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Adhesion of *Streptococcus pneumoniae* to Human Pharyngeal Epithelial Cells In Vitro: Differences in Adhesive Capacity Among Strains Isolated from Subjects with Otitis Media, Septicemia, or Meningitis or from Healthy Carriers

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A method was developed to study the adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells. Epithelial cells from healthy persons, pneumococcal strains from patients with otitis media, meningitis, or septicemia, and pneumococcal cells from the nasopharynx of healthy carriers were used. Adhesion was found to be influenced by changes in the bacterial incubation medium and growth phase, the concentration of bacteria and epithelial cells, the epithelial cell donor, the incubation time and temperature, and the pH and osmolarity of the incubation medium. Pretreatment of bacteria with heat, Formalin, or trypsin decreased adhesion. The highest adhesion was obtained when 10^9 bacteria cultivated for 18 h in streptococcus cultivation broth were added to 10^4 pharyngeal cells and incubated at 37°C for 30 min. *S. pneumoniae* strains from patients with frequent episodes of otitis media and strains from healthy carriers had the highest adhesion values; septicemia and meningitis strains had the lowest. The capsular polysaccharide type did not determine the adhesive capacity of the strains, but otitis strains belonging to the capsular types often associated with otitis media adhered in high numbers. Adhesion may be important for pneumococci colonizing the nasopharynx or inducing otitis media.

About 30% of the episodes of acute otitis media are caused by pneumococci (5, 14). The mechanisms by which these bacteria enter the middle ear cavity and cause infection are not fully understood. The bacteria emanate from the upper pharyngeal mucosa and are thought to ascend through the eustachian tube to the middle ear cavity. The fluid bathing the tubal epithelium and the ciliary movements propagate the bacteria back to the nasopharynx. The bacteria may need to attach to the pharyngeal and tubal epithelial cells to avoid elimination.

It has been suggested that the ability to attach to the mucosal lining of the oral and pharyngeal cavities both influences the colonization pattern of streptococci in the normal flora and determines streptococcal virulence (7, 10, 11, 15). Thus, the distribution of streptococci in different parts of the oral cavity in vivo is reflected by the pattern of adhesion to the same tissue (10) in vitro. Cariogenic streptococci firmly attach to teeth (11), and streptococci causing tonsillitis adhere better to the pharyngeal epithelium than do nonpathogenic variants of the same species (7).

This adhesive capacity has been suggested not to be a virulence factor for pneumonococci (21). The present study, however, describes an in vitro test system which allows the study of pneumococcal adhesion to human pharyngeal epithelial cells and suggests that adhesive capacity is a virulence factor for pneumococci causing otitis media.

(These data were presented in part at the Second International Symposium on Recent Advances in Otitis Media with Effusion, Columbus, Ohio, May 1979 [1].)

MATERIALS AND METHODS

Bacteria. The test system was developed and standardized by using two strains of *Streptococcus pneumoniae*: one isolated from the nasopharynx of a patient with frequent episodes of acute otitis media (strain I, type 4, EF 3296) with high adhesive capacity and one from the blood of a patient with septicemia (strain II, type 9V, EF 1201) with low adhesive capacity. To correlate adhesive capacity with clinical origin, 112 *S. pneumoniae* strains were used. Thirty strains were isolated from the nasopharynx of 30 children with frequent episodes of acute otitis media. Thirty strains were isolated from the blood of patients with

septicemia, 30 strains were isolated from the cerebrospinal fluid of patients with meningitis, and 22 strains were isolated from the nasopharynx of healthy carriers. The strains were collected between 1970 and 1980 and kept lyophilized until used. (Isolation dates of the groups were: frequent otitis, 1973 to 1980; septicemia, 1970 to 1978; meningitis, 1971 to 1977; and healthy carriers, 1980.) For testing, the whole lyophile was suspended in Hanks balanced salt solution (HBSS), and a loopful was transferred to blood agar plates and incubated in CO_2 overnight. Then a loopful of pneumococci was transferred to liquid growth medium in which the bacteria were cultivated in CO_2 without shaking at 37°C for 18 h unless otherwise stated. Bacteria were harvested by centrifugation at $600 \times g$ for 20 min, and the sediment was resuspended in HBSS. The number of bacteria per milliliter was estimated by interference contrast microscopy in a Bürker chamber or by viable counts.

The growth media used were the streptococcus cultivation broth of Holm and Falsen (13), with or without saccharose (10%), nutrient broth (Oxoid CM 67), nutrient broth with addition of glucose (0.75%), brain heart infusion broth (Difco 0037), or minimal salts medium with Casamino Acids as the carbon source. Attempts were made to vary the bacterial adhesion properties before incubation with epithelial cells by boiling, Formalin treatment (0.5%, 1 h at room temperature and 16 h at 4°C), trypsin treatment (from bovine pancreas; Sigma T8253; 5 mg/ml for 4 h at 37°C), or repeated washings in HBSS (zero, two, four, or six times).

Capsule typing. The polysaccharide capsule types were determined at the Statens Seruminstitut, Copenhagen, by the Quellung reaction (17). A few of the recently isolated strains were typed at the Department of Clinical Bacteriology, University of Göteborg, by counter-immunoelectrophoresis (12).

Epithelial cells. Epithelial cells were scraped with a cotton-tipped wooden swab from the pharyngeal wall as high up as possible behind the soft palate. The cells were suspended in HBSS, washed twice, and counted in a Bürker chamber by interference contrast microscopy. All experiments were done with epithelial cells from one test person. Pharyngeal cells from 10 apparently healthy persons were tested and compared with those of the test person.

Adhesion testing. Epithelial cells were mixed with bacteria in various ratios and with HBSS to a final volume of 1 ml. The mixtures were incubated during rotation in a Heto rotor (Birkeröd, Copenhagen, Denmark) (20 rpm at 37°C for 60 min) unless otherwise stated. Immediately after incubation, unattached bacteria were eliminated by repeated washings in HBSS. A drop of trypan blue was added to the cell suspension before counting to allow exclusion of the stained dead epithelial cells. Epithelial cells with most of their surfaces showing were examined in an interference contrast microscope (Ortholux II with interference contrast equipment T; E. Leitz Inc.). Adhesion is given as the mean number of bacteria per cell for 40 epithelial cells after subtracting the background adhesion (i.e., the number of bacteria present on cells incubated with buffer only).

Incubation conditions. The incubation media used were HBSS, NaCl (0.9%), standard phosphate-buffered saline (PBS), and RPMI 1640 with 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) without sodium bicarbonate and without glutamine (Flow Laboratories, Inc.). The influence of pH on adhesion was tested with PBS at pH 5, 6, 7, and 8. The influence of osmolarity was tested with NaCl solutions of 0.3, 0.9, 1.8, 2.7, and 4.5%.

Statistical methods. Standard statistical methods were used. Location and dispersion were estimated by using the arithmetic mean and standard deviation. Different sources of variation were studied by a one-way analysis of variance (1a). The number of bacteria per cell was distributed in a skewed manner. Even in experiments with adhering strains, epithelial cells free of attached bacteria were found. In spite of the skewedness, means and standard deviations were used to describe results. To compare samples of 40 epithelial cells and test for significance, a simple nonparametric test was used. When the adhesion of large groups of pneumococcal strains was compared, the group means were compared by standard parametric methods.

RESULTS

Figure 1 shows the adhesion of one efficiently and one poorly adhering strain as seen by interference contrast microscopy.

Standardization of the adhesion test system (i) Bacterial properties. The adhesive ability of attaching strains was maximal at the end of the logarithmic growth phase and remained high up to 24 h of incubation. Regardless of cultivation time, poorly adhering strains did not attach (not shown). The highest adhesive capacity was seen after growth in streptococcus cultivation broth and nutrient broth. A very small or no difference in adhesive capacity among strains was found after growth in the other media tested (not shown). Boiling or Formalin pretreatment of bacteria decreased adhesion (Table 1). Adhesion decreased with the number of washings of the bacteria before incubation with epithelial cells. Trypsin pretreatment of the bacteria markedly decreased the adhesion of strains with high adhesive capacity (Table 1).

(ii) Epithelial cells. The pharyngeal cell population consisted of about 98% squamous epithelial cells and about 2% columnar respiratory epithelial cells. The average number of bacteria on each of 40 epithelial cells before incubation was 5 (mean of 60 determinations; range, 0 to 21). The effect on adhesion of the viability of the epithelial cells was tested by trypan blue exclusion. Counting only unstained cells gave a mean adhesion value of 18 bacteria per cell (mean of 20 experiments) compared with 17 when both dead and viable cells were counted (mean of 20 experiments). Table 2 demonstrates the varia-

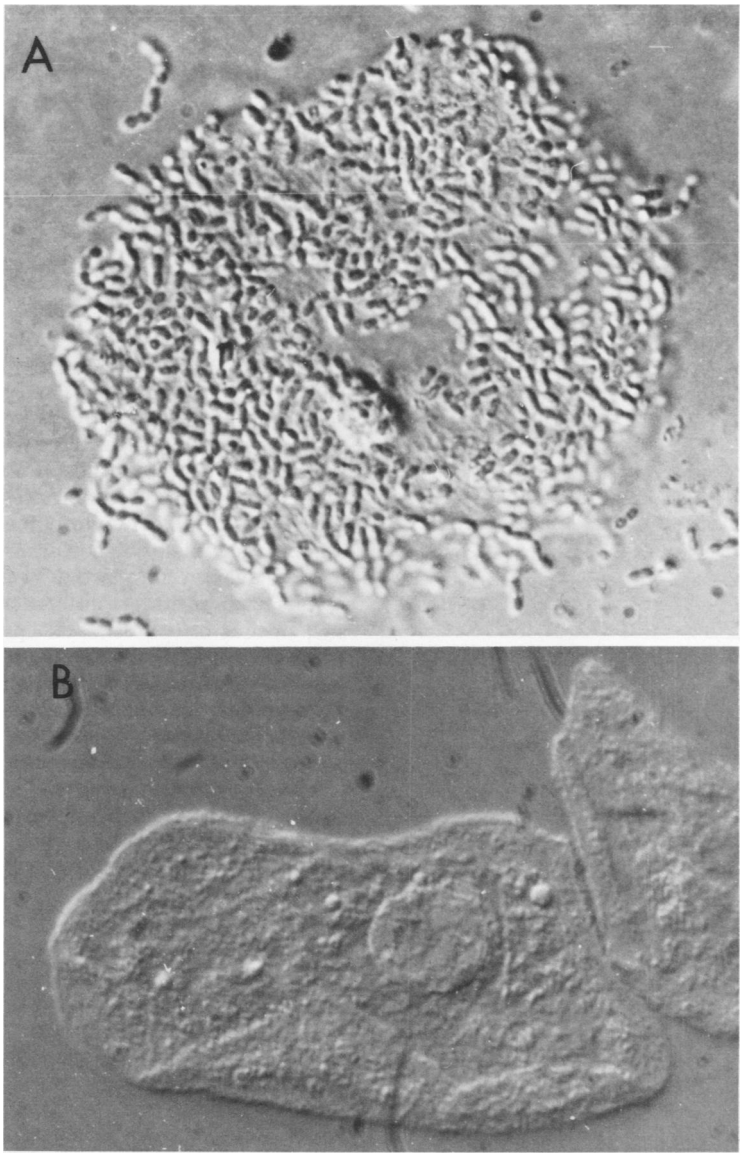

FIG. 1. *Pneumococci attached to human pharyngeal epithelial cells. (A) Efficiently adhering strain (strain I); (B) poorly adhering strain (strain II). SI, 340.*

tion in adhesion to epithelial cells from different subjects.

(iii) Incubation conditions. Figure 2 shows the effect of bacterial concentration on attachment. The adhering strain increased adhesion with increasing bacterial concentrations up to 10^{10} bacteria per ml. The poorly adhering strain attached in low numbers even at 10^{10} bacteria per ml. The optimal epithelial cell concentration was between 10^3 and 10^4 cells per ml (not shown). The influence of incubation time on adhesion was investigated for the adhering and poorly adhering strains. A total of 10^9 bacteria per ml were incubated with 10^4 epithelial cells at 37°C during rotation. Samples were taken after 0, 10, 15, 30, 45, 60, 120, and 180 min. Immediately after incubation, the samples were centrifuged and washed four times. Strain I attached in high numbers from 0 to 120 min, whereas strain II adhered poorly at all times (not shown). Samples which were not immediately centrifuged after incubation had lower adhesion values. In samples that were centrifuged immediately after mixing bacteria and epithelial cells, adhesion

TABLE 1. *Influence of pretreatment of pneumococci on ability to adhere to pharyngeal cells*

Culture condition	Adhesion (bacteria/cell)[a]	
	Strain I	Strain II
Washing with HBSS, 10 ml		
0	90	9
2	47	5
4	18	2
6	13	6
Boiling		
Untreated	79	13
Treated	1	0
Formalin, 0.5%		
Untreated	114	17
Treated	57	4
Trypsin		
Untreated	113	17
Treated	55	8

[a] Mean of two experiments.

TABLE 2. *Adhesion of pneumococci to pharyngeal cells from different subjects*

Age of cell donor (yr)	Adhesion (bacteria/cell)	
	Strain I	Strain II
49	90	0
49	133	3
27	95	0
27	151	0
26	132	0
27	135	0
19	204	0
26	88	0
52	101	0
33	112	0
28	138	0

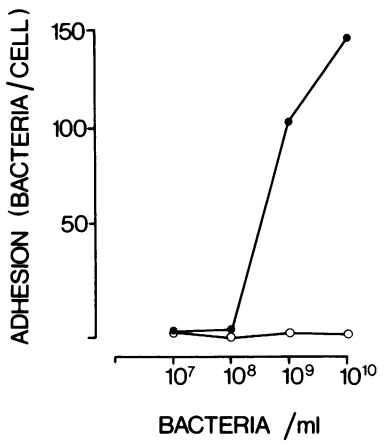

FIG. 2. *Increase in adhesion of pneumococci to pharyngeal epithelial cells in vitro with bacterial concentration. Symbols: (●) strain I (mean of two experiments); (○) strain II (mean of two experiments).*

values similar to those seen with the routine technique were obtained (not shown).

No differences in adhesion were found with PBS, HBSS, NaCl, or RPMI 1640 as the incubation medium. The highest adhesion was obtained at pH 6 and 7. An NaCl concentration of 0.9% produced the highest adhesion. Adhesion was higher at 15, 20, and 37°C than at 4°C and was low at 58°C for both adhering and poorly adhering strains (not shown).

Variation in adhesion within and among preparations of bacteria and epithelial cells. The sources of variation in the adhesion test system were studied in duplicate experiments on 5 consecutive days, using four efficiently adhering strains. The poorly adhering strains were not used for this purpose, since small differences in absolute adhesion give falsely high relative differences. The standard deviation for the number of bacteria per cell among a sample of 40 cells was estimated to be 52. The average standard deviation between the means of duplicate experiments using the same suspensions of bacteria and epithelial cells was estimated to be 15 bacteria per cell. Finally, the average standard deviation among the means for experiments done on different days and by using different bacterial and epithelial cell suspensions was estimated to be 21 bacteria per cell.

The variation measured as the standard deviation for the mean of a given strain may then be estimated as $\sqrt{\frac{21^2}{E} + \frac{15^2}{S} + \frac{52^2}{N}}$ bacteria/cell, where the first term is the variation among experiments done on different days divided by the number E of such experiments, the second term is the variation between duplicates done on the same day divided by the number S of such duplicates, and the third term is the variation among individual cells within one sample divided by the number N of cells counted. The formula may be used to estimate the variation for different numbers of experiments done or epithelial cells counted (Table 3). Maximal variation, 58 bacteria per cell, is obtained if one cell is counted in one experiment. By increasing the number of cells counted to 10, the variation decreases to 30.6. Counting 40 epithelial cells gives a variation of 27.1, which is 1.3 bacteria per cell above the ideal value obtained hypothetically by counting the bacteria attached to all the cells in the sample.

By performing duplicate experiments in which bacteria attached to 40 cells are counted, the variation is decreased to 24.9. By increasing the number of experiments infinitely, a theoretically lower limit for the variation of 22.6 bacteria per cell is obtained. Similarly, the variation obtained

by repeating the experiment on 2 different days is 22.7, and the theoretically lower limit is 17.1 bacteria per cell. The accuracy gained by performing duplicate samples, each consisting of 40 epithelial cells, is 27.1 − 24.9 = 2.2; by repeating the experiment, the accuracy gained is 27.1 − 22.7 = 4.4 bacteria per cell. Our conclusion is that the accuracy gained by these measures does not justify the increase in labor required. Also, little is gained by counting more than 40 cells. Accordingly, a single count of bacteria attached to 40 cells, expressed as the mean number of bacteria per cell, was used as an estimation of the adhesive capacity of each *S. pneumoniae* strain.

Conditions subsequently used for adhesion testing. Bacteria were cultivated overnight on blood agar plates and then in streptococcus cultivation broth for 18 h in a CO_2 atmosphere. A total of 10^9 bacteria and 10^4 pharyngeal epithelial cells, both suspended in HBSS, were mixed and incubated during rotation at 37°C for 30 min. Unattached bacteria were eliminated by washing four times in HBSS. Bacteria attached to each of 40 epithelial cells were counted by interference contrast microscopy in a Bürker chamber. Adhesion is given as the mean number of bacteria per cell after subtracting the background adherence.

TABLE 3. *Analyses of sources of variation in the adhesion test system*[a]

No. of epithelial cells counted	Variation (bacteria/cell)								
	1[b]			2			∞		
	1[c]	2	∞	1	2	∞	1	2	∞
1	58.0								
10	30.6	28.7		26.8					
40	27.1	24.9	22.6	22.7		18.6	17.1		
60	26.7								
∞	25.8			21.1					

[a] See text for details of formula used. Estimated mean adhesion was 107 bacteria per cell.
[b] Number of experiments.
[c] Number of duplicates.

TABLE 5. *Absence of correlation between polysaccharide capsule type and adhesive capacity of pneumococci*

Capsule type[a]	No. of strains	Adhesion (bacteria/cell)			
		Mean			Range
		Frequent otitis	Others	All	
4	5	130	42	64	3–167
6	7	103	24	58	7–152
14	7	40	23	28	14–63
19	8	47	7	22	0–82
23	7	56	20	35	4–99

[a] Danish nomenclature.

Adhesive capacity in relation to the origin of the *S. pneumoniae* strains. The highest mean adhesion was found in the group of strains from patients with frequent acute otitis media and from healthy carriers (Table 4). Strains from patients with septicemia or meningitis showed low mean adhesion. High adhesive capacity (>30 bacteria per cell) was found in 16 of 30 otitis strains, 5 of 30 septicemia strains, 6 of 30 meningitis strains, and 8 of 22 strains from healthy carriers. No relationship between storage time and adhesion was found.

Adhesive capacity in relation to capsule type. No relationship was found between the polysaccharide capsule type and adhesive capacity, as shown by the range of adhesion values within each capsule type (Table 5). Strains belonging to capsule types 4, 6A, 14, 19F, and 23F, types that often cause otitis media (2), had a higher mean adhesion (36 bacteria per cell) than strains of other capsule types (15 bacteria per cell). Within each capsule type, strains from patients with frequent otitis media had higher adhesive capacity than strains of other origins. Poorly adhering strains were, however, also found for strains of all capsular types tested.

TABLE 4. *Adherence to human pharyngeal epithelial cells of pneumococci in relation to clinical origin*

Diagnosis	Site of isolation	No. of strains	Mean adhesion (bacteria/cell)	% of strains with adhesion value >30	Level of significance (*P*)	Range (bacteria/cell)
Frequent acute otitis media	Nasopharynx	30	44	53	<0.01	0–152
Septicemia	Blood	30	18	17		0–167
Meningitis	Cerebrospinal fluid	30	22	20		0–88
Healthy carriers	Nasopharynx	22	39	36		0–342

DISCUSSION

An in vitro test system was developed to study the adhesion of pneumococci to human pharyngeal cells. The system was a modification of the method of Gibbons and van Houte (10) for adhesion studies on streptococci. The method was characterized regarding bacterial parameters, epithelial cell properties, binding kinetics, incubation media, and temperatures. A high number of strains with good adhesive capacity was found among strains from the pharynx of patients with frequent otitis media and from the pharynx of healthy carriers. Strains from patients with septicemia or meningitis attached poorly. Adhesive capacity and polysaccharide capsule type were not strongly related.

Uncontrolled sources of variation remained in spite of the standardization of the method. The variation between duplicates prepared from the same bacterial and epithelial cell suspensions was about 15%. The variation was about 20% among days with the same strain but with a new culture and new pharyngeal cells. Either cellular or bacterial factors were likely responsible for the variation, since incubation media and conditions were kept constant. Epithelial cell parameters such as presence of indigenous flora or differences in viability of epithelial cells did not account for a large part of the variation. The distribution of adhering bacteria on the 40 cells included in each sample was, however, skewed, possibly due to differences in maturation or receptor density of the cells. Such variations in receptivity for attaching bacteria may explain differences in sensitivity to, for instance, rheumatogenic streptococci or urinary tract pathogens (8, 16, 20, 22).

The most important source of variation in the method was, however, the heterogeneity of the bacterial population used for adhesion testing. Since the adhesion-mediating factor on pneumococci has not been identified, one cannot define the optimal culture conditions resulting in a high and constant proportion of the bacterial population with adhesive capacity.

Various mechanisms for bacterial adhesion have been suggested in other experimental systems. Pili are thought to mediate the adhesion of certain salmonellae, gonococci, and *Escherichia coli* strains (C. B. Brinton, Jr., paper presented at the XII U.S.-Japan Conference on Cholera, Atlanta, Ga., September 19–21, 1977). *S. mutans* has glucosyltransferases with the ability to digest sucrose and form glucans that strengthen adhesion to tooth surfaces (9). Group A streptococci adhere through structures containing a complex of M protein and lipoteichoic acid in which the lipoteichoic acid is considered to be the adhesin (3, 4, 18). The pneumococcal C carbohydrate is a choline-containing teichoic acid, the F antigen (cross-reacting with the Forssman antigen) is a choline-containing lipoteichoic acid (6). The role of lipoteichoic acid in pneumococcal adhesion has not yet been investigated. In our system, pretreatment of bacteria with heat or trypsin decreased adhesion, as was described earlier for other streptococci (7, 9, 19). This suggests that a structure containing protein may be involved in the adhesion mechanism.

The polysaccharide capsule is known to be an important virulence factor for pneumococci causing invasive disease (19). The capsule is, however, unlikely to participate in the adhesion reaction, since strongly and poorly adhering strains were found within each capsule type. Strains of capsule types 6A, 14, 19F, and 23F, known to occur very commonly in acute otitis media in children (2), adhered strongly when isolated from the nasopharynx of patients with otitis media, but less strongly when isolated from the blood or cerebrospinal fluid. It was not possible to determine whether the strongly adhering septicemia and meningitis strains originated from middle ear infections.

The high proportion of adhering strains in the otitis media and the healthy carrier groups suggests that attachment is important for colonization of the nasopharynx. The balance between host defense mechanisms and bacterial virulence factors might determine whether infection ensues from colonization. Our results suggest that adhesive capacity is not sufficient to induce infection, since 8 of 22 strains from healthy carriers adhered with >30 bacteria per cell. This may parallel what is found among *E. coli* strains: in those causing urinary tract infections, adhesive ability, certain capsular polysaccharide and endotoxic lipopolysaccharide types, as well as resistance to the bactericidal effect of serum coappear in virulent strains, whereas one or two of the factors but not all are found in normal fecal *E. coli* isolates (C. Svanborg Edén, L. Hagberg, L. Å. Hanson, T. Korhonen, H. Leffler, and S. Olling, Ciba Found. Symp., in press).

The pneumococci causing disease may need different strategies to remain at the site of infection and overcome host defense mechanisms. The results of this study indicate that the ability to attach to the pharyngeal mucosa may be important for the establishment of pneumococci in the nasopharynx preceding otitis media and describe a test system useful for further investigating the interaction with other bacterial factors and host defense mechanisms.

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