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S-carboxymethylcysteine inhibits the attachment of *Streptococcus pneumoniae* to human pharyngeal epithelial cells

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Abstract

Streptococcus pneumoniae causes respiratory and other invasive infections. Increased resistance of this bacterium to antibiotics necessitates new approaches to the treatment of infections. Attachment of bacteria to human pharyngeal epithelial cells is the initial step in the pathogenesis of infection and *S*-carboxymethylcysteine (*S*-CMC) can modulate the attachment of *Moraxella catarrhalis* and nontypable *Haemophilus influenzae* to epithelial cells. Unlike these two, *S. pneumoniae* is gram-positive and has a well-defined capsule. Here we examined the effects of *S*-CMC on the attachment and detachment of *S. pneumoniae* to human pharyngeal epithelial cells in vitro. Treatment of these cells with *S*-CMC significantly reduced the number of attached *S. pneumoniae*. *S*-CMC also resulted in a significant increase in the detachment of already attached *S. pneumoniae* to epithelial cells. In addition, treatment of *S. pneumoniae* with *S*-CMC significantly reduced their ability to attach to epithelial cells, but not the number of viable bacteria. Our study shows that *S*-CMC modulates the attachment of *S. pneumoniae* to human pharyngeal epithelial cells by acting both on cells and bacteria.

Keywords: Respiratory infection; Bacterial attachment; Mucosa; Epithelial cells; Bacterial adherence

1. Introduction

Attachment of bacteria to host epithelial cells is the first step in the pathogenesis of bacterial infections. *Streptococcus pneumoniae* is one of the major groups of bacteria that can cause various invasive and noninvasive infections. Most of these infections are preceded by colonization of the pharynx by *S. pneumoniae*. Then either the bacteria invade the lower respiratory tract causing bronchopneumonia and subsequently invade the blood stream or remain localized and cause infection of the mucosal tissue. In severe cases, the colonized bacterium may invade the upper airways directly to cause meningitis. Therefore, inhibition of bacterial colonization in the pharyngeal area seems to be a useful strategy to prevent infections. This may be achieved by either systemic or local use of anti-attachment agents such as antibodies, receptor analogues or other agents such as xylitol [1], *N*-acetylcysteine [2] and *S*-carboxymethylcysteine (*S*-CMC). Among these, *S*-CMC is perhaps the most promising agent since previous studies have shown that it significantly reduced the number of episodes of acute exacerbations of respiratory infections when administered systemically [3]. Previous studies have also demonstrated that *S*-CMC acts both in vitro and in vivo, by rendering pharyngeal epithelial cells capable of inhibiting the adhesion of *M. catarrhalis* and nontypable *Hemophilus influenzae* (NTHI) [4,5].

S-CMC is a mucolytic agent and has been used in the treatment of different respiratory diseases characterized by abnormal mucus secretion [4,5]. It is also effective for the treatment of otitis media with effusion in children [6]. The precise mode of action of *S*-CMC is unknown; it corrects the intracellular abnormalities of glycoprotein synthesis and normalizes the secretory function of the mucosal epithelium [7].

To our knowledge, the effects of *S*-CMC on gram-positive bacteria and bacteria with a well-defined capsule have not been investigated. These features apply to *S. pneumoniae*, which is also one of the most significant

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pathogens known to cause respiratory tract infections. Furthermore, the increased number of antibiotic-resistant *S. pneumoniae* makes treatment of infections caused by such bacteria difficult to control with commonly used antibiotics. The present study was designed to examine the effects of *S*-CMC on the attachment of *S. pneumoniae* to pharyngeal epithelial cells. Our results indicated that *S*-CMC could modulate the attachment of *S. pneumoniae* to human pharyngeal epithelial cells by acting both on the cells and bacteria. The results suggest that this drug may be potentially useful for the prevention of *S. pneumoniae* infections.

2. Results

2.1. S-CMC inhibits attachment of S. pneumoniae to epithelial cells

Taking all the data of controls into consideration, the mean number of *S. pneumoniae* strain SP-95-19 attached per epithelial cells was 17.0 ± 8.1. The attachment of this strain to epithelial cells significantly decreased following treatment of the cells with *S*-CMC at a concentration of 100, 10, 1 or 0.1 μg/ml ($P < 0.05$, Table 1). However, at 0.01 and 0.001 μg/ml, *S*-CMC had no effect on attachment. Treatment of pharyngeal epithelial cells with *S*-CMC at a concentration of 0.1 μg/ml resulted in a significant ($P < 0.001$) decrease in the attachment of the other three strains of *S. pneumoniae* (SP-95-27, SP-96-29 and SP-95-36) to 33.9 ± 0.8% of the control.

2.2. S-CMC enhances detachment of bacteria from epithelial cells

Following the completion of the attachment assay, epithelial cells with the attached *S. pneumoniae* strain SP-95-19 were treated with 100, 10, 1 or 0.1 μg/ml *S*-CMC. This resulted in a significant decrease in the number of attached bacteria (Table 2). However, no such decrease was noted when cells were treated with 0.01 and 0.001 μg/ml *S*-CMC (Table 2).

The detachment assay also showed a significant ($P < 0.05$) decrease in the proportion of the other three strains of *S. pneumoniae* (SP-95-27, SP-96-29 and SP-95-36) to 44.3 ± 17.3% of the control, when pharyngeal epithelial cells with the attached bacteria were treated with 0.1 μg/ml *S*-CMC.

2.3. Effects of S-CMC treatment of bacteria on attachment to epithelial cells

S-CMC treatment did not change the cell wall or Gram staining pattern of *S. pneumoniae*. Treatment of *S. pneumoniae* strain SP-95-19 with 10 or 1 μg/ml *S*-CMC significantly decreased the number of bacteria attached to epithelial cells (Table 3). However, at concentrations of 0.1 and 0.01 μg /ml, *S*-CMC had no significant effect on the number of attached bacteria.

Treatment of the other three strains of *S. pneumoniae* (SP-95-27, SP-96-29 and SP-95-36) with 10 μg/ml of

Table 1
Effects of *S*-carboxymethylcysteine treatment of pharyngeal epithelial cells on the attachment of *S. pneumoniae* strain SP-95-19

Control	Attachment *S*-CMC (μg/ml)	Decrease in mean attachment (%)	*P* value	Number of experiments
17.8 ± 10.1	5.1 ± 2.6 (100)	71.3	<0.05	6
20.2 ± 10.2	6.9 ± 3.4 (10)	65.8	<0.05	6
19.0 ± 9.9	8.3 ± 4.6 (1)	56.3	<0.05	7
19.0 ± 9.9	8.4 ± 3.8 (0.1)	55.8	<0.05	7
11.3 ± 1.9	8.6 ± 5.8 (0.01)	23.9	NS	4
15.0 ± 5.5	12.7 ± 4.1 (0.001)	16.7	NS	3

Attachment is expressed as number of bacteria attached per pharyngeal epithelial cell. Each experiment was performed in duplicate. For a given concentration of *S*-CMC treatment, experiments were done in separate days. *P* values are for comparisons with the control. NS, not significant.

Table 2
Effects of *S*-carboxymethylcysteine on detachment of *S. pneumoniae* strain SP-95-19 adherent to human pharyngeal epithelial cells

Control	Detachment *S*-CMC (μg/ml)	Decrease in mean detachment (%)	*P* value	Number of experiments
8.9 ± 2.7	3.4 ± 3.1 (100)	69.7	<0.005	5
8.9 ± 2.4	2.6 ± 1.7 (10)	70.8	<0.005	5
8.9 ± 2.4	2.7 ± 1.5 (1)	69.7	<0.005	5
8.2 ± 2.9	2.5 ± 1.4 (0.1)	69.5	<0.05	3
10.4 ± 1.0	11.7 ± 1.7 (0.01)	ND	NS	3
10.4 ± 1.0	9.4 ± 1.0 (0.001)	9.6	NS	3

Detachment is expressed as number of attached bacteria per pharyngeal epithelial cell after detachment assay. Each experiment was performed in duplicate. For a given concentration of *S*-CMC treatment, experiments were done in separate days. *P* values are for comparisons with the control. ND, no decrease; NS, not significant.

Table 3
Effects of *S*-carboxymethylcysteine treatment of *S. pneumoniae* strain SP-95-19 on their attachment to human pharyngeal epithelial cells

S-CMC concentration (μg/ml)	Attachment	Decrease in mean attachment (%)	*P* value
0 (Control)	31.6 ± 8.0		
10	16.8 ± 11.2	46.8%	<0.01
1	19.7 ± 8.5	37.7%	<0.001
0.1	21.1 ± 8.6	33.2%	NS
0.01	37.8 ± 11.4	ND	NS

Attachment is expressed as number of bacteria attached per pharyngeal epithelial cell. A total of four experiments were performed at each concentration. For a given concentration of *S*-CMC treatment, experiments were done in separate days. *P* values are for comparisons with the control. NS, not significant; ND, no decrease.

S-CMC resulted in a significant ($p < 0.001$) decrease in the number of attached bacteria to 37.1 ± 1.7% of the control. However, the effect was variable when these strains when treated with 1 μg/ml of *S*-CMC. In strain SP-95-27, SP-96-29 and SP-95-36, the attachment decreased to 34.4%, 14.6% and 1.1% of the control, respectively.

2.4. S-CMC has no effect on viability of bacteria

Treatment of *S. pneumoniae* with *S*-CMC did not affect the viability of bacteria relative to the control (data not shown).

3. Discussion

Although the pneumococcal polysaccharide vaccine has been licensed for the last 20 years in the US and UK, the effectiveness of this vaccine in preventing infections in the vulnerable population is not satisfactory [8]. In addition, the worldwide spread of penicillin-resistant *S. pneumoniae* emphasizes the need for new approaches to prevent such bacterial infections [9]. The use of a drug that can block *S. pneumoniae* attachment to epithelial cells represents a new approach for the prevention and treatment of infections caused by this microbe. In the present study, we showed that *S*-CMC at a concentration achievable in the sputum [4] inhibited the attachment of *S. pneumoniae* to human pharyngeal epithelial cells in vitro as well the detachment of the already attached *S. pneumoniae* from human pharyngeal epithelial cells. The mechanism by which *S*-CMC prevents the bacterial attachment is not known at present. Since the suppression of attachment was also noted for *M. catarrhalis* and NTHI, it is likely that *S*-CMC acts on a common receptor, or it masks the receptor. Compared to *M. catarrhalis* and NTHI [4,5], significant inhibition of *S. pneumoniae* attachment was achieved when epithelial cells were treated with low concentrations of *S*-CMC. Moreover, detachment was also observed at relatively low concentration of *S*-CMC compared with that of NTHI [5].

A two-step molecular model has been proposed for the attachment of *S. pneumoniae* to eucaryotic cells [10] where the initial encounter between pneumococci and naive host cells is mediated by resting eucaryotic cell receptors. With increasing numbers of bacteria, the induced inflammatory response upregulates new eucaryotic cell receptors that promote addition bacterial attachment. *S*-CMC appears to have an anti-inflammatory function, probably through its effect on sialomucin synthesis and subsequent kinin inhibition [7]. It is still not clear whether *S*-CMC can prevent such receptor upregulation by downregulating the inflammatory response.

The most important and interesting finding of the present study was the significant decrease in the attachment following treatment of *S. pneumoniae* with *S*-CMC. Certainly this effect was not due to any bactericidal effect of *S*-CMC because our quantitative culture assays showed that *S*-CMC had no effect on viable bacterial counts. In addition the reduction in the attachment correlated well with the dose of *S*-CMC used for the treatment of *S. pneumoniae*. Our previous studies showed that *S*-CMC could not reduce the attachment of *M. catarrhalis* and NTHI to epithelial cells [4,5]. The mechanism of *S*-CMC-induced attenuation of *S. pneumoniae* attachment to human pharyngeal epithelial cells certainly deserves further investigation. We previously showed that *S*-CMC could remove carbohydrate from the cell surface [4]. Others have shown that *N*-acetyl-L-cysteine, another mucolytic agent, successfully removed the capsule of *S. pneumoniae* in transformation experiments [11]. It is possible that the effect of *S*-CMC noted in the present study was also due to the same mechanism. *S. pneumoniae* has a well-established capsule in contrast to NTHI and *M. catarrhalis*. In this regard, previous studies showed that the adherence of noncapsulated forms of *S. pneumoniae* to bronchial epithelial cells was stronger than the capsulated parent strain [12]. On the other hand, several proteins on *S. pneumoniae* cell wall, such as choline binding proteins [13,14] and peptide permeases [10] have been reported to act as adhesins assisting bacterial attachment to human bronchial epithelial cell line. It should be noted however, that cultured cells may express aberrant receptor(s) because cell lines are prepared from cancer tissues or modified by virus infection. It may be possible that due to this noncapsulated *S. pneumoniae* attach more to cultured cells than capsulated one. Since we are using cells directly scraped from human pharynx, therefore, the utilization of receptors by *S. pneumoniae* for attachment is possibly different from cultured cells. Recently, Dagan and colleagues [15] reported a significant decrease of nasopharyngeal carriage of *S. pneumoniae* after administration of a 9-valent pneumococcal conjugate vaccine. It seems that anticapsular antibodies prevented attachment and decrease of colonization occurred. Therefore it is possible that the bacterial capsule could play a role in the attachment to human pharyngeal epithelial cells. Studies are currently being conducted in our laboratories to determine the role of *S. pneumoniae* capsule in their attachment to human pharyngeal epithelial cells and the mechanism of action of *S*-CMC on the capsule of *S. pneumoniae*.

The major pathogens causing respiratory infections are *S. pneumoniae*, NTHI and *M. catarrhalis* [16]. It became clear with this study that *S*-CMC reduces the number of episodes of acute exacerbation of respiratory infections by rendering pharyngeal epithelial cells capable of inhibiting the attachment of all these major pathogens.

4. Materials and methods

4.1. Bacteria

The following strains of *S. pneumoniae* were used in this study: strain SP-95-27 of serotype 9V isolated from

post-mortem lung tissue; strain SP-95-19 of serotype 19F isolated from the sputum of a patient with respiratory infection; strain SP-96-29 of serotype 6B isolated from secretion that accumulated inside the endotracheal tube of an unconscious patient; and strain SP-95-36 of serotype 14 isolated from throat swab of a patient. Strain SP-95-19 was used in most experiments unless otherwise stated. All strains were stocked in Mueller-Hinton broth (Merck KgaA, Darmstadt, Germany) containing 5% defibrinated rabbit blood and kept at −20 °C until use. For attachment studies, assay bacteria were cultured overnight on Brain Heart Infusion agar (Merck KgaA, Darmstadt, Germany) containing 7% defibrinated rabbit blood at 37 °C in 5% CO_2 incubator, then the colony was transferred to Todd-Hewitt broth (Difco Laboratories, Detroit, MI, USA) containing 0.5% yeast extract and cultured in an incubator under constant shaking at 37 °C for 6 h. This yielded a bacterial density of 5×10^7 cfu/ml of *S. pneumoniae*. For each experiment this was confirmed by quantitative culture. The bacteria were washed once in phosphate buffered saline (PBS), pH 7.2, at 4 °C, by centrifugation at 3000*g* for 10 min and then used for the attachment assay.

4.2. Pharyngeal epithelial cells

Pharyngeal epithelial cells were collected from a consented normal healthy adult male by scraping the oropharynx with a cotton swab. Cells from the swab were collected in tubes containing 1/15 M phosphate buffer (PB, pH 7.2). Cells were washed three times with PB, by centrifugation at 80*g* for 10 min at room temperature. Finally, pharyngeal epithelial cells were count with a Burker chamber and adjusted to a density of 5×10^4 cells/ml.

4.3. Attachment assay

The attachment assay was performed as described previously [17] with minor modifications. Bacteria and cells were mixed in equal proportions to a volume of 1 ml and incubated for 30 min at 37 °C in a water bath under constant shaking. The Unattached bacteria were then removed by three washings using PBS by centrifugation at 80*g* for 10 min at room temperature. Finally, the cells were collected on a glass slide by Cytospin (Thermo Shandon International, UK). Smears were Gram-stained and viewed under an oil immersion lens of a light microscope to count the number of attached bacteria on 50 consecutive cells. For each slide counting was done blindly.

4.4. Treatment of epithelial cells with S-CMC

For the attachment inhibition assay, pharyngeal epithelial cells were treated with various concentrations of *S*-CMC (100, 10, 1, 0.1, 0.01 and 0.001 μg/ml), and incubated at 37 °C for 30 min in a water bath under constant shaking. Then cells were washed three times with PB at 80*g* for 10 min at room temperature. Control cells were prepared in a similar manner except they were not treated with *S*-CMC. This was followed by conducting the aforementioned attachment assay.

For the detachment assay the following procedure was performed; after completion of the attachment assay, epithelial cells were treated with various concentrations of *S*-CMC (100, 10, 1, 0.1, 0.01 and 0.001 μg/ml) and incubated at 37 °C for 30 min in a water bath under constant shaking. Then cells were washed three times with PBS at 80*g* for 10 min at room temperature, to remove the detached bacteria. Control cells were prepared in a similar manner except they were not treated with *S*-CMC.

4.5. Treatment of bacteria with S-CMC

Bacteria at a density of 5×10^7 cfu/ml were treated with various concentrations of *S*-CMC (10, 1, 0.1, 0.01 and 0.001 μg/ml) at 37 °C for 30 min in a water bath under constant shaking. Then the bacteria were washed three times in PBS at 4 °C by centrifugation at 3000 rpm, each time for 10 min. Bacteria treated similarly but without any drug represented the control sample. Attachment assay was performed as described above and compared with the control. After treatment with *S*-CMC, the viability of *S. pneumoniae* was determined by quantitative cultures on BHI agar containing 7% defibrinated rabbit blood. The following day, the viability of bacteria was compared with that of the control.

4.6. Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using Students *t*-test. Data were considered statistically significant if the *P* value was lower than 0.05.

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