IMMUNOMODULATING ACTIVITY OF NEW MURAMYL DIPEPTIDE DERIVATIVES IN VITRO

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A promising trend in tumor immunotherapy is the use of modifiers of biological reactions and, in particular, of muramyl dipeptide (MDP) derivatives. These substances possess a spectrum of immunomodulator effects: potentiation of the cytotoxicity of natural killer cells [12], stimulation of the tumoricidal and tumoritoxic effect of macrophages [7], induction of the production of interleukin-1 (IL-1) and tumor necrosis factor (TNF) [3, 14], enhancement of lymphocyte proliferation in response to mitogens and alloantigens [6, 13], and others. The marked antitumor action of MDP has been demonstrated when given alone or when used in combination with lipopolysaccharide (LPS), indomethacin, and cyclophosphamide, when it is manifested as inhibition of growth or complete rejection of various transplanted tumors in mice [8]. Meanwhile these substances give rise to various toxic side effects [4]. The creation of MDP derivatives possessing marked immunomodulator and antitumor activity but only minimal toxicity, is therefore an urgent task.

The aim of this investigation was to study the possible stimulating effect of new Russian MDP derivatives on functional activity of T lymphocytes and macrophages.

EXPERIMENTAL METHOD

Original Russian MDP derivatives were synthesized by the Peptide Chemistry Group of the Institute of Bioorganic Chemistry, Russian Academy of Sciences and Simferopol' State University (their code numbers are given in parentheses): MurNAc-L-Ala-D-Glu-$NHC_{18}H_{37}$ (1), dexal-MDP (2), β-$C_{16}H_{33}$MDP (3), β-C_7H_{15}MDP (4), MurNAc-L-Ala-D-iGlu$(OC_{17}H_{33})_2$ (5), and polyacrylamide-MDP (P-MDP; 6). Activity of the above-mentioned compounds was compared against a standard reference MDP (7). Preparations 1, 3, 4, 5, and 7 were dissolved in 96% ethanol, 6 in RPMI-1640 ("Flow Laboratories") containing 0.5% triethylamine, and MDP was dissolved in RPMI-1640. The concentration of the mother solutions of all compounds was 2 mg/ml. Experiments were carried out on male BALB/c and C57BL/6 mice aged 2-4 months. The mice were killed by cervical dislocation, the thymus and spleen were removed aseptically, and the cells were obtained in glass homogenizers and washed three times in Hanks' solution with 10% inactivated (56°C, 30 min) bovine serum (washing medium). The cells were cultured in medium RPMI 1640 containing 5% fetal calf serum ("Flow"), 2 mM L-glutamine ("Flow"), 25 mM HEPES buffer ("Flow"), 2-mercaptoethanol ("Serva," $5 \cdot 10^{-5}$ M), and gentamicin (50 μg/ml). Macrophages were isolated from peritoneal exudate cells (PEC) 3 days after intraperitoneal injection of 5 ml 3% peptone into mice. PEC were introduced into the wells of a 24-well flat-bottomed plate "Linbro") in an amount of $5 \cdot 10^6$ cells/well, they were adsorbed on the plastic for 2 h at 37°C, and nonadherent cells were removed by washing the monolayer 3 times with warm washing medium. To each well of the plate was added 1 ml of culture medium containing the test preparations and the cells

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TABLE 1. Effect of Preparations on Proliferative Activity of C57BL/6 Mouse Splenocytes in B-anti-D MLC (M ± m, n = 5)

Preparation	Dose, μg/ml	PI	AI, %	Dose, μg/ml	PI	AI, %	Dose, μg/ml	PI	AI, %
—		10.4±1.2	—		10.4±1.2	—		10.4±1.2	—
1		12.0±1.0	15		11.6±1.2	12		10.0±3.0	—3
2		11.0±0.8	6		11.6±1.0	12		10.6±2.2	2
3		14.6±0.3*	40		14.4±0.4*	38		13.6±0.7*	31
4	10	17.7±0.6*	69	5	16.2±1.8*	56	2.5	14.4±1.6*	38
5		11.4±1.2	10		14.8±2.4	42		12.4±1.5	19
6		5.6±1.1	—46		12.0±1.8	15		13.0±2.6	25
7		16.1±0.7*	55		14.8±0.8*	42		14.0±1.8	35

Legend. *$p < 0.05$.

were incubated for 18 h at 37°C in an atmosphere of air containing 7% CO_2. At the end of incubation the supernatants were collected to act as the source of IL-1 and TNF and kept at −20°C. A mixed lymphocyte culture (MLC) was set up in the modification of Brondz et al. [1]. The reacting cells were C57BL/6 mouse spleen cells, the stimulators were BALB/c mouse splenocytes, treated with mitomycin C. Treatment of the cells ($20 \cdot 10^6$ cells/ml) with mitomycin C ("Sigma," 50 μg/ml) was carried out for 40 min at 37°C, followed by washing three times to remove the splenocytes. A mixture of 100 μl of responders ($3 \cdot 10^6$ cells/ml) and 50 μl of stimulators ($18 \cdot 10^6$ cells/ml) was introduced into the wells of a 96-well flat-bottomed microplate ("Linbro") and the preparations were added in a volume of 50 μl in the above-mentioned doses (50 μl of culture medium in the control). The microplate was incubated for 5 days at 37°C in an atmosphere of air containing 7% CO_2. Into each well 1 μCi of ^{3}H-thymidine (specific activity 4 Ci/mmole) was added 18 h before the end of culture, and the samples were collected on glass fiber filters ("Flow") with the aid of a harvester and their radioactivity counted on a β-spectrometer ("LKB"). The proliferation index (PI) was calculated by the equation PI = A/B, where A and B denote incorporation of label (cpm) into MLC and monoculture respectively. The effect of the preparations was analyzed with the aid of the activation index (AI), calculated by the equation: AI = (C − D)/D · 100 (%), where C and D denote incorporation of ^{3}H-thymidine (cpm) in the experimental (addition of the preparation) and control (addition of culture medium) samples. Activity of IL-1 was tested by the use of the committogenic platelet test with phytohemagglutinin (final concentration 1 μg/ml) [15]. Activity of the TNF present in the specimens was determined by its cytotoxic action on fibrosarcoma L-929 cells in the photometric test with crystal violet [9]. The results were subjected to statistical analysis, by Student's t test.

EXPERIMENTAL RESULTS

According to data in the literature macrophages and T lymphocytes are among the main effectors of antitumor immunity [2, 10]. These cells exhibit tumoricidal and tumoritoxic effects and produce various cytokines (TNF, IL-1, interferons) which regulate the immune response and can exert a cytocidal action on tumor cells [5, 11]. Accordingly it was decided to study the possible immunomodulating effect of the preparations on functional activity of T lymphocytes and macrophages. Proliferation of T lymphocytes in MLC and production of IL-1 and TNF by macrophages were chosen as the model systems.

In the first series of experiments the effect of MDP derivatives was studied on proliferation of C57BL/6 mouse splenocytes in allo-MLC. It will be clear from the results given in Table 1 that immunomodulators β-$C_{16}H_{33}$ and β-C_7H_{15} MDP (AI 31-69%), in all concentrations used, possessed a marked stimulating effect. Meanwhile, no statistically significant differences were found in the ability of these preparations to activate splenocyte proliferation, by comparison with MDP.

In the second stage of the work the possibility activating action of the preparations was studied on functional activity of BALB/c mouse peritoneal macrophages (treatment with IL-1 and TNF). The effect of these compounds also was tested when they were used in combination with low doses of LPS (20 ng/ml). Data on the effect of the immunomodulators on macrophagal IL-1 production are given in Fig. 1. Clearly the preparations β-$C_{16}H_{33}$MDP

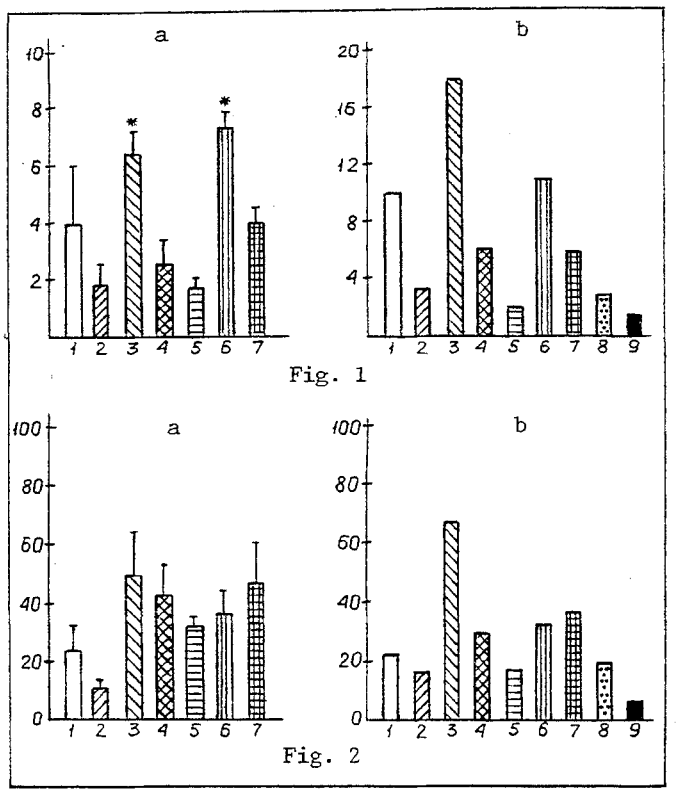

Fig. 1. Effect of MDP derivatives on IL-1 production by BALB/c mouse peritoneal macrophages. Abscissa, variants of macrophage stimulation (a): No. of preparations; b: 1) LPS (20 ng/ml) + preparation 1, 2) LPS + 2, 3) LPS + 3, 4) LPS + 4, 5) LPS + 5, 6) LPS + 6, 7) LPS + 7, 8) MDP, 9) LPS (20 ng/ml). Ordinate, indices of stimulation. MDP derivatives used in a concentration of 20 μg/ml. a) Results of 5 experiments given (M $\pm$ m); b) results of a typical experiment. *$p < 0.05$.

Fig. 2. Stimulation of TNF production by BALB/c mouse macrophages and MDP derivatives. Ordinate, number of cytoxin TNF sample (in %). a) Results of 3 trials given (M $\pm$ m); b) results of typical experiment. The remainder is defined in Fig. 1.

and P-MDP in a concentration of 20 μg/ml had a stronger activating effect on macrophagal IL-1 production than MDP ($p < 0.05$; Fig. 1a). The action of immunomodulators 1 and 3 was similar to that of MDP. On combined treatment of the macrophages with the preparations and LPS, marked stimulation of IL-l synthesis by substances 1, 3, 4, and 6 was observed (Fig. 1b). Incidentally, the activating action of preparations 1, 3, and 6 was stronger than the analogous effect of MDP. In subsequent experiments the ability of the immunomodulators to active TNF production by BALB/c mouse peritoneal mouse macrophages was studied. The preparations were used either as single agents or in combination with LPS. The results showed that preparations 3, 4, and 6 caused the greatest stimulation of TNF production (Fig. 2a). Meanwhile their activating effect did not exceed in magnitude the stimulating effect of MDP. Analysis of the results for the combined use of the preparations with LPS showed that β-$C_{16}H_{33}$ MDP exhibited the strongest activating effect (it was 1.9 times more active than the LPS + MDP combination; Fig. 2b).

It thus follows from the results that preparations 3 and 4 had the strongest immunomodulating effect. In several tests (macrophagal production of IL-1 and TNF) preparations 1 and 6 also exhibited activity. The feature of the chemical structure of these compounds, on account of which more marked activation of the functional activity of the macrophages and T lymphocytes takes place than with MDP, is a particularly interesting question. The possibility cannot be ruled out that incorporation of an aliphatic hydrocarbon chain into the composition of these substances

endows them with special amphiphilic properties and facilitates activator–target cell membrane interaction. Another possibility is that fixation of MDP to the carrier leads to more effective interaction with the corresponding receptors and to intensification of signal transduction. Identification of the molecular biochemical mechanisms of action of MDP derivatives on lymphocytes and macrophages will give the answer to these questions. It is planned to continue the study of the immunomodulating activity of MDP derivatives in vitro and in vivo in the future.

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