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Role of Autolysin in Generating the Pneumococcal Purpura-Producing Principle

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The results of four studies described in this paper support the idea that pneumococcal autolysin (*N*-acetylmuramyl-L-alanine amidase) plays a key role in generating the pneumococcal purpura-producing principle (PPP) from the bacterial cell wall. This conclusion is based on the observation that (i) concentrates of pneumococcal cell-free extracts prepared at a pH which is inhibitory to autolysin activity contained significantly less PPP activity than extract concentrates prepared at a pH which is optimal for autolysin activity; (ii) PPP activity was not detectable in concentrates of cell-free extracts prepared from cells which contained ethanolamine in the cell wall, but a normal amount of activity was found in extract concentrates derived from cells containing choline in the cell wall; (iii) PPP activity was not detectable in concentrates of cell-free extracts prepared from an autolysin-defective mutant, but a normal amount of activity was found in extract concentrates derived from the wild-type strains; and (iv) incubation of purified wild-type strain cell walls with pneumococcal autolysin resulted in a significant increase in the amount of purpurogenic activity in the supernatant fluids of the incubation mixtures.

In 1926, Julianelle and Reimann (6, 16) reported that mice, rabbits, and guinea pigs developed purpura after the injection of cell-free extracts prepared by alternate freezing and thawing of concentrated pneumococcal suspensions. The results of subsequent investigations on the chemical nature and the mode of production of the purpura-producing principle (PPP) were at variance with one another. In 1927, the substrate for pneumococcal autolysin was not known, and Julianelle and Reimann (7) claimed that the PPP was associated with protein breakdown products (primary proteoses) which were generated by the action of the bacterium's autolysin. On the other hand, Sickles and Shaw (20) and Wadsworth and Brown (26) proposed that the activity was associated with a cellular carbohydrate fraction, and Mair (12) claimed that the PPP, rather than being generated by the action of pneumococcal autolysin, was a preformed intracellular constituent which was released after cell disruption. Recently, we isolated the PPP from cell-free extracts of sodium deoxycholate-lysed *Streptococcus pneumoniae* type I, and described some of the physicochemical properties of the purified PPP (2). The results of our studies indicated that the PPP was associated with water-soluble, high-molecular-weight peptidoglycan (PG) fragments, and these results, together with the observation (2) that cell-free extracts prepared from heated cells (100°C for 20 min) did not contain purpurogenic activity (despite the fact that the PPP was heat stable), suggested that pneumococcal autolysin (*N*-acetylmuramyl-L-alanine amidase) plays a key role in generating the PPP from the bacterial cell wall. This communication presents data supporting this hypothesis.

MATERIALS AND METHODS

Bacteria and growth conditions. *Streptococcus pneumoniae* type I (ATCC 6301) was obtained from the American Type Culture Collection (Rockville, Md.). *S. pneumoniae* strain R61, which is unencapsulated, and strain cwl-1, which is an autolysin-defective mutant of strain R61 (4, 10), were kindly supplied by S. Lacks (Biology Department, Brookhaven National Laboratory, Upton, N.Y.). In all experiments except those designed to examine the effect of cell wall choline and ethanolamine on the generation of the PPP, the bacteria were grown in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) as previously described (2).

In experiments comparing the influence of cell wall choline versus ethanolamine on the generation of the PPP, *S. pneumoniae* type I was grown in the maltose medium (M-medium) of Lacks and Hotchkiss (11) modified by elimination of the iron, copper, and zinc salts and by addition of sodium pyruvate (240 mg/liter), sodium chloride (2 g/liter) and either choline (10 mg/liter) or ethanolamine (10 mg/liter). The choline and ethanolamine were of the highest purity obtainable from the Sigma Chemical Co., (St. Louis, Mo.) and Eastman Organic Chemicals (Rochester,

N.Y.), respectively. Cells from a brain heart infusion broth culture (20 ml), in the late logarithmic phase, were harvested by centrifugation, washed once with sterile 0.85% NaCl (20 ml), and suspended in saline (2 ml). Seed cultures were prepared by adding portions (0.1 ml) of the washed cell suspension to choline-containing M-medium and to ethanolamine-containing M-medium (50 ml in 250-ml flasks) and incubating the media, without shaking, for 12 h at 37°C. Portions (2.5 ml) of the seed cultures were added to 6-liter flasks containing 2.5 liters of the same media, and the media were incubated at 37°C without shaking until they showed a turbidity equivalent to that of a McFarland standard (3) having a cell density of ca. 9×10^8 bacteria per ml (ca. 12 h for the choline-containing M-medium and 19 h for the ethanolamine-containing M-medium).

Assay for PPP activity. The assay for PPP activity was performed as previously described (2). Briefly, 8- to 12-week-old, Dub: (ICR) strain, random-bred albino mice (Flow Laboratories, Inc., Dublin, Va.) were injected intraperitoneally, in groups of four mice per group, with portions (0.4 ml) of twofold serial dilutions of the preparations to be tested, and the animals were examined 12 to 24 h postinjection. Occasionally, as observed previously (2), some members of a group of mice injected with identical amounts of a preparation would not exhibit purpura, whereas the rest of the mice would show the typical reaction. One PPP unit was defined as the smallest amount of preparation required to elicit purpura on the ears, tails, feet, or snouts of any of the mice in the group. Despite the occasionally observed variation in response mentioned above, the results of repeated assays of the same PPP preparations never showed more than a twofold difference in specific activity (PPP units per milligram, dry weight). Therefore, differences in purpurogenic activity among the different preparations which were assayed were not considered significant unless there was more than a fourfold difference in activity.

Effect of pH on generation of the PPP. The cells from 5-liter portions of a brain heart infusion broth culture (20 liters) of *S. pneumoniae* type I were washed three times at 4°C with 200 ml of 0.85% NaCl buffered with either 0.05 M acetate (pH 4.5), 0.1 M phosphate (pH 7), 0.05 M Tris-hydrochloride (pH 8.5), or 0.05 M carbonate-bicarbonate (pH 10.5), and the four washed cell preparations were suspended in the different buffered saline solutions (ca. 100 ml) to an optical density at 650 nm (1-cm light path) of ca. 20. The cells were sonically disrupted (Branson Sonifier, model W-185, Branson Sonic Power Co., Danbury, Conn.), at 4°C, with 200-μm glass beads (1 g/100 ml; The Virtis Co., Gardiner, N.Y.) at a power of 90 W for intervals of 10 min, followed by 5-min rest periods, until intact cells were not observed by Gram stain. Mg^{2+} and pancreatic deoxyribonuclease I (1× crystallized, Worthington Biochemical Corp., Freehold, N.J.) were added to each of the four disrupted cell suspensions (final concentrations of 1 mM and 10 μg/ml, respectively), and after incubation (37°C for 4 h) with gentle mixing on a gyratory shaker (model G-25, New Brunswick Scientific Co., New Brunswick, N.J.), debris was removed by centrifugation (13,000 × *g*, 4°C, 20 min).

Concentrates of the four cell-free extracts were prepared by ammonium sulfate precipitation. Enzyme grade (Schwarz/Mann, Orangeburg, N.Y.) ammonium sulfate (39 g) was added slowly to each of the cell-free extracts (ca. 100 ml) with gentle stirring at room temperature (ca. 60% saturation at 25°C). The precipitates were removed by centrifugation (13,000 × *g*, 4°C, 30 min), and additional ammonium sulfate (22.7 g) was added slowly, with gentle stirring at room temperature, to each of the supernatant fluids (final concentration of ca. 90% saturation). After 18 to 24 h at 4°C, the precipitates were sedimented by centrifugation (25,000 × *g*, 4°C, 30 min), the pellets were dissolved in glass-distilled water (10 ml), and the solutions were lyophilized after dialysis for 24 h against 8 liters of glass-distilled water. The four preparations were dissolved in 0.1 M ammonium bicarbonate (5 mg/ml) and were assayed for PPP activity.

A control experiment also was performed to determine whether the PPP is precipitated better by ammonium sulfate at pH 7 than at pH 4.5, 8.5, or 10.5. A cell-free extract was prepared at pH 7, as described above, from cells obtained from 10 liters of a brain heart infusion broth culture, and was lyophilized after being desalted by dialysis against glass-distilled water. Portions (ca. 700 mg) of the lyophilized extract were dissolved in the four buffered saline solutions mentioned above (50 ml), concentrates were prepared as described in the preceding paragraph, and the concentrates were assayed for PPP activity.

Influence of cell wall choline and ethanolamine on generation of the PPP. *S. pneumoniae* type I cells from 5 liters of a choline-containing M-medium culture and from 5 liters of an ethanolamine-containing M-medium culture were washed twice, at 4°C, with 200 ml of 0.85% NaCl buffered with 0.1 M phosphate (pH 7) and were suspended in buffered saline (ca. 100 ml) to an optical density at 650 nm (1-cm light path) of ca. 20. Cell-free extracts of the two suspensions were prepared, as described in the previous section, by sonic disruption followed by deoxyribonuclease treatment and removal of debris by centrifugation. The supernatant fluids were adjusted to pH 7.4 with 1 M NaOH, and the concentrates were prepared, as described in the previous section, by ammonium sulfate precipitation. The two concentrates were dissolved in 0.1 M ammonium bicarbonate (5 mg/ml) and were assayed for PPP activity.

Comparison of PPP activity in cell-free extract concentrates obtained from wild-type pneumococci and autolysin-defective mutant. *S. pneumoniae* type I, strain R61, and strain cwl-1 cells obtained from 5 liters of brain heart infusion broth cultures were processed as described in the previous section, and the cell-free extract concentrates were assayed for PPP activity.

Autolysin-induced generation of purpurogenic activity and characterization of PPP released from the pneumococcal cell wall. Crude autolytic enzyme and purified pneumococcal cell wall preparations were obtained from strain R61 as described by Mosser and Tomasz for strain R 36A (14). Mixtures consisting of cell wall suspension (50 mg in 19 ml of 0.85% NaCl containing 2 mM 2-mercaptoethanol and buffered with 0.01 M K_2HPO_4-HCl, pH 7

[PBS-ME]), crude autolytic enzyme (10 mg, dry weight, in 1 ml of PBS-ME), and a few drops of chloroform were incubated (37°C for 18 to 20 h) with gentle mixing on a gyratory shaker. Debris was removed by centrifugation (25,000 × *g*, 4°C, 20 min), and after dialysis for 1 day against 8 liters of glass-distilled water, the supernatant fluids were lyophilized. The lyophilized preparations were dissolved in 0.1 M ammonium bicarbonate (5 mg/ml) and were assayed for PPP activity. Portions of twofold serial dilutions of the autolysin preparations and samples (2 mg) of the purified cell wall preparations also were examined for PPP activity.

A lyophilized preparation (36 mg in 7 ml of 0.1 M ammonium bicarbonate) of the dialyzed supernatant fluids from a cell wall-autolysin incubation mixture was applied to a column (2.6 by 90 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 0.1 M ammonium bicarbonate at 4°C, and was eluted at a flow rate of 20 ml/h. Fractions (5.0 to 5.5 ml) were assayed for absorbance at 280 and 260 nm and for purpurogenic activity. The active fractions were pooled and lyophilized, and samples (100 μg) of the lyophilized preparation were examined, as previously described (2), for sensitivity to inactivation by lysozyme. Samples also were assayed for total hexosamine (1), which is a component of pneumococcal PG and cell wall teichoic acid, and for choline (21), which is a component of pneumococcal cell wall teichoic acid. For comparative purposes, total hexosamine and choline assays also were performed on PPP obtained from cell-free extracts of strain R61 by the same procedure described for isolating the PPP of *S. pneumoniae* type I (2).

RESULTS

Effect of pH on generation of the PPP. Studies by other investigators have shown that pneumococcal autolysin has optimal activity at pH 6.9 to 7.0 and that the activity is greatly reduced at a pH of <5 or >8 (5). The concentrate of the pneumococcal cell-free extract prepared at pH 7 contained ca. 24-fold, 7-fold, and 230-fold more purpurogenic activity than extract concentrates prepared at pH 4.5, 8.5 and 10.5, respectively (Table 1). In addition, the results of a control experiment to determine whether the PPP is precipitated better by ammonium sulfate at pH 7 than at pH 4.5, 8.5, or 10.5 did not reveal any significant difference between the amount of PPP precipitated at pH 7 and the amount of PPP precipitated at pH 4.5, 8.5, and 10.5. Our results indicate, therefore, that concentrates of pneumococcal cell-free extracts prepared at a pH which is inhibitory to autolysin activity contain significantly less PPP activity than extract concentrates prepared at a pH which is optimal for autolysin activity.

TABLE 1. *Purpurogenic activity of concentrates derived from S. pneumoniae type I cell-free extracts prepared at various pH's*

pH[a]	Yield of concentrate (mg, dry wt)	PPP units per mg	Total PPP units recovered
4.5	41	4	164
7.0	122	32	3,904
8.5	70	8	560
10.5	17	1	17

[a] PPP activity is known to be stable at pH 4.5 to 10.5 (2).

Influence of cell wall choline and ethanolamine on generation of the PPP. *S. pneumoniae* has a nutritional requirement for choline (15) and choline has been reported to be a component of pneumococcal cell wall teichoic acid (14). In addition, if choline is replaced by ethanolamine, the bacteria do not exhibit autolysis (22) because their cell walls are autolysin-resistant and because they produce an abnormal autolysin which has markedly reduced catalytic activity (5, 24). We found that concentrates of cell-free extracts obtained from type I cells grown in choline-containing M-medium had as much purpurogenic activity as previously reported (2) for extract concentrates derived from type I cells grown in brain heart infusion broth. However, purpurogenic activity was not detected in extract concentrates derived from type I cells grown in ethanolamine-containing M-medium (Table 2). Thus, extract concentrates obtained from cells grown in choline-containing M-medium had at least 30-fold more purpurogenic activity than extract concentrates obtained from cells grown in ethanolamine-containing M-medium (Table 2).

Comparison of PPP activity in cell-free extract concentrates obtained from wild-type pneumococci and autolysin-defective mutant. Concentrates of cell-free extracts obtained from the unencapsulated strain R61,

TABLE 2. *Purpurogenic activity of cell-free extract concentrates derived from choline-containing and ethanolamine-containing S. pneumoniae type I*

Source of concentrate	Yield of concentrate (mg, dry wt)	PPP units per mg	Total PPP units recovered
Choline-containing cells	100	16	1,600
Ethanolamine-containing cells	110	<0.5[a]	<55

[a] Half the mice which received 2 mg of the preparation died within 24 h postinjection, presumably due to the toxicity of ethanolamine; however, none of the surviving mice exhibited purpura. In addition, none of the mice injected with 1, 0.5, or 0.25 mg of the preparation died or exhibited purpura.

which produces a normal amount of autolysin, contained as much purpurogenic activity as extract concentrates derived from *S. pneumoniae* type I. However, purpurogenic activity was not detected in extract concentrates prepared from strain cwl-1, the autolysin-defective mutant of strain R61 (Table 3). Thus, extract concentrates obtained from the wild-type strains had at least 61- to 112-fold more purpurogenic activity than extract concentrates obtained from the autolysin-defective mutant strain (Table 3).

Autolysin-induced generation of purpurogenic activity and characterization of PPP released from the pneumococcal cell wall. Purpurogenic activity was not detected in the purified cell wall preparations. The cell wall-autolysin mixtures (50 mg of cell walls and 10 mg of crude autolysin preparation) contained ca. 160 PPP units before incubation, because the crude autolysin preparations were obtained from pneumococcal cell-free extracts and, therefore, also possessed small amounts of the PPP (16 PPP units per mg).

The turbidity of the cell wall-autolysin mixtures (optical density at 650 nm, 1.25) was markedly reduced after incubation at 37°C for 18 to 20 h (optical density at 650 nm, 0.15). The dry weight of the lyophilized preparations obtained from the dialyzed supernatant fluids of the incubation mixtures was ca. 41 mg, which was composed of 31 mg of solubilized cell wall constituents (not sedimented by centrifugation at 25,000 × *g* for 20 min) and 10 mg of crude autolysin preparation. The specific activity of the preparations was ca. 125 PPP units per mg; thus, the dialyzed supernatant fluids of the incubation mixtures contained ca. 5,125 PPP units, which was ca. 32-fold more than that possessed by the cell wall-autolysin mixtures before incubation. The results indicate, therefore, that the PPP is released or generated by the action of autolysin on the pneumococcal cell wall.

TABLE 3. *Purpurogenic activity of concentrates derived from cell-free extracts of S. pneumoniae type I and strain R61 and cwl-1*

Source of concentrate[a]	Yield of concentrate (mg, dry wt)	PPP units per mg	Total PPP units recovered
Type I	115	16	1,840
Strain R61	105	32	3,360
Strain cwl-1	59	<0.5[b]	<30

[a] Type I, Encapsulated and produces normal amount of autolysin; Strain R61, unencapsulated and produces normal amount of autolysin; Strain cwl-1, autolysin-defective mutant of strain R61.

[b] No activity was detected in 2 mg of preparation.

The yield of PPP obtained by Sepharose 6B gel filtration of the supernatant fluids from the cell wall-autolysin incubation mixture was ca. 10 mg, and the smallest amount required to elicit purpura was ca. 4 μg. The crude autolysin preparation contained ca. 6% (wt/wt) PPP, and ca. one-fourth of the dry weight of the preparation applied to the gel column was contributed by the autolysin preparation. We estimate, therefore, that the PPP preparation recovered after gel filtration consisted of ca. 95% (wt/wt) PPP generated by the action of autolysin on the cell wall preparation and of 5% PPP which was present in the autolysin preparation. Activity was eluted at the void volume of the Sepharose 6B gel column (Fig. 1) and was destroyed by incubation with lysozyme, thus indicating that activity was associated with molecules having an apparent molecular weight of at least 1×10^6 to 5×10^6 and requiring intact β-1,4-glucosidic linkages. The preparation contained ca. 22% (wt/wt) total hexosamine and 5% (wt/wt) choline. Samples of PPP isolated, for comparative purposes, from cell-free extracts of sodium deoxycholate-lysed *S. pneumoniae*, strain R61, contained ca. 20% total hexosamine and 5% choline.

DISCUSSION

Recently, we isolated the pneumococcal purpura-producing principle (PPP) from cell-free extracts of *S. pneumoniae* type I and demonstrated that PPP activity was associated with water-soluble, high-molecular-weight PG fragments of the bacterium (2). The present communication is an extension of our earlier studies and presents data which strongly support the idea that the PPP is generated or liberated by the action of the pneumococcal autolysin (*N*-acetylmuramyl-L-alanine amidase) on the bacterial cell wall. Our observation that the PPP is inactivated by incubation with lysozyme (2, and confirmed in this paper) shows that activity requires the intact β-1,4-glucosidic linkages of PG. It is not known at this time, however, whether the soluble PG is the active moiety or whether it serves, when of a certain minimum size, as a carrier for the active moiety. Thus, we should not, at the present time, rule out the possibility that the active moiety of the PPP is a preformed product (i.e., not requiring pneumococcal autolysin for its generation) which is bound to the autolysin-generated, carrier PG fragments. Incubation of pneumococcal cell walls with autolysin has been reported to yield a water-soluble, high-molecular-weight complex of glycan and teichoic acid (14). Furthermore, the results of our assays for choline in PPP isolated from sodium deoxycholate-lysed cells and from cell walls solubilized by autolysin show

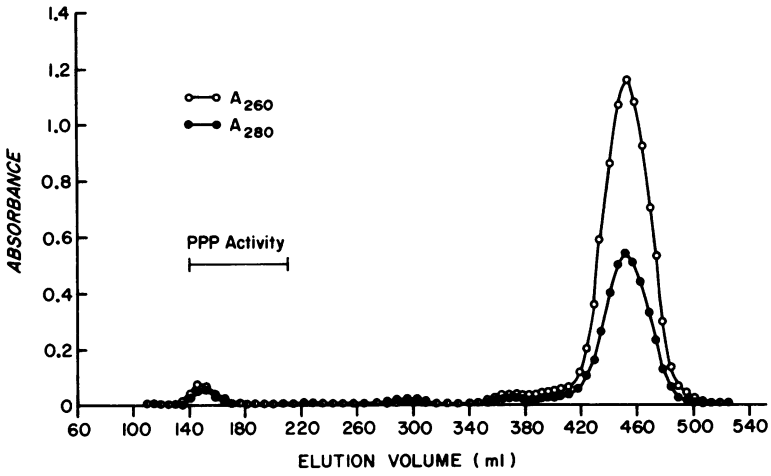

FIG. 1. *Sepharose 6B gel filtration of supernatant fluids from a pneumococcal cell wall-autolysin incubation mixture. The column void volume was approximately 150 ml. Fractions (5.0 to 5.5 ml) were assayed for absorbance at 280* (●) *and 260 nm* (○) *and for purpurogenic activity. The active fractions are enclosed in brackets.*

that both types of preparations contain a significant amount of teichoic acid. It would, therefore, be of interest to determine whether the presence of teichoic acid is required for activity of the pneumococcal PPP.

Water-soluble PG fragments from bacteria other than the pneumococcus have not been found to elicit purpura in experimental animals. However, incubation of *Streptococcus salivarius, S. mutans, S. faecalis, Staphylococcus epidermidis, Bacillus megaterium, Micrococcus lysodeikticus*, and *Lactobacillus plantarum* cell walls with *Streptomyces* sp. enzyme preparations possessing *N*-acetyl muramyl-L-alanine amidase activity and D-alanyl-*meso*-A_2pm endopeptidase activity has been reported to liberate water-soluble PG fragments capable of eliciting adjuvant arthritis in the rat (8–9). On the other hand, incubation of the cell walls with a *Streptomyces* sp. enzyme preparation containing endo-*N*-acetylmuramidase activity (specificity identical to lysozyme) liberated water-soluble PG which lacked (9) or had only weak (8) arthritogenic activity. This finding is relevant to our observation that PPP is inactivated by incubation with lysozyme and suggests that both the purpurogenic activity associated with PG fragments derived from the pneumococcus and the arthritogenic activity associated with PG fragments obtained from the other gram-positive bacteria mentioned above require a certain minimal glycan chain length. The minimal glycan chain length required for PPP activity has not, as yet, been determined. However, Koga et al. (8) found that the minimal glycan chain length required for arthritogenic activity was 2 disaccharide units, but PG with a glycan chain length of more than 5 disaccharide units was more potent than PG with shorter glycan chain lengths. They also reported that the potency of their water-soluble PG preparations was not influenced by the presence of PG-linked teichoic acid and polysaccharide. In addition, Rotta et al. (18) have observed that synthetically prepared, low-molecular-weight analogs of streptococcal PG subunits (i.e., *N*-acetyl muramyl dipeptide and *N*-acetyl muramyl tetrapeptide) possess pyrogenic and thrombocytolytic activities comparable to those of peptidoglycan isolated from group A streptococcal cell walls.

Our finding that pneumococcal autolysin is required for the generation of the PPP is not the first report of a role for the enzyme in biological phenomena involving pneumococci. Pneumococcal autolysin activity has been demonstrated to play a key role in (i) penicillin-induced lysis of pneumococci (23), (ii) unmasking, during the competent state of genetic transformation, of deoxyribonucleic acid binding sites on the cytoplasmic membrane (19), (iii) separation of daughter cells during cell division (25), and (iv) sensitivity to infection, release of progeny phage, and phage-induced lysis of host cells by the pneumococcal Dp-1 bacteriophage (13, 17).

The method to obtain purified PPP which is described in this paper, i.e., Sepharose 6B gel filtration of a cell wall preparation solubilized by pneumococcal autolysin, is simpler and yields ca. sevenfold more PPP than our previously described scheme (2) for isolating PPP from pneumococcal cell-free extracts. In addition, both methods yield PPP having similar specific

activity, sensitivity to lysozyme inactivation, molecular weight, and total hexosamine and choline content. At the present time, we are performing more detailed chemical analyses on the PPP preparations obtained by the two different methods.

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