Thus, the findings do not provide a clear picture of how cholinergic and transmitter receptors interact in the model of spontaneous IgE and IgG production.

REFERENCES

1. T. A. Avdeeva, S. A. Kravchenko, and L. M. Rybchinskaya, *Immunologiya*, № 6, 43-45 (1986).
2. L. V. Bagaeva, V. F. Savel'eva, A. A. Pol'ner, *et al.*, *Ibid.*, № 4, 49-51 (1990).
3. L. V. Bagaeva, *Abstract of Dissertation* [in Russian], Moscow (1993), pp. 53-55.
4. G. F. Del Prete, D. Vercelli, A. Tiri, *et al.*, *J. Allergy Clin. Immunol.*, **79**, 69-77 (1987).
5. S. Grabczewska *et al.*, *Int. J. Tissue React.*, **12**, № 5, 281-289 (1990).
6. J. M. Knutti-Muller, B. M. Stadler, C. M. Magnusson, *et al.*, *Allergy*, **41**, 457-467 (1986).
7. K. J. Turner, N. P. Siemensma, K. D. Krska, *et al.*, *Immunol. Cell Biol.*, **66**, 123-133 (1988).
8. K. J. Turner, P. G. Holt, B. J. Holt, *et al.*, *Clin. Exp. Immunol.*, **68**, 409-417 (1987).
9. S. J. Zalcman, L. M. Neckers, *et al.*, *Life Sci.*, **29**, 69-73 (1981).

Effect of New Muramyl Dipeptide Derivatives on the Major Components of Immunity

O. V. Kalyuzhin, B. B. Fuks, N. V. Bovin, A. E. Zemlyakov, and V. Ya. Chirva

UDC 615.277.3.015.46.07

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 117, № 5, pp. 510-513, May, 1994
Original article submitted October 18, 1993

Immunomodulatory activity of five new synthetic muramyl dipeptide (MDP) derivatives (β-heptylglycoside-MDP, β-hexadecylglycoside-MDP, polyacrylamide-MDP, polyacrylamide-MDP-phosphatidylethanolamine, and dexal-MDP) is studied *in vitro* in different test systems.

Key Words: *muramyl dipeptide derivatives; immunomodulation*

MDP derivatives and glucosamine-MDP have been intensively studied during the last decade, but only a few preparations have been used in clinical practice, notably, in oncology [10]. Monotherapy with MDP derivatives is not effective enough. However, these preparations are of interest as components of complex therapy. The rather high toxicity of MDP, which is due to stimulated production of tumor necrosis factor (TNF), interleukin-1 (IL-1), and prostaglandins, is an obstacle impeding their clinical use. New analogs of natural MDP, which will be less toxic and more effective in the activation of regulator and effector lymphocytes, are required.

Research Institute of Human Morphology, Russian Academy of Medical Sciences, Moscow. (Presented by N. K. Permyakov, Member of the Russian Academy of Medical Sciences)

The aim of our study was to investigate five new MDP derivatives synthesized by us [2,6]: β-heptylglycoside-MDP (C_7H_{15}MDP); β-hexadecylglycoside-MDP ($C_{16}H_{33}$MDP); polyacrylamide-MDP (PMDP); polyacrylamide-MDP-phosphatidylethanolamine (PMDP-PE); and dexal-MDP (DMDP) in different test systems *in vitro*.

MATERIALS AND METHODS

C57Bl/6 and DBA/2 mice of both sexes were used in the study. Medium RPMI-1640 containing 5% fetal calf serum, 50 μg/ml gentamicin, 2 mM L-glutamine, 10 mM HEPES, and 5×10^{-5} M 2-mercaptoethanol was used in the experiments *in vitro*. MDP derivatives were used in the tests in equimolar concentrations: 5, 10, and 20 μM. C57Bl/6

0007-4888/94/0005-0508$12.50

mouse splenocytes (100 ml, $2 \cdot 10^6$ cells/ml) served as responsive cells in a 5-day mixed lymphocyte culture, and DBA/2 mouse splenocytes (50 μl, 15×10^6 cells/ml) treated with mitomycin C (50 μg/ml; 20×10^6 cells/ml; 37°C; 40 min) served as stimulators. Fifty microliters of solution of MDP derivatives were added to the wells in the experiment, and 50 μl of culture medium in the control. Eighteen hours before the completion of culturing 1 μCi of ^{3}H-thymidine (4 Ci/mmol) was added to each well. The index of stimulation of proliferation was calculated. A 3-day blast transformation test (BTT) [14] was performed using 100 μl of suspension (2.5×10^6 cells/ml) of C57Bl/6 mouse splenocytes with 50 μl of concanavalin A (ConA) (Pharmacia) or lipopolysaccharide (LPS, Difco) per well. The final concentration of mitogens was 1 μg/ml. Solutions of MDP derivatives (50 μl) were added to the experimental wells and culture medium was added to the control wells. Four hours before the completion of the test, 1 μCi of ^{3}H-thymidine (24 Ci/mmol) was added to each well. The proliferation index was calculated. Allospecific cytotoxic T lymphocytes (allo-CTL) were generated during 5 days in 24-well plates, the ratio between responsive (C57Bl/6 splenocytes; 2.5×10^6 cells/ml) and stimulating (DBA/2 splenocytes treated with mitomycin C) cells being 5:1. MDP derivatives were added to the experimental wells and culture medium to the control wells. The cytotoxic activity (CTA) was determined in the ^{51}Cr-release assay [8]. The ratio between the effector cells and target cells (P815 strain, 10^5 cells per well) was 30:1, 10:1, and 3:1. CTA was calculated routinely. IL-1 production by C57Bl/6 peritoneal macrophages was induced during 24 h after Lovett *et al.* [11]. Aliquots (1 ml) of peritoneal exudate

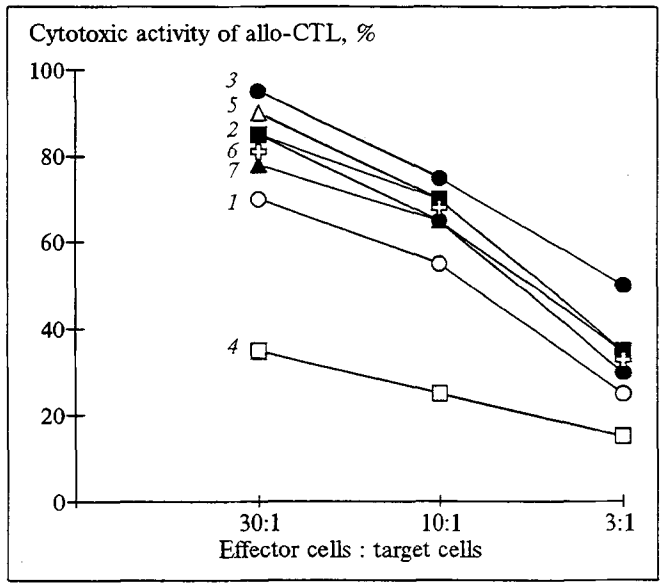

Fig. 1. Effect of MDP and its derivatives (20 μM) on generation of allo-CTL in a mixed lymphocyte culture. *1*) culture medium; *2*) MDP; *3*) C_7H_{15}MDP; *4*) $C_{16}H_{33}$MDP; *5*) PMDP; *6*) PMDP-PE; *7*) DMDP.

cell suspension (5×10^6 cells/ml) were added to the wells of 24-well Linbro plates. After 2 h, nonadherent cells were removed, and 2-ml aliquots of solutions of MDP derivatives (2-ml aliquots of the culture medium in the control) were added to the wells. In studies of synergism between MDP derivatives and LPS, 20 ng/ml LPS (Difco) were added to the cultures. TNF production was induced in the same manner as IL-1 production. IL-1 was tested after Uede *et al.* [15] and TNF after Fish *et al.* [9].

For assessment of the activity of natural killers (NK), C57Bl/6 mouse splenocytes (1 ml, 10^6 cells/ml) were added to the wells of a 24-well plate, and 1-ml aliquots of solutions of MDP de-

TABLE 1. Effect of MDP Derivatives (20 μM) on Splenocyte Proliferation under the Influence of 1 μg/ml LPS or ConA in BTT (A) and on IL-1 Production by Peritoneal Macrophages (B), and Effect of MDP Derivatives (10 μM) on the Cytotoxic Activity of NK (C)

Experimental conditions	A		B	C
	Proliferation index		Index of IL-1-induced thymocyte proliferation	CTA, %
	ConA	LPS		
1. Culture medium	6.7±0.6	3.4±0.2	–	24±4
2. MDP	7.5±0.7	4.1±0.5*	3.9±0.3	28±2
3. C_7H_{15}MDP	7.9±0.5*	4.4±0.3**	4.8±0.2**	31±2**
4. $C_{16}H_{33}$MDP	5.5±0.2**	3.0±1.0	3.9±0.1	21±3
5. PMDP	6.9±0.4	4.1±0.4*	3.9±0.3	34±3**
6. PMDP-PE	6.9±0.6	3.9±0.8	3.1±0.2**	28±4
7. DMDP	8.8±0.5**	4.6±0.3**	3.3±0.3*	31±3**

Note. The reliability of differences between the effect of MDP derivatives and the control (the culture medium for A and C and MDP for B) at $p<0.01$ and $p<0.05$ is denoted by one and two asterisks, respectively.

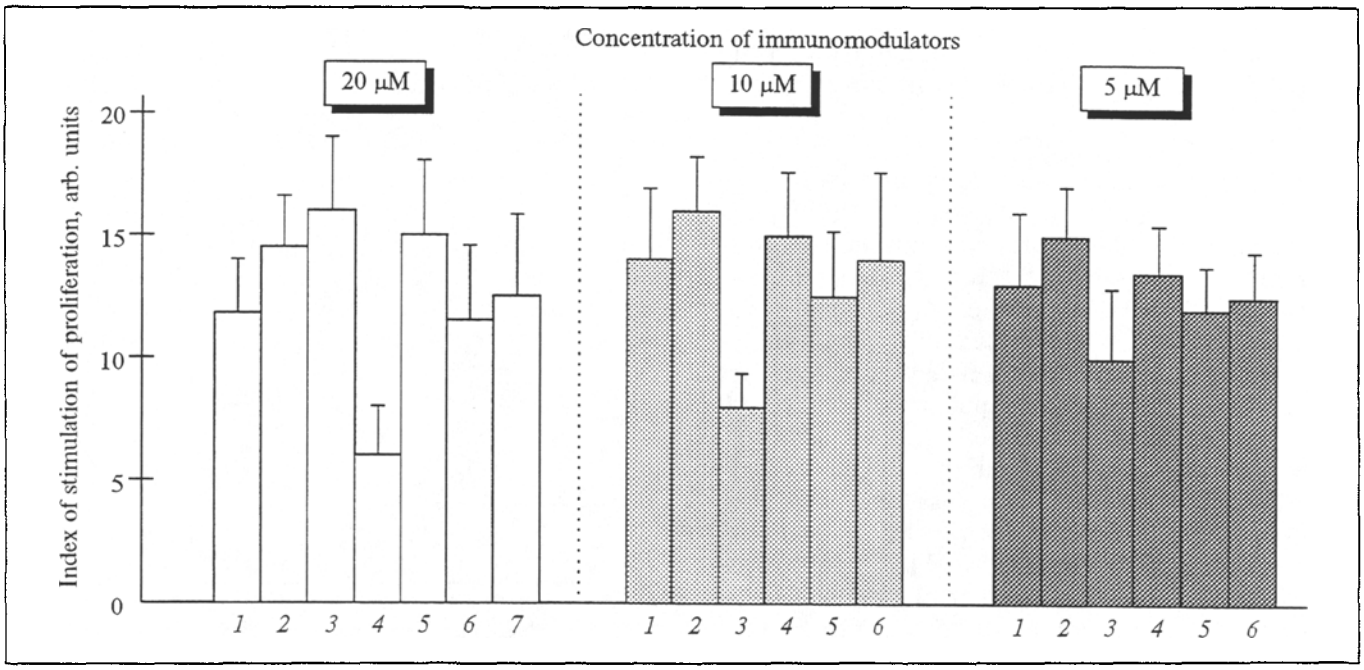

Fig. 2. Effect of MDP and its derivatives on proliferation of murine splenocytes in a mixed lymphocyte culture (rel. units). *1*) culture medium; *2*) MDP; *3*) C_7H_{15}MDP; *4*) $C_{16}H_{33}$MDP; *5*) PMDP; *6*) PMDP–PE; *7*) DMDP.

rivatives (1-ml aliquots of the culture medium in the control) were added to the wells. Forty-eight hours later the cells were washed three times, and CTA was determined in a 4-h ^{51}Cr-release assay, the ratio between effector and target (YAK-1 strain) cells being 100:1, 50:1, and 25:1.

RESULTS

C_7H_{15}MDP was the strongest stimulator of the generation of allo-CTL. In a dose of 20 μM it raised the cytotoxic activity by 32-109%, which was 15-48% higher than in the case of MDP ($p<0.05$) (Fig. 1). The activating effect of the preparation in lower concentrations (10 and 5 μM) dropped slightly. MDP (20 mM) enhanced the cytotoxicity of allo-CTL by 16-41% ($p<0.05$). Similar effects were exerted by PMDP and DMDP. PMDP-PE virtually did not affect the generation of allo-CTL, and $C_{16}H_{33}$MDP in a dose of 20 μM even inhibited it by 51-59% ($p<0.05$), while having no effect in a concentration of 5 μM.

These findings correlated with the results of studies of the effect of MDP analogs on splenocyte proliferation in a mixed lymphocyte culture. C_7H_{15}MDP was the most active stimulator of proliferation (Fig. 2). In a dose of 10 μM it caused a 38% activation of proliferation, this being 16% higher than in the case of MDP ($p<0.05$). PMDP did not differ from MDP, while $C_{16}H_{33}$MDP inhibited proliferation. When the concentration of preparation was lowered from 20 to 5 μM, the inhibition decreased from 50 to 21%. DMDP and PMDP-PE exerted weak effects.

The doses of LPS and ConA in the BTT were chosen so that they constituted 30-50% of the optimal concentration. DMDP in a dose of 20 μM enhanced the ConA-induced proliferation by 31% ($p<0.05$) (Table 1). MDP and C_7H_{15}MDP slightly activated the reaction of blasttransformation, PMDP-PE and PMDP caused no effect, and $C_{16}H_{33}$MDP suppressed proliferation. MDP, C_7H_{15}MDP, and DMDP enhanced the effect of LPS to an approximately equal degree (20-35%, $p<0.05$) (Table 1). PMDP activated blasttransformation only in a dose of 20 μM (20%, $p<0.1$). PMDP-PE had no effect on blasttransformation, and $C_{16}H_{33}$MDP inhibited it.

PMDP in a dose of 10 μM enhanced the spontaneous CTA with respect to NK-sensitive YAK-1 cells (the ratio between effector and target cells being 25:1) by 42% ($p<0.05$) (Table 1). The activity of C_7H_{15}MDP in a dose of 10 μM (29%, $p<0.05$) was somewhat lower. MDP in all doses and its derivatives in concentrations 20 and 5 μM had no significant effect on the activity of NK, except for $C_{16}H_{33}$MDP, which in a dose of 20 μM suppressed the activity of NK by 21-47% ($p<0.05$).

C_7H_{15}MDP in a dose of 20 μM was the strongest activator of IL-1 (25%, $p<0.05$; Table 1) and TNF (38%, $p<0.1$; Fig. 3) production. When its dose was lowered to 5-10 μM, its activity slightly dropped and differed little from the effect of MDP. The activity of the other preparations did not surpass that of MDP, while the effect of

PMDP-PE was inferior to that of MDP. Similar results were obtained in studies of the synergic effect of MDP derivatives and LPS on IL-1 and TNF production. Only $C_{16}H_{33}$MDP in a dose of 20 μM enhanced IL-1 production more actively (by 14%, $p<0.05$) than MDP.

Our findings, for the most part, do not contradict published data [3-5,7,12,13].

In almost all tests the amphiphilic preparation $C_{17}H_{15}$MDP exhibited an activity which was markedly higher than the effect of MDP. It is worthy of note that C_7H_{15}MDP markedly enhanced T-lymphocyte proliferation and the generation of allo-CTL in a mixed culture. These results provide evidence that the regulatory (helper) component in a mixed lymphocyte culture is activated by C_7H_{15}MDP, this leading to reinforcement of the killer component. It is still not clear, how C_7H_{15}MDP activates the production of the important "helper" lymphokines: IL-2 and IL-4. As is well known [10], in some tumors it is precisely these components of the intratumor immune system which are most likely to suffer from immunosuppressive factors of the tumor cell. Such an effect is absent in BTT with ConA and LPS, i.e., this probably occurs under conditions where the proliferation rate depends less on a deficiency of helper functions. The third important component of antitumor immunity, NK, is also considerably activated by this preparation. Finally, C_7H_{15}MDP markedly boosts IL-1 and TNF production.

$C_{16}H_{33}$MDP, a lipophilic analog of the amphiphilic preparation (C_7H_{15}MDP) with a longer aliphatic chain, exerted the opposite effect upon the same components of immunity, except for IL-1 and TNF production. Without an indepth analysis of this fact, we are obliged to conclude that the amphiphilic derivative of MDP, i.e., a substance which more readily migrates from the aqueous phase to the lipid membranes and vice versa, has a more marked effect on the important cytoplasmic and membraneous events leading to lymphocyte activation than its lipophilic homolog $C_{16}H_{33}$MDP, which must be retained by the lipid membranes *a priori*.

In our experiments the use of a polymeric substrate for the MDP groups did not enable us to obtain the same effect as was caused by the presence of the amphiphilic chain. This also applies to the combination of polymeric and lipophilic properties (PMDP-PE). Our findings map out a clear path for further research: the combinaiton in one molecule of physiologically active chemical groups with the amphiphilic nature of the molecule as a whole.

To sum up, we recommend using C_7H_{15}MDP as a modulator of antitumor immunity in further experiments on tumor-bearing animals *in vivo* and, at the next stage, in clinical trials.

PMDP and DMDP are also of some interest. For instance, PMDP in a dose of 10 μM enhanced the cytotoxicity of NK by 42%, and the effect of DMDP with respect to the enhancement of lymphocyte proliferation in BTT with a suboptimal dose of ConA surpassed that of MDP.

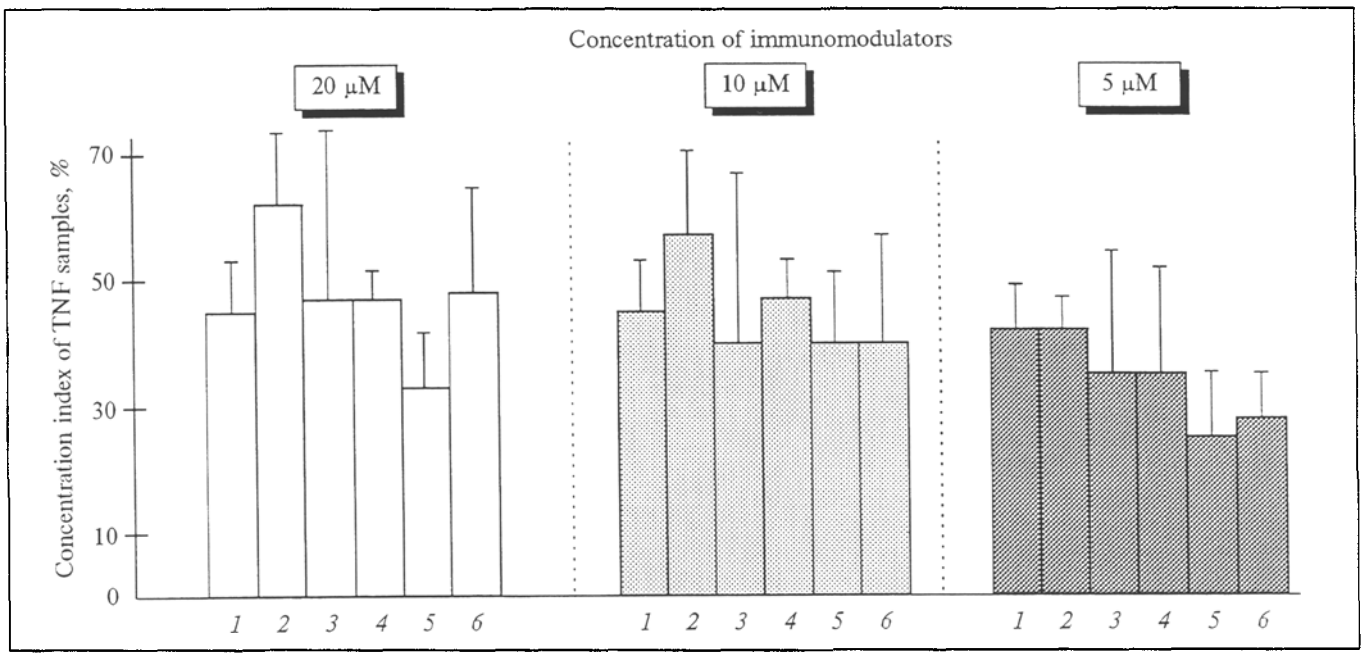

Fig. 3. Effect of MDP and its derivatives on TNF production by murine peritoneal macrophages. *1*) culture medium; *2*) MDP; *3*) C_7H_{15}MDP; *4*) $C_{16}H_{33}$MDP; *5*) PMDP; *6*) PMDP–PE; *7*) DMDP.

$C_{16}H_{33}$MDP induced activation of macrophages to the same degree as MDP, stimulating IL-1 and TNF production. $C_{16}H_{33}$MDP in a dose of 20 μM in combination with a suboptimal dose of LPS (20 ng/ml) was more effective with respect to the induction of IL-1 production than MDP in combination with LPS in the same doses. However, in other tests the preparation exhibited a pronounced inhibitory effect, which diminished when the concentration of the preparation was lowered from 20 to 5 μM.

REFERENCES

1. B. D. Brondz, A. V. Karaulov, and I. F. Abronina, *Molekul. Biol.*, № 6, 1287-1295 (1979).
2. A. E. Zemlyakov and V. Ya. Chirva, *Khimiya Prirod. Soed.*, № 5, 714-718 (1987).
3. A. L. Rakhmilevich, M. S. Rakhimova, and T. M. Andronova, *Antibiotiki Chimioter.*, № 8, 586-589 (1989).
4. B. B. Fuks, A. L. Rakhmilevich, A. A. Pimenov, and A. G. Dubrovskaya, *Byull. Eksp. Biol. Med.*, **104**, № 10, 497-499 (1987).
5. M. Akasaki, T. Takasaki, Y. Kita, and W. Tsukada, *Agents and Actions*, **22**, 144-150 (1987).
6. N. V. Bovin, E. U. Korchagina, T. V. Zemlyanukhina, *et al., Glycoconj. J.*, **10**, 142-151 (1993).
7. E. Brummer and D. A. Stevens, *Cell Immunol.*, **91**, 505-514 (1985).
8. K. T. Brunner, H. D. Engers, and J.-C. Cerrotini, in: *In Vitro Methods in Cell-Mediated and Tumor Immunity* (Ed. B. R. Bloom and J. David), New York (1976), pp. 423-430.
9. H. Fish *et al., Int. J. Cancer*, **32**, 105-112 (1983).
10. B. B. Fuchs and A. L. Rahmilevich, *Sov. Med. Rev. D. Immunol.*, **3**, 1-106 (1991).
11. D. Lovett, B. Kozan, M. Hadam, *et al., J. Immunol.*, **136**, 340-347 (1986).
12. I. Saiki, S. Saito, C. Fujita, *et al., Vaccine*, **6**, 238-244 (1988).
13. S. D. Sharma, V. Tsai, J. L. Krahenbuhl, and J. S. Remington, *Cell Immunol.*, **52**, 101-109 (1981).
14. P. Stromberg, D. N. McMurray, and C. A. Brown, *J. Clin. Lab. Immunol.*, **25**, 89-95 (1988).
15. T. Uede, Y. Koeda, Y. Ibayashi, *et al., J. Immunol.*, **135**, 3243-3251 (1986).