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Bacterial Adherence to Pharyngeal Cells in Smokers, Nonsmokers, and Chronic Bronchitics

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Selective adherence to host mucosal surfaces is probably a requirement for colonization and infection by bacteria. Since pharyngeal colonization may be an important determinant in the pathogenesis of pneumonia, we studied the adherence of 10 different bacteria to pharyngeal cells obtained from nonsmokers, smokers, and chronic bronchitics. Various patterns of adherence among the different groups of subjects were found. Young healthy smokers had increased adherence of *Streptococcus pneumoniae* type I and, to a lesser extent, *S. pneumoniae* type III and *Staphylococcus aureus* when compared with nonsmokers. Middle-aged smokers with a long history of chronic bronchitis had significantly increased adherence only of untypable *Haemophilus influenzae* when compared with age-matched nonsmokers. The acquisition of pneumococcal pneumonia by smokers and the role of nontypable *Haemophilus* species in chronic bronchitis may be determined, in part, by bacterial adherence to pharyngeal cells.

Bacterial adherence to mammalian cells is thought to play an important role in the pathogenesis of infection (8, 16-18, 27, 28). The possible contribution of bacterial adherence to human disease has been studied in dental caries (9), endocarditis (10, 26), diarrheal diseases (4, 6, 18, 24), urinary infection (5, 15, 31, 32), gonorrhea (1, 25), and vaginal infection (21, 22). Colonization of the upper airways by pathogenic bacteria has been documented in a number of disease states (7, 13, 14, 30, 33) and is thought to explain, in part, the predisposition to bacterial pneumonia. The possible relationship between increased bacterial cell adherence and colonization has generally not been examined. In one preliminary report of patients with endotracheal tubes, colonization of the respiratory tract by gram-negative bacteria was associated with increased bacterial adherence to buccal mucosal cells (35). Another study has shown increased bacterial adherence to these cells when they are obtained serially from patients after surgery (J. H. Higuchi, T. Chaudhuri, and W. G. Johanson, Jr., Am. Rev. Respir. Dis. **117**:274, 1978).

The purpose of this study was to investigate adherence of bacteria to pharyngeal cells obtained from cigarette smokers with and without chronic bronchitis and to compare these results with appropriate controls.

MATERIALS AND METHODS

Patient selection. (i) Smokers versus nonsmokers. Two groups of 15 young, healthy adults (10 male and 5 female in each group) were studied. Nonsmokers were defined as patients who did not smoke cigarettes, cigars, or pipes. Cigarette smokers were defined as those who smoked ≥40 cigarettes per day for at least 3 years and were presently smoking this number every day, but who were otherwise healthy, without symptoms or signs of chronic bronchitis.

(ii) Chronic bronchitics versus nonbronchitics. Middle-aged men were studied as follows. (a) Fifteen nonsmokers were considered normal for the purpose of this study if they were free from disease involving the mouth, nasopharynx, or respiratory system. They were identified among hospital visitors or during routine outpatient visits in orthopedics, ophthalmology, or urology clinics. Patients with alcoholism, liver or renal disease, or diabetes were specifically excluded. (b) Fifteen outpatients who were identified as severe chronic bronchitics (23) without exacerbation and who were seen in the chest clinic for routine follow-up visits were studied. These patients had smoked for at least 15 years and were still actively smoking ≥40 cigarettes per day, but were not taking antibiotics and had not been hospitalized recently for any reason.

All subjects lived in Houston, Tex., an urban environment. Subjects were matched for age, race, and sex; there were no significant differences among the groups. Subjects from all groups were studied concurrently, precluding the possibility that different results might reflect slight differences in the assay technique.

In vitro assay. (i) Bacteria. Bacteria used in this study included *Streptococcus pneumoniae* type I and type III, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Haemophilus influenzae* (type B and untypable), *Klebsiella pneumoniae*, *Serratia marcescens*, and *Pseudomonas aeruginosa*. Conventional methods were used to identify gram-positive bacteria. Pneumococci were typed by

the Massachusetts State Laboratories, and *H. influenzae* was typed by the City of Houston, Department of Public Health Laboratories. Enteric bacilli were identified by the API method. Data presented are based on results of studies using only one bacterial isolate for each bacterial strain.

Bacteria were maintained by freezing in broth containing 10% glycerol and storing at −65°C after a number of initial transfers on antibiotic-free medium. For each study, bacteria were grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 37°C; x and v factors (supplement B; Difco Laboratories, Detroit, Mich.) were added to Trypticase soy broth for growth of *H. influenzae*. Serial dilution and culturing of 10-μl samples showed that overnight cultures yielded 5×10^8 to 2×10^9 colony-forming units per ml; for the purpose of this study, the assumption was made that this overnight culture yielded 10^9 colony-forming units per ml. Bacteria were collected by centrifugation ($750 \times g$) for 15 min at 8°C, suspended in phosphate-buffered saline (PBS), pH 7.2, and diluted to yield 10^7 colony-forming units per ml.

(ii) Pharyngeal cells. Pharyngeal cells were obtained by gently scraping the posterior pharynx with a wooden tongue blade. Another tongue blade which was subsequently discarded was held underneath during the collection procedure to avoid contamination with lingual squamous cells. The end of the blade which contained pharyngeal cells was immersed in 5 ml of PBS at pH 7.2 (Sigma Chemical Co., St. Louis, Mo.) at 8°C and agitated for 30 s on a Vortex mixer. Cells were counted with a hemocytometer; the usual yield was about 3×10^5 to 4×10^5 cells for each pharyngeal scraping. Pharyngeal cells were collected and washed over polycarbonate membrane filters (25-mm diameter, 8-μg pore size; Nucleopore Corp., Pleasonton, Calif.), using a total of 10 ml of PBS. Cells were resuspended by placing the entire filter in 5 ml of PBS and agitating for 30 s; this procedure yielded about 10^5 cells in 10 ml, a loss of two-thirds of the initial cells. Recovery of pharyngeal cells was uniformly successful in all patient groups. The same batch of PBS was used throughout this study.

(iii) Cell-bacteria interaction. One milliliter of each suspension (10^7 bacteria and 10^4 cells in PBS; final ratio, 1,000:1) was incubated in plastic tubes in a slow gyrating water bath at 37°C for 1. Cells were again collected, washed through membrane filters, and resuspended in 1 ml of PBS. This suspension was centrifuged (Cytospin, SCA-0030; Shandon Southern Products, Ltd., Cheshire, England) onto glass slides for 20 min at $290 \times g$.

(iv) Staining and examination. Slides were stained by the Gram method, coded to eliminate reader bias, and examined microscopically at a ×1,000 magnification. Bacteria associated with at least 50 pharyngeal cells were counted. Small numbers of bacteria unassociated with cells were regularly present as background, but these were thought not to affect the readings. Control cells that had been incubated without bacteria were included in each study.

(v) Statistical analysis. The mean number of bacteria associated with each of 50 cells was determined for each bacterial species and for each individual subject. These were then averaged for each group of subjects. Results obtained with each bacterium were compared with base-line readings for cells incubated without bacteria, and comparisons were made among the various groups of subjects. Statistical analysis was carried out by the paired-difference *t* test in the biostatistics laboratory of J. Thornby, Veterans Administration Medical Center, Houston, Tex. The level of significance was determined by chi-squared analysis comparing the average number of bacteria per pharyngeal cell for all subjects after incubation with or without a particular bacterium and the average number of bacteria for pharyngeal cells of nonsmokers and smokers after incubation with each species of bacterium.

RESULTS

Normal subjects. When pharyngeal cells from 15 normal subjects (mean age, 32.4 years) were washed over membrane filters and examined without prior incubation with bacteria (controls), 3.5 ± 0.9 cell-associated bacteria, usually gram-positive cocci, were seen. After incubation of pharyngeal cells with streptococci or staphylococci, the control value was subtracted from the observed number of cell-associated bacteria for each subject before statistical comparisons were made. Incubation with all of the bacteria studied yielded a significant increase ($P < 0.01$) in the number of bacteria adherent to pharyngeal cells of all normal subjects. Adherence of gram-negative bacilli was also highly significant ($P < 0.001$), ranging from 7.9 ± 1.4 to 8.6 ± 1.7 bacteria per cell (Table 1).

Smokers. Results obtained in 15 healthy smokers (mean age, 33.6 years) for *S. mutans*, *S. pyogenes*, and *S. marcescens* were nearly identical to those obtained in nonsmokers (Table 1). Adherence of *S. pneumoniae* type I was strikingly greater for smokers than for nonsmokers (21.0 ± 3.1 in smokers versus 1.4 ± 1.2 in nonsmokers, $P < 0.001$). Adherence of *S. pneumoniae* type III and *S. aureus* was increased in smokers, albeit to a lesser degree (3.2 ± 1.5 versus 1.3 ± 1.2, $P < 0.01$; 3.0 ± 2.8 versus 1.2 ± 1.0, $P < 0.05$; respectively). There was less adherence of *Klebsiella* and *Pseudomonas* to pharyngeal cells of smokers than of nonsmokers ($P < 0.01$).

Middle-aged nonsmokers. Results in 15 asymptomatic subjects (mean age, 49.3 years) showed 4.6 ± 1.1 cell-associated bacteria after washing and before incubation with bacteria. A significant increase in adherence ($P < 0.01$) was observed after incubation with all bacteria studied.

Chronic bronchitics. Results in 15 patients with chronic bronchitis (mean age, 50.1 years) showed that bacterial adherence was nearly identical to that observed in middle-aged nonsmokers. The only exception was that adherence of nontypable *H. influenzae* was significantly

greater in bronchitics (10.0 ± 1.1 versus 5.8 ± 1.8, $P < 0.001$). In contrast to results observed in young adults, adherence of *S. pneumoniae* type I was not significantly increased in chronic bronchitics when compared with healthy middle-aged nonsmokers, and a lesser degree of attachment of *S. pyogenes* to pharyngeal cells was observed although there was no difference between results in nonsmokers and in chronic bronchitics.

Reproducibility of assay. The assay system appeared to be highly reproducible. Results of serial studies of four subjects in a 30-day period showed minimal day-to-day variation (Table 2).

TABLE 1. *Adherence of bacteria to pharyngeal cells obtained from nonsmokers, smokers, or chronic bronchitics*[a]

Organism	Adherence			
	Young adults		Middle-aged adults	
	Nonsmokers	Smokers	Nonsmokers	Chronic bronchitics
Control[b]	3.5 ± 0.9	3.5 ± 1.0	4.6 ± 1.1	4.5 ± 1.1
S. pneumoniae type I	1.4 ± 1.2	21.0 ± 3.1[c]	3.3 ± 1.2	3.3 ± 1.2
S. pneumoniae type III	1.3 ± 1.2	3.2 ± 1.5[d]	1.9 ± 0.9	1.9 ± 1.1
S. aureus	1.2 ± 1.0	3.0 ± 2.8[d]	1.2 ± 1.0	2.1 ± 1.2
S. pyogenes	11.3 ± 1.2	10.5 ± 1.4	1.2 ± 1.0	2.1 ± 1.2
S. mutans	3.6 ± 1.4	3.0 ± 2.0	2.0 ± 1.1	1.6 ± 1.0
H. influenzae type B	ND[e]	ND	6.0 ± 1.6	6.7 ± 1.2
H. influenzae nontypable	ND	ND	5.8 ± 1.8	10.0 ± 1.1[f]
P. aeruginosa	7.9 ± 1.4	5.6 ± 1.6[d]	6.9 ± 1.6	6.7 ± 1.2
K. pneumoniae	8.6 ± 1.7	4.9 ± 1.6[d]	6.0 ± 1.8	6.2 ± 1.4
S. marcescens	8.5 ± 2.2	7.9 ± 1.5	6.3 ± 1.2	7.1 ± 1.4

[a] Data are expressed as mean ± standard deviation.
[b] Results obtained when washed pharyngeal cells were incubated without bacteria. Data on adherence of gram-positive cocci were derived by subtracting this control value for each individual subject from the number of cell-associated organisms observed after incubation of the subject's pharyngeal cells with bacteria.
[c] Comparison with results obtained in young adult nonsmokers shows significant difference ($P < 0.001$).
[d] Comparison with results obtained in young adult nonsmokers shows significant difference ($P < 0.01$).
[e] ND, Not done.
[f] Comparison with results obtained in middle-aged nonsmokers shows significant difference ($P < 0.001$).

TABLE 2. *Serial observations of bacterial adherence to pharyngeal cells in four subjects*[a]

Organism	Adherence on day:							
	1	4	7	10	15	20	25	30
S. pneumoniae type I								
Nonsmokers	1.5	0.5	2.5	1.5	1.5	0.5	3.5	0.5
Smokers	17.5	22.5	19.5	18.5	20.5	20.5	18.5	21.5
S. pneumoniae type III								
Nonsmokers	0.5	0	1.5	0.5	0	1.5	1.5	0.5
Smokers	3.5	1.5	0.5	4.5	3.5	3.5	2.5	2.5
S. aureus								
Nonsmokers	0.5	1.5	0	2.5	1.5	0.5	0.5	1.5
Smokers	2.5	3.5	3.5	2.5	4.5	1.5	2.5	2.5
S. pyogenes								
Nonsmokers	3.5	4.5	2.5	3.5	5.5	3.5	4.5	3.5
Smokers	2.5	3.5	1.5	4.5	3.5	2.5	1.5	3.5
K. pneumoniae								
Nonsmokers	5.5	3.5	4.5	3.5	5.5	4.5	2.5	3.5
Smokers	1.5	0.5	2.5	0.5	0	2.5	3.5	1.5

[a] Average of results obtained in two smokers and two nonsmokers.

Similar reproducibility has been found in some individuals who have been studied at various times during a 1-year time period.

DISCUSSION

Adherence of bacteria to cells precedes and is thought to be an important step in the pathogenesis of infection (8, 16–18, 27, 28). This study examined the adherence of a panel of potentially pathogenic bacteria to pharyngeal cells from patients with conditions such as cigarette smoking and chronic bronchitis, which may predispose them to bacterial pneumonia. Our results show various patterns of bacterial adherence among different groups of subjects. Young healthy smokers without chronic bronchitis had increased adherence of *S. pneumoniae* type I and, to a lesser extent, *S. pneumoniae* type III and *S. aureus* when compared with nonsmokers. Middle-aged smokers with a long history of chronic bronchitis had significantly increased adherence only of untypable *H. influenzae* when compared with age-matched nonsmokers without bronchitis.

Results showing increased pneumococcal adherence to pharyngeal cells in smokers may explain the possible contribution of smoking as a risk factor in the susceptibility to bacterial pneumonia. The clinical importance of *H. influenzae* in patients with chronic bronchitis has been demonstrated in previous studies (30). These organisms may be commonly isolated from the sputum in patients with chronic bronchitis and have been implicated in episodes of acute exacerbation or pneumonia (11, 29, 34). The increased adherence of nontypable *H. influenzae* to pharyngeal cells from chronic bronchitics may give insight into the role of nontypable *Haemophilus* species in causing chronic bronchitis.

In our investigation, pharyngeal rather than buccal mucosal cells were used because colonization of the pharynx may be more directly related to the development of bacterial pneumonia than is colonization of the mouth (13). Several studies have suggested a relation between bacterial flora of the lung and pharyngeal flora (2, 12, 19, 20). Organisms such as *S. pneumoniae* and *S. aureus* which are able to colonize the posterior pharynx (3) may be more readily available to cause pneumonia in patients who have an increase in bacterial adherence. Studies currently in progress will attempt to relate the natural carriage of *S. aureus* to adherence of this and other bacteria.

Some findings of this study require further clarification. (i) Adherence of *S. pneumoniae* type I to pharyngeal cells was increased in healthy young smokers, but this same degree of increased adherence was not found with *S. pneumoniae* type III. (ii) In the middle-aged chronic bronchitics no significantly increased adherence of either type of pneumococcus was detected compared with nonsmokers. Why middle-aged heavy smokers who have chronic bronchitis do not have the same increased adherence of *S. pneumoniae* observed in young smokers remains unexplained. Similarly, why *S. pyogenes* should have adhered more vigorously to cells from young than from middle-aged subjects remains unknown. (iii) Adherence of *K. pneumoniae* and *P. aeruginosa* to pharyngeal cells of otherwise healthy young smokers was actually decreased when compared with appropriate controls. Whether this finding helps to explain the relative rarity of gram-negative pneumonia in healthy young smokers remains to be seen. (iv) The data reported herein were obtained by using a single bacterial isolate; different results might be obtained with other isolates. For example, another isolate of *S. pyogenes* was much less adherent to pharyngeal cells of healthy subjects, smokers, and chronic bronchitics than the one shown in Table 1 (data not shown). Nevertheless, the overall result was similar in that significant differences among these groups of subjects were not observed. Similarly, preliminary data suggest that *S. aureus* isolated from the nose of chronic staphylococcal carriers adheres more readily to pharyngeal cells than does *S. aureus* from randomly selected clinical specimens (V. Fainstein and D. Musher, unpublished data); the role of adherence in determining the carrier strain is currently under investigation.

The values obtained for each organism in each group of 15 subjects studied are derived from an average of bacterial counts done in at least 50 cells and are statistically significant. Whether these differences are great enough to indicate a role in the pathogenesis of infection requires further investigation. Recent studies in our laboratory have shown that viral infections may increase adherence of *S. aureus* and, to a lesser extent, *S. pneumoniae* and *H. influenzae* (V. Fainstein, D. Musher, and T. Cate, J. Infect. Dis., in press). The ultimate biological importance of these findings still remains unknown.

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