BACTERIAL PATHOGENICITY

Invasion of murine respiratory epithelial cells in vivo by Burkholderia cepacia

C-H. CHIU*, A. OSTRY† and D.P. SPEERT‡

*Department of Pediatrics, Chang Gung Children’s Hospital, Kweishan 333, Taoyuan, Taiwan, †Department of Pathology and Laboratory Medicine and ‡Department of Pediatrics, University of British Columbia, 960 West 28th Avenue, Vancouver, British Columbia V5Z 4H4, Canada

Pulmonary infections caused by Burkholderia cepacia are an important cause of morbidity and mortality in cystic fibrosis (CF) patients. Several features suggestive of invasion and intracellular sequestration of B. cepacia in CF are persistence of infection in the face of antibiotic therapy and a propensity to cause bacteraemic infections in patients with CF. A mouse respiratory challenge model was used to investigate the invasion phenotype of B. cepacia in vivo. After intratracheal inoculation, epidemic B. cepacia strains translocated from lung to liver and spleen; however, all bacteria were cleared from all organs within 7 days. B. cepacia strains, irrespective of cable piliation, were capable of attaching to and then invading murine respiratory tract epithelial cells. Histopathological examination of lungs showed interstitial infiltrates comprised mainly of polymorphonuclear leukocytes and were associated with widened alveolar septa. Electron microscopy demonstrated B. cepacia within epithelial cells and pulmonary macrophages. This study provides support for in-vitro observations that B. cepacia strains from patients with CF adhere to and then invade respiratory epithelial cells. The invasion phenotype in B. cepacia may be an important virulence factor in CF infections.

Introduction

Burkholderia cepacia is commonly found in soil and water and on plant surfaces. Its ubiquity in nature may contribute to its emergence as an opportunist pathogen of man, particularly those with cystic fibrosis (CF) [1–3]. The clinical outcome for colonised CF patients is variable, but in up to 30% the clinical course is rapidly fatal, usually within a few months following acquisition of the organism [2, 3]. Furthermore, in CF patients who undergo lung transplantation, infection with B. cepacia enhances mortality approximately five-fold [4].

Recent taxonomic studies of isolates of human (CF and non-CF) and environmental origin indicate that the species ‘B. cepacia’ is in fact highly heterogeneous, being composed of many subgroups, some of which might be reclassified as separate species [5]. There are at least six distinct genotypic species in B. cepacia, referred to as genovars I–VI [5, 6]. All six genovars have been isolated from CF patients; most of the epidemic strains are from genovar III [5, 6].


Corresponding author: Dr D.P. Speert.

Despite the progress in taxonomy and a better understanding of the evolving nature of B. cepacia pulmonary infection in patients with CF, very little is known about bacterial features that contribute to pulmonary pathogenicity [7].

One of the most important virulence factors that initiates colonisation and infection may be adhesion of the bacteria to host tissues, which seems to be facilitated by bacterial pili [8]. Recent molecular studies have identified at least five different structural pili types in different B. cepacia strains; one type has been implicated in the enhanced transmissibility of epidemic B. cepacia strains [9]. This unique clone, expressing the specific cable-like pilus (Cbl pilus), has been isolated from CF patients in Canada and the UK [10], many of whom have deteriorated rapidly. This clone exhibits specific in-vitro binding with high affinity to carbohydrates of respiratory mucins [11]; the mucin-binding adhesin is a 22-kDa protein present on the pili [9, 11, 12]. This Cbl pilus is the only genetically well-characterised putative virulence factor associated with an epidemic B. cepacia strain type [10–13].

Identification of B. cepacia within tracheal epithelial
cells from an adolescent female CF patient who died of septicemic infection suggested that B. cepacia might have the ability to invade epithelial cells [14]. This invasion phenotype has been supported by in-vitro observations [15–17]; however, there has been no further in-vivo evidence supporting these findings. The present study was undertaken to evaluate adherence and invasion of epithelial cells by epidemic CF B. cepacia isolates in an in-vivo mouse respiratory challenge model. Both Cbl⁻ and Cbl⁺ B. cepacia strains were studied, to further elucidate the role of the Cbl pili in invasiveness.

Materials and methods

Bacterial strains

Two epidemic strains of B. cepacia were used in this study: C5424 and C6433. Both were isolated from patients with CF and belong to genovar III [18–20]. Isolates were cultured and their identities were confirmed as described previously [18–20]. The presence or absence of the cable pilus gene, cbhA, was determined by a dot-blot hybridisation assay with specific DNA probes [18–20]; strain C5424 was confirmed as Cbl⁻ and strain C6433 as Cbl⁺. Both strains carried the B. cepacia epidemic strain marker [18–20] and were shared by multiple patients.

Mice

Female C57BL/6 mice were purchased from Charles River Breeding Laboratories, St-Constant, Quebec, Canada. Mice were maintained in a specific pathogen-free environment until challenge with B. cepacia, after which they were housed in a biohazard room. Mice were used between 6 and 8 weeks of age. The animal procedures were approved by the University of British Columbia Committee on Animal Care, Vancouver, BC, Canada.

Infection of mice with B. cepacia

Bacterial inocula were prepared by seeding five colonies of each B. cepacia strain into 5 ml of Luria broth (L broth) and allowing them to grow for 18 h at 37°C. Bacteria were collected by centrifugation and resuspended in 1 ml of gelatin 1%-Hanks's Balanced Salts Solution (gel-HBSS; GibcoBRL). The 50% infective dose (ID₅₀) was determined by intratracheal injection of serial dilutions of bacterial suspension [21]. Mice were divided into groups of five; at least four groups were used for each B. cepacia strain. All mice were killed by cervical dislocation at day 3 and haematoxylin and eosin-stained lung sections were examined microscopically for any pathological changes. By intratracheal injection, the ID₅₀ of B. cepacia for C57BL/6 mice, determined by lung histology, was c. 10²–10⁶ cfu. For the following experiments, mice were challenged intratracheally with c. 5 × 10⁶ cfu in 50 µl of gel-HBSS [21]. At predetermined time points after infection, mice were killed, and their spleens, livers and lungs were excised aseptically. Each organ sample was placed in 5 ml of gel-HBSS, homogenised, diluted serially and inoculated on to L agar plates. Viable bacterial counts were determined after incubation at 37°C for 48 h.

Histological examination

A small portion of the lung sample was fixed in sodium phosphate-buffered glutaraldehyde 2.5% and post-fixed in osmium tetroxide 1% for electron microscopy. Another portion was fixed in paraformaldehyde solution, for pathological examination. B. cepacia was also detected in paraffin-embedded, fixed tissue specimens by an immunoperoxidase technique with a modified avidin-biotin-peroxidase method [22]. Briefly, 5-µm sections were mounted on 3-aminopropyltriethoxysilane-coated slides, deparaffinised and hydrated. Slides were washed in PBS and incubated with polyclonal rabbit antiserum to B. cepacia (dilution 1 in 5000) at 25°C for 30 min. Slides were then washed and incubated with biotinylated goat anti-rabbit IgG, followed by the avidin-biotin complex (Signet Laboratories, Dedham, MS, USA). Finally, the substrate diamino-benzidine was applied and the specimens were counterstained with haematoxylin. Sections were covered with aqueous mountant and dried on a warming plate at 60°C until the mountant was polymerised. Slides were then examined by light microscope.

Electron microscopy

The fixed lung samples were dehydrated through a graded alcohol series. The tissues were then embedded in Epon, thin sectioned and stained with oryanyl acetate and lead citrate. Specimens were examined with a Phillips 400 transmission electron microscope.

Results

After intratracheal inoculation with c. 5 × 10⁶ cfu of either Cbl⁻ (strain C6433) (Fig. 1a) or Cbl⁺ (strain C5424) (Fig. 1b) B. cepacia, bacteria translocated from lung to liver and spleen. Nevertheless, no mortality was found. C57BL/6 mice were not susceptible to sustained infection with B. cepacia; all bacteria were cleared from lung, liver and spleen within 7 days following the challenge with a high dose of the bacteria. The results of a representative experiment repeated in triplicate are displayed in Fig. 1. The lung inflammatory response to B. cepacia infection was characterised by cellular infiltration of the bronchioles and adjacent lung tissue and oedema within alveolar spaces. Fig. 2b shows a lung section from a mouse exhibiting interstitial pneumonitis 2 days after infection; Fig. 2c represents a lung section with minimal residual inflammation 7 days after infection. The infiltrating inflammatory cells
were predominantly neutrophil polymorphonuclear leucocytes (PMNLs) (Fig. 2d). The use of immunoperoxidase staining demonstrated B. cepacia, either Cbl– (strain C6433) or Cbl+ (strain C5424), diffusely attached to abnormally thickened alveolar septa 2 h after the infection; both strains adhered in clumps or individually to the epithelial cells (data not shown).

Electron microscopy demonstrated the interaction between B. cepacia strain C6433 and respiratory epithelial cells at 2 h after the intratracheal infection. Large numbers of bacteria were seen in close proximity to the ciliated respiratory tract epithelium (Fig. 3a); some of the organisms appeared to be attached to the epithelial cells. Evidence of attachment (Fig. 3b) and intracellular invasion (Fig. 3c) of type II pneumocytes, a surfactant-secreting epithelial cell type, was demonstrated. A pulmonary macrophage with internalised B. cepacia is shown in Fig. 3d.

Discussion

This study showed the inflammatory response following B. cepacia intratracheal infection in mice; however, rapid clearance of B. cepacia from tissues of mice was observed within 7 days of the infection. The same phenomenon has been reported in mice following intratracheal infection with Pseudomonas aeruginosa [21]. This clearance was associated with an early inflammatory response as expressed by the recruitment of PMNLs in the lungs. The critical role of PMNLs in defence of the lung was further supported by observations that cyclophosphamide treatment, which depleted PMNLs, resulted in increased susceptibility of BALB/c mice to B. cepacia infection (D.P. Speert, unpublished data). On the other hand, a chronic pulmonary P. aeruginosa infection as well as B. cepacia infection in mice, following transoral intrapulmonary inoculation of bacteria enmeshed in agarose beads has been described [23]. It is generally believed that the entrapment of P. aeruginosa and B. cepacia in agar beads induces a delay in the pulmonary recruitment of PMNLs, which thus favours the establishment of a chronic infection [23].

The Cbl pilus has been implicated as one of the adhesins of a specific epidemic B. cepacia clone. Subsequent studies confirmed that a 22-kDa protein of Cbl pili specifically recognises and binds to a
Fig. 2. Haematoxylin and eosin-stained lung sections of mice following intratracheal infection with c. $5 \times 10^5$ cfu of *B. cepacia*. (a) Normal mouse lung 2 days after inoculation of pyrogen-free PBS; (b) infected mouse lung, 2 days after infection, showing cellular infiltration of widened alveolar septa; (c) 7 days after infection, showing minimal cellular infiltration at alveolar septa. (d) The infiltrating inflammatory cells are predominantly PMNLs (→). Magnification = 65 × (a,b,c) and 380 × (d).
Fig. 3. Transmission electron microscopy showing adherence and invasion of *B. cepacia* C6433 to murine respiratory epithelial cells. Large numbers of bacteria (arrows) are in close proximity to the ciliated respiratory tract epithelium (a). Adherence (b) and invasion (c) of a bacterium (→) to a type II pneumocyte (characterised by the presence of lamellar bodies; ➔). *B. cepacia* internalised by a pulmonary macrophage (d). Magnification = 32,500 × (a), 12,000 × (b,c) and 15,000 × (d).
glycolipid receptor on buccal epithelial cells and carbohydrates of respiratory mucins [9, 11, 12]. Despite these in-vitro findings [9], little is known about the contribution of the Cbl pili to bacterial adherence and colonisation in vivo. Sajan et al. used a polyclonal antibody to trace B. cepacia that had adhered to paraffin sections of lung obtained from a CF patient; they found that only strains that express Cbl pili
exhibited adherence to the epithelial cells [24]. Furthermore, *B.cepacia* was noted predominantly attached to the lateral junctions of adjacent epithelial cells, suggesting paracellular invasion of the epithelium [24]. The present study demonstrated that *B. cepacia* isolates, irrespective of cable piliation, are able to attach to mouse respiratory tract epithelial cells in vivo. The binding pattern of the two strains appeared similar, although in an in-vitro study it was found that the Cbl- strains of *B. cepacia* formed clumps when they bound to A549 pneumocytes, whereas the Cbl+ strains attached in a unicellular pattern (Chiu and Speert, unpublished data). These data suggest that the Cbl pilus is not the only factor required for adherence of epidemic *B. cepacia* strains to respiratory tract epithelial cells. Microbial factors of epidemic *B. cepacia* strains responsible for the adherence phenotype remain to be demonstrated, but are clearly more complex than the simple presence or absence of Cbl pil.

Invasion of respiratory epithelial cells in vitro by *B. cepacia* has been described [15, 17]; however, most of the bacteria used in these studies [15, 17] were not genovar III epidemic strains, and the in-vitro tissue culture model does not appear to correlate well with either in-vivo or clinical data [15, 17, 25]. Nevertheless, clinical experience indicates that a substantial percentage of CF patients colonized with *B. cepacia* eventually die of sepsis [4, 14], suggesting that invasion [15, 17] does in fact occur under certain circumstances. The current study demonstrated that *B. cepacia* isolates, either Cbl+ or Cbl-, are capable of crossing respiratory epithelial barriers, resulting in seeding of the liver and spleen. Direct cellular invasion, rather than paracellular invasion [24], by *B. cepacia* was also suggested in this study. *B. cepacia* was demonstrated within type II pneumocytes by electron microscopy.

The finding that *B. cepacia* was internalised by pulmonary macrophages has important clinical implications. It is possible that bacteremic and metastatic infection of *B. cepacia* in CF patients could result directly from transcytosis across endothelial cells, or by transfer from macrophages (‘Trojan horse’ phenomena), or by both mechanisms. Recent studies showed that *B. cepacia* is capable of survival and growth within free-living amoebae, indicating that amoebae may be a reservoir for *B. cepacia* in the environment [26, 27]. The mechanism by which *B. cepacia* survives in amoebae and pulmonary macrophages might be similar [16]. Furthermore, the pathogenic potential of *B. cepacia* for non-CF patients has been illustrated in patients with chronic granulomatous disease. These patients are unable to kill *B. cepacia* via oxidative means, and thus they are at risk for infection with *B. cepacia* which resists non-oxidative killing [28]. Bacteremic spread (from the lungs) has been observed in these patients (Speert, unpublished data). Our data suggest that such spread may occur from direct transit across epithelial cells or within phagocytic cells.

Many bacterial species have demonstrated the ability to invade host epithelial cells. These organisms may simply be sequestered within the invaded host cells or may replicate; some have demonstrated the ability to transcytose across a polarised epithelial cell monolayer [29, 30]. We have observed a phenomenon consistent with invasion of murine respiratory epithelial cells by epidemic *B. cepacia* strains and subsequent transcytosis resulting in bacteremia and metastatic infections in mice. These findings are consistent with the disease caused by the organism in a subpopulation of patients with CF [3, 14].

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References

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