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Stimulation of Nonspecific Resistance to Infection Induced by 6-*O*-Acyl Muramyl Dipeptide Analogs in Mice

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The experimental system utilized in investigating the correlation between the chemical structures of muramyl peptides and their protective activities in the sepsis type of systemic infections caused by *Escherichia coli* was applied in evaluating the enhancement of resistance to infection induced by 32 synthetic glycopeptide analogs, including 6-*O*-acyl derivatives and 1-α-*O*-benzyl derivatives of muramyl dipeptide (*N*-acetyl muramyl-L-alanyl-D-isoglutamine). In assessing the 6-*O*-acyl derivatives of muramyl dipeptide, we found that the degree of protective activity was attributable to the kinds of fatty acids introduced. Acylation of the 6-hydroxy group on the muramic acid moiety in muramyl dipeptide with natural mycolic acid or a synthetic fatty acid possessing either an α-branched or an α-branched, β-hydroxylated group resulted in a decrease in or a disappearance of the protective activity of muramyl dipeptide. Acylation with a normal fatty acid or an iso fatty acid resulted in a retention or enhancement of muramyl dipeptide activity. The activity of acylated derivatives containing linear fatty acids was stimulated by increasing the chain length up to 18 carbon atoms. The highest degree of protective activity occurred with the derivatives acylated with straight-chain fatty acids, particularly with the derivatives acylated with palmitic acid and arachidic acid. Benzylation of the 1-hydroxy group of muramyl dipeptide resulted in a decrease in or a loss of protective activity.

Muramyl dipeptide [MDP(Ala)] has been shown to be the smallest unit responsible for the adjuvant activity of bacterial cell walls (12, 23, 29). Research has been conducted on the biological activities of MDP(Ala), such as adjuvant activity for delayed-type hypersensitivity (DTH) and circulating antibody formation (1, 3, 4, 6, 7, 17, 20), induction of tumor immunity (5), enhancement of resistance to infection (6, 10), pyrogenicity (11, 19, 22), cell-mediated cytotoxicity (15), mitogenicity (37, 38), and arthritogenicity (16, 30). In a study of enhancement of host resistance to infection, Chedid et al. described several active MDP(Ala) analogs and the relationship between the chemical structures and the protective activities of 22 derivatives (10).

We have developed a system for evaluating the protective activities induced by MDP(Ala) derivatives in the sepsis type of systemic infections caused by *Escherichia coli* in STD:ddY mice (K. Matsumoto et al., submitted for publication).

Our current research is directed toward identifying compounds capable of surpassing the protective activity of MDP(Ala) and toward investigating the relationship between the chemical structures and the protective activities of newly synthesized MDP(Ala) derivatives. In this paper, we describe the protective capacities of 6-*O*-acyl derivatives of MDP(Ala).

MATERIALS AND METHODS

Animals. Strain STD:ddY outbred mice were obtained from Shizuoka Cooperative for Experimental Animals, Hamamatsu, Japan, and were maintained on a basal laboratory chow (type F-2; Funabashi Farm). We used 5-week-old male mice weighing 25 to 27 g in all experiments.

Synthetic glycopeptides. In this study, we used 35 compounds, including three different groups of glycopeptides and two acids (Table 1). The first group consisted of compounds 2 and 3 (Table 1), in which the L-alanyl residue of MDP(Ala) was replaced with the residue of another L-amino acid. The second group of compounds, compounds 4 through 27, was composed of 24 6-*O*-acylated derivatives containing several different types of fatty acids. The third group, compounds 28 through 32, was composed of 1-α-*O*-benzyl derivatives of MDP(Ala). We also used two acids, isopentadecanoic acid (compound 33) and stearic acid (compound 34).

MDP(Ala) (compound 1) and the first group of compounds (compounds 2 and 3) were synthesized by the method of Kusumoto et al. (24, 28). In the second

TABLE 1. *Synthetic glycopeptides and related compounds used in this study*

Compound	Abbreviation	Name and/or chemical structures	Amt (μg/mouse)[a]
1	MDP(Ala)	*N*-acetyl muramyl-L-alanyl-D-isoglutamine (MurNAc-L-Ala-D-isoGln)	100
2	MDP(Val)	*N*-acetyl muramyl-L-valyl-D-isoglutamine (MurNAc-L-Val-D-isoGln)	100
3	MDP(Ser)	*N*-acetyl muramyl-L-seryl-D-isoglutamine (MurNAc-L-Ser-D-isoGln)	100
4	S.mycol-MDP(Ala)	6-*O*-smegmamycoloyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (6-*O*-S.mycol-MurNAc-L-Ala-D-isoGln)	333
5	S.mycol-MDP(Ser)	6-*O*-smegmamycoloyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (6-*O*-S.mycol-MurNAc-L-Ser-D-isoGln)	326
6	N.mycol-MDP(Ser)	6-*O*-nocardomycoloyl-*N*-acetyl muramyl-L-seryl-D-isoglutamine (6-*O*-N.mycol-MurNAc-L-Ser-D-isoGln)	237
7	B30-MDP(Ala)	6-*O*-(2-tetradecylhexadecanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine {6-*O*-CH_3-$(CH_2)_{13}$-CH[$(CH_2)_{13}$-CH_3]-CO-MurNAc-L-Ala-D-isoGln}	191
8	BH32-MDP(Ala)	6-*O*-(3-hydroxy-2-tetradecyloctadecanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine {6-*O*-CH_3-$(CH_2)_{14}$CH(OH)-CH[$(CH_2)_{13}$-CH_3]-CO-MurNAc-L-Ala-D-isoGln}	209
9	B46-MDP(Ala)	6-*O*-(2-docosyltetracosanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine {6-*O*-CH_3-$(CH_2)_{21}$-CH-[$(CH_2)_{21}CH_3$]-CO-MurNAc-L-Ala-D-isoGln}	217
10	BH48-MDP(Ala)	6-*O*-(3-hydroxy-2-docosylhexacosanoyl)-*N*-acetyl muramyl-L-alanyl-D-isoglutamine {6-*O*-CH_3-$(CH_2)_{22}$CH(OH)CH[$(CH_2)_{21}$-CH_3]-CO-MurNAc-L-Ala-D-isoGln}	236
11	Iso.15-MDP(Ala)	6-*O*-isopentadecanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-$(CH_3)_2$CH-$(CH_2)_{11}$-CO-MurNAc-L-Ala-D-isoGln]	145
12	L2-MDP(Ala)	6-*O*-acetyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine (6-*O*-CH_3-CO-MurNAc-L-Ala-D-isoGln)	105
13	L4-MDP(Ala)	6-*O*-butyryl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_2$-CO-MurNAc-L-Ala-D-isoGln]	111
14	L8-MDP(Ala)	6-*O*-octanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_6$-CO-MurNAc-L-Ala-D-isoGln]	117
15	L10-MDP(Ala)	6-*O*-decanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_8$-CO-MurNAc-L-Ala-D-isoGln]	122
16	L12-MDP(Ala)	6-*O*-lauroyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{10}$-CO-MurNAc-L-Ala-D-isoGln]	133
17	L14-MDP(Ala)	6-*O*-myristoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{12}$-CO-MurNAc-L-Ala-D-isoGln]	139
18	L16-MDP(Ala)	6-*O*-palmitoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{14}$-CO-MurNAc-L-Ala-D-isoGln]	145
19	L17-MDP(Ala)	6-*O*-heptadecanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{15}$-CO-MurNAc-L-Ala-D-isoGln]	147
20	L18-MDP(Ala)	6-*O*-stearoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{16}$-CO-MurNAc-L-Ala-D-isoGln]	150
21	L19-MDP(Ala)	6-*O*-nonadecanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{17}$-CO-MurNAc-L-Ala-D-isoGln]	153
22	L20-MDP(Ala)	6-*O*-eicosanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{18}$-CO-MurNAc-L-Ala-D-isoGln]	156
23	L24-MDP(Ala)	6-*O*-tetracosanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{22}$-CO-MurNAc-L-Ala-D-isoGln]	167
24	L30-MDP(Ala)	6-*O*-triacontanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{28}$-CO-MurNAc-L-Ala-D-isoGln]	184
25	L18-MDP(Val)	6-*O*-stearoyl-*N*-acetyl muramyl-L-valyl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{16}$-CO-MurNAc-L-Val-D-isoGln]	150
26	L18-MDP(Ser)	6-*O*-stearoyl-*N*-acetyl muramyl-L-seryl-D-isoglutamine [6-*O*-CH_3-$(CH_2)_{16}$-CO-MurNAc-L-Ser-D-isoGln]	150

TABLE 1—*Continued*

Compound	Abbreviation	Name and/or chemical structure	Amt (μg/mouse)[a]
27	L18-MDP(Ala-L-isoGln)	6-*O*-stearoyl-*N*-acetyl muramyl-L-alanyl-L-isoglutamine [6-*O*-CH_3-$(CH_2)_{16}$-CO-MurNAc-L-Ala-L-isoGln]	150
28	α-Bzl-MDP(Ala)	1-α-*O*-benzyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [1-α-*O*-Bzl]-MurNAc-L-Ala-D-isoGln]	118
29	α-Bzl-Iso.15-MDP(Ala)	1-α-*O*-benzyl-6-*O*-isopentadecanoyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine [1-α-*O*-Bzl-6-*O*-$(CH_3)_2CH$-$(CH_2)_{11}$-CO-MurNAc-L-Ala-D-isoGln]	163
30	α-Bzl-MDP(Ala)-*O*Bzl	1-α-*O*-benzyl-*N*-acetyl muramyl-L-alanyl-D-isoglutamine-benzylester [1-α-*O*-Bzl-MurNAc-L-Ala-D-Glu(*O*Bzl)-NH_2]	136
31	α-Bzl-MDP(Val)-*O*Bzl	1-α-*O*-benzyl-*N*-acetyl muramyl-L-valyl-D-isoglutamine-benzylester [1-α-*O*-Bzl-MurNAc-L-Val-D-Glu(*O*Bzl)-NH_2]	135
32	α-Bzl-MDP(Ser-Bzl)-*O*Bzl	1-α-*O*-benzyl-*N*-acetyl muramyl-*O*-benzyl-L-seryl-D-isoglutamine-benzylester [1-α-*O*-Bzl-MurNAc-L-Ser-Bzl-D-Glu(*O*Bzl)-NH_2]	153
33	Iso.15 acid	Isopentadecanoic acid [$(CH_3)_2CH(CH_2)_{11}COOH$]	100
34		Stearic acid [$CH_3(CH_2)_{16}COOH$]	100

[a] Amount administered per mouse [0.2 μmol of each compound; equivalent to 100 μg of MDP(Ala)].

group, three compounds (compounds 4 through 6) were synthesized by the methods of Shiba et al. (36) and Kusumoto et al. (26), five 6-*O*-acylated derivatives containing long-chain fatty acids (compounds 7 through 10 and 24) were synthesized by the method of Kusumoto et al. (25), and the 6-*O*-acylated derivatives containing iso or normal fatty acids (compounds 11 through 23 and 25 through 27) were synthesized by the method of Kusumoto et al. (27). The compounds in the third group (compounds 28 through 32) and the two acids were obtained as synthetic intermediates. Table 1 shows the chemical formulas of the compounds used, their abbreviations, and the quantities administered per mouse.

Bacterial strains. *E. coli* E77156 (O6) was obtained from the Tokyo Metropolitan Research Laboratory of Public Health.

Preparation of bacterial suspension. Nutrient broth (Eiken) inoculated with a suspension of lyophilized *E. coli* E77156 cells was incubated at 37°C for 18 h. A total of 50 nutrient agar slants were inoculated with cell suspension from the preincubated culture and then incubated at 37°C for 18 h. Cells harvested from these slants were suspended in nutrient broth containing 10% horse serum inactivated by heating at 56°C for 30 min, so that the final cell density was 10^8 to 10^9 cells per ml. Portions (3 ml) of the cell suspension were put into 100 sample tubes (5 ml), and the tubes were stored in a deep freezer at −70°C.

Method of infection. At 1 h before infection, one of the sample tubes containing the cell suspension was taken out of the deep freezer. After it was thawed quickly, the cell suspension was diluted to give the desired cell density, which was determined in previous experiments. Each mouse was infected subcutaneously (s.c.) on the back with 0.5 ml of an appropriately diluted suspension 24 h after it was treated with a glycopeptide.

Preparation and administration of synthetic glycopeptide solution or suspension. Synthetic glycopeptides were weighed and suspended or dissolved in calcium-free and magnesium-free phosphate-buffered saline (pH 7.4; Nissui Seiyaku Co., Ltd.). Each solution or suspension was adjusted to a concentration of 1 μmol/ml with phosphate-buffered saline. All synthetic glycopeptide analogs were administered in phosphate-buffered saline either s.c. in the back or intraperitoneally (i.p.) in a volume of 0.2 ml/mouse [equivalent to 100 μg of MDP(Ala)] 24 h before *E. coli* infection.

Determination of protective activity. Survivors were recorded for 7 days after infection. The definitive survival rate was defined as the difference in percent survival between the treated group and its respective control group, as indicated by the following formula: definitive percent survival = [(number of survivors in treated group/number of mice used in treated group) × 100] − [(number of survivors in control group/number of mice used in control group) × 100].

The cumulative results of several comparable experiments are expressed in each figure. *P* values were obtained by using the adjusted chi-square method (14).

RESULTS

Protective activities of 6-*O*-mycoloyl derivatives of MDP(Ala). We investigated the protective activities of 6-*O*-mycoloyl derivatives, which were prepared by coupling MDP(Ala) with natural mycolic acids isolated from *Mycobacterium* or *Nocardia* cells. As Fig. 1 shows, in the control group more than 90% of the mice were killed by a small inoculum (5×10^6 cells), and 100% of the mice infected with a large inoculum (1×10^7 cells) were dead within a few days. Mice treated with MDP(Ala) (compound 1), MDP(Val) (compound 2), or MDP(Ser) (compound 3) were protected significantly ($P < 0.001$) against infection with a small inoculum; however, when they were infected with a large

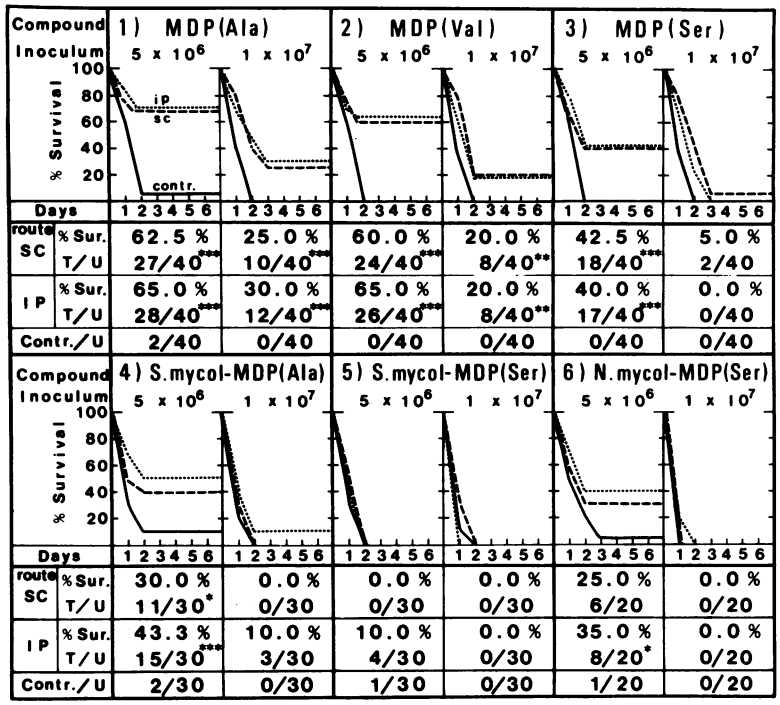

FIG. 1. *Protective activities of MDP(Ala) and 6-O-mycoloyl MDP(Ala) derivatives in mice infected s.c. with E. coli. Each mouse was treated i.p. (dotted lines) or s.c. (dashed lines) with a synthetic glycopeptide at a dose of 0.2 μmol/mouse [equivalent to 100 μg of MDP(Ala)] 24 h before infection with 5 × 10^6 or 1 × 10^7 E. coli E77156 cells. Untreated infected mice (solid lines) served as controls. Abbreviations: % Sur., percent survival, as determined from differences between percentages of survival in treated groups and their respective control groups on day 7: T/U, number of survivors in treated group/number of mice used in treated group; Contr./U, number of survivors in control group/number of mice used in control group. Levels of significance compared with the control group values by the adjusted chi-square method are as follows: one asterisk, P < 0.05; two asterisks, P < 0.01; three asterisks, P < 0.001.*

inoculum, mice treated with compounds 1 and 2 were protected significantly ($P < 0.001$ and $P < 0.01$), but mice treated with compound 3 were not. With the groups of mice treated i.p. or s.c. with S.mycol-MDP(Ala) (compound 4) and i.p. with N.mycol-MDP(Ser) (compound 6), significant but relatively small effects were observed with a small inoculum, but not with a large inoculum. Another mycoloyl-MDP, S.mycol-MDP(Ser) (compound 5), was inactive even with a small inoculum. All of the mycoloyl derivatives (compounds 4 through 6) were much less active than MDP(Ala) (compound 1).

Protective activities of 6-*O*-acylated derivatives containing synthetic branched-chain fatty acids. In order to determine the roles that different kinds of branched-chain fatty acids play with respect to protective activity, MDP(Ala) was acylated with three different types of branched-chain fatty acids (namely, two fatty acids of the α-branched type, two fatty acids of the α-branched, β-hydroxylated type, and one iso fatty acid).

The s.c. administration of the acylated derivatives containing α-branched or α-branched, β-hydroxylated fatty acids (i.e., B30-MDP(Ala) [compound 7], B46-MDP(Ala) [compound 9], and BH48-MDP(Ala) [compound 10]) produced insignificant effects with either inoculum, and the administration of the derivative BH32-MDP(Ala) (compound 8) produced a significant ($P < 0.05$) but marginal effect (Fig. 2). The i.p. administration of the acylated derivatives containing the shorter fatty acids (i.e., B30-MDP(Ala) [compound 7] and BH32-MDP(Ala) [compound 8]) produced significant ($P < 0.01$) but not high activity.

Another derivative of this group, Iso.15-MDP(Ala) (compound 11), which is acylated with isopentadecanoic acid (which is found as a part of the cell wall peptidoglycan structure in *Propionibacterium acnes*), produced considerable protective activity. A statistically significant prolongation of survival in the group of mice treated with compound 11 was observed after infection with either a small or large inoc-

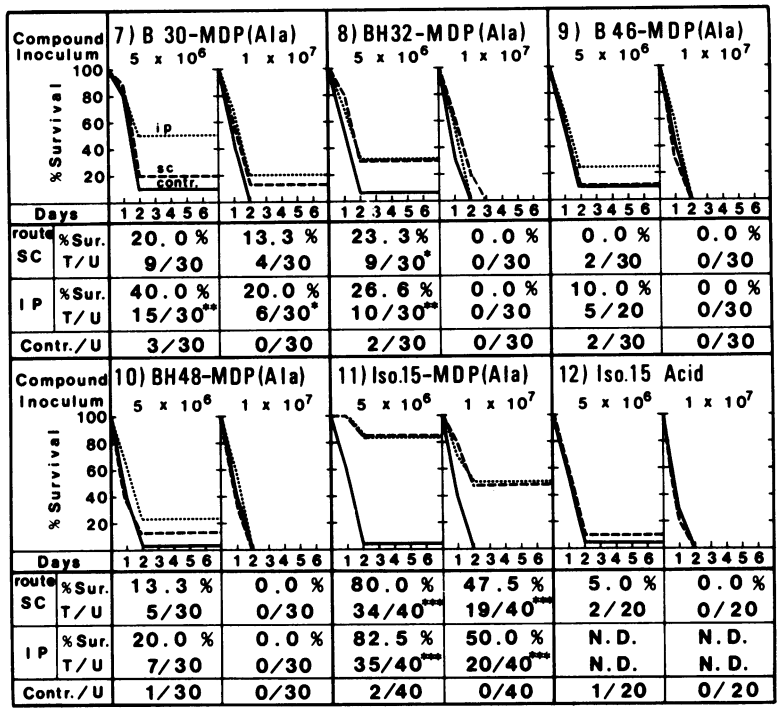

FIG. 2. *Protective activities of 6-O-acylated derivatives containing synthetic branched-chain fatty acids in mice infected s.c. with E. coli. Each mouse was treated i.p. (dotted lines) or s.c. (dashed lines) with a synthetic glycopeptide at a dose of 0.2 μmol/mouse [equivalent to 100 μg of MDP(Ala)] 24 h before infection with 5 × 10^6 or 1 × 10^7 E. coli E77156 cells. Untreated infected mice (solid lines) served as controls. Abbreviations are as described in the legend to Fig. 1. Levels of significance compared with control group values are as follows: one asterisk, P < 0.05; two asterisks, P < 0.01; three asterisks, P < 0.001.*

ulum, whereas isopentadecanoic acid (compound 33) alone was inactive.

Protective activities of 6-*O*-acylated derivatives containing linear fatty acids. We synthesized 13 6-*O*-acylated MDP(Ala) derivatives containing various lengths of straight-chain fatty acids (from acetic acid to triacontanoic acid) to study the effect of lipophilicity and the length of the fatty acid side chain on protective activity. Six analogs [L2-MDP(Ala) (compound 12), L4-MDP(Ala) (compound 13), L8-MDP(Ala) (compound 14), L10-MDP(Ala) (compound 15), L12-MDP(Ala) (compound 16), and L14-MDP(Ala) (compound 17)] protected mice at least as effectively as MDP(Ala) (compound 1) (Fig. 3). However, five compounds possessing longer chains [L16-MDP(Ala) (compound 18), L17-MDP(Ala) (compound 19), L18-MDP(Ala) (compound 20), L19-MDP(Ala) (compound 21), and L20-MDP(Ala) (compound 22) were more effective than MDP(Ala) in both groups of mice infected with either inoculum. The superiority of the acylated derivatives containing linear fatty acids was more clearly demonstrated in the results with the larger inoculum. The greatest protective activities among the 6-*O*-acylated derivatives containing linear fatty acids occurred in compounds 18 through 22. Two compounds which contained longer fatty acids, L24-MDP(Ala) (compound 23) and L30-MDP(Ala) (compound 24), were much less active than L18-MDP(Ala) (compound 20).

Protective activities of 6-*O*-stearoyl MDP(Ala) derivatives. We synthesized two compounds in which the L-alanyl residue in L18-MDP(Ala) was replaced with an L-valyl or L-seryl residue and one compound in which the D-isoglutamyl residue in L18-MDP(Ala) was replaced with an L-isoglutamyl residue to assess the role of the stearoyl group in protective activity. Both L18-MDP(Val) (compound 25) and L18-MDP(Ser) (compound 26) provided efficient protection, as did L18-MDP(Ala) (compound 20), whereas L18-MDP(Ala-L-isoGln) (compound 27) and stearic acid (compound 34) alone were inactive (Fig. 4). Compared with the results shown in Fig. 1, L18-MDP(Val) (compound 25) was more efficient than MDP(Val) (compound 2), and L18-MDP(Ser) (compound 26) was more efficient than MDP(Ser) (compound 3).

Protective activities of 1-α-*O*-benzyl

MDP(Ala) derivatives. In order to clarify the role which the 1-hydroxy group plays, we obtained five compounds possessing benzylated hydroxy groups in the 1-α position of the muramic acid moiety of MDP(Ala) as synthetic intermediates. Two compounds in which one benzyl group was linked to the 1-hydroxy group showed much weaker activity (Fig. 5). Compared with the original compounds, which did not have benzyl groups, α-Bz1-MDP(Ala) (compound 28) demonstrated weaker activity than MDP(Ala) (compound 1), and α-Bz1-Iso.15-MDP(Ala) (compound 29) exhibited less activity than Iso.15-MDP(Ala) (compound 11). No significant protection was observed in mice treated with either the dibenzylated compounds α-Bz1-MurNAc-L-Ala-D-Glu(*O*Bz1)-NH_2 (compound 30) and α-Bz1-MurNAc-L-Val-D-Glu(*O*Bz1)-NH_2 (compound 31) or the tribenzylated compound α-Bz1-MurNAc-L-Ser(Bz1)-D-Glu(*O*Bz1)-NH_2 (compound 32).

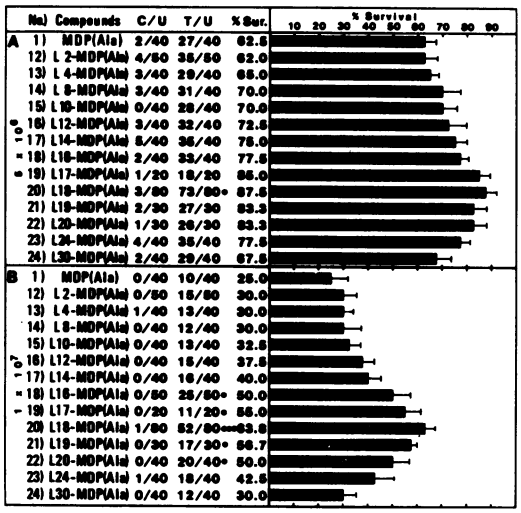

FIG. 3. *Protective activities of 6-O-acylated derivatives containing linear fatty acids in mice infected s.c. with E. coli. Each mouse was treated s.c. with a synthetic glycopeptide at a dose of 0.2 μmol/mouse [equivalent to 100 μg of MDP(Ala)] 24 h before infection with 5 × 10^6 (A) or 1 × 10^7 (B) E. coli E77156 cells. Abbreviations: C/U, number of survivors in control group/number of mice used in control group; T/U, number of survivors in treated group/number of mice used in treated group; % Sur., percent survival determined from the differences between percentages of survival in the treated groups and their respective control groups on day 7 (shown in the histogram). The bars in the histogram indicate the standard errors from several experiments. Levels of significance compared with the group of mice treated s.c. with 100 μg of MDP(Ala) are as follows: one asterisk, P < 0.05; three asterisks, P < 0.001.*

DISCUSSION

Among the various biological activities induced by MDP(Ala) and related compounds, current research has focused particularly on the following two activities: enhancement of resistance to infection and adjuvant activity for DTH and antibody response. In a study of the enhancement of host resistance to infection induced by MDP(Ala) analogs, Chedid et al. identified 5 active compounds among 22 analogs by using a protection test with *Klebsiella* infections. These were MurNAc-L-Ala-D-isoGln, MurNAc-L-Ala-D-Glu (muramyl dipeptide acid), MurNAc-L-Ala-D-Glu-OMe, MurNAc-L-Ala-D-Glu(OMe)-OMe, and MurNAc-D-Glu(L-Lys)-NH_2 (10). As reported elsewhere, the experimental system used in this study was the sepsis type of systemic infection caused by *E. coli* in STD: ddY mice, in contrast to the intramuscular *Klebsiella pneumoniae* infections in (C57BL/6 × AKR)F_1 hybrid mice used by Chedid et al. (12).

As to the correlation between chemical structure and protective activity, it has been reported that the replacement of the L-alanyl residue in MDP(Ala) with a D-amino acid residue and that the replacement of the D-isoglutamyl residue

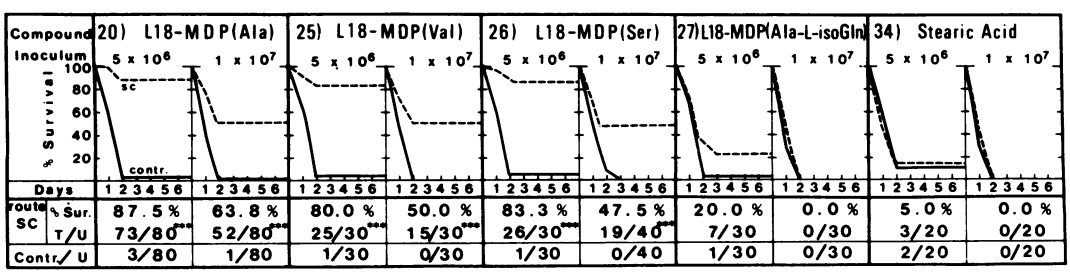

FIG. 4. *Protective activities of 6-O-stearoyl MDP(Ala) derivatives in mice infected s.c. with E. coli. Each mouse was treated s.c. with a synthetic glycopeptide at a dose of 0.2 μmol/mouse [equivalent to 100 μg of MDP(Ala)] 24 h before infection with 5 × 10^6 or 1 × 10^7 E. coli E77156 cells (dashed lines). Untreated infected mice (solid lines) served as controls. Abbreviations are as described in the legend to Fig. 1. Three asterisks indicate a level of significance compared with the control group of P < 0.001.*

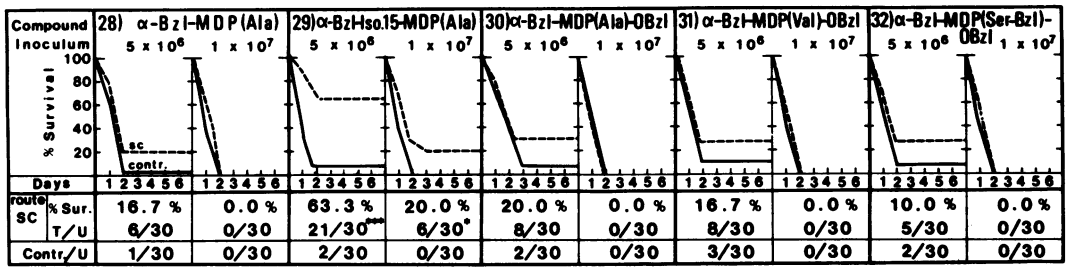

FIG. 5. *Protective activities of 1-α-O-benzylated MDP(Ala) derivatives in mice infected s.c. with E. coli. Each mouse was treated s.c. with a synthetic glycopeptide at a dose of 0.2 μmol/mouse [equivalent to 100 μg of MDP(Ala)] 24 h before infection with 5 × 10⁶ or 1 × 10⁷ cells E. coli E77156 (dashed lines). Untreated infected mice (solid lines) served as controls. Abbreviations are as described in the legend to Fig. 1. Levels of significance compared with control group values are as follows: one asterisk, P < 0.05; three asterisks, P < 0.001.*

with an L-isoglutamyl residue resulted in the disappearance of protective activity, as well as adjuvant activity (10, 12). The stereochemical requirement for both of these amino acid residues in the peptide moiety of MDP(Ala) for adjuvant activity to occur is equivalent to the requirement for protective activity; namely, a correlation was observed between the protective activities and the adjuvant activities for DTH among compounds having different stereochemistries, and the minimal structural unit required for protective activity was MurNAc-L-Ala (or L-Ser)-D-isoGln. This fact was confirmed by our finding which revealed the lack of activity of L18-MDP(Ala-L-isoGln) (compound 27). However, in a recent report, Parant et al. described that desmuramyl peptidolipids, which are devoid of the sugar moiety in MDP(Ala), have antiinfectious activity but not adjuvant activity for DTH and circulating antibody formation (35).

Among the three analogs compounds 1 through 3, MDP(Ala) (compound 1) and MDP(Val) (compound 2) were more efficient than MDP(Ser) (compound 3) in protective activity, whereas MDP(Val) and MDP(Ser) were more active as adjuvants for the development of DTH in guinea pigs than MDP(Ala) (2, 13). Moreover, a series of derivatives have α-branched groups or α-branched, β-hydroxylated groups in their molecules showed inactivity or weak activity in the protection test, whereas all of these compounds revealed adjuvanticity in the DTH reaction (5, 19, 42, 44). We noted particularly that B30-MDP(Ala) (compound 7) showed strong adjuvanticity (18) and demonstrated only weak (nearly negative) activity in the protection test. Therefore, we found that there is no close relationship between the protective activities and the adjuvant properties in the compounds tested (Table 2), and we confirmed that the structural requirement of MDP(Ala) derivatives for enhanced resistance to infection is more specific than that reported by Chedid et al. for the development of adjuvant activity for DTH.

Acylated derivatives with iso fatty acids or linear fatty acids showed significant activity, even with a large inoculum. By increasing the chain lengths of the fatty acids, we enhanced the activity of the acylated derivatives containing linear fatty acids. Five acyl derivatives (compounds 18 through 22) were more efficient than MDP(Ala) (compound 1), and the greatest activity among the acylated derivatives containing linear fatty acids was found in L18-MDP(Ala) (compound 20). Therefore, we clarified that acylation of MDP(Ala) with certain lengths of linear fatty acids or with iso fatty acids stimulates resistance to infection.

Of particular interest was the efficient protective activity of L18-MDP(Ser) (compound 26), in contrast to the inactivity of L18-MDP(Ala-L-isoGln) (compound 27). This fact suggests that the introduction of a stearoyl group results in an increased protective activity in the compounds which possess the same structure and the same sterochemistry, i.e., MurNAc-L-Ala(L-Val or L-Ser)-D-isoGln; however, protective activity was not induced by the compounds which possess a different stereochemistry (e.g., MurNAc-L-Ala-L-isoGln).

With respect to chemical modification of the 1-hydroxy group of the sugar moiety in MDP(Ala), it has been reported that etherification with *para*-aminophenol in the 1-α position eliminates both adjuvant and protective activities (33) and that introduction of a methyl group in the position of the sugar moiety in MDP(Ala) diminishes adjuvant activity (S. Kotani et al., unpublished data). The data described here showed that the presence of a benzyl group in

the 1-α position diminishes the protective activity of MDP(Ala).

Increasing lipophilicity by acylation with linear fatty acids might be one of the factors influencing protective activity, since increasing lipophilicity brings about a prolongation of in vivo drug retention and an increased affinity of the target cells. However, the more lipophilic compounds L24-MDP(Ala) (compound 23) and L30-MDP(Ala) (compound 24) did not demonstrate activity as marked as that of L18-MDP(Ala) (compound 20). This fact may have been due to decreased absorption of the agents administered s.c. Similar results were reported for the stimulation of O_2^- (superoxide) production by macrophages; namely, stimulation was induced by i.p. treatment with B30-MDP(Ala) but not by s.c. treatment, and this difference in activity was due to a trapping of the agents at the site of the s.c. injection (32).

It must be noted that the addition of lipophilicity by acylation causes some additional physicochemical changes in the parent molecule [MDP(Ala)], such as the detergent effect and

TABLE 2. *Correlation between chemical structure of MDP(Ala) analogs and protective activity*

Compound	Name or abbreviation	Protective activity with:		Adjuvant activity for DTH	
		5×10^6 cells[a]	1×10^7 cells[b]	w/o	Reference(s)
1	MDP(Ala)	+[c]	+[c]	+	3–5, 7, 20
2	MDP(Val)	+[c]	±[d]	+ (~++)	2
3	MDP(Ser)	+[c]	−	+	7, 20, 42
4	S.mycol-MDP(Ala)	−[e]	−	+	5, 18
5	S.mycol-MDP(Ser)	−	−	+	42
6	N.mycol-MDP(Ser)	−	−	+	Unpublished data
7	B30-MDP(Ala)	−	−	++	18
8	BH32-MDP(Ala)	−	−	+	18
9	B46-MDP(Ala)	−	−	+	18
10	BH48-MDP(Ala)	−	−	+	18
11	Iso.15-MDP(Ala)	++[c]	++[c]	+	Unpublished data
12	L2-MDP(Ala)	+[c]	+[c]	+	17
13	L4-MDP(Ala)	+[c]	+[c]	+	17
14	L8-MDP(Ala)	+[c]	+[c]	+	17
15	L10-MDP(Ala)	+[c]	+[c]	+	Unpublished data
16	L12-MDP(Ala)	+[c]	+[c]	+	17
17	L14-MDP(Ala)	+[c]	+[c]	+	Unpublished data
18	L16-MDP(Ala)	++[c]	++[c]	+	Unpublished data
19	L17-MDP(Ala)	++[c]	++[c]	+	Unpublished data
20	L18-MDP(Ala)	++[c]	++[c]	+	17, 18
21	L19-MDP(Ala)	++[c]	++[c]	+	Unpublished data
22	L20-MDP(Ala)	++[c]	++[c]	+	Unpublished data
23	L24-MDP(Ala)	++[c]	+[c]	+	Unpublished data
24	L30-MDP(Ala)	+[c]	+[c]	+	18
25	L18-MDP(Val)	++[c]	++[c]	+	Unpublished data
26	L18-MDP(Ser)	++[c]	++[c]	+	Unpublished data
27	L18-MDP(Ala-L-isoGln)	−	−	−	Unpublished data
28	α-Bzl-MDP(Ala)	−	−	ND[f]	
29	α-Bzl-Iso.15-MDP(Ala)	+[c]	±[c]	ND	
30	α-Bzl-MDP(Ala)-*O*Bzl	−	−	ND	
31	α-Bzl-MDP(Val)-*O*Bzl	−	−	ND	
32	α-Bzl-MDP(Ser-Bzl)-*O*Bzl	−	−	ND	
33	Isopentadecanoic acid	−	−	ND	
34	Stearic acid	−	−	ND	
35	*N*-acetyl muramic acid	−[g]	−[g]	−	21

[a] Percent survival after s.c. treatment with MDP(Ala) or an MDP(Ala) derivative and infection with 5×10^6 *E. coli* cells per mouse: −, ≤30%; ±, 31 to 45%; +, 46 to 75%; ++, ≥75%.

[b] Percent survival after s.c. treatment with MDP(Ala) or an MDP(Ala) derivative and infection with 1×10^7 *E. coli* cells per mouse: −, ≤10%; ±, 11 to 20%; +, 21 to 44%; ++, ≥45%.

[c] $P < 0.001$ (level of significance compared with controls, as determined by the adjusted chi-square method).

[d] $P < 0.01$ (level of significance compared with controls, as determined by the adjusted chi-square method).

[e] $P < 0.05$ (level of significance compared with controls, as determined by the adjusted chi-square method).

[f] ND, Not done.

[g] Matsumoto et al., submitted for publication.

ability to form micelles. The detergent effect was exhibited mainly by five compounds (compounds 17 through 21), as determined by the hemolysis test (data not shown). Furthermore, micelle formation in phosphate-buffered saline occurred with certain kinds of 6-*O*-acyl derivatives, but not with MDP(Ala) itself. In order to gain further knowledge of the correlation between the physicochemical properties and the biological activities, Kusumoto et al. measured the partition coefficient ($CHCl_3$/water) of 6-*O*-acyl derivatives of MDP(Ala) (27), and Shiba et al. determined the critical micelle concentrations (in micromoles per milliliter) (unpublished data). The exceedingly small critical micelle concentration (0.013 μmol/ml) obtained for L18-MDP(Ala) (compound 20) seems to be related to its high protective activity. However, this is not always the case because B30-MDP(Ala) (compound 7), which showed only a slight protective activity but had the highest adjuvant activity of all compounds, gave a smaller critical micelle concentration value (0.003 μmol/ml). Moreover, regarding the relationship between critical micelle concentration value and biological activity, Weltzien reported from a comparative study of lysophosphatidylcholine analogs for erythrocytes that the affinity of compounds for biomembranes was stimulated by decreasing the critical micelle concentration (45).

There have been several reports showing that macrophages may play a role in induction of immune response by MDP(Ala); treatment with MDP(Ala) stimulates the phagocytic ability of the reticuloendothelial system in mice (40, 41), inhibits migration of peritoneal macrophages in the absence of lymphocytes (43), stimulates glucosamine incorporation in peritoneal macrophages (39), enhances O_2^- generation which is not mediated by soluble complement (32), and stimulates human mononuclear cells to produce (probably) lymphocyte activating factor, which possesses mitogenic activity toward murine thymocytes (31).

On the other hand, if the mechanism of action of MDP(Ala) analogs in enhancing of resistance to infection depends mainly on stimulation of macrophages, there are also some controversial and conflicting findings concerning the structure-activity relationship and the kinetics of both protective and macrophage-stimulating activities; one of the MDP(Ala) analogs, B30-MDP(Ala) (compound 7), showed a strong capacity for macrophage activation as determined by O_2^- production (32) and had only weak activity as determined by the protection test (Fig. 2). In a comparison of kinetics, the protective activity induced by 100 μg of MDP(Ala) was observed for 3 days after treatment (Matsumoto et al., submitted for publication), but reticuloendothelial system-stimulating activity was observed for only 1 day after treatment with the same amount of MDP(Ala) (32). Moreover, 30 to 100 μg of MDP(Ala) was required to enhance protective activity, but only 1 to 5 μg was needed to stimulate the O_2^--producing ability of macrophages (32).

The studies showing the ability of MDP(Ala) to save thymectomized mice (8) and neonates (34) from death suggested that T lymphocytes do not play the primary role in augmentation of host resistance to infection; it is thought that B lymphocytes do not play a principal role in enhancing resistance to infection, since a comparison of the kinetics of these two activities revealed that the increase in resistance to infection was more rapid than the increase in antibody formation.

Research on the correlation between the chemical structures of different types of newly synthesized MDP(Ala) analogs possessing glutamyl residues in which the carboxy group is substituted and several biological activities, such as enhancement of resistance to infection, induction of tumor immunity, and pyrogenicity, is currently being conducted. In addition, a detailed study of L18-MDP(Ala) (compound 20) with respect to its efficacy as an antiinfectious agent and with respect to its mechanism of action is being carried out. Some MDP(Ala) analogs will be useful for clinical application (9) because they can be given orally and because they have a broad spectrum of activity, including activity against fungi and bacteria resistant to chemotherapeutic agents.

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