cal investigation the morphological pattern of the renal lesion in the experimental group differed from the control in the presence of acute seroproliferative glomerulonephritis. The renal glomeruli were enlarged because of proliferation of mesanginal cells and accumulation of Shumlyanskii's serous exudate in the mesangium and cavity of the capsule, accompanied by cells of desquamated epithelium in some glomeruli. Cloudy-swelling and, in some areas, balloon hydropic dystrophy developed in the convoluted tubules, accompanied by narrowing of the lumen of the tubules. In rats receiving renalin, acute productive glomerulonephritis also developed but was characterized by less marked proliferation of mesangial cells and absence of balloon hydropic dystrophy.

The results are thus evidence that renalin, an alkaline polypeptide from the kidney, can prevent the further development of Masugi nephritis.

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SERUM AND PLASMA ERYTHROPOIETIC DETERMINATION IN MICE WITH PHENYLHYDRAZINE ANEMIA

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KEY WORDS: Erythropoietin, mouse serum and plasma, anemia, method of testing.

Erythropoiesis in vivo is regulated with the participation of the plasma hormone erythropoietin [1]. The study of its properties and the mechanism of its action and synthesis in the body and determination of its concentration in biological fluids cannot be undertaken without the use of adequate methods of assessment of activity of the hormone. Many methods of analysis of erythropoietic activity have been described, but many of them suffer from various disadvantages: low sensitivity, laboriousness, the need to have large quantities of the erythropoietin standard, and sensitivity to impurities.

A micromethod requiring at least 5-10 times smaller doses of erythropoietin than the majority of methods hitherto used has recently been developed [9], and is based on recording incorporation of ^{3}H-thymidine into DNA of erythroid cells under the influence of erythropoietin. In the present investigation a modified method of Krystal [9] was used to determine erythropoietic activity of erythropoietin-enriched mouse serum and plasma. Since the use of any method requires an erythropoietin standard, availability of which is limited, we assessed the possibility of using the serum or plasma of a mouse with phenylhydrazine anemia as the source of erythropoietin, inducing equivalent stimulation of proliferative activity of erythroid cells.

EXPERIMENTAL METHOD

Female CBA and (CBA × $C_{57}BL)F_1$ mice weighing 20-30 g were used in the experiments. Anemia was induced by intraperitoneal injection of phenylhydrazine hydrochloride (PH) in isotonic solution neutralized with NaOH, on two successive days in a dose of 60 mg/kg (0.2-

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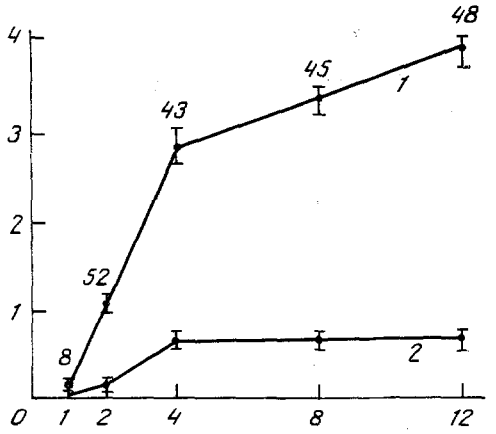

Fig. 1. Dependence of incorporation of ^{3}H-thymidine on cell concentration in culture. Numbers on graph indicate coefficient of proliferative activity. 1) Culture stimulated by anemic serum; 2) control culture without stimulation. Abscissa, cell concentration (in ml·10^6); ordinate, radioactivity (in cpm·10^{-5}).

0.3 ml per mouse). A suspension of spleen cells in α-MEM medium with the addition of 1-2% embryonic calf serum (ECS) was prepared 3 days after the 2nd injection of PH. To obtain a homogeneous suspension, the cell suspension was passed through a nylon filter, washed twice in medium, and the number of nucleated cells was counted. In PH-induced anemia more than 90% of the spleen cells are erythroid [7, 9]. From each spleen on average (5-7) × 10^8 cells were obtained.

The cells were cultured in α-MEM medium without ribonucleosides and deoxyribonucleosides, with the addition to 10-20% ECS, 0.2 mM glutamine, 0.1 mM 2-mercaptoethanol, 20 mM HEPES buffer, and 100 μg/ml of kanamycin sulfate. The cell suspension (in concentrations stated in the text) was seeded into round-bottomed 96-well Linbro plates in a dose of 90 μl per well, to each well 10 μl of test material (solution of erythropoietin, serum or plasma) or α-MEM medium was added, and the plates were cultured at 37°C in an incubator with 5% CO_2 and 100% humidity. After 22-24 h, the cell culture was treated with ^{3}H-thymidine (1 μCi in 10 ml medium per cells) and incubation was continued for 2 h. At each experimental point, 3 to 5 wells were used. After the end of incubation with ^{3}H-thymidine the plates were put on ice and the cells harvested on "Millipore" nitrocellulose filters (pore diameter 0.45 μ, type HA, diameter 25 mm) with the aid of a 30-well vacuum cell harvester. The filters were washed consecutively with isotonic solution (5 ml), and 7.5% TCA (5 ml) and fixed in 96% ethanol (2 ml). The filters, dried in air overnight, were counted on a liquid scintillation beta-counter. The intensity of proliferative activity was estimated as the number of counts per minute.

To prepare erythropoietin-enriched serum or plasma, mice were irradiated on a gamma-ray source in a dose of 6 rads simultaneously, and next day they were injected with a standard dose of PH [10]. Blood was taken 7 days after injection of PH. The serum or plasma obtained was sterilized by filtration, decanted into 1-ml samples, and kept at −20°C.

EXPERIMENTAL RESULTS

To obtain the maximal response to the stimulating action of erythropoietic activity, and also to increase specificity of the method of testing of erythropoietin used, the effect of the conditions of culture and the kinetics of the response of spleen cells from mice with PH-induced anemia was investigated.

The results of one experiment to determine the optimal concentrations in culture of cells obtained from the spleen of mice 3 days after receiving an injection of PH, in response to stimulation by erythropoietin-enriched mouse serum, are shown in Fig. 1; the serum was diluted in α-MEM medium and added to the culture in a volume of 10%. With an increase in the concentration of cells in culture from 10^6 to 12 × 10^6/ml the intensity of incorporation of ^{3}H-thymidine increased, but the ratio of proliferative activity of the cells in stimulated and unstimulated (control) cultures, i.e., the coefficient of proliferative activity (CPA), showed very little change in a range of cell concentrations from 2 × 10^6 to 8 × 10^6/ml. The

TABLE 1. Dependence of Proliferative Activity of Spleen Cells of Anemic Mice on ECS Concentration (M ± m)

ECS concentration, %	Addition of anemic serum	Incorporation of ^{3}H-thymidine, cpm	CPA	Incorporation of ^{3}H-thymidine, cpm	CPA
		expt. I		expt. II	
5	—	8 019±102	25	911±30	16
	+	205 681±35 652		14 829±1 237	
10	—	14 889±285	14	746±100	27
	+	269 478±50 265		20 681±4 288	
20	—	11 550±527	33	949±472	25
	+	382 003±25 694		23 823±3 961	
30	—	15 597±1 467	20	500±36	22
	+	313 740±27 152		11 000±533	
40	—	14 687±324	22	1 484±92	24
	+	332 859±18 956		36 000±2 907	

TABLE 2. Dependence of CPA on Duration of Incubation of Spleen Cells from Anemic Mice with Test Material

Duration of incubation of cells with test material, h	Time after injection of PH, days	CPA during stimulation by anemic serum (I, II) or anemic plasma (III)		
		expt. I	expt. II	expt. III
16	3	10	18	21
	4	56	52	30
24	2	5	6	—
	3	26	87	80
	4	12	48	20
28	4	13	—	—
40	3	1	1	3
	4	1	1	2

use of cells fractionated beforehand in a Ficoll–Verografin gradient (d = 1.077 g/cm^3) gave no advantage over cultures of unfractionated cells; the value of CPA was 26 for fractionated cells and 45 for unfractionated.

Optimal concentrations of different sera for short-term culture of spleen cells from anemic mice also were studied. Bovine serum and calf serum did not maintain growth of the cells in culture. Meanwhile, in medium containing different series of ECS or human serum, the culture responded to the stimulating action by high proliferative activity.

The results of two experiments to determine the optimal concentration of ECS in the culture medium are given in Table 1. When ECS was used in concentrations of between 5 and 40%, CPA in the spleen cell cultures did not change significantly, and accordingly in subsequent investigations, ECS was added to the culture medium in a concentration of 10-20%.

Table 2 gives results reflecting proliferative activity in a culture of spleen cells obtained 2, 3, and 4 days after injection of PH into the mice, depending on the duration of incubation of the cells with the test material. For cells taken from spleens 3 days after the second injection of PH the duration of incubation with the test material ought to be about 24 h, whereas for cells obtained after 4 days a shorter period of incubation is preferable, namely 16 h. Lengthening the incubation times leads to depression of the response of the culture to the stimulating action of erythropoietic activity.

On the basis of the results, spleen cells of mice 3 days after the last injection of PH were used in a concentration of (2-4) × 10^6 cells/ml to test erythropoietic activity in erythropoietin-enriched mouse serum and plasma. After incubation for 24 h in medium containing 10% ECS, 1 μCi of ^{3}H-thymidine was added to each well for 2 h; increasing the duration of incubation of the cells with the label to 4 h caused virtually no change in recorded activity of the isotope.

The character of the proliferative activity of the culture of mouse spleen cells after injection of PH, under the influence of different doses of anemic serum or plasma is shown in Fig. 2. It will be clear from the data presented that the proliferative activity of the cells increased as a linear function with an increase in concentration of the stimulator in

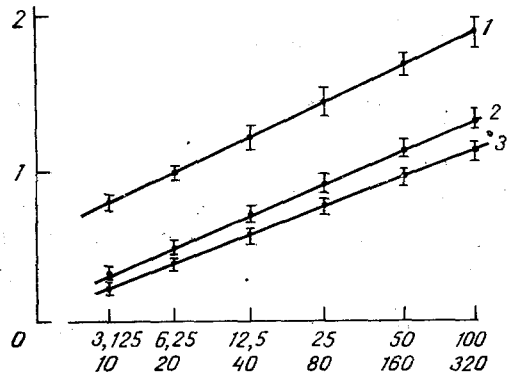

Fig. 2. Dependence of ^{3}H-thymidine incorporation on concentration of erythropoietic activity in culture medium. 1) Culture stimulated by plasma from anemic mice; 2) culture stimulated by serum from anemic mice; 3) culture stimulated by erythropoietin standard. Abscissa, concentration of serum or plasma (in μl/ml); of erythropoietin (in IU/ml of culture). Ordinate, radioactivity (in cpm·10^{-5}).

the medium. Absolute values of incorporation of ^{3}H-thymidine into cells of the culture varied from one experiment to another, but the shape of the curve was unchanged and the ratio of the number of counts recorded in the stimulated culture and the number in the control culture (unstimulated), i.e., CPA, remained between 40 and 80. Dependence of incorporation of ^{3}H-thymidine into spleen cells on the dose of erythropoietin added was similar in character (Fig. 2). When an erythropoietin preparation with activity of 5 U/ml protein was used, it was shown on the basis of linear regression equations that the activity of the anemic serum was equivalent to 7, and that of plasma to 15 U/ml.

A simple micromethod of determination of erythropoietic activity, based on incorporation of ^{3}H-thymidine into dividing hematopoietic cells in short-term suspension cultures has thus been suggested for the determination of erythropoietic activity. The use of erythroid cells from the spleens of anemic mice as target cells enabled many cells responding to erythropoietin stimulation to be obtained and several scores of samples to be tested simultaneously with minimal expenditure of test material. The method is specific and is more sensitive than other methods of biological testing [2, 6, 9]; unlike other methods based on incorporation of ^{59}Fe into heme of cultured hematopoietic cells, the results are independent of the constant of iron, transferrin, and other substances in the test material [3-5, 8]. The level of erythropoietic activity which can be determined is 5-300 IU/ml, which corresponds to the sensitivity of methods of testing erythropoietin in vitro.

The method used in this investigation can be applied for determination of the level of erythropoietic activity in biological fluids, including patients' serum and plasma, if erythropoietin-enriched mouse serum or plasma is used as the erythropoietin standard. To obtain concrete values of the level of erythropoietic activity in unknown samples, in each experiment appropriate dose-effect curves for the erythropoietin standard or for erythropoietin-enriched serum must be plotted.

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