

## TUBERCULOSIS

## Investigation of mycobacterial colonisation and invasion of the respiratory mucosa

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**Background:** The pathogenesis of *Mycobacterium avium* complex and *Mycobacterium tuberculosis* in the respiratory tract is poorly understood, as are the reasons for their differing virulence. We have previously shown that their initial adherence to the mucosa is identical.

**Methods:** The interaction of *M avium* complex, *M tuberculosis*, and *M smegmatis* with human respiratory tissue was investigated in an organ culture model with an air interface. Tissue was infected for intervals up to 14 days and assessed by scanning electron microscopy for adherent bacteria or cultured for recoverable bacteria.

**Results:** The mean number of adherent bacteria/mm<sup>2</sup> (and the viable count of macerated tissue, cfu/ml) at 15 minutes, 3 and 24 hours, 7 and 14 days were: *M avium* complex 168 (153), 209 (136), 289 (344), 193 (313), 14140 (16544); *M tuberculosis* 30 (37), 39 (23), 48 (53), 1 (760), 76 (2186); *M smegmatis* 108 (176), 49 (133), 97 (81), 114 (427), 34 (58), (n=6). There was no significant change in morphology between infected and uninfected tissue or tissue infected with the different species over 14 days. The number of *M avium* complex on the mucosa and recovered from tissue increased over time (p=0.03). *M tuberculosis* decreased on the surface, but recoverable bacteria increased (p=0.01). *M smegmatis* numbers on the mucosa and recovered from tissue decreased. Sectioned tissue showed *M avium* complex and *M tuberculosis* in submucosal mucus glands and *M tuberculosis* penetrating epithelial cells in one experiment.

**Conclusions:** The initial adherence to the mucosa of the three species was similar, but after 14 days they varied in their interaction with the tissue in a manner compatible with their pathogenicity.

*Mycobacterium avium* complex and *Mycobacterium tuberculosis* both cause pulmonary disease in humans. *M avium* complex is generally referred to as an opportunistic pathogen since it usually infects patients with established lung damage, including scarring from previous tuberculosis, cystic fibrosis and other forms of bronchiectasis, pneumococcosis, and chronic obstructive pulmonary disease.<sup>1–4</sup> *M avium* complex can also cause primary infections in patients without any history of respiratory illness,<sup>5–9</sup> and these have been reported with increasing frequency in recent years.<sup>5, 6, 10, 11</sup> In developed countries endobronchial or mediastinal infections<sup>12, 13</sup> and solitary pulmonary nodules<sup>14</sup> are increasingly common. Chronic infection of the airway causes bronchiectasis.<sup>15</sup> In addition, parenchymal infections manifesting as pneumonitis or cavitary disease have been reported.<sup>6</sup>

*M tuberculosis* is an obligate human pathogen transmitted from patient to patient in respiratory secretions and aerosols. Interaction with the alveolar macrophage is the most important mechanism in the disease process but there may be others because, in one study, resident macrophages did not bind *M tuberculosis* efficiently.<sup>16</sup> Endobronchial tuberculosis may develop via mucosal surface infection in smear positive cases by lymphatic extension from a peribronchial focus or by miliary spread.<sup>17</sup> Thompson and Kent concluded that most cases of endobronchial disease were the result of the surface implantation of *M tuberculosis* and that lack of apparent damage seen on bronchoscopy is possibly due to rapid healing and repair of the mucosal surface.<sup>17</sup> An alternative explanation might be uptake of *M tuberculosis* by specialised epithelial cells which might not necessarily result in damage to the cell.<sup>18–20</sup>

The pathogenetic mechanisms of *M avium* complex and *M tuberculosis* in the respiratory tract are poorly understood, as are the reasons for their differing virulence. We have previously reported the initial interaction of *M avium* complex,

*M tuberculosis*, and *M smegmatis* with the respiratory mucosa of human respiratory tissue organ cultures having an air interface.<sup>21, 22</sup> These experiments showed similar adherence of the three species, predominantly to mucus with a fibrous appearance and to fibronectin in extracellular matrix (ECM) exposed when the epithelium was damaged. The results of these studies showed that the virulence of the mycobacterial species did not influence their initial adherence to the respiratory mucosa. We have therefore investigated the adherence to and recovery from organ cultures of *M avium* complex, *M tuberculosis*, and *M smegmatis* at intervals over 14 days incubation.

## METHODS

## Bacteria

A clinical strain of *M avium* complex previously described,<sup>21</sup> *M tuberculosis* H37<sub>Rv</sub> (ATCC 27294), and *M smegmatis* strain Mc<sup>2</sup>155 were used in the study.<sup>23</sup> The mean viable counts of the inocula (2 µl) were calculated as previously described.<sup>21</sup> The sizes of the inocula for *M avium* complex, *M tuberculosis*, and *M smegmatis* were  $9.3 \times 10^7$  cfu/2 µl (range  $6.6 \times 10^7$ – $1.5 \times 10^8$ ),  $7.8 \times 10^7$  cfu/2 µl (range  $7.1 \times 10^7$ – $9.1 \times 10^7$ ), and  $3.8 \times 10^8$  cfu/2 µl (range  $3.1 \times 10^8$ – $5.0 \times 10^8$ ), respectively. There was no significant difference between the number of bacteria in the inocula of the different series of experiments.

## Organ culture

The organ culture with an air interface used in these experiments has been described previously.<sup>21, 24–26</sup> Human nasal turbinate was obtained from patients undergoing surgery for nasal obstruction and human bronchial tissue from patients undergoing tumour resection. Tissue was used in experiments with the written permission of the patients and the protocol of tissue removal and its use in this study was approved by the Royal Brompton Hospital ethics committee.

### Experimental design

Each experiment required 80 pieces of dissected tissue. Organ cultures were studied at five time intervals with the three species matched with an uninfected control; separate organ cultures were processed for scanning electron microscopy (SEM), culture, light microscopy, and transmission electron microscopy (TEM). Two  $\mu\text{l}$  of a washed bacterial suspension (*M. avium* complex, *M. tuberculosis* or *M. smegmatis*) in phosphate buffered saline (PBS) were gently pipetted onto the surface of the organ culture. An uninfected control organ culture, inoculated with PBS alone, was also harvested at each time interval. Uninfected and infected tissues were placed at 37°C in 5% CO<sub>2</sub> for 15 minutes, 3 hours, 24 hours, 7 days, and 14 days. After harvesting from the organ cultures the tissue was washed by pipetting 100  $\mu\text{l}$  ( $\times 3$ ) of sterile PBS onto the mucosal surface in order to remove unattached bacteria.

### Preparation and assessment of tissue for SEM

The method used for tissue preparation and assessment by SEM has been described previously.<sup>21–26</sup> The tissue was assessed for the percentage of the surface occupied by four main mucosal features: mucus, ciliated cells, unciliated cells, and damaged mucosa. The mucosal morphology was compared over the five time intervals and between infected and uninfected tissue. The number of bacteria seen on the tissue was counted for the entire mucosa. The actual area of the mucosa was calculated taking into account the  $\times 25$  magnification of the microscope and the  $\times 2.5$  visual display unit magnification. Using this figure and the number of bacteria adherent to the mucosa, the number of bacterial cells per mm<sup>2</sup> was calculated for each species at each incubation time.

### Calculation of viable counts from macerated tissue

Tissue was removed from the organ culture taking care to remove all surrounding agar. One hundred  $\mu\text{l}$  of sterile PBS was placed in a sterile graduated centrifuge tube and the meniscus was marked on the side of the tube. This procedure was carried out for all centrifuge tubes and for each separate experiment to compensate for variation in tubes and ambient temperature. Tissue was placed in the PBS and the displacement volume was estimated by the amount of PBS required to be removed to return the meniscus to the marked level. The tissue was then placed in 1 ml sterile PBS in a homogeniser grinder tube and homogenised three times for 10 seconds, with a 10 second interval between each spin to prevent

temperature build up in the tissue/PBS due to friction. Ten-fold dilutions of the homogenate were then performed down to 10<sup>-6</sup>. Viable counts were performed as previously described<sup>21</sup> and the number of bacteria recovered per  $\mu\text{l}$  of tissue was calculated using the displacement volume of the tissue.

### Preparation and assessment of tissue for light microscopy

Tissue was fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Sections were made to study the location of the mycobacteria. Five  $\mu\text{m}$  sections were cut and stained with Ziehl-Neelsen stain. Sections from the same tissue were also cut, fixed, and stained with haematoxylin and eosin.

### Preparation and assessment of tissue for TEM

Tissue was initially fixed in 2.5% glutaraldehyde and then post fixed in 1% osmium tetroxide, dehydrated, and embedded in araldite. Ultrathin (80 nm) sections were then cut and used to look for mycobacteria on the surface and within the mucosa by TEM.

### Statistical analysis

Comparison of the percentages of the surface area occupied by mucosal features for infected and control tissue, the number of adherent bacteria, and the number of bacteria recovered from macerated tissue at different time intervals were analysed using Wilcoxon's matched signed rank test. *p* values of  $\leq 0.05$  were judged to be significant.

## RESULTS

### Comparison of the number of mycobacteria observed on the mucosal surface by SEM with the number recovered from macerated tissue

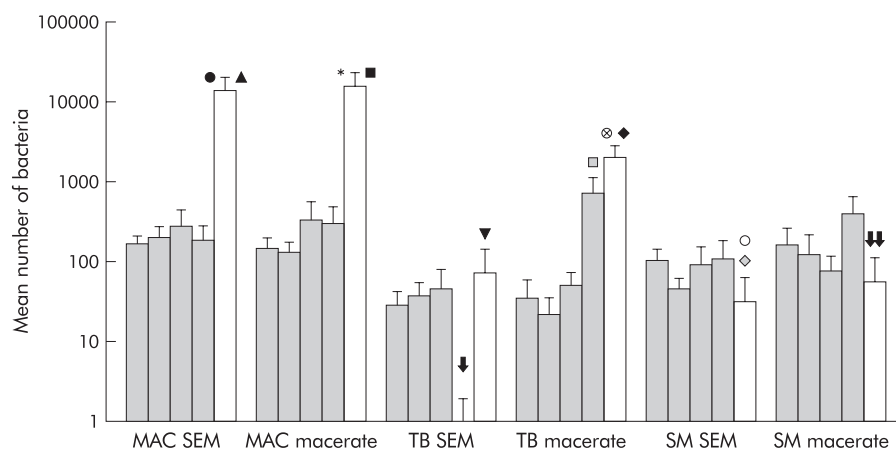
Table 1 shows the morphometric analysis of the four main mucosal features: mucus, damaged epithelium, ciliated, and unciliated cells. Incubation of the organ cultures for up to 14 days had no significant effect on the morphological features of the tissue. There was no significant difference between organ cultures inoculated with the three species of mycobacteria, nor between infected tissue and uninfected controls (data not shown).

Figure 1 shows the mean number of bacteria adhering to the mucosal surface of organ cultures (bacteria/mm<sup>2</sup>) and the

**Table 1** Morphometric analysis of the mucosa of *Mycobacterium avium* complex (MAC), *Mycobacterium tuberculosis* (TB), and *Mycobacterium smegmatis* (SM) infected organ cultures (n=6)

	Mucus					Damaged mucosa					Ciliated mucosa					Unciliated mucosa				
	15 m	3 h	24 h	7 d	14 d	15 m	3 h	24 h	7 d	14 d	15 m	3 h	24 h	7 d	14 d	15 m	3 h	24 h	7 d	14 d
<b>MAC</b>																				
Mean	22.7	19.7	15.1	18.3	10.8	24.0	18.6	20.5	15.3	37.4	14.2	16.9	12.4	19.6	12.2	39.1	44.8	52.0	46.9	39.6
SE	9.2	6.4	7.1	2.4	5.5	12.6	10.3	11.0	6.2	16.9	7.8	7.5	7.5	9.3	4.3	11.9	10.2	12.8	6.8	10.3
Median	15.5	16.2	13.2	19.1	6.1	14.8	12.5	12.7	14.8	17.6	8.9	10.4	9.6	13.4	14.0	40.8	45.7	42.2	42.4	43.7
n	5/6	5/6	5/6	6/6	4/6	5/6	5/6	5/6	4/6	5/6	6/6	6/6	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6
<b>TB</b>																				
Mean	12.8	18.5	17.4	13.5	16.6	19.3	28.7	21.2	25.3	29.0	20.3	21.6	15.8	11.8	16.3	47.6	31.2	45.6	49.4	38.1
SE	5.1	6.5	3.7	4.7	5.7	8.8	13.1	11.4	11.2	19.4	18.6	16.2	14.3	8.5	8.0	7.0	7.8	11.3	6.8	9.0
Median	11.6	16.4	13.6	14.5	11.3	14.7	16.9	14.1	17.8	19.3	11.3	12.8	6.4	0	11.1	46.7	28.5	33.9	44.1	35.1
n	5/6	4/6	4/6	4/6	5/6	5/6	4/6	4/6	4/6	5/6	6/6	5/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
<b>SM</b>																				
Mean	16.8	26.4	19.0	15.1	11.0	24.7	25.9	22.8	26.5	31.3	15.3	19.3	17.9	16.8	16.3	43.2	28.4	40.3	41.6	41.4
SE	2.4	18.4	8.1	3.2	2.7	10.5	9.3	16.2	11.0	25.8	7.4	8.6	8.6	8.3	6.8	6.2	4.7	5.7	7.5	6.5
Median	17.1	14.6	15.5	16.5	11.3	19.4	22.6	12.9	15.6	19.4	12.5	16.7	9.3	11.8	17.1	44.8	30.7	42.4	42.1	38.0
n	5/6	5/6	5/6	5/6	5/6	5/6	5/6	5/6	5/6	6/6	6/6	6/6	5/6	6/6	5/6	6/6	6/6	6/6	6/6	6/6

Figures are the percentage of the mucosal surface surveyed occupied by a particular feature. n=number of experiments in which the mucosal feature was observed out of six.  
15 m=15 minutes; 3 h and 24 h=3 and 24 hours; 7d and 14d = 7 and 14 days incubation time.



**Figure 1** Mean number of *Mycobacterium avium complex* (MAC), *M tuberculosis* (TB), and *M smegmatis* (SM) adhering to the mucosal surface (scanning electron microscopy (SEM); bacteria/mm<sup>2</sup>) or recovered from macerated tissue (macerate; cfu/μl) following incubation for 15 minutes, 3 hours, 24 hours, 7 days and 14 days (from left to right). ●, 15 minutes, 3 and 24 hours v 14 days (p=0.04); ▲, 7 days v 14 days (p=0.03); \*, 5 minutes, 3 and 24 hours v 14 days (p=0.03); ■, 7 days v 14 days (p<0.02); ▼, 15 minutes, 3 and 24 hours v 7 days (p=0.02); □, 15 minutes, 3 and 24 hours v 7 days (p=0.02); ◇, 7 days v 14 days (p=0.02); ↓, 15 minutes, 3 and 24 hours v 14 days (p=0.01); ◇, 7 days v 14 days (p=0.02); ○, 15 minutes, 3 and 24 hours v 14 days (p=0.02); ◇, 7 days v 14 days (p=0.04); ↓↓, 15 minutes, 3 and 24 hours and 7 days v 14 days (p=0.03). Results are expressed as the mean and standard error; n=6 (1 turbinate, 5 bronchial).

mean number of bacteria recovered from macerated tissue (cfu/μl) incubated for 15 minutes to 14 days. At 15 minutes all three mycobacterial species adhered in similar numbers to the same mucosal features, as previously described.<sup>21, 22</sup> Bacteria adhered predominantly to ECM, mucus with a fibrous appearance, and were occasionally seen adhering to areas where extruded epithelial cells had degenerated exposing their contents, open tight junctions between cells, and to healthy unciliated epithelium. Bacteria did not adhere to mucus with a globular appearance, intact extruded cells, collagen fibres, or ciliated cells.

Table 2 shows the number of experiments in which *M avium complex*, *M tuberculosis*, and *M smegmatis* were observed on the mucosal surface by SEM and were recovered from macerated tissue. *M tuberculosis* was seen by SEM in two out of six experiments at 7 days incubation. However, the mean number of *M tuberculosis* (1.2) does not register on fig 1 because of the scale of the y axis. One experiment out of six analysed by SEM showed a large amount of mucosal damage (*M tuberculosis*, 58.3% of the mucosal surface at 14 days incubation; *M smegmatis*, 62.4% at 14 days incubation) which exposed a large amount of ECM (*M tuberculosis*, 19.4% of the mucosal surface at 14 days incubation; *M smegmatis*, 17.3%), and this was the one experiment in which *M tuberculosis* and *M smegmatis* were seen on the mucosal surface at this time point and the one experiment in which *M smegmatis* was recovered from macerated tissue.

*M avium complex* increased in numbers on the mucosal surface with time of incubation. There was a major increase in the numbers of *M avium complex* seen by SEM after 14 days incubation compared with other time points. There was no significant difference in numbers observed by SEM when comparing counts between 15 minutes and 7 days incubation. *M avium complex* recovered from macerated tissue increased with time of incubation. There was an increase in the number of bacteria recovered after 14 days incubation compared with other time points, but there was no significant difference in numbers recovered when comparing counts between 15 minutes and 7 days incubation. *M tuberculosis* numbers decreased on the mucosal surface after 7 and 14 days incubation compared with earlier time points, whereas *M tuberculosis* recovered from macerated tissue increased with time of incubation. There was an increase in the recovery of bacteria after 7 and 14 days incubation compared with earlier time points.

**Table 2** Number of experiments in which *Mycobacterium avium complex* (MAC), *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* were observed by scanning electron microscopy (SEM) and recovered from macerated tissue

Time	MAC	<i>M tuberculosis</i>	<i>M smegmatis</i>
SEM			
15 minutes	6/6	5/6	6/6
3 hours	5/6	5/6	6/6
24 hours	5/6	2/6	6/6
7 days	3/6	2/6	3/6
14 days	5/6	1/6	1/6
Macerated tissue			
15 minutes	6/6	6/6	6/6
3 hours	6/6	6/6	6/6
24 hours	5/6	6/6	6/6
7 days	6/6	6/6	4/6
14 days	6/6	6/6	1/6

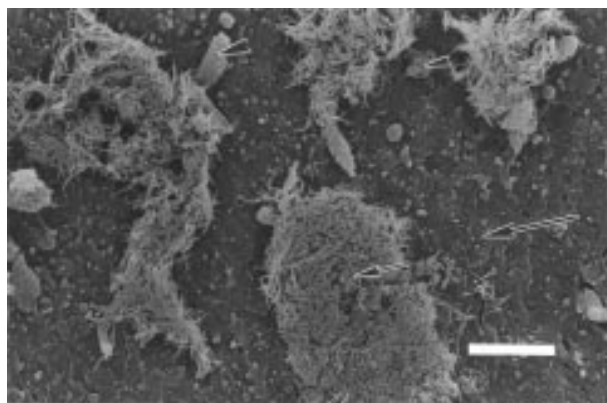
Data provided by a series of six separate experiments using tissue from a different donor for each experiment.

There was a decrease in the number of *M smegmatis* seen on the mucosal surface and recovered from macerated tissue after 14 days compared with other time points.

The numbers of bacteria observed by SEM and recovered from macerated tissue at different times of incubation were compared for *M avium complex*, *M tuberculosis* and *M smegmatis*. The significant differences were: *M avium complex* was seen on the mucosal surface in greater numbers than *M tuberculosis* and *M smegmatis* after 14 days incubation; *M avium complex* was recovered from macerated tissue in greater numbers than *M tuberculosis* and *M smegmatis* after 14 days incubation; and *M tuberculosis* was recovered in greater numbers than *M smegmatis* from macerated tissue after 14 days incubation.

The numbers of bacteria observed by SEM were compared with numbers recovered from macerated tissue for each mycobacterial species. There were more *M tuberculosis* recovered from macerated tissue than adhering to the mucosal surface after 7 and 14 days, but not at earlier time points. There was no significant difference between numbers adhering to the mucosal surface and recovered from macerated tissue for *M avium complex* and *M smegmatis* for all incubation times.



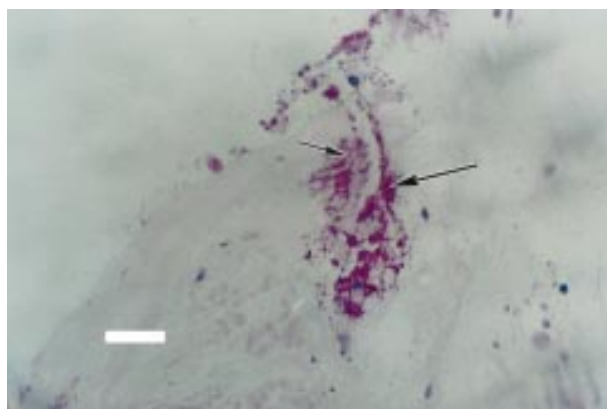


**Figure 2** *Mycobacterium avium* complex adhering to organ culture after incubation for 14 days. The expected size of the mycobacteria used in this study is 1–4  $\mu\text{m} \times 0.5 \mu\text{m}$ . Magnification  $\times 900$ ; scale bar = 20  $\mu\text{m}$ . Large arrow=unciliated mucosa; small arrow=*Mycobacterium avium* complex; large arrowhead=extruded epithelial cell; small arrowhead=extracellular matrix.

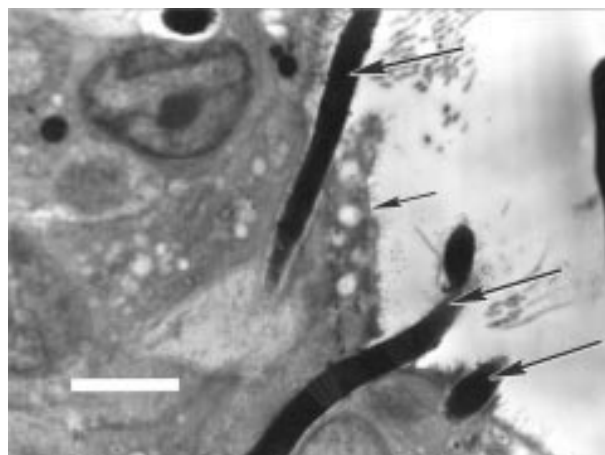
Compared with 15 minutes incubation, SEM counts of *M avium* complex were greater at 14 days indicating multiplication of the organism on the mucosal surface. Although *M avium* complex was seen adhering to ECM and fibrous mucus, large numbers were seen disassociated from these features at 14 days. This suggests that *M avium* complex initially adhered to fibrous mucus and ECM, but either replicated on the mucosal surface independently of them or obscured the features from observation by SEM because of the increased numbers of bacteria. This latter suggestion seems more likely as the bacteria were seen in sporadic “colonies” containing large numbers of organisms (fig 2).

#### Light microscopy sections

*M avium* complex, *M tuberculosis*, and *M smegmatis* were seen adhering to the mucosa at 15 minutes, 3 and 24 hours, and 7 days. *M tuberculosis* and *M smegmatis* were not seen on the mucosal surface after 14 days incubation, whereas *M avium* complex was seen in areas of damaged mucosa in increased numbers compared with other incubation times. *M avium* complex was never observed adhering to intact mucosa in these experiments. *M avium* complex and *M tuberculosis* were seen within submucosal glands underlying damaged mucosa after 7 and 14 days incubation (fig 3). Figure 3 also shows invasion of the epithelium by *M tuberculosis* within the gland.



**Figure 3** Ziehl-Neelsen stained paraffin embedded section showing *Mycobacterium tuberculosis* invasion of the epithelium within a submucosal gland after incubation for 14 days. Magnification  $\times 258$ ; scale bar = 50  $\mu\text{m}$ . Large arrow=*Mycobacterium tuberculosis*; small arrow=*Mycobacterium tuberculosis* within the tissue surrounding the submucosal gland.



**Figure 4** Transmission electron microscopy section showing *Mycobacterium tuberculosis* penetrating unciliated respiratory mucosa. Magnification  $\times 20\,000$ ; scale bar = 1  $\mu\text{m}$ . Large arrow=*Mycobacterium tuberculosis*; small arrow=mucosal surface.

They were, however, not seen in submucosal glands underlying intact mucosa. *M smegmatis* was never seen within submucosal mucus glands.

#### TEM sections

The solitary experiment in which mycobacteria were seen by TEM showed *M tuberculosis* entering a healthy epithelial cell (fig 4). The observation of *M tuberculosis* penetration of epithelial cells was not repeated in paraffin embedded light microscopy sections or by SEM analysis.

#### DISCUSSION

Interaction with the respiratory mucosa is likely to be a key event in the pathogenesis of *M avium* complex which causes chronic airways infection and granulomatous inflammation leading to bronchiectasis, whereas in tuberculosis the primary pathology is in the alveoli and endobronchial disease is less common as the incidence of prolonged smear positive cases declines. *M smegmatis* is an environmental species which is generally considered non-pathogenic. Infection of organ cultures with *M avium* complex and *M tuberculosis* did not affect mucosal morphology over 14 days incubation despite, in the case of *M avium* complex, a large increase in the numbers of bacteria on the mucosal surface and, in the case of *M tuberculosis*, invasion of the tissue. This observation might be because the mycobacteria do not secrete exotoxins, endotoxins, or tissue necrotising enzymes to aid their infectivity. However, it does raise the question as to how *M tuberculosis* in particular invades the tissue. The adherence of *M avium* complex and *M tuberculosis* to open tight cell junctions may represent a potential route of infiltration into submucosal tissues. Since this site of adherence was observed at 15 minutes, it seems likely that mycobacteria took advantage of open tight junctions already present on the mucosa. Phagocytic cells are another potential route of invasion but were not observed on the surface of organ cultures. This could reflect the lack of host circulation from which these cells could migrate, or they may have been washed from the surface during preparation of tissue for microscopic analysis.

Mucosal penetration was not observed by SEM analysis of the mucosal surface of organ cultures, but mycobacteria did adhere to normal unciliated epithelial cells and, in a single experiment (fig 4), endocytosis was observed by TEM. Clark *et al*<sup>27</sup> used confocal microscopy to investigate the invasion of *Yersinia pseudotuberculosis* through murine Peyer's patch M cells with the aim of quantitating the relative contribution of M cells to intestinal invasion by *Y pseudotuberculosis*. We propose

that future studies of mycobacteria could employ a similar technique to investigate invasion of human airway tissue. Labelling of the mycobacteria with luciferase reporter constructs, as described by Snewin and colleagues,<sup>28</sup> may also help to visualise bacteria penetrating the tissue.

The SEM surface counts and recoverable bacteria from macerated tissue showed that there was no difference in the numbers of mycobacteria counted on the mucosal surface and recovered from macerated tissue for the three species at early time points up to 24 hours incubation. This shows that the technique of counting bacteria over the entire mucosal surface correlated well with the total number of viable bacteria on the organ culture, and that the different pathogenicity of the three species was not related to their initial interaction with airway mucosa. The significant increase in numbers of *M avium* complex on the surface over time indicated that it survived and multiplied on the mucosal surface. Bacterial numbers calculated from macerated tissue exceeded SEM counts at 7 and 14 days, which suggests that some invasion of the tissue had also occurred. *M avium* complex formed isolated colony-like patches and did not spread over the surface. These "colonies" were probably located at the sites of initial adherence. This suggests that there were few exposed binding sites on the mucosa for mycobacterial adherence. *M tuberculosis* was recovered from macerated tissue in significantly increased numbers over time, but was seen on the surface of only one of six experiments after 14 days, which shows that it did not survive on the mucosal surface but had invaded the mucosa and multiplied within the tissue. *M smegmatis* did not survive in most organ cultures; it was only seen and recovered from one organ culture after 14 days in small numbers. This organ culture, and the organ culture on which *M tuberculosis* was observed by SEM at 14 days, contained large amounts of damaged mucosa so that large amounts of ECM were exposed, which may have provided a site for more mycobacteria to adhere than was usual.

A large increase in *M avium* complex on the mucosal surface occurred between 7 and 14 days, suggesting that a critical number of these bacteria are required before rapid multiplication occurs, similar to the principle of "quorum sensing" reported for other bacteria.<sup>29-30</sup> Further work is required to investigate the relationship between the initial numbers of *M avium* complex adhering to the mucosa and infection of the mucosa since the infecting dose used in this study was high. This relationship may be important in understanding which patients are vulnerable to infection, since the greater the airway damage, the greater the number of exposed adherence targets and hence the greater the risk of infection with *M avium* complex. Such a study may also offer answers as to why *M smegmatis* was unable to survive in the organ culture after initially adhering in similar numbers to *M avium* complex and *M tuberculosis*. This might relate to nutritional factors, host defences of the tissue or, possibly, the strength of adherence to the tissue.

In our previous studies we have reported that mycobacteria adhere to mucus with a fibrous appearance under SEM.<sup>21-22</sup> The observation of *M avium* complex and *M tuberculosis* within submucosal glands in the present study was not made in sections where glands were seen beneath intact mucosa, suggesting that bacteria could only penetrate those glands associated with mucosal damage. This site may represent a potential route of invasion for mycobacteria. Mycobacteria were not seen within the tissue of the organ culture at other sites by light microscopy, suggesting that invasion is an infrequent event. This is not to say that a rare process is not of potential importance in the pathogenesis of mycobacterial infections, but rather indicates a possible limitation in sensitivity of the techniques employed in this study. The organ culture model well represents the initial interaction of mycobacteria with components of the mucosal surface but, over longer periods of incubation, the absence of a circulation means that the role of

the immune system is limited. For example, granuloma were never observed.

Despite these limitations and those of any in vitro model, this study has made several original observations about the interaction of mycobacteria with the human respiratory mucosa. The initial adherence of each of the three species to the mucosa is similar. Mycobacteria adhere to ECM exposed in sites of mucosal damage, to mucus with a fibrous appearance, and occasionally at other sites including healthy unciliated cells. After 14 days the three species showed differences in their interaction with the tissue. *M avium* complex multiplied on the mucosal surface in discrete colonies, *M tuberculosis* was lost from the surface but multiplied within the tissue, and *M smegmatis* was usually eradicated. An understanding of these differences may give important insights into the pathogenesis of mycobacterial disease. Since the results accord with the clinical features of infection by the three species, this model will be useful in further investigating the virulence of mycobacteria and determining why species differ despite identical initial interactions with the respiratory mucosa.

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## LUNG ALERT .....

### Higher influenza-related paediatric admission rates in the tropics than in temperate regions

▲ Chiu S S, Lung Lau Y, Chan K H, *et al*. Influenza-related hospitalisations among children in Hong Kong. *N Engl J Med* 2002;**347**:2097–103

This retrospective population based study calculated the rate of excess hospital admissions due to influenza in children aged 15 or under with acute respiratory disease. The data were collected from the Hospital Authority of the Hong Kong Special Administrative Region, China. The excess admissions were calculated using an excess hospital admission model from periods of influenza peaks and baseline respiratory syncytial virus rates (7 weeks each in 1998 and 1999). The adjusted rates of influenza-related excess hospital admissions for 1998 and 1999 per 10 000 children were 278.5 and 288.2 for children aged <1 year, 218.4 and 209.3 for those aged 1–<2 years, 125.6 and 77.3 for those aged 2–<5 years, 57.3 and 20.9 for children aged 5–<10 years, and 16.4 and 8.1 for children aged 10–15 years. Influenza-related excess admissions accounted for 38 606 extra bed-days (5.5–8.2% of all paediatric bed-days). These influenza-related hospital admission rates for children are much higher than previously published figures. Possible explanations suggested are the inclusion of high risk patients, differences in admission practice, and higher influenza attack rates in crowded communities.

The reasons for higher influenza-related hospital admission rates in this subtropical climate compared with temperate regions need further investigation. The study highlights the rationale for continued worldwide influenza surveillance, the major impact of this common infection, and the need for effective prevention.

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