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ACTIVATION OF THE ALTERNATIVE COMPLEMENT PATHWAY BY PNEUMOCOCCAL CELL WALL TEICHOIC ACID[1]

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Previous studies have shown that the cell wall plays a major role in the activation of the alternative pathway by the pneumococcus. The present studies were performed in order to identify the biochemical component of the pneumococcal cell wall responsible for activating the alternative pathway.

Teichoic acids were extracted from purified pneumococcal cell walls by three different procedures, and in each case they were able to activate the alternative pathway at concentrations as low as 3 to 10 μg/ml. In contrast, the residual cell walls, which contained the peptidoglycan, lost their activity in proportion to the amount of teichoic acid that had been extracted. Notably, cell walls extracted with periodate lost over 98% of their teichoic acid and the residual peptidoglycan was unable to activate the alternative pathway at concentrations as high as 1 mg/ml.

When an autolysin defective pneumococcal strain is grown in the presence of penicillin, cell wall polymers are secreted into the medium. Crude preparations of secreted cell wall polymers were found to be able to activate the alternative pathway. The activity was removed by absorption with TEPC-15 myeloma with anti-choline specificity. Since both cell wall teichoic acid and lipoteichoic acid contain choline and are removed by TEPC-15 absorption, the activity in the crude preparations of cell wall polymers could have been due to one or both of these compounds. However, purified lipoteichoic acid was unable to activate the alternative pathway. Thus, the ability of crude preparations of cell wall polymers to activate the alternative pathway was due to the presence of cell wall teichoic acid.

The results of these studies demonstrate that cell wall teichoic acid, rather than peptidoglycan, is responsible for pneumococcal activation of the alternative pathway.

Activation of the terminal C components, C3 to 9, plays an important role in the host's defense against bacterial infection (1, 2). Bacteria may activate C3 to 9 by either the classical pathway or the alternative pathway (3, 4). Whereas bacterial activation of the classical pathway is important in acquired immunity, bacterial activation of the alternative pathway plays a significant role in natural immunity.

The biochemical component of Gram-negative bacteria responsible for activating the alternative pathway has been identified as cell wall endotoxic lipopolysaccharide (5). However, the biochemical component of Gram-positive bacteria responsible for activating the alternative pathway has not been identified. Our previous studies on the interaction of pneumococci and the alternative pathway have demonstrated that the cell wall, rather than the cell membrane or capsule, plays a major role in the activation of the alternative pathway (6, 7).

The present studies were performed in order to identify the biochemical component of the pneumococcal cell wall that activates the alternative pathway. The results demonstrate that pneumococcal cell wall teichoic acid, rather than peptidoglycan, is responsible for activating the alternative pathway.

MATERIALS AND METHODS

Buffers. Veronal-buffered saline, pH 7.4, with ionic strength of 0.147, 0.15 mM Ca^{++}, 1 mM Mg^{++}, and 0.1% gelatin (GVB^{++})[3] was prepared as in Reference 8. Veronal-buffered saline, pH 7.4, with ionic strength of 0.074, 0.15 mM Ca^{++}, 1 mM Mg^{++}, 0.1% gelatin, and 2.5% dextrose ($DGVB^{++}$) was prepared as in Reference 9.

Serum. Guinea pig serum deficient in the fourth component of C (C4D GPS) was obtained from animals with a genetically determined complete deficiency of C4 (10), pooled, frozen, and stored in small aliquots at −70°C.

Antiserum. Rabbit antibody to purified guinea pig factor B (11) (Ra-a-GP factor B) was kindly supplied by Dr. Anne Nicholson and was rendered monospecific by absorbing it with 1/8 volume of heated (56°C for 1 hr) GPS for 1 hr at 37°C (12). A purified mouse IgA myeloma, TEPC-15, with anti-choline and anti-phosphocholine specificity was purchased from Linton Bionetics, Bethesda, Maryland.

Purified guinea pig C3. Purified guinea pig C3 was supplied by Dr. Hyun S. Shin (13).

Purified pneumococcal cell walls. Purified pneumococcal cell walls were prepared as previously described (14). Briefly, a strain of unencapsulated pneumococci, R36A, was grown in a chemically defined medium, washed in 0.15 M saline, heated at 70°C for 15 min, and disrupted in a Mickle disintegrator until there were no recognizable bacterial forms. The crude cell walls were isolated by centrifugation, washed twice in

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[3] Abbreviations used in this paper: GVB^{++}, Veronal-buffered saline; $DGVB^{++}$, Veronal-buffered saline with dextrose; C4D GPS, guinea pig serum deficient in C4; Ra-a-GP factor B, rabbit antibody to guinea pig factor B.

acetone and twice in 0.15 M saline, and then treated with DNase and RNase for 4 to 12 hr at 37°C, followed by two sequential 12-hr incubations with trypsin at 37°C. The resultant purified cell walls were washed six times in 0.15 M NaCl, six times in distilled water, heated at 100°C for 30 min, washed once more in distilled water, and lyophilized.

Pneumococcal lipoteichoic acid. Pneumococcal lipoteichoic acid was purified by following its Forssman antigen activity as previously described (15). Briefly, pneumococci were lysed with toluene at 37°C for 3 days. The resultant pellet was separated by centrifugation, washed several times in 0.05 M acetate buffer, pH 4.5, and dialyzed against 0.15 M saline. The material was solubilized by repetitive treatment with trypsin over a period of several days, and then sequentially defatted first in 95% acetone, then in ethanol-ether (3:1) and finally in chloroform-methanol (1:1). The dried residue was then extracted with distilled water, the extract treated with DNase and RNase, and precipitated with acetone.

Secreted pneumococcal cell wall polymers. When an autolysin defective derivative of pneumococcal strain R36A (16) is treated with penicillin, cell wall polymers are secreted into the growth medium (17). Pneumococci in the exponential phase of growth and at a concentration of 3 to 5 × 10^6/ml were treated with benzyl-penicillin at a concentration of 0.05 μg/ml for 90 min. The supernatant fluid was dialyzed extensively against water at 4°C and lyophilized.

Extraction of teichoic acid from pneumococcal cell walls. Teichoic acids were extracted from purified pneumococcal cell walls by three different procedures (14). Pneumococci were grown in the presence of ^{3}H-choline and the radiolabeled choline was incorporated into the teichoic acid. Purified pneumococcal cell walls, containing the ^{3}H-choline, were treated at a concentration of 1 mg/ml with either 0.05 M sodium metaperiodate at 37°C for 45 min, or 100% formamide at 170°C for 20 min, or 5% trichloroacetic acid at 4°C for 7 days. The solubilization of the teichoic acids was followed by measuring the release of ^{3}H-choline into the supernatant fluid. In each case, the solubilized teichoic acids were dialyzed extensively against distilled water and lyophilized, while the residual insoluble cell walls were washed extensively in distilled water and lyophilized.

Absorption of secreted cell wall polymers with TEPC-15 myeloma. Secreted cell wall polymers were incubated at a concentration of 200 μg/ml in TEPC-15 myeloma at a concentration of 800 μg/ml of GVB^{++} for 3 hr at 37°C and then for 18 hr at 4°C. An equal volume of saturated ammonium sulfate was added and after incubation at 4°C for 18 hr the precipitates, containing the TEPC-15 myeloma, were removed by centrifugation. The supernatants were then dialyzed extensively against GVB^{++}. Control secreted cell wall polymers were treated the same way except that they were incubated initially in GVB^{++} without TEPC-15. In some experiments 1 mg/ml of the TEPC-15 myeloma was preincubated with 10 mg/ml of phosphocholine at 37°C for 3 hr and then at 4°C for 18 hr before being used to absorb the secreted cell wall polymers.

C3 consumption. The activation of C3 via the alternative pathway was measured as previously described (4). The test material was suspended to the desired concentration in C4D GPS diluted 1:10 in GVB^{++} and the mixture was incubated at 37°C. After 30 min a sample was obtained and diluted immediately in ice-cold $DGVB^{++}$, and the titer of C3 was determined (9). The consumption of C3 in each test sample was expressed as a percentage of the C3 remaining in a control sample of C4D GPS incubated with buffer alone. C4D GPS was used throughout to insure that the consumption of C3 occurred via the alternative pathway.

In order to confirm that the activation of C3 in C4D GPS occurred via the alternative pathway, each test material was also incubated at a concentration of 100 μg/ml in C4D GPS for 30 min at 37°C and the conversion of factor B from a β- to γ-migrating protein was demonstrated by immunoelectrophoresis. In addition, in order to preclude the direct activation of C3, each test material was incubated at a concentration of 100 μg/ml in 60 units of purified C3 for 30 min at 37°C, and there was no consumption of C3.

RESULTS

Ability of extracted teichoic acids and residual cell walls to activate the alternative pathway. When purified pneumococcal cell walls were treated with periodate, 98% of the ^{3}H-choline was extracted. As can be seen in Figure 1, the extracted teichoic acid was able to consume C3 in C4D GPS. In contrast, the residual cell wall, free of teichoic acid but containing the peptidoglycan, was unable to consume C3 in C4D GPS even at a concentration as high as 1000 μg/ml.

When purified pneumococcal cell walls were treated with hot formamide, 71% of the ^{3}H-choline was extracted. As can be seen in Figure 2, the teichoic acid extracted by this technique was also able to consume C3 in C4D GPS. In contrast, the residual cell wall, containing all of the peptidoglycan and 29% of the original teichoic acid, was significantly less active than either the extracted teichoic acid or purified cell wall, although it did have some residual activity.

Purified pneumococcal cell walls were also treated with cold TCA and only 21% of the ^{3}H-choline was extracted. As can be seen in Figure 3, the teichoic acid extracted by this technique was also able to consume C3 in C4D GPS. The residual cell wall, which still contained 79% of the original teichoic acid in addition to peptidoglycan, was also able to activate C3 in C4D GPS and lost little, if any, of its original activity.

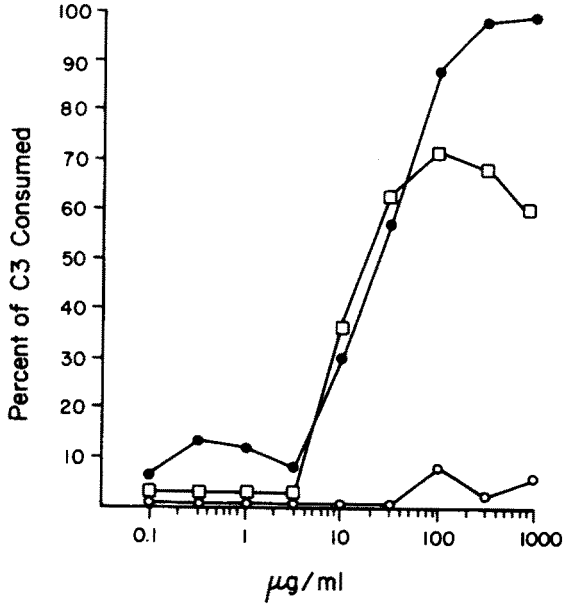

Figure 1. The consumption of C3 in C4D GPS by purified pneumococcal cell walls (●–●), teichoic acids extracted from cell walls using periodate (□–□), and residual cell walls after periodate extraction (○–○). Each preparation was suspended to the desired concentration in C4D GPS diluted 1/10, incubated at 37°C for 30 min, and the consumption of C3 determined.

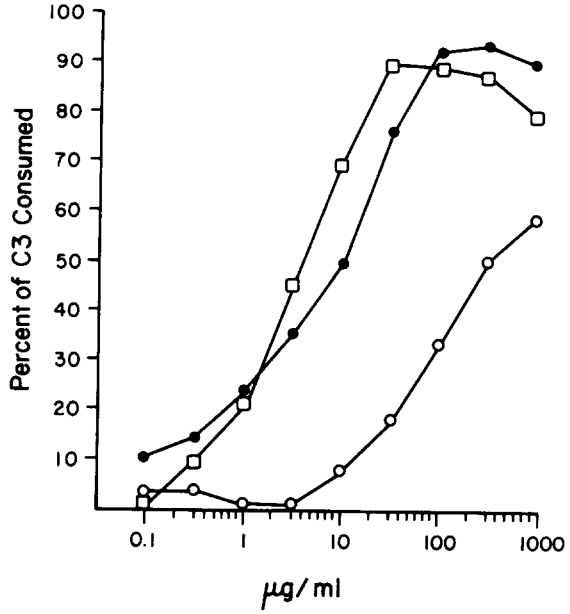

Figure 2. The consumption of C3 in C4D GPS by purified pneumococcal cell walls (●-●), teichoic acids extracted from cell walls using formamide (□-□), and residual cell walls after formamide extraction (○-○). Each preparation was suspended to the desired concentration in C4D GPS diluted 1/10, incubated at 37°C for 30 min, and the consumption of C3 determined.

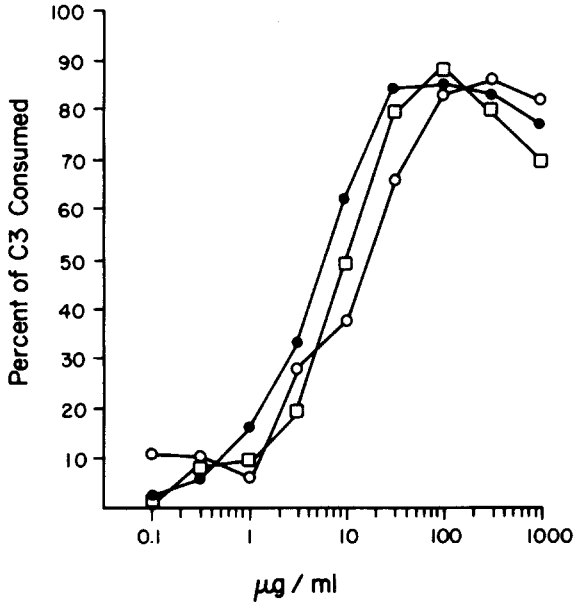

Figure 3. The consumption of C3 in C4D GPS by purified pneumococcal cell walls (●-●), teichoic acids extracted from cell walls using TCA (□-□), and residual cell walls after TCA extraction (○-○). Each preparation was suspended to the desired concentration in C4D GPS diluted 1/10, incubated at 37°C for 30 min, and the consumption of C3 determined.

Thus, teichoic acids extracted by three different techniques from purified pneumococcal cell walls are able to activate the alternative pathway equally as well as the purified cell walls from which they were derived. In contrast, peptidoglycan when free of contaminating teichoic acid is unable to activate the alternative pathway.

Ability of lipoteichoic acid to activate the alternative pathway. When purified pneumococcal lipoteichoic acid was incubated in C4D GPS little, if any, C3 was consumed even at concentrations as high as 1000 μg/ml (Fig. 4).

Ability of secreted pneumococcal cell wall polymers to activate the alternative pathway. As can be seen in Figure 5, a crude preparation of secreted pneumococcal cell wall polymers was able to consume C3 in C4D GPS. Although there was significant activity in these preparations, they were not as active as purified cell walls.

Crude preparations of secreted cell wall polymers contain a variety of cell wall components in addition to teichoic acids (18). In order to determine if teichoic acid was responsible for the ability of secreted cell wall polymers to activate the alternative pathway, a crude preparation of secreted cell wall polymers had its choline-containing teichoic acids removed by

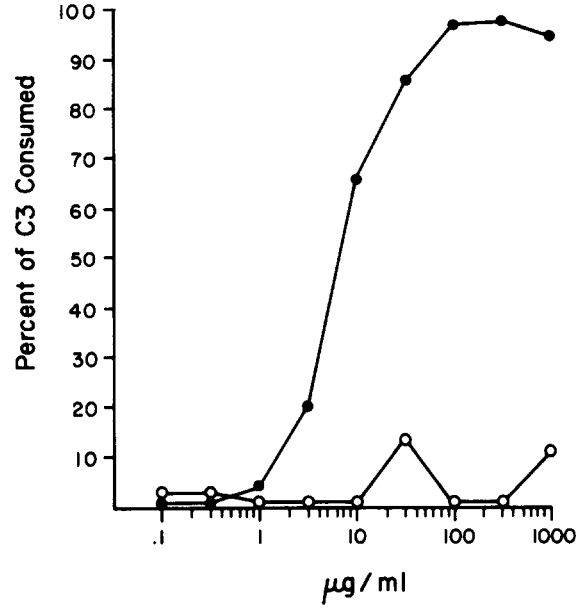

Figure 4. The consumption of C3 in C4D GPS by purified pneumococcal cell walls (●-●), and by lipoteichoic acid (○-○). Each preparation was suspended to the desired concentration in C4D GPS diluted 1/10, incubated at 37°C for 30 min, and the consumption of C3 determined.

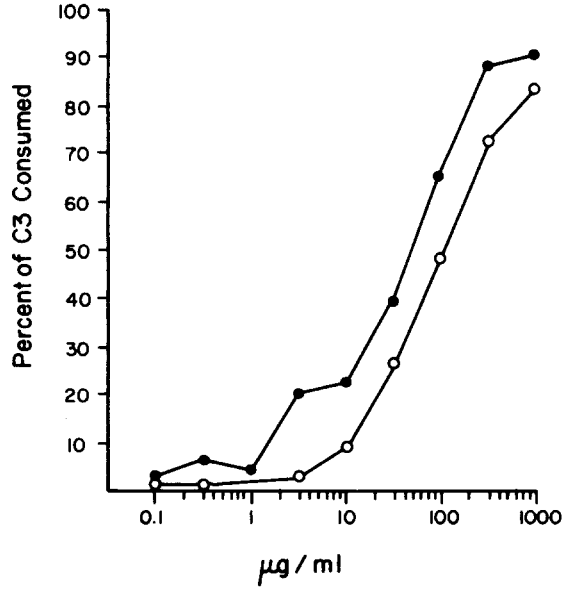

Figure 5. The consumption of C3 in C4D GPS by purified pneumococcal cell walls (●-●), and by secreted cell wall polymers (○-○). Each preparation was suspended to the desired concentration in C4D GPS diluted 1/10, incubated at 37°C for 30 min, and the consumption of C3 determined.

TABLE I

Ability of secreted cell wall polymers to activate the alternative pathway

Treatment of Secreted Cell Wall Polymers	% of C3 Consumed in C4D GPS
None	40
Absorbed with TEPC-15	6
Absorbed with TEPC-15 that had been preincubated in phosphocholine	31

absorption with TEPC-15. As can be seen in Table I, such a preparation of absorbed cell wall polymers had little, if any, ability to consume C3 in C4D GPS. In contrast, when the crude preparation of secreted cell wall polymers was absorbed with TEPC-15 that had first been preincubated with phosphocholine, it retained its ability to activate C3 in C4D GPS.

Thus, the ability of crude preparations of secreted pneumococcal cell wall polymers to activate the alternative pathway is due to the presence of cell wall teichoic acid.

DISCUSSION

Our previous studies have shown that the pneumococcal cell wall is responsible for activating the alternative pathway (6, 7). The cell walls of most Gram-positive bacteria are composed of two repetitive polymers, teichoic acid and peptidoglycan (19). In fact, these two polymers account for approximately 95% of the dry weight of purified pneumococcal cell walls (14). The results of the present study demonstrate that pneumococcal cell wall teichoic acid, rather than peptidoglycan, is responsible for activating the alternative pathway.

Teichoic acids were extracted from pneumococcal cell walls by three different procedures, and in each case, they were able to activate the alternative pathway at concentrations as low as 3 to 10 μg/ml. In contrast, the residual cell walls, which contained the peptidoglycan, lost their activity in proportion to the amount of teichoic acid that had been extracted. Notably, cell walls extracted with periodate lost 98% of their teichoic acid and the residual cell wall peptidoglycan was unable to activate the alternative pathway at a concentration as high as 1 mg/ml. It is possible that the residual cell wall preparations lost their activity because the peptidoglycan had been altered in some way during the different extractions. However, that possibility is unlikely since the three extraction procedures used in these experiments do not solubilize radiolabeled lysine incorporated in cell wall peptidoglycan, indicating that the peptidoglycan is left intact (14). Our finding that pneumococcal peptidoglycan is unable to activate the alternative pathway is in agreement with a previous study showing that peptidoglycans isolated from *Staphylococcus aureus* and from a group A Streptococcus were inefficient in activating the alternative pathway even at concentrations as high as 2 mg/ml (20). In that same study, teichoic acids were not tested for their ability to activate the alternative pathway.

In a second series of experiments, preparations of secreted cell wall polymers were shown to activate the alternative pathway. The activity could be removed by absorption with TEPC-15 myeloma with anti-choline specificity. Since both cell wall teichoic acid and lipoteichoic acid contain choline and both are removed by absorption with TEPC-15, the activity in the preparations of secreted cell wall polymers could have been due to one or both of these compounds. However, in another experiment, purified lipoteichoic acid was unable to activate the alternative pathway. Thus, the ability of crude preparations of secreted cell wall polymers to activate the alternative pathway is due to the presence of cell wall teichoic acid. The activation of the alternative pathway by secreted native cell wall teichoic acid makes it unlikely that the extracted teichoic acids activated the alternative pathway as a result of some change induced in them during the extractions.

The mechanism by which pneumococcal cell wall teichoic acids activate the alternative pathway is unknown. Recently, it has been shown that zymosan and rabbit erythrocytes, both of which activate the alternative pathway, provide privileged sites that protect alternative pathway components deposited on their surfaces from the controlling action of C3b inactivator and β1H (21, 22). In this way, the zymosan and rabbit erythrocytes allow the transition from low grade cleavage of C3 in the fluid phase to amplified cleavage of C3 on their surface. Whether it is by this or another mechanism that pneumococci and other bacteria activate the alternative pathway, and how teichoic acids participate, remain to be investigated.

Although the present study demonstrates that cell wall teichoic acid plays a major role in pneumococcal activation of the alternative pathway, it is possible that the subcellular organelle and/or biochemical component responsible for activating the alternative pathway is not the same for all Gram-positive bacteria. In a study of a group A Streptococcus, the greatest activity was found to be associated with a heat-labile (2 hr at 70°C) protein of the cell membrane (23). Further studies will be necessary to determine whether cell walls and their teichoic acids from a variety of Gram-positive bacteria play a significant role in the activation of the alternative pathway. In this regard, preliminary data from our laboratories have shown that cell walls and teichoic acids from *Baccilus subtilis* are capable of activating the alternative pathway.

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