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Structural Requirements of Muramylpeptides for Induction of Necrosis at Sites Primed with *Mycobacterium tuberculosis* in Guinea Pigs

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Intracutaneous injection of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) in guinea pigs caused an extensive necrotic reaction in footpads prepared by injection of heat-killed *Mycobacterium tuberculosis* in water-in-mineral-oil emulsion. We examined a variety of analogs and derivatives of muramylpeptides for their ability to provoke this reaction. A maximum and a minimum structure responsible for the necrotic reaction were found to be *N*-acetylglycosaminyl-β(1-4)-*N*-acetylmuramyl-tripeptide (GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-A_2pm) and MDP, respectively. An unexpected finding was that GlcNAc-MurNAc-tetrapeptides having L-amino acids at their C termini, unlike comparable compounds having C-terminal D-amino acids, exhibited definite necrosis-inducing activity, probably due to their tendency to undergo in vivo degradation to GlcNAc-MurNAc-tripeptide. Introduction of some acyl groups, especially the stearoyl group, to the 6-O position of the muramic acid or the peptide moiety of muramylpeptides increased the necrosis-inducing activity of the parent molecules. However, this was not observed with 1-thio-muramic acid analogs of MDP. Modification of the α- or γ-carboxyl groups of the glutamic acid residues of muramylpeptides tended to decrease their necrosis-inducing ability. Analogs and derivatives of muramylpeptides which are capable of inducing necrosis at a primed site, with few exceptions, exhibited powerful adjuvanticity against ovalbumin in guinea pigs. However, the reverse was not necessarily true.

N-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyldipeptide; MDP) was demonstrated by synthetic studies to be the minimum structure responsible for immunoadjuvant activity, especially the ability to induce delayed-type hypersensitivity, of Freund complete adjuvant and bacterial cell wall peptidoglycans (6, 27). Since this discovery, MDP was studied by a number of in vivo and in vitro biological assays, and it has been revealed that MDP has an extremely wide range of immunobiological and pharmacological activities (1, 2, 22, 41). Some of them are evidently beneficial from the aspect of a medical application, as exemplified by stimulation of antigen-specific immune responses of hosts to various antigens including protective epitopes, as well as enhancement of nonspecific resistance of animals against pathogenic microbes, viruses, and tumors (1, 22, 41). On the other hand, there are a number of reports describing possible adverse effects of MDP: MDP has induced adjuvant arthritis in susceptible animals (19, 36), caused pyrexia in rabbits (28), and induced distress in guinea pigs (4). MDP was also shown to bring about anaphylactoid shock (early-phase death) in susceptible mice (40a) and to enhance late-phase lethality in mice and guinea pigs (5, 39, 40a) in combination with appropriate endotoxic lipopolysaccharides.

In a previous paper, we described another kind of undesirable effect of MDP in guinea pigs (37). MDP provoked severe hemorrhagic and necrotic inflammation in guinea pigs at a site (the footpad) prepared by injection of heat-killed *Mycobacterium tuberculosis* in water-in-mineral-oil (w/o) emulsion. The typical reaction provoked by intracutaneous injection of MDP in the flank consisted of marked swelling, exudation, hemorrhage, necrosis, and ulceration at the primed footpad. The test animals sometimes succumbed to generalized shock.

In this study, we examined various muramylpeptides and their derivatives, both bacterial and synthetic, for their ability to provoke a necrotic reaction in primed guinea pigs, to elucidate the structure-activity relationship.

MATERIALS AND METHODS

Animals. Female Hartley guinea pigs (closed colony; weighing about 450 g), purchased from Shizuoka Experimental Animal Cooperative (Shizuoka, Japan), were used throughout the study.

Induction of hemorrhagic and necrotic inflammatory reactions (necrotic reaction). Groups of 3 to 12 (usually 4 to 6) guinea pigs were primed by injection at the left hind footpad with 100 μg per animal of heat-killed *M. tuberculosis* H37Rv (cultivated as a pellicle in Sauton medium for 4 weeks) incorporated in 0.2 ml of w/o emulsion prepared from Freund incomplete adjuvant (lot no. 636671; Difco Laboratories, Detroit, Mich.). Four weeks later, the animals were challenged by intracutaneous injection with 400 μg per

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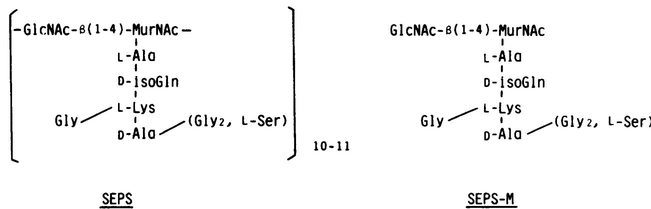


FIG. 1. Proposed chemical structure of SEPS and SEPS-M prepared from *S. epidermidis* peptidoglycans (based on the reports of Kawata et al. [16] and Harada et al. [10]).

animal of test materials in 0.2 ml of phosphate-buffered saline (pH 7.2) at the flank (unless otherwise stated). The thickness of the primed footpad was measured with calipers 24 h after the challenge (provocative) injection, and the values were compared with those measured immediately before the challenge injection. Necrotic reaction at the primed footpad was examined by the naked eye, and the severity of reaction was scored as follows: ++, an extensive necrotic inflammation with marked swelling, exudation, hemorrhage, and ulceration; +, moderate necrotic inflammation; ±, slight necrotic inflammation; −, no changes.

Test compounds. (i) Water-soluble peptidoglycan fragments prepared from enzymatic digests of cell wall peptidoglycans and their acyl derivatives. A "polymer" of peptidoglycan subunits (SEPS) was isolated by gel filtration of the SALE endopeptidase (16) digest of a peptidoglycan specimen that had been prepared by treatment of *Staphylococcus epidermidis* (ATCC 155) cell walls with 10% trichloroacetic acid at 4°C for 48 h to remove the nonpeptidoglycan moiety. A monomer of the peptidoglycan subunit (SEPS-M) was a degradation product of SEPS obtained by treatment with M-1 endo-*N*-acetylmuramidase (15). Proposed chemical structures of SEPS and SEPS-M are given in Fig. 1. Further details of preparation methods and chemical properties of the above two compounds were described previously (10, 16). An LPCM-A specimen (Fig. 2), bisdisaccharide-stem-peptide dimer, was prepared from a digest of *Lactobacillus plantarum* (ATCC 8014) cell walls by treatment with M-1 enzyme as described by Kotani et al. (29). The following disaccharide peptide monomers (Fig. 2) were isolated by combined treatment of *L. plantarum* cell walls with the M-1 endo-*N*-acetylmuramidase enzyme and an AM_3 endopeptidase capable of splitting the inter-stem-peptide linkage between the D-Ala- and the *meso*-A_2pm (13), and by chro-

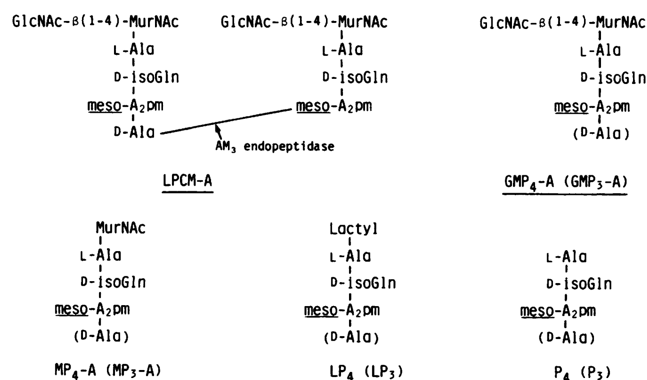


FIG. 2. Chemical structures of LPCM-A, GMP_4-A, lactyltetrapeptide, and tetrapeptide (based on the reports of Kawata et al. [17]).

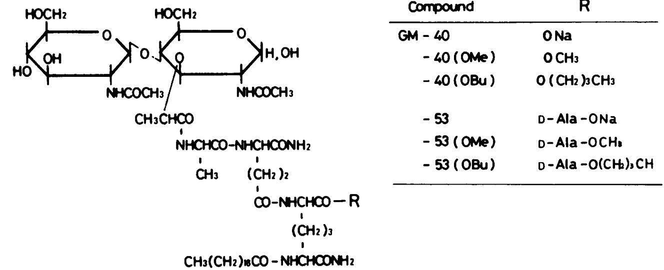


FIG. 3. Chemical structure of GM-40 and GM-53 series compounds.

matography of the digest, as described previously (17): *N*-acetylglucosaminyl-β(1-4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-2,6-diaminopimelic acid-D-alanine (GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-A_2pm-D-Ala; GMP_4-A) and *N*-acetylglucosaminyl-β(1,4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-2,6-diaminopimelic acid (GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-A_2pm; GMP_3-A). Treatment of these disaccharide peptide preparations with exo-β-*N*-acetylglucosaminidase derived from pig epididymis (7) gave MurNAc-L-Ala-D-isoGln-*meso*-A_2pm-D-Ala (MP_4) and MurNAc-L-Ala-D-isoGln-*meso*-A_2pm (MP_3), respectively (17). Lactyltetrapeptide and lactyltripeptide were prepared from GMP_4-A and GMP_3-A, respectively, by treatment with ammonia-water at 30°C for 28 h and by column chromatography of the reaction products. Tetrapeptide and tripeptide specimens were obtained by treatment of GMP_4-A and GMP_3-A, respectively, with *N*-acetylmuramyl-L-alanine amidase (14, 17). The following semisynthetic products also served as test compounds: disaccharide tetrapeptides prepared by using GMP_3-A as a starting material and whose C-terminal amino acids were different from each other (see Table 3), and acyl derivations of GMP_4-A and GMP_3-A (GM-53 and GM-40 series, respectively) (Fig. 3). These compounds were prepared as described elsewhere (8).

Another peptidoglycan monomer, *N*-acetylglucosaminyl-β(1,4)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-2,6-diaminopimelic acid-D-alanyl-D-alanine (GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-A_2pm-D-Ala-D-Ala; GMP_5) that was prepared from water-soluble uncross-linked peptidoglycan fragments liberated from penicillin-treated *Brevibacterium divaricatum* by lysozyme digestion, was a gift from J. Tomašić, Rudjer Bošković Institute, Zagreb, Yugoslavia.

(ii) Synthetic muramylpeptides: MDP and its analogs (muramyldipeptides). MDP and two types of its analogs were synthesized by Kusumoto et al. (33). In one type, the L-alanine residue of MDP was replaced by L-serine or L-valine, and in the other type, the D-isoglutamine residue of MDP was replaced by D-isoasparagine, D-glutamic acid, D-glutamine, L-glutamine, or L-isoglutamine. Another former type of MDP analog, MurNAc-D-Ala-D-isoGln, was a generous gift from E. Lederer, Laboratoire de Biochimie, Centre National de la Recherche Scientifique, Paris, France.

Muramylpeptides other than MDP. The following compounds with structures partly identical to L-lysine-type bacterial peptidoglycan were synthesized by Kusumoto et al. (33, 34); MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala (MP_4), MurNAc-L-Ala-D-isoGln-L-Lys (MP_3), GlcNAc-MurNAc-L-Ala-D-isoGln (GMP_2), MurNAc[β(1-4)GlcNAc]-L-Ala-D-isoGln [M(G)P_2)], MurNAc-L-Ala (MP_1), and GlcNAc-MurNAc-L-Ala (GMP_1). MurNAc-D-Ala-D-isoGln [MDP(D-D)] and the MDP covalently linked with a synthetic-polypeptide [poly(DL-Ala)–poly(L-Lys)], MurNAc-L-Ala-D-isoGln-

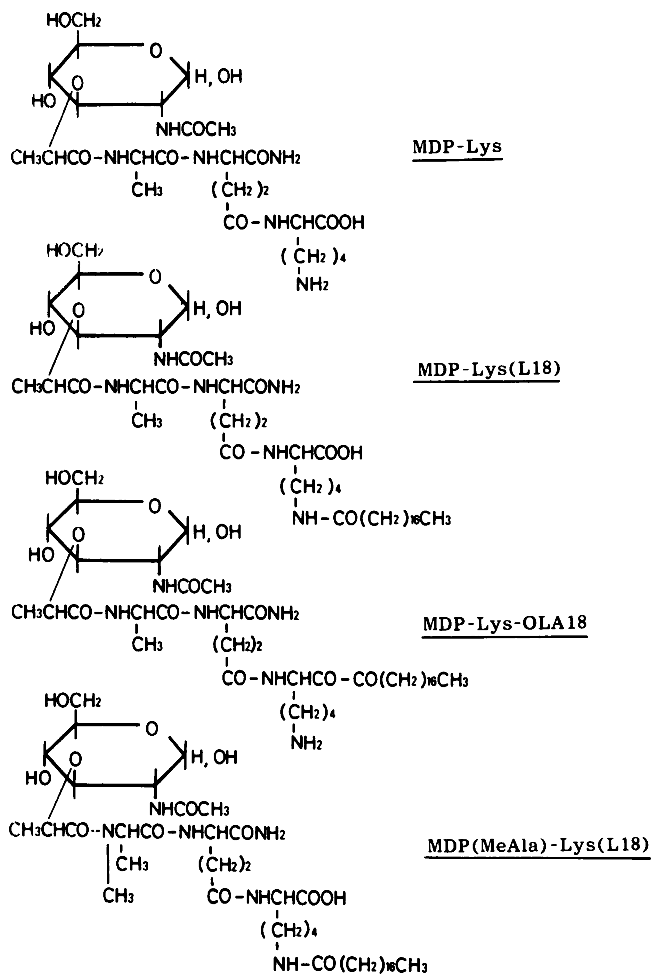
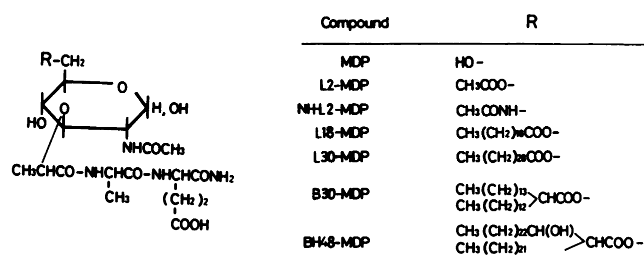


FIG. 4. Chemical structures of MDP-Lys, MDP-Lys(L18), MDP-Lys-OLA18, and MDP(MeAla)-Lys(L18).

A–L (MDP-A–L), and its analog, MurNAc-D-Ala-D-isoGln-A–L [MDP(D-D)-A–L] were generous gifts from L. Chedid, Pasteur Institute, Paris, France.

MDP-Lys(L18) and related compounds. MurNAc-L-Ala-D-isoGln-L-Lys (MDP-Lys), N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-stearoyllysine [MDP-Lys(L18)], its biologically inactive analog, MurNAc-L-Ala-L-isoGln-Lys(L18) [MDP(L-L)-Lys(L18)], a nonpyrogenic analog, MurNAc-*N*-methyl-L-Ala-D-isoGln-Lys(L18) [MDP(MeAla)-Lys(L18)], and a stearyl ester of MDP-Lys (MDP-Lys-OLA18) were kindly supplied by Daiichi Pharmaceutical Co., Ltd. (Tokyo,



Compound	R
MDP	HO-
L2-MDP	CH_3COO-
NH-L2-MDP	CH_3CONH-
L18-MDP	$CH_3(CH_2)_{16}COO$-
L30-MDP	$CH_3(CH_2)_{28}COO$-
B30-MDP	$CH_3(CH_2)_{13}$ / $CH_3(CH_2)_{12}$ >CHCOO-
BH48-MDP	$CH_3(CH_2)_{22}CH(OH)$ / $CH_3(CH_2)_{21}$ >CHCOO-

FIG. 6. Chemical structure of 6-*O*-acyl derivatives of MDP and a related compound.

Japan). Chemical structures of some of the above compounds are shown in Fig. 4.

MDP derivatives substituted in the α-COOH or γ-COOH group and both groups of their D-glutamic acid residue. The compounds listed in Fig. 5 and Table 8 were generously supplied by Daiichi Pharmaceutical Co. Murabutide was prepared for only laboratory use according to the method described by Lefrancier et al. (35).

6-*O*-Acyl-MDPs and related compounds. Various fatty acids with straight chains (2, 18, and 30 carbon atoms; L2, L18, and L30, respectively), an α-branched 30-carbon chain (B30), an α-branched and β-hydroxylated 48-carbon chain (BH48), or nocardomycolic acid were introduced to the 6-*O*-position of MDP as described previously (30-32, 40). 2-*N*-Acyl-MDPs (2-*N*-acetyl-MDP and 2-*N*-stearoyl-MDP) and 4,6-*O*-diacetyl-MDP were prepared by a similar method. Chemical structures of some of the above compounds are shown in Fig. 6.

1-*S*-Acyl derivatives of 1-thio-MDP and 1-thio-MDP-OMe. The compounds listed in Fig. 7 and Table 9 were synthesized by Hasegawa et al. as described previously (11).

Other materials. Bacterial lipopolysaccharide prepared from *Escherichia coli* O127:B8 by the hot phenol-water extraction method was purchased from Difco Laboratories. Bacterial lipid A prepared from lipopolysaccharide of *Salmonella minnesota* R595 was generously provided by C. Galanos, Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany. Synthetic *E. coli*-type lipid A (compound 506; LA-15-PP in new nomenclature) was prepared in T. Shiba's laboratory (12). PPD-RG200-3, an ultrapurified PPD (purified protein derivative of *M. tuberculosis*) which has a high tuberculin activity but lacks other immunobiological activities such as B-cell mitogenicity, was generously given by K. Onoue, Kumamoto University Medical School, Kumamoto, Japan. Partially purified group A streptococcal pyrogenic exotoxin (type A and B) prepared from *Streptococcus pyogenes* [serotype T12, strain THLS(SF-42)] by the method described by Kim and Watson

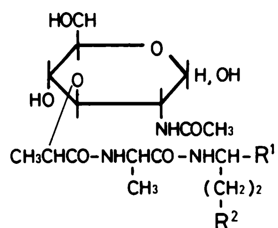


FIG. 5. Chemical structure of MDP derivatives substituted in the α-COOH or γ-COOH group, or both groups, of their D-glutamic acid residue.

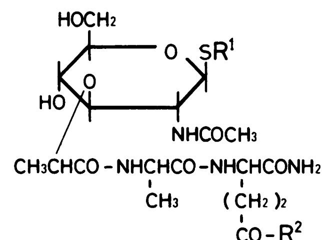


FIG. 7. Chemical structure of 1-*S*-acyl derivatives of 1-thio-MDP and their methyl esters.

TABLE 1. Activity of peptidoglycan partial structures, prepared by enzymatic degradation of *S. epidermidis*, *L. plantarum*, or *B. divaricatum* cell walls, to provoke a necrotic reaction in footpads primed with heat-killed *M. tuberculosis* in w/o emulsion

Test material[a]	Reaction of primed footpads: Avg diam (mm, mean ± SE)		Necrotic reaction		Adjuvanticity[b] (reference)
	Before challenge	After challenge	Individual scores[c]	Incidence[d]	
SEPS	12.8 ± 0.3	13.3 ± 0.2	± ± − − − − − − −	0/9	+ (38)
SEPS-M	12.3 ± 0.2	12.0 ± 0.4	± − − − − −	0/6	+ (38)
LPCM-A	13.0 ± 0.5	13.1 ± 0.3	± − − − − −	0/6	+ (29)
GMP_5	13.5 ± 0.2	13.8 ± 0.3	− − − −	0/4	+ (—[e])
GMP_4-A	13.1 ± 0.5	12.6 ± 0.4	± − − − − − − −	0/8	+ (17)
GMP_3-A	12.3 ± 0.3	14.3 ± 0.4[f]	+ + + + + ± ± − − − − −	5/12	+ (—[e])
GMP_2[g]	12.2 ± 0.3	14.1 ± 0.5[h]	+ + ± ±	2/4	+ (34)
MP_4	12.2 ± 0.1	11.7 ± 0.5	− − − − − −	0/6	+ (17)
MP_3	13.2 ± 0.5	16.0 ± 0.4[f]	+ + + + + ±	5/6	+ (—[e])
MP_2 (MDP)[g]	13.3 ± 0.6	15.5 ± 0.4[h]	++* ++ ++	3/3	+ (27)
LP_4	13.3 ± 0.6	13.3 ± 0.6	− − − − − −	0/6	− (—[e])
LP_3	12.7 ± 0.2	12.5 ± 0.2	− − − − − −	0/6	− (—[e])
P_4	12.0 ± 0.6	13.3 ± 0.2	− − − − − −	0/6	− (—[e])
P_3	12.8 ± 0.1	12.7 ± 0.1	− − − − − −	0/6	− (—[e])

[a] GM, GlcNAc-β(1-4)-MurNAc; M, MurNAc; P_5, L-Ala-D-isoGln-*meso*-A_2pm-D-Ala-D-Ala; P_4, L-Ala-D- isoGln-*meso*-A_2pm-D-Ala; P_3, L-Ala-D-isoGln-*meso*-A_2pm; P_2, L-Ala-D-isoGln; P_1, L-Ala; L, lactyl.
[b] Groups of female albino guinea pigs were immunized by intrafootpad injection of Freund incomplete adjuvant in w/o emulsion containing ovalbumin (100 or 1,000 μg per animal) and test materials (65 to 246 μg per animal). Induction of delayed-type hypersensitivity and stimulation of serum antiovalbumin antibody levels were determined by corneal test and quantitative precipitin reaction 3 and 4 weeks after the immunization, respectively, as previously described (21). No grading of the adjuvanticity was made.
[c] ++, Extensive necrotic reaction with marked swelling, exudation, hemorrhage, and ulceration; +, moderate necrotic inflammation; ±, slight necrotic inflammation; −, no changes. Asterisk shows the animal died of generalized shock.
[d] Number of animals showing necrotic reaction equal to or stronger than +/number of test animals.
[e] Unpublished data (M. Tsujimoto, S. Kotani, et al.).
[f] Significantly different from the respective diameter before challenge ($P < 0.01$).
[g] Synthetic compounds.
[h] Significantly different from the respective diameter before challenge ($P < 0.05$).

(18) was a gift from Seisaburo Kanoh, National Institute of Hygienic Science, Osaka Branch, Osaka, Japan.

All test compounds were dissolved in phosphate-buffered saline or suspended in it as homogeneously as possible by treatment with an ultrasonic oscillator (Sonifier, model W-200P; Branson Instruments Co., Stamford, Conn.) for 30 s to 1 min at 20 kHz.

RESULTS

Induction by partial structures of cell wall peptidoglycans of necrotic reaction at the primed footpad (necrosis-inducing activity). The assay results on the necrosis-inducing activity of bacterial compounds which represent a partial structure of cell wall peptidoglycans (L-lysine and *meso*-A_2pm types) of *L. plantarum*, *S. epidermidis*, and *B. divaricatum* are summarized in Table 1. Both the "polymer" (SEPS) and the monomer (SEPS-M) of disaccharide [GlcNAc-β(1-4)-MurNAc] heptapeptide (Lys type), disaccharide pentapeptide (GMP_5; A_2pm type), disaccharide tetrapeptide (GMP_4; A_2pm type), and MurNAc-tetrapeptide (MP_4; A_2pm type) had little necrosis-inducing activity. In contrast, the test compounds equal to or smaller than disaccharide tripeptide, namely, GlcNAc-MurNAc-tripeptide (GMP_3-A), GlcNAc-MurNAc-dipeptide (GMP_2), MurNAc-tripeptide (MP_3), and MurNAc-dipeptide (MP_2, MDP) provoked the reaction. No significant differences were found in the activity between the tripeptide and dipeptide compounds, although MP_2 (MDP) tended to provoke a more severe necrotic reaction. All the compounds

TABLE 2. Ability of synthetic muramylpeptides possessing partial structures of bacterial peptidoglycans to provoke a necrotic reaction at the primed footpad[a]

Test material	Reaction of primed footpads: Avg diam (mm, mean ± SE)		Necrotic reaction		Adjuvanticity (reference)
	Before challenge	After challenge	Individual scores	Incidence	
M(G)P_2	12.6 ± 0.5	15.0 ± 0.4[b]	+ + + + − − −	4/7	+ (34)
GMP_2	12.2 ± 0.3	14.1 ± 0.5[c]	+ + ± ±	2/4	+ (34)
GMP_1	13.0 ± 0.5	13.3 ± 0.3	− − − −	0/4	− (—[d])
MP_4	12.3 ± 0.3	12.3 ± 0.2	− − − −	0/4	+ (27)
MP_3	11.6 ± 0.3	14.2 ± 0.8[c]	+ + + ±	3/4	+ (27)
MP_2 (MDP)	12.0 ± 0.3	14.1 ± 0.5[c]	++* ++ + +	4/4	+ (27)
MP_1	12.7 ± 0.3	11.3 ± 0.3	− − − −	0/4	− (27)

[a] See Table 1, footnotes *a* through *d*. M(G), MurNAc-β(1-4)-GlcNAc; P_4, L-Ala-D-isoGln-L-Lys-D-Ala; P_3, L-Ala-D-isoGln-L-Lys.
[b] Significantly different from diameter before challenge ($P < 0.01$).
[c] Significantly different from diameter before challenge ($P < 0.05$).
[d] Tsujimoto et al., unpublished data.

TABLE 3. Comparison of the ability of semisynthetic disaccharide tetrapeptides (A_2pm type) possessing D- or L-amino acid at their C termini to provoke a necrotic reaction at the primed footpad[a]

Test material	Reaction of primed footpads				Adjuvanticity (reference)
	Avg diam (mm, mean ± SE)		Necrotic reaction		
	Before challenge	After challenge	Individual scores	Incidence	
MDP (MP_2)[b]	13.5 ± 0.4	17.0 ± 0.4[c]	++* ++* ++ ++ + ±	5/6	+ (27)
GMP_3-A	14.1 ± 0.5	15.5 ± 0.5	++ ++ ± ±	2/4	+ (—[d])
GMP_3-L-Met	13.0 ± 0.3	16.3 ± 0.5[c]	+ + + + + ± −	5/7	+ (24)
GMP_3-L-Phe	13.8 ± 0.2	15.7 ± 0.8[e]	+ + + + + ± ±	5/7	+ (24)
GMP_3-L-Ala	13.2 ± 0.4	15.3 ± 0.7[e]	+ + + + + ± ± ± ± −	5/10	+ (24)
GMP_3-L-Thr	12.8 ± 0.2	14.9 ± 0.4[c]	+ + ± ± ± − −	2/7	+ (24)
GMP_3-D-Met	13.5 ± 0.4	14.3 ± 0.5	− − − − − − −	0/7	+ (24)
GMP_3-D-Phe	14.0 ± 0.4	14.3 ± 0.2	− − − − − − −	0/7	+ (24)
GMP_3-D-Ala (GMP_4-A)	13.6 ± 0.5	13.5 ± 0.6	− − − −	0/4	+ (17)
GMP_3-D-Thr	13.8 ± 0.5	14.3 ± 0.2	− − − − − − − −	0/8	+ (24)

[a] See Table 1, footnotes *a* through *d*.
[b] Synthetic compound.
[c] Significantly different from diameter before challenge ($P < 0.01$).
[d] Tsujimoto et al., unpublished data.
[e] Significantly different from diameter before challenge ($P < 0.05$).

lacking the *N*-acetylmuramic acid residue, namely, lactyltetrapeptide, lactyltripeptide, tetrapeptide and tripeptide, had no necrosis-inducing activity, indicating the important role of the muramic acid residue.

Necrosis-inducing activity of synthetic muramylpeptides possessing a partial structure of the lysine-type peptidoglycan. In agreement with the above results, a synthetic MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala (MP_4) did not provoke the necrotic reaction, whereas MurNAc-L-Ala-D-isoGln-L-Lys (MP_3) and MDP (MP_2) did (Table 2). It was also found that both GlcNAc-MurNAc-L-Ala-D-isoGln (GMP_2) and MurNAc(GlcNAc)-L-Ala-D-isoGln[$M(G)P_2$] provoked the necrotic reaction, whereas MurNAc-L-Ala and GlcNAc-MurNAc-L-Ala had no activity. Among the above active compounds, MDP had the greatest necrosis-inducing activity.

Ability of disaccharide tetrapeptide (A_2pm type) possessing either L- or D-amino acid at the C terminus to provoke the necrotic reaction. The next study was carried out using semisynthetic disaccharide tetrapeptide (A_2pm-type) compounds that were prepared by addition of either L- or D-amino acid as the C terminus to the disaccharide tripeptide (GMP_3-A) obtained from the enzymatic digest of *L. plantarum* cell walls. None of the compounds carrying a D-amino acid at the C terminus provoked the necrotic reaction, whereas the compounds carrying C-terminal L-amino acids did (Table 3).

Activity of acyl derivatives of GMP_3-A and GMP_4-A. An assay was made on semisynthetic compounds carrying a stearoyl group at the A_2pm residue of GMP_3-A and GMP_4-A (GM-40 and GM-53, respectively), their methyl esters [GM-40(OMe) and GM-53(OMe)], and their butyl esters [GM-40(OBu) and GM-53(OBu)]. GM-40 series compounds exhibited stronger activity than MDP and GM-53 series compounds and killed about half of the animals by causing acute generalized shock (Table 4). GM-53 series specimens, unlike the totally inactive parent compound GMP_4-A, also exhibited definite necrosis-inducing activity, though their activity was weaker than that of MDP.

Effects of the addition of acyl group to the peptide moiety of muramylpeptides on their necrosis-inducing activity. Both MDP-Lys(L18) and MDP-Lys-OLA18, in which a stearic acid residue was linked to NH_2 and COOH groups, respectively, of the lysine residue of muramyltripeptide (MDP-Lys), exhibited stronger activity than the parent molecule. On the other hand, neither a stearoyl derivative of the adjuvant-inactive analog MurNAc-L-Ala-L-isoGln-L-Lys, MDP(L-L)-Lys(L18), nor an adjuvant-active analog that had

TABLE 4. Necrotic reaction-inducing activity of semisynthetic acyl derivatives of GMP_3 and GMP_4[a]

Test material[b]		Reaction of primed footpads				Adjuvanticity (reference)
		Avg diam (mm, mean ± SE)		Necrotic reaction		
Abbreviation	R	Before challenge	After challenge	Individual scores	Incidence	
GM-40	ONa	11.7 ± 0.3	15.3 ± 0.3[c]	++* ++* ++ ++ ++ +	6/6	+ (—[d])
GM-40 (OMe)	OCH_3	12.0 ± 0.4	15.2 ± 0.3[c]	++* ++* ++* ++* ++ ++	6/6	+ (—[d])
GM-40 (OBu)	$O(CH_2)_3CH_3$	12.9 ± 0.2	15.8 ± 0.2[c]	++* ++* ++* ++* + + + +	7/7	+ (—[d])
GM-53	D-Ala-ONa	12.5 ± 0.5	14.8 ± 0.4[c]	+ ± ± ± − −	1/6	+ (23)
GM-53 (OMe)	D-Ala-OCH_3	12.0 ± 0.4	14.8 ± 0.4[c]	++ + + + ± −	4/4	+ (—[d])
GM-53 (OBu)	D-Ala-$O(CH_2)_3CH_3$	12.0 ± 0.3	15.1 ± 0.2[c]	+ + + ± ± ± ±	3/7	+ (—[d])
MDP (MP_2)[e]		12.3 ± 0.2	14.0 ± 0.3[c]	++ ++ ++ +	4/4	+ (27)

[a] See Table 1, footnotes *a* through *d*.
[b] See Fig. 3.
[c] Significantly different from diameter before challenge ($P < 0.01$).
[d] Tsujimoto et al., unpublished data.
[e] Synthetic compound.

TABLE 5. Necrotic reaction-inducing activity of synthetic N^{α}-(*N*-MurNAc-L-Ala-D-isoGln)-N^{ϵ}-stearoyl-L-Lys, MDP-Lys(L18), and related compounds[a]

Test material[b]	Reaction of primed footpads				Adjuvanticity (reference)
	Avg diam (mm, mean ± SE)		Necrotic reaction		
	Before challenge	After challenge	Individual scores	Incidence	
MDP-Lys(L18)	12.6 ± 0.2	16.3 ± 0.2[c]	++* ++* ++ ++ + + +	7/7	+ (23)
MDP(L-L)-Lys(L18)	13.6 ± 0.3	13.0 ± 0.4	− − − − − −	0/6	− (—[d])
MDP(MeAla)-Lys(L18)	12.2 ± 0.4	11.8 ± 0.5	− − − − −	0/5	+ (25)
MDP-Lys-OLA18	12.2 ± 0.4	15.3 ± 0.5[c]	++* ++ ++ + + ±	5/6	+ (—[d])
MDP-Lys	12.4 ± 0.4	15.8 ± 0.7[e]	+ + +	3/3	+ (27)
MDP-A–L	12.9 ± 0.5	15.8 ± 0.5[c]	++* ++ ++ ++	4/4	+ (9)
MDP(D-D)-A–L	12.3 ± 0.3	12.3 ± 0.4	− − − −	0/4	− (9)
MDP (MP_2)[f]	13.0 ± 0.5	15.2 ± 0.6[e]	++* ++ ++ +	4/4	+ (27)

[a] See Table 1, footnotes *a* through *d*.
[b] MDP(L-L), MurNAc-L-Ala-L-isoGln; MDP(MeAla), MurNAc-*N*-methyl-L-Ala-D-isoGln; A–L, poly (DL-Ala)–poly(L-Lys); MDP(D-D), MurNAc-D-Ala-D-isoGln.
[c] Significantly different from diameter before challenge ($P < 0.01$).
[d] Tsujimoto et al., unpublished data.
[e] Significantly different from diameter before challenge ($P < 0.05$).
[f] Synthetic compound.

L-methylalanine instead of L-alanine [MDP(MeAla)-Lys(L18)] provoked the necrotic reaction (Table 5). In addition, compound MDP-A–L, in which MDP is coupled to a synthetic peptide, multipoly-(DL-Ala)-poly(L-Lys), exhibited stronger activity than MDP on an equimolar basis (in consideration of the fact that 1 mg of MDP-A–L corresponds to 200 μg of MDP), whereas its stereoisomer, MDP(D-D)-A–L, had no activity, indicating the specificity of the reaction observed with MDP-A–L.

Effect of the addition of acyl groups to the muramic acid residue on the necrosis-inducing ability of MDP. Table 6 shows the effects on necrosis-inducing activity of introducing acyl groups at the 6-*O* position of the muramic acid residue of MDP. The introduction of acetyl (L2) and stearoyl (L18) groups, especially the latter, remarkably increased the necrosis-inducing ability of MDP, while the introduction of higher-molecular-weight fatty acids including nocardomycolic acid, regardless of whether they were the linear (L), α-branched (B), or α-branched and β-hydroxylated (BH) form, abolished the inducing activity of MDP, without impairing their adjuvanticity. In addition, 4,6-L2-MDP, but not NH-L2-MDP, was effective in the induction of necrotic reaction.

Comparison of the necrosis-inducing activities of MDP analogs. Several analogs of MDP, in which the L-alanine residue or D-isoglutamine residue was replaced by other amino acids (I and II group compounds in Table 7, respectively), were assayed for the induction of the necrotic reaction. Concerning group I compounds, all the adjuvant-active analogs exhibited definite provocative activity, and among the active ones the L-serine analog seemed to provoke the strongest necrotic reaction and caused death by generalized shock more frequently than MDP. In contrast, all the group II compounds, including weakly adjuvant-active MDP(D-Glu) and MDP(D-Gln), had no activity.

Effects of modification of α-$CONH_2$ and γ-COOH groups of the D-isoglutamine residue of MDP on necrosis-inducing activity. The finding that the group II compounds in Table 7 lacked necrosis-inducing activity suggests the important role of α-$CONH_2$ and γ-COOH groups of the C-terminal D-

TABLE 6. Necrotic reaction-inducing activity of synthetic 6-*O*-acyl derivatives of MDP and related compounds[a]

Test material[b]	Reaction of primed footpads				Adjuvanticity (reference)
	Avg diam (mm, mean ± SE)		Necrotic reaction		
	Before challenge	After challenge	Individual scores	Incidence	
MDP (MP_2)[c]	13.0 ± 0.5	15.2 ± 0.6[d]	++* ++ ++ +	4/4	+ (27)
L2-MDP	13.8 ± 0.3	17.3 ± 0.7[e]	++ ++ ++ +	4/4	+ (20)
NH-L2-MDP	12.7 ± 0.3	12.0 ± 0.1	− − − −	0/4	ND[f]
4,6-L2-MDP	12.0 ± 0.5	14.7 ± 0.6[d]	++ + + ±	3/4	ND
L18-MDP	12.4 ± 0.6	16.9 ± 0.6[e]	++* ++ ++ ++	4/4	+ (20)
L30-MDP	12.1 ± 0.5	12.8 ± 0.4	− − − −	0/4	+ (43)
B30-MDP	12.3 ± 0.6	12.6 ± 0.4	− − − −	0/4	+ (43)
BH48-MDP	13.8 ± 0.4	14.6 ± 0.5	− − − −	0/4	+ (43)
N-myco-MDP	13.8 ± 0.7	14.3 ± 0.8	− − − −	0/4	+ (3)[g]

[a] See Table 1, footnotes *a* through *d*.
[b] See Fig. 6, except for 4,6-L2-MDP (4,6-diacetyl-MDP) and N-myco-MDP (6-*O*-nocardomycoloyl-MDP).
[c] Synthetic compound.
[d] Significantly different from diameter before challenge ($P < 0.05$).
[e] Significantly different from diameter before challenge ($P < 0.01$).
[f] ND, Not determined.
[g] Adjuvanticity in guinea pigs was determined in terms of skin reaction, using azobenzenearsonate-*N*-acetyl-L-tyrosine as an immunogen and azobenzenearsonate-*N*-acetyl-L-tyrosine–bacterial α-amylase as a test antigen.

TABLE 7. Comparison of the necrotic reaction-inducing activity of synthetic MDP and its analogs[a]

Test material[b]		Reaction of primed footpads				Adjuvanticity (reference)
		Avg diam (mm, mean ± SE)		Necrotic reaction		
Group	X	Before challenge	After challenge	Individual scores	Incidence	
I	L-Ala-D-isoGln (MDP)	12.0 ± 0.3	16.7 ± 0.2[c]	++* ++ ++ + + + + +	8/8	+ (27)
	L-Ser-D-isoGln	12.8 ± 0.3	18.1 ± 0.7[c]	++* ++* ++*	3/3	+ (26)
	L-Val-D-isoGln	13.8 ± 0.4	17.0 ± 0.5[c]	++* + + +	4/4	+ (—[d])
	D-Ala-D-isoGln	13.0 ± 0.5	13.3 ± 0.6	− − − −	0/4	− (26)
II	L-Ala-D-isoAsn	13.1 ± 0.7	13.0 ± 0.5	− − − −	0/4	− (27)
	L-Ala-D-Glu	13.0 ± 0.2	13.3 ± 0.2	− − − − − − − −	0/8	+ (27)
	L-Ala-D-Gln	11.8 ± 0.4	12.2 ± 0.7	− − − −	0/4	− (27); + (42)[e]
	L-Ala-L-Gln	12.0 ± 0.7	12.0 ± 0.7	− − − −	0/4	− (27)
	L-Ala-L-isoGln	11.0 ± 0.5	12.3 ± 0.6	− − − −	0/4	− (27)

[a] See Table 1, footnotes *a* through *d*.
[b] MurNAc-X.
[c] Significantly different from diameter before challenge ($P < 0.01$).
[d] Tsujimoto et al., unpublished data.
[e] Adjuvant activity in mice.

isoglutamine residue of MDP. Thus, we examined the necrosis-inducing activities of various derivatives of MDP in which either the α-$CONH_2$ or the γ-COOH group, or both, of the D-isoglutamine residue of the molecules was substituted with acyl or amide groups. Table 8 shows the importance of both α-$CONH_2$ and γ-COOH groups of MDP in the induction of necrotic reaction: all the test compounds except MDP-OLA8 were found to lack activity, irrespective of their adjuvanticity.

Activity of 1-*S*-acyl derivatives of MDP and their methyl esters. We tested another series of acyl derivatives of MDP. One group includes compounds possessing sulfur at the C-1 position of the muramic acid residue (1-thio-MDP) and their acyl derivatives (1-*S*-acyl-1-thio-MDP). The other group comprises compounds having a methyl ester at the γ-COOH group of their D-isoglutamine residue (1-*S*-acyl-1-thio-MDP-OMe). In contrast to the results with 6-*O*-acyl-MDPs, the introduction of acyl groups larger than the stearoyl group at the 1-*S* position abolished the necrosis-inducing activity of the parent molecules (1-thio-MDP) (Table 9). None of the 1-*S*-acyl-1-thio-MDP-OMe series compounds provoked the necrotic reaction.

Other immunomodulators of bacterial origin. Endotoxic lipopolysaccharide, lipid A (either bacterial or synthetic), PPD, and streptococcal pyrogenic exotoxin did not induce necrotic reaction at the primed footpad of guinea pigs (data not shown).

DISCUSSION

In the present study, we examined various analogs and derivatives of muramylpeptides and related compounds, prepared by enzymatic digestion of bacterial cell wall peptidoglycans or by chemical syntheses, for their ability to induce necrosis in the guinea pig footpad primed by injection of heat-killed *M. tuberculosis* incorporated in w/o emulsion. It was revealed that muramylpeptides of an appropriate size

TABLE 8. Influences of modification of α-$CONH_2$ or γ-COOH group, or both, of the D-glutamic acid residue of MDP on necrotic reaction-inducing activity[a]

Test material[b]			Reaction of primed footpads				Adjuvanticity (reference)
			Avg diam (mm, mean ± SE)		Necrotic reaction		
Abbreviation	R^1	R^2	Before challenge	After challenge	Individual scores	Incidence	
MDP	$CONH_2$	COOH	12.0 ± 0.2	14.5 ± 0.4[c]	++* + + +	4/4	+ (27)
MDP(D-Glu)	COOH	COOH	12.5 ± 0.3	12.8 ± 0.5	− − − −	0/4	+ (27)
MDP-OMe	$CONH_2$	$COOCH_3$	13.2 ± 0.4	13.0 ± 0.5	± − − −	0/4	ND[d]
L18-MDP(OMe)	$CONH_2$	$COOCH_3$	12.5 ± 0.5	14.0 ± 0.6	± ± ± − −	0/4	ND
MDP-OLA8	$CONH_2$	$COO(CH_2)_7CH_3$	12.3 ± 0.3	14.7 ± 0.7[e]	+ + + ±	3/4	ND
MDP-NH_2	$CONH_2$	$CONH_2$	12.3 ± 0.6	12.0 ± 0.5	− − − −	0/4	ND
MDP-NHLA8	$CONH_2$	$CONH(CH_2)_7CH_3$	12.0 ± 0.5	12.8 ± 0.4	− − − −	0/4	ND
MDP-NHLA18	$CONH_2$	$CONH(CH_2)_{17}CH_3$	13.8 ± 0.7	13.7 ± 0.7	− − − −	0/4	ND
MDP(D-Gln)	COOH	$CONH_2$	13.0 ± 0.4	12.9 ± 0.5	− − − −	0/4	− (27); + (42)[f]
Murabutide	$COO(CH_2)_3CH_3$	$CONH_2$	13.7 ± 0.4	13.0 ± 0.1	− − − −	0/4	+ (23)
MDP(OLA8)OMe	$COOCH_3$	$COO(CH_2)_7CH_3$	13.0 ± 0.5	13.7 ± 0.6	− − − −	0/4	ND
MDP(OMe)OLA8	$COO(CH_2)_7CH_3$	$COCH_3$	12.0 ± 0.1	13.5 ± 0.3	± ± − − − −	0/6	ND
MDP(OLA8)OLA8	$COO(CH_2)_7CH_3$	$COO(CH_2)_7CH_3$	12.5 ± 0.2	12.3 ± 0.3	− − − −	0/4	ND

[a] See Table 1, footnotes *b* through *d*.
[b] See Fig. 5.
[c] Significantly different from diameter before challenge ($P < 0.01$).
[d] ND, Not determined.
[e] Significantly different from diameter before challenge ($P < 0.05$).
[f] Adjuvanticity in mice.

TABLE 9. Necrotic reaction-inducing activity of synthetic 1-*S*-acyl derivatives of 1-thio-MDP and 1-thio-MDP methyl ester[a]

Test material[b]		Reaction of primed footpads				Adjuvanticity[c] (reference)
		Avg diam (mm, mean ± SE)		Necrotic reaction		
R^1	R^2	Before challenge	After challenge	Individual scores	Incidence	
–H	–OH	13.5 ± 0.5	16.0 ± 0.6[d]	+ + +	3/3	+ (11)
$–CO(CH_2)_8CH_3$	–OH	12.7 ± 0.4	14.7 ± 0.4[d]	+ ± ±	1/3	+ (11)
$–CO(CH_2)_{10}CH_3$	–OH	12.0 ± 0.3	14.7 ± 0.5[e]	+ + + + + +	6/6	ND[f]
$–CO(CH_2)_{12}CH_3$	–OH	12.0 ± 0.6	14.2 ± 0.3[d]	++ + +	3/3	ND
$–CO(CH_2)_{18}CH_3$	–OH	13.3 ± 0.3	12.8 ± 0.2	– – – –	0/4	ND
$–CO(CH_2)_{28}CH_3$	–OH	13.2 ± 0.4	13.5 ± 0.2	– – – – – –	0/6	+ (11)
–H	$–OCH_3$	12.3 ± 0.8	13.3 ± 0.6	+ + – –	2/4	+ (11)
$–COCH_3$	$–OCH_3$	13.3 ± 0.3	14.0 ± 0.5	– – – –	0/4	+ (11)
$–CO(CH_2)_8CH_3$	$–OCH_3$	12.5 ± 0.8	11.7 ± 0.8	– – – –	0/4	ND
$–CO(CH_2)_{10}CH_3$	$–OCH_3$	13.3 ± 0.3	13.5 ± 0.3	– – – –	0/4	ND
$–CO(CH_2)_{12}CH_3$	$–OCH_3$	13.3 ± 0.3	12.3 ± 0.3	– – – –	0/4	ND
$–CO(CH_2)_{14}CH_3$	$–OCH_3$	12.8 ± 0.2	12.7 ± 0.3	– – – –	0/4	ND
$–CO(CH_2)_{18}CH_3$	$–OCH_3$	12.5 ± 0.3	12.3 ± 0.2	– – – –	0/4	ND
$–CO(CH_2)_{18}CH_3$	$–OCH_3$	13.0 ± 0.5	13.3 ± 0.5	– – – –	0/4	+ (11)

[a] See Table 1, footnotes *b*, *c*, and *d*.
[b] See Fig. 7.
[c] Adjuvanticity in guinea pigs were determined in terms of skin reaction, using azobenzenearsonate-*N*-acetyl-L-tyrosine as an immunogen and azobenzenearsonate-*N*-acetyl-L-tyrosine–bovine serum albumin as a test antigen.
[d] Significantly different from diameter before challenge ($P < 0.05$).
[e] Significantly different from diameter before challenge ($P < 0.01$).
[f] ND, Not determined.

and structure provoke the necrotic reaction, while other bacterial immunomodulators including bacterial lipopolysaccharide and lipid A, which shared many bioactivities with muramylpeptides, did not.

Regarding the structure-activity relationship, compounds capable of inducing the necrotic reaction, with only a few exceptions, were also active in adjuvanticity in terms of the ability to induce delayed-type hypersensitivity and to stimulate serum antibody levels against ovalbumin when administered to guinea pigs in w/o emulsion with the antigen. However, the reverse was not true; many definitely adjuvant-active analogs and derivatives of muramylpeptides were found to lack necrosis-inducing ability. For example, peptidoglycan subunits, either A_2pm type or L-lysine type, larger than muramyltetrapeptides did not induce the necrotic reaction, despite their strong adjuvanticity. It was found that unnatural GlcNAc-MurNAc-tetrapeptides (A_2pm type), which were prepared by the addition of L-amino acid to the C-terminal A_2pm residue of GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-A_2pm (GMP_3-A), unlike MurNAc-L-Ala-D-isoGln-*meso*-A_2pm-D-amino acids, exhibited definite necrosis-inducing ability. This finding can be explained by assuming that disaccharide tetrapeptide possessing C-terminal L-amino acid is readily degraded in vivo to GlcNAc-MurNAc-L-Ala-D-isoGln-*meso*-A_2pm, which is active in necrosis induction. Experimental evidence for the assumption, however, has not yet been obtained.

The present study shows that the introduction of a stearoyl group at the C-6 position of the muramic acid residue of MDP or the L-lysine residue of MDP-L-Lys increased the necrosis-inducing activity of the respective parent molecules, but not of the 1-thio-MDP and 1-thio-MDP-OMe series compounds. The importance of the α-$CONH_2$ and γ-COOH groups of the D-isoglutamine residue of MDP was also shown by experiments using ester and alkylamide derivatives of MurNAc-L-Ala-D-Glu.

Regarding the requirement for priming the induction of the necrotic reaction by muramylpeptides of appropriate structures, a w/o emulsion containing MDP and ovalbumin was found to effectively prepare the injection site in a way similar to heat-killed *M. tuberculosis* in w/o emulsion (37). This finding suggests that combined application of an immunoadjuvant such as MDP and an immunogen such as ovalbumin under proper conditions can prepare the application site, and that the foci of microbial infections where immunogens and adjuvants such as peptidoglycans and endotoxins coexist is vulnerable to the necrotic reaction-inducing activity of muramylpeptides. If this assumption is proved, the ability of some muramylpeptides to induce a necrotic reaction at a primed site can be a disadvantage in the medical application of muramylpeptides, though the necrotic reaction described here has not been observed in mice, rats, or rabbits (37).

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