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The Cell Wall Mediates Pneumococcal Attachment to and Cytopathology in Human Endothelial Cells

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Streptococcus pneumoniae **interacts with vascular endothelial cells during the course of bacteremia. In this study, we characterized the initial attachment of pneumococci to human endothelial cells (EC) and the response of the endothelium to this interaction. Pneumococci adhered to EC in a dose-dependent fashion. Attachment was rapid, with the majority of bacteria attached by 30 min. No difference was found between the attachment of unencapsulated (R6) and encapsulated (SIII) strains. Purified pneumococcal cell wall components competitively inhibited attachment of R6 by a maximum of 60% in a dose-dependent manner. Following attachment of pneumococci or exposure of EC to pneumococcal cell wall, pronounced changes in EC morphology ensued, resulting in striking separation of the cells of the monolayer and, eventually, destruction of the cells. The cytopathic effects of the cell wall were inhibited by antibodies to interleukin-1 but not to tumor necrosis factor. Both antibodies were required to neutralize the cytopathology caused by intact pneumococci. We conclude that pneumococci attach rapidly to human EC and that the cell wall is important in this interaction. Intact pneumococci and pneumococcal cell wall induce profound morphologic changes in human EC, leading to loss of barrier integrity. These cytopathic effects are likely to be cytokine mediated.**

Streptococcus pneumoniae remains an important gram-positive pathogen of humans. This microorganism causes both localized and systemic infections, with otitis media, pneumonia, sepsis, and meningitis being the most frequent clinical syndromes (3). Meningitis caused by *S. pneumoniae* is characterized by a higher mortality than is found with other meningeal pathogens, and neurological sequelae are frequent in survivors (16).

Pneumococci usually enter the host via the nasopharynx, where they can attach to the epithelial cells. The nature of this interaction is not entirely understood. The pneumococcal adhesin has been suggested to be a protein with specificity for GlcNAcβ1-3Gal or GalNAcβ1-4Gal on glycolipids (1, 2, 9).

In order to invade tissues and gain access to the subarachnoid space during the course of bacteremia, pneumococci are assumed to interact with vascular endothelial cells (EC). Before antibiotics, *S. pneumoniae* was also a relatively common (15%) cause of endocarditis, a further indication that pneumococci can associate with peripheral EC (11). We recently showed that pneumococci and the pneumococcal cell wall induce procoagulant activity on cultured human EC (6), and Rubins and coworkers (13) reported toxic effects of purified pneumococcal pneumolysin on cultured bovine pulmonary endothelial cells. These observations provide direct evidence for a disturbance in EC physiology mediated by pneumococci.

Thus, significant evidence has accumulated to indicate that pneumococci interact directly with EC, but the mechanisms of attachment remain unknown. In this study, we investigated the ability of pneumococci to attach directly to human umbilical vein EC (HUVEC). Our results demonstrate that the cell wall plays a role in attachment and that the interaction of EC with intact pneumococci or the pneumococcal cell wall causes pronounced cytopathic effects which are generated through the cytokine cascade.

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MATERIALS AND METHODS

Bacterial components. Two unrelated strains of *Streptococcus pneumoniae*, R6 (unencapsulated) and the encapsulated strain SIII (serotype 3), were grown in semisynthetic medium supplemented with 0.5% yeast extract (Difco, Detroit, Mich.) in a stationary waterbath at 37°C (17). At the mid-logarithmic growth phase, the bacteria were centrifuged, washed, and resuspended to a concentration of 10^8 CFU/ml in phosphate-buffered saline (PBS; Whittaker Bioproducts, Walkersville, Md.). For some experiments, the bacteria were killed by boiling in a waterbath for 10 min.

Purified cell wall components from strain R6 were prepared as described previously (17). Briefly, logarithmically growing pneumococci were heat killed. Crude cell wall was extracted in 5% sodium dodecyl sulfate (SDS) at 100°C for 30 min. After being washed to remove detergent, the cell wall preparation was treated with DNase, RNase, and trypsin. After reprecipitation in SDS, the walls were washed, lyophilized, and stored at room temperature. Ethanolamine-containing cell wall was prepared in the same manner except that pneumococci were grown in defined medium with ethanolamine substituting for choline (17). Other preparations of the cell wall included solubilization with *N*-acetylmuramyl-L-alanine amidase or M1-muramidase (5). Lipoteichoic acid (Forssman antigen) was prepared by an extraction procedure adapted from that of Goebel (17) and purified over an antiphosphorylcholine antibody column (17). For use in bioassays, pneumococcal cell wall suspensions were homogenized by sonification with a Branson

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Sonifier (model 2200; Branson Ultrasonic Corp., Danbury, Conn.).

Labeling of bacteria. Bacteria (10^8 CFU/ml) were mixed for 1 h at 4°C with fluorescein isothiocyanate (FITC) (1 mg/ml; Sigma Chemical Co., St. Louis, Mo.) dissolved in a buffer containing 0.05 M sodium carbonate and 0.1 M sodium chloride. Subsequently, the bacteria were washed three times with PBS and resuspended to a final concentration of 10^8 CFU/ml. In preliminary experiments, the numbers of attached pneumococci detected by gram stain and by fluorescent labeling were equivalent. For ease of counting, fluorescent labeling was used in all subsequent studies.

EC culture. Primary cultures of HUVEC (passage 1; Clonetics Corp., San Diego, Calif.) were added to tissue culture flasks coated with fibronectin (50 μg/ml) and grown in medium 199 (Sigma Chemical Co.) containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma), heparin (10 U/ml; Sigma), EC growth factor (50 μg/ml; Biomedical Technologies Inc., Stoughton, Mass.), human serum (6%; New York Blood Center), fetal calf serum (19%; Sigma), penicillin (100 U/ml), streptomycin (100 μg/ml; both from GIBCO Laboratories, Grand Island, N.Y.), amphotericin B (2.5 μg/ml; Sigma), and L-glutamine (350 μg/ml; GIBCO Laboratories). The EC were grown at 37°C in a 5% CO_2 incubator.

At confluence, the cells were prepared for subculture with trypsin–0.05% EDTA (Sigma). For adherence assays, the EC (passage 1 to 3) were transferred to Terasaki 60-well culture dishes (Robbins Scientific, Sunnyvale, Calif.) and cultured for another 24 to 48 h to form a confluent monolayer. To study cytopathic effects, EC (passage 1 to 3) were transferred to 12-well culture dishes (Becton Dickinson & Co, Lincoln Park, N.J.) and cultured for 3 to 5 days until confluent. Medium free of antibiotics was used at the time of addition of pneumococci. All monolayers were characterized as EC by morphologic criteria and the ability to bind antibody to Von Willebrand factor (Dako Corporation, Carpinteria, Calif.).

Adherence of bacteria to human EC. To study adherence of pneumococci to cultured EC, FITC-labeled pneumococci (10^5 to 10^8 CFU/ml; 5 μl per well) were added to twice-washed EC monolayers. The mixtures were incubated for 0.5 to 6 h at 37°C, and subsequently the cells were washed five times with M199 to remove nonadherent bacteria. In experiments testing pneumococcal cell wall components, cells were incubated with these components (3 μl, 5 to 100 μg/ml) for 30 min at 37°C before the addition of labeled bacteria. Adherent pneumococci were counted visually with an inverted microscope (Diaphot-TMD; Nikon Inc., Melville, N.Y.) equipped for fluorescence with an IF DM-510 filter. Adherence was expressed as the number of attached bacteria per 100 EC counted in a 40× field or as a percentage of the control value. Values for three to five replicate wells were averaged; each experiment was performed at least three times.

Staining of the F-actin cytoskeleton of EC. To stain the F-actin cytoskeleton, the EC in Terasaki plates were fixed with 2% paraformaldehyde–0.1% Triton X-100 for 15 min at room temperature. After 10 washes with PBS, 5 μl of a FITC-phalloidin solution (0.05 mg/ml in PBS–1% dimethyl sulfoxide) was added to each well. The mixture was incubated for 40 min at room temperature in a humid atmosphere. Subsequently, the plates were washed with several changes of PBS. The EC were examined with an inverted microscope (Diaphot-TMD; Nikon Inc.) equipped for fluorescence with an IF DM-510 filter.

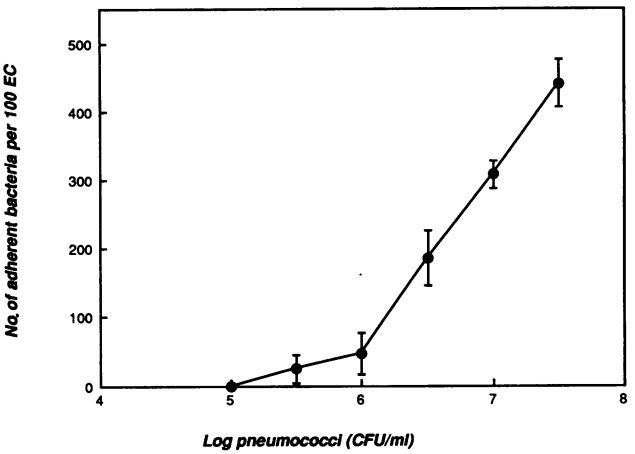

FIG. 1. Dose-response curve for adherence of *S. pneumoniae* to HUVEC. HUVEC were incubated with increasing concentrations of *S. pneumoniae* for 30 min. Adherence is expressed as the number of attached pneumococci per 100 EC. Results are the means ± SD for triplicate wells in at least four independent experiments.

Cytopathic changes in EC. To study morphologic changes in EC after prolonged incubation with intact pneumococci or pneumococcal cell wall, monolayers of EC in 12-well culture dishes were incubated for up to 24 h with bacteria or cell wall with and without anticytokine antibodies dissolved in buffered M199 (pH 7.4). Monolayers were examined by phase-contrast microscopy (Diaphot-TMD). Polymyxin B (1 μg/ml) was added to each well to eliminate the possibility that changes in EC were induced by contaminating endotoxin.

Antibodies. For some experiments, the following anticytokine antibodies were used: goat anti-human interleukin-1α (IL-1α; 10 μg/ml), goat anti-human IL-1β (10 μg/ml), and goat anti-human tumor necrosis factor alpha (TNF-α; 10 μg/ml) (R&D Systems, Minneapolis, Minn.). Goat-anti human factor VIII-related antigen (10 μg/ml) was used as the control antibody.

Electron microscopy. For electron microscopy studies, EC were cultured on glass coverslips (Thermanox; 22 by 22 mm) placed in tissue culture dishes (Nunclon, Intermed, Copenhagen, Denmark). After confluent monolayers had formed, the EC were incubated with pneumococci (5×10^7 CFU/ml) for 1 h at 37°C. Subsequently, the cells were washed with M199 and fixed with K2 buffer (2% paraformaldehyde, 2.5% gluteraldehyde in 0.1 M cacodylate buffer). Staining, embedding, and further processing were carried out as described previously (8).

Statistics. Differences between groups were tested by Student's *t* test. The results are expressed as means and standard deviation (SD).

RESULTS

Kinetics of attachment. Attachment of *S. pneumoniae* to EC occurred in a dose-dependent fashion (Fig. 1). A threshold dose of approximately 10^6 CFU/ml was required to detect attachment. With higher concentrations, adherence increased; at pneumococcus concentrations of more than 10^8 CFU/ml, adherent cells were too numerous to count. The attachment of pneumococci to HUVEC occurred rapidly, reaching a plateau within 30 min (Fig. 2). Incubation times of 1 h or longer were associated with the appearance of an altered morphology (see below). Between 30 and 60 min,

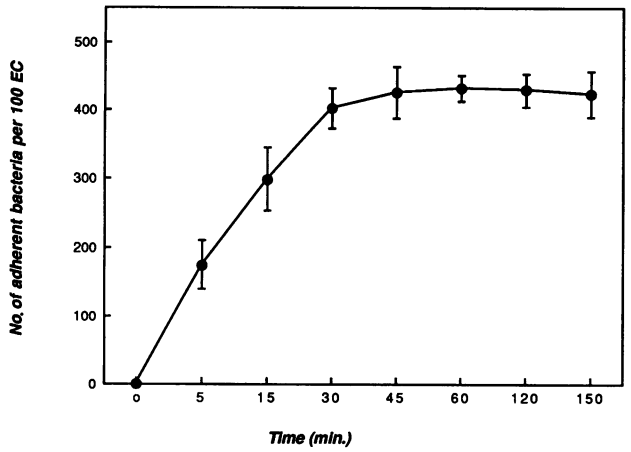

FIG. 2. Time course for adherence of *S. pneumoniae* to EC. EC were incubated with 5×10^7 CFU of *S. pneumoniae* per ml. Adherence is expressed as the number of attached bacteria per 100 EC. Results are the means ± SD for triplicate wells in at least four independent experiments.

pneumococci not only attached to the EC; a portion of the bacterial population appeared to be taken up by the EC and became visible inside vacuoles (Fig. 3).

To investigate whether active metabolism was needed for adherence to EC, live and heat-killed pneumococci were compared in the adherence assay. At an input concentration of 5×10^7 CFU/ml, the number of attached bacteria per 100 EC was 378 ± 57 and 352 ± 57 (mean ± SD, $P > 0.5$) for live and heat-killed R6, respectively.

To determine the effect of the presence of the polysaccharide capsule on adherence of pneumococci to HUVEC, strains R6 (unencapsulated) and SIII (encapsulated) were compared in the adherence assays. At an input concentration of 5×10^7 CFU/ml, the number of attached pneumococci per 100 EC was 402 ± 43 and 388 ± 20 (mean ± SD, $P > 0.5$) for R6 and SIII, respectively.

Role of pneumococcal cell wall in adherence. In order to ascertain the role of the cell wall in adherence, purified cell wall and its soluble subcomponents were tested for their ability to interfere with the binding of intact pneumococci in a competition assay. Pretreatment of EC with purified pneumococcal cell wall decreased the ability of R6 to adhere in a dose-dependent fashion, with a threshold dose of 10 μg/ml (Fig. 4A). Maximum inhibition of attachment to HUVEC of 60% ± 10% occurred with 100 μg of cell wall per ml. Higher doses did not inhibit attachment further. Soluble pneumococcal cell wall subcomponents were also able to inhibit adherence. Treatment of HUVEC with muramidase-digested cell wall (100 μg/ml) or amidase-digested cell wall (100 μg/ml) was as effective as treatment with intact cell wall in competitively inhibiting adherence (Fig. 4B). Lipoteichoic acid (Forssman antigen) (100 μg/ml) was somewhat less inhibitory.

The role of the phosphorylcholine moiety in adherence was assessed by comparing the adherence of pneumococci cultured in medium containing choline or ethanolamine. At an input concentration of 5×10^7 CFU/ml, the number of adherent bacteria was 373 ± 36 and 391 ± 37 (mean ± SD, $P > 0.05$) for pneumococci grown with choline and ethanolamine, respectively.

Morphologic changes in EC induced by intact pneumococci and pneumococcal cell wall. After 1 h of incubation of the EC with intact pneumococci (5×10^7 CFU/ml), the monolayer developed an altered morphology, characterized by progressive widening of the intercellular junctions (Fig. 5). Pneumococci preferentially adhered to the borders of the separating cells (Fig. 6). Early effects were reversible, as monolayers could be reestablished after cells were washed and fresh

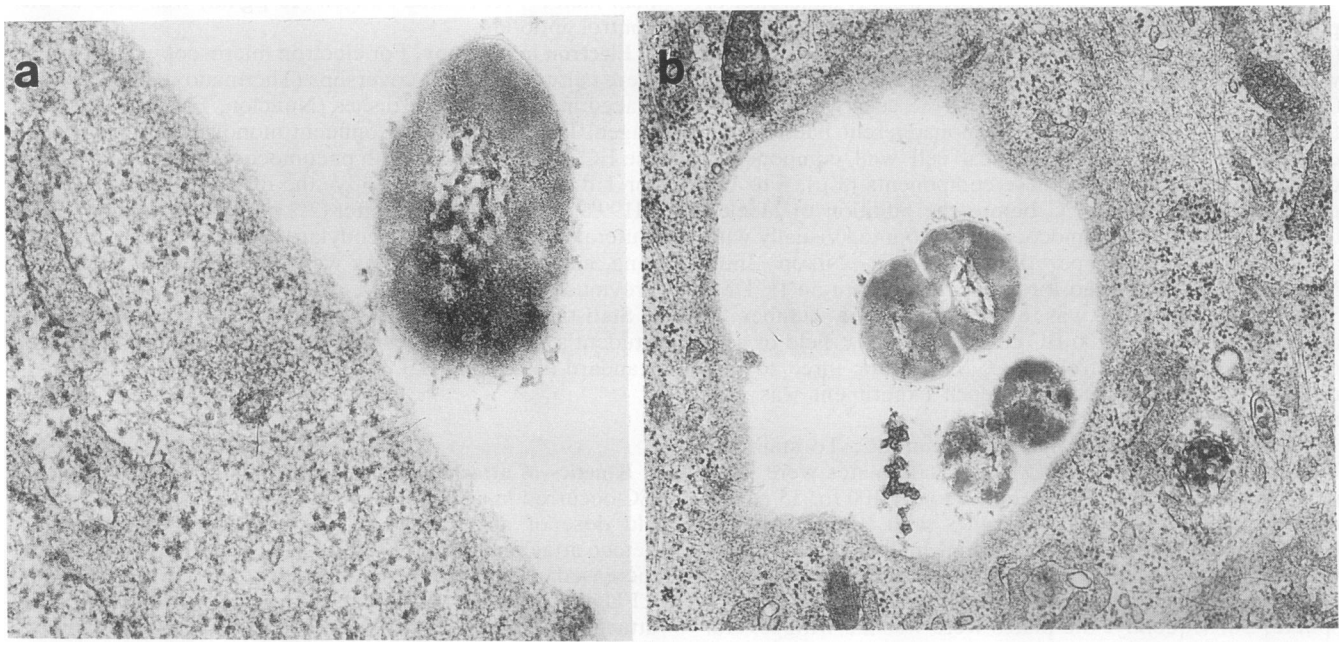

FIG. 3. Transmission electron micrographs of HUVEC after incubation with *S. pneumoniae* (5×10^7 CFU/ml) for 30 min. (a) *S. pneumoniae* adherent to surface of EC. (b) Uptake of *S. pneumoniae* by EC; pneumococci are visible within a cytoplasmic vacuole. Magnification: (A) ×64,000; (B) ×28,000.

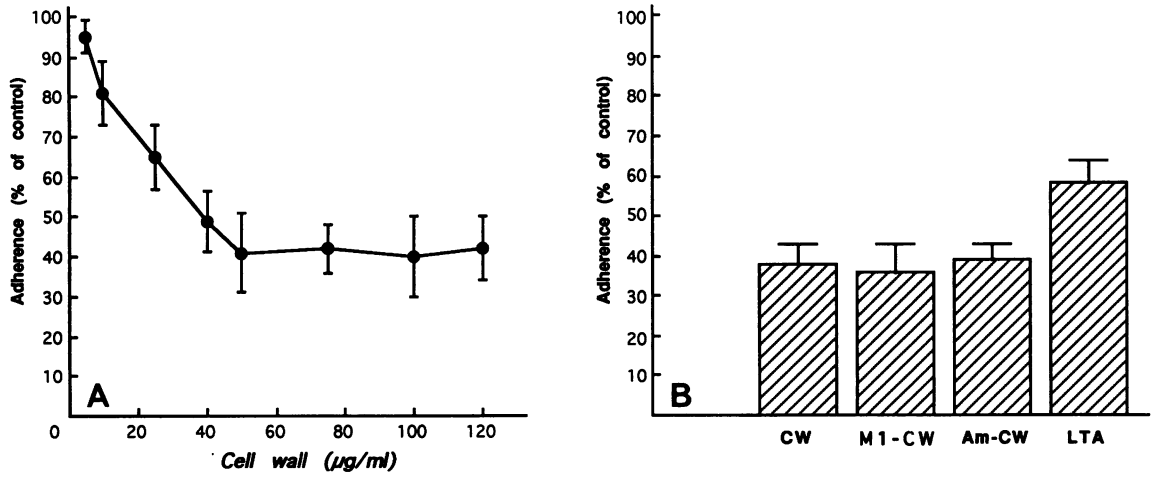

FIG. 4. Inhibition of adherence of *S. pneumoniae* to HUVEC by pneumococcal cell wall (CW) and subcomponents. (A) EC were incubated with increasing concentrations of CW for 30 min prior to addition of intact pneumococci (5×10^7 CFU/ml). (B) EC were incubated with CW or CW treated with muramidase (M1-CW), amidase (Am-CW), or lipoteichoic acid (LTA) for 30 min prior to addition of intact pneumococci (5×10^7 CFU/ml). Results were normalized to adherence of *S. pneumoniae* to control EC not incubated with CW, which was set at 100%. Results are the means ± SD for triplicate wells in at least four independent experiments.

medium was supplied. After 6 h, complete destruction of the monolayer was seen.

Separation of the monolayer could also be induced by purified pneumococcal cell wall (10 to 50 μg/ml), although these changes appeared more gradually, over a period from 6 to 24 h, and were less extensive than with whole pneumococci. Cell wall-induced changes were dose dependent, with high doses (≥25 μg) of cell wall inducing cell damage more rapidly (at 6 h) than lower doses (10 to 25 μg) of cell wall (10 to 12 h). Cell wall subcomponents (25 μg/ml) were not active in this respect.

The role of cytokines in EC damage after incubation with intact pneumococci and pneumococcal cell wall was investigated with anti-cytokine antibodies. Anti-IL-1α and anti-IL-1β but not anti-TNF-α was able to attenuate EC damage induced by pneumococcal cell wall; the combination of these antibodies was more effective. Neither anti-IL-1α, anti-IL-1β, nor anti-TNFα alone could protect EC from damage induced by intact pneumococci, whereas the combination of these antibodies had a strong protective effect (Table 1). *N*-Monomethylarginine (0.25 mM) did not protect EC against cell separation induced by whole pneumococci or pneumococcal cell wall.

DISCUSSION

Previous studies on the pathogenesis of pneumococcal disease have raised convincing evidence that pneumococci and pneumococcal components are likely to interact with endothelial cells (6, 13, 15, 17). However, the mechanisms of these interactions have not been clarified. In this article, we report that pneumococci attach avidly to endothelial cells. Attachment occurred in a dose- and time-dependent fashion, suggesting that the interaction is specific. This property would promote seeding of tissues during pneumococcal disease and provides an opportunity for pneumococcal components to activate EC responses, such as the induction of procoagulant activity (6).

The adherence of the encapsulated strain SIII to HUVEC was similar to that of the unencapsulated strain R6, indicating that the type III polysaccharide capsule did not interfere with adherence. Although small differences in adherence

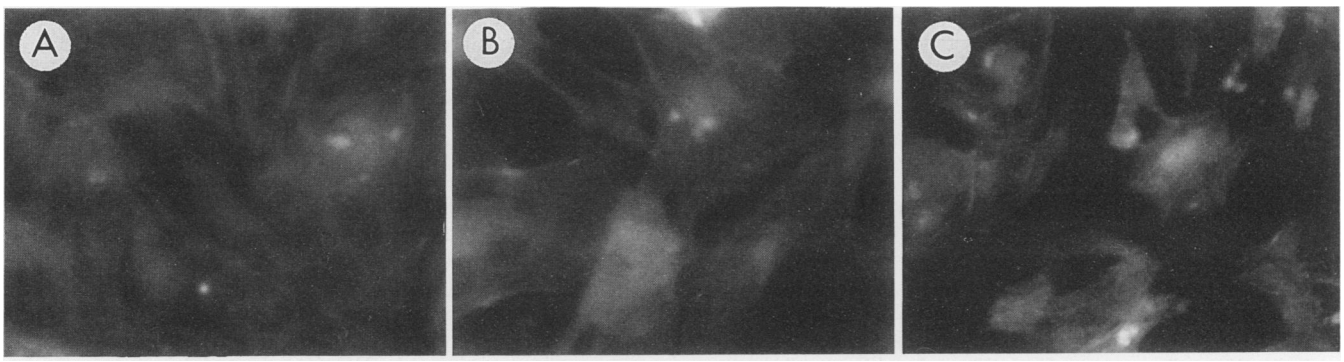

FIG. 5. Immunofluorescence micrographs of cytopathic effects in HUVEC after incubation with *S. pneumoniae* (5×10^7 CFU/ml). (A) Confluent monolayer of HUVEC. (B) Partial disruption of HUVEC monolayer after 2 h, with loss of intercellular junctions. (C) Complete disruption of monolayer after 4 h. Cells were stained with FITC-phalloidin, which stains the F-actin skeleton. (A, B, and C) Magnification, ×336.

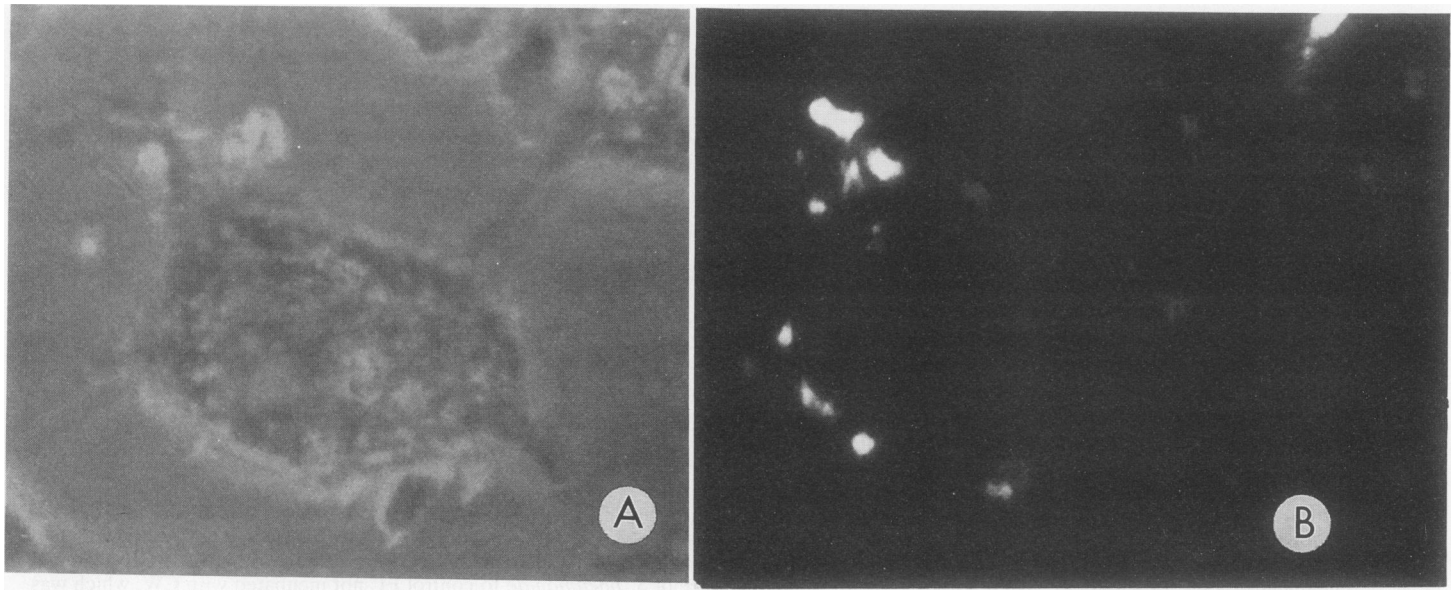

FIG. 6. Light microscope micrograph (A) and fluorescence micrograph (B) of EC after incubation of HUVEC monolayer with *S. pneumoniae* (5×10^7 CFU/ml) for 2 h. Pneumococci seem to preferentially accumulate along the borders of the EC. (A and B) Magnification, ×380.

efficiency cannot be ruled out, this finding is in contrast to the results of studies with *Haemophilus influenzae*, in which capsule-deficient strains associated more rapidly with EC than the encapsulated type b strain (18).

Soluble pneumococcal cell wall and pneumococcal cell wall components interfered with the ability of whole pneumococci to attach. Maximum inhibition was ~60%, indicating that other adherence mechanisms are likely to exist. Competitive inhibition by cell wall may be explained in several ways. One possibility is that soluble cell wall components occupy a receptor(s) for pneumococci on the EC surface. Another possibility is that cell wall induces a change on the EC surface which inhibits subsequent attachment of intact pneumococci. Preliminary studies indicate that the supernatant from EC incubated with pneumococcal cell wall inhibits the attachment of subsequently added bacteria.

TABLE 1. Protective effect of anticytokine antibodies against cytopathic effects induced in EC by intact pneumococci and pneumococcal cell wall[a]

Antibody	Protection[b] against:	
	Intact pneumococci	Purified cell wall
IL-1α	−	+
IL-1β	−	+
TNF-α	−	−
IL-1α + IL-1β	±	+
IL-1α + TNF-α	++	++
IL-1β + TNF-α	++	++
Control	−	−

[a] EC were incubated with intact pneumococci (10^8 CFU/ml) or purified pneumococcal cell wall (25 μg/ml) for up to 24 h. The effect of anticytokine antibodies (10 μg/ml) or control antibody (10 μg/ml) on EC cytopathology was estimated by phase-contrast microscopy. Experiments were performed four times.

[b] −, no protection; ±, little protection; +, clear protection; ++, strong protection.

Since endovascular infection is not a common aspect of pneumococcal disease, it is likely that potent host defense mechanisms operate in vivo either to prevent attachment itself or neutralize toxicity after pneumococcal attachment. The fact that meningitis is a common complication of invasive pneumococcal disease may indicate that these defense mechanisms are less efficient in the area of the blood-brain barrier or that the pneumococci have a preference for these EC.

Our observation that incubation of the EC with pneumococci caused a progressive change in the morphology of the cells, characterized by cell separation and eventually complete destruction of the monolayer, indicates that pneumococci have a profound cytopathic effect on these cells. Rubins et al. (13) have reported the cytotoxic effects of *S. pneumoniae* on bovine pulmonary EC. While they ascribed these effects to pneumolysin, this could not be the case in our experiments with heat-killed pneumococci. The pneumococcal autolysin is inactivated by heating, preventing release of intracellular cytotoxins such as pneumolysin, and pneumolysin itself is heat sensitive (13). Purified pneumococcal cell wall had effects similar to those of whole bacteria on EC. This suggests that the cell wall may harbor a cytotoxin which is responsible for damage to the EC. This would be analogous to the toxic effects of the 1,6-anhydromuramyl peptide derived from gram-negative bacteria, which specifically kills ciliated cells (7). The cytotoxicity of this peptide was suggested to be mediated by IL-1α and involve the generation of nitric oxide (4, 10). The nitric oxide inhibitor *N*-monomethylarginine did not inhibit the cytopathic effects induced in EC by whole pneumococci or pneumococcal cell wall, suggesting that the mechanism of damage differs from that found with the 1,6-anhydromuramyl peptide. Although soluble cell wall particles induced procoagulant activity in EC (6), they did not trigger the cytopathic effects seen with whole pneumococci or pneumococcal cell wall. This finding may indicate that the complexity of particles is important for inducing this phenomenon.

Anti-IL-1 and anti-TNF-α antibodies could protect the EC from injury induced by intact pneumococci or pneumococcal cell wall, indicating that this phenomenon is likely mediated by cytokines. The rapid cell separation and complete destruction of EC monolayers by intact pneumococci was not preventable by either anti-TNF-α or anti-IL-1 alone. However, the combination of antibodies had a strong protective effect, which indicates that this process is likely linked to the production of both TNF-α and IL-1. The observation that anti-IL-1 alone could ameliorate the damage in EC exposed to the cell wall suggests that, with a more gradual progression of the cytopathic process, IL-1 plays a more important role than TNF-α, although the combination remained more effective. This relatively important contribution of IL-1 to cell wall-induced EC cytopathology is compatible with the findings from previous studies on the inflammatory effects of pneumococcal cell wall. IL-1 but not TNF-α was secreted by human macrophages after stimulation with pneumococcal cell wall components, and in the rabbit meningitis model, anti-IL-1 but not anti-TNF-α downmodulated the inflammatory responses induced by pneumococcal cell wall (12, 15).

Interestingly, pneumococci seemed to accumulate along the borders of the EC where separation was occurring (Fig. 6). No specific association of adherent bacteria with underlying cell cytoskeletal elements was discernible with phalloidin labeling of EC. It remains to be determined whether this distribution of bacteria promotes seeding of subendothelial tissue spaces. Some pneumococci were apparently taken up rapidly by the EC, since bacteria were present inside vacuoles within 1 h of incubation (Fig. 3b). Although EC are not considered professional phagocytes, and phagocytic properties may differ between endothelia from different vessels of the body, uptake of microorganisms by EC has been described previously for several microorganisms, such as *Staphylococcus aureus* and *Haemophilus influenzae* (14, 18). The importance of this phenomenon in pneumococcal disease remains to be clarified.

In conclusion, we have shown that pneumococci attach rapidly to EC, a finding consistent with the reported activation of these cells by bacteria and cell walls. The cell wall is important in the interaction of pneumococci with HUVEC. The cytopathic effects of pneumococci and pneumococcal cell wall extend the biological activities of this bacterial component to the activation of the cytokine cascade in EC. The results of this study emphasize the potential need to neutralize the cell wall in order to improve the outcome of inflammation caused by pneumococci.

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