CAN THE ACTION OF TUMOR CELL IMMUNOSUPPRESSION FACTORS BE OVERCOME?

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One of the main causes of ineffectiveness of the immune response with tumors is considered to be the immunosuppressor and suppressor-inducing activity of tumor cells [12]. Humoral products of tumor cells, namely immunosuppressor factors (ISF) — have been obtained in several laboratories and partially characterized. The present writers showed previously that ISF inhibit many functions of cellular immunity in vitro: proliferation of lymphocytes induced by alloantigen or lectin, generation of killer T cells, and production of interleukin-2 (IL-2) and tumor necrosis factor (TNF) [7]. In addition, ISF suppress transplantation immunity when injected into animals and, in particular, the "graft versus host" reaction, and they potentiate tumor growth [7]. Glycoconjugates [7], retrovirus protein p15E [12], and transforming growth factor β (TGF_β) [15] are all ISF. The mechanism of action of ISF on cells of the immune system, however, has not yet been explained. ISF of murine mastocytome P815 contain TGF_β and a macrophage deactivating factor (MDF). Both these factors disturbed activity of protein kinase C cofactor or the ability of protein kinase C to be translocated from the cytosol into membrane fractions of macrophages in response to phorbol diesters. Unlike TGF_β, MDF reduced the affinity of NADPH oxidase for NADPH [17]. The suppressor effect on T on cytokine production was not due to prostaglandins or to an increase in the intracellular cAMP concentration, and was exhibited at the translation level [5]. One approach to the elucidation of the mechanism of action of ISF is to try to overcome their inhibitory effect with the aid of immunostimulators and substances with a known mechanism of action on the cell.

The aim of this investigation was to study the possibility of overcoming the action of the immunodepressor factors of tumor cells. For this purpose we used lipopolysaccharide (LPS), muramyl dipeptide (MDP), and IL-2, as well as the phorbol ester of 12-0-tetradecanoylphorbol-13-acetate (TPA), a protein kinase C activator, ionomycin — a calcium ionophore, and contrykal — an inhibitor of serine-dependent proteolytic enzymes.

METHODS

Tumor cells of murine mastocytma P815 and melanoma B16, maintained by passage in vitro, were used in the experiments. The ISF consisted of 24-h cultural supernatants of B16 cells and 72-h supernatants of P815 cells. To obtain the supernatants the B16 cells were cultured in an initial concentration of $1 \cdot 10^6$/ml, P815 cells in a concentration of $2 \cdot 10^5$/ml. Viability of the tumor cells at the beginning and end of culture exceeded 95% (typan blue test). The cells were cultured in medium RPMI-1640 (Flow Laboratories) with 2 mM L-glutamine (Flow) and 50 μg/ml gentamicin. The resulting supernatants and ascites fluid were passed through filters with a pore diameter of 0.22 (Millipore), and aliquots were obtained and kept at —20°C. The ISF did not contain mycoplasmas, as shown by absence of incorporation of ^{3}H-thymidine into the supernatants [9]. To set up the blast-transformation reaction (BTR) and a mixed lymphocyte

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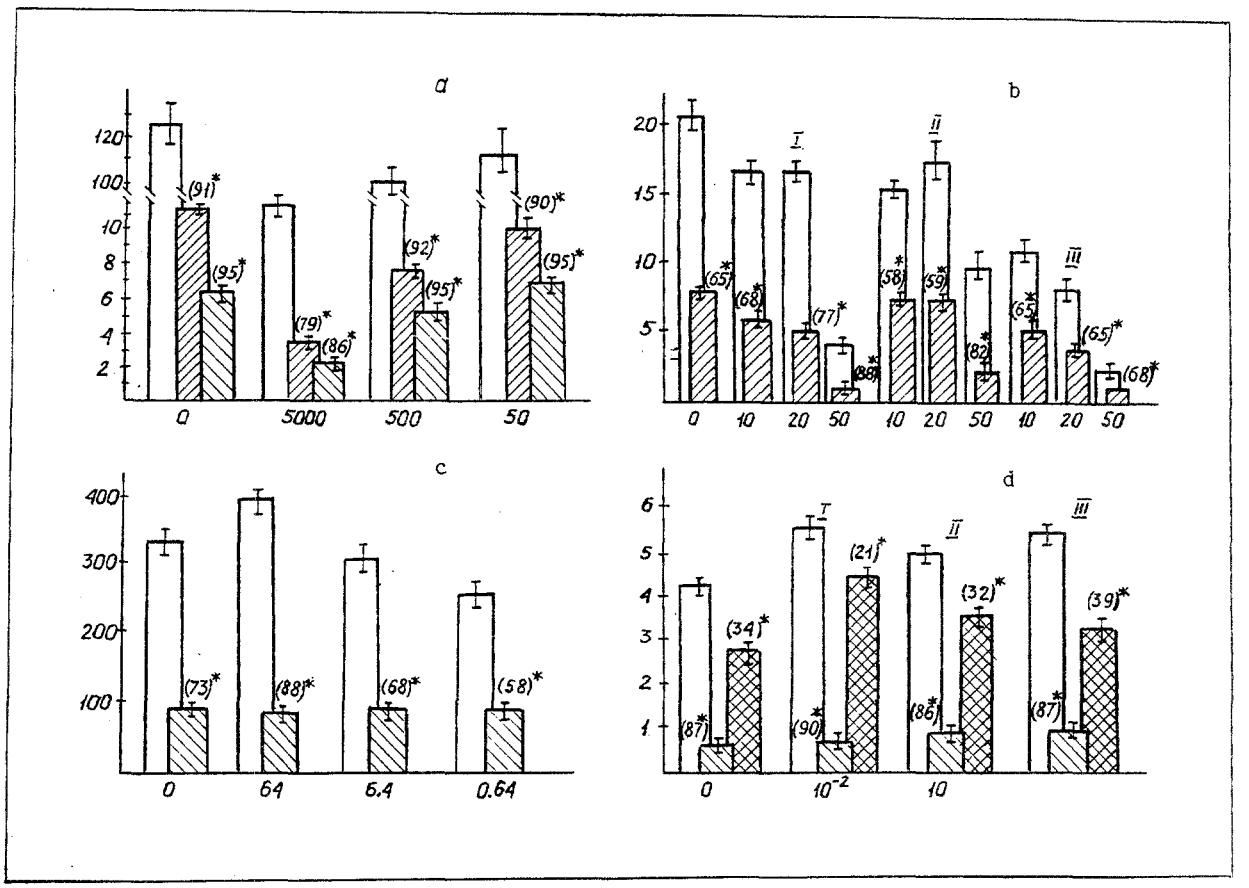

Fig. 1. Effect of recombinant (a) and cultural (c) interleukin-2 LPS and/or MDP (b, d) on action of ISF and DTR (a, b, c) and in MLC (d). Ordinate) incorporation of ^{3}H-thymidine in cpm·10^3. Abscissa) concentration of interleukin-2 (in U/ml — a, c) or LPS and MDP (in μg/ml — b, d). I) LPS; II) MDP; III) LPS + MDP. Unshaded columns — without addition of ISF, obliquely shaded "to the left" — ISF of B16 in a dilution of 1:4; obliquely shaded "to the right" and cross-hatching — ISF of P815 in a dilution of 1:4 and 1:40, respectively. Numbers in parentheses indicate II (in %). Asterisk indicates significance of suppression ≥95%.

culture (MLC), BALB/c and C57B1/6 mice aged 2-4 months, obtained from the "Stolbovaya" Pure-Line Animal Nursery, Academy of Medical Sciences of the USSR, were used. Spleen cells from BALB/c mice (BTR of C57B1/c — MLC) were added to the above-mentioned medium (for MLC — with the addition of $5\cdot10^{-5}$ M 2-mercaptoethanol and 25 mM HEPES buffer) in wells of 96-well plates (Costar), in the number of $3\cdot10^5$/well (MLC) or $2\cdot10^5$/well (BTR), and the activators were then added. In BTR, concanavalin A (con A, from Flow) was added in a final concentration of 5 μg/ml, or in one series of experiments a combination of TPA (10^{-7} M) + ionomycin ($7.5\cdot10^{-8}$ M) was added. In MLC, the stimulators were splenocytes of BALB/c mice, treated with mitomycin C (from Sigma, 50 μg/ml to 10^7 cells), in a number of $9\cdot10^5$/well. Simultaneously with the stimulators, to each well was added 50 μliters of ISF (in BTR with LTS and MDP; the splenocytes were pretreated with ISF, and in the control with medium for 2 h at 37°C, and subsequently washed) and modulators, whereas in the control, culture medium (final volume 200 μliters per well) was added. LPS from E. coli was obtained from Difco, MDP was generously provided by Senior Scientific Assistant N.V. Bovik, recombinant IL-2 came from Biogen, the cultural IL-2 was a 24-h supernatant of BALB/c splenocytes, stimulated with 5 μg/ml of con A, and tested for activity against recombinant IL-2 with the use of 4-day con A-blasts [10], TPA was obtained from Chemsyn, ionomycin from Calbiochem, and contrykal from East Germany. The plates were placed in a CO_2 incubator at 37°C for 72 h. To each well was added 1 μCi of ^{3}H-thymidine (specific activity 25 Ci/mmole) 4 h before the end of incubation. The cells were transferred to filters (Flow) with the aid of

TABLE 1. Abolition of Action of ISF on Con A-Induces Proliferation (incorporation of ^{3}H-thymidine) with the Aid of TPA and/or Ionomycin

TPA, M	Ionomycin, 7.5 × n, M	ISF	II, %	p
		Experiment 1		
—	—	—	47	<0,001
—	—	P815		
10^{-7}	—	—	42	<0,001
10^{-7}	—	P815		
10^{-8}	—	—	5	>0,1
10^{-8}	—	P815		
—	10^{-7}	—	35*	<0,01
—	10^{-7}	P815		
—	10^{-8}	—	51	<0,001
—	10^{-8}	P815		
10^{-7}	10^{-7}	—		
10^{-7}	10^{-7}	P815	11*	>0,1
10^{-7}	10^{-8}	—		
10^{-7}	10^{-8}	P815	26	<0,05
10^{-8}	10^{-7}	—	6*	>0,1
10^{-8}	10^{-7}	P815		
10^{-8}	10^{-8}	—		
10^{-8}	10^{-8}	P815	26	>0,1
		Experiment 2		
—	—	—	75	<0,001
—	—	B16		
10^{-7}	—	—	171*	<0,02
10^{-7}	—	B16		
10^{-8}	—	—	325*	<0,001
10^{-8}	—	B16		
—	10^{-8}	—	5	>0,1
—	10^{-8}	B16		
—	10^{-9}	—	23	>0,1
—	10^{-9}	B16		
10^{-7}	10^{-8}	—	143	<0,1
10^{-7}	10^{-8}	B16		
10^{-7}	10^{-9}	—	358*	<0,02
10^{-7}	10^{-9}	B16		
10^{-8}	10^{-8}	—	11	>0,1
10^{-8}	10^{-8}	B16		
10^{-8}	10^{-9}	—	226*	<0,01
10^{-8}	10^{-9}	B16		

Note. Asterisk indicates stimulation of proliferation compared with control.

a harvester, and radioactivity was determined on a β-counter. The index of inhibition (II) of proliferation was determined by the equation II = [(a — b)/a]·100%, where a and b denote the mean incorporation of ^{3}H-thymidine from three determinations, respectively, in wells with medium and with ISF. The results were subjected to statistical analysis by Student's t test.

RESULTS

It was shown in [8] that are active lymphocytes, contained in nodes of human melanoblastomas, on activation by IL in vitro, were able to produce lysis of all autologous tumor cells. One possible explanation of this phenomenon could be abolition of the action of ISF on lymphocytes located within the tumors, caused by IL-2. To test this hypothesis we added recombinant and cultural murine IL-2 simultaneously with ISF to the BTR. The results of typical experiments are shown in Fig. 1a, c. In a large dose of 5000 U/ml recombinant IL-2 itself had a suppressor action on lymphocyte proliferation, causing an evident reduction of the inhibitory effect of ISF. Neither recombinant nor cultural IL-2, in

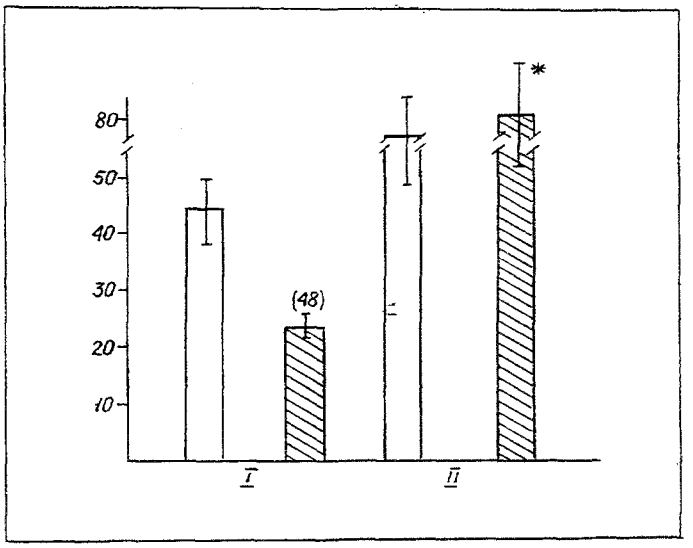

Fig. 2. Effect of con A on action of ISF in BTR induced by TPA and ionomycin. Ordinate) incorporation of ^{3}H-thymidine (in cpm·10^3). I) Without addition of con A; II) with addition of con A, 1 μg/ml. Unshaded columns — without addition of ISF; shaded columns — with addition of ISF of B16 in a dilution of 1:4. Numbers in parentheses give II (in %). Asterisk indicates significant abolition of suppression.

different concentrations, had any significant effect on the inhibitory effect of ISF. Incidentally, in this series of experiments ISF was used in a final dilution of 1:4, which was accompanied by a marked suppressor effect (II = 73, 91, and 95%), and when we used ISF in higher dilutions, recombinant IL-2 could overcome their action partly [4]. The possibility likewise cannot be ruled out that longer culture of the suppressed lymphocytes with IL-2 (more than 10 days in the work cited [8]) would have led to abolition of the action of ISF.

We showed that LPS and MDP synergically activate antitumor immunity, inducing necrosis and regression of syngeneic tumors in mice followed by the development of a lasting immunologic memory [2]. This is evidence that it is possible, in principle, to overcome immunosuppression and to reactive immunity in vivo. It was interesting to study whether LPS and/or MDP in vitro can directly abolish the action of ISF in BTR and MLC. The results of typical experiments are shown in Fig. 1b, d. Clearly, in optimal concentrations for synergic activation of TNF and IL-1 production by more splenocytes in vitro [1], LPS and MDP had virtually no effect on the inhibitory effect of ISF of P815. Only LPS alone (10 ng/ml) partially restored proliferation of lymphocytes in a final dilution of ISF of 1:50 (but not 1:5). Cultural TNF produced in vitro, by the action of MPD and of MPD + LPS [1], consequently, likewise did not affect ISF activity. According to our preliminary data, recombinant TNF did not weaken the inhibitory action of ISF. The contradiction between our results and research in which the action of ISF was partially overcome by recombinant TNF [14, 15] can be explained by the use of different ISF and/or test systems to reveal their action.

It was interesting to discover whether the protein kinase C activator TPA and the calcium ionophore ionomycin abolish the action of ISF. This could shed light on the mechanism of action of tumor products on the two most important pathways of cell activation. Table 1 gives the results of two series of experiments. Clearly, TPA in doses of 10^{-8} M (in cases with ISF of P815 and B16) and 10^{-7} M (with ISF of B16 only) completely overcame the action of ISF and even led to some potentiation of lymphocyte proliferation. Ionomycin in a dose of $7.5 \cdot 10^{-7}$ M itself suppressed lymphocyte proliferation, and in a dose of $7.5 \cdot 10^{-8}$ M it abolished the action of ISF of B16, but not of B18, whereas in a dose of $7.5 \cdot 10^{-9}$ M it significantly reduced the immunosuppressor effect of products of B16 cells. A combination of TCA (10^{-7} M and 10^{-8} M) and ionomycin ($7.5 \cdot 10^{-8}$) caused a significant decrease (almost by half) in the action of ISF of P815 and total abolition of the effect of ISF of B16; in these doses TPA + ionomycin did not themselves

suppress Con A-induced proliferation of splenocytes. In reciprocal experiments, when a combination of TPA (10^{-7} M) and ionomycin ($7.5 \cdot 10^{-8}$ M) was the inducer of splenocyte proliferation, the action of ISF of B16 was suppressed by the addition of con A to the culture (Fig. 2). Incidentally, in these series of experiments con A was used in a final concentration of 1 μg/ml, for in preliminary parallel experiments no effect of TPA and/or ionomycin on the action of ISF could be found in BTR when the dose of con A was 5 μg/ml.

It can be tentatively suggested that ISF, produced by P815 and B16 tumor cells, suppress processes leading to activation of protein kinase C (and, consequently, overcome suppression with the aid of TPA) and, at the same time, suppress certain functions activated by the action of con A. Abolition of the action of ISF of B16 by ionomycin was less marked than when TPA was used, and it could also be due to activation of protein kinase C by the ionophore [6]. Further experiments are needed for the detailed analysis of this fundamental problem.

In the last series of experiments we studied yet another possibility of overcoming the action of ISF. Some tumor cells are known to secrete proteolytic enzymes into the culture medium [13]. Inhibitors of these enzymes possess antitumor activity [16]. It was accordingly decided to study the action of the protease inhibitor contrykal on the effect of ISF in BTR. Contrykal in different doses did not affect ISF activity.

We showed recently [11] that under the influence of ISF on the lymphocyte membrane its permeability for large molecules, such as pancreatic ribonuclease (12 kD) and bacterial endonuclease (30 kDa) is increased. Ribonuclease molecules pass into cell compartments in which protein synthesis is taking place, destroy RNA, and suppress lymphocyte proliferation in BTR and MLC. Concentrations of pancreatic ribonuclease comparable with the concentrations of ribonucleases in the blood were used in the experiments. In the work cited, it is a question of a new mechanism of tumor immunosuppression. We cannot yet answer the question how ISF alters the permeability of the lymphocyte membrane for enzymes.

The results of the present investigation suggest that insufficient activation of protein kinase C is connected with disturbances in the phosphoinositide cycle, linked with the action of ISF on the lymphocyte membrane (for example, with inhibition of the membrane enzyme phospholipase C). We showed previously that the ISF under investigation bind with the lymphocyte membrane strongly even in the cold [3].

It is possible to draw one general conclusion, requiring experimental verification, from the facts described above. It can be tentatively suggested that glycoconjugates, including biantennary and triantennary, bind firmly with certain molecules of the membrane (with endogenous lectins), that perform various (including enzymic) functions. This leads to disturbance of the function of several metabolic systems of the cytoplasm and to a change in membrane permeability for relatively large molecules.

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