Biological Activity of Hemoglobin-Containing Complex Isolated from Blood Serum of Mice with Ehrlich Carcinoma

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Injection of hemoglobin-containing complex of serum proteins isolated from animals with Ehrlich carcinoma led to regression of intraperitoneally and intramuscularly transplanted Ehrlich carcinoma in male C57Bl/6 mice. The hemoglobin-containing complex of serum proteins disturbed cycle distribution of Ehrlich carcinoma cells and caused apoptosis of about 34.3% tumor cells. Addition of hemoglobin-containing serum protein complex into Ehrlich carcinoma incubation medium did not lead to the death of tumor cells and even slightly increased their proliferation.

Key Words: *hemoglobin; serum proteins; Ehrlich carcinoma; apoptosis*

The formation of a specific tissue peptide pool with biological activity was hypothesized [9]. The serum contains proteins (for example, haptoglobin) forming a complex with hemoglobin. This complex modifies the pathway of hemoglobin metabolism and the formation of tissue peptide pool, especially in tissues and organs where hemoglobin is later destroyed. It is also possible that hemoglobin metabolism in animals with tumor differs from that in intact animals. This difference can be due to changes in protein glycosylation during tumor growth [3].

We studied the effects of hemoglobin-containing serum protein complex (HSC) on tumor cell proliferation and lifespan of animals with transplanted Ehrlich carcinoma (EC).

MATERIALS AND METHODS

The study was carried out on male C57Bl/6 mice from Stolbovaya Breeding Center (Moscow region). Ascitic EC (aneuploid ELD strain, Tumor Strain Bank, N. N. Blokhin Cancer Research Center) was transplanted intraperitoneally or intramuscularly into the right thigh (10^6 cells in 0.1 ml RPMI-1640).

Hemoglobin-containing serum complex was isolated from the serum of mice with intramuscularly transplanted EC [4]. Proteins forming the complex with hemoglobin were identified by MALDI-TOF mass spectrometry [8]. The complex included proteins with molecular weights of 100, 68, 65, and 15 kDa identified as haptoglobin, serum albumin, gi|26341396 nameless protein (*Mus musculus*) homologous to α-fetoprotein, and α-hemoglobin, respectively.

Analysis of EC cell ploidy was carried out using flow cytofluorometry (FACS analysis) on a FACScan flow cytometer (Becton Dickinson). The mice with intraperitoneally transplanted EC were injected with HSC or serum (100 μg in 0.4 ml saline) 7 days after transplantation. Aliquots of ascitic fluid were collected 24 h after injection, washed by 10-min centrifugation at 800*g* in saline, and fixed in 70% ethanol at -20°C. The cells were resuspended in saline; propidium iodide was added to a final con-

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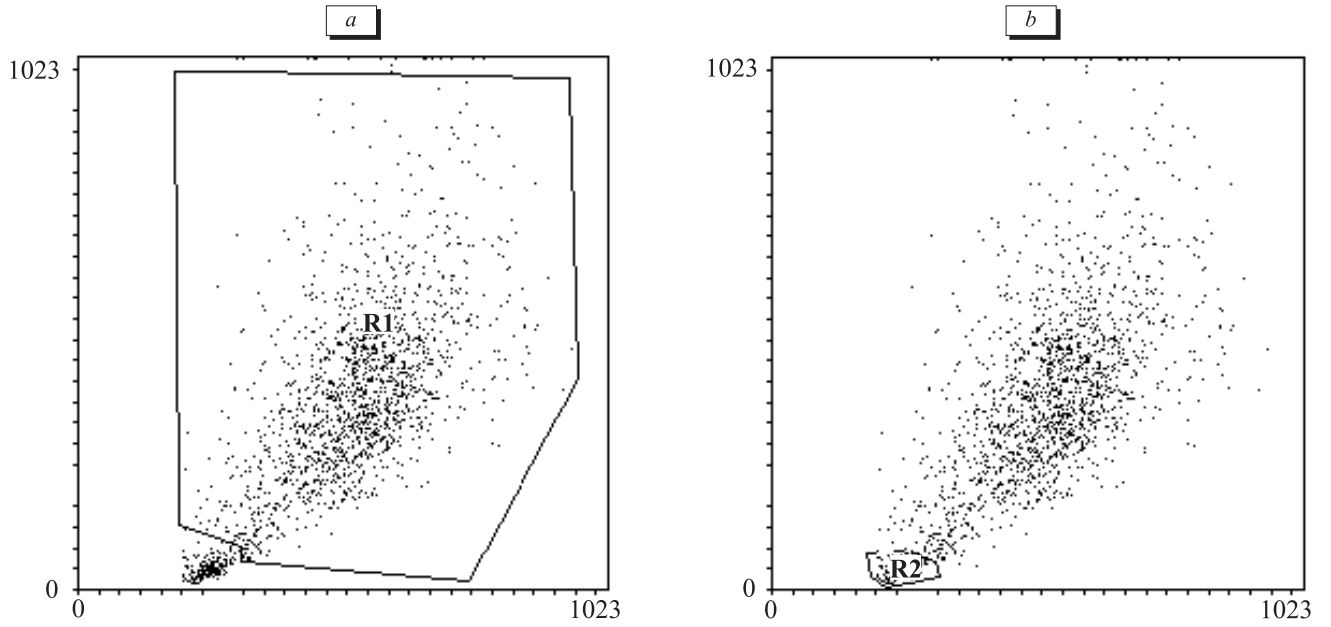

Fig. 1. Dot-blot analysis of ascitic fluid cells from mice with EC. *a*) gate R_1 distinguishes large cell population; *b*) gate R_2 distinguishes small peritoneal cell population. Abscissa: frontal light scattering of cells; ordinate: lateral light scattering of cells.

centration of 1 μg/ml. The gate of cell population was determined by combining direct and lateral light scattering with consideration for cell size. A total of 10,000 events per gate were counted. The data were statistically processed using WINMDI 2.8 software.

In order to evaluate cytotoxicity of HSC, ascitic EC cells (10^4 in 1 ml) were cultured in RPMI-1640 with 10% FCS, 2 mM glutamine, 5000 U/ml streptomycin and 5000 U/ml penicillin in flat-bottom 96-well microplates (Costar) for 18 h in the presence of 100 μg/ml HSC or serum. After culturing, MTT vital dye (Sigma) was added to wells. The resultant diformazan was eluted from cell membranes with dimethyl sulfoxide. The result (percent of cytotoxicity) was evaluated by spectrophotometry at λ=540 nm by optical density measured on a multiscan (Labsystem) [5].

For evaluation of the effect of HSC on animal lifespan, the mice with intraperitoneally or intramuscularly transplanted EC received 5 injections HSC or serum (100 μg/mouse, in 0.4 ml saline) at 48-h intervals. Each group consisted of 8 animals. The data were statistically processed using Fisher-Student's method. The differences were significant at $p<0.05$.

RESULTS

The parameters of lateral and frontal light scattering of ascitic EC cells are presented in Fig. 1. As is seen from the figure, the entire cell population is divided into 2 groups differing by cell size. Gate R_1 distinguishes a population of large cells (carcinoma cells) [3], while gate R_2 distinguishes smaller cells (mononuclear leukocytes, Fig. 1, *b*).

Analysis of EC cell ploidy (Fig. 2) showed that EC cells in animals receiving 100 μg serum contained DNA characteristic of aneuploid chromosome set and were distributed by the cell cycle (Fig. 2, *a*). In 2.3% cells the content of DNA was below diploid. After treatment with 100 μg HSC the distribution of EC cells by the cycle was disturbed and DNA content was below diploid in about 34.3% tumor cells (Fig. 2, *b*), which attested to cell death (apoptosis).

Analysis of mononuclear leukocyte population ploidy showed that the cells contained a diploid DNA set irrespective of protein treatment (Fig. 2, *c*, *d*). The differences in the counts of cells with DNA content less than diploid after treatment with

TABLE 1. Effect of HSC on the Lifespan of Mice with Transplanted EC (*M*±*m*)

Treatment		Mean lifespan of mice, days
EC	intraperitoneally	19.8±1.2
	+serum	20.2±1.4
	+HSC	>365
EC	intramuscularly	59.6±1.7
	+serum	60.1±1.5
	+HSC	>365

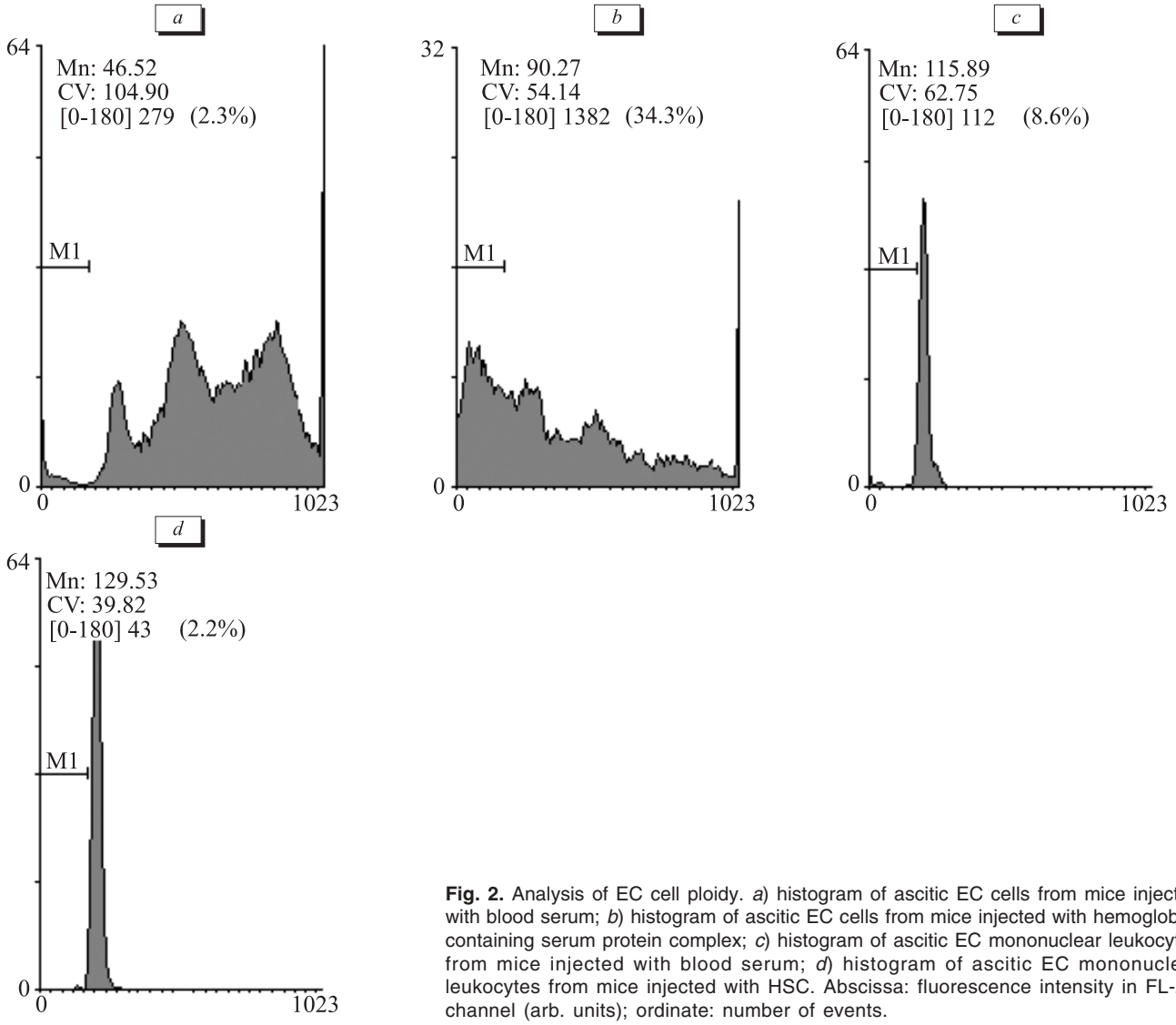

Fig. 2. Analysis of EC cell ploidy. *a*) histogram of ascitic EC cells from mice injected with blood serum; *b*) histogram of ascitic EC cells from mice injected with hemoglobin-containing serum protein complex; *c*) histogram of ascitic EC mononuclear leukocytes from mice injected with blood serum; *d*) histogram of ascitic EC mononuclear leukocytes from mice injected with HSC. Abscissa: fluorescence intensity in FL-2H channel (arb. units); ordinate: number of events.

serum and HSC were negligible (8.6 and 2.2%, respectively).

The study of HSC effect on EC cell viability *in vitro* showed that addition of HSC to the incubation medium did not lead to cell death, but slightly (by 20%) increased cell proliferation. On the other hand, injection of HSC to mice with EC transplanted intraperitoneally or intramuscularly led to complete regression of the tumor (Table 1). Cured animals were resistant to repeated transplantation of the tumor even after transplantation of a greater number of cells (40×10^6/mouse). Autopsy showed no signs of tumor process.

Hence, injection of HSC containing a protein homologous to α-fetoprotein to animals with transplanted EC led to tumor regression without relapses and metastases characteristic of this experimental model [1]. HSC exhibited no direct cytotoxic effect *in vitro*, while its injection to animals caused apoptosis of more than 30% tumor cells. The presence of humoral factors modifying tumor growth in mouse serum was shown previously [2,7]. Our results are in line with previous data on the effects of hemoglobin degradation products and α-fetoprotein inducing apoptosis of tumor cells [6,11]. Presumably, one of the humoral factors, participating in tumor growth, is the complex of the studied proteins. We can conclude that the effect of HSC on the tumor is indirect, because the complex exhibited no cytotoxicity *in vitro*. The described phenomenon of tumor regression without cytotoxic effect is reproduced in experiments on mice with intraperitoneally and intramuscularly transplanted EC. Further thorough studies of this phenomenon are needed, including studies on other experimental models.

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