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STIMULATION OF INTERLEUKIN-2 MEDIATED ACTIVITY OF HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS BY LIPOPOLYSACCHARIDE AND MURAMYL DIPEPTIDE IN VITRO

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Lipopolysaccharide (LPS) and muramyl dipeptide (MDP) are glycoconjugates of bacterial origin which possess immunostimulating properties [11]. The writers showed previously that these substances have a synergic action on production of tumor necrosis factor (TNF) in vitro and in vivo and on regression of syngeneic tumors [2, 5].

It has been suggested that immune rejection of tumors during treatment with LPS and/or TNF takes place in two successive stages: a) necrosis of the tumors, b) the development of antitumor immunity [4, 13]. For the second stage to take place, a sufficient quantity of growth factors must be present and, in particular, of interleukin-2 (IL-2), which induces generation or reactivation of specific and nonspecific antitumor killer cells [8, 16].

IL-2 is widely used in tumor immunotherapy [12]. It is an interesting question whether combined immunotherapy with LPS, MDP, and IL-2 is more effective than treatment with glycoconjugates and lymphokine separately. The aim of the present investigation was to discover how LPS and/or MDP affect the ability of IL-2 to activate the the proliferative and killer functions of human peripheral mononuclear cells in vitro.

EXPERIMENTAL METHOD

Peripheral blood mononuclear cells (PBMS) were obtained from nine healthy male and female blood donors by fractionation of whole blood on a Ficoll—Verografin density gradient [3]. The following reagents were used: *E. coli* 055:B5 LPS were obtained from Difco, USA; MDP was generously provided by N. V. Bovin, of the Institute of Biotechnology, Ministry of the Medical and Biological Industry of the USSR; recombinant human IL-2 was from the Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, Riga.

To study modulation of the proliferative response of PBMS the cells were first incubated for 24 h in a concentration of $2.5 \cdot 10^6$/ml in 2 ml of medium RPMI-1640 (Flow Laboratories, England), enriched with 10% fetal calf serum (Flow Laboratories), 2 mM L-glutamine, 10 mM HEPES buffer, and 50 µl/ml gentamicin, in 24-well panels (Linbro, Flow Laboratories, England) at 37°C in the presence of 5% CO_2 without immunomodulators, and in the presence of LPS (10 ng/ml), MDP (10 µg/ml), or a combination of both.

The cells were washed 3 times, counted, and dropped into 96-well panels (Costar, USA) in a dose of $2 \cdot 10^5$ per well. Meanwhile IL-2 was added to the wells in different concentrations. Each version of the experiment was repeated 3 times. The PBMS were incubated for 48 h at 37°C in the presence of 5% CO_2. ^{3}H-Thymidine (specific activity 25 Ci/mmole) was added to each well 4 h before the end of culture. The cells were transferred to filters (Flow Laboratories) with

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TABLE 1. Intensification of Proliferative Activity of Human Peripheral Blood Mononuclears in Response to IL-2 After Preincubation with LPS and MDP

Donor	Dose of IL-2	Incorporation of ^{3}H-thymidine, cpm (M ± m)			
		Preincubation			
		Medium	MDP	LPS	MDP + LPS
1	—	580±87,94	947±63,13	775±154,34	1083±186,37
	1000 Units/ml	2393±237,17	2925±200,77	1842±304,62	4062±400,20***
	500 Units/ml	2142±432,62	2453±182,33	2196±44,95	3129±274,62
	100 Units/ml	1689±25,25	1877±32,24	1887±73,62	2494±543,46
2	—	1720±94,95	1649±151,23	1372±367,13	1583±82,68
	1000 Units/ml	2241±53,53	2586±215,71	2670±77,78	3559±339,41**
	500 Units/ml	2184±178,28	1872±80,26	2404±309,65	3101±226,27**
	100 Units/ml	1756±354,39	2360±123,26	2600±910,41	3344±245,99***
3	—	640±134,09	1532±345,80	932±207,64	1477±48,66
	1000 Units/ml	1261±138,21	2930±213,08	2965±315,72	4164±415,88*
	500 Units/ml	2211±185,20	2829±34,28	2585±328,25	3506±587,59
	100 Units/ml	1572±327,85	2611±170,86	2466±181,88	3204±309,62***
4	—	550±285,37	429±50,50	727±3,03	746±336,88
	1000 Units/ml	750±224,76	953±41,92	945±136,87	1031±82,83
	500 Units/ml	495±59,09	546±79,80	638±83,33	999±242,43
	100 Units/ml	349±61,62	395±146,47	417±181,82	1169±306,07

Legend. *p<0.01, **p<0.02, ***p<0.05 (significance between groups: preincubation with medium — preincubation with MDP + LPS).

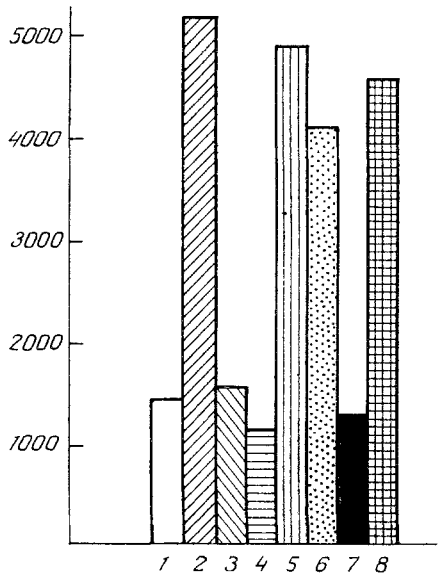

Fig. 1. Effect of LPS, MDP, and IL-2, and also of their combinations on proliferative activity of human PBMS in vitro. Ordinate, incorporation of ^{3}H-thymidine, in cpm. Mononuclears cultured in the presence of: 1) medium, 2) IL-2 (25,000 units/ml), 3) MDP (10 μg/ml), 4) LPS (10 ng/ml), 5) MDP + IL-2, 6) LPS + IL-2, 7) MDP + LPS, 8) MDP + LPS + IL-2.

the aid of a 12-channel system. Radioactivity was measured on a RackBeta β-counter (LKB, Sweden).

In one series of experiments the PBMS were activated simultaneously with LPS, MDP, and IL-2 for 72 h.

To generate cytotoxic lymphocytes the PBMS were incubated in the above-mentioned medium in 24-well panels in a concentration of $2.5 \cdot 10^6$/ml for 72 h with LPS, MDP (in the concentrations given above), and IL-2 (500 U/ml or 2500 U/ml). Human K-562 erythroblastic leukemia cells, human sarcoma HeLa cells, mouse leukemia EL-4 cells, subcultured in vitro and in vivo, and mastocytoma P-815 cells, after passage through DBA/2 mice, were used as the target cells,

TABLE 2. Enhancement of Human Peripheral Blood LAK Activity during Generation with MDP and LPS

Donor	Targets	Cytotoxic index, %							
		Stimulation							
		Medium	IL-2	MDP	LPS	MDP + IL-2	LPS + IL-2	MDP + LPS	MDP + LPS + IL-2
3	K-562	57	90*	85**	67*	100*	100*	78*	88*
	EL-4	<1	10	38	25***	30	23**	5	55**
5	K-562	57	76*	33	54	81***	86*	56	74*
	HeLa	41	77*4	42	50	79***	79**	41	85*4
6	K-562	76	86	86	81	79	89	86	100
	EL-4	<1	10	<1	<1	73**, yy	22	<1	17
7	EL-4	23	73**	13	10	81*, y4	80*	15	83*
	P-815	<1	65*	4	11	72*, yyy	75*, y	14	71*, y
8	K-562	41	84*	30	32	83*	83*	25	86*
	EL-4	<1	8*	<1	<1	14*, yy	9*	<1	16*, yy
	P-815	<1	7*4	<1	<1	14**, yyy	17**, y4	<1	17**, yy

Legend. *p<0.001, **p<0.01, ***p<0.02, ****p<0.05 (significance between groups: stimulation by MDP and/or LPS + IL-2 versus unstimulated cells); y) p<0.001, yy) p<0.01, yyy) p<0.02, y4) p<0.05 (significance between groups: stimulation by MDP and/or LPS + IL-2 versus stimulation by IL-2), EL-4, P-815) target cells maintained by passage in vivo, except donor No. 3.

numbering $(2\text{-}5)\cdot10^6$, were labeled at 37°C for 1 h with $^{51}Na_2CrO_4$ (Amersham International, England, 150 μCi/ml), washed 3 times, and their concentration adjusted to 10^5/ml. The effector and target cells were introduced in a volume of 100 μl into each well of 96-well panels (Linbro, Flow Laboratories). After culture for 4 or 18 h, 100-μl samples of supernatants were taken from each well and their radioactivity measured on a RackGamma γ-counter (LKB, Sweden). The cytotoxic index was determined by the equation $CI = \frac{a-b}{c-b}\cdot100\,\%$ where a denotes radioactivity of the supernatants in wells containing target and effector cells, b denotes radioactivity of the supernatants in wells with target cells and medium, and c the same in wells with target cells and sodium dodecylsulfate. Spontaneous release of the label in most experiments did not exceed 25% of maximal. For each version of effector cells three determinations were used.

The numerical results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

In the first series of experiments the effect of pretreatment of the PBMS with LPS and/or MDP on sensitivity of the cells to IL-2 was studied on the basis of their proliferative response to the lymphokine. Cells from four healthy blood donors were used. As Table 1 shows, pretreatment of the mononuclears for 24 h with a combination of LPS + MDP led in most cases to an enhanced proliferative response of these cells to IL-2 compared with untreated cells or cells treated with each immunomodulator separately.

The writers showed previously that during activation of PBMS for 24 h with LPS and MDP the cells can produce TNF and interleukin-1 (IL-1) [1]. The increase in sensitivity of the cells to IL-2 may thus be associated with intensification of expression of receptors to IL-2, brought about by IL-1 [9] and/or by TNF [14]. We know that LPS alone, when inducing IL-1 production, increase induction of receptors to IL-2 as early as after 20 h of mononuclear activation [10]; in that case, however, microgram doses of the preparation were used. On simultaneous stimulation of PBMS proliferation by LPS, MDP, and IL-2 in different combinations, no activating action of LPS, MDP, or their combination could be observed in addition to that produced by IL-2; the glycoconjugates themselves did not stimulate cell proliferation (Fig. 1).

In the second series of experiments (Table 2) we studied the effect of LPS and/or MDP on generation of IL-2-induced killer cells, i.e., of the so-called lymphokine-activated killer cells (LAK) [15]. As targets a panel of lines of human (K-562, HeLa) and mouse (EL-4, P-815) tumor cells was used; in the last case, cells growing in vivo also were used. Killing of these tumors is a distinguishing feature of LAK [6]. As Table 2 shows, in every case IL-2 enhanced the cytotoxic properties of the PBMS compared with unstimulated cells against K-562, HeLa, and EL-4 targets maintained in vitro, or induced their appearance against EL-4 and P-815 targets maintained in vivo. In most cases MDP and a combination of MDP + LPS, and to a lesser degree, LPS alone, enhanced IL-2-induced cytotoxicity (p<0.01).

In one case MDP exhibited marked synergic action with IL-2 on generation of LAK producing lysis of EL-4 cells (donor No. 6; $p<0.001$). IL-2 in the present experiments enhanced activity of natural killer cells, tested on cells of the standard line K-562, in agreement with data in the literature [7]. The glycoconjugates which we used had a weak but significant action on LAK generation compared with the activating effect of IL-2 alone, but under these circumstances they did not stimulate proliferation of PBMS in vitro.

It must be concluded from these results that in an in vitro system LPS and MDP, in combination, induce increased sensitivity of human peripheral blood mononuclear cells to IL-2 and promote enhanced LAK generation. The results of the present investigation can be used to enhance the process of LAK generation in vitro. However, the possibility of using the principles discovered for generation or reactivation of LAK and of specific antitumor killer T cells in vivo is of significantly greater interest. We are currently investigating this possibility.

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