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Induction of Interleukin 1 Secretion by Adjuvant-Active Peptidoglycans

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The ability of differently structured, purified peptidoglycans (PG) to induce interleukin 1 (IL1) secretion was compared. PG from *Bacillus megaterium* and *Staphylococcus aureus* stimulated the production of IL1 by mouse peritoneal macrophages and human adherent mononuclear cells, whereas PG from *Micrococcus lysodeikticus* and *Corynebacterium poinsettiae* were inactive. There was a correlation between the ability of PG to induce IL1 secretion and previously demonstrated immunoenhancing activities (adjuvant effect, increase of resistance to tumor growth) of PG. PG solubilization by lysozyme decreased but did not abolish the PG effect on IL1 secretion. Active PG induced IL1 production in nude mice and in the C3H/HeJ strain (which is unresponsive to lipopolysaccharides).

Bacterial peptidoglycans (PG) are polymeric cell wall components which possess various immunomodulating properties (21). They exert an adjuvant effect on humoral and cell-mediated immune responses (13), and they can increase resistance to tumor growth (8, 15, 18). Monomeric PG subunits are adjuvant active (14), as are synthetic derivatives of these subunits (2, 5). Bacterial PG are made of glycan strands cross-linked through short peptides (19). The amino acid sequence of the peptide subunit may vary within gram-positive species. Previous studies have shown that there was a correlation between the peptide structure of the PG and their immunomodulating activities. PG from *Bacillus megaterium* and *Staphylococcus aureus* exhibit immunomodulating properties, whereas PG from *Micrococcus lysodeikticus* and *Corynebacterium poinsettiae* are inactive (8, 9). The mechanism of action of bacterial PG is still incompletely understood. PG are able to induce B cell activation (3, 4, 9) and affect several macrophage functions (10, 15, 22). It is also known that various bacterial immunomodulators such as lipopolysaccharide (LPS) and several mycobacterial extracts (6, 17, 23) can induce macrophages to produce interleukin 1 (IL1). This monokine plays an essential role in the immune response by activating T cells (12, 16). Therefore, we have investigated whether PG can induce IL1 production by macrophages. Our results indicate that only adjuvant-active PG can induce IL1 production by mouse macrophages and human mononuclear cells.

MATERIALS AND METHODS

Animals. Female DBA/2 mice, 8 to 12 weeks old, were obtained from Centre de selection et d'élevage d'animaux de laboratoire (Orléans, France). Male athymic (nude) mice (C57BL/6 background), 6 to 8 weeks old, were obtained from the Institut National de Recherches Scientifiques sur le Cancer (Villejuif, France). Male C3H/HeJ mice, 8 to 10 weeks old, were obtained from Institut Pasteur (Paris, France).

Peptidoglycans. The following bacterial strains were used: *Bacillus megaterium* ATCC 14581, *Staphylococcus aureus* Copenhagen, *Micrococcus lysodeikticus* A-270 from Institut Pasteur and *Corynebacterium poinsettiae* NCPP 846. The PG were purified as previously described (9). Their peptide subunit structures are reported in Table 1. Before use, the PG were suspended in saline and heated in a boiling water bath for 5 min. Solubilization of the PG from *B. megaterium* and *M. lysodeikticus* was attained by lysozyme digestion. A suspension of 5 mg of PG per ml was incubated for 24 h at 37°C with 50 μg of egg white lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml in 0.05 M ammonium acetate buffer, pH 6.2. The insoluble residue was eliminated by centrifugation at 35,000 × *g* for 30 min.

Other reagents. Phenol-water-extracted *Escherichia coli* LPS was purchased from Difco Laboratories (Detroit, Mich.); concanavalin A (ConA) was purchased from Pharmacia Fine Chemicals, (Bois d'Arcy, France).

Mouse macrophage cultures. Peritoneal exudate cells were harvested from mice injected 3 days earlier with 1.5 ml of thioglycolate broth (Institut Pasteur). The mice were killed, and the peritoneal cells were collected by washing the peritoneal cavity with 5 ml of Hanks balanced saline solution (HBSS) containing 1% heat-inactivated fetal calf serum (FCS), washed once, and suspended to a density of 0.6 × 10^6 cells per ml in

TABLE 1. Structure of the peptide subunit of the PG used in this study

Bacterium	Peptide sequence
B. megaterium	L-Ala–D-Glu — *m*-A_2pm–D-Ala[a]
S. aureus	L-Ala–D-Glu–NH_2 — L-Lys–D-Ala
M. lysodeikticus	L-Ala–D-Glu–Gly — L-Lys–D-Ala
C. poinsettiae	Gly–D-Glu — L-Hsr–D-Ala

[a] *m*-A_2pm, *meso*-Diaminopimelic acid.

RPMI 1640 medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO Laboratories, Grand Island, N.Y.) with 100 U of penicillin per ml, 10 μg of gentamicin per ml and 5% FCS. The cells were plated in 35-mm petri dishes (BD Labware, Oxnard, Calif.) at 37°C in an atmosphere of 5% CO_2–95% air for 2 h and washed three times with HBSS containing 1% FCS. The adherent cells were incubated in serum-free culture media with or without stimulant for 24 h. Supernatants were collected, centrifuged for 10 min at 1,000 × *g*, and immediately tested or frozen at −20°C until use. The adherent cells consisted of more than 95% macrophages as shown by esterase staining (24).

Human mononuclear cell cultures. Heparinized venous blood was obtained from normal volunteers. The blood was diluted 1:2 with HBSS and centrifuged on Ficoll-Hypaque gradients by the method of Böyum (1). The mononuclear leukocyte (MNL) layer was recovered, washed, and adjusted to a cell density of 5×10^6 MNI per ml in RPMI 1640 containing 25 mM HEPES buffer, antibiotics, and 5% heat-inactivated autologous human serum. Adherent MNL cultures were prepared by incubating 1 ml of this cell suspension in petri dishes for 2 h. The nonadherent cells were removed by three washings with HBSS containing 1% heat-inactivated autologous human serum. The adherent MNL were then incubated for 24 h with or without stimulant, and the supernatants were collected as described above.

Thymocyte proliferation assay. IL1 activity in culture supernatants was assayed by measuring the proliferative response of mouse thymocytes as described by Oppenheim et al. (17). Thymocytes from C3H/HeJ mice were suspended to a density of 1.5×10^7 cells per ml in RPMI 1640–HEPES containing 10% FCS and antibiotics. The cell suspension was seeded under a volume of 0.1 ml into the well of tissue culture plates (Microtest II; BD Labware), and an equal volume of various dilutions of macrophage culture supernatants was added. Cultures were carried out with or without the addition of a submitogenic concentration of ConA (1 μg/ml) (16) and incubated in a humidified CO_2 incubator for 72 h. They were pulsed for the final 6-h

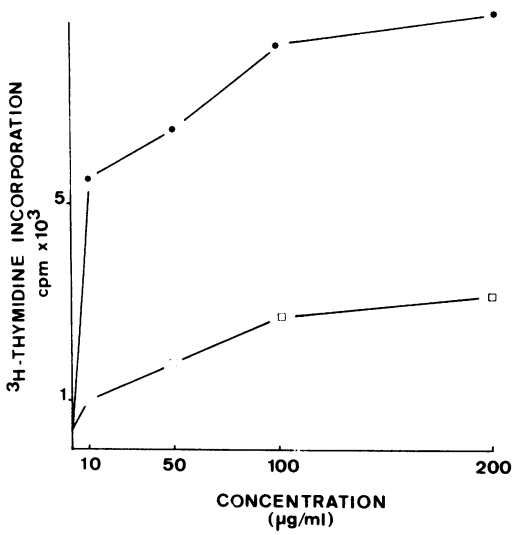

FIG. 1. IL1 activity in culture supernatants of mouse macrophages stimulated with various concentrations of PG from *B. megaterium*. Thymocytes were cultured in the presence of a 1:4 dilution of macrophage culture supernatants with (●) or without (□) ConA.

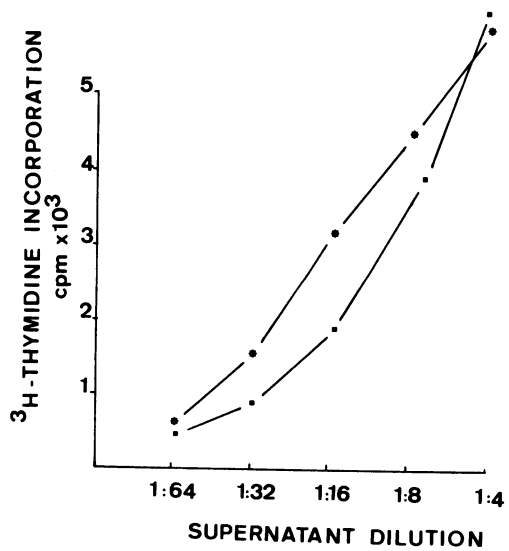

FIG. 2. IL1 activity in various dilutions of culture supernatants of macrophages stimulated by 200 μg of PG from *B. megaterium* per ml (●) or by 25 μg of LPS per ml (■). Thymocytes were cultured in the presence of ConA (1 μg/ml).

culture period with 0.5 μCi of [^{3}H]thymidine (specific activity, 1 Ci/mM). Cells were then harvested onto glass fiber filters with an automatic harvester (Dynatech Laboratories, Alexandria, Va.). Filter disks were dried, and the radioactivity was counted by liquid scintillation spectrophotometry. Results were expressed as the mean counts per minute ± the standard error of the mean (SEM) of quadruplicate samples.

RESULTS

Induction of IL1 secretion by *B. megaterium* PG in normal and nude mice. PG from *B. megaterium* was able to induce mouse peritoneal cells to produce IL1 activity. Optimal response was obtained by a concentration of about 100 μg/ml. Macrophage culture supernatants were directly mitogenic and enhanced the stimulation of C3H/HeJ mice thymocytes by a submitogenic concentration of ConA (1 μg/ml) (Fig. 1).

Supernatants obtained from macrophage cultures stimulated by PG from *B. megaterium* (200 μg/ml) were tested at different dilutions. Significant activity could yet be detected with the dilution 1:32 (Fig. 2) and was similar to that induced by LPS.

To rule out the involvement of contaminating T cell products (i.e., interleukin 2) in the mitogenic effect of macrophage culture supernatants, experiments with athymic nude mice were carried out. These mice are known to be unable to produce interleukin 2 (7). PG from *B. megaterium* induced IL1 production by nude mouse macrophages (Table 2).

Comparative effect of various PG on IL1 production. We compared the ability of differently structured purified PG to induce cultured mouse macrophages to produce IL1 activity. At 100 μg/ml, the activity of PG from *S. aureus* was similar to that of PG from *B. megaterium*, but at 10 μg/ml, the latter was more potent. No IL1 production was observed when PG were used at 1 μg/ml. PG from *M. lysodeikticus* and *C. poinsettiae* were inactive between 1 and 100 μg/ml. Only a slight production was obtained with 200 μg/ml (Fig. 3). None of the PG had any direct stimulatory effect on the murine C3H/HeJ thymocytes.

TABLE 2. Induction of IL1 secretion by PG in nu/nu mice

Stimulant added to macrophages	Concn (μg/ml)	[^{3}H]thymidine incorporation[a]	
		ConA (1 μg/ml)	
		−	+
None		93 ± 9	187 ± 24
PG from *B. megaterium*	100	836 ± 16	2,113 ± 77
PG from *M. lysodeikticus*	200	236 ± 74	465 ± 115
LPS	25	643 ± 107	2,544 ± 417

[a] Mean (±SEM) counts per minute incorporated by C3H/HeJ thymocytes. Thymocytes were incubated with a 1:4 dilution of supernatants of macrophage cultures from nu/nu mice stimulated with the indicated compounds.

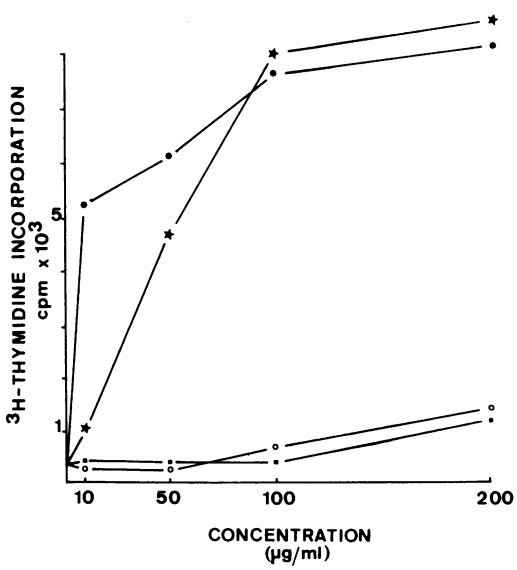

FIG. 3. IL1 activity in supernatants from macrophages stimulated with PG of different structures. Macrophages were cultured with the indicated concentrations of PG from *B. megaterium* (●), *S. aureus* (★), *C. poinsettiae* (■), *M. lysodeikticus* (○). Supernatants were used at a 1:4 dilution in the presence of ConA (1 μg/ml). Control thymocyte cultures incubated with the different PG (100 μg/ml) incorporated, respectively, 258 ± 10, 300 ± 88, 80 ± 22, and 181 ± 22 cpm.

Native PG are polymeric, insoluble materials. Lysozyme digestion produces oligomeric and monomeric soluble subunits (19). Lysozyme digestion of PG from *B. megaterium* decreased the ability of PG to induce IL1 secretion (Fig. 4). Soluble subunits of PG from *M. lysodeikticus* were totally inactive.

Induction of IL1 production in macrophages from C3H/HeJ mice. Macrophages from C3H/HeJ mice are unresponsive to LPS stimulation (11). To determine whether PG stimulate macrophages in the same way as LPS, we studied the effect of PG on C3H/HeJ macrophages. Results reported (Table 3) show that PG from *B. megaterium* and *S. aureus* at 100 μg/ml induced IL1 production in cultures of C3H/HeJ macrophages.

Induction of IL1 production by adherent human mononuclear leucocytes. Results reported (Table 4) show that PG from *B. megaterium* and *S. aureus* induced adherent human MNL to produce IL1 activity. Obvious activity was de-

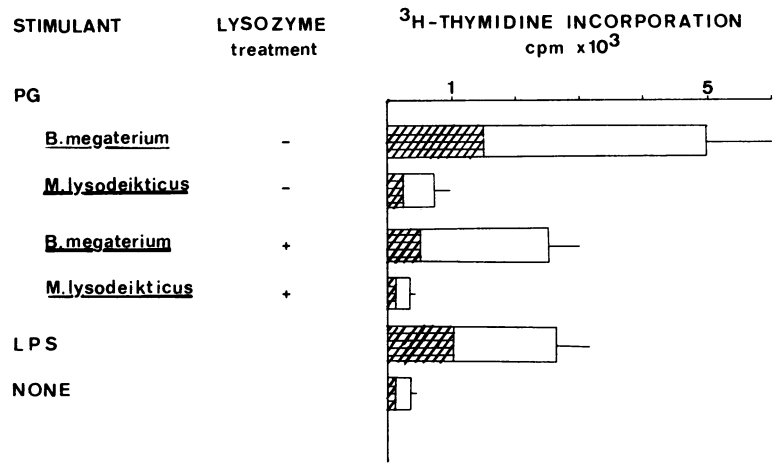

FIG. 4. Effect of lysozyme digestion on PG ability to induce IL1 production. Mouse macrophages were stimulated with lysozyme-solubilized or particulate PG at 100 μg/ml or with LPS (25 μg/ml). Thymocytes were incubated with macrophage culture supernatants at a 1:4 dilution, in the presence (open columns) or in the absence (hatched columns) of ConA. Results are expressed as the mean ± SEM counts per minute incorporated by thymocytes.

tected when human MNL supernatants were used at 1:4 and 1:20 dilutions. PG from *M. lysodeikticus* and from *C. poinsettiae* were found inactive.

DISCUSSION

Data from the present work show that PG extracted from the cell walls of gram-positive bacteria induce IL1 production by mouse macrophages and human MNL. However, this effect is greatly dependent on the chemical structure of the PG. PG from *B. megaterium* and *S. aureus* are potent inducers of IL1 synthesis, whereas those from *M. lysodeikticus* and *C. poinsettiae* have only a weak effect at high concentrations. The comparison of the PG structures showed that active PG possess a peptide sequence beginning with L-alanyl–D-glutamyl residues. In *M. lysodeikticus*, a glycyl residue is bound to the α-carboxyl group of the glutamyl residue. In *C. poinsettiae*, the first amino acid is glycine instead of L-alanine. Moreover, the glutamyl residue is involved in the interpeptide bridge. The critical role of the first two amino acids of the peptide chain was also found in the study of synthetic derivatives of PG (2). The difference in the ability of PG of various structures to induce IL1 secretion showed that this effect is not merely the consequence of the ingestion of particulate material by macrophages. This result suggested rather that IL1 secretion is triggered by a selective interaction between a defined structure of PG and the corresponding target. The mechanism of triggering may be different from the one of LPS, since C3H/HeJ macrophages (which are unresponsive to LPS) produce IL1 after stimulation by PG.

The solubilization of PG by lysozyme digestion decreases its activity, but soluble fragments obtained from active PG are still able to induce IL1 secretion. Soluble synthetic derivatives of PG have also been reported to induce IL1 secretion (17, 23).

An important point is that only PG possessing immunoenhancing effects (adjuvant effect, ability to increase resistance to tumor growth, activation of B lymphocytes) are able to induce IL1 secretion, whereas the PG which do not induce IL1 secretion are also devoid of immunoenhancing effects (8, 9). It is known that various bacterial adjuvants such as LPS (6) and mycobacterial extracts (17, 23) are potent inducers of IL1 secretion. IL1 plays an important role in the

TABLE 3. IL1 secretion induced by PG in C3H/HeJ mice

Stimulant added to macrophages	Concn (μg/ml)	[3H]thymidine incorporation[a]
None		229 ± 104
LPS	25	288 ± 25
B. megaterium PG	100	1,084 ± 64
S. aureus PG	100	1,120 ± 50
M. lysodeikticus PG	100	329 ± 84
C. poinsettiae PG	100	191 ± 149

[a] Mean (±SEM) counts per minute incorporated by C3H/HeJ thymocytes incubated in the presence of 1 μg of ConA per ml and a 1:4 dilution of supernatants of macrophage cultures stimulated with the indicated compounds.

TABLE 4. IL1 activity in supernatants from cultures of adherent human MNL incubated with different PG

PG added to MNL[a]	Dilutions of supernatants	[^{3}H]thymidine incorporation[b]	
		ConA (1 μg/ml)	
		−	+
None	1:4	126 ± 12	114 ± 55
	1:20	296 ± 50	168 ± 44
B. megaterium	1:4	6,479 ± 599	8,993 ± 1,210
	1:20	3,250 ± 528	5,923 ± 764
S. aureus	1:4	2,143 ± 812	3,985 ± 656
	1:20	396 ± 265	2,296 ± 1,029
C. poinsettiae	1:4	249 ± 18	380 ± 142
	1:20	196 ± 28	223 ± 70
M. lysodeikticus	1:4	169 ± 101	202 ± 35
	1:20	161 ± 89	183 ± 53

[a] PG were used at 100 μg/ml.
[b] Mean (±SEM) counts per minute incorporated by C3H/HeJ thymocytes in the presence of supernatants of MNL cultures stimulated with the indicated PG.

initiation of the immune response by activating T cells (12, 16). It has recently been shown that IL1 can exert an adjuvant effect in vivo (20). These findings suggest that one of the major mechanisms of action of bacterial adjuvants could be the stimulation of IL1 synthesis (17). The effect of adjuvant-active PG on IL1 synthesis indicates that in addition to B lymphocytes (3, 4, 9) the macrophage is one of the targets of these compounds. This is in agreement with other reports showing that PG are able to modify various macrophage functions such as migration (10), glucosamine uptake (22), and cytotoxicity (15, 18).

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