

Mitogenic Effects of Bacterial Cell Walls, Their Fragments, and Related Synthetic Compounds on Thymocytes and Splenocytes of Guinea Pigs

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Stimulation of [³H]thymidine incorporation of thymocytes and splenocytes from guinea pigs by various bacterial cell walls and their peptidoglycans, by enzymatic digests, and by synthetic muramyl dipeptides was studied as an indication of mitogenic activity. Cell wall and peptidoglycan preparations, isolated from 19 strains belonging to 18 different species, definitely increased [³H]thymidine incorporation of thymocytes as well as splenocytes, regardless of mycolic acid contents as a non-peptidoglycan component. Both the cell walls from *Nocardia corynebacterioides* (containing mycolic acids) and those from *Streptomyces gardneri* (lacking mycolic acids) showed far stronger mitogenic activities on splenocytes than other cell walls (stimulation index, 25 to 30). Furthermore, water-soluble enzymatic digests, notably the endopeptidase digests, which generally were greater in degree of polymerization of peptidoglycan subunits than the glycosidase digests obtained from representative cell walls, were found to have as distinct a stimulating activity on splenocytes as the original cell walls. In contrast, solubilization of the cell walls by enzymes, irrespective of endopeptidases or glycosidases, was accompanied by disappearance of the mitogenic activity on thymocytes. On the other hand, studies with synthetic 6-*O*-acyl-MurNAc-L-Ala-D-isoGln preparations (6-*O*-acyl-MDPs) revealed that 6-*O*-stearoyl-MDP and 6-*O*-(2-tetradecylhexadecanoyl)-MDP, unlike MDP, had distinct mitogenic activity on thymocytes, whereas their activity on splenocytes was rather weaker than MDP itself. The findings presented here suggest that MDP is the minimal structure for the mitogenic activities of bacterial cell walls on guinea pig splenocytes, but that MDP, though distinctively active by itself, requires a polymerized form to exert effectively its inherent stimulating activities on splenocytes. On the other hand, on thymocytes, MDP, unless it takes a particular form or has appropriate additive groups, cannot exert its mitogenic activities.

Damais et al. reported that cell wall peptidoglycans obtained from *Bacillus megaterium* and *Escherichia coli* exerted mitogenic effects on spleen lymphocytes of nude mice and rabbit splenocytes, but those from *Micrococcus lysodeikticus* and the monomeric subunit of *E. coli* peptidoglycan lacked these activities (8). This study was followed by that of Ciorbaru et al., which demonstrated that a water-soluble polymer of peptidoglycan subunits prepared from *Nocardia rubra* cell walls by use of *Streptomyces albus* G (endo)peptidase exhibited mitogenic activity on murine B cells, but that a

peptidoglycan monomer obtained from the same cell walls digested by lysozyme could not (7). Then we revealed that adjacent-active synthetic MurNAc-L-Ala-D-isoGln (MDP) (1, 11, 21) and MurNAc-L-Ala-D-Glu (21) had weak but significant mitogenic activity on splenocytes of guinea pigs and ICR mice, but none of the adjuvant-inactive analogs, MurNAc-L-Ala-L-isoGln, MurNAc-L-Ala-D-Gln, MurNAc-L-Ala-L-Gln, MurNAc-L-Ala-L-Glu, and MurNAc-L-Ala-D-isoAsn, had such activity (38). Our findings were confirmed by Damais et al., who demonstrated the mitogenic activity of synthetic MDP by cul-

turing splenocytes of DBA/2 mice, a high responder to the mitogenic activity of MDP, in a medium supplemented with 2-mercaptoethanol (10).

With regard to the stimulating activity of cell walls on thymocytes or T cells, Azuma et al. first revealed that cell walls containing mycolic acids prepared from mycobacteria, nocardiae, and corynebacteria exhibited a distinct mitogenic activity on T as well as B cells of C57Bl/6J mice (2, 3).

In this study, we investigated the mitogenic effects of bacterial cell walls and related compounds, either enzymatically obtained or synthetic, upon splenocytes and thymocytes from guinea pigs, to elucidate more clearly the chemical and physical characteristics responsible for the mitogenic activities of bacterial cell walls.

MATERIALS AND METHODS

Cell wall preparations. Cells from which the cell walls were isolated were cultured in conditions as reported previously (18, 38). Harvested cells were disrupted with either a Braun Mechanical Cell Homogenizer (model KMS-100) or a Dyno-Mill, type KDL (Willy A, Bichofen Manufacturing Engineers, Basel, Switzerland). The suspension of disrupted cells was submitted to differential centrifugation, and the crude cell wall fraction thus separated was purified by digestions of contaminants with trypsin and/or pronase as described previously (17, 18, 38). The cell walls of *Actinomyces naeslundii* (ATCC 12104), *A. naeslundii* (human isolated), and *Actinomyces viscosus* (ATCC 19246) were a gift from H. Yamagami, Osaka University Dental School (39). The cell walls of *Staphylococcus aureus* (STA-EMT-P) and *Streptococcus pyogenes* (type 3, strain 0176) were generously given by Y. Hirachi, Osaka University Dental School, and H. Ohkuni, Nippon Medical School (28), respectively. The cell walls from *Ampullariella regularis*, *Corynebacterium betae* (NCPP 373), and *Corynebacterium insidiosum* (NCPP 1110) were kindly supplied by H. R. Perkins, University of Liverpool, England (29-32), and those of *Arthrobacter atrocyaneus* (ATCC 13752) and *Arthrobacter* sp. (NCIB 9423) were a gift from K. H. Schleifer, University of Munich, West Germany (12, 15).

Bacterial peptidoglycans. The peptidoglycans of *S. aureus* (STA-EMT-P) and *Staphylococcus epidermidis* (ATCC 155) were obtained by removal of a non-peptidoglycan moiety from cell walls by extraction with 10% trichloroacetic acid at 60°C for 12 h (14) or with 10% trichloroacetic acid in the cold for 16 h (26), respectively. The peptidoglycans of *Streptococcus mutans* (BHT) and *Lactobacillus plantarum* (ATCC 8014) were similarly obtained by extraction of cell walls with 10% trichloroacetic acid at 60°C for 4 h. The peptidoglycans of *Streptococcus pyogenes*, a gift of H. Ohkuni, were prepared by the hot-formamide method (13).

Enzymatic digests of cell walls. Cell wall lytic

enzymes used in this study were the M-1 endo-N-acetylmuramidase (40), the L-3 D-alanyl-meso- α , ϵ -diaminopimelic acid endopeptidase (16), and the SALE D-alanyl-glycine and glycylglycine endopeptidase (K. Yokogawa et al., manuscript in preparation). The cell walls of *Mycobacterium rhodochrous* (ATCC 184), *Nocardia corallina* (ATCC 14347), *Nocardia corynebacterioides* (ATCC 14898), and *Streptomyces gardneri* (ATCC 23911) were treated with either the M-1 enzyme or L-3 enzyme. The peptidoglycans of *S. epidermidis* were solubilized by the treatment with either the M-1 enzyme or SALE enzyme. Typical experimental conditions for digestion of the cell walls or peptidoglycans with each enzyme are presented in Table 1.

Synthetic muramyldipeptides and 6-O-acylmuramyldipeptides. Syntheses of MDP and its 6-O-acyl derivatives were described elsewhere (22-25, 35). Their chemical structures and solubility in water are shown in Table 2.

Determination of the mitogenic activity. (i) Animals. Randomly bred female guinea pigs, weighing 300 to 600 g, were used.

(ii) Separation of splenocytes and thymocytes. The spleen and thymuses were removed from a guinea pig as aseptically as possible, and were minced on a Cytocieve. The minced tissues were dispersed with a needle (0.65 by 32 mm) to give a homogeneous cell suspension and subjected to Conray 400 (Daichi Pharmaceutical Co., Tokyo)-Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient (specific gravity: 1.09) separation, to purify spleen lymphocytes (splenocytes) or thymus lymphocytes (thymocytes).

(iii) Cell culture. Splenocytes or thymocytes were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (Flow Laboratories, Rockville, Md.) and antibiotics (100 U of penicillin G and 100 μ g of streptomycin per ml) to give 10^6 cells per ml of medium. Samples of 1 ml of the cell suspension were incubated with 0.1-ml portions containing varying doses of test specimen in a CO₂ incubator for 48 h at 37°C.

(iv) Measurement of thymidine incorporation. [³H]thymidine (1 μ Ci; specific activity 2.0 Ci/mmol; Radiochemical Centre, Amersham, Buckinghamshire, England) was added to each culture 24 h before cell harvesting. After completion of the incubation, the cells of each culture were collected on a glass microfibre paper (GF/C; Whatman Ltd., England) and were washed successively with 10 ml of saline, 10 ml of 5% trichloroacetic acid, and 5 ml of ethanol. The glass microfibre paper was dried in a scintillation vial, 10 ml of toluene scintillator was added, and the radiolabeled thymidine incorporated into the cells was measured with a liquid scintillation spectrometer (Aloka LSC-673, Aloka Co., Tokyo). Stimulation of thymidine incorporation by lymphocytes in the presence of test specimen was expressed as stimulation index, i.e., the ratio of counts per minute of a test culture to counts per minute of the control culture without test specimen. In some experiments, phytohemagglutinin (Bacto-PHA-P; Difco Laboratories, Detroit, Mich.) was used as T-cell mitogen, and lipopolysaccharide (Bacto-Lipopolysaccharide B *S. enteritidis*, Difco) was used as B-cell mitogen.

TABLE 1. *Typical experimental conditions for digestion of cell walls with the M-1 endo-N-acetylmuramidase or L-3 or SALE endopeptidase^a*

Enzyme used	Ratio, enzyme/walls (per ml)	Buffer (pH, final concn)	Incubation	
			Temp (°C)	Time
M-1	0.2 mg/10 mg	Na-phosphate (5.9, 0.01 M)	37	5 days
L-3	0.2 mg/10 mg	Sorensen phosphate (7.4, 0.02 M)	37	6 days
SALE ^b	2 µg/10 mg	Na-pyrophosphate (8.0, 0.01 M)	37	6 h

^a Toluene was added as a preservative.

^b After completion of the incubation, a reaction mixture was centrifuged at $1,000 \times g$ for 30 min to remove an insoluble residue. A crude water-soluble peptidoglycan fraction obtained as a supernatant fluid was concentrated under reduced pressure with a rotary evaporator, and the concentrate was subjected to gel filtration with Sephadex G50 and G25 columns connected in series.

TABLE 2. *Chemical structure and solubility in water of synthetic 6-O-acyl-MDPs*

6-O-Acyl-MDP	Abbreviation	Solubility in water
CH ₃ (CH ₂) ₁₆ CO-MDP	[L18]-MDP	Nearly soluble
CH ₃ (CH ₂) ₂₈ CO-MDP	[L30]-MDP	Insoluble
CH ₃ (CH ₂) ₁₃ CHCO-MDP	[B30]-MDP	Insoluble
CH ₃ (CH ₂) ₁₃ CH ₃ (CH ₂) ₂₂ CH(OH) CHCO-MDP	[BH48]-MDP	Insoluble
CH ₃ (CH ₂) ₂₁		

RESULTS

Mitogenic effects of various bacterial cell walls. Figure 1 shows that all tested cell walls, isolated from 18 different bacterial species (19 strains), had definite mitogenic activity on thymocytes from guinea pigs, and that there were no significant differences in strength of the mitogenic activity between the cell walls that were obtained from mycobacteria and nocardiae and contained a variety of mycolic acids as a non-peptidoglycan component and those that were derived from other species and lacked mycolic acids. In addition, even the walls that were inactive as an immunoadjuvant, at least by themselves, i.e., those of *M. lysodeikticus*, *S. epidermidis*, *A. regularis*, *A. atrocyaneus*, *Arthrobacter* sp., *C. betae*, and *C. insidiosum* (18-20), were found to be definitely mitogenic.

Some of the cell wall preparations listed in Fig. 1 were then investigated for their mitogenic activities against both thymocytes and splenocytes from one and the same guinea pig. The walls of *N. corynebacterioides* and those of *S. gardneri* exhibited strikingly strong mitogenic

effects on splenocytes among 11 test cell wall specimens, whereas on thymocytes the walls of these two strains showed mitogenic activities comparable to those of the cell walls from other strains (Fig. 2).

The above findings suggest that bacterial cell walls do not require mycolic acids as a necessary component to exert their mitogenic activity on either thymocytes or splenocytes, and that there are no correlations between the strengths of the mitogenic activities of bacterial cell walls on guinea pig splenocytes and thymocytes.

Comparison of mitogenic effects of cell walls and peptidoglycans. The mitogenic activity of cell wall peptidoglycans of several species on thymocytes and splenocytes was compared with that of the corresponding cell walls. Thymidine uptake by thymocytes was generally more stimulated by cell walls than by the corresponding peptidoglycans, whereas the stimulation of splenocytes by peptidoglycans was slightly stronger than that by the corresponding cell walls (Fig. 3). The results suggest that the mitogenic activities of bacterial cell walls on splenocytes were mainly attributable to a pep-

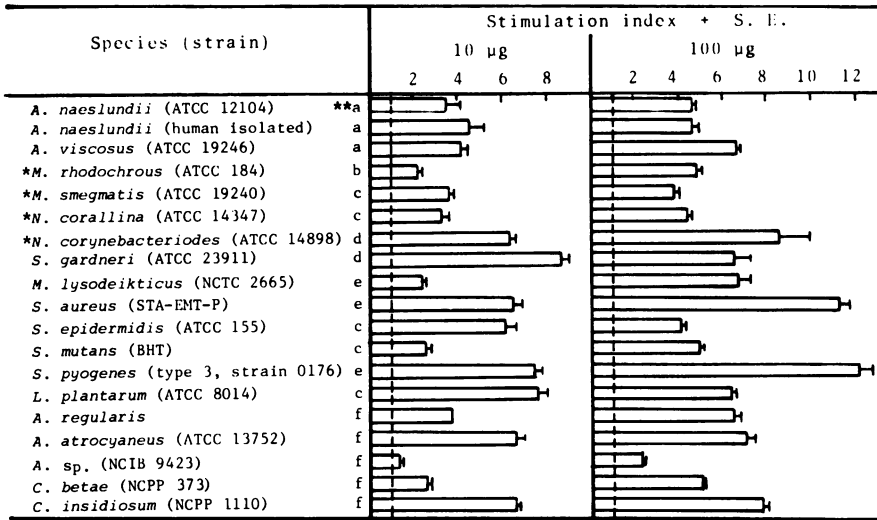


FIG. 1. Mitogenic effects of various bacterial cell walls on thymocytes from female guinea pigs. (*) Cell walls containing mycolic acids. All determinations were carried out in quadruplicate cultures. (**) The incorporation of [³H]thymidine per 10⁶ cells in the control cultures of six different experiments was: 2,768.5 cpm (a), 3,519.8 cpm (b), 7,066.5 cpm (c), 778.8 cpm (d), 1,333.2 cpm (e), and 2,276.3 cpm (f).

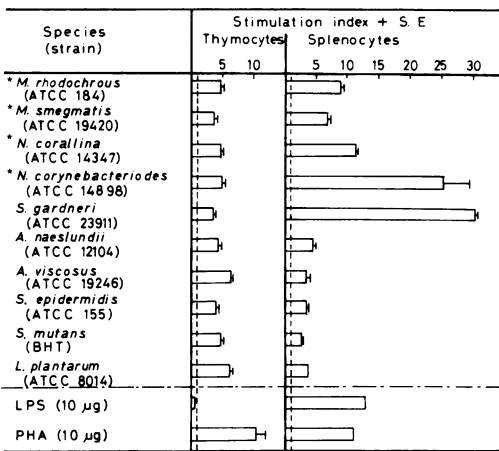


FIG. 2. Mitogenic effects of bacterial cell walls (100 µg) on thymocytes and splenocytes from a female guinea pig. (*) Cell walls containing mycolic acids. All determinations were carried out in quadruplicate cultures. The incorporation of [³H]thymidine per 10⁶ cells in control cultures was: 7,066.5 ± 1,256.3 cpm (standard error [S.E.]) in thymocytes and 868.0 ± 75.0 cpm in splenocytes. LPS, Lipopolysaccharide; PHA, phytohemagglutinin.

tidoglycan moiety, but the stimulating activity on thymocytes might be related to non-peptidoglycan moieties as well as peptidoglycans.

Mitogenic effect of water-soluble, enzymatic digests of cell walls. Cell wall specimens from five different species, containing mycolic acids or not, were submitted to digestions

with either the M-1 endo-*N*-acetylmuramidase, the L-3 endopeptidase, or the SALE endopeptidase. The assay of mitogenicity of the water-soluble specimens thus obtained on thymocytes (Fig. 4A) and splenocytes (Fig. 4B) revealed that the endopeptidase digests have distinct mitogenic activity on splenocytes, comparable to or a little stronger than that of cell walls; that the activity of the M-1 enzyme digest on splenocytes was generally weaker than those of the corresponding walls and endopeptidase digest; and that against thymocytes, on the other hand, none of the test water-soluble digests, regardless of whether they were obtained by use of the endopeptidase or the endo-*N*-acetylmuramidase, exerted any significant mitogenicity.

It may be added here that the water-insoluble residue obtained from the M-1 enzyme digests of the cell walls of *N. corynebacterioides* and *S. gardneri*, amounting to 51 and 32% of the original walls, respectively, exhibited a very strong mitogenicity on splenocytes, although it had a rather weaker stimulating activity on thymocytes than the original walls (data not shown).

Mitogenic effect of synthetic MDP and its 6-*O*-acyl derivatives. Figure 5 indicates that although MDP itself could not exert any significant mitogenic activity on thymocytes, [L18]-MDP and [B30]-MDP exerted a distinct activity, and that on splenocytes, on the other hand, test 6-*O*-acyl-MDP specimens had a rather weaker stimulating activity than MDP. This figure also shows that definite inhibitory effects on thymidine incorporation of both thymocytes

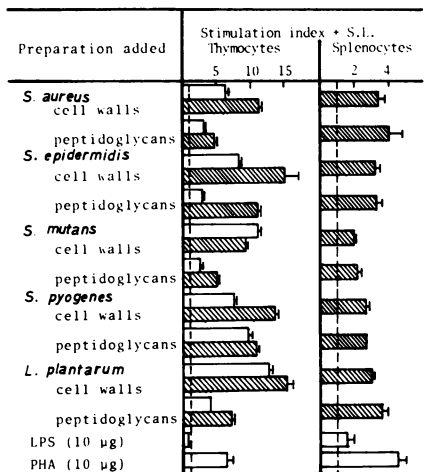


FIG. 3. Comparison of mitogenic effects of cell walls and of peptidoglycans from various bacterial species on thymocytes and splenocytes from a female guinea pig. All determinations were carried out in quadruplicate cultures. The incorporation of [3 H]-thymidine per 10^6 cells in control cultures was: $1,333.2 \pm 56.2$ cpm (standard error [S. E.]) in thymocytes and $1,417.7 \pm 185.7$ cpm in splenocytes. □, 10 µg of preparation added; ▨, 100 µg added. LPS, Lipopolysaccharide; PHA, phytohemagglutinin.

and splenocytes were exerted by [L18]-MDP in a dose equivalent to 100 µg of MDP. Comparison was then made using 6-*o*-acyl-MDP with the same total carbon atom number but different structure. [L30]-MDP was proved to be scarcely active in stimulation on thymocytes under conditions where [B30]-MDP could exert a distinct stimulating activity on thymocytes (Fig. 6).

DISCUSSION

Azuma et al. reported that the cell walls from mycobacteria, nocardiae, and corynebacteria, which contain mycolic acids as one of the principal components of a non-peptidoglycan moiety, exerted mitogenic effects on both T and B cells of C57Bl/6J mice (2, 3). In the present study, we revealed that not only cell walls containing mycolic acids but also those lacking these peculiar, high-molecular-weight fatty acids could exert distinct mitogenic effects on thymocytes as well as splenocytes of guinea pigs. It was also proved that cell walls whose peptidoglycan belonged to group B type (34) and which were shown to be inactive as an immunoadjuvant, unlike the cell walls having group A-type peptidoglycans (20), could show definite stimulating activities on thymocytes. Thus it seems reasonable to assume that mycolic acids were not necessarily required for the mitogenicity of bacterial

cell walls on thymocytes (probably T cells).

We further showed that water-soluble preparations obtained from enzymatically digested cell walls retained mitogenicity of the walls on splenocytes, and that the endopeptidase lysates (polymers of cell wall subunits linked by a fairly long glycan chain) had stronger activity than the endo-*N*-acetylmuramidase digests (at most, oligomers of peptidoglycan subunits, linked by cross-bridges between the neighboring peptide subunits) (Fig. 3), thus confirming in principle the report of Ciorbaru et al. (7). However, there seem to be some discrepancies in the report of Ciorbaru et al. concerning the activity of endo-*N*-acetylmuramidase lysates: i.e., they assumed that the lysozyme lysate of *N. rubra* peptidoglycans did not have mitogenicity. As previously reported, we found that even synthetic MDP had a weak but definite mitogenicity on splenocytes of guinea pigs and ICR mice (37). This finding was confirmed by Damais et al. (9, 10), who emphasized that the mitogenicity of MDP was considerably influenced by cultural conditions and the animal strains from which splenocytes were isolated. The discrepancies observed between the present study and that of Ciorbaru et al., on the mitogenicity of peptidoglycan subunit monomer or oligomers, might thus be due to the use of different animal species in assay.

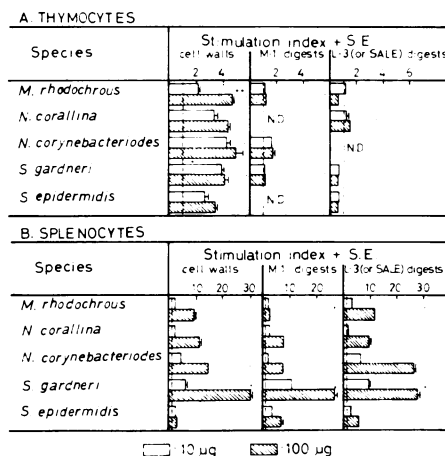


FIG. 4. Mitogenic effects of bacterial cell walls and their enzymatic digests on thymocytes (A) and splenocytes (B) from female guinea pigs. All determinations were carried out in quadruplicate cultures. (**) The incorporation of [3 H]thymidine per 10^6 cells in the control cultures in 11 different experiments was: 3,519.8 cpm (a), 778.8 cpm (b), 1,045.4 cpm (c), 1,310.2 cpm (d), 2,169.8 cpm (e), 1,941.8 cpm (f), 2,094.3 cpm (g), 1,882.8 cpm (h), 2,965.4 cpm (i), 2,301.2 cpm (j), and 1,477.2 cpm (k).

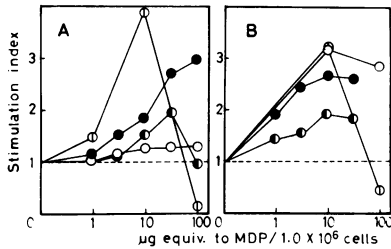


FIG. 5. Mitogenic effects of MDP (○) and its 6-O-acyl derivatives, [L18]-MDP (◇), [B30]-MDP (●), and [BH48]-MDP (◐), on thymocytes (A) and splenocytes (B) from female guinea pigs. All determinations were carried out in quadruplicate cultures. The incorporation of [³H]thymidine per 10⁶ cells in the control cultures was 1,314.8 cpm (for MDP, [B30]-MDP, and [BH48]-MDP in A), 10,052.7 cpm (for [L18]-MDP in A), 5,792.5 cpm (for MDP and [B30]-MDP in B), 463.8 cpm (for [L18]-MDP in B), and 4,288.3 cpm (for [BH48]-MDP in B).

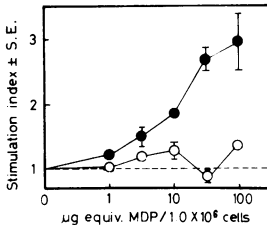


FIG. 6. Mitogenic effects of [B30]-MDP (●) and [L30]-MDP (○) on thymocytes from a female guinea pig. All determinations were carried out in quadruplicate cultures. The incorporation of [³H]thymidine per 10⁶ cells in the control cultures was 1,314.8 ± 55.8 cpm (standard error [S. E.]).

However, we have found no significant differences in the responsiveness of splenocytes from ICR, BALB/c, C3H/He, C57Bl/6J, and DBA/2 mice to the mitogenic activities of bacterial cell walls and their digests, although it is true that we observed that susceptibility to the mitogenic effect of MDP varied from mouse strain to strain. It may be added here that Bona et al. have reported that some fractions obtained from the lysozyme lysate of whole cells of various nocardiae (nocardia water-soluble mitogen) had a very strong mitogenic activity on murine splenocytes (4-6), and that the responsible factors in this fraction might derive from the cytoplasmic membrane components, but not from the cell walls. Thus, the water-soluble preparations examined in the present study might contain non-peptidoglycan mitogen principles similar to that contained in nocardia water-soluble mitogen, although our preparations were obtained from purified cell walls, not from whole cells. In any case, the nature or origin of remarkable potent

mitogenic principle(s) in our water-soluble specimens obtained from the walls of *N. corynebacterioides* and *S. gardneri* deserves further investigation.

It should be emphasized that all of the test water-soluble preparations that were definitely mitogenic on splenocytes were found to be scarcely active on thymocytes (probably T cells) of guinea pigs. However, the study with 6-O-acyl derivatives of MDP showed that both a nearly water-soluble [L18]-MDP and a water-insoluble [B30]-MDP could exhibit distinct mitogenicity on thymocytes of guinea pigs, whereas [B30]-MDP had less activity than MDP itself on splenocytes (Fig. 5). These findings suggest that MDP is essential for mitogenicity of bacterial cell walls on splenocytes (probably B cells), but that mitogenic activity on thymocytes (probably T cells) of cell walls depends greatly on the physical characteristics of the cell walls. The importance of physical characteristics was further suggested by the finding that [L30]-MDP, which was as insoluble in water as [B30]-MDP, was far less active than [B30]-MDP in stimulation of thymocytes (Fig. 6). Therefore, the role of MDP for the manifestation of mitogenic effects of bacterial cell walls on thymocytes is not clear at present.

It is well known that T-cell mitogens, for example, phytohemagglutinin and concanavalin A, require the presence of macrophages to exert their mitogenic activity on murine T cells (27, 33). Recently, Sugimura et al. reported a similar finding with the cell walls of *N. rubra* (36). Thus, there is some possibility that the observed mitogenic activities of test specimens in the present study may emerge through the stimulation of macrophages present in test thymocyte suspensions. Recently, we have demonstrated that MDP is the minimal structure responsible for the stimulating activity of cell walls on guinea pig peritoneal macrophages, but that MDP requires a particular state to exert the activity effectively. Therefore, there seems to be some correlation between stimulating activities on macrophages and those on thymocytes of test cell walls and related preparations. The role of macrophages in manifestation of the mitogenic activity of cell walls and related preparations on thymocytes or vice versa requires further investigation.

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