COMPARATIVE STUDY OF THE ERYTHROPOIETIC ACTIVITY OF PLASMA IN VITRO AND IN VIVO

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The existing tests for detecting the erythropoietic activity of plasma may be divided into two main groups: 1) injection of the plasma into animals, and 2) addition of the plasma to a tissue culture in vitro. In the first case whole or treated plasma is injected into recipient animals, and the indices of erythropoiesis are then studied in these animals: the hemoglobin concentration, erythrocyte and reticulocyte counts, hematocrit index, rate of incorporation of Fe^{59} into erythrocytes, the erythroblastic cells of the marrow, and so on [3, 5, 6, 10]. Several investigators prepare their recipient animals before carrying out these tests, by fasting them or by inducing polycythemia [8, 11]. In investigations in vitro, cultures of bone marrow cells are most frequently used [4, 7], and observations are made of changes in the erythrogram, the mitotic activity of the erythroblasts, hemoglobin synthesis, and so on. The method of leukocyte film culture is less often used.

It must be admitted that each method described above has its limitations when used separately, and only the employment of more than one method will give a more or less objective idea of the erythropoietic activity of the tested substances. The necessity for such an approach has been mentioned by several authors.

Our object was to make a comparative study of the erythropoietins using two parallel methods: injection of a protein-free extract of erythropoietically active plasma into "polycythemic" animals, and addition of the same extracts to a marrow culture in a liquid medium.

EXPERIMENTAL METHOD

Healthy male rabbits were used as donor animals. Erythropoietin formation in these animals was stimulated by the production of acute posthemorrhagic anemia as a result of threefold bleeding. The blood was withdrawn from the heart by puncture without administration of anesthetics. At each bleeding $\frac{1}{3}$ of the blood volume was withdrawn. The erythropoietic activity was investigated 24 h after the first and third bleedings. The blood was stabilized by the addition of one drop of heparin solution, and centrifuged. The plasma obtained from the blood of the individual animals was pooled and from it a protein-free extract was prepared by the method of Gordon and co-workers [9].

Polycythemia was produced in rats and mice by means of a single intraperitoneal injection of an 80% suspension of homologous erythrocytes (rats with a mean weight of 60 g received an injection of 5 ml, and mice with a mean weight of 20 g received 1 ml). On the seventh day complete disappearance of reticulocytes from the peripheral blood was usually observed in these animals. On the same day the animals began to receive subcutaneous injections of plasma extracts, obtained after the first and third bleedings, twice daily for 2 days: mice—1 and 0.5 ml, rats 3 and 1.5 ml. A control group of recipient animals received injections of physiological saline in identical conditions. The indicator of the change in the level of hemopoiesis was the reticulocyte reaction in response to injection of the test substances. Results obtained 24 h after the last injection were taken into consideration.

TABLE 1. Changes in Number of Reticulocytes in Peripheral Blood of "Polycythemic" Animals (in %) 24 h after Last Injection of Tested Substances

Species of animal	Before injection	After injection		
		of physiological saline	of extract of plasma after 1st bleeding	of extract of plasma after 3rd bleeding
Rats	0	0.3 ± 0.1	3.1 ± 1.0	5.0 ± 1.0
Mice	0	0.3 ± 0.2	2.5 ± 0.8	4.2 ± 1.6

TABLE 2. Erythropoietic Action of Extracts of Plasma Detected by Bone Marrow Culture

Marrow cells		Initial figures	Addition of Hanks's solution (control)	Addition of plasma extracts	
				Obtained after 1st bleeding	Obtained after 3rd bleeding
Proerythroblasts		0.2	1.2	0.4	2.4
Erythroblasts	basophilic	3.0	5.0	5.0	14.0
	polychromatophilic	12.0	12.0	25.6	27.6
	oxyphilic	5.0	4.2	11.6	11.2
Percentage of all cells of erythroblastic series		20.2	22.6	42.6	55.2
Absolute number of cells of erythroblastic series in 1 mm^3		780 ± 82	923 ± 18	2280 ± 113	4585 ± 120
Number of megakaryocytes in 1 mm^3		3900 ± 140	4085 ± 65	5350 ± 200	8300 ± 150

Marrow was cultivated by Lajtha's method [12] as modified by S. Yu. Shekhter. About 1 ml of marrow was extracted from both femora of a rabbit by puncture, and shared equally in two test tubes with 5 ml of Hanks's isotonic solution containing heparin. The tubes were centrifuged at 1500 rpm for 10 min, after which the residue of marrow cells from both tubes was transferred to one flask, to which was added sufficient Hanks's solution, solution of antibiotics, and homologous plasma to ensure that plasma amounted to $^2/_3$ of the total volume and that the concentration of antibiotics (penicillin + streptomycin) in the tubes with culture material amounted to 200 units/ml [1]. The contents of the flask were carefully mixed and the marrow suspension was transferred in volumes of 0.5 ml into culture tubes. The extract of the plasma of the anemic rabbits was diluted 1:1 with Hanks's solution and added in volumes of 0.5 ml to the marrow suspension. For control purposes 0.5 ml of Hanks's solution was added to one of the tubes with marrow suspension. All the tubes were then closed with rubber stoppers and incubated at 38° for 20 h. At the conclusion of incubation the total number of myelokaryocytes and the partial erythrogram were determined in all the tubes and the absolute number of cells of the erythroblastic series was calculated. The results thus obtained were compared with the original and control figures, and on this basis the erythropoietic activity of the tested extracts was assessed.

Ten rabbits were used as donors, and 27 mice and 24 rats as recipients. The numerical results were subjected to statistical analysis.

EXPERIMENTAL RESULTS

Injection of the protein-free extract of the plasma of the anemic rabbits into the polycythemia rats and mice led to the appearance of a marked reticulocyte reaction. Meanwhile, injection of physiological saline was followed by only a slight increase in the number of reticulocytes. The plasma extract obtained from the rabbits after the third bleeding caused a statistically more marked ($P < 0.02$) reticulocyte reaction than the plasma extract obtained after the first bleeding (Table 1).

Similar results were obtained when the method of bone marrow culture was used. Cultivation of bone marrow with the addition of Hanks's solution (control) caused a very small (not statistically significant) increase in the total number of myelokaryocytes and in the absolute number of cells of the erythroblastic series. The total number of erythroblasts expressed as a percentage and the erythrogram remained practically unchanged.

The plasma extract obtained from the rabbits 24 h after the first bleeding led to a significant ($P < 0.01$) increase in the absolute number of erythroblasts and in their relative percentage on account of polychromatophilic and oxyphilic forms. The erythropoietic action of the plasma extract obtained 24 h after the third bleeding was analogous but greater in degree ($P < 0.01$). In these experiments, in addition to an increase in the percentage of polychromatophilic and oxyphilic erythroblasts, an increase in the number of proerythroblasts and basophilic erythroblasts was observed (Table 2).

It may be concluded from these results that both methods can be used to assess the erythropoietic activity of the plasma, although each, when used separately, has certain disadvantages and doubtful qualities. In respect to the in vitro methods, for instance, it may be objected that the results obtained are not characteristic of living organisms. As regards the assessment of the erythropoietic activity by the effect of plasma on the reticulocytosis in " polycythemic" animals, as a rule the study of this index alone cannot be regarded as adequate.

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