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PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF HEMOGLOBIN IN MICE WITH EHRLICH'S CARCINOMA

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A special role in the functional system of the blood is played by its hemoglobin component. Hemoglobin is a hemoprotein with an exceptionally wide range of functions; it determines the oxygen balance of the body, and it can respond accurately to various factors in the microenvironment that induce changes in the properties and functional activity of the respiratory protein of the erythrocytes, aimed at directing oxygen-dependent pathological states.

The determinative factors of modulation of the oxygen-binding properties of hemoglobin include the specific features of metabolism in organs and tissues, changes in the endocrine background, etc. Definite changes have been shown to take place in the oxygen transport system in several oncologic diseases [10]. Meanwhile, in order to obtain some idea of the mechanisms of the compensatory reaction of the blood to pathological changes in the body, research embracing both cellular and molecular levels of hemoglobin is essential.

It was accordingly decided to study physicochemical and functional properties of freely soluble and membrane-bound hemoglobin in whole erythrocytes and erythrocytes fractionated in a sucrose density gradient.

EXPERIMENTAL METHOD

Experiments were carried out on mice weighing 18-20 g (50 mice kept under animal house conditions). The animals were divided into two groups: 1) intact, 2) animals with Ehrlich's carcinoma. Inoculation of Ehrlich's tumor cells was carried out by injecting 1 million cells per mouse, suspended in physiological saline. The cells were counted in a Goryaev's chamber. The cells were diluted so that 0.1-0.2 ml of physiological saline contained 1 million cells. Blood was taken from the mice on decapitation on the 10th day after inoculation of the cells. The resistance of the erythrocytes was determined by the acid erythrogram method [9]. Hemoglobin was obtained by Starodub's method [7]. Heparinized blood, after decapitation of the animals, was centrifuged at 1000*g* for 5 min; the plasma was removed and the blood cells washed 3 times with physiological saline. The erythrocytes were hemolyzed with 5 volumes of water, and 2 volumes of 0.1 M Tris-HCl-EDTA buffer, pH 8.9, made up in 1.5 M NaCl, compared with the volume of the erythrocytes, was added to the hemolysate. The mixture was shaken for 20 min and allowed to stand for hemolysis at 4°C for 1 h. The hemolysate was then centrifuged at 15,000*g* for 20 min at 4°C. The soluble hemoglobin fraction was withdrawn by suction with a syringe, and the residue was treated 3 times with cold water to remove the weakly bound and readily soluble hemoglobin; each time, washing was followed by centrifugation at 15,000*g* for

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TABLE 1. Relative Percentages of Mouse Erythrocyte Fractions Separated in a Sucrose Density Gradient

Fraction No.	Sucrose, %	Content of cells, %	
		erythrocytes of intact mice	erythrocytes of mice with Ehrlich's carcinoma
1	30	0,9±0,1	1,0±0,2
2	26	3,5±0,2	2,0±0,3
3	22	5,4±0,4	4,4±0,1
4	18	10,5±0,3	8,6±0,5
5	14	32,9±0,2	49,3±1,2
6	10	21,0±0,7	18,4±1,1
7	6	25,6±0,2	16,2±1,4

20 min. Membrane-bound hemoglobin was isolated from the stroma obtained after hemolysis of the erythrocytes. The stroma was washed repeatedly with cold distilled water. Hemoglobin was obtained from the washed stroma by freezing and thawing 3 times in 0.5 M phosphate buffer, pH 7.4, containing 10 mM EDTA. The hemoglobin concentration was determined by Kushakovskii's method [4]. Preparative separation of the washed erythrocytes from blood plasma was carried out in a sucrose density gradient [5]. The relative percentages of the fractions were calculated from values of scattering of light by the cell suspension, measured on a spectrophotometer at 640 nm. The resistance of the hemoglobin preparations to the action of alkali was studied by recording changes in the absorption spectra. The hemoglobin was dissolved in 5 mM Tris-HCl buffer, pH 8.9, or 5 mM NaCl, equalized for optical density in the region of Cope's band up to 1.0. To 1.8 ml of the hemoglobin thus prepared 0.2 ml of 1 N NaOH was added; the sample was quickly mixed and subjected to photometry every 10 sec for 1 min, after which measurements were repeated at 2, 3, and 5 min. A Specord "UV-VIS" spectrophotometer was used. The rate of alkaline denaturation was determined by the equation

$$V = \frac{\Delta D_{420}}{\Delta t},$$

where V denotes the rate of alkaline denaturation; ΔD_{420} the change in optical density at 420 nm; Δt the change of time. The oxygenation properties of the hemoglobin were determined spectrophotometrically [8].

EXPERIMENTAL RESULTS

The data in Table 1 show that during development of the tumor the relative percentages of erythrocyte fractions, separated in the 14% sucrose zone (fraction No. 5), increased from 32.9% normally to 49.3%. The quantitative increase in this erythrocyte population was evidently connected with more rapid maturation of the erythroblasts and with the appearance of young erythrocytes in the blood stream, that were distributed in zones of lower density than the old cells.

The acid erythrograms of erythrocytes of intact mice and mice with Ehrlich's carcinoma, illustrated in Fig. 1, indicate that during development of the tumor young erythrocytes appear in the peripheral blood, and are characterized by high resistance to the hemolytic. It will be clear from Fig. 1 that the acid erythrogram of mice with Ehrlich's carcinoma is shifted to the right compared with that of intact mouse erythrocytes.

Assuming that the more rapid maturation of the erythrocytes is accompanied by hemoglobinization of their fetal form of hemoglobin, we studied the alkaline resistance of the hemoglobin of mice with Ehrlich's carcinoma. The study of the resistance of hemoglobin to the action of alkali showed that hemoglobin of mice with Ehrlich's carcinoma is more resistant to the action of alkali than hemoglobin from tumor-free mice. As the data in Table 2 show, the resistance to alkali of both free and membrane-bound hemoglobin was increased in mice with the tumor. Accelerated maturation of erythrocytes is evidently linked not only with hemoglobinization of the fetal form of hemoglobin, whose resistance to alkali is higher than that of the adult forms of the respiratory hemoprotein,but also with selective binding of the alkali-resistant form of the respiratory protein with the erythrocyte membrane. Since interaction between hemoglobin and membrane leads to clusterization of the band 3 proteins of the erythrocyte membrane and

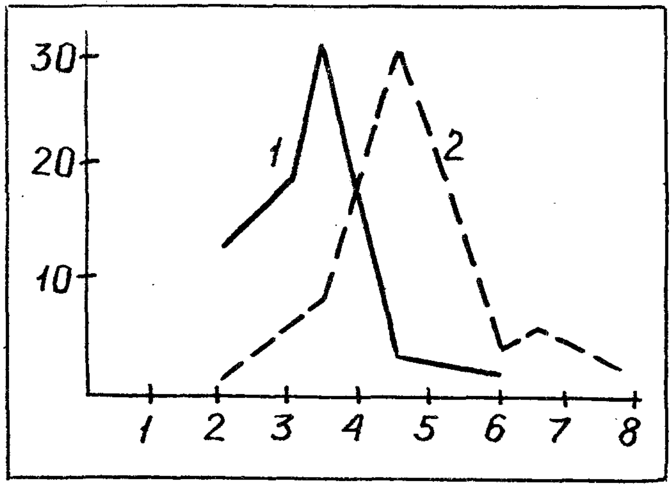

Fig. 1. Acid erythrograms: 1) of intact mice, 2) of mice with Ehrlich's carcinoma. Abscissa, duration of hemolysis (in min); ordinate, % of hemolyzed cells.

to subsequent recognition of these red cells by antibodies, it can be postulated that this is a basic mechanism of recognition of "scrapped" erythrocytes and their ingestion by macrophages [11].

The oxygen-binding properties of hemoglobin constitute its principal functional characteristic. In various diseases (diabetes, carcinoma of the stomach, lung cancer, etc.) marked changes take place in the oxygen-binding properties of hemoglobin [1, 3, 6]. We have shown that the oxyhemoglobin dissociation curve of mice with Ehrlich's carcinoma is shifted to the right: P_{50}, characteristically 33.3 ± 1.4 mm Hg for intact mice, is increased to 38.1 ± 1.9 mm Hg in mice with Ehrlich's carcinoma. Displacement of the oxygenation curve of hemoglobin into the region of high partial pressures of oxygen leads to better dissociation of the HbO_2 complex, and to the more complete giving up of oxygen to the breathing tissues, and enables P_vO_2 of mixed venous blood to be maintained, thereby facilitating pulmonary vasoconstriction. The acute decrease in the affinity of hemoglobin for oxygen is evidently one of the most important compensatory mechanisms enabling the oxygen supply to the tissues to be increased.

Meanwhile, not all erythrocytes respond adequately to changes in metabolic processes in the tissues, and consequently, to changes in oxygen demand. For instance, fraction 5 of erythrocytes, distributed in the 14% sucrose zone, is characterized by a tendency for affinity of hemoglobin for oxygen to be increased. P_{50} of hemoglobin separated from these erythrocytes was reduced from 28 ± 0.9 mm Hg in intact animals to 222 ± 0.7 in animals with Ehrlich's carcinoma. The increase in the affinity of hemoglobin of this blood fraction for oxygen was evidently linked with more rapid hemoglobinization of the hemoglobin of fetal type in this population of red cells, with comparatively high affinity for oxygen, although in fact this is characteristic of developed hypoxia [2].

Characteristically, the same rule is observed for membrane-bound hemoglobin of total blood as for erythrocytes of fraction No. 5. On inoculation of mice with Ehrlich's carcinoma the affinity of hemoglobin bound with the erythrocytic membrane for oxygen increases compared with that of membrane-bound hemoglobin of intact mice. The fraction of hemoglobin bound with the red cell membrane is about 3% of the total hemoprotein. This interaction is electrostatic in character, and the principal binding site is glycophorin. Analysis of data in the literature [12] leads to the conclusion that membrane-bound hemoglobin performs several functions: facilitation of the oxygen flow through the lipid phase of the membrane may affect the gas exchange of O_2 and CO_2 taking place through the membrane, and by transformation into hemochrome, after binding with and free protein, and forming clusters in the erythrocyte membrane, it facilitates the formation of binding sites with receptors of macrophages eliminating old erythrocytes from the blood stream. The increase in affinity of membrane-bound hemoglobin for oxygen which we found in mice with Ehrlich's carcinoma can be regarded as an additional factor in compensatory reactions of the oxygen transport system of the blood in response to developing hypoxia in the tissues, associated with growth of malignant tumors, responsible for easier diffusion of O_2 through the erythrocyte membrane.

TABLE 2. Kinetics of Alkaline Denaturation of Mouse Hemoglobin (Hb) at pH 11.8 (M ± m)

Test object	V, 1 × 10^{-4} sec^{-1}
Soluble Hb of intact mice	4,15±0,90
Membrane-bound Hb of intact mice	4,75±0,76
Soluble Hb of mice with Ehrlich's carcinoma	3,33±0,88
Membrane-bound Hb of mice with Ehrlich's carcinoma	3,92±0,90

The changes observed indicate that changes enabling optimization of the respiratory function of the blood through high oxygen saturation in the alveoli of the lungs take place in the heterogeneous blood system. In this way the conditions are created for compensation of the respiratory function of the blood, when lowering of affinity for oxygen is characteristic of a larger proportion of erythrocytes.

By virtue of their functional properties, an average function is formed from a heterogeneous population of red blood cells, which by and large ensures the accurate tuning of the oxygen transport system to enable it to exhibit its compensatory reserves, and to create a normal gas supply to the tissues should a pathological condition develop.

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