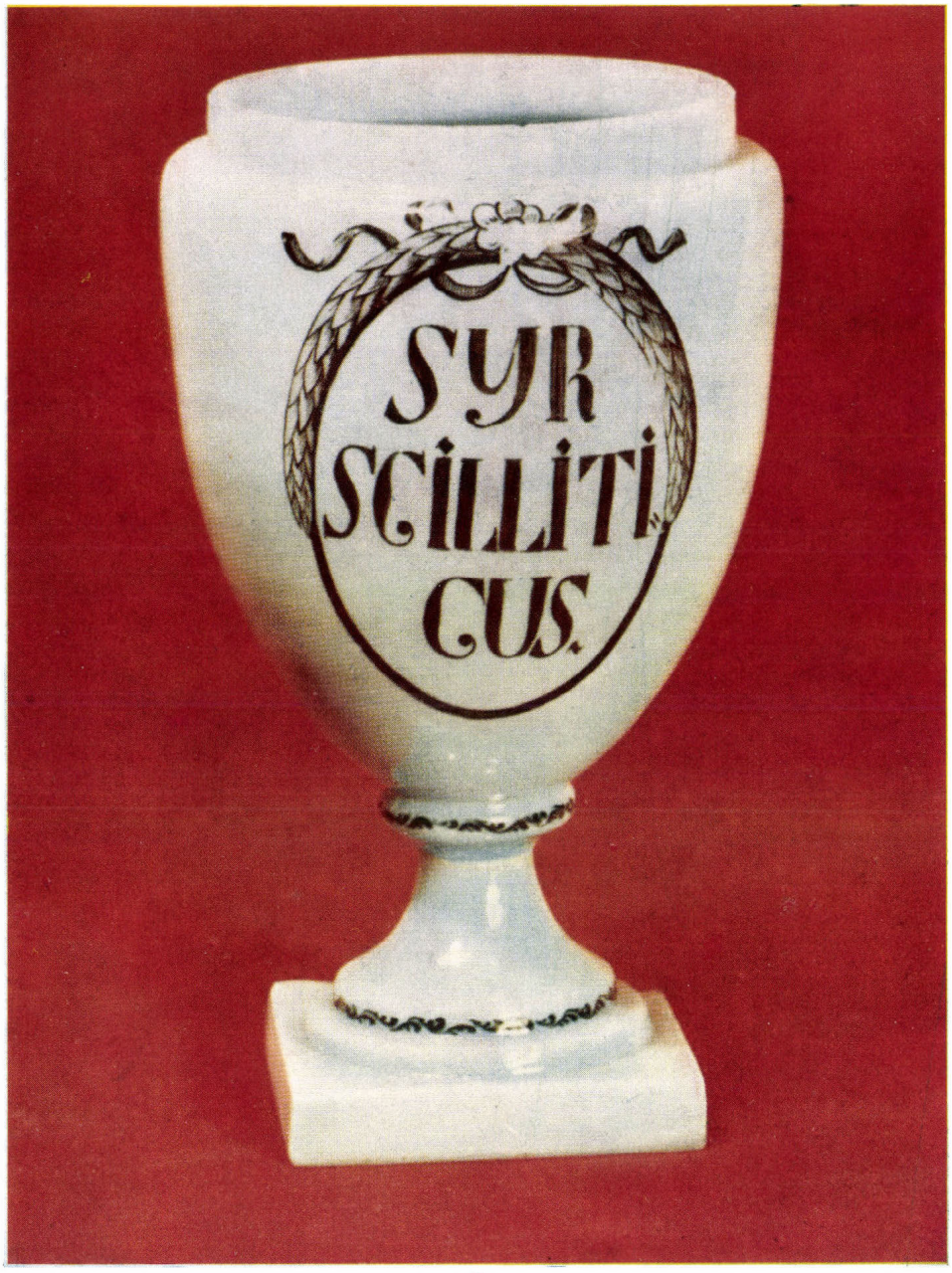

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Card Indexes

E. R. Jaffé, Gertrude Neumann

Effect of diazooxonorleucine and N-ethyl maleimide on the incorporation of nicotinic acid and nicotinamide into the pyridine nucleotides of human erythrocytes in vitro. Haematologia 4, 5 (1970).

Diazooxonorleucine inhibited the incorporation of radioactive nicotinic acid, but not of nicotinamide, into the pyridine nucleotides of human erythrocytes. Inhibition was reflected in an increased accumulation of nicotinic acid adenine dinucleotide, the immediate precursor of NAD. Inhibition of incorporation of nicotinic acid by N-ethyl maleimide and, to a lesser degree, incorporation of nicotinamide paralleled the inhibition of glycolysis produced by this compound. These observations were consistent with the known pathways for the biosynthesis of pyridine nucleotides in mammalian tissues. They also indicated that nicotinamide deamidase activity, if present at all, was extremely limited in human erythrocytes.

Y. Moriyama, H. Saito, Y. Kinoshita

Erythropoietin inhibitor in plasma from patients with chronic renal failure. Haematologia 4, 15 (1970).

In 20 of 23 patients with chronic renal failure no measurable plasma and urinary erythropoietin activity could be detected in spite of the presence of severe anaemia. In addition, plasma from 11 out of 13 anaemic uraemic subjects induced a significant inhibition of ESF, while normal human plasma was enhancing the erythropoietic effects of ESF. The inhibitor was still present after *in vitro* dialysis and was partially inactivated by heating. It was also shown that the inhibitor could be acting during the stage of stem-cell differentiation, but not during the later stages of erythroid cell development.

I. Cornides, Hedvig Medzihradzky, I. Bernát

A new method of preparing nitrogen samples from haem for mass spectrometric isotope analysis. Haematologia 4, 21 (1970).

The life span of a select population of the red cells formed in the bone marrow after thermal injury was studied by means of the ^{15}N stable isotope tracer technique. The adequacy of this method depends on the suitable preparation of nitrogen samples from haem for isotope analysis. A new procedure has been worked out for this purpose utilizing oxidation by cupric oxide in high vacuum at a temperature of about 600 °C. Being much less time consuming and at the same time reasonably accurate, the procedure is superior to the methods used hitherto.

G. G. Tedeschi, D. Amici, M. Paparelli

The uptake of radioactivity of thymidine, uridine, formate, glycine and lysine into cultures of blood of normal human subjects. Relationships with mycoplasma infection. Haematologia 4, 27 (1970).

Experiments have been undertaken to study whether the uptake of radioactive substances into haemocultures of normal human subjects could be related to latent and diffuse mycoplasma infection. Whole blood or washed erythrocytes were incubated in PPLO "Difco" broth, with or without antibiotics, in the presence of 6-^{3}H or 2-^{14}C thymidine, G-^{3}H or 2-^{14}C uridine, ^{14}C Na formate, 2-^{3}H glycine, or G-^{14}C l-lysine. At intervals of several hours or days the centrifuged sediments of the blood cultures were examined for radioactivity in the fractions extractable with warm $HClO_4$ before and after elimination of the acid-soluble fraction, the purines, the nucleic acids, or the protein fraction. In addition, chromatographic fractionation of extracts and autoradiographic examination of smears of incubated erythrocytes were carried out. The findings were qualitatively similar, but quantitatively considerably different. Uptake of thymidine and uridine into the acid-soluble fraction and into the nucleic acids, uptake from formate and glycine into the purine bases and into the nucleic acids, uptake of lysine into the protein fraction were observed. Radioactive or non-radioactive thymidine and uridine, within certain concentration limits, stimulated the uptake of the same nucleosides and of formate. The completed uptake of a certain amount of thymidine radioactivity appeared to exert an inhibitory action on the subsequent uptake. Further, uptake was stimulated under good conditions of oxygenation. In agreement with previous findings it has been concluded that the results may be attributed to a turnover of nucleic acids related to a diffuse and silent infection by mycoplasma or L-forms of schizomycetes and to the multiplication of these micro-organisms in the blood cultures.

J. Armata

Hyperleukocytosis before the beginning of treatment in acute leukaemia in children and its subsequent repetition at the onset of relapses. Haematologia 4, 49 (1970).

The degree of leukocytosis at the beginning of 144 relapses of acute leukaemia, determined by examination of the bone marrow, was studied. In 30 children with leukocytosis 10,000/mm^3 or less before beginning of treatment, 65 relapses were observed, including 4 with leukocytosis above 10,000/mm^3. In the second group of 28 children (leukocytosis over 10,000/mm^3 before treatment), 79 relapses were observed, including 22 with leukocytosis over 10,000/mm^3. In the second group, compared with the first, blastic hyperleukocytosis at the beginning of relapses was significantly more frequent.

K. Krug, L. Stenzel

Contribution to the skeletal manifestations of leukoses, lymphogranulomatoses and retothelsarcomas. Haematologia 4, 55 (1970).

Skeletal manifestations in clinical patients suffering from chronic myeloid leukaemias, leukoses of immature cells, chronic lymphadenoses, lymphogranulomatoses and lymphoreticular sarcomas are described. The characteristics and frequency of osteal islands in the postmortem findings of a pathological institute on patients who died of malignant haematological diseases are compared with the clinical results. Osteolytic islands, circumscript or diffuse osteoscleroses, osteoporoses and quite casually periosteal reactions occurred. Osteal manifestations dependent on the process could be detected more frequently in the autopsy material than in the clinical and roentgenological examinations. In certain cases of the examined disease groups special prognostic and therapeutic conclusions can be drawn from the recognized skeletal manifestations.

S. Tura, M. Baccarani

The management of polycythaemia vera with Dibromomannitol. Haematologia 4, 67 (1970).

Sixteen patients with polycythaemia vera have been treated with Dibromomannitol (DBM) over a 30 month period. Treatment has been cyclic. Dosage regimen included daily doses from 200 mg to 300 mg for 8 to 15 days. Fifty-one cycles are adequate for evaluation: 27 with a total dose of less than 3000 mg and 24 with a total dose of more than 3000 mg. The control of the disease has been achieved in all the sixteen patients, a single cycle every 4 to 6 months being necessary for such a purpose. Leukopenia and thrombocytopenia occurred in 16%, and 18%, respectively of overall treatments. A clear dose-response and dose-toxicity rate are shown. The use of DBM in chemotherapeutic control of polycythaemia vera, both for induction and maintenance therapy, is finally discussed.

R. Farbiszewski, K. Worowski

Inhibition of blood platelet aggregation by serum IgM macroglobulin. Haematologia 4, 75 (1970).

The time of platelet aggregation induced by ADP in platelet rich plasma of a patient with IgM macroglobulinaemia was found to be longer and the aggregates smaller than normal. The patient's platelet poor plasma caused a similar change in the reaction of the platelet rich plasma of a healthy subject. Aggregation time was prolonged by isolated IgM macroglobulin as well.

N. Lahmann

A further case of anti-N in the serum of a person of MN blood group. Haematologia 4, 79 (1970).

A further (fourth) case of anti-N is reported in a person of MN blood group. The MN antigens and anti-N antibody of the 82-year-old German woman behaved regularly in all tests. The antibody obviously belongs to the IgM-class and is to be regarded as a so-called natural antibody.

J. V. Chuba, W. J. Kuhns, R. F. Nigrelli, R. A. Morris, U. E. Friese

B-like blood factor of fur seals and sea lions. Comparative study with D-galactose inhibition, human group B erythrocytes and B-active human, baboon and rhesus monkey saliva. Haematologia 4, 85 (1970).

Fur-seal and sea-lion, but not harbour-seal erythrocytes were shown to possess B-like blood factors. Human anti-**B** serum was shown to possess: 1. a D-galactose-*inhibitable* fraction which cross reacted with both human group B and B-like seal red cells, and 2. a D-galactose-*non*inhibitable fraction, sensitive to 2-mercaptoethanol treatment, which reacted only with human group B red cells. The heteroagglutination of both human group B and B-like seal red cells by catfish anti-**B** heteroagglutinins was specifically inhibited with D-galactose, thus suggesting that immunodeterminant D-galactosyl residues of otherwise dissimilar surface membrane receptor structures provided the basis for the B-like cross reactivity. Variability in the comparative reactivity of human group B and B-like seal red cells with immune plasmas produced by heteroimmunizing catfish with B-active human, baboon and rhesus monkey saliva further illustrated the heterogeneity of: 1. immune responsiveness in the lower vertebrates, and 2. the "blood-group-active" saliva substances of different species of primates.

J. Szücs

Multiple myeloma without paraprotein. Haematologia 4, 97 (1970).

The case history of a patient with multiple myeloma is presented. The diagnosis was based on the typical bone marrow picture and bone deformities. However, paraprotein could not be detected in the blood or urine by either paper electrophoresis or immunoelectrophoresis.

Susan Faragó, I. Oláh

Electron microscopic investigation of the developing forms of megakaryocytes. Haematologia 4, 103 (1970).

The submicroscopic properties of the developing forms of megakaryocytes in mouse spleen have been investigated. The criteria of the classification of the individual developing forms, the formation of the DMS, the supposed double role of the Golgi apparatus, the changes in the chromatin substance of the cell nucleus and its possible functional background are discussed.

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Contents

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Diagnostic Difficulties in a Patient with Jaundice and Anaemia

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(Received December 30, 1969)

In a 40-year-old jaundiced anaemic woman, ill with fever, polyneuropathy and loss of hair, fulminating haemolysis was identified as the cause of the anaemia. The possible causes of this haemolysis have been discussed. The jaundice could not be explained solely on the basis of the haemolysis. Hepatitis was possible; later E. coli sepsis was found, caused probably by cholangitis. Adequate control of the sepsis with antibiotics was followed by improvement of liver function and of the haemolysis. At a subsequent operation, the bile ducts were drained and a liver biopsy was performed. The biopsy specimen showed signs of cholangitis and extrahepatic cholestasis. The patient made a complete recovery after the operation.

A woman aged 40 was admitted to the Utrecht University Clinic for Internal Diseases late in November, 1968. She had previously been hospitalized for ten days in the St. Joseph Hospital, Eindhoven (internist Dr. G. H. W. Jordans).

Since the middle of October, the patient had been tired and nauseated; she vomited nearly every day and became jaundiced after a week. At that time the stools had had a light colour for a few days, while the urine was dark coloured. The patient had been taking Lyndiol tablets for 18 months, and in the course of September she had taken 20 tablets of Irgapyrine (125 mg phenylbutazone + 125 mg aminophenazone) for low back pain. When hospitalized in Eindhoven the patient was jaundiced and severely ill. The skin showed no spider naevi; the mucosa was pale. The temperature varied between 38 and 40°C. Liver and spleen were not palpable and no abnormalities were found. At digital examination of the rectum the faeces on the glove had a brown colour.

Blood picture: ESR 100 mm after 1 hour; Hb 6.7 g/100 ml, later diminishing to 5.2 g/100 ml; haematocrit 18%; red cell count 2.3 million, MCV 79 fl, MCH 29 $\mu\mu$g, MCHC 37%; reticulocytes 2‰; white cell count 11,500; differential count normal; platelet count 227,000.

Blood chemistry: bilirubin 197 mg/100 ml with a 10-minute value of 63%; alkaline phosphatase 10 μmol/min/100 ml; SGOT 123 μmol/min/100 ml; SGPT 210 μmol/min/100 ml. Thymol turbidity test 3.3 U. Serum protein: total protein 4.6 g/100 ml; albumin 46%, α_1-globulin 8%, α_2-globulin 7%, β-globulin 11%, γ-globulin 30%.

Urine: traces of protein; urobilin + + + and bilirubin + + + ; the sediment contained a number of leucocytes. A blood culture was negative.

Clinical course in Eindhoven: to combat the anaemia the patient received packed cells from 11 donors and whole blood from one donor, within a week. The Hb thereupon rose from 5.2 to 7 g/100 ml. The jaundice increased and the bilirubin level rose to 41.6 mg/100 ml. The general condition deteriorated and the patient was transferred to our Department.

After admission, the patient was no longer vomiting but was still nauseated and very tired. She complained of muscular weakness in the arms and legs. Consciousness was unclouded but she gave an impression of being very ill. The temperature was up to 39.4°C. Her hair was conspicuously scanty. Her arms and legs were weak, she was unable to stand. Biceps, patellar and Achilles and plantar reflexes were negative on both sides. The ocular fundi were normal.

Blood picture: ESR 73 mm after 1 hour; Hb 7.0 g/100 ml; haematocrit 20%; reticulocytes initially 1—2‰, later increased to 50—100‰. White cell count 3,200, with normal differential count; platelet count 120,000.

Blood chemistry: creatinine 2.2 mg/100 ml, urea 1.01 mg/100 ml, uric acid 3.7 mg/100 ml. Serum iron 0.16 mg/100 ml, latent iron-binding capacity 0.01 mg/100 ml, saturation 94%. Bilirubin 30 mg/100 ml, 10-minute value 97%. Thymol turbidity test 3.5 U MacLagan. Alkaline phosphatase 7.1 μmol/min/100 ml, amylase 37.6 μmol/min/100 ml; SGOT 1.9 μmol/min/100 ml; SGPT 1.1 μmol/min/100 ml; LDH 145 μmol/min/100 ml.

Other laboratory findings: the bone marrow was poor in cells with occasional megakaryocytes. Erythropoiesis was relatively better represented than granulopoiesis; ratio erythropoiesis : granulopoiesis 1 : 1. The granulopoietic system was shifted to the left and displayed toxic granulation. On iron staining haemosiderin in the RES was slightly more than normal. Later bone marrow punctures revealed many cells, with hyperplastic normoblastic erythropoiesis. The cellular poverty of the bone marrow punctates and the reticulocytopenia in the early phase were probably due to the high fever. After the temperature had returned to normal, bone marrow punctates showed a high cellularity and there was reticulocytosis.

Urine: 4 g protein per 24 hour; urobilin + +, bilirubin +; the sediment still contained a fair number of leucocytes. The urinary culture was negative on one occasion, and disclosed Klebsiella on another.

Since the Hb concentration had risen from 5.2 only to 7 g/100 ml on transfusion of blood from 12 donors, it seemed probable that the patient was suffering from chronic haemolysis because there were no signs of blood loss, nor did the faeces contain blood.

Further laboratory studies showed that syphilis reactions were negative; the serum contained no antinuclear factor; there was no free haptoglobin (the serum protein that selectively binds haemoglobin); there was haemoglobin-haptoglobin complex as well as free haemoglobin: 8 mg/100 ml (normal value up to 5 mg/100 ml). These findings, too were consistent with a severe haemolytic anaemia of not immediately identifiable cause. To identify the causative factor, we reviewed the various possibilities.

The principal causes of haemolytic anaemia are listed in Table 1.

Table 1

A. *Hereditary haemolytic anaemia*

1. hereditary spherocytosis
2. haemoglobinopathies and α- or β-thalassaemia
3. hereditary elliptocytosis
4. non-spherocytic haemolytic anaemias
5. haemolytic anaemia with pigmenturia
6. dyserythropoietic haemolytic anaemias
7. erythropoietic porphyria

B. *Acquired haemolytic anaemia*

1. isoimmune haemolytic anaemia
2. autoimmune haemolytic anaemia
3. haemolytic anaemia from chemical or physical causes
4. haemolytic anaemia associated with infections
5. non-autoimmune haemolytic anaemia associated with systemic diseases, neoplasms, etc.
6. haemolytic anaemia in splenomegaly (hypersplenism)
7. vitamin B_{12} or folic acid deficiency
8. paroxysmal nocturnal haemoglobinuria
9. microangiopathic haemolytic anaemia

re A.1: No spherocytes were found in the blood smear; the osmotic resistance of fresh erythrocytes and erythrocytes incubated for 24 hours was normal. There was no family history of jaundice and/or anaemia. Therefore there were no direct clues to hereditary spherocytosis.

re A.2: Hb electrophoresis yielded no abnormal haemoglobin. The patient had a normal concentration of HbA_2 (3.1%) and a normal HbF concentration of 0.4%. The smear showed no sickle cells or characteristics of thalassaemia with target cells, hypochromasia, etc. Nor did the smear, stained with brilliant cresyl blue, contain inclusion bodies of the type found in α-thalassaemia (haemoglobin H disease). Therefore there were no signs of haemoglobinopathy or thalassaemia.

re A.3: The blood smear showed no elliptocytes; hereditary elliptocytosis was therefore ruled out.

re A.4: Hereditary non-spherocytic haemolytic anaemia usually entails an enzyme deficiency. The enzymes involved can be divided in accordance with the following simplified classification (Fig. 1).

1. *Glycolytic enzymes which occur in the pentose-phosphate pathway:* a) glucose-phosphate dehydrogenase (G-6-PD); b) other enzymes.

(Deficiency of one of the enzymes of this group is often associated with increased autohaemolysis (> 3% haemolysis after 48 hours incubation at 37°C), with diminished autohaemolysis when glucose is added to the blood in advance (autohaemolysis type I of Selwyn and Dacie [4]).

In our patient haemolysis was normal: 1.6%; when glucose was added in advance it was 1.1%. Associated biochemical manifestations of G-6-PD deficiency are GSH instability and the presence of Heinz inclusions (denatured haemoglobin corpuscles) in the erythrocytes. Relevant tests were negative in our patient. The G-6-PD concentration of the erythrocytes was normal: 6.3 μmol/min/g Hb, as was the 6PGD concentration: 3.5 μmol/min/g Hb.

2. *Glycolytic enzymes which occur in the Embden-Meyerhof pathway:* a) pyruvate-kinase (PK); b) other enzymes.

An enzyme deficiency in this anaerobic metabolism is often associated with increased autohaemolysis, which does not diminish when glucose is added to the blood in advance (autohaemolysis type II). The autohaemolysis test was normal in our patient.

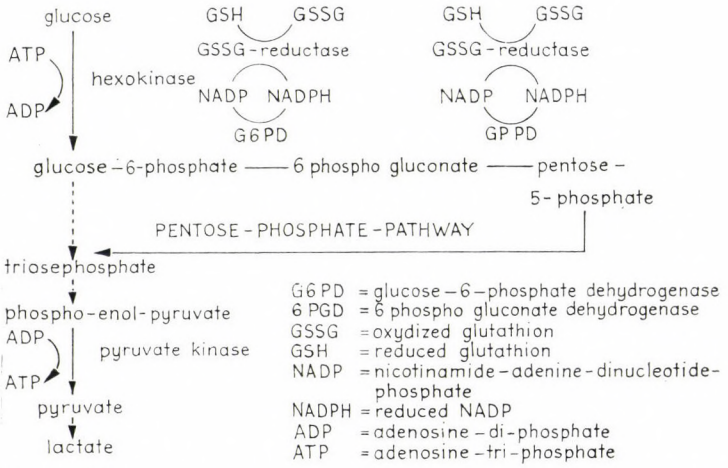

Fig. 1. Schematic survey of glucose metabolism in human erythrocytes

The PK concentration of the erythrocytes was normal: 2.5 μmol/min/g Hb. ATP + 2,3-diphosphoglycerate values were likewise normal, so that the presence of enzyme deficiencies in the Embden-Meyerhof pathway was very unlikely.

3. *Non-glycolytic enzyme deficiencies:* a) glutathione (GSSG) reductase; b) other enzymes.

The study of GSSG reductase in the erythrocytes showed normal values. Nor were there any signs of other non-glycolytic enzyme deficiencies.

Thus, the erythrocyte studies revealed no enzyme deficiency. However, during a haemolytic crisis an enzyme deficiency may be masked by the predominative number of young erythrocytes and most enzymes show a higher concentration in young than in old erythrocytes. At the time of the first investigation, moreover, the blood consisted for a large part of normal donor erythrocytes. A few months later, when the haemolysis had disappeared, enzyme studies of the erythrocytes were therefore repeated; the results obtained were normal.

re A.5: The history yielded no clue to a familial haemolytic anaemia with urinary excretion of abnormal bilirubin metabolites. The urine had never been brown or black. The erythrocytes contained no abnormal inclusion bodies such as those found in particular after splenectomy, which suggested for the condition named hereditary anaemia with Heinz bodies [2]. Thermolabile haemoglobin — another abnormality characteristic of this disease — was likewise absent.

re A.6: There was no sign of any of the rare familial dyserythropoietic haemolytic anaemias [1]. The erythroblasts in the bone marrow were neither binuclear nor abnormally large. The amount of urobilinogen in the faeces (usually much increased in this syndrome) was only slightly increased at a single determination.

re A.7: Patients suffering from erythropoietic porphyria usually show skin changes, particularly after exposure to sunlight. The urine often has a red colour caused by the large amounts of uroporphyrin and coproporphyrin. Our patient never had skin changes and showed good tolerance to sunlight. The colour of the urine was reported never to have been abnormal.

Urine analysis disclosed no porphobilinogen or uroporphyrin. Only a trace of coproporphyrin was found. There were therefore no signs indicating erythropoietic porphyria.

re B.1: Isoimmune haemolytic anaemia is known to occur in erythroblastosis foetalis and in association with transfusion reactions.

Erythroblastosis foetalis was ruled out in this case. Anaemia and jaundice had been present before blood transfusions were given. After the blood transfusions there were no abnormal reactions and no abnormal blood-group antigens were demonstrable.

re B.2: Autoimmune haemolytic anaemia caused by immune antigens against erythrocytes may occur secondarily in systemic diseases such as chronic lymphatic leukaemia, malignant reticulosis and Hodgkin's disease, systemic lupus erythematosus, virus pneumonia, infectious mononucleosis, and syphilis. Whenever no such primary disease is found, the anaemia is called idiopathic. — Our patient showed a negative direct Coombs test with anti-γ-globulin and anti-complement serum. Nor were incomplete warm antibodies found in the serum with the salt technique, the bromelin technique, or the indirect Coombs test. At 16°C no cold agglutinins were present in the serum. Nor were warm or cold haemolysins found in the serum at pH 6.5 and pH 7.5, respectively. The Donath–Landsteiner reaction was negative. There was therefore no autoimmune haemolytic anaemia in this case.

re B.3: Physical or chemical causes of haemolytic anaemia are assumed to play a role in march haemoglobinuria, in haemolysis following heart surgery with insertion of artificial valves, in haemolysis following burns or drowning and in haemolysis following haemodialysis with a dialysing fluid of incorrect composition. None of these causes were present in our case.

Haemolytic anaemia may occur also following ingestion of certain chemicals, toxic substances or drugs. The patient had taken Irgapyrine and Lyndiol tablets; so far, no fulminating haemolysis has been observed in association with these

drugs. There are reports, however, on jaundice due to intrahepatic cholestasis in association with the use of Lyndiol and other contraceptives [5]. No demonstrable amounts of thallium were found in the patient's urine and hairs.

re B.4: Infections which may cause serious haemolysis are as follows:

a) Clostridium welchii infection. Examination of the patient's serum for the relevant lysins and agglutinins had a negative result.

b) Malaria. No malarial parasites were demonstrated.

c) Bartonella bacilliformis infection. This produces the so-called Oraya fever — a disease which occurs in Latin American countries but is unknown in The Netherlands.

Haemolytic anaemia has been described also in association with atypical pneumonia, in infectious mononucleosis and in hepatitis infectiosa. There were no signs of an atypical virus pneumonia. The blood picture showed no features of infectious mononucleosis, and the Paul–Bunnell reaction was negative. Hepatitis, however, was possible.

In view of the persistent fever, blood cultures were made which repeatedly disclosed E. coli. E. coli sepsis was certainly to be considered a factor in the aetiology of the haemolytic anaemia; for example, through a direct effect on the erythrocytes or through indirect agglutination following adsorption of polysaccharides produced by the microorganisms.

re B.5: The bone marrow punctates were consistent with a toxic process and supplied no clue to the presence of a malignant systemic disease. No pathological cells were found in the leucocyte concentrate of the blood either.

— To summarize: there were no signs suggestive of leukaemia, reticulosis or Hodgkin's disease, myelomatosis or any other neoplastic disease.

re B.6: The spleen was not palpable and radiologically not enlarged. Except in the initial phase of the disease, leucocytopenia or thrombocytopenia was not found. Later (in January 1969), the possible degradation of erythrocytes labelled with radioactive chromium was studied. No abnormal sequestration in the spleen was demonstrable. There were therefore no indications of hypersplenism.

re B.7: The bone marrow showed no signs of vitamin B_{12} or folic acid deficiency. The serum vitamin B_{12} and folic acid concentrations were normal, so that haemolysis due to vitamin B_{12} or folic acid deficiency was ruled out.

re B.8: Paroxysmal nocturnal haemoglobinuria (PNH) was unlikely in view of the history. The patient had never voided a dark urine. The acid-haem test was negative. The alkaline phosphatase level of the granulocytes in the smear (usually diminished in PNH) was increased. There were therefore no signs indicative of PNH.

re B.9: No fragmentocytes or acanthocytes were demonstrable in the blood smear. There was no thrombocytopenia nor a disorder of blood coagulation; for example, the serum fibrinogen level, prothrombin time and thrombin time were normal. Microangiopathic haemolytic anaemia was therefore ruled out.

In summary, the most plausible cause of the patient's severe haemolysis was an infectious process, probably hepatitis later followed by E. coli sepsis.

The jaundice, combined with the putty stools and dark urine, the fever and the high transaminase activity in the serum during the initial phase of the disease seemed to suggest an affection of the liver, possibly combined with, or a consequence of, cholestasis. Had the jaundice been due exclusively to the haemolysis, then the serum bilirubin level would not have risen to 41.6 mg/100 ml. A 10-minute value of 97% was likewise unlikely to be caused solely by haemolysis, but was plausible in the case of obstructive jaundice combined with haemolysis. It seemed possible that the patient besides having had hepatitis was suffering from cholangitis; in favour of this suggestion were the fever and the jaundice of the obstructive type. The high transaminase activities might have indicated a damage to the liver parenchyma. A culture was therefore made from the patient's bile, which proved to contain the same organism as the blood, E. coli serotype O_4K_2. Many bacterial colonies were cultured especially from the B and C-bile (serological typing was done by Dr. Heyl of the National Institute of Public Health, Bilthoven).

Meanwhile, the neurologist had established by electromyography a symmetrical polyneuropathy of the arms and legs; this, however, could be consistent with the sepsis.

In view of the serious condition the patient had initially received 60 mg prednisone per 24 hours. During this medication high fever persisted and the jaundice increased further. After nine days, sepsis caused by a Gram-negative rod-shaped microorganism was established, and the patient was treated with kanamycin/cefalotin. Within 24 hours the temperature fell from 40 to 37°C. When the patient was found to suffer from E. coli sepsis, the strain cultured being insensitive to cefalotin and sensitive to tetracycline, the medication was discontinued after two days and tetracycline was prescribed also because cholangitis or cholecystitis was considered possible and tetracycline is usually secreted at high concentrations in the bile. During and after the tetracycline medication the patient remained afebrile and showed distinct clinical improvement. The bilirubin concentration diminished in six weeks from 30 mg/100 ml to 0.68 mg/100 ml (normal value up to 0.8 mg/100 ml). The Hb rose only slightly, from 7.7 to 8.9 g/100 ml, but the patient required no further blood transfusions to maintain the Hb at this level, after having had initially packed cells of 800 ml blood.

After tetracycline medication for a week, a normal amount of free haptoglobin was found in the serum, and the plasma-free haemoglobin level fell to normal values. The fulminating haemolysis seemed to have been arrested. Nevertheless, the survival of the patient's own erythrocytes labelled with ^{51}Cr was still somewhat reduced: the half-life proved to be 20 days (normal half-life 24—36 days). The sequestration of ^{51}Cr-labelled erythrocytes, measured over spleen and liver, was normal. Cholecystography with an oral contrast medium failed to visualize the gall-bladder. After administration of an intravenous contrast medium, a low-contrast gall-bladder of normal shape without concretions was visualized; the contrast medium proved to fill the common duct to a level well into the intestine.

Another bile culture was made after tetracycline treatment. The bile still contained E. coli of the same type as before. After consultation with the surgeon it was decided for a laparotomy to examine and if necessary to drain the gallbladder and bile ducts, and to obtain a liver biopsy specimen.

At operation (University Surgical Clinic, Head: Prof. Dr. J. F. Nuboer), the gallbladder and hepatocystic duct proved to contain no stones; the bile ducts were drained, and a liver biopsy was performed. The patient received 800 ml blood in the course of the operation.

Microscopic examination of the liver specimen (by W. Heyenbrok, Pathological Institute, Head: Prof. Dr. A. de Minjer) showed broadened periportal zones extending far into the surrounding liver tissue. The broadening was caused by an increase in connective tissue, and by an increase in cells. These cells were polynuclear leucocytes and mononuclear cells. The bile passages in the periportal zones contained bile pigment. The entire picture was consistent with extrahepatic cholestasis; there were also signs of cholangitis.

The patient made a slow postoperative recovery. Her arms and legs increased only gradually in strength. The hair growth was restored earlier.

The Hb rose to 12.6 g/100 ml, and the ESR diminished to 28 mm/l hour. Liver function improved, and renal function returned to normal. The patient was discharged on 21st March, 1969.

At follow-up in the out-patient clinic on 24th July, 1969, she was found to have made excellent progress. She was going for long walks, aided by a cane; she looked healthy and had an abundant hairdo.

The ESR was 10 mm after 1 hour; Hb 14.7 g/100 ml, haematocrit 41 %. Red cell count 4.6 million with normal MCV, MCH and MCHC values; reticulocytes 7 ‰; white cell count 7,200, with a normal differential count; platelet count 274,000. The serum free haptoglobin concentration was normal: 60—120 mg/100 ml. Liver function tests were entirely normal (normal protein pattern and thymol turbidity test). E. coli could no longer be cultured from the bile.

In summary, a 40-year-old jaundiced and very ill woman was found to suffer from a severe haemolysis, probably after having had a hepatitis. Later there was found an E. coli sepsis, as a result of cholangitis, perhaps promoted by the treatment with corticosteroids. Besides that the patient suffered from a polyneuropathy and loss of hair. After the condition had been brought under control, the disturbed liver function was restored to normal, haemolysis ceased and the polyneuropathy disappeared gradually.

*

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Unstable Haemoglobin Disease Caused by Hb Santa Ana-β88 (F4) Leu → Pro

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Clinical and haematological data, special red cell characteristics and studies of the haemoglobin structure of an unstable haemoglobin haemolytic anaemia (UHHA) are reported. The instability of the patient's haemoglobin originates from a β88 (F4) Leu → Pro mutation, and thus is together with one observed in France [1] a further example of the unstable Hb Santa Ana. Special red cell studies revealed deranged non-electrolyte permeability, slightly lower GSH, and relatively low ATP content of the patient's red cells. Characteristics of foetal-type erythropoiesis: increased red cell electrophoretic mobility, increased G6PD activity and changes in the lipid composition of the red cell membrane resembling those found in cord blood cell could be detected.

A well-defined group of hereditary non-spherocytic haemolytic anaemias is caused by abnormal haemoglobins (Hb) with reduced molecular stability. The unstable haemoglobin haemolytic anaemias (UHHA) are characterized by a very variable degree of haemolysis and splenomegaly, the presence of red cell inclusion bodies and often a dipyrroluria [1]. Up till now 20 various amino acid substitutions and 3 different amino acid deletions are known to cause overt instability of the Hb molecule.*

Based on the atomic model of Hb it has been possible for Perutz, Kendrew and Watson [2] to predict what kind of substitutions might give rise to pathological effects. These predictions have been confirmed by the structural studies of the unstable haemoglobins and by the evaluation of the detailed atomic interactions of these abnormal Hb-s [3].

Knowledge concerning the UHHA continues to advance. But in spite of the considerable progress in the understanding as to how the Hb instability leads to its intracellular precipitation much less is known in strict biochemical terms about the effect of this intracellular precipitation on the function and viability of the red cell. Notwithstanding the considerable advances in knowledge [7—13] of the pathomechanism of membrane damage, haemolysis and cellular breakdown in UHHA, more clinical, haematological and biochemical data are needed to

* 21 of the 23 unstable Hb-s have been reviewed in reference [4], the structure of the recently described Hb Borås was reported in reference [5] and that of Hb Dakar in reference [6].

elucidate the relationship between the actual structural instability of the haemoglobin molecule and the varying patterns of red cell abnormalities caused by it.

This paper reports clinical and haematological data, results of investigations of haemoglobin, and some data of red cell structure, metabolism and permeability in an UHHA in Hungary.

Methods

Standard methods were used for haematological investigations [14].

Red cell lipids, non-electrolyte permeability, acetylcholinesterase (AchE) activity, glucose-6-phosphate dehydrogenase (G6PD) activity, reduced glutathion (GSH) level, electrophoretic mobility of the patient's red cells were determined according to the methods described in an earlier paper [15].

Investigation of haemoglobin

Hb electrophoresis was carried out in starch gel [16] and on paper [17].

Methaemoglobin was determined immediately after venipuncture by the method of Evelyn and Malloy [18]. Methaemoglobin formation was studied on fresh anticoagulated blood samples, which were incubated at 37°C. Aliquot samples for methaemoglobin determination were taken after 2, 4, 20 and 24 hours of incubation.

Heat denaturation was carried out at pH 6.8 in 0.1 M phosphate buffer at 50°C, and at 65°C respectively, according to the methods of Dacie [19] and Betke [20].

Foetal haemoglobin content was determined by Singer's method modified by Betke [21]. The Hb variant was isolated by paper electrophoresis at pH 8.9 using Tris buffer [17]. After paper electrophoresis the abnormal band was eluted, and the eluate was concentrated by dialysis against vacuum. The haemoglobin solution was then heated for one hour to 50°C at pH 7.4 [21]. A precipitate was formed which was washed repeatedly with buffer at pH 7.4 at 50°C, and then submitted to chemical analysis.

As a first step the remaining haem was removed, and the globin was separated into its α- and β-chains [22]. The β-chains were aminoethylated [23], digested with trypsin and "fingerprinted" by using the methods summarized previously [24].

Potassium and sodium determinations were carried out in a Beckmann flame photometer. The K-content of the erythrocytes was calculated from the K-content of whole blood and plasma, and from the actual haematocrit values. ATP content of the red cells was measured by the Boehringer test.

In vitro iron incorporation was estimated by the method of Belcher and Courtenay [25] using $^{59}Fe^{3+}$ ($FeCl_3$) (Isotope Institute of the Hungarian Academy of Sciences) having a specific activity of 56.2 Ci(g).

The urinary output of dipyrrols was measured according to the method of Siedel and Möller [26] modified by Benedek and Soós [27] in the Department of Clinical Chemistry of the National Institute of Haematology and Blood Transfusion. The absorption spectra were taken by a Perkin Elmer UV 137 spectrophotometer. The reference standard mesobilifuscin was prepared from bilirubin by the method of Siedel [28] and purified according to Benedek and Soós [27].

Case report

J. Z., a 7-year-old Hungarian boy was said to be anaemic since birth, often unduly tired. The mother was 40 years old when the propositus was born. The father is a heavy drinker. Recurrent episodes of disabling fever, jaundice and passage of dark brown urine were observed. He was treated since 1964 at the Second Children's Hospital, Semmelweis University School of Medicine, Budapest.

First admission to the Department of Haematology of the National Institute of Haematology and Blood Transfusion, Budapest, was on May 2, 1968. At that time the normally developed child was only slightly anaemic and had no complaints. The spleen could be palpated 4 cm below the costal margin. Otherwise no abnormality was noted on clinical examination. Haemoglobin 8.9 per cent, PCV 42 per cent, red cell count 3.2 mill/μl, MCHC 27.5 per cent, white cell count 4800/μl, normal distribution and morphology. Platelets 160,000/μl, reticulocytes 16 per cent; no normoblasts; osmotic fragility of the red cells. 0.60—0.20 per cent NaCl. Bone marrow was highly cellular with erythroid hyperplasia (M : E = 1 : 2); serum iron 95 μg per 100 ml; total iron binding capacity 230 μg per 100 ml; 41.3 per cent saturation. Direct and indirect Coombs test and Ham acid test negative; serum bilirubin 1.8 mg per 100 ml (unconjugated 1.3 mg%, direct reacting fraction 0.5 mg%). Red cell sedimentation rate: 4 mm/h; serum haptoglobin: 20 mg%.

Investigation of the red cells

The red cells in the Romanovsky stained blood film were hypochromic, with marked aniso- and moderate poikilocytosis. No inclusion bodies were detectable. In the Nile-blue stained film Heinz-bodies were observed in 3.7 per cent of the red cells. The number of the inclusion body containing erythrocytes increased to 80 per cent after 24 hours of incubation at 37°C.

Non-electrolyte permeability of the erythrocytes was decreased. This is well reflected in the increase of the half-time of haemolysis in isotonic glycerol and thiourea. In addition to the changes in the rate of haemolysis, the slope of the curves differs significantly from the normal (Fig. 1).

Ion permeability: Plasma-Na: 136.0 mEq/l, RBC K : 133.0 mEq/l (normal value).

ATP content = 0.70 μmol/ml erythrocyte (decreased when taking into account the young mean red cell age).

Red cell electrophoretic mobility = $1.62 \cdot 10^{-4}$ cm²/sec/volt (normal range = = $1.20—1.38 \cdot 10^{-4}$ cm²/sec/volt).

G6PD activity = 14.7 Δ OD/min/g Hb (normal range 8.0—12.0 Δ OD/min/g Hb).

GSH = 51 mg/100 ml RBC (normal range = 60—100 mg/100ml RBC).

AchE activity 0.70 Δ pH/h (normal range = 0.60—0.90 Δ pH/h).

Red cell survival = the half-time of disappearance of the propositus' own ^{51}Cr labelled erythrocyte was reduced to 5 days (normal 25 to 30 days).

In vitro ^{59}Fe incorporation considerably increased as compared to the normal control (Fig. 2). In addition to this, an increase was also observed in the non-haem iron binding protein fraction (Fig. 3).

Red cell lipids

Cholesterol	$127 \cdot 10^{-12}$ mg/cell	(normal $\bar{x} = 125.4 \pm 12.62$)
TEFA	$63 \cdot 10^{-12}$ mg/cell	(normal $\bar{x} = 199.8 \pm 11.81$
Lipid-P	$10.8 \cdot 10^{-12}$ mg/cell	(normal $\bar{x} = 11.8 \pm 1.03$)

Fatty acid distribution of the total phospholipids (major components in relative percentage):

$C_{16:0} = 29.1$	$C_{18:2} = 6.9$
$C_{16:1} = 2.6$	$C_{20:4} = 10.3$
$C_{18:0} = 26.5$	
$C_{18:1} = 24.4$	

Urinary dipyrrols

Only trace amounts of mesobilifuscin could be detected in the patient's urine (Fig. 4).

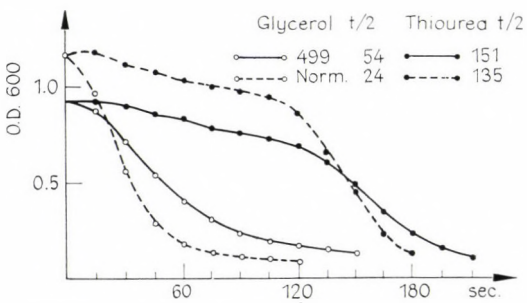

Fig. 1. Non-electrolyte permeability of the erythrocytes of a normal (Norm.) subject and the patient (499)

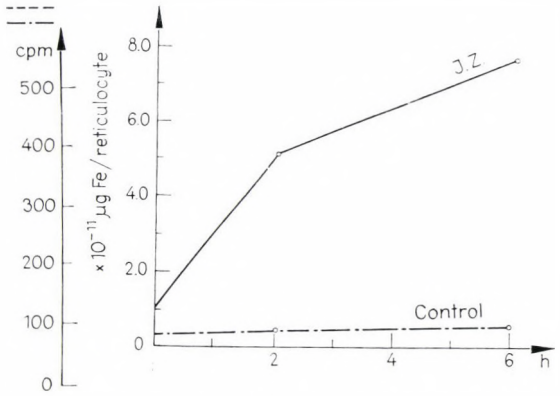

Fig. 2. In vitro ^{59}Fe incorporation into erythrocytes

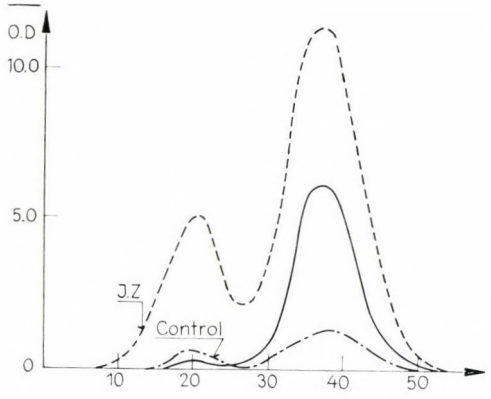

Fig. 3. Column chromatographic analysis of the stroma-free haemolysate of J. Z., and a normal control (first peak: non-haemoglobin iron binding proteins; second peak: haemoglobin)

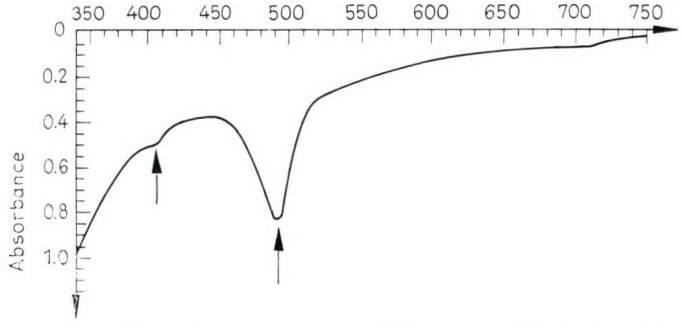

Fig. 4. Spectrum curve of the urine chromogens. 405 mµ : mesobilifuscins; 490 mµ : urobilin and stercobilin

Table 1

Comparative studies of the characteristics of adult and foetal erythrocytes and of the red cell characteristics of the patient with unstable haemoglobin haemolytic anaemia

Characteristics under study		Normal erythrocytes*	Foetal erythrocytes**	Erythrocytes (J.Z.)
Life-span	^{51}Cr t ½ (day)	25—30	14—20	5
Membrane phospholipid fatty acids	relative decrease of unsaturated fatty acids	none	marked	marked
	$C_{18:2}$ (methylester) relative %	12.6 (10.9—14.5)	5.5 (4.3—6.6)	6.9
	$C_{20:3}$ (methylester) relative %	0	2.7 (2.3—3.9)***	0
Electric charge	Elpho-mobility (10^{-4} cm/sec/volt)	1.28 (1.20—1.38)	1.43 (1.34—1.50)	1.62
Structure-enzyme	AchE activity (Δ pH/h)	0.75 (0.60—0.90)	0.42 (0.25—0.58)	0.70
Non-electrolyte permeability	Haemolysis in isotonic glycerol (t ½) (sec) Haemolysis in isotonic thiourea (t ½)	22 (19—26) 123 (115—132)	53 (37—63) 140 (116—175)	54 151
Cell integrity	GSH (mg/100 ml RBC)	69 (60—100)	66.1 (37—81)	51
HMP-shunt	G6PD activity (Δ OD/min/hgb)	10.3 (8.0—12.0)	15.2 (12.7—22.6)	14.7
Erythropoietic activity	erythropoiesis of the bone marrow reticulocyte number ‰ in vivo ^{59}Fe incorporation (10^{-11} μg Fe/reticulocytes)	normal 2—15 0.76—1.09	hyperactive 20—60 48.5 (21.7—82.0)	hyperactive 160 7.9
Haemoglobin F	alkali resistance %	0—1	60—80	1.9

* Results based on the study of 50 blood samples.
** Results based on the study of 30 cord blood samples.
*** Of the 30 cases under study $C_{20:3}$ fatty acid was present only in 17 samples.

Investigations of the haemoglobin

On paper (pH 8.9) and starch gel electrophoresis (pH 8.6) a haemoglobin band was noted which moved between Hb A and A_2 (Figs 5, 6).

Hb F = 1.9 per cent (normal range = 0.6—0.8 per cent)
Hb A_2 = 3.9 per cent (normal range = 2.5—3.5 per cent)
Methb = 2.7 per cent (normal range = 0.5—1.0 per cent)

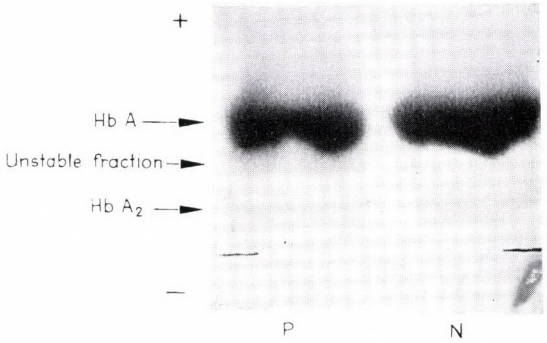

Fig. 5. Paper electrophoresis (pH 8.9) of the haemolysate of a normal subject and the patient. P = patient, N = normal control

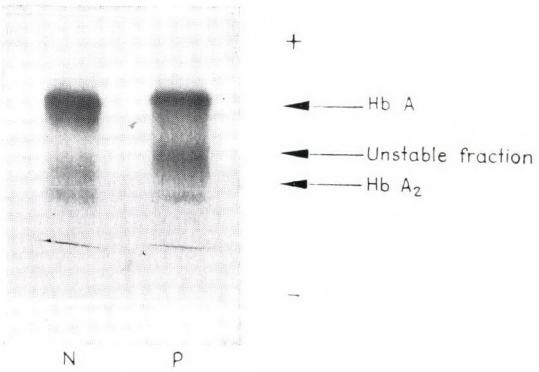

Fig. 6. Starch gel electrophoresis (pH 8.6) of the haemolysate of a normal subject and the patient. N = normal control, P = patient

Methb absorption spectrum (pH 5.5): normal.

Methb formation slightly elevated (Fig. 7).

Heat denaturation at 65°C (pH 7.4) showed an increased lability of J.Z's Hb (Fig. 8).

In the *fingerprint* (peptide chromatogram) of the isolated and aminoethylated abnormal β-chains the tryptic peptides (Tp) consisting of aminoethylated (AE)

βTpX (β 83—95) and βTpXa (β 83—93), both had abnormally low chromatographic mobilities (Fig. 9).

The finding was confirmed by preparing a fingerprint of a mixture of AEβ-chains from haemoglobin A and from the abnormal haemoglobin which showed two spots each corresponding to normal and abnormal AEβTpX and to normal and abnormal AEβTpXa, respectively. All the other tryptic peptides in the fingerprint of the AEβ-chain of the abnormal haemoglobin were in the position expected in haemoglobin A, and gave the staining reactions in normal haemoglobin.

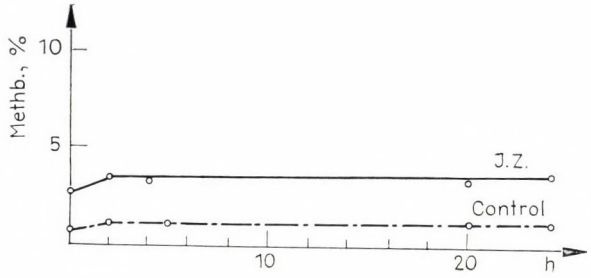

Fig. 7. Methaemoglobin formation of the haemolysate of J. Z. and of the control

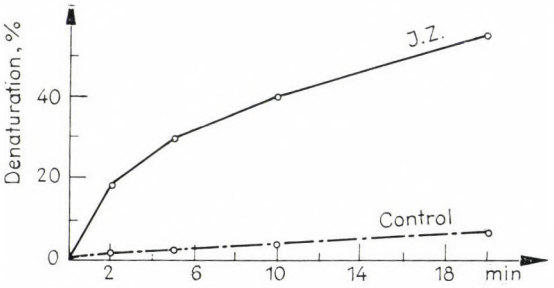

Fig. 8. Heat denaturation of the haemolysate of J. Z. and of the control

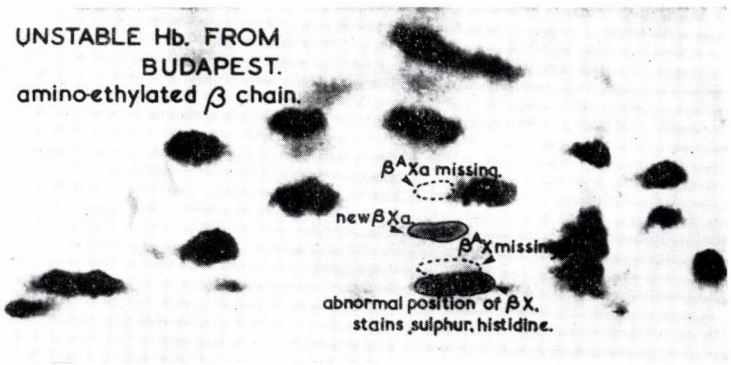

Fig. 9. Fingerprint (peptide map) of the unstable haemoglobin fraction of J. Z.

Table 2

Amino acid sequence of aminoethylated βTpX of haemoglobin A

Sequential number	83	84	85	86	87	88	89	90	91	92	93	94	95
Helical number	EF7 †Gly	8 Thr	Fl Phe	2 Ala	3 Thr	4 Leu	5 Ser	6 Glu	7 Leu	8 His	9 AE-Cys‡	10 Asp	11 Lys†

On tryptic digestion (†), partial hydrolysis (‡) occurs at the carboxyl end of AE cysteine, and in addition to βTpX (β 83—95) β TpXa (β 83—93) and βTpXb (β94—95) are formed.

Table 2 shows the amino acid sequence of AEβTpX of haemoglobin A. It will be seen that the cysteine residue in position 93 is aminoethylated; AE Cys in an amino acid sequence becomes subject to tryptic hydrolysis, hence AEβTpXa (β 83—93) is formed, but the tryptic hydrolysis is usually only partial, and AEβTpX (β 83—95) is found also.

The lower chromatographic mobility together with an unaltered electrophoretic mobility of the peptides from the abnormal haemoglobin suggested the replacement of a neutral amino acid residue by another neutral more hydrophilic residue.

The abnormal AEβTpX was eluted and lyophilised and hydrolysed in 6N HCl at 108°C for 24 hours. The hydrolysate was analysed in an automatic

Table 3

Composition of the abnormal βTpX

Residue	Yield in *n* moles	Found	Residues expected for βATpX
Asp	96.4	1.1	1
Thr	165.0	1.9	2
Ser	87.6	1.0	1
Glu	105.0	1.2	1
Pro	64.0	**0.7**	**0**
Gly	84.0	1.0	1
Ala	101.0	1.2	1
Leu	92.0	1.05	*2*
Phe	83.0	0.95	1
Lys	88.0	**1.05**	**1**
AE-Cys*	56.0	0.7	1
His	80.0	0.95	1

* AE-Cys calculated assuming the same constant as for Lys.

amino acid analyzer. The result is shown in Table 3. The peptide differed from AEβTpX of haemoglobin A by containing one residue of leucine instead of two, and one residue of proline, not present in AEβATpX. The mutation in the haemoglobin of the patient was therefore Leu → Pro.

Two unstable haemoglobins are known which show a Leu → Pro mutation in βTpX, haemoglobin Santa Ana: β 88 [F4] Leu → Pro [29], and haemoglobin Sabine: β 91 (F7) Leu → Pro [30].

To determine whether Leu β 88 or Leu β 91 had been substituted by proline, the abnormal AEβTpX was eluted from preparative fingerprints with 0.5 NH_4OH, the eluate was evaporated to dryness, and the residue was digested with pepsin in 0.05 N HCl at 37°C for 5 hours. Diagnostic and preparative fingerprints were made in the usual way with electrophoretic separation carried out at pH 6.4. The digest yielded three peptides, one negatively charged, one neutral which gave a transient yellow colour with ninhydrin-indicating N-terminal glycine-, and one positively charged, which stained for histidine and for AE-cysteine (with the chloroplatinate reagent).

Table 4

Peptic hydrolysis of β TpX (β 83—95) of haemoglobins Sabine (β 91 Leu → Pro) and Santa Ana (β 88 Leu → Pro)

	Neutral			Neutral			Positive						
	+		—	+		—	+	—		+	+	—	+—
Sabine	Gly	Thr	Phe ↓	Ala	Thr	LEU ↓	Ser	Glu	PRO	His	AE-Cys	Asp	Lys
	83	84	85	86	87	88	89	90	91	92	93	94	95
Santa Ana	Gly	Thr	Phe ↑	Ala	Thr	PRO	Ser	Glu	LEU ↑	His	AE-Cys	Asp	Lys
	+		—	+				—	—	++	+	—	+—
	Neutral			Negative						Positive			

Three peptic peptides are formed from the AE βTpX of both haemoglobins. In haemoglobin Santa Ana one is negatively charged and has the composition shown in Table 5.

Table 5

Composition of the negatively charged peptic peptide from the AE β TpX of the haemoglobin of J. Z.

Residue	Yield in *n* moles	Residue	
		Found	Expected
Thr	25.9	1.0	1
Ser	27.8	1.07	1
Glu	31.2	1.21	1
Pro	20.4	0.79	1
Ala	20.0	0.77	1
Leu	24.4	0.94	1

Table 5 shows the amino acid analysis of the negatively charged peptide. A peptic peptide of this composition could only be produced if the Leu → Pro mutation is at position β 88 (Table 4). Pepsin hydrolyzes the peptide bonds on the carboxy side of residues of leucine and phenylalanine, but not of proline. Table 4 shows the peptic peptides expected to occur in AEβTpX of haemoglobin Sabine and Santa Ana, Leu → Pro β 91 and β 88, respectively. The unstable haemoglobin of our patient is therefore identical with the second of these two, Santa Ana β 88 (F4) Leucine → Proline.

Family studies

Neither of the parents of the propositus had any complaints, nor did they ever have anaemic-icteric episodes. Their haematocrit and Hb values were normal. They had no spleen enlargement. Red cell morphology, reticulocyte stain revealed no abnormalities.

Clinical course and treatment

The child had no complaints and had no severe haemolytic or aplastic crises during the last two years. He seems to be perfectly compensated in a steady state which is characterized by Hb values of 7.0 to 9.0 g%. No transfusion was required, and there was normal physical and mental development. Drugs liable to cause increased oxidative denaturation of the unstable Hb are avoided.

Folic-acid treatment is given when it is indicated by an increased FIGLU excretion, which is being controlled regularly every three months.

Discussion

The case history of the propositus reveals the typical signs and symptoms of UHHA: a well-compensated haemolytic anaemia with bouts of anaemic-icteric crisis presenting themselves in early childhood, splenomegaly and occasional passage of dark urine.

Mesobilifuscinuria is mostly recorded as an essential feature of UHHA. Occasional passage of dark brown urine was mentioned in the case history of our patient, but during our observations only trace amounts or no mesobilifuscin could be detected in the patient's urine. In blood smears from incubated blood samples 80% of the red cells contained Heinz bodies. The instability of the patient's Hb was also reflected in the slightly raised methaemoglobin level, in the increased rate of methaemoglobin formation and heat denaturation.

Paper and starch gel electrophoresis of the haemolysate revealed an abnormal Hb band moving between Hb A and Hb A_2.

Structural studies of the isolated and purified unstable Hb revealed a β 88 (F4) Leu → Pro mutation. The Hb under investigation is thus identical with

Hb Santa Ana [29]. Comparing the case report and laboratory findings of our patient with those recorded of the affected family members in whom the anomaly had been described [29] the similarity is very impressive.

The molecular instability of Hb Santa Ana can be easily explained. The Pro residue β 88 occupies the 4th position in the F helix. According to Perutz and Lehmann [3] Pro-s can be accommodated in the first 3 positions of an α-helix, but in all others short contacts between the pyrrolidine ring and the next turn of the helix would force conformational changes. In fact the mutation B 10 (β28) Leu → Pro in Hb Genova, the mutation F 7 (β91) Leu → Pro in Hb Sabine and the mutation G 19 (β136) Leu → Pro in Hb Bibba all give rise to unstable Hb-s.

In Hb Santa Ana, as well as in Hb Sabine and Hb Bibba a Leu side-chain which is in contact with the haem group is lost. The hydrophobic interactions around the haem group are critical to the function and stability of the molecule. Any change in size or type of the amino acid side-chains in this region causes displacement of the haem group more readily than from a normal subunit [3]. Globin is much more easily denatured and precipitated because the Hb molecule is stabilized by the firm haem-globin binding. The loss of an important hydrophobic haem-globin contact loosens the hold of the globin on the haem. This conformational change permits water to enter into the haem pocket. In the presence of oxygen and water Hb is oxidized to methaemoglobin [31].

It has been suggested [12] that the oxidation of the unstable Hb to methaemoglobin is followed by a subsequent oxidation of the β93 Cys-s. Blockading of the titratable SH groups results in distortion of the molecule giving rise to oxidation of the other SH groups finally causing the precipitation of the molecule. The continuous formation of oxidative products may explain the decreased concentration of GSH found in the red cells of our patient.

In good agreement with the literature [4, 12, 33] the attachment of the Heinz bodies to the red cell membrane has been described in an earlier paper [34]. This attachment is most probably brought about by mixed disulphide bonds between Hb and membrane SH groups. The oxidation of membrane SH groups may cause marked damage to the membrane [12]. This is well reflected in the considerably altered permeability to non-electrolytes of the red cell membrane of our patient.

The relatively low level of the red cell ATP of our patient may be the consequence of excessive demand for ATP by the accelerated cation pump of cells with damaged membranes.

Definite differences were found between the lipid composition of normal red cells and the erythrocytes of our patient, and there is a striking similarity between the lipid structure of the patient's red cells and foetal erythrocytes (Table 1).

Other characteristics of "foetal type" erythropoiesis could be detected: increased G6PD level, considerably increased in vitro iron incorporation and increased electrophoretic mobility of the red cells. In previous papers [15, 35], atten-

tion has been drawn to the fact that cord blood cells resemble in many ways the red cells in certain types of haemolytic anaemia. In both conditions the blood volume expands rapidly with accelerated maturation with possible skipping of some divisions. This foetal type erythropoiesis was found to be very characteristic of UHHA with extremely shortened red cell survival and ineffective red cell production.

The clinical course of the disease revealed that in spite of the greatly increased erythropoiesis complete compensation of the haemolytic process was not achieved. Notwithstanding this fact the patient was well and developed normally. This is often the case in chronic haemolytic anaemias. Bellingham and Huehns [36], Huehns and Bellingham [37] have pointed out in recent papers that one of the factors in compensating for a haemolytic process is an increased ability of the red cells to release oxygen to the tissues. In incompletely compensated anaemia with low red cell counts and low packed cell volumes, the red cells have normal or low oxygen affinity. As a consequence the cells can release more O_2 per g Hb and the patients are equally well off as patients whose red cells have high oxygen affinity and thus need a greater red cell mass to give up the same amount of oxygen to the tissues.

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Immunochemical Studies on Group-Specific Agglutinins of Diverse Origin*

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The agglutinin anti-$\mathbf{A}_{HP}$ from the snail *Helix pomatia* which gives reactions paralleling those of other anti-**A** reagents in tests on human blood was inhibited by N-acetyl-D-galactosamine and to a lesser extent by N-acetyl-D-glucosamine. The agglutinin anti-**Ag** from the snail *Achatina granulata* was inhibited by all three amino sugars, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and N-acetyl-D-mannosamine. Neither anti-$\mathbf{A}_{HP}$ nor anti-**Ag** was inhibited by any of the many other simple sugars tested. Comparative quantitative inhibition tests were carried out with a number of different agglutinating reagents having A specificity in tests on human blood and saliva. The reagents included anti-$\mathbf{A}_{HP}$ snail agglutinin, anti-$\mathbf{A}_1$ lectin *(Dolichos biflorus)*, anti-**A** lectin (lima bean), anti-**A** isoagglutinin of human and rhesus monkey serum, and human anti-**A** serum absorbed with A_2 cells. The results while supporting the predominant role of N-acetyl-D-galactosamine for A specificity, also demonstrated qualitative differences among the reagents. The probable basis for these qualitative differences is discussed.

The most direct approach to the chemical analysis of blood-group specific substances is to isolate each substance in as pure form as possible, then to break the substance down and determine the chemical nature of the various components [1, 2]. Then if the investigator is able to synthesize a substance having the same group-specific activity by building up a chemical compound from the simpler chemical components that he has identified, he may rightly claim that he has established with reasonable certainty the chemical nature of the group-specific substances in question. Other indirect approaches to the problem are to use enzymes having the property of degrading group-specific substances, and to determine the ability of simple chemical compounds of known structure to inhibit the group-specific activity of blood-grouping reagents. In this article will be presented the results of some investigations using the latter approach. Inhibition tests were carried out with simple sugars and blood-group-specific agglutinins derived not only from human serum but also from the serum of monkeys, as well as with hemagglutinins derived from snails and from plants.

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Materials and Methods

The sugars used in the present investigation were obtained from commercial sources. For the inhibition experiments the starting dilution was a 10 per cent solution prepared by dissolving 10 grams of sugar in 100 ml of Bacto hemagglutination buffer. The serial dilutions of each sugar solution for the quantitative inhibition tests were prepared with 0.9 per cent sodium chloride solution.

Powdered albumin gland of *Helix pomatia* was kindly provided by Professor Dr. Otto Prokop, Director of the Institute of Forensic Medicine of the Humboldt University, D. D. R., and the powdered gland of *Achatina granulata* was obtained from Dr. Peter Brain, Natal Blood Transfusion Service, Durban, South Africa. The seeds of *Dolichos biflorus* used for the present study were supplied by Dr. Frank R. Camp, Jr., Director of the Blood Transfusion Division Headquarters US Army Medical Research Laboratory, Fort Knox, Kentucky. The snail powder and powdered seed were mixed with enough 0.9 per cent sodium chloride to make a 10 per cent extract, and cleared of undissolved material by centrifugation. Before use in the inhibition tests, the extracts of the snails and seeds were titrated against a 2 per cent suspension of the testing cells, and then further diluted with saline solution to produce a reagent with a titer of 4 to 8 units.

The agglutination tests, titrations, and inhibition tests were carried out by standard methods, as described in our previous reports and in standard textbooks [3, 4, 5].

Results

In Table 1 are shown the results of inhibition tests with simple sugars using hemagglutinating extracts of the snails *Helix pomatia* and *Achatina granulata*. In direct agglutination tests on human red cells, extracts of *Helix pomatia* give reactions paralleling those of ordinary anti-**A** reagents,* so that this hemagglutinin has been designated anti-$\mathbf{A}_{HP}$ by its discoverers [2, 6], the letters "HP" indicating the source of the reagent. In contrast, the hemagglutinin from *Achatina granulata* clumps red cells of all human beings and mice, but not red cells from non-human primates or other mammals; this antibody, however, is inhibitable by high dilutions of saliva from most non-human primates, and by lower dilutions of many human salivas. The agglutinin from *Achatina granulata* has been assigned the symbol anti-**Ag** to indicate its source [7].

As shown in Table 1, the inhibition tests with saliva from human beings, baboons and *Macaca irus* confirm the **A** specificity of the anti-$\mathbf{A}_{HP}$ agglutinin. Anti-$\mathbf{A}_{HP}$ is inhibited by relatively high dilutions of a solution of N-acetyl-D-

* To avoid ambiguity, symbols for blood factors (serological specificities) and their corresponding antibodies are printed in **boldface** type, symbols for genes and genotypes in *italics*, and symbols for agglutinogens and phenotypes in regular type.

Table 1

Comparison of the immunochemical properties of extracts of two kinds of snails,* *Helix pomatia* and *Achatina granulata*

Inhibitor	Reactions** of *Helix pomatia* agglutinins with human group A cells* after mixture with sugar solution or saliva diluted					Reactions of extracts of *Achatina granulata* with human O red cells, after mixture with sugar solution or saliva diluted				
	Undil.	1 : 4	1 : 16	1 : 64	1 : 256	Undil.	1 : 4	1 : 16	1 : 64	1 : 256
Sugar (10% solutions)										
D-glucose	++	++				++	++			
L-glucose	++	++				++	++			
D-galactose	++	++				++	++			
D-mannose	++	++				++	++			
L-mannose	++	++				++	++			
α-L-fucose	++	++				++	++			
α-D-fucose	++	++				++	++			
D-arabinose	++	++				++	++			
fructose	++	++				++	++			
lactose	++	++				++	++			
sucrose	++	++				++	++			
L-melibiose	++	++				++	++			
D-glucosamine-6-phosphate	++	++				++	++			
N-acetyl-D-mannosamine	±	++				—	—	—	+±	++
N-acetyl-D-galactosamine	—	—	—	—	+	—	—	—	+	++
N-acetyl-D-glucosamine	—	—	±	++	++	—	—	—	+±	++
Saliva										
Human-A Sec	—	—	tr.	+±	++	++	++			
B Sec	++	++				tr.	+	++	++	
O nS	++	++				—	tr.	+	++	
Baboon-group A	—	—	—	—	+	—	—	—	—	tr.
group B	++	++				—	—	—	—	±
M. irus-group A	—	—	—	tr.	++	—	—	—	—	—
group B	++	++				—	—	—	—	+

* Preliminary tests on extracts of a third kind of snail, *Rapana hiamaliana*, kindly provided by Dr. Otto Prokop showed inhibition only by D-glucosamine-6-phosphate and by none of the other sugars listed in the table.

** The degree of agglutination is indicated by the number of plus signs, as follows: ++ = maximal reaction possible, namely one or only a few large clumps with no or hardly any free cells; — = no agglutination; tr. = trace; ± = agglutination visible only under microscope; + = agglutination barely visible to the naked eye, etc.

galactosamine, and to a lesser degree by N-acetyl-D-glucosamine, but not by any of the other sugars listed in the table. In contrast, the tests with saliva confirm the lack of blood-group specificity of the anti-**Ag** agglutinin, which is inhibited about equally by relatively high dilutions of each of the three acetylated amino sugars, N-acetyl-D-mannosamine, N-acetyl-D-galactosamine, and N-acetyl-D-glucosamine.

Table 2

Comparison of the reactions of human anti-**A** serum with those of anti-$\mathbf{A}_{HP}$ agglutinin (*Helix pomatia*) against red cells of man, rabbit and sheep

Test cells	Titers* of human anti-A			Titers of snail anti-$\mathbf{A}_{HP}$			Titers of lectins from	
	Unabsorbed serum	Absorbed with RBC		Unabsorbed	Absorbed with RBC		*Dolichos biflorus*	Lima bean
		Human A_1	Rabbit		Human A_1	Rabbit		
Human O	0	0	0	0	0	0	0	0
A_1	192	0	90	48	0	6	96	20
A_2	160	0	64	48	0	6	4	14
B	0	0	0	0	0	0	0	0
Rabbit								
No. E—1	32	8	0	16	0	0	0	0
No. E—3	16	8	0	12	0	0	0	0
No. E—4	16	8	0	12	0	0	0	0
Sheep	8	4	4	8	0	0	32	0

* Titer value is the reciprocal of the highest dilution of the reagent giving a one-plus reaction. RBC = red blood cells

Further studies were carried out with the anti-$\mathbf{A}_{HP}$ agglutinin in order to obtain insight into the chemical basis for the specificity of the human blood-group substance A. Tests against red cells from rabbits and sheep showed them to be strongly agglutinated by anti-$\mathbf{A}_{HP}$ agglutinin (cf. Table 2), though to a lower titer than human group A cells. This was somewhat surprising because while sheep cells are known to share a specificity with human group A cells (factor $\mathbf{F}_A$), all previous reports had indicated the absence of any A specificity for rabbit red cells. To throw further light on this phenomenon, comparative titration and absorption experiments were carried out on anti-$\mathbf{A}_{HP}$ snail agglutinin in parallel with anti-**A** of human serum origin.

As shown in Table 2, as to be expected, when the human anti-**A** serum was absorbed with rabbit red cells, the agglutinins for human A red cells were only little reduced in titer; similarly, when the serum was absorbed with human A_1 cells, the agglutinins for rabbit cells were but little reduced in titer. This is readily understandable if one postulates that the anti-**A** human serum contains not only the group-specific agglutinins for human A blood, but also species-specific hetero-

agglutinins for rabbit (and sheep) red cells, and, in addition, one cannot exclude the possibility of a small amount of antibody cross reacting with both human A and rabbit (as well as sheep) red cells. In contrast, when the snail anti-$\mathbf{A}_{HP}$ agglutinin was absorbed with human A_1 red cells all activity was removed also for rabbit red cells, while absorption with rabbit red cells removed all but a small fraction of the activity for human A cells. This indicates that all or almost all the agglutinin in *Helix pomatia* extracts is cross reacting not only for human A cells but also for rabbit (and sheep) cells, due to a common specificity shared by those red cells, which is not present in human O or B cells.

To determine the basis for the cross reaction between human A cells and rabbit cells, comparative inhibition tests were carried out with anti-$\mathbf{A}_{HP}$ agglutinin

Table 3

Comparison of immunochemical behavior of snail anti-$\mathbf{A}_{HP}$ agglutinin* *(Helix pomatia)*, in inhibition tests, using different kinds of red cells, human A_1 and rabbit

Inhibitor		Inhibition titers using RBC	
		Human A_1	Rabbit
Saliva			
Baboon — Group	B	0	1
	AB	8	64
	B	0	1
	AB	64	64
	B	0	1
	B	0	1
	AB	32	64
	B	0	8
	B	0	8
	A	32	64
	AB	16	128
Human — Group	O	0	4
	A_1	32	32
	A_2	16	32
	B	0	1
Non-secretor		0	4
Sugars			
D-glucose		0	4
α-D-fucose		0	4
α-L-fucose		0	0
L-mannose		0	0
sucrose		0	4
D-glucosamine-6-phosphate		0	4
N-acetyl-D-mannosamine		1	16
N-acetyl-D-galactosamine		64	256
N-acetyl-D-glucosamine		16	256

* In order to achieve a titer of 4 to 8 units, the extract had to be used more highly diluted when the test cells were human A_1 cells than when they were rabbit cells.

tested against human A_1 cells and against rabbit cells. As shown in Table 3, tests with human saliva and with saliva from baboons and *Macaca irus* monkeys confirm the A specificity of the reactions whether human group A cells (red cells of subgroups A_1 and A_2 give similar reactions with anti-$\mathbf{A}_{HP}$) or rabbit red cells were used in the tests. However, inhibition tests with the sugars showed that when human A_1 test cells were used, N-acetyl-D-galactosamine gave moderately strong inhibition, and N-acetyl-D-glucosamine gave weaker inhibition, while N-acetyl--D-mannosamine hardly inhibited at all. In contrast, when rabbit test cells were used, N-acetyl-D-galactosamine gave very strong inhibition, as did N-acetyl-D-glucosamine, and even N-acetyl-D-mannosamine now gave significant inhibition, while feeble inhibition was obtained also with many of the other simple sugars.

Table 4

Comparison of the immunochemical properties of anti-**A** from *Helix pomatia*, *Dolichos biflorus*, and lima beans

Inhibitor	Inhibition titers for human A_1 cells using agglutinins from			
	Dolichos biflorus	*Helix pomatia*	Lima beans	Human absorbed group B serum
D-glucose	1	0	0	0
L-glucose	1	0	0	0
L-fucose	0	0	0	0
D-fucose	2	0	0	0
D-mannose	0	0	0	0
L-mannose	0	0	0	0
D-galactose	1	0	0	0
l(+)-arabinose	0	0	0	0
sucrose	0	0	0	0
D-glucosamine	0	0	0	0
N-acetyl-D-mannosamine	0	0	0	0
N-acetyl-D-galactosamine	128	64	16	32
N-acetyl-D-glucosamine	1	16	0	2
Saliva				
Human group O	0	0	0	0
A	128	16	128	128
B	0	0	0	0
Non-secretor	0	0	0	0
Baboon group A	128	16	256	128
B	0	0	0	0

In the next experiment, the behavior of anti-$\mathbf{A}_{HP}$ in the inhibition tests were compared with that of anti-$\mathbf{A}_1$ lectin *(Dolichos biflorus)*, that of anti-**A** lectin from lima bean, and that of anti-$\mathbf{A}_1$ prepared by absorbing human group B serum with human red cells of subgroup A_2. For those comparative studies the red cells used were uniformly of human origin and of subgroup A_1. As shown in Table 4,

the anti-$\mathbf{A}_1$ lectin from *Dolichos biflorus* was strongly inhibited by the amino sugar N-acetyl-D-galactosamine, and hardly at all by any of other sugars tried in the experiment. Similarly, the anti-**A** lectin from lima bean was inhibited only by N-acetyl-D-galactosamine, while the anti-A_1 agglutinin from human serum was also inhibited by this amino sugar and to a slight degree also by N-acetyl-D-glucosamine. In contrast, as has already been pointed out, the anti-A_{HP} snail agglutinin was inhibited not only by N-acetyl-D-galactosamine but also to a significant degree by N-acetyl-D-glucosamine.

Table 5

Comparison of inhibition titers of human and rhesus anti-**A** sera using red cells from human A_1 and A_2 and from sheep

Inhibitor	Inhibition titers for human anti-**A** and RBC of			Inhibition titers for rhesus monkey anti-**A** and RBC of	
	Human A_1	Human A_2	Sheep	Human A_1	Human A_2
D-glucose	0	0	0	0	0
D-galactose	0	0	0	0	0
α-L-fucose	0	0	0	0	0
α-D-fucose	0	0	0	0	0
D-mannose	0	0	0	0	0
l(+)-arabinose	0	0	0	0	0
sucrose	0	0	0	0	0
glucosamine-6-phosphate	0	0	0	0	0
N-acetyl-D-mannosamine	0	0	0	0	0
N-acetyl-D-galactosamine	2	4	96	2	8
N-acetyl-D-glucosamine	0	0	2	0	0
Salivas					
Human group O	0	0	0	0	0
A	256	256	2	64	256
B	0	0	0	0	0
Non-secretor	0	0	0	0	0
Baboon group A	256	256	4	64	256
B	0	0	0	0	0

RBC = red blood cells

In the final experiment, the immunochemical behavior of anti-A agglutinins from human serum and monkey serum was compared. As shown in Table 5, whether test cells of subgroup A_1 or subgroup A_2 were used, the anti-**A** agglutinin from both human and rhesus monkey serum was inhibited only by N-acetyl-D-galactosamine, and the degree of inhibition with this amino sugar was considerably less than for the agglutinins from snails and seeds or the human anti-**A** serum that had been absorbed with A_2 cells. However, when instead of human A red cells, sheep red cells were used, the inhibition titers with N-acetyl-D-galactosamine were

markedly increased, and slight inhibition was obtained also with N-acetyl-D-glucosamine. Remarkably, in contrast, the inhibition titers of the group A saliva whether of human origin or from baboons were reduced.

Discussion

The first paper in which the relationship of N-acetyl-D-galactosamine to A specificity was demonstrated was by Morgan and Watkins [8], in 1952. This investigation was by the inhibition technique, also used in the present study, with seed agglutinins from *Vicia cracca* and lima beans. The relationship of D-galactose to B specificity was shown independently by Watkins and Morgan [9] and by Kabat and Leskowitz [10]. Watkins and Morgan demonstrated the inhibition by galactose of the enzymatic destruction of the B substance, while Kabat and Leskowitz used a precipitation inhibition technique. The relationship of L-fucose to H specificity was discovered as early as 1952 by Watkins and Morgan [11], using the method of inhibition of agglutination of group O cells by eel anti-**H** serum. The role of L-fucose in Le^a specificity was first indicated from enzyme inhibition experiments and then confirmed by hemagglutination inhibition, with fucose-containing milk oligosaccharides by Watkins and Morgan [12]. The implication of two fucose residues in Le^b (Le^H) specificity, tentatively deduced from the results given in that paper, has been subsequently confirmed [13].

The findings in inhibition tests with simple sugars, described in the present paper confirm the predominant activity of N-acetyl-D-galactosamine as an inhibitor for anti-**A** reagents. This supports the conclusion that the specificity of the blood group substance A is due to a terminal non-reducing sugar identical with N-acetyl-D-galactosamine. However, a word of caution is necessary. The evidence here is indirect and circumstantial, and the only way to establish the chemical nature of the blood group substance is by actual analysis of the purified substance and then its synthesis [13]. Some experiments along those lines have been carried out [14] and the results to date indicate the validity of the theory that N-acetyl-D-galactosamine is indeed primarily responsible for the specificity of the human blood-group substance A.

The results of the inhibition tests suggest certain inferences also regarding the nature of the combining site on the agglutinin molecule. Since the anti-$\mathbf{A}_{HP}$ agglutinin has a broader specificity than the anti-**A** isoagglutinin, as shown by the reactions of anti-$\mathbf{A}_{HP}$ with rabbit red cells as well as group A cells, and its inhibitability by N-acetyl-D-glucosamine as well as N-acetyl-D-galactosamine, its combining site is presumably smaller and simpler in structure. Uhlenbruck and Gielen [15] and Springer [16] have also found both these sugars to be active in inhibition tests with *Helix pomatia* and *Helix hortensis*. Similarly, anti-**A** lectin *(Dolichos biflorus)* would be expected to have a smaller combining site than the anti-**A** from human serum, though not as small as that of anti-$\mathbf{A}_{HP}$. Again, judging from Tables 4 and 5, the anti-$\mathbf{A}_1$ fraction of human anti-**A** serum probably has a smaller

combining site than the remainder of the spectrum of anti-**A** isoagglutinins. Finally, anti-**Ag** from *Achatina granulata* which has the lowest specificity of any of the reagents described in the present study could be expected to have the smallest and simplest combining site of all. (See also Springer [17].)

Of interest are the different results often obtained with the same single agglutinating reagent when the test cells were changed. Thus, in the inhibition tests with anti-**A** sera, when sheep red cells were used in place of human A cells, the inhibition titers with N-acetyl-D-galactosamine were much higher while the inhibition titers with the salivas were lower, indicating a *qualitative* change in the specificity detected. In contrast, when anti-$\mathbf{A}_{HP}$ was tested against rabbit red cells instead of human A_1 cells parallel results were obtained indicating at most only a quantitative change in the sensitivity of the test. This further supports the idea tha the combining site of the anti-$\mathbf{A}_{HP}$ snail agglutinin is probably smaller and less complex than the combining site of the anti-**A** human serum isoagglutinin.

The findings presented here would be inexplicable were one to adhere to the naive concept of a one-to-one correspondence between antigen and antibody, such as is implicit in the C—D—E and 1—2—3 codes used by some workers for the Rh—Hr blood types. The findings are readily intelligible, however, when one bears in mind that each reagent detects a different specificity of the antigenic substance, and that the specificities are extrinsic attributes of the substance, and are therefore related to it as photographs are related to the object photographed.

Each of the reagents used in the present study of the agglutinogen A was of a different specificity even though they could all be used interchangeably for blood grouping work on human blood, where they identify the same agglutinogen A on the red cells and the same blood-group-specific substance A in the secretions. It would be absurd to maintain that because Helix anti-$\mathbf{A}_{HP}$, anti-**A** lectin and anti-**A** isoagglutinin differ in specificity, as shown by their reactions with simple sugars and red cells from rabbits and sheep and other animals, that they must therefore be detecting different substances in tests carried out on human blood and salivas. (This would be like asserting that, because two photographs, one a profile view and the other full face, are different they cannot be of the same person.) The fact remains that all three reagents give parallel reactions so long as the tests are limited to materials of human origin. Yet, this kind of naive assertion has been made regarding the Rh blood types; namely, that anti-**rhesus** animal sera detect the "animal" Rh antigen while human anti-**Rh** sera detect the "human" Rh factor. If this reasoning were correct, one would have to conclude that anti-$\mathbf{A}_{HP}$ detects the "snail" group A substance of human blood, while anti-**A** lectin detects the "seed" A blood group substance of human blood, etc. — but these are all actually one and the same substance — what differs are only the specificities by which one and the same substance is being recognized.

*

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Haematologia 4 (2), pp. 167 – 176 (1970)

Factors Influencing Growth and Differentiation of Spleen Colonies

II. Erythropoietin and Actinomycin D

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The effect of erythropoietin and Actinomycin D (AD) on the colony-forming ability and differentiation of transplanted colony-forming cells has been studied. When treating the recipients with erythropoietin from the 4th postirradiation day, erythroid colony count and ^{59}Fe-incorporation increased significantly. Erythropoietin given earlier was ineffective. A correlation was found between the dose of erythropoietin and the erythroid colony count. Actinomycin D selectively inhibited the development of erythroid colonies without affecting the granuloid colony count.

Studies in the last years have yielded results which may be interpreted as indicating that the primary effect of erythropoietin (ESF) is to induce the haematopoietic stem cells to differentiate into the erythroid line [1, 2].

For the time being the haematopoietic stem cell cannot be identified morphologically. Fortunately, there are some special indirect methods for the reliable estimation of the haematopoietic stem cell count. For example Till and McCulloch's spleen colony technique determines the stem cell count by the colony-forming ability. The same method allows also the determination of the differentiation ratio of stem cells [3]. Adopting this method, several workers have studied whether or not the colony-forming cell (CFC) is identical with the erythropoietin sensitive cell (ESC). Lately the idea has been accepted that these two cells are not identical. The ESC appears to be a more mature, but morphologically still unrecognizable progeny of the CFC [4—7]. According to another concept the same cell would be the carrier of both erythropoietin responsiveness and colony-forming ability, each of these properties being related to a certain phase of the cell cycle [8, 9].

Several workers have investigated the effect of ESF on the differentiation of colony-forming cells in irradiated polycythaemic mice [10, 11, 12]. It has been demonstrated that erythroid colonies are formed in irradiated polycythaemic mice only if the animals are treated with ESF [10, 11, 12]. On the other hand, the erythroid colony count could not be elevated by ESF [5, 13] in irradiated non-polycythaemic mice. Schooley [5] interpreted the ineffectiveness of exogenous ESF by assuming that the radiation-induced elevated ESF level in the nonpolycythaemic mice proves to be sufficient for the formation of erythroid colonies.

As found in some former, hitherto unpublished studies, the ESF level hardly rose early after irradiation in our mouse strain. This finding has induced us to

assume that ESF would increase erythroid differentiation even in nonpolycythaemic mice of the same strain.

In the present experiments, it has been studied in heavily irradiated mice whether and to what extent the colony-forming ability of the transplanted bone marrow cells and their differentiation ratio changed under the effect of various ESF doses administered at different points of time after irradiation. As a next step, it has been studied how the action of ESF may be influenced by Actinomycin D in the same system.

Material and Methods

Male and female BALB/c mice were used. The conditions under which the animals were kept, the irradiation terms and the bone marrow transplantation method have been described in detail [19].

Colony counting and determination of colony types. The recipient mice were killed 10 days after irradiation, the spleen was removed, cleansed from tissue fragments and weighed on a torsion balance. The colonies were counted under a binocular magnifying glass dissected one by one from the neighbouring tissues, smeared on slides and stained with Giemsa solution. Erythroid, granuloid and mixed colonies could be differentiated. Those colonies were considered mixed ones where both the mature and immature forms of the nonpredominant cell type occurred in at least 5% [14]. In certain experimental groups the spleen was fixed in Bouin's solution and subserially sectioned at 200 μ. The sections stained with haematoxylin-eosin were projected with an ocular enlarger, the picture so obtained was outlined and the colony type was determined. Those colonies were considered macroscopic ones which measured at least 0.3 mm in diameter, in other words which could be seen in at least two sections. Since the qualitative distribution of the colonies was the same in the sections and in the smears, the data yielded by the two methods will be discussed together. Megakaryocyte colonies could not be differentiated in the smears, since megakaryocytes are easily destroyed during the process of smearing and presumably also because they rarely attained the macroscopic size. As to the spleens used for subserial sections, about 0.2 to 0.8 megakaryocyte colonies per spleen were found on the average. Since, however, these colonies failed to attain the macroscopic size and their number found in the experimental groups agreed within the usual standard deviation with that of the control, megakaryocyte colonies were not considered when evaluating the experiments.

Endogenous colony count in the 750 R irradiated, bone marrow transplanted animals amounted to 0.75 to 0.88 per spleen.

Determination of ^{59}Fe incorporation. Twenty-four hours before killing the animals, they were injected with 0.3 μCi ^{59}Fe-citrate (specific activity, 26 Ci/g) intraperitoneally. Activity in the spleen was determined by a scintillation detector. Activity corrected for background was related to the erythroid colony count.

Treatment

Actinomycin D (AD). An AD stock solution in propyleneglycol (1 mg/ml) was prepared from the AD substance (Research Institute for Pharmacology, Budapest). This stock solution was then further diluted with saline to a concentration of 10 μg/ml, and this solution was injected subcutaneously. Only freshly prepared dilutions were used.

Erythropoietin (ESF). The anaemic rabbit serum used in the experiments was obtained from phenylhydrazine treated rabbits. The erythropoietic activity of the sera was assessed by ^{59}Fe-incorporation in polycythaemic mice. One ml of the anaemic rabbit serum contained 3 to 5 Cobalt Units (Co U). The recipient mice were injected with various amounts of the serum subcutaneously at various intervals after irradiation. The amount of the administered ESF was related to Co U per recipient.

Statistical analysis. Statistical analysis of the experiments was performed according to Student's *t* test.

Experimental

Effect of ESF on colony count and qualitative distribution. ESF was given at various times after transplantation; its effect was studied as a function of time.

The mice transplanted with 10^5 bone marrow cells were divided into 7 groups, as follows:

Group C: untreated control; group N I: 0.2 ml normal serum intraperitoneally, 2, 24 and 48 hours after irradiation; group N II: 0.2 ml normal rabbit serum intraperitoneally, daily from the 1st to the 9th day after irradiation; group ESF I: 1 Co U ESF 2, 24 and 48 hours after irradiation; group ESF II: 1 – 1 Co U ESF on days 4, 5 and 6 after irradiation; group ESF III: 1 – 1 Co U ESF on days 7, 8 and 9 after irradiation; group ESF IV. : 1 Co U ESF, daily from the 1st to the 9th day after irradiation.

The macroscopic colony count did not change in the groups treated with normal rabbit serum. In the group treated with normal serum for 9 days the erythroid colony count decreased insignificantly, that of the granuloid colonies increased. Colony count decreased significantly in the group treated with ESF on the first two days after irradiation. This alteration was due to the decrease of the erythroid colony count, since the granuloid and mixed colony counts were unchanged. If the animals were given ESF consecutively on days 4, 5 and 6 or on days 7, 8 and 9, the total colony count increased significantly. When investigating this phenomenon in detail, the increase found in the erythroid colony count was significant also mathematically, while a slight decrease was found in the granuloid colony count. The rise in the erythroid colony count continued when treating the animals with 1 Co U ESF consecutively for nine days. The granuloid and mixed colony count decreased slightly in these animals, too (Table 1).

Table 1

Changes in colony count and qualitative distribution of colonies in mice transplanted with 10^5 bone marrow cells and treated with ESF at different times

Group	Treatment	Time of treatment	Number of animals	Colonies per spleen*				
				Macroscopic	Erythroid	Granuloid	Mixed	Non differentiated
C	—	—	37	7.74±1.61	5.10±0.42	0.78±0.62	1.28±1.04	0.55±0.38
N I	0.2 ml N. S.**	2, 24, 48 h	20	7.36±2.41	4.30±1.90	0.95±0.72	1.45±0.86	0.66±0.42
N II	0.2 ml N. S.**	from day 1 to day 9	9	7.72±2.03	3.58±1.98	1.94±1.46	1.04±0.86	1.17±0.86
ESF I	3×1 Co U ESF	2, 24, 48 h	15	5.99±2.01 $p < 0.05$	3.20±0.47 $p < 0.01$	0.80±0.66	1.13±0.65	0.86±0.46
ESF II	3×1 Co U ESF	days 4, 5, 6	12	9.75±3.17	7.56±2.54 $p < 0.01$	0.17±0.12	1.58±0.85	0.42±0.32
ESF III	3×1 Co U ESF	days 7, 8, 9	14	8.95±3.05	7.00±2.80 $p < 0.05$	0.43±0.36	1.07±0.67	0.45±0.28
ESF IV	9×1 Co U ESF	from day 1 to day 9	16	10.82±2.22 $p < 0.02$	9.12±3.10 $p < 0.01$	0.14±0.32	0.79±0.46	0.77±0.42

* mean ± S. D.
** normal rabbit serum

Endogenous colony count in mice chronically treated with ESF. The initial endogenous colony count in the 750 R irradiated, nontransplanted mice amounted to 0.88. This value did not change appreciably when treating the animals with 1 Co U ESF daily for nine consecutive days. Owing to the low colony count, the qualitative distribution of the colonies could not be evaluated (Table 2).

Table 2

Endogenous colony count in mice irradiated with 750 R and treated with ESF

Group	Number of animals	Mean colony count per spleen	S. D.
Control	17	0.88	0.48
ESF*	14	1.21	0.67

* 1 Co U daily for 9 consecutive days

Effect of various ESF doses on colony count and qualitative distribution of colonies. The effect of 0.1, 0.2 and 2.0 Co U on colony count and qualitative distribution was studied in mice transplanted with 10^5 bone marrow cells. ESF

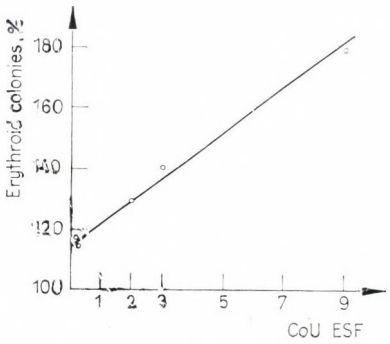

Fig. 1. Effect of ESF on erythroid colony count in percentage of control value

was administered to them in two equal doses on days 4 and 7 after transplantation. The erythroid colony count increased in proportion to the ESF dose. The correlation could not be demonstrated satisfactorily in the total colony count, since the mixed colony count also increased significantly in the group treated with 0.2 Co U ESF. Accordingly, the total colony count was higher than expected. Save the above exception, neither the granuloid nor the mixed colony count differed from the control (Table 3). The erythroid colony count is shown in Fig. 1 as a function of the ESF doses.

Table 3

Effect of various ESF doses on colony count and qualitative distribution of colonies

Group	ESF dose (Co U)	Number of animals	Colonies per spleen				
			Macroscopic	Erythroid	Granuloid	Mixed	Non differentiated
Control	—	19	8.27±2.65	6.36±2.42	0.80±0.46	0.95±0.52	0.16±0.14
ESF I	2×0.05	20	9.78±3.10	7.42±2.54	1.10±0.66	1.10±0.50	0.16±0.12
ESF II	2×0.1	19	10.92±3.16 $p<0.05$	7.24±1.97	1.37±0.71	2.21±0.88	0.10±0.08
ESF III	2×1.0	5	10.40±3.26	8.20±3.11	1.00±0.73	0.80±0.52	0.40±0.18

Effect of various ESF doses on ^{59}Fe incorporation into the spleen and single erythroid colonies. ^{59}Fe incorporation in the spleen of the control animals and of the animals treated with various ESF doses was determined 24 hours after the injection of 0.3 μCi ^{59}Fe. Incorporation in the spleen increased in proportion to the ESF dose, nearly in parallel with the erythroid colony count. Incorporation per erythroid colony was found to be stable in all the experimental groups, within the standard deviation (Table 4).

Table 4

Effect of ESF on 24 hour ^{59}Fe incorporation of spleen and of erythroid colonies

Group	ESF dose (Co U)	Number of animals	Erythroid colony count per spleen	CPM per spleen	CPM per erythroid colony
Control	—	19	6.36±2.42	9.300±1.960	1.470±368
ESF I	2×0.05	20	7.42±2.54	11.000±3.270*	1.500±310
ESF II	2×0.1	19	7.24±1.97	9.720±2.180	1.340±282
ESF III	2×1.0	5	8.20±3.11	12.415±3.360*	1.510±390

* $p < 0.01$

Effect of AD on colony count and qualitative distribution of colonies. Each of the bone marrow transplanted animals was treated subcutaneously with 2 μg AD on days 4 and 7 after irradiation. The macroscopic colony count decreased significantly as compared to the nontreated animals. The decrease followed exclusively from the decrease in the erythroid colony count. The granuloid and mixed colony counts remained unchanged within the standard deviation. If the

animals were given 1 — 1 Co U ESF 1 hour prior to AD administration, AD inhibited the usual effect of ESF; the increase in the colony count failed to occur. The granuloid and mixed colony counts did not change (Table 5).

Table 5

Effect of Actinomycin D (AD) on spleen colony count and qualitative distribution of colonies in mice treated or nontreated with ESF

Group	ESF dose (Co U)	AD dose	Number of animals	Colonies per spleen				
				Macroscopic	Erythroid	Granuloid	Mixed	Non differentiated
Control	—	—	9	8.42±2.55	6.56±2.62	0.56±0.40	1.10±0.78	0.20±0.16
AD	—	2×2 μg	8	3.10±1.42 $p < 0.02$	0.63±0.42 $p < 0.01$	1.25±0.71	1.10±0.40	0.12±0.08
Control	—	—	19	8.27±2.65	6.36±2.42	0.80±0.42	0.95±0.58	0.16±0.14
ESF	2×1	—	5	10.40±3.26	8.20±3.11	1.00±0.73	0.80±0.52	0.40±0.18
ESF + AD	2×1	2×2 μg	10	3.00±1.86 $p < 0.05$	1.10±0.81 $p < 0.01$	0.90±0.62	0.90±0.58	0.10±0.08

Discussion

The erythropoietic response to hypoxia is known considerably to differ in the various mouse strains [15]; the elevation of the ESF level is significantly less in BALB/c mice than in other strains [16]. Our hypothesis that the post-irradiation rise of the ESF level is also strain-dependent was supported by the experiments of O'Grady et al. [11] who also failed to find a rise in the ESF level early after irradiation, and in our own unpublished experiments the ESF level failed to increase significantly in mice irradiated with 750 R before the 7th day after irradiation.

ESF is required for the formation of erythroid spleen colonies; up to now this could be demonstrated for polycythaemic animals only [10, 11, 12].

By administering exogenous ESF we have attempted to influence the differentiation of colonies into the erythroid series in host mice with approximately normal ESF levels. If this treatment was begun later than the 4th day after transplantation, the erythroid colony count increased in every case. A correlation was found between the ESF dose, the erythroid colony count and between the ^{59}Fe uptake of the spleen. The highest colony count was obtained in those animals which had been treated with 1 Co U ESF daily for 9 consecutive days. The phenomenon may be interpreted to mean that the longer the period the more cells

could enter the erythropoietin sensitive phase of the cell cycle. Another interpretation would be that the sensitivity of the target cell is also different and less sensitive cells may also divide if exposed to higher doses.

The number of erythroid cells in a single colony is characterized by its ^{59}Fe incorporation. In the experiments reported, ^{59}Fe incorporation related to the single erythroid colonies was identical in the control and experimental groups. Accordingly, ESF did not seem to accelerate the maturation of differentiated erythroid elements in the spleen colonies.

A correlation was found between the duration of ESF treatment and the erythroid colony count. If treatment was begun on the 4th day after transplantation, the colony count increased in every case. Whenever treatment was begun before the 4th day, the colony count decreased significantly. Similar results were obtained in other experiments where the recipients were treated with various mitogens. Early after transplantation these mitogens were ineffective, but if administered later they induced a significant rise in the colony count [19]. Our results were in a good agreement with those obtained by De Gowin et al. [20] under different experimental conditions. The said authors were of the opinion that replication itself renders the stem cell refractory to ESF. We assume that in the early post-transplantation period colony-forming cells are refractory not only to ESF but to all kinds of mitogenic stimuli.

This hypothesis may account also for Tkadleček and Juraškova [13] not having found any increase in colony count when treating the bone marrow recipients with ESF in the first hours after irradiation. On the other hand, the hypothesis does not explain our observation that the erythroid colony count decreases if ESF is given early after irradiation. The decrease may be explained only by assuming that at the time of treatment part of the still unlodged colony forming cells were in an ESF-sensitive stage, and these cells were differentiating upon the effect of ESF. Since only a non-differentiated colony-forming cell is capable of lodging and of forming colonies, early differentiation results in a decrease of the erythroid colony count.

The erythroid colony count increased also in those groups where ESF treatment was begun 3 to 5 days prior to sacrificing the animals, i.e. when the colonies had developed in a short time. This observation may be interpreted on the basis of the data for polycythaemic animals [21, 22]. According to these, no ESF is required for the lodging of the colony forming stem cells and for their first divisions. Non-differentiated microcolonies consisting of 256 to 512 cells may form in the absence of ESF. It is highly probable that upon the effect of ESF these microcolonies will easily and rapidly grow into macroscopic erythroid colonies.

As known from the literature, AD in low doses selectively inhibits erythropoiesis [17]. Its effect on spleen colonies has not been studied so far.

Indeed, in our experiments AD selectively inhibited the formation of erythroid colonies from transplanted bone marrow cells in both ESF-treated and untreated animals. Since AD is known to inhibit the DNA-dependent RNA synthesis and the effect of ESF may be inhibited by AD, our own experiments support

Krantz and Goldwasser's [18] theory in that the synthesis of a specific, new RNA seems to be the first step in the differentiation of the stem cell into the erythron. AD does not inhibit differentiation into the granuloid series. Thus, it may be assumed that the first step of granuloid differentiation fundamentally differs from the first step of erythroid differentiation.

Our results suggest that all the effects elicited by ESF in polycythaemic mice may be reproduced in animals with a normal or somewhat elevated ESF level. The results further suggest that if the ESF level required for the formation of erythroid colonies is raised by exogenous ESF, both the erythroid colony count and the iron incorporation into the spleen increase in proportion to the dose of ESF.

The slight post-irradiation rise in the ESF level does not seem to require the full capacity of differentiation into the erythron of the colony forming cells.

*

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К выявлению и профилактике дефицита железа у доноров

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Показатели обмена железа изучены у 475 доноров, систематически сдающих кровь, с целью выявления, профилактики и лечения скрытого дефицита железа — обеднения организма железом без развития анемии. Критериями этого состояния были следующие биохимические показатели: снижение концентрации железа в сыворотке крови ниже 80 мкг% при одновременном повышении уровня ненасыщенной железосвязывающей способности сыворотки выше 260 мкг%. Подобное сочетание встретилось у 32% обследованных: чаще у женщин — 40%, реже у мужчин — 12,3%. Выявлена чёткая прямая зависимость между концентрацией ненасыщенной железосвязывающей способностью сыворотки крови и количеством сданной крови у доноров. Клиническая картина скрытого дефицита железа в миниатюре повторяет симптомы железодефицитной анемии и в выраженной форме встречается только у 6% доноров. Обнаружено статистически достоверное снижение активности сукциндегидрогеназы в эпителии слизистой оболочки щеки и содержания цитохромоксидазы в клетках крови у доноров по сравнению с практически здоровыми лицами контрольной группы. Стойкое повышение концентрации ненасыщенной железосвязывающей способности сыворотки и уменьшение содержания цитохромоксидазы в клетках крови являются наиболее точными показателями дефицита железа у доноров. Лечение препаратами железа доноров с клинико-биохимическими признаками дефицита железа позволило полностью излечить это состояние.

Принципиально важным вопросом для практики службы переливания крови является возможность развития дефицита железа у доноров, систематически сдающих кровь. Общепринята оценка достаточности железа в организме доноров по уровню гемоглобина в крови. Однако, в настоящее время не вызывает сомнений существование тканевого дефицита железа, т. е. обеднения организма железом без развития анемии, при котором состав „красной крови" существенно не изменяется [3, 9, 13].

В 1947 году Лаурелл [11], одним из первых изучавший показатели обмена железа у доноров, обнаружил отчётливое снижение уровня железа и повышение — ненасыщенной железосвязывающей способности сыворотки у доноров по сравнению с лицами контрольной группы. Ряд исследователей обмена железа выявили низкий уровень плазменного железа и высокий подъём сидеремии после нагрузки железом у доноров [1, 2, 5, 7, 10], некоторые — повышение общей и ненасыщенной железосвязывающей способности сыворотки [12, 15].

Отмеченные изменения в обмене железа некоторые из перечисленных авторов трактовали как проявления „скрытого дефицита железа". С нашей точки зрения понятие „дефицит железа" включает не столько снижение уровня железа и повышение ненасыщенной железосвязывающей способности сыворотки крови, сколько субъективные и объективные признаки обеднения тканей железом, связанные с нарушением окислительно — восстановительных процессов на клеточном уровне.

Задачей настоящего исследования является изучение клинических симптомов и лабораторных методов, позволяющих рано распознать скрытый дефицит железа у доноров с целью его своевременной профилактики и лечения.

Методы исследования и обследованные группы

Исследование выполнено путём клинического, гематологического и биохимического обследования доноров, систематически и в течение многих лет сдающих кровь. Плазменные показатели обмена железа — концентрация железа (определялась по методу Рамсай) и ненасыщенная железосвязывающая способность (определялась по методу Шаде в нашей модификации) — изучены у 475 доноров. Подробное клиническое (со специальным стоматологическим) осследование выполнено у 230 доноров; оно у 22 человек дополнено биопсией слизистой оболочки щеки с гистологическим и гистохимическим исследованием биоптатов. Выявляли полисахаридный комплекс (реакция Хале на кислые, ПАС реакция — на нейтральные мукополисахариды), по методу Шелтон и Шнейдер изучалась активность фермента сукцин-дегидрогеназы с последующей микрофотометрией на микрофотометре МФ-4.

Обследованы в клинике 30 доноров. Их подробное гематологическое обследование, включавшее исследование костного мозга, дополнялось гистохимическим изучением ферментативной активности пероксидазы и цитохромоксидазы в лейкоцитах крови и определением содержания цитохромоксидазы в клетках (эритроциты и лейкоциты) крови.

Результаты исследования

В соответствии с литературными данными нами обнаружено снижение средних величин концентрации железа и повышение — ненасыщенной железосвязывающей способности сыворотки крови доноров по сравнению с практически здоровыми людьми (в качестве контроля взяты лица того же возраста с нормальными показателями состава крови — у женщин концентрация гемоглобина в крови превышала 12,4 гр%, у мужчин — 12,9 гр%).

Несмотря на одинаковые показатели концентрации гемоглобина в крови у доноров и практически здоровых лиц, у первых (в большей степени у

женщин) изменены показатели обмена железа — повышен уровень ненасыщенной железосвязывающей способности сыворотки и снижен — железа в сыворотке крови, что является свидетельством недостатка железа в организме. Следует отметить, что низкая концентрация железа в сыворотке крови у некоторых доноров может отражать только повышенную потребность в нём красного ростка костного мозга, активизированного кровопусканием. Этот показатель, кроме того, отличается значительной лабильностью даже в течение дня и недели. Повышение концентрации ненасыщенной железосвязывающей способности сыворотки крови более стабильно. Однако, кратковременное повышение уровня ненасыщенной железосвязывающей способности сыворотки также отмечалось нами после острой кровопотери.

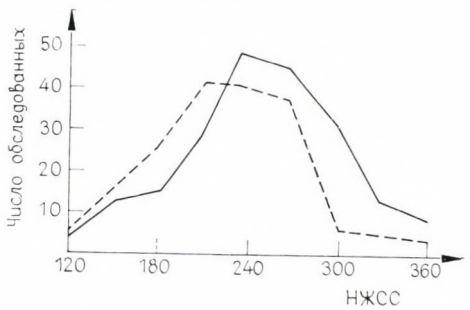

Рис. 1. Кривые распределения концентрации ненасыщенной железосвязывающей способности сыворотки (Н. Ж. С. С.) крови у доноров (сплошная линия) и практически здоровых лиц (пунктирная линия)

Следовательно, только стойкое повышение ненасыщенной железосвязывающей способности сыворотки может рассматриваться как проявление скрытого дефицита железа. Поэтому мы исследовали кровь через 60 и более дней после кровопускания, когда его непосредственное влияние на гемопоэз закончилось.

Концентрация ненасыщенной железосвязывающей способности сыворотки крови оказалась связанной с количеством сданной крови (коэффициент корреляции равен +0,65). Менее отчётливая обратная зависимость выявлена между количеством сданной крови и уровнем железа в сыворотке крови ($\gamma = 0{,}48$).

На рисунке № 1 показано распределение концентрации ненасыщенной железосвязывающей способности сыворотки у практически здоровых лиц контрольной группы и доноров. Заметно отклонение кривой распределения у доноров вправо, что связано с увеличением числа доноров с высоким уровнем ненасыщенной железосвязывающей способности сыворотки по сравнению с практически здоровыми людьми и объясняет более высокие средние величины её у доноров.

Уменьшение концентрации железа в сыворотке крови ниже 80 мкг%

при одновременном повышении уровня ненасыщенной железосвязывающей способности сыворотки более 260 мкг% и, следовательно, значительном снижении процента насыщения железосвязывающей способности сыворотки железом мы считали проявлением скрытого — тканевого — дефицита железа. Подобное сочетание встретилось у 152 из 475 обследованных доноров (32%), чаще у женщин (40%), чем у мужчин (12,3%). Особенно часто — в половине случаев — такие изменения обмена железа отмечены у женщин — многолетних доноров, систематически сдававших кровь.

Отдельные симптомы скрытого дефицита железа встретились у преобладающего большинства доноров с биохимическими проявлениями этого состояния. Полная клиническая картина скрытого дефицита железа выявлена у 30 из 152 доноров (19%) с биохимическими показателями обеднения организма железом. Основными симптомами последнего были: одышка при физической нагрузке, утомляемость, слабость, снижение трудоспособности у всех обследованных, наклонность к обморокам — у 25, дисфагия, головные боли и головокружения — у 18, сухость глотки и полости рта — у 16 обследованных. Типичные для дефицита железа изменения ногтей и волос встретились у 26, бледность и сухость кожи — у 22, ангулярный стоматит — у 13 доноров. Те или иные т. н. „оральные" симптомы дефицита железа — извращение обонятельной и вкусовой чувствительности, сухость, атрофия и бледность слизистой оболочки щёк, жжение и пощипывание языка, атрофия его нитевидных и грибовидных сосочков — при стоматологическом обследовании выявлены у 30% всех обследованных доноров. Обнаружена прямая корреляционная зависимость между выраженностью изменений полости рта и длительностью донорства ($\gamma = 0{,}7$), уровнем ненасыщенной железосвязывающей способности сыворотки ($\gamma = 0{,}6$).

Клинические проявления дефицита железа чаще встречались и были ярче выражены у доноров с многолетним стажем (более 10 лет), уровень железа в сыворотке крови которых был резко снижен, а ненасыщенной железосвязывающей способности сыворотки — значительно повышен (соответственно 52 и 300 мкг%).

Появление „малых симптомов" недостаточности железа при отчётливых биохимических изменениях обмена железа в сыворотке крови позволяет считать несомненным развитие у определённой части доноров синдрома тканевого дефицита железа. Существование изменений желудочной секреции у доноров, которые рассматривались как результат „дистрофических" процессов в слизистой оболочке желудка, известно давно [6]. Однако, неясна была связь этого состояния с дефицитом железа.

Таким образом, скрытый дефицит железа повторяет в миниатюре картину железодефицитной анемии и является её субклинической предстадией. Дополнительные исследования, выполненные у группы доноров в клинических условиях, обнаружили изменения гистохимии лейкоцитов, желудочной секреции, электрокардиограммы, костномозгового кровотворения, характерные для железодефицитной анемии, однако в умеренной степени.

Остановимся коротко на результатах исследований, объясняющих патогенетические механизмы описываемого синдрома. Гистологическое исследование биоптатов слизистой оболочки щеки, выполненное у 22 доноров с выраженными клиническими проявлениями скрытого дефицита железа, свидетельствуют об определённых изменениях в строении эпителия: уменьшении его толщины и числа клеток базального слоя с уменьшением количества митозов последних, появлении двухядерных клеток, явлений паракера-

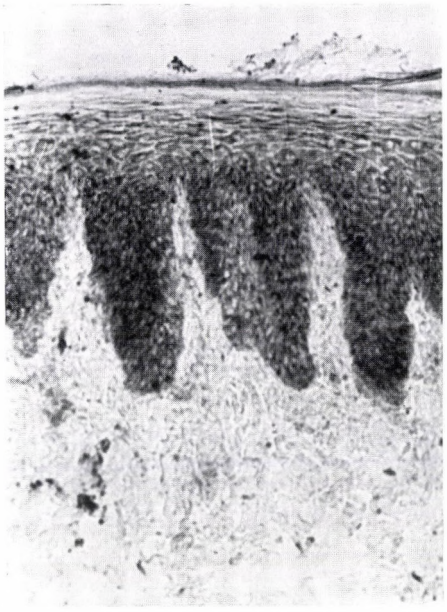

Рис. 2. Ферментативная активность сукциндегидрогеназы в эпителии слизистой оболочки щеки здорового человека с ненарушенными запасами железа. Интенсивное образование гранул формазана, указывающее на сильную активность фермента. Ув. 140

тоза, т. е. нарушении пролиферации и созревания эпителия. Представлены микрофотографии эпителия щеки здорового человека и донора с клинико-биохимическими проявлениями скрытого дефицита железа (рис. 2 и 3).

Отчётливо видно снижение активности фермента сукциндегидрогеназы у донора.

Выявленные нами гистологические изменения могут объяснить механизм возникновения клинических проявлений скрытого дефицита железа — дистрофических нарушений слизистых оболочек, кожи и её придатков. Причиной последних является уменьшение содержания железа и, в частности, железосодержащих ферментов тканевого дыхания в клетках. Изучение активности сукциндегидрогеназы в слизистой оболочке щеки выявило значительное снижение её у доноров по сравнению с лицами контрольной группы.

Таблица 1

Ферментативная активность сукциндегидрогеназы в эпителии слизистой оболочки щеки

Группы	Число наблюдений	Слои эпителия щеки		
		базальный (M ± m)	шиповидный (M ± m)	поверхностный (M ± m)
Доноры	22	79,8±4,7	55,2±4,8	41,2±3,7
Контроль	10	84,6±2,1	64,6±1,7	51,4±6,2

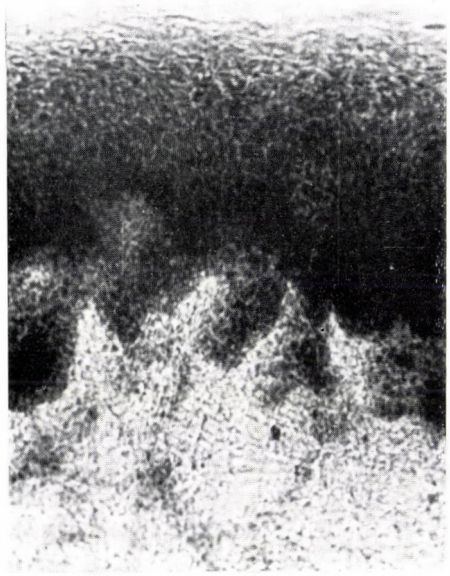

Рис. 3. Ферментативная активность сукциндегидрогеназы в эпителии слизистой оболочки щеки донора с признаками дефицита железа. Незначительное образование гранул формазана, указывающее на слабую активность фермента. Ув. 140

С целью выявления клеточного дефицита железа определялось содержание цитохромоксидазы суммарно в эритроцитах и лейкоцитах крови. Обнаружено статистически достоверное снижение содержания цитохромоксидазы в клетках крови у доноров с клиническими признаками дефицита железа (84 условных единицы) по сравнению с практически здоровыми лицами контрольной группы и донорами с нормальными показателями обмена железа (108 условных единиц).

Что касается причин обеднения организма железом, то следует отметить, что у 25% доноров с клинико-биохимическими признаками дефицита

железа симптомы его существовали до начала донорства, а дополнительное влияние обильных менструальных кровопотерь, частых родов и абортов несомненно у трети женщин. Следовательно, только у половины обследованных женщин-доноров основным этиологическим фактором скрытого дефицита железа явились сдачи крови.

Значение недостатка железа в патогенезе описываемого синдрома подтверждается результатами лечения 30 доноров с несомненными признаками дефицита железа препаратами сернокислого железа с аскорбиновой кислотой (Ferri sulfurici, Acidi ascorbinici — 0,3 г. три раза в день) в течение 2-х месяцев в амбулаторных условиях. В результате лечения у доноров статистически достоверно улучшились показатели обмена железа: концентрация ненасыщенной железосвязывающей способности сыворотки снизилась с 275 до 240 мкг%, а уровень железа повысился с 64 до 78 мкг%. Отмечено и заметное повышение концентрации гемоглобина в крови — от 12,6 до 13,4 гр%. У 22 человек исчезли полностью, а у 8 — частично объективные проявления дефицита железа. Субъективное улучшение отметили 20 доноров.

Всасывание железа в желудочно-кишечном тракте при скрытом дефиците железа повышено [4]. Следовательно нет оснований считать нарушение всасывания причиной дефицита железа у доноров. Речь может идти только о всасывании, неадекватном потерям железа при кровопусканиях и различных кровопотерях. В редких случаях нельзя исключить недостаточное поступление железа с пищей, как одну из причин дефицита железа.

Таким образом, у части доноров развиваются как в связи с донорством, так и в связи с другими факторами клинико-биохимические признаки дефицита железа, патогенетически связанные с недостатком железа в организме. У большей части из них встречаются только биохимические проявления дефицита железа. Необходимо отметить, что скрытый дефицит железа встречается достаточно часто и у практически здоровых женщин с уровнем гемоглобина в крови выше 12 гр% — в 20% случаев [3, 8].

В выраженных случаях распознавание скрытого дефицита железа возможно по его клинической картине, однако для ранней диагностики необходимы лабораторные методы исследования.

Уровень гемоглобина в крови не может быть использован в качестве диагностического теста, а определение концентрации железа в сыворотке крови является недостаточно точным методом выявления скрытого дефицита железа. Наш опыт позволяет рекомендовать для ранней диагностики скрытого дефицита железа определение ненасыщенной железосвязывающей способности сыворотки крови и содержания цитохромоксидазы в клетках крови и широко внедрить этот метод на станциях переливания крови ввиду его простоты и точности. Стойкое повышение концентрации ненасыщенной железосвязывающей способности сыворотки крови выше 260 мкг% следует считать подозрительным, а свыше 280 мкг% — несомненным признаком скрытого дефицита железа. Содержание цитохромоксидазы в клетках крови

(эритроциты и лейкоциты) менее 90 условных единиц является патогномоничным проявлением дефицита железа в тканях.

Положительные результаты терапии железом позволяют считать скрытый дефицит железа полностью излечимым. Продолжительность лечения зависит от выраженности дефицита железа и в лёгких случаях составляет 1,5—2 месяца.

Выводы

1. Скрытый дефицит железа — обеднение организма железом без развития анемии — выявлен у 152 из 475 обследованных доноров (32%). Чаще он встречался у женщин (40%), гораздо реже — у мужчин (12,3%).

2. Основанием для диагностики скрытого дефицита железа явились изменения биохимических показателей — снижение концентрации железа и повышение — ненасыщенной железосвязывающей способности сыворотки крови. Выявлена отчётливая прямая зависимость между уровнем ненасыщенной железосвязывающей способности сыворотки и количеством сданной крови у доноров.

3. Выраженная клиническая картина дефицита железа обнаружена у 19% доноров с биохимическими признаками дефицита железа, т. е. у 6% всех обследованных доноров. У этих лиц обнаружено статистически достоверное снижение активности сукциндегидрогеназы в эпителии слизистой оболочки щеки и содержания цитохромоксидазы в клетках крови по сравнению с практически здоровыми людьми контрольной группы.

4. Наиболее точными методами ранней диагностики этого состояния является определение концентрации ненасыщенной железосвязывающей способности сыворотки крови и содержания цитохромоксидазы в клетках крови. Стойкое повышение первой (свыше 280 мкг%) в крови и снижение второй в клетках крови (ниже 90 условных единиц) является доказательством скрытого дефицита железа.

5. Раннее выявление и лечение скрытого дефицита железа препаратами железа в течение 1,5—2 месяцев позволяет полностью добиться нормализации биохимических и клинических показателей.

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Diagnosis and Prophylaxis of Iron Deficiency in Donors

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The indices of iron metabolism were studied in 475 permanent donors to expose a latent iron deficiency, i.e. the impoverishment of the organism in iron without anaemia. The criteria of this state were, a decrease in serum iron concentration below 80 μg per 100 ml with a simultaneous increase of unsaturated iron-binding capacity to above 260 μg per 100 ml. Such a combination was found in 32% of the examined subjects, in 40% of the women and in 12.30% of the men. A good correlation was found between the unsaturated iron binding capacity and the number of phlebotomies. There was a significant decrease in succinate dehydrogenase activity in the epithelium of the cheek mucous membrane, and in the cytochrome oxidase content of the blood cells of the donors in comparison with control healthy subjects. A stable increase of the unsaturated iron-binding capacity in serum and a decrease of cytochrome oxidase activity in blood cells were the most reliable indices of iron deficiency in the donors. The treatment of these subjects with iron was followed by complete recovery.

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Platelet Adhesiveness and Aggregation in the Final Stage of Pregnancy and in the Puerperium

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The adhesiveness and aggregation of blood platelets has been studied in the final stage of pregnancy and in the puerperium. A statistically significant enhancement of blood platelet adhesiveness in the final stage of pregnancy with an accompanying increase in the beta-lipoprotein level was found. In the early puerperium there was a decrease in platelet adhesiveness with a partial decrease in the beta-lipoprotein level. Similar changes were observed in the ADP induced aggregation time of platelets. The significance of the above findings for the pathogenesis of thrombotic diseases in pregnancy and puerperium and a hypothesis about the interaction of beta-lipoproteins with certain receptor sites on the platelet surface are discussed.

The adhesiveness and aggregation of blood platelets is enhanced in a number of pathological states [7, 8, 13, 14, 18, 19]. Disturbances in fat metabolism may be of great importance in the process of adhesion and aggregation of blood platelets [1]. In essential hyperlipaemia the composition of fatty acids in chylomicrons is altered and the total amount of unsaturated fatty acids in cholesterol ester is reduced and in this state, accelerated blood platelet aggregation has also been observed [15]. According to Mustard et al. [10] an increased blood level of some lipids may enhance the formation of a blood platelet plug, while Nordöy and Chandler [12] observed that a lipid-rich diet increases the thrombotic process induced by ADP infusion in the rabbit.

It has been shown previously that the increased plasma beta-lipoprotein level may be responsible for enhanced aggregation and adhesiveness of blood platelets [4], thus proving that platelet adhesiveness and aggregation are more affected by beta-lipoproteins with an increased amount of lipids in the molecule than normal beta-lipoproteins [5]. Chylomicrons which are lipoproteins with a high proportion of neutral fats, if present in the plasma at high concentrations, may also increase platelet adhesiveness [6]. Since in the last stages of pregnancy there is a considerable increase in the beta-lipoprotein level [2, 3], it seemed interesting to study blood platelet adhesiveness and aggregation in pregnancy and in the early puerperium (Table 1).

Table 1

Platelet adhesiveness and aggregation, and serum beta-lipoprotein level in pregnancy and puerperium

Patient group	Test		
	Per cent of adhesive platelets	Aggregation time, sec. ADP 10^{-6}M	Serum beta-lipoprotein level (mg per 100 ml)
A	39.1* (33.8—47.0)	25 (22—29)	638.6 (532.0—840.0)
B	28.1* (22.1—33.0)	32 (30—36)	502.0 (470.0—530.0)
C	22.9 (20.5—23.5)	38 (35—39)	340.0 (291.0—397.0)

A — final stage of pregnancy
B — early puerperium
C — control
Range of variations are indicated in brackets.
* = statistically significant differences $p < 0.05$

Material and Methods

A total of 15 subjects 15—33 years of age was examined. Blood samples were collected into siliconized glass tubes containing 1/10 volume of 0.1 M trisodium citrate dihydrate and into dry tubes to obtain serum in the final stage of pregnancy and 6—8 days after labour.

Platelet rich plasma was obtained by immediately centrifuging the citrate plasma at 800 r. p. m. for 7 minutes. The platelet count was determined by the method of Rees and Ecker.

Adhesiveness of blood platelets after addition of ADP was estimated *in vitro* at room temperature by the modified method of Stormorken et al. [17].

Platelet aggregation induced by ADP at 10^{-6} M final concentration was recorded by the method of Niewiarowski and Thomas [11], based on the photometric recording of changes in optical density which occurs in platelet rich plasma on the addition of ADP.

Concentration of beta-lipoproteins in plasma was determined by the method of Burstein and Samaille [3]. In a control group of 15 subjects the same tests were performed.

Results and Discussion

Results are presented in Table 1. Mean per cent of adhesive platelets in the final stage of pregnancy was 39.1 and mean aggregation time of blood platelets was 25 sec, with a mean beta-lipoprotein level of 638 mg per 100 ml. Six to eight

days after labour, platelet adhesiveness decreased by 11% and aggregation time increased by 7 sec, with an accompanying decrease to 136.6 mg per 100 ml of the beta-lipoprotein level. Table 1 shows a correlation between adhesiveness and beta-lipoprotein level. In contrast to aggregation the decrease in platelet adhesiveness was statistically significant. On the other hand, the platelet aggregates in subjects with an increased beta-lipoprotein level were solid in contrast to the loose aggregates observed in patients with a decreased beta-lipoprotein level.

Disturbances of blood clotting and haemostatic function of blood platelets in connection with pregnancy and labour represent a great problem in obstetrics. Several studies have shown that in pregnancy there is an increase in some plasmatic blood clotting factors [9, 16] responsible for the formation of thromboses and emboli. The present findings have proved that the increase in the blood lipoprotein fraction is responsible for the enhancement of platelet adhesiveness and aggregation. The decrease in the beta-lipoprotein level occurring during the puerperium leads to a partial normalization of adhesiveness and aggregation. This effect, among others, could explain the tendency to thrombosis and embolism of women during pregnancy and puerperium.

The mechanism of the action of beta-lipoprotein on blood platelets is not fully known. The present findings have confirmed the earlier hypothesis about the interaction of beta-lipoproteins with certain receptor sites on the platelet surface [4]. The beta-lipoproteins, being high molecular substances composed mainly of lipids (phospholipids and cholesterol), seem to change the electrostatic charge on the surface of the platelet membrane, and the coated platelets with changed electrostatic forces might be more reactive to ADP than are normal platelets.

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Mechanism of Thrombocytosis Following Splenectomy in the Mouse

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Following splenectomy in the mouse, the circulating thrombocyte count increased, to exceed the initial count by 71% on the 8th day. Subsequently the count decreased to reach the initial value on the 21st day. In the number of bone marrow giant cells there was a slight decrease on the 5th and 8th days. The serum of splenectomized mice caused no appreciable change of the thrombocyte count in the usual mouse test. Postsplenectomy thrombocytosis is believed to be due to the loss of the spleen's storing function and not to an increased medullary thrombocytopoiesis.

The role of the spleen in the regulation of circulating platelets has been known for long. This regulation could occur by

1. elimination of aged or damaged platelets from the blood [9];
2. storing of thrombocytes [1, 8, 16, 17];
3. a humoral factor inhibiting megakaryocyte production in the bone marrow [7].

After splenectomy there is a rise in the number of circulating platelets. This postsplenectomy thrombocytosis may be due to one or more of three factors, viz. prolongation of the platelet life-span, failure of the spleen's storing function and increased platelet production.

In order to approach the problem, it has been studied in mice whether after splenectomy there was any change in the megakaryocyte content of the bone marrow.

Material and Methods

Three to four months old white mice of both sexes of the BALB/c strain, weighing 20—25 g and kept on a standard diet were used. The thrombocyte count was estimated by Fischer and Germer's [10] direct phase contrast microscopic method, and the number of medullary megakaryocytes by the Bürker chamber direct phase contrast microscopic method of Krizsa et al. [12, 13]. The megakaryocyte content of the bone marrow was expressed by the megakaryocyte index (M. I.). On each day the values for 10 mice were determined.

Serum thrombopoietic activity in intact and splenectomized mice was determined by the mouse test [19]: in five mice the circulating platelet count

was determined before and five days after the intravenous administration of 0.2 ml serum under investigation. The result is regarded as positive if the rise of the thrombocyte level exceeds 30% in at least four of the five animals.

Statistical analysis was carried out by means of Student's *t* test.

Results

The thrombocyte count was determined before splenectomy as well as immediately before sacrificing the animals to estimate the medullary megakaryocyte index. There was no significant change in the platelet count on the second

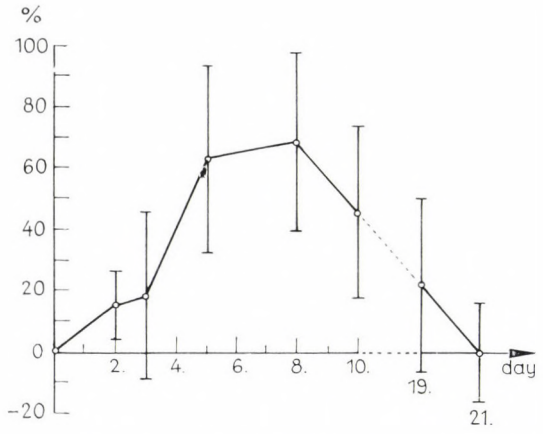

Fig. 1. Thrombocyte count following splenectomy in mice. The mean difference between the initial and the actually determined thrombocyte count of 10 mice is shown in per cents

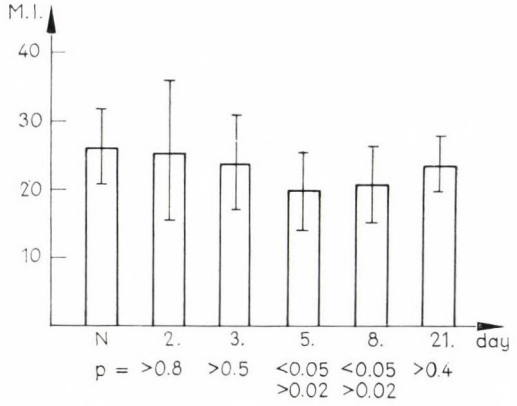

Fig. 2. Medullary giant cells after splenectomy in mice, mean medullary megakaryocyte index (M. I.) in 10 mice, its scattering and statistical significance

and third days, but by the fifth day there was an increase of 62%, and by the eighth day one of 71%. Subsequently, the thrombocyte count decreased and on the 21st day it was the same as initially (Fig. 1).

Similarly, the bone marrow megakaryocyte content showed no appreciable change on the second and third days. The megakaryocyte index on the 5th and 8th days displayed a moderate, but significant decrease; by the 21st day it was identical with the original value (Fig. 2).

Serum thrombopoietic activity was also examined in splenectomized mice. Twenty-four and seventy-two hours after splenectomy, 0.2 ml of the serum of 15 mice, and 48 hours after splenectomy that of 20 mice, collected from groups containing five animals each, were administered to an equal number of recipient animals. The thrombocyte count did not change appreciably till the fifth day.

Discussion

According to Aster's [1, 2] investigations, one third of the human thrombocytes is found in the spleen, and according to Penny et al. [17] the spleen is a thrombocyte pool and its size apparently determines the amount of stored thrombocytes. The isotope studies of Odell et al. [16] and Aster [2] as well as the findings of De Gabrielle and Penington [8] have proved the role of the rat spleen as a thrombocyte store. The findings of Hjort and Paputchis [11] and Aster [2] in the rat, as well as the findings of Castaldi and Firkin [6] and Bosch [3] in humans have shown that the life-span of thrombocytes is not affected by splenectomy.

Buonanno et al. [4] failed to observe an increase in the thrombopoietic activity of the untreated plasma of splenectomized rats, but they found an increased thrombopoietic activity in the acidified and boiled plasma of intact and splenectomized rats. These findings are difficult to evaluate because boiled acid plasma may cause aspecific thrombocytosis owing to the denaturation of some proteins.

According to the present data, thrombocytosis following splenectomy is not a result of the proliferation of megakaryocytes, and the increase in the platelet count after splenectomy is not preceded by a thrombopoietic serum activity: the serum of splenectomized mice administered to intact animals showed no thrombopoietic activity. It is conceivable that the positivity observed earlier by us [18] was connected with the blood loss which occurred at splenectomy; in agreement with the findings of Odell et al. [15] and Buonanno et al. [5] we too demonstrated [13] that bleeding may cause an increase in the thrombopoietic activity. The slight decrease in the medullary megakaryocyte count observed on the fifth and eighth days was most probably due to the negative feed-back effect of the higher peripheral thrombocyte level on the megakaryocyte content [14]. On the basis of our findings, postsplenectomy thrombocytosis in mice is thought to be due to the loss of the storing function of the spleen instead of an increased medullary thrombocytopoiesis. It is not yet known why the platelet count returns

to normal after the third week. There are two possibilities: either the platelet mass is somewhat reduced or the spleen is replaced by the other parts of the reticulo-endothelial system.

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Visual Evidence of Mitosis in Splenic and Peritoneal Macrophages of the Mouse and of the Newt Molge vulgaris

Phase-contrast Cine-micrographic Investigations

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Phase-contrast cine-micrographic recordings yielded visual evidence of mitoses occurring in well-differentiated newt and mice macrophages performing marked phagocytic activity. This is in good agreement with the concept that peritoneal and tissue culture macrophages are not end-cells and may resume their mitotic activity under certain experimental conditions.

In spite of the increasing knowledge of the role of macrophages in immune responses and in inflammation [1, 8], precise information is lacking on the capacity of these cells to perform mitosis.

According to a current view [1] macrophages in culture are end-cells devoid of any proliferative activity. However, recent reports [4, 5, 10] have pointed out the possibility of DNA synthesis in macrophages under some special experimental conditions.

The present investigation conducted by means of phase-contrast micrography presents visual evidence of mitoses occurring *in vitro* in peritoneal and splenic macrophages of the newt *Molge vulgaris* L. and of the mouse.

Methods

Newts *Molge vulgaris* L. of both sexes, weighing 10—15 g, and albino mice of both sexes weighing approximately 50 g were used. In both species peritoneal cells were obtained by a method derived from that of Whitby and Rowley [9] and consisting in washing the peritoneal cavity with medium 199 containing 5 units of heparin/ml. (2 ml per mouse and 1 ml per newt.)

The aspirated fluid was transferred to a sterile cylinder and cells were counted in a haemocytometric chamber. The yield was between 0.8×10^6 and 2.5×10^6 cells/ml in mice and between 0.5×10^6 and 1.5×10^6 cells/ml in newts.

Sterile autologous serum was added to a final concentration of 10%. The medium was dispensed in Leighton tubes (1.5 ml per tube) with coverslips and rubber stoppers. Incubation was performed at 37°C for mouse cells and at 27°C for newt cells. The medium was changed 24 hours later and between 48 and 72

hours the coverslips were removed and placed upside down on concave slides filled with culture medium, with the cell monolayer immersed in the nutritive medium.

The edges of the coverslips were sealed to the slide with melted paraffin. The tissue culture chambers so obtained were ready for phase-contrast observation.

Spleen cultures were prepared by implanting minced tissue fragments in chick plasma embryo extract clots hanging in the concavity of the same slides which had been employed for phase contrast observation of peritoneal macrophages. The concavities were filled with a fluid medium composed of 20% autologous serum and 80% tissue culture medium 199. The fluid medium was changed every 24 hours. Spleen cultures were observed by phase-contrast on the 7th day, by means of a Zeiss microscope enclosed in a thermostatic chamber heated at 37°C for mouse cells and 27°C for newt cells. The phase-contrast microscope was equipped with a Zeiss-Neofluar 100 × oil-immersion lens, oculars 3.6 × and Optovar 1.5 × eyepieces.Cine-recording was performed by means of a Zeiss—Siemens automatic device running at 4—20 photograms/min.

Results

In the present study the macrophagic nature of mononuclear cells was assessed on morphological grounds. Under the phase-contrast microscope, macrophages of both mice and newts could be recognized as large cells with a wide rim of vacuolated cytoplasm and a kidney-shaped or bean-like nucleus provided with a delicate chromatin network.

The cells were performing active phagocytosis by engulfing fragments of effete cells and granules and particles floating in the nutrient medium. No evidence of mitosis was present before two days in either newt or mouse tissue cultures, but after the second-third day a small number (1—2%) of cells of undoubtedly macrophagic aspect appeared to divide. The incidence of divisions was apparently the same in peritoneal and splenic cells.

The morphological aspects of mitosis of newt macrophages are presented in Figs 1, 2 and 3, while in Fig. 4 is reported the karyokinesis of a mouse macrophage.

A common pattern of the division of these cells was the intense emission of cytoplasmic bubbles along the cellular contour during anaphase and telophase. The macrophagic nature of the dividing cells was best evidenced by the abundant phagocyted material contained in the cell shown in Fig. 3.

Active phagocytosis was not performed during karyokinesis; it was suspended at the beginning of the prophase and resumed immediately after the detachment of daughter cells.

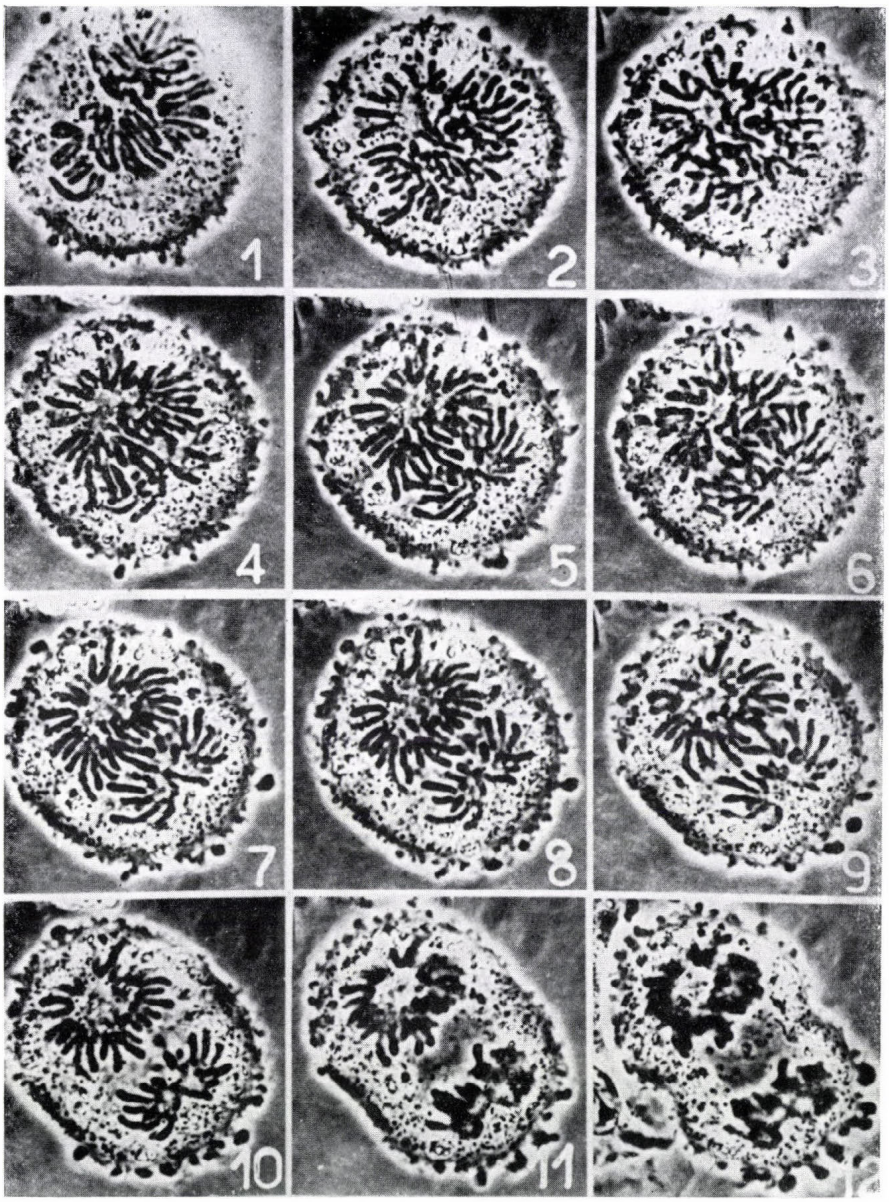

Fig. 1. Metaphase, anaphase and onset of cytokinesis in a newt macrophage, time-lapse cine-recorded by phase-contrast in the living. The bubbling is progressively increasing

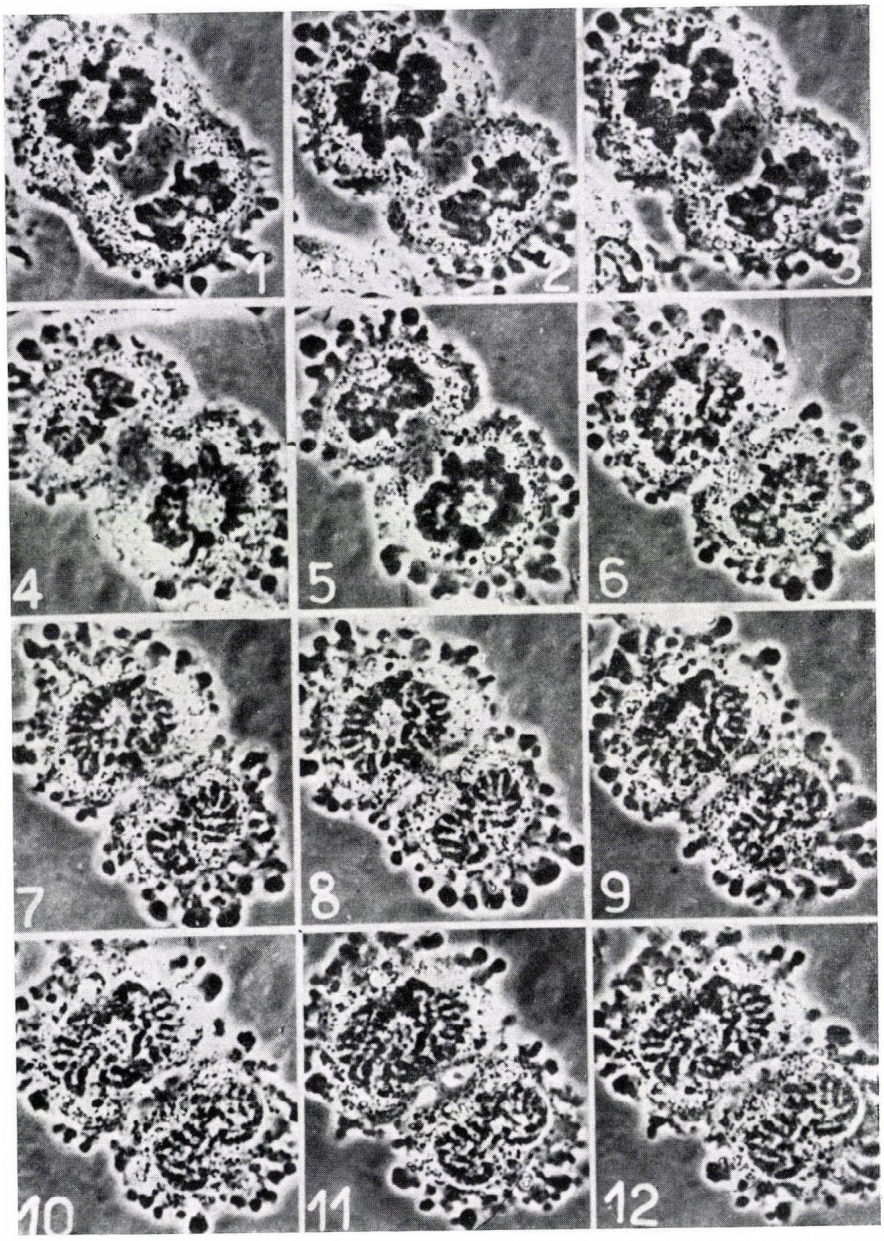

Fig. 2. Completion of cytokinesis in the same newt macrophage with intense cytoplasmatic bubbling

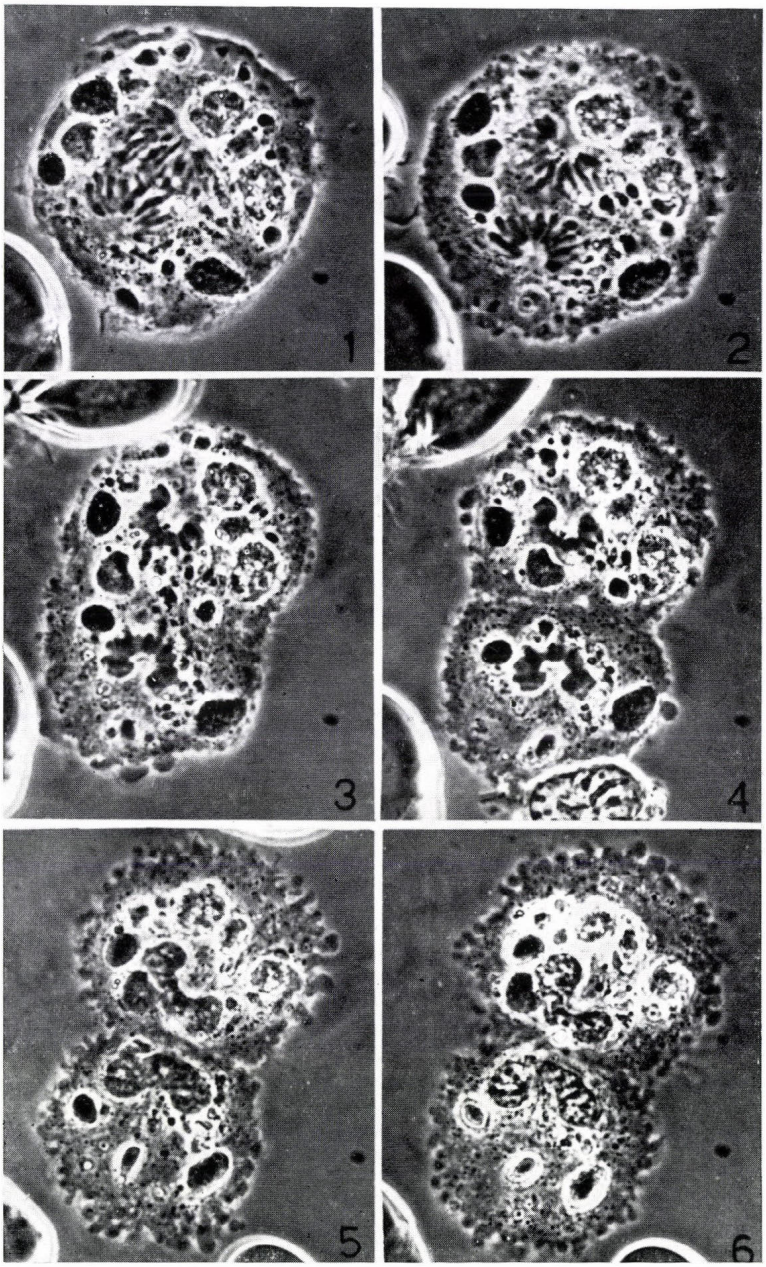

Fig. 3. Anaphase and telophase in a newt macrophage presenting much phagocyted material and a vivid cytoplasmic motility. Phase-contrast photograms from a cine-record in the living

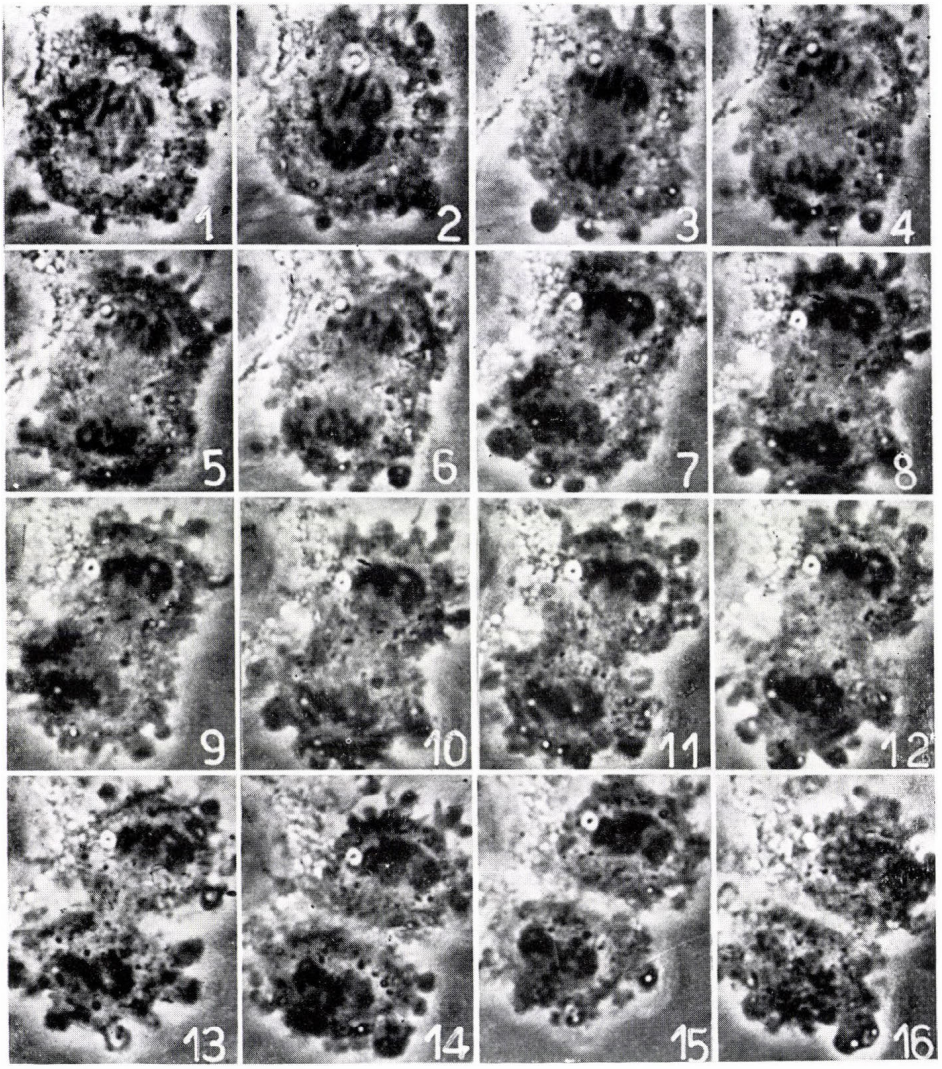

Fig. 4. Anaphase and telophase in a mouse macrophage, time-lapse cine-recorded by phase-contrast in the living. It contains sparse phagocyted material. Very intense cytoplasmic bubbling in the course of cell division. Such a motility is not observed in parenchymal haemopoietic cells in the same environmental conditions

Discussion

Though according to the current view macrophages *in vitro* are end-cells unable to perform mitosis [1], the present investigation yielded visual evidence of cellular divisions of macrophages originating from the peritoneum and the spleen of newts and mice.

The proliferative activity was performed by a consistent, though small number of well differentiated cells exhibiting intense phagocytosis. The first appearance of macrophagic divisions on the second-third day of culturing correlated well with the maximum labelling by ^{3}H-thymidine reported by Khoo and Mackaness [3] on the second day after injection of glycogen into the peritoneal cavity of mice.

It must be stressed that while a certain number of divisions in spleen cultures of newts and mice could be related to cells not fully differentiated, though performing very active phagocytosis, the same was not true for peritoneal macrophages, as the latter had been collected by a simple washing of the peritoneal cavity, without the use of any irritant agent.

The capacity of macrophages to resume a certain degree of mitotic activity under special experimental conditions may thus be assessed. This is in good agreement with the findings of Mackaness [4] who noticed mitoses of macrophages of the peritoneal cavity of mice immune to *Listeria monocytogenes* after a subcutaneous injection of Listeria culture filtrate, and of Forbes and Mackaness [2] who induced vigorous mitosis of mouse peritoneal macrophages by an injection of a purified antigen into previously sensitized mice. In the present study no antigenic stimulation of the animals had been performed, but naturally occurring stimuli might well have unobservedly initiated the mitotic response of macrophages.

It is of some interest that the phagocytic activity of the cells was suspended during division. This could be due to a diversion of energy requirement from phagocytic to mitotic activity, the former being incompatible with the simultaneous occurrence of the latter. On this basis one could interpret the occurrence of multinucleated macrophages in human tuberculin reaction [6, 7] as the consequence of mitoses in macrophages resuming proliferative activity under antigenic stimulation.

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Effect of Haematopoietic Humoral Factors on PHA-Transformed Lymphocytes

I. Haem-synthesis in PHA-stimulated Lymphocyte Cultures. Role of Factors Controlling Erythropoiesis

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Cells displaying the morphological characteristics of polychromatic and orthochromatic normoblasts were found in phytohaemagglutinin-transformed, 72- and 96-hour cultures. In these cultures, supravital staining revealed the presence of reticulocytes in different maturation phases. Haem-synthesis, as assessed by ^{59}Fe-incorporation, was proceeding in the cultures. It was inhibited or stimulated by humoral factors known to inhibit and/or to stimulate erythropoiesis, when added to the cultures 24 hours after phytohaemagglutinin. These same erythropoietic factors failed to interfere with haem-synthesis when added to the cultures simultaneously with phytohaemagglutinin. The mononuclear cell in the circulation did not seem to synthesize haem and was insensitive to the erythropoietic effect. If pretreated with phytohaemagglutinin, it began to synthesize haem and became responsive to erythropoietic factors. The findings did not permit to decide whether all the mononuclear cells or only a certain type of them had the said ability, nor whether the responsive cell will differentiate into a certain or into several lines.

Phytohaemagglutinin (PHA) stimulated peripheral lymphocyte cultures often show such nucleated cells with polychromatic and eosinophilic cytoplasms which resemble intermediate and late normoblasts. The 72 and/or 96 hours duration of cultivation *in se* makes it highly improbable that any normoblast of donor bone marrow origin finding access into the culture with other nucleated elements, should have retained its normoblast character for such a long time. Besides their mere presence, one could also observe the mitosis of these nucleated forms. This latter phenomenon seems to support the possibility that the normoblasts in question originate from the stimulated cells themselves.

The pluripotent theory of the lymphocytes' function was suggested by Maximov [5, 6, 7]. His works, and recently the investigations by Yoffey [11], Vanotti and Cullity [12], Elves [2] and Jones et al. [4] resulted in a series of papers discussing the pluripotent function of circulating lymphocytes. Still, none of these authors have offered a definite standpoint based on direct experimental evidence. The possibility of promoting the differentiation of bone marrow stem cells into the erythron by humoral factors may be considered an established fact. Accordingly, by incubating PHA-stimulated lymphocyte cultures with sera containing various erythropoietic factors, we have attempted to establish the origin

of the microscopically observed morphological elements and thereby to obtain further data on the "lymphocyte-stem cell" problem as well as on the actual mechanism of the erythropoietic factors.

Materials and Methods

Principles of test systems

In the first part of the experiments, lymphocytes derived from the peripheral blood of normal rabbits were cultured with PHA for 24 hours in medium TC 199 enriched by normal rabbit serum. Additional subcultivation for 48 hours was carried out in media with anaemic, hypertransfused and normal sera in the presence of PHA.

In the second part of the experiment, peripheral lymphocytes of normal rabbits were cultured with PHA for 72 hours in media enriched by anaemic, hypertransfused and normal sera, without precultivation in a medium containing normal serum only. ^{59}Fe incorporation into the haemoglobin of the cultures was determined in both types of experiment.

Preparation of sera

Twenty-seven rabbits of both sexes, ranging in weight from 2500 to 3000 g. were used as donors.

Nine rabbits were bled once, through the central auricular artery. To prevent coagulation, 100 I.U./kg heparin was injected intravenously. The quantity of blood collected under sterile conditions was 1 to 1.5% of the body weight. The blood so obtained was washed, resuspended in 9×10 ml saline and subsequently transfused to 9 other rabbits.

A third group, also of 9 rabbits, had no treatment.

Twenty-four hours after treatment and transfusion, the rabbits of all the three groups were exsanguinated *via* the carotid artery under sterile conditions. Their blood was centrifuged at 3000 r. p. m. for 20 minutes at 0°C and the sera were collected and stored at 4°C.

Lymphocyte cultures

Nine normal rabbits of both sexes were given 100 I.U./kg heparin intravenously. Five to ten minutes later the animals were exsanguinated through the carotid artery under sterile conditions. Another 1 to 2 I.U. of heparin was added to the blood per ml; this was allowed to stand for about 20 minutes at room temperature, and then centrifuged at 500 r. p. m. for 5 minutes. The plasma was discarded and the leucocyte-rich supernatant collected for cultivation purposes.

Lymphocyte cultures of 1.5 to 2.3×10^6 nucleated white blood cells were used. Medium TC 199 was mixed with isologous sera (30% v/v) and used in a final volume of 4 ml. Penicillin and streptomycin were added to the cultures in amounts of 80 I.U. and 50 μg per ml, respectively. To initiate blastogenesis, 0.01 ml PHA (Bacto PHA—P, DIFCO) was added to each culture. In each group of the various series, 6 to 10 parallel cultures were made. PHA-nonstimulated control series were also run for each series.

In the first part of the experiment, consisting of 6 series, the lymphocytes were cultured for 24 hours with PHA in a medium with normal serum. After 24 hours of incubation, the cultures were centrifuged at 500 r.p.m. for 3 minutes, and subsequently the medium was changed.

The PHA-nonstimulated control group was supplied with PHA-free medium, containing anaemic serum. The PHA-stimulated cultures were divided into three parts:

— medium with normal serum was added to one part of the tubes;

— medium with serum from the transfused animals was given to the second part; and

— medium enriched with anaemic serum to the third one.

The medium TC 199/serum ratio was 7 : 3 (v/v). The amounts of penicillin and streptomycin and, in the latter groups, that of PHA were the same as mentioned above.

After cultivation for a further 24 hours with different sera, 0.25 μCi—^{59}Fe citrate (Amersham) in 0.3 ml normal serum was added to each culture, and mixed thoroughly by gentle rotation of the tubes.

The cultures were harvested after 72, in some series after 96 hours of incubation and washed twice with medium TC 199 completed with normal rabbit serum. Haemoglobin was isolated according to Thunell [10]. ^{59}Fe activity in the isolated haemoglobins was determined in a well-type scintillation counter; incorporation rate was expressed as cpm/culture haem.

In the first series one culture of each group after washing with medium TC 199 was carefully resuspended in 0.3 ml normal rabbit serum and incubated with 0.5 ml brilliant crezyl blue for another 60 minutes. Next, smears were prepared from each culture for the determination of the reticulocyte count; at least 3000 erythrocytes per smear were counted.

The second part of the experiment, consisting of 3 series, was carried out under identical experimental conditions as the first one except that the sera were added to the cultures simultaneously with PHA, viz. without precultivation with PHA in a medium containing normal serum only. Besides, ^{59}Fe citrate was added to the 24-hour cultures. To the PHA-nonstimulated cultures media with anaemic sera were added.

Two culture series were harvested after 72, and one after 96 hours. Haemoglobin isolation and the determination of ^{59}Fe activity were performed as above.

Results

Intermediate and late normoblasts from a 72-hour lymphocyte culture are shown in Figures 1 and 2, respectively. These forms are difficult to distinguish from degenerating lymphocytes, both in black-and- white pictures and under the microscope. Moreover, the young normoblasts resemble the small lymphocytes to such an extent that their identification is unreliable.

Reticulocytes in various maturation phases, derived from another 72-hour culture, are shown in Figs 3,4 and 5. The reticulocyte seen in Fig. 6 is a large one, twice the normal size. This suggests that this cell had failed to follow the mitotic cycle usual for normoblasts from the bone marrow. Such large reticulo-

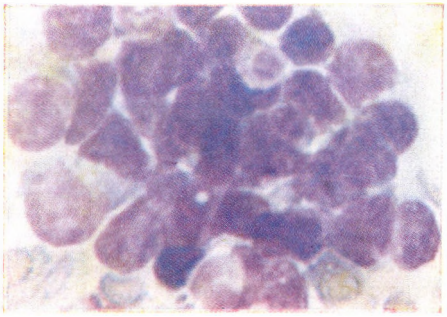

Fig. 1a. Blastoid cells from PHA-stimulated circulating lymphocytes in a 72-hour culture. Among the blasts, a basophilic and an intermediate normoblast. May-Grünwald-Giemsa staining

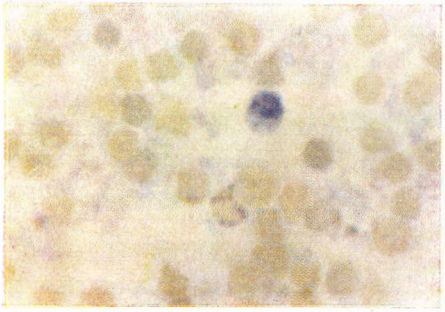

Fig. 1b. Intermediate normoblast from a 72-hour lymphocyte culture. Supravital brilliant crezyl blue staining

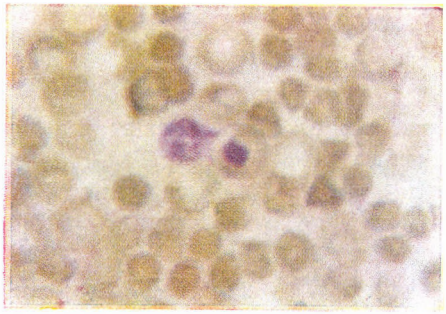

Fig. 2. Normoblast with orthochromatic cytoplasm. The nucleus has lost its characteristic structure. 72-hour culture. May-Grünwald-Giemsa staining

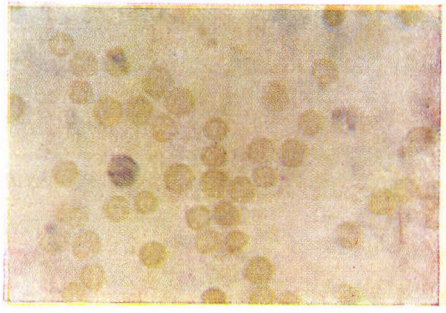

Fig. 3. Four reticulocytes of different stages of maturation, one is still polychromatic. 72-hour culture, brilliant crezyl blue staining

cytes were often seen in these cultures. On the other hand, the reticulocyte in Fig. 7 is small, its size is about half that of a normal erythrocyte. In smears from 72- and 96-hour cultures, stained according to Pappenheim, unequal mitoses in late telophase were observed, resulting in a normal and in a smaller normoblast. Probably, these small reticulocytes originate from unequal mitoses.

The ^{59}Fe-incorporation rates found in the first part of the experiment are shown in Table 1. The most striking feature was that, compared with the PHA-nonstimulated cultures, all the PHA-stimulated cultures displayed an appreciable ^{59}Fe-incorporation into haemoglobin. There was always a slight activity in the haemoglobin of the PHA nonstimulated lymphocyte cultures; since, however, its level failed to hit the counter's background, it was of no importance.

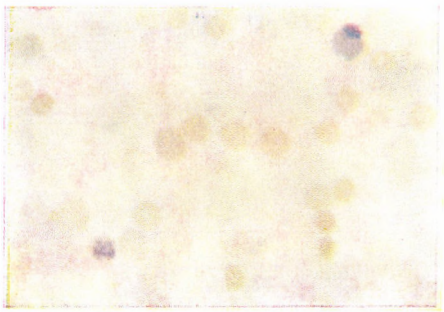

Fig. 4. A reticulocyte and an intermediate nucleated erythrocyte with nucleus extrusion; no network characteristic of reticulocytes is seen. 72-hour culture, brilliant crezyl blue staining

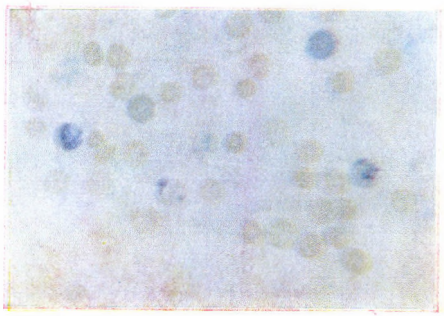

Fig. 5. Reticulocytes and two polychromatic erythrocytes. 72-hour culture, brilliant crezyl blue staining

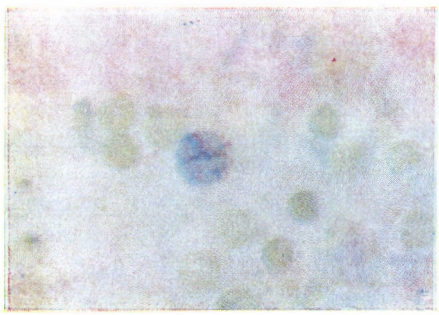

Fig. 6. Large polychromatic reticulocyte. Size about double of normal erythrocytes. 72-hour culture, brilliant crezyl blue staining

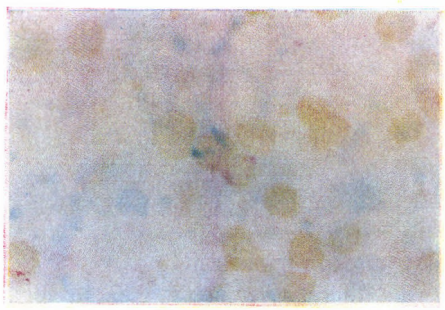

Fig. 7. Small reticulocyte. Size about half of a normal erythrocyte. 72-hour culture, brilliant crezyl blue staining

In 5 out of 6 experimental series, anaemic rabbit serum considerably increased ^{59}Fe-incorporation into the haemoglobin of PHA-stimulated lymphocyte cultures. This stimulatory effect ranged from 56 to 227% in the various series. In one experiment the cultures with anaemic serum showed exactly the same incorporation rate as the cultures with normal serum. Apparently, the donor was ineffectively bled in this case. Namely, control tests in normal mice confirmed that as the anaemic serum failed to contain any erythropoiesis stimulating factor, mice given such serum failed to incorporate into the circulating erythrocytes more ^{59}Fe than the mice treated with normal serum.

On the other hand, the hypertransfused serum reduced ^{59}Fe-incorporation in every experimental series, although inhibition exceeded 20% in two series only.

Table 1

Effect of erythropoiesis stimulating or inhibiting factors on haem-synthesis in PHA-stimulated lymphocyte cultures. The sera were added to the cultures after preincubation with PHA and normal serum for 24 fours

Serial number of experiment	^{59}Fe activity cpm/haem isolated from culture				Cultivation time	Decrease**	Increase**
	PHA-free	Normal serum	Hypertransfused serum	Anaemic serum	hours	per cent	per cent
1	215± 9.8	869±10.6	590±17.5	1570±21.2	96	—32	+80
2	290±10.7	1058±18.9	868±21.9	1716±18.4	96	—18	+62
3*	189±13.2	1013±16.8	768±24.9	1587±11.8	72	—25	+56
4*	180± 9.8	609±10.9	511± 9.3	1992±20.7	72	—17	+227
5*	176± 7.3	1015±13.5	400±12.5	2040±14.7	72	—61	+100
6	207±12.3	892±21.9	528±18.7	928±26.1	96	—41	+4

* From each of these series a culture was prepared for the determination of the reticulocyte count.

** As compared to the control.

Data on the reticulocytes derived from series 3 to 5 of the previous experiment are summarized in Table 2. Similarly as with ^{59}Fe-incorporation, the difference in reticulocyte counts between the PHA-stimulated and nonstimulated cultures was striking. While several thousands of erythrocytes had to be counted prior to finding a single reticulocyte in the cultures without PHA, the reticulocyte count in the PHA-stimulated cultures was high and easy to determine. The results obtained by the two different methods did not seem to be strictly correlated. This was due to the different erythrocyte counts in the different series.

Table 3 summarizes the data for ^{59}Fe-incorporation, recorded after applying anaemic or hypertransfused serum simultaneously with PHA. In these experimental series there seemed to be no difference between the PHA-stimulated and the nonstimulated groups. The anaemic serum failed to increase ^{59}Fe-incorpora-

Table 2

Reticulocyte count in PHA-free and PHA-stimulated lymphocyte cultures; effect of factors stimulating or inhibiting erythropoiesis. The sera were added to the culture after preincubation with PHA and normal serum for 24 hours

Serial number of experiment	Reticulocyte per thousand				Cultivation time	Decrease**	Increase**
	PHA-free	Normal serum	Hypertransfused serum	Anaemic serum	hours	per cent	per cent
3	0.15	8.6	5.1	22.3	96	—45	+155
4	0.33	11.2	8.7	25.9	72	—22	+131
5	1.00	13.8	10.5	37.3	72	—24	+170

** As compared to the control.

tion, as compared either to the normal or the PHA-nonstimulated one. The hypertransfused serum slightly inhibited ^{59}Fe-incorporation. However, the results obtained approached so much the background of the counter that they could hardly be evaluated. The erythroid elements derived from the donor and still capable of haem synthesis seemed to be responsible for the incorporation found in these cultures. This was the more probable, since in these experiments the isotope had been added 24 hours after incubation.

Table 3

Effect of erythropoiesis stimulating or inhibiting factors, given simultaneously with PHA, on haem-synthesis in PHA-stimulated lymphocyte cultures

Serial number of experiment	^{59}Fe activity cpm/haem isolated from culture				Cultivation time, hours
	PHA-free	Normal serum	Hypertransfused serum	Anaemic serum	
1	420±17.3	482±21.7	358±15.8	471±16.6	96
2	456±14.2	443±20.3	436±13.3	497±25.5	72
3	377±16.5	317±27.7	303±19.1	402±24.7	72

Discussion

Humble [3] by the use of PHA achieved recovery in the erythroid status of patients with aplastic anaemia. Bishun et al. [1] observed increased mitosis in the bone marrow of young Wistar rats 27 hours after PHA administration. All these suggest that PHA affects not only mononuclear cells but, directly or indirectly, also the formation of the other elements of the blood, *in vivo*.

According to our results, PHA induced an intensive haem-synthesis in lymphocyte cultures *in vitro*. This synthesis was increased or decreased by humoral

factors known to stimulate or to inhibit erythropoiesis. At the same time, the presence of reticulocytes and nucleated erythroid elements was detected in the 72- and 96-hour cultures. Recently, Saillen et al. have observed porphyrin synthesis in cells of myeloid origin [9, 12] and subsequently in PHA-transformed lymphocyte cultures incubated with delta-amino-laevulinic acid. Although it would be most convenient to have experimental data confirming the presence of myeloblasts among the PHA-transformed blastoid cells, our results do not suggest that eventual myeloid cells present in the culture could be held responsible for the increased porphyrin synthesis, as suggested by Saillen et al. [9].

Our results obtained by the administration of PHA and of erythropoiesis-inhibitory and stimulatory humoral factors have confirmed that the cells incorporating ^{59}Fe in the first experimental series were not erythroid elements originating from the donor. They must have formed in the cultures themselves, i.e. they had become capable of haem-synthesis under the effect of PHA. Accordingly, ^{59}Fe-incorporation into the haem represents not simply haem-synthesis, but haem-synthesis proceeding in the newly formed cells. At the same time, the experiments have also suggested that the erythropoietic serum factor did not affect the peripheral stem cells. The point of attack of the factor has to be such a stem cell which has become responsive to humoral factors controlling erythropoiesis only after a certain transformation, in the present case after stimulation with PHA for 24 hours.

According to our results, this transformation requires more time than the lifetime of the erythropoietic factor present in the sera.

Apparently, the maturation of some of the cells sensitive to the erythropoietic factor is accomplished shortly after differentiation (within a single mitotic cycle), without following the normal mitotic cycle usual for erythroblasts from the bone marrow. The presence in the 72-hour cultures of a high number of reticulocytes with many large ones may be explained solely in the above way. A number of data confirm also the *in vivo* existence of this brief erythropoietic response. However, the conditions of cultivation *in vitro* undoubtedly favour this brief response.

Our results imply that the transformed mononuclear elements PHA-stimulated in lymphocyte cultures are capable of differentiating into the erythron and of responding to humoral eryhtropoietic stimuli. The present stage of the experiments does not, however, permit to answer the problem as to whether all the circulating mononuclear cells or only a certain type are capable of differentiation into the erythron following transformation, furthermore, whether this same mononuclear cell would be capable of transforming into some other cell type upon an adequate stimulus — in compliance with the unitarian theory.

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Thromboplastic Activity of Leukaemic Eosinophils

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The results of haemostatic investigations of the eosinophils isolated from the blood of a patient suffering from eosinophilic reticulosis (eosinophilic leukaemia) are presented. The eosinophil extract was found to possess thromboplastic activity. It shortened the calcium time of plasma as well as the reaction time *r* in the thrombelastogram of normal blood, and it increased prothrombin consumption in serum. The eosinophil extract can be used in the thromboplastin generation test as a substitute for platelets. In the thrombin generation test the extract increased both the thrombin generation rate and the amounts of the generated thrombin. The extract exhibited also a slight antiheparin activity but no antithrombin activity.

In peripheral blood, thromboplastic activity was demonstrated in platelets [14], erythrocytes [7] and leucocytes [16]; it has been proved in normal leucocytes as well as in leukaemic myeloblasts, neutrophils, lymphocytes [17] and monocytes [4]. Due to the technical difficulties of eosinophil isolation from blood [2], the thromboplastic activity of these cells has not been proved. The purpose of the present study was to report on investigations into the thromboplastic activity of eosinophils isolated from the peripheral blood of a patient suffering from eosinophilic reticulosis.

Methods

The eosinophils were isolated from the peripheral blood of a patient with eosinophilic reticulosis (eosinophilic leukaemia) diagnosed on the basis of clinical and histopathological criteria. The total leucocyte count was in the range of 60—100 thousands per cu.mm in consecutive determinations, the eosinophils amounted to 60—84%. The leucocytes were isolated from the blood by differential sedimentation in high molecular weight dextran solution and differential centrifugation [19, 27]. The obtained leucocytes contained about 90% mature eosinophils and a few eosinophil precursors (Fig. 1). From these eosinophils an extract was prepared in Owren's buffer pH 7.35 at a concentration of 1 mg of leucocyte lyophilizate per 1 ml of buffer [17, 18]. All tests were performed at 37°C. The effect of the eosinophil extract on plasma calcium time was studied [17] at 0.25, 0.5 and 1 mg/ml concentration. Altogether 40 determinations were

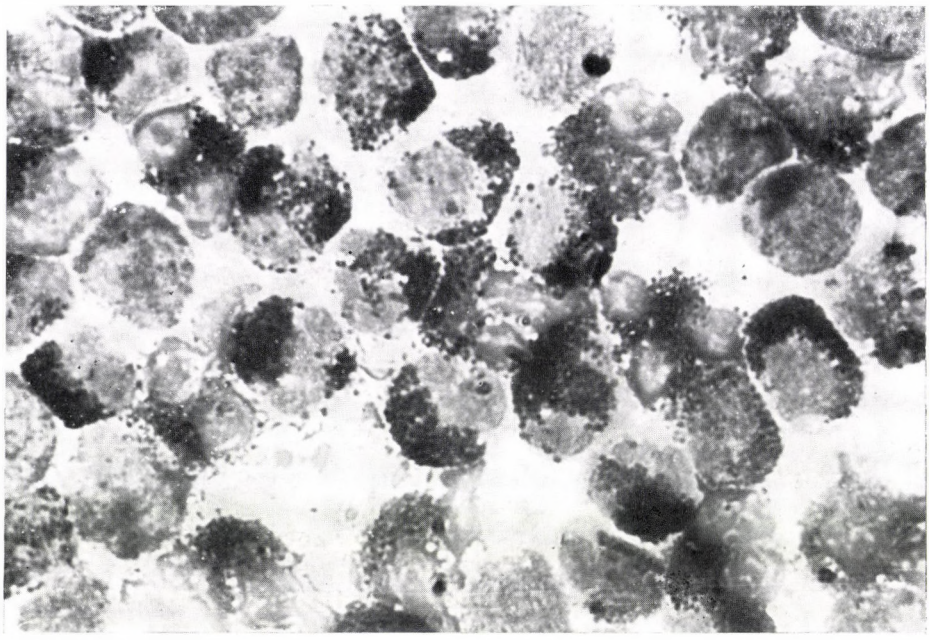

Fig. 1. Eosinophils isolated from the peripheral blood of a patient suffering from eosinophilic reticulosis (eosinophilic leukaemia)

performed. The effect on the thrombelastogram of normal blood was studied by means of Hartert's thrombelastograph; its first cuvette contained 0.05 ml of Owren's buffer (control); the second, 0.05 ml of eosinophil extract at 0.5 mg/ml concentration; the third, 0.05 ml of eosinophil extract at 1.0 mg/ml concentration. To each cuvette 0.3 ml of freshly obtained donor blood was added. In the thrombelastograms, reaction time r, the values k, ma as well as B_1, B_2, B_3 (according to [12]) were calculated. Fifteen thrombelastograms were performed. The effect on prothrombin consumption in serum was investigated by placing 0.2 ml of eosinophil extract at concentrations from 0.125 to 1.0 mg/ml in each of four tubes, and 0.2 ml of Owren's buffer into the fifth control tube, and 3 ml of donor blood was added to each of the test tubes. Prothrombin time in serum was determined by the Quick method 30, 60 and 180 minutes after the blood had clotted. In general 20 determinations were performed.

The effect of eosinophil extract on thromboplastin generation was studied by means of Biggs and MacFarlane's test modified as described elsewhere [16], using instead of a platelet suspension the eosinophil extract at a concentration of 0.5 mg/ml and 1.0 mg/ml. Altogether 40 determinations were performed. The effect of eosinophil extract on thrombin generation in plasma was studied accord-

ing to Pitney and Dacie, adding to the incubation mixture, instead of 0.1 ml Owren's buffer, 0.1 ml of eosinophil extract at 0.25, 0.5 and 1.0 mg/ml concentration [18]. Altogether 82 single determinations were performed. The effect of eosinophil extract on thrombin time of normal and heparinized plasma was studied by a method described previously [18]. Antiheparin activity of 1 mg/ml of eosinophil extract was expressed in μg of inactivated heparin [19]. Altogether 44 determinations were performed. The effect of the extract at 1 mg/ml concentration on thrombin activity was studied by means of a method described elsewhere [18].

Results

The eosinophil extract was markedly shortening the calcium time of plasma (Table 1); higher concentrations produced an effect stronger than small ones. Reaction time *r* in thrombelastogram was also markedly shortened, the values *k*, *ma*, B_1, B_2, B_3, showed no significant change (Table 2, Fig. 2). Prothrombin consumption in serum was increased more effectively at higher than at low concentrations (Fig. 3).

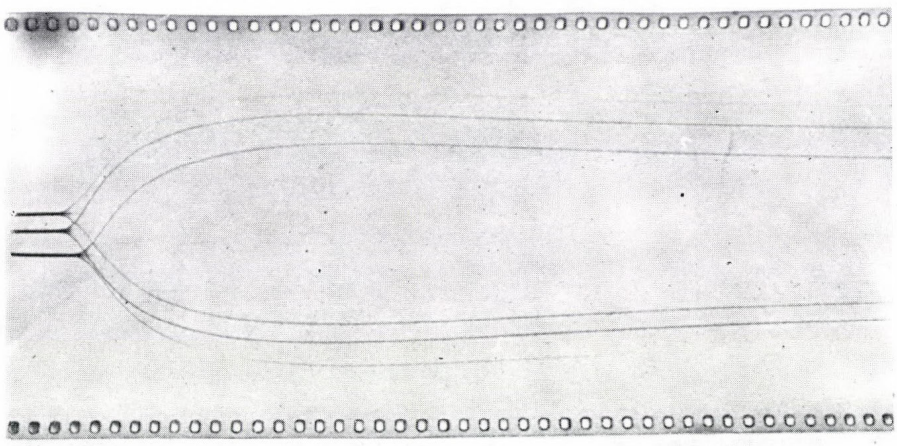

Fig. 2. Effect of eosinophil extract on thrombelastogram of native blood. The two thrombelastograms above: after addition of eosinophil extract to the blood. The thrombelastogram below: control test with buffer

Table 1

Effect of eosinophil extract on calcium time of normal plasma

Eosinophil extract concentration in mg/ml	control	0.25	0.5	1.0
Coagulation time in sec. (arithmetic mean)	97.5	94.7	63.1	57.1
Coefficient of variability, per cent	12.3	8.9	9.6	27.7

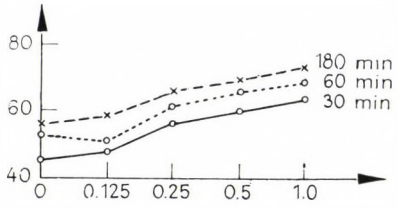

Fig. 3. Effect of eosinophil extract on prothrombin consumption in serum, after 30, 60 and 180 min of blood incubation with eosinophil extract at different concentrations

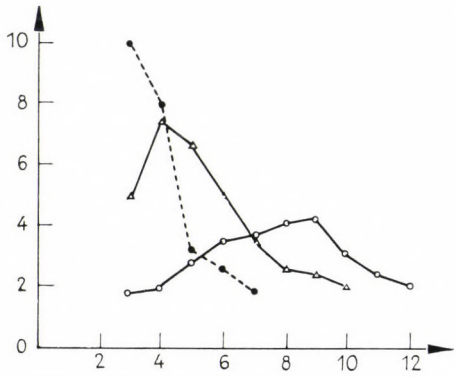

Fig. 4. Effect on thrombin generation in plasma of eosinophil extract at different concentrations: — — — — — — ○ — — — — — — eosinophil extract 1 mg/ml, ——————▲—————— eosinophil extract, 0.5 mg/ml

The eosinophil extract can be used as a substitute for platelet suspension in the incubation mixture of the thromboplastin generation test. The effect of the extract was stronger at higher than at low concentrations (Tables 2 and 3). Thrombin generation was considerably enhanced by the extract both in rate and in quantity (Fig. 4).

The eosinophil extract did not affect the thrombin time of normal plasma and shortened only slightly, statistically not significantly (t = 1.0) the thrombin

Table 2

Effect of eosinophil extract on thrombelastogram of native normal blood

	r sec.	*k* sec.	*ma* mm	B_1 mm	B_2 mm	B_3 mm
control						
Arithmetic mean	360.0	203.0	54.4	50.2	45.2	42.6
Coefficient of variability, per cent	33.7	18.7	7.3	6.0	9.1	9.0
Eosinophil extract 0.5 mg/ml						
Arithmetic mean	360.0	204.0	52.6	48.2	43.4	41.6
Coefficient of variability, per cent	28.4	23.5	7.6	11.0	13.3	16.5
Eosinophil extract 1.0 mg/ml						
Arithmetic mean	337.0	205.0	54.0	52.0	46.6	45.0
Coefficient of variability, per cent	25.6	17.8	6.7	5.2	7.2	2.7

Table 3

Use of eosinophil extract as a substitute for platelet suspension in the thromboplastin generation test

Incubation, min.	2	4	6	8
	coagulation time of substrate plasma in sec.			
Eosinophil extract, 0.5 mg/ml				
Arithmetic mean	51.0	51.25	41.00	27.00
Coefficient of variability, per cent	14.5	3.3	16.6	16.2
Eosinophil extract, 1.0 mg/ml				
Arithmetic mean	52.5	53.0	34.7	26.0
Coefficient of variability, per cent	5.0	4.6	7.2	9.9

time of heparinized plasma (Table 4). The inactivated amount of heparin as calculated from the standard curve [19] was about 0.010 μg. Incubation for 10 to 30 minutes of the eosinophil extract with thrombin solution of known activity caused no change in thrombin activity in comparison with the results obtained after incubation with Owren's buffer.

Table 4

Effect of eosinophil extract on thrombin time of normal and heparinized plasma

Incubation mixture	Coagulation time of substrate plasma, sec.	
	Arithmetic mean	Coefficient of variability, per cent
Plasma–buffer–thrombin	20.5	7.2
Plasma–buffer–heparin–thrombin	44.0	8.3
Plasma–buffer–heparin–eosinophil extract–thrombin	36.9	14.9
Plasma–buffer–eosinophil extract–thrombin	22.0	7.8

Discussion

The role of leucocytes in blood coagulation and in the composition of thrombi has been attracting increasing interest [5, 6, 9, 13, 15, 21, 24]. Neutrophils invade the thrombi and contribute to its disruption and dissolution, the dissolution of fibrin deposits and chemical thrombolysis [10, 21, 23]. The role of lymphocytes and monocytes in thrombus biomorphosis is less known; the fibroblasts in the thrombus might originate after transformation of such cells [10]. Moreover, there are data concerning the influence on blood coagulation of basophil proteolytic enzymes, heparin, histamine, and serotonin [8, 25].

The participation of eosinophils in the evolution of thrombi has become somewhat more clear since profibrinolysin (plasminogen) had been shown to be present in the cytoplasmatic granules of the above cells [3]. Plasminogen is already produced in the eosinophil precursors in the bone marrow, and the activity of the enzyme increases in the course of cell maturation. Fibrinogen and fibrin deposits, but not albumin, are attracting eosinophils into the inflammatory site [23]. It has been suggested that eosinophils migrating to the inflammatory site or into the thrombi are transporting plasminogen to the areas of fibrin collection. This phenomenon may be of significance in wound repair. Eosinophils dissolve also fibrin deposits at the site of an allergic response to allergens in sensitive individuals, as has been shown in the case of an allergic nasal polyp [22].

In the available literature we have found no data indicating the presence in eosinophils of coagulation and fibrinolysis factors other than plasminogen. The present results have supplied evidence of thromboplastic activity in eosinophils. As the extracts of the other leucocyte types [16, 17, 18], the eosinophil extract shortened the recalcification time, increased prothrombin consumption in serum, and proved suitable as a substitute of platelet suspension in the thromboplastin generation test, and increased thrombin generation in plasma. The biochemical character of eosinophil thromboplastic activity and its relation to other known tissue thromboplastins has not yet been appreciated. Our investigations into the

isolation of a purified form of the thromboplastic substance of eosinophils will be presented in another paper.

The conditions of release into the circulating blood of the thromboplastic activity of eosinophils and its role in thrombohaemorrhagic phenomena has not been elucidated. The patient from whom we had obtained the eosinophils died with terminal symptoms of eosinophilic reticulosis. Autopsy revealed in his heart and vessels a number of thrombi developed during life, as well as symptoms of haemorrhagic diathesis in the lungs. The role of thromboplastic activity of eosinophils in the mentioned pathological changes is a difficult question, closely related to the part of leucocytes in the pathogenesis of thrombohaemorrhagic phenomena [6, 11, 26]. There is reason to believe that the thromboplastic activity of leucocytes is of significance in these phenomena.

The present results revealed the presence in eosinophils of some weak antiheparin activity. This activity is high in normal leucocytes and those of chronic granulocytic leukaemia, while weak in the lymphocytes of chronic lymphatic leukaemia and in myeloblasts of acute myelogenous leukaemia [17, 18]. The antiheparin activity of eosinophils is weaker than that of myeloblasts. An evidence of the unspecific character of leucocytic anti-heparin activity has been presented [19]. The activity in question depends on the presence in the cells of many different basic proteins [19] such as ribonucleinase and myeloperoxidase [28]. Thus, the present results allowed to assume that the basic protein content is low in eosinophils.

It was remarkable that the investigated eosinophils contained no antithrombin activity such as was observed previously in normal leucocytes and in leucocytes of chronic granulocytic leukaemia [17, 18]. This last observation is indicative of a difference in leucocytes in respect of their activities and their role in haemostasis and blood coagulation. Of course, our conclusions are of limited value as the investigations were performed on a single patient. The rare occurrence of eosinophilic reticulosis, its controversial nosologic and pathogenetic patterns [1, 20], will make it difficult to generalize our results.

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Вопросы рациональной терапевтической тактики хронического лимфолейкоза

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Проблема лечения хронического лимфолейкоза сводится к выбору момента для начала активной терапии, выбору лечебного препарата, подбору необходимой дозы и определению возможных интервалов в назначении лекарства. Существует группа больных (около 25%), не нуждающихся в активной терапии на протяжении 5—20 лет болезни. При назначении терапии необходимо учитывать не только высоту лейкоцитоза, но и признаки висцеральной патологии, тенденцию к анемизации и тромбоцитопении. Для уменьшения опасности мутагенного действия препарата в больших дозах предложена первично-сдерживающая (профилактическая) терапия, назначаемая при первых признаках агрессивности болезни. После проведения курсового лечения больному назначается поддерживающая терапия. Благодаря такой тактике достигнуто увеличение средней продолжительности жизни больных до 63 месяцев, а в последние годы еще выше. Наиболее удобным химиотерапевтическим препаратом в лечении хронического лимфолейкоза является лейкеран. Им лечились 73 больных. У всех получен хороший непосредственный эффект, а в дальнейшем при проведении поддерживающей терапии-ремиссии продолжительностью 2—4 года. Первично-сдерживающая лейкеранотерапия (10—15 мг 1 раз в 5—7 дней) позволяет предотвратить прогрессирование болезни на 1—3 года. Её получали 57 больных. Стероидные гормоны не должны применяться широко. Они показаны при гемолитическом кризе, выраженной анемии и тромбоцитопении. Рентгенотерапия или телегаммaтерапия применяются при выраженном локальном увеличении лимфоузлов или селезенки. В лечении частых у больных лимфолейкозом пневмоний эффективными оказываются полусинтетические препараты пенициллина (оксациллин, метициллин, цепорин).

Хронический лимфолейкоз представляет особую проблему как в теоретическом, так и в лечебном плане.

При наблюдении за сравнительно большими группами больных хроническим лимфолейкозом обращают на себя внимание те особенности заболевания, которые не оставляют сомнения в его эндогенной природе.

Таблица 1 демонстрирует распределение больных хроническим лимфолейкозом по полу и возрасту.

По наблюдениям разных авторов, в том числе и нашим, охватывающим около 2000 случаев хронического лимфолейкоза, заболевание поражает мужчин в два раза чаще, чем женщин; как правило, хронический лимфолейкоз не поражает лиц моложе 40 лет и в подавляющем большинстве случаев наблюдается в возрасте 50—70 лет.

Таблица 1

Распределение больных хроническим лимфолейкозом по полу и возрасту

Автор	Количество больных	Пол		Возраст			
		м	ж	до 40 лет	41—50 лет	51—70 лет	старше 70 лет
1. Гусейнов Г. А. с соавт.	166	103 (62,2%)	63 (37,8%)	—	—	91 (55%)	—
2. Osgood.	212	2 : 1		13	21	(150 (75%)	47
3. Cook, Romano.	150	96 (64%)	54 (36%)			56,7 средний возраст	
4. Собственные данные	248	180 (72,5%)	68 (27,5%)	14	34	178 (72%)	22

Таблица 2

Случаи заболевания лейкозами кровных родственников

Автор	Хронический лимфолейкоз (число семей)	Хронический миелолейкоз (число семей)
1. Rigby et al., 1966	4	—
2. Gunz, 1957	10	
3. Собственные наблюдения	7 (в двух семьях больны 3 члена семьи)	

Участие наследственного фактора в возникновении хронического лимфолейкоза вытекает из заметной частоты одновременного или последовательного заболевания кровных родственников (таблица 2).

Наконец, немаловажным аргументом в пользу генетической природы хронического лимфолейкоза является определенная зависимость частоты заболевания от национальной принадлежности. Так, многими авторами отмечается редкость хронического лимфолейкоза в странах Востока (Индия, Япония) и довольно большая частота заболевания лиц еврейской национальности [16]. Последнее наблюдение подтверждается и нашими данными.

Констатированное японскими авторами отсутствие роста заболеваемости хроническим лимфолейкозом в районах, подвергшихся действию иони-

зирующей радиации, также свидетельствует об эндогенной, автономной природе данного заболевания.

Перейдем к анализу важнейших моментов терапии хронического лимфолейкоза.

Проблема лечения сводится к трем основным положениям:

1. Определение качества случая.
2. Выбор момента для начала активной терапии.
3. Выбор лечебного препарата.

Теперь уже стало трюизмом, что среди больных хроническим лимфолейкозом можно выделить группу с относительно доброкачественным течением заболевания. У этих больных в течение 5—20 лет от момента установления диагноза наблюдается умеренный лейкоцитоз (20 000—30 000) с лимфоцитозом до 50—70%. Полная сохранность эритро- и тромбоцитопоэза, нерезко выраженная лимфоидная метаплазия костного мозга (40—50% лимфоцитов в пунктате), незначительное увеличение лимфоузлов, отсутствие увеличения печени и селезенки позволяет оценить эти случаи как благоприятные в прогностическом плане и *исключает необходимость активной* цитостатической терапии в течение 10 и даже 20 лет (наши наблюдения).

Под наблюдением клиники находятся 44 больных хроническим лимфолейкозом, у которых заболевание было диагностировано 3—5—10 лет назад. У всех этих больных до сих пор нет необходимости в проведении активной терапии. 4 больных начали лечиться в последние 1—2 года.

Так, у больной П., 68 лет, с давностью заболевания хроническим лимфолейкозом 22 года, за 20 лет количество лейкоцитов увеличилось с 15 000 до 50 000 с нарастанием лимфоцитоза до 70%, красная кровь и тромбоцитопоэз все годы оставались сохранными, гиперплазия периферических лимфоузлов и селезенки отсутствовала. Больная не нуждалась в активной терапии. Лишь в последние два года более быстрое нарастание лейкоцитоза (до 100 000), тенденция к некоторой анемизации (снижение гемоглобина до 65 ед., эритроцитов до 3 000 000), быстрая утомляемость потребовали проведения умеренной сдерживающей лейкерано- и преднизолонотерапии, которая дала быстрый и полный эффект: количество лейкоцитов снизилось до 20 000, восстановилась красная кровь, больная вновь стала трудоспособной.

Начало активной терапии требует специального обсуждения.

Еще сравнительно недавно большинство гематологов считало необходимым как можно дольше воздерживаться от лечения больных хроническим лимфолейкозом, назначая активную терапию лишь в развернутой стадии заболевания при количестве лейкоцитов 350 000—400 000 и выраженных признаках распространенной гиперплазии лимфоидной ткани.

Такая установка обосновывалась двумя важными положениями: 1) относительной незлокачественностью лимфолейкемического процесса, что определяло сохранение соматической компенсации и некоторой трудоспособности больных в течение нескольких лет болезни; 2) опасностью му-

тагенного действия любой цитостатической терапии, что практически, действительно, констатируется нередко убыстрением течения и малигнизацией заболевания после сравнительно короткой (4—6 месяцев) ремиссии при некоторых видах терапии (уретан, эмбихин, рентгенотерапия).

Накопление опыта, однако, привело к пересмотру положений о поздней терапии.

Нужно стремиться не пропустить момента, когда следует начать активную цитостатическую или гормональную терапию. Рационально ли доводить больного до тех резких структурных и функциональных изменений в органах, которые делают эти процессы трудно- или необратимыми?

Поясняя эту мысль, мы хотели бы представить ее конкретно: больной доводится до анемии, вызванной лимфоидной инфильтрацией костного мозга, высокого гиперлейкоцитоза, значительного увеличения лимфоузлов, печени, селезенки и полной потери трудоспособности, а затем лечащий врач ставит перед собой задачу добиться обратного развития процесса. Это требует большого количества времени и лекарственных препаратов в больших дозах, а эти факторы как раз больше всего способствуют мутагенному действию цитостатической терапии.

Мы видим выход из указанных противоречий цитостатической терапии в более раннем начале лечения, которое определяется не только высотой лейкоцитоза, но и учетом признаков висцеральной патологии. Выраженное или быстро прогрессирующее увеличение и уплотнение (!) лимфоузлов, особенно в средостении и брюшной полости, нарастающее увеличение селезенки и печени диктуют необходимость начала активной цитостатической терапии даже при сублейкемической картине крови. Назначение своевременной терапии позволяет сдерживать дистрофогенное действие лейкемического процесса, развитие белковой недостаточности, истощение больных.

Не менее важным моментом, определяющим необходимость терапии, является тенденция к развитию анемии и тромбоцитопении, что указывает на значительную лимфатическую метаплазию костного мозга. Известно же, что после развития анемической и тромбоцитопенической стадии химиотерапия оказывается малоэффективной.

Основной принцип проводимой терапии: максимальное щажение при той степени активности, которая дает возможность добиваться стабилизации процесса (терапия по типу лечения диабета).

Это положение легло в основу предложенной И. А. Кассирским первично-сдерживающей (профилактической) химиотерапии.

Другим, не менее важным принципом, является гибкость терапевтической тактики, постоянный подбор наиболее эффективных доз препарата, стремление не к пропагандируемой французскими авторами эрадикации, которая, очевидно, недостижима, а к своеобразному сосуществованию болезни и препарата, при котором терапия должна предотвратить, насколько возможно, агрессивность болезни, в то же время не являясь амбивалентно действующим фактором.

Говоря о выборе препарата, следует отметить, что разнообразие средств, которые в современных условиях могут быть использованы при лечении больных хроническим лимфолейкозом (различные цитостатики, стероидные гормоны, рентгенотерапия), налагает на врача особую ответственность за выбор наилучшего из возможных препаратов и методов лечения.

Таблица 3

Продолжительность жизни больных хроническим лимфолейкозом при различных видах терапии

Автор	Годы наблюдений	Количество больных (прослеженные до конца жизни отмечены +)	Терапия	Средняя продолжительность жизни в месяцах
1. Leavell	1917—1936	49+	Рентгенотерапия	42
2. Minot and Isaacs	1924	30+ 50+	Симптоматич. Рентгенотерапия	40 42
3. Tivey	1924—1950	685 (данные литературы)	Симптоматич. Рентгенотерапия. Радиофосфор	32
4. Wintrobe, Hasenbush	1926—1938	47+	Мышьяк Рентгенотерапия	28
5. Lawrence	1936—1954	84+	Рентгенотерапия	64
6. Osgood	1941—1962	212	Радиофосфор, рентгенотерапия	64
7. Scott	1957	118+	Рентгенотерапия, химиотерапия	36,7
8. Green, Dixon	1947—1961	125	рентгенотерапия, радиофосфор	48
9. Cook, Romano	1922—1960	138+	Рентгенотерапия	91 б-й — 12 34 б-й — 60 13 б-й — больше 60. Средняя продолжительность 35 месяцев
10. Galton at al,	1961	40+	Лейкеран	64
11. Апасов Г. Н.	1960	54+	Радиофосфор	муж. — 32 жен. — 62
12. Полянская А. М.	1966	275	Гормональная и химиотерапия	56
13. Собственные наблюдения	1948—1966	23 27 73+ 23	Симптоматич. рентгенотерапия в сочетании с химиотерапией Химиотерапия в сочетании с гормонотерапией	31 48,4 63

Главная задача терапии при неизлечимых заболеваниях — продление жизни больных.

Таблица 3 демонстрирует эффективность различных видов терапии с этой точки зрения.

В последние годы выработка более рациональной терапевтической тактики привела к значительному увеличению продолжительности жизни больных. Так, 57 наших больных, находящихся на диспансерном учете, в настоящее время уже пережили наибольшую среднюю продолжительность жизни (63—64 месяца).

Продолжительность их заболевания от момента установления диагноза уже в настоящее время составляет 106,2 мес.

Таблица 4

Продолжительность ремиссий у больных хроническим лимфолейкозом, леченных различными цитостатическими препаратами

Автор	Годы наблюдений	Количество больных	Препараты	Продолжительность ремиссий в месяцах
1. Авралева А. М.	1966	15	Дегранол	2—6
2. Кирик О. Г., Петренко А. М.	1966	78	Дегранол	6—12
3. Плотников Ю. К.	1964	20	ТиоТэф	6—8
Плотников Ю. К.	1965	20	Тиодипин	7,3—7,5
Плотников Ю. К.	1965	11	Рентгенотерапия	6—8
4. Селихова В. В.	1962	43	Рентгенотерапия, радиофосфор	6—20
5. Фрейдзон В. А.	1958—1964	100	Эмбитол, хлорбутин, дегранол, дипин, тиотэф, тиодипин	3—6—12
6. Шерман С. И. с соавт.	1966	24	Преднизолон	3—11
7. Popovic	1962	14	Преднизолон, лейкеран	2—20
8. Erdimili, Stutzman	1965	51	Лейкеран, курсовое лечение	13,2
9. Galton et al.	1961	56	Лейкеран, курсовое лечение	12—24
10. Miller et al.	1959	19	Лейкеран, курсовое лечение	2,5—4,5
11. Cattaneo et al.	1967	12	Дипин	Неполные, только у половины больных; 1—9

Приводимые цифры являются, безусловно, искусственно заниженными, поскольку при медленном и относительно благоприятном течении заболевания установление диагноза может запаздывать по сравнению с истинным началом болезни на несколько лет (2—5 и более лет).

Другим важным критерием оценки эффективности лечебных методов является продолжительность получаемых ремиссий.

Неудовлетворительные результаты курсовой терапии привели нас к выводу о необходимости проведения длительной поддерживающей терапии при хроническом лимфолейкозе, аналогично принятой большинством клиник поддерживающей терапии хронического миелолейкоза.

Учитывая необходимость длительного, по сути, пожизненного лечения больных хроническим лимфолейкозом, следует стремиться, как уже сказано, к назначению максимально эффективного и в то же время наиболее щадящего препарата.

Далеко не все из применяемых в настоящее время препаратов отвечают этому принципу.

Так, при лечении радиоактивным фосфором у многих больных развивается анемия и тромбоцитопения [2]. При лечении тиодипином и тиотэф нередко наступала стойкая тромбоцитопения с геморрагическим синдромом [4, 5]. Оценивая итоги наблюдения и лечения различными цитостатическими препаратами 100 больных хроническим лимфолейкозом, В. А. Фрейдзон приходит к выводу, что эффективность всех указанных препаратов недостаточна (ремиссии после курса лечения продолжались 3—6, редко до 12 месяцев), а их применение затруднено близостью терапевтически эффективных доз к токсическим.

В последние годы в терапевтический арсенал вошел новый препарат из группы хлорэтиламинов — лейкеран (хлорбутин, хлорамбуцил).

На основании нашего опыта мы пришли к выводу, что лейкеран является одним из наилучших цитостатических средств, применяемых в настоящее время в терапии хронического лимфолейкоза.

В большинстве случаев достигается высокая степень соответствия препарата и лейкемической клетки, благодаря чему „малой терапией" (небольшими дозами препарата, назначаемыми с интервалами) удается добиться ремиссии и приостановить прогрессирование болезни на продолжительное время.

В нашей клинике лейкеран систематически применяется с конца 1964 года. За это время им лечились 73 больных. У 16 из них лечение было начато в стационаре. Все больные были в развернутой стадии заболевания продолжительностью 3—10 лет. Отмечалось значительное увеличение периферических лимфоузлов (конгломераты размерами до 5×5—10×15 см), а нередко и наличие плотных конгломератов лимфоузлов в брюшной полости, выраженное увеличение селезенки, выступавшей из-под реберной дуги на 10—25 см, часто увеличение и уплотнение печени, высокий гиперлейкоцитоз (200 000—300 000), как правило, тенденция к анемизации (гемоглобин 60—

65 ед., эритроциты 3 000 000—3 500 000), нередко умеренный гипотромбоцитоз (80 000—120 000). Миелограмма показывала высокую степепень лимфоидной метаплазии костного мозга (70—80% лимфоцитов в пунктате) при выраженной редукции красного ростка. Больные получали ежедневно от 10 до 20 мг лейкерана в течение 2-х—4-х недель в зависимости от быстроты снижения лейкоцитоза и уменьшения размеров лимфоузлов и селезенки с последующим урежением приемов до 1—2 раза в неделю.

После выписки из стационара больные продолжают получать лейкеран в качестве поддерживающей терапии по 12—15 мг 1 раз в 7—10 дней (сроки наблюдения 1—3 года).

Большинству больных, у которых лечение начато в развернутой стадии болезни, поддерживающая терапия дает возможность длительное время сохранять работоспособность при хороших гематологических и клинических показателях: стабилизация лейкоцитоза на уровне 50 000—80 000, гемоглобина не ниже 65 ед., эритроцитов не ниже 3 500 000, отсутствие значительного увеличения лимфоузлов и селезенки.

У некоторых больных, бывших при поступлении в клинику тяжелыми инвалидами, в результате указанной терапии удалось добиться стойкой и практически полной гематологической и клинической компенсации.

Больная С., 65 лет. Хронический лимфолейкоз диагностирован в 1963 г. До 1966 г. терапия не проводилась. В ноябре 1966 г. поступила в клинику. Отмечалось значительное (до 5×10 см) увеличение всех групп периферических лимфоузлов, конгломераты лимфоузлов в брюшной полости. Селезенка выступала из-под реберного края на 15 см, печень на 6 см. Анализ крови: гемоглобин 60 ед., эритроциты — 3 200 000, лейкоциты 256 000, пал. 1%, сегм. 2%, лимф. 97%. В течение двух месяцев в клинике проводилась терапия лейкераном, начиная с дозы 20 мг в день с последующим переходом на поддерживающую терапию — 10 мг 1 раз в 5—7 дней. Одновременно в клинике получала небольшие дозы преднизолона: 20—10 мг в день. После выписки из стационара поддерживающая терапия была продолжена на протяжении двух месяцев. Через 4 месяца от начала лечения периферические лимфоузлы сократились до размеров горошины, лимфоузлы в брюшной полости не пальпируются, селезенка пальпируется в подреберье, печень выступает из-под реберного края на 2 см. Картину крови можно расценивать как показатель хорошей гематологической ремиссии: гемоглобин — 72 ед., эритроциты 4 220 000, тромбоциты 198 000, лейкоциты 6200, эоз. 2%, пал. 1%, сегм. 24% (!), лимф. 68%, мон. 5%. Больная уже в течение года не нуждается в активной терапии.

У 50 больных была применена так называемая первично-сдерживающая (профилактическая) терапия лейкераном. Мы начинали ее при определенных показаниях: относительно высоких цифрах лейкоцитоза, тенденции к анемии и тромбоцитопении, быстром увеличении или уплотнении лимфоузлов (короче говоря, при тенденции к убыстрению темпов прогрессирования заболевания по всем параметрам). При спокойном, замедленном течении заболе-

вания при удовлетворительных указанных показателях мы не считали необходимым проводить терапию.

Первично-сдерживающая терапия заключается в постоянном назначении больному 10—15 мг лейкерана 1 раз в 5—7—10 дней. Цель такой терапии: при помощи малых (но достаточных!) доз препарата добиться прекращения прогрессирования болезни и установления стабилизации процесса при удовлетворительных гематологических показателях и соматической компенсации больного.

Из 50 больных, которым была назначена первично-сдерживающая терапия, 7 получают ее только в течение 1—1,5 месяцев. Результаты их лечения еще не могут быть оценены. У 5 больных лечение проводится 2—4 месяца. Во всех случаях получен хороший непосредственный эффект. Одновременно со снижением количества лейкоцитов уменьшались размеры лимфоузлов и селезенки.

У 38 больных первично-сдерживающая лейкеранотерапия проводится от 6 месяцев до 3,5 лет.

Больные были с разными сроками заболевания: от 3 до 20 лет. До назначения лейкерана активная терапия не проводилась. Часть больных попала под наше наблюдение с развернутой картиной заболевания: лейкоцитоз достигал 100 000—150 000, у некоторых отмечалось умеренное снижение уровня гемоглобина и количества эритроцитов, у всех имела место генерализованная лимфоаденопатия и увеличение размеров селезенки. Другая часть больных наблюдалась нами в течение нескольких лет. Назначение первично-сдерживающей терапии определялось убыстрением темпов прогрессирования заболевания, ранее протекавшего по замедленному типу.

Первично-сдерживающая терапия у всех 38 больных дала положительный эффект: стабилизация клинических и гематологических показателей на удовлетворительном уровне. 29 больных (20 из них уже более 2-х лет) благодаря указанной терапии сохраняют хорошее соматическое состояние и работают. Лимфоузлы и селезенка имеют нормальные размеры или незначительно увеличены. Гематологические показатели также вполне удовлетворительные: при хороших показателях эритроцито- и тромбоцитопоэза лейкоцитоз держится на уровне 40 000—80 000.

Лейкеран у этих 29 больных является единственным применяемым лечебным препаратом. У 9 больных первично-сдерживающая лейкеранотерапия оказалась достаточно эффективной в течение 1—1,5 лет; затем все же наметилось прогрессирование болезни, и больные были госпитализированы для проведения курса лейкеранотерапии более массивными дозами, нередко в сочетании с гормональной терапией и гемотерапией или для проведения терапии другими лечебными средствами. 2 больных госпитализировались по поводу гемолитического криза. Из 9 больных у одной все виды терапии оказались неэффективными, и больная погибла на 11 году болезни. У 2 больных за время наблюдения произошла трансформация болезни в более злокачественную форму (лимфосаркома). Эти больные получают

рентгенотерапию. 6 больных после стационарного лечения продолжают получать поддерживающую лейкеранотерапию.

Мы никогда не стремимся добиться снижения количества лейкоцитов до нормальных цифр, считая это даже небезопасным для больного хроническим лимфолейкозом из-за наступающей в таких случаях глубокой нейтропении.

Таким образом, лейкеран был применен у 73 больных. У 14 не может быть произведена оценка результатов из-за недостаточных сроков наблюдения. У 59 получены хорошие непосредственные результаты. 46 из них за время лечения лейкераном (1—3,5 года) другой активной терапии не получали, проведение лейкеранотерапии дает возможность сохранить состояние больных удовлетворительным по всем параметрам, 35 больных работают. У всех больных нет признаков прогрессирования лимфоидной гиперплазии, уровень гемоглобина, эритроцитов и тромбоцитов нормален, лейкоцитоз держится в пределах 50 000—80 000. Абсолютное содержание нейтрофилов нормально или субнормально. У 7 больных для сохранения соматической компенсации и удовлетворительных гематологических показателей пришлось одновременно с лейкераном периодически назначать стероидные гормоны. 4 больных умерли. У двух наблюдается трансформация лимфолейкоза в лимфосаркому.

Таким образом, поддерживающая лейкеранотерапия позволяет получать ремиссии, превосходящие по своей длительности те, которые могут быть достигнуты при курсовом лечении любыми препаратами.

Никаких осложнений от применения лейкерана, за исключением развития гепатита у одного больного, мы не наблюдали.

В случаях злокачественного течения заболевания, когда применение лейкерана не дает желаемого эффекта, могут быть использованы менее щадящие препараты — дипин, дегранол, циклофосфан.

К рентгенотерапии мы прибегаем по строго определенным показаниям. Ее применение оправдано при наличии резко выраженного избирательного увеличения селезенки или какой-либо одной группы лимфоузлов, иногда с симптомами компрессии. В таких случаях мы прибегаем к местной рентгенотерапии (обычно в дозах 1500—2000 рентгенов) с последующим переводом больного на поддерживающее лечение лейкераном.

Рентгенотерапия, так же как химиотерапия, нами довольно широко применяется амбулаторно. Нередко больные даже не оставляют своей работы.

В последние годы произошла некоторая эволюция во взглядах на применение стероидных гормонов при хроническом лимфолейкозе: прежнее увлечение гормональной терапией сменилось трезвой оценкой возможностей и недостатков этого метода лечения.

Многие годы стремление к длительному воздержанию от цитостатической терапии поддерживалось возможностью лечения больных стероидными гормонами, исходя из их лимфолитического эффекта. Назначение

стероидных гормонов, действительно, ведет к быстрому уменьшению лимфоузлов, некоторому улучшению показателей красной крови, исчезновению адинамии.

Как правило, гормональная терапия весьма импонирует больным: поскольку исчезает адинамия, и больные становятся эйфоричными, они смешивают внешний эффект терапии (уменьшение лимфоузлов) с полноценным гематологическим эффектом.

Гормональная терапия имеет явные отрицательные стороны, главные из которых: 1. Неустойчивость достигнутого терапевтического эффекта, для поддержания которого необходимо прогрессирующее удлинение курсов терапии и повышение применяемых доз. 2. Тенденция к развитию белковой дистрофии в результате катаболического действия стероидных гормонов на белковый обмен. 3. Роль стероидных гормонов в снижении всех видов иммунитета. Наша клиника показала, что снижение иммунитета при хроническом лимфолейкозе имеет место во многих случаях до начала активной терапии и углубляется по мере развития болезни независимо от применения стероидных гормонов. Так, у 30% из 145 обследованных больных уровень сывороточного γ-глобулина был ниже 0,8 г%; у большинства гипогаммаглобулинемия обнаружена до начала какого бы то ни было лечения. Независимо от лечения и содержания γ-глобулинов в сыворотке, у 60% больных хроническим лимфолейкозом обнаружены нарушения в системе иммуноглобулинов (методом иммуноэлектрофореза). У 75% больных не получено образования антител в ответ на иммунизацию препаратом брюшнотифозного Ви-антигена. Среди этих больных были как леченные стероидными гормонами и цитостатическими препаратами, так и не получавшие никакой терапии (Э. Г. Брагина).

Все же следует признать, что длительное, нередко вынужденно беспрерывное, применение стероидных гормонов является нежелательным, так как усугубляет, несомненно, иммунологическое разоружение больных и приводит к повторным бактериальными инфекциям с тяжелым течением (пневмонии, синуситы) и к плохому заживлению самых незначительных повреждений кожи. Общеизвестны и такие неприятные осложнения длительной гормональной терапии как развитие ожирения, стероидного диабета, а иногда и более грозные — язвенные поражения желудочно-кишечного тракта.

Указанные недостатки и возможные осложнения стероидной терапии заставляют соблюдать разумную осторожность при ее применении.

Применение стероидных гормонов имеет совершенно определенные, четкие показания, основное из которых — развитие гемолитического криза, как наиболее тяжелое проявление иммунологической извращенности при хроническом лимфолейкозе. Гемолитический криз, как правило, развивается внезапно и проявляется анемией разной степени тяжести, желтухой с высоким содержанием непрямого билирубина, ретикулоцитозом и высокой лихорадкой. Положительная проба Кумбса доказывает наличие антител к эритроцитам. В таких случаях назначение стероидных гормонов (преднизолон

в дозах 30—60 мг в сутки или адекватные дозы других стероидных препаратов) является единственным эффективным средством помощи больному.

Нередко преднизолон приходится применять у больных и без аутоиммунных расстройств. Иногда под влиянием охлаждения, катара верхних дыхательных путей, а иногда и без видимых причин в очень короткой срок значительно увеличиваются лимфоузлы, чаще в области шеи, с одновременной гиперплазией вальдейерова кольца. Мягкая и тестоватая консистенция лимфоузлов свидетельствует о преобладании отека. Назначение умеренных доз стероидных гормонов (15—20 мг в день на 10—15 дней с последующей постепенной отменой) приводит к быстрому уменьшению лимфоузлов и избавляет больных от тягостных симптомов (затрудненное глотание, снижение слуха).

Некоторым больным мы назначаем преднизолон в связи с тенденцией к анемизации при нормальном количестве лейкоцитов и отсутствии заметной гиперплазии лимфоузлов и селезенки. В последнее время в подобных случаях с успехом назначаются большие дозы анаболических гормонов (метиландростендиол или неробол по 40—100 мг в день).

Наконец, некоторые больные не могут обходиться без стероидных гормонов из-за наступающей при их отмене резкой слабости, подавленности и мучительной потливости. В таких случаях мы назначаем 10—15 мг преднизолона в день на 10—12 дней с последующим снижением дозы до 5 мг, а иногда и полной отменой препарата на короткие сроки (5—10 дней), с постоянным возобновлением указанных курсов.

У многих больных хроническим лимфолейкозом ранним проявлением лейкемического процесса являются частые инфекционные заболевания: катары верхних дыхательных путей, синуситы, отиты, пневмонии. Частота инфекционных осложнений связана с иммунологической беззащитностью больных, резко сниженной их способностью к выработке антител.

Среди наших 248 больных за последние 3 года 18 человек переболели пневмонией, некоторые повторно. Пневмонии нередко носят крайне затяжной характер, имеют склонность к распространению на оба легких и ставят жизнь больных под угрозу.

Пневмонии послужили непосредственной причиной смерти у 22 из 73 больных хроническим лимфолейкозом, умерших в стационаре нашей клиники за последние 20 лет. Мы должны подчеркнуть, что это не были пневмонии терминальных больных, которые нередко имеют место при любом заболевании; некоторые больные погибали на 1—2 году заболевания.

В лечении пневмоний у больных хроническим лимфолейкозом такие антибиотики как пенициллин и стрептомицин не всегда дают желаемый эффект. В таких случаях мы, как правило, с успехом пользуемся полусинтетическими препаратами пенициллина, высокая эффективность которых объясняется отсутствием разрушающего действия на них пенициллиназы. В течение последних двух лет в нашей клинике применяются оксациллин (внутрь по 1500 мг в день), диклоксамицин (внутрь по 1000—1500 мг в день), метициллин

(внутримышечно по 500 мг 3—4 раза в сутки), кефлин (по 500 мг 2 раза в сутки внутримышечно), цепорин (внутримышечно по 500 мг 2—3 раза в сутки).

Как в периоде самого заболевания пневмонией, так и вне его, но при склонности к пневмониям, учитывая характерную для больных гипогаммаглобулинемию, периодически назначаем больным инъекции гаммаглобулина по 3,0—5,0 ежедневно или через день в течение месяца.

Развитие пневмонии не только не является поводом к отмене проводимой лейкеранотерапии, но в некоторых случаях требует ее усиления, поскольку пневмония сопровождается лейкемической инфильтрацией легочной ткани.

Таким образом, в настоящее время мы располагаем довольно широкими возможностями комплексной терапии больных хроническим лимфолейкозом, позволяющими не только избавить их от тягостных ощущений болезни и сделать работоспособными, но и значительно увеличить продолжительность жизни.

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Some Questions of Rational Therapeutical Tactics in Chronic Lymphatic Leukaemia

J. A. Kassirsky, M. A. Volkova

In the treatment of chronic lymphoid leukaemia we are confronted with the following problems: choice of the moment for the beginning of active treatment, choice of the drug, the definition of the necessary dose and possible intervals in administration of the drug. Part of the patients (about 25%) needs no active treatment for 5—20 years of disease. In treatment it is necessary to take into account not only the degree of leucocytosis but also the symptoms of visceral pathology, tendency to anaemia and thrombocytopenia. In order to decrease the danger of mutagenic effect exerted by large doses of the drug, the authors propose a primary restraining (prophylactic) therapy. It is administered during the first severe symptoms of the disease. After the end of the course of active treatment the restraining therapy is administered. Owing to such tactics the average duration of life among patients with chronic lymphoid leukaemia increased to 63 months and even more in the last years. Leukeran is the most convenient chemotherapeutic drug the for treatment of chronic lymphoid leukaemia. A good direct effect was obtained in all 75 patients treated. During the administration of the restraining therapy there were remissions for 2—4 years. The primary restraining therapy with Leukeran (10—15 mg in 5—7 days) protects patients against the progression of the disease for 1—3 years. A total of 57 patients were given such kind of treatment. Steroid hormones should not be used widely. They are indicated in haemolytic crises, considerable anaemia and thrombocytopenia. X-ray therapy and telegamma-therapy are used in severe local enlargement of lymph nodes and spleen. Semisynthetic penicillins (oxacillin, methicillin, ceporin) are effective in treatment of pneumonias which frequently develop in patients with chronic lymphoid leukaemia.

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Diabetes Insipidus Occurring with Acute Leukaemia

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Simultaneous diabetes insipidus and acute myeloid leukaemia case is reported. Cicatrix tissue was found in the neurohypophysis as a morphological basis for diabetes insipidus. In the adrenal cortex leukaemic infiltration and haemorrhage were observed, but the clinical picture and the Metopirone test did not indicate a decreased adrenal cortex function. In the course of the Metopirone test a transitory increase in the specific weight of the urine was observed.

The coincidence of acute leukaemia and diabetes insipidus is rare. Rosenzweig and Kendall [1] have collected ten cases from the literature. In addition to these ten acute myeloid or lymphoid leukaemias, Hungarian authors [2] have reported a case of diabetes insipidus occurring with chronic myeloid leukaemia; however, the symptoms of the former began during the period of the blast transformation.

Case Report

K. J., 27, male, was admitted to our service from a county hospital with the diagnosis of acute leukaemia and diabetes insipidus. In the preceding two months he had consumed 5 or 6 liters of liquid daily, and had lost 22 pounds. He complained of fatigue and low-grade fever.

Pertinent physical findings included skin pallor, enlarged tonsils and generalized adenomegaly. The edge of the spleen was palpable; no hepatomegaly was noted on admission.

Haematologic data: RBC 2.0—2.7 M; Hb, 5.8—8.7 g%; Hct, 20%; serum iron, 112 mcg%; ESR, 4—20 mm in the first hour. Reticulocytes, 38‰ (80,000). WBC, 21,000, with 40—70% blast cells; 2 to 14 nucleated red cells per 100 WBC in the peripheral blood.

Sternal bone marrow showed hypocellularity, with 55% blast cells. The blast forms appeared very immature; no granulation was observed. The ratio of erythroid precursors and the percentage of lymphocytes were elevated. No megakaryocytes were seen.

These data confirmed the diagnosis of acute leukaemia.

Liver function tests were normal. Serum sodium, 134; potassium, 3.8; chloride, 100.1 mEq/liter. Urinanalysis: specific gravity, 1000—1006. Other laboratory data were normal.

Chest X-ray examination showed normal findings. The electrocardiogram was normal.

Ophthalmologic findings: The fundus appeared pigmented in a few spots (posthaemorrhagic?). No haemorrhage could be seen in the retina. Slit-lamp examination showed pale conjunctival vessels and significant sludging. Visual fields were full. Blind spots were normal. The sella was somewhat smaller than usual, and was normal in contour.

To confirm the diagnosis of diabetes insipidus, we performed the following procedures:

1. Measurement of the 24-hour urine output. The volume was 3400—5600 ml per day; specific gravity was 1000—1006.

2. Withholding of fluids did not produce a significant increase in specific gravity (Table 1). After six hours this was discontinued because of his intolerable thirst. On that day the total urine output was 2800 ml.

Table 1

Thirsting test

Time (min)	Specific gravity	Urine output (ml)
0	1000	160
60	1002	100
120	1002	120
180	1004	120
240	1006	160
300	1006	150

3. 40 ml of 10% sodium chloride was administered intravenously. The specific gravity was not elevated, and the urine output did not decrease (Table 2). On that day the total urine output was 4800 ml.

Table 2

Hypertonic saline loading

Time (min)	Specific gravity	Urine output (ml)
0	1000	
60	1001	160
120	1003	150
180	1003	190
240	1003	140

4. Three hours after injection of Pitressin (vasopressin) tannate, the specific gravity increased to 1014. The specific gravity thereafter decreased, but in 12 hours it again became 1004. On that day, the total urine output was 2800 ml.

5. On glucose loading a definitely flat curve was obtained (62—70—60—48 mg% with Somogyi's method).

6. Metopirone test was carried out according to Fraesch [3]. Daily 4 grams were given for two days.

Table 3

Metopirone loading

	Before	During		After	
	"Base"	1st day	2nd day	1st day	2nd day
17-ketosteroid (mg)	17.6	22.4	31.6	28.2	20.4
17-ketogen steroid (mg)	18.0	51.0	66.4	56.4	41.6
Urine output (ml)	3400—5600	3800	2800	3550	4100
Specific gravity	1000	1000	1017	1010	1009

In the course of the investigation keto- and ketogen-steroid excretion was determined according to Norymbersky [4] and the volume and specific weight of urine were controlled. As it can be well seen fromTable 3, during the Metopirone test the excretion of the ketosteroids increased adequately. It was conspicuous, how the specific weight increased (1017) and the urine volume decreased (2800 ml) on the second day of the test. The data agree almost totally with those observed on the day of the administration of Pitressin tannate.

Treatment with 6-mercaptopurine, which had been administered prior to admission, was continued, which caused a decrease in white blood cells from 20,000 to 3000, and finally to 1000. One reason for this severe leukopenia was elimination of the normal elements, because the ratio of blasts remained between 40% and 70%. A few days before the patient's death, the bone marrow aspiration was repeated, and the microscopic examination showed mostly blast cells. Myelopoiesis, erythropoiesis and megakaryopoiesis were significantly diminished.

During the entire period, the patient had a low-grade fever. Transfusions became necessary because of anaemia. In the third week of hospitalization, he complained of visual disturbances. The ophthalmologist consultant found a relative central scotoma in the right field by means of a colored test object. This scotoma later disappeared spontaneously, and the patient claimed improvement in his vision. (We supposed that the visual symptoms were due to leukaemia infiltrates in the optic nerve, choroid, and/or pituitary gland.)

Four days before death, a high-grade fever developed, which could not be controlled by antibiotics and steroids. Three and a half months after the first symptoms of disease, the patient died (May 18, 1966).

Autopsy Findings

There were no changes in the heart, lungs, stomach, kidneys, thyroid gland or pancreas which could be attributed to leukaemia or diabetes insipidus. Leukaemic infiltrates were observed in the lymph nodes, spleen, liver, adrenal glands and testes. The bone marrow contained predominantly reticular elements and fat cells and relatively small amounts of myeloid precursors. Erythropoiesis and megakaryopoiesis were almost completely absent, as described above in the bone marrow aspirate. The anterior lobe of the hypophysis was normal. Gross examination of the neurohypophysis showed normal structure. However, at two points the histologic structure was changed, showing smaller cells than normal, with pyknotic nuclei. The nerve fibers had been replaced by fibrous tissue. The stalk of the hypophysis, the hypothalamus, and the rest of the brain were normal.

The adrenal glands showed the following gross pathology: The inferior portion of the left adrenal gland had diminished in thickness, and the remainder was reddish. The right adrenal gland was normal in size and structure. Microscopically the cortical zone of both adrenals was haemorrhagic. The medulla was hyperaemic and was infiltrated by myeloid cells in small focal areas.

Discussion

The presented case was a leukaemia of acute myeloid type accompanied by the symptoms of diabetes insipidus. In 10 out of the 11 cases reported so far in the literature a massive leukaemic infiltration of the neurohypophysis was detected. In these cases leukaemic infiltrations were found in the other organs as well (adenohypophysis, adrenals, kidney, liver, etc.). In 1 case the symptoms were caused by thrombosis of the small vessels in the hypophysis-hypothalamus, which was presumably a consequence of thrombocytosis, a very rare complication of the acute blastosis.

In our case cicatrix tissue could be detected in the posterior lobe of hypophysis. Leukaemic infiltration was found in the adrenals, tonsils, spleen and lymph nodes. Following features were characteristic of the patient's disease out of his diabetes insipidus symptoms:

1. The 24-hour urine output was high, the spontaneous specific weight was low.

2. On the administration of hypertonic saline (Carter-Robbins test) the diuresis did not decrease, the specific weight did not increase.

3. In the thirsting test the specific weight did not increase, diuresis did not decrease.

4. As an effect of Pitressin tannate the specific weight increased, the daily urine output decreased.

Based on these tests the diagnosis of the psychic and renal diabetes insipidus could be excluded and the existence of a real (according to the recent nomenclature: symptomatic) diabetes insipidus was regarded to be proven.

The increase of the specific weight of urine and decrease of urine output on Metopirone is worth of mentioning. Hankiss [5] carried out the Metopirone test on 5 diabetes insipidus patients and in one case he observed a decrease of diuresis. Metopirone test affects first of all the 11-beta-hydroxylase enzyme in the adrenal cortex. Due to the inhibition of this enzyme the cortisol secretion is stopped or significantly diminished. The precursors: 11-deoxycorticosterone (DOC) and 11-deoxycortisol accumulate. This means on the one hand the actual lack of the "diuretic factor" (cortisol) and on the other the enhancement of the mineralocorticoid effect. Diabetes insipidus develops only if besides the posterior lobe injury the anterior lobe function is normal [6]. The anterior lobe is effective by stimulating the hydrocortison production of the adrenal cortex [7]. The extent of polyuria is determined by the ratio of the antidiuretic hormone and the "diuretic factor" (cortisol). However, certain experimental data [8] seem to support the possibility that Metopirone has a direct effect on the central nervous system. This may also be an explanation for the change in the diuresis.

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Book Review

Handbuch der Mikroskopischen Anatomie des Menschen. VI/6. Die Milz. By F. Tischendorf. 968 pages, 365, in part coloured, illustrations. Springer-Verlag, Berlin—Heidelberg—New York 1969. DM 486.

Everybody who wants to know anything that can be known today of the development, comparative macroscopic and regional anatomy, size, microscopic anatomy, vascular system or innervation of the spleen, can take down from the shelf this bulky volume. The present volume is worthy of its foregoers and reflects faithfully the classical trends of German scientific manuals (the recent member of the traditional Handbuch series).

After a short introduction and a small chapter under the intimately archaic title: "The definition of the spleen", the above mentioned subjects are discussed thoroughly and in great detail. The illustrations, drawings, photographs, X-rays, light and electron microscopic photographs are clear and didactic. 125 pages of references containing nearly 4000 literary data are attached to the work. Orientation is made easy by an author index of 58 pages and a subject index of 89 pages.

The work is totalitarian in its requirements, and in fact it is beyond the expectation based on the title of the series. The author, striving for a relevant, integrated aspect fitting in with modern views, is far from confining the work to microscopic anatomy and descriptive morphology, but extends it time after time over the dimensions of gross anatomy. The work renders an invaluable aid to all those who wish to study the functional histomorphology of the spleen.

J. Forrai

Abstracts

Some effects of pH and temperature upon normal human red cell adenosine triphosphate. G. L. Scott, M. Oates, A. J. Grimes (Department of Haematology, St. Thomas' Hospital Medical School, London). *Scand. J. Haemat. 6*, 227 (1969).

Red cell ATP levels were determined in the fresh blood of 58 normal subjects using an enzymatic method. The values obtained were in the range of 80 to 140 μmoles of ATP per 100 ml of packed red cells. Conditions for obtaining reliable fresh blood ATP values and the effects of different anticoagulants are discussed. It has been confirmed that red cell ATP is pH-sensitive; at pH values below 7.6—7.7 (25°C) it was stable but at values above 7.7 ATP disappeared from the red cells. Kinetic studies of red cell ATP at different temperatures and pH values are described.

I. Árky

Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. R. L. Post, S. Kume, T. Tobin, B. Orcutt, A. K. Sen (Department of Physiology, Vanderbilt University School of Medicine, Nashville, Tenn.) *J. gen. Physiol. 54*, 306s (1969).

In plasma membranes of intact cells an enzymatic pump actively transports sodium ions inward and potassium ions outward. In preparations of broken membranes it appears as an adenosine triphosphatase dependent on magnesium, sodium, and potassium ions together. In this adenosine triphosphatase a phosphorylated intermediate is formed from adenosine triphosphate in the presence of sodium ions and is hydrolyzed with the addition of potassium ions. The normal intermediate was not split by adenosine diphosphate. However, selective poisoning by N-ethylmaleimide or partial inhibition by a low magnesium ion concentration yielded an intermediate split by adenosine diphosphate, insensitive to potassium ions. Pulse experiments on the native enzyme supported the hypothesis of a sequence of phosphorylated forms, the first being formed reversibly from adenosine triphosphate in the presence of sodium ion and the second formed irreversibly from the first and hydrolyzed in the presence of potassium ion. The cardioactive steroid inhibitor, ouabain, appeared to combine preferentially with the second form. Phosphorylation was at the same active site according to electrophoretic patterns of proteolytic phosphorylated fragments of both reactive forms. It is concluded that there is a conformational change in the active center for phosphorylation during the normal reaction sequence. This change may be linked to one required theoretically for active translocation of ions across the cell membrane.

Ilma Szász

The development of hormone sensitivity by the adenyl cyclase of the tadpole erythrocyte. O. M. Rosen, J. Erlichman (Departments of Medicine and Molecular Biology, Albert Einstein College of Medicine, Bronx, N. Y.). *Arch. Biochem. Biophys. 133*, 171 (1969).

Catecholamines have previously been shown to stimulate adenyl cyclase activity in haemolysates of frog erythrocytes but not

in haemolysates prepared from the erythrocytes of premetamorphic tadpoles. This observation has now been confirmed in intact erythrocytes from the frog and tadpole as well as in partially purified, particulate adenyl cyclases prepared from these cells. The development of sensitivity to stimulation by catecholamines was studied in haemolysates prepared from tadpoles undergoing either natural or thyroxine-induced metamorphosis. Catecholamine sensitivity was found to appear in erythrocytes of tadpoles which had developed front legs and were undergoing the process of tail resorption. Acrylamide gel electrophoresis of a haemolysate from this stage of development revealed a new band of haemoglobin which corresponded to the position of adult frog haemoglobin. The sensitivity of tadpole erythrocyte adenyl cyclase to activation by β-adrenergic compounds increased during the period of tail resorption and approached that of the adult frog upon completion of metamorphosis.

I. Árky

The effect of amphotericin B on erythrocyte membrane cation permeability: Its relation to in vivo erythrocyte survival. S. F. Blum, S. B. Shohet, D. G. Nathan, F. H. Gardner (Hematology Research Laboratories of the Presbyterian University of Pennsylvania Medical Center, Philadelphia, Pa.) *J. Lab. clin. Med. 73*, 980 (1969).

The polyene antibiotic amphotericin B causes an increase in the passive efflux of potassium from the human erythrocyte. This is accompanied by hyperactivity of the ouabain-sensitive membrane cation pump which results in an increased rate of active potassium movement into the cells. Adenosine triphosphate stores remain stable in these cells as glycolysis increases with a proportionate increase in lactate production. Membrane fatty acid exchange and total membrane lipid content are unaffected. The action of amphotericin B is reversible by washing the cells with modified Krebs–Henseleit buffer or with plasma. Studies performed on the erythrocytes of a patient receiving an infusion of amphotericin for treatment of Candida endocarditis revealed increased passive efflux of potassium from the cells which was completely compensated by hyperactivity of the membrane cation pump; as a result, there was no net loss of potassium from the cells. Twelve hours after the amphotericin infusion was completed, potassium kinetics had returned almost to normal. These findings of a reversible increase in membrane permeability and an adaptive increase in cation pump rate in response to it provide an explanation for the failure of amphotericin to shorten in vivo erythrocyte life-span.

G. Gárdos

Studies on the lipids of sheep red blood cells: III. The fatty acid composition of phospholipids in HK and LK cells. G. J. Nelson (Bio-Medical Division, Lawrence Radiation Laboratory, University of California, Livermore, Calif.). *Lipids 4*, 350 (1969).

The fatty acid composition of erythrocyte phospholipids has been studied in samples from five high potassium and five low potassium sheep. The total fatty acid composition, including the individual phospholipid composition in the erythrocytes of these animals is reported. There were no significant differences in total fatty acid composition or individual phospholipids between the high potassium and low potassium cells. Sheep red cells had little polyunsaturated fatty acids in their phospholipids. Palmitic, stearic and oleic acids were the major components of glyceryl phospholipids, while nervonic acid accounted for 50% of the fatty acids in the sphingomyelin fraction. The similarity between the fatty acid composition of high potassium and low potassium red cells indicates that quantitative differences in the membrane lipids are not the primary cause of the observed differences in cation levels in the two types of cells. This agrees with conclusions drawn from previous studies.

Ilma Szász

Factors governing the oxygen affinity of human adult and foetal blood. Ch. Bauer, M. Ludwig, I. Ludwig, H. Bartels (Physiologisches Institut der Medizinischen Hochschule, Hannover). *Resp. Physiol. 7*, 271 (1969).

In newborns and pregnant and nonpregnant adults, oxygen half saturation pressure

(P_{50}) of blood and of dialysed haemoglobin solutions with and without added 2,3-diphosphoglycerate (2,3-DPG) or adenosine triphosphate (ATP) has been examined. P_{50} was higher (30.5 mm Hg) in maternal blood than in the blood of the control subjects (26.1 mm Hg) and of the newborns (22.9 mm Hg). In the dialysed haemoglobin solutions of maternal and foetal blood, P_{50} fell to 19.7 mm Hg and after addition of 2,3-DPG rose by 9.7 mm Hg in the maternal dialysate and by 2.7 mm Hg in the foetal dialysate. The effect of ATP on P_{50} was less pronounced. 2,3-DPG, ATP, carbonic anhydrase activity and red cell potassium and sodium concentrations have been examined in the blood of the three groups. In maternal blood these substances were slightly but insignificantly elevated as compared to the blood of the controls. The results are discussed in respect of the different oxygen affinities of the three kinds of blood.

G. Gárdos

Binding of 2,3-diphosphoglycerate to oxyhemoglobin. J. Luque, D. Diederich, S. Grisolia (Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kan.) *Biochem. biophys. Res. Comm. 36*, 1019 (1969).

Extensive binding of 2,3-diphosphoglycerate to oxyhemoglobin has been shown by diafiltration and by Sephadex gel filtration. The average concentration of both 2,3-DPG and hemoglobin in human erythrocytes is about 5 mM; a portion of the 2,3-DPG remains bound to oxyhemoglobin and cannot be removed by Sephadex gel filtration.

G. Gárdos

Current views on histogenesis of haemopoietic tissue under conditions of transplantation. I. L. Chertkov (Labor. mielogemoterapii Centr. Inszt. Gematol. i Pereliv. Krovi, Moskva). *Probl. Gemat. 14*, 5, 14 (1969).

On the basis of data in the literature and personal experimental results the main routes of histogenesis of transplanted haemopoietic tissue are discussed. The direction of differentiation of the parent haemopoietic cells depends on the recipient's induction effect. In the case of autotransplantation, the priority is ensured to the haemopoietic direction of histogenesis, whereas under conditions of allotransplantation, owing to the differences in transplantational antigens, priority is acquired by the lymphoid direction of histogenesis, this causing acute secondary disease. The necessity of the thymic factor for lymphoid differentiation of transplanted haemopoietic cells has been proved. A possible significance of the phenomenon of allogenic inhibition in the reduction of the proliferation of the rate of allogenic haemopoietic grafts is discussed.

J. Dávid

Effect of transplantation of bone marrow preserved at —196°C on haemopoiesis of patients with depressed haemopoiesis. F. E. Fainshtein, M.-P. Bogoyavlenskaya, G. P. Orlov, Ya. S. Sukhanov, N. M. Nemenova, A. G. Fedotenkov, E. A. Zotikov, I. D. Shishkina, R. I. Rodina, R. M. Urinson (Radiol. otd., patolog.-anatom. otd., labor. konszervir. i. kultiviv. tkanei, immunogematol. labor. Centr. Inszt. Gematol. i Pereliv. Krovi, Moskva). *Probl. Gemat. 14*, 5 7 (1969).

Favourable results were obtained with automyelotransplantation in depressed haemopoiesis resulting from treatment with cytotaxic agents, as well as with allomyelotransplantation in subacute hypoplastic anaemia. Attention is called to certain peculiar features of the therapeutic action of transplantation of preserved bone marrow in comparison with freshly obtained bone marrow.

J. Dávid

Transplantation of bone marrow from several donors to dogs irradiated with 1000 r. M. N. Novikova, I. L. Chertkov (Labor. mielogemoterapii osztroj lucsevoj Centr. Inszt. Gematol. i Pereliv. Krovi, Moskva). *Probl. Gemat. 14*, 5, 24 (1969).

Bone marrow obtained from 5 to 8 unrelated donor dogs was injected into dogs

irradiated with 1000 r. In 8 of 9 such experiments there occurred only lymphoid differentiation of the transplanted elements, similarly as with the transplantation of bone marrow from a single donor. Normal haemopoiesis was observed in one of the transplants. Consequently, the occurrence of an acute graft vs. host immune reaction did not prevent normal histogenesis of the most compatible haemopoietic tissue. Only one of 56 donor-recipient combinations proved adequate. Possibilities of the clinical use of a bone marrow mixture from many donors are discussed.

J. Dávid

Survival and restoration of haemopoiesis in irradiated mice following homotransplantation of fresh and cadaveric bone marrow from several genetically different donors. N. V. Butomo (Voenno-med. akad., Leningrad). *Probl. Gemat. 14*, 5, 28 (1969).

The effect of transplantation of homologous fresh and cadaveric bone marrow obtained from one or several genetically different donors on survival and restoration of haemopoiesis in irradiated mice, has been studied. Survival of the animals was longer after transplantation of a mixture of bone marrow cells from several donors. Haemato. logical examination demonstrated ı t in transplantation of bone marrow from many donors, restoration of haemopoiesis may take place at the expense of proliferation of cells from several sources.

J. Dávid

Erythropoetyna u k obiet ciezarnych (Eryhropoietin in pregnant women). F. Przala, M. Bielecki (Katedra Fizjologii Zwierzat WSR, Olsztyn, Poland). *Acta physiol. pol. 20*, 709 (1969).

The erythropoietin level was found to be higher in healthy pregnant women than in non-pregnant ones. In pregnant women suffering from anaemia the level was still higher.

S. Maj

Ilościowa ocena nasilenia erytropoezy w oparciu o metody radioizotopowe i matematyczne (Quantitative evaluation of the intensity of erythropoiesis based on radioisotope and mathematical methods). L. Konopka, K. Rechowicz, S. Pawelski (Instytut Hematologii, Warszawa, Poland). *Pol. Tyg. lek. 24*, 843 1969).

In 45 cases of anaemia of various types and in healthy subjects, a comparative assessment of haemoglobin production was attempted using graphic and mathematical analysis of the ^{59}Fe disappearance curve in the plasma. 24-hour synthesis of haemoglobin calculated by the method of Huff et al. was in every case higher than that determined by the mathematical method of Pollycove and Mortimer.

S. Maj

Wpływ przetwori Fluidex (Dextran 40,000) na hemolize i odczyny potransfuzyjne w nocnej napadowej hemoglobinurii [The effects of Fluidex (Dextran 40,000) on haemolysis and post-transfusion reactions in nocturnal paroxysmal haemoglobinuria]. T. Struzik, M. Kowalczyk, J. Frendo (II Klinika Chorób Wevnetrznych AM, Kraków, Poland). *Pol. Tyg. lek. 24*, 1064 (1969).

Dextran 40,000 was found to reduce haemoglobinaemia, haemoglobinuria and haemosiderinuria in patients with nocturnal paroxysmal haemoglobinuria. It also prevented post-transfusion reactions. In cases displaying severe anaemia, side-effects may appear with a short-lasting increase of haemoglobinaemia and late haemoglobinuria, without any complaint or objective change.

S. Maj

Serum folic acid in patients with rheumatoid arthritis. M. Wartanowicz, M. Płachecka-Gutowska, M. Mikołajew (Institute of Rheumatology, Warsaw, Poland). *Reumatologia pol. 7*, 1 (1969).

In 26.2% of patients with rheumatoid arthritis, serum folic acid level was less than 5 μg/ml.

S. Maj

Obserwacja przeciwciał anty-Gm u chorych na gościec przewlekły postępujący (Observation of anti-Gm antibodies in rheumatoid arthritis patients). G. Wozniczko-Orłowska, J. Weryńska-Przybylska (Instytut Reumatologiczny, Warszawa, Poland). *Reumatologia pol. 7*, 127 (1969).

Anti-Gm antibodies were found in 65.4% of rheumatoid arthritis patients, and in 96.3% of the sera characterized by a positive Waaler-Rose test. In most cases in which anti-Gm antibodies were present, the inflammatory process was expressed and the pathological changes of the locomotor system were advanced.

S. Maj

Wzory linii papilarnych (dermatoglifika) u ludzi chorych na białaczki [Dactyloscopic patterns (dermatoglyphs) of patients with leukaemia]. J. Aleksandrowicz, T. Dębski, Z. Schiffer (III Klinika Chorób Wewnętrznych AM, Kraków, Poland). *Pol. Arch. Med. wewnet. 43*, 991 (1969).

In patients with leukaemia, regardless of its type, radial loops were more frequent than in healthy men and women.

S. Maj

Lactate-dehydrogenase isoenzymes in leukocytes under normal conditions and in leukosis. E. Ya. Ivanova, I. N. Molchanova (Katedr. gospitaln pediatrii Leningradsk. pediatr. medicinszk. inst. i katedr. biochemii voennomed. akademii im. C. M. Kirov, Leningrad). *Probl. Gemat. 14*, 4, 33 (1969).

The lactate-dehydrogenase enzymes of leukocytes were examined in healthy subjects and in patients suffering from various leukoses. Healthy subjects and patients with chronic leukosis contained mostly the cathode fraction of lactate-dehydrogenase. In patients suffering from chronic lympholeukaemia, the majority of the enzyme was contained in the fourth fraction. In children suffering from acute leukosis, lactate-dehydrogenase was found mainly in the third (in myeloleukosis) and in the fourth (in lympholeukosis) fractions.

J. Dávid

Trace element content of blood in chronic lympholeukosis. V. R. Soroka, I. A. Kogan (Katedr. gospitaln. terapii i Central. nautchno-isledovatel. labor. Donec. med. inst.). *Probl. Gemat. 14*, 4, 39 (1969).

Emission spectral analysis was applied for the study of the copper, manganese, silicium, aluminium and titanium content in whole blood, plasma and the formed elements of 20 healthy persons and of 100 individuals suffering from chronic lympholeukosis. In the patients with leukosis the levels were considerably different from the normal and changed in the various stages of the disease, reaching the greatest deviation from normal during the third stage. Under the effect of treatment, a tendency to normalization was noted in the blood and its fractions.

J. Dávid

Active immunizatian af children with living allogenic leukosis cells in the acute period of leukosis. S. V. Skurkovich, N. S. Kislyak, L. A. Mekhanova, S. A. Begunenko (Labor. immunol. i virusol. leikoz, Centr. Inst. Gematol. i Pereliv. Krovi, Moscow). *Probl. Gemat. 14*, 4, 14 (1969).

Assuming that the immunological system of a patient is tolerant to his own leukosis cells, twelve patients with acute leukosis were immunized in the acute period of the disease with living allogenic leukosis cells derived from the peripheral blood and bone marrow of other patients. Immunization was performed repeatedly, both intravenously and intramuscularly. All the immunized patients had leukaemic disease, and were in grave condition characterized by intoxication phenomena, enlargement of peripheral lymph nodes and parenchymatous organs, and a marked haemorrhagic syndrome. A distinct clinico-haematological improvement occurred in 8 patients during the immunization. Complete remission ensued in 3 cases. Antileukaemic treatment was prescribed to all the patients, except one with incomplete and complete remission resulting from immunization. In one child with complete haematological remission after immunization no drug treatment was prescribed. Instead,

active immunization with leukosis cells was continued. This child is still in remission.

J. Dávid

Zmiany hemostatycznych funkcji płytek u królików po diecie cholesterolowej (Changes in haemostatic functions of rabbit platelets after cholesterol diet). R. Farbiszewksi, K. Worowski, M. Szmitkowski (Katedra Chemii Fizjologicznej AM, Białystok, Poland). *Acta physiol. pol. 20*, 719 (1969).

Feeding of rabbits with cholesterol induced changes in the lipid-protein composition of serum, an increase in platelet adhesiveness, and an acceleration of platelet aggregation.

S. Maj

Porównanie wyników reakcji Brdicki z poziomem fibrynogenu i czasem fibrynolizy euglobulin osocza w rozpoznawaniu nowotworów złośliwych (Comparison of the results of Brdička reaction with the plasma fibrinogen level and euglobulin lysis time in the diagnosis of malignant neoplasms). U. Kosmala, S. Niewiadomska, Z. Plamieniak (II Klinika Chorób Wewnętrznych AM, Wrocław, Poland). *Pol. Tyg. lek. 24*, 1112 (1969).

In the majority of 32 patients with various malignant neoplasms there was an increase in the polarographic wave in the reaction of Brdička, a prolongation of euglobulin lysis time, and an increase in fibrinogen concentration.

S. Maj

From the International Litature of Haematology

Acta Haematologica (Basel) **41** (1969) No. 6

The effect of cytosine arabinoside on nucleic acid synthesis in normal and leukaemic human leucocytes *in vitro*. *Chan, B. W. B.* (Dept. of Med., Univ. of Cambridge, Cambridge), p. 321.

On the PAS reaction in acute paraerythroblastic hemoblastoses. *Leder, L.-D.* (Path. Inst. der Univ., 23 Kiel), p. 328.

Glucose-6-phosphate dehydrogenase deficiency in Italy. A study of the distribution and severity of the enzymatic defect. *Salvidio, E., Pannacciulli, I., Tizianello, A., Gaetani, G., Paravidino, G.* (Inst. of Haemat., Univ. of Genoa, 16132 Genoa), p. 331.

Vitamin B_{12} absorption test. *Arkun, S. N., Miller, I. F., Meyer, L. M.* (Long Island Jewish Hosp., Div. of Hemat., Jamaica, N. Y. 11432), p. 341.

Functional and cellular alterations produced by phytohemagglutinin. III. Hematologic findings in mice receiving PHA. *Lozzio, B. B., Machado, E. A., Chernoff, A. I.* (Univ. of Tennessee, Mem. Res. Center and Hosp., Knoxville, Tenn. 37920), p. 349.

Hereditary deficiency of peroxidase and phospholipids in eosinophilic granulocytes. *Presentey, B., Szapiro, L.* (Haemat. Dept., Health Insurance Inst. Kupat Holim, Rehovoth, Israel), p. 359.

Acta Haematologica (Basel) **42** (1969) No. 1

Absorption and excretion of cyanocobalamine after oral administration of a large dose in various conditions. *Raccuglia, G., French, A., Zarafonetis, C. J. D.* (Univ. of Louisville, School of Med., Louisville, Ky.), p. 1

Studies in cryofibrinogenemia. *Zlotnick, A., Shahin, W., Rachmilewitz, E. A.* (Dept. of Med., Hadassah Univ. Hosp. and Med. School, Jerusalem, Israel), p. 8.

Leucocyte alkaline phosphatase. The effect of age and sex. *Ray, P. K., Pinkerton, P. H.* (Dept. of Lab. Haemat., Sunnybrook Hosp., Toronto), p. 18.

Lymphopoietin. *Timson, J.* (Univ. Dept. of Med. Genet., Royal Infirmary, Manchester 13), p. 23.

Le rôle des inhibiteurs faibles du facteur VIII. *Mayer, G., Noel, G., Waitz, R.* (Centre de Transf. Sanguine, 67-Strasbourg), p. 27.

Cell-degeneration pattern in bone marrow and blood of guinea pigs following administration of cyclophosphamide. *Kaul, M., Hudson, G.* (Univ. of Sheffield, Sheffield S10), p. 42.

Histiocytic medullary reticulosis. Case report with electron microscopic study. *Zawadzki, Z. A., Peňa, C. E., Fisher, E. R.* (Depts of Med. and Path., Veterans Adm. Hosp., Pittsburgh, Pa. 15240), p. 50.

A case of acute leukemia with pseudo-Pelger cells containing Auer bodies. *Leder, L.-D.* (Path. Inst. der Univ., D-23 Kiel), p. 58.

Acta Haematologica (Basel) **42** (1969) No. 2

Studies on the pathogenesis and mechanism of hematologic diversification by re-isolation of the myeloid leukemia virus (Graffi). *Fey, F.* (Inst. of Cancer Res., Exp. Sect., 1115 Berlin), p. 65.

Chronology of the mitotic cycle of acute

leukemia cells. *Rondanelli, E. G., Magliulo, E., Pilla, G., Falchi, F., Barigazzi, G. M.* (Dept. of Intern. Med., Univ. of Pavia, Pavia), p. 76.

Distribution of amino acids in urine, plasma leucocytes and erythrocytes of leukaemic and normal subjects. *Lee, M. B., Bolger, C. D., Bridges, J. M.* (Dept. of Clin. Path., Royal Victoria Hosp., Belfast, N. Ireland), p. 86.

Suppression by RNA of human lymphocyte transformation. *Rigby, P. G., Johnson, D. M.* (Univ. of Nebraska Med. Center, Omaha, Nebr. 68105), p. 94.

The starch gel quantitative electrophoresis of haemoglobin as screening in the thalassaemia syndromes. *Ricco, G., Gallo, E., Mazza, U., Bianco, G., Prato, V.* (Ist. di Pat. Speciale Med., 10126 Torino), p. 99.

Effect of lysolecithin on bovine erythrocyte osmotic fragility. *Garfin, S. R., Shea, M. Y., Bernstein, E. F.* (Dept. of Surg., Univ. Hosp. of San Diego County, San Diego, Calif. 92103), p. 106.

Hereditary deficiency of adenylate kinase in red blood cells. *Szeinberg, A., Kahana, D., Gavendo, S., Zaidman, J., Ben-Ezzer, J.* (Dept. of Chem. Path., Gov. Hosp., Tel-Hashomer, Israel), p. 111.

Acta Haematologica (Basel) 42 (1969) No. 3

Blood proliferocytes in human and animal malaria. *Rubini, J. R., Matsui, K., Rane, L.* (Dept. of Med., Univ. of Miami School of Med., Miami, Fla. 33136), p. 129.

Enzymatic and chemical changes in human lymphocyte cultures. *Rozenszajn, L. A., Fischer, D.* (Lab. of Hemat., Meir Hosp., Kfar-Saba, Israel), p. 138.

Transketolase activity of red blood cells from infancy to old age. *Markkanen, T., Heikinheimo, R., Dahl, M.* (Dept. of Med. Microbiol., Univ. of Turku, Turku, Finland), p. 148.

Étude de la fixation non spécifique des immunoglobulines G sur les érythrocytes. I. Influence de la variation de différents paramètres. *Des Gouttes, D., Isliker, H.* (Inst. de Biochim., Univ. de Lausanne, 1000 Lausanne), p. 154.

Haemoglobin L Persian Gulf: α 57 (E6) glycine $\rightarrow$ arginine. *Rahbar, S., Kinderlerer, J. L., Lehmann, H.* (Dept. of Immunol., Univ. of Teh'ran, Teh'ran, Iran), p. 169.

Haptoglobin in thalassaemia. *Yamak, A., Özsoylu, S.* (Dept. of Haemat., Hacettepe Univ., Ankara), p. 176.

Evaluation of a dual radioisotope urinary excretion test in the diagnosis of pernicious anaemia. *Bell, T. K., Lee, D.* (Clin. Path. Lab., Royal Victoria Hosp., Belfast 12, N. Ireland), p. 183.

Blood (New York) **34** (1969) No. 4

The analysis of blood diseases with the electron microprobe. *Beaman, D. R., Nishiyama, R. H., Penner, J. A.* (Dow Chemical Co., Midland, Mich.), p. 401.

Red cell lipids in autoimmune hemolytic anemia. *Brabec, V., Michalec, Č., Palek, J., Kout, M.* (Inst. of Hematol. and Blood Transf., Prague), p. 414.

Impaired pentose phosphate shunt and decreased glycolytic activity in lymphocytes of chronic lymphocytic leukemia. Metabolic pathway? *Brody, J. I., Oski, F. A., Singer, D. E.* (Univ. of Pennsylvania, Dept. of Med., Philadelphia, Pa.), p. 421.

Immunofluorescence studies in human leukemia. *Bates, H. A., Bankole, R. O., Swaim, W. R.* (Swedish St. Barnabas Hosp., Minneapolis, Minn.), p. 430.

Elevated serum iron, low unbound transferrin and candidiasis in acute leukemia. *Caroline, L., Rosner, F., Kozinn, P. J.* (Maimonides Med. Center, Dept. of Pediat., Brooklyn, N. Y.), p. 441.

Isolated defect of folic acid absorption associated with mental retardation and cerebral calcification. *Lanzkowsky, P., Erlandson, M. E., Bezan, A. I.* (Cornell Univ., New York Hosp., Med. Coll., New York, N. Y.), p. 452.

Delayed clearance of chloramphenicol from serum in patients with hematologic toxicity. *Suhrland, L. G., Weisberger, A. S.* (Michigan State Univ., Coll. of Human Med., East Lansing, Mich.), p. 466.

Durable normal erythropoiesis in organotypic cultures of guinea pig foetal liver. *Salvatorelli, G., Gulinati, A. M., Grande, P. D.* (Univ. of Ferrara, Inst. of Comp. Anat., Ferrara, Italy), p. 472.

Regulation of erythropoiesis (XXIV). Studies

on the post-hypoxic "rebound" phase. *Shadduck, R. K., Kubanek, B., Porcellini, A., Ferrari, L., Tyler, W. S., Howard, D., Stohlman, F., Jr.* (St. Elizabeth's Hosp., Boston, Mass.), p. 477.

Studies on iron absorption. IV. Effect of humoral factors in iron absorption. *Höglund, S., Reizenstein, P.* (Karolinska Hosp., Dept. of Intern. Med., Stockholm), p. 488.

Studies on iron absorption. V. Effect of gastrointestinal factors on iron absorption. *Höglund, S., Reizenstein, P.* (Karolinska Hosp., Dept. of Intern. Med., Stockholm), p. 496.

Studies on iron absorption. VI. Transitory effect of oral administration of iron on iron absorption. *Höglund, S.* (Karolinska Hosp., Dept. of Intern. Med., Stockholm), p. 505.

Studies on platelet proteins. IV. Some physical and chemical properties of thrombosthenin. *Ganguly, P.* (Amer. Nat. Red Cross, Blood Res. Lab., Bethesda, Md.), p. 511.

Blood (New York) **34** (1969) No. 5

Serologic specificity of human anti-IgA and its significance in transfusion. *Vyas, G. N., Holmdahl, L., Perkins, H. A., Fudenberg, H. H.* (Univ. of California, School of Med., Los Angeles, Calif., 90024), p. 573.

Intravascular granulocyte kinetics in acute leukemia. *Spivak, J. L., Brubaker, L. H., Perry, S.* (Nat. Cancer Inst., Human Tumor Cell Biol. Branch, Bethesda, Md. 20014), p. 582.

The separation of normal human leukocytes by density and classification by size. *Zucker, R. M., Cassen, B.* (Univ. of California, Lab. of Nuclear Med., Los Angeles, Calif. 90024), p. 591.

Citrate stabilization of chromatographically purified factor VIII. *Hynes, H. E., Owen, C. A., Jr., Bowie, W. E. J., Thompson, J. H., Jr.* (Scott and White Clinic, Sect. Hemat., Temple, Texas 76501), p. 601.

Development of a myeloproliferative disorder in beagles continuously exposed to ^{90}Sr. *Dungworth, D. L., Goldman, M., Switzer, J. W., McKelvie, D. H.* (Univ. of California, School of Vet. Med., Davis, Calif. 95616), p. 610.

Hematopoiesis and serum iron changes following vincristine sulfate. *Nesbit, M. E., Jr., Lowman, J. T.* (Univ. of Minnesota Med. Center, Minneapolis, Minn. 55455), p. 633.

The monovalent cation content and adenosine triphosphatase activity of human normal and leukemic granulocytes and morphologic age. *Lichtman, M. A., Weed, R. I.* (Univ. of Rochester, School of Med. and Dent., Rochester, N. Y. 14627), p. 645.

Red cell iso-antigens of rhesus monkeys. *Hirose, Y., Balner, H.* (Univ. of Tokyo, Dept. of Surg., Shibashirokane, Tokyo), p. 661.

A study of the sickling phenomenon and oxygen dissociation curve in patients with hemoglobins SS, SD, SF and SC. *Cawein, M. J., O'Neill, R. P., Danzer, L. A., Lappat, E. J., Roach, T.* (Univ. of Tennessee, Memorial Res. Center, Knoxville, Tenn. 37916), p. 682.

Brief Report: Prolonged intravenous iron-dextran therapy in a patient with multiple hereditary telangiectasia. *Chernelch, M., Winchell, H. S., Pollycove, M., Sargent, T., Kusubov, N.* (Gen. Hosp., Maribor, Yugoslavia), p. 691.

Radioautographic studies of the lymphocytic bone marrow. *Griffiths, D. A.* (Royal Infirm., Dept. of Surg., Bristol), p. 696.

Brief Report: Plasma activity of antihemophilic globulin (AHG) and other coagulation factors in Swiss type of agammaglobulinemia. *Maurer, H. M., Valdes, O., Shumway, C. N., Massie, F. S.* (Med. Coll. of Virginia, Dept. of Pediat., Richmond, Va. 23219), p. 701.

Brief Report: Cytosine arabinoside therapy for disseminated herpes zoster in a patient with IgG pyroglobulinemia. *McKelvey, E. M., Kwaan, H. C.* (Northwestern Univ., Med. School, Chicago, Ill. 60612), p. 706.

Brief Report: Lipid peroxidation in aging platelets. *Okuma, M., Steiner, M., Baldini, M.* (Memorial Hosp., Div. of Hemat. Res., Pawtucket, R. I.), p. 712.

Blut (München) **19** (1969) No. 8

Autoradiographische und hämatologische Untersuchung zur Zellkinetik der Erythro-

blasten im Kurzzeitversuch nach doppelseitiger Nephrektomie und Ureterligatur bei Mäusen. *Klein, H. O., Lennartz, K. J., Eder, M., Gross, R.* (Med. Univ.-Klinik, 5 Köln-Lindenthal), p. 449.

Beziehungen zwischen enzymatischer Formiataktivierung und Erythrozytenlebensdauer. *Wilmanns, W., Sauer, H., Gelinsky, P.* (Med. Univ.-Klinik, 74 Tübingen), p. 457 .

Ultrastrukturelle Zell- und Organveränderungen nach Verabreichung eines heterologen Antilymphozytenserums bei der Ratte. *Land, W., Rudolph, R., Brendel, W.* (Inst. f. exp. Chirurg., 8 München 15), p. 470.

Chediak—Steinbrinck-Anomalie. *Borsai, G., Szentkirályi, I., Metz, O. B.* (Centrul de Transf., Tirgu Mureş, Rumänien), p. 482.

Über Deduktionen in der formalen Serologie, aufgezeigt am Beispiel der Helix-Agglutinine. *Uhlenbruck, G.* (Med. Univ.-Klinik, Köln), p. 487.

Blut (München) **19** (1969) No. 9

Les inhibiteurs faibles du facteur VIII chez les hémophiles A. *Mayer, G., Noel, G., Kalogjera, V., Waitz, R.* (Inst. d'Hémat., Centre Reg. de Transf. Sanguine, Strasbourg), p. 517.

Eine neue Methode zur Bestimmung der DNS-Synthesegeschwindigkeit von Knochenmarkzellen in vitro. *Brinkmann, W., Dörmer, P., Muschalik, P.* (Inst. f. Hämat., 8 München 15), p. 529.

Changes over time in blood basophil counts of X-irradiated rabbits. *Thonnard-Neumann, E., Taylor, W. L., Nosan, M.* (Saint Elizabeth's Hospital, Washington, D. C. 20032), p. 537.

Untersuchungen zur Bedeutung der Milz im immunologischen Geschehen. *Sundermann, A., Meister, H., Mey, U.* (Med. Klinik der Med. Akad., X 50 Erfurt), p. 544.

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The effects of whole body irradiation on serum colony stimulating factor and *in vitro* colony-forming cells in the bone marrow. *Hall, B. M.* (Cancer Res. Unit., Walter and Eliza Hall Inst., Royal Melbourne Hosp., Victoria, Australia),p. 553.

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Correspondence: Inhibitor of factor VIII in mild haemophilia. *Beck, P., Giddings, J. C., Bloom, A. L.* (Inst. of Path. and Med. Unit, Royal Infirm., Cardiff, Wales), p. 107.

Folia Haematologica (Leipzig) **92** (1969) No. 2

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Untersuchungen über die Stabilität der ABO-Isoagglutinine gegenüber Trypsin. *Haferland, W.* (Inst. f. gerichtl. Med., Humboldt-Univ. zu Berlin, 25 Rostock), p. 227.

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Nouvelle Revue Française d'Hématologie (Paris) **9** (1969) No. 4

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Société Française d'Hématologie — Séance consacrée à la Cytochimie. 16 Juin 1969.

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Société Nationale de Transfusion Sanguine — Séance du 4 Mars 1969.

Scandinavian Journal of Haematology (Copenhagen) **6** (1969) No. 6

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The use of ^{75}Se-methionine for the in vivo study of platelet kinetics. *Najean, Y., Ardaillou, N.* (Inst. de Recherches sur les Maladies du Sang., Hôpital Saint-Louis, Paris 10e), p. 395.

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HL—A genotypes of children with acute leukaemia. A family study. *Thorsby, E., Bratlie, A., Lie, S. O.* (Blood Bank and Dept. of Immunohaemat., Ullevål Hosp., Oslo 1), p. 409.

Protein abnormalities in the cold haemagglutinin syndrome. *Harboe, M., Torsvik, H.* (Inst. for Exp. Med. Res., Ullevål Hosp., Oslo 1), p. 416.

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Introduction. Leukemia and lymphoma, II. *Holland, J. F.* (Roswell Park Memorial Inst., Buffalo, N. Y.), p. 321.

Chemotherapy of chronic myelocytic leukemia. *Galton, D. A. G.* (Chester Beatty Res. Inst., Inst. of Cancer Res., London), p. 323.

The treatment of chronic lymphocytic leukemia. *Silver, R. T.* (Div. of Hemat., New York Hosp., Cornell Med. Center, New York, N. Y.), p. 344.

Modern approaches to the radiotherapy of lymphoma. *Johnson, R. E.* (Nat. Cancer Inst., Nat. Inst. of Hlth., Bethesda, Md.), p. 357.

The therapy of lymphoma. *Ultmann, J. E., Nixon, D. D.* (Dept. of Med., School of Med., Univ. of Chicago, Chicago, Ill.), p. 376.

Thrombosis et Diathesis Haemorrhagica (Stuttgart) **22** (1969) No. 2

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Abnormal fibrin stabilization in renal failure. *Losowsky, M. S., Walls, W. D.* (Dept. of Med., Univ. of Leeds, Leeds, England), p. 216.

The influence of various plasma components on the lysis of dilute human blood clots. *Gallimore, M. J., Shaw, J. T. B.* (Therap. Res. Div., Pfizer Ltd., Sandwich, Kent, England), p. 223

The purification of fibrinogen degradation products by Pevikon block electrophoresis. *Marder, V. J., James, H. L., Sherry, S.* (Dr. Theodore H. Mendell Res. Lab., Temple Univ. School of Med., Philadelphia, Pa. 19140), p. 234.

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Inactivation of human serum plasminogen antiactivator by synthetic fibrinolysis inducers. *Aoki, N., von Kaulla, K. N.* (Dept. of Med., Univ. of Colorado School of Med., Denver, Col.), p. 251.

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Coagulation studies of cord blood, with special reference to "fetal fibrinogen". *von Felten, A., Straub, P. W.* (Dept. of Intern. Med., Kantonspital, Univ. of Zurich, Zurich), p. 273.

The estimation of blood platelet survival. I. General principles of the study of cell survival. *Murphy, E. A., Francis, M. E.* (Depts of Med. and Biostat., Johns Hopkins Univ. School of Med., Baltimore, Md. 21205), p. 281.

Some new observations on glycogenolysis in blood platelets studied by cytochemical scoring method. *Heřmanský, F., Caen, J., Matoušová, O.* (I. Med. Clinic, Charles Univ. of Prague, Prague), p. 296.

Platelet incorporation of labelled adenosine and adenosine diphosphate. *Salzman, E. W., Ashford, T. P., Chambers, D. A., Neri, L. L.* (Dept. of Surg., Harvard Med. School, Boston, Mass.), p. 304.

Congenital combined deficiency of factor V and factor VIII. A case report and the effect of transfusion of normal plasma and hemophilic blood. *Saito, H., Shioya, M., Koie, K., Kamiya, T., Katsumi, O.* (I. Dept. of Intern. Med., Nagoya Univ. School of Med., Tsurumai-cho, Showa-ku, Nagoya, Japan), p. 316.

The coagulation system during the passage of blood through the body. Simultaneous determination of thrombocyte count and 9 plasma activities in samples from 5 different blood vessels in the rabbit. *Schimpf, Kl., Barth, P., Bartman, J., Freienberg, J., Hansen, B., Meier, J.* (Univ. of Heidelberg, Med. Dept., Heidelberg), p. 326.

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Zur Pathogenese obliterierender Arterienprozesse bei Hypercholesterinämie. *Frost, H.* (Med. Poliklin., Univ. München, München,) p. 351.

The disappearance of fibrin from the pulmonary vessels in experimental fat embolism. *Saldeen, T.* (Inst. of Forensic Med., Univ. of Lund, Lund), p. 360.

The effect of chronic administration of lathyrogen (beta-aminopropionitrile) on blood clotting and fibrinolytic system in rabbits. *Bańkowski, E., Worowski, K.* (Dept. of Physiol. Chem., Med. School, Białystok, Poland), p. 372.

Über die fibrinolytische Aktivität von Rinderprothrombinpräparaten nach Seegers. Ein Beitrag zur Frage der Aktivierung von Rinderprothrombin durch Plasmin. *Andrassy, K., Egbring, R., Egli, H.* (Abt. f. Gerinnungsphysiol. u. Bluttransfusionswesen, Univ. Bonn, Bonn), p. 377.

Bleeding time and surgical blood losses in non-hemorrhagic people. *Roskam, J.* (Univ. of Liège, Liège), p. 391.

Ein möglicher Mechanismus der Desaggregation ADP-induzierter Aggregate durch Imipramin und strukturverwandte Verbindungen. *Reuter, H., Linker, H., Gross, R.* (Med. Univ.-Klinik, Köln-Lindenthal), p. 396.

Fibrin crosslinking demonstrated by thrombelastography. *Tyler, H. M.* (Therap. Res. Div., Pfizer Ltd., Sandwich, Kent, England), p. 398.

Demonstration of antiparacoagulating activ-

ity of acid mucopolysaccharides and extracts of the aortic wall. *Lipiński, B.* (Dept. of Biochem., Postgrad. Med. School, Warsaw 45), p. 401.

Have the platelet antiheparin and antiplasmin activities the same support? *Moriau, M.* (Dept. of Intern. Med., Univ. of St-Pierre, Louvain), p. 403.

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Thrombosis et Diathesis Haemorrhagica (Stuttgart) **22** (1969) No. 3

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Studies on blood coagulation factor V. I. The interaction of salts of fatty acids and coagulation factors. *Kahn, M. J. P., Hemker, H. C.* (Lab. for Coagul. Biochem., Univ. Hosp., Leiden), p. 417.

Thrombosthenin. Electron microscopical studies on its localization in human blood platelets and some properties of its subunits. *Bettex-Galland, M., Lüscher, E. F., Weibel, E. R.* (Theodor Kocher Inst., Univ. of Berne, Berne), p. 431.

Platelet reactions and immune processes. I. Activation of complement by several platelet-activating surfaces when coated with gammaglobulin; mechanism of complement fixation. *Jobin, F., Tremblay, F.* (Dépt. de Biochim., Univ. Laval, Hôpital du Saint-Sacrement, Québec, Canada), p. 450.

Platelet reactions and immune processes. II. The inhibition of platelet aggregation by complement inhibitors. *Jobin, F., Tremblay, F.* (Dépt. de Biochim., Univ. Laval, Hôpital du Saint-Sacrement, Québec, Canada), p. 466.

The effect of purified *Vipera palestinae* hemorrhagin on blood coagulation and platelet function. *Grotto, L., Jerushalmy, Z., de Vries, A.* (Rogoff-Wellcome Med. Res. Inst., Tel-Aviv Univ. Med. School. Beilinson Hosp., Petah Tikva, Israel), 482.

Accumulation of macromolecular particles in the reticuloendothelial system (RES) mediated by platelet aggregation and disaggregation. *van Aken, W. G., Vreeken, J.* (Central Lab. of The Netherlands Red Cross Blood Transf. Service, Amsterdam), p. 496.

Reticuloendothelial function and blood coagulation in rats. *Pechet, L., Pechet, G. S., MacDonald, R. A.* (Depts of Path. and Med., Boston Univ. School of Med., Boston, Mass. 02130), p. 508.

Über die Wirkung von Lipidemulsion auf Blutplättchen. I. Zunahme der Plättchenaggregation unter dem Einfluß verschiedener Lipidemulsionen *in vitro*. *Pfleiderer, Th., Morgenstern, E., Weber, E.* (Med. Univ.-Klinik, Heidelberg), p. 513.

Über die Wirkung von Lipidemulsionen auf Blutplättchen. II. Licht- und elektronenmikroskopische Studien. *Morgenstern, E., Pfleiderer, Th., Zebisch, P., Weber, E.* (Inst. f. Zytologie u. Elektronenmikroskopie, Univ. des Saarlandes, Homburg, BRD), p. 525.

On the role of the stable plasminogen activator of the venous wall in occlusion induced fibrinolytic state. *Constantini, R., Spöttl, F., Holzknecht, F., Braunsteiner, H.* (Dept. of Intern. Med., Univ. of Innsbruck, Innsbruck), p. 544.

Die Beeinflussung der Wirkung des Trypsins auf die Blutgerinnung durch natürliche und synthetische Trypsin- und Thrombininhibitoren. *Landmann, H., Markwardt, F., Perlewitz, J.* (Inst. f. Pharmakol. u. Toxikol., Med. Akad., Erfurt), p. 552.

The role of the mast cell in thrombolysis induced by heparin and a heparin-like substance SP 54. *FitzGerald, D. E.* (Dept. of Med., Guy's Hosp. Med. School, London, S. E. 1), p. 569.

Untersuchungen über die Entstehung von Ammoniak im Zitratplasma nach Rekalzifierung. *Wenzel, E., Christ, G.* (Zentrum der Inneren Med., Univ. Frankfurt., Frankfurt. a. M.), p. 575.

Purification of the antihemophilic factor by gel filtration on agarose. *Paulssen, M. M. P., Wouterlood, A. C. M. G. B., Scheffers, H. L. M. A.* (Dept. of Clin. Biochem., St. Clara Hosp., Rotterdam), p. 577.

Transfusion (Philadelphia) **9** (1969) No. 5

Changes in physical properties of stored erythrocytes: relationship to survival *in vivo*. *Haradin, A. R., Weed, R. I., Reed, C. F.* (220 Meyran Ave., Pittsburgh, Pa), p. 229.

Alteration of deformability of the erythrocyte membrane in stored blood. *La Celle, P. L.* (Univ. of Rochester, School of Med. and Dent., Rochester, N. Y.), p. 238.

Clinical evaluation of transfused blood after long-term storage in ACD with adenine. *Shields, C. E., Bunn, H. F., Litwin, S. D., Reed, L. J., Dauber, L. G.* (US Army Med. Res. Lab., Fort Knox, Ky.), p. 246.

Pooled platelet concentrates prepared in a new blood bank centrifuge. *Miller, W. V., Gillem, H. G., Yankee, R. A., Schmidt, P. J.* (Univ. of Missouri, School of Med., Dept. of Pathol., Columbia, Mo.), p. 251.

Aspects of a computer-assisted system for calling blood donors from a specialized panel. *Hopkins, D. F., Milne, G. R.* (Law Hosp., Carluke, Scotland), p. 255.

Cryoprecipitates in treatment of congenital fibrinogen deficiency: case report. *Hattersley, P. G., Dimick, M. L.* (Sacramento Med. Center, Sacramento, Calif.), p. 261.

Anti-Gn^a, a new antibody reacting with a high-incidence erythrocyte antigen. *Fox, J. A., Taswell, H. F.* (Univ. of Minn., Mayo Graduate School of Med., Rochester, Minn.), 265.

Vox Sanguinis (Basel) **17** (1969) No. 4

The En^a antigen and antibody. A genetical modification of human red cells affecting their blood grouping reactions. *Darnborough, J., Dunsford, I., Wallace, J.* (Reg. Transf. and Immunol. Haematol. Centre, Cambridge), p. 241.

The red cell phenotype En(a-) and anti-En*s*: serological and physicochemical aspects. *Furuhjelm, U., Myllylä, G., Nevanlinna, H. R., Nordling, S., Pirkola, A., Gavin, J., Gooch, A., Sanger, R., Tippett, P.* (Finnish Red Cross Blood Transf. Service, Helsinki), p. 256.

Effect of interferon on the course of spontaneous and radiation-induced renal lesions in the RF/Un mouse. *Guttman, P. H., Davis, W., Fudenberg, H. H., Merigan, T. C.* (Univ. of California, Med. Center, San Francisco, Calif.), p. 279.

Solubilized glycoprotein from human erythrocyte membranes possessing blood group A, B and H activity. *Whittemore, N. B., Trabold, N. C., Reed, C. F., Weed, R. I.* (Montreal Gen. Hosp., Dept. of Med., Montreal), p. 289.

N^k and M^g: some serological and physicochemical observations. *Nordling, S., Sanger, R., Gavin, J., Furuhjelm, U., Myllylä, G., Metaxas, M. N.* (Univ. of Helsinki, Third Dept. of Pathol., Helsinki), p. 300.

Pt^a, a new private antigen. *Pinder, L. B., Staveley, J. M., Douglas, R., Kornstad, L.* (Blood Transf. Center, Auckland, New Zealand), p. 303.

Rh antibodies in liquor amnii. *Murray, S.* (Reg. Transf. Center, Newcastle upon Tyne, England), p. 306.

Low incidence of erythrocyte G-6-PD deficiency in aborigines of Taiwan. *Blackwell, R. Q., Blackwell, B.-N., Yen, L., Lee, H.-F.* (U. S. Naval Med. Res. Unit No. 2, APO 14, San Francisco, Calif), p. 310.

Serological findings on quadrizygotic quadruplets. *Mayr, W. R., Pausch, V., Mickerts, D.* (Univ. of Vienna, Inst. of Blood Group Serol., Vienna), p. 314.

New factors and alleles in the M blood-group system of the pig. *Dinklage, H., Major, F.* (Univ. of Göttingen, Inst. of Animal Res., Göttingen, GFR), p. 316.

Vox Sanguinis (Basel) **17** (1969) No. 5

A detailed serological study on the prediction and diagnosis of ABO haemolytic disease of the newborn (ABO HD). *Voak, D., Bowley, C. C.* (Reg. Blood Transf. Centre, Sheffield S10, England), p. 321.

Rapid clearance of Rh positive blood during experimental Rh immunisation. *Woodrow, J. C., Finn, R., Krevans, J. R.* (Nuffield Unit of Med. Genet., Dept. of Med., Univ. of Liverpool, Liverpool), p. 349.

Clearance of Rh-positive foetal erythrocytes from the blood of Rh-negative adults by intravenously applicable immunoglobulin G and anti-D. *Börner, P., Deicher, H., Hoppe, H. H., Pixberg, H. U., Sieg, W.* (Med. Hochschule, D-3000 Hannover, GFR), p. 362.

Value of adenine, various nucleosides, and ouabain in maintenance of integrity of red blood cells under blood bank conditions. *Odake, K., Bishop, C., Warner, W., Ambrus, J. L.* (Roswell Park Mem. Inst., Buffalo, N. Y. 14203), p. 375.

Antigenic markers on cultured human cells. III. The MN antigens. *Dawson, A.* (Dept. of Path., Univ. of Cambridge, Cambridge), p. 393.

Serotyping for homotransplantation XXVIII sib-pair assay for nonHL-A antigens. *Mittal, K. K., Mickey, M. R., Terasaki, P. L.* (Dept. of Microbiol., Baylor Coll. of Med., Houston, Tex. 77025), p. 406.

Stability of immune serum globulin during storage: effects of prior heating. *Art, G. P., Finlayson, J. S.* (Div. of Biol. Stand., Nat. Inst. of Hlth., Bethesda, Md. 20014), p. 419.

Stability of immune serum globulin during storage: effects of modifications in the fractionation scheme. *Painter, R. H., Minta, J. O.* (Dept. of Biochem., Univ. of Toronto, Toronto 181, Ont., Canada), p. 434.

Immunofixation electrophoresis: a technique for the study of protein polymorphism. *Alper, C. A., Johnson, A. M.* (Blood Grouping Lab., Boston, Mass.), p. 445.

A simple method for freezing of lymphocytes with retained viability. *Elgeklint, G., Högman, C. F., Åkerblom, O., Öberg, L.* (Blood Center, Univ. Hosp., S-750, 14 Uppsala), p. 453.

First example of Ny^a blood group antigen in American population. *Pineda, A. A., Taswell, H. F.* (Mayo Clinic and Mayo Found., Rochester, Minn.), p. 459.

Inability of Ag/anti-Ag complexes to fix complement and its significance. *Bütler, R., Brunner, E.* (Central Lab., Blood Transf. Service, Swiss Red Cross, CH-300 Berne), p. 462.

Absence of close linkage between the locus for Xg and the locus for antihydrotic ectodermal dysplasia. *Simpson, J. L., Allen, F. H., Jr., New M., German, J.* (New York Blood Center, New York, N. Y. 10021), p. 465.

Blood group antibody notation. *Bird, G. W.* (Reg. Blood Transf. Service, Birmingham), p. 468.

Vox Sanguinis (Basel) **17** (1969) No. 6

The pathogenesis of ABO haemolytic disease of the newborn. *Voak, D.* (Reg. Blood Transf. Centre, Sheffield S101QT, England), p. 481.

A serum agglutinin inhibited by ionized calcium. *Gunson, H. H.* (Blood Transf. Service, Lancaster Centre, Lancaster, England), p. 514.

Reactivity of human gammaG erythrocyte autoantibodies with fetal, autologous and maternal red cells. *Leddy, J. P.* (Dept. of Med., Univ. of Rochester Med. Center, Rochester, N. Y. 14620), p. 525.

The correlation of the Bg^a blood group with the HL-A7 leucocyte group: demonstration of antigenic sites on red cells and leucocytes. *Morton, J. A., Pickles, M. M., Sutton, L.* (Radcliffe Infirmary, Oxford), p. 536.

Card Indexes

J. Ph. H. B. Sybesma, M. C. Verloop

Diagnostic difficulties in a patient with jaundice. Haematologia 4, 131 – 139 (1970).

In a 40-year-old jaundiced anaemic woman, ill with fever, polyneuropathy and loss of hair, fulminating haemolysis was identified as the cause of anaemia. The possible causes of this haemolysis have been discussed. The jaundice could not be explained solely on the basis of the haemolysis. Hepatitis was possible; later E. coli sepsis was found, caused probably by cholangitis. Adequate control of the sepsis with antibiotics was followed by improvement of liver function and of the haemolysis. At a subsequent operation, the bile ducts were drained and a liver biopsy was performed. The biopsy specimen showed signs of cholangitis and extrahepatic cholestasis. The patient made a complete recovery after the operation.

S. R. Hollán, J. G. Szelényi, M. Miltényi, D. Charlesworth, P. A. Lorkin, H. Lehmann

Unstable haemoglobin disease caused by Hb Santa Ana-β88 (F4) Leu → Pro. Haematologia 4, 141 – 155 (1970).

Clinical and haematological data, special red cell characteristics and studies of the haemoglobin structure of an unstable haemoglobin haemolytic anaemia (UHHA) are reported. The instability of the patient's haemoglobin originates from a β88 (F4) Leu → Pro mutation, and thus is together with one observed in France a further example of the unstable Hb Santa Ana. Special red cell studies revealed deranged non-electrolyte permeability, slightly lower GSH, and relatively low ATP content of the patient's red cells. Characteristics of foetal-type erythropoiesis: increased red cell electrophoretic mobility, increased G6PD activity and changes in the lipid composition of the red cell membrane resembling those found in cord blood cell could be detected.

A. S. Wiener

Immunochemical studies on group-specific agglutinins of diverse origin. Haematologia 4, 157 – 166 (1970).

The agglutinin anti-A_{HP} from the snail *Helix pomatia* which gives reactions paralleling those of other anti-A reagents in tests on human blood was inhibited by N-acetyl-D-galactosamine and to a lesser extent by N-acetyl-D-glucosamine. The agglutinin anti-Ag from the snail *Achatina granulata* was inhibited by all three amino sugars, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and N-acetyl-D-mannosamine. Neither anti A_{HP} nor anti-Ag was inhibited by any of the many other simple sugars tested. Comparative quantitative inhibition tests were carried out with a number of different agglutinating reagents having A specificity in tests on human blood and saliva. The reagents included anti-A_{HP} snail agglutinin, anti-A_1 lectin *(Dolichos biflorus)*, anti-A lectin (lima bean), anti-A isoagglutinin of human and rhesus monkey serum, and human anti-A serum absorbed with A_2 cells. The results while supporting the predominant role of N-acetyl-D-galactosamine for A specificity, also demonstrated qualitative differences among the reagents. The probable basis for these qualitative differences is discussed.

J. Gidáli, I. Fehér

Factors influencing growth and differentiation of spleen colonies. II. Erythropoietin and Actinomycin D. Haematologia 4, 167–176 (1970).

The effect of erythropoietin and Actinomycin D (AD) on the colony-forming ability and differentiation of transplanted colony-forming cells has been studied. When treating the recipients with erythropoietin from the 4th postirradiation day, erythroid colony count and ^{59}Fe-incorporation increased significantly. Erythropoietin given earlier was ineffective. A correlation was found between the dose of erythropoietin and the erythroid colony count. Actinomycin D selectively inhibited the development of erythroid colonies without affecting the granuloid colony count.

A. Ya. Yaroshevsky, V. N. Petrov, M. M. Scherba, V. I. Kalinin, E. N. Mikhajlova

Diagnosis and prophylaxis of iron deficiency in donors. Haematologia 4, 177–185 (1970).

The indices of iron metabolism were studied in 475 permanent donors to expose a latent iron deficiency, i.e. the improvement of the organism in iron without anaemia. The criteria of this state were, a decrease in serum iron concentration below 80 μg per 100 ml with a simultaneous increase of unsaturated iron-binding capacity to above 260 μg per 100 ml. Such a combination was found in 32% of the examined subjects, in 40% of the women and in 12.30% of the men. A good correlation was found between the unsaturated iron binding capacity and the number of phlebotomies. There was a significant decrease in succinate dehydrogenase activity in the epithelium of the cheek mucous membrane, and in the cytochrome oxidase content of the blood cells of the donors in comparison with control healthy subjects. A stable increase of the unsaturated iron-binding capacity in serum and a decrease of cytochrome oxidase activity in blood cells were the most reliable indices of iron deficiency in the donors. The treatment of these subjects with iron was followed by complete recovery.

R. Farbiszewski, Z. Skrzydlewski

Platelet adhesiveness and aggregation in the final stage of pregnancy and in the puerperium. Haematologia 4, 187–190 (1970).

The adhesiveness and aggregation of blood platelets have been studied in the final stage of pregnancy and in the puerperium. A statistically significant enhancement of blood platelet adhesiveness in the final stage of pregnancy with an accompanying increase in the beta-lipoprotein level was found. In the early puerperium there was a decrease in platelet adhesiveness with a partial decrease in the beta-lipoprotein level. Similar changes were observed in the ADP induced aggregation time of platelets. The significance of the above findings for the pathogenesis of thrombotic diseases in pregnancy and puerperium and a hypothesis about the interaction of beta-lipoproteins with certain receptor sites on the platelet surface are discussed.

F. Krizsa, G. Gergely, K. Rák

Mechanism of thrombocytosis following splenectomy in the mouse. Haematologia 4, 191 – 194 (1970).

Following splenectomy in the mouse, the circulating thrombocyte count increased, to exceed the initial count by 71% on the 8th day. Subsequently the count decreased to reach the initial value on the 21st day. In the number of bone marrow giant cells there was a slight decrease on the 5th and 8th days. The serum of splenectomized mice caused no appreciable change of the thrombocyte count in the usual mouse test. Postsplenectomy thrombocytosis is believed to be due to the loss of the spleen's storing function and not to an increased medullary thrombocytopoiesis.

E. G. Rondanelli, E. Magliulo, S. Petrocini, G. C. Fossati

Visual evidence of mitosis in splenic and peritoneal macrophages of the mouse and of the newt Molge vulgaris. Phase-contrast cine-micrographic investigations. Haematologia 4, 195 – 202 (1970).

Phase-contrast cine-micrographic recordings yielded visual evidence of mitoses occurring in well-differentiated newt and mice macrophages performing marked phagocytic activity. This is in good agreement with the concept that peritoneal and tissue culture macrophages are not end-cells and may resume their mitotic activity under certain experimental conditions.

A. Döklen, V. Várterész, L. Varga

Effect of haematopoietic humoral factors on PHA-transformed lymphocytes. I. Haem-synthesis in PHA-stimulated lymphocyte cultures. Role of factors controlling erythropoiesis. Haematologia 4, 203 – 211 (1970).

Cells displaying the morphological characteristics of polychromatic and orthochromatic normoblasts were found in phytohaemagglutinin-transformed, 72- and 96-hour cultures. In these cultures, supravital staining revealed the presence of reticulocytes in different maturation phases. Haem-synthesis, as assessed by ^{59}Fe-incorporation, was proceeding in the cultures. It was inhibited or stimulated by humoral factors known to inhibit and/or to stimulate erythropoiesis, when added to the cultures 24 hours after phytohaemagglutinin. These same erythropoietic factors failed to interfere with haem-synthesis when added to the cultures simultaneously with phytohaemagglutinin. The mononuclear cell in the circulation did not seem to synthesize haem and was insensitive to the erythropoietic effect. If pretreated with phytohaemagglutinin, it began to synthesize haem and became responsive to erythropoietic factors. The findings did not permit to decide whether all the mononuclear cells or only a certain type of them had the said ability, nor whether the responsive cell will differentiate into a certain or into several lines.

J. Lisiewicz, J. Okulski

Thromboplastic activity of leukaemic eosinophils. Haematologia 4 213 – 220 (1970).

The results of haemostatic investigations of the eosinophils isolated from the blood of a patient suffering from eosinophilic reticulosis (eosinophilic leukaemia) are presented. The eosinophil extract was found to possess thromboplastic activity. It shortened the calcium time of plasma as well as the reaction time *r* in the thrombelastogram of normal blood, and it increased prothrombin consumption in serum. The eosinophil extract can be used in the thromboplastin generation test as a substitute for platelets. In the thrombin generation test the extract increased both the thrombin generation rate and the amounts of the generated thrombin. The extract exhibited also a slight antiheparin activity but no antithrombin activity.

I. A. Kassirsky, M. A. Volkova

Some questions of rational therapeutical tactics in chronic lymphatic leukaemia. Haematologia 4, 221 – 234 (1970).

In the treatment of chronic lymphoid leukaemia we are confronted with the following problems: choice of the moment for the beginning of active treatment, choice of the drug, the definition of the necessary dose and possible intervals in administration of the drug. Part of the patients (about 25%) needs no active treatment for 5—20 years of the disease. In treatment it is necessary to take into account not only the degree of leucocytosis but also the symptoms of visceral pathology, tendency to anaemia and thrombocytopenia. In order to decrease the danger of mutagenic effect exerted by large doses of the drug, the authors propose a primary restraining (prophylactic) therapy. It is administered during the first severe symptoms of the disease. After the end of the course of active treatment the restraining therapy is administered. Owing to such tactics the average duration of life among patients with chronic lymphoid leukaemia increased to 63 months and even more in the last years. Leukeran is the most suitable chemotherapeutic drug for the treatment of chronic lymphoid leukaemia. A good direct effect was obtained in all 75 patients treated. During the administration of the restraining therapy there were remissions for 2—4 years. The primary restraining therapy with Leukeran (10—15 mg in 5—7 days) protects patients against the progression of the disease for 1—3 years. A total of 57 patients were given such kind of treatment. Steroid hormones should not be used widely. They are indicated in haemolytic crises, considerable anaemia and thrombocytopenia. X-ray therapy and telegamma-therapy are used in severe local enlargement of lymph nodes and spleen. Semisynthetic penicillins (oxacillin, methicillin, ceporin) are effective in treatment of pneumonias which frequently develop in patients with chronic lymphoid leukaemia.

M. Boga, L. Halmy, P. Rutkai

Diabetes insipidus occurring with acute leukaemia. Haematologia 4, 235—239 (1970).

Simultaneous diabetes insipidus and acute myeloid leukaemia case is reported. Cicatrix tissue was found in the neurohypophysis as a morphological basis for diabetes insipidus. In the adrenal cortex leukaemic infiltration and haemorrhage were observed, but the clinical picture and the Metopirone test did not indicate a decreased adrenal cortex function. In the course of the Metopirone test a transitory increase in the specific weight of the urine was observed.

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Investigations into the Action of 6-Benzimidazole Cobamide Coenzyme (Cobamamide) on Pernicious Anaemia Megaloblasts

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The effect of cobamamide (a coenzymatic form of vitamin B_{12}) on *in vivo* and *in vitro* proliferation and maturation of pernicious anaemia megaloblasts has been studied. Similarly to cyanocobalamine, and possibly with a higher degree of effectiveness, cobamamide normalized the increased proliferation rate of megaloblasts and enhanced the maturative evolution of pernicious anaemia cells. The mechanisms of this action at the biological level are discussed.

In recent years many studies have stressed the importance in cell economy of 6-benzimidazole cobamide coenzyme (cobamamide, CoB_{12} in the following), a naturally occurring and biologically active coenzymatic form of vitamin B_{12} [17]. Some interesting analogies and at the same time some differences in respect of vitamin B_{12} have been reported.

Herbert and Sullivan [18] demonstrated that the physiological uptake of CoB_{12} is dependent on the intrinsic factor. According to Lee and Glass [26] CoB_{12} prior to absorption is bound to the intestinal mucosa more strongly than vitamin B_{12} and, when given to pernicious anaemia patients together with intrinsic factor, it is deposited in the liver in a smaller quantity than is vitamin B_{12}.

In current literature [8, 12, 13, 18, 26—29, 41, 43] we have failed to find more information on the possible qualitative or quantitative analogies (or differences) of CoB_{12} and vitamin B_{12} as far as the action of these two cobamines on two basic activities of red cell precursors, proliferation and maturation, is concerned. As the test material best suited to this type of studies is the megaloblast of pernicious anaemia, a condition in which vitamin B_{12} deficiency is the decisive factor [19], we shall report on investigations into the action of CoB_{12} on the proliferative and maturative activity *in vitro* and *in vivo* of these cells.

Material and Methods

M. M., a 62-year-old Italian male patient was admitted without having had any previous treatment with liver extracts or vitamin B_{12}. He was complaining of weakness, moderate dyspnoea, sore tongue, numbness of lower extremities, slight fever and vague disturbances referred to the gastrointestinal tract. He pre-

sented as a well-nourished, pale individual with a yellowish tint of the sclerae and beefy tongue. Physical signs included a systolic murmur at the base of the heart and a moderate enlargement of the spleen. The haematological findings were as follows:

RBC 1,530,000/mm^3, Hb 46 %, colour index 1.5, haematocrit 14 %, MCV 126 μ^3, reticulocytes 3 %, thrombocytes 110,000/mm^3, WBC 4,200/mm^3, neutrophil granulocytes 24 %, eosinophils 3 %, lymphocytes 66 %, monocytes 7 %. The granulocytes with more than 5 nuclear segments amounted to 15 %. Erythrocytic fragility: haemolysis in 0.50 % NaCl. The bone marrow was hypercellular and intensely megaloblastic. The patient failed to secrete free hydrochloric acid after histamine stimulation. Total urinary excretion of urobilinogen was 2 mg/24 hrs. Total serum bilirubin was 1.98 mg per 100 ml. The vitamin B_{12} level in serum, as measured on Euglena, was 6 $\mu\mu$g/ml whereas serum folate, tested on *L. casei*, was 27 Mμg/ml. FIGLU excretion after histidine load 9 mg/8 hrs. Schilling test 3 % of oral dose excreted in 48 hrs., 8 % after intrinsic factor by mouth.

The patient was treated intramuscularly with 1 mg of CoB_{12} (Trofozim, Crinos, Como, Italy) daily for 15 days. Afterwards the drug was given at a dose of 1 mg twice weekly.

Bone marrow smears. Marrow biopsy was performed before and 15 days after the beginning of cobamamide therapy. Part of the marrow particles was used for experiments to be discussed later, and part was smeared on slides and stained with May-Grünwald-Giemsa, to determine 1) the myelogram; 2) distribution of erythropoietic cells according to maturation stage; 3) distribution of *mitotable* cells (ME), according to maturation stage (thus excluding orthochromatic erythroblasts) [33]; 4) mitotic index (mitoses/1000 cells) of the whole population of mitotable erythroblasts (proerythroblasts plus basophilic and orthochromatic erythroblasts); 5) distribution of mitoses according to maturation stage; 6) specific indices (mitoses/1000 proerythroblasts viz. basophilic or polychromatophilic erythroblasts) according to 3), 4) and 5).

These values were obtained by counting 10,000 cells in each of the samples obtained before and after CoB_{12} treatment.

Maturation in vitro. Part of the marrow particles obtained before treatment was used to prepare 12 cultures in a fluid medium according to a technique derived from the original one of Astaldi, Tolentino and Sacchetti [7] for investigation of maturation activity *in vitro* of haemopoietic cells. The fluid phase contained 80 % TC 199 (Difco) and for the remaining 20 % the following ingredients: autologous serum (AS) (2 cultures); AS + cyanocobalamine (B_{12}) 1 γ/ml (2 cultures); normal homologous serum (NS) (2 cultures); NS + CoB_{12} 1 γ/ml (2 cultures); NS + B_{12} 1 γ/ml (2 cultures). Culture in AS was repeated with the marrow obtained after 15 days of CoB_{12} treatment.

Samples were taken from the cultures at 24, 48 and 72 hours, smeared on slides and stained with May-Grünwald-Giemsa or examined in the native state between slide and coverslip by phase contrast microscopy. In fixed and stained specimens the frequency of elements of the three maturation stages (basophilic,

polychromatophilic and orthochromatic erythroblasts) was determined in 1000 cells.

Proliferation in vitro. Bone marrow samples were cultured as described, for the direct determination of the duration of mitosis [32, 33, 34].

Results

Results are presented in Tables 1—5 and in Figs 1—8.

The curative action of CoB_{12} in pernicious anaemia. Fig. 1 shows that treatment led to a rapid and complete remission.

Cytological studies on bone-marrow smears. Table 1 shows the patient's myelogram while Table 2 the frequency among 1000 mitotable erythroblasts (ME) (oxyphilic forms being thus excluded) of interphase proerythroblasts, basophilic and polychromatophilic erythroblasts (including megaloblasts and intermediate forms). The relative size of the three maturative compartments is also shown together with the 278 erythroblastic mitoses counted before treatment, and the 247 karyokineses found 15 days later, according to maturation stage, in

Table 1

Pre- and post-cobamamide treatment myelograms in a patient suffering from pernicious anaemia

	Before treatment	After treatment
Haemocytoblasts	0.1	0.3
Myeloblasts	0.3	0.4
Neutrophilic promyelocytes	2.5	3.2
Eosinophilic promyelocytes	0.3	1.0
Neutrophilic myelocytes	10.1	11.5
Eosinophilic myelocytes	2.2	5.3
Neutrophilic metamyelocytes	7.0	10.2
Eosinophilic metamyelocytes	0.4	0.5
Neutrophilic granulocytes	3.8	10.5
Eosinophilic granulocytes	0.7	0.5
Basophilic granulocytes	0.1	—
Promegaloblasts	15.0	—
Basophilic megaloblasts	16.4	—
Polychromatophilic megaloblasts	8.5	—
Orthochromatic megaloblasts	3.0	—
Pronormoblasts	4.5	1.3
Basophilic normoblasts	9.8	6.7
Polychromatophilic normoblasts	7.8	26.2
Orthochromatic normoblasts	5.0	20.5
Histiocytic cells	1.1	0.5
Lymphocytes	1.3	0.4

Table 2

Distribution according to maturation of interphasic and mitotic ME before and after treatment with cobamamide

	(1) Frequency/ 1,000 ME	(2) Relative compartment size	(3) Distribution according to maturation of 268 mitoses	(4) Relative frequency of mitoses	(5) Specific mitotic index per 1000
Before treatment					
Proerythroblasts	314.6	1.00	113	1.00	35.91
Basophilic erythroblasts	422.5	1.34	126	1.11	29.82
Polychromatophilic erythroblasts	262.9	0.83	39	0.34	14.83
After treatment			247 mitoses		
Proerythroblasts	38.0	1.00	15	1.00	39.47
Basophilic erythroblasts	195.4	5.14	68	4.53	34.80
Polychromatophilic erythroblasts	766.6	20.17	164	10.93	21.39
Normal values (Rondanelli et al. 1969)			2496 mitoses		
Pronormoblasts	30.3	1.00	115	1.15	37.95
Basophilic normoblasts	210.8	6.95	659	6.59	31.26
Polychromatophilic normoblasts	758.9	25.04	1722	17.22	22.69

Table 3

Frequency in per cents of surviving erythroblasts in

	Basophilic per 100 erythroblasts			
Time of culture	0	24	48	72
Autologous serum (AS)	65.2	35.6	25.0	14.3
AS + cobamamide 1 μg/ml	67.8	40.2	27.0	10.8
AS + cyanocobalamine 1 μg/ml	64.8	34.2	23.0	6.3
Normal homologous serum (NS)	69.9	28.4	14.5	7.8
NS + cobamamide 1 μg/ml	63.8	9.5	3.4	0.6
NS + cyanocobalamine 1 μg/ml	64.5	13.6	4.8	1.1
AS (after 15 days CoB_{12} treatment)	18.8	8.4	1.0	—

agreement with the criteria described previously [33, 34]. The number of mitoses observed among 10,000 ME gave a mitotic index of 27.8‰ prior to and 24.7‰ after treatment. The ratio of the relative frequency of mitoses and the number of mitotic cells among 1000 cells of each compartment allowed to compute the specific mitotic indices of the three maturative stages, as presented in the last column of Table 2.

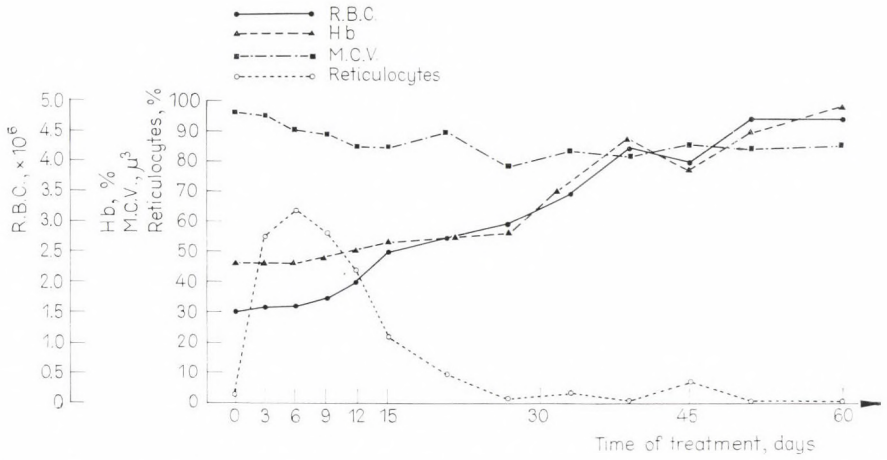

Fig. 1. Haematological data of a patient suffering from pernicious anaemia treated with cobamamide

Tables 1 and 2 show that CoB_{12} treatment induced a disappearance of megaloblasts, a reduction of basophilic forms and an increase in polychromatophilic cells. This led to an almost complete normalization of the relative size of the compartments, with negligible changes in the mitotic index.

different culture media (common component, TC 199)

Polychromatophilic per 100 erythroblasts				Orthochromatic per 100 erythroblasts			
0	24	48	72	0	24	48	72
23.4	43.5	48.3	48.8	11.4	20.9	26.7	36.9
21.3	40.5	39.0	50.3	10.9	19.3	34.2	38.9
26.4	45.3	54.6	53.5	8.8	20.5	22.4	40.2
20.6	49.4	59.1	31.5	9.5	22.2	26.4	60.7
24.5	50.2	39.8	20.6	11.7	40.3	56.8	78.8
24.0	48.6	47.3	28.3	11.5	37.8	47.9	70.6
26.8	40.3	28.9	0.9	54.4	51.3	70.1	99.1

Maturation of erythropoietic cells in vitro. Table 3 and Figs 2, 3 and 4 present the survival *in vitro* of erythroblasts (including megaloblasts, intermediate forms and normoblasts) in the three maturation stages. The frequency of pernicious anaemia basophilic erythroblasts in AS alone or with either CoB_{12} or B_{12} displayed

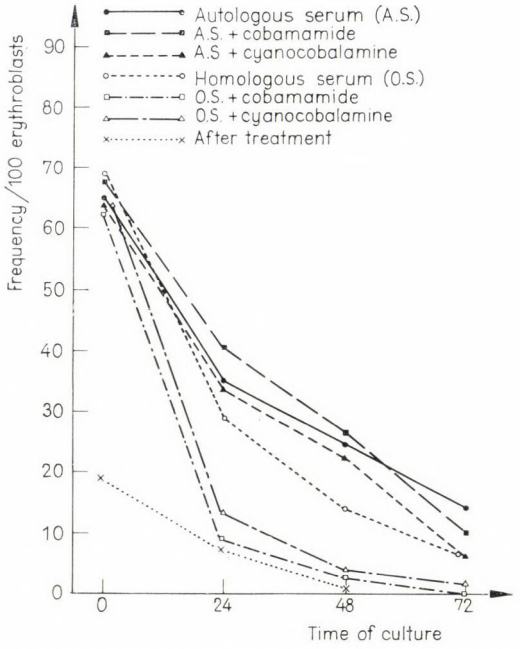

Fig. 2. Maturation *in vitro* of basophilic erythroblasts

a slow decrease, whereas polychromatophils, after an initial increase persisted at a constant level, and orthochromatic cells showed a slow increase. When cultured in NS alone the basophils behaved like in AS, but the polychromatophils initially increased in number, to decreasethen near to their initial value, while the orthochromatic cells reached the sixfold of their initial level by the 72nd hour. The addition of CoB_{12} or B_{12} to NS cultures induced marked changes in the frequency of surviving cells. Basophilic erythroblasts by the 48th hour displayed a steep decrement to 3.4 % in cultures with added CoB_{12}, and to 4.8 % with B_{12}. Polychromatophilic erythroblasts increased steeply until the 24th hour, when they had reached a frequency of 50.2 % when cultured in the presence of CoB_{12} and one of 48.6 % in the presence of B_{12}. After the 48th hour, both frequencies decreased to near the initial one, in cultures with B_{12} to 28.3 %, in those with CoB_{12} to 20.6 %. In NS with added vitamins orthochromatic erythroblasts by the 24th hour multiplied their initial frequency nearly fourfold to 40.3 % in the presence of CoB_{12} and to 37.8 % in the presence of B_{12}. This tendency persisted, so that by the 72nd hour orthochro-

matic cells constituted the largest fraction of surviving cells: 78.8 % with CoB_{12}, and 70.6 % with B_{12}. Table 3 and Figs 2—4 also report the maturative behaviour *in vitro* of erythroblasts from bone marrow samples obtained after the patient had undergone an effective treatment with CoB_{12} and presented normal haematological

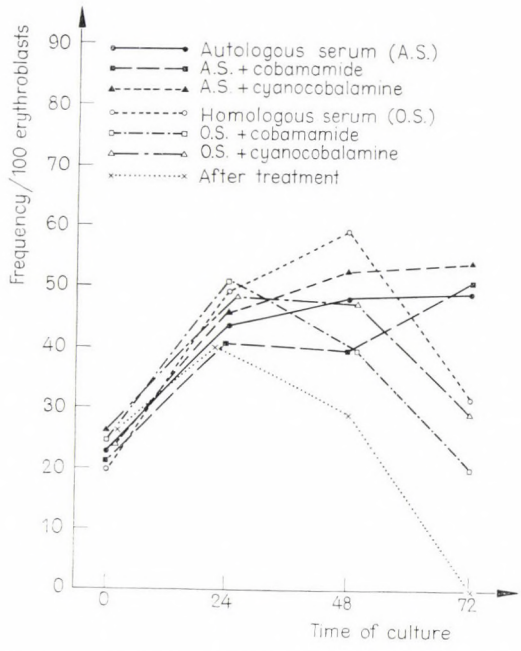

Fig. 3. Maturation *in vitro* of polychromatophilic erythroblasts

values. On this occasion the maturation of erythroblasts displayed a normal pattern, with the disappearance of basophilic cells by the 48th hour, a reduction of polychromatophilic cells to near 0 by the 72nd hour, and with only orthochromatic cells surviving in the culture by the 72nd hour. Thus, whereas erythroblasts cultured in AS, even when cobalamines were added, showed a considerable delay in maturation, cells cultured in NS displayed a more rapid evolution through the successive steps of maturation. This was best achieved by adding cobalamines to the nutrient medium. However, the shift to more mature forms was more marked in cultures with CoB_{12} than in those with B_{12}.

Together with the enhanced maturation *in vitro*, marked morphological modifications were observed, as shown by Figs 5, 6 and 8. In AS medium, megaloblastic erythropoiesis was undisturbed until the 24th hour, being characterized by the presence of large promegaloblasts and basophilic megaloblasts. By the 48th hour smaller elements, though still retaining a megaloblastic appearance, were present in the cultures, in good agreement with the findings of Astaldi and Baldini

Table 4

Mitotic and stathmokinetic index (per thousand) before and after cobamamide treatment (normal values in brackets)

	Before treatment		After treatment	
	Mitotic index	Stathmo-kinetic index	Mitotic index	Stathmo-kinetic index
ME (all)	27.8 (24.9)	193.6 (82.4)	24.7	78.8
Basophilic E.	32.4 (32.1)	315.4 (133.1)	35.5	138.5
Polychromatophilic E.	14.8 (22.6)	82.5 (55.0)	21.39	62.3

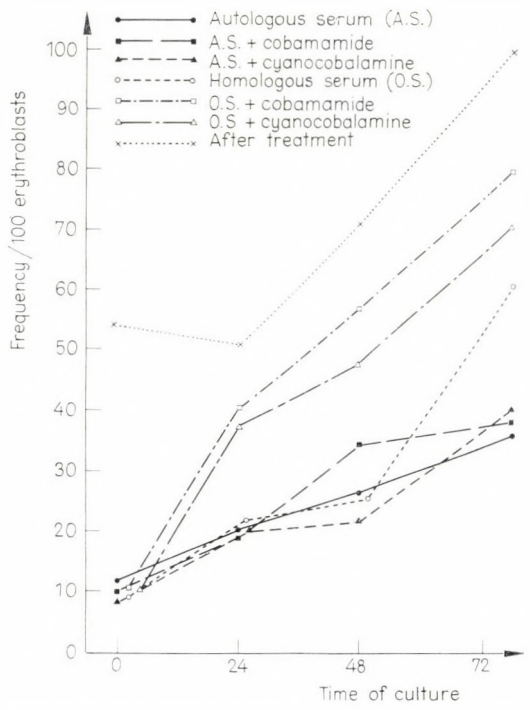

Fig. 4. Maturation *in vitro* of orthochromatic erythroblasts

[2]. At 72 hours, erythropoiesis was still megaloblastic but the smaller cells were increasing in number and their nuclear chromatinic pattern was becoming denser.

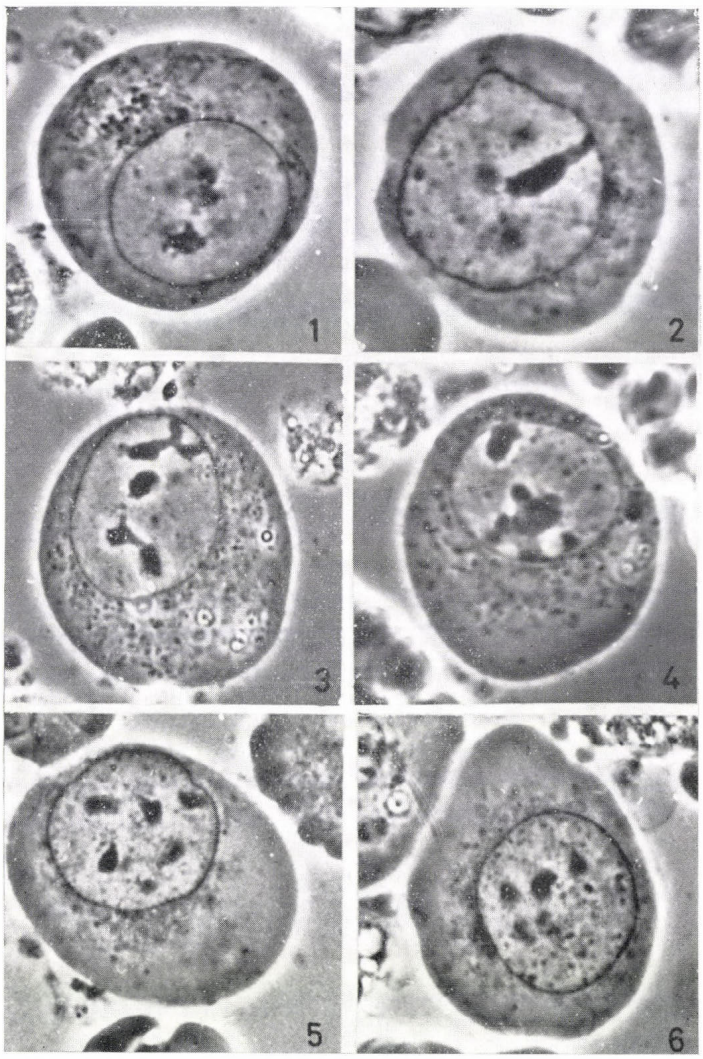

Fig. 5. Pernicious anaemia megaloblasts cultured in fluid medium. Phase-contrast

In NS cultures, especially in those with added CoB_{12}, megalopoiesis was progressively substituted by cells with intermediate morphology, smaller dimensions, higher nucleo-cytoplasmic ratio and a denser chromatinic network interspersed with more definite chromocentres. A rapid shift from megalopoiesis to normoblastopoiesis was then noted.

Proliferation of erythroblasts in vitro. Table 4 and Fig. 8 present the results of the stathmokinetic (colchicine) test applied to pernicious anaemia erythroblasts before and after a therapeutic cycle with CoB_{12}. In agreement with the findings of Astaldi and Mauri [4], the stathmokinetic index of pernicious anaemia erythro-

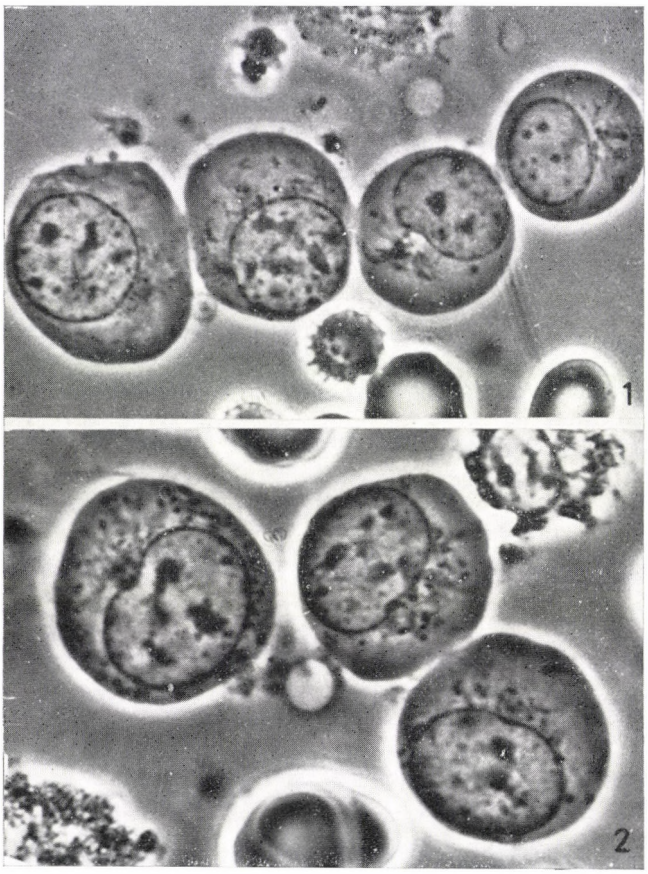

Fig. 6. Intermediate forms after addition of cobamamide to nutrient medium

blasts was higher before than after B_{12} treatment, a result which indicates that pernicious anaemia cells before CoB_{12} treatment have an increased proliferative potential. The control stathmokinetic values for normal subjects [30] were 133.1 $\pm$ $\pm$ 14.30 per thousand in basophilic normoblasts and 55.0 $\pm$ 7.54 per thousand in polychromatophilic normoblasts. CoB_{12} treatment thus appears to have restored to normal the proliferative activity of pernicious anaemia erythroblasts. Table 5 shows the duration of mitosis of pernicious anaemia erythroblasts, as determined on living cells before and after treatment. Before treatment, mitotic time as compared with the normal value [33, 34] was very short in all the three maturation

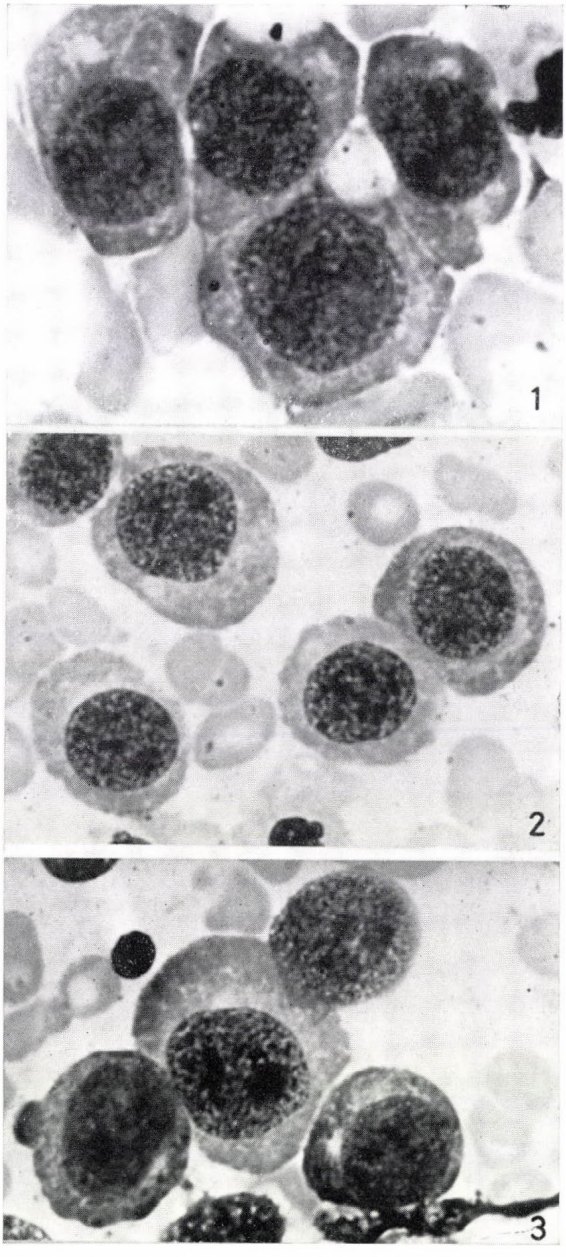

Fig. 7. Top: typical megaloblasts initially present in fluid cultures. May-Grünwald-Giemsa stain. Middle: intermediate forms after addition of cobamamide to the nutrient medium. Bottom: definitive normoblastic forms after addition of cobamamide

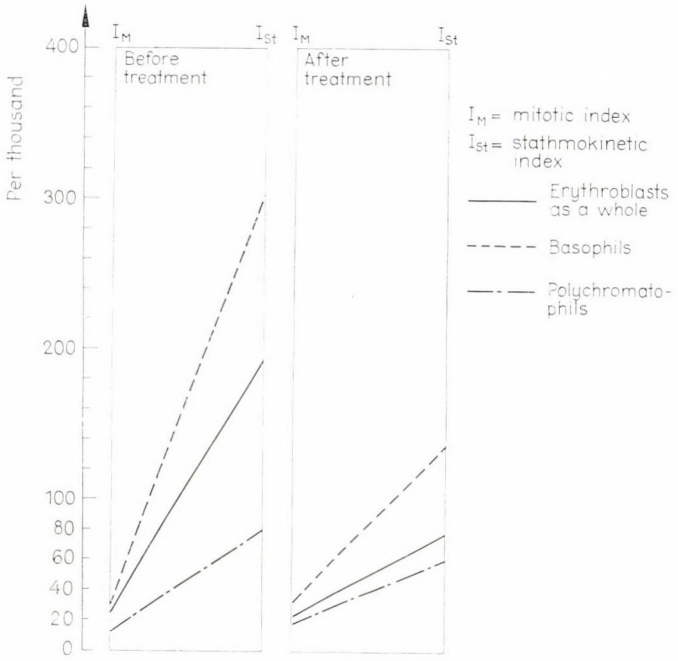

Fig. 8. Stathmokinetic (colchicine) test *in vitro*

Table 5

Mitotic index (hours). Normal values in brackets

	Before treatment			After treatment		
	Pro-	Baso-	Poly-	Pro-	Baso-	Poly-
I	0.52	0.53	0.81	0.65	0.80	1.01
II	0.48	0.70	0.88	0.88	0.84	0.99
III	0.49	0.74	0.80	0.58	0.78	1.10
IV	0.53	0.70	0.72	0.62	0.85	0.89
V	0.39	0.68	0.76	0.64	0.92	0.94
VI	0.49	0.69	0 73	0.63	0.86	1.00
VII	0.48	0.60	0.80	0.62	0.79	1.05
VIII	0.56	0.70	0.81	0.57	0.83	0.98
IX	0.52	0.72	0.81	0.59	0.83	1.05
X	0.50	0.72	0.74	0.59	0.85	1.10
Average	0.496	0.678	0.786	0.627	0.835	1.011
	(0.57)	(0.83)	(0.98)			

stages, having been between a minimum of 0.39 hours and a maximum of 0.62 hours (average 0.516 hours) in promegaloblasts, between 0.63 hours and 0.80 hours (average 0.718 hours) in basophilic megaloblasts and between 0.76 hours and 0.98 hours (average 0.846 hours) in polychromatophilic megaloblasts. The corresponding average duration in normoblasts was much longer, viz. for pronormoblasts 0.70 hours, for basophilic normoblasts 0.85 hours, and for polychromatophilic normoblasts 1.00 hour. After treatment, values very near to those of normal blastopoiesis were obtained: proerythroblasts 0.67—0.78 hours (average 0.717 hours), basophilic erythroblasts 0.78—0.92 hours (average 0.835 hours), and polychromatophilic erythroblasts 0.89—1.10 hours (average 1.011 hours).

Thus one effect of CoB_{12} treatment on the proliferation of erythropoietic cells *in vitro* consisted in a reversal to a normal mitotic time.

Discussion

One of the aims of the present investigation was to study the effect of CoB_{12} on the physiology of red-cell precursors, and to establish whether it was of the same type, though possibly of a different intensity, than reported for cyanocobalamine. The biological basis of the addisonian anomaly of erythroblasts is still a matter of discussion. It has been reported that the inability to regenerate tetrahydrofolate and other active folates in vitamin B_{12} deficiency entails anomalies of nucleic acid synthesis and thus of megaloblastic type of erythropoiesis [44]. According to Beck [9] one of the features of vitamin B_{12} deficiency might be a condition of unbalanced growth with RNA synthesis prevailing on DNA synthesis. Such anomalies might well cause alterations in the proliferative rhythm of pernicious anaemia megaloblasts.

This rhythm is thought by some authors [20, 36] to be depressed, whereas others such as Astaldi et al. [6] found by means of the stathmokinetic (colchicine) test that megaloblasts proliferate more intensely than normoblasts at both the basophilic and the polychromatophilic stage. Recently, Rondanelli et al. [29, 31] have found that mitotic duration and generation time are shorter in megaloblasts than in normoblasts, thus confirming the view of Astaldi et al. More recently, Schmid et al. [37] who performed erythrokinetic and cytoautoradiographic (^{3}H-thymidine, ^{3}H-uridine and ^{3}H-cytidine) studies in pernicious anaemia patients, have found higher than normal synthetic indices and proliferative activity. The cellular defect responsible for the anaemia in Addison–Biermer disease consists not in anomalies of cell proliferation (an activity which is consistently stimulated, probably by a compensatory mechanism), but in the width of ineffective erythropoiesis [24] and in the severity of the maturation defect. The latter, according to studies *in vitro*, is reversible when erythroblasts are cultured in a medium containing normal serum, or serum from pernicious anaemia patients treated with vitamin B_{12}, or folic acid, or folinic acid, or vitamin B_{12} plus gastric juice, or embryo extract, whereas no normalization is obtained by culturing the cells in pernicious

anaemia serum plus vitamin B_{12} alone, or gastric juice, or thymine [1, 3, 5, 11, 14, 40, 42]. Since this action of vitamin B_{12} is exerted only when added to normal serum, Astaldi [1] assumed the existence of a plasmatic binding factor, which is lacking in pernicious anaemia, and which is necessary for the normal utilization of vitamin B_{12} by megaloblasts. It must, however, be stressed that normal serum itself is able to transform megaloblasts, due simply to its content in vitamin B_{12}. Recently, Glass [15] has suggested that Astaldi's plasmatic binding factor might be a circulating form of intrinsic factor, a view previously challenged by Callender and Lajtha [11] and partially confirmed by the immunological investigation of Gullberg and Kistner [16] and by the isotope studies of Rosenthal et al. [35].

The remarkable efficacy of CoB_{12} in our patient might be explained by assuming that in his organism there still was a minimum quantity of plasmatic factor sufficient to bind the parenterally introduced CoB_{12}.

Let us now discuss the effect of CoB_{12} on proliferation and maturation. Proliferation may be evaluated by analyzing the mitotic and stathmokinetic indices and mitotic duration. It has been shown that in pernicious anaemia the mitotic index does not undergo any marked modification, even after successful coenzyme B_{12} therapy. It is, however, well-known that the mitotic index is in relation not only to the proliferative rate, but also to the duration of mitosis [4]. The stathmokinetic test *in vitro*, which allows a partial estimate of the proliferative rate, showed that in our patient before CoB_{12} treatment an increased number of both basophilic and polychromatophilic erythroblasts had begun mitosis per unit of time, having been then arrested in the metaphase by the stathmokinetic action of colchicine. The same test showed that proliferative potentials, so markedly increased in untreated patients, after CoB_{12} treatment had returned to values near to normal [30].

Even if the stathmokinetic index is a poor indicator of proliferative activity, it may be stated that one of the effects of CoB_{12} cobamamide therapy was to reduce the number of cells entering in mitosis per unit of time. The fact that the mitotic index was apparently normal in all conditions might thus be interpreted by assuming that mitotic duration was shortened when proliferative rate was increased. This was wholly confirmed by our direct phase-contrast recording of mitotic time of living pernicious anaemia cells, as in these experiments promegaloblasts, basophilic and polychromatophilic megaloblasts displayed a decrease of mitotic duration corresponding to 14 %, 19 % and 20 %, respectively, of the values for their normal counterparts. CoB_{12} treatment caused mitotic duration to increase to normal (and longer) values. If on the basis of the total incidence of mitoses in bone marrow, of their distribution according to maturation stage, and their duration *in vitro*, the weighted average mitotic time computed for the proliferating pool of mitotable erythropoietic cells was before treatment 0.619 hr, after CoB_{12} treatment, 0.939 hr (normal value, 0.905 hr) [33, 34]. The lengthening of mitotic time after CoB_{12} treatment was approximately 44 % of the pretreatment value.

The knowledge of the mitotic time of the erythroblastic population (I_M) and of the weighted average mitotic time (t_M) allowed to compute the weighted

average generation time (t_G) of the proliferating pool constituted by the sole mitotable cells, with the use of the formula $t_G = t_M/I_M$ of Leblond and Walker [25] and fully considering the limits of such calculations [21, 22, 29, 31–34]. The obtained values for t_G were, before treatment, 22.26 hrs; after treatment, 38.01 hrs; normal value 35 hrs [33, 34].

The t_G of pernicious anaemia erythropoietic cells after treatment increased by 70 % of the pre-treatment t_G, thus reaching a value in the normal range. It must be stressed that generation times computed as above may represent estimates in excess of real values if (1) part of the supposed proliferating erythroblasts are not destined to divide [23, 38]; (2) part of the erythroblasts degenerate or die within the proliferating pool; (3) part of the erythroblasts do not perform all or part of the expected proliferation or maturation sequence; (4) the mitosis terminating the pool is not strictly heteromorphogenic [22]. These possibilities are more easily fulfilled in pernicious anaemia, a disease which is characterized by a high rate of ineffective erythropoiesis, a condition that in this context we prefer to consider a subtraction of mitotable cells from the proliferative and maturative pathway. As a consequence, a still higher level of effectiveness might be attributed to CoB_{12} treatment in normalizing the proliferation of erythropoietic cells.

If the latter is increased in pernicious anaemia, maturation activity is decreased. The maturation defect is, however, easily abolished *in vitro* by normal serum plus cyanocobalamine or, with even higher effectiveness, serum plus CoB_{12}. These effects are remarkably more marked than with homologous serum alone. This study of one single patient might not contribute much to the understanding of the therapeutic effect of CoB_{12} in pernicious anaemia. However, this was coupled with a series of important effects exerted by the B_{12}-analogue on a large cell population in various experimental models.

Thus, we may conclude that the therapeutic value of CoB_{12} in pernicious anaemia is based on the following biological effects on megaloblastic cells:

1. speeding up of the maturative evolution of erythropoietic cells;
2. morphological conversion of megalopoiesis to normoblastopoiesis;
3. normalization of the abnormally increased proliferative activity of erythropoietic cells.

The third mechanism may be interpreted not only as a substitution of megaloblastic by normoblastic tissue which is proliferating at a lower rate, but also as a reduction of the need of reduplicative processes compensating the ineffective erythropoiesis.

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Le polymorphisme de la phosphatase acide érythrocytaire

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Le polymorphisme de la phosphatase acide érythrocytaire a été étudié, par électrophorèse sur gel d'amidon, chez 500 personnes prises au hasard, de la région de Liège. Outre les cinq phénotypes habituels, A, AB, B, AC et BC, le phénotype rare C a été rencontré à deux reprises. Les fréquences géniques calculées d'après les résultats observés, sont: $P^a = 0.349$, $P^b = 0.596$ et $P^c = 0.055$.

Introduction

Il y a maintenant de très nombreux enzymes pour lesquels l'on connaît dans l'espèce humaine des variants génétiques.

Pour la grande majorité de ces polymorphismes génétiques cependant, les variants sont peu fréquents, voire souvent très rares, dans presque toutes les populations étudiées. Ils sont d'ailleurs la cause de maladies souvent graves, ou tout au moins de réactions pathologiques à certains médicaments ou aliments.

Mais depuis quelques années, l'on commence à découvrir des polymorphismes enzymatiques "physiologiques" constitués de deux ou plusieurs gènes assez fréquents et en équilibre, correspondant donc à ce que l'on est en droit d'appeler des groupes sanguins enzymatiques. Citons les polymorphismes des enzymes érythrocytaires: phosphoglucomutase, adénylate kinase, phosphogluconate déhydrogénase, adénosine déaminase, etc . . .

Dans ce travail, nous avons déterminé les fréquences parmi notre population, des différents phénotypes électrophorétiques de la phosphatase acide des hématies dont le polymorphisme a été le premier découvert par Hopkinson, Spencer et Harris [7] en 1963.

Matériel et méthode

Nous avons étudié des membres du personnel et surtout des donneurs de sang se présentant au Centre de Transfusion de Liège, sans aucun ordre préétabli, mais en veillant à ne pas examiner plusieurs personnes d'une même famille, ni des individus manifestement d'origine étrangère.

Les phénotypes de phosphatase acide érythrocytaire ont été déterminés par électrophorèse horizontale des hémolysats en gel d'amidon selon la méthode originale de Hopkinson, Spencer et Harris [7].

Le gel d'amidon est préparé dans un tampon constitué d'acide succinique 0.0025 M et de tris (hydroxyméthyl) aminométhane 0.0046 M de pH 6.0.

La solution dans les cuves à électrodes est un tampon citrate de sodium/acide citrique 0.41 M à pH 6.0.

L'hémolysat est préparé à partir de globules rouges fraîchement prélevés sur A. C. D. ou E. D. T. A., lavés trois fois en NaCl à 0.9 %, additionnés d'un volume égal d'eau distillée et congelés. Après décongélation, les stromas sont éliminés par centrifugation à 13.000 g pendant 20 minutes.

La migration électrophorétique est poursuivie pendant 17 heures sous une tension de 8 volts/cm et à la température de 4° C.

Le gel est ensuite coupé en deux sur son épaisseur et incubé pendant 4 heures à 37° dans un tampon citrate/acide citrique 0.05 M contenant le substrat pour la révélation de l'activité phosphatasique, du diphosphate sodique de phénolphtaléine 0.005 M.

La lecture se fait en alcalinisant la surface du gel à l'aide d'ammoniaque: la phénolphtaléine libérée par l'activité enzymatique colore en rouge les diverses positions électrophorétiques des phosphatases.

D'autres techniques utilisant des tampons différents ont été proposées [2, 3, 9, 16].

Résultats

La figure 1 montre les différents phénotypes. Leurs fréquences observées sont rassemblées dans le tableau 1.

Tableau 1

Fréquence des phénotypes

Phénotypes	A	AB	B	AC	BC	C	Total
Nombre observé	61	209	177	18	33	2	500
Fréquence %	12.2	41.8	35.4	3.6	6.6	0.4	100.0

Fréquences géniques: $P^a = \frac{122 + 209 + 18}{1000} = 0.349$

$$P^b = \frac{209 + 354 + 33}{1000} = 0.596$$

$$P^c = \frac{18 + 33 + 4}{1000} = 0.055$$

Tableau 2

Phénotype	Fréquence génotypique calculée	Nombre calculé	Nombre observé
A	$(P^a)^2 = 0.1218$	60.9	61
AB	$2\ P^aP^b = 0.4160$	208.0	209
B	$(P^b)^2 = 0.3552$	177.6	177
AC	$2\ P^aP^c = 0.0384$	19.2	18
BC	$2\ P^bP^c = 0.0656$	32.8	33
C	$(P^c)^2 = 0.0030$	1.5	2
	$(P^a + P^b + P^c)^2 = 1.0000$	500.0	500

$\chi^2 = 0.250$. Pour 2 degrés de liberté: $0.90 > P > 0.80$

Tableau 3

Groupes sanguins de la famille n°LGS/Inc./1.334

	Sexe	Année de naiss.	Rhésus	MNSs	P_1
I–2	F	1906	Cc D. Ee	Nss	–
II–1	M	1929	CC D. ee	MNss	+
II–2	F	1937	CC D. ee	Mss	+
II–3	M	1947	cc D. EE	MNss	+
III–1	F	1952	CC D. ee	MNss	+
III–2	F	1962	CC D. ee	Mss	+

Gc	Hp	Gm	PAE	PGM_1	AK
I–2	2–2	1, 2, –10	AC	1–1	1–1
II–1	2–1	1, –2, –10	C	2–1	1–1
II–2	2–2	1, –2, 10	B	2–1	1–1
II–3	2–1	1, –2, –10	C	1–1	2–1
III–1	2–2	1, –2, 10	BC	1–1	1–1
III–2	2–1	1, –2, 10	BC	2–2	1–1

Tous sont: A_1, C^w–, K–, Le(a–), Inv(–1), ADA 1–1.
Tous sont: Fy(a+), Jk(a+), sauf II–2 qui n'a pas été testé pour ces facteurs.

Sur la base de la théorie génétique actuellement bien admise de trois gènes codominants, nous avons établi les fréquences géniques: $P^a = 0.349$, $P^b = 0.596$ et $P^c = 0.055$. Sur la base de cette théorie de trois gènes codominants, les fréquences géniques permettent de calculer les fréquences attendues des différents génoty-

pes et donc, dans ce cas, phénotypes, et le tableau 2 montre que les chiffres ainsi calculés sont très proches de ceux que nous avions effectivement observés.

En raison de sa rareté [11] le phénotype C n'a encore été étudié que dans quelques familles. Comme nous l'avons trouvé à deux reprises, nous avons essayé

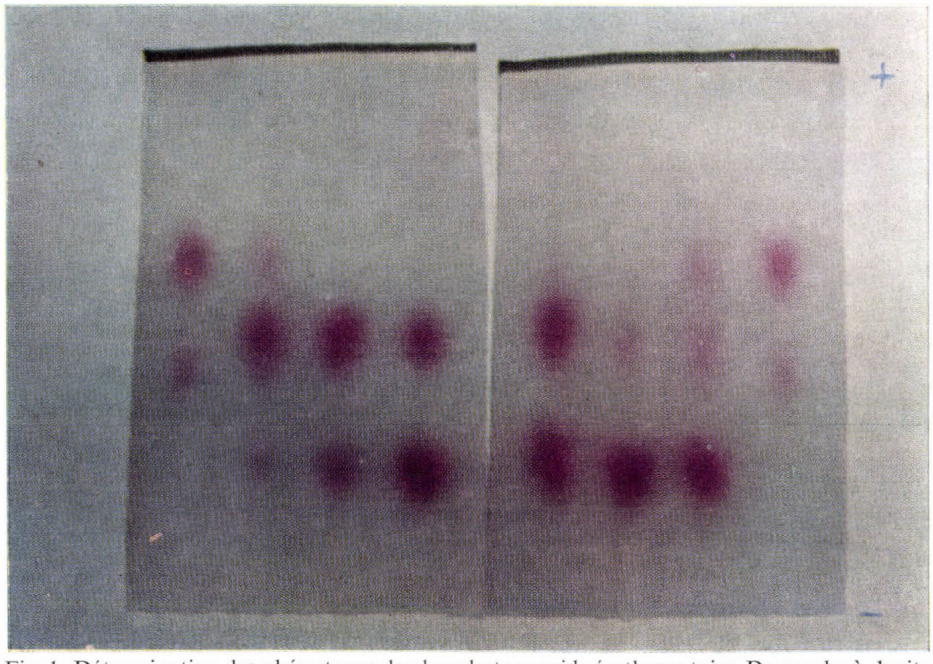

Fig. 1. Détermination des phénotypes de phosphatase acide érythrocytaire. De gauche à droite les phénotypes: A, AB, B, BC (2 fois), C, AC et A

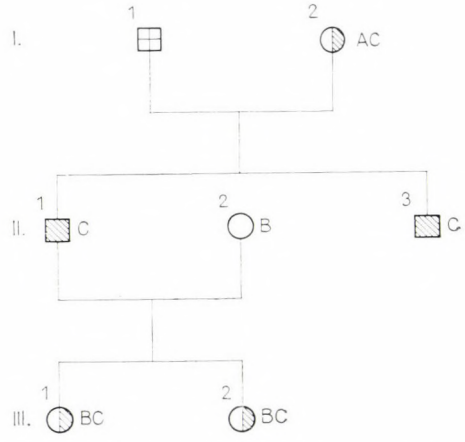

Fig. 2. Arbre généalogique de la famille n° LGS/Inc./I.334.

d'étudier les familles de ces deux individus, mais cela n'a été possible que dans un cas. La figure 2 représente l'arbre généalogique de cette famille, avec les phénotypes de phosphatase acide érythrocytaire. Nous avons ainsi trouvé une autre personne du phénotype C, le frère II-3 du propositus II-1. Ce dernier peut effectivement être homozygote P^cP^c puisque sa mère et ses deux filles ont le caractère C. Mais cette famille était évidemment trop petite pour permettre de démontrer formellement l'homozygotie du propositus. Dans le tableau 3, sont rassemblés les résultats de tous les autres systèmes de groupes sanguins érythrocytaires et plasmatiques étudiés notamment pour vérifier la paternité du propositus.

Discussion

Les fréquences des groupes de phosphatases acides érythrocytaires ont déjà été étudiées dans un certain nombre de populations. Les résultats de ces travaux colligés par certains auteurs [3, 23], montrent que le gène P^c n'existe à une fréquence appréciable que dans les populations blanches. Chez les Noirs et les Jaunes [12], il est très rare ou inexistant: il est vraisemblable que les quelques phénotypes AC ou BC observés dans ces populations, résultent d'un métissage récent.

Chez certains Noirs par contre, Giblett et Scott [2] d'une part et Karp et Sutton [9] d'autre part ont identifié des phénotypes différents des six phénotypes précédemment connus: ils sont déterminés par deux autres gènes allèles codominants du même locus, les gènes P^r et P^d.

Dans le tableau 4, nous avons comparé nos résultats aux fréquences des gènes P^a, P^b et P^c qui, à notre connaissance, ont été étudiés jusqu'à présent parmi des populations blanches. Hormis une fréquence étonnement basse de 0.269 dans l'une des études menées à Fribourg [4] sur un échantillon à vrai dire assez peu nombreux, l'on constate que la fréquence P^a oscille entre 0.3 et 0.4. Mais ces études sont évidemment encore trop peu nombreuses pour que l'on puisse tenter de dresser une carte de la fréquence de ces gènes en Europe.

Il est déjà bien établi que les six phénotypes observés couramment sont déterminés par six génotypes correspondants (tableau 2). résultant de l'appariement de 3 gènes autosomaux codominants. Ce mécanisme génétique est confirmé par plusieurs études familiales portant sur au moins 596 unions avec 1.685 enfants (tableau 5).

Un tel système à 3 gènes codominants est évidemment très utile pour les études de génétique appliquée: diagnostic de mono- ou dizygotie des jumeaux, recherches de linkages, et surtout les expertises médico-légales de filiation. Différents auteurs [1, 5, 10, 14] ont montré l'intérêt de ces groupes pour les recherches d'exclusion de paternité.

Wiener [22] a établi une formule permettant de calculer quelle est la probabilité d'exclusion de paternité a priori pour un système à trois gènes codominants. En appliquant à cette formule les fréquences géniques P^a, P^b et P^c que nous avons trouvées (tableau 1), le calcul montre que dans notre population un homme consi-

Tableau 4

Fréquences des groupes de P.A.E. dans les populations blaches

Population	Auteurs	Année	Réf.	Nombre testé	F^a	F^b	P^c
Allemagne – Berlin	Radam & Strauch	1966	17	1.188	0.362	0.577	0.061
Allemagne – Heidelberg	Kruger & coll.	1968	10	528	0.320	0.626	0.054
Allemagne – Fribourg	Goedde & coll.	1966	4	171	0.269	0.722	0.008
Allemagne – Fribourg	Wille & coll.	1968	23	300	0.310	0.643	0.047
Angleterre	Hopkinson & coll.	1964	8	367	0.36	0.60	0.04
Autriche – Vienne	Speiser & Pausch	1967	19	410	0.365	0.573	0.062
Danemark	Lamm	1968	13	470	0.3415	0.6021	0.0564
France – Paris	Van Cong & Moullec	1967	20	487	0.3214	0.6386	0.0400
Iran	Walter & Bajatzadeh	1968	21	449	0.304	0.666	0.030
Pologne	Schlesinger & Sulich	1968	18	371	0.373	0.553	0.074
Tchécoslovaquie Prague	Herzog & Bohatova	1969	6	307	0.365	0.578	0.057
U.S.A. – Seattle	Giblett & Scott	1965	2	193	0.394	0.547	0.059
Belgique – Liège	présent travail			500	0.349	0.596	0.055

Tableau 5

Etudes familiales des groupes de P.A.E.

Auteurs	Réf. n°	Nombre de familles	Nombre d'enfants
Hopkinson et coll.	8	107	251
Lai et coll.	11	80	395
Giblett et Scott	2	102	279
Goedde et coll.	4	80	118
Van Cong et Moullec	20	134	465
Schlesinger et Sulich	18	93	177
		596	1.685

déré erronément comme le père d'un enfant a 24 % de chances de voir sa paternité exclue par l'étude du seul système des phosphatases acides érythrocytaires.

*

Nous exprimons notre reconnaissance au Professeur Agrégé Ch. Salmon et à Madame J. Seger, du Centre Départemental de Transfusion Sanguine à Paris, qui ont bien voulu accueillir l'un de nous (M. G. G.) dans leur laboratoire.

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Polymorphism of Red Cell Acid Phosphatase

Polymorphism of red cell acid phosphatase has been studied by starch gel electrophoresis, in 500 individuals taken at random, in Liège.

In addition to the five current phenotypes: A, AB, B, AC and BC, the rare C phenotype has twice been found.

Gene frequencies calculated from the observed results are: $P^a = 0.349$, $P^b = 0.596$ and $P^c = 0.055$.

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Vitamin B_{12} Metabolism in Adrenocortical Insufficiency

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The absorption from the digestive tract of labelled vitamin B_{12} has been studied, determining the serum vitamin B_{12} level, the unsaturated binding capacity of ^{57}Co vitamin B_{12} by the serum, and the antibodies to the intrinsic factor, in 16 patients with adrenocortical insufficiency and in 8 adrenalectomized patients. Impaired absorption of vitamin B_{12} was found in 6 cases of primary adrenocortical insufficiency. In the adrenalectomized patients receiving cortisone for substitution no disturbance in vitamin B_{12} metabolism was revealed.

Many observations have suggested the possibility of a simultaneous production of antibodies directed against the adrenal cortex, the thyroid and the gastric mucosa in the same individual; this is, however, not necessarily associated with an anatomical damage to these organs or with an impairment of their function [2, 4, 7, 9]. A clinically clearly defined association of Addison–Biermer's disease with adrenocortical insufficiency (Addison's disease) has rarely been observed [9, 14, 15, 18]. More frequent is the occurrence of atrophic gastritis in the course of Addison's disease and the sera of these patients contained antibodies to the parietal cells in the gastric mucosa, and in some cases also to the intrinsic factor [7, 9, 21].

Since the glucocorticoids exert a favourable effect on the absorption of vitamin B_{12} in cases of Addison–Biermer's disease [1, 6, 11], the hypothesis has been put forward that adrenocortical failure may exist with latent disturbances of the metabolism of vitamin B_{12}, in which substitutive doses of cortisol ensure the absorption of physiologically indispensable amounts of this vitamin and prevent thus the development of its deficiency.

The basic mechanism of Addison–Biermer's disease is an impairment of absorption of exogenous vitamin B_{12}. However, no investigations were done into vitamin B_{12} absorption in patients with adrenocortical failure. In the present study, the metabolism of vitamin B_{12} was investigated in patients with Addison's disease to establish whether a latent deficiency of vitamin B_{12} existed in some of these cases.

Material and Method

Investigations were carried out in 24 patients (18 women and 6 men) aged from 17 to 65 years. In 16 patients symptoms of damage to the adrenal cortex were found; in 15 of these the damage was a primary one and in one case (No. 16) the disturbances were secondary, developing in the course of hypopituitarism. The duration of the disease ranged from 6 months to 21 years. Eight patients had a history of pulmonary tuberculosis; in one case the process was chronic, with fibrotic lesions leading to pulmonary cirrhosis. Two patients had had genitourinary tuberculosis without adrenal calcification. In two women (Cases 2 and 4) manifestations of ovarian dysfunction coexisted, and in Case 1, hypothyroidism was diagnosed (Schmidt's syndrome).

The diagnosis of adrenocortical insufficiency was based on the typical symptoms of the 24-hour urinary excretion of 17-hydroxycorticosteroids under basal conditions [16] and the results of the test of adrenal reserve after administration of sustained-action ACTH. In all cases a lack or a considerable decrease of the adrenocortical reserve was observed.

The second group comprised 8 adrenalectomized patients operated upon for Cushing's disease; in 7 of these cases bilateral total adrenalectomy was carried out and in one case (Case 22) partial adrenalectomy was performed.

In both groups clinical and haematological studies were carried out together with investigation of vitamin B_{12} metabolism including determination of the serum vitamin B_{12} level (normal range from 124 to 664 pg/ml) [13], unsaturated binding capacity of ^{57}Co vitamin B_{12} by the serum (U.B.C.B_{12}) [12], absorption from the digestive tract (Schilling test) of ^{57}Co labelled vitamin B_{12} and antibodies against the intrinsic factor [8]. Vitamin ^{57}Co B_{12} absorption was determined by means of the tests of Miller et al. [17] and of Schilling. An oral dose of 0.5 μc ^{57}Co B_{12} was given and after 2 and 24 hours, 1000 μg non-active vitamin B_{12} was injected intramuscularly. With this method the total dose of vitamin ^{57}Co B_{12} in normal urinary excretion ranged from 15 to 48% (mean, 24 $\pm$ 2 sigma/5.4%).

During these investigations all patients received substitutive doses of cortisone, from 25 to 37.5 mg daily, and 5 mg of DOCA twice weekly.

In the adrenalectomized group the investigations were performed after the disappearance of the clinical manifestations of Cushing's disease (from 3 months to 9 years after the operation). Additionally, the Schilling test was repeated in two cases (Cases 10 and 19) after the administration of 75 mg/24 hrs of cortisone for 5—7 days.

Results and Discussion

Red blood cell, leucocyte and platelet counts and haemoglobin value (11.3 to 16.0 g per 100 ml) were normal in all the patients.

In the group of primary adrenocortical insufficiency (Table 2) in 5 cases (Nos 4, 6, 7, 10, 14) absorption of vitamin B_{12} from the digestive tract was impaired.

A decrease of the serum vitamin B_{12} level to 80 μg/ml was found in one case (No. 4) among patients with impaired vitamin B_{12} absorption; in the remaining four cases the serum vitamin B_{12} level ranged from 140 to 382 μg/ml. In the patient with the lowest vitamin B_{12} absorption rate serum level of this vitamin was within the normal range (382 μg/ml); however, in this case the influence of liver extracts administered six months before could not be ruled out. Antibodies to intrinsic factor were absent in all the patients.

In the group of five patients with primary adrenocortical insufficiency and low vitamin B_{12} absorption inactive pulmonary tuberculous changes were found in two cases (Nos 7 and 10), and one patient had a history of past urinary tuberculosis (Case 14). These, however, could not be regarded as an evidence of adrenal tuberculosis.

In the adrenalectomized group, vitamin B_{12} metabolism was normal (Table 3).

The above tests revealed an asymptomatic vitamin B_{12} deficiency in one case and a slightly impaired vitamin B_{12} absorption from the digestive tract in four cases of idiopathic or probably idiopathic adrenocortical insufficiency. These observations suggested that the impairment of vitamin B_{12} absorption in idiopathic Addison's disease rarely causes a deficiency of vitamin B_{12}, even a latent one.

A comparison of these results with the absence of any disturbance of vitamin B_{12} metabolism in adrenalectomized patients may suggest that mechanisms other than a lack of adrenal activity are responsible for the impairment of intestinal vitamin B_{12} absorption in Addison's disease. Another evidence for this was the improvement of vitamin B_{12} absorption after administration of 75 mg of cortisol daily for 7 days in a patient with primary adrenocortical failure (Case 10), while absorption was not increased after such doses of cortisone in an adrenalectomized patient (Case 19).

Low rates of vitamin B_{12} absorption from the digestive tract and their increase after glucocorticoid treatment have been observed in atrophic gastritis developing in autoimmune processes and associated with Addison–Biermer's disease [1, 11]. Atrophic gastritis occurred in about 50 % of the cases with Addison's disease [21]. In contrast to earlier reports [21], the gastritis does not disappear after substitutive treatment or after correction of electrolyte metabolism, and it is irreversible in part of the patients [10]. Atrophic gastritis occurs more frequently in the idiopathic type of Addison's disease than in cases of tuberculosis of the adrenal cortex [5, 10]. At the same time, in 30—40 % of these patients serum antibodies against the parietal cells of the gastric mucosa are found [5, 7].

In the serum of nearly 50 % of patients with idiopathic adrenocortical insufficiency, antibodies to adrenocortical antigens were found [2, 5]. The presence of antibodies against adrenocortical antigens, the type of histological changes in the adrenals and the association of other autoimmune diseases were the arguments for including the idiopathic type of Addison's disease into the group of autoimmune diseases.

Table 1

Clinical and haematological investigations in cases of adrenocortical insufficiency

Case No.	Initials, age, sex	Duration of disease (years)	Hb g per 100/ml	Serum vitamin B_{12} level pg/ml	U.B.C.B_{12} ^{57}Co* pg/ml	Schilling test (% after 48 hrs)	Antibodies against I. F. in serum (+, −)	Remarks
1	K. J. 21, M	21	14.0	380	1287	23.6	(−)	Schmidt syndrome
2	Sz. S. 38, F	15	11.7	382	447	23.1	(−)	Ovarian insufficiency
3	J. S. 38, F	4.5	13.6	390	398	28.5	(−)	
4	G. H. 41, F	8	14.4	80	1383	13.4	(−)	Ovarian insufficiency
5	K. B. 46, F	11	11.3	428	982	26.0	(−)	
6	B. J. 19, F	4	14.4	232	1248	4.3	(−)	
7	B. B. 17, F	11	12.4	140	1163	12.9	(−)	
8	S. S. 62, F	8	12.8	410	562	29.6	(−)	
9	P. F. 25, M	2	14.8	392	792	21.2	(−)	
10	Sz. F. 38, M	6	12.8	382	1740	8.8	(−)	After corticotherapy, Schilling test 30.9%
11	K. A. 40, F	9	16.0	360	165	22.3	(−)	
12	W. B. 41, F	4	14.0	454	691	29.2	(−)	
13	P. J. 61, F	5	12.0	322	809	19.4	(−)	
14	A. W. 35, M	6 months	16.0	250	1025	12.6	(−)	
15	W. T. 65, M	5	13.6	670	1041	25.8	(−)	
16	Ch. L. 29, F	3	12.8	288	493	25.0	(−)	Pituitary insufficiency
	Normal (6, 12, 13) Mean values and standard deviation		14.0±16.0	380±140 (range from 124 to 664)	1149±135 (range from 879 to 1419)	24±2.7 (range from 15 to 48%)		

* U.B.C.B_{12} ^{57}Co — unsaturated binding capacity of vitamin B_{12} ^{57}Co.

Table 2

Clinical and haematological investigations in adrenalectomized patients

Case No.	Initials, age, sex	Time affer operation (years)	Hb g per 100 ml	Serum vitamin B_{12} level	U.B.C.B_{12} ^{57}Co*	Schilling test (% after 48 hrs)	Antibodies against I. F. in serum (+, −)	Remarks
				pg/ml				
17	D. G. 50, F	9	12.4	370	2009	23.0	(−)	
18	M. N. 58, F	5	14.0	367	1276	31.0	(−)	
19	M. K. 42, F	7	14.4	440	476	15.8	(−)	After corticotherapy, Schilling test 17.6%**
20	S. H. 39, F	1	14.0	266	279	28.3	(−)	
21	M. T. 42, F	1	15.2	300	501	30.4	(−)	
22	P. M. 45, F	4	13.2	400	1702	39.0	(−)	Partial adrenalectomy
23	G. M. 41, M	1.5	16.3	670	1041	25.8	(−)	
24	Z. S. 29, F	3 months	14.8	375	763	33.9	(−)	
Normal (6, 12, 13) Mean values and standard deviation			14.0±16.0	380±140 (range from 124 to 664)	1320±135 (range from 879 to 1414)	24±2.7 (range from 15 to 48%)		

* U.B.C.B_{12} ^{57}Co — unsaturated binding capacity of vitamin B_{12} ^{57}Co.

** in this case Schilling's test was performed on the basis of 24-hour urinary excretion (normal value 10−20%).

Table 3

Results of investigations

Diagnosis	Number of cases	Schilling test		Antibodies to intrinsic factor
		normal	low	
Adrenocortical insufficiency	16	11	5	0
Bilateral adrenalectomy	8	8	0	0

In some cases of atrophic gastritis developing in the course of autoimmune diseases the process of autoimmunization induces a considerable damage to the parietal cells. It is sometimes associated with the appearance of antibodies to the intrinsic factor, impairing its production or causing its neutralization [1, 20]. The absorption of vitamin B_{12} from the digestive tract falls below the indispensable minimum and after exhaustion of the organism's stores, latent or overt Addison–Biermer's disease develops. In the present material in five cases the absorption of vitamin B_{12} ^{57}Co was decreased, while no antibodies to intrinsic factor were found.

The factors (haemorrhage, intoxication, infections) impairing haematopoiesis in certain cases of adrenocortical failure and latent disturbances of vitamin B_{12} metabolism lead to a state in which the amounts of absorbed vitamin B_{12} become too small and inadequate and Addison–Biermer's disease develops additionally.

Conclusions

1. Investigations into vitamin B_{12} metabolism in 16 patients with adrenocortical insufficiency showed in five cases an impairment of intestinal vitamin B_{12} absorption and in one case, a latent Addison–Biermer's disease.

2. Vitamin B_{12} metabolism was normal in eight adrenalectomized patients.

3. Antibodies against the intrinsic factor were absent in all the patients studied.

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Electron Microscopical Study of the Bone Marrow of Burned Patients

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An electron microscopical study carried out in burned patients revealed the following findings. (a) An active rhopheocytotic process takes place in the erythroblasts and reticulocytes of the bone marrow. In spite of this, ferritin is not accumulated in the cytoplasm of the cells but occurs in the form of dispersed granules. The occurrence of ferritin aggregates (siderosomes) is occasional. (b) No morphological change occurs in the mitochondria of erythroblasts and reticulocytes. Iron was only exceptionally detected in them. (c) Active erythrophagocytosis was observed in the reticulum cells of the bone marrow. Ferritin was found in the cytoplasm of the reticulum cells usually in a dispersed form, while within the cytoplasmic cell structures amply, in the form of large clumps.

Light-microscopical cytochemical investigations have shown that while the reticulum cells in the bone marrow contain an increased amount of iron (Fig. 1), the number of sideroblasts (4—18%) is considerably less than usual (20—48%). Following the intravenous injection of iron, 8.1% of the injected metal is excreted in the urine in burned patients, while only 4.3% in healthy subjects [1]. Following the intramuscular injection of desferrioxamine-B (DFO) the iron content of the 24-hour urine of burned patients varies between 1.22—5.70 mg (mean, 2.42 mg), while in healthy subjects it usually does not surpass 1.0 mg (range, 0.1—1.05 mg; mean, 0.67 mg) [2]. These findings indicate that more iron is stored in the iron-depots of burned patients than of healthy individuals.

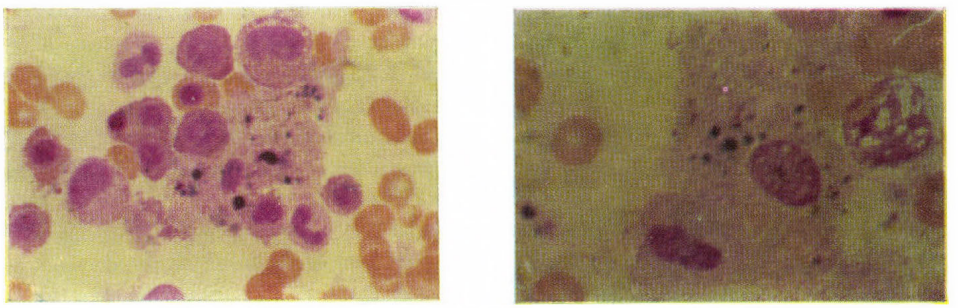

Fig. 1. Iron storage in the cytoplasm of the reticulum cell

Based on this experience we have studied the ultrastructure of the bone-marrow reticulum cells and erythroblasts of burned patients in order to obtain further data on the storage of iron and on the intracellular iron content of erythroblasts, and this the more since to our knowledge such studies have not been carried out in burned patients.

Material and Method

Bone marrow fragments obtained by sternal biopsy were fixed in 6.5 % glutaraldehyde (Millonig buffer, pH 7.2) at room temperature for 4 hours. After washing in buffer overnight, post-fixation was performed in 1 % OsO_4 (s-collidine buffer, pH. 7.2) at room temperature. After a short washing in buffer, the specimens were dehydrated with alcohol and embedded in Durcupan-ACM through propylene oxide. Sections were cut with glass knives on a Porter-Blum MT-1 ultramicrotome, contrasted with uranyl acetate and Reynolds' lead citrate, and examined in a JEM 6-C and Philips EM 300 electron microscope with 80 kv accelerating voltage. The microphotograms were made on AGFA-Gevaert Scientia 23 D 50 slides of 6.5×9 cm.

Results and Discussion

In the bone marrow of burned patients, the granulopoietic elements were predominating, there was a marked shift to the left. Mitotic forms were frequent. Sometimes a considerable asynchrony could be discerned in nuclear and plasma maturation (Fig. 2).

Erythropoiesis was relegated to the background as compared to granulopoiesis. Erythroblastic islands were rare, and if present, they consisted of few cells (Fig. 3).

An active rhopheocytosis could be observed in the erythroblasts and reticulocytes. Its different phases can be seen in Fig. 4. Ferritin granules were present at a distance of a few hundred Å from the surface of the erythroblasts. On the left side of Fig. 4 on the erythroblast membrane a circumscribed invagination can be seen and above its basis, a layer of ferritin molecules. On the right side a just closing invagination can be seen containing iron granules. In Fig. 5 several rhopheocytotic vacuoles are present in the reticulocytes.

Iron for haemoglobin synthesis is transported into the bone marrow mainly by transferrin. This undoubtedly most important way of iron transport does not yet exclude the existence of other transport mechanisms. Rhopheocytosis may belong to the latter. This route of iron transport is obviously insufficient for meeting requirements, as clearly shown by congenital atransferrinaemia, among others. Rhopheocytosis may represent a security mechanism. This conclusion can be drawn, e.g. from its coming into prominence if insufficient amounts of iron are

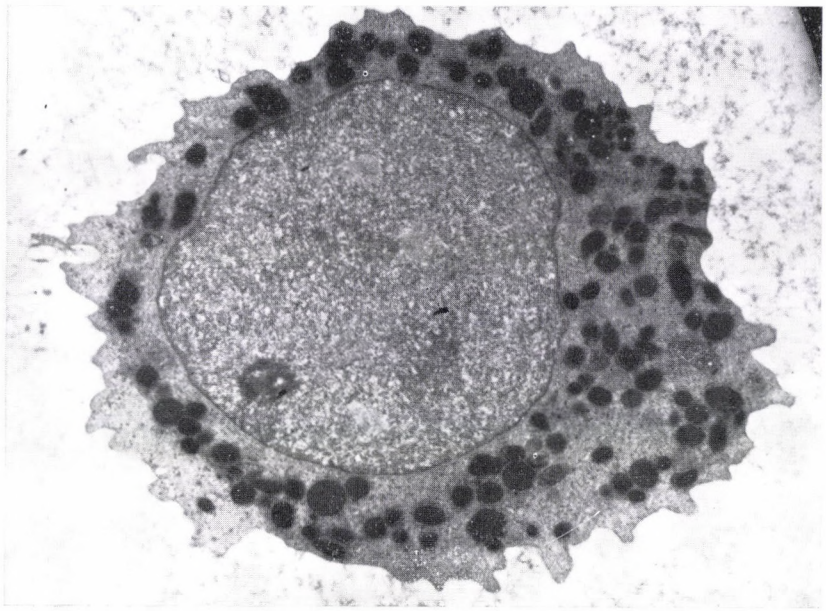

Fig. 2. Granulopoietic cell, asynchronous development of nucleus and cytoplasm. Chromatin-poor nucleus of blast-character with nucleolus. In the cytoplasm a great number of heterogeneous neutrophilic granules, a few azurophilic granules. × 12,000

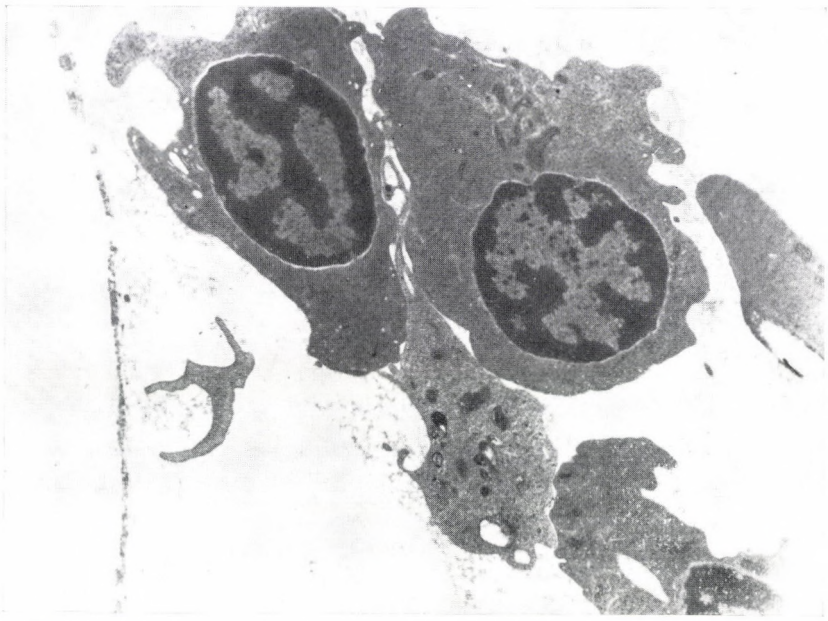

Fig. 3. Erythroblastic island in the bone marrow of a burned patient. Beside the central reticulum cell only few erythroblasts can be seen. × 8940

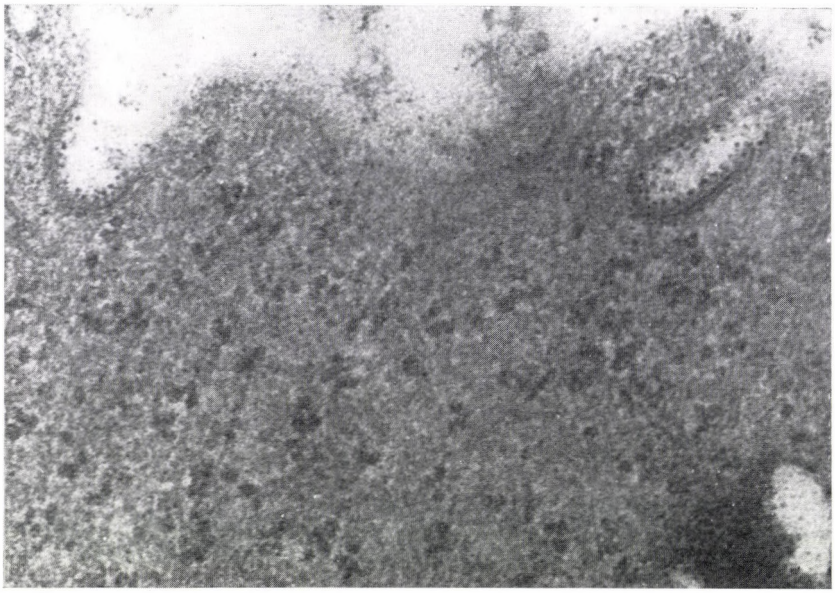

Fig. 4. Rhopheocytosis in burned patient. Invaginations in the erythroblast membrane can be seen with ferritin granules on the surface of the cell membrane and of the invaginations. × 111,700

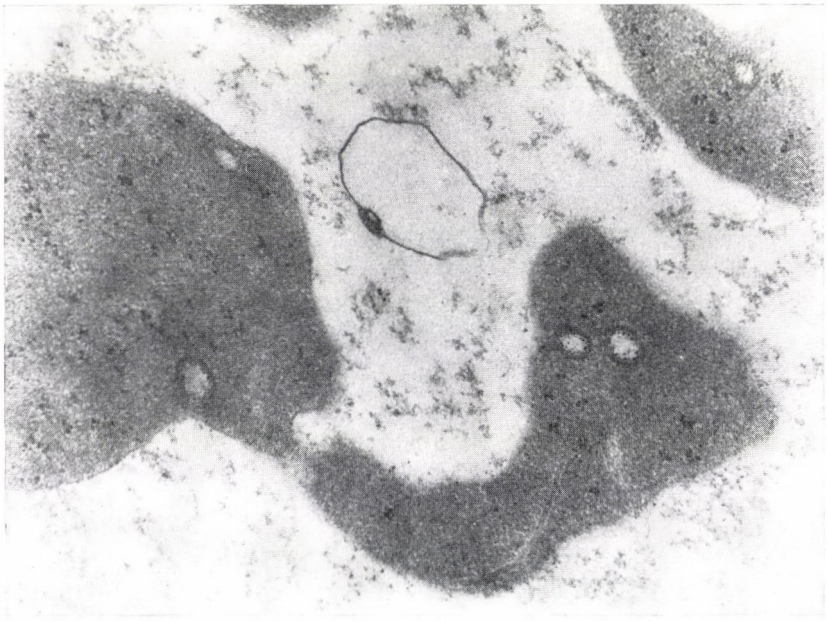

Fig. 5. Rhopheocytosis in burned patient. Several vesicles containing ferritin are seen in the reticulocytes. × 40,100

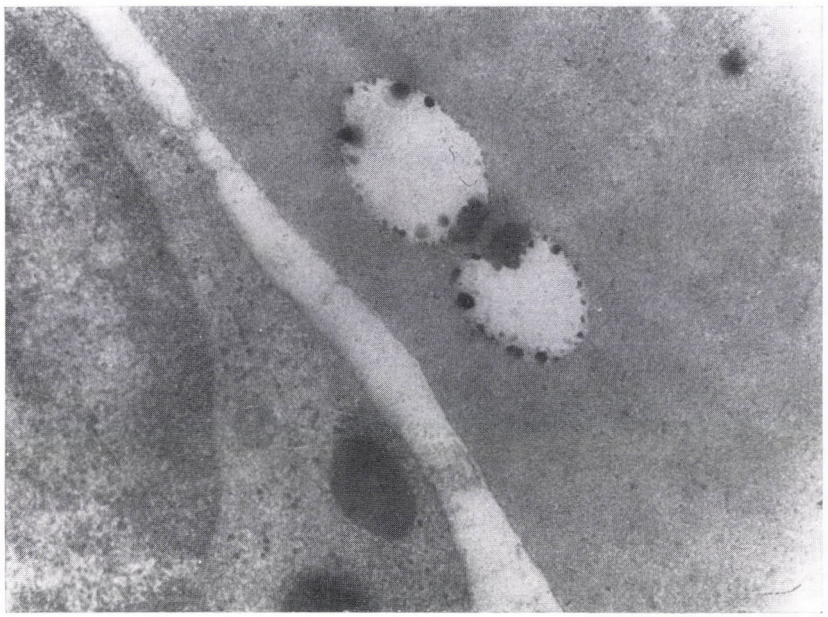

Fig. 6. Vacuoles containing amorphous electron dense material in the cytoplasm of erythroblasts. The electron dense material is located at the margin of the vacuoles. × 57,000

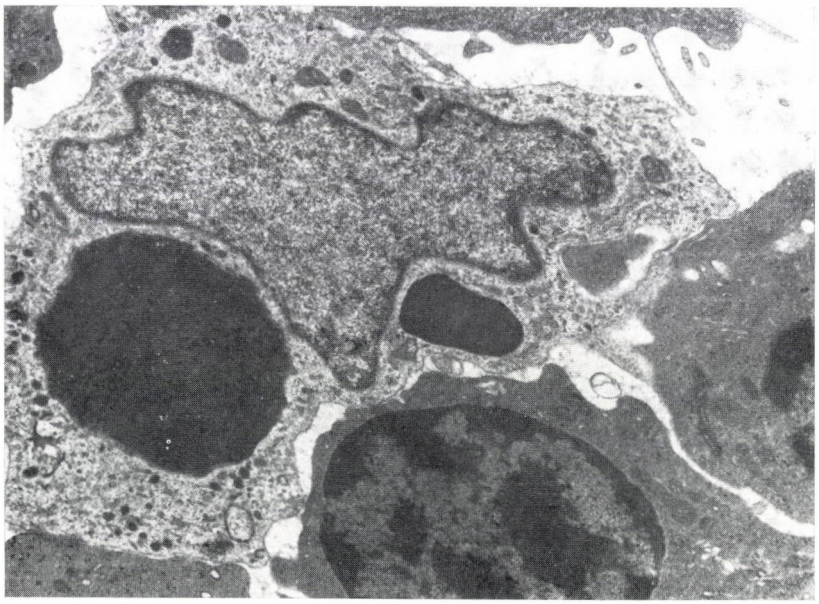

Fig. 7. Erythrophagocytosis in the reticulum cells of the bone marrow in a burned patient. × 13,000

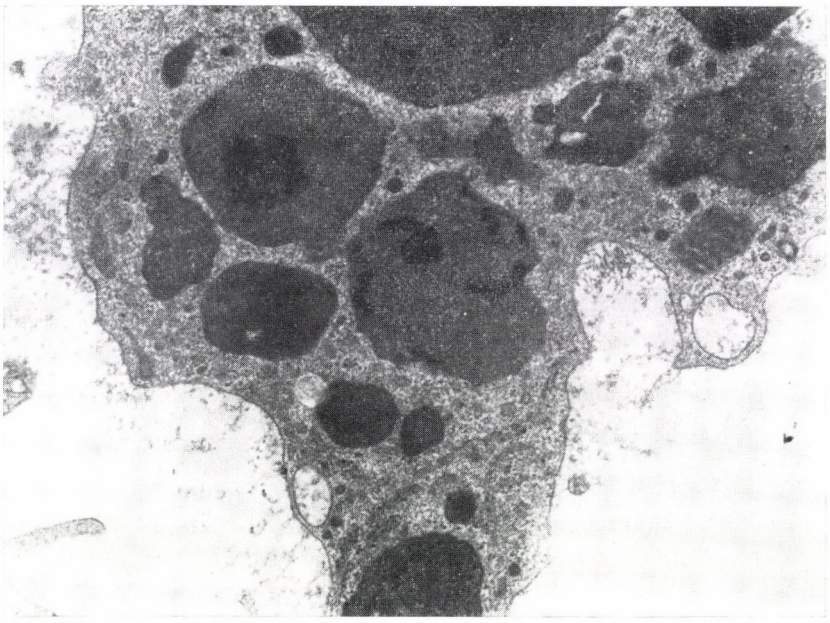

Fig. 8. Erythrocyte fragments in different stages of degradation in the reticulum cell of a burned patient. × 25,500

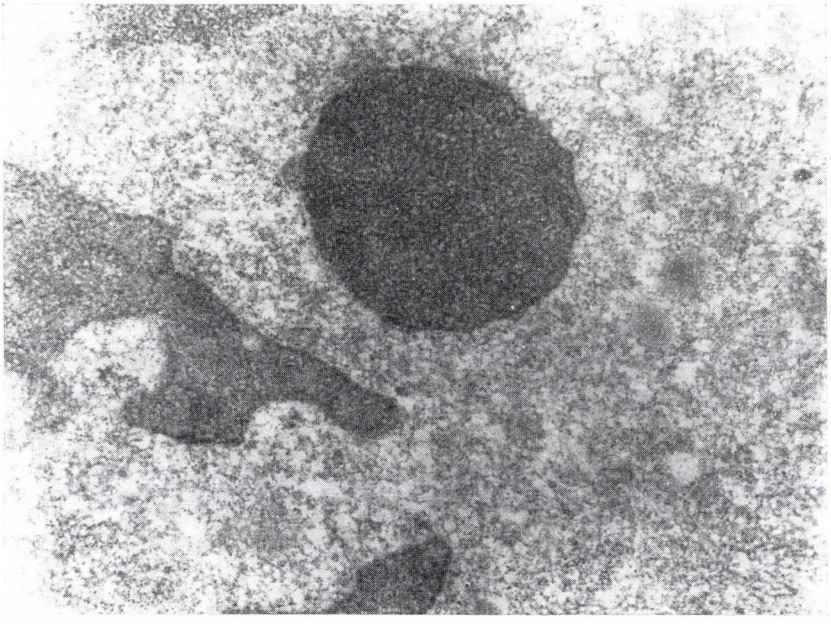

Fig. 9. Dispersed ferritin granules and ferritin aggregates in the reticulum cell of a burned patient. × 57,000

transported into the erythroblast by transferrin. This is the case in the states o- iron deficiency, chronic infections, rheumatoid arthritis, experimental sterile abf scess, etc. [3].

In spite of the active rhopheocytosis, ferritin aggregates (siderosomes) were found in the erythroblasts only exceptionally. It may be assumed that in the burned patient the amount of iron transport into the erythroblasts cannot meet the requirements of haem synthesis, and consequently ferritin, the iron reserve of the cell, cannot be accumulated. According to Bessis and Breton-Gorius [3] in the ripening process of the erythroblast the amount of dispersed ferritin decreases gradually, while the number of iron agglomerates increases. This indicates that in a certain phase of its maturation the erythroblast receives more ferritin than it utilizes for haem synthesis. Thus a portion of the ferritin will be stored in the form of siderosomes.

In the burned patient, vacuoles surrounded by a membrane and containing electron dense amorphous granules can be observed in the cytoplasm of the erythroblasts and reticulocytes (Fig. 6). The nature and composition of these granules are not known but they may contain iron.

The mitochondria of the erythroblasts did not show any morphological changes. They contained iron granules only exceptionally. Such an extensive iron deposition in mitochondria like in sideroachrestic anaemia [6, 7], in sideroblastic refractory anaemia [4, 5], and thalassaemia (micelles ferrugineuses), could not be observed in burned patients.

A few days after the burn there was an active erythrophagocytosis in the reticulum cells of the bone marrow (Figs 7, 8). The erythrocyte fragments were surrounded by a lamellar (myelin) formation around which ferritin granules could be seen.

In the cytoplasm of the reticulum cells, the ferritin was usually dispersed, while in the cytoplasmic structures it was relatively abundant in the form of large clumps (Fig. 9).

The results of the present electron microscopic study have completed and corroborated our cytochemical findings.

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Blood Aspartyl Transcarbamylase Activity in Rauscher Virus Induced Murine Leukemia

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Aspartyl transcarbamylase (2.1.3.2) activity has been studied in hemolysates from normal mice and mice infected with Rauscher leukemia virus. Blood from Rauscher virus-infected mice shows a fourfold increase in specific activity over that found in normal mouse blood. Some of the enzyme properties of the aspartyl transcarbamylase activity in the blood from normal and infected mice were the same. These included apparent Michaelis constants and pH-activity profiles. However, a subtle difference in enzyme properties was detected. There were differential responses of the aspartyl transcarbamylase from the blood of normal and infected mice to the competitive inhibitors, succinic and maleic acids, and the sulfhydryl inhibitors p-hydroxymercuribenzoate and N-ethyl maleimide.

Introduction

The virus induced murine leukemias offer a model system in which to study the biochemical processes of leukemogenesis. Various enzymes have been reported to change after inoculation of mice with murine leukemia viruses [1—4].

Earlier studies of enzyme changes associated with human leukemia have shown that aspartyl transcarbamylase (ATCase), and dihydroorotic acid dehydrogenase levels increase significantly over normal values in patients suffering from leukemia [5].

To determine if there was a comparable change in the virus-induced murine leukemia, aspartyl transcarbamylase activity was studied in the blood of Rauscher virus-infected mice.

Materials and Methods

The blood samples were supplied by Chas. Pfizer and Company, Inc., through Dr. F. E. Durr and Mrs. M. Manousos. Two different lots of normal and Rauscher virus-infected (4 weeks post inoculation) blood samples were used in these experiments.

* Aided by a grant from the American Cancer Society, Florida Division, Inc., the U niversity of South Florida Research Council and a Career Development Award (1-K4CA-1719) from the National Cancer Institute, National Institutes of Health.

The blood samples were diluted with an equal volume of water, and freeze-thawed at least 5 times. The hemolyzed samples were then centrifuged at 20,000 × g for 10—15 minutes at 2—5 °C. The supernatant fluid was used as the enzyme source.

[^{14}C]-aspartic acid (160 mC/mmole in 0.01 N HCl), uniformly labeled, was purchased from International Chemical and Nuclear Corporation. The HCl was removed by lyophilization, and the [^{14}C]-aspartic acid diluted to specific activity of 20 mC/mmole. Carbamyl phosphate, dilithium salt, was purchased from Sigma Chemical Company.

Aspartyl transcarbamylase activity was determined using a radiochemical assay method [6]. The standard incubation mixture contained 25 μl of [^{14}C]-aspartate (20 mC/mmole); 50 μl of carbamyl phosphate, 4×10^{-2} M; 50 μl tris-HCl buffer, pH 8.4, 0.1 M; 25 μl blood (diluted 1 : 1 with H_2O) and H_2O to 200 μl final volume. Blanks were run in which carbamyl phosphate was omitted from the reaction mixture. Controls were run in which [^{14}C]-aspartate and carbamyl phosphate were reacted in the absence of the blood sample. Reactions were carried out at room temperature (23—25 °C). At the end of the reaction period, 1.0 ml of 6 % perchloric acid was added, the sample cooled on ice, and centrifuged to remove the precipitated protein. The supernatant fluid was passed through a column of Dowex 50, H^+ form (a disposable pipet served as a suitable column), and the column washed with 1.5 ml of H_2O. Aliquots (1 ml) of the eluants were taken for radioactivity measurements in a Packard Tri-Carb Liquid Spectrometer, Model 2210, using Bray's solution [7]. Assays were always done in duplicate.

In some experiments, blood samples were treated with Dowex-1, acetate, to remove endogenous carbamyl phosphate. For product analysis, the eluant from the Dowex 50, H^+ columns was neutralized with KOH, the $KClO_4$ removed by centrifugation, and the supernatant fluid lyophilized. Carrier aspartic acid and carbamyl aspartate were added and the samples spotted on Whatman No. 1. The paper chromatograms were developed in a solvent system containing t-butanol–methyl ethyl ketone–water–conc. formic acid (44 : 44 : 11 : 0.26 by volume) [8]. The R_f values for aspartic acid and carbamyl aspartate were 0 and 0.40, respectively. Aspartic acid was detected by ninhydrin and carbamyl aspartate by p-dimethylaminobenzaldehyde [9]. Radioactivity on the chromatograms was detected by use of a Packard Radio-chromatogram Strip Scanner (model 7201).

Protein determinations were made using a standard method [10], with egg albumin (2× crystallized) as the standard.

Results

Under the conditions of our experiments, product formation was linear for at least two hours. The amount of product formed was proportional to the amount of blood added up to 25 μl of 1 : 2 diluted blood sample. After a reaction period of 2 hours, there was more product formed at room temperature than at

37°. Blank values varied with the different lots of [^{14}C]-aspartic acid and also differed depending on whether the blood samples were treated with Dowex-1-acetate prior to use in the reaction. However, the net differences between the experimental sample and the blank were the same. Controls run in the absence of blood gave no evidence of carbamyl aspartate formation, which is contrary to reported results [11].

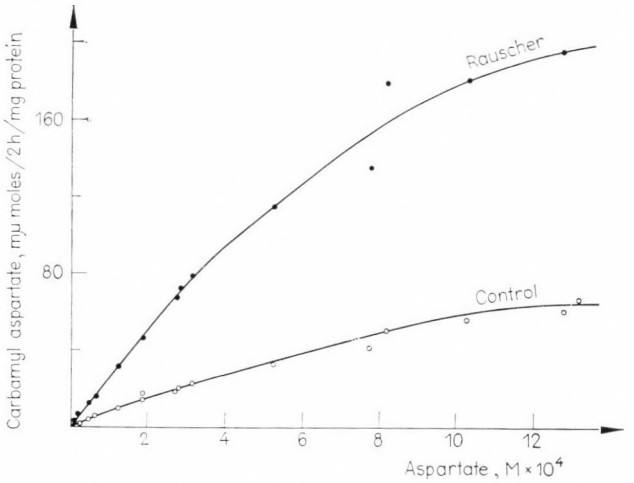

Fig. 1. The effect of varying the aspartate concentration on the velocity of the reaction. The reaction mixture contained [^{14}C]-aspartic acid (0.05 μC) at the concentrations as indicated on the graph; carbamyl phosphate, 2 μmoles, Tris-HCl buffer pH 8.4, 5 μmoles, 25 μl of the enzyme sample in a final volume of 200 μl. Blanks were run at each substrate concentration with the omission of carbamyl phosphate. The assay was carried out as indicated in the Methods section

Product identification of the eluant by paper chromatography showed that the radioactivity coincided exactly with the carrier carbamyl aspartate, and that [^{14}C]-aspartic acid was completely retained by the Dowex 50, H^+ column.

In the blood for Rauscher virus-infected mice (4 weeks post inoculation), there was a 4-fold increase in aspartyl transcarbamylase activity per unit amount of protein when compared to normal mouse blood at various aspartate concentrations (Fig. 1) and at various pH's (Fig. 2). The apparent Michaelis constants, determined from a v *vs.* v/s plot of these data, for the blood from normal and Rauscher virus-infected mice was 1.8×10^{-3} M and 1.4×10^{-3} M, respectively. The V_{max} obtained was 14.4 mμmoles/2 hr/mg protein for normal blood while a value of 41.5 mμmoles/2 hr/mg protein was obtained for the ATCase from the blood of infected mice.

It is apparent from Fig. 1 that the velocity *vs.* aspartate concentration curve does not show sigmoidal properties. CTP was not an allosteric inhibitor and

ATP did not activate in either control or Rauscher virus-infected mice. In fact, at concentrations of ATP greater than 2×10^{-3} M there was inhibition of ATCase activity in both blood samples.

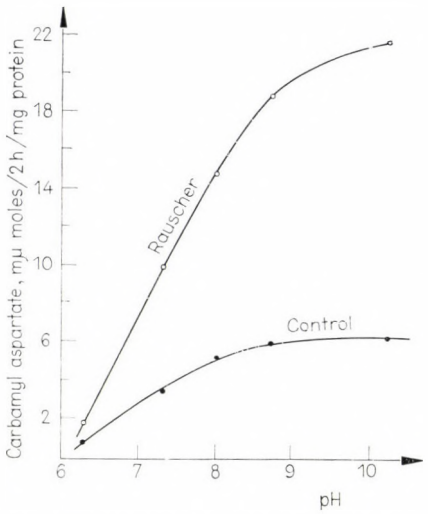

Fig. 2. The effect of pH on ATCase activity. The reaction mixture contained [^{14}C]-aspartic acid, 0.05 μC, 0.1 μmole; carbamyl phosphate, 2 μmoles; Tris-HCl, 5 μmoles, at the pH indicated and 25 μl of the enzyme sample in a final volume of 200 μl. The assay was carried out as indicated in the Methods section

Table 1

Effect of nucleosides and nucleotides on blood ATCase activity

The reaction mixtures contained [^{14}C]-aspartate, 3.3×10^{-5} M, 20 mC/mM; carbamyl phosphate, 1×10^{-2} M; phosphate buffer, pH 8.4, 2.5×10^{-2} M; 25 μl blood sample and the nucleoside or nucleotide at a final concentration of 2×10^{-3} M

Nucleoside or nucleotide	Blood sample	% Activity remaining
CTP	Normal	100
CMP	Normal	100
ATP	Normal	85
AMP	Normal	100
Thymidine	Normal	100
Deoxyadenosine	Normal	100
CTP	Infected	100
CMP	Infected	100
ATP	Infected	83
AMP	Infected	100
Thymidine	Infected	100
Deoxyadenosine	Infected	100

None of the other nucleotides or nucleosides tested on the blood ATCase activity had any effect on this enzyme. These results are summarized in Table 1.

The profile of the pH *vs.* ATCase activity curves (Fig. 2) for the blood ATCase from normal and Rauscher virus-infected mice was very similar although there was a marked difference in specific activity of ATCase at each of the pH's tested.

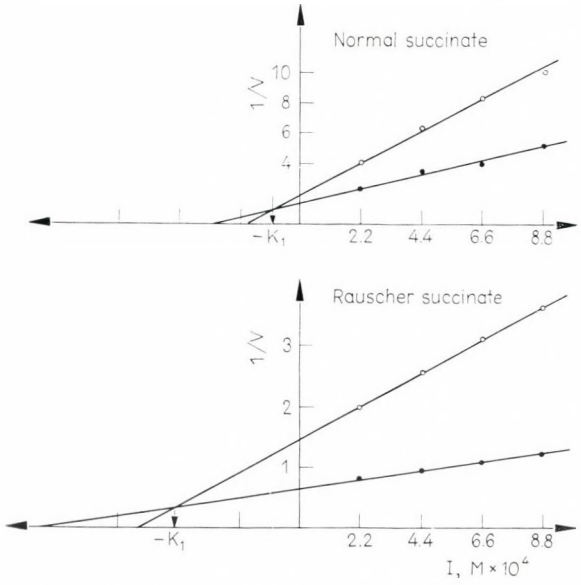

Fig. 3. Dixon plot for the effect of succinate on ATCase activity. The reaction mixture[S] contained [^{14}C]-aspartate, carbamyl phosphate, buffer and succinate, at the indicated final concentration. The final concentrations of aspartate in the curves shown are 3.3×10^{-5} M (●) and 6.6×10^{-5}M (■). The K_I values were determined from the point of intersection, from three independent determinations

While most of the properties of the ATCase from the blood of normal and Rauscher virus-infected mice appeared to be the same, except of course for the 3-fold increase in V_{max}, differences between the two blood ATCase activities have been detected. The response of the ATCase activity to the competitive inhibitors [12] sodium succinate and sodium maleate was different for normal blood ATCase and blood ATCase from infected mice. ATCase activity from infected mice showed less inhibition by succinate and maleate than normal blood ATCase. This difference was reflected by a difference in the inhibitor constants (K_I). The K_I for succinate was approximately 4-fold larger for blood ATCase from infected mice than for normal blood ATCase. K_I values of $1.1 \pm 0.2 \times 10^{-4}$ M and $4.5 \pm 1.0 \times 10^{-4}$ M were determined for the blood from normal and infected mice, respectively. The inhibition was competitive as determined by the graphic method of Dixon [13].

The results are summarized in Figs 3 and 4. The K_I for maleate was approximately 3-fold larger in the blood from virus-infected mice. K_I values of $1.3 \pm 0.2 \times 10^{-4}$ M and $3.6 \pm 0.2 \times 10^{-4}$ M were determined for the blood from normal and infected mice, respectively. The ATCase activity in the blood from infected mice was much

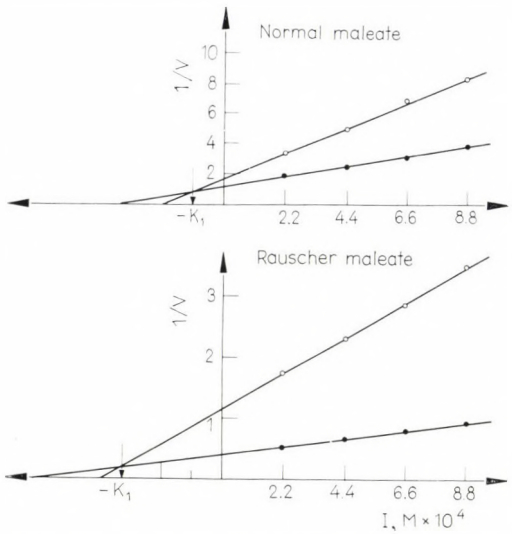

Fig. 4. Dixon plot for the effect of maleate on ATCase activity. Conditions same as shown in Fig. 3

Table 2

Effect of sulfhydryl inhibitors on blood ATCase activity

The inhibitors, p-HMB and N-EM, were incubated with the blood ATCase samples for 30 minutes at the final concentrations listed in the table. Aliquots of these samples were added to reaction mixtures containing [^{14}C]-aspartate, 1.0×10^{-5} M; (20 mC/mM); carbamyl phosphate, 1×10^{-2} M; and phospate buffer, 0.05 M, pH 8.6. Reaction time was 2 hrs. at 24—26°

Enzyme source	Inhibitor	Conc. M × 10^4	Carbamyl aspartate (mμmoles/2 hrs/ mg protein)	% inhi-bition
Normal	—	—	0.22	—
Normal	p-HMB	2.5	0.21	5
Normal	p-HMB	5.0	0.17	18
Normal	N-EM	2.5	0.20	9
Infected	—	—	0.44	—
Infected	p-HMB	2.5	0.33	25
Infected	p-HMB	5.0	0.17	61
Infected	N-EM	2.5	0.14	68

more sensitive to the sulfhydryl inhibitors, p-hydroxymercuribenzoate and N-ethyl maleimide, than was the ATCase activity in normal blood. These results are shown in Table 2. These differences cannot be explained on the basis of differences in sulfhydryl content in the blood samples, since measurements of sulfhydryl concentrations by the indirect N-EM method [14] gave values of 0.102 and 0.101 μmoles of N-EM consumed/mg protein for normal blood and blood from infected mice, respectively.

Discussion

Aspartyl transcarbamylase activity is significantly increased in the blood of mice infected with Rauscher leukemia virus. The spleen, thymus and liver taken from mice infected with Rauscher leukemia virus also show increased ATCase activity [3]. Increases in ATCase activity are seen in human leukemia [5], neoplasms of the colon [15], in rat hepatomas [16], and in HeLa cells following adenovirus infection [17]. Since aspartyl transcarbamylase is involved in the *de novo* synthesis of pyrimidines, the importance of an increase of this enzyme to processes leading to the development of neoplasms can be rationalized. However, it has not yet been shown whether this enzyme increase is directly involved in the leukemogenic process or represents a secondary alteration arising from the pathological state of the animal.

This increase in ATCase activity in blood from Rauscher leukemia virus-infected mice was not accompanied by drastic changes in the pH optimum and the Michaelis constant. The lack of response of the blood ATCase to compounds which act as allosteric effectors in either *E. coli* ATCase [18] or rat liver ATCase [19, 20] agrees with the result observed with ATCase activity from rabbit erythrocytes [6]. There were, however, differences between the normal and virus-infected blood. ATCase from normal blood was more strongly inhibited by succinate and maleate than was ATCase from the blood of Rauscher virus-infected mice. This was reflected in smaller K_I's with respect to succinate and maleate for the normal blood ATCase activity (Figs 3 and 4). ATCase activity in the blood from Rauscher virus-infected mice was much more sensitive to sulfhydryl inhibitors than normal ATCase activity. The increase in specific activity of ATCase in the blood of Rauscher virus-infected mice could be due to an increase in leukocytes. However, the differences in the response of the ATCase from normal and virus-infected blood to two kinds of inhibitors (competitive inhibitors, succinate and maleate; and non-competitive inhibitors, N-EM and p-HMB) indicate that there must be some qualitative difference in the protein molecules. These differences could be the result of slight conformational changes in the ATCase molecule or from differences in the primary structure. Whether this increased ATCase activity results from virus-induced enzyme synthesis, alteration of the control of normal enzyme synthesis, or reflects a change in the cell population of the blood is not known. The role of the increased ATCase activity in the leukemogenic processes is still under investigation.

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Zur Zytochemie der Monozytenleukose*

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Die bei panoptischer Färbung fragwürdigen und unbefriedigenden Differenzierungsmöglichkeiten unreifzelliger Leukosen werden durch den Einsatz verschiedener zytochemischer Methoden entscheidend verbessert. Unter allen Typen unreifzelliger Hämoblastosen läßt sich die Monozytenleukose zytochemisch am zuverlässigsten diagnostizieren. Pathognomonisch für diese Leukoseform ist die hohe Plasmaaktivität an α-Naphthylazetat-Esterase und an durch Natriumfluorid hemmbarer Naphthol-AS-Azetat-Esterase. Bemerkenswert ist die Übereinstimmung des pathologischen Paramonozyten mit dem normalen Monozyten bezüglich des baustein- und fermentzytochemischen Verhaltens. Nicht nur die zytologisch-diagnostischen Möglichkeiten im Einzelfall, sondern auch die Kenntnisse von der nosologischen Stellung der Monozytenleukose werden durch die Zytochemie wesentlich bereichert.

Mit der Weiterentwicklung der zytostatischen Behandlung hat sich das Bedürfnis nach einer zuverlässigen morphologischen Differenzierung unreifzelliger Leukosen verstärkt. In den letzten Jahren ergaben sich Ansatzpunkte für eine Differentialtherapie dieser Leukoseformen in dem Sinne, daß der vorherrschende Zelltyp als ausschlaggebend für die Wahl des einzusetzenden Medikaments angesehen wird [4, 6, 17]. In der Praxis sind Unterteilungsversuche nach den klassischen zytologischen Kriterien aber meist recht unbefriedigend, da sie zu sehr vom subjektiven Eindruck des Untersuchers abhängen [5]. Deshalb blieb auch die große Unsicherheit in der zytologischen Diagnostik der Monozytenleukose seit der Erstbeschreibung durch Reschad und Schilling [22] vor über 50 Jahren bis heute bestehen. Sie äußert sich in den diskrepanten Häufigkeitsangaben dieser Leukoseart, die bei Durchsicht der Literatur immer wieder auffallen (Zusammenstellung bei Löffler [16]) und die keinesfalls echten Verteilungsunterschieden am jeweils untersuchten Material entsprechen können.

Diese Erscheinung wird verständlich, wenn man bedenkt, daß im panoptisch gefärbten Ausstrich an einzelnen Zellexemplaren schon die Abgrenzung des normalen Monozyten vom großen Lymphozyten, der Lymphoidzelle oder der Retikulumzelle schwer fallen kann. Viel schwieriger ist die Identifizierung leukämisch entarteter Monozyten (Abb. 1), die sich durch stärkere Zell- und Kernpolymorphie, Neigung zu erhöhter Kernsegmentierung und Plasmavakuolen von den

* Herrn Prosektor Dr. med. habil. H. Eck zum 65. Geburtstag.

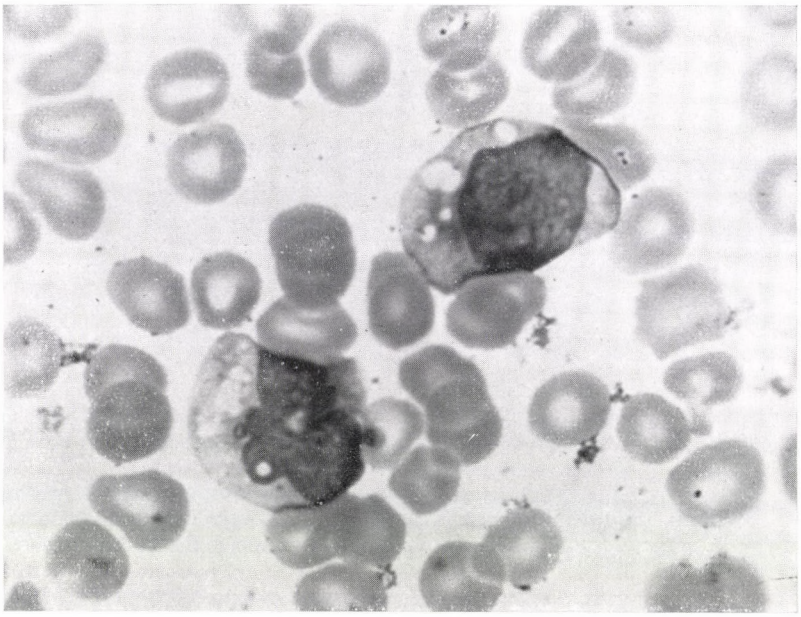


Abb. 1. Monozytenleukose. Peripherer Blutausstrich. Pappenheim

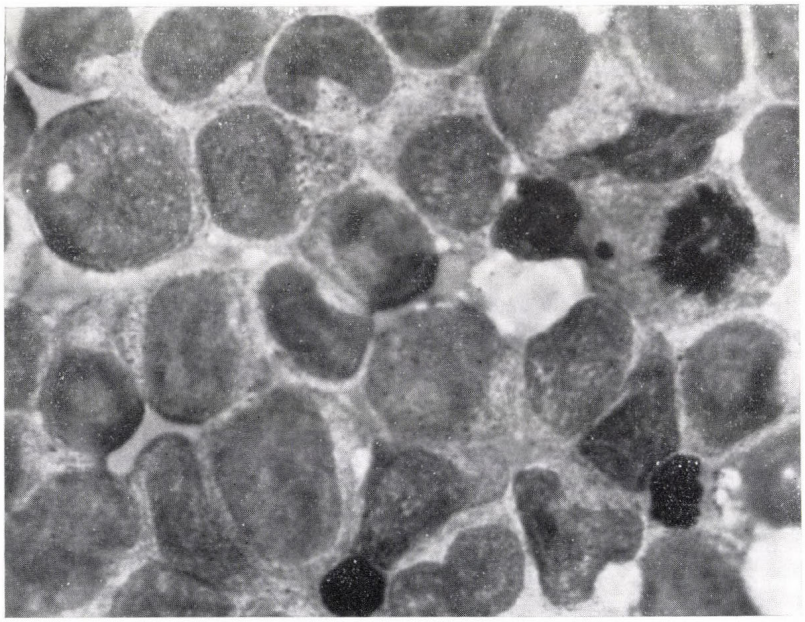


Abb. 2. Monozytenleukose. Sternalmarkausstrich. Pappenheim

Normalzellen unterscheiden. Die im peripheren Blutausstrich noch andeutungsweise erkennbare Ähnlichkeit zu normalen Monozyten geht im Sternalpunktat in der Regel verloren (Abb. 2). Hier erscheinen die pathologischen Zellen meist unreifer und undifferenzierter als im strömenden Blut [16]. Ohne Kenntnis des peripheren Blutausstrichs würde man aus dem Knochenmarkpunktat in manchen Fällen von Monozytenleukose noch nicht einmal einen entsprechenden Verdacht schöpfen. Eine grundlegende differentialdiagnostische Verbesserung brachte die Einführung verschiedener zytochemischer Verfahren, um deren Anwendung zur Definition der Monozytenleukose sich von klinischer Seite Braunsteiner und Schmalzl [3, 24, 25] sowie Löffler [15, 16], von pathologisch-anatomischer Seite Leder [13] verdient machten.

Material und Methodik

An Blut- und Sternalmarkausstrichen von 20 erwachsenen Patienten mit unreifzelligen Leukosen wurden folgende Reaktionen ausgeführt:

Methylenblaufärbung nach Stockinger und Kellner [26],

Toluidinblaufärbung bei pH 4.96 in der Modifikation nach Plenert und Mitarb. [20],

PAS-Färbung nach McManus, Lillie und Hotchkiss,

Sudanschwarz-B-Färbung nach Lison,

α-Naphthylazetat-Esterase nach Löffler [14],

Naphthol-AS-Azetat-Esterase nach Löffler [14],

Naphthol-AS-D-Chlorazetat-Esterase nach Burstone in der Modifikation nach Leder [12],

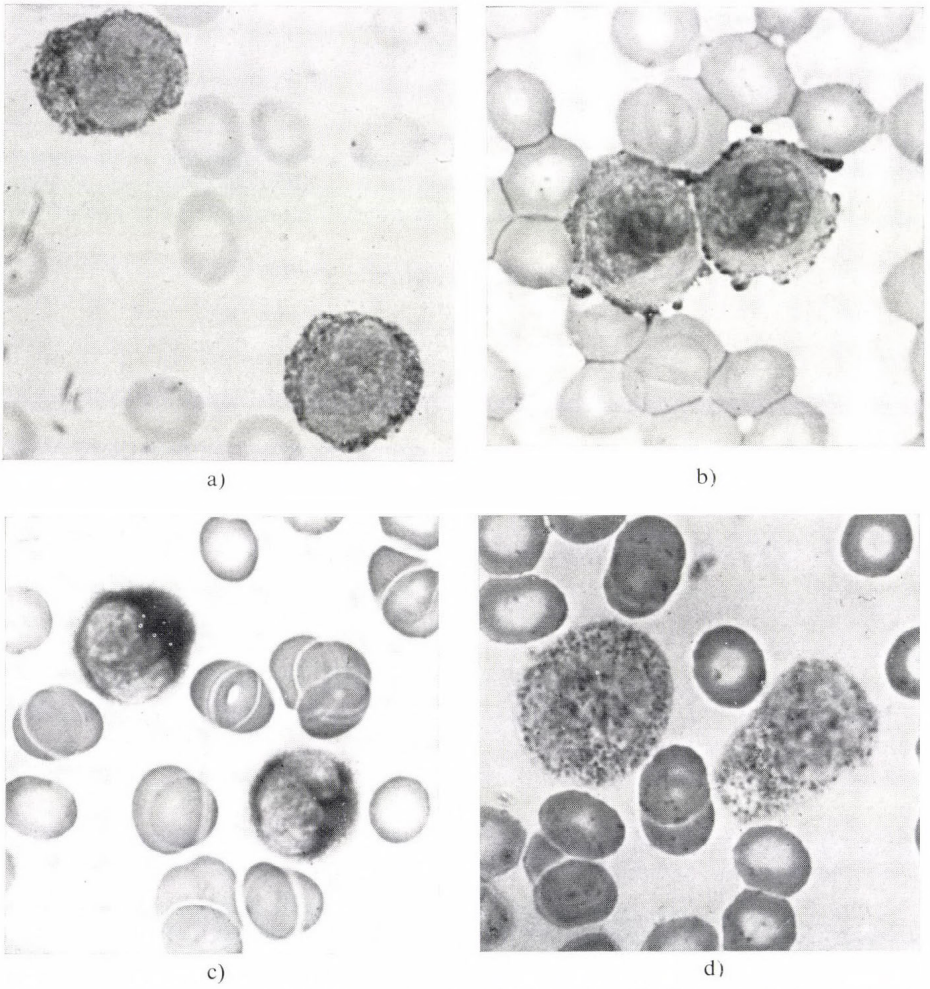
Peroxydase nach Sato und Sekija.

Unter den untersuchten Leukosen befanden sich 2 Fälle, die alle zytochemischen Kriterien der Monozytenleukose, auf die im folgenden eingegangen wird, aufwiesen. Es handelte sich einmal um einen 54jährigen Patienten (Krankenblatt-Nr. 1449/68) mit einem schweren septischen Krankheitsbild und multiplen Hautnekrosen, die im Anschluß an 3 Wofapyrininjektionen aufgetreten und im Sinne eines Sanarelli-Shwartzman-Phänomens zu deuten waren. Bei Gesamtleukozytenzahlen zwischen 8000 und 12 000 pro mm^3 fielen im Differentialausstrich anfangs vereinzelte, später bis 85 % schwer einzuordnende Zellen auf, die in großer Zahl auch im Leukozytenkonzentrat und Sternalpunktat nachgewiesen wurden und die nach der panoptischen Färbung am ehesten als atypische Retikulumzellen anzusprechen waren. Der Patient verstarb nach einem Krankheitsverlauf von knapp 3 Monaten. Autoptisch waren leukotische Infiltrate in Knochenmark, Leber, Milz, Nieren, Lungen, Herzmuskel und Gaumentonsillen nachweisbar. Der andere Fall betraf eine 71jährige Patientin (Krankenblatt-Nr. 21 191/68), die wegen einer Anämie (Hämoglobin 7.6 g %) und Fieberschüben in die Klinik eingewiesen wurde. Bei Leukozytenzahlen um 50 000/mm^3 fanden wir im gefärbten Ausstrich über 60 % atypische Zellen, die nach dem zytologischen Aspekt bereits eine deutliche Ähnlichkeit mit Monozyten erkennen ließen (Abb. 1), wäh-

rend allerdings im Sternalpunktat eine Differenzierungstendenz der leukotischen Zellinfiltrate fehlte (Abb. 2). Unter der kombinierten Behandlung mit Prednison und einem neuen Zytostatikum aus der Gruppe der alkylierenden Substanzen war zunächst eine Teilremission zu erzielen. Die Patientin verstarb 8 Monate nach Krankheitsbeginn zu Hause.

Befunde

Mit den basischen Farbstoffen Methylenblau oder Toluidinblau, die sich zur Nukleolendarstellung lymphatischer Zellen eingebürgert haben, lassen sich



a) b) c) d)

Abb. 3. Monozytenleukose. Periphere Blutausstriche. a) Toluidinblau; b) PAS; c) α-Naphthylazetat-Esterase; d) Naphthol-AS-Azetat-Esterase

sowohl die normalen Monozyten als auch die leukotischen Paramonozyten gut differenzieren. Wir geben dabei grundsätzlich der Toluidinblaufärbung den Vorzug, die eine schärfere Abbildung zytologischer Details ermöglicht. Die monozytoiden Zellen (Abb. 3a) haben ein blaß gefärbtes Zytoplasma und eine zarte filigranartige, mitunter streifige Kerninnenzeichnung, wobei nur selten kleine, fast punktförmige, schwer erkennbare Nukleolen sichtbar sind. Dagegen besitzen andere Spielarten der Paraleukoblasten ein kräftiger angefärbtes Plasma und

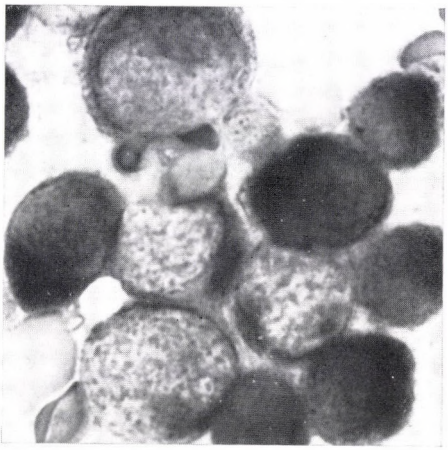


Abb. 4. Monozytenleukose. Sternalmarkausstrich. α-Naphthylazetat-Esterase

große, polymorphe Nukleolen. Auch gegenüber den leukämischen Retikulosen, die bei Pappenheimfärbung mitunter gar nicht von einer Monozytenleukose unterschieden werden können, ergeben sich insofern gewisse Differenzierungsmöglichkeiten, als deren heterogener Zellbestand durch größere Unterschiede der Kern- und Plasmastruktur von Zelle zu Zelle gekennzeichnet ist.

Die gleiche Übereinstimmung von normalen und pathologischen Monozyten besteht auch beim Glykogennachweis mit der PAS-Färbung [4, 7, 10, 11, 15]. Es findet sich eine feinkörnige burgunderrote Granulation der Zellperipherie bei schwacher rötlicher Grundtönung des Zytoplasma (Abb. 3b).

Die für myeloisch differenzierte Zellen typischen Reaktionen (Sudanschwarz B, Naphthol-AS-D-Chlorazetat-Esterase, Peroxydase) fielen in unseren beiden Fällen von Monozytenleukose durchweg negativ aus. Von anderen Autoren wurde eine schwach positive Reaktion an einem Teil der Zellen gesehen [8–11, 15, 19, 20].

Kennzeichnend und beweisend für den normalen und leukämischen Monozyten ist die hohe Plasmaaktivität an den sog. unspezifischen Esterasen. Bei Verwendung von Echtblausalz BB als Diazoniumverbindung läßt sich die α-Naphthylazetat-Esterase in Form einer diffusen braunschwarzen Plasmafärbung (Abb. 3c), die Naphthol-AS-Azetat-Esterase in Gestalt multipler, teilweise auch den Kern überlagernder Granula des blau gefärbten Reaktionsprodukts sichtbar ma-

chen (Abb. 3d). Diagnostisch besonders wertvoll ist, daß auch die zytologisch undifferenziert erscheinenden leukotischen Knochenmarkinfiltrate dank der hohen Zellaktivität an α-Naphthylazetat-Esterase einwandfrei als Monozytenabkömmlinge zu erkennen sind (Abb. 4). Als weiteres Spezifitätsmerkmal des normalen und leukämischen Monozyten fanden Braunsteiner und Schmalzl [3, 24, 25], daß nur in diesen Zellen die Naphthol-AS-Azetat-Esterase durch Zusatz von Natriumfluorid zur Pufferlösung gehemmt werden kann.

Diskussion

Erst durch die Zytochemie wurde die Monozytenleukose exakt diagnostizierbar und zum fest umrissenen nosologischen Begriff. Als entscheidendes zytochemisches Merkmal muß die hohe Zellaktivität der α-Naphthylazetat-Esterase herausgestellt werden [1, 2, 9, 13, 15, 16, 18, 19, 21, 23, 24, 25], die bei keiner anderen Leukoseform auftritt und die damit beweisend für die Monozytenleukose ist. Man sollte die Diagnose deshalb vom Ausfall dieser Reaktion abhängig machen. Besonders hervorzuheben ist der diagnostische Wert der Esteraseaktivitäten an den pathologischen Zellen außerhalb des strömenden Blutes, die in der Regel weniger differenziert wirken und bei den üblichen Standardfärbungen seltener als Monozytenabkömmlinge zu erkennen sind. Das gilt sowohl für das Sternalpunktat und den intravital entnommenen Lymphknoten als auch für das histologische Autopsiepräparat, an dem zytologische Feinheiten infolge verschiedener Einflüsse von Fixations- und Einbettungsverfahren verloren gehen.

Das gleichartige zytochemische Verhalten aller bisher untersuchten Fälle beweist ferner, worauf Leder [13] mit vollem Recht hinweist, daß es sich bei der Monozytenleukose um eine einheitliche Krankheitsform handelt und daß die anfechtbare Einteilung in einen retikulohistiozytären Typ Schilling, einen myeloischen Typ Naegeli und einen Mischtyp Hittmair überflüssig und gegenstandslos geworden ist. Bei zytochemisch völlig homogenen Zellpopulationen wäre es nicht vertretbar, die Erkrankung einmal als Retikuloseform und einmal als Myelosevariante zu definieren.

Zytochemisch ergeben sich außerdem Anhaltspunkte für eine gewisse Sonderstellung der Monozytenleukose unter den Hämoblastosen. Sie weist unter allen Typen unreifzelliger Leukosen die größte Konstanz und Regelmäßigkeit der zytochemischen Befunde auf, sowohl von Fall zu Fall als auch im Einzelfall von Zelle zu Zelle. Dabei besteht eine bemerkenswerte Übereinstimmung im Fermentmuster und im bausteinzytochemischen Verhalten zwischen der normalen "Mutterzelle" und dem leukotischen Paramonozyten [1, 13, 15, 16, 21, 24, 25].

Zytochemische Methoden sind demnach nicht nur für die hämatologisch-zytologische Diagnostik des Einzelfalls unentbehrlich geworden, sondern sie haben zugleich neue Kenntnisse über die nosologische Stellung der Monozytenleukose ermöglicht.

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Contribution to the Cytochemistry of Monocytic Leukaemia

The differentiation of leukaemias of immature-cell type is questionable when based on panoptic staining, the result is significantly improved by the application of different cytochemical methods. The cytochemical differentiation of monocytic leukaemia is the most reliable of all the haemoblastoses of immature-cell type. The high plasma α-naphthylacetate esterase and NaF inhibitable naphthol-AS-acetate-esterase activities are pathognomonic for this form of leukosis. The cytochemical identity of the pathological paramonocytes and of the normal monocytes concerning the structural components and enzymes is worth mentioning. Not only the cytological-diagnostic possibilities in the individual cases, but also the knowledge on the nosology of monocytic leukaemia have been enriched by cytochemistry.

Nachtrag bei der Korrektur:

Während der Drucklegung hatten wir Gelegenheit, 2 weitere Fälle von Monozytenleukose zu beobachten. Es handelt sich um einen 69jährigen Patienten (Kr. Bl. Nr. 13 157/70) und einen 64jährigen Patienten (Kr. Bl. Nr. 13 398/70) mit Krankheitsverläufen von 6 Monaten bzw. 4 Wochen, gerechnet vom Auftreten der ersten Symptome bis zum tödlichen Ausgang. Das zytochemische Verhalten der Leukosezellen entsprach den hier geschilderten Befunden völlig. Zusätzlich wurde die saure Phosphatase mit dem Substrat Naphthol-AS-BI-Phosphat bestimmt. Sie fiel in den pathologischen Zellen ausnahmslos negativ aus, während andere Untersucher auf Aktivitätssteigerungen dieses Ferments in den Zellen der Monozytenleukose hinweisen [15].

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Quantitative Immunglobulinbestimmungen bei Leukämie und Myelofibrose

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Bei 6 an akuter Leukose, 25 an lymphoider Leukose, 21 an myeloider Leukose und 12 an Myelofibrose leidenden Patienten sowie 60 normalen Individuen wurde der Immunglobulinspiegel mit der Radialimmundiffusionsmethode bestimmt. Bei akuter Leukämie konnte bei normalem IgG- und IgM-Spiegel ein herabgesetzter IgA-Spiegel, bei lymphoider Leukämie ein mäßig herabgesetzter IgG-Spiegel sowie ein ausgeprägt gesunkener IgA- und IgM-Spiegel festgestellt werden. Bei myeloider Leukämie war der IgG-Spiegel im allgemeinen normal oder erhöht, der IgA-Spiegel zumeist niedriger und der IgM-Spiegel schwankend. Bei Myelofibrose traten in allen drei Immunglobulinklassen stark schwankende Werte zutage, was zum Teil auf die verschiedenen Krankheitsstadien zurückgeführt wird. Im polyzythämischen Vorstadium der Myelofibrose war die Vermehrung aller drei Immunglobulinformen zu beobachten. Parallel zur Progression der Krankheit konnte eine Verminderung der Immunglobuline wahrgenommen werden.

Löffler [1] hatte 1951 bei einem Leukämiekranken wiederholte Infektionen beobachtet, die er auf Gammaglobulinmangel bzw. auf eine Insuffizienz der Antikörperproduktion zurückführte. Dadurch wurde das Interesse auf die sekundäre Immuninsuffizienz gelenkt und hauptsächlich bei den lymphoproliferativen Krankheitsbildern haben mehrere Autoren Hypo- bzw. Agammaglobulinämie beobachtet [2—6]. Einige Verfasser entdeckten eine Parallele zwischen der Häufigkeit der Infektionen, der Progressivität der Erkrankung und der Hypogammaglobulinämie [7, 8, 9]. Diese Beobachtungen stützten sich fast ausschließlich auf elektrophoretische Untersuchungen [3, 5, 6, 10].

Einen großen Fortschritt bedeutete die Einführung der Immundiffusionsmethoden, welche die Trennung und quantitative Bestimmung einzelner Eiweißkomponenten ermöglichten. Für semiquantitative Bestimmungen eignet sich auch die Immunelektrophorese [10, 11, 12]. Backhausz [13] hat ein quantitatives immunelektrophoretisches Verfahren ausgearbeitet. Für orientierende quantitative Bestimmungen ist auch die Ouchterlonysche [14, 15] radiale doppelte Diffusionsmethode anwendbar. Für quantitative Bestimmungen hat die weiteste Verbreitung das von Oudin [12, 16] eingeführte direkte Diffusionsverfahren gefunden. Diese Methode hat jedoch den Nachteil, daß sie eine große Menge Immunserum benötigt; viel einfacher und wirtschaftlicher ist die 1965 von Mancini und Mitarbeitern [18] ausgearbeitete unmittelbare Radialimmundiffusionsmethode. Diese letz-

tere Methode wandten wir zur Untersuchung der drei wichtigsten Immunglobulinklassen bei Patienten mit akuter bzw. chronischer lymphoider und chronischer myeloider Leukämie oder Myelofibrose an.

Untersuchungsmaterial und Methoden

Das Krankenmaterial wurde in 4 Gruppen eingeteilt.

Die 1. Gruppe umfaßte 6 Patienten mit akuter Leukämie. Die Blutentnahme erfolgte vor Einleitung der Behandlung.

Zur 2. Gruppe zählten 25 an lymphoider Leukämie leidende Kranke. In 19 Fällen dauerte die Krankheit 0—3, in 6 Fällen 3—8 Jahre. Die meisten wurden mit Prednisolon bzw. Chlorambucil, 2 Patienten auch mit Röntgen behandelt.

In die 3. Gruppe gehörten 21 Patienten mit chronischer myeloider Leukämie und 12 mit Myelofibrose. Die myeloide Leukämie dauerte in 16 Fällen 0—3, in 5 Fällen 3—10 Jahre. In 2 Fällen kam vorübergehend Dibrommannit zur Anwendung, die übrigen Kranken wurden mit Busulfan behandelt.

Bei den zur 4. Gruppe gezählten 12 Kranken, die an Myelofibrose leideten, die Dauer der Krankheit (1—14 Jahre) war sehr verschieden.

Die 5. (Kontroll-) Gruppe bestand aus 60 normalen Individuen, und zwar 28 Männern und 32 Frauen mit einem Durchschnittsalter von 46.6 Jahren.

Ein monospezifisches Immunserum, dessen Verdünnung im Vorversuch festgestellt worden war, wurde mit 3 %igem Agar-Gel vermengt und auf Objektträger aufgetragen. Nach Erstarrung wurde ein Antigenbehälter von 3 mm Durchmesser verfertigt und in diesen eine 4fache Verdünnung des zu untersuchenden Serums gegeben. Nach 48stündiger Diffusion bei +4°C wurde das Präparat wiederholt gewaschen, getrocknet, und mit Amidoschwarz gefärbt. Die Dicke der Präzipitationsringe (*h*) wurde in mm angegeben; die einzelnen *h*-Werten entsprechende Antigenkonzentration wurde mit Hilfe einer Kalibrationskurve abgelesen. Diese wurde so konstruiert, daß von einem Serum bekannter Konzentration 3 Verdünnungsreihen 1 : 2 angelegt und aufgrund der Regressions-Durchschnittswerte die zu den gleichen Konzentrationen gehörenden 3 *h*-Werte ermittelt wurden.

Da uns ein internationaler Antigen-Standard nicht zur Verfügung stand, überließ uns zwecks Vergleich unserer Kontrollfälle das Staatl. Impfstoff-Forschungsinstitut Human ein normales Serumgemisch das 1500/100 ml IgG, 200/100 ml IgA und 160/100 ml IgM enthielt.* Bei den Untersuchungen benutzten wir die vom gleichen Institut hergestellten Immunsera anti-IgG-25-Sch (Schaf), anti-IgA-30-Z (Ziege), anti-IgM-1-P (Pferd) und anti-IgM-110-P (Pferd).

* Für das uns zur Verfügung gestellte Serum und die methodologische Hilfe danken wir Herrn Dr. R. Backhausz und Frl. Judit Lajos auch an dieser Stelle.

Ergebnisse

1. Gruppe. Bei akuter Leukämie zeigte der IgG- und IgM-Wert keine wesentlichen Abweichungen. Der IgA-Spiegel war — einen Patienten ausgenommen — bedeutend gesunken. Im Verhältnis zum normalen Durchschnitt (1. Gruppe) war die Senkung signifikant (Tabelle 1).

Tabelle 1

Durchschnittliche Immunglobulinwerte bei den einzelnen Krankengruppen (Streuung, Signifikanz)

Gruppen	IgG mg/100 ml	IgA mg/100 ml	IgM mg/100 ml
Normal n = 60	1570± 52.3	220± 12	193± 15.5
Akute Leukämie n = 6	1630± 141 $p > 0.05$	149± 29 $p < 0.01$	191± 18 $p > 0.05$
Lymphoide Leukämie n = 25	1411± 134 $p > 0.05$	92± 12 $p < 0.001$	64± 8 $p < 0.001$
Myeloide Leukämie n = 21	2070± 138 $p < 0.01$	176± 21 $p > 0.05$	186± 34 $p > 0.05$
Myelofibrose n = 12	2610± 279 $p < 0.001$	243± 61.8 $p > 0.05$	252± 64.3 $p > 0.05$

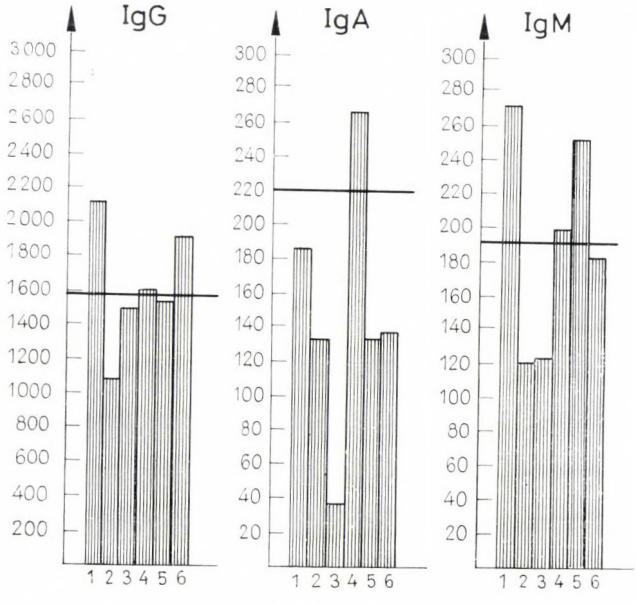

Abb. 1. Der Immunglobulinspiegel von 6 an akuter Leukämie leidenden Patienten

2. Gruppe. Die auffallendsten Abweichungen traten bei den an lymphoider Leukämie leidenden Kranken zutage. Kein einziger IgA- und IgM-Wert erreichte den normalen Durchschnitt; auch totaler Mangel kam vor. Der IgG-Spiegel war zumeist mäßig erniedrigt, einige Werte waren jedoch höher als der normale Durchschnitt. In den seit mehr als 3 Jahren bestehenden Fällen war die Abnahme ausgeprägt. Die Senkung des IgA und IgM war im Vergleich zu den Kontrollwerten stark signifikant (Abb. 2).

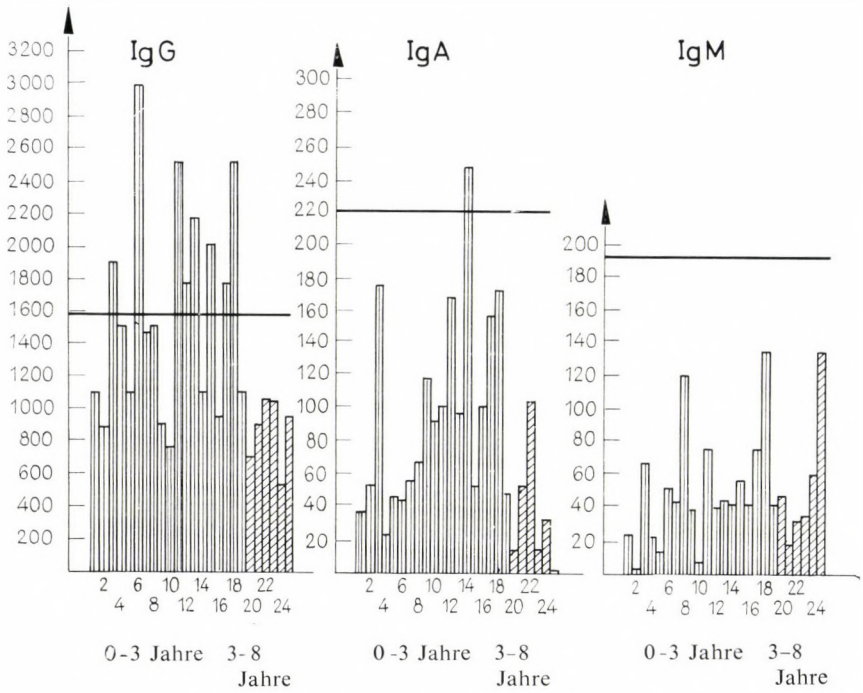

Abb. 2. Der Immunglobulinspiegel von 25 an lymphoider Leukämie leidenden Patienten

3. Gruppe. Bei chronischer myeloider Leukämie war der IgG-Spiegel in den ersten 3 Jahren der Krankheit meistens höher als normal. Eine Senkung trat hauptsächtlich bei dem IgA zutage, und eine auffallende Schwankung ergab sich bei den IgM. Die Verminderung aller drei Ig-Werte bei ein und demselben Kranken kam selten vor, meist handelte es sich um einen isolierten Abfall eines Ig, während der Spiegel der anderen beiden normal oder erhöht war. Mit der Progression der Krankheit konnte eine allgemeine Ig-Abnahme auch hier beobachtet werden. Unter Berücksichtigung des ganzen Krankheitsverlaufs zeigte der IgM-Spiegel im Verhältnis zu den Kontrollen keine Abweichung; der IgG-Spiegel war signifikant erhöht, der IgA-Spiegel demgegenüber signifikant gesunken (Abb. 3).

4. Gruppe. Die größten individuellen Schwankungen wurden bei Myelofibrose beobachtet: bei einem Kranken waren alle drei Ig-Werte bedeutend erhöht, der durchschnittliche IgG-Spiegel war signifikant, der IgM-Spiegel mäßig erhöht (Abb. 4).

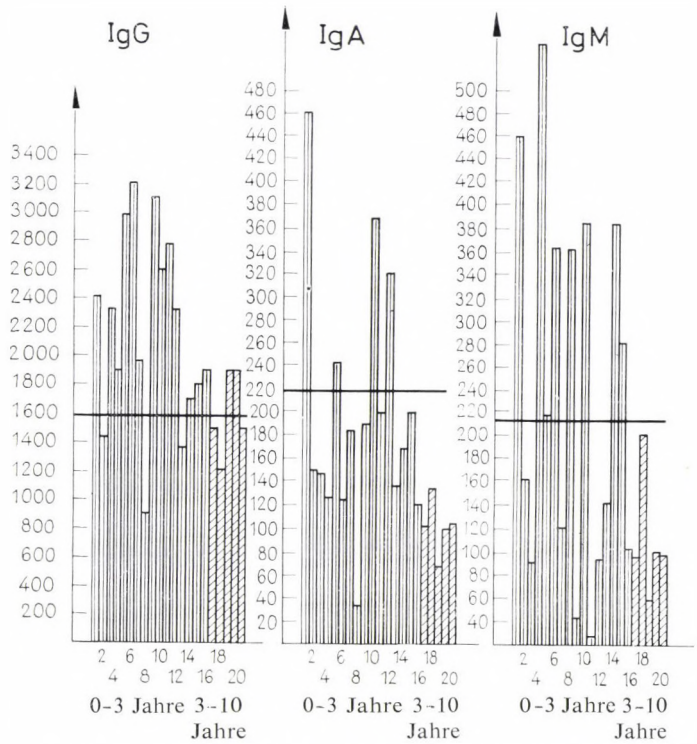

Abb. 3. Der Immunglobulinspiegel von 21 an myeloider Leukämie leidenden Patienten

Tabelle 2

Normale Immunglobulinwerte

	IgG	IgA	IgM
Fahey 1965	1240	280	120
Vergani u. Mitarb. 1967	1150	194	92
Kohler u. Mitarb. 1967	1547	221	78
Grossmann u. Mitarb. 1967	1500	275	123
Rentsch 1969	1240	250	136

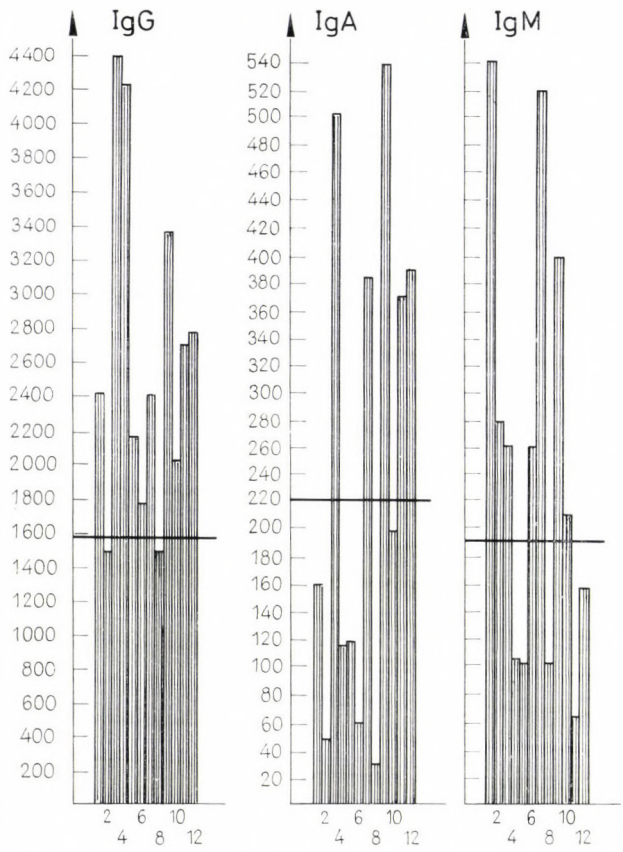

Abb. 4. Der Immunglobulinspiegel von 12 an Myelofibrose leidenden Patienten

5. Gruppe. Die von uns ermittelten normalen Durchschnittswerte sind in Tabelle 1 dargestellt; in den Abbildungen zeigt eine horizontale Linie den Durchschnittsspiegel an. Die von einigen Verfassern angegebenen Normalwerte veranschaulicht Tabelle 2.

Besprechung

Die meisten Autoren machen für die häufigen Infektionen bei akuter Leukämie nicht den humoralen, sondern den zellulären Immundefekt verantwortlich [3, 24]. Die Ergebnisse der quantitativen Immunglobulinbestimmungen deuten ebenfalls darauf hin, daß die Immunglobulinsynthese nicht wesentlich geschädigt ist; unsere bisher unveröffentlichten Resultate scheinen dasselbe zu bestätigen. Die bei lymphoider Leukämie auftretenden häufigen Infektionen können vor

allem mit der Verminderung des IgA und IgG in Zusammenhang gebracht werden. Dies ist auch deshalb wichtig, da die zu therapeutischen Zwecken angewendeten Gammaglobulinpräparate überwiegend IgG enthalten [25]. Bei myeloider Leukämie ist die Ig-Synthese kaum geschädigt; höchstens kommt eine isolierte Ig-Verminderung vor, und dementsprechend sind häufige Infektionen nicht zu beobachten. Die bei Myelofibrose ermittelten Schwankungen sind zum Teil darauf zurückzuführen, daß wir unsere Patienten in verschiedenen Stadien untersucht hatten; die Krankheitsdauer erstreckte sich ja von 1 bis 14 Jahren. Beachtenswerterweise war im polyzythämischen Stadium, in dem alle drei Zellelemente vermehrt sind, auch die Menge der Ig beträchtlich angewachsen, wie z. B. in den Fällen Nr. 3 und 9. (Abb. 4). Schon früher hatten wir angenommen, daß bei Polyzythämie und in der polyzythämischen Vorphase der Myelofibrose das Immunozytensystem parallel mit dem Erythroid-, Myeloid- und Megakaryozytensystem proliferiert.

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Quantitative Immunoglobulin Determination in Leukaemia and Myelofibrosis

The immunoglobulin level of 6 patients with acute, 25 with lymphoid, and 21 with myeloid leukaemia, and 12 with myelofibrosis, as well as of 60 normal individuals was determined by the direct radial immunodiffusion method. In acute leukaemia a decreased IgA level was found together with normal IgG and IgM levels. In lymphoid leukaemia IgG was moderately, IgA and IgM markedly decreased. In myeloid leukaemia the IgG level was usually normal or increased, the IgA level mostly lower and the IgM level variable. In myelofibrosis the deviation was high in every immunoglobulin class, which was partly explained by the different stages of the disease. In the polycythaemic pre-phase of myelofibrosis an increase in all the three immunoglobulin types was observed. With the progress of the disease the immunoglobulin levels were decreasing.

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Présence et spécificité de l' antigène Australie et de l'anticorps correspondant

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La recherche de l'antigène Australie et de l'anticorps correspondant a été effectuée chez 2.339 sujets répartis en 3 groupes: 2.246 examens réalisés sur des sujets hospitalisés pour causes diverses nous ont permis de sélectionner deux sérums porteurs de l'antigène Australie. 12 sérums d'hémodialysés ont été examinés: l'antigène Australie a été décelé une fois, l'anticorps anti-Australie chez 2 malades. 81 malades atteints d'hépatites ont été testés: dans les 4 cas d'hépatite post-transfusionnelle (Virus B), la présence de l'antigène Australie a pu être mis en évidence, mais cette recherche s'est révélée négative chez 68 sujets présentant une hépatite épidémique (Virus A). Pour les deux derniers groupes de malades les résultats de l'immunodiffusion et de la fixation du complément sont comparés. La spécificité de la réaction d'immunodiffusion et le choix d'une méthode de détection sont discutées.

Au cours de ces trois dernières années, de nombreux auteurs [1—5], ont affirmé la corrélation existant entre la présence de l'antigène Australie (Au) ou SH-antigène dans le sérum des malades et la fréquence des hépatites virales. Cet antigène a été identifié à une particule virale [6, 7]. Il est détectable par les techniques d'immunodiffusion et de fixation du complément à l'aide de sérums de sujets polytransfusés.

La recherche sur la présence et la spécificité de l'antigène Australie et de l'anticorps correspondant a fait l'objet de notre étude.

En effet, la spécificité de cet antigène Au demeure mal définie. Ce dernier correspondrait au virus des hépatites pour certains [4, 8] et seulement au virus de l'hépatite post-transfusionnelle pour d'autres [5, 9, 10].

Par ailleurs, la réaction de mise en évidence de l'antigène Au et de l'anticorps anti-Au à l'aide d'un sérum de sujet polytransfusé peut conduire à détecter d'autres systèmes précipitants.

Materiel et méthodes

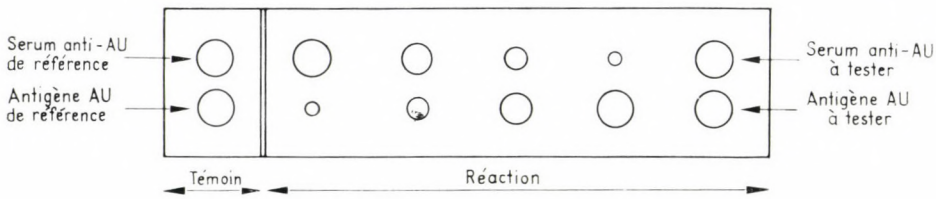
L'antigène Australie et l'anticorps correspondant qui ont permis de débuter ce travail proviennent du Centre National de Transfusion Sanguine.*

Les techniques de base utilisées ont été l'immunodiffusion et la fixation du complément.

* Nous remercions vivement le Professeur Soulier pour son aimable assistance.

Immunodiffusion. Il s'agit d'une microtechnique de double diffusion dans un gel d'agarose à 0.9 %. Le tampon utilisé est conforme à celui de Prince [2]: chlorure de sodium 0.1 M; tri (hydroxyméthyl) aminométhane 0.01 M; E. D. T. A. 0.01 M; pH 7.6 à 22°C. Il n'a pas été ajouté de sulfate de protamine. Le milieu gélosé est coulé sur des lames de verre fixées sur des portoirs de type Gelman. La diffusion se réalise à la température du laboratoire en atmosphère humide durant cinq jours. Avec un éclairage sur fond noir, on peut observer, pour les réactions les plus nettes, de fins arcs de précipitation dès la 24ème heure. Ces derniers sont colorés successivement par l'Amido-Schwarz et le Noir Soudan.

Pour apprécier les concentrations optima réagissantes de l'antigène et de l'anticorps pour un nouveau système précipitant, ces divers réactifs sont disposés dans des alvéoles de différents diamètres suivant le schéma ci-dessous:



Fixation du complément. La réaction de fixation du complément a été réalisée sur plaque de plexiglas modèle O M S. Elle est de type Kolmer avec incubation à +4° pendant 18 heures, semi-quantitative et avec lecture hémolyse 100 %.

Le sérum du malade, qui contient l'antigène, est dilué de 1/1 à 1/512, puis testé en utilisant un sérum anticorps non dilué.

Dans chaque cupule est réparti 0.025 ml de l'antigène, 0.025 ml de l'anticorps et 0.025 ml de complément (2 unités C'H 100), puis après fixation 0.025 ml d'une suspension à 1 % de globules rouges sensibilisés.

Résultats

Les résultats complets de notre étude sont consignés dans le tableau I.

Chez 4 malades sur 4 atteints d'hépatites post-transfusionnelles (virus B), la présence de l'antigène Australie a pu être mis en évidence à la phase préictérique. On constate sa disparition dans les huit premiers jours de la phase ictérique.

Chez 68 sujets présentant une hépatite épidémique (virus A), nous n'avons jamais pu détecter la présence de l'antigène Australie par immunodiffusion; par contre 6 sérums ont présenté une réaction de fixation du complément positive. Nous avons noté la forte activité anticomplémentaire de ces sérums. Il est à noter que le taux de fixation du complément semble nettement inférieur pour les sérums

Tableau 1

Résultats et étude comparative de l'immunodiffusion et de la fixation du complément pour 93 sérums (sujets atteints d'hépatites et hémodialysés)

	Nombre de cas	Immunodiffusion		Fixation du complément	
		* Au +	** Ac anti Au +	Au +	Lecture du degré de fixation
Hépatites d'origine post-transfusionnelle (virus B)	4	4	0	4	++++ au 1/256 ++++ au 1/128 ++++ au 1/256 ++++ au 1/512
d'origine épidémique (virus A)	68	0	0	6	++++ au 1/32 ++++ au 1/4 ++++ au 1/128 ++++ au 1/8 ++++ au 1/8 ++++ au 1/2
d'origine inconnue	6	1	0	1	++++ au 1/256
anictériques	3	1	0	1	++++ au 1/128
hémodialysés	12	1	2	1	++++ au 1/32
hospitalisés pour causes diverses	2246	2	1	technique non utilisée	

* Au + = présence de l'antigène Australie.

** Ac anti Au + = présence de l'anticorps anti-antigène Australie. Le degré de fixation du complément est indiqué par 4 + pour une absence complète d'hémolyse.

provenant de malades faisant une hépatite épidémique ($\leq$1/128) que pour les sujets atteints d'une hépatite d'inoculation ($\leq$1/512).

Sur 12 sérums d'hémodialysés que nous avons examinés, 2 se sont révélés être positifs dans la recherche de l'anticorps anti-Au; chez le malade M . . ., atteint d'une angéite nécrosante avec glomérulonéphrite il a été retrouvé simultanément l'antigène et l'anticorps correspondant.

Les 2246 examens réalisés sur des sujets, hospitalisés pour causes diverses, nous ont permis de sélectionner par immunodiffusion deux sérums porteurs de l'antigène Au et un possédant la spécificité anti-Au.

Discussion

Présence et spécificité de l'antigène Au

Comme certains auteurs (5—9—10), nous détectons la présence de l'antigène Australie uniquement dans les hépatites d'inoculation (virus B) et jamais au cours des hépatites épidémiques (virus A). Il est possible que l'antigène Au soit également spécifique du virus A, mais sa virémie étant très brève, nous le recherchons à une période toujours trop tardive. En s'appuyant sur les hypothèses de

Shulman et Barker [11], il est également possible que le virus A, complexé à l'anticorps, ne soit détectable qu'à l'aide d'artifices immunologiques techniques (excès d'antigène ou excès d'anticorps).

Durant l'hépatite post-transfusionnelle, la mise en évidence de l'antigène Au est positive au cours de la période d'incubation, avant les désordres cellulaires graves avec taux élevé des transaminases (SGOT-SGPT). Dès le 8ème jour de la phase ictérique, il semble que l'antigène ne soit plus présent dans le sérum du malade. Ce fait plaide en faveur de la nature virale de l'antigène Au plutôt que son identité avec un antigène provenant de la lyse cellulaire de l'hépatocyte.

L'antigène Au a pu être mis en évidence chez un parmi trois sujets faisant des hépatites anictériques et chez 2 sur 2246 malades hospitalisés sans antécédents ictériques. Aussi dans la sélection des donneurs de sang le seul critère, absence d'un ictère dans le passé pathologique, ne peut permettre d'écarter un porteur chronique de virus.

Présence et spécificité de l'anticorps anti-Au

L'anticorps anti-Au est surtout rencontré chez les polytransfusés en particulier chez les hémodialysés.

Contrairement à un processus infectieux classique, nous n'avons jamais pu révéler la présence de l'anticorps anti-Au à la phase de convalescence d'une hépatite pour laquelle la mise en évidence de l'antigène Au avait donné un résultat positif. Ainsi, on peut observer une présence indéfinie de l'antigène sans formation d'anticorps chez des porteurs chroniques; de même, au cours d'une hépatite post-transfusionnelle la présence fugace de l'antigène n'est pas suivie d'une réaction immunologique classique avec synthèse d'anticorps à un taux décelable par les techniques utilisées. Ce fait suggère que l'antigène Au possède des qualités immunogènes très faibles. On pourrait également soulever l'hypothèse d'une infection du foetus in utero par l'antigène Au conduisant à un processus de tolérance immunologique et permettant d'expliquer sa présence à l'état latent chez des sujets apparemment sains.

Dans la recherche de l'anticorps anti-Au, nous avons trouvé 19 réactions faussement positives: 12 sérums étaient bactériologiquement contaminés, 4 sérums appartenaient à des sujets du groupe sanguin B, et enfin 3 sérums présentaient une activité anti β lipoprotéinique. Ces réactions faussement positives se colorent très peu par l'Amido-Schwartz, par contre très intensément par le Noir Soudan. Aussi cette double coloration doit être effectuée systématiquement sur les plaques d'immunodiffusion et doit permettre de détecter certaines réactions non spécifiques. Le fait que le sérum possédant l'activité anticorps anti-Au puisse conduire à de fausses réactions n'a rien de surprenant. En effet, un sérum de sujet polytransfusé ayant reçu des sollicitations antigéniques diverses doit logiquement permettre de mettre en évidence d'autres systèmes précipitants et en particulier de révéler des allotypes globuliniques comme ce fut le cas pour Blumberg au début de son expérimentation [12].

Pour l'obtention d'un sérum anti-Au, il est actuellement nécessaire de réaliser une recherche d'anticorps chez des sujets hémophiles polytransfusés, des hémodialysés et chez les membres du personnel hospitalier. Le fait qu'un laboratoire soit tributaire de cette recherche aux résultats incertains, constitue un inconvénient majeur. Seuls les sérums d'animaux hyperimmunisés à l'aide d'un antigène Au adjuvé permettraient peut être de résoudre ce problème. Cette dernière solution aurait également l'avantage de permettre l'obtention d'un sérum qui, après épuisement et purification posséderait une spécificité plus étroite.

Choix de la méthode de détection

La recherche de l'antigène Au s'effectuant actuellement avec des sérums non monospécifiques, il y a intérêt à utiliser une méthode qui ne soit pas très sensible. Aussi, nous pensons que les réactions obtenues par immunodiffusion ont plus de valeur quant à leur spécificité que les résultats obtenus par fixation du complément.

L'immunodiffusion en agarose devrait être réalisée systématiquement avec migration dans un champ électrique selon la méthode de Wewalka (13) afin d'obtenir une réponse rapide (2—4 h). En effet, dans un Centre de Transfusion Sanguine la sélection des donneurs doit être effectuée d'une manière très précoce, le sang étant souvent distribué dans la journée qui suit son prélèvement.

Conclusion

Le diagnostic biologique des hépatites infectieuses par la recherche de l'antigène Au est pour l'instant d'application restreinte. L'antigène Au a pu être mis en évidence dans la phase pré-ictérique et dans les huits premiers jours de la phase ictérique seulement de l'hépatite d'inoculation (virus B); nos recherches s'étant révélées par ailleurs négatives pour l'hépatite épidémique. Une virémie éphémère constitue peut être la cause de cet échec.

Contrairement à un processus infectieux classique nous n'avons jamais pu révéler la présence de l'anticorps anti-Au à la phase de convalescence d'une hépatite pour laquelle la mise en évidence de l'antigène Au avait donné un résultat positif.

Les limites de la spécificité de la réaction sont actuellement mal définies; en effet à l'aide d'un sérum de sujet polytransfusé qui est plurispécifique il est possible de révéler d'autres antigènes que l'antigène Au. Aussi, nous pensons que la réaction d'immunodiffusion en gel d'agarose connue comme peu sensible doit être préférée à la réaction de fixation du complément qui vraisemblablement permet de déceler d'autres spécificités.

Pour la transfusion Sanguine, la sélection des donneurs de sang avec recherche systématique de l'antigène Au s'impose; en effet, le critère fondé sur l'absence d'un ictère dans le passé pathologique ne peut être considéré comme suffisant, l'antigène Au étant retrouvé chez des sujets apparemment sains et chez des malades présentant des hépatites anictériques.

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Presence and Specificity of Australia Antigen and of the Corresponding Antibody

The presence of Australia antigen and of the corresponding antibody has been studied in 2,339 subjects divided in three groups. 1. 2,246 tests made in subjects hospitalized for various causes revealed Australia antigen in two sera. 2. Of 12 patients on maintenance haemodialysis, Australia antigen was found in one, and anti-Australia antigen in two patients. 3. The serum of 81 hepatitis patients has also been tested. 4. The presence of Australia antigen has been detected in four cases of transfusion B virus hepatitis, but in none of the 68 subjects with epidemic A virus hepatitis. Results of the gel diffusion test and complement fixation test in the two last groups have been compared. The specificity of the gel diffusion test and the choice of a detection method are discussed.

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Immunoglobulin Levels in Hodgkin's Disease

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Serum immunoglobulin levels in 11 children and 29 adult patients with Hodgkin's disease were studied by means of immunoelectrophoresis and single radial immunodiffusion. The immunoglobulin leveis were compared to those in a control group of 35 healthy adult individuals. In adult patients with Hodgkin's disease, a significant decrease of IgA and IgM, with a slight elevation of IgG and IgD levels were noted. The findings reflect some humoral reactivity of Hodgkin's disease patients undergoing radiotherapy and chemotherapy.

Introduction

The immunological deficiency associated with Hodgkin's disease [1, 3, 8] manifests itself clearly with a disposition of such patients to mycotic and viral infections, a diminished response of the delayed type and prolonged survival of homografts [8, 14]. Poor transformation of lymphocytes on phytohaemagglutinin stimulation was also reported [2, 12], as well as cytoplasmic processes characteristic of dying cells [4]. The patients' humoral immunity was found to be decreased, especially the capacity for antibody formation in primary response [1, 3, 8, 22, 23].

In this paper, results of our studies of the immunoglobulins in patients with Hodgkin's disease will be presented.

Material and Methods

Fourty patients with histologically confirmed Hodgkin's disease were studied. In most of them the symptoms had appeared two years, in ten patients more than 4 years ago (Table 1).

Treatment usually consisted of two stages. At the onset of the disease patients were treated with radiotherapy (X-ray and in some cases ^{60}Co irradiation) followed later by chemotherapy with Encorton, cyclophosphamide and vinblastine. The response to therapy is shown in Table 1. One patient (No. 32) showed complete regression following X-rays and vinblastine therapy.

The clinical stage of the condition was estimated according to the classification proposed by Peters [19] and Karnofsky [13]. The stages were subdivided

Table 1

Clinical and laboratory data of patients with Hodgkin's disease

Patient No.	Sex	Age	Duration of illness	Total protein, g per 100 ml	Immunoglobulins, mg/ml				Stage of disease	Therapy and response*
					G	M	A	D		
1	2	3	4	5	6	7	8	9	10	11
1	M	3	1 1/2 years	7.1	11.3	0.91	4.70	n.d.**	IVB	Rtg, Enc, Vbl (−)
2	M	6	1 year	6.5	15.7	0.44	1.53	0.22	IIIA	Enc, Vbl (±)
3	M	6	2 years	6.4	17.6	2.04	2.28	n.d.	IVB	R, Enc, Cp, V (±)
4	M	11	1 year	7.5	16.9	1.27	2.40	0.03	IIA	Rtg (+)
5	M	11	7 years	6.9	14.3	0.97	1.00	0.13	IIIA	Rtg (±)
6	M	11	3 years	6.8	10.2	0.60	1.45	0.40	IIIA	R, Enc, Vbl (±)
7	M	12	2 1/2 years	6.5	12.2	1.04	2.46	0.08	IIA	Rtg (+)
8	M	13	9 years	6.5	12.5	0.46	0.90	0.03	IIA	Rtg (+)
9	M	13	3 years	6.5	14.6	0.24	1.84	0.10	IVB	R, Enc, Cp, V (±)
10	F	13	6 1/2 years	7.4	17.0	1.40	1.84	0.18	IIIA	Rtg (+)
11	M	13	5 years	7.2	18.1	1.32	1.48	0.10	IIIB	Rtg, Vbl (+)
12	F	19	3 years	7.7	26.6	2.60	2.80	0.27	IIA	Rtg (+)
13	F	21	1 year	7.1	30.8	1.40	3.14	0.20	IIIA	End, Vbl (+)
14	M	24	8 months	6.1	13.3	0.64	1.30	0.03	IVB	Rtg, Enc, Cp (−)
15	M	25	7 years	8.0	46.0	0.50	1.92	0.13	IVB	Rtg, Enc, Vbl (±)
16	M	26	15 years	7.3	31.2	1.70	1.00	n.d.	IVB	Rtg, ^{60}Co (+)
17	M	28	3 years	3.3	7.3	0.30	0.85	0.01	IIIB	Enc, Cp (−)
18	M	29	10 months	7.3	9.7	1.10	1.62	0.13	IIIA	Rtg, ^{60}Co (+)
19	F	30	6 months	6.1	30.6	0.75	1.92	n.d.	IA	Rtg (+)
20	M	33	1 year	6.6	10.8	1.15	1.90	0.02	IIB	Rtg, Enc (+)
21	F	34	3 years	6.5	30.5	1.40	2.77	n.d.	IIIB	Rtg, Enc, Vbl (+)
22	M	35	4 months	6.3	20.4	0.90	1.75	0.06	IIB	Rtg, Enc (±)
23	M	36	7 years	7.8	28.0	1.94	2.46	0.01	IIB	Rtg, Enc (±)
24	F	37	1 1/2 years	6.4	23.3	2.00	2.05	0.45	IIA	Rtg (+)
25	F	38	8 years	6.0	7.8	0.60	1.24	0.03	IVB	R, Enc, Cp, V (−)
26	F	38	1 year	6.0	11.2	0.64	1.65	n.d.	IA	Rtg (+)
27	M	40	5 months	6.4	7.0	3.45	1.30	n.d.	IA	Rtg (+)
28	M	43	2 years	7.1	19.2	2.05	1.40	0.02	IIIB	R, antibiotics (±)
29	F	43	6 months	5.4	12.5	1.10	1.28	n.d.	IIB	Rtg (+)
30	F	46	4 months	6.2	13.2	1.17	2.30	0.01	IVB	Rtg (+)
31	F	51	2 weeks	6.7	13.0	1.44	1.80	0.23	IIB	Rtg (−)
32	F	52	5 1/2 years	6.6	14.4	1.40	0.95	0.15	IIB	Rtg, Vbl (++)
33	F	60	2 years	6.6	16.5	0.70	3.70	0.01	IIIB	Cp, Vbl, Enc (±)
34	M	61	2 years	6.7	17.4	2.00	3.10	0.14	IIIA	Cp, Vbl, Enc (±)
35	M	64	1 year	7.8	24.8	1.80	6.96	0.02	IA	Rtg, Enc (+)
36	M	65	1 year	7.2	39.0	0.90	3.60	0.01	IIA	Rtg (−)
37	K	66	1 1/2 years	6.0	13.3	0.62	1.45	0.02	IIIB	Rtg, Cp (±)
38	M	67	1 year	7.1	19.3	1.75	3.80	0.10	IIIB	Rtg (±)
39	M	68	1 year	7.3	35.2	0.62	2.22	0.10	IIA	Rtg (+)
40	M	74	4 years	7.3	18.8	2.20	5.30	0.06	IIIB	Rtg (±)

Abbreviations used: R, Rtg — X-rays; Enc — Encorton; Cp — cyclophosphamide; V, Vbl — vinblastine; ^{60}Co — gamma irradiation

* Response to therapy given in parentheses: (++) recovery; (+) good response; (±) weak response; (−) no response

** n.d. — not determined

into A and B, to indicate the absence or presence of systemic symptoms, respectively.

Serum samples after centrifugation from retracted clot were frozen and kept at —20° C. Serum samples from 35 healthy blood donors were taken as a control.

Serum total protein was determined by refractometry (TS-meter, American Optical Company).

Immunoelectrophoretic analysis was performed according to Korngold et al. [15]. Antisera were prepared by immunization of rabbits and goats with immunoglobulin preparations described below. Antiserum anti-immunoglobulin was prepared in a goat by immunizing with a mixture of immunoglobulins. All antisera were rendered specific by means of absorption with cross-reacting antigens. Antisera anti-IgD, anti-BJK and BJL were purchased from the Institute of Sera and Vaccines, Prague.

Single radial immunodiffusion. The method of Mancini et al. [17] was adapted. Serum diluted in 0.15 M buffered saline pH 7.6 was used at dilutions of 1 : 10, 1 : 20, 1 : 40 for IgG determination and 1 : 2, 1 : 4, 1 : 8 for IgA and IgM determination. Undiluted sera were placed in wells on IgD plates, incubated for two days in humid chambers at room temperature and the shadow-graphs were taken in the light of an UV lamp on Brom extra hard paper (111 C, Foton).

The following immunoglobulin preparations were used for immunization of animals and employed as standards. *Immunoglobulin G* was isolated by DEAE-cellulose chromatography from normal human serum [20]; for elution, Tris-NaH_2PO_4 buffer of increasing ionic strength and decreasing pH was applied. *Immunoglobulin A* was a monoclonal myeloma protein isolated from serum by starch-block electrophoresis [16]. *Immunoglobulin M* was a cryomacroglobulin from a case of Waldenström's macroglobulinaemia, isolated by repeated precipitation in 4 °C and re-solution in 37 °C saline, then purified on Sephadex G-200 column at room temperature. *Immunoglobulin D* was isolated from gamma-D myeloma serum by starch-block electrophoresis or by DEAE-cellulose chromatography in phosphate buffer with 0.001 M ε-aminocaproic acid [21].

All immunoglobulin preparations were exhaustively dialyzed against distilled water and lyophilized. Samples at 2 % concentration in buffered saline were checked for purity by immunoelectrophoresis.

Six dilutions of IgG, IgM, IgA and IgD preparations were applied to each series of immunoplates as standards. The diameter of the precipitin rings for standards was plotted on calibrated paper against the concentration.

Statistical significance of differences between values for healthy individuals and patients with Hodgkin's disease was estimated by means of Student's *t* test.

Results

Mean value for serum total protein was 6.96 g per 100 ml in adults and 6.69 g per 100 ml in children with Hodgkin's disease. The highest value was 8.0 g per 100 ml, the lowest 3.3 g per 100 ml (Table 1).

All sera were analyzed by immunoelectrophoresis against antisera anti-human immunoglobulins, anti-immunoglobulin G, anti-kappa chains (BJK) and anti-lambda chains (BJL). In most cases the immunoelectrophoretic patterns displayed a well-developed, distinct and often double IgG line with weaker and faint lines corresponding to IgM and IgA, respectively. In a few cases immunoglobulin M was lacking. In some other cases, however, there was a marked in-

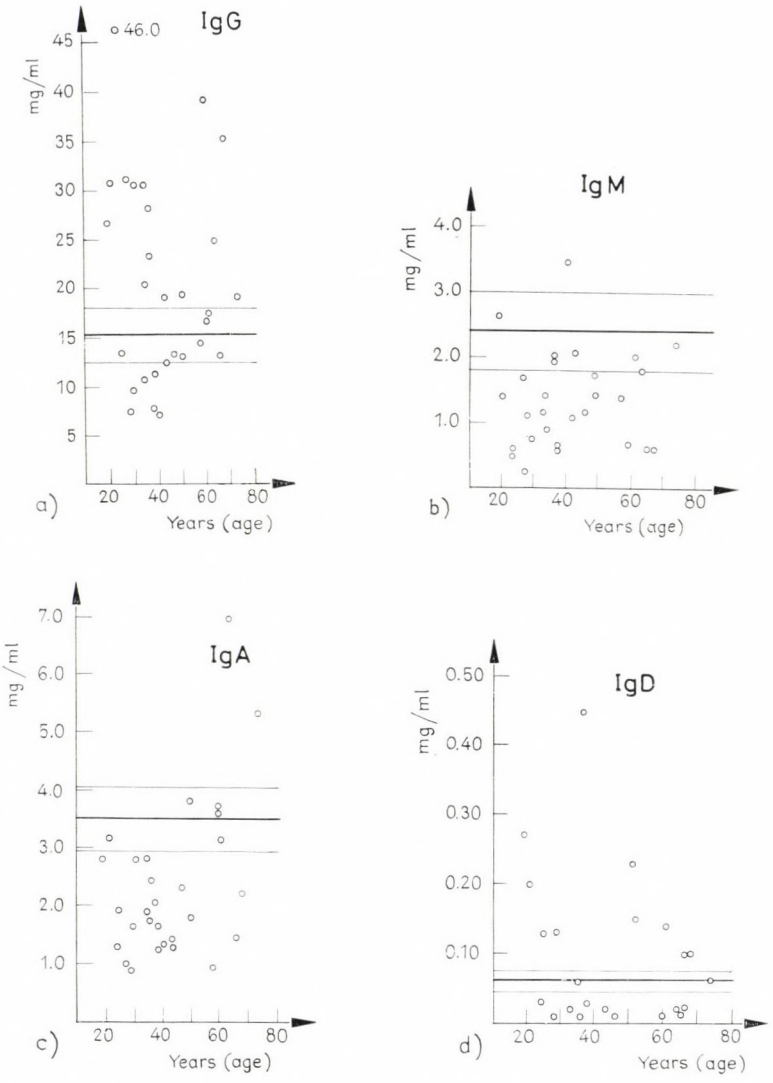

Fig. 1. Serum immunoglobulin levels in patients with Hodgkin's disease in comparison to normal level (middle) $\pm$ standard deviation

crease of all immunoglobulins, as evidenced by the increased intensity and thickness of the precipitin arcs. Monoclonal globulins of either K or L type specificity were not detected in any case.

Immunoglobulin levels in the serum of Hodgkin patients were estimated quantitatively by single radial immunodiffusion and compared with normal values obtained in a group of 35 healthy blood donors of the same age and sex distribution. The differences between mean values and standard deviations (S.D.) of immunoglobulin levels in healthy individuals and adult patients are shown in Table 2.

Table 2

Mean values and standard deviations of serum immunoglobulin levels in healthy individuals and adult patients with Hodgkin's disease

Immunoglobulin class	Healthy individuals		Patients		Significance level (p)
	Mean value, mg/ml	S.D.	Mean value, mg/ml	S.D.	
IgG	15.20	2.80	20.40	10.20	>0.1
IgM	2.40	0.60	1.33	0.72	<0.001
IgA	3.50	0.60	2.25	1.22	<0.001
IgD	0.06	0.03	0.11	0.09	>0.1

Only the differences between the immunoglobulin M and A values were significant statistically ($p < 0.001$). Results for the 4 immunoglobulin classes are presented graphically in Fig. 1 (a—d).

IgM and IgA levels were in most cases below the normal range, while that of IgG was within the normal range, sometimes decreased, but more often increased. Of special interest was the increase of IgD in the patients with Hodgkin's disease. The highest value, 0.45 mg/ml, was found in a patient who responded well to cyclophosphamide and X-rays (No. 24 in Table 1).

In the group of 11 children with Hodgkin's disease the increase of IgG and IgD was even more distinct. Seven patients had elevated IgD; the highest value of 0.40 mg/ml was found in an 11-year-old boy (No. 6 in Table 1).

In addition immunoelectrophoretic patterns of sera containing different amounts of IgM and IgA were compared. This technique revealed no level below 0.5 mg/ml with our antisera. Sera with levels in the 0.5—2.0 mg/ml range gave visible, but faint lines; those in the range of 2.0—6.0 mg/ml gave very distinct and strong lines (Figs 2 and 3). Two sera with the highest IgD value showed faint reaction with the specific anti-IgD antiserum in immunoelectrophoresis.

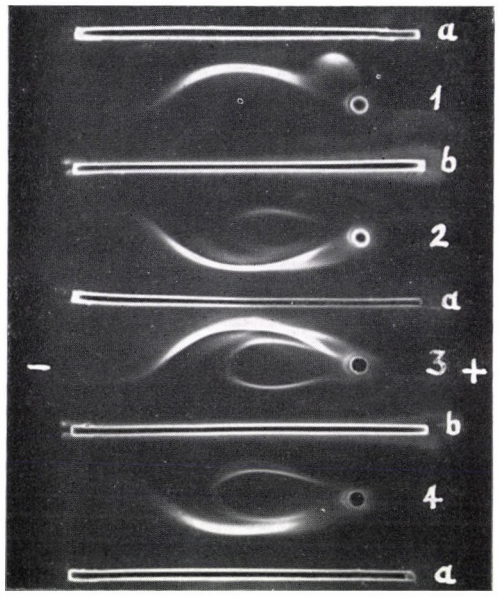

Fig. 2. Immunoelectrophoretic pattern of sera with different IgM concentrations, developed with antisera anti-immunoglobulins (a) and anti-IgM (b). From top to bottom, Hodgkin sera, in Table 1: 1. No. 17 = 0.30 mg/ml; 2. No. 4 = 1.27 mg/ml; 3. No. 12 = 2.60 mg/ml; 4. No. 27 = 3.45 mg/ml

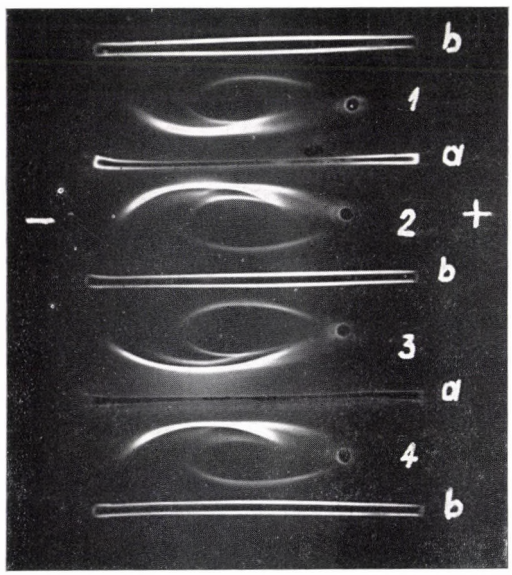

Fig. 3. Immunoelectrophoretic pattern of sera with different IgA concentrations, developed with antisera anti-immunoglobulins (a) and anti-IgA (b). From top to bottom, Hodgkin sera, in Table 1: 1. No. 5 = 1.00 mg/ml; 2. No. 12 = 2.80 mg/ml; 3. No. 40 = 5.30 mg/ml; 4. No. 35 = 6.96 mg/ml

Discussion

Serum proteins in patients with Hodgkin's disease were studied by several authors [5, 6, 8, 10, 18, 25]. They noted an increase in haptoglobins and in protein-bound hexoses exceeding 2.4 times the normal value [8]. CRP, the protein reacting with somatic polysaccharide of pneumococci, was found in the advanced stage of the disease [8]. Alpha$_3$-globulin, an additional band in electrophoresis on cellulose acetate, was identified by Sunderman [25] and was found by Goldman and Hobbs [10] to occur in 62 % of the cases of Hodgkin's disease.

The immunological responsiveness of patients with Hodgkin's disease and their immunoglobulin levels were subjects of special interest. Chase [8] summarized the data for the patients' antibody response; they indicated that antibody production against new antigens was greatly impaired, while the anamnestic response was normal. Aisenberg [1, 3] found diminished synthesis of antibodies against brucellin and tetanus toxoid on primary immunization, but a normal production of antibodies to these antigens on secondary immunization. A significantly decreased primary immune response of Hodgkin patients to a variety of antigens such as tularaemia vaccine [22] and mumps virus, Candida albicans, Trichophyton gypseum and PPD [23] was reported.

Hypogammaglobulinaemia, as detected by electrophoretic or immunoelectrophoretic analysis, was rarely found in Hodgkin's disease and was supposed to occur in the terminal stage of the disease [1, 5, 6, 25]. More detailed studies, using quantitative radial immunodiffusion, disclosed normal or increased IgG levels, normal or decreased IgM levels and decreased IgA levels [10, 18]. The observation of Burtin et al. [7] concerning the elevation of immunoglobulin D in Hodgkin's disease was not confirmed by Goldman and Hobbs [10].

In the present study decreased IgM and IgA levels and normal or increased IgG levels were found. The IgD level was elevated in 10 out of 23 adult patients only, the mean value, however, was almost twice as high as in normal individuals (see Table 2). It was difficult to correlate the immunoglobulin levels with the stage of disease or the response to therapy. Nevertheless, in two cases with general hypoimmunoglobulinaemia (Case No. 17, with severe hypoproteinaemia, and Case No. 25 in Table 1) death occurred in approximately two months after the examination. Another fatal case (No. 36 in Table 1) presented decreased IgM and IgD levels with normal IgA and very elevated IgG.

Decreased IgM levels apparently reflect the diminished ability of patients with Hodgkin's disease to antibody formation during the primary immune response [1, 8, 23]. The same appears to be true for IgA globulins, which have recently been shown to participate in the early phase of the primary immune response [9]. Since all patients with Hodgkin's disease were submitted to X-ray therapy and some to intensive chemotherapy, we are inclined to assume that the effect of this treatment becomes manifest with decreased IgM and IgA levels. It may be presumed that the cells of the lymphatic system responsible for the synthesis of these immunoglobulins are sensitive to such therapy and are suppressed.

Alternatively, the disease may primarily destroy some precursor clones of the cells producing immunoglobulins.

The normal or increased IgG levels probably reflect the known ability of patients with Hodgkin's disease to respond normally to secondary stimulation [1, 3, 8]. The elevation of immunoglobulin G was found to be of the polyclonal type. This polyclonal increase conceivably indicates an immunological reaction to antigenic stimulation. The possible stimuli include antigens of incidental infectious agents or those of the presumed aetiologic agent. Recent studies indicate that these agents may comprise antigens of both exogenous and endogenous origin. Stewart et al. [24] detected two kinds of virus in a continuous cell culture from a lymph node from a patient with Hodgkin's disease, and Grifoni et al. [11] showed the existence of some autoantigens of lymph node origin in Hodgkin's disease. These autoantigens were found to induce antibody production in the course of disease.

Our knowledge of the biological role of IgD is limited. Its increase in some patients with Hodgkin's disease may tentatively be considered to compensate the diminished production of IgM and IgA in response to antigenic stimuli.

*

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Étude statistique de quelques méthodes de mesure de l'activité complémentaire

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On étudie, à partir de valeurs expérimentales, le bien fondé de quatre relations théoriques décrivant la courbe d'hémolyse; on en déduit une méthode explicite d'estimation, par intervalle de confiance, de la dose correspondant à 50% d'hémolyse; les calculs sont systématisés en un programme en langage Fortran.

I. Objet de l'étude statistique

Cette méthode étant purement mathématique, nous ne décrirons pas ici les méthodes et conditions expérimentales. Le lecteur désireux de se documenter à ce sujet peut se référer à [1, 2, ou 3]. Actuellement l'activité complémentaire se mesure en unités 50 % d'hémolyse (U C′ H_{50}), c'est-à-dire, la plus petite quantité de sérum susceptible de lyser la moitié des hématies d'un système hémolytique donné. Le titre d'un sérum se mesure donc par le nombre d'unités 50 % d'hémolyse comprises dans un ml de ce sérum. Les données qui nous ont été fournies sont reprises en appendice. Elles sont du type suivant:

Quantité de sérum dilué au 1/10, en ml	Taux d'hémolyse	
x_1	$U_{1,1}$	$U_{1,2}$
x_2	$U_{2,1}$	$U_{2,2}$
x_3	$U_{3,1}$	$U_{3,2}$
.	.	.
.	.	.
.	.	.
x_s	$U_{s,1}$	$U_{s,2}$

A partir de là, on peut tracer une courbe d'hémolyse en portant en abscisses les quantités de sérum utilisées, en ordonnées les taux d'hémolyse.[1]

[1] Habituellement les utilisateurs portent en ordonnées les quantités de sérum, en abscisses les taux d'hémolyse. Nous avons fait le contraire pour des raisons d'organisation des calculs et des graphiques.

Par interpolation, on peut calculer la quantité de sérum provoquant l'hémolyse 50 %, mais il saute aux yeux que cette méthode n'est pas pratique. Aussi les utilisateurs employent la formule de von Krogh, qui établit entre la dose de complément x et le taux d'hémolyse, u, la relation suivante:

$$x = K\left(\frac{u}{1-u}\right)^{1/n} \quad ; \quad 0 < x < \infty \tag{1,1}$$

ou après transformation

$$u = \frac{1}{1 + \exp\left[-(n \ln x - n \ln K)\right]} ; \tag{1,2}$$

ce qui n'est autre qu'une relation logistique entre $\ln x$ et u. A titre d'expérience, nous avons aussi étudié l'opportunité des relations mathématique suivantes:

a) *distribution gaussienne*

$$u = F_G\left(\frac{\ln x - A}{B}\right) \quad ; \quad 0 < x < \infty \tag{1,3}$$

b) *fonction arctangente*

$$u = \frac{1}{2} + \frac{1}{\pi} \operatorname{arctg}\,(\alpha \ln x + \beta) \; ; \quad 0 < x < \infty \tag{1,4}$$

c) *fonction tangente hyperbolique*

$$u = \operatorname{th}\,\alpha x \; ; \; 0 < x < \infty \, . \tag{1,5}$$

Le problème se pose comme suit: linéariser les courbes mathématiques par des transformations appropriées et ensuite obtenir la relation donnant la quantité de sérum correspondant au taux d'hémolyse 50 %.

a) *Pour la logistique*

En prenant le logarithme népérien des deux membres de la relation (1,1) on a:

$$\ln\left(\frac{u}{1-u}\right) O = n \ln x - n \ln K \, ;$$

en posant

$$y = \ln\left(\frac{u}{1-u}\right),$$

$$z = \ln x,$$

$$n = \alpha,$$

$$-n \ln K = \beta,$$

on obtient

$$y = \alpha z + \beta \ ; \ -\infty < z < \infty \ . \qquad (1,6)$$

b) *Distribution gaussienne*

En prenant le quantile gaussien des deux membres de la relation (1,3) on obtient:

$$Q_G(u) = \frac{\ln x - A}{B} \ ;$$

en posant

$y = Q_G(u)$,

$z = \ln x$,

$\alpha = 1/B$,

$\beta = -\frac{A}{B}$,

on a

$$y = \alpha z + \beta \ ; \ -\infty < z < \infty \ .$$

c) *Fonction arctangente*

La relation (1,4) peut s'écrite:

$$\pi(u - 1/2) = \text{arctg}\,(\alpha \ \ln x + \beta);$$

En prenant la tangente des deux membres on obtient:

$$\text{tg}\,\pi\left(u - \frac{1}{2}\right) = \alpha \ \ln x + \beta \ ,$$

en posant

$y = \text{tg}\ \pi\left(u - \frac{1}{2}\right)$,

$z = \ln x$,

on a

$$y = \alpha z + \beta \ ; \ -\infty < z < \infty \ .$$

d) *Fonction tangente hyperbolique*

Prenons l'arctangente hyperbolique des deux membres de (1,5). On a

$$\text{arcth}\ u = \alpha x$$

ou

$$\frac{1}{2} \ln \left(\frac{1+u}{1-u} \right) = \alpha x \, ;$$

en posant

$$y = \frac{1}{2} \ln \left(\frac{1+u}{1-u} \right),$$

on obtient

$$y = \alpha x \; ; \; 0 < x < \infty \, . \tag{1,7}$$

La quantité de sérum correspondant à $u = 0.5$ correspond, dans le cas des trois premières courbes, à $y = 0$, et à $y = \frac{1}{2} \ln 3$, dans le cas de la tangente hyperbolique.

Cela étant acquis, un certain nombre de problèmes se posent encore.

1°) Les données ainsi linéarisées sont-elles normales?

2°) Sont-elles homoscédastiques, c'est-à-dire, pour un même donneur, la variance de y, pour différentes valurs de z, est-elle constante?

3°) Les courbes mathématiques proposées sont-elles appropriées pour décrire l'expérience? La question revient à étudier, à partir des données, la linéarité des transformées.

4°) Les utilisateurs de la formule de von Krogh supposent, que dans des conditions expérimentales bien déterminées, la pente des droites obtenues est constante. Il convient donc d'étudier le parallélisme des droites obtenues pour les différents donneurs. Nous avons traité ce problème pour les quatre relations ci-dessus, indépendamment de l'étude de linéarité.

5°) Enfin, en vue d'évaluer la précision de la méthode de mesure de l'activité complémentaire, nous calculerons les titres de sérum ainsi que leurs intervalles de confiance.

A la lumière des résultats obtenus, nous essayerons de choisir la courbe qui s'accorde le mieux avec l'expérience.

II. Étude de la normalité des données

Des expériences ont été spécialement réalisées en vue de cette étude.

Pour neuf donneurs différents et pour deux doses de complément différentes, l'expérimentateur a relevé cinq fois les taux d'hémolyse. Les données numériques sont reprises dans l'appendice [3]. Vu le nombre restreint de données que nous possédons pour un même donneur et pour une même dose de complément, nous avons dû nous contenter de la méthode graphique sur papier à anamorphose gaussienne. Nous avons procédé de la façon classique (voir [4]), ordonnant tout d'abord (pour chaque donneur et pour chaque dose) les valeurs des y_i, qui deviennent

$A_1, \ldots, A_5$, et en portant comme ordonnée correspondant à A_i la valeur $(i + 0.7)/5.4$; en principe, sur le diagramme à anamorphose gaussienne, ces cinq points doivent s'aligner sur la "droite de Henry" déterminée par les points $(m; 1/2)$ et $(m + \hat{\sigma}; 0.8413)$ [m étant la moyenne arithmétique des y_i et $\hat{\sigma}$ leur écart-type (diviseur $n - 1 = 4$)].

Les graphiques ayant été construits pour les quatre transformées linéaires des relations mathématiques et pour les neuf donneurs, il s'est avéré que les points expérimentaux ne s'ajustaient que médiocrement aux droites théoriques. Cependant, compte tenu de la robustesse bien connue de la méthode des moindres carrés vis-à-vis des hypothèses de normalité, nous avons poursuivi l'analyse en supposant que les données sont en effet normales.

III. Étude de l'homoscédasticité

La variance des y_i est-elle constante?

Pour répondre à cette question nous avons repris les données de l'appendice [3] et nous avons traité le problème par deux méthodes.

1. *Méthode graphique*

Si l'hypothèse d'homoscédasticité est vraie, pour chaque donneur, les deux droites construites sur papier à anamorphose gaussienne et correspondant chacune à une dose de complément, devraient être parallèles.

Il s'est avéré que les deux droites n'étaient que rarement parallèles.

2. *Méthode des probabilités de dépassement*

Soient

$$s^2_{1,j} \text{ et } s^2_{2,j} \; ; \; (j = 1, \ldots, 9),$$

les variances des y_i correspondant au $j^{\text{ème}}$ donneur et respectivement aux deux doses de complément.

Soient ensuite

$$\left.\begin{aligned} s^2_{(1),j} &= \min\,(s^2_{1,j} \,;\, s^2_{2,j}) \\ s^2_{(2),j} &= \max(s^2_{1,j} \,;\, s^2_{2,j}) \end{aligned}\right\} (j = 1, \ldots, 9).$$

La quantité

$$B_j = \frac{s^2_{(1),j}}{s^2_{(1),j} + s^2_{(2),j}},$$

est une variable aléatoire qui, sous l'hypothèse d'homoscédasticité, possède la distribution bêta avec les degrés de liberté $p = 2$, $q = 2$. On a donc, pour la probabilité de dépassement liée à cette hypothèse (et avec les notations classiques de K. Pearson [5]).

$$PD(B_j) = 2I_{B_j}(2,2)\,.$$

Les probabilités de dépassement individuelles ne nous permettant pas de conclure nettement, nous avons cherché les probabilités de dépassement globales pour chaque type de courbe en calculant

Tableau 1

A) Fonction logistique

Donneur	s_1^2	s_2^2	PD	$-\ln PD$
1	0.00029067	0.00054131	0.562	0.576613
2	0.00075241	0.00022531	0.268	1.310493
3	0.00051934	0.00231781	0.170	1.734307
4	0.00015410	0.00138674	0.056	2.882003
5	0.00082011	0.00075147	0.940	0.067659
6	0.00007480	0.00108223	0.020	3.753262
7	0.00052687	0.00031199	0.618	0.471253
8	0.00055524	0.00094583	0.600	0.480573
9	0.00170199	0.00033432	0.138	1.937734

$$-2\sum_{i=1}^{9} \ln PD_i = 26.427794 \quad PD_{\text{globale}} \simeq 0.091$$

Tableau 1

B) Distribution gaussienne

Donneur	s_1^2	s_2^2	PD	$-\ln PD$
1	0.00029365	0.00054057	0.564	0.564177
2	0.00075918	0.00022369	0.268	1.333019
3	0.00052297	0.00406729	0.066	2.623218
4	0.00015085	0.00138551	0.044	2.917771
5	0.00082491	0.00076320	0.940	0.059212
6	0.00007813	0.00108064	0.028	3.668166
7	0.00052909	0.00030664	0.600	0.493476
8	0.00054693	0.00094681	0.600	0.497132
9	0.00170936	0.00033563	0.136	1.938310

$$-2\sum_{i=1}^{9} \ln PD_i = 28.188962 \quad PD_{\text{globale}} \simeq 0.059$$

Tableau 1

C) Fonction arctangente

Donneur	s_1^2	s_2^2	PD	$-\ln PD$
1	0.00018557	0.00181088	0.046	3.023834
2	0.00047657	0.00085726	0.590	0.538830
3	0.00051242	0.02476368	0.0024	6.015669
4	0.00594239	0.00009665	0.0016	6.404242
5	0.00051158	0.00164859	0.290	1.260731
6	0.00005955	0.00127059	0.009	4.451553
7	0.00035767	0.00036149	0.990	0.079680
8	0.00034411	0.00165217	0.154	1.845932
9	0.00119605	0.00044524	0.359	1.017041

$$-2\sum_{i=1}^{9} \ln PD_i = 49.131620 \quad PD_{\text{globale}} \simeq 0.0001$$

Tableau 1

D) Fonction tangente hyperbolique

Donneur	s_1^2	s_2^2	PD	$-\ln PD$
1	0.00002765	0.00001021	0.358	1.027832
2	0.00007310	0.00000381	0.0144	4.251633
3	0.00002845	0.00001614	0.590	0.517006
4	0.00001542	0.00002122	0.760	0.268499
5	0.00008373	0.00002026	0.188	1.618992
6	0.00001150	0.00005034	0.170	1.697449
7	0.00007252	0.00001469	0.152	1.889944
8	0.00006494	0.00003098	0.484	0.716375
9	0.00024151	0.00001387	0.0140	4.070905

$$-2\sum_{i=1}^{9} \ln PD_i = 32.117270 \quad PD_{\text{globale}} \simeq 0.021$$

$$X = -2\sum_{j=1}^{9} \ln PD_j$$

puis (F_{cc} étant la fonction de répartition chi-carré)

$$PD_{\text{globale}} = 1 - F_{cc}(X, 18)\,.$$

Les résultats obtenus font l'objet du tableau 1.

Les probabilités de dépassement, sans être favorables à l'hypothèse sous épreuve, ne nous permettent cependant pas de la rejeter nettement. En consé-

quence, et sous réserve de plus amples informations, nous nous accomoderons de l'hypothèse d'homoscédasticité. Remarquons aussi que parmi les quatre courbes, la formule de von Krogh et la distribution gaussienne semblent donner les meilleurs résultats.

IV. Étude de linéarité

A) *Logistique, arctangente, distribution gaussienne*

La transformée linéaire de chacune de ces trois courbes se présente sous la forme

$$y = \alpha z + \beta \; ; \; -\infty < z < \infty \, .$$

Nous basant toujours sur l'hypothèse de normalité, nous avons appliqué la méthode classique de péréquation linéaire aux données expérimentales de l'appendice [3].

Pour chaque donneur et pour chaque type de courbe, on a:

$$\text{où} \quad \left.\begin{aligned} y_i &= \alpha z_i + \beta + \varepsilon_i \\ E y_i &= \alpha z_i + \beta \end{aligned}\right\} i = 1, \ldots, 10 \, .$$

Nous avons estimé chaque fois les deux paramètres α et β ainsi que la variance, la somme des carrés de l'erreur SCE et la somme des carrés interne SCINT (résultant des comparaisons entre elles des valeurs de y correspondant à une même valeur de z). Le test de linéarité s'obtient en construisant le rapport

$$X = \frac{\text{SCINT}}{\text{SCE}} \, .$$

La probabilité de dépassement correspondante est

$$PD = \mathrm{I}_{\lambda}(2.5;\ 1.5).$$

Cette fois encore les probabilités de dépassement individuelles ne nous ont pas permis de conclure. Nous avons donc calculé la probabilité de dépassement globale pour chaque courbe, à savoir:

$$PD_{\text{globale}} = 1 - F_{cc}(B\,;\,20)\,,$$

où

$$B = -\,2 \sum_{j=1}^{10} \ln PD_j \,,$$

la sommation portant sur les donneurs. Les résultats obtenus sont repris au tableau 2.

Tableau 2

Donneur	Fonction logistique		Distribution gaussienne		Fonction arctangente	
	PD	$-\ln PD$	*PD*	$-\ln PD$	*PD*	$-\ln PD$
1	0.0111	4.500809	0.0017	6.377067	0.0032	5.744604
2	0.2182	1.522136	0.1979	1.619388	0.0043	5.448029
3	0.0292	3.533394	0.0022	6.119198	0.0030	5.809043
4	0.2119	1.551809	0.0315	3.458208	0.0042	5.472691
5	0.0309	3.478467	0.0048	5.338979	0.0095	4.655813
6	0.0067	5.005958	0.0018	6.319868	0.0065	5 035653
7	0.0309	3.478467	0.0048	5.338979	0.0064	5.051137
8	0.0247	3.701796	0.0053	5.240158	0.0130	4.342766
9	0.0039	5.531411	0.0014	6.571303	0.0991	2.311545
10	0.0412	3.188417	0.0184	3.995261	0.0009	7.013016

$$-2\sum_{i=1}^{10}\ln PD_i = = 70.985528 \qquad PD_{\text{globale}} < 10^{-5}$$

$$-2\sum_{i=1}^{10}\ln PD_i = = 100.756818 \qquad PD_{\text{globale}} < 10^{-5}$$

$$-2\sum_{i=1}^{10}\ln PD_i = = 101.768594 \qquad PD_{\text{globale}} < 10^{-5}$$

Pour les trois formules nous avons obtenu

$$PD_{\text{globale}} < 10^{-5}.$$

Il semblerait donc que l'hypothèse de linéarité, ou ce qui revient au même, l'hypothèse d'adéquation des relations mathématiques étudiées avec la réalité expérimentale, doit être considérée avec une certaine prudence. Mais, répétons-le, nous nous garderons d'émettre un jugement définitif jusqu'à l'obtention de plus amples informations. De plus, certains chercheurs ont constaté après de nombreuses expériences que la relation de von Krogh n'épousait la courbe d'hémolyse obtenue par points expérimentaux, qu'entre 20% et 80% d'hémolyse[6]. Or nos données sont nettement décentrées vers l'extrémité inférieure puisque les taux d'hémolyse varient entre 3.43% et 76.71%.

Il se pourrait que les résultats apparemment négatifs obtenus soient dus en partie à ce fait.

Quoi qu'il en soit, nous nous proposons de reconsidérer le problème dans un article ultérieur.

B) *Fonction tangente hyperbolique*

L'équation

$$y = \alpha x \ ; \ 0 < x < \infty$$

ne comprenant qu'un seul paramètre, nous n'avons pu utiliser les péréquations linéaires mais bien le modèle suivant:

$$Ey_i = \alpha x_i \ ; \ i = 1, \ldots, 10 .$$

Nous avons estimé α par la formule

$$\hat{\alpha} = \frac{\sum_{i=1}^{10} x_i y_i}{\sum_{i=1}^{10} x_i^2} .$$

Ensuite comme au paragraphe 3 A) nous avons calculé la variance, la somme des carrés de l'erreur, la somme des carrés interne et la probabilité de dépassement relative à chaque donneur. Les résultats obtenus sont repris au tableau 3.

Tableau 3

Fonction tangente hyperbolique

Donneur	SCINT	SCE	$X = \frac{\text{SCINT}}{\text{SCE}}$	PD
1	0.00018490	0.17547417	0.0011	$< 2 \cdot 10^{-4}$
2	0.00012130	0.18850708	0.0006	$< 3 \cdot 10^{-5}$
3	0.00023561	0.13659543	0.0017	$< 2 \cdot 10^{-4}$
4	0.00026789	0.23769283	0.0011	$< 2 \cdot 10^{-4}$
5	0.00018721	0.22794437	0.0008	$< 3 \cdot 10^{-5}$
6	0.00028631	0.33906746	0.0008	$< 3 \cdot 10^{-5}$
7	0.00016508	0.24987984	0.0007	$< 3 \cdot 10^{-5}$
8	0.00045676	0.24466610	0.0019	$< 2 \cdot 10^{-4}$
9	0.00045327	0.35222435	0.0013	$< 2 \cdot 10^{-4}$
10	0.00031104	0.27685642	0.0011	$< 2 \cdot 10^{-4}$

Les probabilités de dépassement obtenues sont extrêmement petites si bien que nous pouvons conclure directement que la fonction tangente hyperbolique ne convient pas du tout pour décrire l'expérience.

V. Titres de sérum et leurs intervalles de confiance[1]

A) *Logistique, distribution gaussienne, arctangente*

Nous avons procédé comme suit.
Soit

$$\hat{y} = \hat{\alpha} z + \hat{\beta}$$

l'équation estimée d'une droite.

[1] Nous ne donnons dans ce paragraphe qu'un bref exposé de la méthode. Le lecteur trouvera le détail des calculs dans l'appendice 1.

Pour obtenir la dose de complément correspondant au taux d'hémolyse 50% il suffit de poser

$$\hat{y} = 0.$$

De là on calcule aisément z_{50}, puis x_{50} en passant au cologarithme. D'autre part la courbe délimitant la zone de confiance du lieu estimé est une hyperbole.

L'intervalle de confiance de z_{50} s'obtient en cherchant les points d'intersection de cette hyperbole avec l'axe $y = 0$.

Pour chaque donneur, nous avons aussi calculé la largeur de l'intervalle de confiance en ml de sérum et en unités 50% d'hémolyse ainsi que l'erreur relative. Les résultats obtenus sont repris dans les tableaux 4, 5 et 6 ci-dessous.

Tableau 4

Fonction logistique
Niveau d'incertitude = 0.05

Donneur	En ml de sérum			Erreur relative en %	En unités d'hémolyse 50%		
	X'_{50}	X_{50}	X''_{50}		T'_{50}	T_{50}	T''_{50}
1	0.3577	0.3694	0.3833	5.8	26.09	27.07	27.96
2	0.3592	0.3656	0.3727	3.8	26.83	27.35	27.84
3	0.4063	0.4221	0.4412	8.3	22.66	23.69	24.61
4	0.3548	0.3610	0.3677	3.7	27.19	27.70	28.18
5	0.3433	0.3513	0.3602	4.8	27.76	28.47	29.13
6	0.3044	0.3087	0.3132	2.9	31.93	32.39	32.85
7	0.3260	0.3310	0.3363	3.2	29.74	30.21	30.68
8	0.3346	0.3424	0.3512	4.9	28.47	29.20	29.89
9	0.3076	0.3150	0.3232	5.0	30.94	31.74	32.51
10	0.3171	0.3206	0.3242	2.3	30.85	31.20	31.54

Tableau 5

Distribution gaussienne
Niveau d'incertitude = 0.05

Donneur	En ml de sérum			Erreur relative en %	En unités d'hémolyse 50%		
	X'_{50}	X_{50}	X''_{50}		T'_{50}	T_{50}	T''_{50}
1	0.3563	0.3698	0.3864	8.0	25.88	27.04	28.07
2	0.3609	0.3669	0.3733	3.4	26.79	27.26	27.71
3	0.4062	0.4314	0.4662	13.7	21.45	23.18	24.62
4	0.3533	0.3617	0.3713	4.9	26.93	27.65	28.30
5	0.3410	0.3514	0.3636	6.4	27.50	28.46	29.33
6	0.3028	0.3082	0.3140	3.6	31.85	32.45	33.03
7	0.3229	0.3312	0.3388	4.8	29.52	30.19	30.97
8	0.3319	0.3421	0.3539	6.3	28.26	29.23	30.13
9	0.3074	0.3146	0.3225	4.7	31.01	31.79	32.53
10	0.3158	0.3200	0.3244	2.6	30.83	31.25	31 67

Tableau 6

Fonction arctangente
Niveau d'incertitude = 0.05

Donneur	En ml de sérum			Erreur relative en %	En unités d'hémolyse 50%		
	X'_{50}	X_{50}	X''_{50}		T'_{50}	T_{50}	T''_{50}
1	0.3428	0.3617	0.3874	12.3	25.81	27.65	29.17
2	0.3237	0.3565	0.4171	26.1	24.21	28.05	30.89
3	0.3403	0.3744	0.4376	25.9	22.85	26.71	29.39
4	0.3250	0.3547	0.4052	22.6	24.68	28.19	30.77
5	0.3283	0.3497	0.3795	14.3	26.35	28.60	30.46
6	0.3040	0.3130	0.3207	5.3	31.18	31.95	32.89
7	0.3205	0.3343	0.3511	9.1	28.48	29.91	31.20
8	0.3294	0.3400	0.3628	9.8	27.56	29.41	30.56
9	0.3122	0.3192	0.3268	4.5	30.60	31.33	32.03
10	0.3100	0.3250	0.3444	10.5	29.04	30.77	32.26

L'erreur relative varie entre

2.3% et 8.3% pour la logistique,
2.6% et 13.7% pour la distribution gaussienne,
4.5% et 26.1% pour l'arctangente.

La formule de von Krogh ou logistique semble donc être la plus précise pour l'estimation du titre de sérum.

Cependant, il convient de remarquer que, pour une même transformation mathématique, la précision de l'estimation varie considérablement d'un donneur à l'autre. En fait, deux facteurs influencent la précision des résultats.

Tableau 7

Logistique

Donneur	Ecart-type	Largeurs d'intervalles		Erreur relative en %	X_{50} en ml de sérum
		en ml	En unités 50% d'hémolyse		
3	$1.104 \cdot 10^{-1}$	0.0359	2.01	8.3	0.4238
9	$1.102 \cdot 10^{-1}$	0.0158	1.59	5.0	0.3151
8	$9.107 \cdot 10^{-2}$	0.0170	1.44	4.9	0.3424
1	$8.697 \cdot 10^{-2}$	0.0217	1.59	5.8	0.3688
5	$8.539 \cdot 10^{-2}$	0.0174	1.41	4.8	0.3513
4	$6.992 \cdot 10^{-2}$	0.0136	1.04	3.7	0.3610
6	$6.639 \cdot 10^{-2}$	0.0092	0.91	2.9	0.3087
2	$6.631 \cdot 10^{-2}$	0.0142	1.06	3.8	0.3656
7	$6.208 \cdot 10^{-2}$	0.0107	0.98	3.2	0.3310
10	$4.871 \cdot 10^{-2}$	0.0075	0.73	2.2	0.3206

1°) Plus l'écart-type d'un donneur est grand, plus l'intervalle de confiance est large.

2°) L'imprécision des estimations augmente avec la différence entre la dose de sérum donnant l'hémolyse 50% et la moyenne des concentrations fixes auxquelles se font les observations.

Les tableaux 7, 8 et 9 résument assez bien la situation. Nous y reprenons les écarts-types par ordre de grandeurs décroissantes, les erreurs relatives et les titres de sérum en ml.

Tableau 8

Distribution gaussienne

Donneur	Ecart-type	Largeurs d'intervalles		Erreur relative en %	X_{50} en ml de sérum
		en ml	En unités 50% d'hémolyse		
3	$9.284 \cdot 10^{-2}$	0.0600	3.17	13.7	0.4314
9	$7.679 \cdot 10^{-2}$	0.0151	1.52	4.7	0.3146
8	$6.976 \cdot 10^{-2}$	0.0220	1.87	6.3	0.3421
1	$6.964 \cdot 10^{-2}$	0.0301	2.19	8.0	0.3698
5	$6.539 \cdot 10^{-2}$	0.0226	1.83	6.4	0.3514
4	$5.312 \cdot 10^{-2}$	0.0180	1.37	4.9	0.3617
6	$5.001 \cdot 10^{-2}$	0.0112	1.18	3.6	0.3082
7	$4.978 \cdot 10^{-2}$	0.0159	1.45	4.8	0.3312
10	$3.369 \cdot 10^{-2}$	0.0086	0.84	2.6	0.3200
2	$3.307 \cdot 10^{-2}$	0.0124	0.92	3.4	0.3669

Tableau 9

Arctangente

Donneur	Ecart-type	Largeurs d'intervalles		Erreur relative en %	X_{50} en ml de sérum
		en ml	En unités 50% d'hémolyse		
3	1.037	0.0973	6.54	25.9	0.3744
2	$6.869 \cdot 10^{-1}$	0.0934	6.68	26.1	0.3565
4	$6.545 \cdot 10^{-1}$	0.0802	6.09	22.6	0.3547
5	$3.223 \cdot 10^{-1}$	0.0512	4.11	14.6	0.3497
1	$2.497 \cdot 10^{-1}$	0.0446	3.36	12.3	0.3617
10	$2.275 \cdot 10^{-1}$	0.0344	3.22	10.5	0.3250
8	$2\,093 \cdot 10^{-1}$	0.0334	3.00	9.8	0.3400
7	$1.918 \cdot 10^{-1}$	0.0306	2.72	9.1	0.3343
6	$1.108 \cdot 10^{-1}$	0.0167	1.71	5.3	0.3130
9	$1.058 \cdot 10^{-1}$	0.0146	1.43	4.5	0.3192

Il est assez malaisé de déterminer lequel des deux facteurs d'imprécision est dominant.

Cependant, si on ne peut se débarrasser de l'influence de l'écart-type, il est possible de remédier au deuxième inconvénient.

En effet, il faut remarquer que les titres de sérum sont tous supérieurs à 0.30 ml, moyenne des concentrations fixes. En fait, ils varient en gros entre 0.30 ml et 0.40 ml.

A cet endroit les branches de l'hyperbole de confiance s'écartent nettement du lieu estimé, la zone d'étranglement se situant aux environs immédiats de la moyenne. Par conséquent, on augmenterait la précision des estimations en déplaçant les concentrations fixes. Suggérons, par exemple, les concentrations

0.25 ml; 0.30 ml; 0.35 ml; 0.40 ml; 0.45 ml

dont la moyenne est 0.35 ml.

Cette opération comporte un deuxième avantage: on éviterait ainsi un trop grand nombre d'observations nettement inférieures à 20 % d'hémolyse, celles-ci correspondant en majeure partie à la concentration 0.20 ml.

B) *Fonction tangente hyperbolique*

Pour chaque donneur nous avons calculé le titre de sérum en ml soit

$$x_{50} = \frac{\ln 3}{2\,\hat{\alpha}}.$$

Son intervalle de confiance est donné par les intersections des droites

$$y = \left[\hat{\alpha} - Q_t(1 - \gamma/2,9)\,\frac{\hat{\sigma}}{\sqrt{\sum_{i=1}^{10} x_i^2}}\right] x$$

et

$$y = \left[\hat{\alpha} + Q_t(1 - \gamma/2,9)\,\frac{\hat{\sigma}}{\sqrt{\sum_{i=1}^{10} x_i^2}}\right] x$$

avec la droite

$$y = \frac{1}{2} \ln 3.$$

Au niveau d'incertitude $\gamma = 0.05$, on obtient les résultats du tableau 10 (exprimés en ml de sérum).

Tableau 10

Donneur	x'_{50}	x_{50}	x''_{50}
1	0.3482	0.4383	0.5911
2	0.3487	0.4433	0.6082
3	0.5014	0.6785	1.0489
4	0.3319	0.4299	0.6099
5	0.3103	0.3922	0.5326
6	0.2320	0.2865	0.3745
7	0.2740	0.3395	0.4462
8	0.2929	0.3679	0.4947
9	0.2398	0.2999	0.4002
10	0.2553	0.3148	0.4106

Inutile d'insister : l'imprécision est énorme. Pour la seconde fois, nous pouvons affirmer que la tangente hyperbolique ne convient pas du tout à la description des expériences.

VI. Étude de parallélisme pour la relation de von Krogh, la distribution gaussienne et la fonction arctangente[1, 2]

Nous avons utilisé une méthode de modèles emboîtés. Le modèle M_1 suppose que toutes les pentes des droites sont différentes. Il s'écrit sous la forme

$$Ey_{j,k} = \alpha_j z_{j,k} + \beta_j \ ; \ j = 1, \ldots, 10 \ ; \ k = 1, \ldots, 10 \, .$$

L'indice j porte sur les différents donneurs, l'indice k sur les différentes concentrations pour un même donneur.

Le modèle M_2 suppose que toutes les droites sont parallèles et s'écrit

$$Ey_{j,k} = \alpha z_{j,k} + \beta_j \ ; \ j = 1, \ldots, 10 \ ; \ k = 1, \ldots, 10 \, .$$

Les tables d'analyse de variance et les probabilités de dépassement ont été calculées pour chaque type de courbe.

[1] Ce problème a été traité indépendamment de l'étude de linéarité et des conclusions que nous avons pu en tirer.

[2] Nous avons orthogonalisé les modèles M_1 et M_2 a priori.

a) *Fonction logistique*

		T. A. V.	*dl*
SCT		184.219452	100
SCN		183.686935	20
	Red $[\alpha]$	183.407136	11
	Red $[\alpha_1, \ldots, \alpha_{10}/\alpha]$	0.279800	9
SCE		0.532516	80

$$X = \frac{\text{SCE}}{\text{SCE} + \text{Red } [\alpha_1, \ldots, \alpha_{10}/\alpha]}$$

$$= 0.6556$$

$$PD = I_{0,6556}(40;\ 4.5)$$

$$= 0.000057.$$

b) *Distribution gaussienne*

		T. A. V.	*dl*
SCT		63.227295	100
SCN		62.921692	20
	Red $[\alpha]$	62.842209	11
	Red $[\alpha_1, \ldots, \alpha_{10}/\alpha]$	0.079483	9
SCE		0.305603	80

$$X = 0.7936$$

$$PD = 0.0232.$$

c) *Fonction arctangente*

		T. V. A.	*dl*
SCT		454.027832	100
SCN		435.679199	20
	Red $[\alpha]$	404.577393	11
	Red $[\alpha_1, \ldots, \alpha_{10}/\alpha]$	31.101822	9
SCE		18.348633	80

$$X = 0.3711$$

$$PD < 10^{-7}.$$

Il semble que la distribution gaussienne satisfait le mieux à l'hypothèse de parallélisme ou plutôt que la probabilité de dépassement est suffisante pour ne pas rejeter l'hypothèse.

En ce qui concerne la logistique et l'arctangente les probabilités de dépassement sont tellement faibles que nous devrons considérer l'hypothèse avec une grande prudence.

VII. Conclusions finales

L'analyse globale des résultats obtenus nous amène à rejeter la relation tangente hyperbolique qui ne concorde vraiment pas avec l'expérience.

La relation arctangente ne semble pas fort appropriée non plus. Quant à la formule de von krogh et à la distribution gaussienne, il serait intéressant de pouvoir les étudier à partir de données numériques plus vastes qui permettraient de soumettre l'hypothèse de normalité à un examen approfondi.

Au cas où des tests plus précis nous amèneraient à rejeter cette hypothèse, nous pourrions alors utiliser des méthodes d'approximation reflétant peut-être mieux la réalité.

Quoi qu'il en soit, la formule de von Krogh et la distribution gaussienne s'avèrent être les plus précises pour l'estimation de l'activité complémentaire. Cependant, à notre avis, cette précision pourrait être améliorée comme nous l'avons indiqué au paragraphe V. (Nous nous proposons, dans un article ultérieur, de faire une étude comparative de la méthode de mesure de l'activité complémentaire de R. Kargues.)

Appendice 1

Calcul du titre d'un sérum et de son intervalle de confiance

La première opération à effectuer est de calculer les transformées de u et de x comme il est indiqué au paragraphe I. Ensuite il faut estimer les coefficients de la droite

$$y = \alpha z + \beta.$$

Soit N le nombre d'observations pour un donneur. On a

$$\hat{\alpha} = \frac{\sum_{i=1}^{N} (z_i - \bar{z})(y_i - \bar{y})}{\sum_{i=1}^{N} (z_i - \bar{z})^2}$$

et

$$\hat{\beta} = \frac{\sum_{i=1}^{N} y_i}{N} - \hat{\alpha}\bar{z}.$$

On en tire

$$z_{50} = -\frac{\hat{\beta}}{\hat{\alpha}}$$

et le titre du sérum exprimé en ml est

$$x_{50} = \text{colog}_e\, z_{50};$$

le titre exprimé en unités 50% d'hémolyse est

$$T_{50} = \frac{10}{x_{50}}. \qquad (1)$$

[1] Rappelons que le sérum est dilué au dixième.

Passons au calcul de l'intervalle de confiance.

L'équation de l'hyperbole de confiance est

$$[y - (\hat{\alpha}z + \hat{\beta})]^2 = 2Q_F(1 - \gamma\,;\,2\,;\,N-2)\,\hat{\sigma}^2\left[\frac{1}{N} + \frac{(z-\bar{z})^2}{\sum_{i=1}^{N}(z_i-\bar{z})^2}\right]$$

où γ est le niveau d'incertitude choisi, $Q_F\,(1-\gamma\,;\,2;\,N-2)$ le quantile de la distribution F de SNEDECOR à 2 et N−2 degrés de liberté et correspondant à une probabilité $1-\gamma$ et $\hat{\sigma}^2$ l'estimateur de la variance des y.

Pour estimer cette variance, nous devrons calculer la somme des carrés totale c'est-à-dire

$$\text{SCT} = \sum_{i=1}^{N} y_i^2\,.$$

la somme des carrés normale (c'est-à-dire de l'espace des estimatrices) à savoir

$$\text{SCN} = \frac{\left[\sum_{i=1}^{N} y_i\right]^2}{N} + \frac{\left[\sum_{i=1}^{N}(z_i-\bar{z})(y_i-\bar{y})\right]^2}{\sum_{i=1}^{N}(z_i-\bar{z})^2}$$

et la somme des carrés de l'erreur

$$\text{SCE} = \text{SCT} - \text{SCN}.$$

On obtient enfin

$$\hat{\sigma}^2 = \frac{\text{SCE}}{N-2}\,.$$

Étant en possession de tous les éléments nécessaires, nous pouvons chercher les points d'intersection de l'hyperbole de confiance avec l'axe $y = 0$.

Pour ce faire, nous devons résoudre l'équation du second degré en z suivante

$$(\hat{\alpha}z + \hat{\beta})^2 + 2Q_F\,\hat{\sigma}^2\left[\frac{1}{N} + \frac{(z-\bar{z})^2}{\sum_{i=1}^{N}(z_i-\bar{z})^2}\right]$$

ou encore

$$\left[\hat{\alpha}^2 - \frac{2Q_F\,\hat{\sigma}^2}{\sum_{i=1}^{N}(z_i-\bar{z})^2}\right]z^2 + 2z\left[\hat{\alpha}\hat{\beta} + \frac{2Q_F\,\hat{\sigma}^2\,\bar{z}}{\sum_{i=1}^{N}(z_i-\bar{z})^2}\right] + \hat{\beta}^2 - \frac{2Q_F\,\hat{\sigma}^2}{N} - \left[\frac{2Q_F\,\hat{\sigma}^2\,\bar{z}^2}{\sum_{i=1}^{N}(z_i-\bar{z})^2}\right] = 0\,,$$

Les racines de cette équation sont z'_{50} et z''_{50}.

Par conséquent, x_{50} est compris entre $x'_{50} = \text{colog}_e\, z'_{50}$ et $x''_{50} = \text{colog}_e\, z''_{50}$, x'_{50} et x''_{50} étant exprimés en ml.

La largeur de l'intervalle vaut

$$\lambda\,\gamma = x''_{50} - x'_{50}$$

et l'erreur relative

$$E = \frac{\lambda}{x_{50}}\,.$$

En unités 50 % d'hémolyse, le titre de sérum est compris entre

$$T'_{50} = \frac{10}{x''_{50}} \quad \text{et} \quad T''_{50} = \frac{10}{x'_{50}}\,.$$

La largeur de l'intervalle est $L = T''_{50} - T'_{50}$

et l'erreur relative $\dfrac{L}{T_{50}}$.

Appendice 2

Données expérimentales utilisées aux paragraphes IV, V et VI

Quantités de sérum dilué au 1/10 en ml	Taux d'hémolyse			
	Donneur 1		Donneur 2	
0.40	0.5986	0.5943	0.5829	0.5843
0.35	0.4457	0.4500	0.4643	0.4586
0.30	0.2971	0.2886	0.3086	0.3014
0.25	0.1629	0.1757	0.1586	0.1543
0.20	0.0986	0.0943	0.0686	0.0586
	Donneur 3		Donneur 4	
0.40	0.4657	0.4529	0.6114	0.6186
0.35	0.3043	0.3071	0.4614	0.4714
0.30	0.1529	0.1629	0.3043	0.3086
0.25	0.0771	0.0857	0.1486	0.1371
0.20	0.0386	0.0343	0.0657	0.0571
	Donneur 5		Donneur 6	
0.40	0.6529	0.6557	0.7600	0.7671
0.35	0.4771	0.4700	0.6300	0.6343
0.30	0.3386	0.3300	0.4729	0.4629
0.25	0.1914	0.1971	0.2643	0.2671
0.20	0.0986	0.0871	0.1343	0.1414
	Donneur 7		Donneur 8	
0.40	0.7000	0.6971	0.6714	0.6600
0.35	0.5486	0.5471	0.5300	0.5329
0.30	0.3871	0.3986	0.3571	0.3471
0.25	0.2314	0.2329	0.2029	0.1871
0.20	0.1129	0.1229	0.1086	0.1000
	Donneur 9		Donneur 10	
0.40	0.7529	0.7629	0.7314	0.7243
0.35	0.6043	0.6029	0.5814	0.5771
0.30	0.4329	0.4429	0.4171	0.4314
0.25	0.2371	0.2443	0.2657	0.2629
0.20	0.1386	0.1271	0.1229	0.1171

Appendice 3

Données expérimentales utilisées aux paragraphes II et III

Quantités de sérum en ml	Taux d'hémolyse				
	donneur 1	donneur 2	donneur 3	donneur 4	donneur 5
0.35	0.4471	0.4443	0.3000	0.4586	0.4599
	0.4514	0.4500	0.3014	0.4614	0.4629
	0.4414	0.4543	0.3014	0.4614	0.4671
	0.4414	0.4585	0.3071	0.4657	0.4686
	0.4457	0.4614	0.3114	0.4657	0.4786
0.25	0.1557	0.1471	0.0871	0.1357	0.1914
	0.1571	0.1486	0.0871	0.1414	0.1929
	0.1614	0.1486	0.0914	0.1443	0 1957
	0.1614	0.1514	0.0943	0.1457	0.1999
	0.1629	0.1514	0.0957	0.1471	0.2014
	donneur 6	donneur 7	donneur 8	donneur 9	
0.35	0.6429	0.5829	0.5143	0.5943	
	0.6443	0.5899	0.5143	0.5943	
	0.6471	0.5943	0.5143	0.5957	
	0.6471	0.5943	0.5243	0.6086	
	0.6471	0.5971	0.5257	0.6157	
0.25	0.2671	0.2729	0.2143	0.2514	
	0.2671	0.2757	0.2214	0.2543	
	0.2699	0.2799	0.2243	0 2543	
	0.2729	0.2799	0.2243	0.2586	
	0.2829	0.2814	0.2286	0.2599	

[Ces données nous ont été fournies par le Dr. ANDRÉ, chef de travaux du service de transfusion sanguine de l'Université de Liège; nous le remercions vivement de son obligeance.]

Appendice 4

Programme Fortran calculant LFS titres de plusieurs sérums et leurs intervalles de confiance respectifs

Ce programme a été rédigé pour éviter à l'utilisateur des calculs longs et fastidieux. Il est valable pour

$$1 < N < 100$$

et les transformations préliminaires de u et x sont relatives à la formule de von Krogh.

Toutefois, il serait extrêmement aisé de modifier l'ordre dimension afin de pouvoir considérer un nombre d'observations supérieur à cent par donneur.

De plus, l'utilisateur peut, s'il le désire, choisir une autre solution que celle de von Krogh. Il lui suffira de modifier les ordres 0013 et 0014 en conséquence.

```
DIMENSION X (100), U (100), Z (100), Y (100), EL (100), DIF (100), CAR (100),
ERTY2 (100), ERTY1 (100), CONF (100), HYPBI (100), HYPBS (100)
        NUM = 0
        READ (5, 1) NOMB
     22 NUM = NUM +1
      1 FORMAT (I5)
        READ (5, 2) N, QF
      2 FORMAT (I4, F7. 3)
        READ (5, 3) (U (I), I = 1, N)
        READ (5, 4) (X (I), I = 1, N)
      3 FORMAT (10F 5.4)
      4 FORMAT (10F3.2)

C TRANSFORMATION DES DONNEES
        DO5I = 1, N
        Z (I) = ALOG (X (I) )
        Y (I) = ALOG(U(I)/(1. - U(I) ) )
      5 CONTINUE

C CALCUL DES PARAMETRES DU LIEU
        SYY = 0.
        SY = 0.
        SZ = 0.
        SYZ = 0.
        SZZ = 0.
        DO6I = 1, N
        SYY = SYY+Y(I)*Y(I)
        SY = SY + Y(I)
        SZ = SZ + Z(I)
        SZZ = SZZ + Z(I)*Z(I)
        SYZ = SYZ + Y(I)*Z(I)
      6 CONTINUE
        BCH = SY/N
        ZM = SZ/N
        CP = (N*SYZ - SY*SZ)/N
        SIGZ = (N*SZZ - SZ*SZ)/N
        ALCHA = CP/SIGZ
        BETCHA = BCH - (ZM*ALCHA)
        SCN = (SY*SY)/N + (CP*CP)/SIGZ
        SCE = SYY - SCN
        SICHA2 = SCE/(N-2.)
        SICHA1 = SQRT (SICHA2)
        QUA = 2.*QF*SICHA2
        RAC = SQRT (QUA)

C LIEU ESTIME ET SON HYPERBOLE DE CONFIANCE
        DO7I = 1, N
        EL(I) = BETCHA + (Z(I)*ALCHA)
        DIF(I) = Z(I) - ZM
        CAR(I) = DIF(I)*DIF(I)
        ERTY2(I) = 1./N + CAR(I)/SIGZ
        ERTY1(I) = SQRT (ERTY2(I) )
        CONF(I) = RAC*ERTY1(I)
```

```
      HYPBS(I) = EL(I) + CONF(I)
      HYPBI(I) = EL(I) - CONF(I)
    7 CONTINUE

C CALCUL DE LA DOSE DE COMPLEMENT DONNANT L HEMOLYSE 0, 50
C INTERVALLE DE CONFIANCE
      Z50 = - BETCHA/ALCHA
      X50 = EXP(Z50)
      T50 = 10./X50
      A = ALCHA*ALCHA - QUA/SIGZ
      B = (ALCHA*BETCHA) + (QUA*ZM)/SIGZ
      C = BETCHA*BETCHA - QUA/N - (QUA*ZM*ZM)/SIGZ
      RAU2 = B*B - A*C
      RAU1 = SQRT(RAU2)
      ZI50 = (-B-RAU1)/A
      XI50 = EXP(ZI50)
      ZS50 = (-B+RAU1)/A
      XS50 = EXP(ZS50)
      F = XS50 - XI50
      FERELA = 100.*F/X50
      TI50 = 10./XS50
      TS50 = 10./XI50
      D = TS50 - TI50
      ERELA = 100.*D/T50

C MISE EN PAGE
      WRITE(6, 30) NUM
   30 FORMAT (1H1, 8HPROBLEME, 5X, I5)
      WRITE (6, 9)
    9 FORMAT (1HO)
      WRITE (6, 31)
   31 FORMAT (1H , 70HEQUATION Y = ALPHA*Z+BETA AVEC Y = TRANS-
     FORMEE DU TAUX D HEMOLYSE ET Z = LNX)
      WRITE (6, 9)
      WRITE (6, 8)ZM, SIGZ
    8 FORMAT (1H , 14HMOYENNE DES Z = , F12.8,10X, 30HSOMME DES
     ECARTS QUADRATIQUES = , F12.8)
      WRITE(6, 9)
      WRITE (6, 10) ALCHA, BETCHA
   10 FORMAT (1H, 6HALPHA =, F12.8,10X,5HBETA = ,F12 8)
      WRITE(6,9)
      WRITE(6,11) SICHA2, SICHA1
   11 FORMAT (1H , 12HSIGMA CARRE =, F12.8, 10X, 6HSIGMA =, F12.8)
      WRITE(6,9)
      WRITE(6, 12)
   12 FORMAT (1H, 75HDOSE DE COMPLEMENT DONNANT UNE HEMOLYSE
     0, 50 ET SON INTERVALLE DE CONFIANCE)
      WRITE(6, 9)
      WRITE (6, 13)
   13 FORMAT (1H , 14HEN ML DE SERUM)
      WRITE (6, 14) XI50, X50, XS50
   14 FORMAT (IH, 3 (F8. 6, 10X))
      WRITE (6,9)
      WRITE (6,21) FERELA
```

```
      WRITE (6,9)
      WRITE (6,15)
   15 FORMAT (1H , 25HEN UNITES 0,50 D HEMOLYSE)
      WRITE (6,16) T I50, T50, TS50
   16 FORMAT (1H , 3(F6,3,10X))
      WRITE (6,9)
      WRITE (6,21) ERELA
   21 FORMAT (1H , 16 HERREUR RELATIVE =, F5.29,9HPOUR CENT)
   17 FORMAT (1H , 5X, 9HVAR INDEP, 15X, 7HVAR DEP, 17X, 9HHYPERBOLE,
      15X, 4HLIEU, 2OX, 9HHYPERBOLE)
      WRITE(6,18)
   18 FORMAT(1H , 11X, 1HZ, 23X, 1HY, 17X, 1 1HBRANCHE INF, 13X, 6HES-
      TIME, 18X, 11HBRANCHE SUP)
      WRITE(6,9)
   20 FORMAT(1H , 5X, 5(2X, F9.6,13X) )
      DO19I = 1,N
      WRITE(6, 20)Z(I), Y(I), HYPBI(I), EL(I), HYPBS(I)
   19 CONTINUE
      IF(NUM.GE.NOMB)CALL EXIT
      GO TO22
      END
```

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Estimation of Complement Activity: A Statistical Study of Some Methods

A study has been made the basis of experimental data of four theoretical relations characterizing the curve of haemolysis, and a reliable method has been worked out for the estimation by confidence intervals of the dose corresponding to 50% haemolysis. The computations have been programmed in FORTRAN.

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Coincidence of Autoimmune Diseases and Polycythaemia vera

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The coincidence of polycythaemia vera and autoimmune diseases was observed in three cases. In two cases the autoimmune disease was rheumatoid arthritis, in one case systemic lupus erythematosus. The possible causes of the coincidence of autoimmune disease and malignant cell proliferation are discussed.

If the frequency of the coincidence of two diseases with unknown aetiology cannot be explained by chance, the possibility of a close relationship between the two conditions has to be considered. Such a relationship is assumed to exist between the autoimmune and other malignant diseases. It was the autoimmune haemolytic anaemias accompanying lymphoproliferative diseases which have drawn attention towards these associations [3, 4]. Papers dealing with the associations of other autoimmune diseases and lymphoma have enlarged this perspective [2, 7, 18]. Non-haematological malignant conditions, tumours, are also to occur together with autoimmune diseases, first of all with dermatomyositis [23]. On the other hand, such connections between myeloproliferative diseases and autoimmune phenomena are less known. Apart from a few case reports [5, 14] no data are available as to associations of this type.

In the following we shall present three cases in which a myeloproliferative disease, polycythaemia vera, occurred together with autoimmune diseases.

Case reports

Case No. 1. O. O., 56, female, had had articular complaints since 1958. She had been hospitalized twice with rheumatoid arthritis. She was admitted to our Department for the first time on February 20, 1968. Her joints were painful. Since half a year dizziness, headache, tinnitus and dyspnoea had accompanied the articular complaints.

At admission the patient was plethoric with moderate cyanosis of the lips. The liver was enlarged, the lower pole of the spleen could be reached. The elbow, the wrist and especially the metacarpophalangeal and interphalangeal joints were swollen, sensitive. The fingers were atrophic, in ulnar deviation, the interosseal muscles were atrophic (Fig. 1).

Laboratory results: ESR, 1 mm/h; WBC, 11 800; RBC, 6.8 million; haematocrit, 75 %; platelet count, 47 000. In peripheral blood there was a moderate shift to the left. Marrow biopsy showed hyperplastic, hypercellular bone marrow, enhanced erythro- and myelopoiesis, definite megakaryocytosis (Fig. 2). Serum total

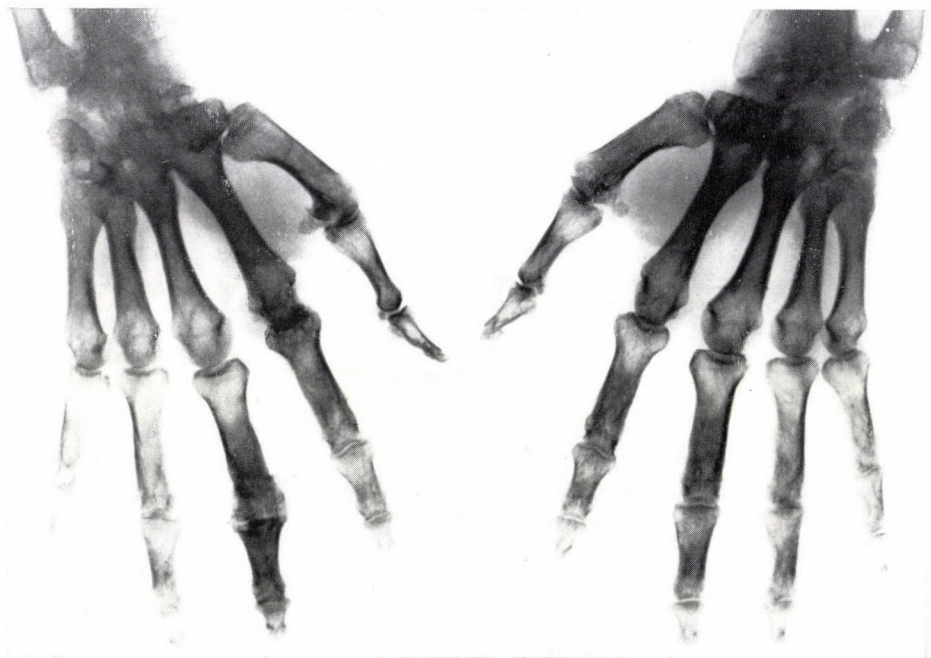

Fig. 1. X-ray of hands. In both hands the carpal bones are ankylosed with each other and with the radius. The interphalangeal spaces are narrow and the third interphalangeal joint of finger III is ankylosed. In the diaphyses there are degenerative cysts. The bones are diffusely osteoporotic

protein, 8.2 %. Quantitative immunoglobulin determination according to Manchini [15]: IgG, 1600 mg per 100 ml, IgA: 300 mg per 100 ml. IgM was moderately increased [19]. Waaler-Rose test, 1 : 256; RF-, Latex-test, + +. LE-cell phenomenon, negative. Cryoglobulin, negative. Sia-test, negative. The diagnosis was polycythaemia vera with rheumatoid arthritis. On prednisolone (30 mg daily) therapy the articular complaints ceased. Against the polycythaemia 5 mCi ^{32}P was prescribed; three months later the blood counts were normal, liver and spleen decreased in size, the polycythaemic complaints had ceased. Waaler-Rose test, 1 : 256; RF-; Latex-test, + +; LE-cell phenomenon, negative; ESR, 2 mm/h.

Case No. 2. In this 70-year-old male patient polycythaemia had been detected in 1965, and in the same year articular complaints had developed; they were localized in the small joints of the hand and manifested themselves with limited motion and

pain. On prednisolone treatment the complaints improved. For the polycythaemia 3 mCi ^{32}P was given in December, 1965. Due to articular complaints and exacerbated polycythaemia, he was admitted to our Department on February 16, 1967. The patient was plethoric, the small joints of the hand were swollen, painful,

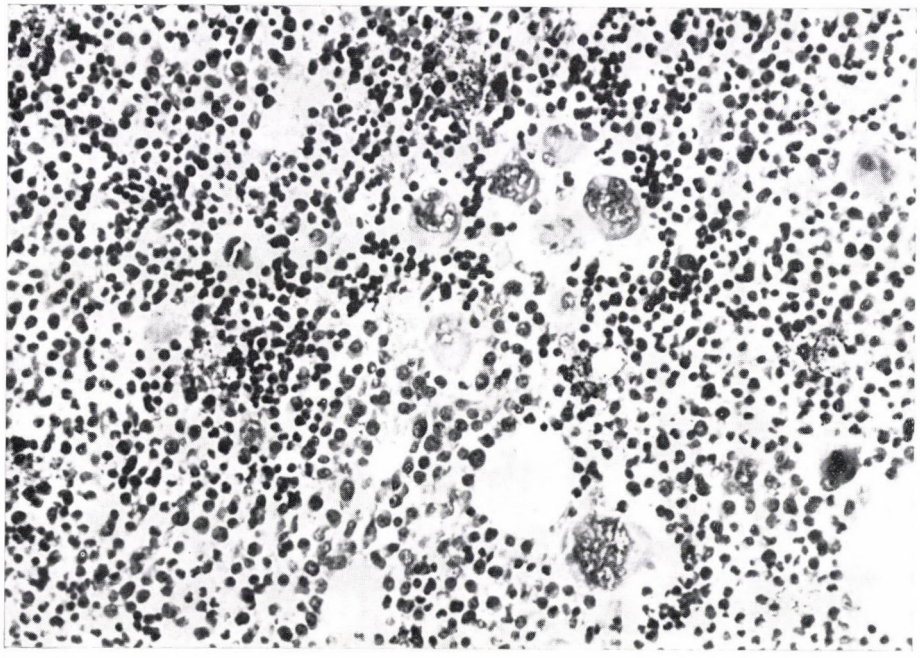

Fig. 2. Bone biopsy. Hyperplastic, hypercellular bone marrow with enhanced erythro- and myelopoiesis and definite megakaryocytosis. ×250.

moderately deformed. Laboratory results: ESR, 2 mm/h; WBC, 12 000; RBC, 6.1 million; haematocrit, 72 %; platelet count, 39 000. Peripheral blood showed a shift to the left. Bone marrow biopsy was characteristic of polycythaemia. Serum total protein, 8.9 g per 100 ml. Electrophoresis showed an increase in gamma globulin, 27.2 %. Waaler-Rose test, 1 : 128. Latex-test, + + +. LE-cell phenomenon, negative. Cryoglobulin, negative. X-rays showed abnormally narrow interphalangeal joint spaces in both hands, cystic degeneration around the joints. The diagnosis was polycythaemia vera with rheumatoid arthritis.

Articular complaints ceased after 10 days on 30 mg prednisolone daily. For the polycythaemia 6 mCi ^{32}P was prescribed; after 3 months the condition showed a total clinical and haematological remission. At present the patient is free from complaints and symptoms, the Waaler-Rose test is 1 : 128; ESR, 1 mm/h.

Case No. 3. R. K., 61, male, was admitted for the first time on 30th October, 1963 with dyspnoea, dizziness, headache, squeezing pains in the chest, and sen-

sitivity below the left costal arch. Earlier he had had malaria, epidemic hepatitis and repeated pleurisies; his tuberculous origin could not be proved. The patient was plethoric, the liver and spleen were enlarged. Laboratory results: ESR, 1 mm/h; WBC, 11 000; RBC, 6.2 million; haematocrit, 70 %; platelet count, 400 000; Wassermann, negative; liver function tests were normal; LE-cell phenomenon was negative. Antinuclear factor (with passive haemagglutination method [17]) was negative. Urine showed normal findings. The diagnosis was polycythaemia vera. 6 mCi ^{32}P was prescribed. Due to recurrent complaints he was given another 6 mCi dose of ^{32}P on September 21, 1964. Owing to the exacerbation of polycythaemia, he was admitted again on June 23, 1966. At admission he had lymph nodes of bean size in the cervical and axillar areas. The liver was enlarged, the lower pole of the spleen could be palpated. Westergreen, 2 mm/h; WBC, 9800; RBC, 6.8 million; haematocrit, 76 %; platelet count, 120 000. The peripheral blood showed a shift to the left. Serum total protein, 9.3 g per 100 ml. Paper electrophoresis: albumin: 49 %, $alfa_1$: 4 %, $alfa_2$: 7.2 %, beta-globulin: 12 %, gamma globulin: 27.8 %. The LE-cell phenomenon was repeatedly positive, the antinuclear factor, 1 : 64, positive; the Waaler-Rose test, 1 : 128; Latex-test, +; Sia-test, positive; cryoglobulin, positive. Ultracentrifugation yielded a normal amount of macroglobulin. The diagnosis was polycythaemia vera with systemic lupus erythematosus.

With regard to the low platelet count, isotope therapy was omitted, and the polyglobulia was reduced by repeated blood lettings (a total of 1500 ml). The patient was discharged with the prescription of 10 mg prednisolone and 0.25 chloroquine daily. A follow-up examination in May, 1968 revealed a negative LE-cell phenomenon, a positive antinuclear factor of 1 : 32 titre. The patient feels well. During the last two years he had pleurisy once and recovered within two weeks.

Discussion

The association of autoimmune diseases with malignant diseases is a frequent occurrence. The study of these associations gave rise to many hypotheses explaining the pathomechanism of the associated diseases with unknown aetiology.

As regards the simultaneous occurrence of dermatomyositis and neoplasm, first of all the changes in antigenicity developing as a consequence of the tumour have been stressed [23]. The coincidence of thymomas and myasthenia gravis points — in addition to the changes in antigenicity — to the pathological function of the antibody-producing system [1]. A similar causal relationship has been postulated by Dameshek [3] between the malignant lymphoproliferative diseases and the autoimmune diseases, first of all the immunohaemolytic conditions [3, 4]. In the cases reported by Howqua and Mackay [7], Petrányi et al. [18] and Cammarata et al. [2] malignant lymphomas were associated with autoimmune diseases. These authors considered the functional disorders (pathological antibodies, protein products) developing as a result of the tumorous proliferation of the immune

system to play an essential role in the association. This, however, is not unidirectional. Cases were described [9, 10, 21], in which the autoimmune disease had preceded the malignant lymphoid proliferation. A high percentage of the natural or artificial immunological abnormalities turns into malignant lymphoproliferation. This is the case, e.g. in NZB/B_1 mice or in Aleutian mink disease [13, 16]. Schwartz and Beldotti [20] and Kaplan and Smithers [8] drew attention to the malignant lymphoproliferation of the homologous disease. These associations refer to an eventual continuity of transmission. The existence of a common aetiological factor (virus?) is indicated especially by experimental data. Furthermore, the pathological function of the immune system may manifest itself in a reduction of the normal resistance including an insufficient protection against the mutations. All this is increasing the chance for neoplastic transformation. Different possibilities may be envisaged in connection with the coincidence of polycythaemia vera and the autoimmune diseases. In our first case, the rheumatoid arthritis had begun 9 years before the onset of polycythaemia. In the second case the two diseases manifested themselves nearly at the same time. The third patient had repeatedly non-tuberculous pleurisies and then abnormal serological reactions of the autoimmune type developed three years after the onset of polycythaemia vera.

In our cases the weakening of the normal functions (defence against mutations) of the damaged immune system may have resulted in an increase in the frequency of tumorous transformation manifesting itself with myeloproliferation.

As to the pathomechanism of the autoimmune diseases, nowadays the intricate counteraction of endogenous (genetic) and exogenous factors are taken into consideration. These (genetic and acquired damage of the immune system, exogenous noxas) become effective from the point of view of the autoimmune disease by supplementing each other. Polycythaemia vera may be a provocative factor in developing the endogenous background. This version may be taken into account mainly in our second and third case. In addition to polycythaemia vera, its therapy, especially that with ^{32}P, may also represent a provocative factor.

A coincidence naturally may appear also by chance. Our three patients were advanced in age. Two of them suffered from rheumatoid arthritis, which — similarly to polycythaemia — occurs more frequently in old than in young subjects.

The coincidence of polycythaemia vera and lymphoma is known from the literature [6]. The occurrence of polycythaemia with tumours of different localization was observed by us several times. In general, the incidence of tumours in polycythaemic patients significantly surpasses the average frequency [11, 12, 22].

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Über die Spezifität von Lektinen mit einem breiten Agglutinationsspektrum

VII. Weitere Untersuchungen mit dem *Maclura aurantiaca* Agglutinin

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Die Spezifität des *Maclura aurantiaca* Agglutinins kann sich sowohl gegen endständige α oder β-Anomere von N-Acetyl-D-Galaktosamin (oder D-Galaktose) als auch gegen die innerhalb einer Kohlenhydratkette gebundenen Galaktosaminstrukturen (wenn C 3 und C 4 frei sind) richten. Es wird empfohlen, kompetitive Hemmteste mit verschiedenen, auch enzymbehandelten Blutkörperchen durchzuführen, um eine mögliche Konkurrenzreaktion zwischen zellständigem Rezeptor und Inhibitor zu erfassen.

In einer vorhergehenden Arbeit [1] haben wir unter einer Reihe von Lektinen, deren Spezifität wir untersuchten, auch auf die mögliche Reaktionsweise des Agglutinins aus *Maclura aurantiaca* hingewiesen und sie als vorwiegend gegen Galaktosylgruppen gerichtet definiert, und zwar in der Regel gegen nicht reduzierend terminal angeordnete.

In dieser Mitteilung wollen wir nun berichten, daß dieses Agglutinin auch gegen in C 2 substituierte D-Galaktose, nämlich N-Acetyl-D-Galaktosamin, gerichtet ist, und zwar vorwiegend wenn es in α (oder β-) glykosidischer Bindung endständig vorliegt oder innerhalb einer (ebenfalls hauptsächlich α-glykosidischen) Heterosaccharidkette angeordnet ist, vorausgesetzt, C 3 und C 4 sind frei. Damit steht neben den schon bekannten Agglutininen aus Schnecken und Pflanzen ein weiteres Reagens zur Erfassung dieses Hexosamins zur Verfügung.

Material und Methoden

1. *Agglutinationsteste, Hemmteste und Enzymbehandlung der Erythrozyten* erfolgten wie in der vorhergehenden Arbeit dieser Reihe [1] beschrieben.

2. *Maclura aurantiaca Lektin.* Wir benutzten einen einfachen Kochsalz-Extrakt aus dem Samen, der nach Quellen der Körner über Nacht und anschließendem Zerkleinern im Starmix und Zentrifugieren als Überstand gewonnen wurde.

3. *Testsubstanzen.* Außer den in den vorhergehenden Mitteilungen bereits beschriebenen Testsubstanzen wurden folgende Präparate mit einbezogen: OSM (Schafsubmaxillarismucin) reinst [2] wurde uns freundlicherweise von Prof. Dr. A.

Gottschalk, Max-Planck-Institut für Virusforschung Tübingen, überlassen. Globosid I gab uns Prof. Dr. E. Klenk, Physiologisch-Chemisches-Institut der Universität Köln. Galaktogen erhielten wir von Prof. Dr. Dr. H. Weinland, Physiologisch-Chemisches-Institut der Universität Erlangen. Schaf-Hydatiden-Cystenflüssigkeit schickte uns liebenswürdigerweise Dr. Anstee, Bristol, Regional Blood Transfusion Service; sie war lyophilisiert, in wenig Wasser aufgenommen und über Sephadex G 25 filtriert worden. Es wurde die Fraktion benutzt, die die stärkste Blutgruppen-P-Aktivität aufwies. Das Material enthielt etwa 9 % Galaktose und 4 % Hexosamin. Die *Shigella flexneri* Lipopolysaccharide (Serotyp 3a, Variante X und eine Rauhmutante vom Chemotyp Rb) überließ uns freundlicherweise Dr. D. A. R. Simmons, Department of Bacteriology and Immunology, University of Glasgow. Azoprotein [3] entspricht p-Aminophenyl-N-Acetyl-β-Galaktosaminid gekuppelt an Ovalbumin. Ein Glykoprotein aus Ovarcysten (Blutgruppe O) ist ebenfalls schon an anderer Stelle beschrieben worden [4]. Es wurde gleichfalls durch Phenol-Kochsalz-Extraktion gewonnen und reagiert nach unserer Auffassung mit Anti-A_{HP} aufgrund von an Threonin und Serin gebundenem (α-glykosidisch) endständigem N-Acetyl-D-Galaktosamin (ähnlich wie OSM).

Ergebnisse und Diskussion

Zunächst sind in Tabelle 1 Agglutinationsteste mit diesem Agglutinin angegeben. Aus dieser Tabelle ist folgendes ersichtlich:

a) Der Titer steigt nach RDE-Behandlung stark an. Hierdurch werden hauptsächlich terminale β-N-Acetyl-Galaktosaminyl-Strukturen frei (Mensch, Schwein, Hund, Taube, Schaf), aber auch β-Galaktosyl-Reste (Rind). Offenbar werden beide durch das Agglutinin erfaßt.

Tabelle 1

Titer der Agglutinationsteste mit *Maclura aurantiaca* Lektin

Zellen	Unbehandelt	Pronase behandelt	Neuraminidase behandelt
Mensch (A)	16	2	64
Mensch (B)	32	∅	128
Mensch (O)	8	∅	64
Mensch (AB)	64	∅	256
Rind	∅	∅	4
Schwein	2	1	16
Hund	32	8	128*
Schaf	2	∅	16
Taube	32	8	128

* Nach anschließender Pronase-Behandlung sinkt der Titer auf 8 ab.

b) Das Agglutinin reagiert auch mit Zellen, die bewiesenermaßen (negative Reaktion mit allen Anti-Galaktosaminyl-Reagentien aus Schnecken) kein terminales N-Acetyl-D-Galaktosamin haben, z. B. Taube und Mensch O und B.

c) Der Rezeptor befindet sich größtenteils in der Mucoidfraktion, denn der Titer wird in der Regel durch Pronase-Behandlung niedriger. Selbst Neuraminidase-behandelte Zellen, die anschließend mit Pronase behandelt werden, zeigen einen stark erniedrigten Titer.

Bei den Hemmtesten in Tabelle 2 zeigt sich folgendes Bild:

a) Es werden Substanzen als inhibitorwirksam aufgeführt, die endständiges N-Acetyl-D-Galaktosamin haben, und zwar α-glykosidisch gebunden (A-Sub-

Tabelle 2

Hämagglutinationshemmteste mit *Maclura aurantiaca* Lektin

Inhibitor	Titer	
	O-Zellen	O-RDE* Zellen
OSM (X)	128,000	∅
A-Substanz aus Pepton	128,000	∅
Pferdeerythrozytenmucoid (X)	1,000	∅
Ovarcyste O	1,000	∅
Hydatidcyste des Schafes	256	∅
Shigella flexneri Typ X Lipopolysaccharid	128	∅
Rindererythrozytenmucoid (X)	64	∅
Melibiose	64	∅
N-Acetyl-D-Galaktosamin	32	∅
Myxosarkommucoid (X)	32	∅
Rinderamnionmucoid (X)	32	∅
Katzenhirngangliosid (X)	16	∅
Disaccharid Gal $\frac{1-3}{\beta}$ N-Ac-Galm	8	∅
D-Galaktosamin	4	∅
Stachyose	4	∅
Hydrolysiertes Rindererythrozytenmucoid	4	∅
D-Galaktose	2	∅
Shigella flexneri Typ Rb Lipopolysaccharid	2	∅
Galaktogen HP	∅	∅
Globosid I	∅	∅
Azoprotein	∅	∅
L-Fucose	∅	∅
L-Arabinose	∅	∅

(X) Substanzen, die kein endständiges N-Acetyl-D-Galaktosamin haben.
*Kristalline Neuraminidase der Behring Werke Marburg.

stanz aus Pepton, Ovarcyste). Azoprotein und Globosid I mit β-glykosidisch gebundenem Hexosamin hemmen nicht.

b) Auch Substanzen mit endständiger Galaktose (oder Glukose), die α-glykosidisch gebunden ist, hemmen gut (Melibiose, Stachyose). Das *Shigella flexneri* Typ X Lipopolysaccharid kann aufgrund der L-Rhamnose-Struktur (C 4 wie in der Galaktose) oder aber mit α-Glucosylresten hemmen, während beim Typ Rb die Reaktion mit terminalen Laktosyl-Gruppen wegen des hydrophoben Charakters nicht zustande kommt. Das stimmt auch mit den Hemmtesten anderer Autoren überein [5].

c) Es werden auch Substanzen erfaßt, die endständig β-glykosidisch gebundene D-Galaktose haben, wie z. B. das Rindererythrozytenmucoid und das Katzenhirngangliosid. Galaktogen (L-Galaktose!) hemmt nicht. Rinderamnionmucoid und das Myxosarkommucoid hemmen wahrscheinlich aus dem gleichen Grunde, ebenso das Pferdeerythrozytenmucoid (β-glykosidische D-Galaktose) sowie das Disaccharid.

d) Es reagieren auch Verbindungen, die kein endständiges N-Acetyl-D-Galaktosamin und keine Galaktose haben (OSM). Dies ist ein Hinweis darauf, daß das Agglutinin auch "innerchain" N-Acetyl-D-Galaktosamin erfaßt, und zwar besonders, wenn C 3 und C 4 bzw. ihre OH-Gruppen noch frei sind. Die Bindung der Neuraminsäure im OSM erfolgt nämlich an C 6 des Galaktosamins [2].

Das *Maclura aurantiaca* Lektin erfaßt also bevorzugt Galaktosederivate, die endständig gebunden sind, und hier insbesondere die α-anomeren Formen unter Bevorzugung der in Stellung 2 durch eine N-Acetyl-Amino-Gruppe substituierten D-Galaktose, also des N-Acetyl-D-Galaktosamins. Dieses wird vor allem auch bevorzugt, wenn es innerhalb einer Kohlenhydratkette α-glykosidisch (an Threonin oder Serin) vorliegt, vorausgesetzt, daß die C-Atome 3 und 4 frei sind, also die Bindung an C 6 erfolgt. Ähnliches mag vielleicht auch für das "innerchain" β Anomere gelten sowie für die nicht-substituierte D-Galaktose, immer unter der Voraussetzung, daß C 3 und C 4 des Rings nicht besetzt sind. Außerdem scheint eine "hydrophile" Nachbarschaft erforderlich (negative Teste mit Shigella Rb LPS und Azoprotein).

Besonders merkwürdig bei diesen Hämagglutinationshemmtesten war, daß sie sich nur mit normalen Zellen des Menschen und der meisten Tiere durchführen ließen, nicht aber, — trotz deutlicher und starker Agglutination — mit den entsprechenden Neuraminidase-behandelten Zellen. Auch bei Mischung verschiedenster (galaktosehaltiger und N-Acetyl-D-galaktosaminhaltiger) Inhibitoren ließ sich keine Hemmung erzielen. Eine Erklärung für dieses merkwürdige Phänomen konnten wir zunächst nicht geben. Die Annahme jedoch, daß die Bindung des Agglutinins an terminales N-Acetyl-D-Galaktosamin (oder an Galaktose) stärker ist und vor allem, daß bei Neuraminidase-behandelten Zellen durch Entfernung der Neuraminsäure (Ladung) auch das hier vorliegende "innerchain" N-Acetyl-D-Galaktosamin (oder Galaktose) besser zur Geltung kommt, führte uns zu einer Erklärung der oben erwähnten paradoxen Ergebnisse (Tab. 2). Nimmt

man nämlich eine ungleich stärkere Affinität des *Maclura aurantiaca* Lektins zu den Neuraminidase-behandelten Zellen (siehe dazu auch Tabelle 1) an, vor allem aufgrund von terminalem und "innerchain" N-Acetyl-D-Galaktosamin, so erscheint es verständlich, daß keine Hemmung eintritt bzw. daß eine initiale Hemmung reversibel gemacht wird (Abb. 1). Bei Verwendung unbehandelter Zellen ist die Affinität des Agglutinins zum Inhibitor stärker als zum zellständigen Re-

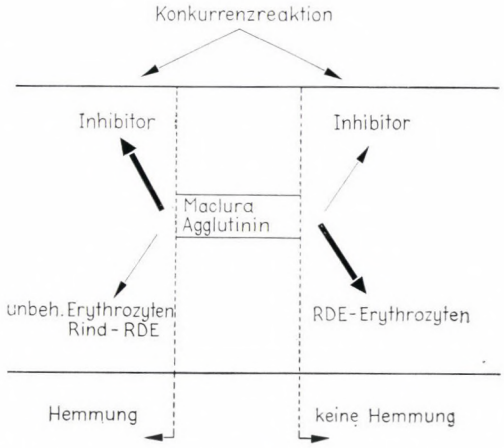

Abb. 1. Kompetitive Reaktion zwischen Inhibitor und verschiedenen Erythrozyten in bezug auf das Maclura Agglutinin. RDE = Neuraminidase

zeptor, es kommt also zur Hemmung. Umgekehrt, unter Verwendung von Neuraminidase-behandelten Zellen, reißt der jetzt sehr starke zellständige Rezeptor das Agglutinin unter Lösung der vorher bereits eingegangenen Bindung zum Inhibitor an sich, die Hemmung kann also nicht zustande kommen. Anders aber ist es, wenn man den sehr schwachen Testerythrozyten "Rind-Neuraminidase-behandelt" (Titer 4, siehe dazu Tabelle 1) einführt. Er ist mit nur terminalen β-Galaktosylresten der schwächere Partner und das Agglutinin wird fast ausschließlich vom Inhibitor gebunden. Der Hemmtiter ist somit unter Benutzung von "Rind-Neuraminidasebehandelten Zellen" weitaus am höchsten, nämlich bei OSM als Inhibitor 256 000, bei A-Substanz aus Pepton ebenfalls 256 000 und bei Ovarcyste 16 000.

*

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On the Specificity of Lectins of a Broad Agglutination Spectrum, VII

The specificity of the agglutinin from *Maclura aurantiaca* may be directed against the terminal α or β-linked anomer of N-acetyl-D-galactosamine (or D-galactose) as well as against inner-carbohydrate chain galactosaminyl structures (if C 3 and C 4 are free). It is suggested to use competitive inhibition tests with different, even enzyme-treated red cells, in order to establish the reaction of the lectin with the inhibitor on the one hand and the cell receptor on the other.

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Effects of Phytohaemagglutinin on the Immune Response of Irradiated Rabbits

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Phytohaemagglutinin injected before primary antigenic stimulation was found to increase secondary antibody production against SRBC in rabbits. This effect was practically abolished by X-ray irradiation and appeared in a slight form in rabbits irradiated before secondary antigenic stimulation.

The effect of phytohaemagglutinin (PHA) *in vivo* has been investigated by a number of authors [1—7]. It is generally accepted that PHA enhances lymphocyte proliferation [8, 9], but as far as the immune response is concerned, its immunosuppressive or stimulatory effect has been questioned [10—18]. The experiments to be reported were designed to elucidate whether PHA, which is known to act on the radiosensitive lymphocytes, would influence the immune response in irradiated rabbits.

Materials and Methods

Thirty-five female chinchilla rabbits of 2 to 2.5 kg body weight were divided into seven groups of 5 animals each. They were given 0.2 ml of Bacto PHA-P (DIFCO) for three consecutive days, the first time intravenously, then intramuscularly. Whole-body irradiation with 500 R (250 kV, 15 mA, 0.5 + 2.0 mm Cu filter, 25 R/min) was delivered 24 hours prior to the primary or secondary immunization. The rabbits were immunized by 2×10^8 sheep red blood cells (SRBC) per kg body weight, injected intravenously on two occasions with an interval of 14 days. Blood samples were collected every 3rd or 4th day, for 5 weeks.

The SRBC haemolysin titres were determined by Takátsy's micro-method [19]. The resistance of the antibodies to 2-mercaptoethanol was investigated according to Hege and Cole [20].

Results and Discussion

Whole-body irradiation with 500 R 24 hours before the first injection of SRBC abolished the primary immune response (Fig. 1). In spite of this the second antigen injection induced antibody production. The haemolysin peak was not

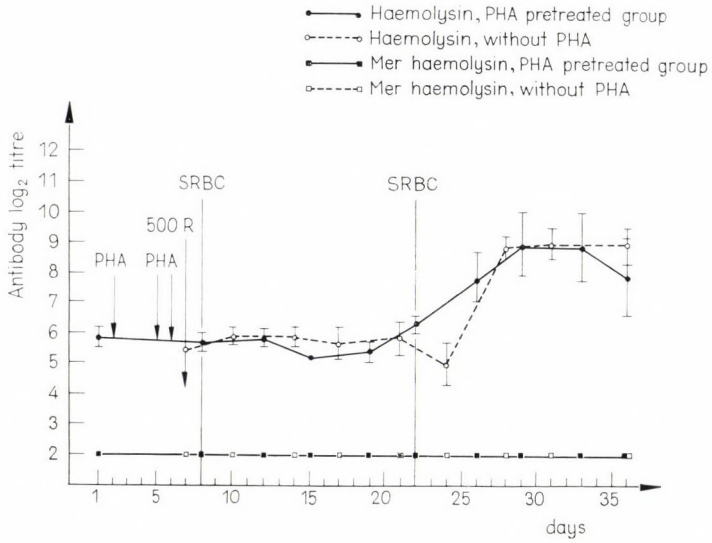

Fig. 1. PHA action on SRBC haemolysin production in rabbits irradiated 24 hours prior to primary antigen stimulus. Antibody titre is represented by base 2 log of the highest dilution still giving a positive reaction. The standard errors are indicated in the Figure

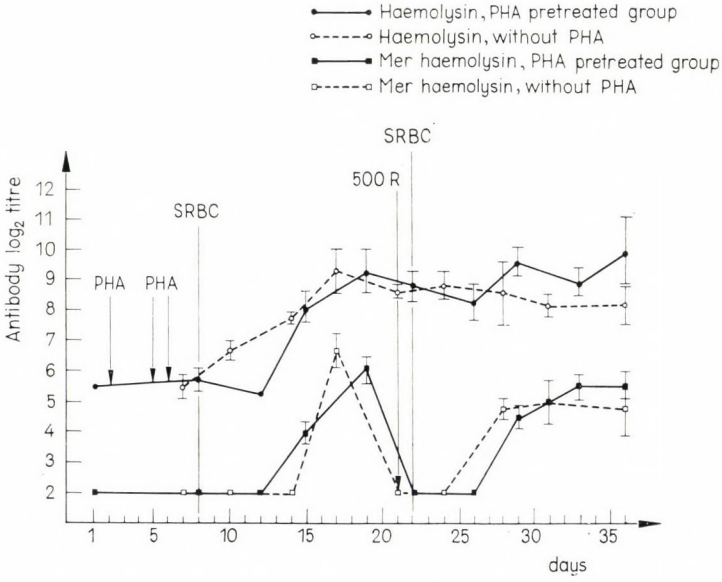

Fig. 2. Influence of PHA on SRBC haemolysin production in rabbits irradiated 24 hours prior to secondary antigen injection. For details, see Fig. 1

higher than the peak during the primary immune response in non-irradiated rabbits (see Fig. 3), whereas no mercaptoethanol-resistant (MER) antibodies could be found in the irradiated animals. PHA pretreatment was ineffective.

The secondary immune response was inhibited in animals subjected to whole-body irradiation with 500 R before the second antigen injection (Fig. 2) and the antibody titre decreased to some extent. The increase of the MER haemolysin titre, however, showed a slight immune reactivity. In irradiated rabbits pretreated with PHA the haemolysin level increased slightly as compared to the level before the secondary stimulation.

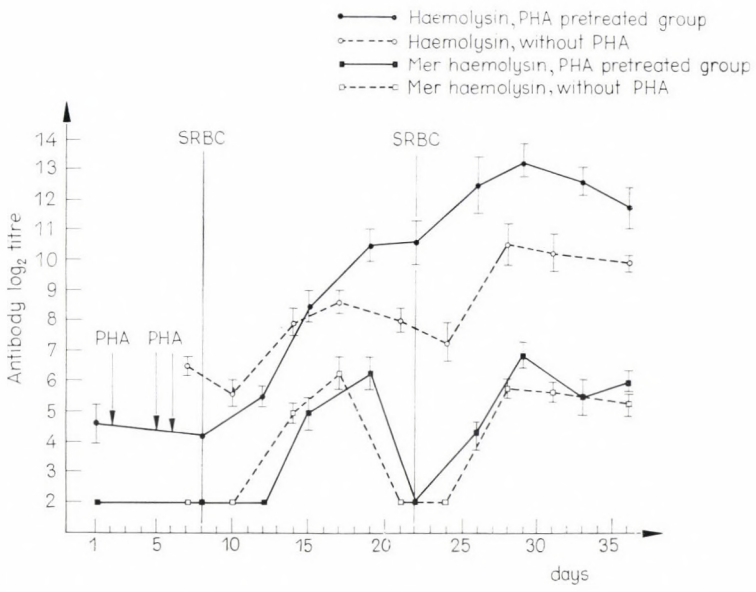

Fig. 3. Stimulatory effect of PHA treatment on immune response of nonirradiated rabbits. For details, see Fig. 1

In nonirradiated rabbits, PHA pretreatment induced a marked increase of the immune response (Fig. 3), mainly during the secondary response.

In another group of animals the effect of PHA on natural SRBC haemolysin has been investigated. The natural haemolysin level did not change following PHA treatment.

The above results indicate that PHA increases the immune response in rabbits. PHA acts probably on 19S antibody production, since the respective MER titres, which apparently correspond to 7S antibody production, have not been affected. The fact that PHA given before the primary antigen injection exerts its effect during the secondary immune response suggests that PHA may act on the memory cell count [21—23].

The results also showed that PHA treatment did not alter the effect of irradiation on the primary immune response. However, antibody production was slightly increased in PHA-treated rabbits irradiated before the secondary antigen stimulation. This again raises the possibility of PHA increasing the memory cell count.

*

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Oxidizing-Reducing Properties of the Intrinsic Prothrombin Activator

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Purified intrinsic prothrombin activator (IA) was investigated during exposure to cysteine, reduced glutathione and N-ethylmaleimide. IA activity was rapidly decreasing in the presence of reducing agents (cysteine, reduced glutathione); the rate of decrease depended on concentration and incubation time. The inhibitory effect was enhanced in nitrogen and reduced in oxygen atmosphere. In order to obtain a similar inhibition with NEM, considerably higher concentrations were needed.

The active form of factor X (X_a) is of decisive importance in the activation of prothrombin to become thrombin. The process of prothrombin activation requires the contribution of active factor V (V_a) and the presence of a phospholipid and calcium. The four contributors form a complex which can be separated from the other clotting factors by centrifugation [7, 12]. According to the suggestion of Straub and Duckert [13] and Müller [7], the complex is called Intrinsic Prothrombin Activator (IA).

The present study deals with the activity of purified IA under exposure to oxidizing and reducing agents used in enzymology.

Material and Methods

1. *Collection of blood*

Blood of healthy subjects and of a patient suffering from haemophilia A (1% of factor VIII) was drawn from a vein with siliconized needles and syringes. 3.3% sodium citrate and 1.34% sodium oxalate 1 : 9 were added as anticoagulants. When no anticoagulant was used, the blood was drawn into common glass tubes, while the blood containing anticoagulants was put into siliconized glass tubes. Plasma was separated from the blood containing anticoagulants by centrifuging at 2000 *g* for 20 min. To obtain serum, blood was incubated at 37 °C for 2 hrs, the clot was cleared off the wall of the test tube and centrifuged at 2000 *g* for 20 min. The serum was removed and incubated at 24° C in tightly stoppered tubes for 24 hrs.

2. *Preparation of barium sulphate adsorbed plasma*

0.10 g of barium sulphate was added to 1 ml of oxalate plasma. The mixture was incubated at 37 °C for 30 min, and shaken every 3 or 4 minutes, then centrifuged at 2000 *g* for 20 min, and the supernatant was removed.

3. *Preparation of bentonite adsorbed plasma according to Hougie [5]*

170 mg of bentonite (Prolabo, Paris) was added to 10 ml of citrate plasma, stirred with a glass rod at room temperature for 10 min and centrifuged at 2000 *g* for 20 min. For the experiments we used the supernatant which contained about 50% of the original prothrombin and 1% of the original factor X concentration.

4. *Barbitone buffer according to Owren [8] pH 7.35, ionic strength 0.154*

20.62 g of sodium diethylbarbiturate was dissolved in 1000 ml distilled water. 570 ml of this stock solution was mixed to 430 ml of 0.1 *N* HCl solution and 5.67 g NaCl was added. Before use the buffer was diluted by an equal amount of physiological saline.

5. *Preparation of IA according to Müller [7]*

A mixture of 2 ml of diluted barium sulphate adsorbed plasma (0.4 ml of adsorbed plasma + 1.6 ml 0.9% saline), 2 ml of diluted serum (0.4 serum + + 1.6 ml 0.9% saline), 2 ml of diluted serum (0.4 ml serum + 1.6 ml 0.9% saline), 2 ml of inosithin suspension (0.0014 g of Inosithin, Associated Concentrates Inc., New York, N. Y., suspended in 2 ml buffer), and 2 ml of 0.025 *M* $CaCl_2$ solution was incubated at 37 °C. After clotting the clot was removed by a sterile stick and discarded. The remaining fluid was centrifuged at 25 000 *g* (Martin Christ Univ. Junior model KS-3) at +4 °C for 30 min. The supernatant was quantitatively discarded and the pellet was resuspended in 8 ml of distilled water and centrifuged again at 25 000 *g*, at +4 °C for 30 min. After repeated washing in distilled water and centrifuging, the pellet was suspended in 2 ml of buffer. In the final suspension no thrombin activity was detected.

6. *Assay of activity of the IA solution*

For IA assay, plasma samples were used which displayed a combined defect of factors VIII and X, thus the rate of prothrombin activation, i.e. of thrombin formation, depended entirely on the amount of IA added. The samples were prepared by mixing equal amounts of plasma obtained from healthy subjects (bentonite adsorbed plasma), and of the barium sulphate adsorbed plasma of the haemophilia A patient. The obtained mixture ("detector plasma") did not coagulate in the presence of calcium for several hours, since the levels of factors VIII

and X were very low. When 0.05 ml of the buffer component of the system, 0.1 ml detector plasma + 0.1 ml buffer + 0.1 ml $CaCl_2$ solution, was substituted by different dilutions of IA, clotting time showed an inverse relationship with the amount of IA added.

7. *Gas manipulation chamber (Labor-MIM, Budapest)*

In the chamber pressure was maintained at 1 atm, but partial pressures could be varied according to the respective amounts of nitrogen and oxygen. Temperature in the chamber was stabilized at 37 °C. Incubation took place in watch-glasses ensuring a sufficiently large surface area. Materials and reagents had to pass an air-lock system to arrive in the chamber. Transfer of reagents and sampling was performed by manipulators. The experiments were performed in pure nitrogen, pure oxygen, or in atmospheric air.

8. *Coagulating effect of IA exposed to oxidizing-reducing agents in the presence of different gases*

0.5 ml of IA was incubated with an equal part of different dilutions of L-glutathione (Fluka), N-ethylmaleimide (NEM) (Sigmachem) or cysteine (Reanal). The redox agents were dissolved in distilled water and monitored by an electrometric pH meter (Radelkis model, Budapest). The solutions were adjusted to pH 7.3 by 0.1 *N* NaOH or 0.1 *N* HCl. 0.1 *M* stock solutions were used to prepare the required dilutions of the respective agents.

At predetermined points of time, 0.1 ml samples were taken of the incubation mixture. 0.1 ml detector plasma and 0.1 ml $CaCl_2$ solution were added to the samples at the same point of time (cf. 6). Clotting proceeded in air in a water bath of 37 °C. Each point of the diagrams represents the mean of two parallel determinations.

Results

1. *Exposure of IA to cysteine, reduced glutathione and NEM in the presence of atmospheric air.* Clotting time was found to increase with the length of incubation with cysteine or reduced glutathione. When reducing agents had not been added to the IA suspension, the increase of clotting time was considerably less. Decrease in IA activity is a function of the concentration of cysteine or glutathione present in the incubation mixture; with growing amounts of the reducing agent the increase of clotting time, i.e. the decrease in IA activity, occurs sooner. The cysteine experiment is shown in Fig. 1. The results were essentially similar with reduced glutathione when the respective molar concentrations were taken into account.

The gradual increase of clotting times is attributable to the effect of cysteine on IA. In this control experiment, initial clotting time was slightly higher when

the amount of cysteine or glutathione was increased, but the curves ran in all instances parallel with that of spontaneous inactivation. Thus, until clotting has become complete, cysteine may effect the IA (and, presumably, also other factors)

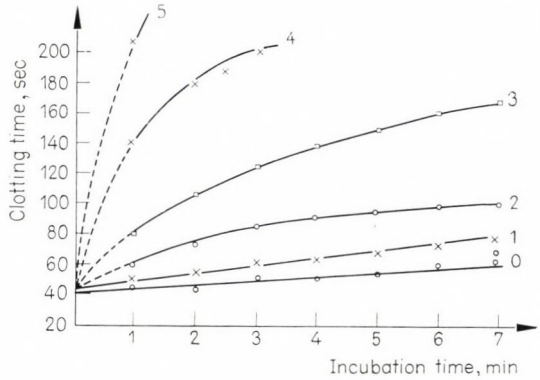

Fig. 1. Effect of cysteine concentration on the IA. Experimental system: 0.5 ml IA stock suspension + 0.5 ml cysteine solution. (Curve 0, buffer: 1, 0.02 *M*; 2, 0.04 *M*; 3, 0.06 *M*; 4, 0.08 *M*; 5, 0.10 *M*). At predetermined times 0.1 ml sample was taken from the incubation mixture and mixed to 0.1 ml detector plasma and 0.1 ml 0.025 *M* $CaCl_2$. Abscissa, incubation time; ordinate, clotting time of detector plasma

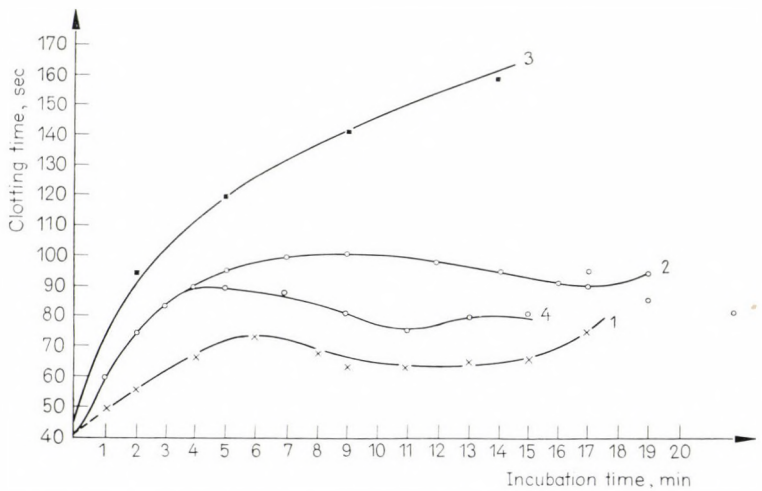

Fig. 2. Effect of cysteine on IA in air, oxygen and nitrogen. Experimental system: 1.0 ml IA stock suspension +1.0 ml cysteine solution (0.04 *M*). Incubation in air (Curve 2), oxygen (Curve 1), and nitrogen (Curve 3). At predetermined times 0.1 ml sample was taken of the incubation mixture and mixed to 0.1 ml detector plasma and 0.1 ml of 0.025 *M* $CaCl_2$. 0.8 ml of the mixture incubated in air was placed into oxygen after 4 min incubation (Curve 4). Abscissa, incubation time; ordinate, clotting time

of the detector plasma; this effect was, however, hardly observable owing to the dilution and rapid clotting.

With NEM, no inhibition of IA could be observed at similar molar concentrations as employed in the cysteine and glutathione experiments. If, however, the concentration of NEM was increased to or above 0.1 *M* in the incubation mixture, clotting time was protracted and independent of incubation time.

2. *IA incubated with cysteine in the presence of nitrogen, air or oxygen.* The relationship between IA activity and incubation time with cysteine was investigated under exposure to gases other than air at a constant temperature. Results are shown in Fig. 2.

The increase of clotting time was considerably faster in nitrogen than in oxygen atmosphere. Clotting time in atmospheric air was intermediate between that found in nitrogen or oxygen. Part of the samples incubated in air was transferred into oxygen atmosphere in the fourth minute: in oxygen, clotting time became definitely shorter.

Discussion

It has been emphasized earlier that certain respiratory, circulatory and clotting events must be regarded as a functional whole; accordingly, it is only natural that the process of blood coagulation should be influenced by redox agents [9, 10].

Redox sensitivity of the coagulation factors is due to the sulfhydryl or disulphide groups contained by them. As shown by our previous experiments [10], further by Carter and Warner [3] and, more recently, also by Caldwell and Seegers [2], cysteine is capable of inactivating prothrombin. Aoki et al. [1] provided evidence that inhibitors affecting the sulfhydryl group inactivated factor V (accelerator globulin) and this has been confirmed by Koppel et al. [6]. It has also been shown [10] that the effect of factor V was not inhibited but rather enhanced by the presence of cysteine.

Since factor V is a contributor to the IA complex and is sensitive against sulfhydryl blocking agents, NEM was suspected to produce an incubation-time-dependent inhibition resembling that found with cysteine. However, when IA activity was studied in its relationship with NEM incubation, clotting was found to be delayed only in the presence of high NEM concentrations. This might be due to the fact that addition of IA to the detector plasma diminishes the activity of factor V in the latter. In our experimental system NEM appeared to have no important inhibitory effect below a concentration of 0.1 *M*. The factor V content of the detector plasma seems to compensate for the inactivation of the factor V component of the complex incubated with NEM.

The results thus suggest that IA might contain some cysteine sensitive structure. The sensitivity of factor X to cysteine was verified by Fantl [4]. According to Seegers [11], autoprothrombin C is a derivative of the prothrombin molecule, and has identical or nearly identical properties with the active form of factor X.

Since activated factor X is a component of the IA complex, the observed sensitivity of IA to cysteine might be due to this relationship.

Whereas in nitrogen atmosphere no IA re-activation was observed during cysteine incubation, activity was found to increase again in other gases, slower in air and faster in oxygen. This allowed the conclusion that the rate of IA re-activation was a function of the partial pressure of oxygen in our experiments.

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Inactivation of Intrinsic Prothrombin Activator in Serum

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The inactivation of IA prothrombin activating complex (phospholipid, active factor V, active factor X, calcium) isolated by centrifugation and washing was studied in serum free from prothrombin. The rate of IA inactivation depends on the quantity of serum present, while in buffer solution IA activity decreases gradually but at a considerably slower rate than in the former case. The factor responsible for inactivation is contained in the alpha-1 globulin fraction; it cannot be detected in the euglobulin fraction. The effect of the inactivating factor present in the test system is particularly conspicuous at low IA activity. It is assumed that, in analogy to the thrombin threshold, there is a minimum amount of IA which can still bring about clotting of the systems containing the inactivating factor.

Examinations carried out in recent years seem to confirm that the enzyme necessary for the activation of prothrombin into the enzyme necessary for the activation of prothrombin into thrombin is the complex of factor V and the active form of factor X, produced with phospholipid and calcium, known also as Intrinsic Prothrombin Activator (IA) [7, 11]. From the other plasma proteins, the complex can be separated by centrifugation [7, 12, 13].

In the course of the examinations to be described it has been studied whether human serum is capable of inactivating the centrifuged and washed IA, and which of the serum protein fractions contains the inactivating factor.

Material and Method

1. *Blood collection*

Blood was drawn from the veins of healthy subjects and a patient suffering from haemophilia A (factor VIII 1%) with siliconised syringe and needle. A solution of 3.3% sodium citrate and 1.34% sodium oxalate at a ratio of 1 ml anticoagulant to 9 ml of blood, was used to prevent clotting. Blood without anticoagulant was collected in common glass tubes, the mixture of blood and anticoagulant in siliconised tubes. Plasma was obtained by centrifugation at 2000 *g* for 20 minutes. To obtain serum, the blood was incubated at 37 °C for 2 hours, the coag-

ulate removed from the glass tube and centrifuged at 2000 *g* for 20 minutes. Subsequently, the separated serum was incubated in tightly stoppered glass tubes at 24 °C for 24 hours.

2. *Barium sulphate adsorbed plasma and serum*

0.10 g barium sulphate was mixed to 1 ml oxalated plasma or 1 ml serum, then incubated at 37 °C for 30 minutes shaking the tube every 3—4 minutes. At the end of the incubation period the mixture was centrifuged at 2000 *g* for 20 minutes and the supernatant syphoned off.

3. *Bentonite adsorbed plasma according to Hougie [4]*

170 mg of bentonite (Bentonite, Pro-Labo, Paris) was added to 10 ml of citrated plasma and stirred with a glass rod at room temperature for 10 minutes, then centrifuged at 2000 *g* for 20 minutes. The supernatant was used for the experiments. The plasma so obtained contained about 50 % of the original amount of prothrombin and about 1 % of the original factor X content.

4. *Sodium veronal-veronal buffer according to Owren [8]*

20.62 g Na veronal is dissolved in 1000 ml distilled water. In a mixture consisting of 750 ml of this stock suspension and 430 ml 0.1 *n* HCl, 5.67 g of NaCl is dissolved and the obtained buffer is mixed with an equal amount of physiological saline before use. The pH value of the buffer is 7.35; its ionic strength, 0.154.

5. *Preparation of IA according to Müller [7]*

To 2 ml of barium sulphate diluted plasma (0.4 ml adsorbed plasma + + 1.6 ml of 0.9 % NaCl solution), 2 ml diluted serum (0.4 ml serum + 1.6 ml of 0.9 % NaCl solution), 2 ml 0.9 % NaCl solution, 2 ml Inosithin* suspension (0.0014 g of Inosithin suspended in 2 ml of buffer and 2 ml of M/40 $CaCl_2$ solution) were mixed and incubated at 37 °C. After clotting the coagulate was removed by a sterile wooden stick and the residual fluid was centrifuged (Martin Christ Univ. Junior, KS-3) at 25 000 *g*, at +4 °C, for 30 minutes. The supernatant was discarded and the sediment suspended in 8 ml of distilled water and the suspension centrifuged at 25 000 *g*, at +4 °C, for 30 minutes.

6. *Estimation of the IA suspension's activity*

To measure the activity of the IA suspension we used the mixed standard citrated plasma of healthy subjects (Warner Chilcott Diagnostic Normal Plasma)

* Inosithin, Associated Concentrates Inc., New York, N. Y.

to each 0.1 ml of which 0.1 ml IA suspension and simultaneously 0.1 ml of 0.025 *M* $CaCl_2$ solution was added. As to the activity of the suspension, conclusions were drawn from the clotting time. After centrifuging and washing, IA suspended in 2 ml buffer yielded a clotting time of about 15 sec in this system. An 0.1 ml amount of the IA suspension was considered to represent 10 units of IA activity if it caused to clot 0.1 ml of the standard normal plasma with 0.025 *M* of $CaCl_2$ solution at 37 °C, in 17 sec. In the experiments, the stock suspension was adjusted to this activity and was then kept at +4 °C.

7. *Preparation of the euglobulin suspension*

Adding 14 ml of distilled water to 1 ml of plasma (bentonite adsorbed plasma, serum, etc.) it was exposed to CO_2 at +4 °C for 10 min, and centrifuged at 2000 *g* for 20 min. The supernatant was discarded and the sediment dissolved in 1 ml of buffer, equal to the original amount of plasma. The euglobulin suspension so obtained contained more than 80 % of the original amount of plasma prothrombin and fibrinogen.

8. *Starch block electrophoresis [5]*

Common household starch was added to sodium veronal-veronal buffer until a thick paste-like mass was obtained. This mass was filled into a 32 cm by 20 cm, 1 cm high plexiglass dish. After 30 min the paste was covered with 10—15 layers of filter paper which was pressed down by a plexiglass plate closely fitting into the dish and weighted with 5 kg. Except the sheet in direct contact with the gel, the filter sheets were changed twice until the paste hardened. Prior to filling the paste into the plexiglass dish, three Whatman No. 1 filter paper sheets were placed in its two ends for connection to the buffer vats. Using a suitable template, an 18 by 1 cm hole was cut in the centre of the starch block into which we poured the viscous mass of human serum and starch powder. Electrophoresis at 80 mA and 110 V was continued for four hours. Then a wet strip of Whatman No. 1 filter paper was placed upon the paste and left there for half an hour. Then it was removed, dried as usual and stained. The stained paper strip showed clearly the location of the different protein fractions (albumin, alpha-1, alpha-2, beta and gamma globulin) in the gel. These were cut out, put into large centrifuge tubes and, adding one part of physiological saline to about four parts of paste, mixed and centrifuged at 3000 *g* for 30 minutes. The supernatant was analysed by immune-electrophoresis. This yielded beside pure albumin and gamma globulin, multicomponent alpha-1, alpha-2 and beta fractions.

The protein content of the individual protein fraction was established by the biuret reaction [1] and during the estimation of inactivation, on its basis the degree of dilution was taken into consideration.

Results

1. *Preparation of detector plasma*

The normal citrated plasma used for determination of the activity of the IA stock suspension is not suitable for measurement of the activity of dilute suspensions. Due to the effect of $CaCl_2$, citrated plasma would coagulate about 120 sec even without IA. In order to determine smaller IA activities, a test system had to be evolved in which clotting time depended solely on the added quantity of IA. Such a sensitive test system ("detector plasma") was obtained by mixing

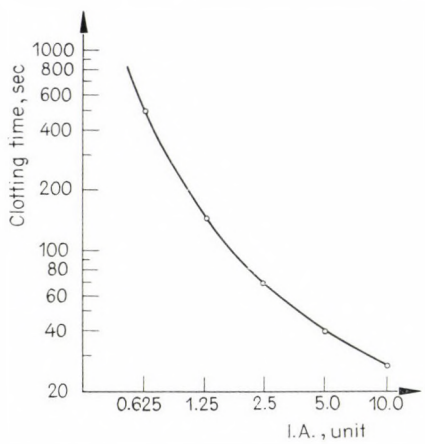

Fig. 1. Effect of IA upon the clotting time of detector plasma. Reaction mixture: 0.1 ml detector plasma + 0.1 ml of various dilutions of the IA stock suspension + 0.1 ml of 0.025 *M* $CaCl_2$. 0.1 ml of non-diluted stock suspension contained 10 I.A. units as determined with normal plasma (cf. Methods). Abscissa: different dilutions of the stock suspension expressed in terms of IA units. Ordinate: clotting time in seconds

the bentonite adsorbed plasma of healthy subjects with an equal amount of barium sulfate adsorbed plasma of "A" haemophilic patients. This plasma mixture, due to its very low factor X and VIII content, when mixed with calcium did not coagulate for several hours. The low factor X content of the detector plasma so produced ensured a high degree of specificity to IA activity. Although, in principle, clotting might occur in the system due to thrombin contamination, this could be eliminated by repeated washings in distilled water of the IA stock suspension, whereafter clotting became dependent solely on the effect of IA, i.e. the generation of thrombin from the prothrombin present in the detector plasma. Since clotting of the detector plasma depends, within wide limits, on the amount of IA admixed to it, it can be used to advantage for the plotting of a calibration curve. A dilution series was prepared from the stock suspension with buffer (adjusted to an activity

of 10 units) and 0.1 ml of diluted IA suspension was added to 0.1 ml detector plasma simultaneously with 0.1 ml of 0.025 *M* $CaCl_2$ solution (see Fig. 1).

As appears from Fig. 1 the stock suspension of 10 units/0.1 ml activity showed a longer clotting time with normal citrated plasma due to the fact that the quantity of prothrombin diminished in the course of the process. Since the quantity of prothrombin could not be standardised during the preparation of the detector plasma, normal standard plasma was used for estimation of the stock suspension's activity.

In Fig. 1, each point corresponds to the mean of two parallel measurements, with a deviation of less than 10%.

2. *Inactivation of IA in serum, barium sulphate-adsorbed serum, and buffer*

1.2 ml serum or 1.2 ml barium sulphate adsorbed serum or 1.2 ml buffer was added to 0.4 ml of IA stock suspension and incubated at 37 °C. Samples were taken at intervals, and the IA activity measured in the detector plasma (0.1 ml of the sample +0.1 ml of the detector plasma +0.1 ml of 0.025 *M* $CaCl_2$). Using the calibration curve plotted at the beginning of the test, the IA activity of the incubated mixture was calculated from the clotting times.

It was found that in the presence of serum, IA activity decreased sooner than on incubation with buffer only. The examination of inactivation in serum was, however, hampered by the thrombin generated in the incubated system.

Thus, to eliminate the disturbance of thrombin generation, barium sulphate adsorbed serum was used in the subsequent experiments.

3. *Examination of IA inactivation in the presence of different amounts of adsorbed serum*

The aim was to establish whether the rate of inactivation was proportional to the amount of barium sulphate adsorbed serum present in the incubated sys-

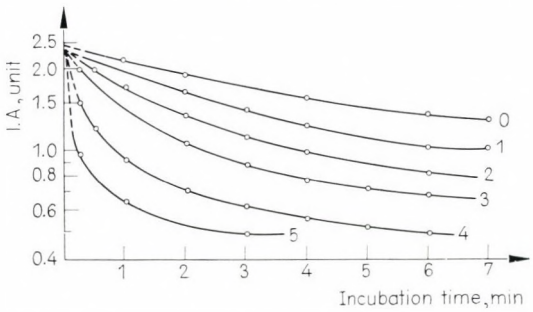

Fig. 2. IA inactivation in the presence of different amounts of serum. Incubation mixture: to 0.4 ml stock suspension, serum and buffer were added in a final volume of 1.6 ml. Curve 0: 0.00 ml serum; curve 1: 0.24 ml serum; curve 2: 0.48 ml serum; curve 3: 0.72 ml serum; curve 4: 0.96 ml serum and curve 5: 1.20 ml serum. Samples were withdrawn at intervals indicated on the abscissa. IA activity was determined with detector plasma (cf. legend to Fig. 1). The IA units given on the ordinate were calculated from the clotting times using the calibration curve presented in Fig. 1

tem. It was found that in the initial stage of incubation the rate of inactivation depended on the quantity of serum in the mixture (Fig. 2).

4. *Inactivation with serum protein fractions*

Barium sulphate adsorbed serum was fractionated by starch block electrophoresis. The fraction which had an inactivating capacity different from that of the control was the one corresponding to alpha-1 globulin. The IA inactivating capacity of the euglobulin fraction was equal to that of the control.

5. *Effect of IA on the coagulation of detector plasma and its euglobulin fraction*

From a mixture of bentonite adsorbed plasma and the barium sulphate adsorbed plasma of "A" haemophilic patients (detector plasma), an euglobulin fraction was prepared. Its comparison with the original mixture showed that the euglobulin contained the original amounts of prothrombin and fibrinogen in nearly the same measure but did no longer contain any IA inactivating factor.

Fig. 3 shows an experiment in which different dilutions of the IA stock suspension were made to act on the detector plasma and the euglobulin solution prepared from it. At low IA activity, the euglobulin system clotted considerably sooner than did the plasma system containing the inactivating factor.

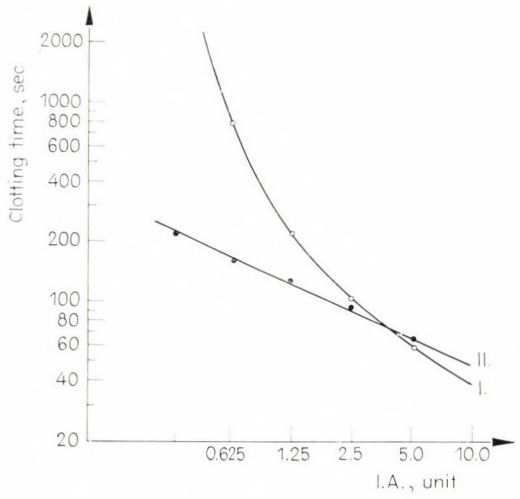

Fig. 3. Comparison of the clotting of detector plasma (I) and of euglobulin solution (II) prepared from detector plasma, in the presence of different quantities of IA. Test system: 0.1 ml detector plasma (or euglobulin suspension) + 0.1 ml IA suspension of different activity +0.1 ml of 0.025 *M* $CaCl_2$ solution. The IA content of the stock suspension was determined with normal plasma

Discussion

Although several investigators have dealt with the specific inactivation in serum of the "blood thromboplastin" obtained in the thromboplastic generating system [2, 3, 14], this inactivation cannot be considered equivalent with the inactivation of the phospholipid, active factor V, active factor X, and the calcium complex (IA) which converts prothrombin into thrombin, since the thromboplastin generating system contains also some other factors which enhance coagulation (intermediary product I, active factor IX, thrombin, etc.). Other researchers studied the inactivation in serum of the active form of the factor X isolated with gel-filtration, carrying out the partial isolation of the inactivating factor as well [15]. The active form of factor X, however, is not identical with the IA isolated by centrifuging, since the active factor X is only one of the fractions of the IA complex.

IA can be isolated by centrifugation from those other clotting factors present in the thromboplastin generating system and capable of activating prothrombin into thrombin even without any other clotting factor present. However, the IA separated by centrifugation contains in addition some components in small quantities — thrombin activity in the first place. After two washings in distilled water and re-centrifuging, no thrombin could be detected; the washing procedure caused a 30—40% drop in IA activity.

We have examined the inactivation of IA in serum and for this purpose barium sulphate adsorbed serum was used. It was found that the rate of inactivation depended on the amount of serum added. IA was inactivated even in the absence of serum. According to Müller, the drop of activity in the control suspension can be attributed to a gradual dissociation of the complex [7]. However, in serum some factors must be present which cause the IA activity to disappear at a rate faster than on spontaneous inactivation. This factor was found in the alpha-1 globulin fraction — the fraction which caused the IA to inactivate faster than the buffer.

It is difficult to determine the order of the reaction in the inactivation of IA on the basis of our experiments. One of the difficulties is due to the fact that in buffer IA loses its activity at a fast rate even spontaneously. Another problem is that the process of inactivating continues after the IA and serum have been added to the detector plasma. Due to the dilution this latter process is of a lesser degree than the former. This is the reason why the IA values are slightly below the real ones. This effect might contribute to the apparent increase in the rate of inactivation if the process is studied in the presence of higher serum concentrations. Even so, our data indicate that the inactivation of IA in serum proceeds according to a higher than first order of reaction.

Previously we have dealt in considerable detail with the reaction kinetics of thrombin inactivation [9, 10] and computed the reaction kinetics of the thrombin-fibrinogen interaction in the case in which antithrombin, too, was present in the system. The experiments and calculations indicated that with given quantities of fibrinogen and antithrombin, a thrombin threshold exists; while this is

still causing clotting smaller amounts of thrombin are no longer capable to induce clotting.

In the present studies a phenomenon similar to the thrombin threshold has been observed. Gradually decreasing the quantity of IA in an euglobulin test system in which no IA inactivating factor is present, we have observed the classic Legler law [6], according to which in a double logarithmic system the values for clotting time fall on a straight line. On the other hand, when a detector plasma containing the inactivating factor was used, the deviations from the values obtained with euglobulin increased steadily at low IA activities. We therefore assume that in the presence of the IA inactivating factor, the reduction of the quantity of IA will allow to reach the minimum amount of IA still sufficient for causing clotting. If lesser quantity of IA were present in the experimental system of a given volume and a given fibrinogen, prothrombin and antithrombin content, the inactivating factor would render it completely inactive before the threshold quantity of thrombin needed for the induction of clotting would have arisen.

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Haematologia 4 (3—4), pp. 399—403 (1970)

Plasma Fibrinolytic System and Blood Pressure in Patients with Hypertensive Cardiovascular Disease Treated with Reserpine* and Phenformin**

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The fibrinolytic system has been studied in patients with hypertensive cardiovascular disease treated with reserpine and phenformin. Increase of plasma euglobulin activity and decrease of blood pressure were found, with a slight decrease of the fibrinogen level and plasminogen and antiplasmin activity. A considerable increase of the urinary urokinase level and of plasminogen activator in blood was observed. The findings may suggest a role of the fibrinolytic system in the pathogenesis of hypertension and thus drugs enhancing fibrinolysis might be useful in the treatment of this disease.

Since the experiments of Goldblatt et al. [7] it is generally accepted that the kidneys play a decisive role in the pathogenesis of hypertension. Several authors [1, 13, 20] regard the disturbance of renal function as the main cause of hypertension. On the other hand, the kidney is known to produce plasminogen activator [4, 14, 15]. Data in the literature [e.g. 1, 3] indicate some interrelationship between the fibrinolytic and the arterial blood pressure regulating system.

Therefore, an interrelationship is assumed to exist between blood pressure, renal function and plasma fibrinolytic activity in patients with hypertensive cardiovascular disease. Previously we observed a distinct inhibition of plasma fibrinolytic activity in clinical and experimental hypertension [16, 17] and, on the other hand, phenformin is known to enhance plasma fibrinolytic activity [6]. The purpose of the present study was to investigate the effect of reserpine and phenformin on the plasma fibrinolytic system and on blood pressure in patients with hypertensive cardiovascular disease.

Twenty-one patients (10 females and 11 males, aged from 25 to 74 years) were involved in the study. The diagnosis was established on the basis of routine examinations. In 10 patients renal hypertension and in 11 patients hypertension with atherosclerosis were diagnosed; all of them were in stage II or II/III. The control group consisted of 25 normal subjects (15 females and 10 males, aged from 25 to 75 years). Blood samples obtained from the antecubital vein were mixed with sodium citrate 1 : 10. Citrate plasma was obtained by centrifugation at 3000 r.p.m. for 10 min. All determinations were performed using fresh plasma or plasma stored at —20 °C for a few days.

* Raupasil® — "Polfa", Poland
** Phenformin® — Winthrop, England

The parameters determined were: plasma euglobulin fibrinolysis [9], fibrinogen level [18], plasminogen [11], antiplasmin [12], and activity of plasminogen activator in blood [8]. Plasminogen activator activity is expressed as an index $I = \frac{1000}{t_{min}}$ (t = time of lysis). Urokinase in urine was determined according to Boomgard [3].

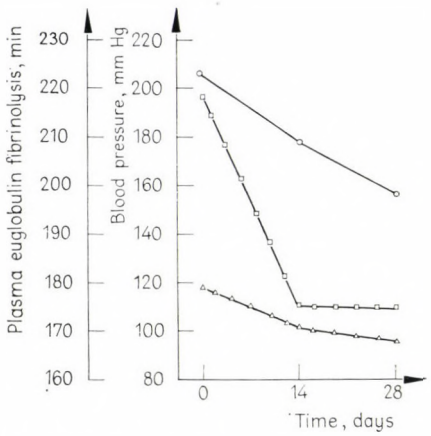

Fig. 1. Blood pressure and plasma euglobulin fibrinolysis time in patients with hypertensive cardiovascular disease treated with reserpine and phenformin (mean values). ⊙——⊙ systolic blood pressure; △—△ diastolic blood pressure; □—□ plasma euglobulin fibrinolysis time

The patients were hospitalized for 4 weeks and treated with reserpine 0.25 mg t.i.d. (12 persons) or phenformin 50 mg b.i.d. (9 persons). Treatment was started after 2—3 days observation without any hypotensive drug.

The results from all patients are summarized because we did not find any significant differences in the tested parameters induced with both drugs and in both groups of patients. No significant changes were noted in the control group. Zero day indicates the data obtained before the onset of treatment.

Figure 1 presents the values for blood pressure and plasma euglobulin fibrinolysis. It can be seen that systolic and diastolic blood pressure decreased significantly in the course of treatment. Plasma euglobulin fibrinolysis reached the highest level in the second week and did not change subsequently. An increase in plasma fibrinolysis euglobulin activity was accompanied with a small decrease of the fibrinogen, plasminogen and antiplasmin levels in most patients (Table 1).

The UK level increased about threefold, reached its maximum in the second week and then decreased slightly. Simultaneously, there was a significant increase in the activity of plasminogen activator in blood (Fig. 2), and a considerable clinical improvement in all the patients.

Table 1

Fibrinogen, plasminogen and antiplasmin level in patients with hypertensive cardiovascular disease treated with reserpine and phenformin (mean values). 100% represents initial value before treatment. Numbers in brackets denote variation range

Test	Time, days				
	0	7	14	21	28
Fibrinogen, mg/100 ml	353 (216–552)	306 (216–408)	293 (192–492)	307 (210–432)	298 (228–408)
Plasminogen, %	100	85 (43–134)	85 (51–118)	74 (60–114)	63 (50–80)
Antiplasmin, %	100	90 (50–130)	77 (50–112)	86 (45–125)	78 (30–120)

It is commonly accepted that there is a relationship between certain features of hypertension and the severity of renal failure [1, 10, 13, 20]. The hypotensive drugs do not affect renal filtration. On the other hand, reserpine has been shown to enhance plasma fibrinolytic activity in the rat [2] and during long-term therapy with big doses of reserpine the patients often display haemorrhages. Fearnley et al. [6] showed that phenformin has a potent activating action on the fibrinolytic system and may safely be applied for long-term therapy. Previously we have shown that plasma fibrinolytic activity was decreased in hypertensive patients [16, 17]. The present results may indicate that reserpine enhances the activity of the plasma fibrinolytic system and the same was observed in patients treated with phenformin. In addition, with the activation of the plasma fibrinolytic system a decrease of blood pressure occurred.

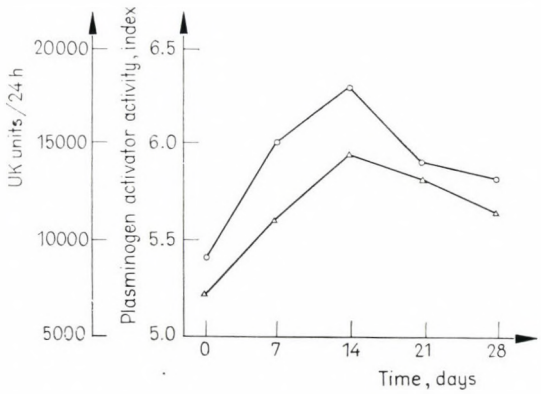

Fig. 2. Plasminogen activator activity in blood and urokinase level in patients with hypertensive cardiovascular disease treated with reserpine and phenformin (mean values). ○——○ plasminogen activator activity index; △——△ urokinase level in Ploug units/24 hrs

The activity of plasminogen activator in blood increased but slightly in comparison to the UK level. On the other hand, Boomgard [3] found low UK levels in patients with hypertension. We have observed that during the improvement of the patient's condition, the decrease in blood pressure was correlated with an increase in the UK level. This fact may indicate an improvement of renal blood flow despite the decrease of blood pressure. This is supported by the increase in diuresis and by functional tests which yielded normal results in our patients in the course of therapy (unpublished results). The second mechanism responsible for the effect of the applied drugs is a decrease of plasma lipids and beta-lipoproteins which are known to inhibit fibrinolysis [19, 21] and to enhance platelet aggregation [5].

Thus, the present results may support the assumption that the fibrinolytic system has a role in the genesis of hypertension and that drugs which enhance fibrinolysis may be useful in the treatment of hypertension.

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Platelet Metabolism and Function

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The literature on normal platelet energy metabolism and the effect of stimuli is reviewed. Particular emphasis has been given to outlining the problems associated with the study of platelet enzymes and platelet metabolism in isolated cells.

Introduction

When platelets interact with surfaces such as collagen in injured vessels, they adhere, release ADP and other cellular components and aggregate under the influence of ADP [1, 2]. There is now good evidence that these reactions and the response of platelets to other biological stimuli, e.g. thrombin and antigen-antibody complexes, are of fundamental importance in thrombosis and haemostasis [3] and perhaps also in the pathogenesis of the organ rejection phenomenon [4]. Platelet aggregation requires energy [5, 6] and for this reason there has been considerable interest in the metabolic basis of platelet reactivity. In this report it is proposed to briefly review the literature on platelet energy metabolism.

Activities of platelet enzymes

There has been increasing interest in the study of platelet enzymes in recent years, especially those concerned with energy metabolism. Such studies have been performed in an attempt to provide a biochemical basis for platelet functional disorders, and more recently to seek a possible relationship between platelet aging and changes in platelet energy metabolism.

Limitations of enzyme assays. Although most of the enzymes of the glycolytic pathway, tricarboxylic acid cycle and pentose phosphate pathway have been measured [7—11] the significance of the results obtained is limited because 1. the preparation of cell extracts for assay may modify enzyme activity and 2. the optimal conditions used in the assay procedures are unlikely to exist *in vivo*.

The effect of extraction procedures on enzyme activity has been investigated by comparing the effects of different methods of cellular disruption on activity of 11 glycolytic enzymes, on glucose-6-phosphate dehydrogenase (G-6-PD) and on

6-phosphogluconate dehydrogenase [8]. The following procedures were used; freezing and thawing, ultrasonic disintegration, treatment with digitonin and treatment with Triton X-100. Freezing and thawing markedly inactivated phosphofructokinase and reduced hexokinase and G-6-PD activity by approximately 10 %. In addition, rapid inactivation of G-6-PD activity occurred in cell lysates prepared by the four different procedures so that approximately half of the original activity was lost within 80 minutes. This could be prevented by the addition of the cofactor NADP to the lysing medium.

Assay of hexokinase activity was found to present a special problem since this enzyme unlike the other glycolytic enzymes in partly bound to intracellular structures [12], probably mitochondria [13]. It is noteworthy in this regard that most previous reports of platelet hexokinase activity were based on assays performed on a particle-free supernatant fraction of cell lysates and therefore represent soluble activity only [7, 9, 10, 11, 14].

Activities of the glycolytic enzymes as measured *in vitro* give little indication of the control points in this pathway *in vivo*. Thus aldolase is the glycolytic enzyme with lowest activity [8] but calculations based on glucose and glycogen utilization in intact platelets* indicated that this enzyme functions at less than 15 % of its activity in cell lysates; a finding which suggests that the absolute levels of any of the glycolytic enzymes are unlikely to be rate-limiting even under conditions of high glycolytic rate.

Enzyme levels and platelet function. Platelet enzyme abnormalities have been reported in some patients who were considered to have had thrombasthenia. A deficiency of both glyceraldehyde phosphate dehydrogenase and pyruvate kinase was found in a group of 5 patients and it was suggested that the defect in platelet function resulted from impairment of energy production consequent on the enzyme defect [16]. A similar defect in platelet function has been found in association with a deficiency of an enzyme not directly involved in ATP production. Thus two siblings with defective clot retraction and long bleeding time were found to have a severe deficiency of glutathione reductase [14]. A defect in platelet energy metabolism has also been found in patients with the Wiskott-Aldrich syndrome, in which there is reduced platelet survival associated with defective aggregation to ADP [17]. In a study of the platelets of three brothers with this condition there was a 38 to 55 % deficiency of hexokinase activity and decreased capacity to utilize glucose [17].

Abnormal levels of platelet enzymes have been reported in association with a number of other diseases in which platelet function is apparently normal. A decrease of 70 % to 80 % in platelet G-6-PD activity was found in Caucasian

* Our value for maximal glycolytic rate is 1.8 μmoles of glucose glycogen utilized per minute per $10^{\prime\prime}$ platelets [15] which would require an aldolase activity of 1.8 μmoles of substrate per minute per $10^{\prime\prime}$ platelets. This is less than 15 % of the value we obtain for aldolase activity assayed at 37° C *in vitro*.

patients with a complete deficiency in erythrocyte G-6-PD [18, 19, 20]. In muscle glucose-6-phosphatase (G-6-Pase) deficiency (Type 1 glycogenosis) there is a complete deficiency of this enzyme in platelets which are the only blood cells which normally contain G-6-Pase [21]. Since measurements on platelets are more convenient than performing a muscle biopsy, assay of the platelet enzyme could provide a simple diagnostic method for Type 1 glycogenosis [22]. Similarly the assay of enzymes in blood cells may provide a simple means of detecting enzyme abnormalities in other metabolic diseases.

A recent study of carbohydrate enzymes in platelets suggests that as in the red cell a decline in the activity of regulator enzymes may contribute to cell senescence. Thus phosphorylase, glycogen synthetase, fructose-1-6-diphosphatase, hexokinase, phosphoglucomutase and phosphohexose isomerase were significantly reduced in small light (old) platelets as compared to large heavy (young) platelets [23]. The large heavy platelets also showed a 4—6-fold greater rate of glycogenolysis, glycolysis, and glycogen synthesis [24].

Platelet energy metabolism

Problems associated with metabolic studies in isolated platelets. Ideally studies on platelet energy metabolism should be carried out in plasma. However, such studies are complicated by relatively low platelet counts and by variability in the concentration of glucose, fatty acids and metabolites in the medium. There is also the need for anticoagulants such as citrate or ethylene diamine tetra-acetic acid (EDTA) both of which have been found to influence glycolysis and oxidative metabolism [25, 26, 27]. Because of these difficulties most studies on platelet metabolism have been performed on washed suspensions but this has the disadvantage that the platelets are inevitably altered by the isolation procedure.

There has been wide variation in the published values of indices of metabolism. Thus values for oxygen uptake have varied from 37 to 379 μmoles/hour/10$''$ platelets [28, 29], lactate production from 15 to 320 μmoles/hour/10$''$ platelets [25, 30] and ATP levels from 2.0 to 52.5* μmoles/10$''$ platelets [31, 32].

It is likely that different techniques of isolation as well as differences in incubation conditions are the major causes of this wide variation. The degree of aeration of the incubation medium is particularly important, since glycolysis under anaerobic conditions is more than double the aerobic rate [15]. Many investigators have failed to consider this problem and in most reported studies the degree of aerobiosis is either uncontrolled or inadequately described. The exposure of platelets to foreign surfaces during isolation and incubation may also contribute to the variation in results reported above. Platelets have been found to interact with glass, siliconised glass and plastic surfaces and release ADP [33]; a reaction

* Results, originally express as μmoles/g wet weight, were converted to μmoles/10$''$ platelets assuming 1 g wet weight = 4.5×10^{10} platelets.

(which is associated with an increase in energy metabolism. It is of interest in this regard that albumin is effective in both reducing platelet surface interactions and in decreasing glycolysis when added to platelet suspensions in glass vessels [15, 33]. The finding that resuspension of platelets in artificial media (not containing plasma proteins) results in stimulation of glycogen breakdown [34] and glycolysis [35] is also consistent with the possibility that certain plasma proteins protect platelets from surface stimulation [33].

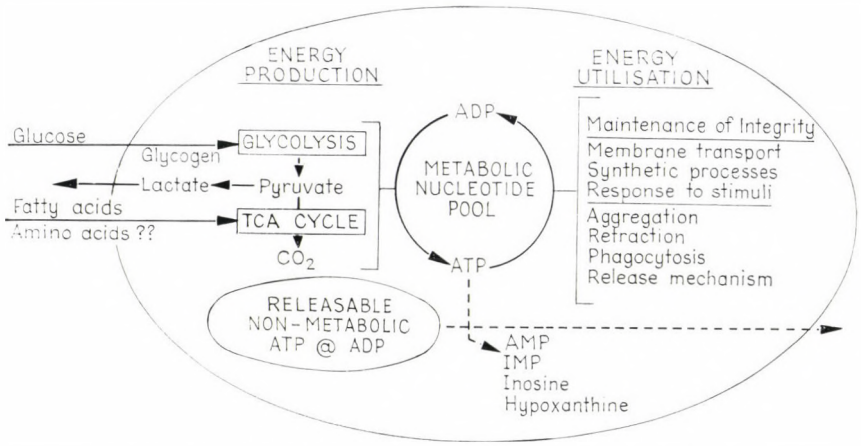

Fig. 1. Scheme relating platelet energy production to utilization

In view of these factors, it would seem likely that most of the published values for rates of platelet energy production in washed suspension differ from those which occur under physiological conditions.

In Fig. 1 is shown a scheme relating platelet energy production to utilization.

Platelet ATP and ADP. Platelets contain a high level of ATP and ADP which is distributed between at least two pools [36–38].

Approximately 60 % of total ATP and ADP is present in granules as a releasable pool which does not participate in intermediary metabolism. The remaining 40 % of ATP and ADP is in a state of continuous turnover and has been referred to as the 'metabolic pool' [38]. Approximately a quarter of this metabolic pool of nucleotide, presumably consisting of ATP only is utilized during the release reaction and irreversibly converted to inosine monophosphate, inosine and hypoxanthine. It is suggested that both energy rich phosphoryl groups of ATP are utilized during release by a combination of an ATPase (possibly thrombosthenin) adenylate kinase and 5′-AMP deaminase [38]. Following release there is a 50 % drop in the ATP/ADP ratio, a more than 2-fold increase in AMP in the metabolic nucleotide pool, and a rise in intracellular orthophosphate levels [39, 40]. These changes would be expected to lead to an increase in activity of the three regulator

enzymes of glycolysis. Phosphorylase because it requires AMP for activation [41] and orthophosphate as one of its substrates; hexokinase because inhibition of this enzyme by glucose-6-phosphate would be relieved by a rise in the orthophosphate level [42]; and phosphofructokinase because inhibition of this enzyme by the high levels of ATP normally present in the cell would be reduced by the fall in concentration of this nucleotide. Furthermore reversal of this inhibitory effect of ATP is enhanced by orthophosphate and AMP [43].

Energy utilization and production. Platelet energy is required for the active uptake of ions, substrates [44, 45] and serotonin [46, 47] and endergonic processes such as synthesis of lipids [48—55] and proteins [56, 57, 58]. Platelet ATP is also utilized during the thrombin-induced release reaction [38, 59—61], ADP-induced aggregation [5, 6, 61] clot retraction [5, 62, 63, 64] and phagocytosis [65, 66]. Energy may also be utilized following release and aggregation for repair mechanisms such as the resynthesis of phospholipids [67].

Platelets metabolise glycogen and extracellular glucose by aerobic glycolysis to form lactate and CO_2 [7, 15, 26, 63, 68]. Although only a small proportion of glucose is oxidized by the TCA cycle [15] the role of this pathway is an important one because the energy yield from the complete oxidation of 1 mole of glucose is 19 times as great as that from anaerobic glycolysis. In addition to glucose other substrates such as palmitic acid are oxidized by the TCA cycle [15, 69, 70]. The relative contribution of oxidative metabolism and glycolysis to platelet energy production under physiological conditions is uncertain. However, studies in washed suspensions suggest that both pathways contribute approximately equal amounts [15, 26] and that either pathway is able to support release and aggregation [5].

The production of ATP by oxidative metabolism depends upon the existence of coupled mitochondria. Although it has been suggested that oxidative phosphorylation in washed platelets resuspended in the absence of plasma, may be uncoupled [71] so that mitochondria do not produce ATP, most evidence is to the contrary. Thus several investigators have observed an increase in glycolysis under anaerobic conditions — a Pasteur effect [15, 35, 72], and an increase in oxygen uptake in the absence of glucose — a Crabtree effect [73]. In addition, glucose oxidation is increased approximately 4-fold by the uncoupling agent 2,4-dinitrophenol [15].

Effect of stimuli on energy metabolism. The exposure of platelets to stimuli such as collagen [27], thrombin [26, 30, 63, 68, 74], ADP [61], epinephrine [61], heterologous antibodies [75] and latex particles [65] is associated with increased glycolysis. A sustained increase in glucose oxidation via the citric acid cycle has also been reported following exposure of platelets to thrombin or latex particles [65, 68]. On the other hand a transient increase, or "burst" in oxygen uptake has been observed by some investigators [59, 76] but not by others [73, 77] following exposure of platelets to thrombin or latex particles. In contrast to the sustained increase referred to above this burst in oxygen uptake is cyanide and antimycin insensitive, suggesting that it is not the result of a stimulation of mitochondrial-linked respiration [59]. An increase in oxygen uptake which is cyanide insensitive

is also found in leukocytes during phagocytosis [78, 79], and it has been suggested that this may be due to activation of NADPH oxidases in the early stages of phagocytosis [80].

Conclusion

Considerable progress has now been made in our understanding of the relationship between platelet energy metabolism and platelet function. However, several major problems remain. The first is that platelets because of their very nature are highly sensitive to environmental factors (e.g., temperature, composition of the medium, nature of the container surface) and it is therefore difficult to prepare platelets in washed suspensions which have not undergone structural or biochemical changes and which still retain normal reactivity to biological stimuli. The second major problem is to elucidate the biochemical reactions which form the basis of the response of platelets to stimuli. The solution of these problems should facilitate the development of therapeutic measures for the manipulation of platelet reactivity.

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Tissue Mast Cells in Human Blood

Preliminary Report

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In the course of earlier experiments, tissue mast cells were detected in the blood of rats [4]. Following cortisone treatment the number of these cells increased [4], while after thymectomy they disappeared from the blood [5]. It has been assumed that — the primary site of mast cell production being the thymus [2] — the different forms of the developing mast cells are transported in the blood stream towards the periphery. Thus the question arose of the presence of tissue mast cells in human blood and of their behaviour in diseases, especially in those involving the thymus.

A total of 125 blood smears of 39 patients suffering from autoimmune diseases, degenerative processes of the locomotor system, as well as tumours, and those of 10 healthy subjects were fixed in Carnoy's fluid and stained with iron-alaun alcian-blue safranine [3, 6], the dye used for the detection of mast cells in rat blood. The advantage of the method is that it reveals the developing forms of the mast cells. For comparison May-Grünwald-Giemsa staining was applied. Mast cells were counted in 1500 microscopic fields of each smear under 600-fold magnification.

Mast cells were present in the blood of each examined subject, whether healthy or ill. 98 % of the cells were of lymphoid character (Fig. 1a, b, c, e), similar in size to the erythrocytes. Their nucleus was round and they had a thin plasma border containing alcian-blue-positive granules (Fig. 1a, b, c). This cell type corresponds to the young form of developing mast cells in the rats [1, 2]. In a few cases safranine positive mast cells were found (Fig. 1d). The amount of the mast cells was about 1% of all white blood cells in the healthy subjects, whereas in several pathological cases their proportion amounted to 2 to 4 % of the total white blood cell count. The mast cells were easy to distinguish from the basophil granulocytes by their size and the shape of their nucleus. Their incidence was remarkably higher than that of the basophils, sometimes there were two of them in one microscopic field (Fig. 1c).

In different diseases the blood mast cell count varied. The results have shown an increase in the number of blood mast cells in autoimmune diseases involving the thymus, as compared to the controls or other subjects suffering from degenerative diseases. In contrast, in subjects with tumour the mast cell count

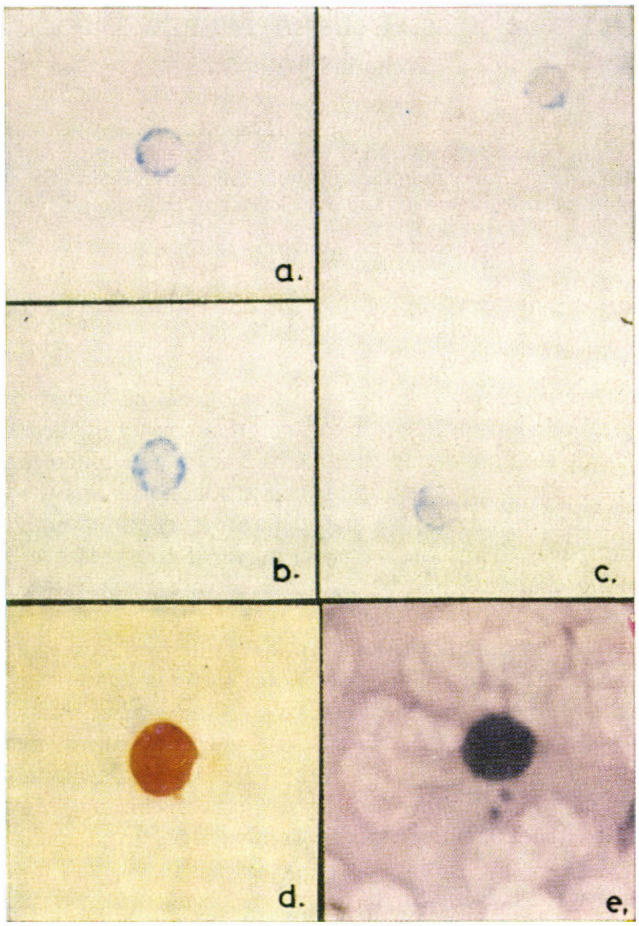

Fig. 1a, b, c. Lymphoid mast cells in human blood with a round nucleus and a thin plasma border containing alcian-blue positive granules. c) Two mast cells are visible in one microscopic field. Alcian blue-safranine, ×1000, oil immersion; d) Mature, safranine positive mast cell in human blood, a rare finding. Alcian blue-safranine, ×1000, oil immersion; e) Lymphoid mast cells are similar in size to the erythrocytes. The dense granulation covers the nucleus of the lymphoid mast cell, when May-Grünwald-Giemsa's dye is applied, ×1000, oil immersion

displayed a decreasing trend. Mediastinal X-ray irradiation, by affecting the thymus, was found to influence the blood mast cell count as well. The eventual diagnostic importance of the blood mast cell count is indicated by the aforementioned findings. However, in order to evaluate correctly the shifts, further examinations of a large patient material are needed. Such investigations are in progress.

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Haematologia 4 (3—4), pp. 419—425 (1970)

Open Forum

Editorial note. There is suggestive evidence that spontaneous bleeding episodes of hemophiliacs may be precipitated by emotional factors. It is also claimed that the success achieved by some "preventive" measures may depend on psychic influence especially if the patients treated require a new outlook on life. In spite of this hematologists have given little emphasis to the psychological aspects of hemophilia. With this in mind, although it does not fully meet the requirements set forth by Haematologia, this paper has been accepted for publication and is being presented for open discussion. Critical contributions are invited to discuss the theoretical and practical value of the general use of the advocated or related psychiatric measures in hemophiliac care.

Regular Use of Suggestibility by Pediatric Bleedings*

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The usefulness of suggestive therapy with bleeders has been long recognized. This pilot study documents the validity of encouraging the use of their suggestibility by these children more routinely than is documented, rather than reserving it only for urgent problems. The employment of trance by patients by themselves to achieve diminished morbidity was the end desired and realized. While the primary clotting defect is not modified by the mobilization of his suggestibility by a bleeder, other additives to it are minimized with resulting lower total morbidity.

Introduction

There are several reports on the usefulness of encouraging children who have a bleeding diathesis to reach within themselves for remedy through the utilization of their inherent suggestibility. These reports are nearly all concerned with the advantage which the hemophiliac may garner for himself by his use of suggestion in urgent situations. Since dental surgery is such an emergency in those with a bleeding tendency, there has been thoughtful consideration of the problems attending this frequently encountered crisis [1, 2]. That this interest is warranted is evident from the fact that such patients have succumbed to tooth extraction [3, 4]. In contrast, the successful employment by a hemophiliac of suggestion in his recovery, after gastrectomy had resulted in otherwise unassailable hemorrhage, has recently been documented [5].

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Such experience with suggestive therapy of hemophilic children has a long tradition. The most celebrated case has been the management by Rasputin of Tsarevitch Alexis of Imperial Russia who was bequeathed his defective X chromosome from his great-grandmother, Queen Victoria of England, it was apparently in her father, Edward, where the mutation had originated [6, 7]. While naturally employed frequently in routine affairs, suggestion was seldom identified as an operative factor in human transactions by anyone. What was then inaccountable is today more comprehensible.

Most efforts to induce bleeders to enlist their suggestibility in countering untoward effects of their disorders have been expedient means of coping with emergency situations. Since these have been notably successful, it is conjectured that the routine use of their trance capability could be as useful. Urgent matters could then be managed by them within the framework of a comfortably established way of life embellished preliminarily by patients' acquisition of greater command of their suggestibility. The urgency of emergency could then be handled with more alacrity.

Coaching in the autonomous employment of trance to encourage its use by the patient harrassed by his ailment whenever and wherever the need arises, independent of his suggestive therapist, is a paramount feature of great value in contemporary suggestive therapy. This was anticipated in bleeders. Another useful feature sought was the return of a modicum of control of his malady directly to the patient, thereby erasing some of his hopelessness and ensuing helplessness.

This partial alleviation of anxiety and depression accompanying the usual feeling of impotence in bleeders was hoped to diminish the overt manifestations of the bleeding diathesis. For along with other precipitants of hemorrhage, emotional stress has been recognized [8, 9]. Bleeding bouts have been temporally related to hospital visiting hours [1], when children are excited about communicating with parents, to name one variable. A member of this medical center has documented the hypnotic control of nosebleed [10]. The emotionally stressed bleeder who presents a relatively benign hematological picture may conversely present a disastrous clinical image [11]. Oppositely, the calm patient with a severe problem projects a more radiant portrait than his hematological disability predicts sometimes.

While the importance of emotional factors in hereditary bleeding diseases was often dismissed until about fifteen years ago by investigators overwhelmed by their chemical elusiveness, their social, behavioral, and psychological aspects are now being documented [12–14]. This is fortunate as the clinical manifestations prescribed by seemingly immutable innate ordinance can actually be thwarted by external controls [15]. While the biochemistry translating emotional stress to increased hemorrhage is not yet conclusively known, it is being probed. Fibrinolytic activity and blood coagulability have been found to be influenced by anxiety [16]. Spontaneous fibrinolysis associated with the specific anxieties of surgery and trauma has been recorded [17].

Procedure

The opportunity for a pilot study on the utilization of their trance capabilities by pediatric bleeders presented, due to the allegiance of a number of families manifesting bleeding disorders, to the hematological program of a university medical center.* The clinical records of these children dating back over years permitted each patient to be his own control in evaluating his progress before and after his use of suggestive therapy as an adjunct to many established features of his care. Following weekly meetings over six months of an exploratory group of the parents of afflicted children led by a social worker concerned with common attitudes and singular points of view peculiar to our particular families, those parents were oriented by the child psychiatrist on the plan for him to institute weekly group meetings with the youngsters. The parents' and children's groups were then continued simultaneously for a brief transitional period before the adults disbanded. A degree of independence of the children was thus at once a feature of their endeavor. The outcome of this phase will be reported elsewhere [18].

The children's introductory group met weekly for three months to explore the shared and unique thoughts and feelings, hopes and fears, and basic attitudes of the victims of bleeding disorders. During this preliminary period, group and individual rapport was established between the patients and the therapist. This relationship is a cornerstone of successful suggestive therapy. A group identity was fostered thereby, as well. The group was finally composed of seven individuals ranging in age from latency to adolescence. An initial failure to interest teenagers was overcome in some instances by proposing that they align themselves with the therapist to assist him to understand and effectively deal with younger members not so experienced in the ups and downs of their mutual ailment as the older fellows. Required of the therapist was the tolerance of an unusual amount of absenteeism to group gatherings, due largely to the frequent morbidity of the youngsters. The phenomenon of the defensively overprotective mother of a hemophiliac sometimes fancying morbidity where none flourished deference was accorded, for some of those ladies are painfully guilty in the knowledge that they transmitted the disorder to their sons. Bleeders whose problems reached inpatient proportions were visited in the hospital.

Following our preliminary meetings, suggestive therapy was proposed as a means of relaxation and diminution of psychic tension which might reduce the patients' morbidity. We learned the acquisition of greater control of trance ability. As soon as familiarity had bred skill, the achievement of trance acquisition at home alone was coached and universally accomplished, the stage having been set for this in our personal confrontations.

Also, patients were frequently reached by telephone when they could not appear in person, a useful variation of approach in trance therapy [19]. Especially

* This effort of the University of Colorado Medical Center is expedited by John H. Githens, M.D., and William E. Hathaway, M.D., Professor and Asst. Professor of Pediatric Hematology.

in children, who are more than normally dependent when disabled, this is a fortunate method, even though the distraction and distress of many medical problems in adults, also, may temporarily steal their established ability with autonomous trance. One telephone trance induced by the therapist is then often a useful way to restore the patient's independence.

The coherence of our group was also possible to maintain in this way. It was possible for the absentee to participate with the assembled others over the open circuit, useful trance being capitalized upon by individuals thirty miles apart. This had added importance to the distant member, clearly the most needful at that moment. Small talk with others preceding the trance with the rhythmical reality of the suggestive session provided the absentee with a close continuing relationship with us at a time when it was most useful to him.

Another opportunity for useful exploitation of our inclusiveness came when a member was hospitalized. We others then decamped to his room and there held our mutual suggestive session.

Case report No. 1

A 7-year-old boy whose family were incorrigible deniers of his hemophilic problem was admitted when in a fistic encounter with an unimpaired sibling, he had acquired two black eyes. Hemorrhage into both anterior chambers as a result had quickly blinded him. The terror this induced in him atop his basic fear of his underlying problem was massive. His response was to become in the hospital a plaintively wailing shadow of his former self who was too hyperactive to conform to postures recommended by his ophthalmological consultants. His oral intake of food and drink was greatly diminished as a result of his depression with its rapidly advancing hopelessness. The calmative trance to which he readily subscribed was an effective instrument in assisting him to cope and to adopt behavior which helped him leave the hospital.

Case report No. 2

A 9-year-old boy who had assiduously mastered his lessons in our group was approaching with trepidation his appointment with his dentist. The dentist was familiar with the use of suggestion with hemophiliacs. The lad was unable to attend the meeting of our group just prior to his dental visit as he had developed bleeding into a knee joint. While this discomfited him in itself, he had placed unnecessary importance upon that just prior suggestive session, as well. His dilemma was solved when he joined us by telephone. He soon had a root canal procedure performed using his suggestion prompted by his dentist without any other analgesia. His pain and bleeding was minimal and controlled. He was

pleased with his accomplishment, but retained his spoken belief that his trance ability served him best in more routine matters. Settling himself in the face of stress and preparing himself for sleep were the ends to which he put his internal ally.

Case report No. 3

A 19-year-old lad whose hemophilia was discovered when he was five months of age has a similarly afflicted brother four years his senior. Our patient has suffered multiple severe disabilities from his disease over the years. Besides a usual array of hemarthroses, he has had gastric and splenic hemorrhages, and one

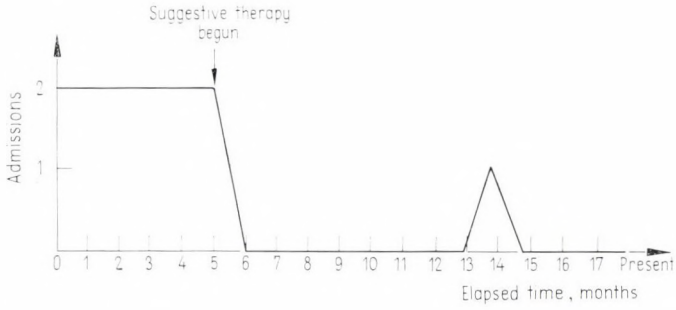

Fig. 1. Hospital admission rate over last eighteen months — Case No. 3

proven and another suspected cerebral vascular accident. Biting his tongue once precipitated a 17-day hospitalization. In the eastern state of his birth and early rearing, he survived over three dozen stints of inpatient care. Since being in Colorado, he has accumulated as many more. His rate of hospitalization had about doubled in the few months just prior to joining our suggestive group. The events left him with a halting gait, a mild speech impediment, and other motor deficits. A bright boy with a Full Scale IQ now diminished only to a still quite respectable 113, he was not thriving in school or elsewhere due primarily to his distress over his infirmity. He sorely needed to regain a sense of personal worth.

He felt more useful when recruited to help younger ones sharing his malady, but had another primary motivation in joining us. His many blood transfusions over the years have left him unable to accept more, so ominous have become his multiple sensitivities. Another source of succor was sorely needed.

He learned to mobilize his idle trance capability, becoming an ardent disciple of that method of relaxing. His academic performance began to mirror his intellect, greatly improving as he lowered his level of stress by using his suggestibility.

Many weeks passed without his need for the hospital. A coagulation time near normal was eventually obtained, the lowest in his record.

When he faced taking a college entrance examination which provoked him greatly due to some of its sections being a race against time and to his desire for a sterling performance, he felt great tension. As he walked away from his school after that test, he felt pain in his right knee which by nightfall forced him to the hospital. He was admitted for the first time in eight months and fourteen days, by far his longest remission in nineteen years of struggle. This remission represented roughly an 800% improvement over the five just previous ones (see Fig. 1). During a week in the hospital, other forms of treatment than transfusion, including suggestive therapy, were successful.

Up to the time of this report, he has not required another inpatient stint, having been in the hospital one time in the past thirteen months. Though bleeding tendencies are known to improve spontaneously, this patient's use of his suggestibility is thought to deserve part of the credit for his startling remission. He accomplished it by minimizing the effect of the "psychophysiological mechanism superimposed upon the physical defect" which is recognized in bleeders [20].

Comment

This study indicated that their suggestibility may be used by bleeders effectively on a routine basis, this not being only a crisis tool. In view of the fact that some primary expectations were realized in this pilot endeavor, a program wider in scope over a greater time including more children with bleeding tendencies is being promulgated. Collaborating specialists will attempt to start elucidation of the chemical correlaries of the phenomenon of decreased morbidity accompanying diminished psychic tension. Children not included among those ordinarily managed in the university medical center are being encouraged to participate.

It is felt that the report of these preliminary results can alert others to the possible institution of similar programs. Details of satisfactory suggestive techniques with children have been delineated elsewhere [21–23].

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Book Reviews

Gerinnungsstörungen in der Geburtshilfe. Ed. by Walther Kuhn, Henner Graeff, Uwe Bleyl. Georg Thieme, Stuttgart 1970. 156 pages with 53 figures and 4 tables. Price DM 39.

The development of the obstetrical defibrination syndromes, their pathophysiology, correct diagnosis and adequate treatment have drawn the attention of many experts during the last years. As a result of intensive research work in this field a great deal of information has been accumulated, in the possession of which this disease developing with dramatic suddenness and leading often to death may be recognized rapidly and treated adequately.

These results, however, are not yet generally known and it is mainly haematologists, pathologists and coagulation experts who are familiar with them. Thus, the endeavour of the authors to give a systematic summing up of current knowledge on disseminated intravascular coagulation is to be welcomed. The book will be of use even to general practitioners.

As an introduction, the process of blood clotting and fibrinolysis, the way of action of anticoagulants (heparin, coumarin) and fibrinolysis inhibitors (EACA, AMCHA, PAMBA) are described and the changes of the blood coagulation and fibrinolysis system during pregnancy are outlined. In the chapter dealing with the pathophysiology of disseminated intravascular coagulation and intravascular fibrinolysis, the dissolution of the fibrin clot, the changes in the formed elements, consumption of the coagulation factors and the appearance of fibrin degradation products are discussed.

The book interprets in detail the changes in circulation and metabolism, and the lesions of the kidneys, lungs, heart, skin, hypophysis, liver, pancreas, etc. brought about by disseminated intravascular coagulation (DIC). A separate chapter deals with the foetal and newborn cases of DIC.

From the obstetric point of view, the chapter discussing the obstetrical cases where DIC may develop (placenta praevia, amniotic fluid embolism, septic abortion, chorion amnionitis, intrauterine death of the foetus) is of paramount importance. Aetiology, pathogenesis, general symptoms, incidence, characteristics of the coagulopathy, the problems of clinical and laboratory diagnosis, as well as the therapeutical possibilities and the eventual prophylaxis of each disease are systematically described.

For haematologists, the chapter discussing the care of mothers with haemorrhagic diathesis (thrombocytopenia, von Willebrand's disease, coagulopathies) in the course of pregnancy, the preparation for delivery, the tasks at labour and the principles of puerperal care are especially instructive.

The book closes with a short methodical part containing the basic laboratory methods aiming at the diagnosis of DIC.

The excellent construction of the monograph, its illustrations, the clinical experience and experimental work of the authors ensure that the readers obtain up-to-date information on the obstetrical defibrination syndrome in a clearly written concise book.

Susan Elődi

Die Milz. Struktur, Funktion, Pathologie, Klinik, Therapie. Ed. by Karl Lenner, Dieter Harms. Springer Verlag, Berlin—Heidelberg—New York 1970. 465 pages with 176 figures. Price DM 128.

The enhanced interest for the spleen is shown by the facts that three years ago a spleen symposium was organized in Erfurt and last year the 14th German Haematological Congress in Kiel had been focused on the problems connected with the spleen. The book under review represents the proceedings of this congress, containing 47 papers of 107 authors, including the discussions and references. Excellent figures, tables and microphotograms contribute to the value of the papers. The high standard and the actuality of the book are ensured by its authors being excellent experts and well-known research workers. Professor Lennert took care of the important prerequisites of the success of any publication of this kind, the proportional construction, which is exemplary in this case.

Part I deals with the methods of spleen diagnostics, part II describes the morphological and functional basis of lienal diseases, the red and white pulp, as well as the immunological role of the spleen. Part III discusses the relations of the different blood cell systems to lienal function and diseases, part IV describes hypersplenism, part V splenectomy and its consequences, part VI the tumour metastases in the spleen while part VII is the record of a round table conference, the topic of which was splenopathic bone marrow inhibition and Banti's disease. The papers have not revealed any new surprising results; they rather reflected the progress and the current views developed on the basis of recent data and observations.

Only certain parts of the given topics are discussed in the book. The reader when turning its pages may miss the discussion of questions included in the title, e.g. the role of the spleen in some acute and first of all chronic infectious diseases, in thesaurismoses, in the disturbance of porphyrin metabolism, in proliferative processes, in the diseases of the reticular system, in collagenoses, in preblastomatosis, etc. The mosaic-like structure involves the lack of a general view concerning the spleen's relation to the whole organism, although the function of an organ represents a characteristic unit and is not only the sum of part-functions. This shortcoming, however, is a natural consequence of any publication that cannot aim at totality.

Reading this book gave special pleasure to the reviewer whose monograph, *The Spleen in Internal Medicine* is just being published in German.

The book under review is a collection of fine papers. It will be a most efficient aid for experts and for those who are closely interested in these problems.

I. Barta

L. H. Criep, *Clinical Immunology and Allergy*. Grune and Stratton, New York and London 1969. 2nd ed. 962 pages with 197 figures.

The first edition of this work dates back to 1962. This new edition represents a new book rather than a mere enlargement and partial revision of the first one. The difference between the two editions is a good indicator of the recent advances in immunology. The book's construction does not follow the usual pattern of immunological manuals; the chosen form will facilitate the survey of the described information.

The book is divided into three main parts.

Part I (Fundamentals of Immunology) deals with the essentials of modern immunology. The author does not strive for completeness, still the reader will find all knowledge indispensable for the understanding of the clinical part. Beside those discussing the antigens and immunoglobulins, chapters interpreting the immune response and thymic function are presented. Although the immunochemical aspect is not neglected, clinical relations are predominating in the text.

The second and largest part (pp. 116–836) describes the hypersensitivity reactions. It is divided into four main chapters. The hypersensitivity reactions connected with circulating antibodies are discussed in the first one, the hypersensitivity reactions connected with the cellular immune response in the second.

Following the description of the basic phenomena of hypersensitivity reactions connected with early humoral immune response, several chapters deal with the physiological, pathological, immunological and clinical relations of atopy. Great clinical experience and versatile knowledge

are characteristic of the chapters discussing the clinical aspects of allergy. Among others, pollen allergy, gastrointestinal, cardiovascular, articular, neurological, etc. allergy and bronchial asthma are surveyed in detail. The dermatological, urological, ophthalmological and otological relations of allergy are dealt with as well.

Of the processes based on the late hyperimmune response, eczematic contact dermatitis of allergic origin, the homograft reaction and the states connected with bacterial, fungal and viral hypersensitivity reactions are discussed.

Autoimmune diseases are discussed in the third chapter and diffuse connective tissue diseases in the fourth.

The last part deals with veterinary allergy.

The book offers a comprehensive view of modern clinical immunology for those working in each special field. Based on current knowledge, it reveals the immunopathological relations of the diseases. The methodological and therapeutical parts are of great help in practical work. The rich documentation contributes to the value of the book and renders it even more useful.

J Gergely

Rubidomycin. Ed. by J. Bernard, R. Paul, M. Boiron, Cl. Jacquillat and R. Maral. Recent Results in Cancer Research, Vol. 20. Springer, Berlin—Heidelberg—New York 1969. 181 pages, 69 figures. Price: DM 48.

The year of the drug's birth is 1962. It was discovered by two independent research groups, a French team (Rubidomycin) and an Italian (Daunomycin) one. Soon it turned out that the two drugs are identical. The drug was produced and its anti-tumour effect demonstrated in the Soviet Union too, between 1964 and 1967. The first clinical study with Rubidomycin was done in Paris (1965—66), with Daunomycin in New York (1965). The 1967 Symposium in Paris summarized the experiences. The history of the drug consists of three phases: 1. Recognition of the antimitotic effect of some antibiotics; since actinomycin (1940), the drug in question is the most effective. 2. Isolation of *Rubidomycin* from streptomyces strains. 3. The phase of the first clinical investigations. Alone in Paris more than 800 patients were treated with Rubidomycin between 1965 and 1968. Daunomycin — which was not applied clinically by the Italians — was tested in the Sloane Kettering Institute.

The first part of the monograph deals with the preparation of the drug and with experimental investigations. The chemical structure is practically known. The drug is a glycoside belonging to the anthracyclin group; it forms a red crystalline powder. Its antitumour activity was proved on solid tumours (sarcoma, cancer), on Ehrlich ascites tumour, tumours of viral origin and on leukaemia. It was the first antibiotic effective against some transplantable mouse leukaemias. Prolonged administration induces testicular and myocardial lesions in experimental animals. The drug has a strong mitostatic effect *in vitro* even in small doses. It changes the chromosome structure, disturbs the metabolism of DNA and later even that of RNA. It has an immune depressive effect. Much information has been obtained in experiments using tritium as a label.

The second part of the book summarizes the clinical and therapeutical investigations. So far mainly leukaemias, in smaller number sarcomas and certain epitheliomas have been treated with the drug. Its dose is 0.5—2.0 mg/kg daily, the rate of dosage varies according to indication. The total dose must not surpass 25—30 mg/kg in six months. Treatment may cause 1. bone marrow aplasia which, however, is a prerequisite of the remission, but it is difficult to counterbalance sometimes even by a most active substitution therapy; 2. cardiac complications; 3. rarely some other (hepatic, renal) toxic effects.

The effect of the drug is rapid. There is no cross-resistance. There are great individual differences in sensitivity. It is not easy to establish the correct dose. The bone marrow effect, which is advantageous from the point of view of medullar leukoblastosis, is disadvantageous concerning haemopoiesis. In both untreated and previously treated lymphoblastic leukaemia, the drug causes a complete remission in 60% of the cases. During treatment, frequent cytological control is recommended. Of myeloblastic leukaemia patients 35—50% can be brought into total remission. Acute

promyelocytic leukaemia is also sensitive to the drug. Applied in combination with vincristine and prednisolone, Rubidomycin brought about a total remission in 97% of the infantile and in 74% of the adult lymphoblastoses, generally between the first and third week. It is effective even in the tumorous and hyperleucocytic forms. In acute leukaemia of the myeloblastic type the drug is given in combination with ARA-C, 6-MP, prednisolone and MGGH; for the time being, all that can be said is that this medication is tolerated by the patients. Rubidomycin may be prescribed even in granulocytic leukaemia in combination with other drugs, if a rapid effect is needed in the busulfan-resistant myelocytic or in the myeloblastic phase. The drug is ineffective in chronic lymphoid leukaemia. In lymphosarcoma it may be given as a trial; the way of administration is like in lymphoblastic leukaemia. It is not indicated in lymphogranulomatosis, reticulosarcoma and carcinoma.

The excellently edited, easily readable monograph offers almost complete information on an effective drug with a perspective. The book contains a list of 274 references, including those published in 1968.

K. Rák

Recent Results in Cancer Research. Vol. 21: Scientific Basis of Cancer Chemotherapy. Ed. by G. Mathé. Springer-Verlag, Berlin—Heidelberg—New York 1969. 60 figures. IX + 96 pages. Cloth. Price: DM 28.—

The book contains the eight lectures of a symposium held in Paris, March 1968. The authors are well-known authorities. The screening tests for anti-tumour agents are dealt with by *Connors*, the extracellular factors influencing the tumour-chemotherapeutical drugs by *Double*, the intracellular factors by *Ball*, the immune reactions connected with chemotherapy by *Amiel*, the dosage and way of administration of chemotherapeutical drugs by *Kenis*, the methods of controlling clinical effects by *Schwartz*, the biological basis of hormonal cancer therapy by *Tagnon* and the strategy of the chemotherapy of malignant tumours by *Mathé*.

The only criticism that may befall the book is practically valid for all similar publications: when it becomes available, the presented information is not sufficiently fresh for the experts acquainted with the problems.

E. Kelemen

Abstracts

Aktivität der Glutathion-Peroxidase in Erythrocyten vom Fetal- bis zum Erwachsenenalter (Erythrocyte glutathione-peroxidase activity from foetal to adult age). M. Vetrella, W. Barthelmai, J. Rietkötter (Universitäts-Kinderklinik, Marburg a. d. Lahn) *Klin. Wschr. 48*, 85 (1970).

Glutathione peroxidase activity in erythrocytes reaches its normal level at the 25th week of pregnancy, to remain constant during the whole life. Studies in cell populations with increased and decreased amounts of reticulocytes showed that activity of the enzyme does not decrease even in old erythrocytes. Other enzymes such as glutathione reductase, G-6-P-dehydrogenase and 6-phospho-gluconate dehydrogenase which are closely involved in glutathione metabolism, and the whole quantity of reduced glutathione are present in the same amount in the erythrocytes of the foetus and of the full-term newborn. The increased fragility and the formation of Heinz-bodies in the erythrocytes of newborns might be due to the instability of thiol-groups in the erythrocyte membrane.

I. Árky

The glucose-6-phosphate dehydrogenase activity (G-6-PD) of the red blood cells in hyperthyroidism and hypothyroidism. M. Viherkoski, B. A. Lamberg (Third Department of Medicine, University of Helsinki, Helsinki) *Scand. J. clin. Lab. Invest. 25*, 137 (1970).

The mean value for the G-6-PD of the red blood cells was significantly increased in patients with mild and with moderate to severe hyperthyroidism. During treatment the values gradually decreased but reached the normal level only after the patients had been euthyroid for 1 to 2 months. The mean value for G-6-PD in hypothyroidism differed significantly from that in the control groups, but the individual values were within the normal range. In pregnancy, G-6-PD was normal. G-6-PD is of diagnostic value in hyperthyroidism after iodine contamination and during pregnancy.

G. Gárdos

The acid phosphatase group system of erythrocytes in the diagnosis of zygosity of twins. B. Wyslouchowa (Department of Anthropology, Polish Academy of Sciences, Wrocław) *Acta med. pol. 11*, 73 (1970).

Demonstration of concordant acid phosphatase groups of erythrocytes in twins increases the probability of their being monozygotic. A method for calculating the probability on the basis of the frequency of phosphatase genes in the Polish population is described.

G. Gárdos

Studies on ATPase in sheared micro vesicles of human erythrocyte membranes. S. L. Schrier, E. Giberman, D. Danon, E. Katchalski (Stanford University School of Medicine, Stanford, Calif., and Weizmann Institute of Science, Rehovoth, Israel) *Biochim. biophys. Acta (Amst.) 196*, 263 (1970).

Human erythrocyte membranes were disrupted by shearing. The resulting membrane vesicles could be separated by sucrose density centrifugation into 2—3 distinct classes which differed in their content of $(Na^+ - K^+)$-activated ATPase and Mg^{2+}-activated ATPase. Relatively high specific activities of $(Na^+ - K^+)$-ac-

tivated ATPase were found in the class of vesicles that retained most of their trilamellar structure as visualized by electron microscopy. Furthermore, the $(Na^+ - K^+)$-activated ATPase of the separated vesicles was not completely inhibited by concentrations of oligomycin that completely inhibited $(Na^+ - K^+)$-activated ATPase of unseparated membrane particles. It is proposed that membranes sheared at relatively low pressures are cleaved at structurally weak loci. The resulting vesicles have different proportions of $(Na^+ - K^+)$-activated ATPase and Mg^{2+}-activated ATPase and have probably lost a binding site for oligomycin.

Ilma Szász

Sodium-activated adenosine triphosphatase activity of the erythrocyte membrane. R. Blostein (Department of Experimental Medicine, McGill University, Montreal, Quebec) *J. biol. Chem. 245*, 270 (1970).

Evidence for a phosphorylated intermediate of Na^+-activated ATPase of the erythrocyte membrane has been presented previously in studies of a Na^+-stimulated component of membrane phosphorylation at low ATP concentrations. In this investigation, the effects of ouabain and oligomycin on Na^+-activated membrane phosphorylation and (^{14}C)ADP-ATP exchange have been studied in relation to effects on Na^+-activated ATP hydrolysis. In agreement with findings with other particulate preparations, ouabain interaction with membranes resulted in inhibition of *(a)* Na^+-activated ATP hydrolysis; *(b)* Na^+-stimulated increase and K^+-stimulated decrease in the steady state level of phosphorylated intermediate; and *(c)* Na^+-activated (^{14}C)ADP-ATP exchange. Ouabain inhibition of hydrolysis was diminished by addition of potassium ions. The ratio of Na^+-stimulated (^{14}C)ADP-ATP exchange activity to ATPase activity was lower at 37°C than at 0°C. The exchange reaction was stimulated by oligomycin, but the degree of stimulation relative to the degree of inhibition of ATP hydrolysis was also low at 37°C as compared to that at 0°C. Oligomycin inhibited Na^+-activated ATP hydrolysis without affecting the level of ^{32}P bound in the presence of Na ions alone. K^+-stimulated dephosphorylation was decreased by oligomycin. With increasing concentrations of ATP, the catalytic centre activity of Na^+, K^+-activated ATPase increased to 1980 per min determined at 0.05 m*M* ATP and to approximately 6000 per min estimated at 2 m*M* ATP.

G. Gárdos

Synthesis of adenosine triphosphate at the expense of downhill cation movements in intact human red cells. I. M. Glynn, V. L. Lew (Physiological Laboratory, University of Cambridge, Cambridge) *J. Physiol. (Lond.) 207*, 393 (1970).

When red cells starved for about 6 hrs are loaded with inorganic phosphate and incubated in sodium-rich potassium-free media, the ouabain-sensitive efflux of potassium from the cells is accompanied by a ouabain-sensitive incorporation of inorganic phosphate into ATP. The degree of incorporation varies roughly linearly with the concentration of sodium in the medium. The ratio of ouabain-sensitive potassium efflux/ouabain-sensitive ATP synthesis is probably not much less than 2 nor much greater than 3. Potassium in the medium inhibits the ouabain-sensitive incorporation of phosphate. The concentration of potassium necessary for half-maximum inhibition is about the same as the concentration at which, under similar conditions, ouabain-sensitive potassium influx and the stimulation of ouabain-sensitive potassium efflux are both half-maximal. These observations suggest that the ouabain-sensitive efflux of potassium from red cells incubated in sodium-rich potassium-free media is associated with a reversal of the entire pump cycle. In media containing sufficient potassium to saturate the pump, the efflux appears to involve the reversal of only part of the cycle.

G. Gárdos

Structure of the erythrocyte. J. A. Sirs (Department of Physics, St. Mary's Hospital Medical School, University of London, London) *J. theor. Biol. 27*, 107 (1970).

To account for the discoidal shape of the erythrocyte a structure is proposed,

based on a uniform membrane consisting of rigid units, probably disks, bonded together by flexible and elastic bonds, at both the inner and the outer surface. The rigid units are in direct contact and cover the whole surface area. The discoid shape is then formed by the outer elastic bonds being stronger than those on the inner surface, over the concave regions, and the reverse arrangement around the convex periphery. A simplified model has been built using this system which is mechanically and osmotically stable in the discoid form. Disk-to-sphere transformations can be made and the model returns to the discoid form without external assistance. No change of thickness or stiffness of the membrane is required and no internal structure or force is involved.

Ilma Szász

The protein components of the erythrocyte membrane and their molecular weights. H. Demus, E. Mehl (Max-Planck Institute of Psychiatry, Division of Neurochemistry, Munich) *Biochim. biophys. Acta (Amst.) 203*, 291 (1970).

Membrane proteins from pig erythrocytes were solubilized by a phenol–formic acid–water solvent. Such treatment yielded monomers which were subjected to electrophoresis across a polyacrylamide gel gradient. The heterogeneity of the protein components was demonstrated by the appearance of 14—16 zones. The major components comprising about 30 and 13% of the total protein had molecular weights of 48.000 and 27.000, respectively. With the exception of one zone, the other components, which each contributed about 5% or less to the total protein mass, ranged in molecular weight from 26.000 to 65.000. One zone accounting for about 8% of the protein may have had a molecular weight as high as 180.000, but it could not be proved to be a monomeric protein. The variation of results reported by other authors is explained by difficulties in completely dissolving the membrane proteins to monomers rather than by species differences.

Ilma Szász

Studies on the lipids of sheep red blood cells. IV. The identification of a new phospholipid, N-acyl phosphatidyl serine. G. J. Nelson (Bio-Medical Division, Lawrence Radiation Laboratory, University of California, Livermore, Calif.) *Biochem. biophys. Res. Comm. 38*, 261 (1970).

A new phospholipid has been isolated from the erythrocytes of sheep. It has been tentatively identified as N-acyl phosphatidyl serine. It amounts to between 2.0 and 4.0% of the total phospholipids in sheep erythrocytes. The same phospholipid has been detected in the red cells of other ruminants such as the cow and the goat.

G. Gárdos

Cross-linking of erythrocyte membranes with dimethyl adipimidate. W. G. Niehaus, Jr., F. Wold (Department of Biochemistry, Pennsylvania State University, University Park, Pa.) *Biochim. biophys. Acta (Amst.) 196*, 170 (1970).

Dimethyl adipimidate reacts with lysine residues of proteins to form covalent cross-links. When human erythrocyte membranes are treated with dimethyl adipimidate the percentage of protein which can subsequently be solubilized by treatment with aqueous pyridine is reduced. Evidence is presented that this is due to the formation of cross-links between soluble and insoluble protein molecules. The solubility distribution of protein-bound sialic acid, hexose, and hexosamine is altered in a parallel manner. The effect is not produced by treatment of the membranes with methyl butyroimidate, a monofunctional analogue.

I. Árky

Erythrocyte membrane lipid composition in exogenous and endogenous hypertriglyceridemia. J. D. Bagdade, P. O. Ways (Department of Medicine, University of Washington School of Medicine, Seattle, Wash.) *J. Lab. clin. Med. 75*, 53 (1970).

Erythrocyte membrane lipid composition and ^{51}Cr survival were examined in patients with exogenous (dietary) and endogenous (hepatic) forms of hypertriglyceridemia. Despite increased concentrations in plasma, erythrocyte cholesterol and phos-

pholipid levels were decreased in both types of lipoprotein abnormality. Autologous and homologous erythrocyte survival was normal in both groups of patients. The results indicate that increased plasma levels of triglyceride-rich lipoprotein of both endogenous and exogenous origin are associated with alterations in red cell membrane lipid composition. These changes, however, do not appear to predispose the erythrocyte to premature destruction.

Ilma Szász

Protoporphyrin-induced photohemolysis in protoporphyria and in normal red blood cells. A. A. Schothorst, J. Van Steveninck, L. N. Went, D. Suurmond (Laboratory for Medical Chemistry, Leiden) *Clin. chim. Acta 28*, 41 (1970).

Irradiation of erythrocytes of patients with erythropoietic protoporphyria with light of about 410 nm wavelength in the presence of oxygen was found to induce haemolysis. In a nitrogen atmosphere, photohaemolysis was inhibited completely, indicating that an oxidative step is involved in the process. Several redox reagents inhibited the photohaemolytic response. Similar photohaemolysis could be observed with normal red cells, after adding protoporphyrine-IX-dimethyl ester to the medium. The haemolytic process itself could be characterized as a colloid osmotic haemolysis.

I. Árky

The identical effect of electrolyte and non-electrolyte on the osmotic fragility of mammalian erythrocytes. M. F. Coldman, M. Gent, W. Good (Department of Pharmacy and Mathematics, University of Bradford, Bradford) *Comp. Biochem. Physiol. 33*, 157 (1970).

Experimentally determined fragility of mammalian erythrocytes was compared in sodium chloride and mannitol. It was found that cell fragility depended almost entirely on the osmotic properties of the suspending medium. When allowance is made for the relative impermeability of erythrocytes to sodium ions, their free permeability to chloride and the unequal distribution of chloride between cells and suspending medium, the osmotic effects of sodium chloride and mannitol are practically identical.

I. Árky

Influence of hematocrit and colloid on whole blood viscosity during volume expansion. R. W. Schrier, K. M. McDonald, R. E. Wells, D. P. Lauler (Department of Medicine, Peter Bent Brigham Hospital and Harvard Medical School, Boston, Mass.) *Amer. J. Physiol. 218*, 346 (1970).

The effect on whole blood viscosity of changes in haematocrit and plasma colloid concentration during volume expansion was measured by Couette viscosimetry in anaesthetized dogs. Volume expansion was induced by the infusion of each of three red blood cell-free solutions, namely colloid-free Ringer-lactate, 6% dextran, and 25% dextran in isotonic saline. The decline in haematocrit with all three infusates was associated with a marked diminution in whole blood viscosity. An independent influence of colloidal dextran on whole blood viscosity was also readily apparent. During the infusion of colloid-free Ringer solution the range of viscosity measurements at low shear rates (0.2 and 2 sec^{-1}) was well below the range found during the 6 and 25% dextran infusions. Some 25% dextran infusions increased viscosity above control levels in the face of a diminished haematocrit. As with haematocrit, the effect of colloid on whole blood viscosity was most marked at low shear rates. Lastly, the degree of the natriuretic response to volume expansion by the infusates correlated most closely with the interrelated factors of whole blood viscosity, colloid content of the infusate, and interstitial fluid volume expansion.

I. Árky

The etiology, pathogenesis and prophylaxis of leukemias in humans and animals in the light of epidemiologic studies. J. Aleksandrowicz (3rd Clinic of Internal Diseases, Medical Academy, Cracow) *Acta med. pol. 11*, 1 (1970).

A theory of the pathogenesis of leukaemia is proposed; it is based on the assumption that genetic predisposition to harmful eco-

logic factors causes neoplastic proliferation in the haematopoietic system. Genetic predisposition is indicated by the results of anthropometric and dactyloscopic studies. Extracorporeal leukosogenic factors were demonstrated by previous epidemiologic investigations. Further investigation of the environmental leukosogens is planned, and enrichment of the soil with magnesium is postulated as a method of leukaemia prophylaxis.

G. Gárdos

Leukocyte energy metabolism. III. Anaerobic and aerobic ATP production and related enzymes. M. Jemelin, J. Frei (Laboratoire Central de Chimie Clinique de l'Hôpital Cantonal de Lausanne, Lausanne) *Enzym. biol. clin. 11*, 289 (1970).

Anaerobic and aerobic energy metabolism has been estimated in leukocytes from human and animal blood, using several methods. Special care was taken to correct the results for an unavoidable contamination with thrombocytes, which has been neglected in all previous work. White cells were isolated by a method combining sedimentation in dextran at low speed centrifugation. A pellet of thrombocytes was prepared in parallel from the same blood. Mitochondria were isolated by differential centrifugation in 0.25 *M* sucrose. First, two cytosolic glycolytic enzymes, phosphoglycerate kinase and pyruvate kinase were studied, both of which catalyze a reaction producing ATP. These two enzymes are very active in leukocytes. Other nucleotides such as CDP, GDP and UDP can also be phosphorylated. Second, the activity of several mitochondrial enzymes was measured; the most active was α-glycerophosphate oxidase. Using an enzymatic test, the amount of ATP produced in oxidative phosphorylation was estimated. In these experiments it was necessary to consider the activity of adenylate kinase as well as correcting for thrombocyte contamination. Several metabolites in direct or indirect relation with the Krebs cycle were used as oxidizable substrates: glutamate, succinate and α-glycerophosphate gave the best results. Except for amytal, inhibitors of oxidative phosphorylation also inhibit ATP production in leukocyte mitochondria. Thus, oxidative phosphorylation exists in leukocytes, but the phosphorylation steps in glycolysis produce 1000 to 10,000 times more ATP than does aerobic energy metabolism.

I. Árky

Untersuchungen über den Leukocytenstoffwechsel. V. NAD und ATP isolierter Leukocyten und Erythrocyten bei menschlichen Leukämien unter dem Einfluss alkylierender cytostatischer Verbindungen C 73, Busulfan und Cyclophosphamid (Investigations on leucocyte metabolism. V. NAD and ATP of isolated leucocytes and erythrocytes in human leukaemia under the influence of the alkylating cytostatic compounds C 73, Busulfan and Cyclophosphamide). H. Ehrhart, W. Hörmann, G. Scheffel, E. Armbröster (I. Medizinische Klinik der Universität München, München) *Klin. Wschr. 48*, 204 (1970).

Investigations into the cytostatic mechanism of action of alkylating agents were made to establish the behaviour of NAD and ATP in the leucocytes of chronic myeloid and chronic lymphatic leukaemia patients treated with C 73, Busulfan and Cyclophosphamide. C 73 treatment induced a marked decrease of the typically elevated leucocyte NAD content and of ATP in 48 hours. After Busulfan therapy NAD and ATP decreased but to a slighter extent and later. Under successful Cyclophosphamide treatment no change in leucocytic NAD and ATP content was noted.

Ilma Szász

Amoeboid movements and cytoplasmic fragmentation of glycerinated leukocytes induced by ATP. B. Norberg (Department of Pharmacology, University of Lund, Lund) *Exp. Cell Res. 59*, 11 (1970).

Lymphocytes and monocytes from peripheral blood may be mounted in a fibrin coagulum between slide and coverslip, then glycerinated and subjected to contraction studies. In the presence of ATP and calcium and magnesium ions, these cell models contracted and showed amoeboid movements or formation of free cytoplasmic fragments. The cell area could be reduced by 30—50%

during contraction associated with cytoplasmic fragmentation. In this way a large or medium-sized lymphocyte turned into a small one. The possible physiological significance of the contractile events induced in glycerinated mononucleates is discussed.

Ilma Szász

Proteolytic activity of human blood leukocytes towards synthetic trypsin substrate. I. Method. A. Szczeklik, T. Teresiak (IIIrd Clinic of Internal Diseases, Medical Academy, Wrocław, Poland) *Acta med. Pol. 10*, 383 (1969).

A new method has been developed for assaying the proteolytic activity of human blood leukocytes using Nz-carbobenzoxy-diglycyl-1-arginyl-β-naphthylamide. The method is based on the colorimetric determination of β-naphthylamine liberated from the substrate during its incubation with isolated and homogenized leukocytes. Human leukocytes were found to possess activity hydrolyzing the amide linkage in the substrate. Normal values yielded by the method for healthy persons have been determined.

S. Maj

Zachowanie się aktywności fosfatazy zasadowej granulocytów w wirusowym zapaleniu wątroby (Alkaline phosphatase of granulocytes in virus hepatitis). F. Kusch (Oddział B Chorób Wewnętrznych, Szpital Wojewódzki, Opole, Poland) *Pol. Arch. Med. wewnęt. 44*, 25 (1970).

The modified diazo method of Kaplow was used for histochemical determination of the alkaline phosphatase activity of granulocytes in 30 patients with virus hepatitis and in 30 healthy subjects. The results showed a slight but statistically significant increase of enzyme activity in virus hepatitis and a characteristic dynamic development of this activity in the course of the disease.

S. Maj

Lysosomes in lymphocytes of peripheral blood in certain cases of connective tissue diseases. H. Chwalińska-Sadowska (Instytut Reumatologiczny, Warszawa, Poland) *Reum. pol. 7*, 305 (1969).

Using a histochemical method the presence of lysosomes was demonstrated in peripheral blood lymphocytes. Differences were observed in their behaviour in patients with rheumatoid arthritis and systemic lupus erythematosus as compared with control subjects.

S. Maj

Badania nad haptoglobinami u chorych na nowotwory układu limfatycznego (Studies on haptoglobins in patients with neoplasms of the lymphatic system). H. Glińska, K. Jaegermann, B. Turowska, A. Urban, M. Pawlicki (Instytut Onkologii, Kraków, Poland) *Pol. Tyg. lek. 24*, 1767 (1969).

An increased level of haptoglobins was found in patients with Hodgkin's disease and lymphosarcoma. A tendency for a further rise of this level with the progress of the disease was noted. Control haptoglobin determinations in patients during and after treatment had no prognostic significance.

S. Maj

Próba oceny jakościowej i aktywności biologicznej krwinek białych krwi konserwowanej (Qualitative evaluation of the biological activity of white blood cells in stored blood). Z. Traczyk, J. Daszyński (Instytut Hematologii, Warszawa, Poland) *Pol. Tyg. lek. 24*, 1192 (1969).

The average number of white blood cells after 3 weeks of storage was about 61% of the initial count and 36.6% were viable cells. Blastic transformation was observed in cultures of recently stored blood and of blood stored for 5 and 10 days.

S. Maj

Ocena wskazań do klinicznego stosowania dekstranu na podstawie doświadczenia Instytutu Hematologii (Evaluation of indications for clinical use of dextran in the light of the experience of the Institute of Haematology). W. Rudowski (Instytut Hematologii, Warszawa, Poland) *Pol. Tyg. lek. 24*, 1044 (1969).

In recent years the use of dextran has considerably increased in the Institute of Haematology, particularly in its Surgical

Section. The indications for medium-molecular dextran were shock, severe burns, thrombophlebitis, prevention of surgical shock, thrombotic complications. Low-molecular dextran (Fluidex) was infused intravenously in cases of haemorrhagic shock and burns with disturbances of microcirculation, for the treatment of thrombophlebitis, in preoperative preparation of patients with polycythaemia vera, in thrombotic complications in anaemic and thrombocytopenic patients and in thrombocytosis developing after splenectomy.

S. Maj

Przeżycie ponad 10-letnie u chorych z ziarnica leczonych napromienianiem (Survival beyond 10 years of irradiated patients with Hodgkin's disease). T. Zieliński, E. Jordan (Klinika Radiotherapii AM, Gdańsk, Poland) *Nowotwory 19*, 153 (1969).

Of 105 patients with Hodgkin's disease treated during 1953—1958, 16 patients (15.2%) survived beyond 10 years. Of 31 patients with localized Hodgkin's disease, 13 (41.9%) survived 10 years, including 9 patients (25.8%) without any pathological symptom.

S. Maj

Obraz białek surowicy krwi u chorych na ziarnicę (Serum protein pattern in patients with Hodgkin's disease). H. Glińska, B. Adamczyk (Instytut Onkologii, Kraków, Poland) *Pol. Tyg. lek. 24*, 993 (1969).

In 76 patients with Hodgkin's disease electrophoretic investigation of serum proteins was carried out prior to treatment. Changes in the serum protein pattern in the whole group of patients consisted in decrease of the albumin level and an increase of the globulin level. The intensity of protein pattern disturbance was found to be correlated with the dynamics and progression of the malignant process.

S. Maj

Badania elektroforetyczne białek surowicy krwi u chorych z mięsakami limfatycznymi (Electrophoretic investigations of serum proteins of patients with lymphosarcoma). H. Glińska, B. Adamczyk (Instytut Onkologii, Kraków, Poland) *Nowotwory 19*, 257 (1969).

Hypoalbuminaemia and hyper-alpha$_2$ globulinaemia were observed in all of 32 patients with microscopically confirmed lymphosarcoma. A correlation was found between the degree of dysproteinaemia and the clinical course as well as the presence of liver metastases.

S. Maj

Niejednorodność antygenowa globulin szpiczakowych gamma G. I. Wyodrębnienie podgrup antygenowych. (Antigenic heterogeneity of gamma G myeloma globulins. I. Distinction of antigenic subgroups). K. Madaliński (Zakład Immunopatologii PZH, Warszawa, Poland) *Med. dośw. Mikrobiol. 21*, 315 (1969).

By immunization of rabbits with type K and L of human myeloma globulins, nine different antisera were obtained. After absorption, two kinds of antibody were separated, which served for analysis of the antigenic structure of myeloma globulins.

Antibodies were divided into 1. type-nonspecific, i.e. reacting with a certain number of monoclonal globulins belonging to both (K and L) antigenic types; 2. type-specific, i.e. reacting only with globulins of the same type of light chain as an antigen used for the immunization.

Type-nonspecific antibodies served for distinction of four antigenic subgroups among isolated myeloma globulins.

S. Maj

Niejednorodność antygenowa szpiczakowych globulin gamma G. II. Lokalizacja determinant antygenowych (Antigenic heterogeneity of gamma G myeloma globulins. II. Localization of antigenic determinants). K. Madaliński (Zakład Immunopatologii PZH, Warszawa, Poland) *Med. dośw Mikrobiol. 21*, 385 (1969).

Light and heavy polypeptide chains were isolated from 14 gamma G myeloma globulins and a normal globulin, to determine the localization of antigenic determinants responsible for immunochemical differences between these globulins. The major

part of the determinants by which four antigenic subgroups of gamma G globulins are distinguished was found to be localized probably in the region of heavy-light chain combination, as indicated by the disappearance of these determinants after mercaptoethanol treatment.

Type-specific antibody reactions suggest that heavy chains of myeloma globulins may contain determinants characteristic for the antigenic type of light chain. The results indicated a certain analogy between the molecule of monoclonal globulin from myeloma cases and normal globulin acting as antibody.

S. Maj

Preparatyka koncentratu globuliny przeciwhemofilowej (czynnika VIII) metodą krioprecypitacji i niektóre jego właściwości [Preparatory procedures for obtaining antihaemophilic globulin (factor VIII) by the method of cryoprecipitation and certain properties of this globulin]. S. Łopaciuk, A. Nałęczyńska, Z. Klenowska, J. Daszyński, S. Pawelski (Instytut Hematologii, Warszawa, Poland) *Pol. Tyg. lek. 24*, 826 (1969).

Highly active antihaemophilic globulin concentrates were obtained from fresh plasma using Pool's slightly modified method. Recovery of factor VIII *in vitro* amounted to 62% on the average, both from ACD and CPD plasma. Factor VIII was concentrated about 6 times in relation to volume and about 12 times in relation to protein. The cryoprecipitate could be stored at -25° for at least 10 weeks without a loss of activity. Recovery of factor VIII *in vivo* after transfusion of cryoprecipitate was similar to that observed after transfusion of freshly cooled plasma.

S. Maj

Wartość lecznicza krioprecypitatu w hemofilii A (Therapeutic value of cryoprecipitate in haemophilia A). S. Łopaciuk, M. Szczepański, Z. Klenowska, J. Daszyński, S. Pawelski (Instytut Hematologii, Warszawa, Poland) *Pol. Tyg. lek. 24*, 829 (1969).

Transfusion of cryoprecipitate maintained the level of factor VIII above 25% of normal value through a desired period of time in patients with haemophilia A. Clinical results were good in all cases. No side-effects were observed apart from a fever reaction in one case. The observations concerned 206 transfusions performed in 38 patients.

S. Maj

Analiza dwuletnich obserwacji pracowni prób krzyżowych Instytutu Hematologii (Analysis of two years experience with cross-matching in the Institute of Haematology). K. Frankowska, B. Górska, E. Kacperska, B. Mączyńska-Rusiniak, H. Seyfriedowa (Instytut Hematologii, Warszawa, Poland) *Pol. Tyg. lek. 24*, 838 (1969).

In 64 (7.8%) out of 830 blood recipients, isoantibodies have been detected. In this group, 34% constituted Rh-negative recipients who produced anti-D antibodies after transfusions of Rh-positive blood. Analyzing the usefulness of individual tests in cross-matching for detection of incompatibility between blood donor and recipient, it was found necessary to include the papain and antiglobulin tests into the group of obligatory tests.

S. Maj

Badanie układu krzepnięcia krwi i fibrynolizy w mucoviscidosis u dorosłych (Studies on the blood-clotting and fibrinolysis systems in mucoviscidosis in adults). M. Dziewolska, S. Witoszyński (I Klinika Chorób Wewnętrznych AM, Poznań, Poland) *Pol. Arch. Med. wewnęt. 43*, 1453 (1969).

An evident inhibition of fibrinolysis and a decrease of the platelet count were observed in most patients with mucoviscidosis.

S. Maj

Zaburzenia hemostazy we wtórnej nadpłytkowości w świetle badania agregacji płytek krwi (Disturbances of haemostasis in secondary thrombocythaemia in the light of investigations of platelet aggregation). W. Ławkowicz, R. Michałowska, M. Słomkowski (Klinika Hematologiczna Instytutu Hematologii, Warszawa, Poland) *Pol. Arch. Med. wewnęt. 43*, 1555 (1969).

Clinical data of 11 cases of secondary thrombocythaemia are presented. Frequent occurrence of haemorrhagic purpura was observed, while thrombotic complications were less frequent. In each case platelet aggregation induced by ADP was studied. Disturbances of aggregation were found with reversibility of aggregation with ADP at concentrations of the order of 0.25 μg/ml of platelet-rich plasma in 5 patients and inhibition of aggregation in 1 patient.

S. Maj

Wpływ transkardialnej galwanizacji na układ fibrynolityczny krwi (The effect of transcardiac galvanic current on blood fibrinolytic activity). M. Bielawiec, H. Łukcjan, A. Poniecki, J. Darewicz, M. Gabrylewicz (I Klinika Chorób Wewnętrznych AM, Białystok, Poland) *Pol. Tyg. lek. 25*, 249 (1970).

Transcardiac galvanic current was found to introduce an increase of fibrinolytic activity in patients with chronic coronary failure as well as in healthy subjects. A transient fall of arterial blood pressure was also observed.

S. Maj

Pathomorphology of experimental influenza. III. Spleen. Z. Radwański (State Institute of Hygiene, Warsaw, Poland). *Acta med. Pol. 10*, 363 (1969).

Influenza viruses injected parenterally to mice were observed after 10 minutes in the nuclei and cytoplasm of most of the reticulum cells in the peripheral part of the lymph nodules surrounded by exudate containing gamma globulins and fibrin. In the spleen of mice which had died 30—60 hours after infection, influenza viruses were detected also in some of the reticulum cells within the granules and in numerous leukocytes of the red pulp. Production of gamma globulins, probably specific for influenza virus antigens, was observed in cells of the plasmatic series 48 and 144 hours after infection.

S. Maj

From the International Literature of Haematology

Acta Haematologica (Basel) **42** (1969) No. 4

Inter-relations between iron stores, general factors and intestinal iron absorption. *Hausmann, K., Kuse, R., Sonnenberg, O. W., Bartels, H., Heinrich, H. C.* (Hämat. Abt., Allg. Krankenhaus St. Georg, 2 Hamburg 1), p. 193.

Zytologische Untersuchungen zur Diagnostik und Ätiologie der Erythroleukämie. *Müller, D.* (Med. Univ.-Klinik, 74 Tübingen), p. 208.

Étude de la fixation non spécifique des immunoglobulines G sur les érythrocytes. II. Essais d'inhibition par des immunoglobulines G natives ou modifiées et par d'autres protéines. *Des Gouttes, D., Isliker, H.* (Inst. de Biochimie, Univ. de Lausanne, 1000 Lausanne), p. 219.

Der zytologische Immunoglobulinnachweis in lymphatischen Reizzellen. *Asamer H., Braunsteiner, H.* (Med. Univ.-Klinik, Innsbruck), p. 230.

Exercise-induced thrombocytosis. *Dawson, A. A., Ogston, D.* (Dept. of Med., Univ. of Aberdeen, Aberdeen, Scotland), p. 241.

Severe megaloblastic anemia due to nutritional vitamin B_{12} deficiency. *Ledbetter, R. B. Jr., Del Pozo, E.* (US Army Hosp., Fort Gordon, Ga.), p. 247.

Acta Haematologica (Basel) **42** (1969) No. 5

Electron microscopic study of the granules in guinea-pig bone marrow basophils. *Chan, N. S. T.* (Univ. of Hong Kong, Dept. of Anatomy, Hong Kong), p. 258.

Periodic acid-Schiff reaction in human basophilic leucocytes. *Kaung, D. T.* (Med. Serv., Vet. Adm. Hosp., Iowa City, Ia. 52240), p. 269.

Ultrastructural localization of alkaline phosphatase in human neutrophils. *Nakatsui, T.* (Res. Inst. for Nuclear Med. and Biol., Hiroshima Univ., Kasumi-Cho, Hiroshima, Japan), p. 275.

Über die Wirkung des Mittelgebirgsklimas und des Wetters auf das Blutbild. *Schulte-Wissermann, H., Hille, H., Jesdinsky, H. J., Göpfert, H., v. Boroviczény, K. G.* (Inst. f. Klimaphysiol. d. Univ., D-78 Freiburg), p. 281.

Der Einfluß dysproteinämischer Seren auf die Resistenz normaler Erythrozyten. *Gramlich, F., Kirchhoff, F.* (I. Med. Abt. des Städtischen Rudolf-Virchow-Krankenhauses, 1 Berlin 65), p. 296.

May-Hegglinsche Anomalie. Beschreibung einer neuen Familie und Untersuchungen zur Funktion, Biochemie und Ultrastruktur der Thrombozyten. *Lechner, K., Breddin, K., Moser, K., Stockinger, L., Wenzel, E.* (I. Med. Klinik der Univ., A-1090 Wien), p. 303.

Acta Haematologica (Basel) **42** (1969) No. 6

Urinary and faecal iron excretion in thalassaemia syndromes. *Malamos, B., Constantoulakis, M., Gyftaki, E., Kesse-Elias, M., Augoustaki, O.* (Dept. of Clin. Therap., Univ. of Athens, Alexandra Hosp., Athens), p. 321.

Megakaryocytes and platelets in central venous and arterial blood. *Kallinikos-Maniatis, A.* (Blood Res. Lab., New England Med. Center, Boston, Mass. 02111), p. 330.

Panmyelophthise und Virushepatitis. Experimentelle Untersuchungen mit dem Mäusehepatitis-Virus MHV_3. *Hunstein, W., Perings, E., Eggeling, B., Uhl, N.* (Med. Univ.-Klinik, D-34 Göttingen), p. 336.

Quantitative study of the hemopoietic recovery after a sublethal X-irradiation in the mouse. *Haot, J., Barakina, N. F.* (Inst. de Path., Univ. de Liège, 4000 Liège), p. 347.

Presence of two Phl chromosomes in cells with 49 clone from patient in blast crisis of granulocytic leukaemia. *Khan, M. H., Martin, H.* (Johann-Wolfgang-Goethe-Univ., D-6 Frankfurt/M. 70), p. 357.

Acta Haematologica (Basel) **43** (1970) No. 1

Characteristics of the RNA synthesized *in vitro* by human erythroblasts. *Torelli, U. L., Vaccari, G. L., Mauri, C.* (Inst. of Med. Path., Univ. of Modena, Modena), p. 1.

Reticulocyte generation time and rate of discharge from the marrow. *Griffiths, D. A., Rosse, C., Edwards, A. E., Gaches, C. G. C., Long, A. H. L., Wright, J. L. W., Yoffey, J. M.* (Dept. of Anat., Univ. of Bristol, School of Med., Bristol, England), p. 13.

Pre-leukaemic acute myelogenous leukaemia. *Kumar, S., Bhargava, M.* (Dept. of Path., Lady Hardings Med. Coll., New Delhi), p. 21.

Eosinophil leucocytes and recovery from severe hypoxia. *Sibley, Y. D. L., Hudson, G.* (Univ. of Sheffield, Sheffield S 10 2TN, England), p. 31.

Haemoglobin 0 Indonesia (α116 Glu → Lys) in an Italian family. *Sansone, G., Centa, A., Sciarratta, V., Gallo, E., Lehmann, H.* (Ospedale Pediat. S. Filippo, Genova), p. 40.

Zellkinetik der Granulopoese und des Neutrophilensystems bei einem Fall von zyklischer Neutropenie. *Meuret, G., Fliedner, T. M.* (Abt. f. klin. Physiol., Univ. Ulm, Ulm), p. 48.

Acta Haematologica (Basel) **43** (1970) No. 2

Disturbances of hemostasis in acute myeloblastic leukemia. *Rosner, F., Dobbs, J. V., Ritz, N. D., Lee, S. L.* (Dept. of Med., Maimonides Med. Center, Brooklyn, N. Y. 11219), p. 65.

Fine structural features of lymphocyte transformation induced by mercuric salts. *Schöpf, E., Nagy, G.* (Dept. of Dermatol., Univ. of Hamburg, 2 Hamburg 20), p. 73.

Identity of erythroblast precursors in bone marrow. *Rosse, C., Griffiths, D. A., Edwards, A. E., Gaches, C. G. C., Long, A. H. L., Wright, J. L. W., Yoffey, J. M.* (Dept. of Biol. Struct., Univ. of Washington, School of Med., Seattle, Wash. 98105), p. 80.

Sickle cell disease in Saudi Arabs. *Gelpi, A. P.* (1849 Webster Street, Palo Alto, Calif. 94301), p. 89.

A Ghanaian adult, homozygous for hereditary persistence of foetal haemoglobin and heterozygous for elliptocytosis. *Ringelhann, B., Konotey-Ahulu, F. I. D., Lehmann, H., Lorkin, P. A.* (Ghana Med. School, P. O. Box 4236, Accra, Ghana), p. 100.

Tumorartige intrathorakale extramedulläre Hämatopoese bei hämolytischer Anämie. *Cohnen, G., Scholz, N., Messer, B., König, E., Zimmerschitt, E., Brittinger, G.* (Med. Klin., Klin. der Ruhr-Univ., D-43 Essen-Holsterhausen, BRD), p. 111.

Congenital deficiency of fibrinogen in two sisters. A clinical and haematological study. *Dube, B., Agarwal, S. P., Gupta, M. M., Chawla, S. C.* (Dept. of Paediat., Coll. of Med. Sci., Banaras Hindu Univ., Varanasi, India), p. 120.

Acta Haematologica (Basel) **43** (1970) No. 3

A study of the effects of possible toxic metabolites of uraemia on red cell metabolism. *Wardle, E. N.* (Royal Victoria Infirm., Wellcome Res. Labs., Newcastle upon Tyne, England), p. 129.

Die makrozytäre Regeneration der Erythropoese. Tierexperimentelle Untersuchungen bei der Phenylhydrazinhämolyse. *Becker, Hj., Knecht, L. H., Straschil, F.* (Zentralspital Inn. Med., Abt. Hämatol., Frankfurt-M.), p. 144.

Incorporation of ^{3}H-uridine and ^{3}H-leucine in erythroid cells of normal and anemic rabbits. *Lo Curto, M.* (Univ. of Palermo, Clin. Pediat., Palermo), p. 172.

Hereditary methemoglobinemia due to diaphorase deficiency. Report of a case of heterozygote presenting with cyanosis after birth. *Lo, S. S., Hitzig, W. H., Marti, H. R.* (Univ. of Zurich, Dept. of Pediat., Zurich), p. 177.

Swiss type hereditary persistence of foetal haemoglobin in a case of acquired haemolytic anaemia. *Pawlak, A. L., Kozłowska, F.* (Zaklad Genet. Czlowieka, Poznan), p. 184.

Acta Haematologica (Basel) **43** (1970) No. 4

Experimental studies on leukemia in guinea pigs. *Gross, L., Dreyfuss, Y., Ehrenreich, Th., Moore, L. A.* (Cancer Research Unit, Veterans Administration Hospital, Bronx, N. Y. 10468), p. 193.

Wirkung von L-Asparaginase aus *Escherichia coli* auf die Stimulation von Lymphozytenkulturen. *Kirchner, H., Bauer, S.* (Hämatologische Abteilung, Klinikum Steglitz der Freien Universität Berlin, D-1 Berlin 45), p. 210.

Cytochemical studies of acute leukemias. *Leinonen, E. A.* (Simpson Memorial Institute, University of Michigan, Ann Arbor, Mich. 48105), p. 219.

Gastric mucosa in iron deficiency anaemia. Results of follow-up examinations. *Ikkala, E., Salmi, H. J., Siurala, M.* (Second Department of Medicine, Meilahti Hospital, Helsinki 29), p. 228.

Zur Proliferationskinetik der Lebererythropoese heranwachsender Ratten. *Haas, R. J., Sparrer, E., Fliedner, T. M.* (Abteilung für Klinische Physiologie, Universität Ulm, D-79 Ulm, BRD), p. 232.

The osmotic fragility and density distribution of erythrocytes in the newborn. *Danon, Y., Kleimann, A., Danon, D.* (Israel Defence Forces, The Weizmann Institute of Science, Rehovot, Israel), p. 242.

The osmotic fragility of red cells in newborns and infants. *Luzzatto, L., Esan, G. J. F., Ogiemudia, S. E.* (Sub-Department of Haematology, University College Hospital, Ibadan, Nigeria), p. 248.

Bibliotheca Haematologica (Basel) (1970) No. 34

Transfusion in hemophilia. *Van Creveld, S.* (Hemofilie Klin., Huizen, The Netherlands), p. 1.

In vitro and *in vivo* recovery of cryoprecipitated factor VIII. *Verstraete, M., Lust, A., Vermylen, J.* (Univ. Louvain, Dept. of Med., Lab. of Blood Coagul., Louvain), p. 9.

The P. P. S. B. fraction. *Josso, F., Ménaché, D., Steinbuch, M., Blatrix, C., Soulier, J. P.* (National Centre of Blood Transf., Paris 15e), p. 18.

Cryoprecipitated factor VIII concentrate. *Pool, J.* (Stanford Univ. School of Med., Palo Alto, Calif. 94304), p. 23.

Purification and clinical evaluation of a factor VIII concentrate. *Johnson, A. J., Karpatkin, M. H., Newman, J.* (New York Univ. Med. Center, New York, N. Y. 10016), p. 30.

Christmas factor concentrates. The clinical use of several preparations. *Tullis, J. L., Breen, F. A., Jr.* (New England and Deaconess Hosp., Dept. of Med., Boston, Mass. 02215), p. 40.

Rapid correction of AHF deficiency by antihemophilic factor-method four, with special reference to inhibitors. *Shanbrom, E.* (Hyland Labs., Los Angeles, Calif.), p. 52.

Steroids and hemophilia. *Gourdeau, R., Denton, R. L.* (Montreal Children's Hosp., Montreal, 25, Quebec), p. 65.

Choline and hemophilia. *Boudreaux, H. B.* (Louisiana State Univ., Dept. of Entomol., Baton Rouge, La. 70803), p. 69.

Hypnosis and stress in hemophilia. *Lucas, O. N.* (Univ. of Oregon Dental School, Dept. of Physiol., Portland, Ore. 97201), p. 73.

Aspects psychologiques de l'hémophilie. *Alby, N.* (Hôpital St. Louis, Paris 10e), p. 83.

Psychiatric factors in hemophilia. Methods of parental adaptation. *Agle, D. P., Mattsson, A.* (Univ. Hosp., Cleveland, Ohio 44106), p. 89.

Introductory remarks. *Harris, C. E. C.* (St. Mary's Hosp., Montreal 26, Quebec), p. 97.

Prophylaxis in hemophilia. *Britten, A. F. H.* (New England Med. Center Hosp., Dept. of Med., Boston, Mass. 02111), p. 104.

Our experience in Sweden with prophylaxis of haemophilia. *Nilsson, I. M., Blombeck, M., Ahlberg, A.* (Almanna Sjukhuset, Koagul. Lab., Malmö), p. 111.

Inhibitors in haemophilia. *Biggs, R.* (Churchill Hosp., Oxford), p. 125.

Electroencephalographic patterns in hemophilia. *Denton, R. L., Gourdeau, R.* (Montreal Children's Hosp., Montreal 25, Quebec), p. 135.

Dental treatment of patients with hemorrhagic disorders. *Webster, W. P., Roberts, H. P.* (Univ. of N. Carolina, School of Med. and Dental Res. Center, Chapel Hill, N. C. 27514), p. 139.

Problems related to anticoagulants in hemophiliacs. *Strauss, H. S.* (Children's Hosp. Med. Center, Boston, Mass. 02115), p. 149.

Introduction of topic. *Jordan, H. H.* (Lenox Hill Hosp., Hemophilia Center, New York, N. Y.), p. 159.

Treatment of contractures in haemophilia. *Kingma, M. J.* (Hemofilic Clinic, Huizen, The Netherlands), p. 162.

The management of acute haemarthroses and muscle haemorrhages, *Dormandy, K. M., Madgwick, J. C. A.* (Royal Free Hosp., Haemophilia Centre, London, N. W. 3), p. 164.

Orthopaedic surgery in bleeders. *Ahlberg, A.* (Malmö Almanna Sjukhuset, Orthopaedic Dept., Malmö), p. 170.

Aspects du traitement non-spécifique chez les hémophiles. *Cosma, A., Ionescu, V.* (Inst. d'Évaluation du Capacité de Travail, Bucharest), p. 177.

La chirurgie chez l'hémophile. *Sultan, Y.* (Hôpital St. Louis, Paris 10e), p. 182.

Experimental transplantation of the spleen for classical hemophilia A. Rationales and long-term results. *Norman, J. C., Covelli, V. H., Sise, H. S.* (Boston City Hosp., Sears Surg. Lab., Boston, Mass. 02118), p. 187.

Hemophilia. Some vocational aspects. *Katz, A. H.* (Univ. of California, Los Angeles, Calif. 90024), p. 203.

Problems in the vocational development of the hemophilic individual. *Rusalem, H.* (No address), p. 209.

Blood (New York) **34** (1969) No. 6

Determinants of erythrocyte size in chronic liver disease. *Kilbridge, T. M., Heller, P.* (Univ. of Illinois, Coll. of Med., Chicago, Ill. 60680), p. 739.

Role of copper in iron localization in developing erythrocytes. *Goodman, J. R., Dallman, P. R.* (Vet. Adm. Hosp., San Francisco, Calif. 94720), p. 747.

Circulating siderocytes in human subjects. *Kurth, D., Deiss, A., Cartwright, G. E.* (Univ. of Utah, Coll. of Med., Salt Lake City, Utah 84112), p. 754.

Immunologic memory of phytohemagglutinin-stimulated lymphocytes. *Brody, J. I., Soltys, H. D.* (Univ. of Pennsylvania, School of Med., Philadelphia, Penna. 19104), p. 765.

Gel filtration studies of serum B_{12} binding: an abnormal pattern in patients with vitamin B_{12} deficiency. *Lawrence, C.* (Albert Einstein Coll. of Med., Municipal Hosp. Center, Bronx, N. Y. 10461), p. 774.

The effect of leukocyte transfusions on experimental bacteremia in the dog. *Epstein, R. B., Clift, R. A., Donnall Thomas, E,* (Univ. of Washington, School of Med.. Seattle, Wash. 98105), p. 782.

On the development of slowly-turning-over cell types in neonatal rat bone marrow (Studies utilizing the complete tritiated thymidine labeling method complemented by C-14 thymidine administration). *Haas, R. J., Bohne, F., Fliedner, T. M.* (Univ. of Ulm, Center for Basic Clin. Res., Ulm-D., D 79), p. 791.

Fatal transfusion malaria. *Brooks, M. H., Barry, K. G.* (Cook City Hosp., Dept. of Endocr., Chicago, Ill.), p. 806.

Blood (New York) **35** (1970) No. 1

Tribute to Dr. William Dameshek. *Tullis, J. L.* (No address)

A personal remembrance. *Stratton, H. M.* (No address)

Acute leukemia occurring during chronic lymphocytic leukemia. *McPhedran, P., Heath, C. W., Jr.* (Yale Univ., New Haven Hosp., New Haven, Conn. 06520), p. 7.

Electrophoretic mobility and N-acetyl neuraminic acid content of human normal

and leukemic lymphocytes and granulocytes. *Lichtman, M. A., Weed, R. I.* (Univ. of Rochester, School of Med. and Dent., Rochester, N. Y. 14627), p. 12.

Identification of the Philadelphia (Ph-1) chromosome. *Prieto, F., Egozcue, J., Forteza, G., Marco, F.* (Inst. of Invest. Cytol., Dept. of Cytogenet., Valencia, Spain), p. 23.

Chromosomal aberrations in polycythemia vera. *Modan, B., Padeh, B., Kallner, H., Akstein, E., Meytes, D., Czerniak, P., Ramot, B., Pinkhas, J., Modan, M.* (Tel Hashomer Govt. Hosp., Dept. of Clin. Epidemiol., Tel Aviv), p. 28.

A closed system for large-scale lymphocyte purification. *Woods, A. H.* (Vet. Adm. Hosp., Tucson, Ariz. 85713), p. 39.

Preparation and purification of tritiated phytohemagglutinin and studies of cellular localization in human leukocyte cultures. *Conard, R. A., Demoise, Ch. F.* (Brookhaven Nat. Lab., Upton, N. Y. 11973), p. 44.

Radiation sensitivity of human bone marrow cells measured by a cell culture method. *Senn, J. S., McCulloch, E. A.* (Univ. of Toronto, Dept. of Med., Toronto, Ont.), p. 56.

In vitro growth of myeloid colonies from bone marrow of patients with acute leukemia in remission. *Harris, J., Freireich, E. J.* (Univ. of Ottawa, Faculty of Med., Ottawa, Ont.), p. 61.

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Chlorambucil treatment of patients with cold agglutinin syndrome. *Hippe, E., Jensen, K. B., Olesen, H., Lind, K., Thomsen, P. E. B.* (Univ. of Copenhagen, Copenhagen), p. 68.

Erythrocyte vitamin B_{12} activity in health, polycythemia, and in deficiency of vitamin B_{12} and folate. *Omer, A., Finlayson, N. D. C., Shearman, D. J. C., Samson, R. R., Girdwood, R. H.* (Univ. of Khartoum, Khartoum, Sudan), p. 73.

β-Melanocyte stimulating hormone levels in subjects with hyperpigmentation associated with megaloblastic anemia. *Baker, S. J., Mathan, V. I., Abe, K.* (Christian Med. Coll. Hosp., Wellcome Res. Unit, Vellore, India), p. 83.

Lack of effect of L-methionine ingestion on $^{14}CO_2$ excretion from L-histidine (Imidazole-2-^{14}C) in folic acid and vitamin B_{12} deficient humans. *Stahelin, H. B., Winchell, H. S., Kusubov, N.* (Univ. of Calif. Donner Lab., Berkeley, Calif. 94720), p. 86.

Diagnosis of erythrocyte glucose-6-phosphate dehydrogenase deficiency in the Negro male despite hemolytic crisis. *Herz, F., Kaplan, E., Scheye, E. S.* (Sinai Hosp. of Baltimore, Div. of Pediat. Res., Baltimore, Md. 21215), p. 90.

Absorption of hemoglobin iron: the role of xanthine oxidase in the intestinal heme-splitting reaction. *Dawson, R. B., Rafal, S., Weintraub, L. R.* (Tufts Univ., New England Hosp., Boston, Mass. 02155), p. 94.

The production of schistocytes by fibrin strands (a scanning electron microscope study). *Bull, B. S., Kuhn, I. N.* (Loma Linda Univ., School of Med., Dept. of Path., Loma Linda, Calif. 92505), p. 104.

Surface ultrastructure of the human marrow. A brief note. *Trubowitz, S., Broers, A., Pease, R. F. W.* (New Jersey Coll. Med., E. Orange, N. J.), p. 112.

Clinical profile: hereditary hemolytic disorders and enzymatic deficiencies of human erythrocytes. *Jaffé, E. R.* (Albert Einstein Coll. of Med., Dept. of Med., Bronx, N. Y. 10461), p. 116.

Blood (New York) **35** (1970) No. 2

A possible mechanism for the erythrocytosis associated with hepatocellular carcinoma in man. *Gordon, A. S., Zanjani, E. D., Zalusky, R.* (Dept. of Biol., Graduate School of Arts and Science, New York Univ., New York, N. Y.), p. 151.

A family with three β^{γ}-thalassemia homozygotes. *Ramot, B., Ben-Bassat, I., Gafni, D., Zaanoon, R.* (Dept. of Hemat., Tel-Hashomer Gov. Hosp., Gaza, Israel), p. 158.

Reduction of oxidized glutathione in normal and glucose-6-phosphate dehydrogenase deficient erythrocytes and their hemo-

lysates. *Rieber, E. E., Jaffé, E. R.* (Dept. of Med., Albert Einstein Coll. of Med., Bronx Municipal Hosp. Center, Bronx, N. Y.), p. 166.

Megakaryocytopoiesis in experimentally induced immune thrombocytopenia. *Rolovic, Z., Baldini, M., Dameshek, W.* (Memorial Hosp., Pawtucket, R. I.), p. 173.

Prophylactic treatment of factor VIII deficiency. *Hirschman, R. J., Itscoitz, S. B., Shulman, N. R.* (Nat. Inst. of Arthritis and Metabolic Diseases, Nat. Inst. of Hlth, Bethesda, Md.), p. 189.

Hypofibrinogenemia due to L-asparaginase: studies of fibrinogen survival using autologous ^{131}I-fibrinogen. *Bettigole, R. E., Himelstein, E. S., Oettgen, H. F., Clifford, G. O.* (Dept. of Med., Memorial Hosp. for Cancer and Allied Diseases, New York, N. Y.), p. 195.

Electron microscopic studies on the normal human spleen: especially on the red pulp and the reticulo-endothelial cells. *Hirasawa, Y., Tokuhiro, H.* (First Dept. of Intern. Med., Chiba Univ., Chiba, Japan), p. 201.

Paraproteinemia in a child with leukemia. *Lindqvist, K. J., Ragab, A. H., Osterland, C. K.* (Depts of Preventive Med. and Med., Washington Univ. School of Med., St. Louis, Mo.), p. 213.

C group trisomy in identical twins with acute leukemia. *Hilton, H. B., Lewis, I. C., Trowell, H. R.* (Dept. of Path., Princess Margaret Hosp. for Children, Perth, W. Australia), p. 222.

Chromosomes in the spinal fluid: evidence for metastatic origin of meningeal leukemia. *Mastrangelo, R., Zuelzer, W. W., Ecklund, P. S., Thompson, R. I.* (Univ. Cattolica del S. Cuore, Rome), p. 227.

Effect of phytohemagglutinin on enzymes of thymidine salvage pathway of cultured chronic lymphatic leukemic lymphocytes. *Rabinowitz, Y., Wong, P., Wilhite, B. A.* (Vet. Adm. Hosp., Hines, Ill.) p. 236.

Fibrillar bundles in the nucleus of blood lymphocytes from leukemic and nonleukemic patients. *Stefani, S. S., Tonaki, H.* (Therap. Radiol. Serv., Vet. Adm. Hosp., Hines, Ill.), p. 243.

The inhibition of lymphocyte blastogenesis by L-asparaginase. *Ohno, R., Hersh, E. M.* (Develop. Therap., Univ. Texas M. D. Anderson Hosp., Houston, Texas), p. 250.

Prevalence of precipitating antibody to antigens derived from Burkitt lymphoma cultures infected with herpes-type virus (EB virus). *Stevens, D. A., Pry, T. W., Blackham, E. A.* (Microbiol. Sect., Viral Biol. Branch, Nat. Cancer Inst., Bethesda, Md.), p. 263.

Blood (New York) **35** (1970) No. 3

Testosterone-induced depletion of thymus and marrow lymphocytes as related to lymphopoiesis and hematopoiesis. *Frey-Wettstein, M., Craddock, C. G.* (Dept. of Intern. Med., Univ. of Zurich, Zurich), p. 257.

The use of homogenized human leukocytes for the preparation of anti-lymphocyte serum. *Jehn, U. W., Renner, H. H.*, (Tufts Univ. School of Med., Boston, Mass.), p. 272.

Immunologic aspects of the biogenesis of erythropoietin. *Schooley, J. C., Zanjani, E. D., Gordon, A. S.* (Lawrence Radiation Lab., Univ. of California, Berkeley, Calif.), p. 276.

Chromosome studies in an infant with acute erythremic myelosis. *Kroll, W., Schlesinger, K.* (Dept. of Pediat., Ruprecht-Karl Univ., Heidelberg), p. 282.

Development of aplastic anemia and the exposure to Stoddard solvent. *Prager, D., Peters, C.* (Dept. of Med., Div. of Hemat., Allentown Hosp., Allentown, Penna.), p. 286.

Uracil mustard in the treatment of thrombocythemia. *Robertson, J. H.* (Belfast City Hosp., Belfast, N. Ireland), p. 288.

DNA metabolism and development of organelles in guinea-pig megakaryocytes: a combined ultrastructural, autoradiographic and cytophotometric study. *Paulus, J.-M.* (Inst. de Méd., Dépt. de Clinique et Path. Méd., Univ. of Liège, Liège), p. 298.

Studies on the regulation of granulopoiesis. I. The response to neutropenia. *Morley, A., Stohlman, F., Jr.* (St. Elizabeth's Hosp., Boston, Mass.), p. 312.

Structural changes in the deoxyribonucleoprotein (DNP) complex of leukocytes

from patients with infectious mononucleosis. *Bolund, L., Gahrton, G., Killander, D., Rigler, R., Wahren, B.* (Inst. for Med. Cell Res. and Genet., Med. Nobel Inst., Stockholm), p. 322.

Aspirin ingestion compared with bleeding disorders. Search for a useful platelet antiaggregant. *Weiss, H. J.* (Columbia Univ. Coll. of Physicians and Surgeons, New York, N. Y.), p. 333.

Evaluation of a standard dosage schedule with streptokinase. *Hirsh, J., O'Sullivan, E. F., Martin, M.* (Depts of Path. and Med., McMaster Univ., Hamilton, Ont.), p. 341.

Improvement of leukocyte bactericidal activity in chronic granulomatous disease. *Johnston, R. B., Jr., Baehner, R. B.* (Children's Hosp. Med. Center, Boston, Mass.), p. 350.

Bone marrow lymphocytosis in acute leukemia: a second look. *Skeel, R. T., Bennett, J. M., Henderson, E. S.* (Yale Univ. School of Med., New Haven, Conn.), p. 356.

Granulocytic sarcoma (chloroma). *Wiernik, P. H., Serpick, A. A.* (Baltimore Cancer Res. Center, Baltimore, Md.), p. 361.

A macroglobulin with inhibitory activity against coagulation factor VIII. *Castaldi, P. A., Penny, R.* (Haemat. Dept. Austin Hosp., Heidelberg, Victoria, Australia), p. 370.

The use of heparin in autoimmune hemolytic disease. *Bardana, E. J., Jr., Bayrakci, C., Pirofsky, B., Henjyoji, H.* (Div. of Immunol., Allergy and Infectious Diseases, Univ. of Oregon Med. School, Portland, Ore.), p. 377.

Ultrastructural aspects of antibody plaque-forming cells from clinically normal and overtly autoimmune NZB mice. *Siegel, B. V., Brooks, R. E., Morton, J. I.* (Dept. of Path., Univ. of Oregon Med. School, Portland, Ore.), p. 386.

Waldenström's macroglobulinemia: correlation between expanded plasma volume and increased serum viscosity. *MacKenzie, M. R., Brown, E., Fudenberg, H. H., Goodenday, L.* (Dept. of Med., Univ. of California, School of Med., San Francisco, Calif.), p. 394.

Blood (New York) **35** (1970) No. 4

Morphology of normal erythrocyte and acanthocyte using Nomarski optics and the scanning electron microscope. *Kayden, H. J., Bessis, M.* (New York Medical Center, Department of Medicine, New York, N. Y. 10003), p. 427.

Effects of hypertonic stress of the fine structure of sickled erythrocytes. *White, J. G.* (University of Minnesota, Medical Center, Department of Pediatrics, Minneapolis, Minn. 55455) p. 437.

Inheritable differences in the electrophoretic pattern of hemoglobin revealed in a local random-bred colony of albino rats. *Martinovic, J. V., Marinkovic, D. V., Kanazir, D. T., Martinovic, P. N.* (Boris Kidrič Institute of Nuclear Science, Belgrade), p. 447.

Brief report: Absence of special fetal hemoglobin in beagle dogs. *Lecrone, C. N.* (University of Washington, School of Medicine, Department of Pathology, Seattle, Wash. 98105), p. 451.

Evidence for abnormal red cell population in refractory sideroblastic anemia. *Ben-Bassat, I., Brok Simoni, F., Ramot, B.* (Tel Hashomer Government Hospital, Department of Hematology, Tel Aviv), p. 453.

Diagnostic specificity of sucrose hemolysis test for paroxysmal nocturnal hemoglobinuria. *Hartmann, R. C., Jenkins, D. E., Jr., Arnold, A. B.* (Vanderbilt University, School of Medicine and Hospital, Nashville, Tenn. 37203), p. 462.

Studies on the mechanism of the increased dose-response of erythropoietin after stimulation with erythropoietin. *Fogh, J.* (Rigshospital, Department of Nuclear Medicine, Copenhagen), p. 476.

Ferrioxamine iron in rabbit reticulocyte heme synthesis. *Gerlanc, M. D., Zapolski, E. J., Rubin, M., Princiotto, J. V.* (Georgetown University, School of Medicine and Dentistry, Department of Physiology, Washington, D. C. 20007), p. 493.

Enlargement of mitochondrial compartment in iron and copper deficiency. *Dallman, P. R., Goodman, J. R.* (University of California, San Francisco Medical Center, Department of Pediatrics, San Francisco, Calif. 94122), p. 496.

A Philippino glucose-6-phosphate dehydrogenase variant (G6PD Union) with enzyme deficiency and altered substrate specificity. *Yoshida, A., Baur, E. W., Moutlsky, A. G.* (University of Washington, Department of Medicine, Seattle, Wash. 98105), p. 506.

Brief Note: Effects of prostaglandins, epinephrine and NaF on human leukocyte, platelet and liver adenyl cyclase. *Scott, R. E.* (NIAMO, Laboratory of Experimental Pathology, Bethesda, Md. 20014), p. 514.

Glycogen in human blood platelets, isolation by ultracentrifugation and characteristics of the isolated particles. *Scott, R. B., Still, W. J. S.* (Virginia Communal University, Medical College, Richmond, Va. 23219), p. 517.

Rare variant of lipid storage disorders. *Saidi, P., Azizi, S. P., Sarlati, R., Sayar, N.* (Rutgers State University, Medical School, Department of Medicine, New Brunswick, N. J. 08903), p. 533.

Brief report: Establishment of long-term lines from small aliquots of normal lymphocytes. *Broder, S. W., Glade, P. R., Hirschhorn, K.* (Mount Sinai School of Medicine, Department of Pediatrics, New York, N. Y. 10029), p. 539.

Bullous eruption and elevated leukocyte alkaline phosphatase in the course of busulfan-treated chronic granulocytic leukemia. *Dosik, H., Hurewitz, D. J., Rosner, F., Schwartz, J. M.* (Maimonides Medical Center, Department of Medicine, Brooklyn, N. Y. 11219), p. 543.

Storage of platelet concentrates at 22°C. *Murphy, S., Sayar, S. N., Gardner, F. H.* (University of Pennsylvania, Presbyterian Hospital, Philadelphia, Penna. 19104), p. 549.

Blood (New York) **35** (1970) No. 5

Introduction. *Schwartz, R.* p. 575. No address.

Cold reacting antibody in NZB/B1 mice. *Costea, N., Yakulis, V., Heller, P.* (University of Illinois, Department of Medicine, Chicago, Ill. 60637), p. 583.

Genetic and biological aspects of the HL-A system of human histocompatibility. *Dausset, J., Colombani, J., Legrand, L., Feingold, N., Rapaport, F. T.* (University of Paris, St. Louis Hospital, Paris), p. 591.

DFP labeling of platelets during recovery from thrombocytopenia. *Ebbe, S., Sapienza, P., Duffy, P., Stohlman, F., Jr.* (St. Elizabeth Hospital, Boston, Mass. 02135), p. 613.

Autoradiography of diffusible compounds in human nonnucleated erythrocytes: studies with tritiated glucose and adenine. *Greenwalt, T. J., Paige, J. C., Etoh, N., Steane, E. A.* (American National Red Cross, Blood Research Laboratory, Washington, D. C. 20006), p. 624.

Chromatin clumping in mature leukocytes: a hitherto unrecognized abnormality. *Gustke, S. S., Becker, G. A., Garancis, J. C., Geimer, N. F., Pisciotta, A. V.* (Marquette University, Milwaukee City General Hospital, Milwaukee, Wis. 53233), p. 637.

The effect of chromium on platelet function in vitro. *Kattlove, H. E., Spaet, T. H.* (New York Blood Center, New York, N. Y. 10021), p. 659.

Effect of amino acids on iron absorption from a staple vegetable food. *Martinez-Torres, C., Layrisse, M.* (Institut Venezolano Invest. Cient, Caracas, Venezuela), p. 669.

The lymphocyte in immunological reactions in vitro: ultrastructural studies. *McFarland, W., Schechter, G. P.* (Veterans Administration Hospital, Hematological Research Section, Washington, D. C. 20422), p. 683.

In vitro effect of chloramphenicol on chromosomes. *Mitus, W. J., Coleman, N.* (New England Medical Center Hospital, Blood Research Laboratory, Boston, Mass. 02111), p. 689.

Hemostatic defects in dysproteinemias. *Perkins, H. A., MacKenzie, M. R., Fudenberg, H. H.* (San Francisco Medical Society, Irwin Memorial Blood Bank, San Francisco, Calif. 94118), p. 695.

Ribosome biosynthesis in cultured lymphocytes: II. The role of ribosomal RNA production in the initiation and maintenance of lymphocyte growth. *Rubin, A. D.* (Mt. Sinai School of Medicine, Depart-

ment of Medicine, New York, N. Y. 10029), p. 708.

Hemolysis and erythropoiesis. VI. A comparative study of the utilization of hemoglobin iron and transferrin iron by the erythropoietic tissue. *Sanchez-Medal, L., Duarte, L., Labardini, J.* (Institute of National Nutrition, Department of Hematology, Mexico City, Mexico), p. 721.

Subcellular distribution of ^{51}Cr and characterization of its binding sites in human platelets. *Steiner, M., Baldini, M.* (Brown University Institute for Health Sciences, The Memorial Hospital, Pawtucket, R. I.), p. 727.

Blut (München) **20** (1970) No. 1

Kristallisation von Haptoglobin 1—1 aus Humanserum. *Haupt, H., Heide, K.* (Behringwerke AG, 355 Marburg/Lahn, BRD), p. 1.

Zur Diagnostik seltener Paraproteine. *Schneider, W.* (DRK-Blutspendedienst, 58 Hagen/Westf., BRD), p. 4.

IgG-Paraproteinämien im Immunoelektrophoresebild. *Kühn, R. A.* (Med. Klinik d. Friedrich-Schiller-Univ., X 69 Jena), p. 14.

Makroglobulinämie Waldenström mit außergewöhnlicher pulmonaler Tumorbildung. *Beil, E.* (Path. Inst. d. Univ., 8 München 15), p. 25.

Beziehungen zwischen Plättchenfaktor 3 und Adenosindiphosphat und dem Kontaktaktivierungsprodukt des Gerinnungssystems. *Kommerell, B., Barth, P.* (Med. Univ.-Klinik, 69 Heidelberg), p. 31.

Die Wirkung von Antilymphozytenserum allein oder in Verbindung mit einem Zytostatikum auf eine Mäuseleukämie. *Mempel, W., Thierfelder, St.* (Hämat. Inst. d. Ges. f. Strahlenforschung, 8 München 15), p. 37.

Toxische Nebenwirkungen des Daunomycins bei Kindern. *Massimo, L.* (Kinderklinik "G. Gaslini" der Univ., Genua), p. 44.

Blut (München) **20** (1970) No. 2

Hematological study of a case with cerebellar hemangioblastoma associated with erythrocytosis, with special reference to an erythropoiesis-stimulating activity present in the fluid of the cyst of tumor. *Kawafuchi, J., Shirakura, T., Azuma, M., Tsunoda, T., Maekawa, T.* (Neurochirurg. Klinik, Univ. Gunma, Gunma, Japan), p. 69.

Klinische und elektronenmikroskopische Untersuchungen bei lymphoid-plasmazellulärer Retikulose. *Pietschmann, H., Stockinger, L.* (2. Med. Univ.-Klinik, A-1090 Wien), p. 76.

Na- und Nb-Antigen-Substanzen im Serum des Schweines. *Brucks, R., Hojný, J.* (Inst. f. Tierzucht u. Haustiergenet., Universität, 34 Göttingen), p. 86.

Feinstruktur gefriergeätzter neutrophiler Granulozyten. *Steidle, Ch., Huhn, D.* (Inst. f. Hämat., 8 München), p. 90.

Ultrastrukturelle Zell- und Organveränderungen nach Verabreichung eines heterologen Antilymphozytenserums (ALS) bei der Ratte. II. ALS-Inkubation in vitro von Ductus-thoracicus-Zellen ALS-behandelter Ratten. *Rudolph, R., Land, W., Brendel, W.* (Veterinär-Path. Inst., Univ., 63 Gießen), p. 105.

Apparative Volumenverteilungs-Analyse von Blutzellen. *Wechselberger, E.* (Med. Univ.-Klinik, 74 Tübingen), p. 112.

Blut (München) **20** (1970) No. 3

Karyometrische Untersuchungen im Schnittpräparat des normalen menschlichen Knochenmarks. *Bodenstein, H.* (Path. Inst. der Univ., 23 Kiel), p. 137.

Untersuchungen zur Tiefkühlkonservierung von Blut. I. Die Verwendung niedriger Glyzerin-Konzentrationen bei Temperaturen von −196°C. *Seidl, S., von der Heyden, V., Knoch, H., Sonneborn, R.* (Blutspendedienst Hessen, 6 Frankfurt/Main), p. 148.

Der Einfluß eines 4wöchigen Trainings in der Unterdruckkammer (2250 und 3450 m) auf das rote Blutbild in Körperruhe und während maximaler Ergometer-Belastung. *Samek, L., Roskamm, H., Weidemann, H., Meier-Liehl, T., von Boroviczény, K. G., Renemann, H., Reindell, H.* (Inst. f. Sportmed., Praha 2), p. 156.

Leucocytes in the peripheral blood of *Stenella styx* and *Delphinus delphis* (*Cetacea*,

Delphiniadae) and a haemomyelogram of *Delphinus delphis*. *De Monte, T., Pilleri, G.* (Lab. for Med.-Biochem. Res., 34125 Trieste), p. 165.

Elektronenoptische Untersuchungen der Lymphozyten bei verschiedenen Formen von Antikörpermangel im Kindesalter. *Huhn, D., Tympner, K.-D.* (Univ.-Kinderklin., 8000 München), p. 169.

Transitorische Hämolyse, Hyperlipämie und unspezifische Leberveränderung bei Alkoholabusus (*Zieve*-Syndrom). *Rüdiger, H. W., Blume, K. G., Esselborn, H., Glogner, P., Kaffarnik, H., Finke, J., Löhr, G. W.* (Med. Univ. Klinik, 78 Freiburg i. Br.), p. 178.

Die hereditäre Sphärozytose. *Esselborn, H., Löhr, G. W.* (Med. Univ.-Klin., 78 Freiburg i. Br.), p. 185.

Blut (München) **20** (1970) No. 4

Chromosomale und zytochemische Befunde bei Osteomyelosklerose. *Müller, D., Haberlandt, W.* (Med. Univ. Klinik, 74 Tübingen), p. 205.

Basophilic leukocytes in the hyperlipemic rabbit. *Thonnard-Neumann, E., Fox, R. R., Taylor, W. L.* (St. Elizabeth Hosp., Washington, D. C. 20032), p. 214.

Über das Vorkommen von Hämagglutininen bei Landlungenschnecken. *Schnitzler, St., Kilias, R.* (Inst. f. gerichtl. Med., Humboldt-Univ., X 104 Berlin), p. 221.

Zur Ultrastruktur der Lymphozyten des Haushuhns. *Kriesten, K., Enbergs, H.* (Inst. f. Anat. u. Physiol. der Haustiere, Univ. Bonn, 53 Bonn), p. 229.

The acridine-orange fluorescent method in PHA-stimulated lymphocytes in culture. *Urbanczyk, J., Kujawski, J.* (Dept. of Intern. Med. "B", County Hosp. Opole, Poland), p. 233.

Spätresultate der Radiophosphor-Therapie der Polycythaemia vera. *Höfer, R., Kühböck, J.* (II. Med. Univ-Klinik, A 1090-Wien), p. 237.

Über das Auftreten von diploiden Megakaryoblasten, Promegakaryozyten, und Megakaryozyten bei schweren Hämopathien, ein Rückgriff in die Phylogenese. *Undritz, E., Nusselt-Bohaumilitzky, K.* (Neubadstrasse 83, CH-4000, Basel 4), p. 242.

Blut (München) **20** (1970) No. 5

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Senkungsaktivität nach Kreuzen von Bluten Neugeborener und ihrer Mütter. *Krauß, A., Wolf, H.-J.* (Frauenklin. der Karl-Marx-Univ., 701 Leipzig), p. 288.

Hemoglobinopathies in Campania with particular reference to the rare and new types. *Quattrin, N., Ventruto, V., De Rosa, L.* (Center for Studies on Microcythemias and Genotypical Hemoglobinopathies, Naples), p. 292.

Nachweis des C-reaktiven Proteins in normalen Seren. *Scheiffarth, F., Pérez-Miranda, M., Götz, H.* (Abt. f. klin. Immunol., Univ.-Krankenhaus, 852 Erlangen, BRD), p. 296.

Diagnostic procedures as a guide for the treatment of severe Rhesus disease. *Degnan, T. J., Karasik, S.* (Dept. of Hemat., North Shore Hosp., Manhasset, N. Y. 11030), p. 306.

2,3-Diphosphoglyceratstoffwechsel und 2,3--Diphosphoglyceratmutase-Mangel in Erythrozyten. *Schröter, W.* (Univ.-Kinderklin., 2 Hamburg 20), p. 311.

Blut (München) **20** (1970) No. 6

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On the activity of glucose-6-phosphate phosphohydrolase in the red blood cells. *Aba-*

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Verminderte Phytohämagglutinin-Stimulation von menschlichen Lymphozytenkulturen bei Leberzirrhose: Untersuchungen mit ^{3}H-Thymidin und ^{125}J-Desoxyuridin. *Surianu, P., Heininger, H. J., Feinendegen, L. E.* (Institut für Medizin, 517 Jülich 1 KFA, BRD), p. 370.

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Blut (München) **21** (1970) No. 1

Diagnostic experience with the naphthol AS-D chloroacetate esterase reaction. *Leder, L.-D.* (Institute of Pathology, University of Kiel, 23 Kiel), p. 1.

Die Bestimmung der Thrombozytenlebenszeit mit ^{51}Cr. II. Untersuchungen über die Lebenszeit autologer Thrombozyten bei Patienten mit ausgeprägter Thrombozytopenie. *Adam, W., Heimpel, H., Kratt, E.* (Abt. f. Hämatologie u. Gerinnungsforschung, Zentrum f. Innere Medizin u. Kinderheilkunde der Universität, 79 Ulm/Donau), p. 9.

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British Journal of Haematology (Oxford) **18** (1970) No. 2

Annotation: Glucose-6-phosphate dehydrogenase deficiency. *Beutler, E.* (City of Hope Med. Center, Duarte, Calif. 91010), p. 117.

Annotation: Oral iron therapy. *Callender, S. T.* (Nuffield Dept. of Clin. Med., Radcliffe Infirmary, Oxford), p. 123.

Increased prothrombin conversion: a new activity of lysed fibrinogen. *Triantaphyllopoulos, D. C., Brunetti, A. J., Triantaphyllopoulos, E.* (Dept. of Physiol. and Pharmacol., Wayne State Univ. School of Med., Detroit, Mich.), p. 127.

Blood coagulation and fibrinolysis in acute leukaemia. *Brakman, P., Snyder, J., Henderson, E. S., Astrup, T.* (James F. Mitchell Fdn., Washington, D. C.), p. 135.

Coagulation factor IX in normal and haemophilia B plasma. *Somer, J. B., Castaldi, P. A.* (Dept. of Med., Univ. of Sydney, Sydney), p. 147.

The relation of "Fletcher factor" to factors XI and XII. *Hathaway, W. E., Alsever, J.* (Dept. of Pediat., Univ. of Colorado Med. Center, Denver, Col. 80220), p. 161.

Familial autoimmune haemolytic anaemia associated with rheumatoid arthritis and pernicious anaemia. *Pollock, J. G., Fenton, E., Barrett, K. E.* (Ottawa Civic Hosp., Dept. of Haemat., Ottawa, Canada), p. 171.

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Defective synthesis of an unstable haemoglobin: haemoglobin Köln (β^{98} Val→Met). *White, J. M., Brain, M. C.* (M.R.C.

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Iron metabolism and absorption studies in the X-linked anaemia of mice. *Pinkerton, P. H., Bannerman, R. M., Doeblin, T. D., Benisch, B. M., Edwards, J. A.* (Dept. of Med., State Univ. of New York at Buffalo, Buffalo, N.Y.), p. 211.

Tissue typing using a routine one-step lymphocyte separation procedure. *Harris, R., Ukaejiofo, E. O.* (Univ. Dept. of Med. Genet., The Royal Infirmary, Manchester 13, England), p. 229.

The osmotic fragility of human leucocytes in normal and in some pathological conditions. *Nir, E., Efrati, P., Danon, D.* (Lab. for Blood Morph. and Cytol., Kaplan Hosp., Rehovoth), p. 237.

British Journal of Haematology (Oxford) **18** (1970) No. 3

Annotation: The role of complement in antibody-mediated red-cell destruction. *Mollison, P. L.* (M.R.C. Exp. Haemat. Unit, St. Mary's Hosp. Med. School, London, W. 2), p. 249.

Megakaryocytopoiesis in splenectomized and "hypersplenic" rats. *Rolovic, Z., Baldini, M.* (Univ. Hosp. "B", Belgrade), p. 257.

Effects of sulphydryl inhibition on the erythrocyte in paroxysmal nocturnal haemoglobinuria. *Canellos, G. P., Brain, M. C., Dacie, J. V.* (Dept. of Haemat., Postgraduate Med. School, London, W. 2), p. 269.

Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. *Moore, M. A. S., Metcalf, D.* (Walter and Eliza Hall Inst., Royal Melbourne Hosp., Melbourne), p. 279.

Absorption and excretion of an oral dose of tritiated vitamin K_1 in man. *Shearer, M. J., Barkhan, P., Webster, G. R.* (Dept. of Haemat. and Chem. Path., Guy's Hosp., Med. School, London, S.E. 1), p. 297.

An example of polyagglutinability due to the Tn antigen. *Gunson, H. H., Stratton, F., Mullard, G. W.* (Reg. Blood Transf. Serv., Manchester), p. 309.

The factor-X defect: recognition of abnormal forms of factor X. *Denson, K. W. E., Lurie, A., De Cataldo, F., Mannucci, P. M.* (M.R.C. Lab., Oxford Haemophil. Centre, Churchill Hosp., Oxford), p. 317.

Intestinal absorption of reduced folate compounds in man. *Perry, J., Chanarin, I.* (St. Mary's Hosp. Med. School, London W. 2), p. 329.

Simultaneous fibrinogen and platelet survival with ^{75}Se selenomethionine in man. Studies in diseases with normal coagulation and in hepatocellular disease with abnormal coagulation. *Brodsky, I., Siegel, N. H., Benham, S., Kahn, S. B., Ross, E. M., Petkov, G.* (Dept. of Med., Hahnemann Med. Coll., Philadelphia, P.), p. 341.

The haemoglobin constitution of infants with the haemoglobin Bart's hydrops foetalis syndrome. *Weatherall, D. J., Clegg, J. B., Wong Hock Boon* (Nuffield Unit. of Med. Genet., Dept. of Med., Univ. of Liverpool, Liverpool), p 357.

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British Journal of Haematology (Oxford) **18** (1970) No. 4

Annotation: Methylmalonic aciduria. *Gompertz, D., Hoffbrand, A. V.* (Dept. of Haemat., Royal Postgraduate Med. School, London, W. 12), p. 377.

Abnormal kinetics of red cell membrane cholesterol in acanthocytes: studies in genetic and experimental betalipoproteinaemia and in spur cell anaemia. *McBride, J. A., Jacob, H. S.* (Dept. of Lab., Henderson Gen. Hosp., Hamilton, Ontario), p. 383.

Factor XIII assay by an isotope method. I. Factor XIII (transamidase) in plasma, serum, leucocytes, erythrocytes and platelets and evaluation of screening tests of clot solubility. *Dvilansky, A., Brittein, A. F. H., Loewy, A. G.* (New England Med. Center Hosp., Tufts Univ. School of Med., Boston, Mass.), p. 399.

Fletcher factor deficiency: a report of three unrelated cases. *Hattersley, P. G., Hayse, D.*

(Sacramento Clin. Lab., Sutter Commun. Hosp., Sacramento, Calif.), p. 411.

Haematological changes in heat-acclimated golden hamsters. *Meyerstein, N., Cassuto, Y.* (Negev Inst. for Arid Zone Res., Beer-Sheva, Israel), p. 417.

The content of vitamin B_{12} in adult and foetal tissue: a comparative study. *Rappazzo, M. E., Salmi, H. A., Hall, C. A.* (Dept. of Physiol., Univ. of Turku, Turku, Finland), p. 425.

Idiopathic Heinz body anaemia: Hb-Bristol (β67 (Ell) val → asp). *Steadman, J. H., Yates, A., Huehns, E. R.* (Dept. of Clin. Haemat., Univ. Coll. Hosp. Med. School, London), p. 435.

Megakaryocytes in states of altered platelet production: cell numbers, size and DNA content. *Penington, D. G., Olsen, T. E.* (Melbourne Univ. Dept. of Med., St. Vincent's Hosp., Fitzroy 3065, Australia), p. 447.

Lysis by anti-I in dyserythropoietic anaemias: role of increased uptake of antibody. *Lewis, S. M., Grammaticos, P., Dacie, J. V.* (Royal Postgraduate Med. School, London, W. 12), p. 465.

The haematological and histological findings in 18 patients with clinical features resembling those of myelofibrosis. *Pegrum, G. D., Risdon, R. A.* (Depts of Haemat. and Histopath., Charing Cross Hosp. Med. School, London, W.C. 2), p. 475.

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British Journal of Haematology (Oxford) **18** (1970) No. 5

Annotation: Heparin therapy and its laboratory control. *Pitney, W. R.* (Dept. of Haemat., Royal Postgraduate Med. School, London, W. 12), p. 499.

The haemoglobin concentration of peripheral and central blood of the laboratory rat. *Doell, B. H., Hegarty, P. V. J.* (Unilever Res. Lab., Colworth House, Sharnbrook, Bedford, England), p. 503.

Lymphocyte kinetics in chronic lymphocytic leukaemia. *Spivak, J. L., Perry, S.* (Human Tumor Cell Biol. Branch, Nat. Cancer Inst., Bethesda, Md. 20014), p. 511.

Some variations in the normal haemoglobin concentration. *Cruickshank, J. M.* (Group Path. Lab., Warwick, England), p. 523.

The effects of parity on the leucocyte count in pregnant and non-pregnant women. *Cruickshank, J. M.* (Group Path. Lab., Warwick, England), p. 531.

The effect of age, sex, parity, haemoglobin level, and oral contraceptive preparations on the normal leucocyte count. *Cruickshank, J. M., Alexander, M. K.* (Group Path. Lab., Warwick, England), p. 541.

On the mechanism of the *in vitro* production of haem-positive red cells by sulphydryl compounds: role of pH and free-SH groups. *De Sandre, G., Cortesi, S., Corrocher, R., Falezza, G., Perona, G.* (Clin. Med. Gen. dell'Univ. di Padova, Padova), p. 551.

Haemolysis in cold agglutinin disease: the role of C' and cell age in red cell destruction. *Dudok de Wit, C., van Gastel, C.* (Majella Hosp., Bussum, Netherlands), p. 557.

The effect of neomycin on iron absorption in germfree rats. *Geever, E. F., Crowley, L. V., Levenson, S. M.* (Depts of Surg. and Path., Albert Einstein Coll. of Med., Bronx, N.Y. 10461), p. 567.

The role of platelets in fibrinolysis. Studies on the plasminogen activator and antiplasmin activity of platelets. *Ekert, H., Friedlander, I., Hardisty, R. M.* (Dept. of Haemat., Inst. of Child Hlth and Hosp. for Sick Children, Great Ormond Street, London, W.C. 1), p. 575.

Antibody production to the factor in human urine stimulating colony formation *in vitro* by bone marrow cells. *Stanley, E. R., McNeill, T. A., Chan, S. H.* (Dept. of Microbiol., The Queen's Univ. of Belfast, Belfast, BT12 6BN, N. Ireland), p. 585.

British Journal of Haematology (Oxford) **18** (1970) No. 6

Annotation: Surface counting in the assessment of sites of red cell destruction. *Szur, L.* (Hammersmith Hospital, Royal Post-

graduate Medical School, London, W. 12), p. 591.

Excretion of endogenous non-haem iron by the gastrointestinal tract in human subjects and its influence on the size of body store of iron with particular reference to coeliac disease. *Singh, A. K.* (Department of Haematology, St. Thomas' Hospital and Medical School, London, S.E. 1), p. 597.

Shortened red cell survival in chronic post-haemorrhagic anaemia: effect of iron and prednisone. *Dyrkorn, K., Magnus, E. M.* (Krohgstotten Department, Oslo City Hospital, Oslo), p. 611.

Lyophilized plasma as an assay standard for factor VIII: calibration and control with fresh plasma samples. *Hills, M., Ingram, G. I. C., Matchett, M. O.* (Department of Haematology, St. Thomas' Hospital, London, S.E. 1), p. 617.

Abnormal fibrin ultrastructure, polymerization, and clot retraction in multiple myeloma. *Lackner, H., Hunt, V., Zucker, M. B., Pearson, J.* (New York University Medical Center, New York, N.Y. 10016), p. 625.

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The coagulation mechanism in acute bacterial infection. *Goldenfarb, P. B., Zucker, S., Corrigan, J. J. Jr., Cathey, M. H.* (National Communicable Disease Center, Atlanta, Ga.), p. 643.

The relationship between body iron stores and ferritin turnover in rat liver and intestinal mucosa. *Cumming, R. L. C., Smith, J. A., Millar, J. A., Goldberg, A.* (M.R.C. Group in Iron and Porphyrin Metabolism, University Department of Medicine, Western Infirmary, Glasgow, W. 1, Scotland), p. 653.

Continuous intravenous infusion of factor VIII in classic haemophilia. *McMillan, C. W., Webster, W. P., Roberts, H. R., Blythe, W. B.* (Departments of Pediatrics, Pathology and Medicine, University of North Carolina, Chapel Hill, N.C. 27514), p. 659.

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The lysosomal nature of the anomalous granules and chromosome aberrations in cultures of peripheral blood in Chediak-Higashi syndrome. *Rozenszajn, L. A., Radnay, J.* (Clinical Laboratories, Meir Hospital, Kfar-Saba, Israel), p. 683.

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Folia Haematologica (Leipzig) **92** (1969) No. 3

Die Protectine und ein neues Protectin: Anti-P_{rut}. *Prokop, O.* (Inst. f. Gerichtl. Med., Humboldt-Univ. 104 Berlin), p. 266.

Untersuchungen der sauren Erythrozytenphosphatase bei Tieren. *Radam, G., Strauch, H.* (Inst. f. Gerichtl. Med., Humboldt-Univ., 104 Berlin), p. 276.

Untersuchungen über den Zeitpunkt der Stabilität des Lp(a)-Faktors. *Thomas, K., Hofmann, F.* (Bezirks-Inst. f. Blutspende- u. Transfusionswesen Dresden, 8016 Dresden), p. 281.

Über die Bedeutung der Gm-Serumgruppen in der Bluttransfusionspraxis. *Kurtewa, B.* (Inst. f. Hämat. u. Bluttransf., Sofia), p. 287.

Durch anti-Inv^{1a} und anti-Gm^a-Antikörper bedingte Bluttransfusionsunzulänglichkeit. *Velvartová, M., Hrubiško, M.* (Inst. f. Hämat. u. Bluttransf., Bratislava), p. 295.

On the spread of the blood-group-incompatible married couples in Bulgaria. *Ilieva, V. A.* (Central Lab. of Isoserol., Immunohaemat. and Blood Transf., Sofia), p. 299.

Minus-minus-varianten am S/s-locus. *Spielmann, W.* (Blutspendedienst Hessen, 6 Frankfurt-Süd), p. 304.

Serologische und karyologische Veränderungen bei Kranken mit Blutkrankheiten und bösartigen Geschwülsten. *Májsky, A., Křeček, M., Jakuobková, J., Macháčková,*

M. (Inst. f. Hämat. u. Bluttransf., Prag), p. 311.

Untersuchungen zur Verteilung von Wr^a. *Blauärmel, O., Liesegang, R.* (Bezirksinst. f. Blutspende- u. Transfusionswesen, 1134 Berlin), p. 320.

False blood group determination after massive incompatible blood transfusions. *Doichinova, N., Kourteva, B.* (Inst. of Haemat. and Blood Transf., Dept. of Immunohaemat. and Microbiol., Sofia), p. 323.

Serologische Untersuchungen nach homologer Knochenmarktransfusion. *Stinner, G., Boden, B., Fünfhausen, G.* (I. Med. Klinik, Humboldt-Univ. [Charité], 104 Berlin), p. 326.

Unsere Erfahrungen mit Knochenmarktransfusionen bei Aplasie der Hämopoese. *Šteruska, M., Hrubiško, M.* (Reg. Zentrum f. Hämat. u. Bluttransf., Bratislava), p. 332.

Unverträglichkeit im ABO- und Rh(D)-System bei Frauen mit einer Bluttransfusionsanamnese. *Dzierzkowa-Borodej, W., Olearczyk, J.* (Blutspendestation Wrocław, Wrocław), p. 341.

Etiology and prophylaxis of immunological haemolytic transfusion reactions. *Doichinova, N.* (Inst. of Haemat. and Blood Transf., Sofia 56), p. 344.

Bemerkungen zur "germfree education". *Kirst, R.* (Inst. f. gerichtl. Med., 104 Berlin), p. 349.

Die Organisation der Früherfassung des Morbus hämolyticus neonatorum im Bezirksinstitut für Blutspende- u. Transfusionswesen Leipzig. *Thierbach, V., Berge, H.* (Bezirksinst. f. Blutspende- u. Transfusionswesen Leipzig, 7021 Leipzig), p. 353.

Serologische Untersuchungsmethodik und deren Ergebnisse bei der Früherfassung des Morbus haemolyticus neonatorum (Mhn). *Berge, H.* (Bezirksinst. f. Blutspende- u. Transfusionswesen Leipzig, Leipzig), p. 359.

Die Austauschtransfusion beim Morbus haemolyticus neonatorum infolge ABO-Unverträglichkeit. *Olearczyk, J.* (Blutspendestation Wrocław, Wrocław), p. 364.

Morbus haemolyticus neonatorum bei seltener vorkommenden Blutgruppenantikörpern. *Dzierzkowa-Borodej, W., Olearczyk, J.* (Blutspendestation Wrocław, Wrocław), p. 366.

Die Dextran-Austauschsenkung in der serologischen Diagnostik unter besonderer Berücksichtigung des Molekulargewichtes. *Brandstädter, W., Riechers, F.* (Bezirksinst. f. Blutspende- u. Transfusionswesen Magdeburg, 30 Magdeburg), p. 369.

Anti-Ce/e (Anti-rh_i/hr'') im Blut einer Schwangeren. *Tänzer, S., Scheffler, W.* (Bezirksinst. f. Blutspende- u. Transfusionswesen, 901 Karl-Marx-Stadt), p. 376.

Eine Modifikation des Antiglobulintests zur Diagnose inkompletter Antikörper bei der ABO-Erythroblastose. *Wegrzecki, T.* (Blutspendestation Lublin, Lublin), p. 381.

Untersuchungen über den Nachweis zellständiger Antikörper in Human-Leukozyten. *Bundschuh, G.* (Inst. f. Gerichtl. Med., Humboldt-Univ. 104 Berlin), p. 384.

Zur Charakterisierung der Isoagglutinine in Abhängigkeit von der Untersuchungstechnik. *Keßler, M.* (Bezirksinst. f. Blutspende- u. Transfusionswesen, 30 Magdeburg), p. 387.

Das Erscheinen der Anti-A- und Anti-B-Antikörper in Trans- und Exsudaten im Vergleich zu ihrem Serumtiter und zur Speichelausscheidung. *Buttchereit, M., Moeller, H.-G.* (Städt. Krankenhaus Friedrichshain, Frauenklinik, 10 Berlin), p. 393.

Der Einfluß der Pockenschutzimpfung auf die Ausscheidung der Anti-A- und Anti-B-Antikörper in den Speichel. *Buttchereit, D., Moeller, H.-G.* (Städt. Krankenhaus Friedrichshain, Frauenklinik, 10 Berlin), p. 398.

Untersuchungen zur Diagnostik von D^u. *Brandstädter, W., Fünfhausen, G., Krause, U.* (Bezirksinst. f. Blutspende- u. Transfusionswesen, 30 Magdeburg), p. 402.

Die Bedeutung der Plasmafraktionierung für die Herstellung diagnostischer Blutgruppenseren. *Malaska, Z., Edl, M., Frajtová, J.* (Ustav sér a ockovacich látek, Praha), p. 408.

Agglutinationspasten — eine neue Methode zur Stabilisierung. *Wegrzecki, T.* (Blutspendestation Lublin, Lublin), p. 415.

Die Herstellung von thermostabilen Antiglobulinseren. *Boczar, A., Wegrzecka, R.* (Blutspendestation Lublin, Lublin), p. 418.

Herstellung von diagnostischen AGH-(Coombs-)Seren aus Seren verschiedener Tierarten. *Kattošová, E., Kubiček R.*, (Ustav sér a ockovacich látek, Praha), p. 421.

Folia Haematologica (Leipzig) **92** (1970) No. 4

Die Physiologie der Thrombozytenfunktion. *Lüscher, E. F.* (Theodor-Kocher-Inst. der Univ. Bern, Bern), p. 433.

Über die medikamentöse Beeinflussung der Plättchenfunktion. *Markwardt, F.* (Pharmakol. Inst. der Med. Akad., 50 Erfurt), p. 448.

Die Hemmung der Thrombozytenaggregation in vitro und in vivo. *Breddin, K.* (I. Med. Klinik der Univ. Frankfurt, 6 Frankfurt/M.-Süd), p. 460.

Einfluß von verschiedenen Arzneimitteln auf die Thrombozytenaggregation und die Freisetzung des Blutplättchenfaktors 4 (Antiheparinfaktor). *Farbiszewski, R., Niewiarowski, St., Poplawski, A.* (Dept. of Physiol. Chem. Med. School, Białystok), p. 470.

Der Einfluß der bei Polyarthritis angewandten Medikamente auf die Funktion der Thrombozyten. *Pazdur, J.* (Rheumatol. Inst., Hämat. Klinik, Warszawa), p. 477.

Das Verhalten einiger Thrombozytenfunktionen in Gegenwart verschiedener Volumenersatzmittel. *Fischer, M.* (I. Med. Univ.-Klinik, 1090 Wien), p. 485.

Untersuchungen über Plättchenfunktionen nach Dextranzufuhr. *Cronberg, S.* (Koagul.-Labor., Allmänne Sjukhuset, Malmö), p. 491.

Über den Einfluß von Pharmaka auf die Aminspeicherfunktion der Blutplättchen. *Barthel, W.* (Pharmakol. Inst. der Med. Akad., 50 Erfurt), p. 497.

Untersuchungen über den Energiestoffwechsel menschlicher Blutplättchen. *Hoffmann, A., Körbs, E.* (Pharmakol. Inst. der Med. Akad. Erfurt, 50 Erfurt), p. 510.

Untersuchungen über den Einfluß einiger klinisch gebräuchlicher Medikamente auf thrombozytäre Funktionen. *Vogel, G., Krusch, G.* (Med. Klinik der. Med. Akad. Erfurt, 50 Erfurt), p. 518.

Über den Einfluß der Antifibrinolytika auf die Thrombozytenaggregation in vitro und in vivo. *Hladovec, J.* (Irst. f. Kreislaufforsch., Praha), p. 524.

Experimentelle Untersuchung über die Wirkung von Antikoagulantien auf die Thrombozytenagglutination. *Merkulov, M. Ph., Lakin, K. M., Feldbaum, W. A., Efimov, W. S.* (2. Med. Abt., Pirogow-Inst., Moskau), p. 529.

Kollagen und Thrombozyten. *Perlick, E.* (Med. Univ. Klinik, 701 Leipzig), p. 533.

Die Wirkung von Schlangentoxin (Haemarctin) auf die Thrombozytenfunktion. *Rogner, G., Maak, B.* (Univ.-Kinderklinik, 69 Jena), p. 536.

Zur Stimulierung der Thrombozytenfunktion durch ein neues Adrenochromderivat. *Rex, J. O., Bach, G.* (I. Med. Klinik der Charité, 104 Berlin), p. 546.

Untersuchungen über die Beeinflussung der Thrombozytenfunktion durch Flavonderivate. *Zürn, H.* (Bezirksinst. f. Blutspende- u. Transfusionswesen Dresden, 80 Dresden), p. 550.

Fibrinogensynthese in Blutplättchen. *Rosiek, O., Wegrzynowicz, Z., Sawicki, Z., Kopeć, M.* (Inst. f. Kernforsch., Abt. f. Radiobiol. u. Gesundheitsschutz, Warszawa), p. 553.

Die periphere vasodilatatorische Aktivität des Thrombins. *Delin, A., Olsson, P., Swedenborg, J., Teger-Nilsson, A.-C.* (Karolinska Sjukhuset, 104 01 Stockholm 60), p. 558.

Das Verhalten der Thrombozytenüberlebenszeit während der Behandlung der essentiellen Thrombopenie. *Schulz, K., Preussner, S., Frick, G., Kröger, W.* (Med. Univ.-Klinik, 25 Rostock), p. 567.

Probleme der Präparation und der Konservierung von Thrombozytenkonzentraten. *Stein, P., Karl, M.* (Bezirks-Inst. f. Blutspende- u. Transfusionswesen, 50 Erfurt), p. 577.

Präparation und therapeutische Anwendung von Thrombozyten-Konzentraten. *Wegner, H., Breidenbach, H.* (Blutspendedienst, Med. Fakultät Leipzig, 701 Leipzig), p. 582.

Nouvelle Revue Française d'Hématologie (Paris) **9** (1969) No. 6

Génétique du système HL-A. Fréquences génétique, haplotypique et génotypique observées dans 113 familles. *Dausset, J., Colombani, J., Colombani, M., Legrand, L., Feingold, N.* (Hôpital St. Louis, Inst. Rech. Leucémies, Paris 10e), p. 749.

Analyse des anomalies chromosomiques dans l'anémie pernicieuse. *Castoldi, G. L., Scapoli, G. L., Dallapiccola, B., Spanedda, P.* (Univ. Ferrara, Clin. Med., Ferrara, Italie), p. 769.

Leucoses après greffes de tissus rénaux ou pulmonaires chez des souris C57B1. *Rudali, Jullien, P., Silberman, Cl.* (Fdn. Curie, Inst. Radium, Lab. Genet., Paris 5e), p. 783.

Aspect ultrastructural particulier du réticulum endoplasmique dans un cas de leucémie lymphoïde chronique. *Foa, R., Foa, C., Muratore, R.* (Clin. Med. Therapeut., Lab. Hémat., Marseille 5e), p. 795.

Étude au microscope électronique de l'îlot érythroblastique par la technique de cryodécapage. *Keyhani, E., Bessis, M.* (Univ. of Pennsylvania, Johnson Res. Fdn., Philadelphia, Penna. 10104), p. 803.

Dysfibrinogénémie congénitale et familiale sans tendance hémorragique. *Samama, M., Soria, J., Soria, C., Bousser, J.* (Hôtel Dieu, Lab. Hémostase, Serv. Hémat., Paris 04), p. 817.

Société Française d'Hématologie — Séance du lundi 17 mars 1969, p. 833.

Nouvelle Revue Française d'Hématologie (Paris) **10** (1970) No. 1

Les érythrocytes crénelés. *Bessis, M.* (Inst. de Path. Cell., Hôpital de Bicêtre, F.-94-Le Kremlin-Bicêtre), p. 5.

L'anémie hémolytique aiguë transitoire $C'\gamma_M$ chez l'enfant après rhino-pharyngite. *Homberg, J.-C., Bonnet-Gajdos, M., Salmon, C.* (Centre dépt. de Transf. Sanguine, Serv. d'immunol., 75-Paris 12e), p. 9.

Étude quantitative du système de groupes sanguins ABO. I. Étude quantitative des antigènes A_1, A_i, A_2, B normaux et des types d'interactions entre eux. *Prodanov, P.* (Clin. d'Hémat. et de Transf. Sanguine, Bratislava), p. 19.

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Hémoglobine instable au cours d'une anémie hémolytique à inclusions érythrocytaires et urines noires. Hémoglobine trouvée à Lille, analogue à l'hémoglobine

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L'histologie de la moelle osseuse dans la maladie de Vaquez et le problème de la myélosclérose. A propos de 70 biopsies. *Duhamel, G., Najman, A., André, R.* (Hôpital Saint-Antoine, F. 75-Paris, 12e), p. 209.

Étude des différentes étapes de l'érythrophagocytose par microcinématographie et microscopie électronique à balayage. *Bessis, M., Boisfleury, A. de.* (Inst. de Path. Cell., Hôpital de Bicêtre, 94-Le Kremlin-Bicêtre, France), p. 223.

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L'hétérogenéité fonctionelle normale ou pathologique du locus ABO. *Salmon, Ch.* (Centre Départemental de Transfusion Sanguine, Service d'Immunologie, F. 75-Paris 12e), p. 321.

Données récentes sur les immunoglobulines monoclonales. *Seligmann, M.* (Laboratoire d'Immunochimie, Hôpital Saint-Louis, F. 75-Paris 10e), p. 329.

Les protéines spécifiques des cancers humains. *Burtin, P.* (Laboratoire d'Immunochimie, Institut de Recherches Scientifiques sur le Cancer, F. 94-Villejuif, France), p. 335.

Les anticorps antitissues en pathologie humaine. *Homberg, J. C.* (Service d'Immunologie du Centre Départemental de Transfusion Sanguine de la Seine, F. 75-Paris 12e), p. 339.

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Cryoprécipités riches en facteur VIII. Etude *in vitro* et *in vivo*. *Parquet-Gernez, A., Bauters, F.* (Centre Régional de Transf. Sanguine, 59-Lille, France), p. 439.

Élimination urinaire de molécules différentes des chaînes légères au cours des dysglobulinémies "monoclonales". *Fine, J.-M.* (Centre Nat. de Transf. Sanguine, 75-Paris 15e), p. 451.

Précipitation réversible des alpha lipoprotéines sériques par le polyvinylpyrrolidone à pH 5,00. *Burstein, M., Gouzi, M.-M.* (Centre Nat. de Transf. Sanguine, 75-Paris 15e), p. 463.

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Accidents transfusionnels dus à du sang contaminé par des bacilles à pigment jaune. *Thuillier, M., Gandrille, M. C.* (Poste de Transf. Sanguine, Hôpital Boucicaut, Paris 15e), p. 477

Les gamma-globulines antivaccinales et antitétaniques d'origine plasmatique humaine. *Herzog, F., Gaiffe, M., Badillet, M.* (Lab. de Séroprophylaxie, Hôpital Necker — Enfants Malades, Paris 15e), p. 481.

Scandinavian Journal of Haematology (Copenhagen) 7 (1970) No. 1

Slow activation of factor VIII by thrombin and other enzymes at low temperature. *Özge-Anwar, A. H.* (Sunnybrook Hosp., Dept. of Lab. Haemat., Univ. of Toronto Clinic, Toronto), p. 5.

Immunological studies on human antithrombin. III. Influence of age, sex and use of oral contraceptives on serum concentration. *Fagerhol, M. K., Abildgaard, U.* (Nat. Inst. of Public Hlth, Oslo 1), p. 10.

Intestinal mucosa in children with geophagia and iron-deficiency anaemia. *Berkel, I., Say, B., Kiran, Ö.* (Dept. of Paediat., Inst. of Child Hlth, Hacettepe Univ. of Med., Ankara), p. 18.

Pernicious anaemia and acute leukaemia. A case report with cytogenetic studies. *Nielsen, V. G., Jensen, M. K.* (Depts of Med. TA and A., Rigshosp., Copenhagen), p. 26.

Cytogenetic studies in a case of erythroleukaemia. *Nichols, W. W., Nordén, Å., Bradt, C., Berg, B., Peluse, M.* (Inst. for Med. Res., Camden, N. J. 08103), p. 32.

Prenatal assessment of blood group antibodies against "public" antigens. An example of anti-Ve (Vel) in pregnancy. *Drachmann, O., Lundsgaard, A.* (Blood Bank, Århus Kommunenhosp., DK-8000 Århus C, Denmark), p. 37.

An inhibitor to fibrin stabilizing factor (FSF, factor XIII). *Godal, H. C.* (Dept. IX, Ullevål Hosp., Univ. Clinic, Oslo), p. 43.

A simple test for detecting disorders of thromboplastin generation. *Benedek, E., Soós, E.* (Central Inst. of Haemat. and Blood Transf., Clin. Lab., Budapest XI), p. 49.

Site of iron absorption in man. *Wheby, M. S.* (Univ. of Virginia School of Med., Box 185, Charlottesville, Va. 22901), p. 56.

Treatment of congenital hypoplastic anaemia with prednisone. *Sjöln, S., Wranne, L.* (Dept. of Paediat., Univ. Hosp., S-901 85 Umeå, Sweden), p. 63.

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Pure red-cell anaemia with pro-erythroblast maturation arrest. *Wranne, L., Bonnevier, J. O., Killander, A., Killander, J.* (Regionsjukhuset, Örebro, Sweden), p. 73.

Transient erythroblastopenia in infancy and childhood. *Wranne, L.* (Regionsjukhuset, Örebro, Sweden), p. 76.

Another genetic variant of haemophilia B: haemophilia B Leyden. *Veltkamp, J. J., Meilof, J., Remmelts, H. F., van der Vlerk, D., Loeliger, E. A.* (Dept. of Med., Univ. Hosp., Leyden), p. 82.

Congenital haemorrhagic condition similar but not identical to factor X deficiency. A haemorrhagic state due to an abnormal factor X? *Girolami, A., Molaro, G., Lazzarin, M., Scarpa, R., Brunetti, A.* (Blood Coagul. Center, Inst. of Semeiotica Medica, Padua), p. 91.

Subbands of lactate dehydrogenase isoenzymes in human thrombocytes. *Ursin Knudsen, F., Gormsen, J.* (Koagul.-Lab., Sundby Hosp., DK-2300 Copenhagen), p. 100.

Release of splenic cells into the blood of guinea-pigs of different ages. *Sandberg, G.* (Dept. of Histol., Karolinska Inst., 104 01 Stockholm 60), p. 104.

Apparent C trisomy in bone marrow cells. Report of two cases. *De la Chapelle, A., Wennström, J., Wasastjerna, C., Knutar, F., Stenman, U.-H., Weber, Th. H.* (Folkhälsan Inst. of Genet., Helsinki 10, Finland), p. 112.

Effects of some chemicals on blood platelet microtubules, platelet shape and some platelet functions in vitro. *Behnke, O.* (Anat. Dept. C, Univ. of Copenhagen, DK-2200 Copenhagen N), p. 112.

Haemolytic anaemia in infectious mononucleosis due to inapparent congenital spherocytosis. *Gehlbach, S. H., Cooper, B. A.* (Dept. of Med., Royal Victoria Hosp., Montreal 112), p. 141.

Scandinavian Journal of Haematology (Copenhagen) **7** (1970) No. 3

A biphasic response of platelets to serotonin. *White, J. G.* (Dept. of Paediat., Univ. of Minnesota Med. Center, Minneapolis, Minn. 55455), p. 145.

Effect of the blood sampling procedure on the fibrinogen estimate in rabbit plasma. *Grüner, O. P. N.* (Dept. of Path., Ullevål Hosp., Oslo 2), p. 152.

Platelet enumeration in rabbits. *Grüner, O. P. N.* (Dept. of Path., Ullevål Hosp., Oslo 2), p. 157.

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Erythrophagocytosis in the human bone marrow. *Marton, P. F.* (Pat. Anat. Lab., Aker Sykehus, Oslo 5), p. 177.

Progressive multifocal leukoencephalopathy in chronic lymphatic leukaemia caused by polyoma virus? *Rausing, A., Axelsson, U.* (Path. Inst., Malmö Allmänne Sjukhus, Malmö), p. 184.

The HL-A system: evidence of a third sublocus. *Thorsby, E., Sandberg, L., Lindholm, A., Kissmeyer-Nielsen, F.* (Tissue Typing Lab., Blood Bank, Ullevål Hosp., Oslo 1), p. 195.

Stem cell leukaemia in myelomatosis. *Andersen, E., Videbaek, A.* (Med. Dept. C., Gentofte Hosp., DK-2900 Hellerup, Denmark), p. 201.

The relationship between mucosal iron and iron absorption in the guinea pig. *Pollack, S., Campana, T.* (Dept. of Med., Lincoln Hosp., Bronx, N. Y. 10454), p. 208.

Effect of prednisolone on the coagulation and fibrinolytic systems. *Isacson, S.* (Coagul. Lab., Malmö Allmänne Sjukhus, 21401 Malmö), p. 212.

Seminars in Hematology (New York) **7** (1970) No. 1

In memoriam. *Jaffé, E. R., Miescher, P. A. Stratton, H. M.* (Albert Einstein Coll. of Med., Dept. of Med., Bronx, N. Y. 10461), p. 1.

Introduction to the nutritional anemias. *Herbert, V.* (No address), p. 2.

Vitamin B_{12} metabolism and megaloblastic anemia. *Sullivan, L. W.* (Boston Univ., School of Med., Sect. Hemat., Boston, Mass. 02215), p. 6.

Folic acid deficiency anemia. *Streiff, R. R.* (Univ. of Florida, Coll. of Med., Gainesville, Fla. 32601), p. 23.

Vitamin E and the hematopoietic system. *Silber, R., Goldstein, B. D.* (New York Univ. School of Med., New York, N. Y. 10003), p. 40.

The effect of riboflavin deficiency on erythropoiesis. *Alfrey, C. P., Lane, M.* (Baylor Coll. of Med., Dept. of Med., Houston, Tex. 77025), p. 49.

Anemia associated with protein deficiency. *Adams, E. B.* (Univ. Natal, Durban, S. Africa), p. 55.

Iron deficiency anemia. *Charlton, R. W., Bothwell, T. H.* (Univ. of Witwatersrand Med. School, Johannesburg, S. Africa), p. 67.

The sideroblastic anemias. *Hines, J. D., Grasso, J. A.* (No address).

Seminars in Hematology (New York) **7** (1970) No. 2

Introduction. *Cohn, Z. A.* (Rockefeller Univ., New York, N. Y. 10021), p. 107.

Structure of monocytes and macrophages. *Fedorko, M. E., Hirsch, J. G.* (Rockefeller Univ., New York, N. Y. 10021), p. 109.

Origin and kinetics of monocytes and macrophages. *Furth, R. van* (Univ. Hosp. of Leiden, Dept. of Microbiol. Dis., Leiden), p. 125.

Functional biochemistry of the macrophage. *Axline, S. G.* (Stanford Univ. School of Med., Stanford, Calif. 9430), p. 142.

Endocytosis. *North, R. J.* (Trudeau Inst., Saranac Lake, N. Y. 12982), p. 161.

The monocyte in cellular immunity. *Mackaness, G. B.* (Trudeau Inst., Saranac Lake, N. Y. 12982), p. 172.

Macrophages and viral immunity. *Silverstein, S.* (Rockefeller Univ., Dept. of Cellular Immunol., New York, N. Y. 10021), p. 185.

Macrophages and delayed-typed hypersensitivity. *Bloom, B. R., Bennett, B.* (Albert Einstein Coll. of Med., Bronx, N. Y. 10461), p. 215.

The function of macrophages in the immune response. *Unanue, E. R., Cerottini, J.-Ch.* (Scripps Clin. and Res. Found. Dept. of Exp. Path., La Jolla, Calif. 92037), p. 225.

Thrombosis et Diathesis Haemorrhagica (Stuttgart) **23** (1970) No. 1

Virus infection and blood coagulation. *Gagel, Ch., Linder, M., Müller-Berghaus, G., Lasch, H. G.* (Dept. of Med., Justus-Liebig-Univ., Gießen), p. 1.

Simplification of the concept of coagulation by revival of the inhibitor theory. *Mann, F. D.* (Good Samaritan Hosp., Phoenix, Ariz.), p. 12.

A simple procedure for accurate quantitation of factor VIII inhibitors. *Tse, D., Fekete, L., Shanbrom, E.* (HYLAND Div. Travenol Lab., Inc., Costa Mesa, Calif. 92626), p. 19.

Structural changes in prothrombin during activation: a theory. *Seegers, W. H., Murano, G., McCoy, L.* (Dept. of Physiol. and Pharmacol., Wayne State Univ. School of Med., Detroit, Mich. 48207), p. 26.

Further studies on thrombin-coagulase. *Soulier, J. P., Prou, O., Hallé, L.* (Centre Nat. de Transf. Sanguine, Paris 15e), p. 37.

Recovery of fibrinogen in fibrinolytic dogs. Influence of split products. *Young, M. C., Kolmen, S. N.* (Dept. of Physiol., Shriners' Hosp. for Crippled Children, Galveston, Tex.), p. 50.

Fibrinogen recovery in fibrinolytic dogs. Influence of vitamin K. *Dulock, M. A., Kolmen, S. N.* (Dept. of Physiol., Univ. of Texas Med. Branch, Galveston, Tex.), p. 58.

Isolation and purification of highly enriched tissue plasminogen activator from pig heart. *Rickli, E. E., Zaugg, H.* (Theodor Kocher Inst., Univ. of Berne, Berne), p. 64.

Protection by catatoxic steroids against phenindione overdosage. *Selye, H.* (Inst. de Méd., Univ. de Montréal, Montréal), p. 77.

Blutgerinnung und Fibrinolyse im Nabelschnurblut. I. Vergleichende Untersuchungen bei verschiedenen Entnahmetechniken. *Gmür, J. P., Straub, P. W.* (Med. Univ.-Klinik, Zürich), p. 82.

Inhibition of platelet aggregation by dialysable fibrinogen degradation products (FDP). *Stachurska, J., Latałło, Z., Kopeć, M.* (Dept. of Biochem., Inst. of Rheumatol., Warsaw), p. 91.

Über die Wirkung von Lipidemulsionen auf Blutplättchen. III. Verhalten von ATP, Glykogen und Glukoseverbrauch. *Weber, E., Pfleiderer, Th., Feder, U., Morgenstern, E.* (Med. Univ.-Klinik (Ludolf-Krehl-Klinik, Heidelberg), p. 99.

Platelet reactions and immune processes. III. The inhibition of platelet aggregation by chymotrypsin substrates and inhibitors. *Jobin, F., Tremblay, F., Morisette, M.* (Dépt. de Biochim., Univ. Laval, Québec), p. 110.

The interaction of platelets with polymer surfaces. I. Uncharged hydrophobic polymer surfaces. *Lyman, D. J., Klein, K. G., Brash, J. L., Fritzinger, B. K.* (Polymer Chem. Dept., Stanford Res. Inst., Menlo Park, Calif. 94025), p. 120.

Species-dependent inhibition of platelet aggregation by adenosine and dipyridamole: paradoxical potentiation in the rat. *Philip, R. B.* (Dept. of Pharmacol., Univ. of Western Ontario, London, Canada), p. 129.

Amino acid transport into human platelets and subsequent incorporation into protein. *Cooper, I. A., Firkin, B. G.* (Cancer Inst., Melbourne, Victoria 3000), p. 140.

The effect of adenosine monophosphate, arcaine and anti-inflammatory agents on thrombosis and platelet function in rabbits. *Peterson, J., Zucker, M. B.* (Amer. Nat. Red. Cross Res. Lab., New York, N. Y.), p. 148.

Second phase platelet aggregation induced by adenosine diphosphate in patients with cerebral vascular disease and in control subjects. *Danta, G.* (Dept. of Neurol., North Staffordshire Royal Infirmary, Stoke-on-Trent, England), p. 159.

Some characteristics of the clottable protein of *Limulus polyphemus* blood cells. *Solum, N. O.* (Inst. for Thrombos. Res., Univ. of Oslo, Rikshosp., Oslo 1), p. 170.

A method for automatic recording of the osmotic fragility of blood platelets. *Zahavi, J., Danon, D., Dreyfuss, F., Nelken, D.* (Univ. Dept. of Med. "B", Ichilov Municipal Hosp., Tel Aviv), p. 182.

Pre- and postoperative platelet adhesiveness in pheochromocytoma. *Danta, G.* (Dept. of Neurol., North Staffordshire Hosp. Centre, Stoke-on-Trent, England), p. 189.

Transfusion (Philadelphia) **9** (1969) No. 6

Recovery of glycerolized red blood cells frozen in liquid nitrogen. *Runck, A. H., Valeri, C. R.* (U. S. Naval Blood Res. Lab., Chelsea, Mass. 02150), p. 297.

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Studies of the metabolic integrity of human red blood cells after cryopreservation. I. Effects of low-glycerol-rapid-freeze preservation on energy status and intracellular sodium and potassium. *Derrick, J. B., Lind, M., Rowe, A. W.* (New York Blood Center, Lab. of Cryobiol., New York, N. Y. 10021), p. 317.

Mechanism whereby platelets support the endothelium. *Wojcik, J. D., Van Horn, D. L., Webber, A. J., Johnson, S. A.* (Vet. Adm. Hosp., Thrombos. Res. Lab., Washington, D. C. 20422), p. 324.

The reference laboratories program of the American Association of Blood Banks. *Grove-Rasmussen, M., Huestis, D. W.* (Massachusetts Gen. Hosp., Blood Bank, Boston, Mass. 02114), p. 336.

Evaluation of automated multichannel blood-grouping apparatus. I. Use in mass blood grouping, including comparison with "card typing methods". *Shields, C. E., Camp, F. R. Jr., Adams, J., Tabor, W. G., Gates, S. S.* (U. S. Army Med. Res. Lab., Fort Knox, Ky. 40121), p. 348.

Anti-A_1, lectins from Crotalaria. *Bloch, M.* (Hosp. Rosales, Dept. of Clin. Path., San Salvador, El Salvador), p. 355.

Transfusion (Philadelphia) **10** (1970) No. 1

Statement on laboratory screening tests for identifying carriers of viral hepatitis in blood-banking and transfusion services. *Sgouris, J. T.* (Dept. of Public Health, Lansing, Mich.), p. 1.

Changes in strength of A antigen in children with acute leukemia. *Starling, K. A., Fernbach, D. J.* (Baylor Coll. of Med., Houston, Texas 77025), p. 3.

Familial occurrence of increased activity of blood-group A in serum. *Ottensooser, F., de Araujo, J. T., Rosales, T.* (Dept. of Physiol., Univ. of São Paulo, Brazil), p. 6.

Polycythemic donors. Are they useful and safe? *Hyman, G. A., Carlsen, E.* (Presbyterian Hosp., Columbia-Presbyterian Med. Center, New York, N. Y. 10032), p. 10.

Complement preservation in citrated human blood. *Reich, M. L., Heilweil, L., Fischel, E. E.* (Dept. of Med., Bronx-Lebanon Hosp. Center, Bronx, N. Y. 10456), p. 14.

International Symposium on modern problems of blood preservation. *Bishop, C. W.* (Biochem. Lab., Buffalo Gen. Hosp., Buffalo, N. Y. 14203), p. 17.

Separation and prolongation of viability of human lymphocytes for tissue typing. Evaluation of a technic. *Collins, Z., Shepherd, L. P., Tarris, R., Walford, R. L.* (Hyland Lab., Los Angeles, Calif. 90039), p. 21.

Plasma exchange transfusion for hepatic coma. New technic. *Graw, R. G. Jr., Buckner, C. D., Eisel, R.* (Nat. Cancer Inst., Bethesda, Md. 20014), p. 26.

Microtitration of serum cold agglutinins. *Feizi, T., Monger, E.* (Rockefeller Univ., New York, N. Y. 10021), p. 33.

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Circulation of concentrated one-day-old platelets *in vivo*. *Roy, A., Cavins, J., Djerassi, I.* (Children's Cancer Research Foundation, Boston, Mass. 02115), p. 52.

Safety and long-term effects of plasmapheresis. *Cohen, M. A., Oberman, H. A.* (Department of Pathology, University of Michigan, Ann Arbor, Mich.), p. 58.

The nature of Rh blocking antibodies. *Prager, M. D., Reed, J.* (University of Texas Southwestern Medical School, Dallas, Texas 75235), p. 67

Antenatal transplacental hemorrhage. *Grobbelaar, B. G., Mostert, D.* (Natal Institute of Immunology, Durban, S. Africa), p. 76.

Social psychology of blood donors. *Condie, S., Maxwell, N.* (Brigham Young University, Provo, Utah 84601), p. 79.

Comparative demographic profiles. Voluntary and paid blood donors. *Condie, S., Maxwell, N.* (Brigham Young University, Provo, Utah 84601), p. 84.

Another example of phenotype Rh_{null}. *Senhauser, D. A., Mitchell, M. W., Gault, D. B., Owens, J. H.* (University of Missouri School of Medicine, Columbia, Mo. 65201), p. 89.

Vox Sanguinis (Basel) **18** (1970) No. 1

The automated screening of irregular blood group antibodies. *Myhre, B. A., Reilly, F. C.* (Los Angeles Orange Counties Red Cross Blood Program, Los Angeles, Calif. 90006), p. 1.

Complement-fixing platelet iso-antibodies. V. HL-A typing. *Svejgaard, A., Kissmeyer-Nielsen, F.* (Blood Bank and Blood-Grouping Lab., Univ. Hosp., 8000 Århus), p. 12.

A high frequency of cold agglutinins of anti-IA specificity in a New Guinea highland population. *Baumgarten, A., Curtain, C. C.* (School of Path., Univ. of New South Wales, Kensington, N. S. W. 2033), p. 21.

Action of Pronase and Neuraminidase on the electrophoretic mobility of erythrocytes from normal cats and those with spontaneous tumours. *Mehrishi, J. N.* (Dept. of Radiotherap., Univ. of Cambridge, Cambridge), p. 27.

On erythrocyte agglutinability. I. Variation in the membrane protein. *Maddy, A. H., Spooner, R. L.* (Dept. of Zool., Univ. of Edinburgh, Edinburgh EH9 3JT, Scotland), p. 34.

Automatically controlled continuous recovery of plasma protein fractions for clinical use. A preliminary report. *Watt, J. G.* (Blood Products Unit, Scottish Nat. Blood Transf. Ass., Edinburgh, EH3 9YW, Scotland), p. 42.

An example of anti-Co^a found in pregnancy. *Smith, D. S., Stratton, F., Howell, O., Riches, R.* (Wessex Reg. Transf. Centre, Path. Dept., Southampton Gen. Hosp., Shirley, Southampton, England), p. 62.

Haemolytic transfusion reaction due to anti-Sd^a. *Peetermans, M. E., Cole-Dergent, J.* (Blood Transf. Centre, Antwerp.), p. 67.

Blood group A reactive substance in the common limpet *(Patella vulgata)*. *Pemberton, R. T.* (Reg. Transf. Centre, Leeds LS15 7TW, England), p. 71.

Haemagglutinins from the slug *Limax flavus*. *Pemberton, R. T.* (Reg. Transf. Centre, Leeds LS15 7TW, England), p. 74.

Inability of fava bean extract to agglutinate tannic acid coated erythrocytes. *Suescin, E., Pachtman, E. A., Frumin, A. M.* (Dept. of Lab., Albert Einstein Med. Center, Daroff Div., Philadelphia, Penna. 19147), p. 77.

Human sera as source of IgG subclass-specific reagents. *van Loghem, E., de Lange, G.* (Central Lab. of the Netherlands Red Cross Blood Transf. Service, Amsterdam), p. 81.

A new factor of the Ag-system: Ag(g). *Bütler, R., Brunner, E., Morganti, G., Vierucci, A., Scaloumbacas, N., Politis, E.* (Central Lab. of the Blood Transf. Service, Swiss Red Cross, 3000 Berne), p. 85.

Ag(d), a further factor of the Ag-system. *Bütler, R., Brunner, E., Scaloumbacas, N., Politis, E., Morganti, G., Vierucci, A.* (Central Lab. of the Blood Transf. Service, Swiss Red Cross, 3000 Berne), p. 90.

Vox Sanguinis (Basel) **18** (1970) No. 2

Genetics of the HL-A system. A population and family study. *Svejgaard, A., Thorsby, E., Hauge, M., Kissmeyer-Nielsen, F.* (Univ. Hosp. of Århus, Århus), p. 97.

New HL-A alleles. Identification by planned immunization. *Thorsby, E., Kissmeyer-Nielsen, F.* (Blood Bank and Dept. of Immunohaemat., Ullevål Hosp., Oslo), p. 134.

Inhibition of anti-I sera by human milk. *Marsh, W. L., Nichols, M. E., Allen, F. H. Jr.* (Serol. and Genet. Lab., New York Blood Center, New York, N. Y. 10021), p. 149.

Anti-steroid factors in normal and pathological human sera. *Pinto, M., Rimon, A.* (Dept. of Microbiol., Tel-Aviv Univ., Tel-Aviv), p. 155.

The influence of modifying the surface properties of sheep red cells upon hemolytic antibody production in mice. *Wilkins, D. J., Bliznakov, E. G.* (Inst. Battelle, 1227 Carouge, Geneva), p. 163.

The HL-A antigen, KN−HN (=HL-A13). *Kissmeyer-Nielsen, F., Kjerbye, K. E., Mayr, W., Thulstrup, H.* (Blood Bank and Blood Group. Lab., Århus Kommunehosp., 8000 Århus), p. 176.

MaKi, a new leukocyte antigen. Preliminary report. *Mayr, W. R.* (Inst. f. Blutgruppenserol., Univ. Wien, A-1090 Wien), p. 180.

Severe reactions after cryoprecipitated human factor VIII. *Ahrons, S., Glavind-Kristensen, S., Drachmann, O., Kissmeyer-Nielsen, F.* (Blood Bank and Blood-Group. Lab., Århus Kommunehosp., 8000 Århus), p. 182.

Antigenic composition of amyloid in relation to its ultrastructure. *Haferkamp, O., Schäfer, H., Schultz, R. T., Milgrom, F.* (Dept. of Path. I, Univ. of Ulm, Ulm), p. 185.

Anti-P_1 associated with liver-fluke infection. *Bevan, B., Hammond, W., Clarke, R. L.* (Reg. Blood Transf. Centre, Cardiff, Wales), p. 188.

Autoantibodies to vitreous body in sheep. *Bash, J. A., Milgrom, F., Witebsky, E.*, (Dept. of Microbiol., State Univ. of New York at Buffalo, Buffalo, N. Y. 14214), p. 190.

Vox Sanguinis (Basel) **18** (1970) No. 3

Definition of a new HL-A antigen: Da18. *Colombani, M., Colombani, J., Legrand, L., Dausset, J.* (Lab. d'Immuno-Hémat. Hôpital Saint Louis, Paris), p. 193.

The effect of storage on adhesion and aggregation of platelets. *Shively, J. A., Gott, C. L., de Jongh, D. S.* (Path. Dept., Univ. of Missouri School of Med., Columbia, Mo.), p. 204.

Variant of group B lacking the B antigen on the red cells. *Gundolf, F., Andersen, J.* (Statens Seruminst., Blood Bank and Blood Group. Dept., 2300 Copenhagen S.), p. 216.

The recognition of water-soluble I blood group substance. *Dzierzkowa-Borodej, W., Seyfried, H., Nichols, M., Reid, M., Marsh, W. L.* (Lab. Dept., District Blood Transf. Centre, Wrocław), p. 222.

Agglutinins for antigens of two different human blood group systems in the seeds of *Moluccella laevis*. *Bird, G. W. G.*,

Wingham, J. (Reg. Blood Transf. Service, Birmingham), p. 235.

N-acetylneuraminic (sialic) acid and human blood group antigen structure. *Bird, G. W. G., Wingham, J.* (Reg. Blood Transf. Service, Birmingham), p. 240.

On the genetic interpretation of the 6-phosphogluconate-dehydrogenase-isozymes in man. *Ropers, H., Op't Hof, J.* (Inst. f. Humangenet u. Anthropol., Univ. Freiburg i. Br., D-78 Freiburg), p. 244.

Ox erythrocyte agglutinability. 2. Differential agglutinability. *Spooner, R. L., Cowpertwait, J. L., Maddy, A. H.* (Animal Breeding Res. Organisation, Edinburgh), p. 251.

Pig A-O hypothesis. I. Ontogenic variation in A-coating ability of porcine serum. *Lang, B. G.* (Blood Group Res. Unit, Vet. Field Station, Easter Bush, Roslin, Scotland), p. 257.

A family showing independent segregation of Bu^a and Yt^b. *Giles, C. M., Bevan, B., Hughes, R. M.* (M. R. C. Blood Group Ref. Lab., London S. W. 1), p. 265.

A case of idiopathic autoimmune haemolytic anaemia in an infant treated with chloroquin. *Forrester, R. M., Stratton, F.* (Wigan and Leigh Hosp. Group, Manchester, England), p. 267.

Heterophile antibodies in mice grafted with syngeneic tumors. *Thunold, S., Müller-Ruchholtz, W., Milgrom, F.* (Gade Inst., Dept. of Path., Univ. of Bergen, 5000 Bergen, Norway), p. 270.

The Gm and Inv factors of the Thai people. *Phansomboon, S., Singhprasert, P.* (Dept. of Path., Siriraj Hosp., Mahidol Univ., Bangkok, Thailand), p. 274.

Compound antigens in the porcine E and L systems. *Lang, B. G.* (Blood Group Res. Unit., Vet. Field Station, Easter Bush, Roslin, Scotland), p. 277.

The detection of Chagas' disease. A rapid haemagglutination test for special use in blood banks and epidemiological studies. *Knierim, F., Rubinstein, P.* (Dept. of Microbiol. and Parasitol., Univ. of Chile, Santiago, Chile), p. 280.

Pepsin "A" substance. Further uses. *Stapleton, R. R.* (Reg. Transf. Centre, Sheffield, England), p. 287.

Vox Sanguinis (Basel) **18** (1970) No. 4

Australia antigen, transfusion and hepatitis. *Okochi, K., Murakami, S., Ninomiya, K., Kaneko, M.* (Tokyo University Hospital, Blood Transfusion Service, Tokyo), p. 289.

Studies on the secretion of blood group substances. I. Observations on the red cell phenotype Le(a+b+x+). *Sturgeon, Ph., Arcilla, M. B.* (University of California, School of Medicine, Department of Pediatrics, Los Angeles, Calif. 90024), p. 301.

Cytotoxic HL-A antibodies. Studies of synergism and gene-dose effect. *Ahrons, S., Thorsby, E.* (Århus Kommune Hospital, Århus, Denmark), p. 323.

The micro-complement fixation test. I. A solution of the technical problems of HL-A typing? *D'Amaro, J.* (University Hospital of Leiden, Department of Immunohaematology, Leiden), p. 334.

A two-stage agglutination test for the detection of antithrombocyte antibodies. *Hanna, N., Nelken, D.* (Hebrew University, Hadassah Medical School, Department of Immunology, Jerusalem), p. 342.

Immunoglobulin components in crystalline ceruloplasmin and anticomplementary activity. *Fisher, G. B., Deutsch, H. F.* (University of Wisconsin, Department of Physiological Chemistry, Madison, Wis. 53706), p. 349.

A case of haemolytic disease of the newborn due to a new red cell antigen, Zd. *Švanda, M., Procházka, R.* (District Hospital, Transfusion Department, Bundějovice, Czechoslovakia), p. 366.

A new interpretation of the serum factor 'Ag(e)'. *Bütler, R., Brunner, E.* (Swiss Red Cross, Blood Transfusion Service, Berne), p. 370.

Cross-reactive HL-A antibodies: absorption and immunization studies. *Thorsby, E., Kjerbye, K. E., Bratlie, A.* (Ullevål Hospital, Department of Immunohaematology, Oslo), p. 373.

Demonstration of I-anti-I interaction in a precipitin system. *Feizi, T., Marsh, W. L.* (Rockefeller University, New York, N. Y. 94122), p. 379.

News Items

5th Congress of the Hungarian Haematological Society

Budapest, May 26–28, 1971

Apart from plenary sessions at which invited speakers will deliver special lectures in English, there will be English-speaking symposia on platelet kinetics and physiopathology, immunologically functioning cells, and the structure and function of red cells.
Further information is obtainable from:

Haematological Congress
MOTESZ, Budapest I, Apród u. 1–3
Hungary

E. Kelemen, M.D., D.Sc.
President of the Congress

First Announcement

The First Meeting of the European Division of the International Society of Haematology will be held from 10 to 12 September 1971 in Milan (Italy).
Programme committee: E. E. Polli, Milano (President); A. Fieschi, Genova; G. Astaldi, Tortona.
The official language will be English.

The main subjects will be: *Haemolytic Anaemias* and *Lymphocytes.*
Apart from plenary sessions where speakers will be invited, there will be simultaneous symposia on related topics, as well as free communications and workshop sessions.

For further information, registration and abstract forms, please contact **A. T. Maiolo**, Secretary of the First Meeting of the European Division of I. S. H., Istituto di Patologia Medica, Via Pace 15, 20112 Milano (Italy).

Contents

Volume 4

Author Index

Subject Index

MEDICOR
BUDAPEST

СОВРЕМЕННЫЕ СРЕДСТВА ПЕРЕЛИВАНИЯ КРОВИ

Применение переливания крови разрешило ряд таких проблем биологии, физиологии, генетики, иммунологии и антропологии, на которые долгое время мы не могли дать ответа.

Как известно, прошло 303 года с тех пор, что придворный врач французского короля Людовика XIV-го, Жан Денис, сообщил о переливании крови. 10 лет раньше Ричард Лоуер на основании опыта, приобретенного в ходе экспериментов на собаках, указал на то, что можно замещать потерянную кровь. На основании сведений первую трансфузию от человека на человека провел в 1818 году Джеймс Бланделл, открывая этим путь практическому терапевтическому переливанию крови и его широкому распространению.

В Венгрии первое сообщение о переливании крови опубликовано в 1861 году в журнале Орвоши Гетилап.

В настоящее время, можно сказать, нет такой области клинической практики, где бы не применялись переливания крови.

Открытием консервирования крови был решен вопрос сохранения крови в жидком состоянии. Исследование консервирования крови привело к новым достижениям в деле познания процессов обмена веществ форменных элементов крови, белков крови и факторов свертывания крови. Связанные с этим технические методы проделали большое развитие. Строгим требованием при переливании крови как и при любом вмешательстве, является асептика. Асептика обеспечивается только непрямым переливанием крови в закрытой системе. Под этим подразумевается тот способ переливания консервированной крови, когда применением соответствующей трансфузионной аппаратуры кровь вводится в сосудистую систему больного таким образом, что она при этом не соприкасается с внешней средой. Применение несоответствующего средства или отклонения от предписания могут быть причиной вредных симптомов. Наиболее частой причиной так называемой пирогенной реакции является неправильная очистка трансфузионной аппаратуры.

Реакция может быть предупреждена тщательной, соответствующей предписаниям очисткой и стерилизацией средств, аппаратуры.

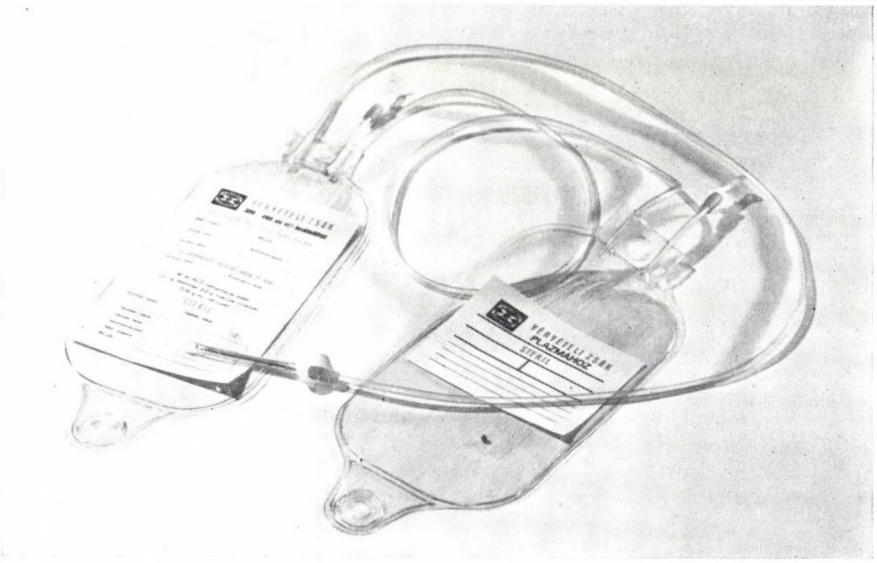
Наиболее надежными являются поступающие в потребление пластмассовые средства, в стерильной упаковке предназначенные для однократного потребления. Эти средства являются в настоящее время самыми современными и они обладают многочисленными приемуществами по сравнению со средствами из резины. Пластмассовые средства прозрачные и таким образом можно следить за течением крови из бутылки до сосудистой системы больного.

Венгерская медицина получила только в 1949 году возможность ознакомиться с системой наилучше организованных институтов переливания крови в мире, с здравоохранением Советского Союза. Венгерское здравоохранение вслед за этим обратило внимание на проблематические вопросы переливания крови и приступило к устранению недочетов. В наших больницах было введено переливание крови консервированной кровью.

Как мы уже упоминали в современной терапии кровь уже незаменима. В стране ежегодно проводится около 200 000 трансфузий.

Мы считаем полезным ознакомить читателя со средствами взятия крови венгерского производства (Медикор), удовлетворяющими современные требования:

Двухсекционный пластмассовый мешок для взятия крови, для переливания взрослым

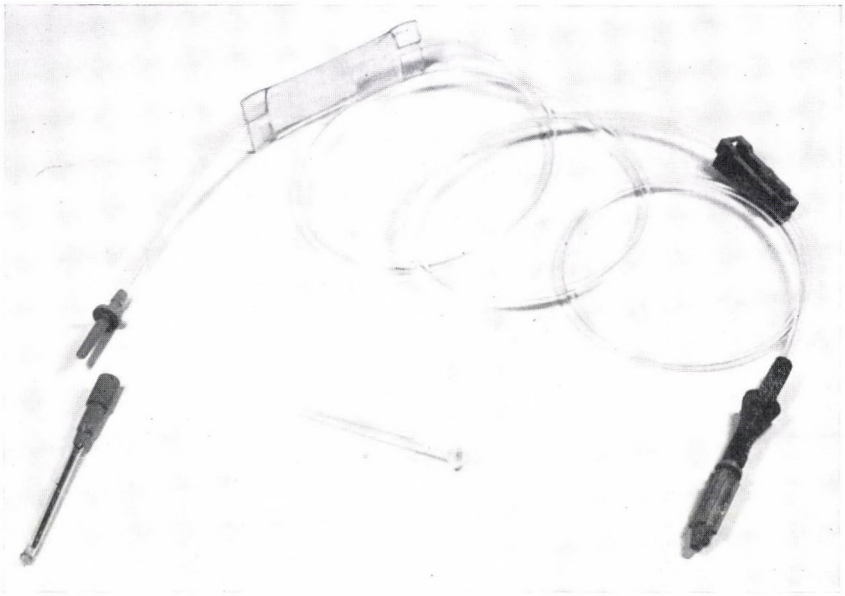


Изготовленные из прозрачного, непирогенного, нетоксичного мягкого поливинилхлорида два 500 мл-вые мешка — один для взятия крови, другой для плазмы соединяются при помощи поливинилхлоридовой трубки. В кровеотборной трубке мешка для взятия крови имеется крыльчатая игла взятия крови, спрессованная из полипропилена. Мешок содержит 100 мл раствора для консервирования крови, состоящего из лимонной кислоты и глюкозы. На обоих мешках имеются таблички для занесения необходимых данных. Приспособление предназначено для однократного использования и поступает в торговую сеть в стерильном состоянии.

Преимущества: отделенная путем центрифугирования после взятия крови плазма может быть в закрытой системе продавлена во второй мешок и таким образом может храниться и использоваться отдельно. При старом методе взятия крови в стеклян-

ную посуду вышеупомянутые манипуляции могут быть произведены только в стерильных лабораторных условиях, так как составные компоненты крови не могут быть в закрытой системе перенесены из одной посуды в другую.

Универсальные пластмассовые узлы для переливания крови



Узлы состоят из непирогенных, нетоксичных, мягких поливинилхлоридовых и из твердых шприцованных полипропиленовых и силиконо-резиновых деталей. Подключаемая к телу часть снабжена двойной металло-пластмассовой иглой с целью прободения пробки обычной стеклянной склянки. После удаления металлической иглы эта часть подключается к мешку для взятия крови. Узел содержит фильтрующий мешок для предотвращения проскальзывания тромбов. В конце узла, снабженном окончанием для прикрепления иглы, имеется удлинние из силиконовой резины для введения в сосудистое русло медикаментов. Регулировка скорости потока крови обеспечизается роликовым краном. Узел поставляется в стерильной форме вместе с венной иглой.

Преимущества: по сравнению с обычными узлами данная конструкция используется только один раз и поэтому исключается опасность инфекции. Благодаря своей универсальности узел с успехом применяется и в тех местах, где переход к более современной технике взятия крови с помощью пластмассовых мешков осуществлен только частично.

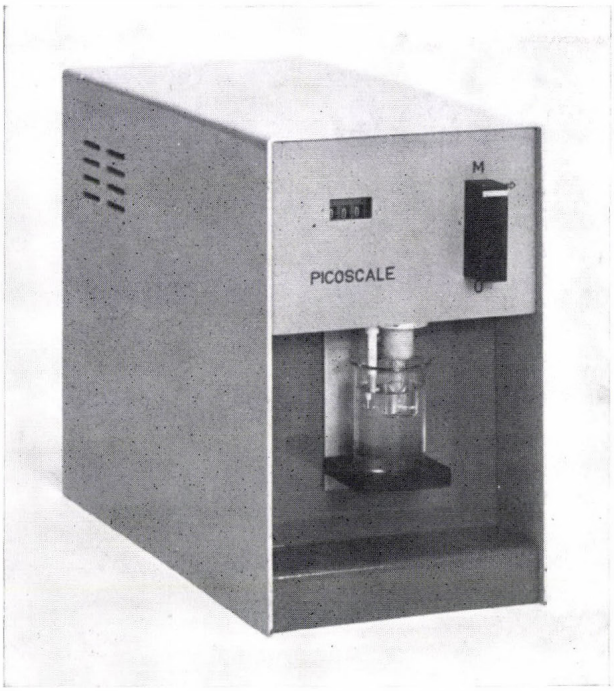
Плазмоэкстрактор

Плазмоэкстрактор имеет пружинную опрокидывающуюся конструкцию. Мешок для взятия крови можно повесить на вилочную вешалку, которая поворачивается по дуге и размещена на задней стенке. Откидная передняя стенка изготовлена из прозрачной пластмассы, что обеспечивает легкий контроль манипуляций и их видимость. Прибор представляет собой легкую стальную конструкцию, он эстетичен и пригоден для любой гематологической лаборатории.

Посредством центрифугирования крови, взятую в двухсекционный пластмассовый мешок, можно разделить на плазму и на осажденные форменные элементы. Поместив мешок для взятия крови в плазмоэкстрактор, плазму можно выдавить в другой мешок. Таким образом плазма и форменные элементы могут храниться в закрытой системе в отдельных мешках.

Пикоскел

Автоматический счетно-вычислительный прибор для подсчета форменных элементов крови



Прибор пикоскел предназначен для подсчета телец дисперсных систем, в особенности в таких областях, где проведение анализов проводится в большом количестве (например, в больничных и поликлинических лабораториях).

Автобус для взятия крови

Донорство становится во всем мире таким массовым движением, что взятие крови не может уже ограничиваться на один центральный донорский пункт. Взятие крови проводится отчасти на предприятиях, отчасти же — в соответствии с потребностями —-вдали от донорских пунктов.

В интересах осуществления этого был сконструирован такой автобус для взятия крови, в котором можно у 300 доноров брать кровь и который должным образом оснащен. Для транспорта бригады медицинских работников для взятия крови требуются две машины.

Первый автобус.

Автобус приспособлен к перевозке 35—40 лиц, отчасти медицинской бригады, состоящей из 22 человек, с другой стороны для перевозки доноров на место и обратно домой.

Второй автобус.

Автобус перевозит на место назначения оборудование для взятия крови и взятую кровь.

Наиболее важной частью оснащения является холодильник, вмещающий 300 банок крови и содержащий их при соответствующей температуре.

Область применения

В первую очередь прибор применяется для подсчета эритоцитов и лейкоцитов, но он пригоден и для проведения нижеследующих исследований:

— подсчета тромбоцитов,
— подсчета дроммевых грибков,
— исследования размножения микроорганизмов,
— исследования способности пленок пропускать бактерии,
— исследования взвесей,
— исследования газовых включений в жидкостях,
— исследования осадков.

В различных областях здравоохранения и биологии в настоящее время широко применяются счетно-вычислительные приборы для подсчета телец, с которыми в отношении быстроты и точности не могут соперничать другие методы подсчета.

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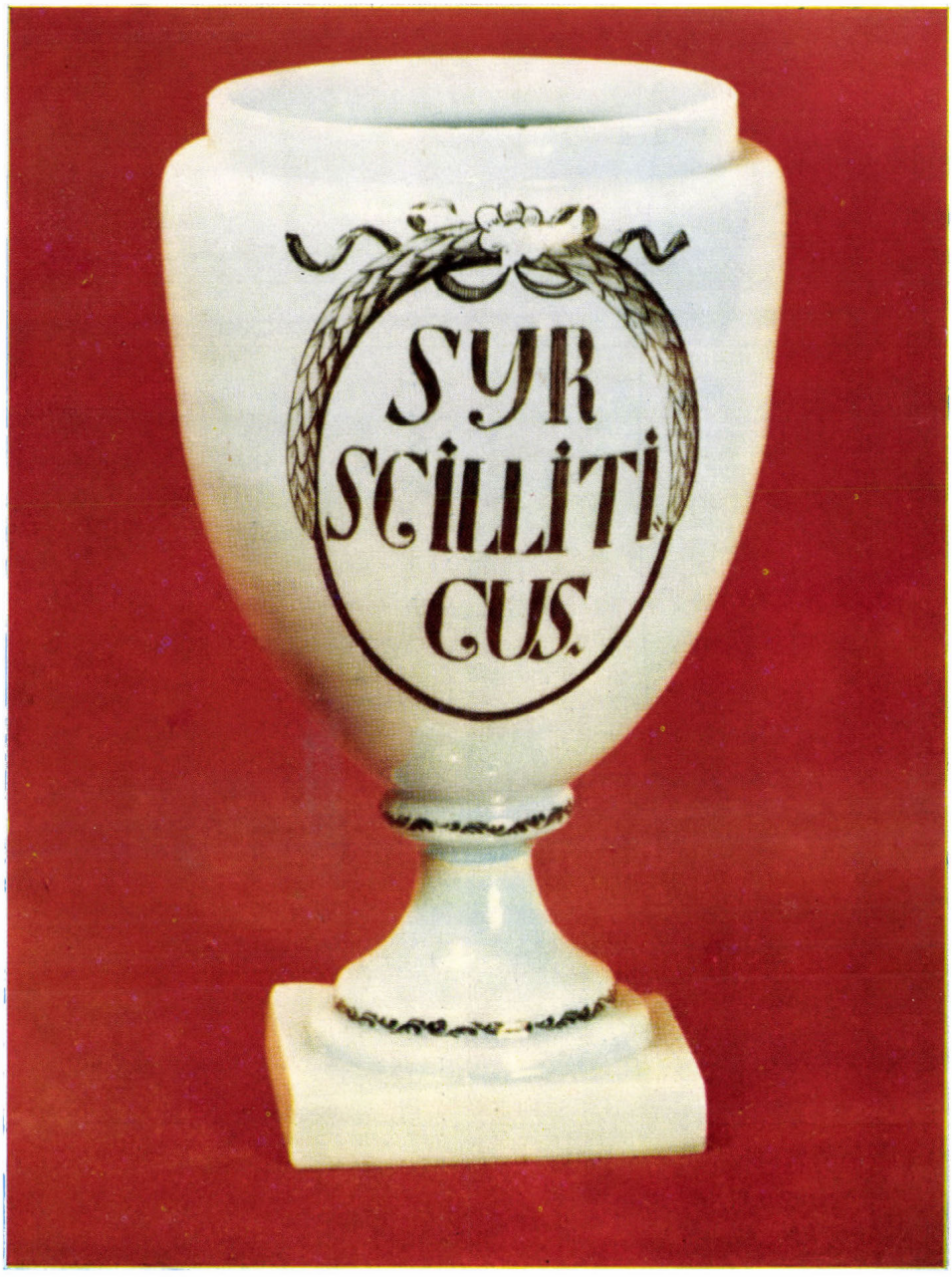

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Card Indexes

E. Rondanelli, E. Magliulo, F. P. Carco, E. Cerra

Investigations into the action of 6-benzimidazole cobamide coenzyme (Cobamamide) on pernicious anaemia megaloblasts. Haematologia 4, 261 (1970).

The effect of cobamamide (a coenzymatic form of vitamin B_{12}) on *in vivo* and *in vitro* proliferation and maturation of pernicious anaemia megaloblasts has been studied. Similarly to cyanocobalamine, and possibly with a higher degree of effectiveness, cobamamide normalized the increased proliferation rate of megaloblasts and enhanced the maturative evolution of pernicious anaemia cells. The mechanisms of this action at the biological level are discussed.

J. Brocteur, M. Gilissen-Gottschalk, A. André

Polymorphism of red cell acid phosphatase. Haematologia 4, 279 (1970).

Polymorphism of red cell phosphatase has been studied by starch gel electrophoresis, in 500 individuals taken at random, in Liège. In addition to the five current phenotypes: A, AB, B, AC and BC, the rare C phenotype has twice been found. Gene frequencies calculated from the observed results are: $P^a = 0.349$, $P^b = 0.596$ and P^c 2 0.055.

S. Pawelski, W. Hartwig, K. Rechowicz, L. Konopka, Anna Kasperlik-Załuska

Vitamin B_{12} metabolism in adrenocortical insufficiency. Haematologia 4, 287 (1970).

The absorption from the digestive tract of labelled vitamin B_{12} has been studied, determining the serum vitamin B_{12} level, the unsaturated binding capacity of ^{57}Co vitamin B_{12} by the serum, and the antibodies to the intrinsic factor, in 16 patients with adrenocortical insufficiency and in 8 adrenalectomized patients. Impaired absorption of vitamin B_{12} was found in 6 cases of primary adrenocortical insufficiency. In the adrenalectomized patients receiving cortisone for substitution no disturbance in vitamin B_{12} metabolism was revealed.

G. Lelkes, I. Bernát

Electron microscopical study of the bone marrow of burned patients Haematologia 4, 295 (1970).

An electron microscopical study carried out in burned patients revealed the following findings. (a) An active rhopheocytotic process takes place in the erythroblasts and reticulocytes of the bone marrow. In spite of this, ferritin is not accumulated in the cytoplasm of the cells but occurs in the form of dispersed granules. The occurrence of ferritin aggregates (siderosomes) is occasional. (b) No morphological change occurs in the mitochondria of erythroblasts and reticulocytes. Iron was only exceptionally detected in them. (c) Active erythrophagocytosis was observed in the reticulum cells of the bone marrow. Ferritin was found in the cytoplasm of the reticulum cells usually in a dispersed form, while within the cytoplasmic cell structures amply, in the form of large clumps.

J. G. Cory

Blood aspartyl transcarbamylase activity in Rauscher virus induced murine leukemia. Haematologia 4, 303 (1970).

Aspartyl transcarbamylase (2.1.3.2) activity has been studied in hemolysates from normal mice and mice infected with Rauscher leukemia virus. Blood from Rauscher virus-infected mice shows a fourfold increase in specific activity over that found in normal mouse blood. Some of the enzyme properties of the aspartyl transcarbamylase activity in the blood from normal and infected mice were the same. These included apparent Michaelis constants and pH-activity profiles. However, a subtle difference in enzyme properties was detected. There were differential responses of the aspartyl transcarbamylase from the blood of normal and infected mice to the competitive inhibitors, succinic and maleic acids, and the sulfhydryl inhibitors p-hydroxy-mercuribenzoate and N-ethyl maleimide.

H. Meister

Contribution to the cytochemistry of monocytic leukaemia. Haematologia 4, 311 (1970).

The differentiation of leukaemias of immature-cell type is questionable when based on panoptic staining, the result is significantly improved by the application of different cytochemical methods. The cytochemical differentiation of monocytic leukaemia is the most reliable of all the haemoblastoses of immature-cell type. The high plasma α-naphthyl-acetate esterase and NaF inhibitable naphthol-AS-acetate-esterase activities are pathognomonic for this form of leukosis. The cytochemical identity of the pathological paramonocytes and of the normal monocytes concerning the structural components and enzymes is worth mentioning. Not only the cytological-diagnostic possibilities in the individual cases, but also the knowledge on the nosology of monocytic leukaemia have been enriched by cytochemistry.

T. Horváth, A. Patakfalvi, G. Böhm, E. Kajtsa

Quantitative immunoglobulin determination in leukaemia and myelofibrosis. Haematologia 4, 319 (1970).

The immunoglobulin level of 6 patients with acute, 25 with lymphoid, and 21 with myeloid leukaemia, and 12 with myelofibrosis, as well as of 60 healthy individuals was determined by the direct radial immunodiffusion method. In acute leukaemia a decreased IgA level was found together with normal IgG and IgM levels. In lymphoid leukaemia IgG was moderately, IgA and IgM markedly decreased. In myeloid leukaemia the IgG level was usually normal or increased, the IgA level mostly lower and the IgM level variable. In myelofibrosis the deviation was high in every immunoglobulin class, which was partly explained by the different stages of the disease. In the polycythaemic pre-phase of myelofibrosis an increase in all the three immunoglobulin types was observed. With the progress of the disease the immunoglobulin levels were decreasing.

R. Arnaud, P. Maupas, A. Audurier, J. Avril

Presence and specificity of Australia antigen and of the corresponding antibody. Haematologia 4, 327 (1970).

The presence of Australia antigen and of the corresponding antibody has been studied in 2,339 subjects divided in three groups. 1. 2,246 tests made in subjects hospitalized for various causes revealed Australia antigen in two sera. 2. Of 12 patients on maintenance haemodialysis, Australia antigen was found in one, and anti Australia antigen in two patients. 3. The serum of 81 hepatitis patients has also been tested. 4. The presence of Australia antigen has been detected in four cases of transfusion B virus hepatitis, but in none of the 68 subjects with epidemic A virus hepatitis. Results of the gel diffusion test and complement fixation test in the two last groups have been compared. The specificity of the gel diffusion test and the choice of a detection method are discussed.

K. Madaliński, W. J. Brzosko, M. Zuławski

Immunoglobulin levels in Hodgkin's disease. Haematologia 4, 333 (1970).

Serum immunoglobulin levels in 11 children and 29 adult patients with Hodgkin's disease were studied by means of immunoelectrophoresis and single radial immunodiffusion. The immunoglobulin levels were compared to those in a control group of 35 healthy adult individuals. In adult patients with Hodgkin's disease, a significant decrease of IgA and IgM, with a slight elevation of IgG and IgD levels was noted. The findings reflect some humoral reactivity of Hodgkin's disease patients undergoing radiotherapy and chemotherapy.

G. Mersch

Estimation of complement activity: A statistical study of some methods. Haematologia 4, 343 (1970).

A study has been made on the basis of experimental data of four theoretical relations characterizing the curve of haemolysis, and a reliable method has been worked out for the estimation by confidence intervals of the dose corresponding to 50% haemolysis. The computations have been programmed in FORTAN.

G. Szegedi, G. Nagy

Coincidence of autoimmune diseases and polycythaemia vera. Haematologia 4, 367 (1970).

The coincidence of polycythaemia vera and autoimmune diseases was observed in three cases. In two cases the autoimmune disease was rheumatoid arthritis, in one case systemic lupus erythematosus. The possible causes of the coincidence of autoimmune disease and malignant cell proliferation are discussed.

I. Sprenger, G. Uhlenbruck, G. I. Pardoe

On the specificity of lectins with a broad agglutination spectrum. VII. Further Investigation with Maclura aurantiaca Agglutinin. Haematologia 4, 373 (1970).

The specificity of the agglutinin from *Maclura aurantiaca* may be directed against the terminal α or β-linked anomer of N-acetyl-D-galactosamine (or D-galactose) as well as against inner-carbohydrate chain galactosaminyl structures (if C 3 and C 4 are free). It is suggested to use competitive inhibition tests with different, even enzyme-treated red cells, in order to establish the reaction of the lectin with the inhibitor on the one hand and the cell receptor on the other.

V. Kutas, K. Merétey, E. Elekes, L. Kocsár

Effect of phytohaemagglutinin on the immune response of irradiated rabbits. Haematologia 4, 379 (1970).

Phytohaemagglutinin injected before primary antigenic stimulation was found to increase secondary antibody production against SRBC in rabbits. This effect was practically abolished by X-ray irradiation and appeared in a slight form in rabbits irradiated before secondary antigenic stimulation.

L. Á. Pálos, G. Sas, J. Jákó, C. László

Oxidizing-reducing properties of the intrinsic prothrombin activator. Haematologia 4, 385 (1970).

Purified intrinsic prothrombin activator (IA) was investigated during exposure to cysteine, reduced glutathione and N-ethylmaleimide. IA activity was rapidly decreasing in the presence of reducing agents (cysteine, reduced glutathione); the rate of decrease depended on concentration and incubation time. The inhibitory effect was enhanced in nitrogen and reduced in oxygen atmosphere. In order to obtain a similar inhibition with NEM, considerably higher concentrations were needed.

G. Sas, J. Jákó, C. László, I. Pető

Inactivation of intrinsic prothrombin activator in serum. Haematologia 4, 391 (1970).

The inactivation of IA prothrombin activating complex (phospholipid, active factor V, active factor X, calcium) isolated by centrifugation and washing was studied in serum free from prothrombin. The rate of IA inactivation depends on the quantity of serum present, while in buffer solution IA activity decreases gradually but at a considerably slower rate than in the former case. The factor responsible for inactivation is contained in the alpha-1 globulin fraction; it cannot be detected in the euglobulin fraction. The effect of the inactivating factor present in the test system is particularly conspicuous at low IA activity. It is assumed that, in analogy to the thrombin threshold, there is a minimum amount of IA which can still bring about clotting of the systems containing the inactivating factor.

K. Worowski, J. Prokopowicz, A. Gabryelewicz

Plasma fibrinolytic system and blood pressure in patients with hypertensive cardiovascular disease treated with reserpine and phenformin. Haematologia 4, 399 (1970).

The fibrinolytic system has been studied in patients with hypertensive cardiovascular disease treated with reserpine and phenformin. Increase of plasma euglobulin activity and decrease of blood pressure were found, with a slight decrease of the fibrinogen level and plasminogen and antiplasmin activity. A considerable increase of the urinary urokinase level and of plasminogen activator in blood was observed. The findings may suggest a role of the fibrinolytic system in the pathogenesis of hypertension and thus drugs enhancing fibrinolysis might be useful in the treatment of this disease.

J. C. G. Doery, J. Hirsh, G. C. de Gruchy

Platelet metabolism and function. Haematologia 4, 405 (1970).

The literature on normal platelet energy metabolism and the effect of stimuli is reviewed. Particular emphasis has been given to outlining the problems associated with the study of platelet enzymes and platelet metabolism in isolated cells.

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