Ciliostasis is a key early event during colonization of canine tracheal tissue by *Bordetella bronchiseptica*

Tracy L. Anderton, Duncan J. Maskell and Andrew Preston†

Centre for Veterinary Science, Department of Clinical Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK

The primary site of infection for *Bordetella bronchiseptica*, *Bordetella pertussis* and *Bordetella parapertussis* is the ciliated respiratory epithelium. Previous studies have implicated adherence of bacteria to cilia, induction of mucus production, induction of ciliostasis and damage to the ciliated epithelium in *Bordetella* pathogenesis. This paper describes the use of an air-interface organ culture system using canine tracheal tissue infected with *B. bronchiseptica* to assess the temporal relationship between these pathologies. Ciliostasis occurs very early during the host tissue–pathogen interaction, before mucus production and obvious signs of epithelial damage occur. A *B. bronchiseptica* bvg mutant does not colonize the organ culture model, induce ciliostasis or cause damage to the epithelial cell layer, but it does induce similar amounts of mucus release as does infection by wild-type bacteria. The authors propose that ciliostasis is a key early event during the *B. bronchiseptica*–host tissue interaction that abrogates the mucociliary defences of the host tissue, renders it susceptible to colonization by the bacteria and allows subsequent damage to the epithelium. The organ culture model described offers a physiologically relevant tool with which to characterize the molecular basis for interactions between *Bordetella* and its primary site of infection, the ciliated respiratory epithelium.

INTRODUCTION

There are eight species in the genus *Bordetella*, with *Bordetella bronchiseptica*, *Bordetella pertussis* and *Bordetella parapertussis* being the most-studied species. *B. pertussis* and *B. parapertussis* cause whooping cough in children (Amano et al., 1998; Cherry, 1996) and *B. pertussis* is now recognized as a cause of chronic cough in adults (Nennig et al., 1996). A different lineage of *B. parapertussis* can also infect sheep (Porter et al., 1996). In contrast to the restricted host range of these species, *B. bronchiseptica* can infect a wide range of mammals with consequences ranging from no clinical signs through to acute respiratory tract pathology (Goodnow, 1980). In canines, *B. bronchiseptica* causes infectious tracheobronchitis characterized by an acute-onset hacking cough, giving the disease its common name: kennel cough (Bemis et al., 1977). The disease is usually self-limiting and uncomplicated cases resolve within a few weeks, but *B. bronchiseptica* infection may render the host susceptible to secondary infections.

Little is known about what determines the host range or the differing pathologies caused by different *Bordetella* species. The genomes of a representative strain of each of these bacteria have recently been sequenced and analysed (Parkhill et al., 2003; Preston et al., 2004). This information allows the generation of new hypotheses to explain the molecular basis for *Bordetella* pathogenesis.

The bordetellae are known to initially infect the cilia of the respiratory epithelium and it is likely that this is the major, and possibly only, site of infection. Our current understanding is that *Bordetella* pathogenesis involves adherence of bacteria to the cilia of the respiratory tract, induction of ciliostasis, mucus production, damage to the epithelium and induction of an inflammatory response in the respiratory tract (Bemis et al., 1977; Bemis & Kennedy, 1981; Bemis & Wilson, 1985; Funnell & Robinson, 1993; Matsuyama, 1977; Muse et al., 1977; Thompson et al., 1976). However, these observations have been made in separate systems, many of them highly artificial, that have not allowed the temporal relationship between these pathologies to be investigated.

We report here the use of air-interface organ culture from canine tracheal tissue, infected with *B. bronchiseptica*, to model the interaction between these bacteria and ciliated respiratory tissue. We have identified ciliostasis as a very early event in the host tissue–pathogen interaction that occurs before obvious damage to the epithelial surface and...
mucus production. We thus hypothesize that ciliostasis occurs very early during *B. bronchiseptica* infection of the respiratory tract and that this prevents clearance of the bacteria and allows further colonization of the tissue. It is also likely to prevent clearance of the mucus and extruded cells that are subsequently induced, and accumulation of this material in the respiratory tract is likely to contribute to the pathology associated with *Bordetella* infections.

**METHODS**

**Bacterial strains and growth conditions.** *B. bronchiseptica* strain RB50 and its *bvg* mutant, RB54, were kindly donated by Professor J. F. Miller, UCLA, USA, and have been reported previously (Cotter & Miller, 1994). Strain SB796, isolated from a dog with kennel cough, was kindly donated by Professor R. Gaskell, University of Liverpool, UK. SB799 is the isogenic *bvg* mutant of SB796, and was generated by our laboratory using methods previously described (Martinez de Tejada et al., 1996). The expected genomic rearrangement in the mutant was confirmed by Southern analysis. SB799 grew on Bordet–Gengou (BG) agar as flat, grey, non-haemolytic colonies typical of *bvg* mutants, and as expected, was deficient in the expression of pertactin as determined by Western blot analysis (data not shown). Bacteria were grown on BG agar (Becton Dickenson), supplemented with 10 % defibrinated horse blood (TCS Cellworks) at 37 °C.

**Construction of air-interface organ culture.** Canine tracheae were obtained from beagles used as naive controls in other research experimental studies with the cooperation of Glaxo-SmithKline, Welwyn Garden City, UK, and other research groups at the University of Cambridge.

Organ cultures were constructed based on the method developed for human tissues by Jackson et al. (1996). The dogs that were used in this study did not have signs of clinical disease and had not received treatment with a pharmacological agent. Tracheae were taken from both male and female dogs whose age ranged from 9 months to 4 years. The age and sex of the dog was not considered when constructing organ cultures. The whole trachea, from the larynx to the carina, was removed from the euthanased dog by surgical excision. Connective tissue that was adherent to the trachea was removed by blunt dissection. The trachea was placed, intact, into a 400 ml capped bottle filled with pre-warmed Dulbecco’s modified Eagle’s medium (DMEM) containing 10 mM glutamine, 2.5 μg amphotericin ml⁻¹, 100 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ and incubated at 37 °C for 48 h and the resultant colonies counted to ensure that the inocula contained the expected number of bacteria. All inocula were within 0-5 log c.f.u. of the expected value. Organ cultures were inoculated with 10 μl of the appropriate suspension. Ten-microlitre volumes of PBS were inoculated onto the surface of organ cultures to act as uninfected controls. To check for contamination of organ cultures, a bacteriological loop was run along one edge of the tissue piece and streaked onto horse-blood agar and BG agar plates to check that uninfected controls remained sterile and that a pure growth of *B. bronchiseptica* was obtained from experimentally infected organ cultures.

**Scanning electron microscopy (SEM).** Tissue pieces were fixed in 5 % glutaraldehyde in PIPES buffer, post-fixed in 1 % osmium tetroxide, dehydrated in a graded ethanol series and subjected to critical-point drying. Samples were mounted on aluminium stubs and sputter-coated with 15 nm of gold. Samples were examined on a Philips XL30 FEG scanning electron microscope at an accelerating voltage of 5 kV. SEM analysis was performed at the Multi-Imaging Centre, Department of Anatomy, University of Cambridge.

**Morphometric analysis.** Organ cultures were analysed as described previously (Jackson et al., 1996). Briefly, the proportion of organ culture surface covered by particular features was estimated. The presence of four features was recorded: cilia, mucus, cellular damage and processing damage. Processing damage consisted of tissue shearing. Cellular damage included the presence of extracellular matrix, extruded ciliated epithelial cells or the presence of denuded basement membrane. Twenty predetermined squares were examined at ×2000 magnification. Each field had an edge of 30 μm and typically contained 325 cells. A transparent acetate sheet with 100 equal squares was placed over the video display unit to allow scoring of these fields. The predominant cell type in each square was recorded. Each score represented 1 % of the total area of that field. The occurrence of each feature was expressed as a percentage of the visible epithelium in each image. Percentages were adjusted to account for tissue damaged by processing. To determine the effect of infection on the percentage of tissue covered by mucus, the percentage of mucus present on uninfected organ cultures was subtracted from the percentage of mucus present on the infected samples. Comparisons were made between samples from individual tracheae, which negated variation between individuals. Data represent the mean and standard contamination. Organ cultures were incubated at 37 °C in a humidified atmosphere containing 5 % CO₂. The ciliary function of organ cultures was tested (see below) before use in experiments, and any organ cultures failing to clear beads were discarded.

**Inoculation of organ cultures.** *B. bronchiseptica* were grown on BG agar for 48 h and then suspended in DMEM to OD₅₅₀ 1-0. The suspensions were serially diluted to concentrations of 10⁵ and 10² c.f.u. per 10 μl. Dilutions of these inocula were plated on to BG agar, incubated at 37 °C for 48 h and the resultant colonies counted to ensure that the inocula contained the expected number of bacteria. All inocula were within 0-5 log c.f.u. of the expected value. Organ cultures were inoculated with 10 μl of the appropriate suspension. Ten-microlitre volumes of PBS were inoculated onto the surface of organ cultures to act as uninfected controls. To check for contamination of organ cultures, a bacteriological loop was run along one edge of the tissue piece and streaked onto horse-blood agar and BG agar plates to check that uninfected controls remained sterile and that a pure growth of *B. bronchiseptica* was obtained from experimentally infected organ cultures.
deviation of six independent experiments obtained from organ cultures prepared from separate animals. The significance of differences between samples was assessed using Mann–Whitney analysis. Comparisons between uninfected and infected cultures, wild-type and bvg mutant infections, and RB50 and SB796 infections were made. All differences discussed in the text are significant at the 5% level.

**Light microscopy.** Samples were submerged in 10% formalin in PIPES buffer and fixed for between 24 and 72 h. The majority of tissue cartilage was removed using a scalpel, to leave only a thin section attached to the mucosa. Samples were placed in histology cassettes and processed using a Shandon Citadel 2000 automatic processor. Samples were dehydrated in a series of graded methylated spirits of increasing concentration, submerged in chloroform and then embedded in molten Tissue-TEK III embedding centre wax (Raymond Lamb Laboratory Supplies). Samples were correctly oriented in molten wax before cooling to allow the hardening of wax and ‘setting’ of tissue. Then 5 μm transverse sections of tissue were cut along a lateral edge of the organ culture on a Leica Ultrcut E microtome, dried overnight, taken through a series of graded methylated spirits to remove wax, cleared in xylene and stained.

**Staining of sections.** Sections were stained with haematoxylin and eosin by standard methods (Bancroft & Cook, 1994), using a Linistain GLX Staining Machine (Shandon), in methylene blue for 2 min or with PAS/alcian blue using standard methods (Bancroft & Cook, 1994). Schiff reagent was purchased from Fisher Scientific. Staining reagents were purchased from Surgipath Europe. Sections were mounted using SubX mounting media (Surgipath) and examined using a Nikon Optiphot microscope.

**Quantification of mucus on canine organ cultures.** Mucus on organ cultures was quantified as described previously (Thornton et al., 1989). Mucus was gently scraped from the mucosal surface using a plastic loop and placed into 1 ml PBS. Samples were shaken vigorously and stored at −20 °C. For assay, samples were thawed at room temperature and heated for 30 min at 37 °C. Then 50 μl was transferred to a nitrocellulose membrane (0.2-μm pore size) using a dot-blot apparatus. Mucin from bovine submaxillary gland (1 mg ml⁻¹; Sigma Aldrich, catalogue no. M3895) was used as a positive control and water as a negative control. Following transfer the membrane was removed and dried in a fume hood. It was then washed in distilled water for 2 min, incubated in a freshly prepared solution of 1% (v/v) periodic acid in 3% (v/v) acetic acid for 30 min and then rinsed twice for 2 min in freshly prepared 0.1% (w/v) sodium metabisulphite in 1 mM HCl. Schiff’s reagent was added and the colour allowed to develop for 15 min, after which time the membrane was rinsed in 0.1% sodium metabisulphite in 1 mM HCl and then dried in a fume hood.

The quantity of mucin contained within each sample was calculated by measurement of the reflectance density of each blot using the Bio-Rad Quantity One Quantification Software program and a Bio-Rad GelDoc 2000 imaging system. Each sample was positioned in a small rectangular region of analysis of 0.5 cm² and the volume of pixels within this region was measured. The 'show saturated pixel' feature was turned on during measurements to ensure that saturation had not been reached. The amount of mucin in samples correlates to the volume of pixels contained within the analysed region, and was calculated by $V = T - B$, where $V$ is the corrected volume of pixels of a sample, $T$ is the total volume of pixels of the analysed rectangle and $B$ is the volume of background pixels. The relative levels of mucin within samples were compared. Data represent the mean and standard deviation of nine independent experiments obtained from organ cultures prepared from three dogs.

**Latex bead clearance assay.** Ten microlitres of 1 μm diameter polystyrene microsphere beads (latex beads; Polyscience) was pipetted onto the ciliated surface of organ cultures such that the beads covered the entire mucosal surface. Cultures were incubated for 30 min at 37 °C and 5% CO₂ and then assessed by visual inspection. Ciliary-mediated clearance was assessed to be intact if the latex beads had been cleared to one edge of the organ culture piece. Preliminary experiments determined that for normal tissue, this occurred within 30 min (data not shown).

**RESULTS**

**Development of canine air-interface tracheal organ culture**

In order for infection-induced pathology to be studied in the organ culture model, it was imperative that changes that occur to the tissue were not induced by its maintenance in vitro. Therefore tissue taken at post-mortem was processed immediately and compared to tissue that was maintained in organ culture for 24 h. Tissue was analysed by SEM in order to assess the major features constituting the surface of tissue pieces. Morphometric analysis (see Methods) was performed to record the presence of cilia, mucus and damage arising from processing of samples for SEM analysis; as these were the most abundant features noted in preliminary studies (data not shown).

Both post-mortem and organ-culture samples predominantly comprised ciliated tissue, with very few non-ciliated areas, although post-mortem tissue often had mucus that obscured the underlying cilia (Fig. 2). A mean of 65% (range 32–96%) or 95% (range 89–98%) of the surface area was ciliated in post-mortem or organ culture samples, respectively. Mucus covered 32% (range 2–68%) or 3% (range 0–9%) of the surface areas of the post-mortem or organ culture samples, respectively, and where present it obscured cilia from view (Fig. 2). The post-mortem samples, on average, contained a higher level of mucus and showed wider variation in mucus levels than did organ culture samples. It is likely that mucus that was present on the tracheal tissue at the time of post-mortem was removed during the washing steps that were performed during the setting up of the organ cultures, resulting in the lower level of mucus observed in organ culture tissue. These data also suggested that maintenance of the tissue in vitro did not induce large amounts of mucus expression onto the tissue surface. Some splits in the tissue were observed; these were classed as processing damage that occurred during sample preparation for SEM. This affected up to 10% of the surface of samples in both post-mortem and organ culture samples. In experiments with extended incubation periods the appearance of tissue surface remained unchanged for up to 72 h. Beyond this time, cilia became less organized and some were not upright (data not shown). Therefore organ cultures were maintained for 24 h or less in subsequent experiments.

The data from these preliminary experiments suggested that
organ culture of the tracheal tissue provided a tissue surface that maintained its close resemblance to in vivo tissue for at least 24 h. Thus this model provided a system in which to study the interaction between B. bronchiseptica and a physiologically relevant host tissue surface over a number of hours.

**Pathology induced by B. bronchiseptica**

To investigate changes in tracheal tissue induced by B. bronchiseptica infection, organ cultures were inoculated with different strains of B. bronchiseptica, incubated for 24 h and then analysed by SEM/morphometric analysis. The surface area of the tissue that comprised cilia, mucus or epithelial cell damage was recorded and compared with uninfected controls. Two wild-type (WT) strains were used: RB50, an isolate from a rabbit and the genome sequence strain, and SB796, a clinical isolate from a case of kennel cough. Strain RB50 was used because it is a well-characterized strain for which a large number of well-defined mutants are available. Strain SB796 was used in order to ensure that the interaction between a clinical isolate and the cognate host tissue was being studied. In addition, organ cultures were infected with isogenic bvg mutants of each strain, RB54 and SB799, respectively. These mutants are locked in the Bvg-minus phase and thus do not express any of the protein adhesins and toxins previously implicated in B. bronchiseptica pathogenesis (Cotter & Miller, 1994). Organ culture pieces were inoculated with $10^8$, $10^5$ or $10^2$ c.f.u. In all experiments, pure cultures of B. bronchiseptica were recovered from infected organ cultures, while uninfected pieces remained sterile.

Organ cultures inoculated with $10^8$ c.f.u. of WT bacteria were covered in sheets of mucus to which numerous rod-shaped bacteria were adhered. Underlying surface structures were obscured from view by the mucus (Fig. 3). Extruded cells were not observed. Uninfected control organ culture pieces were not covered with mucus, consistent with previous results (see above). Both of the WT strains induced a similar increase in the surface area of tissue that was covered by mucus when compared to uninfected controls. Organ culture samples infected with RB50 or SB796 had a mean of 84% (±20%) or 91% (±9%), respectively, more tissue surface covered in mucus than uninfected controls (Fig. 4). Infection with $10^5$ or $10^2$ c.f.u. of WT bacteria did not induce any changes in surface morphology when compared to uninfected controls (data not shown). Some mucus was observed on the surface of these samples although the level of mucus was not significantly different from that in uninfected controls (Fig. 4). These data suggested that infection by WT bacteria induced mucus expression by the organ culture tissue, that a certain number of bacteria were required for this induction and that this...
A threshold level was reached by inoculation with somewhere between $10^5$ and $10^8$ bacteria.

In contrast to the WT bacteria, inoculation of organ cultures with the \textit{bvg} mutants did not cause a large increase in the coverage of the tissue surface by mucus (Fig. 4). No bacteria were observed on any of the \textit{bvg}-mutant-infected organ cultures (data not shown). It was possible that induction of large amounts of mucus by \textit{B. bronchiseptica} required factors whose expression was \textit{Bvg}-dependent. However, it was also possible that induction of mucus expression required colonization of the tissue by bacteria and it was this colonization that was dependent on \textit{Bvg}. Our data do not allow discrimination between these scenarios.

**Induction of ciliostasis**

Coordinated, directional ciliary beat is a fundamental property of respiratory tract physiology. Previous reports suggested that \textit{B. bronchiseptica} induced ciliostasis of tracheal cells cultured as explant outgrowths (Bemis & Kennedy, 1981; Bemis & Wilson, 1985). To assay the ability of \textit{B. bronchiseptica} to induce ciliostasis in air-interface organ culture tissue, a simple assay was developed that measured the ability of the organ culture to clear a suspension of latex beads from its surface. When inoculated on to the surface of the organ cultures, a drop of latex bead suspension spread to form a white layer on the surface of the tissues (Fig. 5). Clearance of the beads by the organ cultures was observed in real time as the beads were moved by ciliary beat action to one edge of the tissue piece (Fig. 5; see also the movie file available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). Clearance of the beads indicated that the cilia were beating and doing so in a coordinated fashion. By noting the orientation of the tissue piece in relation to its original anatomical orientation it was possible to establish that ciliary-mediated clearance was in a distal to proximal (lungs-to-larynx) direction, and that the organ culture tissue maintained the same direction of ciliary beat that occurred \textit{in vivo}. Organ culture samples continued to clear beads for 48 h. After this time, the number of organ cultures clearing beads dropped (data not shown). Thus, during the 24 h time period of the experiments reported here, organ culture pieces maintained coordinated and functional ciliary beat.

The effect of \textit{B. bronchiseptica} infection on ciliary beat was investigated. Organ cultures were inoculated with $10^8$ c.f.u. of WT or \textit{bvg} mutant bacteria and ciliary clearance of latex beads was assessed 24 h later. Uninfected controls

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**Fig. 3.** SEM micrographs of organ cultures infected with $10^8$ c.f.u. of WT bacteria for 24 h. (a) The tissue surface is covered in sheets of mucus, obscuring underlying tissue from view. (b) Numerous rod-shaped bacteria, approximately 1 $\mu$m in length, are adhered to mucus. Scale bars, 10 $\mu$m.

**Fig. 4.** Percentage of the tissue surface covered with mucus as determined by morphometric analyses for samples 24 h following inoculation with $10^8$, $10^5$ or $10^2$ c.f.u. of WT bacteria (SB796 and RB50) or \textit{bvg} mutant bacteria (SB799 and RB64). Percentages were adjusted to account for tissue damaged by processing. The percentage of tissue covered in mucus measured in uninfected organ cultures was subtracted from that measured in infected organ cultures to give the level of mucus induced by infection alone. Comparisons were made between samples from the same trachea in order to eliminate variation between dogs. *Statistically significant difference ($P<0.05$) between WT and the corresponding \textit{bvg} mutant at $10^8$ c.f.u. inoculum.
continued to clear the latex beads as expected. None of the organ cultures inoculated with WT bacteria continued to clear beads (Fig. 6). Inoculation of organ cultures with $10^5$ c.f.u. of WT bacteria also resulted in loss of bead clearance in some cases (Fig. 6): 18% of RB50-infected and 35% of SB796-infected organ cultures failed to clear beads. No inhibition of bead clearance was observed following inoculation of organ cultures with $10^2$ c.f.u. of WT bacteria (Fig. 6). That inoculation with $10^5$ c.f.u. of bacteria was able to disrupt ciliary-mediated bead clearance suggested that this phenomenon was not solely due to mucus blocking access of the cilia to the beads, as no mucus was observed in response to this dose of bacteria (see above).

Organ cultures were inoculated with $10^8$ c.f.u. of $bvg$ mutant bacteria and bead clearance was assessed 24 h later. RB54 and SB799 inhibited bead clearance in only 15% or 25% of organ cultures, respectively, in contrast to the 100% inhibition of clearance observed with the same inocula of WT bacteria (Fig. 6). Inoculation of organ cultures with either $10^5$ or $10^2$ c.f.u. of the $bvg$ mutants resulted in inhibition of bead clearance in only a single organ culture (Fig. 6).

Fig. 5. Ciliary-mediated clearance of latex beads. A suspension of 1 μm diameter latex beads was pipetted on to the surface of an organ culture piece. The suspension covered the tissue surface and was visible as a white layer (a). Ciliary-mediated clearance of the beads occurred over a period of approximately 15 min (b, 7 min; c, 10 min) and occurred in a ‘lung to larynx’ direction. The arrow in (a) indicates the direction of clearance. After 15 min the beads are cleared to one edge of the tissue piece (d). Bead clearance was recorded by video (see the movie file available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). Individual still-images from different time points during the video are shown here.

Fig. 6. Percentage of organ culture samples still clearing latex beads 24 h after inoculation with $10^8$, $10^5$ or $10^2$ c.f.u. of WT bacteria (SB796 and RB50) or $bvg$ mutant bacteria (SB799 and RB54). More than 20 organ culture pieces were examined for each inoculum. U, uninfected control.
**Time-course of infection by WT bacteria**

The results above demonstrated that WT bacteria, at an inoculum size of $10^8$ c.f.u., induced mucus expression and abrogation of ciliary-mediated clearance by 24 h post-inoculation. In order to define further the time-course of these events, organ cultures were inoculated with $10^8$ c.f.u. of SB796 and analysed at 1, 2, 4, 8 and 12 h post-inoculation. At 1 h all of the organ cultures tested failed to clear beads (and thus were not tested for bead clearance at later time points). In SEM analysis, very few bacteria were visible adherent to cilia at this point; the cilia appeared healthy and there was little mucus present, suggesting that ciliostasis occurred without extensive colonization of or damage to the cilia. At each subsequent time point there was an increase in the number of bacteria observed on the tissue surface. By 12 h, much of the tissue surface was covered by mucus, obscuring the bacteria adherent to cilia. In regions not covered by mucus, large numbers of bacteria were visible. Representative images for each time point are shown in Fig. 7. At 1 and 2 h post-inoculation no mucus was observed. By 4 h, some fibrous mucus was evident on the tissue surface, which increased at 8 h. By 12 h much of the tissue surface was covered by mucus; therefore much of the mucus observed at 24 h (see above) was expressed on to the tissue surface by 12 h. Representative images of this analysis are shown in Fig. 8. Thus bacteria adhered to the cilia of the organ cultures and proliferated on tissue in organ culture before significant mucus expression. This suggested that mucus expression occurred in response to colonization of the tissue by bacteria. These data also demonstrated that inhibition of bead clearance was an early event following inoculation of the organ culture with bacteria and that it occurred prior to mucus expression on the tissue surface and prior to obvious damage to the ciliated epithelium.

**Quantification of mucus**

SEM imaging and morphometric analysis determined the percentage of the organ culture surface that was covered in mucus but did not calculate the quantity of mucus present on the culture. Therefore organ cultures were inoculated with $10^8$ c.f.u. of WT or bvg mutant bacteria and incubated for 0, 2, 8 or 24 h, after which mucus was scraped from the organ culture surface and quantified.

Incubation of tissue pieces in organ culture induced a significant increase in mucus compared to cultures at the start of the incubation period (i.e. immediately following inoculation) in all cases, including uninfected controls (Fig. 9), demonstrating that incubation of tissue in organ culture induced expression of mucus onto the tissue surface. At 2 h post-inoculation, there was no difference in the quantity of mucus measured in infected and uninfected cultures.

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**Fig. 7.** SEM micrographs depicting the time-course of bacterial proliferation on organ culture tissue. Organ cultures were inoculated with $10^8$ c.f.u. of SB796 bacteria, incubated for 1 (a), 4 (b), 8 (c) or 12 (d) h and then analysed by SEM. Increasing numbers of bacteria on the tissue at each time point suggested that bacteria proliferated on the tissue in organ culture. Fibrous mucus (F) was also observed. Scale bars: 2 $\mu$m (a, b); 10 $\mu$m (c, d).
organ cultures. Thus, during this time period the bacteria did not stimulate mucus expression on to the tissue surface. However, by 8 h post-inoculation significantly more mucus was measured in infected organ cultures than in controls, suggesting that bacteria induced expression of mucus on to the tissue surface between 2 and 8 h post-inoculation. The same trend was also apparent at 24 h post-inoculation, although the quantity of mucus was not significantly different on infected and control organ cultures. There was no significant difference between the amount of mucus produced in response to WT or bvg mutant bacteria at any time point. Thus, the incubation conditions employed induced the expression of some mucus on to the tissue surface in the absence of infection by bacteria, but infection by WT or bvg mutant bacteria increased mucus expression.

Morphometric analysis demonstrated that organ culture conditions or bvg mutant bacteria did not induce mucus production, whereas WT bacteria did. The results above contradicted this, as the amount of mucus measured in response to WT or bvg mutant bacteria did not differ, and at 24 h in organ culture the same amount of mucus was measured in uninfected and infected organ cultures. A third method to investigate mucus release onto the tissue surface was used to try to explain this contradiction.

Fig. 8. SEM micrographs depicting the time-course of mucus expression on to the tissue surface in organ cultures inoculated with 10^8 c.f.u. of SB796 bacteria. Organ cultures were inoculated with bacteria, incubated for 2 (a), 4 (b), 8 (c) or 12 (d) h and then analysed by SEM. Coverage of the tissue surface increased with time. Scale bars: 5 µm (a); 10 µm (b–d).

Fig. 9. Quantification of mucus. Organ cultures were inoculated with 10^8 c.f.u. of WT (SB796 and RB50) or bvg mutant (SB799 and RB54) bacteria and incubated for 0, 2, 8 or 24 h. Mucus was then scraped from the surface of the organ cultures and quantified as described in Methods. Uninfected (U) and infected samples at 2, 8 or 24 h contained significantly more mucus than samples at 0 h. Infected samples contained significantly more mucus (*P<0.05) than uninfected samples at 8 h.
Organ cultures were inoculated with $10^8$ c.f.u. of WT or $bvg$ mutant bacteria, and incubated for either 0 or 24 h. Sections of tissue samples were cut, stained by PAS/alcian blue and examined by light microscopy. In this procedure neutral mucus was stained magenta, acid mucus was stained blue and goblet cells containing mucus not yet released onto the tissue surface were easily identified.

Organ cultures incubated for 24 h without infection contained numerous goblet cells that stained magenta, indicating that they still contained mucus (Fig. 10a). The number of stained goblet cells was less than at 0 h incubation (data not shown), suggesting that organ culture conditions stimulate some release of mucus from goblet cells. Organ cultures infected with WT bacteria did not contain any stained goblet cells (Fig. 10b), indicating that these bacteria stimulated the release of mucus from all of the goblet cells in the tissue piece. Organ cultures infected with $bvg$ mutant bacteria contained a small number of stained goblet cells (Fig. 10c) but not as many as uninfected cultures, suggesting that these bacteria stimulated mucus release, but not to as great an extent as did WT bacteria.

A layer of mucus was observed on the tissue surface of infected organ cultures (Fig. 10), stained blue (suggesting that the mucus was acidified either by the tissue or by the incubation conditions following its release from the goblet cells). However, in $bvg$-mutant-infected organ cultures, this mucus layer was thin and did not cover the cilia whereas in WT-infected cultures the mucus layer was thick and covered the cilia. No mucus was observed on the tissue surface of uninfected organ cultures (Fig. 10a). This suggested that the tissue was unable to clear the mucus that was released in response to WT bacteria whereas that released in control or $bvg$-mutant-infected organ cultures was cleared from the tissue surface, presumably by ciliary beat. It was possible that mucus released in response to WT bacteria was qualitatively different from that released by control or $bvg$-mutant-infected organ cultures and that this accounted for the difference in clearance. However, taking into account the results of bead clearance assays, these data suggested that mucus was released in response to both WT and $bvg$ mutant bacteria but that WT bacteria abrogated ciliary-mediated clearance, and this prevented clearance of mucus released in response to infection, whereas $bvg$ mutant bacteria did not. When sections of organ cultures were examined at lower magnification by light microscopy, large amounts of mucus were observed at one edge of the tissue in organ cultures infected with $bvg$ mutant bacteria (Fig. 11). This supported the idea that $bvg$ mutant bacteria induced mucus release but that the mucus was cleared from the tissue surface, to one edge of the organ culture, by ciliary beat.

**DISCUSSION**

The study of *Bordetella* pathogenesis has been hindered by a lack of suitable, readily available infection models. Human volunteer studies are currently not permitted for studying *B. pertussis* and *B. parapertussis* infection. *B. bronchiseptica* does infect a wide range of mammals that might provide suitable models but it only causes overt disease in large or companion animals, making large-scale studies logistically difficult. Thus, there have been few experimental studies of *B. bronchiseptica* infection in canines (Bemis et al., 1977; Thompson et al., 1976) demonstrating that the bacteria bind to cilia of the respiratory tract and induce an influx of inflammatory cells into the respiratory tract. While experimental infections of mice have yielded much information, these infections do not cause signs of disease.

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**Fig. 10.** Representative light micrograph sections of organ cultures incubated for 24 h, in which neutral mucus within goblet cells was stained magenta and released mucus on the tissue surface was stained blue. (a) Uninfected organ culture; (b) WT (SB796)-infected organ culture; (c) $bvg$ mutant (SB799)-infected organ culture. Scale bars, 100 μm.
and thus interpretations regarding pathogenesis must be made with caution. A number of studies have utilized tissue from relevant hosts as either tracheal tissue outgrowths or submerged organ cultures to study the interactions between Bordetella and host tissue (Bemis & Kennedy, 1981; Bemis & Wilson, 1985; Funnell & Robinson, 1993; Matsuyama, 1977; Muse et al., 1977). These studies demonstrated that colonization of the epithelial tissue by Bordetella induced loss of cilia from the tissue surface, at least in part through extrusion of ciliated cells, that ciliostasis was induced within hours of adherence of B. bronchiseptica to canine tracheal cells and that this required live, metabolizing bacteria and an intimate association between bacteria and cilia. However, limitations to these studies are that respiratory tract tissue does not exist in vivo in a submerged state and this may affect its behaviour. Furthermore, mucus release on to the tissue surface is not observed in submerged systems. Mucus release from goblet cells and mucus glands in response to various stimuli is an important element of respiratory tract physiology and defence (Rogers, 1994). Particulate matter is embedded in the released mucus and then cleared from the respiratory tract by the ciliary beat.

A previous study investigated interactions between the exclusively human pathogen B. pertussis and human adenoidal tissue or nasal turbinate tissue maintained in organ culture at an air interface (Soane et al., 2000). In this system, B. pertussis infection resulted in damage to the ciliated tissue surface and release of mucus on to the tissue surface. This model was thus proposed as a physiologically relevant one in which to analyse the interaction between B. pertussis and host tissue. However, amounts of human tissue similar to those used in this study, particularly if that tissue is tracheal or bronchial, are rarely available, making it difficult to fully develop the model for humans.

We decided to follow on from this study with human tissue by using B. bronchiseptica infection of canine air-interface tracheal organ culture. This choice retains a natural pathogen–host combination but utilizes a tissue that is far more readily available than human tissue. Our data demonstrate that for at least 24 h, the tracheal tissue is maintained in a state that closely resembles that of the tissue in vivo. Beyond this time, some deterioration of the tissue was observed. It is likely that the tissue starts to deteriorate as soon as it is harvested from the host. In some 24 h organ culture samples examined by transmission electron microscopy, some tight-cell junctions had come apart and there was evidence of heterochromatin in the nuclei of some cells (data not shown), indicative of some cell death. However, these changes did not appear to materially affect the tissue, as even these samples retained bead clearance activity and their tissue surface appeared normal by SEM (data not shown). Nevertheless, it is likely that beyond 24 h in organ culture, natural tissue deterioration will make the use of samples less attractive for the study of infection-induced pathology.

Infection of the organ culture tissue with B. bronchiseptica resulted in changes that are consistent with pathology observed in vivo and previous studies: B. bronchiseptica adhered to cilia, induced mucus expression on to the tissue surface, resulted in damage to the epithelial layer and induced ciliostasis. Thus, we propose that this model is suitable for the study of the interaction between B. bronchiseptica and host ciliated respiratory tract tissue. We consider that this interaction is likely to be one of the very early steps during B. bronchiseptica infection and that the outcome of this interaction is likely to influence greatly whether infection is established or the bacteria are cleared from the host.

We consider that the model used here has several advantages over the submerged organ cultures or submerged ciliated cell outgrowths that had been used previously to study Bordetella pathology. Mucus expression on to the tissue surface was not observed in the submerged systems. Our data, and those of Soane et al. (2000), suggest that this response is an important component of the host tissue response to Bordetella infection.

Submerged organ cultures systems did not permit an easy assessment of ciliary activity. Previous studies measured a reduction of ciliary beat frequency (CBF) of individual cells, grown as tracheal explant outgrowths, when infected by B. bronchiseptica (Bemis & Kennedy, 1981; Bemis & Wilson, 1985). However, ciliary clearance function is dependent not only on ciliary beat, but also on the coordination of ciliary beat across the entire tissue. Ciliary beat of cells is coordinated to produce a wave-like motion (metachronal waves) on the tissue surface that provides clearance of material in a lungs-to-larynx direction. The simple bead-clearance assay used here measures this functional clearance activity of the tissue. Furthermore, the need to use a microscope to focus on a small region of cells for...
CBF measurement means that simultaneous measurement of multiple samples is not possible. The bead clearance assay provides a ‘high-throughput’ analysis in which ciliary-mediated clearance can be assessed in many samples simultaneously. It is also possible to perform the assay multiple times on the same organ culture sample with no apparent loss of function.

Our data demonstrated that ciliary-mediated clearance was abrogated very soon after inoculation of the organ cultures with bacteria. This occurred before the expression of mucus on to the tissue surface and before obvious damage to the epithelial layer. It was not possible using the bead clearance assay to determine whether ciliary beat had been completely halted or whether its coordinated nature had been interrupted to prevent bead clearance. However, clumps of ciliated cells were removed by gentle scraping from organ cultures 1 h after inoculation with bacteria and thus at a time when bead clearance had been halted. These clumps were viewed by light microscopy and cilia were observed to be clearly motionless (data not shown). When cells were viewed in this way from uninfected control samples, cilia were observed to be clearly beating. This strongly suggests that infection had induced ciliostasis rather than disorganization of ciliary beat. Thus, we propose that B. bronchiseptica induces ciliostasis very soon after interacting with the ciliated epithelium.

An elegant series of previous studies demonstrated that B. pertussis released a soluble peptidoglycan fragment, termed tracheal cytotoxin (TCT), during normal growth and that TCT exhibited cytotoxicity to submerged hamster ciliated tracheal organ cultures (Heiss et al., 1993). Furthermore, it was demonstrated that TCT-mediated cytotoxicity was due to induction of nitric oxide (NO) synthesis via interleukin-1-induced expression of type II NO synthase (Goldman et al., 1994). More recently, it was demonstrated that TCT acted in synergy with LPS to cause cytotoxicity, that NO synthesis occurred exclusively in non-ciliated cells of the hamster organ culture and that the NO diffused to neighbouring ciliated cells, causing their death (Flak & Goldman, 1999; Flak et al., 2000). Thus, it was proposed that TCT and LPS induction of NO synthesis is a major contributor to loss of ciliary function during B. pertussis infection. In our studies ciliostasis occurred within 1 h of infection, whereas TCT/LPS-induced cytotoxicity occurred at least 36 h after infection of the organ cultures with B. pertussis. Furthermore, addition of TCT (a kind gift from W. E. Goldman, Washington University, MO, USA) in combination with LPS to organ cultures failed to abrogate bead clearance in our model (data not shown). A low level of ciliostasis was observed following infection of organ cultures with bvg mutants (Fig. 6). TCT secretion and LPS expression occur in the absence of Bvg stimulation and thus it is possible that TCT/LPS contribute, albeit at a low level, to the ciliostasis observed in our studies, although the effect of Bvg-mediated LPS variation (Preston et al., 2003) on TCT/LPS-induced cytotoxicity is not clear. However, the ciliostasis demonstrated in our studies probably occurs through a different mechanism than TCT/LPS-induced synthesis of NO.

Although bvg mutants did not abrogate bead clearance to a large extent they did not adhere to the cilia; thus it was not possible to determine whether induction of ciliostasis is a Bvg-dependent event, or whether ciliostasis requires prior adherence of the bacteria to the cilia, and the adherence is a Bvg-dependent event. Previously, it was demonstrated that heat-killed or chloramphenicol-inhibited B. bronchiseptica adhered to ciliated canine tracheal cells without inducing ciliostasis, suggesting that adherence and induction of ciliostasis were independent events (Bemis & Wilson, 1985). However, this study also demonstrated that induction of ciliostasis required an intimate association between bacteria and cilia. Recently, it was demonstrated that adherence of B. bronchiseptica to ciliated rabbit tracheal cells, grown as explant outgrowths, induced calcium transients within the cells and that this required a functional Bvg system (Groathouse et al., 2003). Induction of calcium transients occurred within seconds of adherence of the bacteria to the cilia. It is possible that in this study the Bvg-dependence for induction of calcium transients was due to the need for adherence to the cilia for this phenomenon and that adherence was Bvg-dependent. These data suggest that calcium-mediated signal transduction is stimulated within ciliated cells by adherence of B. bronchiseptica. It is tempting to speculate that these signal transduction events are involved in the induction of ciliostasis.

In the studies reported here, induction of mucus expression on to the tissue surface occurred hours after inoculation of the organ cultures with bacteria. Uninfected controls also released mucus, suggesting that culture conditions stimulated mucus release from goblet cells. However, at 8 h post-inoculation, mucus levels were higher on infected tissue than on uninfected controls, suggesting that the bacteria were also a stimulus to release mucus. Interestingly, bvg mutants stimulated the same level of mucus release as WT bacteria, suggesting that this stimulation is Bvg-independent and that it does not require adherence of the bacteria to host cells. Mucin secretion can be stimulated by a variety of molecules, including bacterial components such as LPS (Kim et al., 2003). The amount of mucus measured in our studies was highly variable between samples. Mucus within granules in goblet cells is highly condensed and expands up to 600-fold following release on to the tissue surface (Rogers, 2003). Thus, a small variation in the number of goblet cells stimulated to release mucus may result in large differences in measurable mucus volume on the organ cultures. Therefore it is likely that the measurement of the number of goblet cells that contain mucus is a more accurate estimate of mucus release than is direct measurement of mucus volume. In this analysis, infection by B. bronchiseptica resulted in the release of mucus from more goblet cells than did culture conditions alone, but with no significant difference between WT- and
bvB mutant-induced mucus release. Our data thus suggest that induction of mucus expression onto the tissue surface and ciliated cell death and damage to the epithelial layer all occur after abrogation of ciliary-mediated clearance. We thus hypothesize that induction of ciliostasis by *B. bronchiseptica* is a key early step during infection. Induction of ciliostasis abrogates the clearance activity of the mucociliary escalator and thus disables this important respiratory tract defence mechanism. This in turn renders the respiratory tract susceptible to further colonization by *B. bronchiseptica* bacteria that proliferate on the surface of the respiratory epithelium. Clearance from the respiratory tract of the mucus, cell debris and inflammatory-mediated influx that is induced after ciliostasis by *B. bronchiseptica* infection is likely to be impaired and accumulation of this material in the respiratory tract is likely to contribute to the pathology associated with *B. bronchiseptica* infection.

A limitation of the organ culture system is the lack of immune cells in this model. Thus, a key aspect of the response to *B. bronchiseptica* infection, induction of an influx of inflammatory cells into the lumen of the respiratory tract, is missing. Studies of this aspect of the host response are still limited to animal models of infection. However, it is very likely that induction of the inflammatory response occurs after the initial interaction between *B. bronchiseptica* and ciliated tissue, and thus after the induction of ciliostasis described here.

Organ cultures maintained at an air interface more closely resemble the in vivo respiratory tissue environment than do submerged organ cultures, and have been used to study the interaction between respiratory tract tissue and a number of pathogens (Dowling et al., 1998; Middleton et al., 2003; Wilson et al., 1992). Here, the use of the air-interface organ culture model allowed the assessment of adherence of *B. bronchiseptica* to canine tracheal cilia, induction of mucus expression on to the tissue surface, induction of ciliostasis and damage to the ciliated epithelium in a single model system. To our knowledge, this is the first time that the temporal sequence of induction of ciliostasis, induction of mucus release and ciliated epithelial damage in response to *Bordetella* infection has been conclusively determined.

ACKNOWLEDGEMENTS

A Wellcome Trust Programme Grant, no. 054588, supported this work. A Wellcome Trust Prize Studentship supported T. L. A. We thank Dr Jeremy Skepper and Mr Tony Burgess of the Multi-Imaging Centre, Department of Anatomy, University of Cambridge, for invaluable help with processing and imaging of samples for electron microscopy.

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