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# Liberation of Serotonin from Rabbit Blood Platelets by Bacterial Cell Walls and Related Compounds

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A study was made on the activity of various bacterial cell walls and peptidoglycans to liberate serotonin from rabbit blood platelets. All of the test cell walls or peptidoglycans prepared from 27 strains of 21 bacterial species were shown to cause a marked release of serotonin, regardless of differences in types of peptidoglycan and non-peptidoglycan moieties and in some biological properties. The assay made with the water-soluble "digests" of *Staphylococcus epidermidis* cell wall peptidoglycans, which were prepared by use of appropriate enzymes, revealed that a polymer of peptidoglycan subunits (a disaccharide-stempeptide) was definitely active in the release of serotonin, but a structural unit monomer was inactive. Among a variety of synthetic muramylpeptides and their 6-*O*-acyl derivatives, only 6-*O*-(3-hydroxy-2-docosylhexacosanoyl)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine was found to hold a strong serotonin-liberating activity.

Rašková et al. (12) and Rýc and Rotta (14) reported that peptidoglycans of *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* lysed blood platelets of rabbits and rats and liberated serotonin (5-hydroxytryptamine). In the present study, we have investigated whether or not this activity is common to bacterial cell walls or peptidoglycans in general, by use of a number of cell walls which differ in the types of peptidoglycans and a non-peptidoglycan moiety and in some biological properties. A study was also made of serotonin-releasing activity by water-soluble enzymatic digests of *Staphylococcus epidermidis* peptidoglycans, synthetic muramylpeptides, and their 6-*O*-acyl derivatives to elucidate the chemical structure responsible for the serotonin-liberating activity of bacterial cell walls.

## MATERIALS AND METHODS

**Cell walls and peptidoglycans.** Cell wall specimens employed in the present study are listed in Table 1. Among them, the cell walls of *Corynebacterium betae*, *Corynebacterium insidiosum*, and *Ampullariella regularis* were a gift from H. R. Perkins (Department of Microbiology, University of Liverpool, U.K.), and those of *Arthrobacter atrocyaneus* and *Arthrobacter* sp. were generously given by K. H. Schleifer (Lehrstuhl für Mikrobiologie, Universität München, West Germany). Other cell walls were prepared as described in previous papers (5, 20, 21). Cell wall peptidoglycans were obtained by the conventional methods, namely, by extraction of isolated cell walls with 10% trichloroacetic acid at 4°C for 18 h with *S. epidermidis* and at 60°C for 4 h with *Lactobacillus plantarum* to remove non-peptidoglycan moieties. *Treponema pallidum* cell wall peptidoglycans were prepared from whole cells, which were anaerobically grown in Spirolate Broth (Difco Laboratories) by extraction with aqueous sodium dodecyl sulfate as described in a separate paper (24).

**Cytoplasmic membrane fractions.** Cytoplasmic membrane fractions were prepared from *S. aureus* (strain Tazaki) and its L-forms (strain STA-EMT-1) by the method previously described (19) and served as a reference specimen.

**Cell wall lytic (peptidoglycan-degrading) enzymes.** Two enzyme specimens were used for preparation of water-soluble digests of *S. epidermidis* cell wall peptidoglycan. One of them was obtained in the following way. A *Cytophaga* sp. strain B-30 was cultured in an 80-liter portion of a liquid medium (pH 6.0) consisting of 1.5% yeast extract and 1.0% mannitol in a 100-liter fermentor, with forced aeration at 30°C for 2 to 3 days. The culture supernatant obtained by centrifugation was submitted to salting out with ammonium sulfate. A fraction precipitating between 45 and 80% saturation with the salt was collected and dissolved in 1 liter of 0.05 M phosphate buffer, pH 7.0. The solution, after

TABLE 1. Release of serotonin from rabbit blood platelets by bacterial cell walls and peptidoglycans[a]

| Species (strain) | % Release | |
|---|---|---|
| | Mean | SE |
| *Micrococcus lysodeikticus* (NCTC 2665) | 60 | 11.7 |
| *Micrococcus lysodeikticus* (IFO 3333) | 69 | 6.8 |
| *Staphylococcus epidermidis* (ATCC 155) | 85 | 4.5 |
| *Staphylococcus epidermidis* (ATCC 155) [PG][b] | 68 | 9.0 |
| *Staphylococcus aureus* (Tazaki) | 75 | 8.6 |
| *Staphylococcus aureus* (Tazaki) [CM][b] | 1 | 5.5 |
| *Staphylococcus aureus* (STA-EMT-1) [CM] | −14 | 1.0 |
| *Streptococcus pyogenes* (Sv, type 3) | 76 | 7.9 |
| *Streptococcus pyogenes* (Su, type 3) | 84 | 1.5 |
| *Streptococcus pyogenes* (T12, type 12) | 75 | 7.3 |
| *Lactobacillus plantarum* (ATCC 8014) | 80 | 3.2 |
| *Lactobacillus plantarum* (ATCC 8014) [PG] | 71 | 9.1 |
| *Corynebacterium diphtheriae* (PW 8) | 66 | 8.3 |
| *Rhodococcus rhodochrous* (ATCC 184) | 54 | 13.3 |
| *Actinomyces viscosus* (ATCC 15987) | 76 | 4.9 |
| *Actinomyces viscosus* (ATCC 15988) | 71 | 7.7 |
| *Actinomyces viscosus* (ATCC 19246) | 66 | 8.9 |
| *Actinomyces naeslundii* (ATCC 12104) | 78 | 4.7 |
| *Actinomyces naeslundii* (human isolate) | 80 | 3.5 |
| *Nocardia canicruria* (ATCC 17896) | 69 | 5.7 |
| *Nocardia corynebacteriodes* (ATCC 14898) | 75 | 5.1 |
| *Nocardia polychromogenes* | 65 | 10.4 |
| *Streptomyces gardneri* (ATCC 23911) | 81 | 7.0 |
| *Streptomyces albus* (IAM 0057) | 70 | 3.5 |
| *Streptomyces aureofaciens* (IAM 0087) | 78 | 8.1 |
| *Arthrobacter atrocyaneus* (ATCC 13752) | 96 | 3.5 |
| *Arthrobacter sp.* (NCIB 9423) | 76 | 7.5 |
| *Corynebacterium betae* (NCPP 373) | 79 | 3.6 |
| *Corynebacterium insidiosum* (NCPP 1110) | 86 | 1.5 |
| *Ampullariella regularis* | 65 | 2.0 |
| *Treponema pallidum* (kazan) [PG] | 52 | 10.0 |

[a] Dose = 100 μg per ml of reaction system.
[b] PG, Peptidoglycans; CM, cytoplasmic membranes.

being dialyzed against the phosphate buffer overnight in the cold, was subjected to fractionation with cold acetone. A precipitate obtained in a concentration of acetone between 45 and 80% (vol/vol) was dissolved in 100 ml of the phosphate buffer. The crude enzyme preparation thus obtained was purified by negative adsorption with DEAE-cellulose chromatography on a hydroxyapatite column and gel filtration with a Sephadex G-75 column. The purified preparation, *S. aureus* lytic enzyme (SALE), was essentially free of caseinase activity, gave a single protein band in disc electrophoresis, and exhibited powerful lytic activity towards the cell walls of *S. aureus* and *S. epidermidis*. Further details of the preparation method of SALE are described in the patent specification of Japan Patent Application no. 108481.

The mode of lytic action of SALE on staphylococcal cell walls was analyzed by the methods described previously with L-11 enzyme (2, 4). It has been proved that this enzyme exerts its staphylolytic activity by the cleavage of pentapeptide cross-links between neighboring stempeptide subunits of the peptidoglycan.

The other of the peptidoglycan-degrading enzymes used in the present study, M-1 endo-*N*-acetylmuramidase, was prepared as described in a previous paper (25).

**Water-soluble enzymatic digests of *S. epidermidis* cell wall peptidoglycan.** A sample (2.526 g) of *S. epidermidis* (ATCC 155) peptidoglycan was incubated with 5 mg of the SALE in a 185-ml portion of 0.01 M sodium pyrophosphate solution at 37°C for 6 h. The materials solubilized were recovered by centrifugation at 1,000 × *g* for 30 min, concentrated by evaporation under reduced pressure, and lyophilized (1.8 g). The solubilized material was then submitted to gel filtration with columns of Sephadex G-50 (5 cm diameter by 100 cm long) and Sephadex G-25 (13 by 100 cm) connected in series (Fig. 1). Higher-molecular-weight fractions (tube no. 49 to 54) with high contents of free amino groups and low reducing power were pooled, concentrated, and lyophilized (1.5 g, referred to as SEPS in the following description).

Samples (1 g) of SEPS were subjected to hydrolysis with 5 mg of the M-1 enzyme in 95 ml of 0.01 M sodium acetate buffer (pH 6.5). After the completion of hydrolysis of a glycan chain of SEPS (as monitored by determination of reducing power), the reaction mixture was boiled for 2 min to stop the reaction and centrifuged at 10,000 × *g* for 30 min to remove a very small amount of insoluble residue. Solubilized material was concentrated by evaporation and applied on connecting columns (1.8 by 90 cm each) of Sephadex G-50/G-25. Elution patterns in terms of free amino groups and reducing power are shown in Fig. 2, with those of SEPS applied on the same columns but in a different gel filtration. Fractions of tube no. 65 to 80 showing high contents of both free amino and reducing groups were pooled, concentrated, and lyophilized. The preparation thus obtained (SEPS-M) weighed 868 mg.

**Chemical analyses.** Qualitative and quantitative analyses of amino sugars and amino acids were performed with an amino acid analyzer (model KLA-5, Hitachi Ltd., Tokyo, Japan) on test specimens which had been hydrolyzed in a sealed glass tube with 6 N HCl in an oil bath at 100°C for 14 h. The temperature of the analyzer resin column was kept at 40°C. Reducing groups were estimated by the method of Park and Johnson (11).

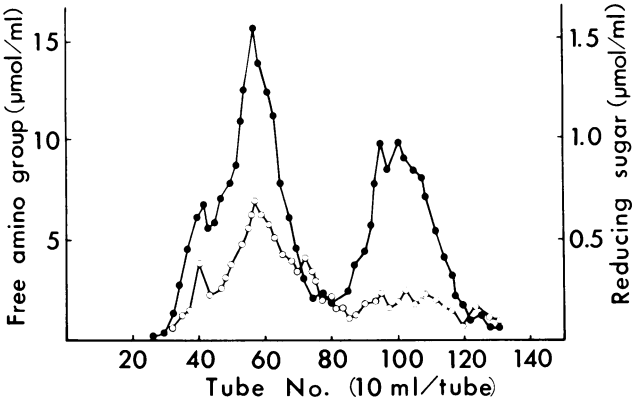



FIG. 1. Gel filtration pattern of a SALE enzyme digest (SEPS) of *S. epidermidis* cell wall peptidoglycan with columns of Sephadex G-50 and G-25 connected in series. Symbols: ●, free amino groups; ○, reducing groups.

Free and total amino groups and N-terminal amino acids were determined by the method described by Ghuysen et al. (1). The average chain length of a glycan portion was estimated by the method described in a previous paper (3).

**Synthetic muramylpeptides and their 6-*O*-acyl derivatives.** *N*-Acetylmuramyl-L-alanyl-D-isoglutamine (MDP), *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (MDP-L-Lys-D-Ala), and the peptide portion of the latter were synthesized as described previously (9). Lipophilic derivatives of MDP, in which the hydroxyl group at the C-6 position of the muramic acid residues was acylated with various fatty acids, were prepared in one of the following two ways. In the first method, 6-*O*-stearoyl- (L18-), 6-*O*-tetracosanoyl- (L24-), and 6-*O*-(2-tetradecylhexadecanoyl)- (B30-) MDPs were prepared by using the acid chloride method as described previously (7, 8, 16). The other 6-*O*-acyl-MDPs, i.e., 6-*O*-triacontanoyl- (L30-), 6-*O*-(2-docosyltetracosanoyl)- (B46-), and 6-*O*-(3-hydroxy-2-docosylhexacosanoyl)- (BH48-) MDPs, were synthesized by the reaction of the corresponding 6-*O*-acylmuramic acid derivatives with L-alanyl-D-isoglutamine benzyl ester, followed by hydrogenolytic deprotection as described in a previous paper (7). We prepared 6-*O*-(3-hydroxy-2-docosylhexacosanoyl)-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (BH48-MDP-L-Lys-D-Ala), mp 171 to 175°C, in a similar manner to that for BH48-MDP by using L-alanyl-D-isoglutaminyl-ε-*N*-benzyloxycarbonyl-L-lysyl-D-alanine benzyl ester (9) in place of the dipeptide derivative. The 6-*O*-nocardomycoloyl-MDP was obtained as described previously (17), using a natural nocardomycolic acid isolated from cells of *Nocardia asteroides* 131.

**Preparation of blood platelets.** Blood specimens

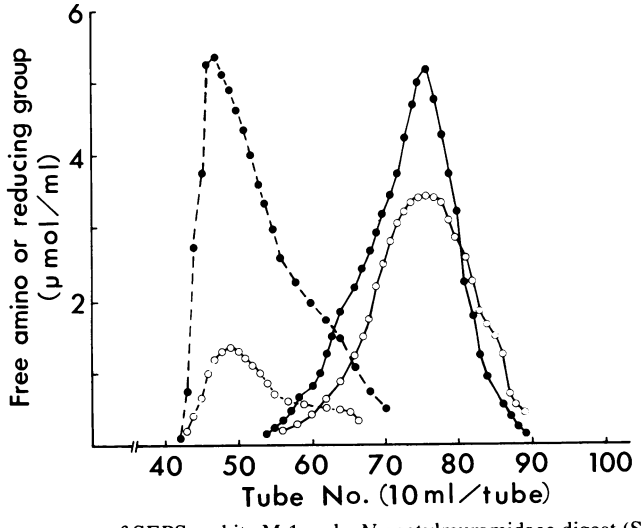



FIG. 2. Gel filtration patterns of SEPS and its M-1 endo-*N*-acetylmuramidase digest (SEPS-M) with columns of Sephadex G-50 and G-25 connected in series. Symbols: ●—●, free amino groups (SEPS-M); ○—○, reducing groups (SEPS-M); ●---●, free amino groups (SEPS); ○---○, reducing groups (SEPS).

were drawn from the carotid arteries of Japanese domestic rabbits weighing around 3 kg and collected in siliconized tubes containing 3.8% sodium citrate solution (the volume ratio of the citrate solution to blood was adjusted to 1:9). The citrated blood was centrifuged at 130 × *g* for 30 min at 4°C to separate plasma containing platelets from erythrocytes and leukocytes. In some experiments, the plasma containing platelets was centrifuged at 720 × *g* for 20 min at 4°C, and sedimented platelets were washed three times with a physiological saline solution. Washed platelets were suspended in the original volume of either a citrated plasma heated at 56°C for 30 min, an EDTA plasma which was obtained by the use of EDTA (final concentration, 0.01 M) as an anticoagulant, or 0.38% citrated physiological saline.

**Assay for the activity of test specimens to release serotonin from rabbit blood platelets.** Portions (1 ml) of the platelet suspension in citrated plasma or other vehicles were incubated with varying doses (none in a control experiment) of test specimens which were suspended or dissolved in 1 ml of 0.38% citrated physiological saline. Test and control mixtures incubated at 37°C for 60 min (unless otherwise noted) were centrifuged at 720 × *g* at 4°C for 20 min, and sedimented platelets were subjected to determination of serotonin by the method of Snyder et al. (18), with minor modifications. The fluorescence was read on a fluorescence spectrophotometer (model FP-4, Japan Spectroscopic Co. Ltd., Tokyo, Japan) at a wavelength of 490 nm. The percentage of release of serotonin was calculated by the following formula: $(1 - OD_{490}$ in a test experiment/$OD_{490}$ in a control) × 100, where $OD_{490}$ is the optical density at 490 nm. With few exceptions, where an available amount of test specimens was very limited, assays were triplicated with each specimen and each dose unless otherwise specified. Variations inherent in the method adopted for serotonin determination are on the order of 3 to 6% in terms of the ratios of the mean to a standard error.

**Electron microscopy.** Test reaction mixtures were centrifuged at 1,000 × *g* for 20 min, and the sediment was prefixed in cold 4% glutaraldehyde–5% glucose–0.1 M phosphate buffer (pH 7.4) at room temperature for 1 h. The material was rinsed with 5% glucose-phosphate buffer by centrifugation. The washed sediment was then fixed in 2% osmium tetraoxide–5% glucose-phosphate buffer, rinsed with 5% glucose-phosphate buffer, and dehydrated with ethyl alcohol in the usual manner. The dehydrated specimen was embedded in Epoxy resin. Ultrathin sections (60 to 90 nm thick) were prepared with a Porter and Blum ultramicrotorm (model MT-1, Ivan Sorvall, Inc., Norwalk, Conn.) and contrasted with Watson uranyl acetate for 15 min and Reynolds lead stain solution for 3 min. Stained sections were examined in a Hitachi model HU-11D-S electron microscope at 75 kV.

## RESULTS

**Serotonin-releasing activity of bacterial cell walls and cell wall peptidoglycans.** All of the test cell walls isolated from 26 strains of 20 bacterial species caused a definite liberation of serotonin from rabbit blood platelets at a dose of 100 μg per ml of reaction system (Table 1). Thus, it has been revealed that the release of serotonin from platelets is a property common to all bacterial cell walls tested, regardless of whether the peptidoglycan is of the group A or group B type (15) and in spite of considerable differences in the non-peptidoglycan moieties, which are well known to be genus-, species-, or even group specific, unlike the peptidoglycan moiety. The peptidoglycans isolated from the cell walls of *S. epidermidis*, *L. plantarum*, and *T. pallidum* were also active in serotonin liberation. No liberation of serotonin was observed when rabbit platelets were incubated in the cold with the cell walls of *S. pyogenes* (strain Su) and of *A. atrocyaneus* (data not shown). This indicates that the serotonin liberation by cell walls is temperature dependent. Cytoplasmic membrane preparations of *S. aureus*, unlike its cell walls, were inactive.

Dose-response relationships were examined with the cell walls of several bacterial species (*S. epidermidis*, *S. aureus*, *S. pyogenes*, *L. plantarum*, *Nocardia corynebacterioides*, *Streptomyces gardneri*, and *C. insidiosum*) and the peptidoglycan of *T. pallidum*. There were appreciable differences in minimum effective dose; from 0.4 μg/ml with the cell walls of *S. pyogenes* (strain Sv), which were the most active, to 25 μg/ml with the *N. corynebacterioides* walls, the least effective.

**Serotonin liberation by a polymer (SEPS) but not a monomer (SEPS-M) of peptidoglycan subunits prepared from S. epidermidis cell walls.** Table 2 summarizes the analytical data of SEPS and SEPS-M. There were no essential differences in the constituent amino sugars and amino acids between SEPS and SEPS-M. Although there was no significant difference in the ratio of total amino groups to free amino groups (6.5 with SEPS and 6.3 with SEPS-M), a marked difference was noticed in the ratio of total to free reducing groups (17.9 with SEPS and 2.5 with SEPS-M). The average chain length of a glycan portion was found to be 10 to 11 disaccharide units with SEPS and 1 unit with SEPS-M. The N-terminal amino acid of SEPS was estimated to be glycine 0.59, serine 0.20, alanine 0.16, and ε-amino-lysine 0.05 (molar ratios to total glutamic acid residue). SEPS-M gave a similar analytical result in this respect.

The analytical results described above, taking together a possible structure of *S. epidermidis* cell wall peptidoglycan which was proposed by Tipper and Berman (22, 23) and the mode of hydrolytic action of the SALE and M-1 enzymes (21), indicate that SEPS is a polymer which consists of disaccharide-stempeptides with some fragment derived from cleaved cross-links and is assembled through an intact glycan chain, whereas SEPS-M is a monomer which was

TABLE 2. Analytical data of water-soluble enzymatic digests (SEPS and SEPS-M) of *S. epidermidis* cell wall peptidoglycans[a]

| Group | Analysis | SEPS | SEPS-M |
|---|---|---|---|
| Amino sugar and amino acid | Glucosamine | 570 (0.91)[b] | 580 (0.97) |
| | Muramic acid | 773 (1.23) | 730 (1.22) |
| | Alanine | 1,495 (2.39) | 1,300 (2.17) |
| | Glutamic acid | 626 (1.00) | 600 (1.00) |
| | Lysine | 733 (1.17) | 740 (1.23) |
| | Glycine | 851 (1.36) | 840 (1.40) |
| | Serine | 157 (0.25) | 160 (0.27) |
| Reducing | Total | 2,430 | 2,430 |
| | Free | 136 | 939 |
| | Total/free | 17.9 | 2.5 |
| Amino | Total | 7,110 | 7,000 |
| | Free | 1,090 | 1,100 |
| | Total/free | 6.5 | 6.3 |
| | Average chain length[c] | $(X\text{-}Y)_{10\text{-}11}$ | (X-Y) |

[a] Results are expressed as micromoles per gram unless otherwise specified.
[b] Numbers in parentheses indicate molar ratio to glutamic acid residue.
[c] Estimated by the previously described (3). (X-Y):(*N*-acetylglucosamine-*N*-acetylmuramic acid).

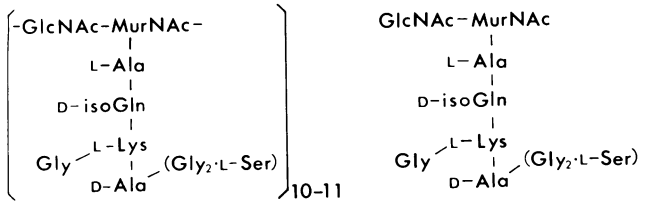
formed by hydrolysis of the glycan chain of SEPS into disaccharide units with the M-1 enzyme (Fig. 3).

The serotonin-liberating activity of SEPS was compared with that of the cell walls of *S. epidermidis* and *S. pyogenes* (strain Sv) (Table 3). SEPS, a polymer of peptidoglycan subunits, was as active as *S. epidermidis* cell walls, though less active than *S. pyogenes* walls, in terms of minimum effective dose. SEPS-M, a structural unit monomer, was inactive at a high dosage of 400 μg/ml, and 100 μg of SEPS did not cause serotonin release when incubated with blood platelets at 4°C (data not shown).

**Assay of serotonin-releasing activity of synthetic muramylpeptides and their 6-*O*-acyl derivatives.** Neither MDP (500 μg/ml) nor any of the nine 6-*O*-acyl-muramyldipeptides cited above (at the concentration of 202 nmol/ml, or 100 μg of MDP per ml) exhibited any activity to release serotonin from rabbit blood platelets. A noteworthy finding is that BH48-MDP-L-Lys-D-Ala, in contrast to BH48-MDP, induced a distinct release of serotonin. Assays on dose-response relationship (Table 4) indicated that a minimum effective dose of BH48-muramyltetrapeptide was around 0.8 μg per ml of reaction system, not differing significantly from that of *S. pyogenes* cell walls (0.4 μg/ml). The muramyltetrapeptide portion of the above-mentioned 6-*O*-acyl compound lacked the activity to liberate serotonin (Table 4).

Electron microscopy revealed that the serotonin release caused by the BH48-muramyltetrapeptide was due to the lysis of rabbit blood platelets (Fig. 4), as was the case with *S. pyogenes* cell walls (12–14).

**Effect of media suspending blood platelets on their susceptibility to serotonin liberation by cell walls and related compounds.** The cell walls of *S.*




SEPS SEPS-M

FIG. 3. One of the possible chemical structures of SEPS and SEPS-M. Abbreviations: MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; D-isoGln, D-isoglutamine.

TABLE 3. Comparison of the serotonin-liberating activity of a polymer of the peptidoglycan subunits (SEPS) isolated from *S. epidermidis* peptidoglycan with that of *S. epidermidis* and *S. pyogenes* cell walls

| Dose of test material (μg/ml) | % Release of serotonin | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | SEPS expt. | | *S. epidermidis* (ATCC 155) cell walls expt. | | | | | *S. pyogenes* (Sv. type 3) cell walls expt. | | | |
| | I | II | I | II | III | IV | V | I | II | III | IV |
| 2,000 | | | 72 | | | | | | | | |
| 500 | | | 87 | | | | | | | | |
| 125 | | | 77 | | | | | | | | |
| 100 | 85 | | | 80 | | | | | | | |
| 31.3 | | | 71 | | | | | | | | |
| 25.0 | 67 | | | 60 | | | | | | | |
| 12.5 | | 67 | | | 81 | | | | | | |
| 7.8 | | | 36 | | | | | | | | |
| 6.3 | 23 | | | 29 | | 68 | 49 | 77 | 90 | | |
| 3.2 | | 39 | | | 32 | | | | 85 | | |
| 1.6 | –[a] | | | | | 45 | 21 | 41 | 80 | 56 | 60 |
| 0.8 | | 25 | | – | 29 | | | | | | |
| 0.4 | | | | | | – | – | 26 | | 32 | 20 |
| 0.1 | | | | | | | | | | – | |

[a] –, Less than 10% release of serotonin.

*epidermidis* and *S. pyogenes*, SEPS, and the BH48-muramyltetrapeptide (50, 50, 12.5, and 36.4 μg/ml, respectively) did not cause the liberation of serotonin from blood platelets when the incubation was made in citrated blood plasma inactivated by heating at 56°C for 1 h, a plasma specimen which was obtained by using EDTA in place of sodium citrate as an anticoagulant, and a physiological saline solution supplemented with 0.38% citrate, in sharp contrast to the marked serotonin release by the test materials incubated with citrated plasma.

TABLE 4. Dose-response relationship of the activity of BH48-MDP-L-Lys-D-Ala to release serotonin from rabbit blood platelets

| Test material | Dose[a] | % Release of serotonin | | | | |
|---|---|---|---|---|---|---|
| | | Expt. | | | | |
| | | I | II | III | IV | V |
| BH48-MDP | 100 | –[b] | | – | | |
| BH48-MDP-L-Lys-D-Ala | 100 | 88 | 79 | | | |
| | 50 | | 83 | | | |
| | 25 | | 76 | | | |
| | 12.5 | | 88 | 91 | | |
| | 6.3 | | | 84 | | |
| | 3.2 | | 48 | 96 | 53 | |
| | 1.6 | | | 98 | 50 | 62 |
| | 0.8 | | | 31 | 64 | 13 |
| | 0.4 | | | | – | 25 |
| MDP-L-Lys-D-Ala | 100 | | | 20 | 27 | – |

[a] Given in micrograms of MDP equivalent per milliliter.

[b] –, Less than 10% release of serotonin.

## DISCUSSION

The studies of Rašková et al. (12) and of Rýc and Rotta (14) on the release of serotonin from rabbit blood platelets by bacterial cell wall peptidoglycans were confirmed and extended to include cell walls or peptidoglycans obtained from 21 bacterial species (27 strains). All of the test cell walls, including the walls of *Micrococcus lysodeikticus*, *S. epidermidis*, *A. atrocyaneus*, *Arthrobacter* sp., *C. insidiosum*, *C. betae*, and *A. regularis* and the peptidoglycan of *T. pallidum*, which had been shown to be inactive or only weakly active in the stimulation of cellular as well as humoral immune response to ovalbumin in guinea pigs (5, 6, 23), were definitely active with respect to the liberation of serotonin from rabbit blood platelets. It may be added here that the cell walls of *M. lysodeikticus*, *S. epidermidis*, *A. atrocyaneus*, *C. insidiosum*, and *A. regularis*, unlike those of *S. pyogenes*, *L. plantarum*, *S. gardneri*, and *N. corynebacterioides*, were found to be scarcely active in causing a slowly developing and long-lasting contraction of a strip of isolated guinea pig ileum (10).

The study was advanced to elucidate a chemical entity carrying the serotonin-releasing activity of cell walls, namely, water-soluble peptidoglycan "fragments" obtained by enzymatic degradation of *S. epidermidis* cell wall peptidoglycan, synthetic muramylpeptides, and their lipophilic derivatives. It was proved that a polymer of structural units of the peptidoglycan and BH48-MDP-L-Lys-D-Ala was as active as whole cell walls or peptidoglycans in causing liberation of serotonin from rabbit platelets. In contrast, neither SEPS-M, a monomer of peptidoglycan

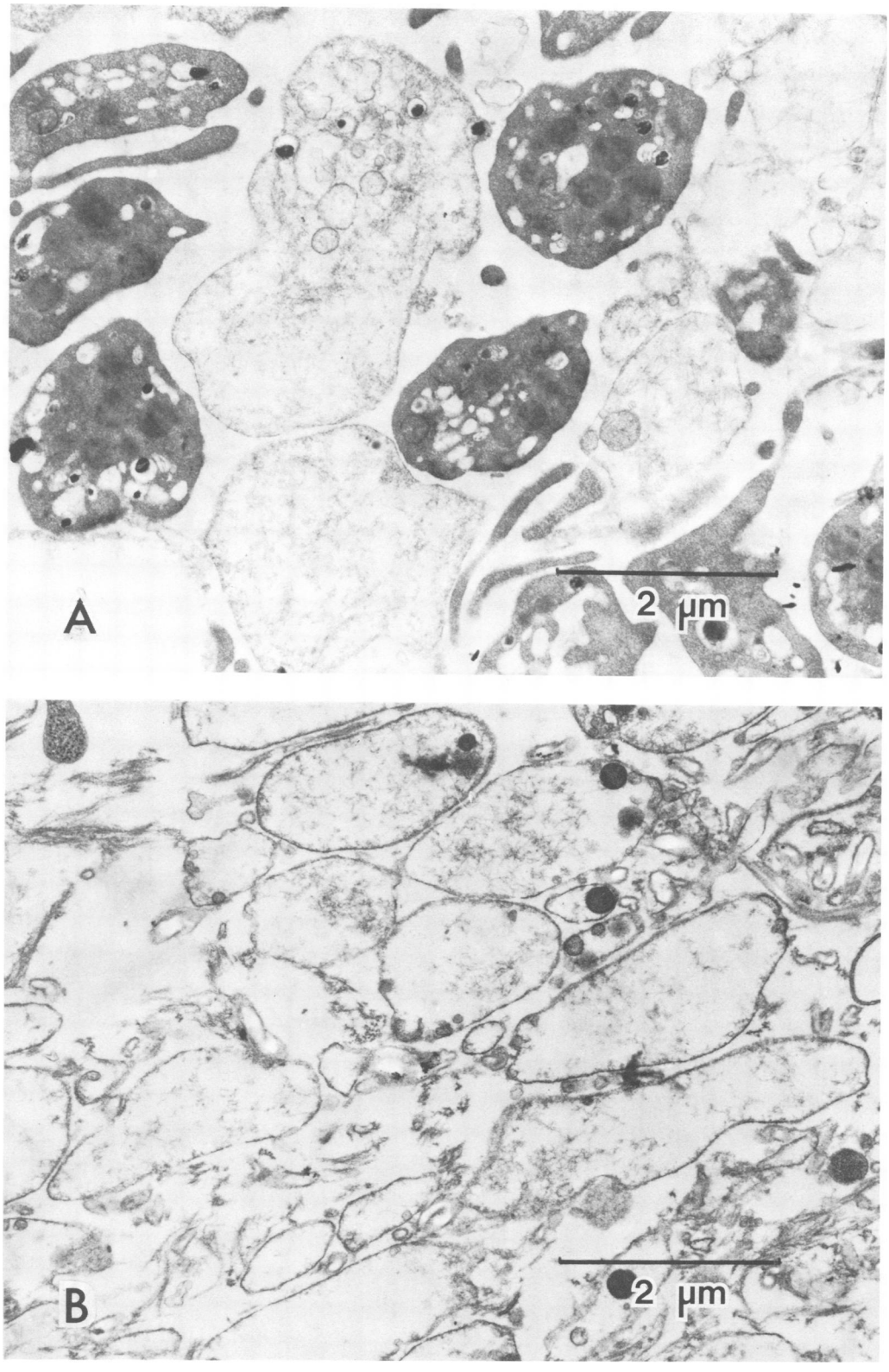



FIG. 4. Electron micrographs of a thin section of rabbit blood platelets incubated with (A) BH48-*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-L-alanine (10 μg/ml), (B) *S. pyogenes* cell walls (2 μg/ml), (C) SEPS (100 μg/ml) , and (D) none.

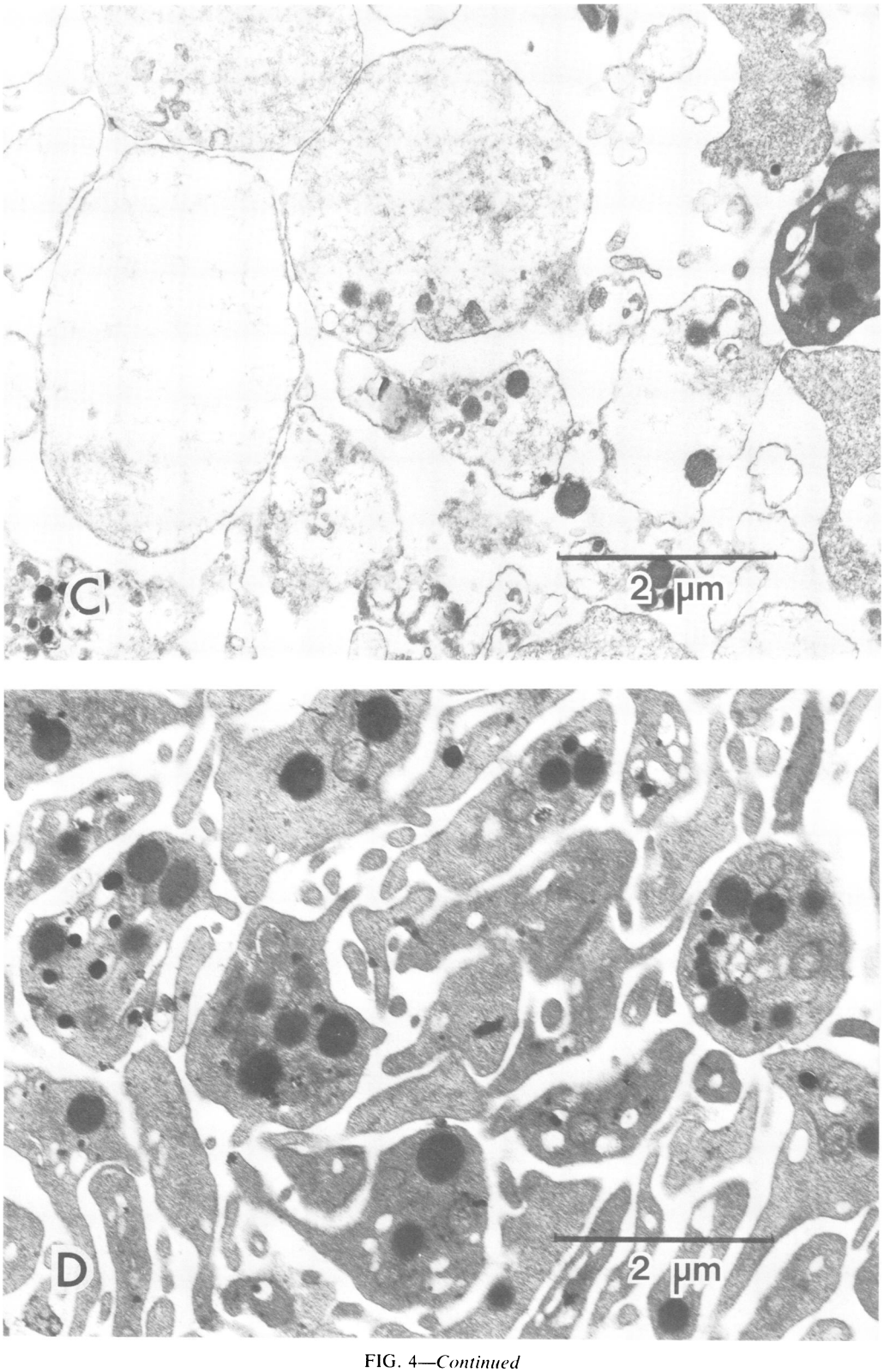



FIG. 4—*Continued*

subunits, nor synthetic muramyldipeptides, including BH48-MDP and 6-*O*-nocardomycoloyl-MDP, were shown to be effective in the serotonin release.

In a recent study, Rotta et al. (13) have shown by electron microscopic examination that MDP and muramyltetrapeptide cause morphologically evident lysis of rabbit platelets, but that the di- or tetrapeptide portion alone does not lead to any platelet lysis. The definite ultrastructural changes were only observed after platelets were subjected to muramyldipeptide and muramyltetrapeptide at a concentration as high as 500 μg/ml. This finding seems to be inconsistent with the result reported here, that MDP and SEPS-M, a disaccharide-heptapeptide, did not cause serotonin release at concentrations of 500 and 400 μg/ml, respectively (the limited amount of MDP-L-Lys-D-Ala available did not permit us to use dosages higher than 100 μg in the assay). The reason for this discrepancy is not known at present. One possible explanation is the difference in sensitivity of the assay methods adopted: ultrastructural changes of blood platelets by test specimens might be a more sensitive indicator of platelet damage than liberation of serotonin.

At present, we cannot draw a definite conclusion on what structure in cell wall peptidoglycans is responsible for the activity causing serotonin release in rabbit blood platelets. The findings reported here suggest that physical characteristics as well as chemical structures are involved in determining the serotonin-releasing activity of test specimens.

The present study confirmed the finding reported by Rašková et al. (12), that the release of serotonin from blood platelets by bacterial cell walls and related compounds is apparently calcium dependent, and that the presence of fresh plasma is necessary to initiate the release of serotonin. This fact suggests that the observed serotonin liberation may be a complement-dependent phenomenon. It is pertinent to quote the recent finding of Kotani et al. (5a) that cell walls isolated from a number of bacterial species, including those examined here, activate the human complement system through the alternative pathway, as well as, depending upon the bacterial species, via the classical pathway, and that SEPS and BH48-MDP-L-Lys-D-Ala cause a definite activation of the complement system, principally via the alternative pathway, whereas SEPS-M, MDP, and their 6-*O*-acyl derivatives, including BH48-MDP and MDP-L-Lys-D-Ala, were inactive in this respect. B30-MDP, which was proved in this study to be ineffective in serotonin release, activates the human complement mainly through the classical pathway, but by a mechanism different from that by antigen-antibody complex. Thus there is a correlation between the activity to release serotonin from rabbit blood platelets and that to activate the human complement system through the alternative pathway. As shown with B30-MDP, the activation of the complement system by the pathway other than the alternative one does not seem to be associated with the liberation of platelet serotonin.

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