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Adherence of *Streptococcus pneumoniae* to Polystyrene Plates and Epithelial Cells and the Antiadhesive Potential of Albumin and Xylitol

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ABSTRACT: Aimed to prevent *Streptococcus pneumoniae* biofilm infections, we studied the adherence of nine pneumococcal strains to polystyrene plates and on epithelial cells and the antiadhesive effect of albumin and xylitol. The adherence was variable among strains, but there was a good correlation between their adherent ability and binding to abiotic material and cells. Strains of serotypes 6B and 23F were the most adherent organisms, whereas serotype 3 strains were the least adherent. Human serum albumin (HSA) enhanced bacterial growth at low concentrations (0.5–2.5%) but inhibited it at 10%. Xylitol inhibited bacterial growth of all strains at concentrations ranging from 5 to 15%. Exposure to 0.5–5% HSA in solubilized form and to 5% HSA precoating of plates diminished adherence to polystyrene >80% for all strains, except for serotype 3 strains. Contrarily, 0.5 and 5% xylitol did not diminish significantly pneumococcal adherence to polystyrene plates or on epithelial cells. Our results suggest that 1) the potential application of HSA coatings on medical devices to inhibit pneumococcal adherence and 2) the possible beneficial effect of xylitol in preventing some pneumococcal infections could be because of its antimicrobial activity rather than to an antiadhesive effect. (***Pediatr Res* 69: 23–27, 2011**)

Streptococcus pneumoniae is a common colonizer of the human upper respiratory tract and a leading cause of acute otitis media, community-acquired pneumonia, bacteremia, and bacterial meningitis. Bacterial adherence allows the organism to bind to eukaryotic cells and inert material; thus, it may be considered the first step for pathogenicity. Bacterial adhesion to inert material is probably mediated by physicochemical interaction, wherein many factors are involved, including cell envelope plasticity of bacteria, the target substratum properties, and environmental features (1). Once bacterial cells adhere to any surface, accretion of bacteria can ensue with bacterial cluster formation and further biofilm development (2).

Pneumococcal adherence to clinically used abiotic material has not been deeply investigated (3,4), but pneumococcal binding to certain devices (*i.e.* tympanostomy tubes and cochlear implants) may lead to difficult-to-treat complications (5,6). To decrease bacterial adherence to abiotic surfaces, several compounds, including plasma, serum, albumin, bacterial proteins, heparin, gelatin, pepsin, and saliva, have been tested (1). Because bacterial adhesion to inert material is diminished in the presence of serum and albumin, their use as coatings for foreign medical devices has been advocated (7,8). However, few studies have searched compounds with antiadhesive properties against pneumococci (4). Some reports have suggested that xylitol can prevent pneumococcal acute otitis media (9,10), although the mechanisms underlying such beneficial effect have not been completely elucidated (11–14), but a potential interference with bacterial adherence was proposed (12).

The aims of this study were, first, to determine the binding properties of different pneumococcal strains to polystyrene plates and on epithelial cells under different experimental conditions, and second, to evaluate the effect of albumin and xylitol on the adherence phenomenon.

MATERIALS AND METHODS

Bacterial strains. All the strains used in this study are listed in Table 1. These strains were selected in basis of the serotypes that are commonly involved in human infections. Hereinafter, the strains are described by the numbers followed by their serotype.

Compounds and effects on bacterial strains. Human serum albumin (HSA) and xylitol were purchased from Sigma Chemical Co.-Aldrich (St. Louis, MO). Minimum inhibitory concentrations (MICs) of HSA and xylitol were determined for the nine strains by broth microdilution methodology (15) using cation-adjusted Mueller Hinton broth (CA-MHB; Becton Dickinson, CO, Sparks, MD) enriched with 5% lysed horse blood.

Adherence to polystyrene (microtiter) plates and effects of solubilized albumin and xylitol. Isolated colonies from blood agar plates incubated for 20 h at 35°C in a 5% CO_2 atmosphere were used to prepare inocula. A bacterial suspension was prepared in prewarmed CA-MHB adjusted to an OD equivalent to 10^8 colony-forming units (CFU)/mL, diluted 1/2 in CA-MHB, and then 200 μL per well were transferred to flat-bottom polystyrene, nontissue culture-treated 96-well microtiter plates (Greiner Bio-one, Frickenhausen, Germany). After 1-h incubation at 35°C in air, the liquid was removed

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Abbreviations: CA-MHB, cation-adjusted Mueller Hinton broth; **CFU,** colony-forming units; **HSA,** human serum albumin; **MIC,** minimum inhibitory concentration

from the wells, and the wells were gently washed 10 times with saline. After the last washing, 50 μL of CA-MHB was added to each well and sonicated at 35 kHz (Bandelin Sonorex TK2; Schaltec GMBH, Morffelden-Waldorf, Germany) for 2 min. Material removed was resuspended in 150 μL of CA-MHB, diluted, and plated on blood agar plates for colony counting.

The effect of HSA and xylitol both at 0.5 and 5% (wt/vol) on the adherence to polystyrene plates was also studied following the previously described method. The adherence rate for each strain unexposed (control) and exposed to the compounds was arbitrarily expressed as CFU recovered per 10^6 CFU of initial inoculum, because no bacterial growth was detected during the incubation period. All experiments were done five times by duplicate, and means (SD) were calculated.

Adherence to albumin pretreated polystyrene plates. Wells of two polystyrene plates were pretreated by adding 200 μL of 5% HSA, and then, one plate was incubated at 35°C for 2 h and the second plate at 30°C for 24 h. Control wells containing only CA-MHB were also included. Later, both plates were washed aseptically with the same quantity of CA-MHB and dried at 37°C for 20 min. The adherence of all pneumococcal strains was studied following the same methodology as above. All experiments were done five times by duplicate, and means (SD) were calculated.

Adherence to epithelial cells and effect of xylitol. The adherence to epithelial cells was studied using a human lung alveolar carcinoma epithelial cell line A549 (ATCC CCL-185) and prepared as previously described (16). Briefly, cells were grown in DMEM (GIBCO, NY) with 10% FCS, penicillin G (100 U/mL), and streptomycin (100 μg/mL) and maintained in DMEM with 2% FCS. Washed A459 monolayers preformed in 24-well tissue culture trays (Greiner Bio-one) were exposed to 500 μL of midlogarithmic phase cultures of each pneumococcal strain with ~10^6 CFU/mL and diluted in DMEM plus 10% FCS in presence or absence of xylitol at 0.5 and 5%. After incubation for 2 h at 37°C in 5% CO_2 atmosphere, the culture fluid from each well was removed, diluted, and plated on blood agar plates for counting of final bacterial concentrations. Then, the monolayers were washed four times with PBS pH 7.5 and then detached from plate by treatment with 100 μL of 0.25% trypsin and 0.02% EDTA (Sigma Chemical Co.). Epithelial cells were next lysed by addition of 400 μL of 0.025% Triton X-100 (Sigma Chemical Co.), and 100-μL aliquots (and serial 10-fold dilutions thereof) were plated on blood agar to determine the total number of adherent bacteria. The adherence rate for each strain was arbitrarily expressed as CFU recovered per 10^6 CFU of final bacterial concentration. Assays were performed in duplicate, and results presented are the means (SD) of 10 wells.

Statistical analysis. The significance of differences between means was analyzed by the one-way ANOVA test. Levene's test was used to assess the equality of variances, and Welch's test was used because the variances differed statistically significant from each other. Comparisons between two groups (control *versus* any treated group or among two treated groups) were performed by Bonferroni's post test. All tests were two tailed. Differences with p values < 0.05 were considered significant. SPSS version 14.0 statistical package was used (SPSS Inc., Chicago, IL). When organisms showed no adherence, a value at the detection limit (1 CFU) was used for the calculation of mean values, and this value is the minimum value represented in the figures.

Table 1. *Pneumococcal strains, serotypes, sources, and* in vitro *susceptibility to HSA and xylitol*

No. strain			MIC (%, wt/vol)	
Reference	Serotype	Source	HSA	Xylitol
1–AR33118	3	Respiratory tract	10	5
2–FL2812	3	Blood	10	10
3–FL5629	3	Blood	10	10
4–MJD1225	6B	Blood	10	15
5–AR06016	9V	Respiratory tract	10	15
6–ATCC49619	19F	Sputum	10	15
7–FJD60	23F	Blood	10	15
8–AR30118	23F	Respiratory tract	10	15
9–MJD573	23F	Cerebrospinal fluid	10	15

RESULTS

Effect of albumin and xylitol on pneumococcal growth. Table 1 presents the serotypes, sources, and susceptibility of nine pneumococcal strains to HSA and xylitol after 22-h exposure. Bacterial growth was inhibited for all strains by 10% HSA concentration (MIC), but it was stimulated by concentrations of 0.5–2.5%. For all strains, growth was inhibited by 15% xylitol, showing the serotype 3 strains the lower MIC values (5–10%), and particularly, bacterial growth was inhibited at 0.5% xylitol for strain number 3 (serotype 3).

Adherence of pneumococcal strains to polystyrene and effects of solubilized albumin and xylitol. Because no bacterial growth was detected during the incubation period (1 h), values were expressed in number of adhered CFU per 10^6 CFU of initial inoculum (Fig. 1). The less adherent strains belonged to serotype 3, whereas the most adherent belonged to serotypes 6B and 23F. After exposure to any HSA concentration, the adherence diminished significantly (>90%) for all strains ($p < 0.0001$), except for all serotype 3 strains. The adherence changes of all serotype 3 isolates were difficult to evaluate because of the poor adherence of their controls, which was close to the detection limit. The decreased effect on adherence after HSA exposure seems to be concentration dependent for five of the six valuable strains. Xylitol at 0.5 and 5% diminished the adherence in two and four strains, respectively, but again the differences were not significant.

Adherence of pneumococcal strains to polystyrene with and without 5% albumin coating (pretreatment) for 2 or 24 h. As in the experiments with solubilized HSA, no bacterial growth was detected in any wells after the 1-h incubation period; thus, values were also expressed in number of adhered CFU per 10^6 CFU of initial inoculum (Fig. 2). The adherence diminished significantly ($p < 0.0001$) in >80% for all strains

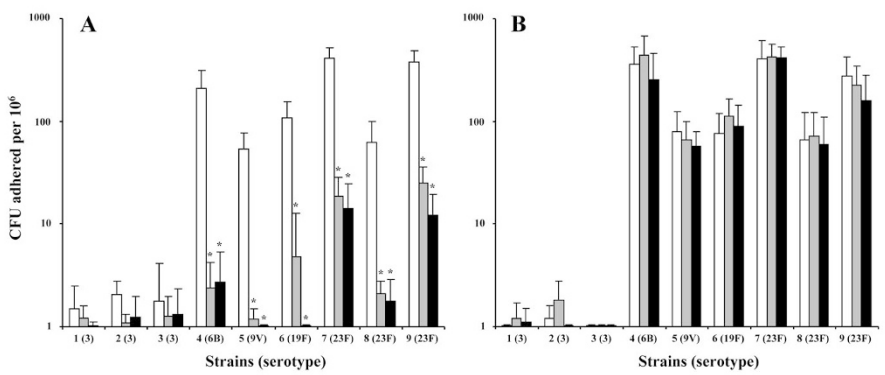

Figure 1. Adherence of pneumococcal strains to polystyrene alone (control; □) and the effect of solubilized HSA (*A*) and xylitol (*B*) both at 0.5% (▨) and 5% (■) (wt/vol). Figure represents means with SD (*positive value*) of CFU adhered per 10^6 CFU of inoculum ($n = 10$): *$p <$ 0.0001, 0.5, or 5% *vs* control; one-way ANOVA with Bonferroni's multiple comparison test.

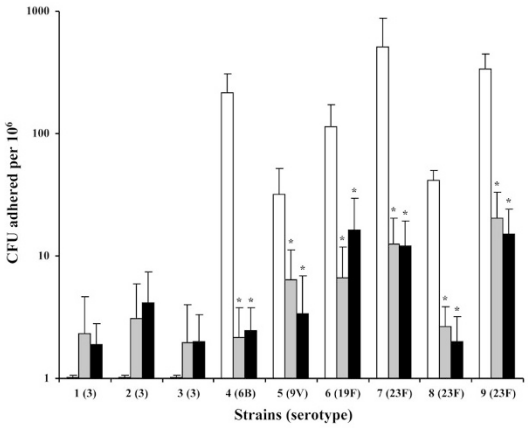

Figure 2. Adherence of pneumococcal strains to polystyrene without (control; □) and with pretreatment of plates with 5% HSA (wt/vol) for 2 h at 35°C (▨) and for 24 h at 30°C (■). Figure represents means with SD (*positive value*) of CFU adhered per 10^6 CFU of inoculum ($n = 10$): *$p < 0.0001$, 5% HSA for 2 h or for 24 h *vs* control; one-way ANOVA with Bonferroni's multiple comparison test.

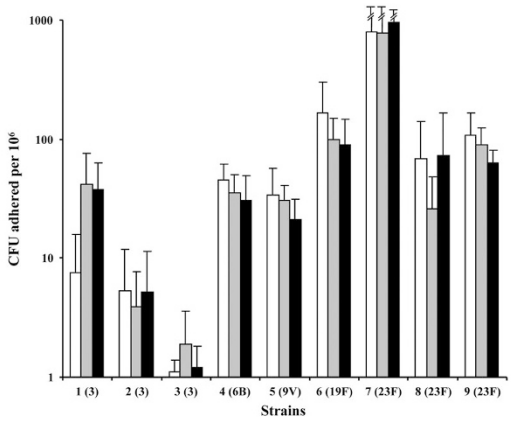

Figure 3. Adherence of pneumococcal strains to epithelial cell A549 monolayers alone (control; □) and the effect of 0.5% (▨) and 5% (■) (wt/vol) xylitol exposure. Adherence is given as means with SD (*positive value*) of CFU recovered per 10^6 CFU of final bacterial count ($n = 10$). No significant differences were found between control and xylitol groups ($p > 0.05$).

at 2 and 24 h, except those of serotype 3. Again, no significant differences on adherence were observed for serotype 3 strains. The effect on bacterial adherence of 5% HSA both in solubilized form (Fig. 1) and as pretreatment of wells (Fig. 2) was similar.

Effect of xylitol on pneumococcal adherence to epithelial cells. Contrarily to the previous experimental conditions, bacterial growth was detected during the 2-h incubation period. Xylitol at 0.5 and 5% slowed bacterial growth in one and six of the nine strains tested, respectively (Fig. 3). Therefore, the adherence values were expressed in number of adhered CFU per 10^6 CFU of final bacterial concentrations in the same well.

There was a good correlation between adherence to epithelial cells and adherence to polystyrene for the strains studied. Strains of serotype 3 were the less adherent bacteria to both epithelial cells and polystyrene, whereas two serotype 23F strains were the most adherent ones. Xylitol at 0.5 and 5% diminished, although not significantly, bacterial adherence in six and four strains, respectively.

DISCUSSION

The adherence of *S. pneumoniae* to epithelial cells has been widely studied. However, studies on the adherence ability of this organism to abiotic materials are scarce (3,4,17,18), but the pneumococcal adherence properties may be of interest in clinical situations where inert material is implanted (tympanostomy tubes, cochlear implants, ventriculostomy derivations, and so on) (5). Furthermore, bacterial adherence is considered to be the first step in biofilm development (19). We have observed notable differences in the adherence ability of pneumococcal strains. Two serotype 23F strains and one serotype 6B strain were the most adherent strains. However, all serotype 3 strains showed the lowest adherence ability, which confirms previously published results (3,4).

Pneumococcal adherence to epithelial cells involves the recognition of host cell receptor glycoconjugates by pneumococcal surface structures (20), but the bacterial adhesins have not been well characterized so far, except for PsaA (16). A pneumococcal adherence and virulence factor A (PavA), displayed to the cell outer surface of pneumococcus, could modulate expression or function of important virulence determinants (21). It has been reported the low ability of serotype 3 strains to bind epithelial cells, which could be related to their great amount of capsular polysaccharide (22,23), and possibly to the opaque phenotype, characteristic of the majority of capsulated strains (24,25). The low adherence observed in the three serotype 3 strains tested here also confirms such observations. However, nonencapsulated strains or isogenic nonencapsulated derivatives of capsulated pneumococci that were usually of transparent phenotype showed greater binding ability to epithelial cells than that of the capsulated WT strains (26). Pneumococcal adherence to epithelial cells not only depends on the bacteria but also on the host cells. It seems that some host receptors are implicated in such binding and that certain host genes are specifically involved (26). In addition, host cell modifications induced by external agents, such as coinfections with respiratory syncitial virus, can enhance pneumococcal adhesion (27). Our results show an interesting relationship between pneumococcal adherence to epithelial cells and polystyrene material, suggesting that some mechanisms for bacterial adhesion could be common for binding both biotic and abiotic material.

We previously reported the ability of gerbil sera to diminish pneumococcal adhesion to polystyrene plates (4), and other authors have described the inhibitory effect of C-reactive protein (28), sialylated oligosaccharides alone or covalently coupled to HSA (29), *N*-acetylcysteine, and hydrocortisone (30) on adhesion of pneumococcal strains to host cells. The results of this study show that albumin in solubilized form or on pretreated polystyrene plates diminishes pneumococcal adherence in >80% among the most adherent strains. However, for the least adherent strains (all

serotype 3 strains), the adherence on pretreated plates increased slightly.

The effects of serum and albumin on bacterial adhesion have been widely investigated, and most studies show that exposure to both compounds persistently diminish adherence of many organisms to a variety of abiotic materials (1,4), but to the best of our knowledge, no information regarding the effect of albumin on pneumococcus has been published. Our results might be of interest for preventing pneumococcal adhesion to some inert materials, such as tympanostomy tubes, cochlear implants, and ventriculostomy derivations, devices that may be associated with serious pneumococcal infections. The antiadhesive effect of albumin may be because of its acidic structure that raises the surface net negative charge increasing the repulsion between electric double layers adjacent to the organism and the polystyrene plate (1,4).

The role of xylitol in preventing acute otitis media in children has been investigated. In two clinical trials, regular consumption of xylitol after each meal five times a day was effective in preventing acute otitis media by 30–40% (9,10), but such beneficial effect has not been shown in children who received xylitol only when they had an acute respiratory infection (31), during the respiratory infection season (32), or if they had tympanostomy tubes (10). Nevertheless, the possible beneficial effect of xylitol for preventing otitis media in children could be due to a direct antimicrobial effect (11,12), interference with bacterial adherence (12), effect on the oxidative burst and bacterial killing in polymorphonuclear leukocytes (14), or alterations in capsule gene expression of pneumococci (33). Our results demonstrate a direct antimicrobial effect of xylitol on the pneumococcal strains tested with MIC values of 5–15%, but bacterial growth of one strain was diminished even with concentrations of 0.5%, which can be easily achieved in saliva, although transitorily, in children who received xylitol chewing gum (34). We found an inhibitory effect of xylitol on the adherence of some pneumococci to both abiotic material and epithelial cells, although such antiadhesive effect was not statistically significant using strict statistical analysis. Nevertheless, a previous published work showed the inhibitory effect of xylitol on pneumococcal adherence (12). Therefore, the possible beneficial effect of xylitol in preventing otitis media in children could be multifactorial highlighting its antimicrobial effect, which was showed in *in vitro* studies.

A remarkable limitation to this study is the relative low number of isolates that were tested. Hence, it will be necessary to confirm the adhesion findings by testing a larger number of clinical isolates.

In summary, pneumococcal adherence to polystyrene plates and epithelial cells was variable among strains, but there was a good correlation between ability of the different strains for binding both surfaces, being serotype 3 strains the less adherent organisms. Because albumin, but not xylitol, diminished pneumococcal adherence in different experimental conditions, this study supports the case for the use of albumin as coatings for medical devices to inhibit pneumococcal adherence on abiotic materials. However, we suggest that the possible beneficial effect of xylitol for preventing some pneumococcal infections may be because of its antimicrobial activity rather than to its effect on adherence.

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