BACTERIAL PATHOGENESIS

Immunolocalisation of *Burkholderia cepacia* in the lungs of cystic fibrosis patients

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Infection by *Burkholderia cepacia* is sometimes fatal in patients with cystic fibrosis (CF), as the organism can cause necrotising pneumonia and septicaemia (the cepacia syndrome), and is resistant to antibiotics. To increase knowledge of the pathogenesis of lung infection, the present study investigated the distribution of *B. cepacia* in lung explants from nine CF recipients of double lung transplants, of which six were colonised with both *B. cepacia* and *Pseudomonas aeruginosa* and the other three with *P. aeruginosa* only. In one case, explants of the donor lung (allograft) became available after the patient succumbed post-operatively to the cepacia syndrome. Further autopsy sections were examined from two patients who had chronic and then acute infection with *B. cepacia*. A specific antibody reactive with all five genomovars of the *B. cepacia* complex and another antibody specific for the 22-kDa adhesin of cable pili, were used to localise bacteria *in situ*. In chronic infection, the organisms were diffusely distributed, but most concentrated in hyperplastic bronchiolar epithelium, inflamed peribronchial and perivascular areas, between adjacent airway epithelial cells and in pathologically thickened alveolar septae and luminal macrophages. In acute infections the distribution was more focal, with *B. cepacia* on injured airway surfaces and in sites of pneumonia and abscess formation. In autopsy sections from one of the patients with chronic, then acute infection, *B. cepacia* was also observed in the lumen of blood capillaries. These results suggest that *B. cepacia* has the capacity to be highly invasive, migrating from the airways across the epithelial barrier to invade the lung parenchyma and capillaries, thereby initiating septicaemia.

Introduction

The aim of the present study was to determine the pattern of distribution of *Burkholderia cepacia* in the lungs of cystic fibrosis (CF) patients with chronic and acute infections. Valuable insights about the pathogenesis of lung infections due to other organisms have been gained by similarly studying the distribution of bacteria in relation to disease-affected lung structures. For example, it has been demonstrated [1, 2] that the location of mucoid *Pseudomonas aeruginosa* in CF lung is generally endobronchiolar and associated with obliterative changes in small bronchioles. The bacteria were found in regions in which pulmonary damage was

most severe, thus emphasising the association of mucoid *P. aeruginosa* with pulmonary deterioration. *P. aeruginosa* was rarely seen in extraluminal areas, even though inflammatory changes were evident as chronic interstitial infiltration or fibrosis, or both. *Staphylococcus aureus*, one of the early colonisers in CF patients, is found mainly in mucus exudates of obstructed airways and not on airway epithelium or in lung parenchyma [3]. In contrast, *Haemophilus influenzae* is diffusely present in the epithelial and subepithelial layers of bronchi, in large and small bronchioles, in the alveolar epithelium, interstitial tissue, pulmonary macrophages and visceral pleura, but not in the lumen of airways. This widespread distribution is thought to reflect the invasiveness and persistence of *H. influenzae* in CF patients [4]. To date there are no reports documenting the distribution of *B. cepacia*, an opportunist micro-organism that is associated with chronic and acute lung disease in adult and

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adolescent CF patients [5]. At the outset of this study it was not known whether *B. cepacia* colonises indiscriminately throughout the lung or in focal clusters, in large or small airways, or whether it invades the parenchyma or adjacent extrapulmonary tissues such as pleura or cartilage. All the lung sections for this study came from patients followed in the Toronto CF clinic. In this centre, almost all *B. cepacia*-infected CF patients harbour one hypertransmissible clonal lineage [6] which belongs to genomovar III of the *B. cepacia* complex [7]. This strain is known as the ET 12, or RAPD-type 2 strain, and it expresses surface cable pili [8]. Thus, it was anticipated that the distribution patterns of *B. cepacia* in the CF lung would be specific for this strain.

Because *B. cepacia* is an opportunist pathogen, the development of lung infection in CF patients probably depends on both host and bacterial factors. A study published several years ago described the adherence of cable pili/adhesin-positive genomovar III isolates to mucins [9], implying that hypersecretion of mucus or luminal mucostasis in CF may predispose to chronic colonisation. Prior lung damage due to *P. aeruginosa* infection may also be a determinant, as it is primarily adolescents and adults with long-standing *P. aeruginosa* lung disease who acquire *B. cepacia*. Various bacterial factors have also been proposed as potential virulence factors, including a potent haemolysin [10], a siderophore [11], surface lipopolysaccharide [12, 13], an extracellular protease [14], lipases [15], a melanin-like pigment [16], as well as the cable pili-associated 22-kDa adhesin which has now been shown to mediate bacterial binding to mucin, cells and lung sections of CF patients [17–20]. The ability of some *B. cepacia* to invade and survive in epithelial cells and macrophages [21–23] and the resistance of this micro-organism to airway epithelial cell antimicrobial activity and to oxidative killing by phagocytes [21, 24] may also contribute to virulence.

The present study examined the morphology of lung explants from CF patients undergoing double lung transplantation (chronic infection), lung allografts from a patient who died due to re-infection with *B. cepacia*

shortly after lung transplantation (acute infection) and autopsy specimens from the lungs of patients who were colonised for 5–10 years but were otherwise clinically stable, and then unexpectedly succumbed over a period of 2–3 months to the cepacia syndrome (chronic plus acute infection). *B. cepacia* was localised in lung sections by immunofluorescent techniques, and its distribution correlated with pathological changes observed in the sections.

Materials and methods

Patients and tissue samples

As shown in Table 1, nine CF patients, age-range 14–38 years, underwent double lung transplantation at the Toronto Hospital, Ontario between 1989 and 1998. All had been colonised with *P. aeruginosa* at some stage before receiving the transplant and six of them were also colonised with *B. cepacia*. Of these six, two patients (nos. 5 and 6) had had no culturable *P. aeruginosa* in their sputum for 6 and 2 years, respectively, before lung transplantation. Lung explants from patients nos. 1–4 and 7–9 were obtained as paraffin blocks from the Pathology tissue library at the Toronto Hospital. For patients nos. 5 and 6, samples of lung tissue (1 cm²) from both left and right lungs were obtained within 30 min of explantation and fixed in buffered formalin. Autopsy samples (1 cm²) of lung allografts from patient no. 5, who died with the cepacia syndrome 11 days after transplantation, were processed in the same fashion. Autopsy lung specimens were obtained from two CF patients (nos. 10 and 11, Table 1) who were colonised with *B. cepacia* for >5 years, then unexpectedly developed the cepacia syndrome and died within a few months. Collection and utilisation of lung samples from patients were approved by the hospital human ethics committee. Paraffin sections (5 µm thick) were used to determine morphology by haematoxylin and eosin staining.

Bacteria and culture conditions

All *B. cepacia* isolates except BC7 and BC45 were kindly provided from the *B. cepacia* research panel of

Table 1. Details of patients investigated

Patient information					Year transplanted	First BC cultured after transplantation	Survival time post-transplant	Clinical diagnosis at death
No.	Age	Sex	BC	PA				
1	21	M	+	+	1989	5 days	11 days	Sepsis/pneumonia
2	19	F	+	+	1990	2.1 years	2.3 years	Sepsis/pneumonia
3	22	M	+	+	1990	24 days	30 days	Sepsis/pneumonia
4	35	M	+	+	1997	5 days	3 months	Sepsis/pneumonia
5	27	M	+	–	1998	9 days	11 days	Sepsis/pneumonia
6	38	M	+	–	1998	6 days	Alive	N/A
7	23	M	–	+	1990	No BC	Alive	N/A
8	24	F	–	+	1996	No BC	Alive	N/A
9	14	F	–	+	1996	No BC	Lost to follow-up	
10	25	F	+	+	Died with cepacia syndrome 5 years after <i>B. cepacia</i> colonisation (1999)			
11	23	M	+	+	Died with cepacia syndrome 10 years after <i>B. cepacia</i> colonisation (1997)			

BC, *B. cepacia*; PA, *P. aeruginosa*; N/A, not applicable.

isolates by Dr Mahenthiralingam, School of Biosciences, Cardiff University, UK and have been described previously [20]. Isolates BC7 and BC45 were cultured from the sputum of CF patients at the Hospital for Sick Children and have been characterised previously [9, 17–20]. Mucoid and non-mucoid *P. aeruginosa*, *H. influenzae* and *S. aureus* were clinical isolates from CF patients, and were identified and provided by the Department of Clinical Microbiology at the Hospital for Sick Children.

Antibodies

A rabbit polyclonal antibody (R418) to *B. cepacia* and a mouse polyclonal antibody (designated anti-adhesin) to the 22-kDa adhesin associated with cable pili of *B. cepacia* have been described previously [17, 20, 25].

Western blot analyses were done as follows. Bacterial isolates were grown in tryptic soy broth, centrifuged, washed once with phosphate-buffered saline (PBS) and suspended to give 10^9 cfu/ml. Ten μ l from each bacterial suspension were mixed with an equal volume of reducing buffer, boiled for 10 min, centrifuged and the supernate was subjected to SDS-PAGE. Separated proteins were then transferred to a nitrocellulose membrane, blocked with bovine serum albumin (BSA) 3% in 10 mM Tris-buffered saline, pH 7.6 (TBS) for 2 h at room temperature and incubated with the R418 antibody (1 in 5000 dilution) overnight at 4°C. The blot was washed with TBS containing Tween 20 0.05% and incubated with a second antibody (anti-rabbit IgG) conjugated to alkaline phosphatase. For detection, the substrate NBT-BCIP (Boehringer Mannheim, Burlington, Ont., Canada) was used. Specificity of the anti-adhesin antibody was established with crude preparations of outer-membrane proteins from each bacterial species, as described previously [20]. To establish further the specificity of R418 and the anti-adhesin antibody, and to ensure that both antibodies would detect bacterial cells in lung sections, bacterial smears prepared on glass slides were incubated with 1 in 1000 diluted R418 antibody or 1 in 100 diluted anti-adhesin antibody for 1 h at 37°C. Slides were washed three times with TBS and incubated with appropriate anti-rabbit or anti-mouse IgG conjugated to CY3 fluorophore (1 in 250 dilution). Bacteria were observed by light and fluorescence microscopy (Leica Dialux 22, Willowdale, Ont., Canada). Bacterial smears and lung sections treated with non-immune serum or immune serum pre-absorbed with cable pili/adhesin-positive *B. cepacia* served as controls.

Immunolocalisation of *B. cepacia* in lung sections

Paraffin sections of lung tissue were mounted on silanated slides, deparaffinised by xylene treatment, rehydrated in graded alcohols, washed twice with PBS and treated with Triton X-100 0.1% in PBS for 10 min at room temperature. Some sections required proces-

sing by microwave heating (DAKO Diagnostics Canada, Mississauga, Ont., Canada) for 1.5 min in 10 mM citrate buffer, pH 6.0 [26] for detection of *B. cepacia* in the airway lumen. Sections were rinsed with water, equilibrated with TBS, blocked with goat serum 5% in TBS for 1 h at room temperature and then incubated for 1 h with the R418 (1 in 1000 dilution) or anti-adhesin (1 in 100 dilution) antibody. Unbound antibody was removed by washing with TBS three times (10 min each) and sections were then incubated with anti-rabbit or anti-mouse IgG conjugated to CY3 fluorophore as appropriate. Slides were washed three times with TBS as before, counter-stained with Mayer's haematoxylin (Sigma) and examined with a fluorescence microscope or by confocal microscopy (Leica TCS 4D equipped with Argon-Krypton laser scanner; Heidelberg, Germany). Optical sectioning (*c.* 1 μ m thickness) of lung sections was performed in the X–Y plane. The bacterial load of *B. cepacia*-colonised lung was semi-quantified by observing 15 random fields in each section and grading each section 1–4 depending on the number of fields that were positive for *B. cepacia*. The presence of *B. cepacia* in 11–15 fields was given a value of 4; in 6–10 fields a value of 3; in 3–5 fields a value of 2; and in 1–2 fields a value of 1. Scores from all sections for each patient were averaged to arrive at a final score. Control samples were treated with non-immune rabbit or mouse serum, or primary antibody absorbed with *B. cepacia* isolate BC7.

Immuno-electron microscopy

Lung allografts fixed in formalin 10% were washed with PBS, fixed in paraformaldehyde 4% and glutaraldehyde 0.1% in 0.1 M phosphate buffer and embedded in Lowicryl (K4M). Thin sections were taken and mounted on formvar-coated nickel grids, blocked with PBS containing glycerol 0.15% and BSA 2% and then incubated with 1 in 1000 diluted R418 or 1 in 100 diluted anti-adhesin antibody for 1 h at room temperature. Sections were rinsed with PBS containing BSA to remove unbound antibody and then incubated with anti-rabbit or anti-mouse immunoglobulin conjugated with 10 nm colloidal gold (1 in 20 dilution) for 1 h at room temperature. Sections were rinsed with distilled water, counter-stained with uranyl acetate and lead acetate, and observed with a JEOL 1200 II transmission electron microscope (JEOL USA, Peabody, MA, USA) fitted with a lanthanum hexaboride cathode and operated at 60 kV accelerating voltage.

Results

Specificity of R418 and 22-kDa adhesin antibodies

Western blot analyses were performed on extracts (lysates) and crude outer-membrane preparations of *B. cepacia* isolates belonging to all five genomovars, and clinical isolates of other common CF pathogens (Fig.

1). The R418 antibody reacted strongly with numerous bands in all *B. cepacia* isolates tested (Fig. 1a) but not with *P. aeruginosa*, *H. influenzae* or *S. aureus*. The anti-adhesin antibody reacted only with genomovar III isolates BC7 and C5424 (Fig. 1b). The other three genomovar III isolates lack the adhesin [17, 20] and did not react with the antibody.

The reactivity of both antibodies with intact bacteria was also tested by immunofluorescent assays of bacterial smears (data not shown). The R418 antibody reacted strongly with all *B. cepacia* isolates but not with other micro-organisms, indicating that the R418 antibody would be appropriate for detection of *B. cepacia* in sections of lung tissue. The anti-adhesin antibody reacted strongly with the two cable pili/adhesin-positive *B. cepacia* isolates BC7 and C5424,

very faintly with all other *B. cepacia* isolates and not at all with other micro-organisms. These results predicted that the antibody would detect cable pili-positive isolates expressing the 22-kDa adhesin in lung tissue sections.

Lung explants of CF patients (chronic infection with B. cepacia)

At least one section from each lung transplant patient except patient no. 3 (Table 1) was taken from the proximal part of the lung, which showed second to fifth generation bronchi and large bronchioles. Other sections (four-to-eight per patient) were representative of peripheral lungs, showing numerous small, terminal and respiratory bronchioles and adjacent parenchyma. Some sections also contained pleural membrane. All

Fig. 1. Immunoreactivity of R418 and anti-adhesin (22 kDa) antibody with *B. cepacia* and other CF lung pathogens. Bacterial extracts (a) or crude outer-membrane preparations (b) from *B. cepacia* (genomovars I, II, III, IV and V) and other organisms (10^7 cfu) were subjected to SDS-PAGE and proteins were transferred to a nitrocellulose membrane. The membrane was incubated with antibody R418 (a) or the anti-adhesin antibody (b). Lane 1, molecular mass standards; 2, *B. cepacia* ATCC 25416 (genomovar I); 3, C5393 (genomovar II); 4, BC7; 5, C5424; 6, BC45; 7, J2315; 8, CEP138 (genomovar III); 9, FC473 (genomovar IV); 10, CEP040 (genomovar V); 11–14, clinical isolates of non-mucoid *P. aeruginosa*, mucoid *P. aeruginosa*, *S. aureus* and *H. influenzae*, respectively.

lung explants showed evidence of end-stage lung disease, including marked bronchiectasis and extensive mucus plugging (Fig. 2a). Within the mucus plugs, a dense infiltrate of acute and chronic inflammatory cells, plasma cells and exfoliated epithelial cells was noted. Often neutrophils were also seen within the bronchial/bronchiolar epithelial layer. Hyperplasia, squamous metaplasia, partial loss of cilia or exfoliation of epithelia, or both, were observed in many small bronchioles. Peribronchiolar and perivascular fibrosis with focal interstitial extension was seen in many sections. Many alveolar septae were abnormally thickened (Fig. 2b) with loose fibrous connective tissue and infiltrating macrophages. In some sections interlobular septae had dilated lymphovascular spaces. Many of the air spaces contained scattered macrophages. Samples from all the patients showed many regions of pneumonic consolidation with heavy infiltration by neutrophils. By visual inspection no difference was observed in morphology between patients colonised with *P. aeruginosa* alone (patients nos. 7, 8 and 9, Table 1) and those colonised with both *P. aeruginosa* (either in the past or at the time of transplant) and *B. cepacia* (patients nos. 1–6, Table 1).

Immunofluorescence of *B. cepacia* in lung explants was performed with antibody R418 and the anti-adhesin antibody. Two different methods were used to detect the colonised organisms in paraffin-embedded tissue sections. In the first method, sections were treated with Triton X-100 0.1% which permeabilises tissue and exposes antigenic sites. In the second method, sections were heated in a microwave oven to 'retrieve' antigens [26]. Both methods gave comparable results in all patients except where indicated below. Both antibodies gave positive signals only in explants from *B. cepacia*-infected patients (nos. 1–6) and not in explants from patients who were colonised with only *P. aeruginosa* (nos. 7–9). The load of colonised *B. cepacia* differed between patients (Table 2), but no correlations could be discerned between the apparent load, the degree of pathology or the subsequent outcome of double lung transplantation in these patients. Lung explant sections treated with non-immune serum, or R418 antibody pre-incubated with *B. cepacia* (controls), were negative.

B. cepacia was found in both the airways and the lung parenchyma of patients nos. 1–6, principally in

Fig. 2. Haematoxylin and eosin-stained sections of a CF lung explant from a *B. cepacia* colonised patient. (a) Mucus exudate plugging a small bronchiole; (b) thickened alveolar septa. Both sections show extensive inflammatory cell infiltration.

Table 2. Distribution of *B. cepacia* in CF explant and autopsy lungs

Patient no.	Grade of <i>B. cepacia</i> colonisation detected by immunofluorescence in									
	Bronchiolar (small) epithelium		Alveolar septum		Bronchial epithelium		Airway (bronchi) lumen		Cartilage	
	R418	22 kDa	R418	22 kDa	R418	22 kDa	R418	22 kDa	R418	22 kDa
1	4	4	4	4	1	1	2	2	2	0
2	4	1	4	1	4	1	4	1	4	0
3	3	3	4	4	N/A	N/A	N/A	N/A	N/A	N/A
4	4	4	4	4	1	1	1	1	0	0
5	2	2	3	3	1	1	1	1	0	0
6	2	2	2	2	1	1	1	1	0	0
10	4	4	4	4	N/A	N/A	N/A	N/A	N/A	N/A
11	4	4	4	4	4	4	4	4	4	4

Bacteria detected by immunofluorescence with R418 or anti-adhesin (22 kDa) antibody. Localisation of *B. cepacia* was scored over 15 random high-power fields. Scoring: presence of *B. cepacia* in 11–15 fields = 4; 6–10 fields = 3; 3–5 fields = 2 and 1–2 fields = 1. Patients nos. 7–9 are not presented as they were colonised only by *P. aeruginosa* and gave no reactivity in any location with anti-*B. cepacia* antibodies. N/A, not available.

peripheral rather than proximal airways. In the larger bronchi (second to fifth generation), *B. cepacia* clusters were occasionally observed associated with epithelium and luminal exudates, as judged by the tissue permeabilisation method. In small bronchi (Fig. 3a and b), *B. cepacia* was found in surface epithelial cells, but even more prominently in the deeper layers of hyperplastic thickened respiratory epithelium. This preferential deeper distribution has not been described for other CF pathogens. Patient no. 2 showed abundant immunoreactive *B. cepacia* in the epithelium of small bronchi, but very few bacteria in the lumen, even though numerous gram-negative rods were identified by Gram's staining. This prompted the use of the antigen retrieval technique instead of Triton X-100 permeabilisation of tissue sections, before incubating with the R418 antibody. With antigen retrieval, numerous *B.*

cepacia were detected in the exudate of the bronchial lumen (Fig. 3c and d), but bacteria in the epithelium were less well detected by this technique. It was concluded that permeabilisation of the tissue is necessary to visualise bacteria associated with the epithelium, whereas the antigen retrieval technique optimises visualisation of *B. cepacia* in the lumen. Proximal lung sections from several patients were then re-tested by the latter technique and, in most sections, very few *B. cepacia* were found in the lumen of large or small bronchi. However, positive signals were observed in the lumen of bronchioles that also contained mucopurulent exudates.

Colonisation was not confined to grossly inflamed bronchioles, which is the situation reported for *P. aeruginosa* [1, 2]. Frequently, *B. cepacia* (or, possibly,

Fig. 3. Distribution of *B. cepacia* in small bronchi of CF lung explants. Treated paraffin sections were incubated with R418 antibody followed by anti-rabbit IgG conjugated with fluorophore. (a), (c) and (e) show immunoreactive *B. cepacia* in the bronchial epithelium, lumen and cartilage, respectively; (b), (d) and (f) show haematoxylin-stained sections corresponding to (a), (c) and (e) respectively.

immunoreactive degradation products of the organism) were observed in the intercellular junctions of epithelial cells of relatively normal-looking bronchioles (Fig. 4a and b). This was best revealed by high resolution confocal microscopy with optical sections taken every micrometer, and reconstruction of the image (Fig. 4c). The bacteria were detected by both antibodies, indicating that they belonged to the cable/adhesin-positive ET 12 strain. Intercellular distribution has not been described for other CF pathogens and suggests an invasive phenotype. *B. cepacia* was also detected in peribronchiolar regions containing inflammatory cell infiltrates (Fig. 4d and e), suggesting that the bacteria may be able to migrate from the lumen *via* intercellular pathways to the deeper parenchyma.

Unlike the reported distribution of *P. aeruginosa* in CF lungs, *B. cepacia* was prominent in abnormally thickened alveolar septae in explants from all six patients (Fig. 5a and b) and was associated with alveolar epithelial cells, infiltrating and luminal macrophages (Fig. 5c and d). Pulmonary tissue that had been replaced by consolidated inflammatory cells frequently showed abundant immunoreactive *B. cepacia* (Fig. 5e and f), although fibrotic tissue was spared.

Surprisingly, explants from patients nos. 1 and 2 also showed *B. cepacia* in the cartilage adjacent to small bronchi. The micro-organisms were associated with calcified chondrocytes (Fig. 3e and f) and, in patient no. 1, *B. cepacia* was also noted in the submucosal

Fig. 4. Distribution of *B. cepacia* in terminal bronchioles and peribronchiolar areas of CF lung explants. Deparaffinised and rehydrated sections incubated with antibody R418 (a) and (d) or anti-adhesin antibody (c). (a) and (d) *B. cepacia* in a terminal bronchiole and a peribronchiolar area; (b) and (e) haematoxylin-stained sections corresponding to (a) and (d) respectively; (c) high resolution confocal image of a terminal bronchiole showing *B. cepacia* distributed in the junctions between adjacent epithelial cells and in the parenchyma.

Fig. 5. Distribution of *B. cepacia* in the parenchyma of CF lung explants reactive with anti-adhesin antibody. (a) *B. cepacia* in alveolar septa, (c) association of *B. cepacia* with alveolar macrophage, (e) *B. cepacia* distribution in consolidated area; (b) (d) and (f) are haematoxylin-stained sections corresponding to (a), (c) and (e), respectively; (d) *B. cepacia* associated with alveolar macrophages are shown by arrows.

glands. These are unusual locations for a pulmonary pathogen, but recently *H. influenzae* has also been detected in cartilage cells and submucosal glands of CF patients [4].

By visual inspection, the distribution and load of *B. cepacia* revealed by the anti-adhesin antibody were the same as those revealed by the R418 antibody in the lung explants of all but two *B. cepacia*-colonised patients. The anti-adhesin antibody failed to detect *B. cepacia* in chondrocytes (patients nos. 1 and 2) and the anti-adhesin signal was much lower than the R418 antibody signal in all tissues of patient no. 2. This suggests a loss or masking of the adhesin of *B. cepacia* once the bacteria migrate to cartilage cells and a similar loss of adhesin in the *B. cepacia* of patient no.

2. Alternatively there may have been colonisation of the lungs of patient no. 2 by more than one strain (or genomovar), with the dominant strain lacking cable pili. Unfortunately, sputum cultures were not available to distinguish between these alternatives.

Taken together, these results suggest that the majority of *B. cepacia* in the lungs of chronically infected Toronto patients (nos. 1–6) belonged to genomovar III, and were the ET 12 or cable pili/adhesin-positive strain. The bacteria were mainly distributed distally, on the surface and between epithelial cells of small bronchioles, in the deeper layers of hyperplastic epithelia, and within the chronically inflamed alveolar septae. This distribution is clearly consistent with an invasive profile.

Lung allografts of a CF patient (acute infection of the donor lung)

Allograft tissue was available from only one patient (no. 5). The broncho-alveolar lavage fluid was *B. cepacia* culture-positive 2 days after double lung transplantation. Subsequently, the patient developed bronchopneumonia and sepsis, and died 9 days later. Gross pathological observation of the lungs indicated focal areas of necrotising bronchopneumonia and pleural effusion, but many regions of both lungs appeared normal.

Sections from infected regions showed necrotising pneumonia, consolidation, micro-abscesses and no normal airway or parenchymal structures. A bronchial section close to the site of surgical anastomosis showed partly exfoliated epithelium with some inflammatory cells in the lumen. Sections obtained from minimally inflamed areas also showed partially exfoliated epithelium in bronchioles and cellular material in the lumen. Although alveolar septae appeared normal, the alveolar air space was filled with inflammatory cells, mainly foamy macrophages. Non-inflamed, normal-appearing areas showed normal lung histology. The alveolar septae were also normal, but the alveolar airspaces frequently showed foamy alveolar macrophages, and occasionally neutrophils, suggestive of a low grade inflammatory reaction.

Immunofluorescent localisation of *B. cepacia* produced strong signals from both antibodies in regions of pneumonia, exfoliating epithelium close to the anastomosis site (Fig. 6a and b) and in perivascular areas containing inflammatory infiltrates (Fig. 6c and d). Signals were not seen in the lumen, on the surface of bronchiolar epithelia, or in alveolar septae in normal areas of the lung. However, micro-organisms reactive with both antibodies were found associated with foamy macrophages in otherwise normal-appearing alveolar airspaces (Fig. 6e). Electron microscopy confirmed that *B. cepacia* cells were inside macrophages (Fig. 6f, shown by arrows), and had retained the immunoreactive adhesin (Fig. 6 inset, gold labelling shown by arrowheads).

Lungs from CF patients who died with a 'delayed' cepacia syndrome (chronic plus acute infection with B. cepacia)

In this group there were two patients (nos. 10 and 11) who were infected chronically with *B. cepacia* for >5 years, then developed the cepacia syndrome and died within 3 months. Their lungs showed extensive necrotising pneumonia and abscess formation, diffuse alveolar damage and interstitial pneumonia. In most sections the airways and parenchyma were not easily recognised because of massive tissue necrosis. Some sections showed recognisable airways, but they were denuded of epithelium and the lumen was filled with

exudate and inflammatory cells. Alveolar septae were thickened and infiltrated with foamy macrophages. The air spaces also often contained macrophages. Tomaszewski *et al.* [27] reported similar histopathological changes in the lungs of CF patients who had a rapid decline with fatal necrotising pneumonia and septicaemia shortly after *B. cepacia* colonisation.

Both antibodies gave an identical pattern of distribution. The bacterial load was scored as 4 in the same scoring system as that used for explants (Table 2). Numerous *B. cepacia* cells were detected in regions of interstitial pneumonia and abscesses, in damaged epithelial cell layers of bronchi and in alveolar macrophages in air spaces (not shown). *B. cepacia* was also found in small, terminal and respiratory bronchioles and in alveolar epithelia. One of the patients (no. 10) also had *B. cepacia* in capillaries adjacent to alveoli (Fig. 7), a location not seen either in lung explants or allograft sections. Thus the autopsy sections exhibited *B. cepacia* distribution characteristics that were a combination of acute and chronically infected lungs, but in addition, there was evidence of intravascular invasion.

Discussion

Although the prevalence of *B. cepacia* lung infection in CF patients is estimated to be only about 3% on a world-wide scale [5], it is much higher in selected geographic regions, including Toronto, Canada, where it has been found in sputum cultures of >40% of the adult CF population [28]. The clinical outcome after acquisition is variable and unpredictable, ranging from no apparent change to a rapidly fatal necrotising pneumonia and septicaemia (the cepacia syndrome) [29–31]. Infected patients who undergo double lung transplantation have a particularly high risk of infection in the donor lung and death due to the cepacia syndrome during the early post-operative period, when they are maintained on heavy doses of immunosuppressive drugs. The result is a much higher mortality rate in the first 3 months post-operatively (64%) than for transplant patients previously colonised by *P. aeruginosa* alone (19%) [32, 33]. Results from the present study indicate that cable pili/adhesin-positive *B. cepacia* can be highly invasive, migrating from the airways across the epithelial barrier, to invade the lung parenchyma and blood vessels. This may help to explain why CF patients colonised with *B. cepacia* before undergoing double lung transplantation are at high risk for post-operative mortality.

The distribution of *B. cepacia* was determined in explants from patients with chronic and acute infections, with and without concomitant *P. aeruginosa* infections. It was considered important to use antisera with multiple recognition epitopes to prevent misidentification of *B. cepacia*, or lack of recognition of *B.*

Fig. 6. *B. cepacia* distribution in lung allografts detected with anti-adhesin antibody. (a) *B. cepacia* in a small bronchus with exfoliating epithelia; (c) *B. cepacia* in an inflamed perivascular area; (b) and (d) haematoxylin-stained sections corresponding to (a) and (c), respectively; (e) *B. cepacia* in alveolar macrophages (confocal microscopy); (f) transmission electron microscopy of a lung allograft section with bacteria (arrows) in a macrophage. The inset is a portion of one bacterium (magnification 14 $\times$) showing colloidal gold immunoreactivity with the anti-adhesin antibody (arrowheads).

cepacia that might have lost antigenic epitopes during in-vivo colonisation. The R418 antibody recognised multiple protein bands in all five genomovars tested, whereas the anti-adhesin antibody was highly specific for the 22-kDa doublet previously identified in cable pili-positive genomovar III organisms [20]. In all but one patient (no. 2), the immunoreactivities of *B. cepacia* detected in lung tissue by both antisera were identical, suggesting that the strain responsible for infection was the cable pili/adhesin-positive genomovar

III strain. Further specificity was confirmed by demonstrating that other common respiratory pathogens did not react with the antibodies, that the antibodies did not react with lung explants taken from patients colonised by *P. aeruginosa* alone, and that pre-adsorption of R418 antibody with isolate BC7 (cable/adhesin-positive *B. cepacia*) abrogated reactivity. In the case of patient no. 2, *B. cepacia* cells reactive with antibody R418 were more numerous than those reactive with the anti-adhesin antibody. It is likely that this

Fig. 7. *B. cepacia* in an alveolar capillary of a CF patient who died with the delayed cepacia syndrome. Bacteria were detected with the anti-adhesin antibody.

reflects colonisation by more than one strain of *B. cepacia*, an unusual but previously recognised occurrence in CF [5, 34]. Alternatively, some members of the cable pili-positive strain may have undergone phase variation with a loss of the pili and associated adhesin. Loss of pili is a frequent occurrence in selected strains of *Escherichia coli* and *H. influenzae* once they have colonised host tissues [35, 36]. As five of the six *B. cepacia*-infected patients did not appear to lose adhesin reactivity, phase variation seems an unlikely explanation. One possible exception might be in the case of the *B. cepacia* noted in calcified chondrocytes of both patients nos. 1 and 2, as neither reacted with the anti-adhesin antibody in that location.

The morphology of CF lung tissues was similar in all nine explants studied, with no distinctive features peculiar to *B. cepacia*-infected patients. This is not surprising considering that the patients all had end-stage lung disease and widespread destruction of normal lung structure at the time the explants were obtained. However, the distribution of *B. cepacia* was much more widespread and invasive in all cases than that reported for *P. aeruginosa* [1, 2]. *B. cepacia* was found mainly in epithelia of the terminal and respiratory bronchioles, and exhibited intercellular migration, presumably allowing it to penetrate the respiratory epithelium. The mechanism permitting penetration was not explored, but it might be speculated that bacterial exoproducts (proteolytic enzymes) could be involved. Organisms were also numerous in peribronchiolar and perivascular regions, in inflammatory cell infiltrates, in thickened alveolar septae and in luminal and parenchymal macrophages. These are not typical locations for *P. aeruginosa*,

which tend to localise to luminal positions but do not persist in phagocytes. Overall, the *B. cepacia* distribution was closer to that described by others for *H. influenzae* [4], although the intercellular location of *B. cepacia* appears to be unique.

The allograft of patient no. 5 who died 11 days after transplantation, and the autopsy sections of patients nos. 10 and 11, who exhibited a 'delayed' cepacia syndrome, showed areas of necrotising pneumonia, unlike the explant tissues from chronically infected patients. This is consistent with previous descriptions of lung tissue from patients who succumbed to the cepacia syndrome shortly after colonisation [27]. *B. cepacia* was prominent in areas of necrotising pneumonia, and a special feature of acute infections was the high number of 'foamy' macrophages in alveolar spaces, many of which contained cable pili/adhesin-positive organisms. *B. cepacia* is known to be able to persist in CF macrophages, at least under in-vitro conditions [21, 22], and to replicate and resist killing by an oxidative burst. This may be an important mechanism *in vivo* to allow *B. cepacia* to escape the normal immune clearance process.

The septicaemia characteristic of the cepacia syndrome was dramatically revealed by autopsy sections from patient no. 10 in which immunoreactive organisms were seen to fill the capillary lumen, and by the allograft sections from patient no. 5 in which perivascular areas were particularly prominent sites of bacterial clusters. Thus, it is speculated that cable pili/adhesin-positive *B. cepacia* can be highly invasive, especially in immunocompromised patients, moving from the airways through the epithelial barrier to parenchyma and into blood capillaries of the donor lung within a few days post-operatively. Other CF lung pathogens rarely, if ever, exhibit such rapidly invasive behaviour, or lead to such devastating results as those seen in the cepacia syndrome.

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