BACTERIAL PATHOGENICITY

Ultrastructural study of *Mycobacterium avium* infection of HT-29 human intestinal epithelial cells

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Mycobacterium avium is a common pathogen in AIDS patients and, in a large percentage of those patients, *M. avium* infection appears to be acquired via the gastrointestinal tract. *M. avium* is able to bind to and enter human and murine intestinal epithelial cells in vitro and in vivo. The invasion by and intracellular fate of *M. avium* in the HT-29 intestinal epithelial cell line was examined in an ultrastructural study. Bacterial contact with polarised cells was observed 10–15 min after monolayer infection and in polarised monolayers this always occurred in areas lacking microvilli. Contact with HT-29 cells did not appear to take place in a preferential area on the bacterial cell. Following invasion, *M. avium* was encountered within vacuoles containing either single or multiple bacteria; the latter evolved to contain only an individual bacterium. Vacuoles containing more than one bacterium were seen early in the infection and eventually underwent segmentation, with each bacterium occupying a vacuole. No bacteria were observed outside vacuoles up to 5 days after infection.

Introduction

Mycobacterium avium can cause disseminated disease in patients with AIDS and pulmonary disease in non-AIDS patients [1–3]. Sufficient evidence exists to suggest that the gastrointestinal (GI) tract is the major route of infection in patients with AIDS [4], although the respiratory tract appears to be the route of infection in a small percentage of cases [5]. Therefore, a critical step in the pathogenesis of *M. avium* infection is the crossing of the intestinal mucosa. Previous studies have shown that *M. avium* can bind to and enter intestinal mucosal cells in vitro and in vivo [6–8]. Investigations in mice have determined that *M. avium* invades the intestinal mucosa primarily through enterocytes, although the role of M cells in the interaction of the bacterium with the intestinal mucosa cannot be ruled out [9]. In contrast, it has been shown previously that other mycobacteria, such as *M. bovis* BCG, invade the intestinal mucosa by interacting with M cells [10]. It has also been demonstrated that *M. avium* can multiply intracellularly in intestinal cells in vitro [6]; however, how *M. avium* enters intestinal cells and the fate of the organism once within intestinal epithelial cells is unclear. Studies in macrophages have determined that

M. avium lives within a vacuole that fails to fuse with a lysosome or to acidify [11].

The HT-29 human intestinal cell line is an enterocyte cell line that expresses typical enterocyte differentiation and polarisation when cultured in the presence of galactose [8, 12]. The use of differentiated cell lines has been very useful in understanding the attachment and invasion mechanisms of intestinal pathogens such as *Salmonella* [13], *Shigella* [14] and *Listeria monocytogenes* [15]. The present study investigated the ultrastructural process of *M. avium* invasion and intracellular fate within HT-29 intestinal epithelial cells in vitro.

Materials and methods

Mycobacteria

M. avium strains 101 (serovar 1) and 104 were used in the studies. Strain 101 was originally isolated from the blood of an AIDS patient and has been well characterised and used in several laboratories worldwide [16]. The *M. avium* strains were cultured on plates of Middlebrook 7H11 medium supplemented with oleic acid, albumin, dextrose and catalase for 10 days. Transparent colonies were obtained, suspended in 7H9 broth and incubated for 5 days to the logarithmic phase of growth, as reported previously [8]. Bacteria

were then washed twice in Hanks's Balanced Salts Solution (HBSS) and the final suspension was adjusted to 5×10^7 bacteria/ml by the McFarland turbidimetric standard. The bacterial suspension was then passed through a 27-gauge needle 10 times and vortex mixed for 2 min. The suspension was allowed to settle at room temperature for 5 min and the upper third was collected, stained by acid-fast stain and observed by light microscopy to ensure bacterial dispersion. Viability of the bacterial suspension was determined by plating the inoculum on to 7H11 agar plates.

HT-29 cells and invasion assay

HT-29 intestinal cells were maintained in McCoy's tissue culture medium (Difco Laboratories, Detroit, MI, USA) supplemented with galactose 1%, 2 mM glutamine and fetal bovine serum (Sigma) 10% and incubated at 37°C with CO₂ 5%. Cells were used between passage 20 and 30. Six-well tissue culture plates were seeded with HT-29 cells at 1×10^5 cells/well and the cells were allowed to grow until 80% semi-confluence.

The HT-29 monolayers were exposed to 1×10^7 bacteria suspended in HBSS for 1 h and the monolayers were then washed extensively to remove extracellular bacteria. Monolayers were incubated for different periods of time and then fixed with glutaraldehyde, as described below, for thin-section transmission electron microscopy [17, 18].

In some studies, HT-29 cells (10^5 cells) were seeded on a transwell membrane with pores of 3 µm diameter. Cells were allowed to grow to confluence and the transmembrane resistance of the monolayers was monitored daily. Monolayers were used when resistance reached c. 400 ohm $\times$ cm². Monolayers were infected by adding 1×10^7 *M. avium* to the top chamber for 1 h, followed by washing to remove the extracellular bacteria.

Transmission electron microscopy

A 1-ml bacterial suspension was added to each well of a six-well tissue culture plate (Costar, Cambridge, MA, USA) of the top chamber of a transwell monolayer. These plates were then incubated at 37°C in the presence of CO₂ 5%. At 30 min, 1 h, 1, 2, 5 and 10 days, cells were washed with HBSS, fixed in buffered glutaraldehyde 2.5% for 1 h, post-fixed for 1 h at 4°C with osmium tetroxide 2% and washed with distilled water. Small segments were then dehydrated through ethyl alcohol 50 and 80% at room temperature, embedded in LR white resin [17, 18] and polymerised at 52°C. Thin sections were cut and stained with uranyl acetate and lead citrate. Electron micrographs were obtained with a Philips 201 transmission electron microscope. To infect the transwell monolayer, 10^7 *M. avium* cells were added to the top chamber and at 15 min, 30 min and 1 h the transwell membrane was fixed with glutaraldehyde 2% for 30 min, cut out of the

plastic, embedded in resin, and processed for electron microscopy as described previously [18].

Results

All the figures show results with *M. avium* strain 101 because the results with strain 104 were indistinguishable from those obtained with strain 101. Fig. 1a and b show the interaction between *M. avium* and polarised HT-29 cells 15 min after infection. *M. avium* was first observed invading HT-29 cells after incubation for 30 min. (Fig. 1c). Bacterial internalisation followed a response from the epithelial cells (cell pit formation, Fig. 1a, b and c). *M. avium* was seen in contact with epithelial cells only in areas lacking microvilli, an observation that agrees with an observation previously made in vivo [7, 9] (unpublished observations).

M. avium infection of HT-29 cells was followed for up to 5 days. At all the time points, bacteria were contained within well-defined vacuoles. At 24 h after uptake, a large number of mitochondria and other cell organelles were lying close to the vacuole membrane, polarised towards the site where the bacterium was encountered in the cell (Fig. 2). This finding could be a consequence of adherence of mitochondria to the bacterial vacuole, thus explaining why mitochondria were encountered in close proximity to the bacterial vacuole in the cytoplasm. This phenomenon was observed in approximately two-thirds of HT-29 cells infected for 24 and 48 h, but was not seen in cells infected for 5 days. A number of intracellular organelles other than mitochondria were seen also in the same area of the cells where bacterial vacuoles were located.

In HT-29 cells infected for 48 h, bacterial vacuoles were seen undergoing segmentation. Large vacuoles, initially with more than one bacterium, divided into several small vacuoles containing a single bacterium within 48 h after infection (Fig. 3a, b and c).

Five days after infection, large numbers of bacteria were observed intracellularly, although vacuoles with more than one organism were rarely seen (Fig. 4a, b). Some bacteria were observed to be undergoing replication (Fig. 4c).

During the 5-day infection period, *M. avium* was always seen within vacuoles and not free in the cytoplasm. Close visualisation of the *M. avium* vacuole in some cases (Fig. 5) shows the presence of intravacuolar material, which is probably similar to that reported previously.

Discussion

These results demonstrate that *M. avium* makes initial contact with intestinal epithelial cells by triggering

Fig. 1. Electron micrographs illustrating *M. avium* uptake by HT-29 cells. *M. avium* are in contact with transmembrane polarised HT-29 cells (a, 52 000 $\times$; b, 78 000 $\times$) and being engulfed by an HT-29 cell cultured on plastic (c) (8250 $\times$). The protrusions of the intestinal cell were observed to be asymmetrical. No microvilli were observed in the area of contact between *M. avium* and HT-29 cells. Arrow-heads show (c) the bacteria being engulfed by HT-29 cells.

Fig. 1. (continued)

Fig. 2. Polarisation of organelles to the bacterial site within HT-29 cells was observed 24 h after infection of HT-29 cell monolayers. *M. avium* was seen within peri-nuclear vacuoles with a number of cell organelles surrounding it (12 400 $\times$).

Fig. 3. *M. avium* within HT-29 cells 48 h after infection, showing: (a) segmentation of the vacuoles containing bacteria (arrows) and polarisation of organelles was similar to the observation in cells infected for 24 h, although in a small number of cells (12 400 $\times$); (b) less marked organelle polarisation (12 400 $\times$); (c) segmentation of vacuoles at higher magnification (55 000 $\times$).

Fig. 3. (continued)

predominantly membrane protrusions that engulf the bacterium (Fig. 1a, b and c). Based on the present observations, the contact between the bacterium and the HT-29 cell can involve either only one tip or the entire body of the bacterium, suggesting that putative bacterial adhesin(s) must be expressed on the whole bacterial surface and not in a localised fashion as shown in recent studies on the mechanism of invasion of *Listeria monocytogenes* into epithelial cells. These studies demonstrated that the interaction between *L. monocytogenes* and epithelial cells occurs through the bacterial tip [20]. It has been shown also that internalin, an *L. monocytogenes* invasive protein, is located in only one pole of the bacterium and does not spread on the bacterial envelope [20].

In the present study, a typical clathrin-mediated uptake of the bacteria, such as that observed with *Chlamydia psittaci*, was not observed [21]. The in-vitro studies showed that invasion of epithelial cells by *M. avium* results in re-arrangement of the cytoskeleton with actin polymerisation (data not shown).

Following uptake by intestinal epithelial cells, *M. avium* was encountered within cytoplasmic vacuoles, which underwent segmentation during the course of infection and tended to become single vacuoles containing only one bacterium by 5 days after infection. The vacuole was found close to the nucleus in most of the infected cells. This feature of mycobacterial vacuoles has also been described pre-

viously in macrophages [22, 23]; the explanation for this special characteristic remains to be elucidated. Another feature of the *M. avium* lifestyle in epithelial cells is the size of the vacuole, i.e., *M. avium* lives intracellularly within large vacuoles. In contrast, *M. avium* vacuoles in macrophages have been observed to be smaller (sometimes just opposed to the bacterium) than the vacuoles in epithelial cells [19, 23]. The reason for the difference in size between the vacuoles is currently unknown. Studies with other organisms such as *Salmonella typhimurium* have shown that this pathogen also lives inside large vacuoles [24]. It has been argued that large vacuoles, in contrast to tight vacuoles, are advantageous for the bacterium because they decrease the concentration of any intravacuolar bactericidal product delivery by the host cell.

In the present study, no extra-vacuolar bacteria were observed (in the cytoplasm). Two recent communications investigating intracellular *M. tuberculosis* (but not *M. avium*) within macrophages and type II alveolar epithelial cells have shown that the bacterium is capable of lysing the vacuole membrane and escaping into the cytoplasm [25, 26]. However, these observations are controversial [17, 19, 22, 23] and the issue requires further investigation.

The fate of *M. avium* within epithelial cells in vitro is not known. A recent study of *M. avium* infection of macrophages has shown that apoptosis is a common event in infected cells [18]. Furthermore, it has been

Fig. 4. *M. avium* apparently actively multiplying 5 days after infection. Organisms are seen within vacuoles that are undergoing division (a and b) (27 500 $\times$ and 25 250 $\times$ respectively). (c) A bacterium apparently undergoing division is seen in a large vacuole (19 250 $\times$).

Fig. 4. (continued)

Fig. 5. *M. avium* vacuole within an HT-29 cell. The dark material suggests either bacterial or host cellular matter (55 000 $\times$).

shown that *M. avium* organisms that leave macrophages undergoing apoptosis are capable of invading a second macrophage with increased efficiency [18]. A similar invasive bacterial phenotype was observed after passage within intestinal epithelial cells, although strong correlation with apoptosis cannot be made (F. Sangari, L. E. Bermudez, unpublished observations). However, epithelial cell death and detachment following *M. avium* infection have been documented in the intestinal mucosa [27]. This finding, if confirmed by further studies, may be important in the understanding of the pathogenesis of *M. avium* infection of intestinal epithelial cells. *Shigella*, but not *Listeria*, cells have been demonstrated to invade epithelial cells by the basolateral pole [28, 29] and *Shigella* spp., as well as *L. monocytogenes*, have been shown to escape from infected epithelial cells [30, 31].

The observation that by 48 h the mycobacterial vacuoles contained detached matter is in agreement with previous results [32], although the significance of this finding is currently unknown.

These results suggest a specific interaction between *M. avium* and epithelial mucosal cells, showing that *M. avium* is well adapted to survive and replicate in, and perhaps escape from, intestinal mucosal cells.

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