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Staphylococcus aureus Peptidoglycan Induces Histamine Release from Basophil Human Leukocytes In Vitro

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Whole killed cells, cell walls, and peptidoglycans of *Staphylococcus aureus* were found to release histamine from human leukocytes and isolated rat mast cells in vitro. The histamine-releasing capability increased in the order of whole bacteria, cell walls, and peptidoglycans. Peptidoglycan was found to release histamine by a nonimmunological mechanism, as demonstrated by release in cells deprived of surface immunoglobulins, whereas whole bacteria and cell walls seemed to operate both by immunological and nonimmunological mechanisms. Histamine release was not a specific property of *S. aureus*; a wide range of whole bacterial species had this activity. We suggest that peptidoglycan may be a common factor responsible for histamine release by different bacteria.

Staphylococcus aureus Wood 46 has been demonstrated to induce release of histamine from human basophil leukocytes in vitro (12, 18). Whole killed bacteria and cell walls of Wood 46 have this property (6). It seems that at least two different mechanisms are involved in this bacterial histamine release: an immunoglobulin E (IgE)-dependent mechanism, in which removal of IgE from the basophils abolished or reduced the bacteria-induced histamine release, and a nonimmunological mechanism, in which this was not the case (6). We found that bacterial ultrasonicates of several strains from the normal flora of the upper respiratory tract caused release of histamine from basophil leukocytes in both intrinsic asthma patients and normal individuals (8, 13). This release might be a pathogenic mechanism in intrinsic asthma and in bacterial infections.

In this study we examined the histamine-releasing capability of whole cells from several bacteria and focused on the activity in the bacterial cell wall by examining the effect of *S. aureus* peptidoglycan.

MATERIALS AND METHODS

Bacterial species and culture conditions. *S. aureus* E2371 (protein A containing) and strain E1369 (protein A poor) have both been described previously (3, 15). *S. aureus* Wood 46 (protein A free) was kindly provided by K. Rosendal, Statens Seruminstitut, Copenhagen, Denmark. *Staphylococcus epidermidis* strains A1271/76, A1388/76, A1394/76, and A1389/76 were all isolated from blood cultures and have recently been characterized (4). *Streptococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus mitior*, group A streptococcus, group B streptococcus, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, *Proteus vulgaris*, *Enterobacter cloacae*, and *Haemophilus influenzae* were all recent clinical isolates. All strains were stored freeze-dried, and when reconstituted, they were maintained on blood agar at 4°C. Several colonies were inoculated into 100 ml of Truche medium consisting of 40 g peptone per liter (Orthana, Copenhagen, Denmark), with NaCl and glucose added to final concentrations of 5 g and 2 g per liter, respectively. The bacteria were grown for 18 h at 37°C with shaking and harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. After this treatment, the bacteria were washed three times in saline.

Preparation of whole Formalin-killed bacteria. Whole washed bacteria were suspended in saline containing 1.5% Formalin incubated for 1 h at 37°C and washed six times in saline. Finally, the bacteria were suspended in saline and adjusted to 100 mg/ml (wet weight).

Preparation of *S. aureus* undigested cell walls. Cell walls were prepared from *S. aureus* strains according to the method of Yoshida et al. (24). Whole non-Formalin-treated and washed bacteria suspended in distilled water (400 g per liter) were broken after freezing by three passes through an X-press (D-25, AB Biox, Nacka, Sweden). Whole unbroken bacteria were removed by repeated centrifugations at 3,000 × *g* for 15 min at 4°C. Cell walls were harvested from the final supernatant by centrifugation at 10,000 × *g* for 25 min at 4°C. After this treatment, the cell walls were washed six times with distilled water at 10,000 × *g* for 25 min at 4°C and lyophilized, and finally they were suspended in saline and adjusted to 100 mg/ml. Cell walls (62 to 110 mg) were obtained from 20 g (wet weight), of whole bacteria. By electron microscopy after negative staining (5), a typical picture of cell walls was seen consisting of empty shells with only a few whole cells present.

Preparation of *S. aureus* peptidoglycan. Peptidoglycan from cell walls of *S. aureus* strains E2371 and E1369 was prepared by the method described previously (1). Briefly, cell walls were extracted in 10% trichloroacetic acid for 72 h at 4°C. After centrifugation (4,000 × *g* for 10 min at 4°C), the pellet was further extracted with 5% trichloroacetic acid for 10 min at 90°C. After an identical centrifugation, the pellet was digested for 18 h at 37°C by crystalline trypsin (Difco Laboratories, Detroit, Mich.) at 1 mg/ml in buffer (pH 8.2) containing 0.05 M NH_4HCO_3 and 0.005 M NH_4OH. Insoluble peptidoglycan was harvested by centrifugation at 4,000 × *g* for 10 min at 4°C, washed six times in distilled water and lyophilized. Finally it was dissolved in saline and adjusted to 100 mg/ml. From 50 mg of cell walls, 14 and 17 mg of insoluble peptidoglycan were obtained from strain E2371 and strain E1369, respectively.

Solubilization of *S. aureus* peptidoglycan. Peptidoglycan from both strains was solubilized either by sonication (1) or

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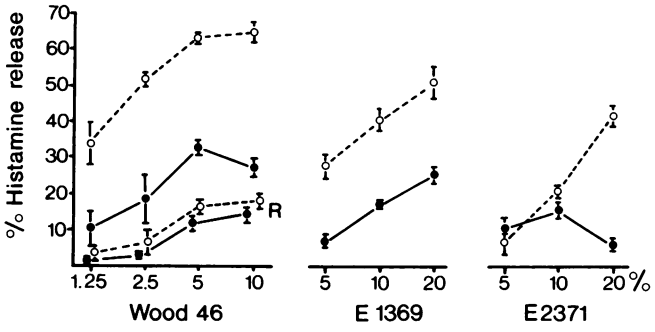

FIG. 1. Basophil histamine release from human leukocytes caused by whole bacteria and cell wall preparations of the *S. aureus* strains Wood 46, E1369, and E2371. Histamine release in mast cells from germ-free rats (R), whole bacteria (●) and bacterial cell walls (○). Final concentrations of bacterial preparations are given. The experiments included four individuals. Results show the mean ± the standard error of the mean.

treatment with lysozyme or lysostaphin as described in detail previously (2). All solubilized preparations gave two immunochemically identical immunoprecipitates when investigated by crossed immunoelectrophoresis for polyspecific rabbit antibodies against *S. aureus* cell walls (2).

Histamine release assay. Blood was obtained from six normal consenting volunteers, aged 23 to 60 years. Human leukocyte suspensions containing ca. 2% basophilocytes obtained by Ficoll-Hypaque gradient centrifugation were washed twice and suspended in a glucose-free Tris-AMC buffer containing 25 mM Tris at pH 7.6, 0.12 M NaCl, 5 mM KCl, 0.6 mM $CaCl_2$, 1.1 mM $MgCl_2$, and 0.3 mg of human serum albumin per ml (19). A 98% pure population of peritoneal mast cells from germ-free Pan:Wist rats or non-germ-free Wistar rats sensitized to horse serum was obtained as described by Norn and Stahl Skov (11). The cells were suspended in a glucose-free Tris-AMC buffer. The use of washed cells in the absence of serum excludes the possibility of histamine release by complement activation. Histamine release by whole bacteria, bacterial cell wall, and peptidoglycan was performed by incubation of 80 μl of cell suspension for 40 min at 37°C with 20 μl of the undiluted and diluted preparations. The bacterial products were omitted for spontaneous histamine release and for determination of the total histamine content; in the latter case the samples were left at 4°C. After this, 3 ml of ice-cold Tris-AMC was added to all of the samples, and they were centrifuged at 2,000 × *g* for 10 min. In addition to released histamine, the supernatant also contains protein, the bacterial products, and other substances which interfere with histamine analysis. The supernatant was therefore discarded, and the residual histamine was determined in the cell sediment by the spectrofluorometrical OPT method (19). The release of histamine was expressed as a percentage of the total histamine content of the sample. In the absence of bacterial products, the spontaneous histamine release amounted to 6 to 10% in all of the experiments. Therefore, it should be noted that only release of >10% is considered significant.

To examine whether the histamine release was caused by type I allergic reactions or nonimmunological mechanisms, cell-bound IgE and IgG were removed from the basophil leukocytes by exposure of the cell suspension to pH 3.8 for 5 min at 4°C (20) before challenge of the cells with the bacterial products. The removal of these immunoglobulins was controlled functionally by a decrease in anti-IgE- and anti-IgG-induced histamine release.

TABLE 1. Basophil histamine release from human leukocytes caused by peptidoglycan from *S. aureus* strains E1369 and E2371

Peptidoglycan	Cells[a]	% Histamine release at a peptidoglycan concn (%) of:[b]			
		0.6	1.25	2.5	5.0
Strain E1369	Intact	4 ± 1	14 ± 2	31 ± 5	63 ± 6
	Immunoglobulin deprived	5 ± 1	10 ± 3	41 ± 7	72 ± 6
Strain E2371	Intact	5 ± 2	15 ± 2	24 ± 4	
	Immunoglobulin deprived	8 ± 2	17 ± 1	30 ± 3	

[a] Intact cells and cells deprived of immunoglobulins. The experiments were done with four individuals, and results are the mean ± the standard error of the mean. No significant difference in histamine release between intact cells and cells deprived of immunoglobulin ($P > 0.05$, by Student's *t*-test).
[b] Final concentration of peptidoglycan preparations.

RESULTS

Histamine release caused by *S. aureus*. Whole killed staphylococci and staphylococcal cell walls all released histamine from human leukocytes (Fig. 1). Cell walls showed a higher maximal release of histamine than did whole cells (Fig. 1). To examine whether the induced histamine release depended on the presence of immunoglobulins on the cell surface, histamine release was also investigated in leukocytes after the immunoglobulins had been removed. Both whole bacteria and bacterial cell walls induced release of histamine in immunoglobulin-deprived leukocytes, but the release was lower in two of the four individuals, compared with that obtained in intact leukocytes (data not shown). Whole killed strain Wood 46 cells and cell walls of this strain were also tested in rat mast cells and showed histamine release (Fig. 1). In this case the difference between cell walls and whole bacteria was not significant (Fig. 1).

Table 1 shows the histamine release from human leukocytes by peptidoglycan from *S. aureus*. Both peptidoglycans were able to release histamine in a dose-dependent manner (Table 1). No difference was found in histamine release between intact cells and cells deprived of immunoglobulins (Table 1). Release was also obtained in mast cells from germ-free rats, and the dose-response curves were similar to those obtained in non-germ-free sensitized rats (Fig. 2). Examination of the solubilized peptidoglycan preparations in final concentrations of up to 1% did not show release of histamine.

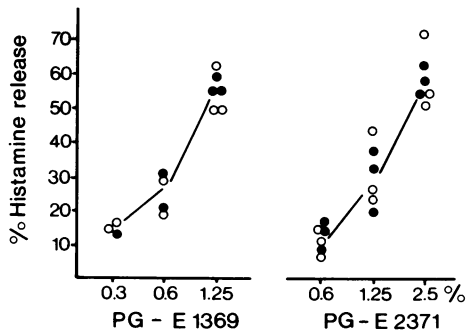

FIG. 2. Mast cell histamine release caused by peptidoglycan (PG) from *S. aureus* strains E1369 and E2371. Symbols: ○, Germ-free rats; ●, nongerm-free, sensitized rats. Final concentrations of peptidoglycan preparations are given. Each point indicates triple determinations.

TABLE 2. Basophil histamine release from human leukocytes caused by whole Formalin-killed bacteria

Bacterial species	No. of persons tested	No. of positive responders[a]	No. of negative responders[a]
Staphylococcus epidermidis A1271/76	6	5	1
Staphylococcus epidermidis A1388/76	6	6	0
Staphylococcus epidermidis A1394/76	5	5	0
Staphylococcus epidermidis A1389/76	6	6	0
Streptococcus faecalis	6	1	5
Streptococcus pneumoniae	6	0	6
Streptococcus mitior	6	0	6
Group A streptococcus	6	0	6
Group B streptococcus	6	0	6
Escherichia coli	6	5	1
Klebsiella pneumoniae	6	6	0
Klebsiella oxytoca	4	4	0
Proteus mirabilis	3	2	1
Proteus vulgaris	6	5	1
Enterobacter cloacae	6	6	0
Haemophilus influenzae	6	0	6

[a] In positive and negative responders the maximal release of histamine amounted to 18 to 43 and 6 to 10%, respectively.

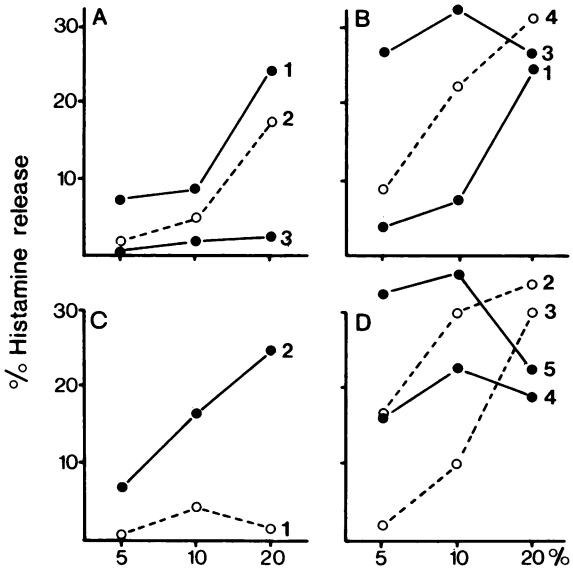

FIG. 3. Basophil histamine release caused by whole bacteria in normal individuals. (A) *S. epidermidis* A1271/76, (B) *S. epidermidis* A1389/76, (C) *P. vulgaris*, and (D) *K. pneumoniae*. Final concentrations of bacterial preparations are given. Each point indicates triple determinations in five different subjects.

Histamine release caused by other bacterial species. Leukocyte suspension from six normal individuals were challenged with whole killed bacteria. Several strains, both gram positive and gram negative, released histamine in leukocytes from almost all of the individuals (Table 2). Histamine release was thus induced by four strains of *Staphylococcus epidermidis*, by *Escherichia coli*, *K. pneumoniae*, *K. oxytoca*, *P. mirabilis*, *P. vulgaris*, and *Enterobacter cloacae*. No release was induced by *Streptococcus pneumoniae*, *Streptococcus mitior*, group A streptococcus, group B streptococcus, or *H. influenzae*, and only one of six individuals responded to *Streptococcus faecalis*. The same differentiation between release-inducing and non-release-inducing bacteria was obtained when mast cells from germ-free rats were challenged with the whole bacteria (data not shown). The dose-response curves for four of the bacteria are shown in Fig. 3, and it can be seen that leukocytes from different persons show individual variations in histamine release.

In five of the six individuals, no difference was found in the bacterial histamine release between intact cells and cells deprived of immunoglobulins. However, one of the six individuals showed lower release in immunoglobulin-deprived cells compared with that obtained in intact leukocytes by four of the bacteria (*Staphylococcus epidermidis* A1271/76, *K. pneumoniae*, *K. oxytoca*, and *Enterobacter cloacae*) but not with the others. An example from each of the two groups is shown in Fig. 4.

DISCUSSION

In the present study we demonstrated that, in addition to whole cells and cell walls, *S. aureus* peptidoglycan has the capacity to release histamine from human leukocytes in vitro. The concentrations of whole cells, cell walls, and peptidoglycan necessary to induce histamine release are relatively high, i.e., in the range of 0.3 to 20 mg/ml; however, similar concentrations were used to demonstrate complement activation and chemotaxigenesis in vitro (14, 17, 23). When comparing the histamine release obtained by peptidoglycan (Table 1) with the release obtained by cell walls and whole cells (Fig. 1), it can be seen that peptidoglycan is more potent than cell walls and that cell walls show a higher histamine release than whole cells. Interestingly, it seems that only intact peptidoglycan can induce histamine release, since peptidoglycan fragments prepared either by sonication or digestion with lysozyme or lysostaphin showed no activity.

Peptidoglycan represents ca. 50 to 60% of the dry weight of *S. aureus* (16). It is composed of glycan strains of alternating beta-(1,4)-glycosidically linked *N*-acetylmuramic acid and *N*-acetylglucosamine residues, which are cross-linked by penta- or hexaglycine interpeptide bridges (16). Lately, *S. aureus* peptidoglycan has been shown to have a wide range of biological properties, which include suppression of chemotactic and phagocytotic activity and activation of the classic and alternative complement pathway (10, 14). These effects and the present finding of histamine release suggest a modulating function of peptidoglycan in the inflammatory process.

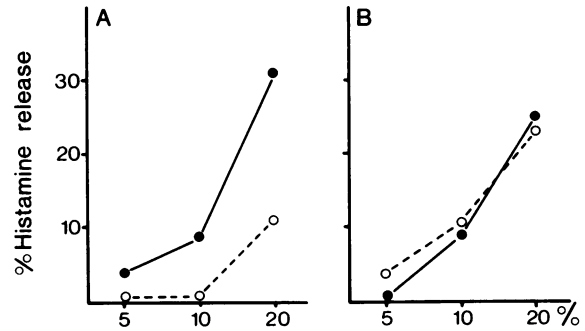

FIG. 4. Basophil histamine release induced by whole bacteria of (A) *S. epidermidis* A1271/76 and (B) *P. vulgaris*. Symbols: ●, Intact cells; ○, cells deprived of immunoglobulins. Final concentrations of bacterial preparations are given. Each point indicates triple determinations.

Peptidoglycan antibodies have been demonstrated in serum from normal individuals (1, 21, 22), and the titer is enhanced during severe *S. aureus* infections (1, 21, 22). To examine whether peptidoglycan causes histamine release by an IgE-mediated reaction, the immunoglobulins were removed from the basophil cell surface before challenge with peptidoglycan. No difference was, however, found in histamine release between intact cells and cells deprived of immunoglobulins. Furthermore, release was also obtained in mast cells from germ-free rats, and the dose-response curve was similar to that obtained in non-germ-free sensitized rats. These findings indicate that the release was caused by a nonimmunological mechanism. However, the possibility of an IgE-dependent mechanism cannot be excluded until an increased number of individuals have been investigated.

When whole killed *S. aureus* cells and preparations of the cell walls were tested, we found, in two of the four individuals, a lower release of histamine in immunoglobulin-deprived cells than in intact cells, which indicates the possibility of both an IgE-dependent mechanism and a nonimmunological mechanism. The nonimmunological mechanism might depend on the cell wall peptidoglycan content, since as mentioned, peptidoglycan was found to act nonimmunologically. This is in agreement with our suggestion of lectin-carbohydrate-mediated reactions in bacteria-induced histamine release, in which bacterial sugars interact with lectins on the target cell leading to histamine release (7). The *S. aureus* surface component which induces the IgE-dependent histamine release is unknown. Since both protein A-positive and -negative strains are active, protein A cannot be involved in all cases, but it might be active in some cases, since protein A has been demonstrated to induce release of histamine in vivo (9). Probably, the immunological reaction is caused by different surface components in different individuals. Whether peptidoglycan also has the capacity to induce immunological histamine release remains to be investigated.

To elucidate the possibility of peptidoglycan acting as a common factor responsible for histamine release induced by different strains of bacteria, a variety of gram-positive and gram-negative bacteria were examined. All four *S. epidermidis* strains showed histamine-releasing activity (Table 2). In most cases the activity was caused by a nonimmunological mechanism. Since the composition of peptidoglycan in *S. aureus* and *S. epidermidis* only shows minor differences (16), the release by *S. epidermidis* might be caused by the peptidoglycan content. The streptococci investigated showed no activity (Table 2). The reason for this result might be that a histamine-releasing effect of the peptidoglycan is masked by the polysaccharide capsule of the streptococci. All the gram-negative bacteria, except *H. influenzae*, released histamine. If peptidoglycan accounts for the activity, the polysaccharide capsule of *H. influenzae* might explain the negative response by this strain. However, the release obtained by gram-negative bacteria may also depend on other factors, i.e., lipopolysaccharides or other components of the outer envelope.

Our results suggest a role of bacterial peptidoglycan in the bacteria-induced histamine release. However, investigations of peptidoglycan from different strains are needed to clarify whether peptidoglycan is a common factor in bacterial histamine release.

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