Macrophage Activation by Bacterial Cell Walls and Related Synthetic Compounds

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Activation of peritoneal macrophages from guinea pigs by various bacterial cell walls, M-1 endo-N-acetylmuramidase enzymatically digested bacterial cell walls and synthetic muramyl dipeptides was studied in terms of stimulation of [14C]glucosamine incorporation. All test bacterial cell wall preparations significantly increased a [14C]glucosamine uptake by the macrophages. Some of the water-soluble M-1 enzyme digests also exerted stimulating effects on macrophages, although the activity of the digests was found to be weaker than those of original cell walls. Furthermore, an adjuvant-active synthetic MurNAC-L-Ala-D-isoGln (MDP) showed a weak but significant activity, whereas an adjuvant-inactive analog, MurNAC-L-Ala-L-isoGln, did not show a significant activity, at least with the dose of 100 µg. Additional studies with 6-O-acyl derivatives of MDP revealed that 6-O-(2-tetradecylhexadecanoyl)-MDP and 6-O-(3-hydroxy-2-tetradecyl-octadecanoyl)-MDP exhibit stronger macrophage-stimulating effects than MDP. It can be concluded from the above findings that MDP is the essential structure responsible for stimulating the activity of cell walls on guinea pig peritoneal macrophages, but it requires a particle state, which results from an additive character of lipophilicity, to exert the activity fully and effectively.

Stimulation of radiolabeled glucosamine incorporation by macrophages as one expression of macrophage activation was first shown by Hammond and Dvorak (8, 9) on the reaction of peritoneal macrophages from antigen-primed guinea pigs with a specific antigen in vitro. This study preceded the finding that plant lectins, concanavalin A, and phytohemagglutinin stimulated macrophages from nonimmunized animals to increase glucosamine uptake (9) and that BCG cell walls had a similar activity (11).

In this study, we investigated the stimulating effects of bacterial cell walls and related compounds, either enzymatically obtained or synthetic, upon peritoneal macrophages from guinea pigs in terms of elevation of glucosamine uptake. The study was undertaken to determine the chemical structure and physical state required for the macrophage activation as determined by the increase of glucosamine incorporation.

MATERIALS AND METHODS

Cell wall preparations. Cells as a starting material for isolation of the walls were cultured under the following conditions. Mycobacterium smegmatis ATCC 19420 was grown as a pellicle on 250 ml of Sauton medium in Roux flasks at 30°C for 14 days, Nocardia corynebacteriodes ATCC 14898 was grown in a medium (pH 7.2) containing 1% maltose, 1% meat extract, 1% polypeptone, 0.5% NaCl, and the deforming agents (0.2% silicone KM-70 and 0.01% Karaline 102). Streptomyces gardneri ATCC 23911 was grown with aeration in a medium (pH 7.2) consisting of 1% glucose, 0.5% polypeptone, 0.3% malt extract, and the above deforming agents. Cultivations of N. corynebacteriodes and S. gardneri were made in 20-liter Jar fermentors containing 10-liter portions of the above medium by incubating each 100 ml of a seed culture at 30°C for 3 days. Cultivation of Lactobacillus plantarum ATCC 8014 was carried out without aeration in 10-liter portions of a medium (pH 7.4) composed of 2% glucose, 1% polypeptone, 1% meat extract, 0.5% NaCl, 0.2% yeast extract, 1% sodium acetate, and 10-4 M MnSO4 at 37°C for 2 days.

Harvested and washed cells were suspended at a concentration of 1 g (wet weight) in 15 ml of water. The cells in suspension were subjected to vigorous agitation by passing through a 600-ml container, to which was added 500 ml of glass beads (0.1-mm diameter), and the suspension was rotated at 3,000 rpm under cooling in a DYNO-Mill type KDL (Willy A,
Bachofen Manufacturing Engineers, Basel, Switzerland) at a flow rate of 80 ml/min. The disrupted cell suspension was diluted two times with distilled water; after the glass beads were removed, the suspension was subjected to differential centrifugation to yield crude cell walls. The crude cell walls thus obtained were thoroughly washed with 1 M NaCl solution and water successively and then digested fully with trypsin (2,000 U/g; E. Merck, Darmstadt, West Germany). The trypsin-digested walls were lyophilized after thorough washing with water. The cells of *N. corynebacterioides* were then submitted to delipidation by successive extraction with acetone, ethanol-ether (1:1, vol/vol), and chloroform at room temperature. The delipidated walls were resuspended in acetone and dried. Isolated cell wall preparations were checked by electron microscopy and by determination of ultraviolet absorption at 260 and 290 nm for detection and estimation of contaminated proteins and nucleic acids. *Corynebacterium diphtheriae* Park-Williams no. 8 walls were obtained as reported previously (12, 19).

**Enzymatic digests of cell walls.** The method of preparation and properties of the M-1 endo-N-acetylmuramidase used here were reported elsewhere (27).

Cell walls of *N. corynebacterioides* (1,800 mg) were incubated with the M-1 enzyme (36 mg) in 180 ml of 0.01 M sodium acetate buffer, pH 5.9, for 4 days at 37°C. The reaction mixture was then centrifuged at 100,000 × g for 1 h to remove an insoluble residue (51% of the original walls). The concentrate was recentrifuged at 100,000 × g for 1 h to provide a resulting insoluble fraction (3.4%). The supernatant fluid obtained was applied on Sephadex G50 and G25 connecting columns equilibrated with water and eluted with water. Two fractions were separated on the basis of determinations of eluted fractions on total hexosamine, total amino groups, reducing groups, pentose, and hexose. They were named NCCM-1 (18.7%) and NCCM-2 (10.8%), respectively. The chemical analyses showed that NCCM-1 was a complex of degraded peptidoglycan moiety and many acidoic acids-arabinogalactan, containing approximately 1% mycolic acids (I. Yano, unpublished data). On the other hand, NCCM-2 was composed mainly of degraded peptidoglycan.

*S. gardneri* cell walls (3,000 mg) were similarly solubilized with 60 mg of M-1 enzyme. The supernatant fluid obtained by two successive centrifugations (the insoluble residues were 31.9 and 10.2%, respectively) was subjected to the gel filtration described above, giving only one broad fraction, in terms of total hexosamine, total amino groups, reducing groups, pentose, and hexose determinations. This fraction (SGCM) was found to be a complex of degraded peptidoglycan moiety and unidentified special structures consisting of organic phosphorous, galactosamine, hexose, pentose, and methyl pentose. The detailed analytical data of NCCM-1, NCCM-2, and SGCM will be reported elsewhere.

**Synthetic muramyl dipeptides and 6-O-acetyl muramyl dipeptides.** Synthesis of an immunoadjuvant-active MurNAc-L-Ala-D-isoGln (MPD), was described elsewhere (18). For the preparation of 6-O-acetyl muramyl dipeptide, two different synthetic routes were used depending on the nature of the acyl groups as reported previously (15-17). Thus, 6-O-stearoyl- and 6-O-(2-tetradecylhexadecanoyl)-MDP ([L18]-MDP and [B30]-MDP, respectively) were prepared by selective acylation of the 6-hydroxyl group in 1-O-benzyl-MDP benzyl ester with corresponding acid chlorides followed by hydrolytolytic deprotection. On the other hand, introduction of triantennary and 6-hydroxy-3-tetradecyloctadecanoyl groups was performed by means of substitution reaction of 6-O-tosyl groups in 1-O-benzyl-6-O-tosyl-N-acetylmuramic acid diphenylmethyl ester with potassium salts of the corresponding acids. Selective removal of the diphenylmethyl group in the resulting 6-O-acetyl derivatives followed by coupling with L-alamyl-d-isoglutamine benzyl ester and final hydrolytolytic deprotection resulted in 6-O-triantennaryl-MDP ([L30]-MDP) and 6-O-(3-hydroxy-2-tetradecyloctadecanoyl)-MDP ([BH32]-MDP). All 6-O-acetyl-MDP specimens thus obtained showed a single spot on thin-layer chromatography and satisfactory elemental analyses. The molecular formulas and solubility in water of these synthetic preparations are shown in Table 1.

**Animals.** Randomly bred female albino guinea pigs weighing 300 to 500 g were used.

**Macrophages.** A guinea pig was injected intraperitoneally with 50 ml of sterile thioglycollate medium (Nissui Seiyaku Co., Tokyo, Japan). After 3 days, the peritoneal cavity of the guinea pig was washed with 100 ml of Hanks balanced salt solution (HBSS; Nissui Seiyaku Co., Tokyo, Japan) to extract exudates. The peritoneal wash was centrifuged at 150 × g for 10 min at 4°C to collect exudate cells. The cells deposited were then washed three times with HBSS. Washed cells were resuspended in a complete medium composed of RPMI 1640 medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 15% fetal bovine serum (Flow Laboratories, Rockville, Md.) and antibiotics (100 U of penicillin and 100 μg of streptomycin per ml) to give 1.0 × 10⁶ cells per ml of medium. One milliliter portions of the cell suspension in the medium were distributed to each well of a plastic culture tray (24 flat-bottom wells, ca. 1.7 by 1.6 cm; Linbro Scientific, Inc., Subsidiary of Flow Laboratories, Inc., Hamden, Conn.) and incubated in an atmosphere of 96% air–5% CO₃ at 37°C. After 2 h, the cells of the wells were washed three times with HBSS to remove nonadherent cells. The monolayer consisting almost exclusively of macrophage (at least 98% pure on the basis of a microscopic check) was thus prepared.

**Cell culture.** Each well containing a macrophage monolayer was added with 1 ml of the complete medium containing test materials, and the macrophages were cultured in an atmosphere of 95% air–5% CO₂ at 37°C for 72 h. Duplicate to quintuplicate cultures were performed.

**Glucosamine incorporation.** During the final 8 h of the above cell cultures, macrophages in each well were pulsed with 0.25 μCi of D-[¹⁴C]glucosamine (specific activity, 60.8 mCi/mmol, Radiochemical Centre, Amersham, England). Macrophage monolayers were then washed three times with warm HBSS and then incubated with 0.5 ml of 4% sodium lauryl sulfate at 60°C for 30 min. The cells were removed from the wells and dispersed into the suspension. The suspensions were transferred to scintillation vials and added with 11 ml of scintillation fluid containing 600 ml of

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Table 1. Chemical structure and solubility in water of [L18]-MDP, [L30]-MDP, [B30]-MDP, and [BH32]-MDP

<table>
<thead>
<tr>
<th>6-O-acyl-MDP</th>
<th>Chemical structure of acyl group</th>
<th>Solubility in water*</th>
</tr>
</thead>
<tbody>
<tr>
<td>[L18]-MDP</td>
<td>CH₅(CH₂)₆CO—</td>
<td>Nearly soluble; in a concentrated solution, gives a micelle</td>
</tr>
<tr>
<td>[L30]-MDP</td>
<td>CH₅(CH₂)₅CO—</td>
<td>Insoluble; gives a less coarse suspension</td>
</tr>
<tr>
<td>[B30]-MDP</td>
<td>CH₅(CH₂)₁₃</td>
<td>Insoluble; gives a coarse suspension</td>
</tr>
<tr>
<td></td>
<td>CH₅(CH₂)₁₃, CH(OH)</td>
<td>Insoluble; gives a fine suspension</td>
</tr>
<tr>
<td>[BH32]-MDP</td>
<td>CH₅(CH₂)₁₃</td>
<td></td>
</tr>
</tbody>
</table>

*6-O-acyl-MDP specimens were added with RPMI 1640 medium at a concentration of a 1-mg equivalent of MDP/ml and the mixtures were sonicated.

xylene, 400 ml of Nonion NS-210 (Nihonyushi Inc., Tokyo, Japan), 100 ml of 10% ethanol, 4 g of PPO (2,5-diphenyloxazol), and 0.1 g of POP (1,4-bis-[2]-[5-phenyloxazolyl]benzene) in a total volume of 1,100 ml. Radioactivity was measured in a liquid scintillation spectrometer (ALOKA LSC-673, Alok Co., Tokyo, Japan). The representative results of a series of assays are shown.

RESULTS

Increased glucosamine incorporation of macrophages incubated with cell walls. Figure 1 shows that all of the test cell walls significantly stimulated glucosamine incorporation of peritoneal macrophages from a guinea pig. Among the cell walls containing mycolic acids as one of the components of non-peptidoglycan moiety, those from M. smegmatis and N. corynebacterioides (the mycolic acid contents were 35 and 3%, respectively) exerted remarkable stimulating effects, but the cell walls from C. diphtheriae, which are known to contain corynomycolic acids, showed only weak activity. On the other hand, the walls from S. gardneri and L. plantarum, both of which contained no mycolic acids, exhibited rather strong activity. These results suggested that bacterial cell walls, either mycolic acid containing or mycolic acid free, have macrophage-stimulating activity in terms of increasing glucosamine incorporation.

Macrophage-stimulating activity of enzymatic digests of the cell walls from N. corynebacterioides and S. gardneri. Both water-soluble fractions obtained from the M-1 endo-N-acetyluramidase digests of N. corynebacterioides cell walls, including NCCM-2 containing no mycolic acids, showed weak but definite stimulating activity, whereas a water-soluble fraction from the M-1 digests of S. gardneri cell walls had no stimulating activity on macrophages (Fig. 2). The findings suggest that cell wall components can stimulate glucosamine uptake of macrophages, even in a water-soluble form, but they need a particle state to exert the stimulating effects fully and effectively.

Macrophage-stimulating effects of synthetic muramylpeptide derivatives. Figure 3 shows that the adjuvant-active MDP exhibits weak but significant stimulating effects on glucosamine incorporation of guinea pig peritoneal macrophages, but adjuvant-inactive analog MurNAc-L-Ala-L-isoGln lacked the activity. Figure 4 further indicates that [B30]-MDP and

![Fig. 1. Stimulation of glucosamine incorporation of peritoneal macrophages from a female guinea pig by various bacterial cell walls. (A) C. diphtheriae Park-Williams no. 8; (B) M. smegmatis ATCC 19420; (C) N. corynebacterioides ATCC 14898; (D) S. gardneri ATCC 23911; and (E) L. plantarum ATCC 8014. Results are the mean of triplicate cultures ± standard error. The mean of the [¹⁴C]glucosamine incorporation in control cultures was 341.6 ± 18.2 dpm.](http://iai.asm.org/)

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Macrophage Activation by [B30]-MDP

**Figure 2.** Stimulation of glucosamine incorporation of peritoneal macrophages from a female guinea pig by water-soluble fractions from the M-1 endo-N-acetyl-muramidase digests of N. corynebacterioides and S. gardneri cell walls. (A) Cell walls of N. corynebacterioides; (B) NCCM-1; (C) NCCM-2; (D) cell walls of S. gardneri; and (E) SGCM. Results are the mean of duplicate cultures ± standard error. The mean of [14C]glucosamine incorporation in control cultures was 371.9 ± 9.1 dpm in A, B, and C, and 125.8 ± 49.4 dpm in D and E.

**Figure 3.** Stimulation of glucosamine incorporation of peritoneal macrophages from a female guinea pig by MDP (●) and its adjuvant-inactive analog, MurNAC-l-Ala-L-isoGln (○). The results are the mean of quintuplicate cultures ± standard error. The mean of [14C]glucosamine incorporation in control cultures was 265.2 ± 44.3 dpm. The difference between the counts in the test and the control cultures was significant at a level of 10% (*) or 5% (**) by the Student t test.

[BH32]-MDP exerted significantly stronger stimulating activity than MDP; however, the stimulating effects of [L18]-MDP and [L30]-MDP were not found to be different from those of MDP, although with [L18]-MDP, the high dose led to inhibitory effects. These results suggest that the physical state is one of the factors in expression of macrophage-activating activity of bacterial cell wall components, in view of the fact that 6-O-acyl-MDPs showed various hydrophobic characteristics, as shown in Table 1.

**DISCUSSION**

Kelly reported that BCG cell walls induced in vitro activation of peritoneal macrophage from nonspecifically sensitized guinea pigs, as determined by an increase in glucosamine incorporation (11). In the present study, we found that glucosamine incorporation of guinea pig peritoneal macrophages was stimulated by bacterial cell walls regardless of their mycolic acids content and that some water-soluble fractions obtained by using the endo-N-acetyl muramidase exerted weak, but definitive stimulating effects.

Our study further showed that even synthetic MDP, the minimal structure responsible for the immunoadjuvancy of bacterial cell walls (2, 3, 6, 13, 14, 23, 26) could stimulate macrophage definitely, whereas its adjuvant-inactive stereoisomer, MurNAC-l-Ala-L-isoGln, could not. This finding is in accord with the studies of Tanaka et al. (22), Yamamoto et al. (25), Adam et al. (1), and Nagao et al. (20; S. Nagao, A. Tanaka, Y. Tamamoto, T. Koga, K. Shiba, S. Kotani. Infect. Immun. in press), which showed that MDP activated the reticuloendothelial system of mice in vivo and inhibited the migration of peritoneal macrophages of guinea pigs in vitro as the expressions of macrophage activation. More recently, Tanaka et al. reported that the macrophages induced into guinea pig peritoneal cavities by mineral oil injection, when cultured with MDP, showed stronger adherence and spreading onto a plastic surface than those cultured with-

In 1975, Juy and Chedid (10) reported that MDP could activate murine macrophages in vitro to inhibit the growth of tumor target cells. Another study showing that macrophages are the target cells toward which MDP exerts its immunopotentiating activity was reported by Fevrier et al., who presented evidence that factors liberated from macrophages under the action of MDP ultimately acted on B cells through T-cell mediation (7). In addition, Dinarello et al. (5) showed that MDP and its analogs release leukocytic pyrogen from either rabbit or human phagocytic cells in vitro. More recently, Togawa et al. (Abstr. Fed. Proc. Fed. Am. Soc. Exp. Biol., Abstr. 1589, 1978) reported that MDP stimulated human monocytes to produce lymphocyte-activating factor, which increased the responsiveness of T lymphocytes to mitogens. On the other hand, we reported previously that MDP and the adjuvant-active analog, MurNAc-L-Ala-D-Glu, stimulated splenocytes of guinea pigs and ICR mice, whereas the adjuvant-inactive analogs did not (21). Recently, a similar finding was reported by Damais et al. (4) on splenocytes of DBA/2 mice, indicating that the presence of 2-mercaptoethanol in the assay system raised the stimulation. Furthermore, the cell wall preparations and water-soluble fractions of some of them examined here were found to be strong mitogens for splenocytes (and thymocytes in the case of cell walls themselves) of guinea pigs (H. Takada, M. Tsujimoto, I. Morisaki, T. Okunaga, K. Kato, S. Kotani, S. Kusumoto, K. Inage, T. Shiba, S. Kawata, K. Yokogawa, S. Nagao, and I. Yano, Abstr. Annu. Meet. Jpn. Soc. Immunol., Abstr. no. 1755, 1978). Therefore, there is some possibility that the macrophage activation observed in this study may be mediated by lymphocytes contaminating macrophage preparations. Actually, bacterial lipopolysaccharides stimulate macrophages to increase glucosamine incorporation only when macrophages are cultured with lymphocytes (24), and BCG cell walls have also been found to exert their macrophage-stimulating activity effectively under the coexistence of lymphocytes (11). However, the fact that SGCM, a strong mitogen for guinea pig splenocytes, could not stimulate macrophage at all clearly indicates that the participation of lymphocytes in macrophage stimulation may be excluded in our assay system.

As mentioned above, MDP was found to enhance adhering and spreading of guinea pig macrophages. Thus, the increase in glucosamine incorporation might be only a result due to the difference in macrophage number between cultures with stimulants and those without stimulants. In our assay, however, the difference in macrophage numbers between control and test cultures was not significant to explain the difference in glucosamine incorporation between them.


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LITERATURE CITED