IMMUNOLOGY AND MICROBIOLOGY

Effects of Muramyl Dipeptide Glycosides on Lymphocyte Proliferation and Production of Interleukin-2

O. V. Kalyuzhin, A. E. Zemlyakov, E. V. Kalyuzhina, M. V. Shkalev, and M. V. Nelyubov

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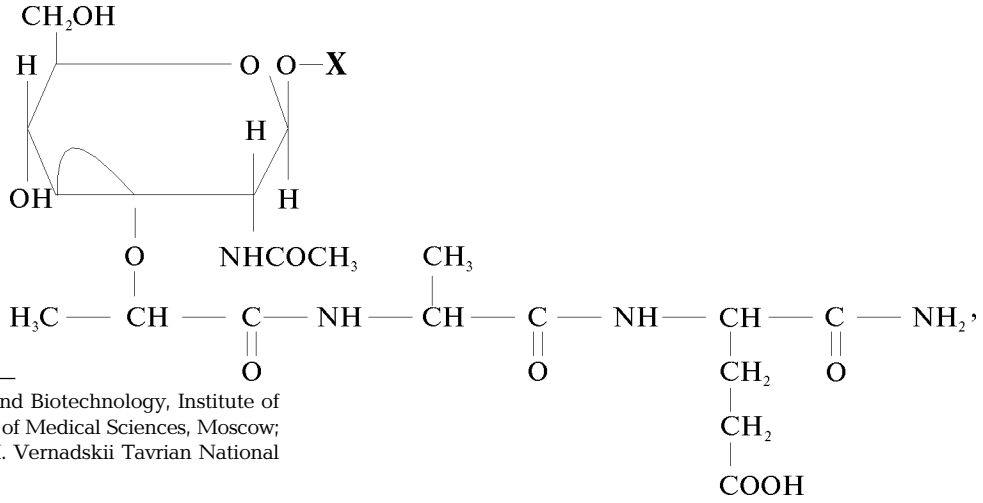
We studied the effect of new muramyl dipeptide glycosides with different structure of aglycon and configuration glycoside bond on spontaneous and mitogen-induced proliferation of mouse splenocytes and production of interleukin-2 by these cells. Biological activity of muramyl dipeptide β-glycosides with aliphatic, carbocyclic, adamantile, and phenol aglycons is higher than that of the original compound, while α-glycosylation decreases the immunostimulating effect of this glycopeptide.

Key Words: *muramyl dipeptide glycosides; proliferation; interleukin-2*

N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) is a minimal structure determining immunomodulating effects of complete Freund adjuvant, which can replace mycobacterium in its content [7]. Attempts at increasing biological activity of MDP via modulation of its structure were undertaken [3-5,9]. We used the glycoside core of the molecule for introduction of modifying components in MDP structure. Glycoside modification fixes the C_1 configuration and prevents the formation of anomeric mixture characteristic of MDP and its derivatives with free hydroxyl forms in aqueous solutions. This makes unnecessary temporary protection of the anomeric hydroxyl, thus simplifying glycopeptide synthesis, and increases stability of muramyl peptides with lipophilic aglycons in biological media compared to lipophilic MDP esters [2].

The structural formula of MDP β-glycosides is the follows:



Laboratory of Cell Immunopathology and Biotechnology, Institute of Human Morphology, Russian Academy of Medical Sciences, Moscow; Department of Organic Chemistry, V. I. Vernadskii Tavrian National University, Simferopol

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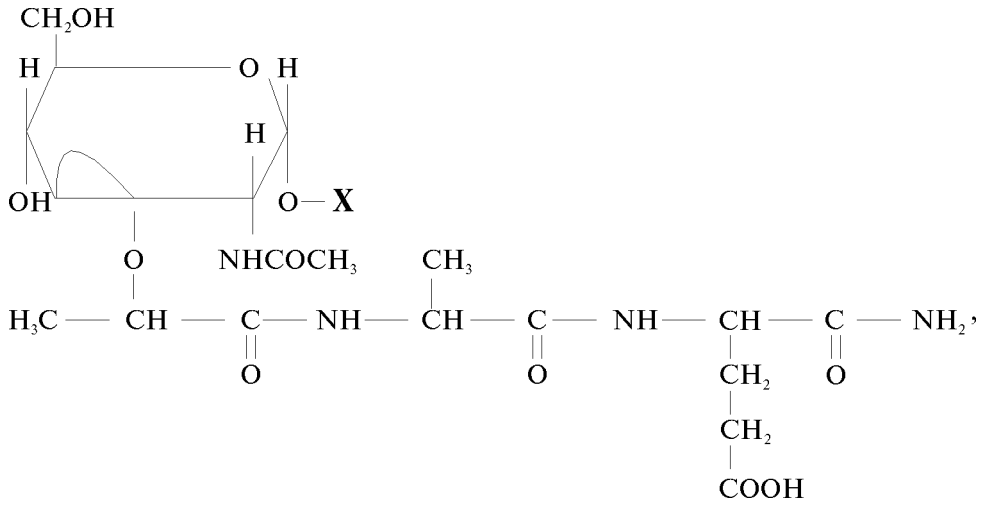
X (aglycon)	β-Glycoside	
	name	abbreviation
C_4-alkyl	MDP β-butylglycoside	MDP β-C_4
C_6-alkyl	MDP β-hexylglycoside	MDP β-C_6
C_7-alkyl	MDP β-heptylglycoside	MDP β-C_7
C_8-alkyl	MDP β-octylglycoside	MDP β-C_8
C_6-cycloalkyl	MDP β-cyclohexylglycoside	MDP β-cycl
Adamantyl	MDP β-adamantylglycoside	MDP β-ada
Phenyl	MDP β-phenylglycoside	MDP β-phen
Phenethyl	MDP β-phenethylglycoside	MDP β-pheneth
Naphthyl	MDP β-naphthylglycoside	MDP β-naphth

The structural formula of MDP α-glycosides is the follows:



X (aglycon)	α-Glycoside	
	name	abbreviation
C_7-alkyl	MDP α-heptylglycoside	MDP α-C_7
C_6-cycloalkyl	MDP α-cyclohexylglycoside	MDP α-cycl

We studied biological activity of some original MDP glycosides with different aglycon structures and glycoside bond configurations. To this end, we used test-systems allowing evaluation of the effects of test preparations on lymphocyte proliferation and production of interleukin-2 (IL-2), the key events in the series of homeostatic and defense immune reactions.

MATERIALS AND METHODS

MDP α- and β-glycosides were synthesized as described previously [2]. The cells were cultured in RPMI-1640 (Flow Lab.) supplemented with 5% inactivated fetal calf serum (Flow Lab.), 2 mM L-glutamine, 10 mM HEPES buffer (Flow Lab.), 5×10^{-5} M 2-mercaptoethanol (Serva), and 50 μg/ml gentamycin (Unique) at 37°C in humid air with 5% CO_2. The effects of MDP derivatives (1, 10, and 100 μg/ml) on spontaneous and mitogen-induced proliferation of splenocytes in 6-8-week-old C57Bl/6 mice (from Center for Breeding of Experimental Animals, Kryukovo Department) were evaluated as described previously [8,11]. Concanavalin A (Pharmacia Fine Chemicals) and *Escherichia coli* lipopolysaccharide 0111:B4 (Difco Lab.) in final concentrations of 1 μg/ml were used as standard mitogens. Proliferative activity (proliferation

TABLE 1. Effects of MDP Glycosides (10 μg/ml) on Lymphocyte Proliferation (*M*±*m*)

Experimental series	Proliferation index, arb. units	
	spontaneous	conA-stimulated
Control	1.0±0.2	11.8±1.1
MDP	3.1±0.3*	19.1±1.2*
MDP β-C_4	3.2±0.4*	19.3±1.8
MDP β-C_6	3.8±0.3*	20.5±2.9*
MDP β-C_7	4.2±0.3*+	25.2±1.7*+
MDP β-C_8	4.3±0.4*+	24.8±2.0*+
MDP α-C_7	1.8±0.2*+	13.0±1.8+
MDP β-ada	3.9±0.2*+	25.0±2.2*+
MDP β-cycl	2.9±0.4*	19.9±2.6
MDP α-cycl	1.9±0.2*+	13.8±1.7+
MDP β-phen	4.0±0.3*+	23.7±2.1*+
MDP β-pheneth	3.0±0.3*	20.6±2.4*
MDP β-naphth	2.6±0.4*	19.8±1.8*

Note. Here and in Table 2: $p<0.05$ *compared to the control, +compared to MDP.

index) was evaluated by the ratio of ^{3}H-thymidine incorporation (cpm) in the experiment and control cultures.

The production of IL-2 was induced by a modified method [10]. In brief, C57Bl/6 mouse splenocytes were suspended (5×10^6 cells/ml) in a medium containing muramyl peptides (1, 10, and 100 μg/ml) and incubated for 24 h. For evaluation of the effects of MDP glycosides on mitogen-stimulated production of IL-2 the test agents were added in cultures simultaneously with concanavalin A (1 μg/ml). After incubation the cells were washed 3 times, resuspended in medium without mitogen and test drugs to a concentration of 5×10^6 cells/ml, and cultured in 24-well plates (Linbro) for 24 h. After incubation the cells were precipitated by centrifugation and the supernatants were collected and stored at -20°C. The activity of IL-2 (IL-2-like factors) in supernatants was evaluated using IL-2-dependent CTLL T-cell strain [6]. MDP served as the reference control in all test systems.

RESULTS

All test agents in a wide dose range activated spontaneous lymphocyte proliferation *in vitro*. The concentration of 10 μg/ml was optimum for the majority of drugs (Table 1). MDP α-glycosides were less active than β-glycoside anomers and original MDP. β-Glycosides MDP β-C_7, MDP β-C_8, MDP β-ada, and MDP β-phen most effectively stimulated cell proliferation. Their activity in the majority of experiments surpassed that of MDP.

The same MDP derivatives showed a pronounced comitogenic effect in combination with lipopolysaccharides and concanavalin A in suboptimal concentrations (1 μg/ml, Table 1). Other β-glycosides activated mitogen-induced cell division similarly to MDP. MDP β-glycosides in all doses did not appreciably modulate lymphocyte proliferation stimulated by B- and T-cell mitogens. Only a trend to activation of lymphocyte proliferation was observed at a concentration of 10 μg/ml (Table 1).

All MDP derivatives enhanced concanavalin A-induced production of IL-2-like factors (Table 2). β-Glycosides were most active in this respect. The stimulatory effect of these compounds (except MDP

TABLE 2. Effects of MDP Glycosides on Spontaneous and Concanavalin A-Stimulated Production of IL-2 by Splenocytes of C57Bl/6 Mice (*M*±*m*, *n*=3)

MDP derivatives	Concentrations of MDP derivatives, μg/ml		
	1	10	100
Control	5.1±1.1 / 12.7±1.5	— / —	— / —
MDP	18.1±2.2* / 6.3±0.8	22.8±2.8* / 8.4±1.6*	19.5±2.0* / 6.9±1.0
MDP β-C_4	6.8±1.2 / 17.6±2.0*	10.5±1.5* / 28.9±1.8*+	7.0±1.3 / 24.9±2.3*+
MDP β-C_6	8.9±1.5* / 21.8±1.8*	12.8±1.2*+ / 29.7±2.1*+	10.3±1.1*+ / 25.6±3.1*+
MDP β-C_7	9.3±1.1*+ / 20.9±1.8*	12.7±1.0*+ / 30.3±3.3*+	10.6±1.4*+ / 26.4±3.2*+
MDP β-C_8	9.1±1.3*+ / 22.1±2.5*	13.1±1.6* / 29.5±2.7*+	10.1±1.3*+ / 25.5±2.5*+
MDP α-C_7	5.3±0.8 / 14.7±1.7	5.6±0.9 / 16.9±2.1*+	6.3±1.2 / 18.1±2.6*
MDP β-ada	7.6±0.8* / 21.9±1.8*	13.5±2.1*+ / 31.1±3.9*+	9.9±1.2*+ / 26.4±2.9*+
MDP β-cycl	7.9±1.1* / 22.3±2.1*	11.3±2.0* / 29.8±2.8*+	7.5±0.8* / 24.7±3.3*
MDP α-cycl	4.9±0.7 / 15.0±1.6	4.7±1.0+ / 18.4±2.5*	5.9±1.1 / 16.9±2.7
MDP β-phen	8.2±0.9* / 20.8±2.2*	9.6±1.1* / 29.9±3.3*+	10.3±1.2*+ / 25.7±2.4*+
MDP β-pheneth	6.4±0.8 / 19.7±2.0*	8.5±0.9* / 30.7±3.5*+	7.6±0.8* / 23.9±1.5*+
MDP β-naphth	7.1±1.1 / 16.9±1.6*	7.9±1.3* / 25.3±3.7*	6.8±1.0 / 19.1±2.4*

β-naphth) on the production of IL-2 significantly ($p<0.05$) surpassed that of original MDP. α-Glycosides produced a less pronounced effect compared to that of MDP (Table 2).

Splenocyte pretreatment with nonmodified MDP in all doses only slightly induced IL-2 synthesis (Table 2). Similar effect was produced by MDP α-glycosides in high concentrations (100 μg/ml). In lower concentrations α-glycosides had no effect on the basal IL-2 production by intact lymphocytes. β-Glycosides MDP β-C_6, MDP β-C_7, MDP β-C_8, MDP β-cycl, MDP β-ada, MDP β-phen, and MDP β-pheneth in most cases stimulated IL-2 production and it significantly surpassed the basal production of this lymphokine in the control (Table 2). MDP β-C_4 and MDP β-naphth only slightly stimulated IL-2 production. The following glycosides stimulated the production of IL-2 more actively than MDP: MDP β-C_6 in concentrations of 10 and 100 μg/ml, MDP β-C_7 (1, 10, and 100 μg/ml), MDP β-C_8 (1 and 100 μg/ml), MDP β-ada (10 and 100 μg/ml), and MDP β-phen (100 μg/ml).

These data agree with the results of evaluation of the effects of these MDP derivatives on proliferative activity of lymphocytes. This is not surprising, because proliferative response of lymphocytes to mitogens includes production and reception of IL-2 [1]. These events play the key role in lymphocyte recruitment in the proliferation cycle. We conclude that stimulation of spontaneous and mitogen-induced lymphocyte proliferation by MDP derivatives is associated with stimulation of IL-2 production by Th1 lymphocytes. Activation of lymphokine production and cell proliferation in our test systems induced by muramyl peptides resulted from both their direct effect on lymphocytes and their effect on macrophages. Macrophages produce IL-1 (among other things) stimulating not only IL-2 production, but also reception of this cytokine by lymphocytes.

Hence, MDP derivatives induce and stimulate IL-2 production and lymphocyte proliferation. This is true primarily for MDP β-glycosides with aliphatic (MDP β-C_6, MDP β-C_7, MDP β-C_8), carbocyclic (MDP β-cycl, MDP β-ada), and phenol (MDP β-phen) aglycons, whose immunostimulating effect surpassed that of nonmodified MDP, *i.e.* structural modification and synthesis of β-glycosides increased biological activity of MDP, while α-glycosylation attenuates the immunotropic effects of MDP.

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