Pneumococcal Adherence to the Buccal Epithelial Cells of Cigarette Smokers*

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Adherence to mucosal surfaces is necessary for bacterial colonization. The in-vitro adherence of type 25 Streptococcus pneumoniae to buccal epithelial cells was studied in 15 smokers, 15 nonsmokers, and 21 exsmokers. Background adherence in smokers and nonsmokers was similar, but smokers had a markedly increased pneumococcal adherence compared to nonsmokers (12.3 ± 6.9 vs 0.7 ± 0.4). This increase was not related to subject age or duration of cigarette use. Pneumococcal adherence in some exsmokers remained elevated for up to three years after smoking cessation. Incubation of nonsmokers’ cells with smoker’s saliva resulted in increased pneumococcal adherence to the nonsmokers’ cells (1.1 ± 0.9 to 8.2 ± 4.4), suggesting mediation of pneumococcal adherence by a noncellular constituent of smokers’ saliva. The increased pneumococcal adherence in cigarette smokers may promote oropharyngeal colonization and contribute to the increased risk of respiratory infection in cigarette smokers.

Assay

An in-vitro assay was performed concurrently on all experimental groups to determine the adherence of S pneumoniae type 25 to buccal epithelial cells. The buccal epithelial cells (BEC) were obtained by gently scraping the inner aspect of both cheeks with a blunt metal spatula, avoiding lingual and dental contamination. The cells were collected in phosphate buffered saline (PBS), washed twice with PBS and harvested by differential centrifugation at 2.4 G. Equal volumes of the bacterial (10⁵ bacteria/ml) and buccal epithelial cell (10⁵ cells/ml) suspensions were mixed and incubated in a shaking bath both at 37°C for 36 minutes. After incubation, bacteria unattached to BEC were removed by four ten-minute differential centrifugations at 2.4 G. The BEC were then stained with crystal violet to visualize adherent bacteria. Background adherence was measured simultaneously for each subject using BEC incubated with buffer solution alone. The number of bacteria adherent to 50 consecutive cells from both background and S pneumoniae incubated groups were counted. The total background count was divided by 50 to yield mean background adherence per cell. The total background count for each subject was subtracted from the total count of the cells that had been incubated with S pneumoniae and the difference divided by 50 to yield mean pneumococcal adherence per cell.

All slides were read by two independent observers in a blinded fashion. Five smoking and six nonsmoking subjects had adherence measurements repeated 14 weeks after the first determination. Buccal cell maturation was graded by accepted cytologic criteria for all subjects.

Effect of Saliva

Unstimulated fasting morning whole saliva was collected by pipette from the anterior lower gingival gutter of randomly chosen subjects in the smoking and nonsmoking groups. The saliva was centrifuged and the supernatant filtered to remove all cells and bacteria. The BEC were obtained from six randomly selected nonsmokers and four randomly selected smokers. The BEC from the nonsmoking subjects were then mixed with (A) the nonsmoker’s own saliva, (B) another nonsmoker’s saliva, and (C) a smoker’s saliva. The BEC from the smoking subjects were mixed with (A) the smoker’s own saliva, and (B) a nonsmoker’s saliva. Saliva-BEC mixtures were incubated in a shaking water bath at 37°C for 30 minutes, then

METHODS

Study Groups

Three groups of volunteer, nonpaid subjects were studied: 15 nonsmokers (NS), ages 20 to 51, who had never smoked tobacco or other substances; 15 smokers (S), ages 25 to 65, who currently smoke >10 cigarettes a day and have smoked for at least five years; and 21 exsmokers (ES), ages 24 to 58, who stopped all smoking at least six months prior to testing. This study was approved by the Rochester General Hospital Human Investigations Committee. All volunteers gave informed consent.

No subject had respiratory symptoms or poor oral hygiene. Serum thiocyanate levels were measured in exsmokers to confirm abstinence from smoking. A cotton applicator was used to obtain cultures for S pneumoniae from the inner aspect of the cheek and pharynx in ten randomly chosen subjects from each group.

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Table 1—Repeat Measurements of Pneumococcal Adherence in Smokers and Nonsmokers

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<td>Smokers</td>
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<td>25.1</td>
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<td>11.7</td>
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<td>12.0</td>
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<tr>
<td>MEAN±SD</td>
<td>14.1±7.1</td>
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<td>Nonsmokers</td>
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<td>0.6</td>
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<tr>
<td>MEAN±SD</td>
<td>0.5±0.4</td>
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Results

Background adherence values were not significantly different in smokers (9.1 ± 9.1), exsmokers (4.8 ± 6.4), and nonsmokers (8.5 ± 21.1). However, after incubation with S pneumoniae type 25, the pneumococcal adherence to BEC of smokers (12.3 ± 6.9) was significantly greater (p < 0.001) than that of nonsmokers (0.7 ± 0.4), as shown in Figure 1. There was poor correlation between background adherence and pneumococcal adherence in nonsmokers (r = 0.5) and smokers (r = 0.68). Smokers’ and nonsmokers’ BEC were indistinguishable in terms of maturation as determined microscopically. The correlation coefficient for the blinded adherence estimates between the two readers was 0.95, 0.90 and 0.85 for S, ES, and NS, respectively.

The reproducibility of the elevated pneumococcal adherence estimates is shown in Table 1. The mean pneumococcal adherence for the repeat adherence
measurements is not significantly different from the original value, and the reproducibility for each individual subject also appears to be good.

There is no predictable relationship between either smoker's age (r = 0.01) or pack years of smoking (r = 0.03) to pneumococcal adherence.

Three years after cessation of smoking, all subjects have adherence values (0.5 ± 0.4) which fall in the range of the nonsmokers, as shown in Figure 2. Prior to the three years, six of 13 had elevated values but the degree of elevation in these six (4.5 ± 2.1) is significantly (p<0.05) less than that of current smokers (12.3 ± 6.9). All exsmokers with elevated adherence had serum thiocyanate levels which were within the normal range.

No pneumococci were isolated from any smoking or nonsmoking subjects.

The effect of smokers' saliva on pneumococcal adherence to BEC of nonsmokers is shown in Table 2. Pneumococcal adherence to BEC of nonsmokers is similar when the cells are incubated with either the nonsmoker's own saliva (1.1 ± 0.9) or with saliva of another nonsmoker (0.9 ± 0.2) but is significantly increased (p<0.001) to 8.2 ± 4.4 by incubation of these cells in smoker's saliva. The pneumococcal adherence to BEC of smokers, on the other hand, is not significantly changed by incubating their cells in their own saliva or in nonsmoker's saliva (21.0 ± 13.8 to 16.2 ± 6.2).

The salivary amylase in smokers was elevated compared to nonsmokers (135,800 vs 69,300 μg/ml) and the SGOT of smokers was lower (10 vs 27 μg/ml).

**DISCUSSION**

Bacterial adherence to cells is tissue- as well as host-specific and it is an essentially irreversible molecular interaction between cell surfaces. It is believed to determine the ability of bacteria to colonize and to perhaps infect and invade many mucosal surfaces.

Several studies have shown a relationship between increased bacterial adherence to buccal or pharyngeal mucosal cells and the development of pneumonia. Johanson et al have shown that increased in-vitro adherence of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E coli*, and *Proteus mirabilis* to buccal epithelial cells is associated with in-vivo colonization, which in turn has been correlated with increased risk of nosocomial pneumonia. Influenza infection has been shown to increase the adherence of *Staphylococcus aureus*, *S pneumoniae*, and *Hemophilus influenzae* to pharyngeal epithelial cells. These organisms cause pneumonias which complicate influenza respiratory illness.

This finding of increased adherence of type 25 *S pneumoniae* to smokers' buccal epithelial cells is similar to the increased adherence demonstrated by Fainstein and Musher77 for type 1 and type 3 *S pneumoniae*. In both studies, there was no difference in background adherence values between smokers and nonsmokers. This suggests that the increased adherence in smokers is mediated by some specific mechanism rather than a generalized increase in avidity of bacteria for smokers' buccal epithelial cells. Pneumococcal adherence in smokers remained constantly elevated over a 14-week interval. Thus, day-to-day variations in cigarette consumption, oral hygiene, or other acute effects of cigarette smoking are probably not important factors in determining adherence of *S pneumoniae* to buccal epithelial cells.

Adherence does not vary with age or duration of smoking. That is, it appears that pneumococcal adherence in smokers is fully expressed after a few years of smoking and does not intensify thereafter. Therefore, if adverse clinical consequences accrue from an increased adherence of Pneumococcus to oral epithelial cells, young smokers are at risk early in their smoking history. In this regard, the increased incidence of pneumonia in adolescent and young adult cigarette smokers is of interest.

The persistent adherence in exsmokers might be due to continued smoking, but the observation that these subjects had serum thiocyanate levels (half-life of 14 days) within the nonsmokers' range gives us confidence that the history of smoking cessation given by these nonpaid volunteers was correct.

Using routine clinical laboratory techniques, none of our subjects had positive pharyngeal cultures for *S pneumoniae*. Similarly, the enhanced in-vitro adherence of *S pneumoniae* following experimentally-induced influenza infection was not associated with pneumococcal colonization. This inability to culture *S pneumoniae* in asymptomatic cigarette smokers may be in part due to technical difficulties in identifying small numbers of colonized organisms. In any event, these observations do not refute the relation of adherence to colonization, but rather emphasize the multifactorial nature of the interaction of bacteria with their environment. Local defense mechanisms such as bacterial antagonism, surface immunoglobulins, salivary flow, surface fluids chemistry, and cell...
desquamation exist in the upper airway in-vivo and provide protection against surface colonization. If these are unfavorably altered in the host, the tendency for increased pneumococcal adherence may manifest itself in colonization and subsequent disease may then take place.

The mechanism by which smoking raises adherence and by which the increased adherence was maintained after smoking cessation in some subjects is not known. The elevation of nonsmoker’s adherence after incubation of their epithelial cells in a cell-free filtrate of smoker’s saliva suggests that a constituent of smoker’s saliva alters the surface properties of buccal epithelial cells in a manner that enhances their avidity for Pneumococcus, perhaps by making binding sites on buccal epithelial cells available to type 25 S pneumoniae. Although this study was not designed to determine how this takes place, one possibility is the removal of an antiadherence factor from the epithelial cell. Such a mechanism exists in the urinary tract where adherence of Gram-negative organisms to bladder mucosal cells rises when surface glycosaminoglycans are removed, and in the oral cavity where removal of fibronectin by trypsin enhances the adherence of Pseudomonas aeruginosa to the buccal cells. Whole saliva contains many enzymes including amylase and trypsin, which may act upon cell surfaces in an analogous fashion to increase pneumococcal adherence. Salivary amylase was elevated in the three of the smoking subjects tested.

It has been shown that in-vitro adherence of S pneumoniae to buccal epithelial cells of cigarette smokers is increased. This increased adherence remains constant over a period of several months in active cigarette smokers and may persist for up to three years after smoking cessation. Since increased adherence of other bacteria to surface cells is an established pathogenetic step in infection in both the lung and other organs, this observation may explain the increased risk of lower respiratory infection that exists in cigarette smokers. This increased adherence is mediated by a factor found in the cell-free filtrate of smoker’s saliva.

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