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MITOGENIC EFFECTS OF BACTERIAL CELL WALLS AND THEIR COMPONENTS ON MURINE SPLENOCYTES

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SUMMARY Stimulation of splenocytes from ICR mice by cell walls of 17 species of gram-positive bacteria, and peptidoglycans and water-soluble enzymatic digests prepared from some of them, and by synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) was studied in terms of mitogenic activity.

All the cell walls tested except those of *Micrococcus lysodeikticus*, regardless of their mycolic acid content and immunopotentiating activity, had definite stimulatory activity on splenocytes. In general, the cell walls of mycobacteria, nocardia, and stretomyces had stronger mitogenic activity than other cell walls. The mitogenic activity of cell walls was mainly attributable to a peptidoglycan moiety, and the activity was retained even when the peptidoglycan had been degraded into a monomer or a dimer of its subunit, though a polymerized form of the subunits exhibited stronger activity than the monomer or dimer. Definite mitogenicity of synthetic MDP on splenocytes from ICR mice was also confirmed.

The origin of the unknown principle(s) that is found in the cell walls of nocardia, mycobacteria and streptomyces and is responsible for their potent mitogenic activity on murine splenocytes was discussed.

INTRODUCTION

There have been many studies on the mechanism of the immunopotentiating actions of bacterial cell walls, and of the minimal effective structure, MurNAc-L-Ala-D-isoGln (MDP) (Ellouz et al., 1974; Kotani et al.,

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1975b). The mitogenic activities of cell walls and related compounds in bacteria have been extensively investigated, and cell walls (Azuma et al., 1976b) and peptidoglycans (Damais et al., 1975; Dziarski and Dziarski, 1979) were shown to have strong mitogenic effects on murine B cells. However, there is some controversy on the stimulation of B cells by monomers of peptidoglycan subunits and synthetic muramyl peptides. While there are several reports that neither peptidoglycan monomers nor MDP have mitogenic effects on murine lymphocytes (Damais et al., 1975; Specter et al., 1977; 1978; Watson and Whitlock, 1978), we (Takada et al., 1977) demonstrated that MDP, but not its adjuvant-inactive analogues, exhibited definite mitogenic effects on splenocytes of ICR mice and guinea pigs. Igarashi et al. (1977) also showed that MDP had a mitogenic effect on splenocytes of DBA/2 mice, and Damais et al. (1977, 1978) found that MDP is mitogenic on murine B cells in the presence of 2-mercaptoethanol instead of fetal calf serum, but that adjuvant-inactive analogues of MDP are not active under the same conditions. Furthermore, they showed that there were considerable differences in the responsiveness of lymphocytes from different strains of mice to the mitogenic effect of MDP.

In view of these findings and the controversial state of information on the minimum structure of the cell walls required for mitogenic activity, we carried out further studies on the mitogenic activities on murine splenocytes of cell walls and their components, prepared enzymatically or synthetically.

MATERIALS AND METHODS

1. *Test materials*

1) Cell wall preparations

Test specimens were prepared as described previously (Kotani et al., 1970, 1975a, 1975c; Takada et al., 1979a, 1979b) from the following bacterial species: *Mycobacterium rhodochrous* (ATCC 184), *Mycobacterium smegmatis* (ATCC 19420), *Mycobacterium tuberculosis* ($H_{37}Rv$), *Nocardia corallina* (ATCC 14347), *Nocardia corynebacteriodes* (ATCC 14898), *Nocardia erythropolis* (ATCC 4277), *Nocardia gardneri* (IFO 3385), *Streptomyces gardneri* (ATCC 23911), *Micrococcus lysodeikticus* (NCTC 2665), *Staphylococcus aureus* (FDA 209P), *Staphylococcus epidermidis* (ATCC 155), *Streptococcus mutans* (BHT), *Streptococcus salivarius* (IFO 3350), and *Lactobacillus plantarum* (ATCC 8014). The cell walls of *Actinomyces viscosus* (ATCC 15987) and *Streptococcus sanguis* (ATCC 10556) were generously given by Dr. H. Yamagami, Osaka University Dental School (Yamagami, 1978). The cell walls of *Corynebacterium poinsettiae* (NCPP 177) were kindly supplied by Dr. H. R. Perkins, University of Liverpool, England (Perkins and Nieto, 1970).

2) Cell wall peptidoglycans

Peptidoglycans of *S. epidermidis*, *S. mutans*, and *L. plantarum* were prepared by extracting the cell walls with cold or hot trichloroacetic acid to remove a nonpeptidoglycan moiety as described in the previous paper (Takada et al., 1979b).

3) Enzymatic digests of cell walls

The cell wall lytic enzymes used were M-1 endo-*N*-acetylmuramidase (Yokogawa et al., 1975), L-3 D-alanyl-*meso*-2,6-diaminopimeric acid endopeptidase (Katayama et al., 1976), and SALE D-alanylglycine and glycylglycine endopeptidase (unpublished). The cell walls of *M. rhodochrous*, *M. smegmatis*, *N. corallina*, *N. corynebacteriodes*, *S. gardneri*, and *L. plantarum* were digested with either the M-1 enzyme or the L-3 enzyme. The peptidoglycans of *S. epidermidis* were solubilized by treatment with the M-1 or SALE enzyme. Details of experimental conditions for digestion of the cell walls and peptidoglycans were described previously (Takada et al., 1979b).

4) *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP)

A specimen synthesized by the method reported previously (Kusumoto et al., 1976) was kindly supplied by Dr. A. Inoue (Daiichi Seiyaku Co., Tokyo).

2. *Measurement of the mycolic acid content of cell walls*

The mycolic acid contents of cell walls of bacteria belonging to the order *Actinomycetales*, *i.e.*, *A. viscosus*, *M. rhodochrous*, *M. smegmatis*, *N. corallina*, *N. corynebacteriodes*, *N. erythropolis*, *N. gardneri*, and *S. gardneri*, were measured by gas chromatography mass spectrometry as reported previously

(Yano et al., 1978).

3. *Determination of mitogenic effects*

1) Animals

Male ICR mice of 6–12 weeks old from closed colony were purchased from CLEA Japan Incorp., Tokyo.

2) Separation of splenocytes

A lymphocyte-rich fraction was separated from the spleens of 4–7 mice. For this, the spleens were removed as aseptically as possible, and cells were separated on a Ficoll gradient (specific gravity 1.09) as described previously (Takada et al., 1979b). This fraction is hereafter referred to as splenocytes. In some experiments, splenocytes obtained from individual animals were used to avoid the possible influence of allogeneic effects in the assay of mitogenicity using pooled splenocytes.

3) Determination of mitogenic effects.

Mitogenic effects was assayed as described previously (Takada et al., 1979b). Splenocytes (1.0×10^6 cells) suspended in 1.0 ml of RPMI 1640 medium (Nissui Seiyaku Co., Tokyo) supplemented with 10% fetal bovine serum (Flow Laboratories, Rockville, Md., USA) and antibiotics (100 U/ml of penicillin G and 100 μg/ml of streptomycin) were mixed with the test material suspended or solubilized in 0.1 ml of RPMI medium. The splenocytes were cultured for 48 hr at 37°C in a humdified CO_2-incubator, and during the final 24 hr of cultivation, 1 μCi of [^{3}H]thymidine was added to each culture. Then the [^{3}H]thymidine incorporated into the cells was measured with a liquid scintillation spectrometer (Aloka LSC-673, Aloka Co., Tokyo). The extent of stimulation of incorporation of thymidine into splenocytes by a test material was expressed as the stimulation index (SI), *i.e.*, the ratio of cpm in the test culture to that in the control culture. Four replicate cultures were used for each test.

RESULTS

1. *Mitogenic effects of bacterial cell walls*

Figure 1 shows that the cell walls isolated from all 17 bacterial species tested, except *M. lysodeikticus*, including cell walls that themselves had no immunoadjuvant activity, *i.e.*, those of *S. epidermidis* and *C. poinsettiae* (Kotani et al., 1975a, 1977), had definite mitogenic effects on splenocytes of ICR mice. Figure 1 also shows that in general cell walls containing mycolic acids as constituents of the nonpeptidoglycan moiety, *i.e.*, those of mycobacteria and nocardia exhibited stronger mitogenic activity than those of other bacterial

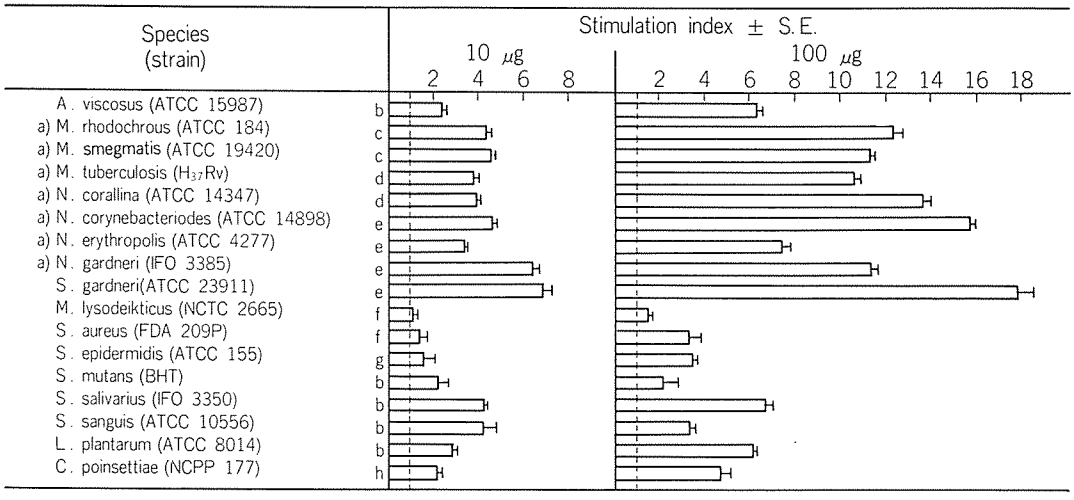

FIGURE 1. Mitogenic effects of various bacterial cell walls on splenocytes from male ICR mice. a) Cell walls containing mycolic acids. The mean incorporations of [^{3}H]thymidine per 10^6 cells in control cultures in the seven different experiments were 2,444 cpm (b), 7,330 cpm (c), 3,267 cpm (d), 3,389 cpm (e), 3,075 cpm (f), 15,049 cpm (g), and 2,766 cpm (h).

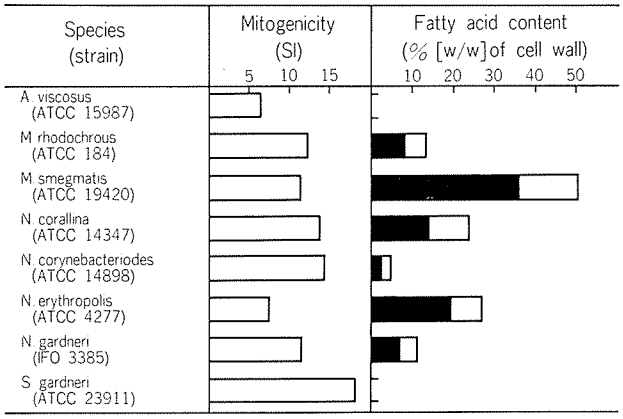

FIGURE 2. Correlation between the mitogenic effect and mycolic acid content of cell walls isolated from bacteria of *Actinomycetales*. Mitogenicity was expressed as the stimulation index (SI) when cultures were treated with 100 μg cell wall preparation. Open and closed columns show the content of total fatty acids and mycolic acids, respectively.

species. However, the cell walls of *S. gardneri* (belonging to the order *Actinomycetales* together with mycobacteria and nocardia) had the strongest activity among cell walls tested, although they contained no mycolic acids.

So we studied the correlation between the mycolic acid content and the mitogenicity of several cell walls isolated from bacteria of the order *Actinomycetales*. Figure 2 shows that there was no significant correlation between the mitogenicity and the mycolic acid content.

2. *Comparison of the mitogenicities of cell walls and peptidoglycans*

The mitogenic activities of peptidoglycans from *S. epidermidis*, *S. mutans*, and *L. plantarum* were compared with those of the corresponding cell walls (Fig. 3). The peptidoglycans had stronger mitogenic activity than the cell walls on a weight basis. However, considering the yields of peptidoglycans from the cell walls tested (86%, 51%, and 39% for *S. epidermidis*, *S. mutans*, and *L. plantarum*, respectively), there were no significant differences between the mitogenicities of the cell walls and the peptidoglycans. Thus the results suggest that the mitogenic effects of bacterial cell walls mainly depend on their peptidoglycan moiety.

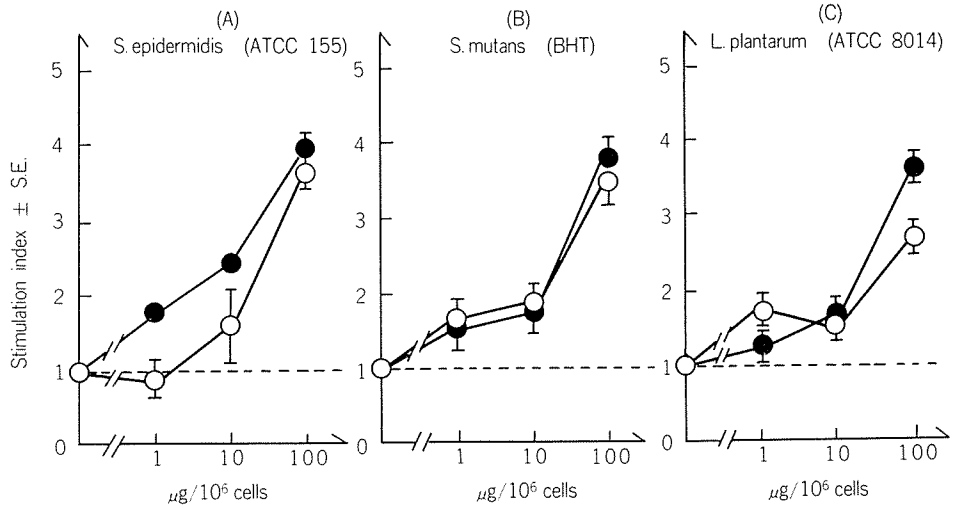

FIGURE 3. Comparison of the mitogenicities of cell walls (○) and peptidoglycans (●) isolated from *S. epidermidis* (ATCC 155), *S. mutans* (BHT), and *L. plantarum* (ATCC 8014), respectively. The incorporations of [^{3}H]thymidine per 10^6 cells in control cultures were 15,049 cpm (mean)$\pm$1,648 cpm (standard error) in (A) and 11,777 cpm$\pm$1,536 cpm in (B) and (C).

3. *Mitogenic activity of water-soluble, enzymatic digests of cell walls*

The cell walls of seven species were digested with either M-1 endo-*N*-acetylmuramidase (glycosidase), L-3 endopeptidase or SALE endopeptidase. On the basis of the modes of action of these enzymes, the endopeptidase digests were assumed to consist of polymerized forms of cell wall subunits linked through a glycan chain, and the glycosidase digests were assumed to consist of either the monomer or dimer form (Katayama et al., 1976; Yokogawa et al., 1975). In general, the endopeptidase digests exhibited stronger mitogenic activity than the glycosidase digests on splenocytes (Fig. 4).

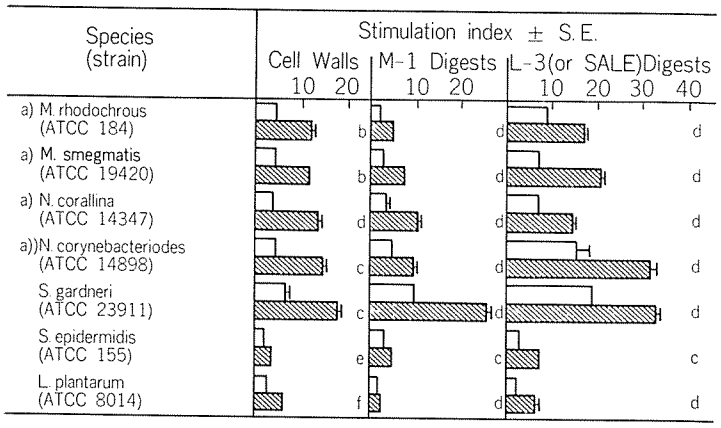

FIGURE 4. Mitogenic activity of water-soluble cell wall digests on splenocytes from male ICR mice. a) Cell walls containing mycolic acids. The mean incorporations of [^{3}H]thymidine per 10^6 cells in control cultures in the five different experiments were 7,330 cpm (b), 3,889 cpm (c), 3,267 cpm (d), 15,049 cpm (e), and 2,244 cpm (f). 100 μg (▨) or 10 μg (□) of test specimen was added to each culcure.

TABLE 1. *Mitogenic activity of MDP on splenocytes from ICR mice*

Experimental number	Stimulation index±S.E. (Mean [^{3}H]thymidine uptake in control cultures, cpm)	Dose[b] exhibiting maximum stimulation
1	3.35±0.06 (2,686)	100
2	3.03±0.47 (2,766)	100
3	2.62±0.29 (8,136)	10
4	2.55±0.39 (33,233)	10
5[a]	2.53±0.18 (2,693)	100
6	2.49±0.21 (13.257)	10
7	2.46±0.09 (3,420)	10
8	2.42±0.22 (1,914)	10
9[a]	2.35±0.12 (3,047)	100
10	2.24±0.32 (1,406)	100
11	2.14±0.19 (7,509)	100
12	2.07±0.11 (7,330)	100
13	2.04±0.35 (3,936)	10
14	1.99±0.28 (3,075)	100
15	1.97±0.10 (12,479)	100
16	1.89±0.12 (734)	10
17	1.72±0.07 (6,665)	10
18	1.69±0.22 (13,402)	100
19	1.67±0.17 (38,252)	100
Mean	2.27±0.44	

[a] Spleen cells obtained from an individual ICR mouse were used in the determination to avoid possible allogeneic stimulation.

[b] Dose (μg) of MDP added to the cultures exerted the maximal stimulating effect. Various doses (0.1, 1.0, 10, and 100 μg) of MDP were used in this assay.

4. *Mitogenic effect of synthetic MDP*

We attempted to confirm our previous finding that MDP exhibited mitogenic effect on splenocytes of ICR mice as well as guinea pigs (Takada et al., 1977). Table 1 shows that splenocytes of ICR mice incubated with MDP show, on the average, 2 to 3 times higher incorporation of [^{3}H]thymidine than control splenocytes incubated without MDP, though some variation in the activity was observed.

DISCUSSION

Among the cell walls tested, all those capable of immunopotentiation, irrespective of their mycolic acid content, exhibited definite mitogenic activity on splenocytes from ICR mice. Among the cell walls lacking immunoadjuvancy by themselves, on the other hand, those of *M. lysodeikticus* lacked mitogenicity, but those of *S. epidermidis* and *C. poinsettiae* showed definite mitogenicity. The lack of mitogenicity of *M. lysodeikticus* cell walls was in accord with the report by Damais et al. (1975). Cell walls containing mycolic acid from mycobacteria and nocardia in general showed stronger mitogenic activity than cell wall of other gram-positive bacteria. But the finding that *S. gardneri* cell walls, which contain no mycolic acids, had the strongest mitogenic effect of all the cell walls tested, and the observation that peptidoglycans from three bacterial species had stronger mitogenicity than the corresponding cell walls, suggest that the non-peptidoglycan moieties of bacterial cell walls, including mycolic acids, were not necessarily required for manifestation of mitogenic activity on splenocytes from ICR mice. It is pertinent to add here that the above statement does not exclude the possibility that mycolic acids themselves have mitogenic activity as reported by Azuma et al. (1977) and also allow peptidoglycans to exert their mitogenicity more effectively.

The present study clearly showed that the mitogenic activity of bacterial cell walls was not lost on solubilization of the walls with peptidoglycan-degrading enzymes. Polymerized forms of cell wall subunits (endopeptidase digests) had more mitogenic activity than monomer or dimer forms (glycosidase digests). This finding seems to be consist with the report of Ciorbaru et al. (1976) that a *Streptomyces albus* G endopeptidase digest of peptidoglycan of *Nocardia rubra* was definitely mitogenic on murine splenocytes, but that a lysozyme (glycosidase) digest had no mitogenicity in their assay.

The conclusions reported in the previous paper (Takada et al., 1977) were that synthetic MDP stimulated the incorporation of [^{3}H] thymidine by splenocytes from ICR mice and that MDP is the minimal structure for the mitogenic activities of bacterial cell walls on splenocytes from ICR mice, as observed with guinea pig splenocytes (Takada et al., 1979b). These conclusions, however, do not seem to agree with reports from other laboratories, that MDP has no mitogenicity (Azuma et al., 1976a; Specter et al., 1977; 1978; Watson and Whitlock, 1978). The discrepancy is probably attributable to differences in the responsiveness of the mouse strains used, because in preliminary studies we found that splenocytes from BALB/c, C3H/He, and C57BL/6J mice did not respond significantly to MDP (unpublished data).

Another observation worthy of discussion is that water-soluble cell wall digests of bacteria of the order *Actinomycetales* in general had stronger mitogenic effects than those of other bacteria. There may be some mitogen(s) other than common peptidoglycan components in cell walls with very strong mitogenicity, and further studies are needed to clarify this point. Our finding may be relevent to the observation of French investigators (Bona et al., 1974a; 1974b; Ciorbaru et al., 1975) that some fractions obtained from lysozyme digests of whole cells of nocardia (NWSM) exerted very strong mitogenicity on splenocytes. These workers suggested that a mitogenic principle derived from membrane components might be involved in the strong mitogenicity of NWSM (Ciorbaru et al., 1976).

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