

## Effect of salmeterol on *Haemophilus influenzae* infection of respiratory mucosa *in vitro*

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*Effect of salmeterol on Haemophilus influenzae infection of respiratory mucosa in vitro.* R.B. Dowling, M. Johnson, P.J. Cole, R. Wilson. ©ERS Journals Ltd 1998.

**ABSTRACT:** *Haemophilus influenzae* is a common bacterial pathogen causing human respiratory tract infections. We have previously shown that the  $\beta_2$ -agonist salmeterol reduces damage to the respiratory mucosa caused by *Pseudomonas aeruginosa* *in vitro*. We have now investigated the effect of salmeterol on *H. influenzae* infection of adenoid tissue in an organ culture by scanning electron microscopy.

Tissue was preincubated with or without salmeterol ( $4 \times 10^{-7}$ M), prior to infection with *H. influenzae* and incubated for 12 or 24 h.

Infected organ cultures had increased epithelial damage and decreased numbers of both ciliated and unciliated cells at 12h, which were significantly different ( $p < 0.01$ ) from the controls at 24 h. Salmeterol ( $4 \times 10^{-7}$ M) significantly ( $p < 0.03$ ) reduced damage and loss of ciliated cells in infected organ cultures at both 12 and 24, and significantly ( $p < 0.03$ ) reduced loss of unciliated cells at 24 h. Salmeterol had no effect on the density of bacteria adhering to each individual mucosal feature or the total number of bacteria adhering to the organ culture.

These results suggest that salmeterol protects the respiratory epithelium against *Haemophilus influenzae*-induced damage. The mechanism of salmeterol cytoprotection and its potential clinical relevance remain to be investigated.

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Nontypable *Haemophilus influenzae* is a common commensal bacterium in the upper respiratory tract, present in up to three quarters of normal subjects [1]. However, under permissive conditions it may become pathogenic, and frequently causes upper and lower respiratory tract infections, including otitis media, sinusitis, pneumonia and infective exacerbations of chronic obstructive pulmonary disease. *H. influenzae* is also commonly isolated from purulent sputum of patients with cystic fibrosis and bronchiectasis [2–4].

Studies of bacterial interactions with respiratory cells or intact mucosa *in vitro* have provided important information about the pathogenesis of *H. influenzae* infections, which may in the future lead to the development of new treatments. Infection causes patchy, and sometimes confluent, damage to epithelium [5, 6] and bacteria adhere predominantly to mucus, damaged cells and exposed collagen. *H. influenzae* endotoxin induces the release of pro-inflammatory mediators (interleukin (IL)-6, IL-8, tumour necrosis factor (TNF) $\alpha$  and intercellular adhesion molecule (ICAM)-1) from human bronchial epithelial cells [7]. *H. influenzae* also releases uncharacterized factors which impair mucociliary clearance by stimulating mucus secretion [8], causing ciliary beat slowing and dyskinesia, and damaging epithelial cells [9, 10]. Immunoglobulin (Ig)A1 proteases reduce mucosal defence mechanisms [11]. These effects all promote bacterial persistence on the mucosa [12].

Salmeterol is a potent  $\beta_2$ -agonist with a prolonged action [13–15]. We have recently shown that salmeterol

( $4 \times 10^{-7}$ M) reduced the damage that occurred to respiratory epithelium during *Pseudomonas aeruginosa* infection of nasal turbinate organ cultures, and decreased ultrastructural damage to epithelial cells caused by the *P. aeruginosa* toxins pyocyanin and elastase [16]. In separate experiments [17, 18] it was observed, using light microscopy, that the disruption of epithelial integrity caused by pyocyanin was delayed in the presence of salmeterol ( $2 \times 10^{-7}$ M) and other agents that increased intracellular cyclic adenosine monophosphate (cAMP). Selective antagonists were used to demonstrate that salmeterol reduced epithelial damage *via* stimulation of  $\beta_2$ -adrenoceptors. These data suggest that agents that elevate intracellular cAMP may protect respiratory epithelium from damage caused by bacterial infection.

Having established that salmeterol reduces *P. aeruginosa*-induced epithelial damage of nasal turbinate organ cultures [16], we wished to determine whether this effect was species and tissue specific. We have now investigated the effect of salmeterol on the interaction of *H. influenzae* with the mucosa of an adenoid organ culture with an air-mucosal interface using scanning electron microscopy.

### Materials and methods

#### Bacteriology

*H. influenzae* strain SH9 is a nontypable clinical isolate that has been studied previously in our laboratory [6]. SH9

was stored at  $-70^{\circ}\text{C}$  in a haemophilus broth (brain heart infusion broth with haemin  $1\text{ mg}\cdot\text{mL}^{-1}$  and NAD  $1\text{ mg}\cdot\text{mL}^{-1}$ ) and glycerol mixture (80:20), then retrieved onto levinthal agar. After overnight culture, 2–3 colonies were dispersed in 5 mL of haemophilus broth and incubated overnight at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  with agitation. Two millilitres of the culture, which previous experiments had shown corresponded to approximately  $1\times 10^9$  colony forming units (cfu) $\cdot\text{mL}^{-1}$ , were washed twice with 10 mL of phosphate-buffered saline (PBS; Oxoid, Basingstoke, UK). The bacterial pellet was resuspended in 150  $\mu\text{L}$  of PBS, mixed and viable counts performed.

#### Preparation of salmeterol

Salmeterol hydroxynaphthoate (Glaxo Wellcome, Uxbridge, UK) (6.03 mg) was dissolved in the minimum amount of glacial acetic acid and then diluted with PBS to give a concentration of  $1\times 10^{-5}\text{M}$ . This was further diluted with minimal essential medium (MEM, Gibco, Paisley, UK) to yield a final concentration of  $4\times 10^{-7}\text{M}$ .

#### Organ cultures

This method has been described previously [16, 19–21]. Briefly, human adenoid tissue resected from patients was transported to the laboratory in MEM containing antibiotics ( $50\text{ }\mu\text{g}\cdot\text{mL}^{-1}$  streptomycin,  $50\text{ IU}\cdot\text{mL}^{-1}$  penicillin and  $50\text{ }\mu\text{g}\cdot\text{mL}^{-1}$  gentamicin). Dissection was performed in antibiotic medium to yield small squares approximately  $3\text{ mm}^2$  in area and 2–3 mm thick. The tissue was screened for ciliary activity in order to select squares with at least one fully ciliated edge. These were immersed in antibiotic medium for at least 4 h in order to remove commensal bacteria and then in antibiotic-free medium for at least 1 h in order to remove antibiotics.

A sterile 3.5 cm diameter petri dish (Sterilin, Stone, UK) was placed aseptically within a 6.0 cm petri dish. A strip of sterile filter paper (No. 1; Whatman, Maidstone, UK) with dimensions approximately  $5\times 70\text{ mm}$  was soaked in MEM without antibiotics and placed aseptically across the diameter of the inner petri dish. The filter paper strip adhered to the base of the inner petri dish and each of its moistened ends adhered to the base of the outer petri dish. A single tissue square was placed, ciliated surface uppermost, on the centre of the filter paper strip in the inner petri dish, and its edges were sealed with agar (30  $\mu\text{L}$ ). Four millilitres of antibiotic-free medium were pipetted into the outer petri dish. The filter paper strip acted as a wick to draw medium from the outer petri dish to the underside of the tissue.

#### Experimental design

For each experiment ( $n=6$ ), four organ cultures were prepared: control; tissue infected with *H. influenzae*; tissue preincubated with salmeterol; and tissue preincubated with salmeterol and then infected with *H. influenzae*. Appropriate tissue squares were preincubated with 4 mL of salmeterol ( $4\times 10^{-7}\text{M}$ ) for 30 min prior to assembly of organ cultures. During this time, the other tissue squares

were incubated in MEM alone. Two microlitres of the washed bacterial suspension in PBS were gently pipetted onto the surface of the appropriate tissue squares immediately after organ culture construction. The other organ cultures were inoculated with 2  $\mu\text{L}$  of PBS. The organ cultures were incubated in a humidified atmosphere at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  for 12 h (protocol A). Tissue from the same patients was used to construct four more organ cultures where the experimental end point was increased to 24 h (Protocol B). At 12 h in Protocol B, 20  $\mu\text{L}$  of salmeterol ( $4\times 10^{-7}\text{M}$ ) was gently pipetted onto the surface of those organ cultures that had previously been incubated with salmeterol. Twenty microlitres of PBS was pipetted onto the surface of the other tissue squares. The organ cultures were then incubated for a further 12 h. At the end of each experiment (Protocol A or Protocol B), the four edges of each organ culture were touched with a sterile loop and plated onto levinthal agar in order to assess the sterility of uninfected organ cultures, and the purity of *H. influenzae* growth in infected organ cultures. The filter paper strip was then cut near the tissue with a sterile blade, removed with the tissue attached and fixed for scanning electron microscopy as previously described [16, 19–21].

#### Assessment of tissue by scanning electron microscopy

At the end of each experiment, tissue squares were given a coded number by an independent observer so that the original identity of the samples was unknown during analysis. Each tissue square was examined using a scanning electron microscope (S-4000; Hitachi, Katsuta-shi, Ibaraki-Ken, Japan) by the same observer. The tissue was initially viewed at a magnification of  $\times 50$ . A transparent acetate sheet with 100 equal squares was placed over the screen of the visual display unit. A predetermined pattern of 40 grid squares were selected for further viewing and analysis at  $\times 3,000$  magnification. This pattern involved the horizontal, vertical and two diagonal axes, so giving a representative survey of the mucosal surface measuring  $1.42\times 10^4\text{ }\mu\text{m}^2$ . Care was taken to ensure that there was no overlap of squares in the centre of the organ culture. Each of the 40 squares, at a magnification of  $\times 3,000$ , was assessed for percentage of the surface area occupied by four mucosal features: mucus; ciliated cells; unciliated cells; and damaged epithelium. Extruding cells, cell debris, dead cells and loss of epithelium were scored together in the category of damaged epithelium. Unciliated areas were defined as areas not covered by cilia, with or without microvilli. Summation of the scores allowed assessment of the percentage of each field that was occupied by each mucosal feature.

The number of bacteria associated with each of the four mucosal features was counted. An approximation was made when large numbers of bacteria were present in sheets. In these instances it was difficult to determine which mucosal feature(s) the bacteria were adhering to, but observation of the tissue surrounding the bacteria enabled an estimate to be made. The total number of bacteria adhering to each organ culture was compared. In order to overcome the difficulty caused by different proportions of the organ culture surface being occupied by each mucosal feature, which made comparison between organ cultures difficult, the total number of bacteria adher-

ing to a mucosal feature was divided by the proportion of the surface of the organ culture occupied by that feature [16, 21]. This was referred to as the density of bacteria adherent to a mucosal feature.

#### Statistical analysis

All values are presented as the mean $\pm$ SEM. The mean percentage surface area occupied by each of the four mucosal features was compared using the Mann-Whitney test. Total bacterial numbers and bacterial densities associated with each mucosal feature were analysed using the Wilcoxon signed rank pairs test. A p-value of less than or equal to 0.05 was considered significant.

## Results

### Bacteria

The mean inoculum of *H. influenzae* in 2  $\mu$ L of PBS used for the infected organ cultures was  $1.0 \times 10^8 \pm 0.4 \times 10^8$  cfu. At 12 and 24 h all uninfected organ cultures were sterile and all *H. influenzae* infected organ cultures gave a pure growth.

#### Scanning electron microscopy (table 1)

Control organ cultures were quite well ciliated, but exhibited some mucosal damage at both 12 and 24 h. The amount of mucosal damage did not increase with time suggesting that the damage was present at the beginning of the experiment. Salmeterol ( $4 \times 10^{-7}$ M) had no effect on the mucosal features at 12 or 24 h. *H. influenzae* infection of organ cultures caused a significant ( $p \leq 0.01$ ) increase in mucosal damage and a significant ( $p \leq 0.01$ ) decrease in the number of both ciliated and unciliated cells at 24 h compared to control. Ciliated cells were particularly reduced indicating that these cells may be preferentially damaged by infection (fig. 1). The same trend of increased mucosal damage accompanied by decreased numbers of ciliated and unciliated cells was evident at 12 h, but was not significantly different from control tissue.

Table 1. – Effect of salmeterol on *Haemophilus influenzae* infection of respiratory mucosa (n=6)

| Method                              | Mucus          | Damaged mucosa              | Ciliated epithelium         | Unciliated epithelium       |
|-------------------------------------|----------------|-----------------------------|-----------------------------|-----------------------------|
| <b>Protocol A (12 h)</b>            |                |                             |                             |                             |
| Control                             | 18.4 $\pm$ 6.0 | 33.8 $\pm$ 8.4              | 11.9 $\pm$ 4.4              | 35.9 $\pm$ 5.3              |
| Salmeterol                          | 13.1 $\pm$ 3.4 | 27.0 $\pm$ 2.5              | 15.7 $\pm$ 3.4              | 44.2 $\pm$ 2.6              |
| <i>H. influenzae</i>                | 14.3 $\pm$ 7.2 | 68.2 $\pm$ 11.0             | 3.0 $\pm$ 1.5               | 14.5 $\pm$ 7.0              |
| <i>H. influenzae</i> and salmeterol | 19.0 $\pm$ 3.7 | 28.7 $\pm$ 5.9 <sup>+</sup> | 22.9 $\pm$ 7.7 <sup>+</sup> | 29.4 $\pm$ 8.4              |
| <b>Protocol B (24 h)</b>            |                |                             |                             |                             |
| Control                             | 20.9 $\pm$ 4.7 | 20.0 $\pm$ 4.4              | 22.5 $\pm$ 3.3              | 36.6 $\pm$ 7.9              |
| Salmeterol                          | 11.3 $\pm$ 3.8 | 26.5 $\pm$ 7.8              | 27.4 $\pm$ 7.8              | 24.8 $\pm$ 7.2              |
| <i>H. influenzae</i>                | 6.2 $\pm$ 2.8  | 89.5 $\pm$ 4.8 <sup>†</sup> | 1.2 $\pm$ 1.2 <sup>†</sup>  | 3.1 $\pm$ 1.8 <sup>†</sup>  |
| <i>H. influenzae</i> and salmeterol | 22.5 $\pm$ 7.2 | 46.4 $\pm$ 5.8 <sup>‡</sup> | 9.2 $\pm$ 4.6 <sup>‡</sup>  | 21.9 $\pm$ 5.2 <sup>‡</sup> |

Results are of six experiments in each protocol and are presented as mean $\pm$ SEM. Values indicate the per cent surface area of organ culture occupied by each mucosal feature. <sup>+</sup>:  $p \leq 0.03$  versus *H. influenzae* 12 h; <sup>†</sup>:  $p \leq 0.01$  versus control 24 h; <sup>‡</sup>:  $p \leq 0.03$  versus *H. influenzae* 24 h.

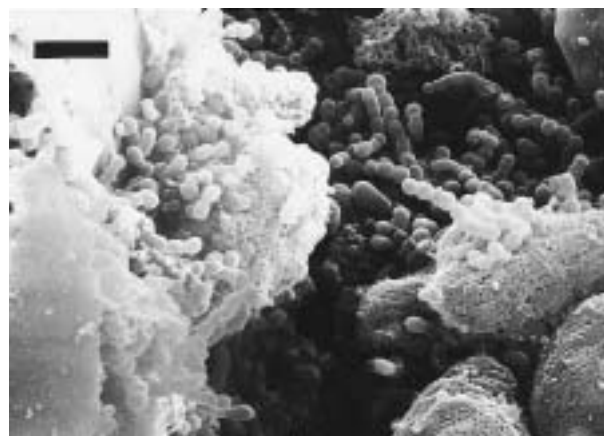


Fig. 1. – *Haemophilus influenzae* infection of human respiratory adenoid tissue caused mucosal damage and loss of both ciliated and unciliated cells at 24 h. Bacteria adhered preferentially to mucus and damaged cells. Internal scale bar = 2.36  $\mu$ m.

Infected tissue that had been treated with salmeterol ( $4 \times 10^{-7}$ M), had significantly ( $p \leq 0.03$ ) less mucosal damage and more ciliated cells compared with *H. influenzae* infection alone, at both 12 and 24 h. At 24 h, salmeterol also significantly ( $p \leq 0.03$ ) reduced the loss of unciliated cells in infected organ cultures (fig. 2). However, infected tissue treated with salmeterol still had significantly ( $p \leq 0.01$ ) elevated levels of mucosal damage at 24 h compared with control, indicating that salmeterol did not completely protect the epithelium at this time point.

#### *H. influenzae* adherence to organ cultures (table 2)

The interaction of *H. influenzae* with the organ culture was similar to that reported previously [5, 6, 9]. Bacteria were commonly seen adhering to mucus and damaged epithelium in preference to both ciliated and unciliated cells. *H. influenzae* commonly adhered to gaps between separated epithelial cells. There was no significant difference in the density of bacteria adhering to each individual mucosal feature in the presence or absence of salmeterol at 12 or 24 h or in the total number of bacteria adhering to the organ culture.



Fig. 2. – Prior incubation of the tissue with salmeterol ( $4 \times 10^{-7}$ M) for 30 min followed by reapplication after 12 h reduced the amount of mucosal damage caused by *Haemophilus influenzae* infection at 24 h but did not affect the density or total number of bacteria adhering to the organ culture. Internal scale bar = 4.72  $\mu$ m.

Table 2. – Effect of salmeterol on the density of *Haemophilus influenzae* adhering to each mucosal feature and the total number of bacteria adhering to the organ culture (n=6)

| Method                              | Mucus | Damaged mucosa | Ciliated cells | Unciliated | Total bacteria |
|-------------------------------------|-------|----------------|----------------|------------|----------------|
| <b>Protocol A (12 h)</b>            |       |                |                |            |                |
| <i>H. influenzae</i>                | 10.2  | 10.6           | 0.3            | 0.1        | 293            |
|                                     | ±5.5  | ±6.4           | ±0.2           | ±0.1       | ±159           |
| <i>H. influenzae</i> and salmeterol | 28.1  | 15.0           | 0.3            | 0.1        | 353            |
|                                     | ±20.1 | ±10.7          | ±0.2           | ±0.1       | ±267           |
| <b>Protocol B (24 h)</b>            |       |                |                |            |                |
| <i>H. influenzae</i>                | 15.4  | 20.2           | 0.0            | 0.0        | 788            |
|                                     | ±10.9 | ±6.8           |                |            | ±276           |
| <i>H. influenzae</i> and salmeterol | 4.3   | 24.7           | 0.1            | 0.1        | 567            |

Results are of six experiments in each protocol and are presented as mean±SEM. Values indicate the total number of bacteria adhering per unit area  $3.55 \times 10^2 \mu\text{m}^2$  to each mucosal feature and the total number of bacteria adhering to the organ culture in the area surveyed (*i.e.* number of bacteria per  $1.42 \times 10^4 \mu\text{m}^2$  of mucosal surface at  $\times 3,000$  magnification).

### Discussion

The interaction of nontypable *H. influenzae* with the respiratory mucosa of adenoid tissue organ cultures with an air interface, used in the present study, was similar to those previously reported with both nasal turbinate and adenoid tissue organ cultures submerged in medium [5, 6, 9]. This result is in contrast to *H. influenzae* type B, in which adherence to the mucosa was reduced in the presence of cell culture medium [21]. The poor adherence to epithelial cells of capsulated compared to unencapsulated strains may explain this difference [10]. The amount of mucosal damage present in the control tissue was greater with adenoid tissue compared to our previous study with nasal turbinates [16]. This is likely to reflect damage already present, or inflicted during resection, rather than developing during the experiment. *H. influenzae* infection caused extensive mucosal damage, which increased between 12 and 24 h, and bacteria predominantly adhered to mucus and damaged epithelium. The tight junctions between epithelial cells were frequently separated and *H. influenzae* adhered preferentially to these areas that might serve as a route of invasion. When epithelial damage was severe the epithelium was stripped away exposing basement membrane and occasionally the collagen layer. *H. influenzae* adhered particularly strongly to these areas in large numbers, often forming bacterial sheets.

Salmeterol is a long-acting  $\beta_2$ -adrenoreceptor agonist, and is an effective bronchodilator used in the treatment of asthma [13–15]. The mechanism of action of salmeterol has been the subject of much debate [22, 23] but it has now been shown to bind a specific exosite domain of the  $\beta_2$ -receptor, thus remaining available to activate the receptor in a continuous manner [24, 25]. The concentration of salmeterol ( $4 \times 10^{-7}\text{M}$ ) used in these experiments was selected on the basis of our previous studies [16]. The salmeterol concentration that is likely to be achieved at the level of the epithelium *in vivo* is unknown, but total lung tissue concentrations, following an inhaled dose of 50  $\mu\text{g}$ , have been estimated as 4 nM suggesting that these findings with salmeterol may have *in vivo* relevance [26].

Salmeterol alone did not alter the appearance of the tissue. Incubation of tissue with salmeterol prior to bacterial infection significantly ( $p < 0.03$ ) reduced the damage caused to the respiratory epithelium by *H. influenzae* at 12 h. However since mucosal damage was not significant at 12 h compared with control (table 1), we also carried out experiments over 24 h. The pharmacodynamics of salmeterol action suggested that further treatment of the tissue would be necessary [24]. Our previous study [16] showed that salmeterol was effective in reducing the damage caused by infection when it was pipetted onto the organ culture surface. Therefore, further salmeterol was applied at 12 h in Protocol B. At 24 h, tissue exposed to *H. influenzae* and salmeterol had significantly ( $p < 0.03$ ) less damage than tissue infected with *H. influenzae* alone. However, the levels of damage were elevated compared with control, showing that salmeterol did not give complete protection at this time point.

The mechanism of salmeterol protection is not known. *H. influenzae* produces toxins that slow ciliary beat [9] and salmeterol has been shown to increase ciliary beat frequency [13]. Maintenance of ciliary beating of infected organ cultures in the presence of salmeterol could protect the epithelium against *H. influenzae*-induced damage by reducing the concentration of toxins in the microenvironment of the mucosal surface. However, the concentration of salmeterol ( $4 \times 10^{-7}\text{M}$ ) used in these experiments has previously been shown not to affect ciliary beat frequency of epithelial cell monolayers [13] or nasal epithelial cells [17]. Salmeterol also increases intracellular cAMP [13]. We have previously shown that *P. aeruginosa* and its toxins pyocyanin and elastase damage human epithelial cells and that this damage is reduced by salmeterol [16]. Pyocyanin is a toxin whose effect is mediated *via* cAMP and we have previously shown that salmeterol attenuates pyocyanin-induced decreases in intracellular cAMP [17]. However, salmeterol also protected human nasal epithelial cells against *P. aeruginosa* elastase-induced damage, where the activity of the enzyme is not mediated *via* cAMP [16]. These results suggest that the effect of salmeterol on intracellular cAMP protects epithelial cells against bacterial toxin-induced damage. Salmeterol did not affect the total number or density of bacteria adhering to the respiratory mucosa. Similar results were obtained with *P. aeruginosa* [16], and suggest that the beneficial effects of salmeterol do not depend on suppressing bacterial growth or adherence. How increasing the level of intracellular cAMP reduces the damage caused by bacterial infection is not known.

The results of the present and our previous study [16] indicate that salmeterol is effective in reducing damage to the epithelium from varying sites in the upper respiratory tract caused by different bacterial species *in vitro*. Salmeterol may benefit patients with mucosal infections not only by its bronchodilator properties, but also by a cytoprotective action in minimizing epithelial damage caused by bacteria. Epithelial damage is an important feature of the pathogenesis of mucosal infections, since it impairs mucociliary clearance and provides a major site for bacterial adherence [12]. Further work is needed to elucidate the mechanism of salmeterol cytoprotection and its clinical relevance.

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