TUMOR CELL IMMUNOSUPPRESSOR FACTORS ARE SPECIES-NON-SPECIFIC IN ACTION AND DO NOT COMPETE WITH RECOMBINANT INTERLEUKIN-2 AND PHYTOHEMAGGLUTININ FOR LYMPHOCYTE MEMBRANE RECEPTORS

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The immunosuppressor action of tumor cells is considered one of the main causes of the inefficacy of immunity in tumor growth [6]. This action may be determined by immunosuppressor factors (ISF) – humeral products secreted by tumor cells into the culture medium in vitro or in vivo [6]. We showed previously that ISF of cells of mouse mastocytoma P 815 and leukemia EL-4 suppress many functions of cellular immunity of mice in vitro [3] and the graft versus host reaction in vivo [4]. The fundamental mechanisms of the action of ISF on cells of the immune system remain completely unstudied. Only the phenomenon of membrane toxicity of ISF has been demonstrated [3].

The aim of this investigation was to determine whether ISF can compete for receptors to a mitogen or to interleukin-2 (IL-2) on the surface of the lymphocyte membrane, whether ISF possess species-specificity of action, and whether they act on already activated lymphocytes.

EXPERIMENTAL METHOD

Human peripheral blood mononuclears were isolated from blood from healthy individuals on a Ficoll–Hypaque gradient ("Flow Laboratories"). Whole blood taken from the finger (0.2 ml to 2 ml of complete medium) was used in some experiments. BALB/c mice, male and female aged 2-3 months, were obtained from the "Stolbovaya" Inbred Animals Nursery, Academy of Medical Sciences of the USSR, and kept on a high-caloric diet. The ISF used consisted of 3-day cultural supernatants of the following tumor cell lines: a) mouse mastocytoma P 815, leukemia EL-4, melanoma B-16, and transformed fibroblasts L-929; b) human erythromyeloleucosis K-562. The supernatants did not contain mycoplasmas, as was shown by the absence of incorporation of ^{3}H-thymidine after culture for 72 h [5]. The blast-transformation reaction (BTR) with concanavalin A (con A, "Flow Laboratories," final concentration 5 μg/ml) or with phytohemagglutinin (PHA, "Difco" final concentration 5 μg/ml) was set up as follows. Peripheral blood mononuclears from normal blood donors or spleen cells of BALB/c mice were suspended in medium RPMI-1640 with 10% fetal calf serum, 2 mM L-glutamine (all reagents from "Flow Laboratories"), and gentamicin 50 μg/ml, and distributed 0.1 ml at a time into wells of 96-well round-bottomed plates ("Kostar"), at the rate of $2 \cdot 10^5$/well. In the case of whole blood, 0.1 ml of a 10% solution of blood was added to each well. The mitogens and ISF were added in a volume of 50 μl. The plates were incubated at 37°C and in an atmosphere with 6% CO_2 for 72 h. ^{3}H-thymidine (specific radioactivity 25 Ci/mmole) was added to each well in a dose of 1 μCi 4 h before the end of culture. The cells were transferred to filters with the aid of a harvester ("Flow Laboratories"). Radioactivity was counted on a Beta-spectrometer. Tests with each version of the lymphocytes

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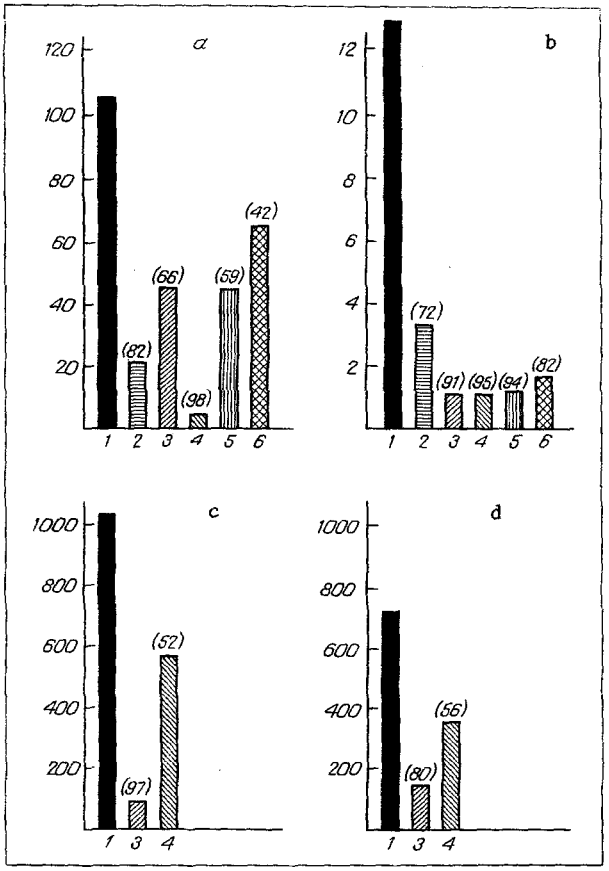

Fig. 1. Species nonspecificity of action of ISF of tumor cells. Ordinate, index of stimulation of proliferation (ratio of value of cell proliferation with mitogen to spontaneous proliferation) of isolated human peripheral blood mononuclears (a, b) or present in whole blood (c, d). PHA (a, c, d) or con A (b) was used as mitogen. Medium (1) or ISF of cells EL-4 (2), B 16 (3), P 815 (4), L-929 (5), and K-562 (6) were added to the cells. II of proliferation given in parentheses.

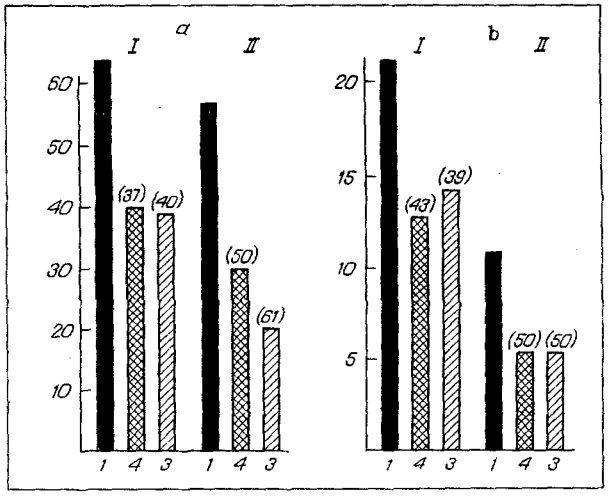

Fig. 2. Action of ISF of mouse tumor on human blood mononuclears, pretreated with PHA (absence of competition for receptors to PHA). Mononuclears isolated were pretreated for 2 h in vitro with medium (I) or with PHA 50 μg/ml (II) at 4°C (a) or at 37°C (b) and their proliferation in response to PHA (5 μg/ml) was estimated. Remainder of legend as to Fig. 1.

TABLE 1. Absence of Competition for Receptors to IL-2 (I) and of Inhibition of Proliferation of Lymphocytes Activated by con A in vivo (II) during the Action of ISF from P 815 Cells ($M \pm m$)

Series of experiments	Pretreatment of spleen cells		Incorporation of ^{3}H-thymidine		
			ISF		
	4 °C	37 °C	medium	1:4	1:40
I	—	—	30 050±2115	7 257±773 (76)	16 516±597 (45)
	Medium	—	38 308±2976	7 640±261 (80)	20 281±52 (47)
	IL-2	—	22 985±424	6 104±517 (73)	14 326±1070 (37)
	—	IL-2	16 890±451	5 743±274 (66)	10 409±629 (38)
	ISF 1:4	—	2 059±488 (94)	n.d.	n.d.
II	Medium	—	68 029±1874	75 206±7554	n.d.
	ISF 1:4	—	74 958±2295	n.d.	n.d.

Legend. I) Proliferation in response to action of IL-2 for 72 h, II) spontaneous proliferation in course of 4 h. Percentage of inhibition given in parentheses. Significance of difference between control group and addition of ISF > 99%. Here and in Table 2, results of one typical experiment from three series of experiments; n.d.) not done.

TABLE 2. Functional Heterogeneity of ISF ($M \pm m$)

Pretreatment followed by washing at 4°C	Incorporation of ^{3}H-thymidine in presence of con A, CPM				
	medium	1:4	1:40	1:400	1:4000
Without treat.	67 274±1721	9 012±294(86)	52 065±5017(22)	81 900±4315*	77 353±5775
Medium	71 005±3230	12 156±45(83)	n.d.	n.d.	n.d.
ISF					
1:400	76 829±1410**	13 108±1227(83)	n.d.	n.d.	n.d.
1:4000	87 117±3918***	12 686±1510(84)	n.d.	n.d.	n.d.

Legend. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.02$.

were carried out in triplicate. As a first step in some experiments the cells were treated with PHA (50 μg/ml), recombinant IL-2 ("Biogen," 500 U/ml), or ISF 1:4 at 4 or 37°C for 2 h, followed by washing 3 times. To determine the ability of ISF to compete for receptors to IL-2 the experimental model described by the writers previously [1] was used. Spleen cells were obtained from BALB/c mice, into which con A in a dose of 200 μg had been injected intravenously 18 h beforehand. These cells had acquired the ability to proliferate intensively in response to stimulation by IL-2 and they were characterized by a high spontaneous proliferative response. In the first case, splenocytes ($2 \cdot 10^5$/well) were cultured with IL-2 (500 U/ml) for 72 h, with the addition of ^{3}H-thymidine 4 h beforehand, whereas in the second case ^{3}H-thymidine was added to the splenocytes at once and the cells were transferred to filters after culture for 4 h at 37°C in an atmosphere with 6% CO_2. The index of inhibition (II) of proliferation during the action of ISF was calculated by the formula:

$$II = 1 - \frac{\text{incorporation of } ^3\text{H-thymidine in wells with ISF}}{\text{incorporation of } ^3\text{H-thymidine in wells with medium}} \cdot 100\%.$$

the results were subjected to statistical analysis by Student's t-test.

EXPERIMENTAL RESULTS

In the experiments of series I the species-specificity of action of ISF was studied. The results show that ISF of mouse tumors inhibits the BTR of human mononuclears in vitro (Fig. 1). The model of testing ISF in the BTR with whole blood proved to be convenient and sufficiently reproducible: ISF from two donors gave a comparable effect (Fig. 1c, d). Thus the ISF tested exhibit species-specificity of action. The question arises of the mechanism of action of ISF on cells of the immune system. At least three possibilities can be tentatively suggested: 1) ISF interact with receptors on the membrane, covering access to activating molecules such as mitogens, alloantigens, and so on; 2) ISF interact with receptors on the membrane that differ from receptors for activating molecules; 3) ISF act, not at the membrane level, but by penetrating into the cell and disturbing its me-

tabolism. We demonstrated this mechanism previously [3]. In the present study we investigated the first possibility. Pretreatment of peripheral blood mononuclears with a large dose of PHA at 4 or 37°C for 2 h, followed by careful washing, leading to attachment of the mitogen to the corresponding receptors, did not abolish or did not even reduce the suppressor action of ISF of P 815 and B 16 cells on the BTR (Fig. 2). Pretreatment of mouse splenocytes, expressing receptors to IL-2 [2], activated with con A in vivo in the same way with recombinant IL-2 likewise did not abolish subsequent suppression of their proliferative response to IL-2 on the addition of ISF of P 815 cells (Table 1, I). This elates with data [7] according to which ISF of glioblastomas (β growth transforming factor) does not suppress T-cell proliferation without disturbance of interaction of IL-2 with its own receptors. It is important to note that pretreatment of splenocytes with ISF in the cold for 2 h followed by triple washing was accompanied by the same inhibition of the proliferative response to IL-2 as in the case of the presence of ISF in the culture for 72 h. This is evidence of the high affinity of interaction of ISF with unknown receptors on the cell surface. In addition, the results of pretreatment with ISF are evidence against the possibility of direct interaction of ISF with the mitogen or IL-2, which could depress the immune response due to a decrease in concentration of the stimulator.

ISF of P 815 cells did not affect proliferation of already activated splenocytes, whether the cells were pretreated with it or after its addition to the culture (Table 1, II). In the course of the experiments we noted that in high dilutions the cultural supernatants of the tumor cells had a stimulating, and not an inhibitory, action on lymphocyte proliferation in the BTR. The question arises whether the same substance may have a suppressor and activating effect, or whether these are different substances; moreover, the action of the latter was not exhibited because of the stronger effect of the suppressor factors in low dilutions of the supernatant. To answer this question, we pretreated mouse spleen cells with ISF from P 815 cells in high dilutions at 4°C for 2 h, and then washed the cells 3 times and set up the BTR in the presence of low dilutions of ISF (1:4). It will be clear from Table 2 that pretreatment of the splenocytes with high dilutions of ISF in the cold led to subsequent and equally significant strengthening of their response in BTR, just as when ISF was present in the culture for 72 h. Consequently, activating factors act by attachment to receptors on spleen cells. ISF in low dilutions, however, suppressed proliferation both of cells pretreated with activating factors and of cells not pretreated (or pretreated with ordinary culture medium, II = 83-86%), just as actively. It can be tentatively suggested that the activating and suppressor components of ISF are attached to different receptors, i.e., that they are different substances. A more penetrating study of this problem is necessary. We showed previously that ISF of P 815 cells are glycoproteins differing in molecular weight [3]. The aim of future research will be to determine the more detailed biochemical characteristics of the suppressor and activating components of the supernatant of tumor cells and to examine the mechanism of action of ISF on the different biochemical mechanisms of activation of cells of the immune system.

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