

## Role of the Bacterial Cell Wall in Middle Ear Inflammation Caused by *Streptococcus pneumoniae*

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The pathogenesis of middle ear inflammation caused by *Streptococcus pneumoniae* was explored in the chinchilla model with different pneumococcal cell wall (CW) preparations, including isolated native CW, M1 muramidase CW (M1-CW) digest, amidase CW digest, and M1 peptidoglycan (M1-PG) digest. Inflammatory cell and lysozyme concentrations in middle ear fluid (MEF) were measured between 6 and 72 h after the middle ears were inoculated with one of the preparations or sterile saline. Middle ear histopathology was measured quantitatively at 72 h. Native CW, M1-CW digest, and amidase-CW digest caused significantly more inflammatory cell influx and lysozyme accumulation in MEF than saline did. M1-PG digest also caused more inflammatory cell influx and lysozyme accumulation in MEF than saline did but caused less inflammation than native CW or either CW digest. Epithelial metaplasia was significantly greater in ears inoculated with native CW than in ears inoculated with the CW or PG digest or with saline. Pneumococcal CW is, therefore, the principal factor that initiates middle ear inflammation in acute pneumococcal otitis media, and CW teichoic acid seems to be important in initiating this response.

*Streptococcus pneumoniae* is the most frequent cause of acute otitis media. It has consistently been cultured from about 30% of acute middle ear effusions (3, 6). In addition, pneumococcal capsular polysaccharide has been detected in 60% of culture-negative acute effusions, which make up about 30% of all effusions (13). Pneumococcus, therefore, causes about 50% of all acute otitis media episodes.

In 5 to 10% of the children affected, acute otitis media progresses to chronic otitis media with effusion, which has been associated with conductive hearing loss and irreversible middle ear pathology. Like acute middle ear effusions, culture-negative chronic effusions show evidence of bacteria and bacterial antigens (4, 7, 12), suggesting that killed bacteria may be retained in the middle ear.

The mechanism by which bacteria produce acute otitis media and their role in the pathogenesis of chronic otitis media with effusion are not well understood. However, the presence of nonviable bacteria and their envelope components in middle ear fluid (MEF) has led to the hypothesis that nonviable pneumococci or their subcellular envelope components contribute to both the initiation or persistence of middle ear inflammation and the transition from acute to chronic otitis media.

Isolated pneumococcal cell wall has been shown to invoke inflammation in several experimental animal models (27, 28), and heat-killed encapsulated and nonencapsulated pneumococci, as well as isolated pneumococcal cell wall, induce acute middle ear inflammation in chinchillas (16, 20). In the present study, different pneumococcal cell wall preparations were utilized in the chinchilla otitis media model to further investigate the molecular basis of middle ear inflammation produced by pneumococci.

### MATERIALS AND METHODS

Cell wall preparations were isolated, as previously described (5, 15), from R36A, an unencapsulated *S. pneumoniae* strain originally derived from a type 2 encapsulated strain. Four different preparations were used: isolated native cell wall, M1 muramidase cell wall digest, amidase cell wall digest, and M1 muramidase digest of the peptidoglycan cell wall fraction (M1-PG digest). Digestion was stopped by boiling the preparation to inactivate the enzyme, and neither preparation treated with muramidase had detectable lysozyme activity (Lysozyme Quantiplates Test Kits; Kallestad, Chaska, Minn.). All preparations were diluted in sterile 0.01 M phosphate-buffered saline, pH 7.4 (PBS). The isolated cell wall preparation was sonicated for 30 s at 6 W to disperse the material before dilution, and adequate dispersion of the sample was confirmed by light microscopy. The cell wall digests were in solution. Native-cell wall inoculum sizes were 10.0, 1.0, and 0.1  $\mu\text{g}$ , and the cell wall digest inoculum size was 10.0  $\mu\text{g}$ ; the 10- $\mu\text{g}$  inoculum is approximately  $10^8$  cell equivalents, which equals the concentration of pneumococci in chinchilla middle ear effusion 24 to 48 h after 40 CFU are injected into the ear (11).

Thirty healthy chinchillas 1 to 2 years old, weighing 400 to 600 g, with normal middle ears (as ascertained by otoscopy and tympanometry) were selected. Animals were given food and water ad lib. All procedures were performed with ketamine hydrochloride anesthesia. Bilateral eustachian tube obstruction was performed with Silastic sponges, as previously described (1). The hypotympanic middle ear bullae of each chinchilla were inoculated approximately 24 h after eustachian tube obstruction, as previously described (16). The right ear of each chinchilla was inoculated with 0.5 ml of one of the cell wall preparations; the left ear was inoculated with 0.5 ml of PBS and served as the control.

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MEF was aspirated via the superior bulla 6, 24, 48, and 72 h after inoculation; 50 to 100  $\mu$ l of fluid was obtained with each aspiration. A 10- to 20- $\mu$ l aliquot of MEF was cultured on 5% sheep blood agar. Data obtained with ears that developed culture-positive MEF were excluded from analysis. Total leukocyte counts and lysozyme assays of MEF were performed as previously described (16).

All animals were killed 72 h after inoculation. Temporal bones were harvested and fixed in 10% buffered formalin, decalcified in trichloroacetic acid, and subjected to dehydration in a graded series of alcohols. Specimens were cleared with xylene, impregnated, and embedded in paraffin. Samples were cut to a thickness of 7  $\mu$ m and stained with hematoxylin and eosin. Histopathology was scored by using a modification of a previously described semiquantitative method (20). Parameters scored were middle ear mucoperiosteum thickness, number of inflammatory cells and fibroblasts, hemorrhage, edema, osteoneogenesis, vascularization of the subepithelial space, epithelial type, and goblet cells. Morphologic measurements were made by light microscopy with the aid of a calibrated grid.

Each parameter was evaluated at three anatomic locations in the middle ear cleft: the promontory, the cochlear apex, and the anterior bulla. Three adjacent areas were scored at each location, and the relative scores were averaged. Relative scores were assigned as follows: for middle ear mucosa thickness, 1 = normal (<10  $\mu$ m), 2 = 10 to 80  $\mu$ m, 3 = 81 to 140  $\mu$ m, 4 = 141 to 200  $\mu$ m, and 5 = >200  $\mu$ m; for number of inflammatory cells and fibroblasts, 1 = none, 2 = 1 to 25, 3 = 26 to 50, 4 = 51 to 100, 5 = 101 to 200, 6 = 201 to 400, and 7 = >400 cells per 625  $\mu$ m<sup>2</sup>; for hemorrhage and edema in the subepithelial space under a 100- $\mu$ m length of epithelium, 1 = none, 2 = focal, and 3 = diffuse; for vascularization in the subepithelial space along a 100- $\mu$ m length of epithelium occupied by vasculature, 1 = <10%, 2 = 10 to 20%, 3 = 21 to 30%, 4 = 31 to 40%, and 5 = >40%; for epithelial type along the 100- $\mu$ m length of epithelium, 1 = squamous, 2 = cuboidal, 3 = squamous and cuboidal, 4 = columnar, and 5 = squamous or cuboidal and columnar; for osteogenesis along the 100- $\mu$ m length of epithelium, 1 = none, 2 = <25  $\mu$ m, 3 = 25 to 50  $\mu$ m, and 4 = >50  $\mu$ m; and for goblet cells along the 100- $\mu$ m length of epithelium, 1 = none, 2 = focal, and 3 = diffuse.

Inflammatory cell and lysozyme data were logarithmically transformed, and log means were analyzed by Student's *t* test for nonpaired data (two-tailed) and by analysis of variance. Significance was defined as  $P < 0.05$ . The numbers of ears analyzed in each inoculum group were as follows: native cell wall,  $n = 7$ ; M1 cell wall digest,  $n = 10$ ; amidase cell wall digest,  $n = 7$ ; and M1-PG digest,  $n = 6$ .

Histopathologic data represent the average of the mean scores for each parameter at each of the three anatomical sets, with four to five temporal bones in each inoculum group. Means were analyzed by Student's *t* test for nonpaired data with equal variance (two-tailed), and significance was defined as  $P < 0.05$ .

This study was performed in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and the Animal Welfare Act (Public Law 89-144 as amended); the animal use protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee.

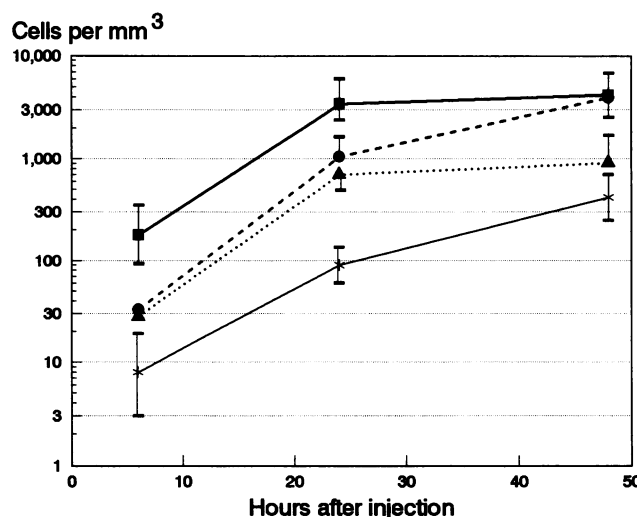


FIG. 1. Inflammatory cell concentration in MEF between 6 and 48 h after native pneumococcal cell wall was inoculated into the right middle ears of chinchillas. Cell wall inoculum sizes were 10  $\mu$ g (■), 1.0  $\mu$ g (●), and 0.1  $\mu$ g (▲). PBS was inoculated into the left ears (×), which served as controls. Data are geometric means and 95% CIs.

## RESULTS

A dose-response study was performed with 10.0-, 1.0-, and 0.1- $\mu$ g doses of native cell wall inoculated into the middle ear. All three doses induced a significant increase in the concentration of MEF inflammatory cells compared with concentrations in paired contralateral PBS-inoculated ears at all three sampling times (Fig. 1). However, 10  $\mu$ g of cell wall induced significantly more inflammatory cells than 1  $\mu$ g of cell wall at 6 h ( $t = 2.215$ ,  $P < 0.05$ ) and at 24 h ( $t = 4.202$ ,  $P < 0.01$ ), but not at 48 h. The 1- and 0.1- $\mu$ g doses induced concentrations of inflammatory cells that were not significantly different at 6 and 24 h but that were significantly different at 48 h ( $t = 4.172$ ,  $P < 0.01$ ).

Native cell wall, M1 cell wall digest, and amidase cell wall digest caused a significant increase ( $P < 0.01$ ) in MEF inflammatory cell concentration between 6 and 72 h after inoculation (Table 1) compared with that caused by PBS. The M1-PG digest, which lacked teichoic acid, caused a significant increase in MEF inflammatory cells at 6 and 24 h ( $P < 0.01$ ), but not after 24 h, compared with that caused by PBS. The M1-PG digest induced significantly fewer inflammatory cells at 24 h ( $t = 5.44$ ,  $P < 0.01$ ) than native cell wall and also induced fewer cells than M1 cell wall or amidase cell wall digests at 24 h ( $F = 5.03$ ,  $P < 0.05$ ); however, inflammatory cell concentration differences among ears inoculated with the three cell wall fractions after 24 h were not significant. Differences among ears inoculated with native cell wall, the M1 cell wall digest, and the amidase cell wall digest were not significant at any time.

Native cell wall, M1 cell wall digest, and amidase cell wall digest also caused a significant increase ( $P < 0.01$ ) in MEF lysozyme concentration compared with that obtained with PBS between 6 and 72 h after inoculation (Table 2). The M1-PG digest caused a significant increase in MEF lysozyme compared with that obtained with PBS between 6 and 48 h, but not at 72 h. The M1 cell wall digest and the M1-PG digest induced a significantly lower lysozyme response than native cell wall between 6 and 48 h. Although the lysozyme

TABLE 1. Inflammatory cell concentrations in MEF of chinchillas

Middle ear inoculum <sup>a</sup> (no. of ears tested)	Inflammatory cell concn (cells/mm <sup>3</sup> ) at <sup>b</sup> :			
	6 h	24 h	48 h	72 h
Native cell wall (7)	179 (94–340)	3,431 (2,354–5,002)	4,160 (2,770–6,247)	4,866 (3,260–7,265)
M1 cell wall digest (10)	246 (138–439)	3,281 (1,959–5,496)	3,891 (2,869–5,277)	6,279 (4,248–9,882)
Amidase cell wall digest (7)	584 (258–1,322)	2,931 (1,275–6,738)	4,351 (2,147–8,818)	6,629 (3,827–10,137)
M1-PG digest (6)	436 (198–959)	725 (466–1,129)	2,044 (1,067–3,916)	3,016 (1,312–6,932)
PBS (26)	41 (32–51)	133 (93–191)	875 (609–1,255)	1,520 (974–2,373)

<sup>a</sup> Each inoculum size was 10 µg.

<sup>b</sup> Data are geometric means (95% CIs).

concentration in ears inoculated with M1 cell wall digest reached that of native cell wall-inoculated ears by 72 h, significantly less lysozyme persisted in ears inoculated with M1-PG. Lysozyme differences between ears inoculated with native cell wall and the amidase cell wall digest were not significant at any time.

Middle ear histopathology produced by native cell wall and the cell wall digests was minimal in this acute experiment. However, significantly more epithelial metaplasia was caused by native cell wall (mean score at cochlear apex = 3.9; 95% confidence interval [CI] = 3.5 to 4.3) than by PBS (mean score at cochlear apex = 2.3; 95% CI = 1.9 to 2.8); none of the three cell wall digests caused significantly more metaplasia than PBS.

## DISCUSSION

Pneumococcal infections are characterized by an intense inflammatory reaction in infected tissues, and they cause considerable morbidity and mortality despite standard antimicrobial therapy. The pathogenesis of these infections is not well understood, but the role of the organism's structural components in host inflammatory responses has been an area of active investigation. It is well known that the polysaccharide capsule conveys virulence to pneumococcus through its antiphagocytic properties, yet it reacts poorly with complement and C-reactive protein.

The pneumococcal wall lies just under the capsular polysaccharide and consists of two major components, a peptidoglycan polymer, common to all bacterial cells, and teichoic acid, a polysaccharide covalently linked to peptidoglycan. While teichoic acid is found in many gram-positive cell walls, pneumococcal teichoic acid is unique in that it contains phosphorylcholine (15, 21). This phosphorus-containing teichoic acid is designated C-polysaccharide and is a recognition site for activation of the alternative complement pathway (10, 30) and the binding of C-reactive proteins (14),

certain myeloma proteins (10, 22, 30), and pneumococcal autolysin (8, 15).

The precise nature of the host response to pneumococcal envelope components is not known, but there is accumulating evidence that subcapsular components can evoke an inflammatory reaction, including an antibody response (9, 29). Inoculation of pneumococcal cell wall into the rabbit subarachnoid space results in cerebrospinal fluid leukocytosis and biochemical changes consistent with meningitis (24, 26, 28). The presence of teichoic acid polymers seems to be important for this inflammatory activity (23). Pneumococcal cell wall is also a potent pulmonary inflammatory agent in a rabbit model of experimental pneumococcal pneumonia (27). In addition, a purpura producing factor has been generated by the hydrolysis of pneumococcal cell wall (2). More recently, pneumococcal cell wall was shown to induce the production of interleukin-1, but not tumor necrosis factor, from isolated human monocytes (19). These studies strongly suggest that pneumococcal cell wall contributes to the pathogenesis of pneumococcal infection. Cell walls from *Bacillus subtilis*, *Micrococcus lysodeikticus*, and *Escherichia coli* also are capable of inducing inflammation in the rabbit meningitis and pneumonia models (25), and *Haemophilus influenzae* type b endotoxin is capable of inducing middle ear effusion in guinea pigs (18).

We previously demonstrated that pneumococcal cell wall, when presented to the middle ear as nonviable, nonencapsulated intact organisms, was capable of triggering the entire cellular, biochemical, and histopathologic inflammatory cascade associated with otitis media (16, 17, 20). In addition, isolated pneumococcal cell wall inoculated into the middle ears of chinchillas 5 to 7 days after eustachian tube obstruction caused histopathologic changes of acute otitis media (20). The dose response to killed encapsulated and nonencapsulated pneumococci previously reported (16) is consistent with the dose response to native cell wall reported here. Inflammation was greater with 10<sup>8</sup> killed pneumococci (10 µg

TABLE 2. Lysozyme concentrations in MEF of chinchillas

Middle ear inoculum <sup>a</sup> (no. of ears tested)	Lysozyme concn (µg/ml) at <sup>b</sup> :			
	6 h	24 h	48 h	72 h
Native cell wall (7)	7.9 (5.3–11.8)	23.5 (18.3–30.2)	30.4 (20.4–45.1)	30.9 (20.9–45.8)
M1 cell wall digest (10)	3.7 (2.8–5.0)	11.7 (10.1–13.6)	20.2 (16.8–24.2)	25.8 (20.8–31.9)
Amidase cell wall digest (7)	4.9 (3.8–6.2)	15.4 (11.7–20.2)	31.6 (25.4–39.4)	39.6 (29.6–53.1)
M1-PG digest (6)	4.1 (3.2–5.2)	7.6 (6.3–9.3)	12.5 (10.9–14.5)	15.2 (11.6–19.8)
PBS (26)	2.9 (2.7–3.2)	5.0 (4.6–5.5)	9.0 (7.8–10.4)	12.3 (10.8–14.1)

<sup>a</sup> Each inoculum size was 10 µg.

<sup>b</sup> Data are geometric means (95% CIs).

of cell wall) than with  $10^6$  pneumococci (1.0  $\mu\text{g}$  of cell wall), and the MEF inflammatory cell response to  $10^5$  pneumococci was not significantly greater than the response to PBS. The concentration of pneumococci in middle ear effusion from children with acute otitis media has not been measured.

Although this study was performed with animals within 24 h following eustachian tube obstruction, killed pneumococci also induce significant middle ear histopathology in nonobstructed chinchilla ears (20). Tubal obstruction is employed in studying pneumococcal cell wall-induced inflammation in chinchillas for the following reasons. (i) It models otitis media with effusion, a condition that persists in 60% of children after treatment of acute otitis media, when bacterial cell envelope products are likely to be present in middle ear fluid. (ii) It prevents drainage of the inoculum from the middle ear via the eustachian tube, and thus permits samples of MEF to be obtained soon after injection. (iii) In acute experiments, in which samples are obtained within 72 h after tubal obstruction, middle ear mucoperiosteal histopathology caused by obstruction alone is virtually nonexistent.

These data confirm both the ability of pneumococcal cell wall to induce acute middle ear inflammation and the importance of teichoic acid in initiating this response. Although middle ear tissue histopathology was assessed only 72 h after inoculation, native cell wall had already produced significant epithelial metaplasia. A differential effect on middle ear mucoperiosteum was not observed among the three cell wall digests, probably because of the short duration of the experiment.

Presently, pneumococcal infections are treated preferentially with  $\beta$ -lactam and cephalosporin antibiotics, whose primary target is the bacterial cell wall. While these agents effectively kill the bacteria, cell lysis results in the release of cell wall fragments, which may increase inflammation. Persistence of these fragments may lead to chronic inflammation. Tuomanen et al. (24) showed that ampicillin treatment of rabbits with pneumococcal meningitis led to a transient but significant increase in meningeal inflammation. We recently made a similar observation by using the chinchilla otitis media model (11). If, indeed, pneumococcal cell wall fragments induce and sustain middle ear inflammation, understanding the mechanisms by which these components are cleared from the middle ear becomes important and may have implications for future antipneumococcal treatments.

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