Differential persistence of *Burkholderia multivorans* and *Burkholderia cenocepacia* in the mouse

by

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Abstract

Members of the *Burkholderia cepacia* complex (BCC) of organisms are important opportunistic pathogens, particularly in cystic fibrosis (CF) patients. Infection with members of the BCC is associated with poor prognosis. There is speculation that the BCC are intracellular pathogens, surviving and growing inside epithelial cells or professional phagocytes. *B. cenocepacia* strains are the predominant Canadian BCC-CF pathogens in that members of this species cause the most numerous infections, are the most highly transmissible, and are associated with the highest rates of systemic illness and mortality. *B. multivorans* infections, though prevalent, are associated with lower rates of transmissibility and mortality. The apparent differential pathogenic ability of these two species has not yet been studied in detail in any model systems. The purpose of these studies was to determine the mechanisms of differential pathogenicity between these two related bacterial species.

Three different models of murine infection were used to evaluate *B. multivorans* and *B. cenocepacia* infections: an intraperitoneal model of systemic infection, a leukopenic model of pulmonary infection, and an immunocompetent model of pulmonary infection. Differences in the infection kinetics of *B. multivorans* and *B. cenocepacia* were observed in all three systems. These potential differences in the pathogenic capability of *B. multivorans* and *B. cenocepacia* were further characterized in the immunocompetent model of infection.

In the immunocompetent model of intranasal infection, mice were challenged with a single dose of bacteria in stationary phase that had been adjusted to 10^7 CFU. *B. cenocepacia* strain C6433 caused a greater degree of systemic illness in mice despite speedy clearance from the lung; persistent *B. multivorans* strain C5568 caused no systemic illness in mice. The differential infection kinetics and host toxicity demonstrated in this model

mirrored observations in a leukopenic model of pulmonary infection, as well as an intraperitoneal infection model.

The pulmonary host response in the high-dose intranasal model was evaluated. Bronchoalveolar lavage fluid (BALF) was collected for cytological profiling and immunoassay for pro-inflammatory cytokines. Effective clearance of *B. cenocepacia* C6433 infection was associated with a more pronounced interleukin (IL)-1β induction and neutrophil response, on Day 1 of infection. *B. multivorans* C5568 infection was associated with a gradual increase in the level of neutrophils in the lung, which peaked on Day 2, and a delayed IL-1β response. There was no difference in pulmonary levels of tumour necrosis factor (TNF)-α and macrophage inflammatory protein (MIP)-2 in response to infection with either strain; challenge with both C5568 and C6433 was associated with a rapid increase of TNF-α and MIP-2, followed by a rapid decline.

Bacterial persistence in the lung was also examined in the high-dose intranasal model. Immunofluorescent localization of bacteria in day 4-infected lung tissues detected bacteria in association with Mac-3+ mononuclear cells, likely macrophages, in both alveolar space and in lymphoid aggregates. Transmission electron microscopy showed bacteria in membrane-bound vacuoles of alveolar macrophages of day 4-infected lungs. *In vitro* experiments with primary alveolar macrophages as well as the murine alveolar macrophage cell line, MH-S, showed that *B. multivorans* strain C5568 had a greater association index with host macrophages than *B. cenocepacia* strain C6433. These results suggest that *B. multivorans* strain C5568 may persist in the mouse by virtue of establishing intracellular infection in host macrophages, while *B. cenocepacia* strain C6433 may be cleared by eliciting a vigorous host response.

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List of Abbreviations

BAL(F)	bronchoalveolar lavage (fluid)
BCC	*Burkholderia cepacia* complex
cAMP	cyclic adenosine monophosphate
cc	cubic centimeter = millilitre
CD11c	cell differentiation antigen 11c
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
CFU	colony-forming units
CGD	chronic granulomatous disease
CPA	cyclophosphamide
CR1	complement component 1 receptor
CR3	complement component 3 receptor
dH_2O	de-ionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPS	exopolysaccharide
EtOH	ethanol
FCS	fetal calf serum
gHBSS	Hanks Balanced Salt Solution with 1% v/v gelatin
GM-CSF	granulocyte-macrophage colony-stimulating factor
GTP	guanosine triphosphate
H. influenzae	*Haemophilus influenzae*
H & E	hematoxylin and eosin staining
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
HRP	horseradish peroxidase
IL-1β	interleukin-1 beta

IL-4	interleukin-4
IL-8	interleukin 8
IL-10	interelukin-10
I.P.	intraperitoneal
IS	insertion sequence
IV	intravenous
kb	kilo base pair
kda	kilo Dalton
Kdo	3-deoxy-D-manno-oct-2-ulosonic acid
kg	kilogram
Ko	D-glycero-D-talo-oct-2-ulosonic acid
LB	Luria-Bertani
LPS	lipopolysaccharide
M	molar
MB	mega base pair
MDM	monocyte-derived macrophages
MIP-2	macrophage inflammatory protein-2
mg	milligram
mL	milliliter
mM	millimolar
MOI	multiplicity of infection
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NFκB	nuclear factor kappa B
O_2^-	superoxide anion
P. aeruginosa	*Pseudomonas aeruginosa*
PALS	periarteriolar lymphatic sheath
PAS-H	periodic acid-Schiff Hematoxylin staining
PBS	phosphate-buffered saline
PFA	paraformaldehyde
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid

R10 medium	RPMI 1640 medium containing 10% FCS, 10 mM sodium pyruvate, 10 mM L-glutamine
S. aureus	*Staphylococcus aureus*
SCV	small colony variant
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	standard error of the mean
TEM	transmission electron microscopy
TSA	tryptic soy agar
TNF-α	tumour necrosis factor-alpha
v/v	volume/volume
w/v	weight/volume

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Rationale and Road Map

My interest in the following story was generated by a trend seen in the Canadian cystic fibrosis (CF) experience. Members of the *Burkholderia cepacia* complex are problematic opportunists in this group of immune-compromised individuals, and infection outcome appears dependent on infection with particular species. In Canada, *B. cenocepacia* strains cause the greatest number of infections and are associated with the highest levels of mortality and transmission. *B. multivorans* infections, while prevalent in North American CF clinics, are associated with lower rates of transmission and mortality. This apparent difference in the pathogenic abilities of these two species became the basis of my project.

I sought to answer the following questions:

- Can an *in vivo* model of chronic BCC infection be established that shows differences between *B. multivorans* and *B. cenocepacia* infections?
- Where are persistent BCC organisms localizing *in vivo*?
- What are the underlying mechanisms/determinants that dictate the disparate nature of *B. multivorans* and *B. cenocepacia* infection kinetics and corollary host responses?

My initial hypothesis for the project focused on the fact that BCC infections in CF individuals are chronic in nature. I hypothesized that persistent strains do so by establishing intracellular infection. The objectives were to model chronic infection in a mouse and to localize the site of persistence – implying that a host cell would be identified. A side aim was to determine whether differences between *B. multivorans* and *B. cenocepacia* infections could be discerned in the model(s) used.

Ironically, I was able to address the last aim first. Differences in the kinetics of *B. multivorans* and *B. cenocepacia* infections in mice were observed in three different models of

chronic infection. Table 1 presents a summary of the model systems used in this thesis, and particulars of infection with either *B. multivorans* or *B. cenocepacia.*

The first model examined was an intraperitoneal model of systemic challenge. A high dose of bacteria was injected in the peritoneal cavity, and tissue from lung, liver and spleen were harvested at various timepoints to monitor the bacterial load over time. While the *B. multivorans* strain persisted in the spleen for up to 8 weeks post challenge, the *B. cenocepacia* strain was cleared within one week.

This result was reproducible in a leukopenic intranasal model of infection. In this system, mice were rendered mildly leukopenic with low dose cyclophosphamide during the course of infection. Under these circumstances, they were challenged intranasally with a low bacterial dose. Viable counts of *B. multivorans* were recovered from the organ of interest, the lung, to 16 days, but *B. cenocepacia* strains were cleared from the lungs within 4 days. Furthermore, mice given the *B. multivorans* strains appeared healthy despite prolonged carriage of a low bacterial load, whereas mice given the *B. cenocepacia* strains appeared ill. This leukopenic model was the basis of a matrix experiment that evaluated the infection dynamics of 17 strains, representing 5 BCC species, in the lung.

Physiological relevance aside, the low challenge dose and use of immune-suppression in the leukopenic intranasal model complicated rather than simplified the issues of where persistent bacteria might be hiding, and whether the health of animals was a result of the drug or a reflection of bacterial virulence. A simplified intranasal model was used to circumvent these problems. Immune-competent mice were intranasally challenged with a single, high dose of bacteria. The observations of previous experiments were again reproducible in this model. Mice challenged with *B. multivorans* strains were unable to clear them from the lung

by day 16, whereas mice given *B. cenocepacia* strains showed infection resolution by day 4, and in addition, a degree of illness not seen in any *B.multivorans*-infected mice.

In summary, two very distinct patterns of infection, caused by two different species in the BCC, were observed in three different models of infection. This helped focus the hypothesis for the project: *B. multivorans* strains persist in the mouse because they are able to establish intracellular infection in a host cell, and *B. cenocepacia* strains are cleared from the mouse because they are more inflammatory. The following thesis describes work that was done to generate the original observations, and to test the two assertions of the subsequent hypothesis.

Table 1. Mouse models described in this thesis: inbred strains, route and degree of challenge, and particulars of infection with *B. multivorans* and *B. cenocepacia*.

Model/Route of challenge	Challenge dose (CFU)	*B. multivorans* infection	*B. cenocepacia* infection
C57BL/6 Intraperitoneal	10^7	Persistent in spleen for 8 weeks	Cleared from all organs in 7 days
		1 strain evaluated	1 strain evaluated
BALB/c Leukopenic Intranasal	10^4	Persistent in lung for 16 days	Cleared from all organs in 4-16 days
		Mice look healthy	Mice look ill
		6 strains evaluated	7 strains evaluated
BALB/c Immunocompetent Intranasal	10^7	Persistent in lung for 16 days	Cleared from all organs in 4 days
		Mice look healthy	Mice look ill
		2 strains evaluated	2 strains evaluated

1 Introduction

1.1 The Burkholderia cepacia complex (BCC)

Members of the *Burkholderia cepacia* complex (BCC) are Gram-negative bacilli typically measuring 0.5-1.0 μm in width and 1.5-5.0 μm in length. The BCC are obligate aerobes which do not form spores, and motility is facilitated by peritrichous pili. These organisms demonstrate weak oxidase activity and most strains are catalase positive. Optimal growth temperature for the BCC is between 30°C and 35°C, although some strains have been found to grow in temperatures as high as 47°C.

1.1.1 History and organization of the BCC

The organism which would eventually be known as *Burkholderia cepacia* was first described in 1950 as the causative agent of a bacterial onion rot that was characterized by sour skin (22). Based on its phenotype, the organism was placed in the genus *Pseudomonas* and was named *Pseudomonas cepacia*. In 1975, the ribosomal ribonucleic acid (rRNA) homologies within the genus *Pseudomonas* were re-examined and the genus was re-organized into five distinct groups based on their homologies; *P. cepacia* was placed in group II (170). *P. cepacia* and six other species in rRNA group II were assigned to the genus *Burkholderia* in 1992, after 16S rRNA homology, DNA-DNA homology, cellular lipid analysis, fatty acid composition and phenotypic profiling demonstrated that these organisms merited their own genus (247). The genus *Burkholderia*, then comprised of *B. solanacearum, B. pickettii, B. gladioli, B. mallei, B. pseudomallei, B. caryophylli* and *B. cepacia*, was also transferred from the γ-subdivision of the *Proteobacteria* phylum (where classic Pseudomonads belong), to the β-subdivision (126).

Taxonomic evaluation of multiple strains of *B. cepacia* in 1997 showed that five distinct genomes could be discerned within the group; *B. cepacia* was expanded to a complex, comprised of five genomovars (241). Genomovars represent novospecies; although phenotypically similar, they are genotypically distinct species, demonstrating low DNA cross-hybridization levels (50-70%) (239). Recognition of different species within the BCC began in 1995 with the proposal of *B. vietnamiensis* for genomovar V in the complex, a group of nitrogen-fixing strains found in rice paddies of Vietnam (80). The BCC is now comprised of nine genomovars, each of which has its own species name. Genomovar I within the complex retains the name *B. cepacia*, and includes the reference strain 25416. *B. cepacia* strains are typically environmental isolates, although some clinical isolates may be found within the group (151). Genomovar II strains were renamed *B. multivorans* in 1997; this group is comprised mainly of clinical isolates (241). *B. cenocepacia*, formerly genomovar III, was traditionally comprised of clinical isolates only, until the isolation of members of the species from various plant rhizospheres (9). Members of *B. cenocepacia*, which only recently acquired its own species name after re-examination of DNA-DNA hybridization levels with *B. cepacia*, are the dominant BCC opportunistic pathogens in cystic fibrosis (242). The rest of the BCC, typically composed of a combination of environmental (soil and biocontrol) and clinical isolates, are: *B. stabilis* (genomovar IV), *B. dolosa* (genomovar VI), *B. ambifaria* (genomovar VII), *B. anthina* (genomovar VIII) and *B. pyrrocinia* (genomovar IX) (42-43, 240, 243).

1.1.2 The BCC genome

Members of the BCC have unusually large genomes, ranging from 4 to 8 MB in size (141). This genome is peculiarly arranged as multiple, circular replicons. BCC genomes may be divided into two to four large replicons (>500 bp), and may also include smaller replicons, or plasmids (33,

245). The genome of *B. cepacia* reference strain 25416 is 8.1 MB in size and organized into three large replicons of 3.65 MB, 3.17 MB and 1.07 MB, and a 200 kb plasmid (183).

Another important aspect of the BCC genome is that it is abundant in insertion sequences (IS). *IS931*, a repeated sequence comprised of long terminal inverted repeats flanked by shorter direct repeats, was isolated during the cloning and sequencing of genes from the *tft* operons of *B. cepacia* strain AC1100, that are responsible for the degradation pathway of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), (232). 2,4,5-T is a toxic chlorinated aromatic component of herbicides that is often retained in the environment long after initial use. *B. cepacia* strain AC1100 was isolated from a chemostat after several months of growth on 2,4,5-T as the sole carbon and energy source (128). *IS931* is upstream of operons *tftAB* and *tftEFGH*, its amino acid product shows homology with the phage Mu transposase, and it has been shown to transpose from the AC1100 genome into a plasmid kPT240 (55-56, 92, 232). Two other IS elements have been found in strain AC1100; both *IS932* and *IS1490* are adjacent to the *tft* gene clusters, and IS1490 has been implicated in the activation of its adjacent *tft* operon (107). The clustered organization of IS elements on BCC replicons creates hot spots for homologous recombination and results in the deletion and rearrangement of adjacent genes. Sequence analysis of DNA surrounding the IS elements and *tft* operons suggests that the 2,4,5-T degradation pathway may have evolved in strain AC1100 through IS element-mediated events (106). These observations have led to the speculation that by facilitating the horizontal transfer of genetic information, IS elements are an important means of bringing genes into the BCC genome, thereby contributing to its complexity and its plasticity.

1.1.3 Environmental significance of the BCC

While taxonomists and molecular biologists have been sorting out the organization and genetics of the BCC, applied and environmental microbiologists have been studying the BCC as

prominent environmental organisms. The nutritional versatility and metabolic capabilities of members of the BCC have been well documented, and consequently there has been immense interest in developing this group of organisms for bioremediation and biologic control purposes.

As mentioned earlier, insertion sequence elements were discovered in the BCC during the characterization of genes involved in 2,4,5-T degradation. Diclofop-methyl, the active ingredient of another herbicide (Hoegrass284), can serve as a carbon and energy source for *B. cepacia* (208). The BCC are also capable of degrading polycyclic aromatic hydrocarbons (PAH), a group of crude oil and coal by-products found to be toxic and carcinogenic. *B. cepacia* F297 was found to degrade not only fluorene, a PAH constituent of coal derivatives, but other members of the PAH family, including naphthalene (88).

In concert with the ability to catabolize noxious compounds, the BCC are capable of producing their own anti-fungal compounds, making them attractive candidates for "plant-growth-promoting rhizobacteria" (98). *B. cepacia* protects pea plants from the damping-off phenomenon caused by the fungal pathogen *Pythium aphanidermatum,* and from root rot caused by another fungus, *Aphanomyces euteiches* (172). Other fungal diseases the BCC have been documented to control include sunflower wilt, ginseng leaf blight, and apple blue-gray mold (117, 122, 157). One well-studied antimicrobial compound made by the BCC is pyrrolnitrin, which inhibits electron transfer in the respiratory chain, and was found to be active against the fungus *Neurospora crassa* and the Gram-positive bacterium *Streptomyces antibioticus* (65).

1.1.4 Clinical significance of the BCC

Prior to the 1980's, the only clinical reports describing the BCC involved catheter-associated urinary tract infections and intravenous catheter-associated bacteremia and pseudo-bacteremia. In the last 20 years, the number of nosocomial infections with *B. cepacia* has increased (98). The broad

nutritional range that the BCC demonstrate in the environment helps them survive well in water-based environments in the hospital, such as intravenous solutions, disinfectants and even nebulizing devices for the delivery of prophylactic antibiotics (98). The latter were the contaminated source of an epidemic outbreak of *B. cepacia* respiratory tract infections in an oncology ward in Japan (248). Another outbreak, of *B. cepacia* bacteremia, occurred in an oncology ward in Alabama as a result of contaminated central venous catheters that had been flushed with heparin solution from the same bag (175). A single dominant clone of *B. cepacia* was isolated from 245 patients in the intensive care unit of a hospital following the long-term ventilation of one patient with tubing contaminated with *B. cepacia* (99).

One feature of the BCC that has substantial clinical impact is the highly antibiotic resistant nature of the complex. The BCC not only make many of their own antimicrobial products, but are intrinsically resistant to most of the ones humans have developed for therapy. Infection management has become increasingly difficult as the BCC are inherently resistant to a wide range of antibiotics (25, 186, 219). The diminishingly few pharmaceuticals still effective against the BCC worries physicians caring for the two groups of immunocompromised individuals for which the BCC have become increasingly problematic: cystic fibrosis and chronic granulomatous disease patients.

1.2 Cystic fibrosis

Cystic fibrosis (CF) is the most common autosomal recessive disorder among Caucasians, with a frequency in North America of 1 in 3000 live births. The CF gene has been identified, cloned and sequenced (125, 182, 184). CF results from each of over 1,000 mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride ion channel and regulator expressed on the apical surface of epithelial cells of the intestine, airways, secretory glands, bile ducts and epididymis (5, 12, 144, 195, 198). Absence of functional CFTR on epithelia arrests Cl^-

transport and confounds the regulation of ion transport via other channels (112, 144). This dysregulation in electrolyte transport affects all of the organs in which the CFTR is expressed, but most importantly the pancreas and the lungs. Individuals with CF suffer from malabsorption due to pancreatic insufficiency and chronic progressive lung disease; the major cause of morbidity and death in CF is pulmonary failure.

1.2.1 CF lung disease

Although all individuals with CF endure chronic lung infections and suffer progressive pulmonary disease, a wide range of prognoses exists (237). The inability to predict a CF phenotype with any given CF genotype has supported the idea that factors such as modifier genes and environment play large roles in the pathogenesis of CF lung disease (150, 237. 252). The existence of many different hypotheses describing the path from aberrant CFTR expression to failed lung defense has generated often controversial debate over the pathogenesis of CF lung disease. Some of the current hypotheses are based on the role of the CFTR in ion transport and regulation and describes altered airway surface liquid (ASL) properties conducive to bacterial colonization, while some are based on the idea that the CFTR could serve as a bacterial receptor. The high salt hypothesis predicts that normal airways maintain a hypotonic ASL via Cl^- transport-driven Na^+ reabsorption by epithelia; in CF airways, the lack of Cl^- transport-driven Na^+ reabsorption effects a higher NaCl content in the ASL of CF lungs, which is inhibitory to the natural antimicrobial activity found therein (82, 207). The low volume hypothesis predicts that in normal airways, the CFTR regulates Na^+ absorption by epithelia to maintain the volume of the periciliary liquid layer; in CF airways, the absence of CFTR-regulated Na^+ absorption results in increased Na^+ and fluid movement out of the airways, thereby dehydrating the mucus therein (17, 155). The viscosity of this mucus would impede the mucociliary clearance of inhaled particles and organisms from the lung. The low

pH hypothesis also predicts that failure of Cl^- transport results in impaired mucociliary clearance of inhaled matter; the higher salt content in CF ASL could result in acidification of the airway surface liquid and affect pH-dependent elements such as mucin and airway cell responses (40-41). Another hypothesis suggests that while wild type CFTR serves as a bacterial receptor that triggers endocytosis and subsequent elimination of bacteria via shedding, mutant CFTR is altered in this function, compromising lung defense (81, 177). None of these hypotheses needs to be mutually exclusive; indeed, some of them show overlaps in describing the sequence of events that help set the stage for an attractive environment for incoming microbes. The end result, however, is reduced pulmonary defense capabilities facilitating colonization by opportunistic pathogens.

1.2.2 Host response in the CF lung

Innate immunity in the CF lung is disadvantaged in both constitutive defense mechanisms – as alluded to above – and recruited defense mediators. The low volume hypothesis predicts impairment in physical removal of inhaled organisms via mucociliary clearance, while the high salt hypothesis predicts inactivation of bactericidal defensins within the mucus itself. The absence of an effective constitutive response to remove the majority of incoming microbes allows the accumulation and growth of microbes in the lung, which affect the production of inflammatory mediators that recruit neutrophils to the site of infection. What is surprising about this response is that it is unable to eradicate infection despite an excessively vigorous inflammation that is characterized by secretion of high levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-8, and the influx of highly active neutrophils (10). Moreover, the high levels of proteases, oxidants and defensins released by the incoming neutrophils appear to be more effective at injuring host tissues and contributing to the immunopathology that ultimately arrests lung function, than killing the microbes they were intended to remove (202).

The question of whether inflammation in the CF lung arises in response to infection, or precedes encounter with pathogens as another clinical manifestation of CF, has emerged from examination of inflammatory mediators and cells in the CF bronchoalveolar lavage fluid (BALF). *In vitro* cultures of unstimulated CF epithelial cells produce more of the pro-inflammatory cytokines IL-6 and IL-8, and less of the anti-inflammatory cytokine IL-10, than cultures of healthy control epithelial cells, and BALF from CF infants as young as 4 weeks of age contain higher concentrations of IL-8 and higher numbers of neutrophils and free elastase activity than healthy control babies (16, 127). These higher basal cytokine output levels are a result of exaggerated and prolonged NFκB nuclear activity, possibly in the absence of cytosolic IκBα (227, 244). That infection resolution is not achieved in the face of chronic pro-inflammatory cytokine production in response to high levels of NFκB activation is worrisome in view of the apparent lack of an effective negative feedback loop. Whatever the aetiology, long-term exposure to neutrophil products and cachexia, as mediated by expression of IL-1β, IL-8 and TNF-α, seems to harm, rather than help, the CF host (136).

1.2.3 Microbiology of the CF lung

The microbiology of the CF lung is restricted to a surprisingly limited group of organisms, and a progression of opportunists is usually isolated from CF sputa, bronchoalveolar lavage and lung specimens. *Staphylococcus aureus* is commonly isolated in infancy, followed closely by *Haemophilus influenzae* during early childhood (110). *Pseudomonas aeruginosa* colonization is usually detected by the teen years and goes on to infect >80% of CF adults by age 26, although *P. aeruginosa* has been isolated from the bronchoalveolar lavage fluid of CF patients as young as 3 years of age (23, 73). *P. aeruginosa* is by far the most dominant CF pathogen; chronic infection, in concert with episodes of pulmonary exacerbation generated by immunopathology of the CF lung, are the major factors contributing to morbidity and mortality (86).

S. aureus is a Gram-positive, non-motile, non-spore forming human commensal found frequently in nasal passages and in the upper respiratory tract. More importantly, this organism has very high pathogenic capacity in normal hosts and frequently causes disease in immunologically healthy children (63, 79). Three forms of *S. aureus* have been isolated from CF sputum: mucoid, non-mucoid and small colony variant (SCV). All three forms adhere to human mucins, although the mucoid phenotype has demonstrated higher binding to mucus proteins than the others (235). *S. aureus* has also been shown to bind to the asialoganglioside (aGM_1) of CF epithelial cells, this adherence being attributed to interaction with teichoic acids (4, 114). The most persistent *S. aureus* infections are associated with the SCV type. These strains are also more resistant to antibiotics including aminoglycosides, trimethoprim sulphamethoxazole and gentamicin (110). Control of *S. aureus* infection, however, has been relatively straightforward until the recent emergence of methicillin-resistant (MRSA) strains. Putative virulence factors include haemolysin and enterotoxin, although a role in pathogenesis of CF lung disease is unclear (44). One interesting finding is that the transcription of putative virulence factors is downregulated in response to high salt concentrations, which in the context of the CF lung generates questions surrounding the significance of *S. aureus* virulence determinants in CF lung disease (31).

H. influenzae is a Gram-negative commensal of the mammalian upper respiratory tract. Strains isolated from CF patients are non-encapsulated and therefore have been classed as non-typeable strains, as the typing system for *H. influenzae* is based on antigenic specificity to capsule proteins (110). Infection management with fluoroquinolones has occasionally been ineffective due to mutations in *gyrA* and *parC* (27). *H. influenzae* LPS and toxins in culture supernatant filtrates exert damage to mammalian epithelial cells (236). Although the exact role of *H. influenzae* in CF lung disease is still unclear, the above factors could certainly contribute to CF lung injury.

P. aeruginosa is by far the predominant CF pathogen, and carries with it the worst prognosis upon colonization (110). The problems generated by chronic colonization and hyperimmune responses are compounded by the organism's high level resistance to antibiotic treatment (86). The hallmark of *P. aeruginosa* infection of the CF lung is a unique relationship in which the incoming motile, non-mucoid bacteria expressing the smooth form of lipopolysaccharide (LPS) effect a phenotypic switch to a sessile, mucoid form expressing rough LPS upon colonization (91). The initial report of the frequent isolation of an atypical *P. aeruginosa* from the lungs of CF patients was made by Doggett in the 1960's, and described strains producing a substance resembling algal alginate which lost their mucoidy on subculture (61). This mucoidy is a result of bacterial production of mucoid exopolysaccharide, also known as alginate, an acetylated co-polymer of mannuronic and guluronic acids (201). Alginate is a component of the matrix in which sessile *P. aeruginosa* grow as microcolonies in the CF lung, also known as the biofilm mode of growth (51, 138). *P. aeruginosa*'s conversion to a mucoid, sessile morphotype in the lung has been described as the key event leading to poor clinical condition and a hyperimmune response (171, 174). Bacteria growing in biofilms are much less susceptible to antibiotics than their planktonic counterparts, and this has in part been responsible for the inability to eradicate chronic *P. aeruginosa* infections in CF (97). Other roles for alginate in the progression of CF lung disease include the inhibition of both non-opsonic and opsonic phagocytosis by human neutrophils and macrophages, and the scavenging of the reactive oxygen intermediate hypochlorite (26, 135, 140, 203). The persistent infections that result from this multi-tiered evasion of the immune response and resistance to antibiotic treatment make management of *P. aeruginosa* infections a formidable task.

The spectrum of CF pathogens is rounded out by *Aspergillus* spp., *Ralstonia pickettii*, *Alcaligenes xylosoxidans* and *Stenotrophomonas maltophilia.* Isolated with increasing frequency,

and of great concern due to their resistance to antimicrobial treatment, are *Burkholderia cepacia* complex organisms.

1.2.4 BCC infection of the CF lung

The emergence of the BCC as problematic CF opportunists was documented by two seminal papers in the 1980's (116, 231). These papers described an increase in incidence in the number of patients from whom the BCC (at the time named *P. cepacia*) was being isolated, and a higher mortality rate associated with *P. cepacia* colonization. An aggressive pneumonia accompanied with high fever, and occasional septicemia, the "*B. cepacia* syndrome," was also described. The view that colonization by the BCC was a poor prognostic indicator for CF patients was reinforced by later reports of longer periods of hospitalization and increased mortality, irrespective of lung function (49, 228). Generally, infection by the BCC is followed by one of three clinical outcomes: chronic, asymptomatic carriage, progressive deterioration over many months, or the fatally rapid deterioration of the "*B. cepacia* syndrome," the first of a trio of clinical CF concerns in the 1990's (87). The second concern was the BCC's intrinsic multiple antibiotic resistance. This is still an issue of great concern as the list of antibiotics to which the BCC are still susceptible is markedly short: carbapenems, some cephalosporins, quinolones and trimethoprim-sulfamethoxazole (215). The third concern, which evolved with the growing competence of strain typing methods, was the high rate of person-to-person spread (85, 145). The combination of these three concerns mandated segregation of CF patients infected with the BCC from those who were culture-negative, a policy with potentially devastating social impact (121).

With the evolution of the taxonomy of the BCC to include genomovars, species-specific trends could be discerned in the areas of patient-to-patient transmission, strain acquisition and post-transplantation survival. Strains belonging to genomovar III, or *B. cenocepacia*, not only show the

highest potential for transmissibility between patients, but also the ability to replace pre-existing *B. multivorans* infections in the CF host (152, 215). Preoperative infection with *B. cenocepacia*, but no other BCC species, of CF patients receiving lung transplants is associated with poor survival post-transplantation in the immediate and mid-term time categories (7). In North America, *B. cenocepacia* is the dominant BCC CF pathogen, infecting the highest proportion of CF patients in the United States and Canada (146, 215). The underlying pathogenetic mechanisms that differentiate *B. cenocepacia* from other BCC species are still unclear.

1.3 Chronic granulomatous disease (CGD)

Chronic granulomatous disease (CGD) is a rare immunodeficiency syndrome, affecting 1 in 250,000 live births (158). Individuals with CGD suffer from severe fungal and bacterial infections of the respiratory tract, skin and soft tissues (75). The biochemical basis of the disease lies in the inability of host phagocytic cells – neutrophils, eosinophils, monocytes and macrophages – to generate reactive oxygen intermediates in response to infection (53). NADPH oxidase, the enzyme responsible for the production of superoxide anion (O_2^-), the first reactive oxygen species, is a multi-component system that is activated after phagocytosis. Five main proteins and two accessory proteins comprise the NADPH oxidase: the two-component cytochrome b_{558} and a GTP-binding protein are located in the membranes of specific granules and secretory vesicles, while a trio of oxidase proteins and another GTP-binding protein are located in the cytoplasm. Upon ingestion of particles, the cytosolic components migrate to the plasma membrane to cooperate with cytochrome b_{558} and form the redox center that transfers an electron from NADPH to molecular oxygen, producing O_2^-, H_2O_2 and HOCl (158). A variety of mutations in any of four of the five NADPH oxidase components results in a profound reduction in oxidative activity that ranges from 70-100% (185).

Although CGD phagocytes are capable of chemotaxis, phagocytosis and degranulation, they are unable to kill microorganisms oxidatively and consequently provide a niche for a specific subset of organisms. Catalase-positive organisms, with the means to detoxify their own oxygen by-products, and organisms resistant to non-oxidative killing mechanisms are commonly isolated from CGD respiratory tract infections and wounds (158). A recent registry of CGD patients reports a shift from the traditional set of CGD pathogens, *Staphylococcus* species and enteric bacteria, to *Aspergillus* species and *Burkholderia cepacia* (246). BCC sepsis and pneumonia was the second most lethal infection in patients of that registry. The same registry reports that the BCC are the most virulent Gram-negative bacterial pathogens in CGD. BCC organisms are particularly successful CGD pathogens because they are both catalase-positive and extremely resistant to non-oxidative killing (216). BCC infections in CGD are aggressive; pneumonia is the most common phenomenon and sepsis may occur (215). The ability of the BCC to invade the bloodstream from the initial site of infection, an extremely rare occurrence with other CGD organisms, has generated speculation about the invasive and/or pathogenic capabilities of the BCC (215).

1.4 BCC virulence factors

The success of members of the BCC as CF and CGD pathogens has fuelled the search for virulence determinants in the last decade and a half. The first reports of possible BCC virulence factors described the production of extracellular products with impressive abilities: a lipase that inhibited the phagocytic ability of rat macrophages, a protease that produced a vigorous pulmonary inflammatory response in rats, and cepalysin, a hemolytic substance that also demonstrated antifungal properties (1, 147, 156, 223). An extracellular toxic complex comprised of carbohydrate, polysaccharide and protein, expressed from early log phase through stationary phase, and toxic to mice when administered intraperitoneally has also been described (224). Some BCC products cause

cell death: *B. cenocepacia* produces a hemolysin that causes human neutrophils to degranulate and undergo apoptosis, while a clinical BCC isolate has been found to secrete ATP-converting enzymes that produce agonists for the purinergic receptors on eukaryotic cells that trigger apoptosis (111, 159). Some other BCC products appear to protect them from the punishing host immune response: a melanin produced by a *B. cenocepacia* strain is able to scavenge superoxide (253). While all of these products offer tantalizing chapters in the BCC-CF story, their importance in the virulence of BCC infection *per se* has yet to be demonstrated.

One group of potential BCC virulence factors shown to be important in promoting the growth of BCC organisms are the iron-chelating enzymes, siderophores. The BCC produce four different siderophores: pyochelin, cepabactin, salicylic acid (formerly azurechelin) and ornibactin (163, 210, 211, 220). While all four show high binding affinity for iron, possible roles in virulence have also been explored. BCC isolates from patients with the most aggressive infections are pyochelin-positive; pyochelin-negative strains are associated with mild infections (210). In a rat model of pulmonary BCC infection, the addition of pyochelin to the lungs of rats infected with pyochelin-negative strains increased the virulence of the infection (214). Transposon mutagenesis of genes involved in ornibactin biosynthesis and the ornibactin receptor affects the colonization and persistence of *B. cenocepacia* in both acute and chronic models of pulmonary infection (212-213).

Adherence as an important step in establishing a chronic pulmonary infection was addressed in the finding that BCC pili mediate binding to both respiratory mucins and epithelial cells. Approximately 60% of BCC strains express peritrichous pili. One particular unique arrangement of pili, assembled from one major 17 kDa subunit, in a cable-like morphology was observed in all isolates associated with an epidemic in a large North American center (137, 191). A 22 kDa protein expressed along the length of these cable pilus fibers has been identified as the adhesin that binds to

N-acetylglucosamine and *N*-acetylgalactosamine in human mucin and to cytokeratin 13 on buccal epithelial cells (189-190, 192). A role for the cable pilus in lung disease has also been investigated. Pulmonary challenge to $CFTR^{-/-}$ mice with a hyper-piliated strain of epidemic origin produced a florid and severe bronchopneumonia similar to that seen in clinical CF, and association of the cable pilus with cytokeratin 13 on squamous epithelium resulted in host cell cytotoxicity and IL-8 release (188, 193). The low frequency of the gene encoding the cable pilus, *cblA*, among epidemic strains in North America and Europe, in concert with the fact that all reported *cblA*-positive strains to date belong to the one epidemic lineage, however, has diminished the popularity of the cable pilus as an epidemic strain marker (14, 32, 146).

BCC exopolysaccharide (EPS) production has been investigated in light of the clinical ramifications of infections caused by alginate-producing *P. aeruginosa*. The BCC produce four different EPS, one major species being expressed by mucoid and non-mucoid clinical BCC isolates (28, 30). This representative species is an acidic, highly branched heptasaccharide containing one rhamnosyl, three galactosyl, one mannosyl, one glucosyl and one glucuronosyl residues (29). A possible role for EPS in BCC virulence was elaborated when the spontaneous conversion of a non-persistent *B. cenocepacia* strain to persistence in a leukopenic mouse model was accompanied by an increase in the EPS production that changed colonial morphology (37). The clinical implications suggested by this study and the finding that BCC EPS production is optimal during growth on physiological salt and at physiological temperature beg further investigation in the timing and regulation of EPS production by BCC, as well as its role in pathogenesis (3).

Quorum sensing, the cell-to-cell population communications system regulating gene transcription, is a new and exciting field of study in CF microbial pathogenesis. Quorum sensing molecules have been found in the sputa of CF patients infected with *P. aeruginosa* and have been

implicated in biofilm formation in the CF lung (164, 204). The unresponsiveness of *P. aeruginosa* growing in microcolonies to antibiotic treatment and host adaptive immunity and the production of exopolysaccharides by the BCC have generated immense interest in the quorum sensing and biofilm forming capabilities of the BCC. Two BCC quorum sensing systems have been identified; genes for the *cep* system have been amplified from the genomes of *B. cepacia*, *B. cenocepacia* and *B. stabilis* and *B. vietnamiensis*, while genes for the *bvi* system are found only in *B. vietnamiensis* isolates (47, 142, 149). BCC strains using the *cep* system produce two signaling molecules, *N*-octanoyl-L-homoserine lactone, and *N*-hexanoyl-L-homoserine lactone. The *cep* system regulates BCC ornibactin, protease and lipase synthesis, biofilm maturation, swarming motility and killing in a nematode model of pathogenesis (104-105, 131). The BCC appear to be responsive to quorum sensing molecules made by the *P. aeruginosa*, indicating a unidirectional signaling between these two strains (143, 181). Biofilm formation has also been reported as a precursor for host cell invasion by the BCC (199).

The discovery of genes encoding a type three secretion system in an epidemic strain of *B. cenocepacia* offers a mechanism for the export of virulence factors (173). Many Gram-negative bacterial pathogens use this conserved secretory system to deliver effector proteins into plant and mammalian host cells (108). Some virulence determinants dependent on type III secretion for delivery include: invasion proteins of *Salmonella enterica* serovar Typhimurium and *Shigella flexneri*, attachment and effacement proteins of enteropathogenic *Escherichia coli*, cytotoxicity factors of *P. aeruginosa*, and proteins involved in host immune manipulation by *Bordetella bronchiseptica* (45, 72, 118, 162, 249). A type III secretion mutant of an epidemic *B. cenocepacia* isolate is less virulent in an agar bead model of pulmonary infection in mice in that it is cleared more

rapidly from infected organs and produces a less vigorous inflammatory response, suggesting that a functional type III system may play an important role in the delivery of BCC effectors (233).

1.5 The pro-inflammatory nature of the BCC and BCC products

The antigenic capacity of BCC products, including that of LPS, became an area of intense examination following one CF multicenter study from the United Kingdom that reported higher serum levels of the inflammatory markers C-reactive protein and neutrophil elastase α1-antiproteinase complex during BCC-associated exacerbations than those caused by *P. aeruginosa* (66). BCC LPS is more endotoxic than LPS from *P. aeruginosa*, and is more potent than *P. aeruginosa* LPS in its capacity to induce epithelial cells to express IL-8, to prime human neutrophil respiratory burst activity and to induce human monocytes to express TNF-α (109,169, 180, 200, 254). Infection of CF mice by repeated aerosol exposure to *B. cenocepacia* produces a vigorous inflammatory response and lung disease (57, 193). No correlation, however, was found between LPS chemotype (rough or smooth) and virulence in a polyacrylamide gel electrophoresis analysis of LPS from three BCC species representative of epidemic and non-epidemic strains (69). To date, no studies have been undertaken to compare the biologically active portion of LPS, lipid A-3-deoxy-D-manno-2-oct-ulosonic acid (Kdo), from different species and from epidemic and non-epidemic strains from within the BCC. In light of the constitutively inflamed environment in the CF lung and the highly pro-inflammatory potential of BCC, the elucidation of the structure, composition and variations therein of the active core of BCC endotoxin is paramount and still in progress.

1.6 Animal models of BCC infection

The creation of animal systems to model and dissect the mechanisms behind the pathogenesis of CF is an interesting, many-pronged history. No fewer than 13 genetic mouse models of CF have

been developed since the identification and sequencing of the human CF gene (58). Despite the large selection of CF mice, a wide spectrum of phenotypes and survival levels, in concert with the cost of developing and maintaining a colony large enough for experimentation, has engendered the contemporary use of non-CF models to study BCC infection (58).

1.6.1 CF mouse models of BCC infection

The first mouse model of CF, generated via gene replacement of exon 10 of the mouse *Cftr* gene, was accomplished three years after the identification of the human CF gene (209). These mice initially failed to survive past 40 days of age due to intestinal obstruction, but the development of special liquid diets allowed maturation of these animals for experimentation (64). CF mice of two different genotypes have been infected with members of the BCC: the $Cftr^{tm1Hgu}/Cftr^{tm1Hgu}$ mouse is a result of targeted insertional mutagenesis in exon 10 of murine *Cftr*, and the $Cftr^{tm1Unc}/Cftr^{tm1Unc}$ mouse is "the original CF mouse," a product of exon 10 replacement in *Cftr* (62, 209).

The $Cftr^{tm1Hgu}/Cftr^{tm1Hgu}$ mice were challenged with an epidemic isolate of *B. cenocepacia* via repeated aerosol exposure (57). These mice showed reduced clearance of *B. cenocepacia* from their lungs compared with their non-CF littermates, and aspects of greater pulmonary pathology in the form of a greater incidence of pneumonia and edema. The $Cftr^{tm1Unc}/Cftr^{tm1Unc}$ mice were challenged by a *B. cenocepacia* isolate from the same epidemic lineage as the one used in the 1995 study, via repeated instillation of bacteria in the nares (193). *B. cenocepacia* developed persistent infections in these mice, and caused severe bronchopneumonia. Interestingly, there was little difference in the pro-inflammatory cytokines recovered in BAL fluid and the level of neutrophil activation between infected $Cftr^{tm1Unc}/Cftr^{tm1Unc}$ mice and their infected wild-type littermates despite a significant difference in the size of the inflammatory infiltrate. While both these models are significant achievements in the search for an *in vivo* system of studying BCC infection in the context

of the CF lung, the lack of chronic lung inflammatory pathology in both naïve and BCC-infected mice, a hallmark of the CF lung, begs further examination and development.

1.6.2 Non-CF models of BCC infection

The biggest problem encountered by investigators using both CF and non-CF mouse models to study BCC infection is establishing a chronic bacterial infection. One way to circumvent this obstacle is to challenge animals repeatedly. This has worked well with CF mice, which show reduced ability to clear the infection after repeated exposure to high doses of aerosolized organisms (57, 193). Non-CF mouse models have traditionally used bacterial entrapment or host immune suppression to artificially develop chronic pulmonary infection (37, 39, 212, 214, 225, 233). The route of challenge also varies; investigators using bacterial encasement in agar beads prior to infection prefer the intratracheal route of infection to ensure accurate deposition of the challenge titer, while those using immune-suppressed mice challenge by aerosol exposure or intranasal instillation. Both systems have their drawbacks: while encasement of bacteria in agar beads prevents host-pathogen interaction, and the route of introduction is highly invasive, pharmacological manipulation of the host immune system undermines evaluation of host immune response to infection. Despite these caveats non-CF models have been used to examine potential BCC virulence factors and invasion.

Inclusion of pyochelin in the agar beads encasing pyochelin-negative BCC mutants, while failing to increase bacterial persistence, effected a greater foci of inflammation and more lung pathology in a rat agar bead model (214). BCC mutants in ornibactin biosynthesis are cleared faster than wild-type BCC organisms from a rat agar bead model, and a type III secretion mutant of a clinical *B. cenocepacia* strain is less invasive than the wild-type in a mouse agar bead model (212, 233). These studies report that the respective BCC mutants elicited less of a pulmonary

inflammatory infiltrate post-infection than their wild-type counterparts, suggesting diffusibility of bacterial products despite encasement in agar. Another group found no correlation between the ability of BCC to invade human epithelial cell line A549 and to invade and cause systemic infection in a mouse agar bead model (39).

Administration of low doses of the immunosuppressive drug cyclophosphamide renders mice mildly leukopenic for short windows of time, mainly affecting the B lymphocytes (94, 221). No explanation of how or why this elimination of cells of the adaptive immune system helps *P. aeruginosa* or BCC organisms establish infection yet exists. BCC pyochelin mutants have been assessed in a leukopenic mouse model (212). A spontaneous colonial variant exhibiting increased EPS production and heavy piliation was recovered from lungs of leukopenic mice after challenge with a *B. cenocepacia* parent strain that was cleared readily from lungs and demonstrated scant EPS production (37). While the leukopenic mouse is a useful system for evaluating some aspects of microbial pathogenesis, the significance of certain findings, especially those involving the host response, need to be further evaluated in models that are less reliant on immunosuppression, and more directly representative of CF.

1.7 The BCC as intracellular organisms

The invasive capacity of the BCC has been examined by many different groups in the interest of elucidating the mechanisms behind the uniquely invasive nature of BCC infections in CF, which is not demonstrated by any other persistent CF pathogen. Several groups using short term modified antibiotic protection in *in vitro* assays have shown viable clinical BCC isolates inside the human epithelial cell line A549, human type II pneumocytes and the human macrophage cell line U937 (24, 123-124, 154). All reports with micrographic evidence describe the containment of intracellular bacteria in membrane-bound vacuoles. Differential invasion of A549 cells among BCC species has

been demonstrated; interestingly, clinical *B. multivorans* and *B. cenocepacia* strains are more invasive than environmental isolates from their own species and strains from other species (124). To date, only one report documents the *in vivo* localization of a clinical *B. cenocepacia* isolate inside host murine pulmonary epithelial cells and alveolar macrophages (34).

Intracellular BCC can also survive for up to 72 hours in mammalian and trophozoitic hosts (139, 153-154, 187). Clinical *B. vietnamiensis* and *B. dolosa* isolates localize in membrane-bound vacuoles of murine macrophages 24 hours post-infection, despite host cell release of TNF-α and reactive oxygen intermediates (187). Viable counts from human and murine macrophages indicate that while the BCC are able to evade host killing mechanisms, intracellular replication is limited (154, 187). Interestingly, species-specific host tropism was demonstrated in the ability of BCC organisms to parasitize three different species of the free-living amoebae *Acanthamoeba*; *B. cepacia* and *B. vietnamiensis*, two BCC species found routinely in the environment, consistently infected and survived in host trophozoites, while clinical *B. multivorans* and *B. cenocepacia* strains either could not parasitize amoebae, or did so inconsistently across amoebal species (153). Species-specific persistence was also suggested in the survival of a clinical *B. cenocepacia* strain in human macrophage and epithelial cell lines concomitant to the rapid killing of an environmental *B. cepacia* isolate in the same systems (154). While studies of invasion and intracellular survival focusing on the pathogenic ability of clinical epidemic isolates substantiate many of the theories behind BCC virulence, questions of species-specific virulence, virulence determinants and host tropism need to be addressed in view of the fact that more than one BCC species infects the CF lung, with varying infection outcomes.

1.8 Thesis objectives

The BCC are an important family of opportunistic pathogens in both CF and CGD. In North America, *B. cenocepacia* organisms are the dominant BCC infectious agents in CF, are highly transmissible and associated with poor and possibly devastating clinical outcome. *B. multivorans* infections in CF, while prevalent, are associated with lower rates of transmission, morbidity and mortality. The unresponsiveness of the BCC to clinical antibiotic treatment, in concert with the potentially invasive nature of BCC infections, has generated speculation that the BCC are intracellular pathogens. Research to evaluate intracellular parasitism and possible virulence determinants of the BCC has focused predominantly on clinical epidemic *B.cenocepacia* isolates, cross-species comparisons of pathogenic capacity is limited in the existing body of information.

The work described in this thesis was undertaken with the following questions in mind:

Can an *in vivo* model of chronic BCC infection be established that shows differences between *B. multivorans* and *B. cenocepacia* infections?

Where are persistent BCC organisms localizing *in vivo*?

What are the underlying mechanisms/determinants that dictate the disparate nature of *B. multivorans* and *B. cenocepacia* infection kinetics and corollary host responses?

I hypothesize that the BCC, specifically *B. multivorans*, are persistent in the murine lung by virtue of their ability to establish intracellular infection in host macrophages, and that *B. cenocepacia* infections in CF may be more problematic by virtue of a greater pro-inflammatory capacity as yet not fully characterized.

2 Materials and Methods

2.1 Bacterial strains

The bacterial strains used in this study and their characteristics are listed in Table 2. They were stored at –70°C in Mueller-Hinton broth with 8.0% dimethylsulfoxide (DMSO). All strains were subcultured on blood agar plates (PML Microbiologicals, Richmond, Canada) and cultured in Luria-Bertani (LB) broth (Miller formulation). Viable counts were enumerated on tryptic soy agar (TSA) plates (Becton Dickinson, Cockeysville, Md.).

2.2 Animals

All studies were approved by the University of British Columbia Animal Care Committee (UBC-ACC #A98-0130 and UBC-ACC #A00-0138). The C57BL/6 and BALB/c strains of mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). All mice were housed and cared for in accordance with regulations of the UBC-ACC and Canadian Council on Animal Care. Females weighing 15-20 grams and aged 6-8 weeks were used in each experiment. General health of animals studied in both intranasal models of infection was assessed daily on a three point system based upon the following: weight, food and water consumption and general appearance. Animals were determined unwell when two of the three following observations were made: >10% weight loss, <3 gram water or food consumption daily, or general ill appearance (ruffled coats, huddled position, lack of retreat in handler's presence). Mice were randomly assigned into various experimental groups and sacrificed for data analysis in these groups exclusively of their individual health status. No mice were ever observed to be morbid in any experiments conducted in these studies.

Table 2. BCC strains evaluated in this thesis.

Strain name	Source[a]	Model of Study[b]
B. multivorans		
C1576	CF-e, UK	IN-CPA
FC147	CGD, USA	IP
C5568	CF, Canada	IN-CPA, IN
B. cenocepacia		
C1394	CF-e, UK	IN-CPA
C6433	CF-e, Canada	IN-CPA, IN
B. vietnamiensis		
FC811	CF, N. America	IN-CPA

[a] CF, cystic fibrosis. e, epidemic strain. CGD, chronic granulomatous disease. UK, United Kingdom. USA, United States of America.
[b] IN-CPA, leukopenic model of intranasal infection. IP, intraperitoneal model of systemic infection. IN, intranasal model of infection.

2.3 *Infections*

2.3.1 Intraperitoneal Infection

Bacteria were cultured from frozen stocks for 24 hours on blood agar plates at 37°C. Isolated colonies were picked and grown in LB broth for 16 hours; cells were harvested by centrifugation (10 minutes at 30,000x*g*) and resuspended in Hanks' Balanced Salt Solution containing 1% v/v gelatin (gHBSS, Difco, Detroit). Cultures were adjusted to ~8 X 10^8 CFU/mL on a Beckman Du spectrophotometer, at a wavelength of 620 nm (~OD_{620} = 0.600). Bacteria were then diluted in gHBSS to concentrations ranging from 8 X 10^6 CFU/mL to 1 X 10^8 CFU/mL. C67BL/6 mice were challenged intraperitoneally (I.P.) with final doses between 4 X 10^6 CFU and 5 X 10^7 CFU/mL in a 500 µL volume. At pre-selected time points after infection, groups of three mice were killed by cervical dislocation. Spleen, liver and lung were excised and weighed, homogenized, serially diluted 1:10 in gHBSS and plated onto TSA plates. Viable bacterial counts were determined after 24-48 hours of incubation at 37°C.

2.3.2 Immunosuppression of Animals

BALB/c mice were anaesthetized with gaseous methoxyflurane (Janssen, Toronto, Canada). Cyclophosphamide (150 mg/kg; CPA, Bristol, Montreal, Canada) was then administered I.P. CPA injections were given on days –1, 4, 9 and 14 of each experiment. At pre-selected time points during the experiment, groups of mice were anaesthetized I.P. with 65 mg/kg sodium pentobarbital (MTC Pharmaceuticals, Cambridge, Canada) and exsanguinated via cardiac puncture. Total peripheral leukocyte counts were determined on a Sysmex Toa 9500 hematology system; differential counts were determined microscopically from Giemsa-stained blood smears. Spleens were also excised and weighed.

2.3.3 Intranasal Infection

Bacteria were cultured from frozen stocks for 24 hours on blood agar plates at 37°C. Isolated colonies were picked and grown in LB broth for 16 hours; cells were harvested by centrifugation (10 minutes at 30,000xg) and resuspended in gHBSS, or phosphate-buffered saline (PBS). Cultures were adjusted to ~8 X 10^8 CFU/mL on a Beckman Du spectrophotometer, at a wavelength of 620 nm. In the immunosuppressed model of intranasal infection, bacteria were diluted in gHBSS to 4 X 10^5 CFU/mL. BALB/c mice were challenged intranasally with a final dose of 1.6 X 10^4 CFU in a 40 μL volume as follows: mice were anaesthetized with 60 mg/kg ketamine hydrochloride (MTC Pharmaceuticals) administered intraperitoneally, and the infectious dose was given by placing drops on alternate nares while animals were in a supine position. In the higher dose model of intranasal infection, bacteria were diluted in PBS to 2.5 X 10^8 CFU/mL, and BALB/c mice were challenged intranasally, as described above, with a final dose of 1 X 10^7 CFU/mL in a 40 μL volume. At pre-selected time points after infection, groups of three mice were killed by either cervical dislocation or an overdose of sodium pentobarbital. Lungs were excised and weighed, homogenized, serially diluted 1:10 in gHBSS or PBS and plated onto TSA plates. Viable bacterial counts were determined after 24-48 hours of incubation at 37°C.

2.4 Tissue histology

2.4.1 Processing of tissues

Intraperitoneal model: mice were killed by cervical dislocation prior to excision of spleens. Spleens were fixed overnight in 4% paraformaldehyde (PFA, Sigma Canada); the next day samples were enclosed in histology cassettes and dehydrated by serial 15 minute immersions in 70%, 95%, 95%, 100%, 100% ethanol, and 2 immersions in xylene, prior to being embedded in paraffin

(Paraplast X-tra tissue embedding medium). Spleen sections were cut to 4 μm, fixed onto glass slides (Superfrost^R slides, VWR) and allowed to air dry. Samples were stained with hematoxylin and eosin (H & E), and slides from each group were examined for histopathology by blinded observation. Intranasal models: mice were killed by overdose with sodium pentobarbital prior to excision of lungs and trachea. Excised lungs were inflated with either 2% PFA or 1:1 acetone methanol (all Sigma Canada Ltd.) via cannulation of the trachea, and immersed in more fixative. Following overnight (PFA) or one hour (acetone methanol) fixation, lungs were also dehydrated, embedded in paraffin and sectioned (5 μm). Lung tissue was stained with H & E and periodic acid-Schiff hematoxylin stains (PAS-H). Slides from each group were examined for histopathology by an observer blinded to their identity.

2.4.2 Hematoxylin and eosin stain

Paraffin sections on slides were rehydrated through serial 10 minute immersions in xylene, 100%, 95%, 95%, 70% ethanol and dH_2O. Slides were then subjected to the following series prior to mounting under Permount:

Hematoxylin	5 minutes
Running water	2 minutes
Acid alcohol (1% HCl in 70% EtOH)	10 seconds
Running water	2 minutes
Saturated lithium carbonate (0.5% w/v)	10 seconds
Running water	2 minutes
Eosin	2 minutes
95% ethanol	10 seconds
100% ethanol	15 seconds

100% ethanol	1 minute
Xylene	1 minute

2.4.3 Periodic acid-Schiff hematoxylin stain

Paraffin sections on slides were rehydrated through serial 10 minute immersions in xylene, 100%, 95%, 95%, 70% ethanol and dH_2O. Slides were then subjected to the following series prior to mounting under Permount:

Periodic acid (1% w/v)	5 minutes
Running water	2 minutes
Schiff's reagent (Sigma)	10 minutes
Running water	2 minutes
Hematoxylin	5 minutes
Running water	2 minutes
Acid alcohol	10 seconds
Running water	2 minutes
Saturated lithium carbonate	10 seconds
Running water	2 minutes

2.5 Bronchoalveolar lavage

2.5.1 Bronchoalveolar lavage

Mice were killed by overdose with I.P.-administered sodium pentobarbital prior to BAL. BAL was performed *in situ* by exposing the trachea, inserting a 22-gauge I.V. catheter (Johnson and Johnson, Gargrave, U.K.) into the proximal end and securing it with a 3-0 silk suture. The lungs were then alternately insufflated and deflated with cold sterile PBS. The use of cold PBS, instead of

pre-warmed PBS containing lidocaine, was previously determined not to affect host cell viability and morphology, or bacterial viability. A total volume of ~1.5 mL was collected by 1 cc syringe. Cells were removed from the BAL fluid by centrifugation (5 minutes at 250x*g*) and resuspended in 200 μL PBS with 0.1% v/v gelatin and 10% v/v fetal calf serum (Cansera, Canada). BAL fluid supernatants were aliquoted and frozen at –70°C until ELISA analysis was performed.

2.5.2 Lavage cellular analysis

20 uL aliquots of cellular suspension were diluted in 0.4% Trypan blue exclusion dye and subjected to hemocytometer counts for enumeration of cells recovered by lavage. The concentration of the resuspended lavage cells was calculated as follows:

(no. cells in central 25 squares of hemocytometer) X (dilution factor) X (10^4 cells/mL)

Absolute numbers of cells recovered by lavage were determined by multiplying the concentration by 0.2 mL. Additional 100 μL aliquots of the lavage cell suspensions were deposited onto glass slides by cytocentrifuge (Shandon, Sewickley, PA), and stained with Hemacolor (EM Diagnostic Systems, Gibbstown, NJ) for differential counts.

2.5.3 Bronchoalveolar lavage fluid supernatant cytokine analysis via enzyme-linked immunosorbent assay (ELISA)

BAL supernatants were frozen in aliquots until analysis by ELISA. Assays for mouse TNF-α, IL-1β and MIP-2 (R&D Systems, Minneapolis, MN) were performed according to manufacturer's directions. All steps were performed at room temperature. 96-well plates were coated overnight with 100 μL of capture antibody diluted in PBS to working concentrations of 0.8 – 4 μg/mL. The

next day, plates were washed vigorously 3 times with wash buffer (0.05% Tween 20 in PBS, pH 7.2) prior to blocking for one hour in 1% bovine serum albumin (BSA), 5% sucrose in PBS. Plates were washed 3 times and standards and diluted samples were added to wells and incubated for 2 hours. After another wash series, 100 µL of detection antibody diluted in 1% BSA in PBS (0.2 µm filtered) to working concentrations of 200-400 ng/mL was added to wells and incubated for 2 hours. Plates were washed and 100 µL of streptavidin-horseradish peroxidase was added to wells and plates were further incubated for 20 minutes in the dark. Plates were washed, 100 µL of substrate solution (1:1 H_2O_2 and tetramethylbenzidine) was added to wells and plates were incubated for 20 minutes in the dark. Substrate reactions were stopped with the addition of 50 µL stop solution (2 N H_2SO_4) to wells. Plates were read at a wavelength of 450 nm on a Biorad Model 3550 microplate reader within 30 minutes of being developed.

2.5.4 Lavage viable count analysis

In some experiments using the intranasal model, the viable counts recovered by bronchoalveolar lavage was compared with the viable counts recoverable from homogenized lungs. Mice were killed by I.P.-administered sodium pentobarbital overdose and bronchoalveolar lavage was performed. BAL fluid was first diluted 1:10 in 0.5% Triton X-100 in PBS, allowed to sit for 10 minutes, and then mixed vigorously by vortex to ensure permeabilization of all lavage fluid cells and even distribution of bacteria within fluid. This dilution was then serially diluted 1:10 in PBS and plated onto TSA plates. Lavaged lungs were excised, homogenized, serially diluted 1:10 in PBS, and plated onto TSA plates. Viable counts were enumerated after incubation at 37°C for 24-48 hours.

2.6 Antibodies

The antibodies used in this study were: rabbit polyclonal serum against *B. multivorans* strain FC147, rat anti-mouse Mac-3 antigen, hamster anti-mouse CD11c (BD Pharmingen, Burlington, ON, Canada). Secondary antibodies used in this study were: Alexa 488-conjugated goat anti-rabbit, Alexa 594-conjugated goat anti-rat (Molecular Probes, Eugene, OR). Primary antibodies were diluted 1/50 to 1/1000 in blocking buffer (PBS, 0.5% v/v Tween-20, 5% v/v normal goat serum); secondary antibodies were diluted 1/500 in blocking buffer.

2.7 Immunohistology

Permeabilization and blocking of histology sections was achieved with a 60-minute incubation of sections in PBS containing 1% normal goat serum and 0.5% Tween-20 (Sigma, Oakville, ON, Canada). Primary antibodies were incubated with samples for one hour at room temperature. Sections were washed 3 times in PBS, 0.05% v/v Tween-20 prior to addition of secondary antibody. Sections were incubated with the secondary antibody for 1 hour at room temperature, in the dark, prior to another three washes in blocking buffer. Where indicated, sections were counterstained with Mayer's hematoxylin (EM Diagnostic Systems) for 45 seconds, rinsed with distilled water and air-dried in the dark. Samples were mounted with Mowiol (Calbiochem, La Jolla, CA) and viewed with a Zeiss Axioplan 2 imaging system with Z HBO 103 fluorescence unit, by an observer blinded to their identity. Northern Eclipse 5.0 imaging software was used to view sections at 40X and 100X power, with cube filters #1 and #2.

2.8 Transmission electron microscopy (TEM)

On day 4 of pulmonary infection, mice were killed by overdose with sodium pentobarbital prior to excision of lungs and trachea. Excised lungs were inflated with 1%

(w/v) glutaraldehyde (Sigma) in 0.1 M sodium cacodylate buffer (pH 7.3) via cannulation of the trachea. Lungs were immersed into more fixative and diced into 1 mm^3 cubes with a sterile scalpel. Cubed lung samples were fixed for 1.5 hours in glutaraldehyde while on ice, washed three times in cacodylate buffer, and post-fixed with 1% osmium tetroxide (OsO_4, Canemco, Quebec, Canada) in 0.1 cacodylate buffer for 1.5 hours. Following three 10 minute washes in double distilled H_2O, samples were dehydrated through a graded ethanol series and immersed in 100% propylene oxide (Canemco). Samples were infiltrated in a 1:1 mixture of propylene oxide and Epon/Araldite resin (15.5 g Epon, 28.5 g DDSA, 8.8 g Araldite, 0.95 g dibutyl phthalate, catalyzed with 48 drops of DMP-3 catalyst, all Canemco) for 45 minutes followed by infiltration in 100% resin for 30 minutes at regular pressure and for 6 minutes under vacuum. Full resin infiltration was manifested in the sinking of high density samples to the bottom of the container. Samples were placed in plastic moulds containing fresh resin which polymerized overnight at 60°C.

Blocks were trimmed and faced with glass knives on an ultramicrotome (both Leica, Vienna, Austria). Semithin sections (0.5 μm) were cut in alternation with 90-nm ultrathin sections with a diamond knife throughout each specimen block; 10 semi-thin sections were generated per 10-20 ultrathin sections through any 5 μm depth examined. Semithin sections were mounted on glass slides (Fisher Scientific) and stained with toluidine blue for viewing by light microscopy and selection of areas for viewing by electron microscopy. Ultrathin sections (~90 nm) were mounted on 100-mesh Formvar-coated copper grids (Canemco). Sections were stained with 2% w/v aqueous uranyl acetate (Canemco) for 20 minutes, washed with dH_2O, stained with 0.1% w/v lead citrate (Fisher Scientific) for 10 minutes, washed again with dH_2O, and viewed on a Hitachi H7600 transmission electron microscope.

2.9 *In vitro assays*

2.9.1 Preparation of primary alveolar macrophages

Mice were killed by sodium pentobarbital overdose prior to cannulation of trachea with a 22-gauge I.V. catheter. Lungs were subjected to vigorous bronchoalveolar lavage with ten X 1 mL aliquots of ice cold, sterile 1 mM EDTA in PBS. Lavages were pooled and pulmonary cells were pelleted by centrifugation at 250xg for 5 minutes at room temperature. Pelleted cells were resuspended in 1 mL RPMI 1640 medium (GIBCO) and enumerated on a hemocytometer. The cellular suspension was adjusted by volume to 1 X 10^5 cells/mL. 100 µL aliquots were then placed onto acid-treated, 1 mm round coverslips in a 24-well plate and allowed to adhere at 37°C in 95% air/5% CO_2 for 2 hours. At the end of the incubation, coverslips were gently washed twice with 0.5 mL of R10 medium (RPMI 1640 containing 10% FCS, 10 mM sodium pyruvate and 10 mM L-glutamine). One mL of R10 medium was added to wells containing adherent macrophages and plates were incubated at 37°C in 95% air/5% CO_2 for an additional 72-96 hours prior to use in binding experiments.

2.9.2 MH-S cells

The MH-S cell line was derived by SV40 transformation of an adherent cell enriched population of mouse alveolar macrophages (BALB/c). Cells (ATCC CRL-2019), purchased from the American Type Culture Collection in September 1997, were maintained frozen in 1.8 mL aliquots of RPMI 1640 medium containing 10% DMSO. To prepare for use, each vial of frozen culture was thawed in a 37°C water bath until a pea-sized chunk of cells remained frozen in the vial. Vials were removed from water bath, sprayed down with 70% EtOH and transferred to a biosafety hood for the remainder of cell line preparation. All liquid in the vial was transferred to a 50 mL

Falcon conical tube. Five mL of cold (4°C) R10 medium was slowly added to the conical tube; an additional 5 mL of R10 medium was used to wash out the frozen chunk of cells remaining in the storage vial, and these washes were added to the conical tube. Cells were gently washed by pipetting for 30 seconds prior to being pelleted by centrifugation at 250x*g* for five minutes. The pellet was resuspended in 10 mL of fresh R10 medium and transferred to vented tissue culture flasks with 0.2 μm pore size, for incubation at 37°C in 95% air/5% CO_2 for 72 hours prior to use in binding experiments. The day before use in binding experiments, cells were harvested from tissue culture flasks by loosening them from the bottom of the tissue culture flask with a cell scraper. Cells were washed in R10 and adjusted by volume to 5 $X10^5$ cells/mL. 100 μL aliquots were seeded onto acid-treated, 1 mm round coverslips in a 24-well plate and cells were allowed to adhere at 37°C in 95% air/5% CO_2 for 2 hours. At the end of the incubation, coverslips were gently washed twice with 0.5 mL of R10 medium. One mL of R10 medium was added to wells containing adherent macrophages and plates were incubated at 37°C in 95% air/5% CO_2 for an additional 24 hours prior to use in binding experiments.

2.9.3 Association/binding assay with adherent cells

Wells were gently washed twice with 1 mL aliquots of R10 medium. Bacteria or zymosan particles were then added to wells at a multiplicity of infection (MOI) of 50:1 in a final volume of 0.5 mL RPMI 1640 medium. Plates were incubated stationary for 3 hours at 37°C in 95% air/5% CO_2. Following three 1 mL washes with RPMI 1640, cells were subjected to acetone-methanol fixation and staining with Hemacolor. Coverslips were air-dried and mounted cell side down onto glass slides with Permount. Slides were viewed at 60x magnification for enumeration of bound particles. One hundred macrophages per condition per experiment were counted in a randomized manner.

2.9.4 Preparation of monocyte-derived macrophages from whole human venous blood

The day before harvest of whole human venous blood, sheep erythrocytes were prepared for selection and elimination of T lymphocytes from blood. Briefly, 5 mL of sheep erythrocytes were washed three times in 10 mL volumes of RPMI 1640 medium, taking care to gently resuspend cells by tube inversion. After the third wash, 0.5 mL of sheep erythrocytes were taken from the pellet and added to 50 mL RPMI 1640 medium containing 100 μL *V. cholerae* neuraminidase (Calbiochem, La Jolla, CA). This suspension was incubated at 37°C for thirty minutes. The sheep erythrocytes were then washed three times in 10 mL volumes of R10 medium prior to resuspension in 50 mL of R10 medium. On the day of harvest, 100 mL of venous blood was collected into heparinised tubes from volunteers, and mixed 1:1 with R10 medium. 20 mL volumes of the blood mixture were layered over 10 mL volumes of Ficoll Paque Plus (Amersham, Baie d'Urfe, Quebec, Canada) in separate 50 mL Falcon tubes. Separation was achieved by centrifugation at 1450 rpm (~450X*g*) on a Jouan CR312 centrifuge, for twenty minutes at room temperature, without brake-assisted deceleration. The platelet-containing upper phase was aspirated and disposed of, the leukocyte-containing intermediate layer was collected using a wide bore Pasteur pipet and mixed 1:1 with PBS. The leukocytes were washed twice with PBS prior to being resuspended in a 1:1 mixture of R10 medium and neuraminidase-treated sheep erythrocytes. The suspension was pelleted via centrifugation and allowed to incubate at room temperature for one hour. Following incubation, the cells were gently resuspended by inversion and again separated on a Ficoll Paque Plus gradient. The upper phase was again aspirated and discarded and the monocyte-containing intermediate phase was collected and washed twice with PBS. The pellet was sampled and viewed microscopically to verify rosetting of T lymphocytes by sheep erythrocytes. Monocytes were resuspended in R10 and enumerated on a

hemacytometer, diluted 1/10 in 0.5% Trypan blue. Monocytes were diluted in R10 medium and seeded in 6 well plates at a concentration of 4 X 10^6 cells/well in 3 mL medium. Plates were incubated for one hour at 37°C with 5% CO_2 for monocyte adherence, after which medium was removed and wells washed gently with PBS. For differentiation into macrophages, monocytes were resuspended in 4-6 mL of R10 medium containing 10% autologous serum and transferred to Teflon pots. Teflon pots were incubated at 37°C with 5% CO_2 for 5 days to allow monocyte differentiation.

2.9.5 Association/binding assay with cells in suspension

Five-day old monocyte-derived macrophages were harvested from Teflon pots, resuspended in sterile gHBSS and enumerated on a hemocytometer. Macrophages were adjusted by volume to 1-5 X 10^5 cell/mL in gHBSS and 1 mL aliquots were placed in 6 mL polypropylene tubes (BD Falcon, Franklin Lakes, NJ). Overnight cultures of bacteria were washed, resuspended and adjusted to yield MOI's of 100:1 in a final volume of 100 µL of gHBSS, and added to tubes. Tubes were tumbled end-on-end at 37°C for one hour, after which macrophages were washed twice with 2 mL volumes of sterile cold PBS. Macrophages were resuspended in 250 µL PBS with 0.1% v/v gelatin and 10% v/v FCS and two 100 µL aliquots were deposited onto glass slides by cytocentrifuge. Cytospots were stained with Hemacolor and viewed under 60x magnification to count associated bacteria. Fifty macrophages were counted per cytospot per experiment in a randomized manner.

2.10 Crude LPS extraction and analysis

The proteinase K digestion method of Hitchcock and Brown (95) was used for the crude extraction of lipopolysaccharide from bacterial cultures. Bacteria were grown overnight in LB broth and collected via centrifugation. Cultures were washed in an equal volume of PBS prior to spectrophotometric adjustment to achieve $A_{525} = 0.500$. 1.5 mL of absorbance-adjusted culture was

sedimented in a 1.6 mL Eppendorf tube. The pellet was resuspended in 50 μL sample buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue in 1 M Tris-HCl buffer, pH 6.8) and heated to 100°C for ten minutes. 25 μg of proteinase K (Protease Type XI, Sigma) in 10 μL sample buffer was added and the mixture allowed to incubate at 60°C for one hour. 15 μL of sample was loaded into a discontinuous polyacrylamide gel system (Hitchcock and Brown) with 14% separating gel. Samples were electrophoresed at 160 volts for one hour (~ 90 milliamps), stained with the Pro-Q Emerald 300 LPS gel stain kit (Molecular Probes) and visualized on the Gel Doc 1000 (Biorad) with the help of Molecular Analyst software.

2.11 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). Due to the small sample sizes used in all groups ($N < 20$), all data were analyzed by the Mann-Whitney test for nonparametric, unpaired values. P values < 0.05 were considered statistically significant.

2.12 Definition of Infection

In this thesis, "infection" is defined as the colonization of a host's otherwise sterile tissues by a microorganism which is not found therein under normal conditions. Therefore an animal or organ system is considered "infected" when viable counts of the infective agent are isolated from that animal or organ system. Gross tissue pathology is not a requisite feature of infection, as there are many situations where infection is established without any evidence of disease (e.g. primary asymptomatic tuberculosis). "Virulence" is defined as the ability to provoke host damage, which can be manifested as systemic illness, gross pathology, or inflammation.

3 Results

3.1 *Intraperitoneal model of systemic BCC infection*

3.1.1 Objective

The invasive potential of BCC infections in CF and CGD prompted the development of an intraperitoneal model of systemic infection to examine the relative virulence of strains within the BCC. In this model, a CGD isolate of *B. multivorans*, strain FC147, persists in the spleens of C57BL/6 mice for up to 35 days after challenge, while all three *B. cenocepacia* strains examined (of CF origin) are cleared from spleen, liver and lung by the 35^{th} day after infection (218). This section describes work done in an attempt to determine where persistent *B. multivorans* organisms are being harboured in the spleens of C57BL/6 mice.

3.1.2 *B. multivorans* strain FC147 persistence in the spleen

An abbreviated infection period was developed from the original model in order to expedite examination of tissue histology. Infection kinetics of *B. multivorans* strain FC147 were established for the first 7 days of infection. C57BL/6 mice were challenged intraperitoneally with ~5 X 10^7 CFU in 0.5 mL of gHBSS, with a deposition efficiency of approximately 1%. Viable counts were determined from serially diluted samples of spleen harvested on days 0, 1, 2, 3, 4, 5, 6 and 7 (Figure 1). This daily assessment of the bacterial load in the spleen (Figure 1) shows that strain FC147 establishes and maintains a persistent infection to day 7.

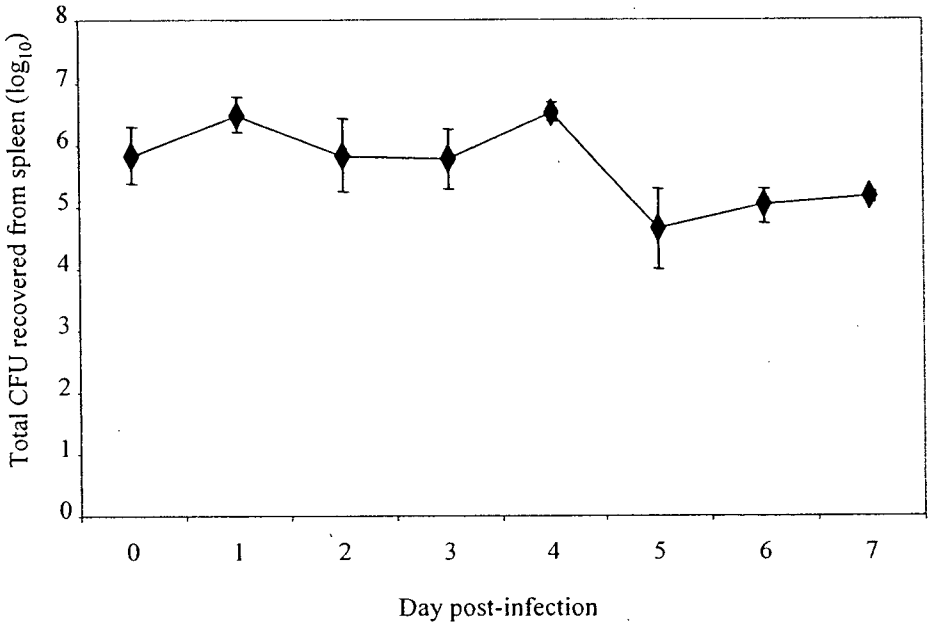

Figure 1. Daily splenic bacterial load after infection with *B. multivorans* strain FC147. C57BL/6 mice were intraperitoneally challenged with ~5.0 X 10^7 CFU. Quantitative bacteriology of the spleen was assessed at 0 (3 hours), 1, 2, 3, 4, 5, 6 and 7 days. Data are the mean $\pm$ SEM from six animals at each time point, and are representative of two separate experiments.

3.1.3 Association of persistent *B. multivorans* FC147 with mononuclear cells of the white pulp of the spleen

Tissue histology was taken from infected spleens on the 7th day after bacterial challenge with *B. multivorans* strain FC147 to investigate the site of persistence. Five-micron thick sections were incubated with rabbit polyclonal sera generated against strain FC147, and with an Alexa 488-conjugated anti-rabbit secondary monoclonal antibody. This antibody was previously determined to be specific for *B. cepacia* complex strains, and sensitive to 1/1000 dilution when used as part of an indirect immunofluorescence protocol, yielding a strong signal at this dilution. Splenic sections were counterstained with Mayer's hematoxylin prior to viewing. All fluorescently stained bacteria were localized to the white pulp, in association with mononuclear cells. A total of 40 stained bacteria were observed in 18 of 69 sections cut from 6 spleens from three separate 7-day infection experiments. The number of fluorescently stained bacteria observed per section of spleen ranged from 0 to 6. Morphometry predicts that cutting 5 μm-thick sections across a 10 mm-long spleen would yield 2000 sections; in a splenic infection sustaining a total bacterial load of ~10^5 CFU, assuming that the bacterial load is evenly distributed throughout the organ, up to 50 bacteria should be viewed per section. The observation of only 40 bacteria in 69 sections, a frequency of 0.58, suggests that persistent bacteria may have been localized in foci of infection on day 7. While the quality of staining may have been affected by the presence of bacterial exopolysaccharide, lack of permeabilization or quality of the primary antibody, pilot experiments showed that these effects were unlikely. Figure 2 is a representative diptych of fluorescently stained bacteria (Fig. 2a) amid mononuclear cells of the white pulp (Fig. 2b). Identification of these cells by

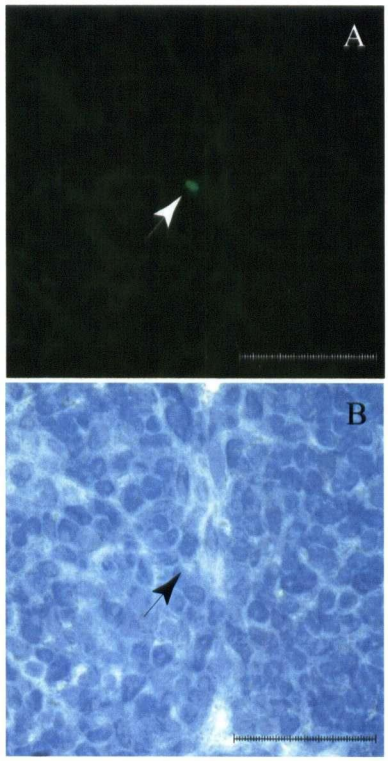

Figure 2. Immunolocalization of *B. multivorans* strain FC147 in day 7 infected spleen tissue. Sections were stained with rabbit polyclonal sera generated against FC147, Alexa 488-conjugated secondary monoclonal antibody and counterstained with Mayer's hematoxylin. A: green channel; B: same field of view under light microscopy. 1000x magnification. Micrographs are representative of immunohistology of five spleens from three separate experiments. Bar = 25 μm.

morphology was unsuccessful due to the crowded architecture of the spleen. Analysis via immunofluorescent staining of candidate host cell surface markers was complicated by erythrocyte-generated autofluorescence native to the spleen. No evidence of bacterial replication, in the form of aggregates of bacteria localized to one host cell, was observed.

3.1.4 Discussion

The largest secondary lymphoid organ, the spleen is a dynamic center of activity, serving the dual purposes of removal of dead cells and particles from the blood and generation of adaptive immune responses. These activities are handled by the various mononuclear cells of the red pulp and the white pulp, respectively. The white pulp of the spleen is comprised of a central arteriole, through which incoming blood cells enter. This is immediately surrounded by the T lymphocytes that make up the periarteriolar lymphatic sheath (PALS), site of naïve and memory T cells. Dendritic cells reside and present antigen to these T cells on the outer edge of the PALS; B lymphocytes form follicles around dendritic cells in the main body of the white pulp (76, 167-168). The marginal zone, which surrounds the white pulp and separates it from the red pulp, is composed of marginal zone B cells and two different kinds of macrophages, marginal zone macrophages and metallophilic macrophages, which engulf foreign antigen and dead blood cells and present antigen to marginal zone B cells (2, 21, 251). The sheer number and variety of mononuclear cells in the white pulp provide a plethora of potential host cells within which *B. multivorans* strain FC147 might persist.

B. multivorans persisted in the spleen, but not the liver or lung, after intraperitoneal challenge of C57BL/6 mice (218). It is unclear whether *B. multivorans* was transported to the spleen by peritoneal macrophages, or whether bacteria actively invaded this organ. What is clear is that the bacteria were able to establish a chronic infection in the white pulp of the spleen despite the presence

of so many cells of the host immune response. One possible explanation is that while the spleen is an active site for the development of adaptive immune response, it can also be an immune-privileged site in the absence of granulocytes (167). The macrophages of the marginal zone primarily scavenge dead erythrocytes, present antigen and produce cytokines in response to viral infection (67, 132-133). Marginal zone macrophages have been identified as the splenic host cell for intracellular *S. enterica* serovar Typhimurium infection in an intraperitoneal challenge model, and BCC isolates can survive without replication in activated macrophage cell lines for up to 96 hours (187, 194).

In view of this evidence it is tempting to suggest that the macrophage is the host cell in which *B. multivorans* persists for over a month. However, the macrophages of the spleen are far outnumbered by the B and T lymphocytes. While viruses are the dominant intracellular parasites for these two cell types, a Gram-negative pathogen, *S. enterica* serovar *enteriditis*, is capable of entering and slowly replicating in B and T lymphocytes (134). Dendritic cells, as antigen-presenting cells, also have the capacity to harbour intracellular pathogens; *Legionella* and *Mycobacterium* species are able to slowly replicate within this cell type (148, 166). An examination of the relationship between the BCC and lymphocytes and dendritic cells has not been described, and while these cell types can support intracellular parasitism by other bacteria, their candidacy as host cells for *B. multivorans* in the spleen was not examined (134, 148, 166).

The splenic marginal zone macrophage was the dominant candidate host cell in which *B. multivorans* might be persisting, in spite of the problems encountered in identifying it. Macrophages serve as host cells for the intracellular pathogens *Legionella pneumophila* and *Mycobacterium tuberculosis* in the lung (38, 130). In the spleen, marginal zone macrophages harbour persistent *Salmonella enterica* serovar Typhimurium (194). Members of the BCC have been shown to invade and survive within activated human macrophage cell lines (154, 187). Moreover, internalization of

B. cenocepacia by alveolar macrophages has been observed after intratracheal challenge in a model of acute pulmonary infection (34).

3.2 Intranasal models of pulmonary BCC infection

3.2.1 Leukopenic model

3.2.1.1 Objectives

The discovery of marked differences in the infection kinetics of strains from different species within the BCC in the intraperitoneal model of infection prompted the development of a more physiologically relevant model – reflecting the pulmonary nature of CF infections - to further examine differences between these species. In the absence of a well-established and characterized CF mouse colony, a leukopenic model of intranasal infection was chosen. This system was chosen over the agar bead model of intratracheal infection because it allowed for bacterial challenge by a non-invasive route, and greater opportunity for bacterial-host interaction. An initial matrix evaluation of 16 BCC strains representing 5 species revealed that *B. multivorans* strains tended to persist in the lungs of immune-suppressed mice, while *B. cenocepacia* strains were cleared from the lungs of healthy and CPA-treated mice. The objectives of the work done with the leukopenic model were: 1) to replicate the infection kinetics differences observed in the intraperitoneal model, in the lung; 2) to evaluate whether a corollary difference in the pulmonary host response would also occur; and 3) to identify site of persistence in the lung.

3.2.1.2 Effect of cyclophosphamide (CPA) treatment on animal health, peripheral leukocyte counts and splenic weight

The efficacy of CPA as an inducer of mild leukopenia was evaluated. Mice were injected I.P. with either sterile PBS or CPA (150 mg/kg) prior to, and every fifth day during the course of, infection or gHBSS-challenge (control). Animals were then monitored daily for general health and signs of illness were recorded. Figure 3 demonstrates that CPA treatment prior to intranasal introduction of a gHBSS bolus caused minimal systemic illness in BALB/c mice, when compared to sterile PBS treatment prior to gHBSS bolus. Systemic ill health (as determined by parameters detailed in Materials and Methods) was only observed one day after the third and fourth CPA injections, respectively, and animals appeared to recover from this state of ill health within 24 hours. No healthy, infected control animals demonstrated signs of systemic ill health, but immune-compromised, infected animals appeared sicker than uninfected CPA-treated animals. Figure 4 shows that mice treated with PBS and instilled with *B. vietnamiensis* strain FC811 did not appear sick, while signs of systemic illness were observed in mice treated with CPA and instilled with the same strain. These observations suggest that CPA treatment alone rendered BALB/c mice mildly ill, but recovery was swift. CPA treatment followed by bacterial challenge rendered the animals consistently sicker, for longer periods of time, than CPA alone.

Total peripheral leukocyte counts were measured in the CPA-treated and PBS-treated mice on days 0, 2, 4 and 16. CPA affected a decrease in the total peripheral leukocyte count, although none of the measured differences were significant except for day 16 (5277 ± 947 cells/mm^3 in PBS-treated controls compared to 995 ± 325 cells/mm^3 in CPA-treated mice, $P = 0.0043$). Differential counts of blood smears demonstrated that CPA, as used in this model, elicited mild pan-leukopenia rather than simple neutropenia: Table 3 lists the total leukocyte population and proportion of

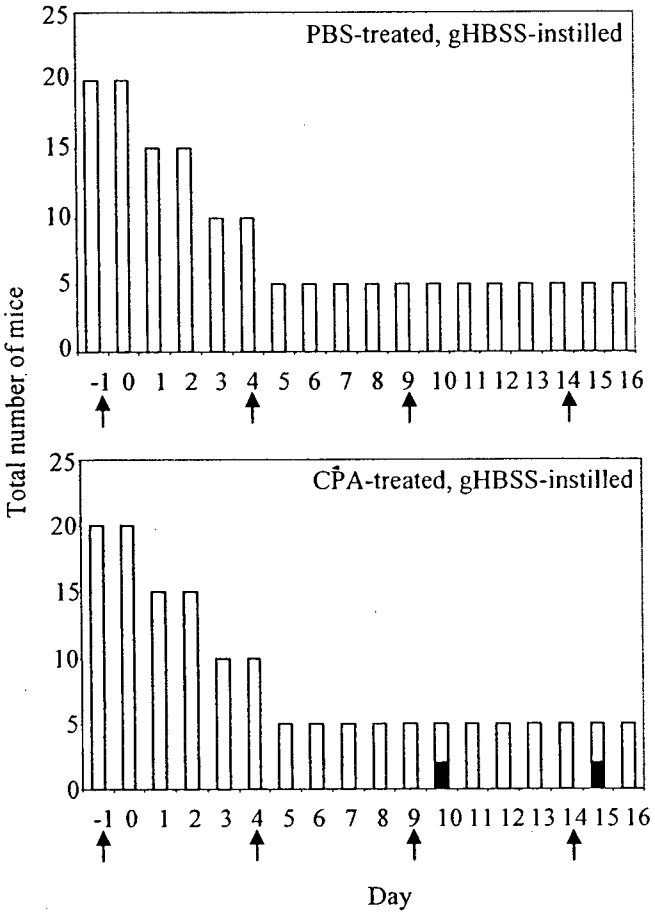

Figure 3. Effect of CPA treatment on animal health over time. PBS-treated and CPA-treated mice were challenged with gHBSS only. Animals were monitored daily for the duration of the experiment and assessed on a three point system for ill health. Unhealthy animals (black bars) and healthy animals (white bars) are shown at each day. The total number of animals studied decreased over time as animals were removed for analysis. Mice were sacrificed in the groups to which they were randomly pre-assigned, with no preference for euthanization of sick-looking animals. Arrows denote administration of either CPA or PBS.

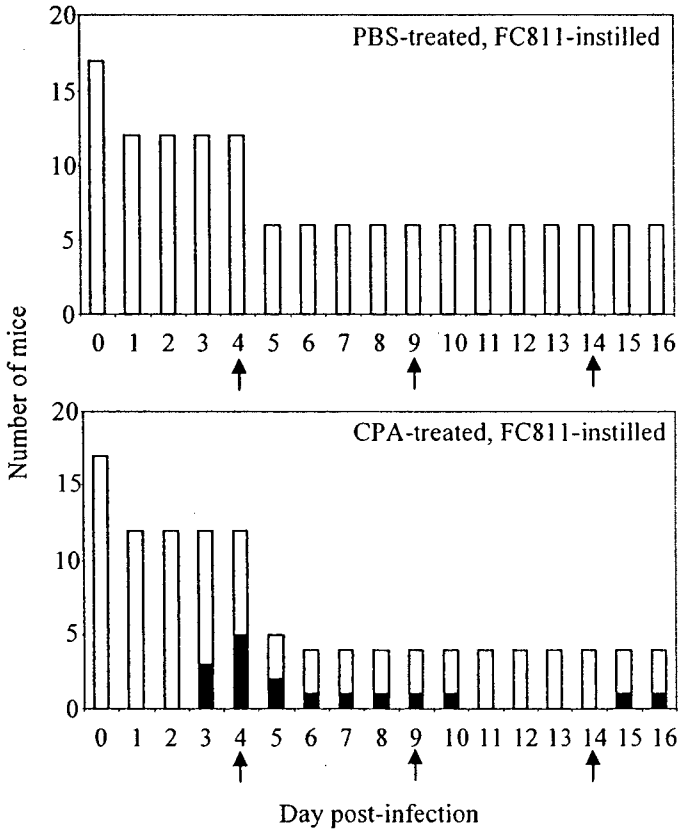

Figure 4. Effect of CPA treatment and bacterial infection on animal health over time. PBS-treated and CPA-treated mice were challenged with ~1.6 X 10^4 *B. vietnamiensis* strain FC811. Animals were monitored daily for the duration of the experiment and assessed on a three point system for ill health. Unhealthy animals (black bars) and healthy animals (white bars) are shown for each day. The total number of animals studied decreased over time as animals were removed for analysis. Mice were sacrificed in the groups to which they were randomly pre-assigned, with no preference for euthanization of sick-looking animals.

Table 3. Peripheral blood profile of mice subjected to various treatments. BALB/c mice that had been either given anaesthesia only, I.P. PBS or I.P. CPA, as detailed in Materials and Methods, were bled on day 16 for blood analysis. Data are the mean and standard error of the mean for three animals at each time point. Asterisk denotes a significant differerence ($P < 0.05$) between the PBS-treated and CPA-treated groups, as well as between the Anaesthesia only and CPA-treated groups.

Condition	Total WBC/mL ± SEM (x 1000)	% Monocytes ± SEM	% PMN ± SEM
Anaesthesia only	5197.5 ± 811.5	82.3 ± 2.7	17.8 ± 2.7
PBS-treated	5276.7 ± 947.0	87.3 ± 1.5	12.7 ± 1.5
CPA-treated	995.0 ± 325.5*	79.0 ± 3.2	21.0 ± 3.2

mononuclear and polymorphonuclear cells in peripheral blood on day 16 after various treatments. One group of mice was given anaesthesia only and handled daily, another group was subjected to four PBS treatments, and the last group was subjected to four CPA treatments. Monocyte and polymorphonuclear cell populations were depressed equally by drug administration and the percentages of each group in the peripheral blood did not change. CPA treatment also correlated with a significant drop in splenic weight on days 0 ($P = 0.004$), 4 ($P = 0.04$) and 16 ($P = 0.02$) of treatment, with an average of 37.3% decrease.

3.2.1.3 Infection kinetics of BCC members in the lung

Table 4 lists the 16 BCC strains from the Canadian *B. cepacia* Research and Referral Repository initially evaluated for infection kinetics in the mouse model. In five out of the six *B. multivorans* strains tested (C1576, C5274, C5393, C5568, FC147), immune-compromised mice sustained the infection in the lungs to day 16, whereas healthy controls infected with the same organisms cleared the infection by day 16. None of the *B. multivorans* strains caused illness in any of the mice. In contrast, none of the *B. cenocepacia* strains persisted in either immune-compromised or healthy mice to day 16. Moreover, five of the seven *B. cenocepacia* strains (C1394, C4455, C5424, C6433, Cep511) were cleared by day 4 of the infection, while causing a greater degree of illness in infected animals than *B. multivorans*. The *B. stabilis* strain (C7322) was cleared by day 16 in both groups. Immune-compromised mice infected with either the *B. vietnamiensis* (FC811) or *B. dolosa* (Cep873) strains had a slight increase in pulmonary bacterial load, above the initial dose delivered, by days 10 and 14, respectively. Figure 5 shows that a greater number of CPA-treated mice challenged with *B. cenocepacia* strain C6433 appeared ill early in the course of infection, while few CPA-treated mice challenged with *B.multivorans* strain C1576 show signs of systemic illness until late in the course of infection, after the second and third CPA treatments. In light of these

Table 4. BCC strains evaluated in matrix pilot experiment of leukopenic model.

Strain name	Strain type[a]	Source and location[b]	Infection kinetics in CPA-treated mice, IN-CPA model
B. multivorans			
C5393	03	CF, Canada	Sustained infection, day 16
C3430	07	CF, Canada	Clearance by day 16
C1576	10	CF-e, UK	Sustained infection, day 16
C5274	12	CF, Canada	Sustained infection, day 16
FC147	12	CGD, USA	Sustained infection, day 16
C5568	19	CF, Canada	Sustained infection, day 16
B. cenocepacia			
C1257	01	CF, N. America	Clearance by day 16
C5424	02	CF-e, Canada	Clearance by day 4
K56-2	02	CF-e, Canada	Clearance by day 16
C6433	04	CF-e, Canada	Clearance by day 4
Cep511	05	CF-e, Australia	Clearance by day 4
C4455	06	CF, Canada	Clearance by day 4
C1394	13	CF-e, UK	Clearance by day 4
B. stabilis			
C7322	16	CF, Canada	Clearance by day 16
B. vietnamiensis			
FC811	08	CF, N. America	Sustained infection, day 16
B. dolosa			
Cep873	10	CF, Canada	Sustained infection, day 16

[a]Strain type assigned for this study correlates to the numerical RAPD type assigned previously to this fingerprint pattern.
[b]CF, cystic fibrosis; CGD, chronic granulomatous disease; e, epidemic strain.

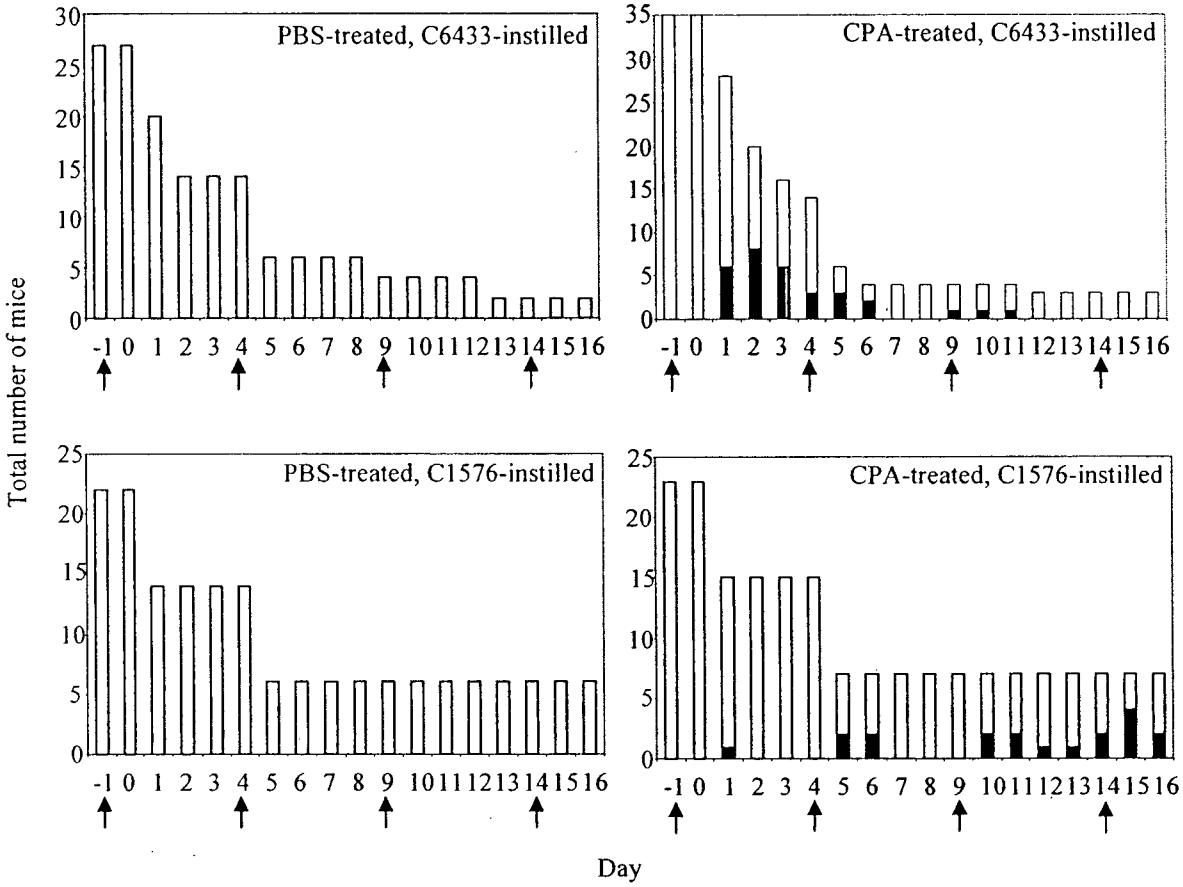

Figure 5. Effect of CPA treatment and bacterial infection on animal health over time. PBS-treated and CPA-treated mice were challenged with ~1.6 X 10^4 *B. cenocepacia* strain C6433 or *B. multivorans* strain C1576. Animals were monitored daily for the duration of the experiment and assessed on a three point system for ill health. Unhealthy animals (black bars) and healthy animals (white bars) are shown for each day. The total number of animals studied decreased over time as animals were removed for analysis. Mice were sacrificed in the groups to which they were randomly pre-assigned, with no preference for euthanization of sick-looking animals. Arrows denote administration of either CPA or PBS.

observations and particularly the apparent differential virulence between *B. multivorans* and *B. cenocepacia* strains within the model, further studies were conducted with five strains: *B. multivorans* C1576 and C5568, *B. cenocepacia* strains C1394 and C6433 and *B. vietnamiensis* FC811.

3.2.1.4 Effect of CPA treatment on pulmonary bacterial load

Figure 6 displays the kinetics of infection with five BCC strains in both healthy and immunocompromised mice. Animals were injected intraperitoneally with either sterile PBS or CPA (150 mg/kg) prior to and every fifth day during the course of infection. Mice were challenged with ~1.6 X 10^4 CFU intranasally. Viable counts were obtained from diluted lung homogenates on days 0, 4 and 16 of the infection. Immune-compromised mice infected with either of the two *B. multivorans* strains (C1576 and C5568) or *B. vietnamiensis* strain FC811 sustained a pulmonary bacterial infection at high titres for up to 16 days. Healthy control mice infected with strains C1576 and C5568 demonstrated slow clearance of the infection from their lungs, while healthy controls infected with strain FC811 displayed rapid clearance. In contrast, both *B. cenocepacia* strains (C1394 and C6433) were cleared by day 4 from healthy and immune-compromised lungs alike. CPA treatment therefore impeded pulmonary clearance of the *B. multivorans* and *B. vietnamiensis* strains but not *B. cenocepacia* strains.

3.2.1.5 Cellular analysis of bronchoalveolar lavage (BAL) fluid of infected lungs

Figure 7 shows the effect of CPA treatment on the BAL fluid macrophage population of mice challenged with a gHBSS bolus only or infected with *B. multivorans* strains C1576 or C5568, *B. cenocepacia* strains C1394 or C6433 or *B. vietnamiensis* strain FC811. CPA treatment with a sterile gHBSS bolus resulted in a decrease in absolute macrophage numbers recovered in the BAL fluid by

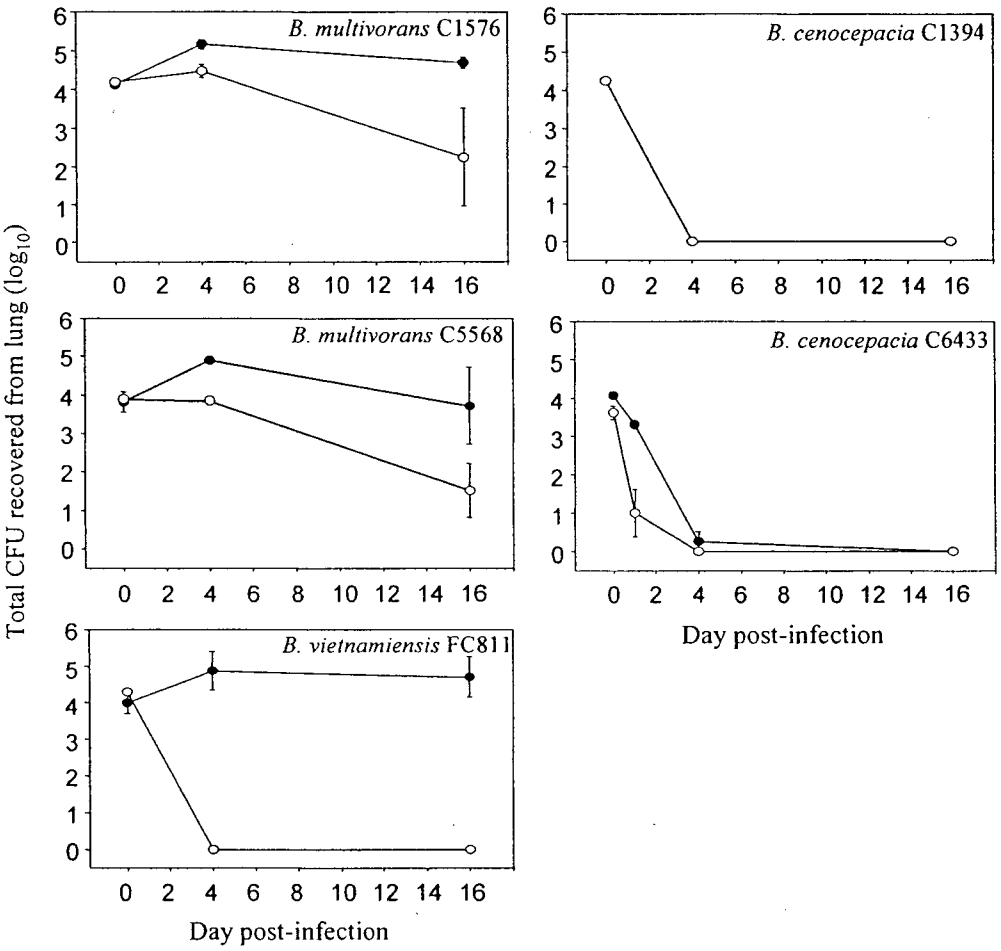

Figure 6. Effect of CPA treatment on pulmonary bacterial load after infection with five BCC strains. CPA-treated (closed symbols) and PBS-treated (open symbols) BALB/c mice were intranasally challenged with ~1.6 X 10^4 CFU. Quantitative bacteriology of the lung was assessed at 0 (3 hours), 4 and 16 days. Values for C1394-infected, CPA group were identical to those for the PBS-treated group. Data are the mean $\pm$ SEM for three animals at each time point.

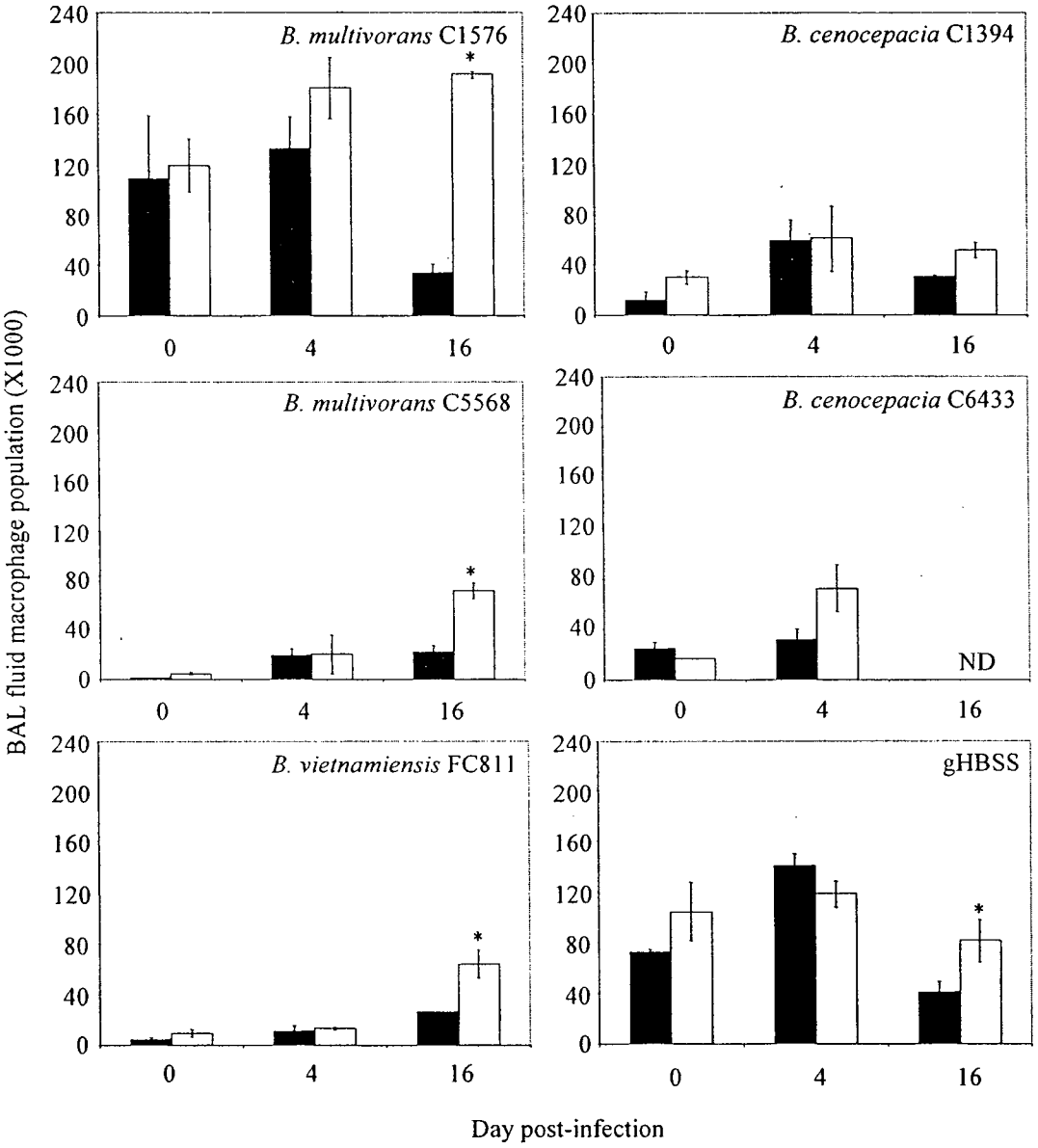

Figure 7. Effect of CPA treatment on BAL fluid macrophage population in response to BCC infection. BAL was performed on CPA-treated (black bars) and PBS-treated (white bars) mice infected with five BCC strains at 0, 4 and 16 days. Macrophages were enumerated from haemocytometer counts and Wright-stained cytocentrifuge preparations. Data represent the mean $\pm$ SEM for three mice at each time point. Asterisks denote a significant difference ($P < 0.05$) between CPA-treated and PBS-treated groups. ND = not done.

day 16 with a significant difference ($P = 0.009$) observed between the CPA-treated and PBS-treated groups at this time point. Despite changes in absolute macrophage numbers in the BAL fluid between time points, macrophages as a percent of the BAL fluid cell population remained high in all groups examined with a parallel change in total BAL fluid cell count observed, Table 5. A difference in the absolute number of BAL fluid macrophages recovered at day 16 was also observed between the CPA-treated and PBS-treated mice infected with either strain C1576, C5568 or FC811 (C1576 $P = 0.002$, C5568 $P = 0.04$, FC811 $P = 0.03$). This difference between the CPA-treated and PBS-treated mice in the number of macrophages recovered from BAL coincided with the greatest difference between CPA-treated and PBS-treated groups in terms of pulmonary bacterial load (Figures 6 and 7). Furthermore, dramatically lower absolute numbers of macrophages were recovered in the BAL fluid of mice infected with strains C5568 and FC811, than from mice infected with other strains or challenged with gHBSS, regardless of whether or not they received CPA treatment. No significant difference in macrophage number was observed between CPA and normal mice infected with either *B. cenocepacia* strain at any time point, although the absolute numbers recovered in the BAL of CPA-treated mice were low for both.

No significant difference was observed in the BAL neutrophil population from any group of mice. Figure 8 illustrates that the number of neutrophils recovered in BAL of PBS- and CPA-treated mice challenged with gHBSS or BCC strains alike was neither significantly elevated in response to infection in the absence of CPA treatment, nor proportionately less when subjected to CPA treatment. These observations suggest that BCC strains were not particularly pro-inflammatory at the challenge doses used.

Table 5. Macrophages as a proportion of the total BAL fluid cell population. BAL was performed on three mice from each group, and cellular analysis performed as described in Materials and Methods. Data are the mean and standard error of the mean for three to six mice for each time point. Asterisks denote a significant difference ($P < 0.05$) between CPA-treated and PBS-treated mice. ND, not done.

Condition	Day 0	Day 4	Day 16
PBS - gHBSS	94.75 ± 0.25	96.17 ± 0.60	95.25 ± 0.44
CPA - gHBSS	87.50 ± 4.19	98.50 ± 0.87	89.75 ± 2.89
PBS - C1576	94.00 ± 0.20	95.17 ± 1.01	95.17 ± 0.67
CPA - C1576	88.83 ± 4.42	98.67 ± 0.17	71.25 ± 10.25*
PBS - C5568	80.00 ± 4.25	87.33 ± 3.09	90.50 ± 1.76
CPA - C5568	64.00 ± 20.00	78.67 ± 3.56	75.83 ± 5.80*
PBS - C1394	82.17 ± 1.76	92.67 ± 1.76	ND
CPA - C1394	88.26 ± 1.06	97.50 ± 2.12	ND
PBS - C6433	91.70 ± 1.50	89.27 ± 2.89	ND
CPA - C6433	86.93 ± 1.47	88.10 ± 1.90	ND
PBS - FC811	82.75 ± 3.75	90.50 ± 2.29	89.17 ± 3.35
CPA - FC811	80.33 ± 1.59	84.75 ± 4.99	81.00 ± 0.06*

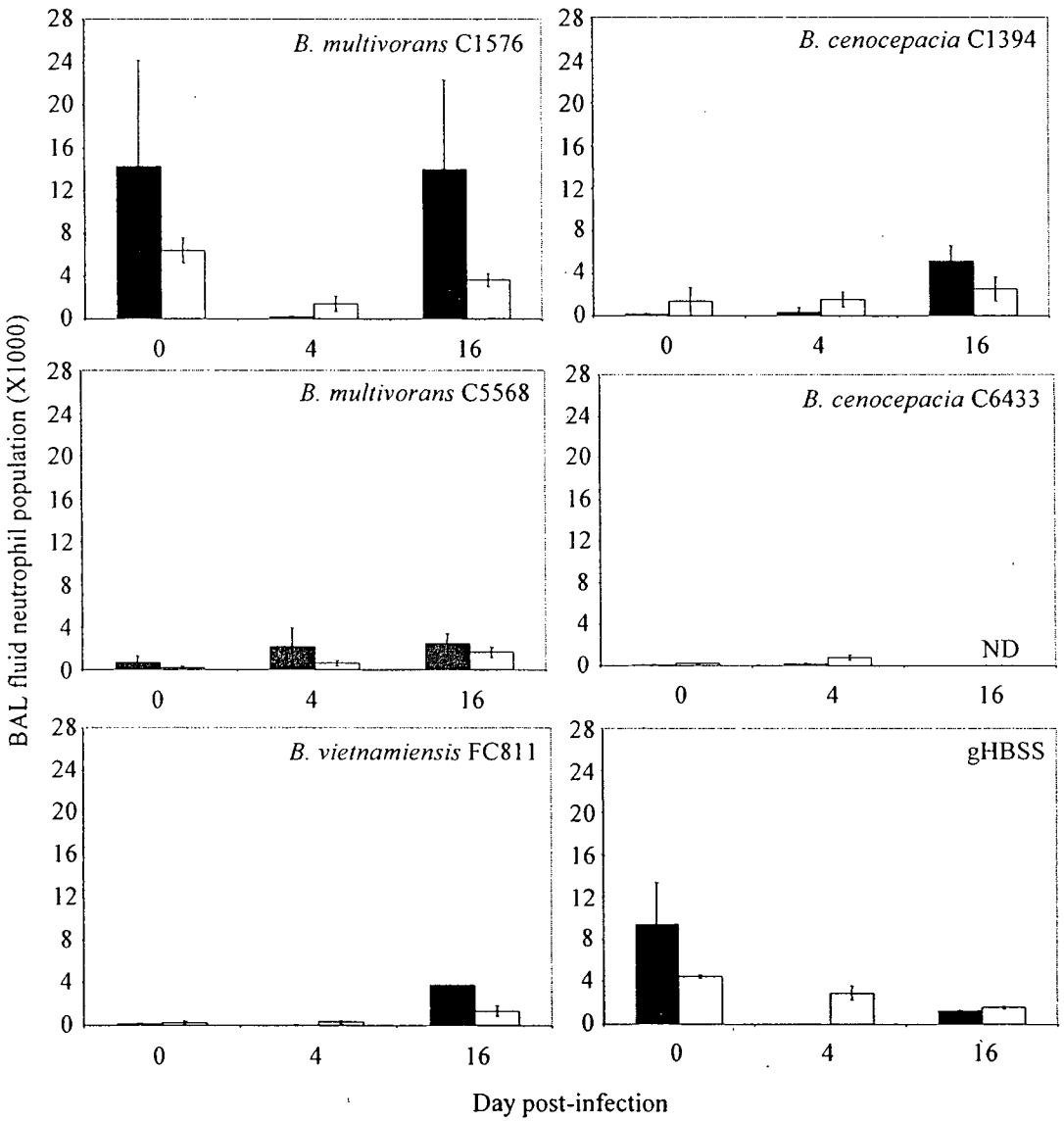

Figure 8. Effect of CPA treatment on BAL fluid neutrophil population in response to BCC infection. BAL was performed at 0, 4 and 16 days on CPA-treated (black bars) and PBS-treated (white bars) mice infected with five BCC strains. Neutrophils were enumerated from haemocytometer counts and Wright-stained cytocentrifuge preparations. Data represent the mean ± SEM for three mice at each time point. ND = not done.

3.2.1.6 Cytokine analysis of BAL fluid of infected lungs

Bronchoaveolar lavage was performed on all mice and cell-free supernatants from cytocentrifuged preparations were tested by ELISA for the pro-inflammatory cytokines TNF-α and MIP-2. Low levels of both cytokines were detected in the BAL fluid of mice infected with *B. multivorans* C5568, *B. cenocepacia* C6433 and *B. vietnamiensis* FC811. No significant difference was observed between infected mice and untreated, uninfected controls, Figures 9 and 10.

3.2.1.7 Immunofluorescent detection of persistent BCC organisms in lung tissue

Lung tissue that had been processed for histology was stained for bacteria with anti-*B. multivorans* polyclonal sera and Alexa 488 secondary antibody, prior to counterstaining with Mayer's hematoxylin. Tissue taken on days 0 and 16 of the experiment, from CPA-treated, PBS-challenged mice and all CPA-treated, bacterially-challenged mice, were examined. Aside from day 0-infected sections, which served as positive controls for bacterial staining, bacteria were observed only in sections from CPA-treated mice sustaining infections on day 16. A total of 21 fluorescently stained bacteria were observed in 20 sections from three different infections (18 in 5 of 7 sections from two lungs infected with *B. multivorans* C5568, 2 in 1 of 3 sections from one lung infected with *B. multivorans* C1576, and 1 in 1 of 10 sections from one lung infected with *B. vietnamiensis* FC811). The number of bacteria observed per section of lung ranged from 0 to 9. Morphometry predicts that cutting 5 μm-thick sections along the depth of a 5 mm lobe of lung would yield 1000 sections; in a pulmonary infection sustaining a total bacterial load of ~10^4 CFU, assuming that the bacterial load is evenly distributed throughout the 2 major lobes of this organ, up to 5 bacteria should be viewed per section. The observation of only 21 bacteria in 20 sections, a frequency of 1.05, suggests that persistent bacteria may have been localized in foci of infection on day 16. Figure 11

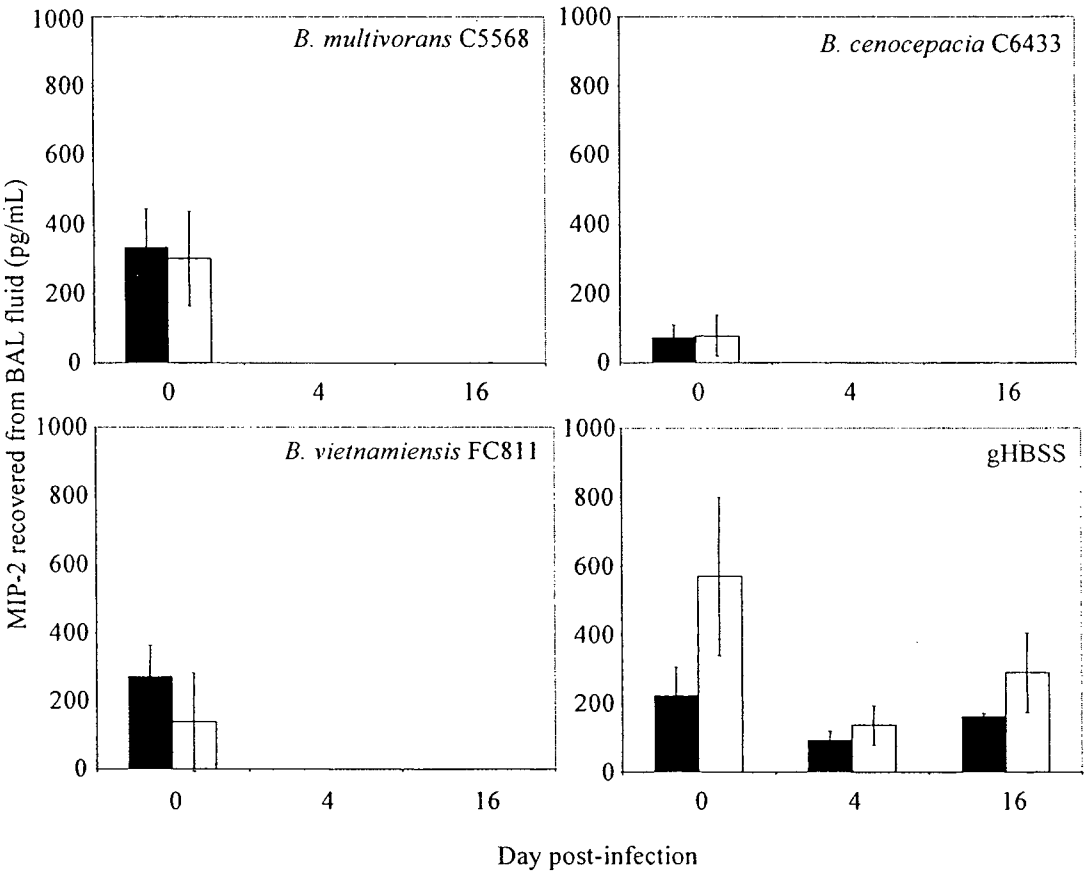

Figure 9. Pulmonary MIP-2 content following bacterial infection. CPA-treated (black bars) or PBS-treated (white bars) BALB/c mice were intranasally challenged with ~1.6 X 10^4 CFU of bacteria or sterile gHBSS. Bronchoalveolar lavages were performed performed *in situ* on days 0 (3 hours), 4 and 16 post-infection, and supernatants were evaluated for MIP-2 via ELISA as described in Materials and Methods. Data are the mean $\pm$ SEM for three animals at each time point.

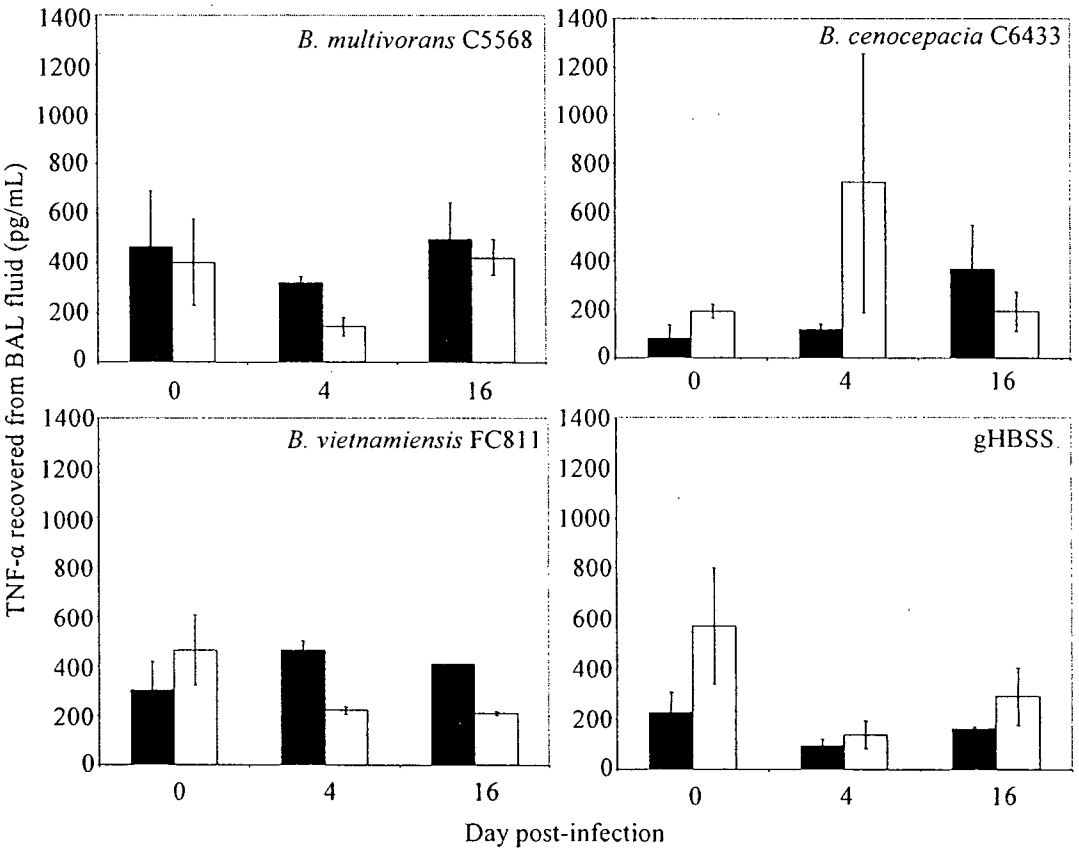

Figure 10. Pulmonary TNF-α content following bacterial infection. CPA-treated (black bars) or PBS-treated (white bars) BALB/c mice were intranasally challenged with ~1.6 X 10^4 CFU of bacteria or sterile gHBSS. Bronchoalveolar lavages were performed performed *in situ* on days 0 (3 hours), 4 and 16 post-infection, and supernatants were evaluated for TNF-α via ELISA as described in Materials and Methods. Data are the mean ± SEM for three animals at each time point.

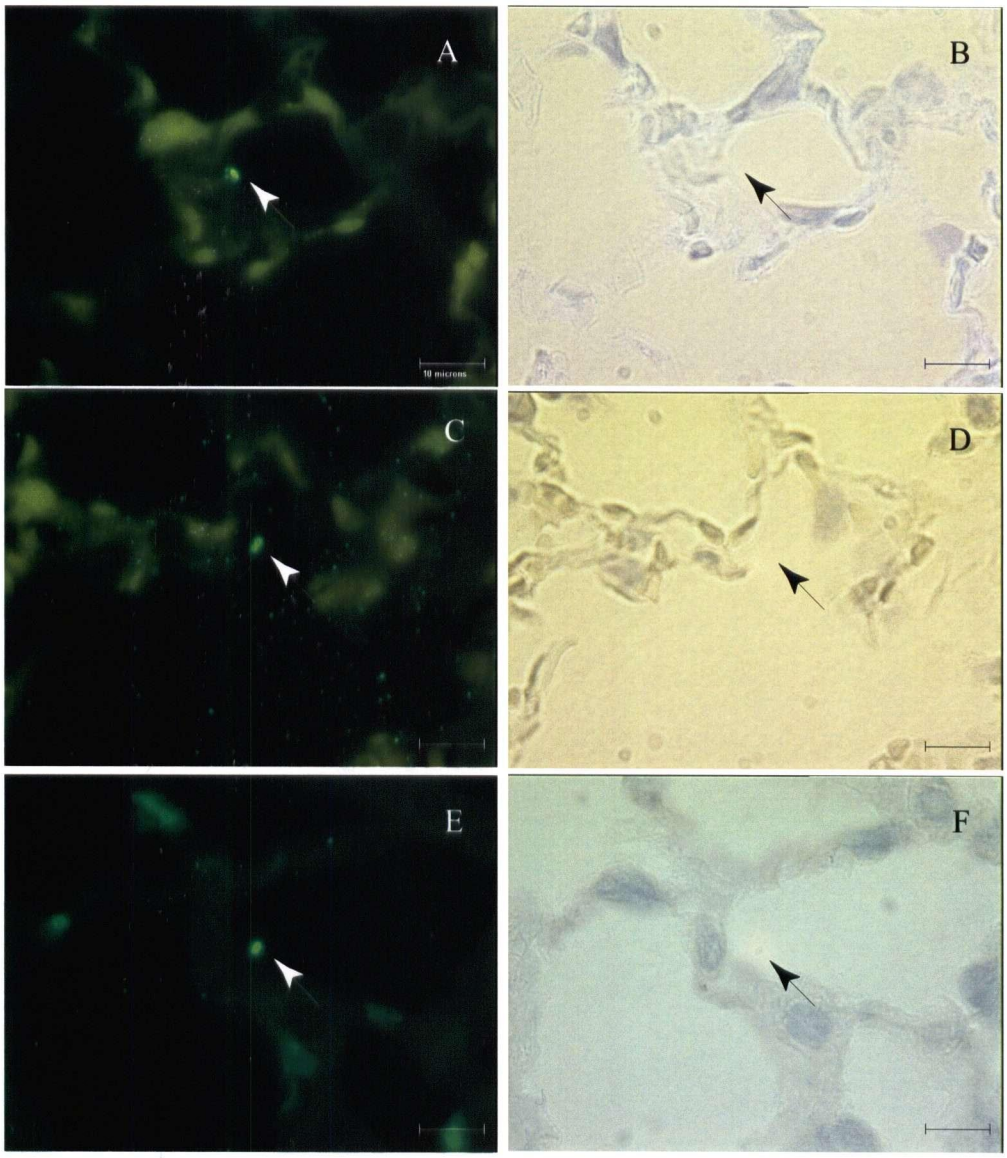

Figure 11. Indirect immunofluorescent detection of BCC organisms in the lung at day 16. Sections from *B. multivorans* C5568 (A, B), C1576 (C, D) and *B. vietnamiensis* FC811 (E, F) infected lungs were labeled with anti-*B. multivorans*-Alexa 488 (A, C. E) and counterstained with Mayer's hematoxylin (B. D. F). Fluorescently stained bacteria shown in green; arrows point to localization of BCC organisms in alveolar space. All micrographs taken at 1000x magnification. Bar = 10 μm.

shows sections of lung from CPA-treated mice infected with strain C5568, C1576 or FC811. All images were generated from lungs sampled on day 16. All bacteria were observed in the alveolar spaces or in loose association with alveolar epithelium. Extensive examination of immunofluorescent sections failed to reveal bacteria within host macrophages or epithelial cells.

3.2.1.8 Discussion

The purpose of these studies was to develop a physiologically relevant animal model that would permit investigation of bacterial virulence determinants or host responses conducive to the persistent infections that are characteristic of BCC infection of the immune-compromised host. The goals were to determine the species specificity of *in vivo* responses and to evaluate their relative contributions to infection outcome. Several animal models have already been adapted to, and developed for, the study of BCC *in vivo*. Two CF mouse models have been extensively examined in the context of lung disease during *B. cenocepacia* infection (57, 193). While these models are useful for exploring host response to, and localization of, acute BCC infection in the CF lung, they do not serve the focus of this thesis, the apparent species-specific difference in infection outcome in the mouse as a model of the human.

The system used in this study is similar to that described by Cryz (52), and employs CPA (150 mg/kg intraperitoneally, every five days) to render animals mildly leukopenic prior to bacterial infection. The non-invasive, mildly immunosuppressive nature of the model allowed sustained infection without causing extreme ill health in the animals. Infection dynamics were also highly reproducible in the model, which was able to discern differences between *B. multivorans* and *B. cenocepacia* in this respect. CPA has been previously cited as an agent of reduction in both peripheral blood neutrophils and lymphocytes (46, 94). CPA treatment resulted in a mild leukopenia, rather than acute neutropenia, in BALB/c mice, as confirmed by differential counts

performed on peripheral blood smears. This condition was manifested physiologically as a mild state of ill health (weight loss, decreased grooming) from which the animals recovered within 72 hours post drug administration, and decrease in splenic mass. Histology and ELISA analysis done on BAL fluid from mice receiving drug or saline controls showed that CPA provoked neither histopathology nor inflammatory cytokine release in the lung. CPA treatment thus allowed persistence of certain strains from the BCC in the lungs of immune-suppressed mice, but was mild enough to allow this persistence to be monitored for over 2 weeks in the model.

Aspects of this model are indirectly similar to CGD, another genetic disease in which BCC is the predominant Gram-negative bacterial pathogen (246). Both CGD patients and the corresponding transgenic murine model lack functional NADPH oxidase and are unable to mount a respiratory burst response to fungal and bacterial infection (165, 246). Because the NADPH oxidase is found largely in phagocytic cells, this renders that population of host cells incapable of oxidative killing, and solely reliant on non-oxidative killing mechanisms to control infection. Since the BCC is resistant to non-oxidative killing mechanisms, CGD host cells are virtually ineffective against infection with the organism (216). The use of CPA mimics the CGD scenario in part because of the drug's effect on the same cell population; whereas CGD phagocytes are unable to mount a host response, phagocytes from a mouse treated with CPA are probably reduced in number.

The general aim was to discern differences among species in the BCC, particularly differences which may elucidate factors or mechanisms controlling the disparate outcomes associated with *B. multivorans* versus *B. cenocepacia* infection. Substantial differences in the infection kinetics between *B. multivorans* and *B. cenocepacia* were observed. Both *B. multivorans* strains tested persisted for up to 16 days in the lung in immune-compromised mice, whereas in contrast neither of the *B. cenocepacia* strains persisted in the lung until that time point, but were

cleared by day 4 of infection. These observations were encouraging as they were not only similar to infection kinetics in the intraperitoneal model, but also able to discriminate between BCC species in a separate system of experimentation. The persistence of *B. multivorans* and *B. vietnamiensis* strains in immune-compromised animals despite the single, low infectious dose, in contrast with the clearance of *B. cenocepacia* strains in the corresponding controls, warranted a more detailed examination of host response to infection by these strains.

No evidence of a vigorous host inflammatory response was observed in histological sections or immunoassay of BAL fluid from mice infected with any of the five strains. While the lack of gross pathological evidence of a pulmonary leukocyte influx is inconsistent with previous observations of BCC infection in the lung (193, 212), the low infectious dose and use of a leukopenic agent may have contributed to this phenomenon. The absence of a significant difference between PBS-treated, infected mice, and their CPA-treated, infected cohorts, in the pro-inflammatory cytokines TNF-α and MIP-2, especially at 3 hours post-infection, suggests that the BCC strains were non-inflammatory at the given dose. While only two cytokines from a long list of molecules associated with pulmonary inflammation were evaluated from BAL fluid, the pleiotropic nature and early induction of TNF-α and MIP-2 suggest that had any of the BCC strains any inflammatory potential, it would have been detected in the TNF-α and MIP-2 levels in the BAL three hours after infection (226, 238).

Evaluation of host cell populations within collected BAL samples revealed that although the BCC strains might not induce a pro-inflammatory response, effective clearance of bacteria may be macrophage-dependent. Repeated CPA treatment resulted in a gradual decrease in the total number of cells; macrophages, comprising the majority of the BAL fluid population, were affected proportionately to the other cells but decreased greatest in absolute number. In concert with these

observations, healthy mice infected with any of the BCC organisms cleared their infections better than their immune-suppressed cohorts. Moreover, the greatest difference between CPA-treated mice and saline-treated controls in terms of macrophage population in the lung coincided with the greatest difference between the same groups in terms of bacterial load. This suggested that resident macrophages played a critical role in control of pulmonary infection with BCC.

While the number of neutrophils recovered in the BAL fluid of infected mice was also markedly low, it is consistent with the low levels of both TNF-α and MIP-2 detected in the BAL fluid. This observation is inconsistent with the typical shift in observed host cell population from a macrophage-dominant one in BAL fluid of uninfected $Cftr^{-/-}$ mice, to one composed predominantly of neutrophils and lymphocytes when animals are repeatedly infected with high doses of *B. cenocepacia* (193). The infectious dose used in that study, however, was three logs greater than the one used in the leukopenic model. While the unconventionally low bacterial challenge titer used in this study was chosen in an effort to mimic a physiologically relevant level of exposure, it may not have provided enough antigenic stimulation for vigorous, differential and measurable immune responses to occur.

Extremely low absolute numbers of cells were recovered in BAL of mice infected with *B. multivorans* C5568 and *B. vietnamiensis* FC811, particularly at early time points in the experiment when CPA effects would not have been yet manifest; this phenomenon was observed in both healthy-infected and immune-suppressed-infected groups alike. The numbers of cells recovered in the BAL from these mice were lower even than that recovered from control mice which received a sterile gHBSS bolus. This suggested that although CPA may have an effect on macrophage presence and function in the lung, these two isolates themselves may also have played roles in modulating host defense to establish persistent infections.

Indirect immunofluorescence of lung sections from all infected mice yielded no evidence that chronic infection was due to invasion of, and survival within, either pulmonary epithelial cells or alveolar macrophages. All visualized bacteria were localized in the alveolar space or in loose association with type II pneumocytes. It is possible that the small fraction of cells visualized by this method is not representative of the infection scenario if foci of infection occur. The effect of CPA on macrophage presence and function in the lung may also be an important factor in the equation; previous studies have shown that CPA treatment reduces not only the number of peripheral blood phagocytes, but phagocytic activity and release of reactive intermediates by these cells (93, 202). A decrease in the resident and recruited macrophage population, coinciding with defect in phagocytic activity in the surviving cells, would contribute to a paucity of macrophages able to internalize bacteria.

3.2.2 Intranasal model

3.2.2.1 Objectives

The differential persistence of BCC strains within the leukopenic model of pulmonary infection, without a corollary difference in host response, prompted the development of a 2nd model of pulmonary infection that would help achieve the original objectives. A simplified version of the leukopenic model was developed to yield the intranasal model of infection. BALB/c mice are subjected to one single, high challenge dose of bacteria in the absence of immune suppression prior to tissue harvest for evaluation of the pulmonary immune response and localization of persistent bacteria on days 1 through 4. The number of animals required for the scope of examination necessitated narrowing the focus of inter-species differences to one representative *B. multivorans* strain C5568 and one *B. cenocepacia* strain C6433.

3.2.2.2 Infection kinetics

Figure 12 displays the infection kinetics of *B. multivorans* strain C5568 and *B. cenocepacia* strain C6433 in the lungs of immunologically normal BALB/c mice. Mice were challenged with ~1.0 X 10^7 CFU intranasally, and viable counts were obtained from diluted lung homogenates at 3 hours, 24 hours, 48 hours and 96 hours of infection. Mice infected with strain C5568 sustained their pulmonary bacterial infection for 4 days. In contrast, strain C6433 was cleared quickly from the lungs of infected mice. A significant difference was observed in the pulmonary bacterial loads at 24 hours ($P = 0.0043$), 48 hours ($P = 0.0022$) and 96 hours ($P = 0.0022$) of infection.

3.2.2.3 Effect of pulmonary infection on animal health

Figure 13 shows the average daily weight change of mice challenged intranasally with *B. multivorans*, *B. cenocepacia* or PBS. The mean weight loss in animals infected with *B. cenocepacia* strain C6433 was significantly greater ($P = 0.004$) than those infected with *B. multivorans* strain C5568 on every day of the experiment (24 hours $P < 0.0001$, 48 hours $P = 0.0005$, 72 hours $P = 0.0397$). At 24 and 48 hours post-infection, mice infected with C6433 experienced mean levels of weight loss of 7.75 $\pm$ 0.34%, and 8.09 $\pm$ 0.57%, respectively. In contrast, although a few animals challenged with C5568 experienced weight loss greater than 5%, at no time during the 4-day infection did the mean weight loss for these animals exceed 5.28 $\pm$ 0.31% total change. In addition to significantly greater weight loss, a higher proportion of C6433 infected animals displayed other signs of systemic illness 24, 48 and 72 hours post-infection (Figure 14). These animals consumed less food, and displayed ruffled fur, cachexia and loss of social behaviour to a greater degree than animals given C5568. Moreover, a greater number of C6433-challenged animals experienced a combination of all three criteria for systemic illness than C5568-challenged animals.

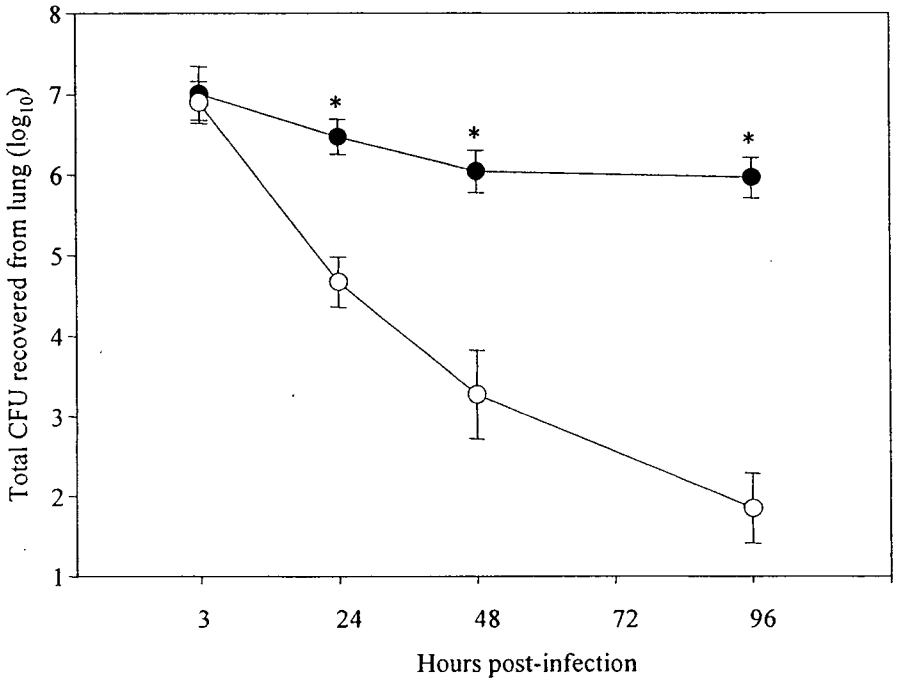

Figure 12. Pulmonary bacterial load after infection with *B. multivorans* strain C5568 (closed symbols) and *B. cenocepacia* strain C6433 (open symbols). BALB/c mice were intranasally challenged with ~1.0 X 10^7 CFU. Quantitative bacteriology of the lung was assessed at 3, 24, 48 and 96 hours. Data are the mean $\pm$ SEM for six animals at each time point, and are representative of two separate experiments. Asterisks denote a significant difference ($P < 0.05$) between C5568- and C6433-challenged groups.

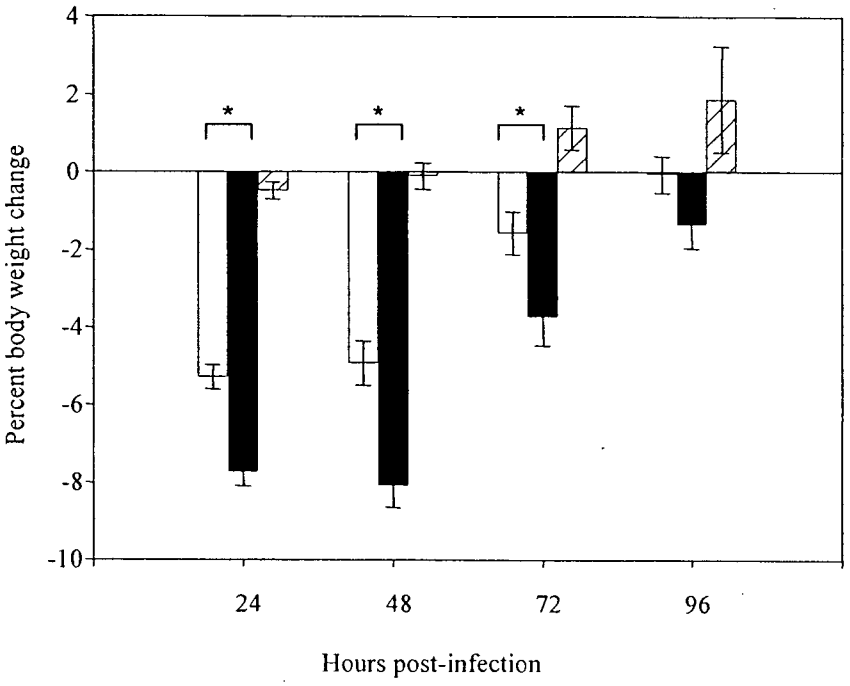

Figure 13. Effect of bacterial infection on body weight fluctuation over time. BALB/c mice were intranasally challenged with ~1.0 X 10^7 CFU of *B. multivorans* strain C5568 (white bars), *B. cenocepacia* strain C6433 (black bars) or sterile PBS (hatched bars). Animals were weighed daily for the duration of the experiment; bars represent body weight change from pre-infection values at time 0. Data are the mean ± SEM for 47 (24 hours), 33 (48 hours) and 18 (72 and 96 hours) animals. Asterisks denote a significant difference ($P < 0.05$) between C5568- and C6433-challenged groups. The total number of animals studied decreased over time as animals were removed for analysis. Mice were sacrificed in the groups to which they were randomly pre-assigned, with no preference for euthanization of sick-looking animals.

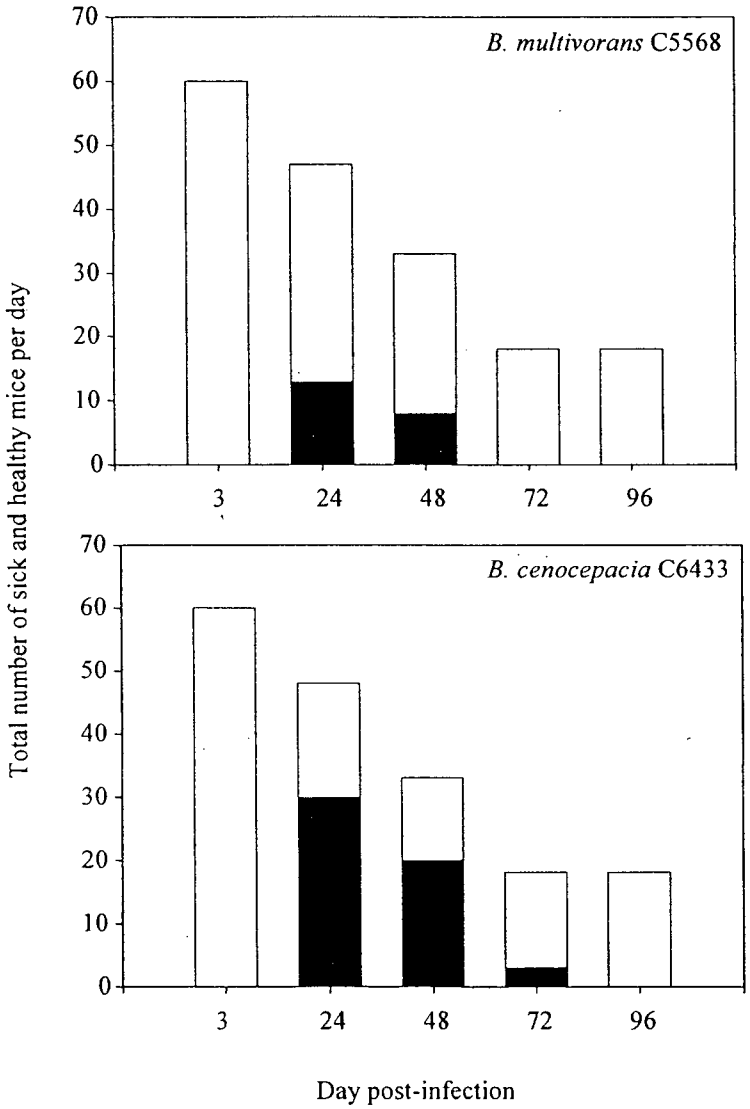

Figure 14. Effect of bacterial infection on animal health over time. BALB/c mice were intranasally challenged with ~1.0 X 10^7 CFU of *B. multivorans* strain C5568, or *B. cenocepacia* strain C6433. Animals were monitored daily for the duration of the experiment and assessed on a three point system for ill health. Unhealthy animals (dark bars) and healthy animals (light bars) are shown for each day. The total number of animals studied decreased over time as animals were removed for analysis. Mice were sacrificed in the groups to which they were randomly pre-assigned, with no preference for euthanization of sick-looking animals.

3.2.2.4 Recovery of viable challenge titer in BAL of infected mice

Quantitative bacteriology of BAL fluid from *B. multivorans* and *B. cenocepacia*-infected mice was performed 3 and 24 hours post infection and compared to bacteriology from lung homogenates. A smaller fraction of the *B. multivorans* strain C5568 bacterial challenge titer was recovered in lavage than the C6433 titer at both time points, Figure 15. At 3 hours post infection, 4.7% $\pm$ 3.6% of the total lung bacterial load was recovered from the lavage of C5568-infected animals, compared with 15.8% $\pm$ 7.8% recovered from C6433-infected animals ($P = 0.0022$). This difference was even greater at 24 hours post infection: only 7.7% $\pm$ 4.2% of the C5568 bacterial load was recovered by lavage, compared with 33.9% $\pm$ 14.8% of the C6433 bacterial ($P = 0.0022$).

3.2.2.5 Cellular analysis of bronchoalveolar lavage fluid of infected lungs

Pulmonary neutrophil and macrophage populations were quantified by Hemacolor stained cytospin of BAL from mice challenged with C5568, C6433, or PBS as a vehicle control, Figure 16. The number of neutrophils in the lungs of infected mice peaked on day 1 of infection for both C5568- and C6433-challenged groups. This maximal number of neutrophils was significantly greater in the BAL fluid of mice infected with C6433 than those infected with C5568 ($P = 0.0173$). The timing of this maximally differential lung cellular response correlated with the point of greatest difference in overall health of infected animals (Figure 14). The number of neutrophils in the lungs of both groups diminished quickly thereafter, with no difference between C5568- and C6433-challenged mice in the recovery phase. The number of macrophages recovered from the BAL of infected mice increased on day 1, peaked on day 2 of infection, and was sustained at day 4. While the number of macrophages recovered in the BAL fluid of infected mice was significantly greater than that recovered from PBS-challenged mice on days 1, 2 and 4 (day 1: $P = 0.0195$ both

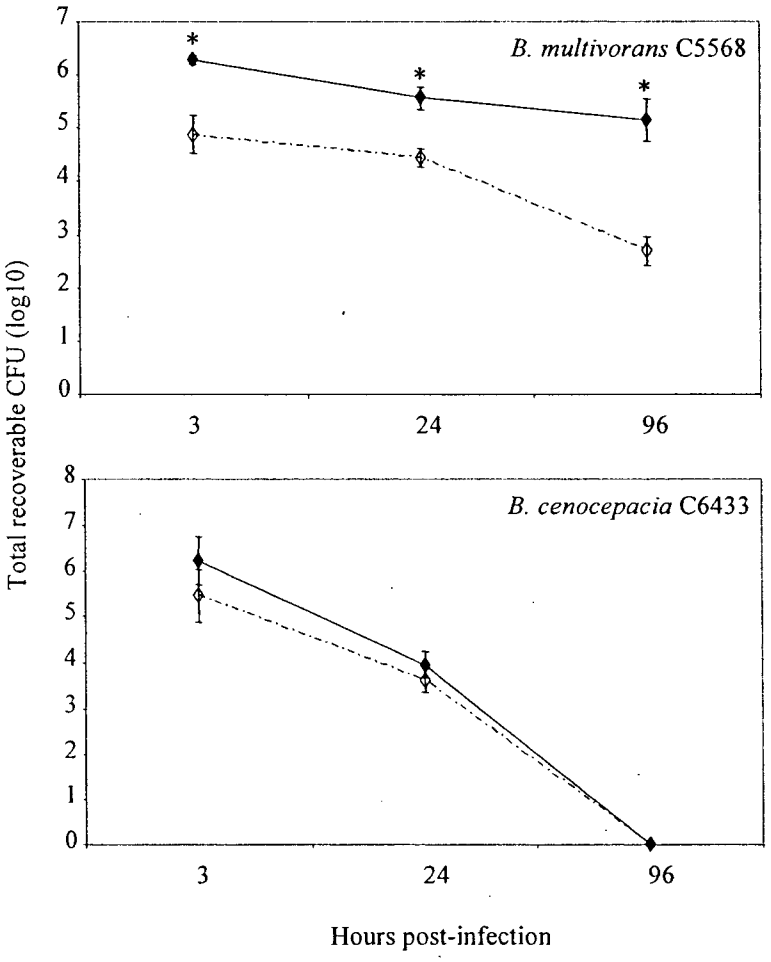

Figure 15. Pulmonary bacterial load recovered after infection with *B. multivorans* and *B. cenocepacia*. BALB/c mice were intranasally challenged with ~1.0 X 10^7 CFU of *B. multivorans* strain C5568 or *B. cenocepacia* strain C6433. Quantitative bacteriology of BAL fluid (open symbols) and homogenates of lavaged lungs (closed symbols) was assessed at 3, 24 and 96 hours post-infection. Data are the mean $\pm$ SEM from six animals at each time point. Asterisks denote a significant difference ($P = 0.0022$, all comparisons) in recovered viable counts between lung lavage and lung homogenate.

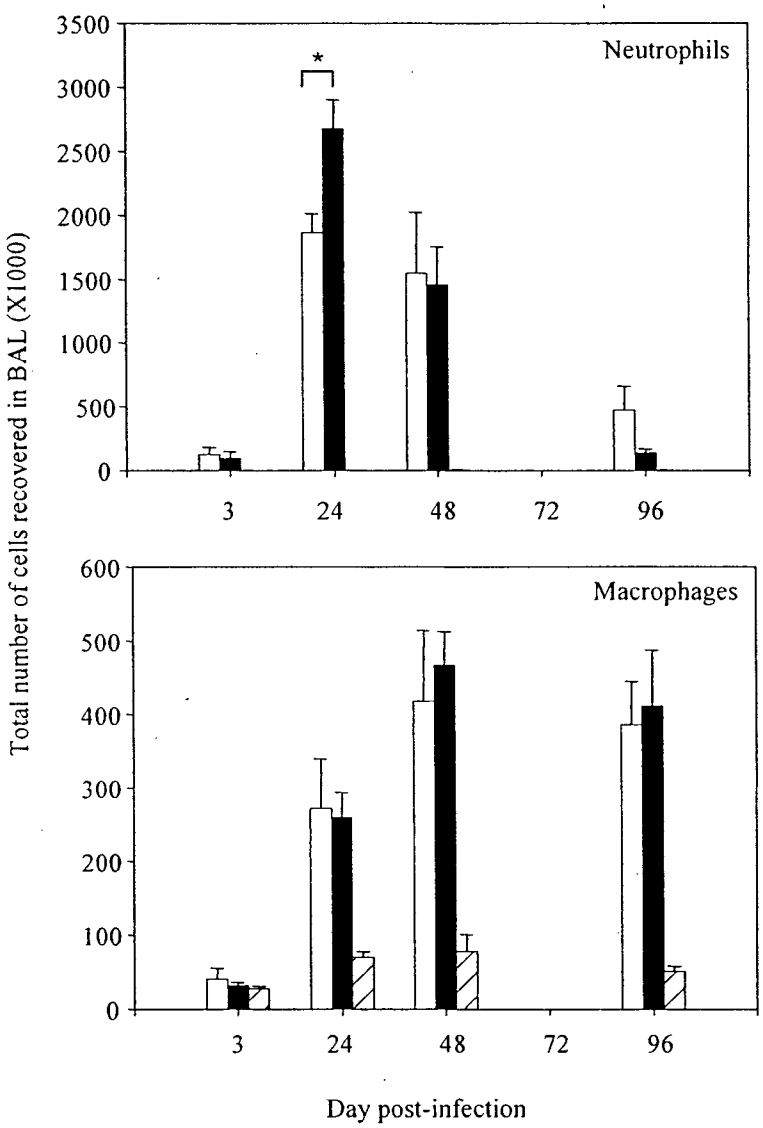

Figure 16. Pulmonary cellular infiltrate following bacterial infection. BALB/c mice were intranasally challenged with ~1.0 X 10^7 CFU of *B. multivorans* strain C5568 (white bars), *B. cenocepacia* strain C6433 (black bars) or sterile PBS (hatched bars). Bronchoalveolar lavages were performed *in situ* at 3, 24, 48 and 96 hours post-infection, and total and differential counts performed. Data are the mean $\pm$ SEM for six animals at each time point. Asterisk denotes a significant difference ($P = 0.0173$) between C5568- and C6433-challenged groups.

comparisons, days 2 and 4: $P = 0.0238$ all four comparisons), there was no difference in the number of macrophages recovered from the BAL of mice infected with each of the two strains.

3.2.2.6 Cytokine analysis of bronchoalveolar lavage fluid of infected lungs

Supernatants of BAL from mice challenged with C5568, C6433 or PBS as a control, were analyzed for the pro-inflammatory cytokines IL-1β, MIP-2 and TNF-α by ELISA, Figure 17. All three cytokines exhibited peak recoverable levels from lung lavage three hours post-challenge, with a quick decline in detectable levels thereafter. The level of IL-1β in C6433-challenged mice was significantly higher ($P < 0.0001$) than the levels detected in C5568-challenged mice three hours post-infection. This difference was resolved by 24 hours post-infection as IL-1β levels quickly dropped, and was not significantly different from levels detected in PBS-challenged mice by 48 hours after infection. The levels of MIP-2 and TNF-α in C6433-challenged mice did not differ significantly from those in C5568-challenged mice on any day of the infection.

3.2.2.7 Immunofluorescent detection of persistent *B. multivorans* C5568 in the lung

Infected lung tissue sections were either immunostained for bacteria prior to Mayer's hematoxylin counterstain, or immunostained for both bacteria and Mac-3 antigen (Figure 18). Mac-3 is a 110 kDa glycoprotein expressed on mouse mononuclear phagocytes, similar to CD107b/LAMP-2 (96). In the lung, Mac-3 is expressed on alveolar macrophages, bronchial epithelium and alveolar lining cells which are most likely interdigitating dendritic cells (74). Lung tissue from both C5568- and C6433- challenged mice were taken for analysis on day 4 of infection, but bacteria were only observed in C5568 infected lungs, in accordance with CFU counts. A total of 98 fluorescently stained bacteria were observed in 44 sections from six lungs infected with strain C5568 (39 in 27 of 28 sections from 3 lungs in one experiment, and 59 in 16 sections from 3 lungs in

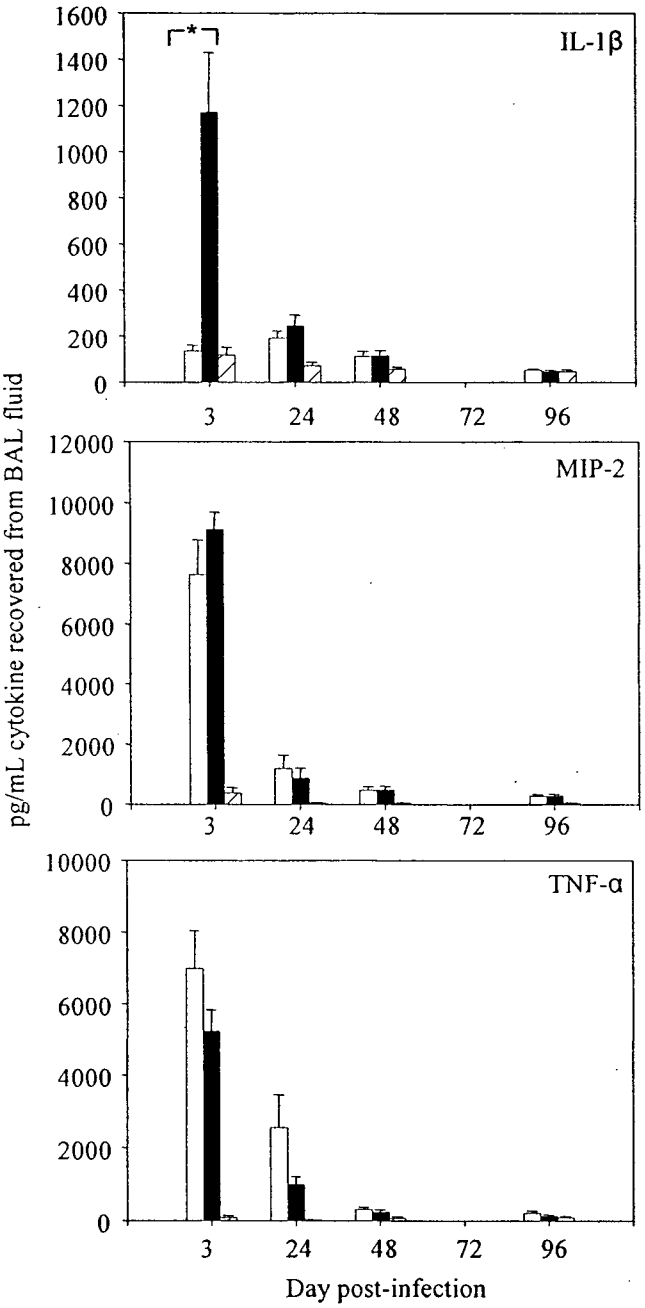

Figure 17. Pulmonary inflammatory cytokine profiles following bacterial infection. BALB/c mice were intranasally challenged with ~1.0 X 10^7 CFU of *B. multivorans* strain C5568 (white bars), *B. cenocepacia* strain C6433 (black bars) or sterile PBS (hatched bars). BAL were performed performed *in situ* at 3, 24, 48 and 96 hours post-infection, and supernatants were evaluated for pro-inflammatory cytokines IL-1β, MIP-2 and TNF-α. Data are the mean ± SEM for six animals at each time point. Asterisk denotes a signficant difference ($P < 0.0001$) between C5568- and C6433-challenged groups.

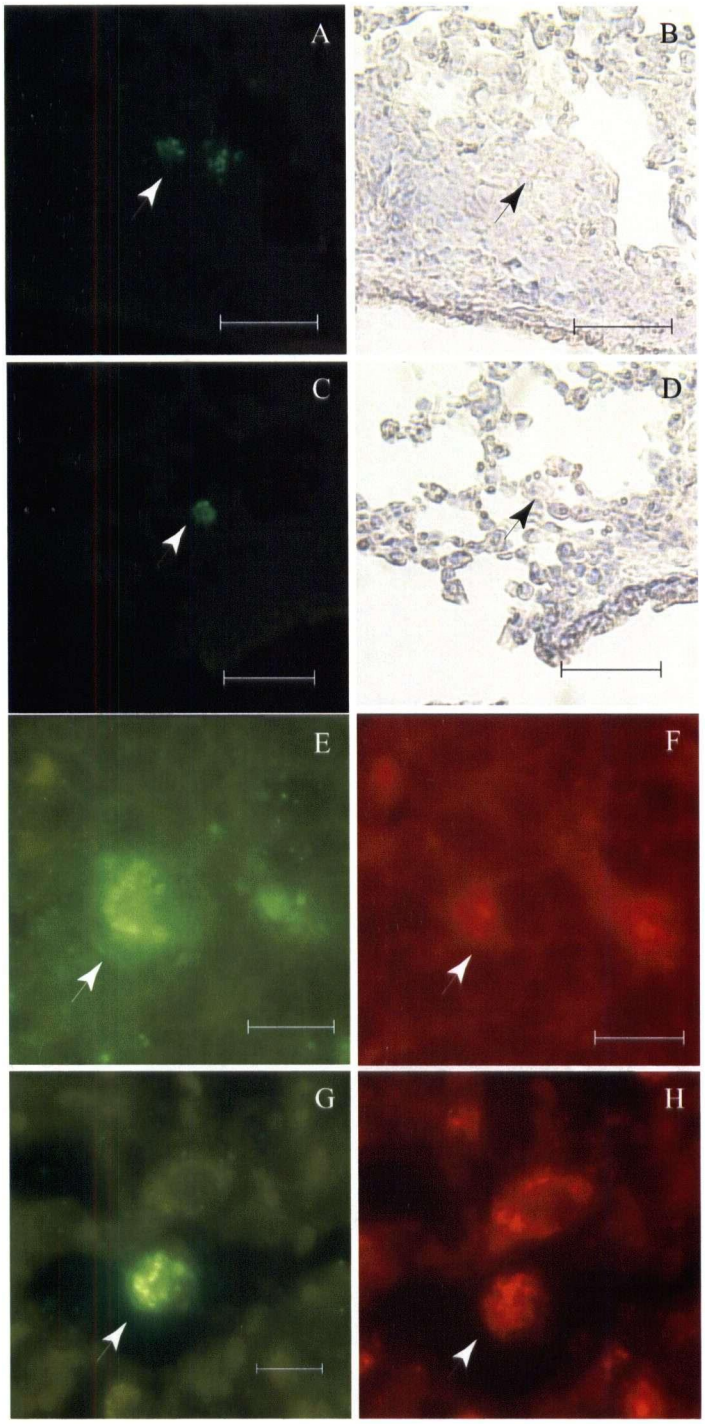

Figure 18. Indirect immunofluorescent detection of persistent *B. multivorans*. Sections were either stained for bacteria prior to hematoxylin counterstain (A-D), or double immunostained for bacteria and the Mac-3 antigen (E-H). Bacteria (green) localized primarily in lymphoid aggregates (A, B), or with mononuclear cells in alveolar space (C, D). Double staining showed bacteria co-localized with Mac-3 positive cells (red) in these areas (E, F lymphoid aggregate, G, H macrophage). Bars A-D = 25 µm, bars E-H = 10 µm.

a second experiment). The number of bacteria observed per section ranged from 0 to 7. Morphometry predicts that cutting 5 μm-thick sections along the depth of a 5 mm lobe of lung would yield 1000 sections; in a pulmonary infection sustaining a total bacterial load of ~10^6 CFU, assuming that the bacterial load is evenly distributed throughout the 2 major lobes of this organ, up to 500 bacteria should be viewed per section. The observation of only 98 fluorscently stained bacteria in 44 sections, a frequency of 2.22, indicates that persistent bacteria may have been localized in foci of infection on day 4. Sections immunostained with a polyclonal antibody specific for BCC showed bacteria localized to lymphoid aggregates in 39.0% of analyses, 7.3% of which were organized as BALT (A & B). 56.1% of fluorescently stained bacteria localized with macrophages (C & D), and 4.9% localized with bronchiolar epithelium (not shown), as assessed by morphology after Hematoxylin counterstain. Similar patterns of bacterial-host association were observed in the sections subjected to double immunostaining. In these sections, 76.5% of bacteria localized to areas also positive for the Mac-3 antigen. 28.8% of these double-positive loci were cells within lymphoid aggregates (E & F), and 51.9% were identified as alveolar macrophages on the basis of their location within alveolar space (G & H). 5.8% of double positive cells appeared to be bronchiolar epithelia, whereas 13.5% of double-positive cells occurred in areas of collapse or excessive erythrocyte penetration, obfuscating cellular identification. Of the 23.5% fluorescently stained bacteria that did not occur in Mac-3 positive loci, over half (13.2%) were in lymphoid aggregates, while the rest occurred in collapsed areas obscuring cellular identity. These observations all strongly suggest that on the fourth day of a persistent infection in an immune-competent mouse, *B. multivorans* C5568 localized primarily with macrophages in the lung.

3.2.2.8 Transmission electron microscopy

Lung samples were processed from mice on the 4th day after intranasal challenge with *B. multivorans* strain C5568 to investigate the site of persistence, with PBS-challenged lung as a control. Semithin sections (0.5 μm) stained with toluidine blue were viewed by light microscopy to gain a general orientation of the section, and to help select areas of examination by electron microscopy. Observations from lung histology indicated that one out of every 300 viewed macrophages would likely be associated with bacteria. A total of 1000 macrophages were viewed in 30 semithin sections from 3 different *B. multivorans*-infected tissue blocks. Four macrophages containing bacterium-like structures were observed in 1 semithin section, and these macrophages were further examined in ultrathin sections on 2 different grids by transmission electron microscopy (TEM). Figure 19 shows TEM of *B. multivorans* C5568-challenged and PBS-challenged lung on day 4. Electron-dense bacteriomorphic structures were observed in membrane-bound vacuoles of macrophages in the alveolar space (A-D). Macrophages in *B. multivorans*-challenged lung samples, regardless of whether or not they contained bacteria, were more numerous and slightly larger than macrophages in PBS-challenged lung (C). Many of these cells also had large vacuoles containing membranous material (asterisks in C); although the identity of these structures was not confirmed with colloidal gold staining, acid phosphatase activity staining or late phagosomal markers, the presence of intraphagosomal vesicular material suggests that these structures were phagolysosomes (6, 71, 113). No bacteria were observed in conducting airways or lymphoid aggregates by TEM. One hundred macrophages were examined in 25 semithin sections from 1 tissue block from a PBS-challenged lung, a decreased number of cells per section compared with infected tissue. Macrophages from

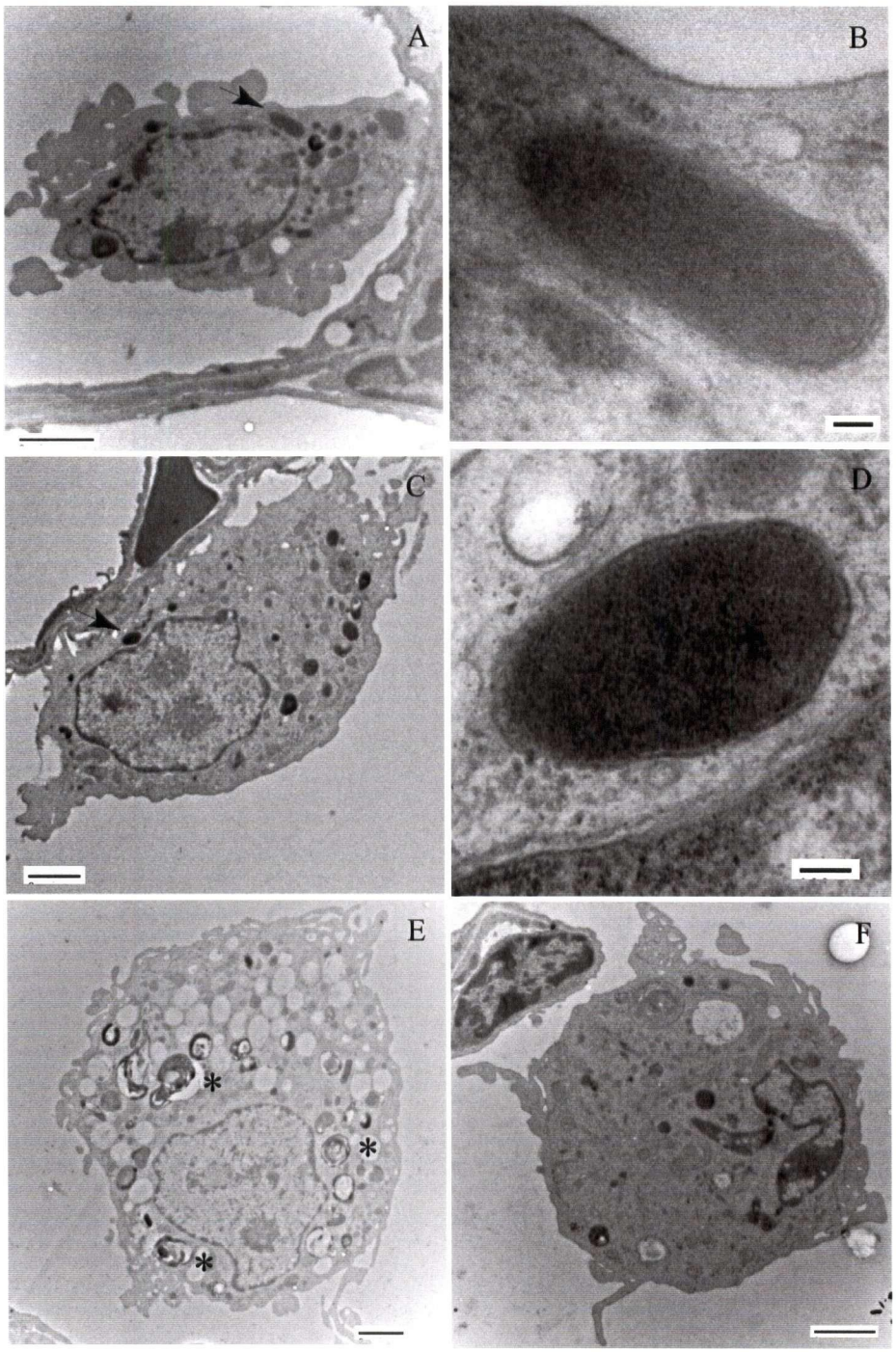

Figure 19. Transmission electron micrographs of *B. multivorans*-infected lung (A-E) and PBS-challenged lung (F) at day 4. Electron-dense bacteriomorphic structures (B, D) were found in vacuoles of alveolar macrophages (arrows in A, C). B & D are high magnification micrographs of the structures in A & C, respectively. E: macrophages in infected lung were often vacuolized and some contained phagolysosomes (asterisks). F: alveolar macrophages from PBS-challenged lung on day 4. Lower magnification micrographs taken at 8,830-15,100x magnification, higher magnification micrographs taken at 101,000x magnification. A, C, E, F bar = 2 μm; B, D bar = 100 nm.

PBS-challenged lungs appeared slightly smaller than macrophages from *B. multivorans*-infected lungs, and had few cytoplasmic vacuoles and no phagolysosomes (D). No bacteriomorphic electron-dense entities were observed in macrophages or any other cell types of PBS-challenged lungs.

3.2.2.9 LPS gel electrophoresis

The rapid clearance of *B. cenocepacia* strain C6433, coinciding with a more pronounced host response in the form of IL-1β output and neutrophil influx into the lung, suggested that strain C6433 was more pro-inflammatory than *B. multivorans* C5568. The endotoxic capacity of LPS, particularly LPS produced by members of the BCC, has been documented (169, 180, 200). While no correlation was found between LPS chemotype and virulence in a previous study (69), crude LPS extracts from strains C5568 and C6433 were compared in view of the differential inflammatory potential of these strains. LPS from *B. multivorans* C5568 and *B. cenocepacia* C6433 was extracted from overnight cultures by the proteinase K digestion method of Hitchcock and Brown (95). Samples were electrophoresed on a 12% polyacrylamide gel and stained with a commercial kit prior to viewing. While both strains C5568 and C6433 expressed the smooth form of LPS, the banding patterns were different between the two strains, Figure 20. The slower migration of the outer core sugars from strain C6433 suggests higher molecular weight polysaccharides on strain C6433 compared with strain C5568.

3.2.2.10 Discussion

A non-invasive, pulmonary model of BCC infection in an immune-competent murine host, which differentiates members of two clinically relevant BCC species by virtue of *in*

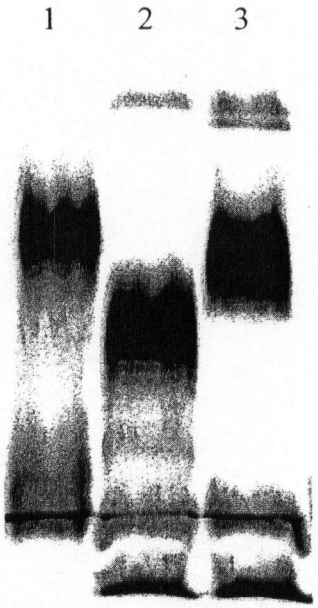

Figure 20. Analysis of lipopolysaccharide (LPS) by polyacrylamide gel electrophoresis. Crude LPS extracts were isolated by the proteinase K method of Hitchcock and Brown. Samples (5-10 μg) were separated through a 12% acrylamide gel and stained with the Molecular Probes LPS Gel Stain Kit. Lane 1, smooth LPS from *E. coli* serotype O55:B5, lane 2, LPS from *B. multivorans* strain C5568, lane 3, LPS from *B. cenocepacia* strain C6433.

vivo persistence and toxicity, has been described. In this model, persistent *B. multivorans* was found in association with pulmonary macrophages, while *B. cenocepacia* was cleared from the lung by inducing a strong inflammatory response from host cells. The differential infection kinetics and host health consequences observed in this system were consistent with those made in an earlier model of pulmonary infection, the leukopenic intranasal model. That both systems show persistence of one species and clearance of another, despite the differences in host immune status and challenge dose, substantiates the observations made about these two species.

The current model of pulmonary infection proved more informative than the leukopenic model of pulmonary infection with regard to the host response and its role in persistence or clearance of infection. Bronchoalveolar lavages demonstrated significant differences in host cytokine and cellular responses, and tissue samples retained sufficient numbers of bacteria for immunolocalization of persistent species. Observed differences in animal health and host response could be correlated with different bacterial challenge species, and were not obscured by possible effects of immunosuppression, as was the case in the earlier model. Moreover, the latter model yielded important *in vivo* evidence for intracellular survival as a mechanism of pathogenicity for the BCC.

A significant difference in host response to the two BCC strains was observed within hours of pulmonary instillation. Both *B. multivorans* strain C5568 and *B. cenocepacia* strain C6433 induced peak levels of pro-inflammatory cytokines in the lung by three hours post-challenge, but the level of IL-1β induced by C6433 was significantly higher than that induced by C5568. This difference preceded a significantly greater neutrophil recovery from the lungs of C6433-infected mice than from C5568-infected mice at day 1. Although MIP-2

(mouse homologue of IL-8) is associated with neutrophil recruitment (20), no appreciable difference was observed in the level of MIP-2 in the lungs of mice infected with either C5568 or C6433. Associated with a broad repertoire of biological activity, IL-1β is also chemotactic for neutrophils *in vivo* (176, 196). It is possible that the difference in the number of neutrophils in the lung 24 hours post infection was a reflection of the IL-1β levels at three hours post-challenge, likely generated by resident alveolar macrophages in response to the high level of C6433 antigen exposure (70). IL-1β has been described as "the prototypic pro-inflammatory cytokine", because of its broad spectrum of biological activities (60). The rapid clearance of C6433 from the lung could be attributed to the increased expression of IL-1β, which plays a role not only in neutrophil mobilization, but also in granule exocytosis and elastase release, thereby presenting an amplified neutrophil activation in response to C6433 (18, 206). C5568, which also elicited production of the chemokine MIP-2, induced much less of an IL-1β response than C6433; this may have benefited C5568 in that both resident and incoming host cells lacked a second level of activation that IL-1β may have provided.

These differences in host response suggest that C6433 is more pro-inflammatory than C5568. The vigorous inflammatory response to C6433 may reflect the expression or up-regulation of bacterial products with greater pro-inflammatory capacity than those expressed by strain C5568. While the pro-inflammatory nature of BCC LPS has been well studied, all published reports of BCC LPS inflammatory potential have been conducted with BCC strains belonging to *B. cenocepacia.* LPS gel electrophoresis of crude extracts from C5568 and C6433 demonstrated that these two strains expressed different types of the smooth form of LPS, suggesting a possible factor for the differential host responses. While lipid A is the biologically active component of LPS (77, 250), the O-polysaccharide has been shown to

play a role in host-bacteria interactions as well. Expression of O-polysaccharide, and the resulting smooth form of LPS, in many members of the Enterobacteriaceae, confers resistance to complement-mediated killing and phagocytosis (11, 119). These biological activities have also been correlated to length of the O-polysaccharide and its distribution within the outer membrane (83, 90, 120). A more detailed examination of the O-polysaccharides produced by C5568 and C6433 is necessary in order to determine whether differences in this LPS component could contribute to virulence.

Additional virulence factors have also been described for *B. cenocepacia*, including a hemolysin that induces apoptosis in human neutrophils, and a type III secretion system with a role in pathogenesis in the mouse and the capacity to export other virulence determinants (111, 233). Alternatively, C6433 may be more pro-inflammatory than C5568 because antigens, such as flagella, are constantly exposed to responsive host cells, since it does not establish an intracellular infection. *In vitro* experiments involving the exposure of different host cell types to both whole bacteria and bacterial products to examine whether C6433 truly elicits more of a cytokine response than C5568 could provide more insight in to the primary source and nature of the host response. The current model also provides a valuable system in which to evaluate pathogenesis of, and host response to, BCC virulence determinant mutants.

The observation that *B. cenocepacia* C6433 was rapidly cleared from the murine lung appeared initially counter-intuitive and inconsistent with the clinical observation that *B. cenocepacia* infections in CF patients are chronic and notoriously difficult to eradicate (84). This inconsistency may relate to the choice of inbred mouse strain in these studies, which is resistant to infection with *Pseudomonas*, a closely related pathogen, and the absence of *Cftr* mutation and consequent complicated immune scenario in the CF lung (222). In the context

of the CF lung, a more pronounced inflammatory response does not correlate with infection resolution (35). It is possible that CF neutrophils, which are present in the lung in both high numbers and at high levels of activation, may not be able to effectively respond to the immune response induced by *B. cenocepacia* strains, whereas the ability of *B. multivorans* strains to gain an intracellular niche may shield them from the host response (50, 229). Nevertheless, the pathogenic differences between species revealed in this model may highlight key elements of virulence and host responses, which may be relevant in BCC infection in CF.

Despite induction of a pro-inflammatory response in the host, strain C5568 was able to persist. The inflammatory response induced by C5568 was less vigorous than that induced by C6433, as evidenced by the lower levels of IL-1β and neutrophils in the lung early in the course of infection, and resulted in less severe systemic symptoms. Quantitative bacteriology of BAL fluid from C5568 and C6433-infected mice showed that a smaller proportion of the C5568 pulmonary bacterial load was recovered in lavage, than the C6433 titer, early in infection. These observations suggest enhanced cell association of C5568 from an early stage, and are supportive of a model in which C5568 persists in the lung by surviving and replicating in host phagocytes. The majority of these host cells may be located in interstitial compartments of the lung and therefore would be difficult to access via lavage.

Indirect immunfluorescent staining of the infected lung showed that persistent C5568 bacteria were found in association with host macrophages on the fourth day of infection. Transmission electron micrographs of similarly infected lungs provided further evidence that the alveolar macrophage acts as a host for *B. multivorans*. Both observations strongly indicate that intracellular localization may be protective for the bacteria and have a role in

diminishing the host response. Whether C5568 actively invades host cells, as in the case of the coiling phagocytosis induced by *Legionella pneumophila* strain Philadelphia 1, or is simply amenable to phagocytosis by macrophages and evades host-mediated killing thereafter, as in the cases of *Mycobacterium* and *Legionella*-induced arrest of phagosomal maturation, is unclear (78, 101-102). The immunofluorescent localization of persistent bacteria in lymphoid aggregates of the lung suggests that host macrophages or dendritic cells migrate to these areas to present antigen after internalization of bacteria (19, 129). Whether or not antigen presentation actually takes place in these areas is unclear. Studies of the host response to *Mycobacterium* infection have shown that bacilli are taken up in the BALT of rabbits by phagocytes prior to transport to lymph nodes, while aerosol infection of guinea pigs with *Mycobacterium* has demonstrated tubercle formation in BALT late in the course of disease; evidence that pulmonary lymphoid tissue is a site that pathogens do encounter on lung infection (148, 179).

Alternatively, persistent bacteria in lymphoid aggregates may have been carried there by professional antigen presenting cells such as dendritic cells, and delivered to resident macrophages within these structures (205, 234). The role of these sites as immune-privileged niches for strain C5568 could be explored by future localization of bacteria in these structures at a later infection time point. The persistence of strain C5568 in the murine lung might therefore be aided in part by this strain's ability to survive within host macrophages, and in part by the compartmentalization and processing of the host response itself (15, 205, 234).

3.3 *In vitro association*

3.3.1 Objectives

The immunofluorescent and electron microscopic localization of persistent *B. multivorans* C5568 to pulmonary macrophages initiated a more extensive investigation into the degree of association between the BCC and this putative host cell. The objective of this series of studies was to examine and compare the association of *B. multivorans* C5568 and *B. cenocepacia* C6433 with macrophages from three different sources and levels of differentiation. Bacterial binding and adherence to an immortalized murine alveolar macrophage cell line, primary murine alveolar macrophages obtained via BAL, and human monocyte-derived macrophages were assessed.

3.3.2 Association of *B. multivorans* and *B. cenocepacia* with primary murine alveolar macrophages

Primary alveolar macrophages were evaluated for their ability to bind zymosan, *B. multivorans* C5568 and *B. cenocepacia* C6433. BAL was performed on BALB/c mice and macrophages were cultured on glass coverslips for 72-96 hours prior to incubation with particles. The degree of adherence was measured and reported as the percent of macrophages binding particles, and the mean number of particles bound by 100 macrophages (association index). Primary alveolar macrophages bound zymosan particles extremely well, with the majority of cells (65.5% $\pm$ 2.5%) binding more than 10 zymosan particles and a mean of 12.6 $\pm$ 0.1 zymosan particles per cell, Figures 21 and 22.

B. multivorans strain C5568 bound to primary alveolar macrophages better than *B. cenocepacia* C6433 after a three hour incubation. While the majority of macrophages

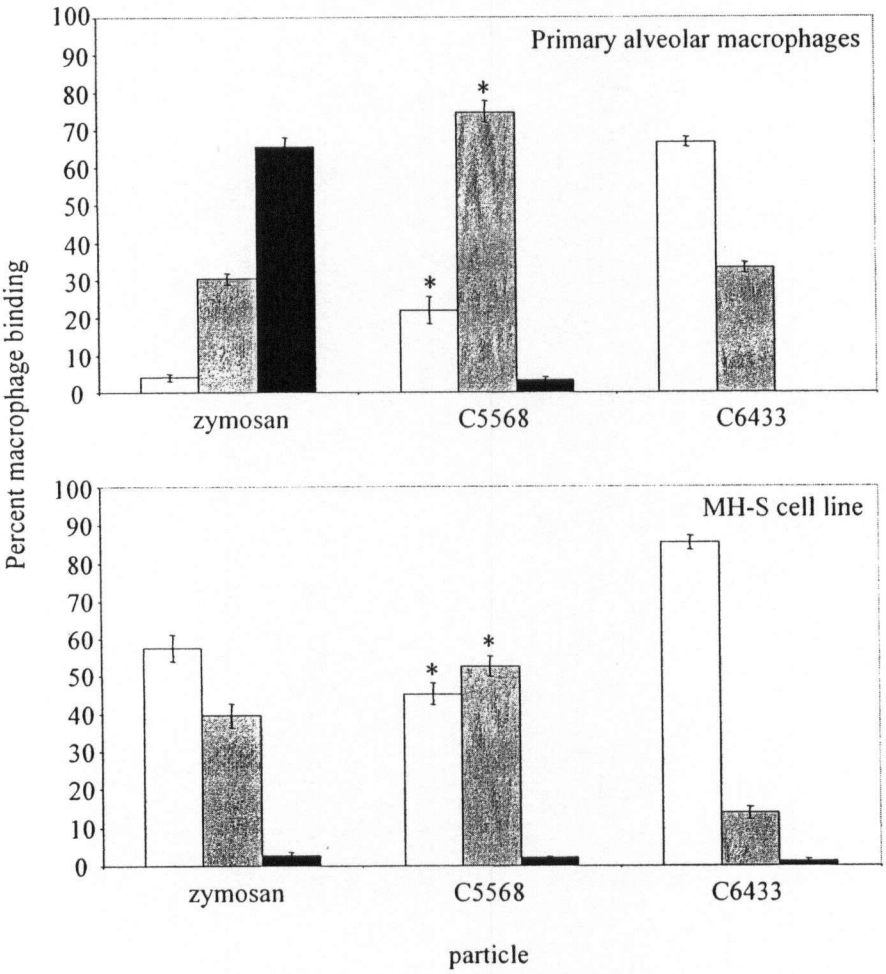

Figure 21. Association of primary alveolar macrophages and the alveolar macrophage cell line, MH-S, with zymosan, *B. multivorans* C5568 and *B. cenocepacia* C6433. Macrophage cultures on glass coverslips were incubated with particles for three hours at 37°C in the absence of serum, at an MOI of 50:1. The mean percentage of macrophages binding 0 particles (white bars), 1-10 particles (grey bars) and >10 particles (black bars) is reported. Data are the mean ± SEM for $n = 6$ (3 separate experiments with duplicate coverslips in each experiment). Asterisk denotes a significant difference ($P < 0.0001$) in each evaluation group between macrophages exposed to C5568 and those exposed to C6433.

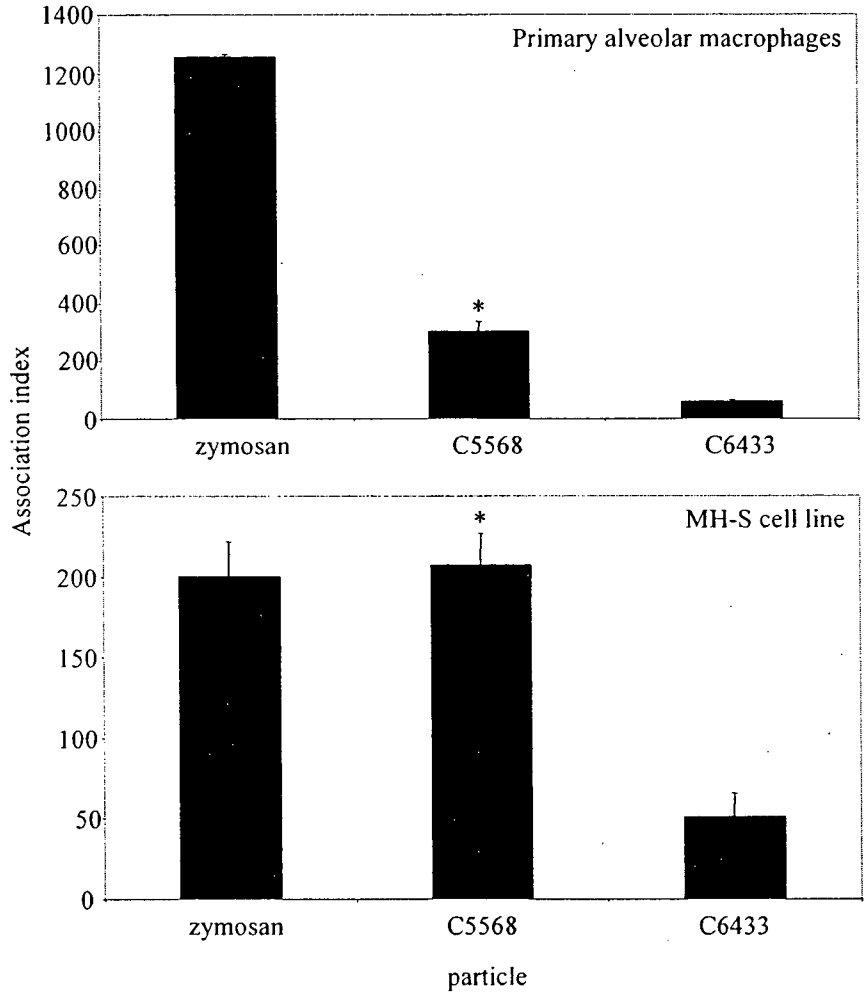

Figure 22. Binding of zymosan, *B. multivorans* C5568 and *B. cenocepacia* C6433 to primary alveolar macrophages and the alveolar macrophage cell line, MH-S. Macrophage cultures on glass coverslips were incubated with particles for three hours at 37°C in the absence of serum, at an MOI of 50:1. Binding was assessed as the number of particles associated with 100 macrophages (association index). Data are the mean ± SEM of n=6 (3 separate experiments with duplicate coverslips in each experiment). Asterisks denote a significant difference ($P < 0.0001$) between C5568- and C6433-exposed groups.

exposed to C5568 were associated with 1-10 bacteria (74.7% ±2.9%), the majority of those exposed to C6433 (66.7% ± 1.3%, $P = 0.002$) did not bind any particles; only 33.3% ± 1.3% of the latter group were associated with 1-10 bacteria, Figure 21. The mean number of bacteria bound per 100 macrophages was also higher for macrophages incubated with C5568 than with C6433, Figure 22. Primary alveolar macrophages bound almost five times as many C5568 bacteria as C6433 bacteria (association index 304.3 ± 33.6 for C5568 versus 63.3 ± 2.7 for C6433, $P = 0.002$).

3.3.3 Association of *B. multivorans* and *B. cenocepacia* with the murine alveolar macrophage cell line, MH-S

The murine alveolar macrophage cell line, MH-S, was also evaluated for association with zymosan, *B. multivorans* C5568 and *B. cenocepacia* C6433. Macrophages cultured on glass coverslips for 72-96 hours were incubated with particles for three hours prior to enumeration of adherent particles. While MH-S cells did not bind zymosan or bacteria as well as primary alveolar macrophages, a similar pattern of association was observed with C5568 and C6433. 52.6% ± 2.7% of macrophages incubated with C5568 bound 1-10 bacteria, while only 13.7% ± 1.6% of macrophages incubated with C6433 bound 1-10 bacteria ($P < 0.0001$); the majority (85.3% ± 1.7%) of the latter group bound no bacteria at all, Figure 21. The mean number of bacteria bound per 100 macrophages was also higher for MH-S cells incubated with C5568, association index 207.2 ± 20.2, than cells incubated with C6433, association index 50.9 ± 14.7, $P < 0.0001$ (Figure 22).

3.3.4 Association of *B. multivorans* and *B. cenocepacia* with human monocyte-derived macrophages

Human monocytes were isolated from peripheral blood and cultured for five days prior to incubation with *B. multivorans* C5568 and *B. cenocepacia* C6433. Macrophages in suspension were co-incubated with bacteria in tumbling polypropylene tubes at 37°C for one hour in the absence of serum, at an MOI of 100:1. 100 μL aliquots were cytocentrifuged onto glass slides and stained with Hemacolor to facilitate enumeration of bound bacteria. MDM association with *B. multivorans* C5568 was not significantly different from association with *B. cenocepacia* C6433, Figure 23. Nonetheless, human MDM incubated with C5568 associated with an apparently greater mean number of bacteria than MDM incubated with C6433 (Figure 23). Calculation of power (β error = 0.20) indicated that a sample size of n = 54 for this particular set of experiments could have yielded significantly different results for C5568 and C6433; the large donor to donor variation likely yielded the high SEM. While this number is within the realm of testability, the amount of time needed to bleed and culture MDM from 54 donors prohibited this scope of study.

3.3.5 Discussion

Binding experiments with both primary alveolar macrophages and MH-S cells showed that both groups of murine macrophages bound strain C5568 better than C6433, suggesting that C5568 may express a ligand for a host receptor that augments binding and uptake of this strain by macrophages. Whether this initial difference in bacterial uptake contributes to the apparent long-term intracellular survival of *B. multivorans* strain C5568 is unclear. It is tempting to suggest that strain C5568 has the capacity to modulate its own

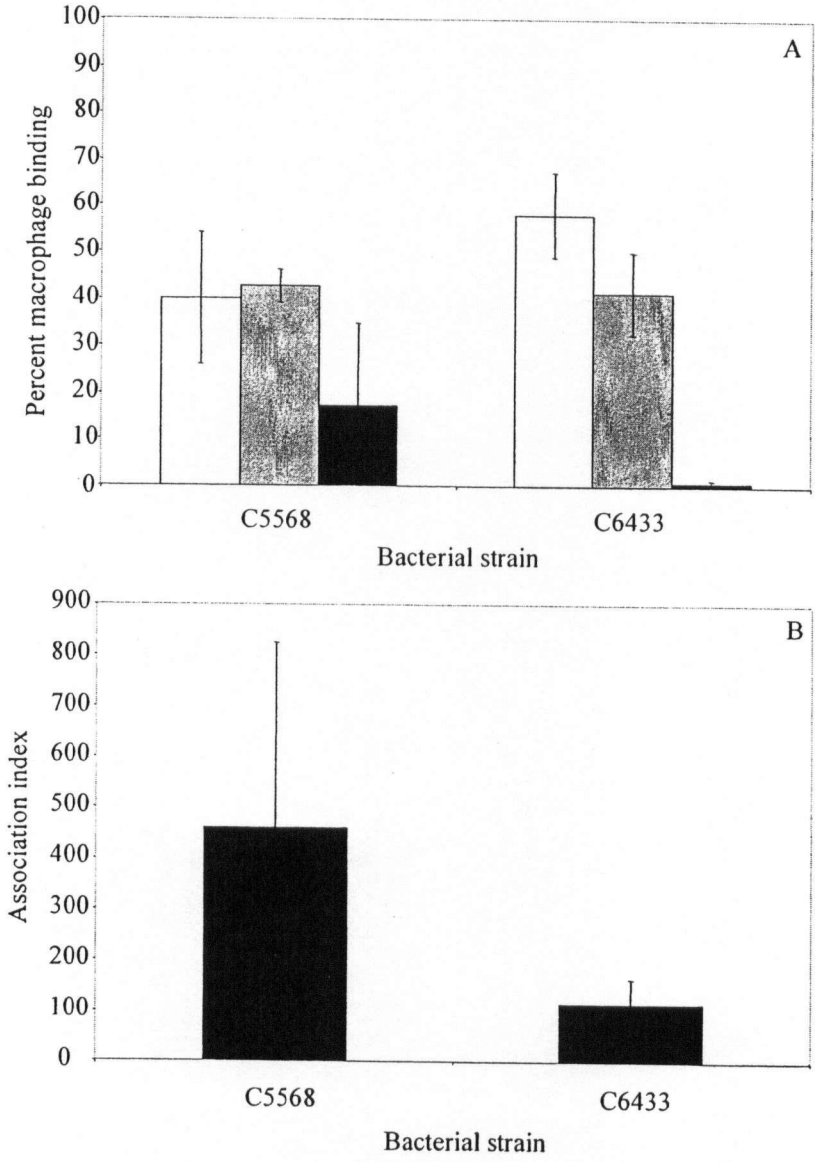

Figure 23. Association of human monocyte-derived macrophages (MDM) with *B. multivorans* strain C5568 and *B. cenocepacia* strain C6433. MDM in suspension were incubated with particles for one hour at 37°C in the absence of serum, at an MOI of 100:1. The mean percentage of macrophages binding 0 bacteria (white bars), 1-10 bacteria (grey bars) and >10 bacteria (black bars) is reported, A. Binding was also assessed as the number of bacteria associated with 100 macrophages (association index), B. Data are the mean ± SEM for three experiments.

phagocytosis via a pathway that is conducive to its own survival in the host macrophage, as do *Salmonella typhi* and *Leishmania major*. Entry of *S. typhi* via the complement receptor 3 (CR3) results in normal maturation of the phagolysosome, while phagosomes formed after complement receptor 1 (CR1)-mediated uptake of *S. typhi* are permissive to survival and intracellular replication (115). The promastigote parasite *L. major* also exploits CR1-mediated phagocytosis to enter and replicate inside macrophages that do not make superoxide (54).

Examples of receptor-ligand interactions of pulmonary intracellular pathogens that modulate phagosome maturation and trafficking, however, are scarce. A host of mycobacterial receptors on human macrophages include CR1, CR3, CR4, the mannose receptor (MR), CD14 and the surfactant protein A receptor; of these CR3 and MR interactions have been most extensively studied (68). While *M. tuberculosis* is able to associate with and gain entry into host macrophages via binding of its capsular polysaccharides to the β-glucan portion of the CR3, internalization of the bacteria by this pathway is not related to subsequent survival and intracellular replication, nor is it related to host disease (103, 160). Human macrophages that internalize particles via the MR do not produce superoxide, nor do their phagosomes accumulate the lysosomal marker Hck, suggesting that uptake of mycobacteria via binding of lipoarabinomannan in the bacterial cell wall by MR is conducive to intracellular survival by virtue of maturational arrest of phagosomes (8).

Differences in association with human MDM between *B. multivorans* and *B. cenocepacia* were not as dramatic as the trends demonstrated with murine alveolar cells. This may be due to differences in the source of macrophages used; the inferior binding of

zymosan by MH-S cells in comparison with primary murine alveolar and peritoneal macrophages has been described (161). Different phagocytic capacities have been reported for macrophages isolated from guinea pig, rat, rabbit and human in an acid-induced model of uptake; particulate uptake by alveolar macrophages from rat, dog and monkey are also dissimilar (197, 230). Differential bacterial uptake by macrophages from different sources may also indicate host-specific tropism, evidenced in the species-specific uptake and survival of various BCC species within amoebae and macrophages alike (153-154). Another consideration lies in the congenic nature of the source of the primary alveolar macrophages and the MH-S cells, the BALB/c mouse, in contrast with the highly heterogeneous humans from which monocytes were isolated. The phenotypic variability of monocytes from different human individual sources would presumably be greater than that of macrophages from a group of congenic mice, which may have been reflected in the binding experiments in this study.

4 General Discussion

Three general sets of questions were iterated during the course of this thesis, reflected in the thesis objectives. 1) Can the apparently species-specific disparate infection outcomes observed in chronic BCC infections of CF individuals be directly or indirectly modeled *in vivo*? 2) Should such a model be successfully established, could long-term survival in the host be proven to be a product of intracellular parasitism? 3) Can the ultra-inflammatory nature of clinical BCC isolates be manifested in the model system, and is it truly restricted to *B. cenocepacia* strains?

In Canada and the United States, *B. cenocepacia* numerically dominates all BCC infections of CF patients (146, 215). In Canada, *B. cenocepacia* infections are associated with the highest rates of transmission, morbidity and mortality, while *B. multivorans* infections are associated with much lower corresponding figures (217). The first objective was to determine whether an *in vivo* system could be developed that might directly or indirectly reflect these differences. The establishment of chronic infection in these models would facilitate the evaluation of pathogenic differences and site of long-term bacterial survival.

Three different animal models were explored during the course of investigation. Chronic colonization was established in all of them, and a distinct species-specific pattern of infection was manifest in each. *B. multivorans* strains persisted in the spleen in a systemic model of infection, and in the lung in two different pulmonary models of infection. *B. cenocepacia* strains were cleared from all organs in all models. This reproducibility in the kinetics in three separate animal models supports the concept of differential virulence of *B.*

multivorans and *B. cenocepacia* infections. Localization of persistent bacteria to a host cell was the obvious next step. In addition, the apparently asymptomatic nature of *B. multivorans* infections, in contrast with the systemic illness caused by *B. cenocepacia* infections, provided a platform for comparing the inflammatory potential of different BCC species.

The rapid clearance of *B. cenocepacia* strains from all infection models appears discordant with the clinical observation that *B. cenocepacia* infections in CF are extremely difficult to eradicate (84). Mice are inherently resistant to infection with pseudomonads, and the choice of the BALB/c mouse, an inbred strain shown to be resistant to the closely related species *Pseudomonas* species, as a model host for pulmonary infection is a definite factor in this discordance (222). It is possible that *B. cenocepacia* persistence in the CF lung is as much a product of the hyperactive but ineffective immune response against bacteria growing in biofilms as of the invasive capacity of these organisms (48, 199). While previous studies have provided *in vitro* and *in vivo* evidence of short-term survival of *B. cenocepacia* strains within mammalian host cells, these observations were made immediately after challenge with high bacterial titres (34, 154). Invasion and intracellular survival in host cells by *B. cenocepacia* strains in the pulmonary infection models in this study may not have been manifest in the face of immune-competent hosts with the ability to clear infection, particularly in response to pro-inflammatory strains.

The chronic infections established by *B. multivorans* in both the spleen and the lung provided an *in vivo* scenario in which intracellular parasitism as a pathogenic mechanism could be explored. A professional phagocyte that performs the dual role of scavenger and antigen-presenting cell, the macrophage is a host for the pulmonary pathogens *M. tuberculosis* and *L. pneumophila*, the close BCC relative *B. pseudomallei*, and thus is a prime

candidate host cell for the BCC (78, 100-101). *B. multivorans* associated with mononuclear cells in the white pulp of the spleen, and with mononuclear cells – lymphocytes and macrophages – in the lung. The parity in the observations made in spleen and lung, in concert with the identification of pulmonary cells associated with persistent bacteria via Mac-3 positivity in immunohistology and morphology in electron microscopy, endorses the role of the macrophage as primary host target for members of the BCC. While the observation that *B. multivorans*, but not *B. cenocepacia*, is able to persist in host macrophages is inconsistent with previous studies that focused on the invasive nature of CF epidemic strains (24, 34, 154, 187), the findings in this study lend solid evidence that members of the BCC can indeed be internalized by host cells, and be identified in a morphologically whole manner in a relatively chronic *in vivo* scenario.

Persistence of bacteria in lymphoid structures in both spleen and lung also highlights their possible role as immune-privileged sites for opportunists. As discussed, mycobacteria take advantage of pulmonary lymphoid tissue during chronic infection (148, 179). What is still not entirely clear is whether strain C5568 actively invaded these tissues, or whether the bacteria were transported there by macrophages or dendritic cells upon engulfment, for antigen presentation. The concomitant localization of persistent bacteria in alveolar macrophages by TEM suggests that the latter may be the case: *B. multivorans* may use the macrophage as its Trojan horse into the lymphoid aggregate. The role of the macrophage as a host cell and lymphoid tissue as site of chronic infection, however, needs to be further investigated by examination of chronically infected tissues beyond the fourth day of challenge. The role of other mononuclear leukocytes in the spleen and lung in *B. multivorans* evasion of rapid clearance, however, cannot be dismissed. Lack of positive

evidence does not exclude the dendritic cell or T lymphocyte, both cells present in the white pulp of the spleen and in the lung, from bacterial interaction. As a professional antigen presenting cell, the dendritic cell in particular may play an important part in persistent BCC infection.

Identification of the macrophage as a host cell for persistent bacterial infection calls for a better understanding of the association therein. *In vitro* binding assays showed that *B. multivorans* strain C5568 bound better to this cell type than *B. cenocepacia* C6433. As the assay does not allow discrimination between internalized and adherent bacteria, it is unclear whether this initial association leads to increased internalization, and whether this process is controlled by the bacteria or the macrophage. In any case, the identification of the bacterial ligand-host receptor complex that mediates uptake could further clarify the fate of cells after this interaction.

Other questions immediate to enhanced macrophage-C5568 binding involve the fate of both cells after bacterial uptake: Are the bacteria actively replicating, or merely surviving in a slow cycle of replication and killing, as previously demonstrated? The observations of both single bacteria and bacterial aggregates in pulmonary macrophages by immunohistology, and of single bacterial cells only within membrane-bound vacuoles of alveolar macrophages by TEM, in concert with the lack of increase in pulmonary bacterial load over time, suggests that the latter is the case. A second set of questions involves the fate of the macrophage after bacterial internalization: does the macrophage undergo phagosomal maturation and activation, or are any of these processes arrested? While *Mycobacteria* and *Legionella* benefit by hijacking normal host processes to ensure intracellular survival, a

clinical BCC strain has demonstrated survival, but not replication, within an activated macrophage cell line (78, 101, 187).

The observation that *B. cenocepacia* elicits a more vigorous inflammatory response in the host than *B. multivorans* supports of previous reports of the ultra-inflammatory nature of the former species, and promotes speculation about the different mechanisms of pathogenesis of these two species. *B. multivorans* may persist in the murine lung by provoking a less vigorous host response that allows bacterial ingestion by, and intracellular survival within, macrophages, while *B. cenocepacia* clearance from the lung could be effected by the aggressive immune response that it provokes. *B. multivorans* infection of the CF lung may follow a similar course, which could explain the long-term carriage of this BCC species with a low incidence of adverse clinical outcome. *B. cenocepacia* infections in the CF lung may be problematic by virtue of the combined hyper-inflammatory nature of the bacteria, inaccessibility of bacteria growing in biofilm, and the inefficacy of and immunopathology caused by the resulting immune response.

The differential inflammatory capacity between C5568 and C6433 should be further characterized by measuring the chemokine, cytokine and cell surface activation marker expression profiles of host macrophages and epithelial cells upon exposure to bacteria *in vitro*. In addition, the general health of host cells after incubation with bacteria can also be assessed by measuring degree of apoptosis and necrosis in response to bacterial interaction. *B. cenocepacia* is capable of inducing apoptosis in human neutrophils (111). The paucity of *B. cenocepacia*-binding MH-S cells and primary alveolar macrophages in the *in vitro* studies could be as much a product of bacterial toxicity to host cells upon ingestion, as lower binding affinity. *B. cenocepacia* in the CF lung may be able to gain a foothold in macrophages by

promoting neutrophil apoptosis post-ingestion, and surviving within vacuoles of macrophages that scavenge those apoptotic neutrophils.

The molecular basis for the differential antigenic potential between C5568 and C6433, however, is still unknown. While LPS offers an obvious beginning to the search for molecular differences, a limited investigation of the LPS from C5568 and C6433 only indicated that they possessed different forms of smooth LPS. A deep rough mutant of the *B. cepacia* type strain 25416 revealed modifications to the inner core and lipid A that discourage recognition and binding by mammalian cationic peptides (89). Gronow found that the substitution of a D-glycero-D-talo-oct-2-ulosonic acid (Ko) for one of the two Kdo residues, and the addition of 4-amino-4-deoxyarabinose (Ara4N) residues on both phosphates of the lipid A backbone both lower the anionic charge of the molecule and subsequent association with antimicrobial peptides, but a role in pathogenesis is not yet clear. The absence of the LPS O-polysaccharides, however, did not affect internalization by macrophages and the ability to induce a TNF-α response. A more comprehensive examination of the lipid A-Ko portion of the LPS, and any differences therein between *B. multivorans* and *B. cenocepacia*, is needed to clarify whether LPS plays any role in the pathogenesis of disease.

In conclusion, differential persistence between *B. multivorans* and *B. cenocepacia* was observed in three models of chronic infection. In a model of intranasal infection, two observations that endorse long-held ideas on pathogenic mechanisms of the BCC were made: persistent *B. multivorans* strain C5568 survives in pulmonary macrophages on the fourth day of infection, while inflammatory *B. cenocepacia* strain C6433 was cleared immediately after promoting a vigorous host immune response. Although the mechanisms behind these

phenomena are still unclear, these findings provide important *in vivo* evidence for BCC species as intracellular opportunists and as aggressive CF pathogens, and introduces another animal system in which to evaluate putative virulence determinants of the BCC.

References

1. Abe, M. and T. Nakazawa. 1994. Characterization of hemolytic and antifungal substance, cepalycin, from *Pseudomonas cepacia*. Microbiol. Immunol. 38: 1-9.

2. Aichele, P., J. Zinke, L. Grode, R. A. Schwendener, S. H. Kaufmann and P. Seiler. 2003. Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses. J. Immunol. 171: 1148-55.

3. Allison, D. G. and M. J. Goldsbrough. 1994. Polysaccharide production in *Pseudomonas cepacia*. J. Basic Microbiol. 34: 3-10.

4. Aly, R. and S. Levit. 1987. Adherence of *Staphylococcus aureus* to squamous epithelium: role of fibronectin and teichoic acid. Rev. Infect. Dis. 9(Suppl 4): S341-50.

5. Anderson, M. P., D. P. Rich, R. J. Gregory, A. E. Smith and M. J. Welsh. 1991. Generation of cAMP-activated chloride currents by expression of CFTR. Science. 251: 679-682.

6. Arenas, G. N., A. S. Staskevich, A. Aballay, L. S. Mayorga. 2000. Intracellular trafficking of *Brucella abortus* in J774 macrophages. Infect. Immun. 68: 4255-63.

7. Aris, R. M., J. C. Routh, J. J. LiPuma, D. G. Heath and P. H. Gilligan. 2001. Lung transplantation for cystic fibrosis patients with *Burkholderia cepacia* complex. Survival linked to genomovar type. Am.J.Respir.Crit.Care Med. 64: 2102-6.

8. Astarie-Dequeker, C., E. N. N'Diaye, V. Le Cabec, M. G. Rittig, J. Prandi and I. Maridonneau-Parini. 1999. The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages. Infect. Immun. 67: 469-77.

9. Balandreau, J., V. Viallard, B. Cournoyer, T. Coenye, S. Laevens and P. Vandamme. 2001. *Burkholderia cepacia* genomovar III is a common plant-associated bacterium. Appl. Environ. Microbiol. 67: 982-985.

10. Bals, R., D. J. Weiner and J. M. Wilson. 1999. The innate immune system in cystic fibrosis lung disease. J. Clin. Invest. 103: 303-7.

11. Batchelor, R. A., P. Alifano, E. Biffali, S. I. Hull and R. A. Hull. 1992. Nucleotide sequences of the genes regulating O-polysaccharide antigen chain length (rol) from *Escherichia coli* and *Salmonella typhimurium*: protein homology and functional complementation. J. Bacteriol. 174: 5228-36.

12. Bear, C. E., C. H. Li, N. Cartner, R. J. Bridges, T. J. Jensen, M. Ramjeesingh and J. R. Riordan. 1992. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell. 68: 809-18.

13. Berger, M. 2002. Inflammatory mediators in cystic fibrosis lung disease. Allergy Asthma Proc. 23: 19-25.

14. Bevivino, A., C. Dalmastri, S. Tabacchioni, L. Chiarini, M. L. Belli, S. Piana, A. Materazzo, P. Vandamme and G. Manno. 2002. *Burkholderia cepacia* complex bacteria from clinical and environmental sources in Italy: genomovar status and distribution of traits related to virulence and transmissibility. J. Clin. Microbiol. 40: 846-51.

15. Bienenstock, J. 1982. Gut and bronchus associated lymphoid tissue: an overview. Adv. Exp. Med. Biol. 149: 471-7.

16. Bonfield, T. L., M. W. Konstan and M. Berger. 1999. Altered respiratory epithelial cell cytokine production in cystic fibrosis. J. Allergy Clin. Immunol. 104: 72-8.

17. Boucher, R. C. 2002. An overview of the pathogenesis of cystic fibrosis lung disease. Adv. Drug Deliv. Rev. 54: 1359-71.

18. Brandolini, L., R. Bertini, C. Bizzarri, R. Sergi, G. Caselli, D. Zhou, M. Locati and S. Sozzani. 1996. IL-1 beta primes IL-8-activated human neutrophils for elastase release, phospholipase D activity, and calcium flux. J. Leukoc. Biol. 59: 427-34.

19. Breel, M., M. Van der Ende, T. Sminia and G. Kraal. 1988. Subpopulations of lymphoid and non-lymphoid cells in bronchus-associated lymphoid tissue (BALT) of the mouse. Immunology. 63: 657-62.

20. Broxmeyer, H. E., S. Cooper, G. Cacalano, N. L. Hague, E. Bailish and M. W. Moore. 1996. Involvement of Interleukin (IL) 8 receptor in negative regulation of myeloid progenitor cells in vivo: evidence from mice lacking the murine IL-8 receptor homologue. J. Exp. Med. 184: 1825-32.

21. Buiting, A. M., Z. De Rover, G. Kraal and N. Van Rooijen. 1996. Humoral immune responses against particulate bacterial antigens are dependent on marginal metallophilic macrophages in the spleen. Scand. J. Immunol. 43: 398-405.

22. Burkholder, W. H. 1950. Sour skin. A bacterial rot of onion bulbs. Phytopathology. 40: 115-117.

23. Burns, J. L., R. L. Gibson, S. McNamara, D. Yim, J. Emerson, M. Rosenfeld, P. Hiatt, K. McCoy, R. Castile, A. L. Smith and B. W. Ramsey. 2001. Longitudinal assessment of *Pseudomonas aeruginosa* in young children with cystic fibrosis. J. Infect. Dis. 183: 444-452.

24. Burns, J. L., M. Jonas, E. Y. Chi, D. A. Clark, A. Berger and A. Griffith. 1996. Invasion of respiratory epithelial cells by *Burkholderia (Pseudomonas) cepacia*. Infect. Immun. 64: 4054-4059.

25. Burns, J. L. and L. Saiman. 1999. *Burkholderia cepacia* infections in cystic fibrosis. Pediatr. Infect. Dis. J. 18: 155-6.

26. Cabral, D. A., B. A. Loh, and D. P. Speert. 1987. Mucoid *Pseudomonas aeruginosa* resists nonopsonic phagocytosis by human neutrophils and macrophages. Pediatr. Res. 22: 429-31.

27. Campos, J., F. Roman, M. Georgiou, C. Garcia, R. Gomez-Lus, R. Canton, H. Escobar and F. Baquero. 1996. Long-term persistence of ciprofloxacin-resistant *Haemophilus influenzae* in patients with cystic fibrosis. J. Infect. Dis. 174: 1345-7.

28. Cerantola, S., J. Bounery, C. Segonds, N. Marty and H. Montrozier. 2000. Exopolysaccharide production by mucoid and non-mucoid strains of *Burkholderia cepacia*. FEMS Microbiol. Lett. 185: 243-6.

29. Cerantola, S., A. Lemassu-Jacquier and H. Montrozier. 1999. Structural elucidation of a novel exopolysaccharide produced by a mucoid clinical isolate of *Burkholderia cepacia*. Characterization of a trisubstituted glucuronic acid residue in a heptasaccharide repeating unit. Eur. J. Biochem. 260: 373-83.

30. Cescutti, P., G. Impallomeni, D. Garozzo, L. Sturiale, Y. Herasimenka, C. Lagatolla and R. Rizzo. 2003. Exopolysaccharides produced by a clinical strain of Burkholderia cepacia isolated from a cystic fibrosis patient. Carbohydr. Res. 338: 2687-95.

31. Chan, P. F. and S. J. Foster. 1998. The role of environmental factors in the regulation of virulence-determinant expression in *Staphylococcus aureus* 8325-4. Microbiology 144: 2469-79.

32. Chen, J. S., K. A. Witzmann, T. Spilker, R. J. Fink and J. J. LiPuma. 2001. Endemicity and inter-city spread of *Burkholderia cepacia* genomovar III in cystic fibrosis. J. Pediatr. 139: 643-9.

33. Cheng, H. P. and T. G. Lessie. 1994. Multiple replicons constituting the genome of *Pseudomonas cepacia* 17616. J. Bacteriol. 176: 4034-42.

34. Chiu, C. H., A. Ostry and D. P. Speert. 2001. Invasion of murine respiratory epithelial cells *in vivo* by *Burkholderia cepacia*. J. Med. Microbiol. 50: 594-601.

35. Chmiel, J. F., M. Berger and M. W. Konstan. 2002. The role of inflammation in the pathophysiology of CF lung disease. Clin. Rev. Allergy Immunol. 23: 5-27.

36. Chu, K. K., D. J. Davidson, T. K. Halsey, J. W. Chung and D. P. Speert. 2002. Differential persistence among genomovars of the *Burkholderia cepacia* complex in a murine model of pulmonary infection. Infect. Immun. 70: 2715-20.

37. Chung, J. W., E. Altman, T. J. Beveridge and D. P. Speert. 2003. Colonial morphology of *Burkholderia cepacia* complex genomovar III: implications in exopolysaccharide production, pilus expression, and persistence in the mouse. Infect. Immun. 71: 904-9.

38. Cianciotto, N. P. 2001. Pathogenicity of *Legionella pneumophila*. Int. J. Med. Microbiol. 291: 331-43.

39. Cieri, M. V., N. Mayer-Hamblett, A. Griffith and J. L. Burns. 2002. Correlation between an in vitro invasion assay and a murine model of *Burkholderia cepacia* lung infection. Infect. Immun. 70: 1081-6.

40. Clary-Meinesz, C., J. Mouroux, J. Cosson, P. Huitorel and B. Blave. 1998. Influence of external pH on ciliary beat frequency in human bronchi and bronchioles. Eur. Respir. J. 11: 330-3.

41. Coakley, R. D. and R. C. Boucher. 2001. Regulation and functional significance of airway surface liquid pH. J. Physiol. 2(4 Suppl): 294-300.

42. Coenye, T., J. J. LiPuma, D. Henry, B. Hoste, K. Vandemeulebroecke, M. Gillis, D. P. Speert and P. Vandamme. 2001a. *Burkholderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. Int. J. Syst. Evol. Microbiol. 51: 271-9.

43. Coenye, T., E. Mahenthiralingam, D. Henry, J. J. LiPuma, S. Laevens, M. Gillis, D. P. Speert and P. Vandamme. 2001b. *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. Int. J. Syst. Evol. Microbiol. 51: 1481-90.

44. Coia, J. E., L. Browning, L. Haines, T. H. Birkbeck and D. J. Platt. 1992. Comparison of enterotoxins and haemolysins produced by methicillin-resistant (MRSA) and sensitive (MSSA) *Staphylococcus aureus*. J. Med. Microbiol. 36: 164-71.

45. Collazo, C. M. and J. E. Galan. 1997. The invasion-associated type-III protein secretion system in *Salmonella*--a review. Gene. 192: 51-9.

46. Colvin, O. M. 1999. An overview of cyclophosphamide development and clinical applications. Curr. Pharmaceut. Design. 5: 555-560.

47. Conway, B. A. and E. P. Greenberg. 2002a. Quorum-sensing signals and quorum-sensing genes in *Burkholderia vietnamiensis*. J. Bacteriol. 184: 1187-91.

48. Conway, B. D., V. Venu and D. P. Speert. 2002b. Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. J. Bacteriol. 184: 5678-5685.

49. Corey, M. and V. Farewell. 1996. Determinants of mortality from cystic fibrosis in Canada, 1970-1989. Am. J. Epidemiol. 143: 1007-17.

50. Corvol, H., C. Fitting, K. Chadelat, J. Jacquot, O. Tabary, M. Boule, J. M. Cavaillon and J. Clement. 2003. Distinct cytokine production by lung and blood neutrophils from children with cystic fibrosis. Am. J. Physiol. Lung Cell. Mol. Physiol. 284: L997-L1003.

51. Costerton, J. W., P. S. Stewart and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. Science. 284: 1318-22.

52. Cryz, S. J., E. Furer and R. Germanier. 1983. Simple model for the study of *Pseudomonas aeruginosa* infections in leukopenic mice. Infect. Immun. 39: 1067-1071.

53. Curnutte, J. T., D. M. Whitten and B. M. Babioer. 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. N. Engl. J. Med. 290: 593-7.

54. Da Silva, R. P., B. F. Hall, K. A. Joiner and D. L. Sacks. 1989. CR1, the C3b receptor, mediates binding of infective *Leishmania major* metacyclic promastigotes to human macrophages. J. Immunol. 143: 617-22.

55. Daubaras, D. L., C. E. Danganan, A. Huber, R. W. Ye, W. Hendrickson and A. M. Chakrabarty. 1996. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by *Burkholderia cepacia* strain AC1100: evolutionary insight. Gene. 179: 1-8.

56. Daubaras, D. L., C. D. Hershberger, K. Kitano and A. M. Chakrabarty. 1995. Sequence analysis of a gene cluster involved in metabolism of 2,4,5-trichlorophenoxyacetic acid by *Burkholderia cepacia* strain AC1100. Appl. Environ. Microbiol. 61: 1279-1289.

57. Davidson, D. J., J. R. Dorin, G. McLachlan, V. Ranaldi, D. Lamb, C. Doherty, J. Govan and D. J. Porteous. 1995. Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. Nat. Genet. 9: 351-7.

58. Davidson, D. J. and J. R. Dorin. 2001. The CF mouse: an important tool for studying cystic fibrosis, Expert Reviews in Molecular Medicine. 2001.

59. Davidson, D. J. and M. Rolfe. 2001. Mouse models of cystic fibrosis. Trends Genet. 17: S29-37.

60. Dinarello, C. A. 1997. Interleukin-1. Cytokine Growth Factor Rev. 8: 253-65.

61. Doggett, R. G., G. M. Harrison, R. N. Stillwell and E. S. Wallis. 1966. An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. J. Pediatr. 68: 215-221.

62. Dorin, J. R., P. Dickinson, E. W. Alton, S. N. Smith, D. M. Geddes, B. J. Stevenson, W. L. Kimber, S. Fleming, A. R. Clarke et. al. 1992. Cystic fibrosis in the mouse by targeted insertional mutagenesis. Nature. 359: 211-5.

63. Eady, E. A. and J. H. Cove. 2003. Staphylococcal resistance revisited: community-acquired methicillin resistant *Staphylococcus aureus*--an emerging problem for the management of skin and soft tissue infections. Curr. Opin. Infect. Dis. 16: 103-24.

64. Eckman, E. A., C. U. Cotton, D. M. Kube and P. B. Davis. 1995. Dietary changes improve survival of CFTR S489X homozygous mutant mouse. Am. J. Physiol. 269: L625-30.

65. el-Banna, N. and G. Winkelmann. 1998. Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against streptomycetes. J. Appl. Microbiol. 85: 69-78.

66. Elborn, J. S., M. Dodd and J. Maddison. 1994. Clinical and inflammatory responses in CF patients infected with *Pseudomonas aeruginosa* and *Pseudomonas cepacia*. Pediatr. Pulmonol. 10: 287.

67. Eloranta, M. L. and G. V. Alm. 1999. Splenic marginal metallophilic macrophages and marginal zone macrophages are the major interferon-alpha/beta producers in mice upon intravenous challenge with herpes simplex virus. Scand. J. Immunol. 49: 391-4.

68. Ernst, J. D. 1998. Macrophage receptors for *Mycobacterium tuberculosis*. Infect. Immun. 66: 1277-81.

69. Evans, E., I. R. Poxton and J. R. Govan. 1999. Lipopolysaccharide chemotypes of *Burkholderia cepacia*. J. Med. Microbiol. 48: 825-32.

70. Faccioli, L. H., G. E. Souza, F. Q. Cunha, S. Poole and S. H. Ferreira. 1990. Recombinant interleukin-1 and tumor necrosis factor induce neutrophil migration *in vivo* by indirect mechanisms. Agents Actions 30: 344-9.

71. Feldmesser, M., Y. Kress, P. Novikoff and A. Casavadell. 2000. *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. Infect. Immun. 68: 4225-37.

72. Finck-Barbancon, V., J. Goranson, L. Zhu, T. Sawa, J. P. Wiener-Kronish, S. M. Fleiszig, C. Wu, L. Mende-Mueller and D. W. Frank. 1997. *ExoU* expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol. Microbiol. 25: 547-57.

73. FitzSimmons, S. C. 1993. The changing epidemiology of cystic fibrosis. J. Pediatr. 122: 1-9.

74. Flotte, T. J., T. A. Springer and G. J. Thorbecke. 1983. Dendritic cell and macrophage staining by monoclonal antibodies in tissue sections and epidermal sheets. Am. J. Pathol. 111: 112-24.

75. Forrest, C. B., J. R. Forehand, R. A. Axtell, R. L. Roberts and R. B. Johnston, Jr. 1988. Clinical features and current management of chronic granulomatous disease. Hematol. Oncol. Clin. North Am. 2: 253-66.

76. Fu, Y. X. and D. D. Chaplin. 1999. Development and maturation of secondary lymphoid tissues. Annu. Rev. Immunol. 17: 399-433.

77. Galanos, C., E. T. Rietschel, O. Luderitz, O. Westphal, Y. B. Kim and D. W. Watson. 1972. Biological activities of lipid A complexed with bovine serum albumin. Eur. J. Biochem. 31: 230-233.

78. Gatfield, J. and J. Pieters. 2000. Essential role for cholesterol in entry of mycobacteria into macrophages. Science 288: 1647-50.

79. Ghaffar, F., L. S. Muniz, K. Katz, J. L. Smith, T. Shouse, P. Davis and G. H. McCracken Jr. 2002. Effects of large dosages of amoxicillin/clavulanate or azithromycin on nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae*, nonpneumococcal alpha-hemolytic streptococci, and *Staphylococcus aureus* in children with acute otitis media. Clin. Infect. Dis. 34: 1301-9.

80. Gillis, M., T. Van Van and R. Bardin. 1995. Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis*. sp. nov. for N_2-fixing isolates from rice in Vietman. Int. J. System. Bacteriol. 45: 274-289.

81. Goldberg, J. B. and G. B. Pier. 2000. The role of the CFTR in susceptibility to *Pseudomonas aeruginosa* infections in cystic fibrosis. Trends Microbiol. 8: 514-20.

82. Goldman, M. J., G. M. Anderson, E. D. Stolzenberg, U. P. Kari, M. Zasloff and J. M. Wilson. 1997. Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. Cell. 88: 553-60.

83. Goldman, R. C., K. Joiner and L. Leive. 1984. Serum-resistant mutants of *Escherichia coli* O111 contain increased lipopolysaccharide, lack an O antigen-containing capsule, and cover more of their lipid A core with O antigen. J. Bacteriol. 159: 877-82.

84. Govan, J. R. 2000. Infection control in cystic fibrosis: methicillin-resistant *Staphylococcus aureus, Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex. J. R. Soc. Med. 93(Suppl 38): 40-5.

85. Govan, J. R., P. H. Brown, J. Maddison, C. J. Doherty, J. W. Nelson, M. Dodd, A. P. Greenberg and A. K. Webb. 1993. Evidence for transmission of Pseudomonas cepacia by social contact in cystic fibrosis. Lancet 342: 15-9.

86. Govan, J. R. and V. Deretic. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiological Reviews 60: 539-574.

87. Govan, J. R. and J. W. Nelson. 1992. Microbiology of lung infection in cystic fibrosis. Br. Med. Bull. 48: 912-30.

88. Grifoll, M. A., S. A. Selifonov, C. V. Gatlin and P. J. Chapman. 1995. Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds. Appl. Environ. Microbiol. 61: 3711-3723.

89. Gronow, S., C. Noah, A. Blumenthal, B. Lindner and H. Brade. 2003. Construction of a deep-rough mutant of *Burkholderia cepacia* ATCC 25416 and characterization of its chemical and biological properties. J. Biol. Chem. 278: 1647-55.

90. Grossman, N., M. A. Schmetz, J. Foulds, E. N. Klima, V. E. Jiminez-Lucho, L. L. Leive, K. A. Joiner and V. Jiminez. 1987. Lipopolysaccharide size and distribution determine serum resistance in *Salmonella montevideo*. J. Bacteriol. 169: 856-63.

91. Hancock, R. E., L. M. Mutharia, L. Chan, R P. Darveau, D. P. Speert and G. B. Pier. 1983. *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis: a class of serum-sensitive, nontypeable strains deficient in lipopolysaccharide O side chains. Infect. Immun. 42: 170-177.

92. Haugland, R. A., U. M. Sangodkar and A. M. Chakrabarty. 1990. Repeated sequences including RS1100 from *Pseudomonas cepacia* AC1100 function as IS elements. Mol. Gen. Genet. 220: 222-8.

93. Hickman-Davis, J. M., J. R. Lindsey and S. Matalon. 2001. Cyclophosphamide decreases nitrotyrosine formation and inhibits nitric oxide production by alveolar macrophages in mycoplasmosis. Infect. Immun. 69: 6401-10.

94. Hirakata, Y., N. Furuya, T. Matsumoto, K. Tateda and K. Yamaguchi. 1995. The influence of immunosuppressive agents on the immune system of mice. Immunol. Infect. Dis. 5: 88-93.

95. Hitchcock, P. J. and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. 154: 269-277.

96. Ho, M. K. and T. A. Springer. 1983. Tissue distribution, structural characterization, and biosynthesis of Mac-3, a macrophage surface glycoprotein exhibiting molecular weight heterogeneity. J. Biol. Chem. 258: 636-42.

97. Hoiby, N., H. Krogh Johansen, C. Moser, Z. Song, O. Ciofu and A. Kharazmi. 2001. *Pseudomonas aeruginosa* and the *in vitro* and *in vivo* biofilm mode of growth. Microbes Infect. 3: 23-35.

98. Holmes, A., J. Govan and R. Goldstein. 1998. Agricultural use of *Burkholderia (Pseudomonas) cepacia:* a threat to human health? Emerg. Infect. Dis. 4: 221-7.

99. Holmes, A., R. Nolan, R. Taylor, R. Finley, M. Riley, R. Z. Jiang, S. Steinbach, R. Goldstein. 1999. An epidemic of *Burkholderia cepacia* transmitted between patients with and without cystic fibrosis. J. Infect. Dis. 179: 1197-205.

100. Hoppe, I., B. Brenneke, M. Rohde, A. Kreft, S. Haussler, A. Reganzerowski and I. Steinmetz. 1999. Characterization of a murine model of melioidosis: comparison of different strains of mice. Infect. Immun. 67: 2891-900.

101. Horwitz, M. A. 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. 158: 2108-26.

102. Horwitz, M. A. 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell 36: 27-33.

103. Hu, C., T. Mayadas-Norton, K. Tanaka, J. Chan and P. Salgame. 2000. *Mycobacterium tuberculosis* infection in complement receptor 3-deficient mice. J. Immunol. 165: 2596-602.

104. Huber, B., K. Riedel, M. Hentzer, A. Heydorn, A. Gotschlich, M. Givskov, S. Molin and L. Eberl. 2001. The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology 147: 2517-28.

105. Huber, B., K. Riedel, M. Kothe, M. Givskov, S. Molin and L. Eberl. 2002. Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. Mol. Microbiol. 46: 411-26.

106. Hubner, A., C. E. Danganan, L. Xun, A. M. Chakrabarty and W. Hendrickson. 1998. Genes for 2,4,5-trichlorophenoxyacetic acid metabolism in *Burkholderia cepacia* AC1100: characterization of the *tftC* and *tftD* genes and locations of the tft operons on multiple replicons. Appl. Environ. Microbiol. 64: 2086-93.

107. Hubner, A. and W. Hendrickson. 1997. A fusion promoter created by a new insertion sequence, IS1490, activates transcription of 2,4,5-trichlorophenoxyacetic acid catabolic genes in *Burkholderia cepacia* AC1100. J. Bacteriol. 179: 2717-23.

108. Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. 62: 379-433.

109. Hughes, J. E., J. Stewart, G. R. Barclay and J. R. Govan. 1997. Priming of neutrophil respiratory burst activity by lipopolysaccharide from Burkholderia *cepacia*. Infect. Immun. 65: 4281-7.

110. Hutchison, M. L. and J. R. Govan. 1999. Pathogenicity of microbes associated with cystic fibrosis. Microbes Infect. 1: 1005-1014.

111. Hutchison, M. L., I. R. Poxton and J. R. Govan. 1998. *Burkholderia cepacia* produces a hemolysin that is capable of inducing apoptosis and degranulation of mammalian phagocytes. Infect. Immun. 66: 2033-9.

112. Hwang, T. C., L. Lu, P. L. Zeitlin, D. C. Gruenert, R. Huganir, W. B. Guggino. 1988. Cl channels in CF: lack of activation by protein kinase C and cAMP-dependent protein kinase. Science 244: 1351-1353.

113. Ibrahim-Granet, O., B. Philippe, H. Boleti, E. Boisvieux-Ulrich, D. Grenet, M. Stern and J. P. Latge. 2003. Phagocytosis and intracellular fate of *Aspergillus fumigatus* conidia in alveolar macrophages. Infect. Immun. 71: 891-903.

114. Imundo, L., J. Barasch, A. Prince and Q. Al-Awqati. 1995. Cystic fibrosis epithelial cells have a receptor for pathogenic bacteria on their apical surface. Proc. Natl. Acad. Sci. U S A 92: 3019-23.

115. Ishibashi, Y. and T. Arai. 1990. Roles of the complement receptor type 1 (CR1) and type 3 (CR3) on phagocytosis and subsequent phagosome-lysosome fusion in *Salmonella*-infected murine macrophages. FEMS Microbiol. Immunol. 2: 89-96.

116. Isles, A., M. I., I. Maclusky, M. Corey, R. Gold, C. Prober, P. Fleming and H. Levison. 1984. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. J. Pediatr. 104: 206-210.

117. Janisiewicz, W. J. and J. Roitman. 1988. Biological control of blue mold and gray mold on apple and pear with *Pseudomonas cepacia*. Phytopathol. 78: 1697-1700.

118. Jarvis, K. G., J. A. Giron, A. E. Jerse, T. K. McDaniel, M. S. Donnenberg and J. B. Kaper. 1995. Enteropathogenic *Escherichia coli* contains a putative type III secretion

system necessary for the export of proteins involved in attaching and effacing lesion formation. Proc. Natl. Acad. Sci. U S A 92: 7996-8000.

119. Joiner, K. A. 1985. Studies on the mechanism of bacterial resistance to complement-mediated killing and on the mechanism of action of bactericidal antibody. Curr. Top. Microbiol. Immunol. 121: 99-133.

120. Joiner, K. A., N. Grossman, M. Schmetz and L. L. Leive. 1986. C3 binds preferentially to long-chain lipopolysaccharide during alternative pathway activation by *Salmonella montevideo*. J. Immunol. 136: 710-5.

121. Jones, A. M., M. E. Dodd and A. K. Webb. 2001. *Burkholderia cepacia*: current clinical issues, environmental controversies and ethical dilemmas. Eur. Respir. J. 17: 295-301.

122. Joy, A. E. and J. L. Parke. 1994. Biocontrol of *Alternaria* leaf blight on American ginseng by *Burkholderia cepacia* AMMD. Challenges of the 21st century. Proceedings of the International Ginseng Conference, Vancouver, British Columbia, Canada.

123. Keig, P. M., E. Ingham and K. G. Kerr. 2001. Invasion of human type II pneumocytes by *Burkholderia cepacia*. Microbial Pathogenesis 30: 167-170.

124. Keig, P. M., E. Ingham, P. A. Vandamme and K. G. Kerr. 2002. Differential invasion of respiratory epithelial cells by members of the *Burkholderia cepacia* complex. Clin. Microbiol. Infect. 8: 47-9.

125. Kerem, B., J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M. Buchwald and L. C. Tsui. 1989. Identification of the cystic fibrosis gene: genetic analysis. Science 245: 1073-80.

126. Kersters, K., W. Ludwig, M, Vancanneyt, P. De Vos, M. Gillis and K. H. Schleifer. 1996. Recent changes in the classification of the pseudomonads: an overview. Syst. Appl. Microbiol. 19: 465-477.

127. Khan, T. Z., J. S. Wagener, T. Bost, J. Martinez, F. J. Accurso and D. W. Riches. 1995. Early pulmonary inflammation in infants with cystic fibrosis. Am. J. Respir. Crit. Care Med. 151: 1075-1082.

128. Kilbane, J. J., D. K. Chatterjee, J. S. Karns, S. T. Kellogg and A. M. Chakrabarty. 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. 44: 72-8.

129. Kolopp-Sarda, M. N., M. C. Bene, N. Massin, J. J. Moulin and G. C. Faure. 1994. Immunohistological analysis of macrophages, B-cells and T-cells in the mouse lung. Anat. Rec. 239: 150-157.

130. Kondo, E., T. Yasuda and K. Kanai. 1982. Electron microscopic demonstration of close contact between intracellular mycobacteria and the phagosomal membrane. Jpn. J. Med. Sci. Biol. 35: 197-201.

131. Kothe, M., M. Antl, B. Huber, K. Stoecker, D. Ebrecht, I. Steinmetz and L. Eberl. 2003. Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the cep quorum-sensing system. Cell. Microbiol. 5: 343-51.

132. Kraal, G. 1992. Cells in the marginal zone of the spleen. Int. Rev. Cytol. 132: 31-74.

133. Kraal, G., H. Ter Hart, C. Meelhuizen, G. Venneker and E. Claassen. 1989. Marginal zone macrophages and their role in the immune response against T-independent type 2 antigens: modulation of the cells with specific antibody. Eur. J. Immunol. 19: 675-80.

134. Kramer, J., A. H. Visscher, J. A. Wagenaar and S. H. Jeurissen. 2003. Entry and survival of *Salmonella enterica* serotype *Enteritidis* PT4 in chicken macrophage and lymphocyte cell lines. Vet. Microbiol. 91: 147-55.

135. Krieg, D. P., R. J. Helmke, V. F. German and J. A. Mangos. 1988. Resistance of mucoid *Pseudomonas aeruginosa* to nonopsonic phagocytosis by alveolar macrophages *in vitro*. Infect. Immun. 56: 3173-9.

136. Kronborg, G., M. B. Hansen, M. Svenson, A. Fomsgaard, N. Hoiby and K. Brentzen. 1993. Cytokines in sputum and serum from patients with cystic fibrosis and chronic *Pseudomonas aeruginosa* infection as markers of destructive inflammation in the lungs. Pediatr. Pulmonol. 15: 292-297.

137. Kuehn, M., K. Lent, J. Haas, J. Hagenzieker, M. Cervin and A. L. Smith. 1992. Fimbriation of *Pseudomonas cepacia*. Infect. Immun. 60: 2002-7.

138. Lam, J., R. Chan, K. Lam and J. W. Costerton. 1980. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infect. Immun. 28: 546-56.

139. Landers, P., K. G. Kerr, T. J. Rowbotham, J. L. Tipper, P. M. Keig, E. Ingham and M. Denton. 2000. Survival and growth of *Burkholderia cepacia* within the free-living amoeba *Acanthamoeba polyphaga*. Eur. J. Clin. Microbiol. Infect. Dis. 19: 121-3.

140. Learn, D. B., E. P. Brestel and S. Seetharama. 1987. Hypochlorite scavenging by *Pseudomonas aeruginosa* alginate. Infect. Immun. 55: 1813-8.

141. Lessie, T. G., W. Hendrickson, B. D. Manning and R. Devereux. 1996. Genomic complexity and plasticity of *Burkholderia cepacia*. FEMS Microbiol. Lett. 144: 117-128.

142. Lewenza, S., B. Conway, E. P. Greenberg and P. A. Sokol. 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. 181: 748-56.

143. Lewenza, S., M. B. Visser and P. A. Sokol. 2002. Interspecies communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*. Can. J. Microbiol. 48: 707-16.

144. Li, M., J. D. McCann, C. M. Liedtke, A. C. Nairn, P. Greengard and M. J. Welsh. 1988. Cyclic AMP-dependent protein kinase opens chloride channels in normal but not cystic fibrosis airway epithelium. Nature 331: 358-360.

145. LiPuma, J. J., J. E. Mortensen, S. E. Desin, T. D. Edlind, D. V. Schidlow, J. L. Burns and T. L. Stull. 1988. Ribotype analysis of *Pseudomonas cepacia* from cystic fibrosis treatment centers. J. Pediatr. 113: 859-62.

146. LiPuma, J. J., T. Spilker, L. H. Gill, P. W. Campbell, L. Liu and E. Mahenthiralingam. 2001. Disproportionate distribution of *Burkholderia cepacia* complex species and transmissibility markers in cystic fibrosis. Am. J. Respir. Crit. Care Med. 164: 92-6.

147. Lonon, M. K., D. E. Woods and D. C. Straus. 1988. Production of lipase by clinical isolates of *Pseudomonas cepacia*. J. Clin. Microbiol. 26: 979-84.

148. Lugton, I. 1999. Mucosa-associated lymphoid tissues as sites for uptake, carriage and excretion of tubercle bacilli and other pathogenic mycobacteria. Immunol. Cell Biol. 77: 364-72.

149. Lutter, E., S. Lewenza, J. J. Dennis, M. B. Visser and P. A. Sokol. 2001. Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex. Infect. Immun. 69: 4661-6.

150. Mahadeva, R., L. Sharples, R. I. Ross-Russell, A. K. Webb, D. Bilton and D. A. Lomas. 2001. Association of alpha(1)-antichymotrypsin deficiency with milder lung disease in patients with cystic fibrosis. Thorax 56: 53-8.

151. Mahenthiralingam, E., T. Coenye, J. W. Chung, D. P. Speert, J. R. Govan, P.Taylor and P. Vandamme. 2000. Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. J. Clin. Microbiol. 38: 910-3.

152. Mahenthiralingam, E., P. Vandamme, M. E. Campbell, D. A. Henry, A. M. Gravelle, L. T. Wong, A. G. Davidson, P. G. Wilcox, B. Nakielna and D. P. Speert. 2001. Infection with *Burkholderia cepacia* complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace *Burkholderia multivorans*. Clin. Infect. Dis. 33: 1469-75.

153. Marolda, C. L., B. Hauroder, M. A. John, R. Michel and M. A. Valvano. 1999. Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae. Microbiology 145: 1509-17.

154. Martin, D. W. and C. D. Mohr. 2000. Invasion and intracellular survival of *Burkholderia cepacia*. Infect. Immun. 68: 24-29.

155. Matsui, H., B. R. Grubb, R. Tarran, S. H. Randell, J. T. Gatzy, C. W. Davis and R. C. Boucher. 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. Cell 95: 1005-15.

156. McKevitt, A. I., S. Bajaksouzian, J. D. Klinger and D. E. Woods. 1989. Purification and characterization of an extracellular protease from *Pseudomonas cepacia*. Infect. Immun. 57: 771-8.

157. McLoughlin, T. J., J. P. Quinn, A. Bettermann and R. Bookland. 1992. *Pseudomonas cepacia* suppression of sunflower wilt fungus and role of antifungal compounds in controlling the disease. Appl. Environ. Microbiol. 58: 1760-3.

158. Meischl, C. and D. Roos. 1998. The molecular basis of chronic granulomatous disease. Springer Semin. Immunopathol. 19: 417-34.

159. Melnikov, A., O. Zaborina, N. Dhiman, B. S. Prabhakar, A. M. Chakrabarty and W. Hendrickson. 2000. Clinical and environmental isolates of *Burkholderia cepacia* exhibit differential cytotoxicity towards macrophages and mast cells. Molec. Microbiol. 36: 1481-1493.

160. Melo, M. D., I. R. Catchpole, G. Haggar and R. W.Stokes. 2000a. Utilization of CD11b knockout mice to characterize the role of complement receptor 3 (CR3, CD11b/CD18) in the growth of *Mycobacterium tuberculosis* in macrophages. Cell. Immunol. 205: 13-23.

161. Melo, M. D. and R. W. Stokes. 2000b. Interaction of *Mycobacterium tuberculosis* with MH-S, an immortalized murine alveolar macrophage cell line: a comparison with primary murine macrophages. Tubercle Lung Dis. 80: 35-46.

162. Menard, R., C. Dehio and P. J. Sansonetti. 1996. Bacterial entry into epithelial cells: the paradigm of *Shigella*. Trends Microbiol. 4: 220-6.

163. Meyer, J. M., D. Hohnadel and F. Halle. 1989. Cepabactin from *Pseudomonas cepacia*, a new type of siderophore. J. Gen. Microbiol. 135: 1479-87.

164. Middleton, B., H. C. Rodgers, M. Camara, A. J. Knox, P. Williams and A. Hardman. 2002. Direct detection of N-acylhomoserine lactones in cystic fibrosis sputum. FEMS Microbiol. Lett. 207: 1-7.

165. Morgenstern, D. E., M. A. Gifford, L. L. Li, C. M. Doerschuk and M. C. Dinauer. 1997. Absence of respiratory burst in X-linked chronic granulomatous disease mice leads to abnormalities in both host defense and inflammatory response to *Aspergillus fumigatus*. J. Exp. Med. 185: 207-18.

166. Neild, A. L. and C. R. Roy. 2003. *Legionella* reveal dendritic cell functions that facilitate selection of antigens for MHC class II presentation. Immunity 18: 813-23.

167. Nolte, M. A., E. N. Hoen, A. van Stijn, G. Kraal and R. E. Mebius. 2000. Isolation of the intact white pulp. Quantitative and qualitative analysis of the cellular composition of the splenic compartments. Eur. J. Immunol. 30: 626-34.

168. Pabst, R. and J. Westermann. 1991. The unique role of the spleen and its compartments in lymphocyte migration. Res. Immunol. 142: 339-42.

169. Palfreyman, R. W., M. L. Watson, C. Eden, A. W. Smith. 1997. Induction of biologically active interleukin-8 from lung epithelial cells by *Burkholderia (Pseudomonas) cepacia* products. Infect. Immun. 65: 617-622.

170. Palleroni, N. J., R. Kunisawa, R. Contopoulou and M. Doudoroff. 1973. Nucleic acid homologies in the genus *Pseudomonas*. Int. J. System. Bacteriol. 23: 333-339.

171. Parad, R. B., C. J. Gerard, D. Zurakowski, D. P. Nichols and G. B. Pier. 1999. Pulmonary outcome in cystic fibrosis is influenced primarily by mucoid *Pseudomonas aeruginosa* infection and immune status and only modestly by genotype. Infect. Immun. 67: 4744-4750.

172. Parke, J. L., R. E. Rand, A. E. Joy and E. B. King. 1991. Biological control of Pythium damping-off and Aphanomyces root rot of peas by application of *Pseudomonas cepacia* or *Pseudomonas fluorescens* to seed. Plant Dis. 75: 987-992.

173. Parsons, Y. N., K. J. Glendinning, V. Thornton, B. A. Hales, C. A. Hart, C. Winstanley. 2001. A putative type III secretion gene cluster is widely distributed in the *Burkholderia cepacia* complex but absent from genomovar I. FEMS Microbiol. Lett. 203: 103-8.

174. Pedersen, S. S., N. Hoiby, F. Espersen and C. Koch. 1992. Role of alginate in infection with mucoid *Pseudomonas aeruginosa* in cystic fibrosis. Thorax 47: 6-13.

175. Pegues, D. A., L. A. Carson, R. L. Anderson, M. J. Norgard, T. A. Argent, W. R. Jarvis and C. H. Woernle. 1993. Outbreak of *Pseudomonas cepacia* bacteremia in oncology patients. Clin. Infect. Dis. 16: 407-11.

176. Perretti, M., E. Solito and L. Parente. 1992. Evidence that endogenous interleukin-1 is involved in leukocyte migration in acute experimental inflammation in rats and mice. Agents Actions 35: 71-8.

177. Pier, G. B., M. Grout and T. S. Zaidi. 1997. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the lung. Proc. Natl. Acad. Sci. U S A 94: 12088-93.

178. Quinton, P. M. 1999. Physiological basis of cystic fibrosis: a historical perspective. Physiol. Rev. 79: S3-S22.

179. Racz, P., K. Tenner-Racz, Q. N. Myrvik, B. T. Shannon and S. H. Love. 1978. Sinus reactions in the hilar lymph node complex of rabbits undergoing a pulmonary cell-mediated immune response: sinus macrophage clumping reaction, sinus lymphocytosis and immature sinus histiocytosis. J. Reticuloendothel. Soc. 24: 499-525.

180. Reddi, K., S. B. Phagoo, K. D. Anderson and D. Warburton. 2003. *Burkholderia cepacia*-induced IL-8 gene expression in an alveolar epithelial cell line: signalling through CD14 and mitogen-activated protein kinase. Pediatr. Res. 21: 21.

181. Riedel, K., M. Hentzer, O. Geisenberger, B. Huber, A. Steidle, H. Wu, N. Hoiby, M. Givskov, S. Molin and L. Eberl. 2001. N-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology 147: 3249-62.

182. Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou et al. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245: 1066-73.

183. Rodley, P. D., U. Romling and B. Tummler. 1995. A physical genome map of the *Burkholderia cepacia* type strain. Mol. Microbiol. 17: 57-67.

184. Rommens, J. M., M. C. Iannuzzi, B. Kerem, M. L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J. L. Cole, D. Kennedy, D. Hidaka et al. 1989. Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 245: 1059-65.

185. Roos, D., M. de Boer, F. Kuribayashi, C. Meischl, R. S. Weening, A. W. Segal, A. Ahlin, K. Nemet, J. P. Hossle, E. Bernatowska-Matuszkiewicz and H. Middleton-Price. 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood 87: 1663-81.

186. Saiman, L., S. Tabibi, T. D. Starner, P. San Gabriel, P. L. Winokur, H. P. Jia, P. B. McCray and B. F. Tack. 2001. Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis. Antimicrob. Agents Chemother. 45: 2838-44.

187. Saini, L. S., S. B. Galsworthy, M. A. John and M. A. Valvano. 1999. Intracellular survival of *Burkholderia cepacia* complex isolates in the presence of macrophage cell activation. Microbiology 145: 3465-3475.

188. Sajjan, U., C. Ackerley and J. Forstner. 2002. Interaction of cblA/adhesin-positive *Burkholderia cepacia* with squamous epithelium. Cell. Microbiol. 4: 73-86.

189. Sajjan, U. and J. F. Forstner. 1992. Identification of the mucin-binding adhesin of *Pseudomonas cepacia* isolated from patients with cystic fibrosis. Infect. Immun. 60: 1434-1440.

190. Sajjan, U. S. and J. F. Forstner. 1993. Role of a 22-kilodalton pilin protein in binding of *Pseudomonas cepacia* to buccal epithelial cells. Infect. Immun. 61: 3157-63.

191. Sajjan, U. S., L. Sun, R. Goldstein and J. F. Forstner. 1995. Cable (cbl) type II pili of cystic fibrosis-associated *Burkholderia* (*Pseudomonas*) *cepacia*: nucleotide sequence of the cblA major subunit pilin gene and novel morphology of the assembled appendage fibers. J. Bacteriol. 177: 1030-8.

192. Sajjan, U. S., F. A. Sylvester and J. F. Forstner. 2000. Cable-piliated *Burkholderia cepacia* binds to cytokeratin 13 of epithelial cells. Infect. Immun 68: 1787-95.

193. Sajjan, U., G. Thanassoulis, V. Cherapanov, A. Lu, C. Sjolin, B. Steer, Y. J. Yu, O. D. Rotstein, G. Kent, C. McKerlie, J. Forstner and G. P. Downey. 2001. Enhanced susceptibility to pulmonary infection with *Burkholderia cepacia* in *Cftr*$^{-/-}$ mice. Infect. Immun. 69: 5138-50.

194. Salcedo, S. P., M. Noursadeghi, J. Cohen and D. W. Holden. 2001. Intracellular replication of *Salmonella typhimurium* strains in specific subsets of splenic macrophages *in vivo*. Cell. Microbiol. 3: 587-97.

195. Sato, K. and F. Sato. 1984. Defective beta adrenergic response of cystic fibrosis sweat glands *in vivo* and *in vitro*. J. Clin. Invest. 73: 1763-1771.

196. Sayers, T. J., T. A. Wiltrout, C. A. Bull, A. C. Denn, A. M. Pilaro and B. Lokesh. 1988. Effect of cytokines on polymorphonuclear neutrophil infiltration in the mouse. Prostaglandin- and leukotriene-independent induction of infiltration by IL-1 and tumor necrosis factor. J. Immunol. 141: 1670-7.

197. Schlesinger, R. B., J. M. Fine and L. C. Chen. 1992. Interspecies differences in the phagocytic activity of pulmonary macrophages subjected to acidic challenge. Fundam. Appl. Toxicol. 19: 584-9.

198. Schoumacher, R. A., R. L. Shoemaker, D. R. Halm, E. A. Tallant, R. W. Wallace and R. A. Frizzell. 1987. Phosphorylation fails to activate chloride channels from cystic fibrosis airway cells. Nature 330: 752-754.

199. Schwab, U., M. Leigh, C. Ribeiro, J. Yankaskas, K. Burns, P. Gilligan, P. Sokol and R. Boucher. 2002. Patterns of epithelial cell invasion by different species of the

Burkholderia cepacia complex in well-differentiated human airway epithelia. Infect. Immun. 70: 4547-55.

200. Shaw, D., I. R. Poxton and J. R. Govan. 1995. Biological activity of *Burkholderia (Pseudomonas) cepacia* lipopolysaccharide. FEMS Immunol. Med. Microbiol. 11: 99-106.

201. Sherbrock-Cox, V., N. J. Russell and P. Gacesa. 1984. The purification and chemical characterisation of the alginate present in extracellular material produced by mucoid strains of *Pseudomonas aeruginosa*. Carbohydr. Res. 135: 147-154.

202. Shirai, R., J. Kadota, K. Tomono, K. Ogawa, K. Iida, K. Kawakami and S. Kohno. 1997. Protective effect of granulocyte colony-stimulating factor (G-CSF) in a granulocytopenic mouse model of *Pseudomonas aeruginosa* lung infection through enhanced phagocytosis and killing by alveolar macrophages through priming tumour necrosis factor-alpha (TNF-alpha) production. Clin. Exp. Immunol. 109: 73-79.

203. Simpson, J. A., S. E. Smith and R. T. Dean. 1988. Alginate inhibition of the uptake of *Pseudomonas aeruginosa* by macrophages. J. Gen. Microbiol. 134(Pt 1): 29-36.

204. Singh, P. K., A. L. Schaefer, M. R. Parsek, T. O. Moninger, M. J. Welsh and E. P. Greenberg. 2000. Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407: 762-4.

205. Sminia, T., G. J. van der Brugge-Gamelkoorn and S. H. Jeurissen. 1989. Structure and function of bronchus-associated lymphoid tissue (BALT). Crit. Rev. Immunol. 9: 119-50.

206. Smith, R. J., B. J. Bowman and S. C. Speziale. 1986. Interleukin-1 stimulates granule exocytosis from human neutrophils. Int. J. Immunopharmacol. 8: 33-40.

207. Smith, J. J., S. M. Travis, E. P. Greenberg and M. J. Welsh. 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell **85**: 229-36.

208. Smith-Grenier, L. L. and A. Adkins. 1996. Isolation and characterization of soil microorganisms capable of utilizing the herbicide diclofop-methyl as a sole source of carbon and energy. Can. J. Microbiol. 42: 221-6.

209. Snouwaert, J. N., K. K. Brigman, A. M. Latour, N. N. Malouf, R. C. Boucher, O. Smithies and B. H. Koller. 1992. An animal model for cystic fibrosis made by gene targeting. Science 257: 1083-8.

210. Sokol, P. A. 1986. Production and utilization of pyochelin by clinical isolates of *Pseudomonas cepacia*. J. Clin. Microbiol. 23: 560-2.

211. Sokol, P. A., C. J. Lewis and J. J. Dennis. 1992. Isolation of a novel siderophore from *Pseudomonas cepacia*. J. Med. Microbiol. 36: 184-9.

212. Sokol, P. A., P. Darling, D. E. Woods, E. Mahenthiralingam and C. Kooi. 1999. Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-ornithine N^5-oxygenase. Infect. Immun. 67: 4443-4455.

213. Sokol, P. A., P. Darling, S. Lewenza, C. R. Corbett and C. D. Kooi. 2000. Identification of a siderophore receptor required for ferric ornibactin uptake in *Burkholderia cepacia*. Infect. Immun. 68: 6554-60.

214. Sokol, P. A. and D. E. Woods. 1988. Effect of pyochelin on *Pseudomonas cepacia* respiratory infections. Microb. Pathog. 5: 197-205.

215. Speert, D. P. 2002. Advances in *Burkholderia cepacia* complex. Paediatr. Respir. Rev. 3: 230-5.

216. Speert, D. P., M. Bond, R. C. Woodman and J. T. Curnutte. 1994. Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of nonoxidative killing by neutrophils in host defense. J. Infect. Dis. 170: 1524-1531.

217. Speert, D. P., D. Henry, P. Vandamme, M. Corey and E. Mahenthiralingam. 2002. Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada. Emerg. Infect. Dis. 8: 181-7.

218. Speert, D. P., B. Steen, K. Halsey and E. Kwan. 1999. A murine model for infection with *Burkholderia cepacia* with sustained persistence in the spleen. Infect. Immun. 67: 4027-4032.

219. Spencer, R. C. 1995. The emergence of epidemic, multiple-antibiotic-resistant *Stenotrophomonas (Xanthomonas) maltophilia* and *Burkholderia (Pseudomonas) cepacia*. J. Hosp. Infect. 30(Suppl): 453-64.

220. Stephan, H., S. Freund, W. Beck, G. Jung, J. M. Meyer and G. Winkelmann. 1993. Ornibactins--a new family of siderophores from *Pseudomonas*. Biometals 6: 93-100.

221. Stockman, G. D., L. R. Heim, M. A. South and J. J. Trentin. 1973. Differential effects of cyclophosphamide on the B and T cell compartments of adult mice. J. Immunol. 110: 277-282.

222. Stotland, P. K., D. Radzioch and M. M. Stevenson. 2000. Mouse models of chronic lung infection with *Pseudomonas aeruginosa*: models for the study of cystic fibrosis. Pediatr. Pulmonol. 30: 413-24.

223. Straus, D. C., M. K. Lonon and J. C. Hutson. 1992. Inhibition of rat alveolar macrophage phagocytic function by a *Pseudomonas cepacia* lipase. J. Med. Microbiol. 37: 335-40.

224. Straus, D. C., M. K. Lonon, D. E. Woods and C. W. Garner. 1989. Production of an extracellular toxic complex by various strains of *Pseudomonas cepacia*. J. Med. Microbiol. 30: 17-22.

225. Straus, D. C., D. E. Woods, M. K. Lonon and C. W. Garner. 1988. The importance of extracellular antigens in *Pseudomonas cepacia* infections. J. Med. Microbiol. 26: 269-80.

226. Strieter, R. M., J. A. Belperio and M. P. Keane. 2003. Host innate defenses in the lung: the role of cytokines. Curr. Opin. Infect. Dis. 16: 193-8.

227. Tabary, O., S. Escotte, J. P. Couetil, D. Hubert, D. Husser, E. Puchelle and J. Jacquot. 2001. Relationship between IkappaBalpha deficiency, NFkappaB activity and interleukin-8 production in CF human airway epithelial cells. Pflugers Arch. 443: S40-4.

228. Tablan, O. C., W. J. Martone, C. F. Doershuk, R. C. Stern, M. J. Thomassen, J. D. Klinger, J. W. White, L. A. Carson and W. R. Jarvis. 1987. Colonization of the respiratory tract with *Pseudomonas cepacia* in cystic fibrosis - risk factors and outcomes. Chest 91: 527-532.

229. Taggart, C., R. J. Coakley, P. Greally, G. Canny, S. J. O'Neill and N. G. McElvaney. 2000. Increased elastase release by CF neutrophils is mediated by tumor necrosis factor-alpha and interleukin-8. Am. J. Physiol. Lung Cell Mol. Physiol. 278: L33-41.

230. Taya, A., D. B. Carmack, B. A. Muggenburg and J. A. Mewhinney. 1992. An interspecies comparison of the phagocytosis and dissolution of 241AmO2 particles by rat, dog and monkey alveolar macrophages *in vitro*. Int. J. Radiat. Biol. 62: 89-95.

231. Thomassen, M. J., C. A. Demko, J. D. Klinger and R. C. Stern. 1985. *Pseudomonas cepacia* colonization among patients with cystic fibrosis. A new opportunist. Am. Rev. Respir. Dis. 131: 791-6.

232. Tomasek, P. H., B. Frantz, U. M. Sangodkar, R. A. Haugland and A. M. Chakrabarty. 1989. Characterization and nucleotide sequence determination of a repeat element isolated from a 2,4,5-T degrading strain of *Pseudomonas cepacia*. Gene 76: 227-38.

233. Tomich, M., A. Griffith, C. A. Herfst, J. L. Burns and C. D. Mohr. 2003. Attenuated virulence of a *Burkholderia cepacia* type III secretion mutant in a murine model of infection. Infect. Immun. 71: 1405-1415.

234. Toyoshima, M., K. Chida and A. Sato. 2000. Antigen uptake and subsequent cell kinetics in bronchus-associated lymphoid tissue. Respirology 5: 141-145.

235. Trivier, D., N. Houdret, R. J. Courcol, G. Lamblin, P. Roussel and M. Davril. 1997. The binding of surface proteins from *Staphylococcus aureus* to human bronchial mucins. Eur. Respir. J. 10: 804-10.

236. Tsang, K. W., A. Rutman, K. Kanthakumar, A. Palmer, D. Roberts, G. Tillotson, P. J. Cole and R. Wilson. 1995. The effects of low concentrations of antibiotics on epithelial damage caused by non-typable *Haemophilus influenzae* and bacterial morphology. J. Antimicrob. Chemother. 36: 545-9.

237. Tummler, B. and C. Kiewitz. 1999. Cystic fibrosis: an inherited susceptibility to bacterial respiratory infections. Mol. Med. Today 5: 351-8.

238. Ulich, T. R., L. R. Watson, S. M. Yin, K. Z. Guo, P. Wang, H. Thang and J. del Castillo. 1991. The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate. Am. J. Pathol. 138: 1485-96.

239. Ursing, J. B., R. A. Rosello-Mora, E. Garcia-Valdes and J. Lalucat. 1995. Taxonomic note: a pragmatic approach to the nomenclature of phenotypically similar genomic groups. Int. J. System. Bacteriol. 45: 604.

240. Vandamme, P., D. Henry, T. Coenye, S. Nzula, M. Vancanneyt, J. J. LiPuma, D. P. Speert, J. R. Govan and E. Mahenthiralingam. 2002. *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools. FEMS Immunol. Med. Microbiol. 33: 143-9.

241. Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters and J. R. Govan. 1997. Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. Int. J. Syst. Bacteriol. 47: 1188-200.

242. Vandamme, P., B. Holmes, T. Coenye, J. Goris, E. Mahenthiralingam, J. J. LiPuma and J. R. Govan. 2003. *Burkholderia cenocepacia* sp. nov.-a new twist to an old story. Res. Microbiol. 154: 91-6.

243. Vandamme, P., E. Mahenthiralingam, B. Holmes, T. Coenye, B. Hoste, P. De Vos, D. Henry and D. P. Speert. 2000. Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). J. Clin. Microbiol. 38: 1042-7.

244. Venkatakrishnan, A., A. A. Stecenko, G. King, T. R. Blackwell, K. L. Brigham, J. W. Christman, T. S. Blackwell. 2000. Exaggerated activation of nuclear factor-kappaB and

altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 23: 396-403.

245. Wigley, P. and N. F. Burton. 2000. Multiple chromosomes in *Burkholderia cepacia* and *B. gladioli* and their distribution in clinical and environmental strains of *B. cepacia*. J. Appl. Microbiol. 88: 914-8.

246. Winkelstein, J. A., M. C. Marino, R. B. Johnston, Jr., J. Boyle, J. Curnutte, J. I. Gallin, H. L. Malech, S. M. Holland, H. Ochs, P. Quie, R. H. Buckley, C. B. Foster, S. J. Chanock and H. Dickler. 2000. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore) 79: 155-69.

247. Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki and M. Arakawa. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. 36: 1251-1275.

248. Yamagishi, Y., J. Fujita, K. Takagawa, K. Negayama, T. Nakazawa and J. Takahara. 1993. Clinical features of *Pseudomonas cepacia* pneumonia in an epidemic among immunocompromised patients. Chest 103: 1706-9.

249. Yuk, M. H., E. T. Harvill, P. A. Cotter and J. F. Miller. 2000. Modulation of host immune responses, induction of apoptosis and inhibition of NF-kappaB activation by the *Bordetella* type III secretion system. Mol. Microbiol. 35: 991-1004.

250. Zahringer, U., B. Lindner and E. T. Rietschel. 1994. Molecular structure of lipid A, the endotoxic center of bacterial lipopolysaccharides. Adv. Carbohydr. Chem. Biochem. 50: 211-76.

251. Zandvoort, A. and W. Timens. 2002. The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. Clin. Exp. Immunol. 130: 4-11.

252. Zielenski, J., M. Corey, R. Rozmahel, D. Markiewicz, I. Aznarez, T. Casals, S. Larriba, B. Mercier, G. R. Cutting, A. Krebsova, M. Macek, Jr., E. Langfelder-Schwind, B. C. Marshall, J. DeCelie-Germana, M. Claustres, A. Palacio, J. Bal, A. Nowakowska, C. Ferec, X. Estivill, P. Durie, and L. C. Tsui. 1999. Detection of a cystic fibrosis modifier locus for meconium ileus on human chromosome 19q13. Nat. Genet. 22: 128-9.

253. Zughaier, S. M., H. C. Ryley and S. K. Jackson. 1999a. A melanin pigment purified from an epidemic strain of *Burkholderia cepacia* attenuates monocyte respiratory burst activity by scavenging superoxide anion. Infect. Immun. 67: 908-913.

254. Zughaier, S. M., H. C. Ryley and S. K. Jackson. 1999b. Lipopolysaccharide (LPS) from *Burkholderia cepacia* is more active than LPS from *Pseudomonas aeruginosa* and

Stenotrophomonas maltophilia in stimulating tumor necrosis factor alpha from human monocytes." Infect. Immun. 67: 1505-1507.