

Bacterial adhesion to oropharyngeal and bronchial epithelial cells in smokers with chronic bronchitis and in healthy nonsmokers

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Bacterial adhesion to oropharyngeal and bronchial epithelial cells in smokers with chronic bronchitis and in healthy nonsmokers. G.C. Riise, S. Larsson, B.A. Andersson. ©ERS Journals Ltd 1994.

ABSTRACT: Bacterial adhesion is probably a prerequisite for colonization of mucous membranes, but adhesion to the bronchial mucosa has not been studied in detail. We investigated adhesion of respiratory pathogens to bronchial epithelial cells, and asked whether chronic bronchitis had an influence on bacterial adhesion.

Oropharyngeal and bronchial cells were collected during bronchoscopy from 14 healthy nonsmokers, 22 smokers with nonobstructive chronic bronchitis, and 19 smokers with chronic bronchitis and chronic obstructive pulmonary disease (COPD). Patients with a forced expiratory volume in one second (FEV₁) less than 50% predicted were excluded. Adhesion of highly adherent test strains of *H. influenzae* and *S. pneumoniae* to these cells were studied.

The test strains of *H. influenzae* and *S. pneumoniae* were found to adhere well to both oropharyngeal and bronchial cells. *H. influenzae* showed a higher degree of adhesion both to ciliated and goblet cells from the patients with nonobstructive bronchitis than to cells from the healthy nonsmokers. No corresponding difference was found for *S. pneumoniae*. The patients with COPD did not differ from the controls in their adhesion values.

Our results indicate that bacterial adhesion is of importance for the colonization and retention of *H. influenzae* in the human airways. For *S. pneumoniae* the role of adhesion is more uncertain.

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Bacterial adhesion may be an important factor favouring bacterial persistence and colonization of the respiratory tract [1–3]. Most studies have investigated bacterial adhesion of *Streptococcus pneumoniae* and *Haemophilus influenzae* to oropharyngeal and buccal cells, either to exfoliated cells or to nasopharyngeal mucosal biopsies. Adhesion has also been demonstrated to exfoliated human bronchial cells for the airway pathogens *Bordetella pertussis* [4], *Pseudomonas aeruginosa* [5], and *Staphylococcus aureus* [6].

Smokers have been shown to have an enhanced adherence of *S. pneumoniae* to buccal cells [7, 8], and smokers with chronic bronchitis appear to have increased adherence of *H. influenzae* to their pharyngeal cells [9]. However, the effect of the smoke-induced airway disease, chronic bronchitis, on bacterial adhesion to bronchial epithelial cells is not known.

Chronic bronchitis is characterized by bronchial hypersecretion and productive cough. Some patients with chronic bronchitis are prone to recurrent infectious exacerbations with sputum purulence, and some develop airway obstruction as part of the disease entity, chronic obstructive pulmonary disease (COPD) [10]. Patients with chronic bronchitis have a higher frequency of positive bacterial cultures from the lower respiratory tract

than healthy individuals [11, 12]. Studies in chronic bronchitis have shown that, both at acute infectious exacerbations and at intervals free from exacerbations, about half of the patients are colonized intrabronchially with bacteria belonging to the nasopharyngeal flora [13, 14]. The species cultured include α -haemolytic streptococci, *S. pneumoniae*, *H. influenzae* and coagulase negative staphylococci [15, 16]. Why only some, and not all, patients with chronic bronchitis develop exacerbations is not known.

The first object of the present investigation was to study adhesion of *S. pneumoniae* and *H. influenzae* to human respiratory cells. Secondly, to determine whether the smoke-induced airway diseases, chronic bronchitis and COPD, change the ability of bronchial cells to adhere *S. pneumoniae* and *H. influenzae*. Thirdly, to investigate whether changed adhesion could be a factor promoting recurrent infectious exacerbations in chronic bronchitis.

Material and methods

Design

Bacterial adhesion of test strains of *S. pneumoniae* and *H. influenzae* to epithelial cells from the upper and

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lower respiratory tract was evaluated. Adhesion to cells sampled from 41 patients with chronic bronchitis were compared to cells taken from 14 healthy nonsmoking controls.

Subjects

The control group comprised 14 healthy nonsmoking volunteers with normal spirometry and no signs of infectious respiratory disease during the previous 4 weeks. The patient group comprised 41 patients with chronic bronchitis. All were current smokers with productive daily cough for at least three consecutive months each year during the past 2 yrs. They were subdivided into four groups, according to presence or absence of airway obstruction, and presence or absence of recurrent infectious exacerbations:

1. Twelve patients with nonobstructive chronic bronchitis with less than two recurrent infectious exacerbations in the past 2 years, as defined by BOMAN *et al.* [17]. They had normal spirometry (defined as a forced expiratory volume in one second (FEV₁) >80% of predicted and no reversibility on β_2 -agonists).
2. Ten patients with nonobstructive chronic bronchitis with at least three or more infectious exacerbations in the past two yrs.
3. Eleven patients with chronic bronchitis and COPD (defined as a FEV₁ <80% of predicted and no reversibility on β_2 -agonists) and with less than two exacerbations in the past 2 yrs.
4. Eight patients with chronic bronchitis and COPD with at least three or more infectious exacerbations in the past 2 yrs.

Eight nonobstructive chronic bronchitis patients used β_2 -agonists for inhalation at irregular intervals. Of the patients with chronic bronchitis and COPD, eight used oral N-acetylcysteine (NAC) regularly (200 mg at least twice daily), eight used β_2 -agonists for inhalation at irregular intervals, and four used inhaled steroids regularly. None used theophylline preparations or oral steroids. The duration of the bronchitis symptoms in the patient group varied from 2.5 to 24 yrs, with a mean of 12.5 yrs.

Criteria for exclusion were abnormal chest X-ray, bronchial hypersecretion caused by factors other than cigarette smoking, a history of asthma or reversibility >10 % on β_2 -agonists, and for safety reasons a FEV₁ less than 50% predicted or age more than 70 yrs. Ventilatory lung function (FEV₁) was measured with a Bernstein spirometer or Vitalograph.

The mean age and the mean maximum FEV₁ (in percentage of predicted normal) did not differ substantially between the healthy controls and the patients with nonobstructive chronic bronchitis. The patients with chronic bronchitis and COPD were significantly older than the healthy controls, and had a lower FEV₁ than the controls and the patients with nonobstructive chronic bronchitis. The smoking history of the four subgroups with chronic bronchitis was comparable (table 1).

The study design was approved by the local Ethics Committee and the volunteers gave their consent after written and oral information.

Bronchoscopy

Premedication was given with haloperidol, 5 mg orally followed by 0.5–1 ml morphine-scopolamine *i.m.* Five millilitres 1% tetracaine, without preservative, was nebulized with a pressurized nebulizer (Plug-in inhalator, Aiolos systems, Karlstad, Sweden) and inhaled in an upright position for local anaesthesia. All bronchoscopies were performed transorally by one of two experienced bronchoscopists, and with the patient in the supine position. Olympus flexible fiberoptic bronchoscopes of several models were used.

Collection of samples

Epithelial cells were scraped from the oropharynx with a cotton-tipped wooden swab and suspended in phosphate-buffered saline.

Bronchial brush biopsies for adhesion studies were taken during bronchoscopy using a standard cytology brush (Olympus BC 9C-26101). Three consecutive brushings from an approximately 2–4 cm² intrabronchial area were sampled from two levels in the bronchial tree: from the carina level and from the subsegmental bronchial level in the right lower lobe. Two separate brushes were used for the two levels. The samples were immediately fixed in buffered 5% formaldehyde. A pilot study using airway cells taken from patients, mostly smokers, undergoing routine bronchoscopy for investigation of various airway diseases had previously shown that formaldehyde fixation caused no interference with bacterial adhesion (data not shown). The cells were stained with a drop of Sedistain (Clay Adams, Becton Dickinson, Parsippany, NJ 07054, USA) and

Table 1. – Age, FEV₁ and cigarette consumption of healthy controls, patients with chronic bronchitis, and patients with chronic bronchitis and COPD

	n	Age* yrs	FEV ₁ ** % pred	Pack-years**
Healthy controls	14	46 (26–66)	101 (1)	0
Chronic bronchitis without infectious exacerbations	12	49 (36–62)	96 (2)	35 (2)
Chronic bronchitis with infectious exacerbations	10	55 (48–68)	87 (2)	36 (2)
Bronchitis and COPD without infectious exacerbations	11	60 (44–70)	63 (3)	43 (2)
Bronchitis and COPD with infectious exacerbations	8	54 (38–66)	63 (3)	45 (2)

*: data are presented as mean and range in parenthesis; **: data are presented as mean and SEM in parenthesis. FEV₁: forced expiratory volume in one second; COPD: chronic obstructive pulmonary disease; % pred: percentage of predicted normal.

morphologically analysed using an interference contrast microscope (Optophot II, Nikon, Fuji Bldg, 2-3, Marunouchi 3-chome, Chiyoda-ku, Tokyo 100, Japan).

Bacteria

The ability of epithelial cells sampled from the oropharynx and the bronchi of patients with chronic bronchitis and healthy controls to bind bacteria was analysed by using two highly adherent bacterial test strains: nontypable *H. influenzae* CCUG (culture collection of the University of Göteborg) 21594 isolated from the nasopharynx of a child with acute otitis media, and *S. pneumoniae* CCUG 10175, type 19F, isolated from the nasopharynx of a healthy child. All bacteria were kept lyophilized until used for the adhesion analysis.

Adhesion assay

Bacteria were cultivated on solid medium over night, and then in liquid medium for 10 h as described previously [18, 19]. After washing, 10^4 cells·ml⁻¹ were mixed with 10^9 bacteria·ml⁻¹ in a total volume of 200 µl. The mixture was centrifuged at 1,500×G for 10 min and incubated at 37°C for 30 min. Unattached bacteria were separated from the epithelial cells by two cycles of suspension in a buffer of physiological saline (0.9%) with 10 mg·ml⁻¹ choline chloride and centrifugation at 500×G for 10 min. The mean number of adhering bacteria per epithelial cell was counted using an interference contrast microscope. The analysis was performed without any knowledge of which subject group the cells had been taken from. In the bronchial brush biopsy samples, each cell type with at least 20 analysable cells was included. In samples containing a high number of cells, 40 cells were analysed.

For oropharyngeal cells, adhesion was arbitrarily defined as high at ≥ 40 bacteria·cell⁻¹, intermediate at 10–40 bacteria·cell⁻¹ and low at < 10 bacteria·cell⁻¹. For the smaller bronchial epithelial cells, high adhesion was arbitrarily defined as > 10 bacteria·cell⁻¹.

For technical reasons, it cannot be excluded that some of the cells classified as goblet cells covered with

bacteria were damaged columnar epithelial cells with oedema and without cilia. Similar methodological difficulties have been described previously by other investigators [20].

To estimate the natural variation in bacterial adhesion over time for the two test strains, we used cells from a nonsmoking healthy individual (BA) as a reference for each adhesion experiment. The mean adhesion values for the *H. influenzae* test strain to the BA cells did not differ statistically when used for the study of healthy subjects 32 (SD 14), chronic bronchitis patients 27 (SD 18) and COPD patients 29 (SD 18). Nor did the mean adhesion values for the *S. pneumoniae* test strain to the oropharyngeal cells of BA differ statistically when used parallel to the study of healthy subjects 110 (SD 49), chronic bronchitis 90 (SD 30) and COPD 84 (SD 42).

Statistical analysis

The bacterial adhesion values were calculated using the arithmetic mean and standard deviation. The Student's t-test was used for the comparison of the distribution of the bacterial adhesion values between groups of subjects. Multiple regression analysis was used to determine whether patient factors such as age, smoking habits, duration of bronchitis symptoms, FEV₁ values or serum levels of immunoglobulin (Ig) A, M, G and IgG-subclasses had an influence on the bacterial adhesion.

Results

Adhesion of bacterial test strains to oropharyngeal epithelial cells

A pronounced adhesion of *H. influenzae* CCUG 21594 and *S. pneumoniae* CCUG 10175 to the oropharyngeal epithelial cells both from patients and controls is shown in table 2. No statistically significant

Table 2. – Adhesion of *H. influenzae* (CCUG 21594) and *S. pneumoniae* (CCUG 10175) to oropharyngeal epithelial cells from healthy controls, patients with chronic bronchitis, and patients with chronic bronchitis and COPD, and with and without exacerbations, respectively

Subject category	Adhesion					
	<i>H. influenzae</i>			<i>S. pneumoniae</i>		
	n	bacteria·cell ⁻¹	p	bacteria·cell ⁻¹	p	
Healthy controls	14	55 (38)		149 (67)		
Chronic bronchitis, total group	22	55 (26)	NS	184 (78)	NS	
Without exacerbations	12	61 (23)	NS	176 (70)	NS	
With exacerbations	10	47 (28)	NS	194 (88)	NS	
Chronic bronchitis and COPD, total group	19	66 (50)	NS	135 (67)	NS	
Without exacerbations	11	66 (50)	NS	147 (67)	NS	
With exacerbations	8	61 (34)	NS	116 (68)	NS	

Data are presented as mean and SD in parenthesis. CCUG: culture collection of the University of Göteborg; COPD: chronic obstructive pulmonary disease; NS: nonsignificant.

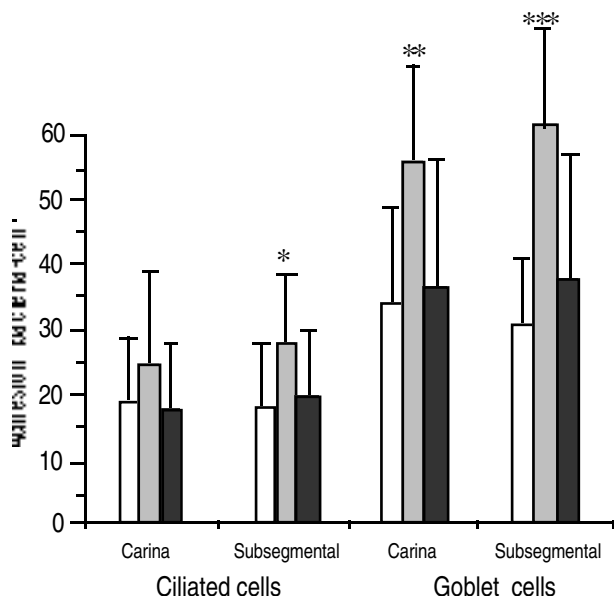


Fig. 1. – Adhesion of *H. influenzae* (CCUG 21594) to bronchial epithelial cells from the carina and subsegmental levels in healthy non-smoking controls (n=13), smokers with nonobstructive chronic bronchitis (n=19), and smokers with chronic bronchitis and COPD (n=18). Data are presented as mean and sd. Significantly higher adhesion compared to corresponding cells from healthy controls. *: p<0.05; **: p<0.01; ***: p<0.001. □: healthy controls; □: nonobstructive chronic bronchitis; ■: chronic bronchitis and COPD. COPD: chronic obstructive pulmonary disease.

difference was found between cells from bronchitic patients and controls, nor between nonobstructive chronic bronchitis patients and patients with chronic bronchitis and COPD.

Adhesion of bacterial test strains to bronchial epithelial cells

There was a significantly higher adhesion of *H. influenzae* CCUG 21594 to ciliated columnar epithelial cells from the subsegmental level of patients with nonobstructive chronic bronchitis compared to the corresponding cells from healthy controls (p<0.05). Furthermore, there was a significantly higher adhesion to goblet cells both from the carina level (p<0.001) and the subsegmental level (p<0.01). The patients with chronic bronchitis and COPD had no significant differences

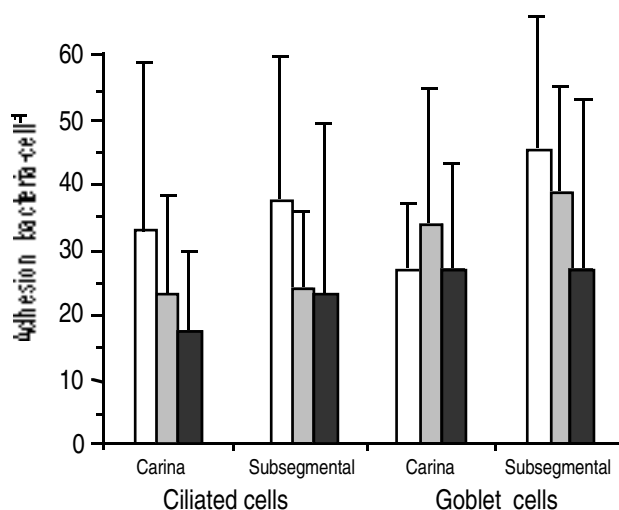


Fig. 2. – Adhesion of *S. pneumoniae* (CCUG 10175) to bronchial epithelial cells from the carina and subsegmental levels in healthy non-smoking controls (n=13), smokers with nonobstructive chronic bronchitis (n=19), and smokers with chronic bronchitis and COPD (n=18). Data are presented as mean and sd. □: healthy controls; □: nonobstructive chronic bronchitis; ■: chronic bronchitis and COPD.

in adherence of *H. influenzae* CCUG 21594 to any bronchial cell type compared to the controls (fig. 1).

There was a high bacterial binding of *S. pneumoniae* CCUG 10175 to bronchial epithelial cells both from patients and controls. No statistically significant difference was found between cells from bronchitis patients and controls, or between cells from the nonobstructive chronic bronchitis patients and the patients with chronic bronchitis and COPD (fig. 2).

No significant difference in adhesion could be demonstrated for *H. influenzae* CCUG 21594 or *S. pneumoniae* CCUG 10175 to bronchial epithelial cells from patients with and without infectious exacerbations (table 3). Nor was any difference seen in adhesion between cells from the upper and lower sampling levels in the bronchial tree.

Multiple regression analysis was used to determine whether patient factors such as age, smoking habits, duration of bronchitis symptoms, FEV₁ values, and serum levels of immunoglobulin A, M, G and IgG subclasses had an influence on the bacterial adhesion. No significant covariation was found (data not shown).

Table 3. – Adhesion of *H. influenzae* (CCUG 21594) to ciliated epithelial cells and goblet cells from all patients with chronic bronchitis, with and without infectious exacerbations

Cell type	Adhesion						
	With exacerbations			Without exacerbations			
	bacteria-cell ⁻¹	n	p	bacteria-cell ⁻¹	n	p	
Ciliated epithelial cells							
Carina level	23	(12)	17	27	(19)	23	NS
Subsegmental level	25	(15)	17	29	(17)	22	NS
Goblet cells							
Carina levels	21	(27)	13	22	(26)	18	NS
Subsegmental level	22	(29)	12	21	(29)	15	NS

Data are presented as mean and sd in parenthesis. For abbreviations see legend to table 2.

The differing subject numbers for bacterial adhesion to bronchial cells in figures 1, and 2 and table 3 are due to exclusion of subjects with too few analysable cells.

Discussion

For *H. influenzae*, the following picture of bacterial adhesion emerges. Both oropharyngeal and bronchial epithelial cells showed a high binding capacity for the *H. influenzae* test strain in the present study. We also found that two strains of *H. influenzae* isolated from the bronchi of the patients in the present study adhered well to oropharyngeal and bronchial epithelial cells (unpublished data). In chronic bronchitis, the ability of epithelial cells from the respiratory tract to bind *H. influenzae* seems to be increased. FAINSTAIN and MUSER [9] have previously demonstrated a higher adhesion of *H. influenzae* to oropharyngeal epithelial cells from patients with chronic bronchitis than to cells from healthy controls [9]. This could not be confirmed in our study. However, a significantly higher binding of *H. influenzae* was found to bronchial goblet and ciliated cells from patients with nonobstructive chronic bronchitis as compared to cells from the healthy controls. These results are consistent with the idea that adherence of *H. influenzae* is possibly an important factor in the colonization of the airways, and may help provide an explanation for the reported tendency for patients with chronic bronchitis to retain *H. influenzae* on their bronchial epithelial surfaces [21].

For *S. pneumoniae* the situation is more complex. The pneumococcal test strain, used in the present study, with a known high adhesive capacity, adhered well to both the oropharyngeal and bronchial epithelial cells. We have previously demonstrated that a high frequency of pneumococcal strains adhering well to oropharyngeal cells can be found in isolates from the middle ear, in acute otitis media (AOM), and from the nasopharyngeal mucosa [18, 22]. However, approximately 45% of the isolates from these sites proved to be poorly adherent to oropharyngeal cells [18, 22]. We have also found that strains demonstrating poor adherence are over-represented among certain common serotypes. In 20 blood isolates of pneumococcus type 3 from adults with septicaemia, we found a mean adhesion of only 2 bacteria-cell⁻¹ (99% confidence interval (99% CI) 1–4 bacteria-cell⁻¹), and in 26 nasopharyngeal isolates of pneumococcus type 3 from children with AOM a mean adhesion of 3 bacteria-cell⁻¹ (99% CI 0–8 bacteria-cell⁻¹ (unpublished data). This may explain why earlier studies using pneumococcal strains of capsular type 3 were unable to show adhesion [23–25]. The data from the present study demonstrate that strains adhering to epithelial cells from both the upper and the lower airways exist. It is, however, unclear if bacterial adhesion to bronchial epithelial cells is of importance for the colonization of pneumococci.

Smoking has been found to increase the tendency of oropharyngeal cells to bind *S. pneumoniae* [7, 8]. A similar, although not statistically significant, trend was

seen for the test strain in our study. However, no increased adhesion of *S. pneumoniae* was seen to bronchial cells from the patients with chronic bronchitis as compared to cells from the controls.

No statistically significant difference in bacterial binding could be demonstrated for either test strain to cells from the patients with chronic bronchitis and COPD as compared to the controls. This is surprising, as a convincing difference was found between the non-obstructive patients and the controls for *H. influenzae*. Furthermore, as we reported previously [12], the patients with COPD showed an intrabronchial bacterial flora comparable to that of the nonobstructive patients. As the cells from the COPD patients were collected somewhat later in time, we investigated whether the adhesive properties of the test strains had changed, but no evidence for this could be found. There was, however, a greater range of the adhesion data for the COPD patients than for the other groups, and in some COPD patients a very high bacterial adhesion to the bronchial epithelial cells was demonstrated.

No differences in adhesive properties were found for cells taken from patients with and without recurrent infectious exacerbations. We chose to use highly adherent test strains in the present study to optimize the conditions for a possible demonstration of bacterial adhesion to bronchial cells. However, it is possible that such test strains could also cause saturation of adhering bacteria to cells which have a low tendency for bacterial adhesion. Thus, subtle differences between subject groups, such as the patients with and without infectious exacerbations, could have been disguised.

There are some additional methodological issues in our study that need to be addressed. It has been claimed that the binding of bacteria to exfoliated cells, as performed in our assay, is an *in vitro* artifact of no clinical importance [25]. However, at present there are no other methods that enable studies of the influence of bronchial diseases on bacterial adhesion. Furthermore, our finding of different adhesion properties for cells from different subject groups shows that the assay is influenced by events occurring before exfoliation, *e.g.* disease processes, such as chronic bronchitis, and thus have a clinical implication. We cannot rule out the possibility that our bacterial test strains could bind to mucus as well as to exfoliated cells, especially since we focused on subjects with chronic bronchitis known to have a production of excess mucus and bacterial infections. Earlier studies using organ culture of respiratory mucosa have shown a binding of *S. pneumoniae* [24] and *H. influenzae* [26] to mucus. However, we did not observe any mucus strands associated with the exfoliated cells, or bacteria adhering to mucus, and we believe that most mucus had been washed away during the adhesion assay.

In summary, adhesion of *H. influenzae* to ciliated columnar cells and goblet cells from the bronchi was demonstrated, and increased adherence was found to bronchial cells, especially goblet cells, from patients with nonobstructive chronic bronchitis. Bacterial adhesion to airway epithelial cells may be a factor of importance

for colonization and retention of *H. influenzae* intra-bronchially. The role of adhesion for colonization with *S. pneumoniae* intrabronchially seems, however, to be less clear. We found no evidence that increased bacterial adhesion can explain why some patients with chronic bronchitis develop recurrent infectious exacerbations.

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