in the numerical composition of the individual cell populations in the adenohypophysis evidently arise after long-term exposure to regulators. For example, as a result of the long-term hormonal restructuring taking place during pregnancy, profound changes in adenohypophyseal function occur with redistribution of individual types of cells.

The methods of quantitative estimation of changes in DNA synthesis in hormone-secreting cells developed by the present authors may find application in the study of the genesis of hormone-secreting tumors of the adenohypophysis and of other glands of internal secretion, in the search for new preparations and in the designing of rational schedules of hormone therapy.

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ACTIVATION OF CYTOTOXIC FACTOR PRODUCTION OF MOUSE SPLEEN CELLS THROUGH IN-VITRO STIMULATION BY LIPOPOLYSACCHARIDE AND MURAMYL DIPEPTIDE

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UDC 612.411.017.4.014.46:579.222; 547.915.5'458

KEY WORDS: lipopolysaccharide; muramyl dipeptide; tumor necrosis factor; interleukin-1.

Lipopolysaccharides (LPS) and muramyl dipeptide (MDP) are bacterial products which stimulate antitumor immunity in vitro [9, 14] and in vivo [11, 13]. There is evidence of synergism in the action of these substances on activation of the tumoricidal function of macrophages in vitro [9] and on regression of syngeneic tumors in mice [1, 2]. A tumor necrosis factor (TNF), or cytokine, to which an important role in antitumor immunity is ascribed, appears in the serum of animals sensitized by bacteria containing MDP, or by MDP itself [6], in response to injection of LPS.

The aim of this investigation was to determine the conditions of synergic action of LPS and MDP on cytotoxin production by mouse splenocytes in vitro, revealed by lysis of L-929 target cells. This property is known to be a feature of TNF produced mainly by activated macrophages [15] and, to a lesser degree, by natural killer cells [12] and T lymphocytes [7], and also of lymphotoxins (LT), secreted by activated T and B lymphocytes [5]. For convenience these factors will be described by the general name of "cytotoxic factors" (CTF).

EXPERIMENTAL METHODS

DBA/2 ($H\text{-}2^d$) and C57Bl/6 ($H\text{-}2^b$) mice of both sexes, aged 2-3 months, were used. Spleen cells were suspended (5.5×10^6/ml) in medium RPMI-1640 (Flow Laboratories, England), containing

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(Presented by Academician of the Academy of Medical Sciences of the USSR A. P. Avtsyn.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 105, No. 4, pp. 483-486, April, 1988. Original article submitted June 9, 1987.

0007-4888/88/0004-0582$12.50

heated 10% fetal calf serum (Flow Laboratories), 20 mM HEPES buffer, 2 mM L-glutamine, and gentamicin (50 μg/ml). The same medium was used in the remaining tests. The cell suspension was poured in a volume of 1.8 ml into the wells of 24-well panels (Flow Laboratories). To it was added 100 μl of LPS from E. coli 0.55:B5 (Difco, USA) and/or MDP in different concentrations (the MDP was supplied by N. V. Bovin, on the staff of the Institute of Biotechnology, Ministry of the Medical Industry of the USSR). Medium or immunomodulators without splenocytes were added to the control wells. After 24 h (in some experiments after 1, 2, and 6 h also) the contents of each well were transferred to test tubes and centrifuged, and the supernatants were used in tests or were frozen to −20°C. CTF activity was determined by the method in [4]. For this purpose 100 μl of each supernatant was added to the wells of a flat-bottomed 96-well plate (Linbro, USA), into which $5 \cdot 10^4$ L-929 cells had been poured 24 h previously. Medium was added to the control wells. The experimental and control samples were treated with 100 μl of actinomycin D (serva, West Germany) in a final concentration of 2 μg/ml. Three wells were used to test each supernatant. The plates were incubated in an atmosphere of 5% CO_2 at 37°C. After 18 h the supernatant was removed and the monolayer stained with a 0.2% solution of crystal violet in 2% ethanol for 10 min. The plates were washed with water and dried. The optical density was measured on a Multiscan (Titertek, England) densitometer at a wavelength of 540 nm. The cytotoxic index (CTI) was determined by the formula $CTI = \frac{a-b}{a} \cdot 100$, where a is the optical density of the wells with supernatants.

Interleukin-1 (IL-1) activity in the supernatants was determined by the method in [10]. 50 μl of supernatants (medium in the control) and 150 μl of thymocytes from C57Bl/6 mice ($1.5 \cdot 10^6$ cells in each well) were introduced into the wells of a round-bottomed 96-well panel and incubated in an atmosphere containing 5% CO_2 at 37°C for 3 days. To each well 1 μCi of ^{3}H-thymidine (specific activity 25 Ci/mmole) was added 6 h before the end of incubation. The cells were transferred to filters (Flow Laboratories) by means of a 12-channel device, added to scintillation fluid, and their radioactivity was measured on a β-counter (Packard, USA). Three tests were carried out with each supernatant. IL-1 activity was judged by the increase in the thymocyte proliferation stimulation index (SI);

$$SI = \frac{\text{Incorporation of } ^3\text{H-thymidine in experimental wells (cpm)}}{\text{Incorporation of } ^2\text{H-thymidine in wells with medium (cpm)}}$$

To remove macrophages from the suspension of splenocytes $6 \cdot 10^7$ spleen cells were introduced into plastic Petri dishes 14 cm in diameter (Flow Laboratories) for 2-2.5 h at 37°C and the nonadherent cells were collected into test tubes. To obtain a cell population rich in macrophages, after removal of the nonadherent cells 3 times with warm Eagle's medium containing 10% fetal calf serum, the adherent cells were incubated in Versene solution (for 20 min at 37°C) and collected after careful pipeting.

To remove T lymphocytes from the population of nonadherent cells, the cells were treated with anti-Thy-1.2 monoclonal antibodies (Cederlane, Canada) in a dilution of 1:1000 for 30 min at 20°C and with nontoxic rabbit complement (1:12) at 37°C for 30 min. The results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

In the experiments of series I the minimal quantity of LPS required to exert a synergic action with MDP on CTF production by mouse splenocytes in vitro was determined. Reduction of the dose of LPS, but with maintenance of its immunostimulating activity, is important from the practical point of view, for large doses of LPS (alone or together with MDP) are toxic when used for tumor immunotherapy [8]. We showed previously that the synergic action of LPS and MDP in vitro is exhibited by LPS in a concentration of between 10 ng/ml and 10 μg/ml [1]. with a decrease in the dose of LPS to 0.1 pg/ml, accompanied by MDP in a dose of 10 μg/ml, synergic action of the two substances on CTF production by splenocytes was not observed. LPS alone did not stimulate the splenocytes, and the immunomodulators themselves were not toxic for L-929 cells (Fig. 1).

In the next series of experiments the time course of CTF production by mouse splenocytes in vitro was determined in response to stimulation by LPS, MDP, or a combination of both. CTF activity appeared in the supernatants 2 h after stimulation of the splenocytes and reached a maximum after 6 h (CTI = 76-86%). Synergism of action of LPS and MDP on CTF formation, however, did not appear until 24 h after stimulation ($p < 0.01$). After culture for 6 h marked activation of the spleen cells was observed both by LPS and by MDP (CTI 62 and 86%,

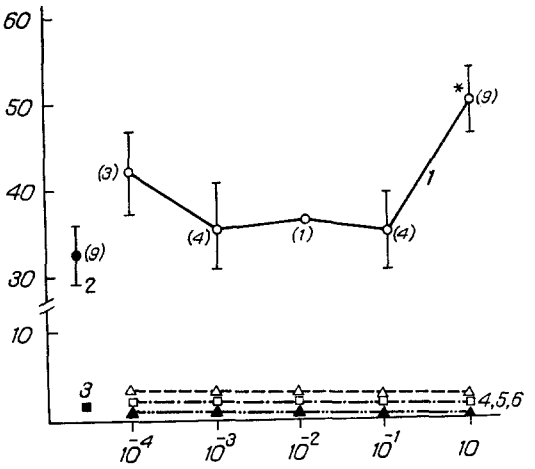

Fig. 1

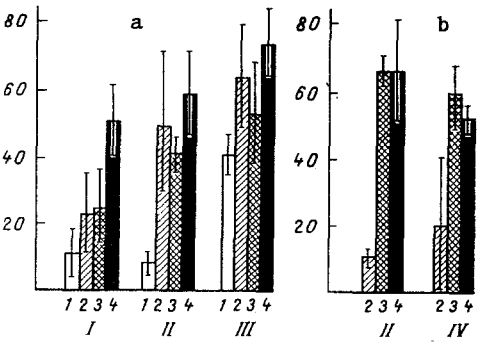

Fig. 2

Fig. 1. Synergism of action of LPS and MDP on CTF production by mouse splenocytes in vitro. MDP concentration 10 μg/ml. Ordinate, CTI (in %) of supernatants of splenocytes activated by LPS (4), MDP (2), LPS + MDP (1), or of the immunomodulators themselves: LPS (5), MDP (3), LPS + MDP (6). Abscissa, LPS concentration in culture (in ng/ml); numbers in parentheses give number of experiments. *$p < 0.01$.

Fig. 2. CTF production by "adherent" and "nonadherent" spleen cells (a) and lymphocytes exhausted on T cells (b). Ordinate, CTI (in %) of supernatants of unfractionated splenocytes (I), of splenocytes not adherent to plastic without treatment by antibodies (II) or treated by monoclonal antibodies to Thy-1.2-antigen with complement (IV), and adherent to plastic (III). Cells not stimulated (1) or activated by LPS, 10 mg/ml (2), by MDP, 10 μg/ml (3), or by LPS + MDP (4). Results of three or four independent experiments (a) and of two experiments (b).

TABLE 1. Synergism of Action of LPS and MDP on IL-1 Production by Mouse Splenocytes in Vitro (M ± m, n = 3)

Splenocytes	Immunomodulators	SI of thymocyte proliferation
+	Medium	0,88±0,47
+	LPS, 10 ng/ml	1,67±0,20
+	MDP, 10 μg/ml	1,21±0,32
+	LPS + MDP	4,22±0,45*
—	Medium	1,00±0,00
—	LPS, 10 ng/ml	1,11±0,42
—	MDP, 10 μg/ml	1,64±0,36
—	LPS + MDP	1,66±0,65

Legend. *$p < 0.01$.

respectively), whereas after 24 h stimulation of the splenocytes by LPS did not induce CTF formation (CTI 2%). The results are in agreement with data in the literature [3] on maximal activity of TNF in monocyte supernatants 6 h after stimulation by LPS; it will be recalled, however, that at this time spontaneous secretion of CTF by splenocytes was observed in the present experiments (CTI 28%).

An answer was required to the question of which cells in our system were responsible for CTF production during stimulation by LPS and MDP. By using the method of adsorption on plastic, we obtained a splenocyte population rich in macrophages ("adherent" cells) and in lymphocytes ("nonadherent" cells) and we stimulated each population by LPS and MDP separately and in combination. The results of these experiments are given in Fig. 2a. Clearly both macrophages and lymphocytes were able to respond by CTF production to stimulation by LPS and MDP and by both substances together, and they did so to a greater degree than the initial unfractionated cell population. Under these circumstances there was no synergism

of the action of LPS and MDP. During the action of the immunomodulators chosen, TNF production evidently takes place both by macrophages and by LT lymphocytes. In a whole population of spleen cells, lymphocytes and macrophages may perhaps suppress the ability of each other to respond to LPS and MDP.

CTF production in response to stimulation of lymphocytes by immunomodulators was not a function predominantly of T lymphocytes, for treatment of spleen cells unattached to plastic by monoclonal antibodies against Thy-1.2-antigen in the presence of complement did not affect CTF production by the remaining cells (Fig. 2b).

Since LPS and MDP activate macrophagal production of IL-1 [14], which has cytotoxicity relative to L-929 cells, it was interesting to study whether lysis of the targets in these experiments was due to TNF and LT only or to IL-1 also. For this purpose, activity of IL-1 with respect to stimualtion of thymocyte proliferation was determined in supernatants obtained 24 h after stimulation of the splenocytes by LPS and MDP (Table 1). Synergism of action of LPS and MDP on splenocytes also was manifested as activation of IL-1 synthesis (SI = 4.22; $p < 0.01$). MDP alone did not induce IL-1 production by the cells (SI = 1.21), but it led to CTF production (CTI 32%). Consequently, cytotoxicity of the supernatants to L-929 cells was not due only to IL-1.

The investigation thus showed that LPS (10 ng/ml) and MDP (10 μg/ml), separately and in combination with each other, stimulate CTF production by mouse macrophages and lymphocytes, including T cells. Synergism of action of a combination of the substances on CTF and IL-1 production by the mouse splenocytes was observed 24 h after activation. CTF production began after 2 h of culture and reached a maximum after 6 h.

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