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Processing of *Bacillus subtilis* Peptidoglycan by a Mouse Macrophage Cell Line

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It has previously been established that muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) is an effective immunostimulant whose primary target cell type is the macrophage. Muramyl dipeptide is known to be structurally identical to a portion of the monomer unit of peptidoglycan, a nearly ubiquitous component of bacterial cell walls. To establish whether muramyl dipeptide or glycopeptides structurally related to it are formed as a result of macrophage processing of peptidoglycan, *Bacillus subtilis* cell walls radiolabeled in the muramic acid, glucosamine, and alanine residues of the constituent peptidoglycan were incubated in the presence of the cultured macrophage-like cell line RAW264, and the glycopeptides which were released into the medium were fractionated and analyzed. Although muramyl dipeptide was not found in the culture medium, at least three glycopeptides structurally related to it were found, namely GlcNAc-MurNAc-Ala-isoGln-Dap-Ala, GlcNAc-MurNAc-Ala-isoGln-Dap, and GlcNAc-MurNAc-Ala-isoGln.

N-Acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide [MDP]), the minimal adjuvant-active structure of bacterial peptidoglycan (6, 16), is capable of enhancing both cellular and humoral immunity (10, 30). The primary target cell type upon which MDP acts is thought to be the macrophage (7, 15), which is well known for its functions in the phagocytosis of foreign materials, presentation of antigen, and secretion of immunoregulatory molecules such as interleukin-1 (19, 26, 29).

The mechanism by which MDP or any other adjuvant acts is poorly understood. Because MDP structurally represents a fragment of the monomer unit of bacterial peptidoglycan, we hypothesized that macrophages which phagocytize bacteria might partially degrade peptidoglycan and release small glycopeptides such as MDP into the extracellular milieu. If so, this processing of bacterial cell walls would directly lead to the production of signals for amplification of the immune system. To test this hypothesis, we cultured the macrophage-like cell line RAW264 (23) in the presence of *Bacillus subtilis* cell walls that were strategically radiolabeled in the peptidoglycan component, and analyzed the radiolabeled material released by these macrophages. We present evidence which is consistent with the release by RAW264 cells of at least three small glycopeptides derived from the bacterial peptidoglycan.

MATERIALS AND METHODS

Radiolabeled *B. subtilis* cell walls. *B. subtilis* Porton was cultured in Penassay broth (Difco Laboratories) to which either D-[1-^{3}H]glucosamine (17 Ci/mmol, 100 to 500 μCi/liter) or L-[*U*-^{14}C]alanine (150 mCi/mmol, 100 to 500 μCi/liter) was added in the early log phase of growth. After culture for 18 h at 37°C, cells were harvested by centrifugation at 4,000 × *g* for 30 min at 4°C and washed by centrifugation from sterile Hanks balanced salt solution (HBSS), pH 7.2. The bacteria were subsequently killed by autoclaving and were then mechanically disrupted at 40,000 lb/in^2 at 5 to 10°C in a Ribi Sorvall cell fractionator (Ivan Sorvall, Inc.). The resulting slurry was centrifuged, and the pelleted cell walls were washed by centrifugation from HBSS before sterilization by autoclaving. Sterile cell wall pellets were stored at −20°C before use. Several different cell wall preparations were used in this study. Their specific activities varied somewhat, but typical values were 2.16 × 10^4 to 7.64 × 10^4 dpm/mg for walls labeled by growth in the presence of D-[1-^{3}H]glucosamine and 0.16 × 10^5 to 1.07 × 10^5 dpm/mg for walls labeled by growth in the presence of L-[*U*-^{14}C]alanine.

MurNAc pentapeptide. Radiolabeled *N*-acetylmuramyl pentapeptide (MurNAc pentapeptide), a biosynthetic precursor of peptidoglycan, was prepared by a modification of the procedure detailed by Bracha et al. (2). The vancomycin-containing medium used by Bracha et al. was modified by the deletion of all divalent cations and the addition of chloramphenicol (50 μg/ml) and D-[1-^{3}H]glucosamine (17 Ci/mmol). The resultant UDP-MurNAc pentapeptide was purified as previously described (2) and identified by the presence of radiolabel in material which absorbed at 260 nm. Cleavage of the phosphodiester linkage between UDP and MurNAc pentapeptide was accomplished by mild acid hydrolysis (0.1 M HCl for 2 h at 60°C), and the MurNAc pentapeptide was recovered by gel permeation chromatography on a column (2 by 179 cm) of Bio-Gel P-2 (Bio-Rad Laboratories) in 0.1 M acetic acid. Amino acid analysis indicated that muramic acid, alanine, glutamic acid (Glx), and diaminopimelic acid (Dap) were present in molar ratios of 1.00, 3.16, 1.07, and 1.07, respectively (values normalized to muramic acid). In addition, traces of the following components were observed in this preparation (in moles relative to muramic acid): glucosamine (0.04), aspartic acid (Asx) (0.07), serine (0.01), glycine (0.03), unknown at t = 31 min (0.03), and unknown at t = 78 min (0.11). The molar ratios of muramic acid and of the major amino acids are those expected.

Cell culture and endocytosis experiments. RAW264 cells were maintained in pyrogen-free Eagle minimal essential medium (MEM) supplemented with 2.0 mM L-glutamine (GIBCO Laboratories), penicillin G (100 U/ml; GIBCO), streptomycin (100 μg/ml; GIBCO), and 10% fetal bovine serum (Hyclone). Endocytosis experiments were performed in serum-free medium, supplemented as above plus 50 μM 2-

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mercaptoethanol (Eastman Chemical Products, Inc.). Adherent cells were detached by incubation for 5 to 10 min at 37°C in a solution of 2 mM EDTA in divalent cation-free phosphate-buffered saline.

The time course of uptake of *B. subtilis* cell walls by RAW264 macrophages was determined by incubating 2×10^7 RAW264 cells in 20 ml of serum-free medium in the presence of radiolabeled cell walls. Portions of the culture supernatant were removed periodically for scintillation counting. To determine whether opsonization would alter the time course of uptake of cell walls by RAW264 cells, the radiolabeled cell walls (21 mg) were first incubated at 37°C for 30 min in 5 ml of human serum from a healthy donor. After centrifugation, the pellet was used as the inoculum in the time-course assay described above. The supernatant from the opsonization was assayed for the presence of C5a based on the chemotactic response it engendered in polymorphonuclear leukocytes in the laboratory of Robert D. Nelson (Department of Surgery, University of Minnesota, Minneapolis) (18). This assay established that active chemotactic proteins were present in the supernatant from the opsonization and that opsonization of the cell walls had therefore occurred.

Preparative endocytosis experiments were carried out in the multilevel culture vessels (cell factories; GIBCO) since fairly large quantities of material were required for subsequent purification. Vessels with 6,000 cm^2 of surface area were inoculated with 10^9 RAW264 cells in 1 liter of serum-containing MEM, and after a 2-day period to allow for cell attachment and reproduction (cell doubling time was ca. 13 h), the medium was decanted. Adherent cells were rinsed with warm HBSS, and serum-free MEM (1 liter) which contained ca. 600 mg of *B. subtilis* cell walls labeled separately by growth in the presence of [^{3}H]glucosamine and [^{14}C]alanine as described above, was then added. After a 2-h incubation at 37°C, the medium was decanted and saved as post-pulse supernatant. The cells were then washed four times with 250-ml portions of HBSS, which were pooled and saved. Fresh serum-free medium containing neither bacterial cell walls nor radiolabel was then added to the cell factory and incubation was continued for 6 h at 37°C. Medium from this incubation, referred to as catabolite accumulation medium, was also decanted and saved. Finally, the cells were detached by treatment with the EDTA solution described above, and the resulting suspension was subjected to centrifugation at $800 \times g$ for 10 min. Triplicate portions were obtained at each stage of the experiment for liquid scintillation counting.

Fractionation of radiolabeled catabolites. The catabolite accumulation medium and post-pulse supernatant medium were first reduced ca. 15-fold in volume by evaporation under vacuum and were then dialyzed against distilled water (membrane cutoff, 12,000 to 14,000 daltons). Since low-molecular-weight glycopeptides were sought, the dialysate was retained, concentrated, and then subjected to gel-permeation chromatography on a column (2.0 by 179 cm) of Bio-Gel P-2 in 0.1 M acetic acid. (In one early experiment, a 2.5 by 108-cm column of Bio-Gel P-6 was used instead.) The radiolabeled fractions were pooled and evaporated to dryness under vacuum to remove acetic acid, and each pool was chromatographed separately on a column (2.0 by 11 cm) of DEAE-Sephadex by a modification of the procedure described by Krueger et al. (13) for purification of a mammalian sleep glycopeptide. The column was eluted first with 50 mM ammonium acetate (pH 5.0) and then with a 0 to 1.0 M gradient of aqueous NaCl. Radiolabeled fractions were desalted by elution through a small column (2.5 by 33 cm) of Bio-Gel P-2 in 0.1 M acetic acid.

Amino acid analyses. Amino acid analyses were performed with a Beckman 118BL amino acid analyzer equipped with a column of W-3H resin (0.9 by 34 cm). The column was eluted sequentially with sodium citrate buffers at pH 3.25, 3.77, and 6.40; the buffers were changed at 50 and 85 min. Samples to be analyzed were hydrolyzed for 18 to 20 h in 6 N HCl at 110°C in evacuated tubes. After hydrolysis, samples were evaporated to dryness and dissolved in a small amount of 0.2 N citrate buffer (pH 2.2) for application to the analyzer column. Total amino acid analyses were performed on equal amounts of samples before and after hydrolysis, and the individual amino acids observed in unhydrolyzed samples were subtracted from their hydrolyzed counterparts. (The free amino acids present in unhydrolyzed samples were presumed to be contaminants arising from MEM.)

Standards of dried, carefully weighed muramic acid, alanine, and *N*-acetylglucosamine (GlcNAc) were hydrolyzed for 22 h in 6 N HCl as described above. Amino acid analysis indicated that 57.7% of the muramic acid and 77.0% of the glucosamine were present after hydrolysis. These results agree well with those reported by Waxman et al. (32), i.e., that 57% of muramic acid and 83% of glucosamine remained after standard hydrolysis. Standard Dap was also analyzed and found to have a color yield of 1.28 relative to alanine. The integrator values obtained for muramic acid, glucosamine, and Dap were corrected accordingly.

Analytical methods. Radioactivity was assessed by liquid scintillation counting in an LS330 counter (Beckman Instruments, Inc.) in vials containing 3.0 ml of Ready-Solv EP (Beckman Instruments, Inc.) Quench curve determinations, performed according to the external standard channels ratio method, indicated counting efficiencies of 89% for ^{14}C and 48% for ^{3}H. Appropriate quench curves were used to calculate the disintegrations per minute in double-label experiments. Interleukin-1 was quantified by its ability to enhance the phytohemagglutinin-stimulated uptake of [^{3}H]thymidine by thymocytes derived from C3H/HeJ mice. Assays of various RAW264 cell culture fluids were performed in the laboratory of Joel Taurog, Department of Medicine, University of Minnesota, Minneapolis, by the method of Mizel et al. (17).

RESULTS

Amino acid composition and distribution of radiolabels in *B. subtilis* cell walls. Amino acid analyses of hydrolysates from radiolabeled *B. subtilis* cell walls were performed by using the stream-splitter attachment, and fractions were collected at 2-min intervals and analyzed for radioactivity. Each of the cell wall preparations gave identical amino acid analysis profiles. The amino acids and amino sugars observed in these cell walls (relative to 1.00 muramic acid) and the percentage of total radioactivity associated with each component in the two preparations are given in Table 1. The components in this table are grouped into three sections: the first group consists of amino acids and amino sugars expected as constituents of peptidoglycan, the second group consists of amino acids known to substitute for those in the first group, and the third group consists of components not expected as constituents of peptidoglycan. Clearly, although peptidoglycan constituents predominate, these cell walls contain substantial amounts of nonpeptidoglycan amino acids. The distribution of the radiolabels among cell wall constituents indicated that the radiolabeled substrates were being utilized by *B. subtilis* for metabolic purposes in

TABLE 1. Amino acid and amino sugar composition and distribution of radiolabel in *B. subtilis* cell walls labeled by growth in the presence of L-[*U*-^{14}C]alanine and D-[1-^{3}H]glucosamine

Retention time (min)	Component	Molar ratio[a]	% Radioactivity	
			[^{14}C]alanine[b]	[^{3}H]glucosamine[c]
86	Glucosamine	1.08	7.9	33.6
42	Muramic acid	1.00	8.9	48.1
65	Alanine	2.75	63.8	
47	Glx	3.52	5.8	
78	Dap	0.78		
34	Asx	1.50		
38	Threonine	0.83		
39	Serine	0.79		
59	Glycine	1.13		
15	—[d]	0.06	1.8	3.5
32	—[d]	0.09	3.3	6.0
81	Methionine	1.54	6.8	
83	Isoleucine	0.30		
89	Leucine	0.87		
95	—[d]	1.23		7.4
108	Tyrosine	0.49		
110	Phenylalanine	0.60		
125	Histidine	1.27		

[a] Normalized to muramic acid.
[b] A ninhydrin-negative component that made up 1.7% of the total radioactivity was also observed at a retention time of 23 min.
[c] A ninhydrin-negative component that made up 1.4% of the total radioactivity was also observed at a retention time of 122 min.
[d] —, Identity unknown.

addition to peptidoglycan biosynthesis. Since radioactivity was being used only as a sensitive tracer by which to detect materials of interest before chemical analysis, the presence of radiolabel was not interpreted as evidence for any particular amino acid or amino sugar. However, since 81.7% of the D-[1-^{3}H]glucosamine and 86.4% of the L-[*U*-^{14}C]alanine substrates ended up in compounds associated with peptidoglycan in the cell walls, the likelihood of finding radiolabel primarily associated with peptidoglycan fragments was favorable.

Time course of removal of *B. subtilis* cell walls from culture medium. The time course of removal of radiolabeled *B. subtilis* cell walls from medium by RAW264 cells is shown in Fig. 1. Virtually no difference could be detected in the initial phase of the experiment, regardless of whether the cell walls were opsonized (Fig. 1). In both cases, rapid removal of radiolabel from solution was followed by a gradual return to initial levels. Our interpretation of this result is that a rapid initial uptake of cell walls by macrophages is followed by a period of metabolic processing and, finally, by release of catabolites into the culture medium. A control experiment (without macrophages) showed that the observed removal of radioactivity from solution was not due to simple adherence of the cell walls to the surface of the plastic culture dish.

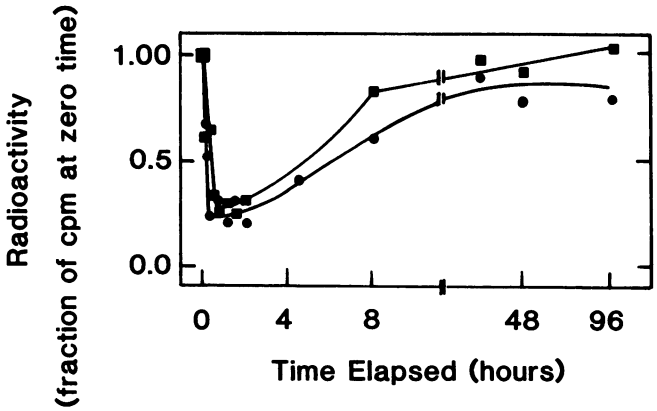

FIG. 1. Results of time-course experiments in which 2 × 10^7 RAW264 macrophages in 20 ml of serum-free MEM were incubated with 10 mg of radiolabeled *B. subtilis* cell walls which had (■) or had not (●) been opsonized.

Interleukin-1 production by RAW264 cells. Since activated macrophages are known to produce interleukin-1 (8, 19), the culture medium derived from RAW264 cells exposed to *B. subtilis* cell walls was assayed for interleukin-1 to establish whether activation had occurred. Measurements were made on supernatants from cultures of identical numbers of RAW264 cells (3 × 10^7) which had been incubated for 6 h at 37°C in the presence or absence of 5 mg of nonopsonized *B. subtilis* cell walls. In these experiments, a 4.3-fold increase in interleukin-1 production relative to the amount produced constitutively by an unstimulated control culture was noted.

Distribution of radiolabel in endocytosis experiments. For preparative endocytosis experiments, 10-level culture vessels with 6,000 cm^2 of surface area were inoculated with 10^9 RAW264 cells in 1 liter of MEM containing 5 to 10% fetal bovine serum, and after 2 days of attachment and reproduction the medium was decanted and the cells were pulsed for 2 h with radiolabeled *B. subtilis* cell walls. The pulse inoculum consisted of a mixture of two cell wall preparations, each derived from a 500-ml culture of *B. subtilis* grown in the presence of D-[1-^{3}H]glucosamine or L-[*U*-^{14}C]alanine. The pulse inoculum was then decanted, and the cells were carefully rinsed four times with 250-ml portions of HBSS. The postpulse supernatant and rinses typically contained ca. 94% of the total radioactivity (Table 2). The adherent cells were then incubated in serum-free MEM for 6 h to allow catabolites to accumulate in the medium. Typically, 1 to 2% of the total radioactivity was present in the catabolite accumulation medium. The adherent cells were then detached with EDTA and pelleted by centrifugation at 800 × *g* for 10 min; ca. 1% of the total radioactivity was found in the EDTA solution, whereas ca. 3% of the radioactivity remained with the pelleted cells (Table 2).

Control endocytosis experiments. Before the preparative generation of cell wall catabolites, several control experiments were performed to assess the degree to which catabolism was macrophage-mediated. Specifically, flasks containing only serum-free MEM and *B. subtilis* cell walls (no macrophages) were prepared and incubated at 37°C for various intervals. It was found that, even after 48 h with constant agitation, only 2.1% of the inoculated radiolabel was present in fragments small enough to be included on Bio-Gel P-6. Thus, the cell walls were not especially suscep-

TABLE 2. Distribution of radiolabel in endocytosis experiments with RAW264 cells

Fraction	% Radioactivity[a]
Post-pulse supernatant	76.4 ± 5.6
Rinses	17.9 ± 6.5
Catabolite accumulation medium	1.3 ± 0.8
EDTA supernatant	0.8 ± 0.9
Cell pellet	3.5 ± 2.0

[a] Mean of four experiments; ^{3}H + ^{14}C ±1 standard deviation.

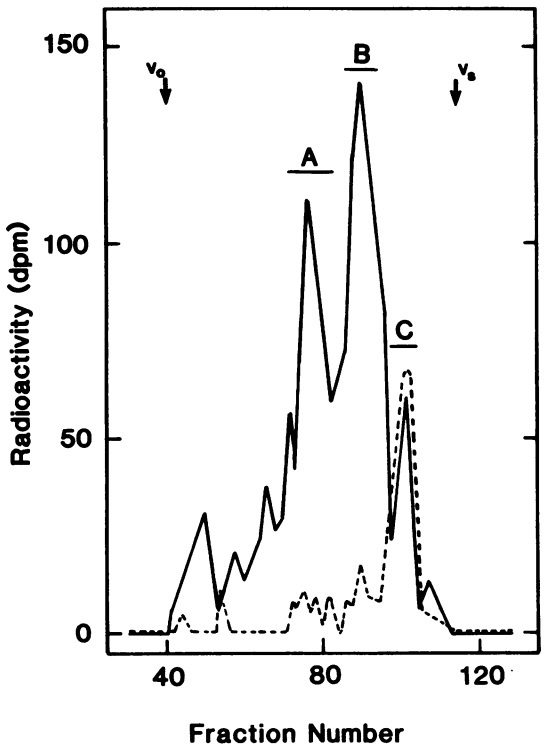

FIG. 2. Gel-permeation chromatographic profile of the catabolite accumulation medium fractionated on a column (2.5 by 108 cm) of Bio-Gel P-6 equilibrated in 0.1 M acetic acid. Fractions (2.8 ml) were collected and portions (150 μl) were analyzed for ^{14}C (– – –) and ^{3}H (———). (The endocytosis experiment used *B. subtilis* cell walls radiolabeled by growth in the presence of L-[*U*-^{14}C]alanine and D-[1-^{3}H]glucosamine.). V_0, Voided volume; V_s, included volume of salt.

tible to autolysis or simple disintegration in the culture medium. It was also of interest to determine what catabolic effect would be found by incubating cell walls with cell-free culture medium which had previously been in contact with unstimulated or stimulated RAW264 cells. To establish the former condition, two flasks were prepared, each containing 3×10^7 RAW264 cells in MEM containing 50 μM 2-mercaptoethanol. The cells were incubated for 5.5 h at 37°C, and the media were then decanted and filtered through a 0.22-μm-pore-size filter. The filtered media were then mixed with radiolabeled cell walls and incubated at 37°C with occasional agitation for 5.5 h. It was found that an average of 7.1% of the radiolabel in the duplicate experiments was in fragments small enough to be included on Bio-Gel P-6. Thus, some degradative activity was associated with constitutively released macrophage enzymes. A final control involved incubating conditioned medium from RAW264 cells with bacterial cell walls. A flask containing 3×10^7 RAW264 cells was pulsed with unlabeled *B. subtilis* cell walls (ca. 4 mg) for 5.5 h at 37°C. The medium was then decanted and filtered (0.22-μm-pore-size filter) before a 5.5-h incubation period (at 37°C, with agitation) with a similar quantity of radiolabeled *B. subtilis* cell walls. In this experiment, 8.0% of the radiolabel was present in fragments small enough to be included on Bio-Gel P-6. Stimulated macrophages may therefore release into their milieu some entity, presumably one or more enzymes, capable of degrading *B. subtilis* cell walls. The amount of degradation observed in these control experiments is low compared with that observed when RAW264 cells were present; i.e., incubation of 3×10^7 RAW264 cells for 5.5 h in the presence of 4 mg of radiolabeled *B. subtilis* cell walls resulted in degradation to the extent that 24% of the total radioactivity was present in fragments small enough to be included on Bio-Gel P-6.

Isolation and amino acid analysis of peptidoglycan catabolites. Catabolites released by macrophages into the post-pulse supernatant and catabolite accumulation medium were fractionated as described above and then characterized by amino acid analysis. The MurNAc-pentapeptide, prepared from the Park nucleotide, was used as a chromatographic standard since we anticipated that this compound would be chemically and chromatographically similar to small glycopeptide catabolites of peptidoglycan. Given the large number of radiolabeled fractions resulting from the purification process, the chromatographic properties of MurNAc-pentapeptide guided the selection of pools for further analysis.

The purification protocol used in this work represents the final refinement from a series of attempts. Earlier purifications had involved gel-permeation chromatography on Bio-Gel P-6 rather than Bio-Gel P-2, but when preliminary results indicated the small size of the compounds of interest, we anticipated (and later found) that chromatography on Bio-Gel P-2 provided superior separation. Some important glycopeptides, however, were isolated from the catabolite accumulation medium by the Bio-Gel P-6 step, and so a representative profile is included here (Fig. 2). The three indicated fractions (A, B, and C) were collected and subjected to anion-exchange chromatography on DEAE-Sephadex (Fig. 3), and after being desalted by passage through a small column (2.5 by 33 cm) of Bio-Gel P-2, the individual fractions were subjected to total amino acid analysis. The quantities of amino acids and amino sugars which were found in these fractions are expressed in Table 3 both as absolute nanomoles analyzed and as values normalized relative to 1.00 muramic acid residue. The fractions derived from Bio-Gel P-6 (Fig. 2 and 3, pool C) were found to consist of mixtures of individual amino acids (not peptides), and so their compositions are not listed in Table 3.

Because it appeared that gel-permeation chromatography on Bio-Gel P-2 was more effective than on Bio-Gel P-6 in resolving these low-molecular-weight glycopeptides, the post-pulse supernatant was subsequently fractionated on Bio-Gel P-2 (Fig. 4). The two major radiolabeled fractions (Fig. 4, fractions B and C) were then subjected to anion-exchange chromatography (Fig. 5), and the components which were eluted with the salt gradient (Fig. 5, B-3, 4, 5 and C-3) were desalted, hydrolyzed, and subjected to total amino acid analysis. The amino acid and amino sugar compositions of these components are given in Table 4.

DISCUSSION

The present study was undertaken as part of an effort to further understand macrophage immunoregulatory functions. The facts that (i) MDP is effective as an immunostimulant and (ii) acts upon the macrophage suggested to us a mechanism for macrophage involvement in the recognition of bacterial infections and in immune amplification. The hypothesis underlying this work was that the macrophage would phagocytize and catabolize the invading bacterium, releasing immunologically informational glycopeptides, such as MDP, derived from the cell wall peptidoglycan. These glycopeptides, in turn, would serve to recruit other macrophages (7, 15) as well as, perhaps, polymorphonuclear leukocytes (20). Additionally, the concomitant release of interleukin-1 by the macrophage could serve to recruit and

activate lymphocytes. Because of the ubiquitous occurrence of peptidoglycan in bacterial cell walls and the highly conserved structure of the cross-link region, of which MDP is a part, such a mechanism for recognition and defense would be quite general.

To test this hypothesis, a model system was constructed to simplify the many complex events inherent in immunoregulatory functions. A macrophage-like cell line, RAW264, was chosen over primary cell cultures to ensure large quantities of homogeneous cells. The cell line chosen has been reported to express many characteristics of macrophages (21, 23, 27). Simplifying factors were also considered in choosing a particular bacterium as a source of cell walls. Since the hypothesis to be tested centered on peptidoglycan processing, we chose an organism with a structurally simple cell wall, rich in peptidoglycan. The exquisite sensitivity of macrophages to gram-negative endotoxin (9, 12, 22) ruled out this entire group of bacteria even though they also contain peptidoglycan. A gram-positive organism (*B. subtilis*), whose peptidoglycan structure is well established and is consistent with immunomodulatory activity, was therefore chosen (31). This bacterium was therefore cultured in the presence of two radiolabeled substrates known to be constituents of peptidoglycan (L-[*U*-^{14}C]alanine and D-[1-^{3}H]glucosamine), and the radiolabeled cell walls derived from these

TABLE 3. Composition of glycopeptides found in the catabolite accumulation medium by sequential chromatography on Bio-Gel P-6 (Fig. 2) and DEAE-Sephadex (Fig. 3)

Amino acid or sugar	Amt (nmol) of acid or sugar (ratio)[b] in fraction:			
	A-1	A-2	B-1a[a]	B-1b[a]
Glucosamine	26.0 (1.00)	27.1 (1.22)	7.76 (0.92)	4.11 (1.45)
Muramic acid	26.0 (1.00)	22.2 (1.00)	8.47 (1.00)	2.83 (1.00)
Alanine	38.8 (1.49)	26.2 (1.18)	9.08 (1.07)	5.17 (1.83)
Glx	30.9 (1.19)	22.1 (1.00)	9.18 (1.08)	4.42 (1.56)
Dap	30.3 (1.17)	15.2 (0.68)		6.22 (2.20)
Asx	3.04 (0.12)	0.86 (0.04)		1.37 (0.48)
Serine		2.13 (0.10)		1.54 (0.54)
Unknown, t = 15 min				2.13 (0.75)

[a] Material in pool B-1 was separated into two fractions, a and b, during the desalt step (Bio-Gel P-2). Pool B-1a eluted first.

[b] Values in parentheses are normalized relative to 1.00 muramic acid residue.

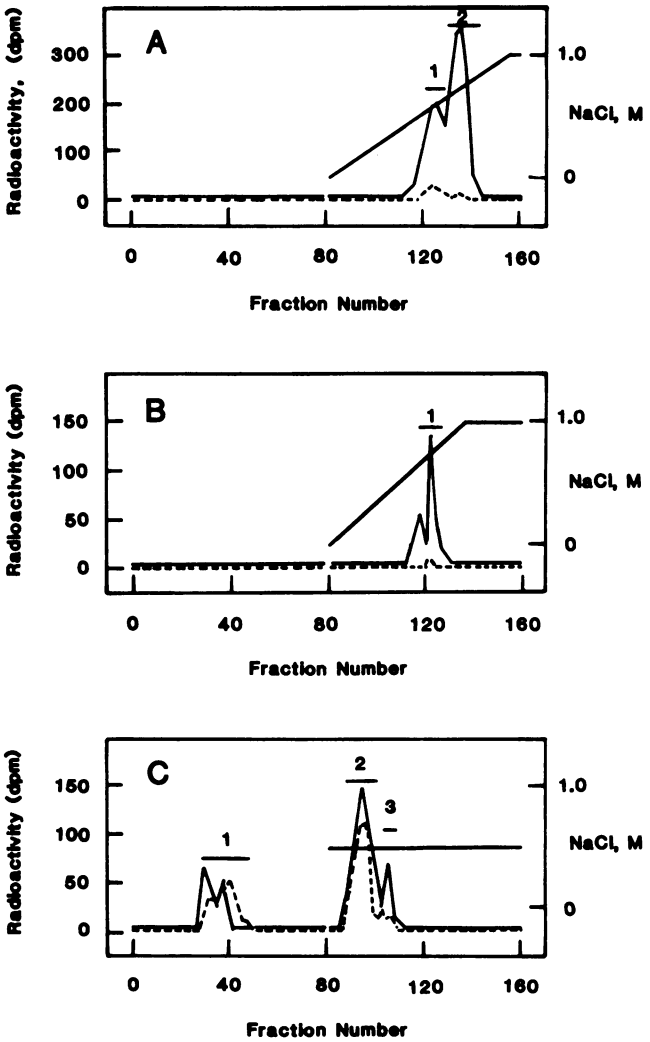

FIG. 3. Anion-exchange chromatographic profiles of Bio-Gel P-6 pools A, B, and C (Fig. 2) fractionated on a column (2.0 by 11 cm) of DEAE-Sephadex. The column was eluted first with 50 mM ammonium acetate (pH 5.0) and then with a linear gradient of 0.0 to 1.0 M NaCl (pool C was eluted isocratically with 0.5 M NaCl instead of with the linear gradient described above). Fractions (2.3 ml) were collected, and portions (A, 150 μl; B, 100 μl; C, 300 μl) were analyzed for ^{14}C (– – –) and ^{3}H (———). Under the elution conditions used for pools A and B, the MurNAc-pentapeptide eluted at fractions 108 to 116.

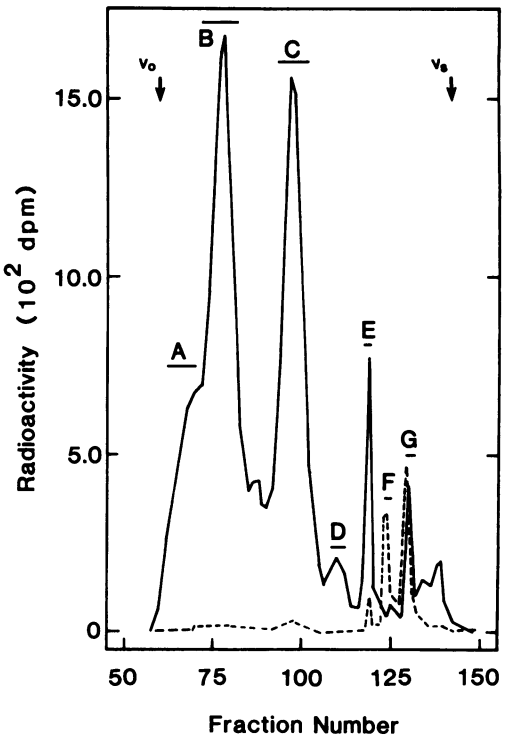

FIG. 4. Gel-permeation chromatographic profiles of the post-pulse supernatant fractionated on a column (2.0 by 179 cm) of Bio-Gel P-2 equilibrated with 0.1 M acetic acid. Fractions (3.8 ml) were collected and portions (100 μl) were analyzed for ^{14}C (– – –) and ^{3}H (———). With the same column under identical elution conditions, the MurNAc-pentapeptide eluted at fractions 83 to 89. V_o, Voided volume; V_s, included volume of salt.

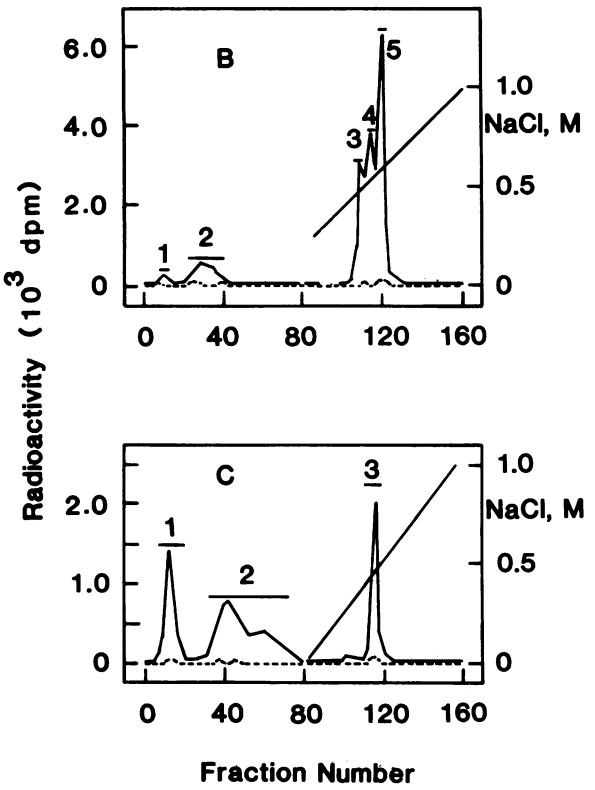

FIG. 5. Anion-exchange chromatographic profiles of Bio-Gel P-2 pools B and C (Fig. 4) fractionated on DEAE-Sephadex. The column and elution conditions are the same as described in the legend to Fig. 3. Portions (100 μl) of each fraction were analyzed for ^{14}C (– – –) and ^{3}H (———).

cultures were fed to macrophages. The intention in using crude cell walls and not purified, biosynthetic fragments was to mimic as closely as possible a normal macrophage encounter with the organism. The use of live *B. subtilis* organisms was precluded on the basis that bacteria used in sufficient numbers to provide a suitable amount of material for subsequent analysis were sometimes capable of overgrowing the macrophages.

Incubation of the radiolabeled cell walls with RAW264 cells led to the rapid removal of radiolabel from solution (Fig. 1). Under the conditions used, a difference in the rates of uptake could not be detected if the cell walls were opsonized. Since RAW264 cells are known to possess receptors for complement protein C3b (21, 23), it is highly probable that the opsonized cell walls were internalized via these receptors. The similarly rapid rate by which nonopsonized cell walls were removed from the medium suggests a receptor-mediated process, but it by no means constitutes proof. It is also not known whether *B. subtilis* cell walls were actually internalized by RAW264 macrophages, but circumstantial evidence (see below) as well as previous studies showing that other particulates are internalized (23) suggests that internalization of the bacterial cell walls occurred. If cell wall degradation had occurred at the surface of RAW264 cells or in solution by secreted enzymes, we would expect to observe a heterogeneous mixture of partially degraded fragments, especially during early stages of incubation. Macrophages are well known for their ability to secrete lysozyme and other hydrolytic enzymes (5, 11), and, indeed, some degradation of radiolabeled cell walls occurred upon incubation with culture medium derived unstimulated RAW264 cells or cells previously stimulated by the addition of unlabeled cell walls. Importantly, however, the gel-permeation chromatographic profiles of the catabolite accumulation medium (Fig. 2) and post-pulse supernatant (Fig. 4) showed that the released glycopeptides were fairly homogeneous in size, i.e., a continuum of distribution of soluble, oligomeric peptidoglycan fragments was not observed. It should also be noted that subsequent examination of the RAW264 cells showed that radiolabel was associated with both the purified (14) membranes and the cytoplasmic fraction, again suggesting that internalization had occurred (experiments not reported).

Three major steps were used in the purification of the released peptidoglycan catabolites, namely sequential dialysis, gel-permeation chromatography, and anion-exchange chromatography. The conditions chosen for anion-exchange chromatography on DEAE-Sephadex were very similar to those used by Krueger et al. (13) for purification from human urine of a sleep-promoting substance which was found to have a composition very similar to the peptidoglycan catabolites reported here. Stringent precautions were taken to avoid airborne amino acid contaminants. Correction for free amino acid contaminants could be made by subtraction of those compounds which were observed in both unhydrolyzed and hydrolyzed samples; however, peptide contaminants could not be subtracted by this process. Considering the numerous secretory functions of macrophages, it is not unreasonable to expect contamination by peptides, not derived from peptidoglycan, which would have chromatographic properties similar to those of the glycopeptides of interest. Despite these difficulties, a few glycoprotein catabolites were isolated in apparently homogeneous form. In cases in which contamination was observed, a reasonable guess of the composition of the glycopeptide could be made on the basis of its gel-permeation and anion-exchange elution profiles.

Inspection of Tables 3 and 4 reveals that, indeed, glycopeptides with compositions characteristic of the cross-link region of peptidoglycan are present in both the catabolite accumulation medium and post-pulse supernatant fractions. The following proposed structures for these glycopeptides are based on their amino acid and amino sugar compositions

TABLE 4. Composition of glycopeptides found in the post-pulse supernatant by sequential chromatography on Bio-Gel P-2 (Fig. 4) and DEAE-Sephadex (Fig. 5)

Amino acid or sugar	Amt (nmol) of acid or sugar (ratio)[b] in fraction[a]			
	B-3	B-4	B-5	C-3
Glucosamine	10.4 (1.08)	5.25 (1.11)	1.57 (0.89)	18.6 (0.88)
Muramic acid	9.66 (1.00)	4.72 (1.00)	1.76 (1.00)	21.2 (1.00)
Alanine	8.12 (0.84)	3.70 (0.78)	2.51 (1.43)	31.4 (1.48)
Glx	28.3 (2.93)	5.46 (1.16)	2.92 (1.66)	34.1 (1.61)
Dap	3.19 (0.33)	1.08 (0.23)		2.88 (0.14)
Asx		0.59 (0.13)	0.46 (0.26)	1.70 (0.08)
Threonine	0.13 (0.01)			0.31 (0.01)
Serine	0.59 (0.06)	0.21 (0.04)	0.91 (0.52)	1.07 (0.05)
Glycine	1.69 (0.17)	0.91 (0.19)	1.41 (0.80)	3.17 (0.15)
Unknown t = 15 min		0.39 (0.08)	0.38 (0.22)	

[a] Insufficient amounts of fractions A, D, E, F, and G (Fig. 4) were available for analysis.

[b] Values in parentheses are normalized relative to 1.00 muramic acid residue.

and are consistent with the previously established structure of the cell wall peptidoglycan (31, 32). It should be emphasized, however, that no sequence data were obtained and, moreover, since these proposed structures are based on the results of amino acid analysis, it is not clear whether the glutamic and pimelic acid residues remain amidated or are present in their free carboxyl form, or whether amino sugars remain *N*-acetylated.

Considering first the glycopeptides resulting from fractionation of the catabolite accumulation medium by sequential gel-permeation chromatography on Bio-Gel P-6 (Fig. 2) and anion-exchange chromatography on DEAE-Sephadex (Fig. 3), the following proposals can be advanced (Table 3). Fraction A-1 most likely represents a mixture of the disaccharide-tetrapeptide (GlcNAc-MurNAc-Ala-isoGln-Dap-Ala) and the disaccharide-tripeptide (GlcNAc-MurNAc-Ala-isoGln-Dap); the former contains two alanines and the latter one alanine, giving an intermediate ratio (1.49) relative to muramic acid. Fraction A-2 is probably a mixture of the disaccharide-tripeptide and the disaccharide-dipeptide (GlcNAc-MurNAc-Ala-isoGln), because the ratio for Dap (0.68) is lower than unity (1.0). Fraction B-1a, in contrast, appears to be the pure disaccharide-dipeptide, whereas fraction B-1b, which was resolved slightly from fraction B-1a by subsequent desalting of fraction B-1 (Fig. 2) on Bio-Gel P-2, is most likely a mixture of the disaccharide-dipeptide and other low-molecular-weight peptides originating from the peptidoglycan cross-link (note the high ratios for alanine, Glx, and Dap).

Fractionation of the post-pulse supernatant gave glycopeptides of the same composition as those derived from the catabolite accumulation medium (Table 4). Fractions B-3 and B-4 appear to be mixtures of the disaccharide-tripeptide and the disaccharide-dipeptide because of the low ratio for Dap. Fraction B-5 lacks Dap and is therefore probably the disaccharide-dipeptide. The composition of fraction C-3, which, except for contaminants, is essentially the same as for fraction B-5, is indicative of the disaccharide-dipeptide. The smaller size of fraction C relative to fraction B on Bio-Gel P-2 (Fig. 4) may indicate that fractions C-3 and B-5 represent different structurally modified forms of the disaccharide-dipeptide or that fraction C-3 is actually GlcNAc-MurNAc-Ala and the Glx observed in this fraction is due to contamination. The latter interpretation may be reasonable, since high ratios for Glx were also observed in fractions B-3 and B-5.

Clearly, despite the fact that such small amounts of material were available for analysis, the data presented above are consistent with the presence of at least three peptidoglycan-derived glycopeptides in the catabolite accumulation medium and post-pulse supernatant, namely the disaccharide-tetrapeptide, disaccharide-tripeptide, and disaccharide-dipeptide. It is also clear that these glycopeptides could not have been derived solely from lysozyme digestion. Although Waxman et al. (32) have shown that small amounts of the disaccharide-tripeptide are present in lysozyme digests of the cell walls of this same *B. subtilis* strain, the major products of lysozyme action were shown to be various cell wall oligomers and the bis(disaccharide-peptide) representing the entire cross-link region. These products were not observed in the experiments reported here.

Although MDP itself was not detected in the endocytosis experiments, its structure is contained within the glycopeptide catabolites that were identified. The disaccharide-dipeptide, for example, differs only in its content of the β-1,4-linked GlcNAc residue. The disaccharide-dipeptide has, in fact, previously been shown to be 3 to 7 times more active than MDP in tests of adjuvancy (28). Other peptidoglycan-derived glycopeptides larger than MDP, including the disaccharide-tripeptide and disaccharide-tetrapeptide, have also been found to be active as adjuvants (1, 6). The three glycopeptides formed as a result of processing of *B. subtilis* peptidoglycan by RAW264 macrophages, therefore, are all known to be active as adjuvants.

The results of these experiments are therefore consistent with our initial proposal that the processing of bacterial peptidoglycan by macrophages would result in the release of immunologically informational glycopeptides. The rapid release of these glycopeptides into the post-pulse supernatant and the concomitant release of interleukin-1 suggest that processing plays a direct role in activation of the immune system. Although *B. subtilis* was used as the source of cell walls for these experiments, the results should be applicable to more infectious bacteria because of the ubiquitous occurrence of peptidoglycan in bacterial cell walls. The fragments formed by processing are of special interest, and so their precise structures and biological activities must be established if this proposal is to be fully verified. Although others have examined the fate of bacteria endocytosed by macrophages, their investigations included only limited analysis of the products of degradation, such as solubility in trichloroacetic acid or alcohol-ether (3, 4), resistance to lysozyme (24), or ability to pass through a 10-μm-pore-size filter (25). The results reported here are therefore thought to constitute the first detailed analytical-biochemical study in this important area.

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