ACTIVATION OF TUMOR NECROSIS FACTOR PRODUCTION BY THE COMBINED ACTION OF LIPOPOLYSACCHARIDE AND MURAMYL DIPEPTIDE IN VITRO AND IN VIVO

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Tumor necrosis factor (TNF) assumes an important role in tumor immunotherapy [6]. It is produced by activated macrophages [9] and by natural cytotoxic cells [8]. Lipopolysaccharides (LPS) induce TNF synthesis in vitro [5] and in vivo [2]. Muramyl dipeptide (MDP) and its derivatives have a similar action in vitro [7]. TNF production in vivo increased significantly if LPS is injected into animals sensitized by certain bacteria (*Corynebacterium parvum, Mycobacterium*), for which MDP is an adjuvant glycopeptide [3]. Administration of MDP and, in particular, of its lipophilic derivative (MTP-PE) into B6 mice prevents them from developing lung metastases of myeloma B16 [4]. Synergism of action of LPS and MDP in vivo has recently been demonstrated [1]. The authors cited obtained complete regression of small (7.5 mm) syngeneic sarcomas in BALB/c mice. However, the combined action of LPS and MDP was not analyzed with respect to the most important criterion, namely TNF production.

The aim of this investigation was to study synergism of action of LPS and MDP on TNF production by splenocytes in vitro and on growth of sarcoma SA-1 in A/Sn mice in vivo.

EXPERIMENTAL METHOD

Two variants of sarcoma SA-1 (H-2^a), differing in antigens associated with the tumor cell, were used. Sarcoma cells (10^6-3×10^6) were injected subcutaneously into A/Sn (H-2^a) mice of both sexes weighing 15-18 g. The preparations of LPS from *E. coli* (055:B5, Difco, USA) and (or) MDP were injected intravenously or intraperitoneally into the mice in doses of 10 and 30 μg, respectively, 3-4 times at intervals of 2-3 days. An alternative version of LPS administration also was used. Splenocytes of A/Sn mice (5×10^7/ml) were treated, in a siliconized vessel to prevent loss of macrophages (Silicon-Lösung, Serva, West Germany), with LPS in a final concentration of 50 μg/ml for 2 h in medium RPMI-1640 (Flow Laboratories, England) at 37°C with periodic shaking. The splenocytes were then washed 3 times and injected intravenously in a dose of 15×10^6-20×10^6 per mouse. Two perpendicular diameters of the subcutaneous tumors were measured by means of calipers. TNF was tested in the sera obtained from A/Sn mice 2 h after the last injection of the preparation, by the method described below.

The experiments in vitro were carried out on splenocytes of DBA/2 mice. The splenocytes were cultured in 24-well plates (Flow Laboratories) in a concentration of 5×10^6/ml in medium RPMI-1640 with 10% fetal calf serum (Flow Laboratories), 2 mM L-glutamine, 10 mM HEPES buffer, and 50 μg/ml of gentamicin. LPS and MDP were added to the culture medium in different concentrations. The supernatants were collected 24 h (in some experiments 48 h) after the beginning of culture and their TNF concentration was determined by the method described previously [5]. Transformed cells of C3H mouse cell line L-929, subcultured in vitro, were used as the targets. The target cells were suspended in the above medium and transferred to 96-well flat-bottomed plates (Titertek, from Flow Laboratories) in a dose of 5×10^4 per well to obtain a dense monolayer. Next day tenfold dilutions of the test sera or supernatants in a volume of 100 μl and actinomycin D (Serva) in a final concentration of 2 μg/ml and in a volume of 100 μl were added to the monolayer. After 18 h the supernatant was removed and the

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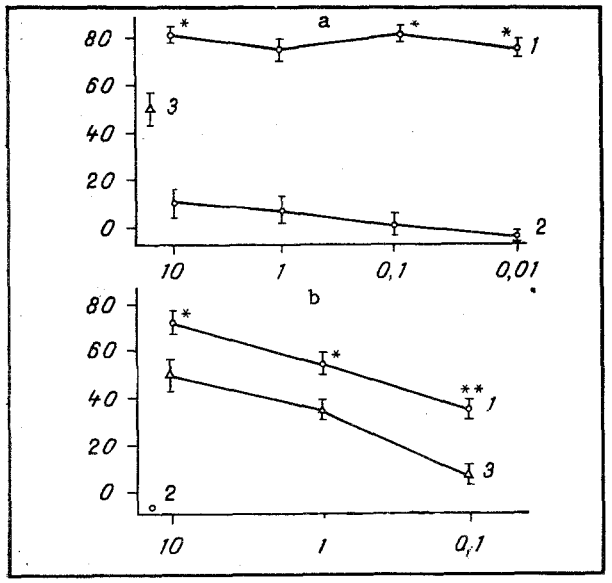

Fig. 1. Synergism of action of LPS and MDP on TNF production by mouse splenocytes in vitro. Ordinate, CTI (in %). a) LPS concentration in culture (in μg/ml) with constant MDP concentration (10 μg/ml). Mean results of 3-6 experiments (M ± m); b) MDP concentration in culture (in μg/ml) with constant LPS concentration (10 ng/ml). Mean results of 4-6 experiments (M ± m). 1) LPS + MDP; 2) LPS; 3) MDP. Dilution of supernatants 1:2. *p < 0.01, **p < 0.05.

plates were stained for 10 min with crystal violet (0.2% solution in 2% ethanol), after which they were washed with water and dried. The optical density of the dye in the surviving monolayer was determined on a Multiscan apparatus at a wavelength of 540 nm. The cytotoxicity index (CTI) was determined by the equation:

$$CTI = \frac{a - b}{a} \times 100\%,$$

where *a* denotes the optical density of the dye in the wells with medium (control) and b the optical density of the dye in wells with the experimental supernatant for serum. To calculate CTI mean values of three determinations of optical density were used. The results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

Data on TNF production in response to the combined action of LPS and MDP on splenocytes of DBA/2 mice in vitro are given in Fig. 1. The combination of these preparations had a more marked effect on TNF production than each preparation separately. The effective MDP concentration was lowered under these circumstances from 10 μg/ml to 100 ng/ml, if the LPS concentration remained constant at 10 ng/ml (Fig. 1b; $p < 0.05$). Incidentally, the chief active principle of LPS is lipid A, but in the LPS preparation from *E. coli* 055 which we used there were many R-forms of the molecules, in which lipid A is not screened by carbohydrates [10].

There were three series of experiments with treatment of subcutaneous SA-1 tumor nodes in A/Sn mice (Fig. 2). Both variants of sarcoma SA-1 grew relatively quickly, to reach a diameter of 1 cm in the course of 7-12 days. Growth then became significantly slower. In some mice the diameter of the tumor reached 3-4 cm and the mice died in the course of 10-12 weeks. In about 50% of control mice the tumors were spontaneously rejected after 5-7 weeks. Analysis of killer cell generation on the two variants of the tumors in vitro showed that the sarcomas used differed from one another antigenically. It can thus be concluded that we were dealing with immunogenic variants of sarcoma SA-1

When the A/Sn mice with one (Fig. 2a, c) or other (Fig. 2b) variant of sarcoma SA-1 were treated, necrosis of the subcutaneous tumor nodes measuring 1 cm or more in diameter took

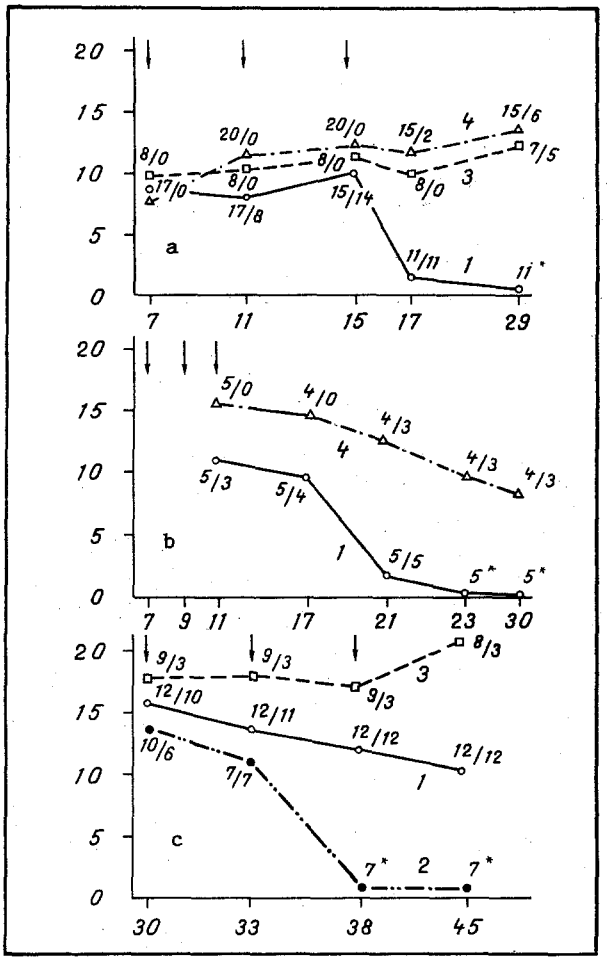

Fig. 2. Immunotherapy of A/Sn mice with subcutaneous nodes of sarcoma SA-1 with the LPS + MDP combination. Abscissa, time after injection of 10^6-3 × 10^6 tumor cells (in days); ordinate, mean diameter of tumor (in mm). a, b, c) Series of experiments. 1) LPS-treated splenocytes + MDP; 2) LPS + MDP; 3) nothing injected; 4) untreated splenocytes injected. Numerator gives number of mice living at that particular time; denominator, number of mice with necrosis of the tumor. *) Regression of tumors in all mice. Arrows indicate injection of preparations and splenocytes.

place. The tumor in all mice disappeared 2 weeks after the 3rd injection of LPS-splenocytes and MDP. After 1 month, the recurrent nodes appeared in two mice in the experiments of series I, and in one mouse the recurrent node regressed spontaneously.

In the experiments of series III (Fig. 2c) the mice were selected for progressive growth of the tumor for 30 days, after which they received immunotherapy. The effectiveness of a combination of LPS-splenocytes + MDP and of LPS + MDP was compared. It will be clear from Fig. 2c that only 2 days after the first injection of LPS and MDP total necrosis of the tumor nodes appeared in all the mice. After 1 week the tumor disappeared in all mice (the preparations were injected only once into the mice of this group). When three injections of LPS-splenocytes with MDP were given, regression of the tumors took place more slowly than after injection of LPS with MDP. However, this last combination was toxic (three of the 10 mice died on the day after injection of the preparation). Later two mice treated with LPS and MDP developed recurrent tumors, from which they died. In the control, spontaneous regression of the tumors took place in two of the eight mice after 8 weeks.

Analysis of the TNF concentration in serum of the A/Sn mice with a subcutaneous SA-1 tumor 2 h after the 3rd injection of the preparations showed the following (Fig. 3). Activity of TNF was observed only after injection of both preparations. LPS and MDP separately had no such action. Injection of LPS on cells was far less effective than without cells.

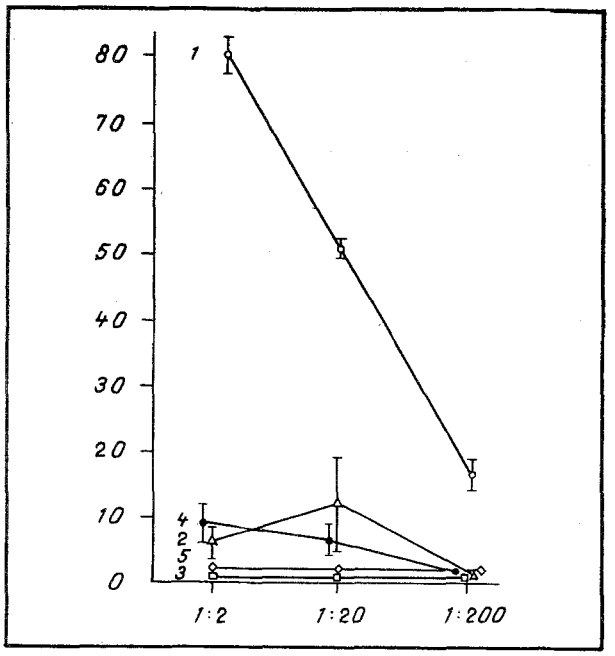

Fig. 3. Active TNF production in serum of A/Sn mice with subcutaneous nodes of sarcoma SA-1 as a result of three injections of a combination of LPS and MDP. Abscissa, dilution of serum; ordinate, CTI (in %). 1) LPS + MDP; 2) LPS; 3) MDP; 4) LPS-treated splenocytes + MDP; 5) no treatment. Mean results of one typical experiment are shown (M ± m).

Thus three facts were discovered: synergism of action of LPS and MDP on TNF production in vitro and in vivo and effectiveness of a combination of these preparations in the treatment of syngeneic sarcoma SA-1 in A/Sn mice. The mechanism of the synergic action of LPO and MDP will be a subject for our future study. The combination of preparations tested will evidently be effective in the immunotherapy of various types of tumors. For instance, according to preliminary data, total necrosis of mammary gland carcinoma nodules, developing spontaneously in noninbred mice, has been achieved. We need to know how the effect depends on the TNF concentration within the tumor and on activation of other functions of the antitumor immune system. The cellular version of injection (adoptive immunotherapy with LPS-treated splenocytes) is less toxic, although less effective, than injection of the preparations themselves. Whatever the case, the problem arises of targeted delivery of a combination of lipid A and MDP into the tumor, and this is evidently tantamount to the use of cells, monoclonal antibodies and, possibly, glycosphingolipids, for this purpose.

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