

**THE PATHOGENICITY OF
BURKHOLDERIA CEPACIA
IN CYSTIC FIBROSIS**

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Thesis presented for the Degree of Doctor of Philosophy

University of Edinburgh

1997



TABLE OF CONTENTS

Table Of Contents	i
Abbreviations	vi
Abstract	viii
Acknowledgements	x
Declaration	xii
Chapter 1 Introduction	1
1.1 Cystic Fibrosis	1
1.1.1 Clinical presentation of CF	1
1.1.2 Genetic basis of CF	3
1.1.3 CFTR structure and function	5
1.1.4 The microbiology of CF lung disease	7
<i>P. aeruginosa in CF</i>	8
<i>Why P. aeruginosa?</i>	9
1.1.5 Recent developments in CF	12
<i>CF mouse models</i>	12
<i>Gene therapy in CF</i>	15
1.2 Inflammation In Cystic Fibrosis	18
1.2.1 CF as an inflammatory disease	18
1.2.2 Neutrophils: an overview	20
<i>Neutrophil granules and their constituents</i>	21
<i>Neutrophil recruitment</i>	24
<i>Neutrophil phagocytosis and activation</i>	26
<i>Respiratory burst activity and ROS generation</i>	30
<i>Neutrophil priming</i>	33
<i>Cellular mechanisms controlling neutrophil priming and activation</i>	35
1.2.3 Neutrophil-mediated damage in the CF lung	41
<i>ROS-mediated damage</i>	42
<i>Proteolytic enzymes</i>	44
1.3 <i>Burkholderia cepacia</i>	45
1.3.1 Background	45
1.3.2 Clinical impact of <i>B. cepacia</i>	47
1.3.3 Characteristics and identification of <i>B. cepacia</i>	48
1.3.4 Biotechnological exploitation of <i>B. cepacia</i>	50
1.4 <i>Burkholderia cepacia</i> In Cystic Fibrosis	52
1.4.1 Background	52
1.4.2 <i>B. cepacia</i> and transmissibility	53

<i>Highly transmissible B. cepacia lineages</i>	54
<i>B. cepacia transmissibility markers</i>	57
1.4.3 Is <i>B. cepacia</i> a pathogen in CF?	58
<i>Genomovar status and pathogenicity</i>	60
1.4.4 <i>B. cepacia</i> cell surface structures	61
<i>Exopolysaccharide production</i>	62
<i>Outer membrane protein expression</i>	62
<i>Lipopolysaccharide</i>	64
1.4.5 Production of extracellular virulence factors by <i>B. cepacia</i>	65
<i>Protease, lipase and haemolysin production</i>	66
<i>Siderophore production</i>	68
1.4.6 <i>B. cepacia</i> and intracellularity	69
1.4.7 The immune response to <i>B. cepacia</i> infection in CF	70
<i>Humoral immune responses</i>	70
<i>Inflammatory damage</i>	72
Chapter 2 Methods	74
2.1 Materials And Equipment	74
2.1.1 Bacterial strains	74
2.1.2 Serum and antibodies	74
2.1.3 Mice	76
2.1.4 Chemicals and media	77
<i>Solutions</i>	77
<i>Bacteriological media</i>	77
<i>Chemicals</i>	79
2.1.5 Equipment	80
2.2 General Bacteriological Methods	80
2.2.1 Storage, recovery and growth of bacteria	80
2.2.2 Extracellular products of <i>B. cepacia</i> isolates	80
2.2.3 Growth in iron-rich and iron-deficient media	81
2.2.4 Extraction of outer membrane proteins	82
2.2.5 Phenol water extraction of lipopolysaccharide	83
2.2.6 SDS-PAGE gel analysis of OMP and LPS extractions	84
<i>Solutions</i>	84
<i>LPS preparations</i>	85
<i>OMP preparations</i>	85
<i>SDS Polyacrylamide gel electrophoresis</i>	85
<i>Silver staining for LPS</i>	86
<i>Coomassie blue staining for protein</i>	87
2.2.7 Bacterial pre-opsonisation	88
2.3 Extraction And Preparation Of Phagocytic Cells	89
2.3.1 Preparation of monocytes	89
2.3.2 Neutrophil preparation using the plasma-Percoll method	90
2.3.3 Neutrophil preparation using the Histopaque method	91
2.3.4 Preparation of washed blood	92

2.4 Studies Of Bacteria:Phagocyte Interactions	92
2.4.1 Washing assay for intracellular survival in monocytes	92
2.4.2 Uptake of bacteria by monocytes using acridine orange/crystal violet staining	93
2.4.3 Killing of bacteria by neutrophils	95
2.4.4 Chemiluminescence assay of neutrophil respiratory burst activity	96
2.4.5 Cytochrome c reduction assay for superoxide production	97
2.4.6 Flow cytometric analysis of neutrophil surface marker expression	99
2.4.7 Intracellular H ₂ O ₂ production by neutrophils	100
2.5 Studies In The Edinburgh CF Mouse Model	102
2.5.1 Bacterial inoculation of mice	102
2.5.2. Preparation of mice lungs	103
2.6 Statistical Analysis	104
RESULTS	
Chapter 3 Characteristics Of Clinical And Environmental <i>B. cepacia</i>	106
3.1 Production Of Extracellular Toxins	106
3.2 Effect Of Iron On Growth Of <i>B. cepacia</i>	109
Chapter 4 Intracellularity Of <i>B. cepacia</i>	112
4.1 Uptake By Human Monocytes	112
4.2 Survival Of Intracellular Bacteria	114
Chapter 5 <i>B. cepacia</i> And Neutrophils: Bacterial Killing And Effects On The Neutrophil Cell Surface	116
5.1 Bacterial Killing By Neutrophils	116
5.2 Flow Cytometric Analyses Of Neutrophil:Bacteria Interactions	119
5.2.1 Comparison of neutrophil preparation techniques on neutrophil:bacteria interactions	119
5.2.2 Induction of CR3 by non-opsonised bacteria on neutrophils in washed blood	125
Chapter 6 Neutrophil Respiratory Burst Responses To <i>B. cepacia</i>	126
6.1 Chemiluminescence Responses To Non-Opsonised <i>B. cepacia</i> And <i>P. aeruginosa</i>	126
6.1.1 Luminol chemiluminescence responses to non-opsonised bacteria	126
6.1.2 Comparison of clinical and environmental <i>B. cepacia</i>	128
6.1.3 Comparison of <i>B. cepacia</i> and <i>P. aeruginosa</i> isolated from CF patients	128
6.1.4 Effect of growth conditions on LCL induction	132
6.1.5 Comparison of luminol and lucigenin chemiluminescence responses	132

6.2	Effect Of Opsonisation On Chemiluminescence Responses	137
6.2.1	Effect of complement and serum on LCL and LuCL responses to the Edinburgh epidemic strain of <i>B. cepacia</i>	137
6.2.2	Chemiluminescence induction following pre-opsonisation of a range of <i>B. cepacia</i> strains	140
6.2.3	Comparison of sera from <i>B. cepacia</i> + and <i>B. cepacia</i> - CF patients	142
6.3	Production Of Superoxide Anions By Neutrophils	149
6.4	Production Of Intracellular H ₂ O ₂ By Neutrophils Stimulated With <i>B. cepacia</i> And <i>P. aeruginosa</i>	158
Chapter 7 Effect Of <i>B. cepacia</i> Products On Neutrophils		164
7.1	Effect Of Lipopolysaccharide On Neutrophil CR3 Expression	164
7.1.1	Lipopolysaccharide extracts	164
7.1.2	Induction of CR3 on neutrophils stimulated with LPS from representative strains.	164
7.1.3	Neutrophil CR3 induction by LPS from a range of bacterial strains	172
7.2	Priming Of Fmlp-Induced Respiratory Burst Activity By Bacterial Products	174
7.2.1	Priming by LPS from <i>B. cepacia</i> J2315 and <i>E. coli</i> O18K-	174
7.2.2	Priming of neutrophil respiratory burst responses by a panel of representative strains	176
7.2.3	Analysis of neutrophil subpopulations	179
7.3	Priming Of Neutrophil Respiratory Burst Responses To Whole Bacteria	183
Chapter 8 Studies Of <i>B. cepacia</i> In A Mouse Model		185
8.1	Background Flora And Delivery Of Bacteria	185
8.1.1	Background flora	185
8.1.2	Intratracheal inoculation of bacteria: delivery and bacterial recovery	189
8.2	Clearance Of <i>B. cepacia</i> J2315 In CF And Non-CF Mice	190
8.3	Clearance Of Environmental And Clinical <i>B. cepacia</i>	195
Chapter 9 Discussion		197
9.1	The pathogenicity of <i>B. cepacia</i>	197
9.2	<i>B. cepacia</i> Exoproducts	198
9.3	<i>B. cepacia</i> And Intracellularity	203
9.4	Interaction Of <i>B. cepacia</i> With Neutrophils	206
	<i>Killing of B. cepacia by neutrophils</i>	206
	<i>Respiratory burst activity</i>	208

9.5 Bacterial Opsonisation And Respiratory Burst Induction	213
<i>The role of opsonic activity in disease progression</i>	215
<i>Effect of atmospheric conditions</i>	217
<i>Opsonic immunoglobulin and B. cepacia colonisation</i>	218
<i>Relationship to other B. cepacia strains</i>	219
9.6 <i>B. cepacia</i> And The Respiratory Burst Pathway	221
9.7 The Pro-Inflammatory Effect Of <i>B. cepacia</i>	223
<i>Pro-inflammatory effects of whole bacteria</i>	225
<i>B. cepacia products in neutrophil activation processes</i>	226
9.8 Studies In The CF Mouse	231
9.9 Genomovar Status And Bacterial Origin: Are Environmental Strains Of <i>B. cepacia</i> Pathogenic?	233
Chapter 10 Conclusions	238
References	240

ABBREVIATIONS

α 1-PI	α 1-proteinase inhibitor (α 1-antitrypsin)
ACFA	Association for Cystic Fibrosis Adults
ANOVA	Analysis of variance
AO	Acridine orange
ASF	Airways surface fluid
BAL	Bronchoalveolar lavage
BCESM	<i>B. cepacia</i> epidemic strain marker
BSA	Bovine serum albumen
CF	Cystic fibrosis
CFL	Cystic Fibrosis Laboratory
CFTR	Cystic fibrosis transmembrane regulator
cfu	Colony forming units
CGD	Chronic granulomatous disease
CK	Chemokine
CR1, 3	Complement receptor 1, 3
CRP	C-reactive protein
CV	Crystal violet
DHR	Dihydrorhodamine
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulphoxide
Fc γ R	Immunoglobulin G Fc receptor
FCS	Fetal calf serum
FEV ₁	Forced expiratory volume in 1 minute
FITC	Fluorescein isothiocyanate
FMLP	fMet-Leu-Phe
FSC	Forward scatter
GM-CSF	Granulocyte/macrophage colony stimulating factor
GPC	Guinea pig complement
GPI	Glycosylphosphatidylinositol
hBD-1	Human β -defensin-1
HBSS	Hanks balanced salt solution
ICAM	Intercellular adhesion molecule
ip	intraperitoneal
IS	Insertion sequence
LBP	LPS-binding protein
LCL	Luminol-enhanced chemiluminescence
LFA-1	Lymphocyte function associated antigen-1
LPS	Lipopolysaccharide
LuCL	Lucigenin-enhanced chemiluminescence
MCF	Mean channel fluorescence
MPA	Mucoid <i>P. Aeruginosa</i>

MPO	Myeloperoxidase
MPRL	Microbial Pathogenicity Research Laboratory
MRC HGU	Medical Research Council Human Genetics Unit
MSD	Membrane spanning domain
NBD	Nucleotide binding domain
NEAP	Neutrophil elastase antiproteinase complex
NMPA	Non-mucoid <i>P. Aeruginosa</i>
O ₂ ⁻	Superoxide ion
OMP	Outer membrane protein
ORCC	Outward rectifying chloride channel
OZ	Opsonised zymosan
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
pH ₂ O	Pyrogen-free water
PHS	Pooled human serum
PI	Pancreatic insufficiency
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PS	Pancreatic sufficiency
RBC	Red blood cells
RBGE	Royal Botanic Gardens, Edinburgh
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SEBTS	South East Blood Transfusion Service
SEM	Standard error of the mean
SOD	Superoxide dismutase
SSC	Side scatter
TF	Transferrin
TGU	Transgenic Unit
TNF α	Tumour necrosis factor α

ABSTRACT

Although colonisation with multiresistant *Burkholderia cepacia* is associated with increased morbidity and mortality in cystic fibrosis (CF) patients, little is known about the pathogenic mechanisms underlying *B. cepacia* infection. The aim of this project was to determine factors which enable *B. cepacia* to colonise the CF lung and to induce the marked inflammatory response characteristic of *B. cepacia* colonisation.

This project has focused on the interaction of *B. cepacia* with neutrophils, the predominant immune cell in CF. As *B. cepacia* is resistant to neutrophil non-oxidative killing mechanisms, respiratory burst induction by *B. cepacia* was investigated using another CF pathogen, *Pseudomonas aeruginosa*, as a comparison. In general, non-opsonised CF isolates of *B. cepacia* induced little respiratory burst activity. By contrast, *P. aeruginosa* strains induced a greater range of responses, with a subset of strains inducing considerable activity. Opsonisation with specific immune sera increased neutrophil responses to both *B. cepacia* and *P. aeruginosa*. However, in view of the cleavage of opsonins and opsonin receptors within the CF lung, opsonisation may have little impact on host defences to *B. cepacia* within the respiratory tract.

Lipopolysaccharide (LPS) extracted from *B. cepacia* was shown to upregulate neutrophil expression of complement receptor 3 (CR3) and to prime respiratory burst responses to fMet-Leu-Phe and to whole bacteria. Significantly LPS from the major epidemic strain of *B. cepacia*, ET12, increased neutrophil respiratory burst responses to *P. aeruginosa* but not to the ET12 strain itself. Thus it was speculated that

B. cepacia may establish a foothold in the *P. aeruginosa*-infected lung by selectively upregulating immune responses to *P. aeruginosa*.

In view of the development of environmental *B. cepacia* as biocontrol agents, environmental and clinical isolates were compared for potential virulence determinants. Few obvious differences were found between CF, non-CF clinical and environmental strains. Significantly LPS from two environmental strains of *B. cepacia* primed neutrophil responses to a similar degree as LPS from CF strains of *B. cepacia*. A surprising result was that an environmental *B. cepacia* strain was less effectively cleared in both CF and non-CF mice than the ET12 strain.

This project has provided evidence to support the hypothesis that *B. cepacia* colonisation is associated with a marked and damaging immune response in CF patients. The avoidance of respiratory burst induction may allow *B. cepacia* to survive in the hostile milieu of the CF lung, whilst increased neutrophil CR3 expression may promote increased neutrophil recruitment to the lung. Furthermore, neutrophil priming promotes increased damage to lung tissue by activated neutrophils. Finally, the evidence for potential pathogenicity in environmental *B. cepacia* supports calls for the tighter control of *B. cepacia* biocontrol programmes, including an application presently before the US Environmental Protection Agency to release *B. cepacia* in large scale agricultural trials.

ACKNOWLEDGEMENTS

To acknowledge everyone who has been of assistance in the preparation of this PhD is a difficult task, particularly as so many have helped me in many different ways. First mention must go to Dr John Govan, who initially employed me despite my lack of funding and experience, spent many a month drafting grant applications in the hunt for a Fellowship, and without whose support (and occasional almond cakes!) little progress would have been made towards this thesis. Thanks must also go to Dr John Stewart, my second supervisor, whose advice, encouragement, and understanding has been invaluable, particularly in the last year of this thesis. Much of the work towards this thesis was only possible because of the generous provision of equipment and advice by Dr Robin Barclay, SBTS, to whom I am deeply indebted. Laboratory colleagues past and present have played a major role, particularly Cathy Doherty and Mike Kerr who have provided excellent technical advice and assistance from the beginning of my laboratory career. I am also indebted to Sarah Butler and Debbie Shaw who first initiated me into the ways of PhD students, as well as my ever patient laboratory colleagues, Wendy, Alison and Mike, who have put up with my endless requests for blood donations and often untidy approach to the lab and office with remarkable good humour!

Many other colleagues have provided advice, assistance and even blood donations, including Dr Ian Poxton, Robert Brown, Dr Ian Laurenson, Ian Hodgson, Dr Cathy Taylor, Prof Sebastian Amyes, Malcolm Baldock, Bill Neil, Bill Adams and Angus MacAulay, Dr Ian Dransfield and Dr Andy Greening. Thanks must also go to Dr Kathy Liddell at the adult CF unit, Western General Hospital who has provided many

of the serum samples used in this thesis. Studies involving the CF mouse have all been conducted with a great deal of help from staff at the MRC Human Genetics Unit, including Dr Julia Dorin, Dr Gerry McLachlan and, particularly, Dr Donald Davidson. The generous provision of a travelling Fellowship by the CF Trust enabled me to spend a very successful two weeks in the Tübingen, working with Dr Dieter Worlitzsch in the laboratory of Prof Gerd Döring ; the warm welcome and continuing encouragement offered by both Gerd and Dieter has been very much appreciated.

Finally I wish to thank all of those without whom completion of this PhD would have been impossible; the MRC who provided funding, my lab colleagues, especially Cathy and Katrina whose support and friendship has made all the difference during difficult times and finally my husband Dave, without whose patience, love and encouragement none of this would have been achieved.

DECLARATION

All of the experiments and procedures presented in this thesis were carried out by the author unless otherwise stated in the text.

CHAPTER 1 INTRODUCTION

1.1 CYSTIC FIBROSIS

Cystic fibrosis (CF) is the commonest life-threatening, autosomal recessive condition in Caucasian populations, with an incidence of approximately 1 in 2500 live births, thus affecting approximately 350 children born in the UK each year (Elborn, 1994). CF presents as a multisystem disorder of exocrine organs, including the lungs, gastrointestinal tract, pancreas, liver, vas deferens and sweat glands. Research into the pathophysiological mechanisms underlying the many clinical manifestations of CF has progressed rapidly since the identification of the CF gene in 1989. Based on present evidence, disease is known to be a consequence of a defect in a single protein, the cystic fibrosis trans-membrane conductance regulator (CFTR), which functions as a chloride channel on the apical surface of secretory epithelial cells within a number of organ systems. Until recently the outlook for affected children was poor but sophisticated treatment regimes, including physiotherapy programmes, the aggressive use of antibiotic therapy and careful nutritional management, have led to many patients surviving into adulthood, with, for example, an average life expectancy of 28 years of age in 1990 (Fitzsimmons, 1993).

1.1.1 Clinical presentation of CF

The basic defect in CF results in disordered fluid and electrolyte balance in secretory epithelia throughout the body (Elborn 1994; Noone et al, 1994; Frizzell, 1995).

Although many organ systems are affected, lung disease accounts for the greatest

morbidity and mortality, particularly in individuals surviving infancy (Welsh and Smith, 1993). Classically, patients produce copious amounts of thick mucous within the bronchioles. An inability of muco-ciliary clearance mechanisms to remove these secretions leads to bacterial colonisation and the blockage of small airways by plugs of mucous. Infective exacerbations result in inflammation and damage to healthy lung tissue, leading to the development of bronchiectasis. Thus, patients enter a vicious circle whereby lung damage leads to bacterial colonisation and hence more lung damage. Clinically, CF pathophysiology is associated with slow respiratory decline punctuated by periods of more rapid decline associated with infective exacerbations, followed eventually by respiratory failure and death (Elborn, 1994). Intensive physiotherapy may clear congested airways and aggressive antibiotic therapy can slow disease progression but ultimately, treatment is unable to prevent decline in the majority of patients. Lung transplantation has been successful in many patients but is not suitable, nor available, for all and is itself associated with significant mortality (Moss, 1995).

Gastrointestinal manifestations frequently play a role in CF disease progression.

Approximately 16% patients will present at birth with gastrointestinal blockage due to thickened intestinal secretions, a condition termed 'meconium ileus'

(Fitzsimmons, 1993). Furthermore, approximately 90% of patients will show poor

pancreatic function termed pancreatic insufficiency (PI) due to the production of viscid secretions which block pancreatic ducts (Kubesch et al, 1993). The

subsequent lack of pancreatic enzymes leads to digestive problems and children with

CF may present with failure to thrive (Fitzsimmons, 1993). Although pancreatic

deficiency can be treated with enzyme supplementation, patients are at risk of being clinically undernourished, compromising their resistance to respiratory infection still further. Indeed the minority of patients who are pancreatic sufficient (PS) generally have a less severe course of lung disease than PI patients (Kubesch et al, 1993).

Other manifestations of CF include the production of a characteristic salty sweat, which is the basis of the 'sweat test' used to diagnose CF, and male infertility due to blocked or absent vas deferens. In over 10% of older patients, pancreatic damage leads to diabetes, whilst 1-2% of patients develop cirrhosis as a result of chronic damage within the liver (Fitzsimmons, 1993). Indeed, as radical new treatments are developed for respiratory disease, it is likely that these latter gastrointestinal manifestations will become more important.

1.1.2 Genetic basis of CF

In 1983, Quinton described a defect in Cl⁻ secretion at the apical surface of CF epithelial cells and proposed that aberrant chloride channel production was responsible for the many manifestations of CF disease. By 1985, investigation of affected and non-affected siblings in families with CF by restriction fragment length polymorphisms identified chromosome 7 as the site of the CF gene (Tsui et al, 1985). This breakthrough was followed by the discovery of the putative CF gene in 1989, using the combined techniques of linkage analysis and chromosome 'walking' and 'jumping' (Rommens et al, 1989; Riordan et al, 1989; Kerem et al, 1989). Confirmation that this was the correct gene came with the identification of a mutant form in CF patients, with approximately 70% of CF patients showing a three base

pair deletion, resulting in the loss of a phenylalanine residue at position 508, termed the $\Delta F508$ mutation (Riordan et al, 1989; Kerem et al, 1989).

The CF gene spans more than 250kb and includes 27 exons, coding for the 1480 amino acid protein termed CFTR (reviewed in Frizzell, 1995). Protein production includes extensive mRNA splicing and complex post-translational modifications, including glycosylation within the Golgi apparatus. Although the $\Delta F508$ mutation is by far the most frequent mutant allele found in CF patients, over 400 other mutations have been described, including deletions, nonsense mutations encoding early stop codons, frameshift mutations, splice mutations at intron/exon boundaries affecting mRNA processing and missense mutations resulting in substitution of single amino acids (Welsh and Smith, 1993). Individual mutations have varying effects on CFTR production and function (Table 1.1); for example the $\Delta F508$ mutation results in a defect in post-translational modification and intracellular protein transport; consequently CFTR is arrested and degraded within the endoplasmic reticulum and not expressed at the cell surface (Tsui, 1995).

Table 1.1 Classes of CFTR mutations (adapted from Welsh and Smith, 1993)

Class	Defect	Result	Example	Frequency	PI/PS
I	Protein production	Production of no or truncated protein	G542X	3.4	PI
II	Processing	Failure to traffic to the correct cellular location	ΔF508	67.2	PI
III	Regulation	Mutations in the NBD leading to altered interactions with ATP and decreased channel activity	G551D	2.4	PI
IV	Conduction	Mutations in MSD reducing the rate of Cl ⁻ flow through the open channel	R117H	0.8	PS

Abbreviations used: PI, pancreatic insufficient; PS, pancreatic sufficient; NBD, nucleotide binding domain; MSD, membrane spanning domain

1.1.3 CFTR structure and function

CFTR is structurally homologous to a family of proteins termed the transport ATPases and comprises 1480 amino acids within 15 domains (Frizzell, 1995).

Twelve transmembrane domains are arranged as 2 hydrophobic regions which form the basis of the ion channel. The remaining domains consist of a regulatory region (R), containing several sites for phosphorylation, and two flanking ATP-binding regions termed the nucleotide binding domains (NBD-1, NBD-2). Activation of CFTR depends upon phosphorylation of the R region followed by binding of ATP to one or both of the NBD regions. The open probability of CFTR is a simple saturable function of ATP concentration with a maximum P_o of 0.44; thus even at saturating

concentrations of ATP, CFTR is open for less than 50% of time (Venglarik et al, 1994).

The identification of CFTR and its gene has led to several attempts to link genotype with the varied phenotypic presentations of the disease. In one large Canadian study, the majority of PS patients were found to express at least one missense mutation affecting amino acids within the transmembrane regions encoding the actual chloride channel, presumably because such mutations are associated with some residual CFTR function (Kristidis et al, 1992). By contrast null mutations, mutations resulting in intracellular arrest of CFTR post-translational modification, such as $\Delta F508$, and missense mutations affecting the regulatory regions of CFTR were usually associated with the more severe PI form of disease (Table 1.1).

Homozygosity for the $\Delta F508$ mutation has also been shown to correlate with more severe respiratory disease, as assessed by measurements of respiratory function such as forced expiratory volume in 1 min (FEV_1) or by age at colonisation with the CF pathogen *P. aeruginosa* (Kubesch et al, 1993). However variation between patients is too great to allow prognosis to be predicted for any individual from a knowledge of their genotype.

Whilst knowledge of the genetic basis of CF has increased, our understanding of the role of CFTR within respiratory epithelia is still far from complete, and is complicated by the observation that CFTR in the lung is mainly expressed in the submucosal glands rather than in respiratory epithelium *per se* (Engelhardt et al, 1992). Studies of ion transport in normal and CF epithelia have demonstrated that CFTR functions both as a chloride channel and as a regulatory molecule for other

transport proteins, including the Ca^{2+} -dependent outwardly rectifying chloride channel (ORCC) (Noone et al, 1994; Gabriel et al, 1993). Although a limited ability to secrete Cl^- ions is characteristic of CF cells, the dominant abnormality of affected epithelia is the hyperabsorption of Na^+ ions mediated via a selective sodium channel also situated on the apical surface of the epithelial cell (Noone et al, 1994). The net result is a flow of ions, and hence fluid, out of the respiratory tract, leading to the production of dehydrated viscid respiratory secretions. It seems likely that CFTR may play a regulatory role with respect to the Na^+ channel as well as ORCC (Noone et al, 1994). CFTR has also been implicated in cAMP-dependent regulation of endocytosis and exocytosis and in the acidification of intracellular organelles during post-translational modification processes (Cheng et al, 1990; Barasch and Al-Awqati, 1993; Dunn et al, 1994). Thus a lack of CFTR may effect cellular processes other than ion transport; for example, sialylation and sulphation of cell surface macromolecules is reduced in CF epithelia (Al-Awqati, 1995).

1.1.4 The microbiology of CF lung disease

A striking feature of pulmonary infection in CF is the limited spectrum of microbial pathogens involved, although this spectrum is evolving as more effective treatment regimes have increased the life expectancy and hence the age range of CF patients. In the earliest microbiological study of post-mortem cultures of lung tissue from CF infants, *Staphylococcus aureus* was the predominant colonising organism, found in 12 out of 14 CF patients (Di Sant Agnese and Anderson, 1946). Subsequently *S. aureus* and *Haemophilus influenzae* were recognised as important CF pathogens;

however, improved antibiotic therapy against both of these organisms facilitated the emergence of *P. aeruginosa* as a major CF pathogen. Other respiratory pathogens have been implicated in CF, including mycobacterial species, influenza and respiratory syncytial viruses, and the fungal pathogen, *Aspergillus fumigatus* (Govan and Nelson, 1993). In recent years, several previously uncommon respiratory organisms have also emerged, including *Stenotrophomonas (Xanthomonas) maltophilia*, *Alcaligenes xylosoxidans* (VuThien et al, 1996; Karpati et al, 1994), *Burkholderia (Pseudomonas) cepacia* (Govan and Deretic, 1996) and *Burkholderia gladioli* (Wilsher et al, 1997).

P. aeruginosa in CF

Although both *S. aureus* and *H. influenzae* are associated with repeated and often severe infective exacerbations, *P. aeruginosa* is generally regarded as the major CF pathogen, responsible for the greatest morbidity and mortality amongst CF populations. Colonisation with *P. aeruginosa* usually occurs after a succession of other CF pathogens and the incidence of *P. aeruginosa* increases amongst CF patients with age (Pedersen et al, 1986; Fitzsimmons, 1993). However a recent epidemiological study demonstrated an incidence of 21% in patients under 1 year of age, suggesting that *P. aeruginosa* colonisation in younger patients may be increasing (Fitzsimmons, 1993).

Colonisation with *P. aeruginosa* is characterised by the dramatic adaptation of this versatile opportunistic pathogen to the unusual environment of the CF lung.

P. aeruginosa is initially acquired in a non-mucoid form, expressing a range of virulence factors including pyocyanin, elastase, exotoxin A and exoenzyme S.

However, within a few months of colonisation, mucoid variants emerge as a result of 'muc' mutations in genes regulating the production of a mannuronic acid- and guluronic acid-rich polysaccharide termed alginate (Govan and Deretic, 1996). The resulting constitutive production of viscous exopolysaccharide, which gels rapidly in physiological concentrations of electrolytes, leads to biofilm formation and allows *P. aeruginosa* to evade host defences by surviving as microcolonies within an alginate matrix. Conversion to mucoidy is usually associated with the down-regulation of exotoxin production and with a switch from smooth to rough LPS, reflecting the metabolic demands of alginate production. Furthermore, some mucoid variants may also contain mutations rendering them extremely sensitive to the action of β -lactam antibiotics. Such mutations give rise to increased outer membrane permeability and thus improve bacterial survival by promoting nutrient uptake within the nutrient-poor environment of the alginate biofilm. Any disadvantage conferred by increased antibiotic sensitivity is outweighed by the protective effects of alginate, which is largely impermeable to antibiotic therapy.

Why P. aeruginosa?

The ability of *P. aeruginosa* to colonise the CF lung so effectively is surprising in view of the rarity of *P. aeruginosa* lung infections in non-CF individuals, including individuals with other chronic respiratory diseases. Recent developments have shed some light on the possible mechanisms underlying this remarkable predilection.

P. aeruginosa has been shown both to bind to tracheobronchial mucin (Ramphal et al, 1987) and to induce enhanced production of mucin by human goblet cells (Jian-Dong, 1996). Coupled with the inhibition of ciliary function by the *P. aeruginosa*

exoproducts pyocyanin and rhamnolipid (Kanthakamur et al, 1993; 1994), such an upregulation of human mucin production may lead to a further increase in the quantity and viscosity of respiratory secretions. Binding of non-mucoid *P. aeruginosa* to the epithelial glycolipid asialo GM₁ has also been suggested as an initial step in colonisation processes (Krivan et al, 1988). In contrast to normal epithelial cells, which express the sialylated form of GM₁, CF epithelial cells have been shown to express primarily asialo GM₁ (Imundo et al, 1995), possibly through defective intracellular acidification of the trans Golgi network and subsequent suboptimal activity of the sialyl transferases. Finally, recent studies have demonstrated enhanced binding of *P. aeruginosa* to both exposed basement membrane components in damaged epithelia and to the apical surface of repairing epithelial cells (de Bentzmann et al, 1996), suggesting that damage to the CF lung initiated by earlier pathogens may expose suitable binding sites for *P. aeruginosa* and hence promote colonisation. The increased binding of *P. aeruginosa* to repairing epithelial cells has been linked to the enhanced expression of asialo GM₁ on these cells, particularly in CF epithelia, where asialo GM₁ levels are increased still further (Girod de Bentzmann et al, 1995).

In an innovative study, Pier et al (1996) have suggested an alternative explanation for *P. aeruginosa* colonisation in the CF lung. Cultured normal respiratory cells were found to bind and internalise *P. aeruginosa* via intact CFTR, expressed at the cell surface. The authors speculate that such uptake of *P. aeruginosa*, followed by epithelial cell desquamation, protects the host from colonisation. In individuals expressing no surface CFTR, *P. aeruginosa* would remain adherent to

tracheobronchial mucin and thus set up a persistent infection. Interestingly, binding appeared to be mediated by complete LPS, with rough mutants showing diminished internalisation. Thus the conversion of *P. aeruginosa* from smooth to rough forms in vivo may contribute to *P. aeruginosa* survival in the CF host. Indirect evidence to support the hypothesis that *P. aeruginosa* internalisation via CFTR is protective, is provided by the observation of *P. aeruginosa* colonisation at an earlier age in individuals homozygous for the $\Delta F508$ mutation (Kubesch et al, 1993), since this mutation is associated with intracellular arrest of CFTR trafficking to the cell surface. Further studies will be required, however, to demonstrate whether patients expressing non-functional CFTR at the apical cell membrane are less likely to be colonised by *P. aeruginosa* than patients with no surface CFTR expression.

Recent interest has also focused on the possible role of 'defensins' present in respiratory secretions in susceptibility to both *S. aureus* and *P. aeruginosa* infection. 'Defensin' is a general term for small cationic peptides possessing antimicrobial properties and produced in a range of biological systems, including phagocytic cells and epithelial secretions. Smith et al (1996) demonstrated intrinsic anti-pseudomonal activity in airways surface fluid (ASF) secreted by primary cultures of normal human respiratory epithelial cells, but not in ASF from cultured CF respiratory epithelial cells. Activity was dependent on the salt content in ASF and could be restored in CF ASF by dilution in hypotonic salt solutions. Goldman et al (1997) have identified a β -defensin, termed human β -defensin-1 (hBD-1) in ASF from a human bronchial xenograft in athymic mice. Following cloning of the hBD-1 gene, molecular studies identified expression of hBD-1 throughout respiratory epithelial in both CF and non-

CF lungs and demonstrated that hBD-1 was solely responsible for ASF anti-pseudomonal activity. However, as in the studies of Smith et al (1996), activity was highly dependent on salt concentration, suggesting that the hypertonic nature of ASF in CF individuals abrogates any hBD-1 antimicrobial activity and thus predisposes the host to infection with *P. aeruginosa*.

In recent years, the emergence of new CF pathogens has provoked concern amongst patients and carers in the CF community. Of these *B. cepacia*, which will be discussed in detail in sections 1.3 and 1.4, has undoubtedly had the greatest impact, both in terms of pathogenicity and epidemic spread within and between CF centres. However disturbing reports have recently emerged of potential epidemic spread of *Stenotrophomonas maltophilia* and *Burkholderia gladioli*, associated with serious infections in CF patients, (Karpati et al 1994; Wilsher et al, 1997; C Doherty, personal communication). Ominously, both of these opportunistic pathogens display the multiresistant phenotype, characteristic of many emerging infective organisms during the latter years of the twentieth century

1.1.5 Recent developments in CF

CF mouse models

As CF is unique to man, basic research into the physiology and pathology of CF has been hampered by the lack of a suitable animal model. However the discovery and

cloning of the CF gene in humans (Riordan et al, 1989) and subsequently mice (Tata et al, 1991) has enabled the development of a number of murine models of CF. Depending on the nature of the underlying mutation, considerable differences in survival and the severity of CF-type disease are apparent in the various mouse models. For example the UNC knock-out mice express no normal CFTR and display a severe phenotype with less than 20% of mice surviving beyond weaning (Snouwaert et al, 1992). By contrast, the Edinburgh CF mouse, which was developed by insertional mutagenesis, possesses a 'leaky' mutation due to exon skipping and aberrant splicing (Dorin et al, 1992). The resultant low level production of CFTR (~10% wildtype levels of CFTR mRNA expression; Dorin et al, 1994) is associated with a milder phenotype and approximately 95% of mice survive beyond weaning, enabling further studies to be conducted. Recently mice have been developed to mimic human CF more closely by replacement of murine CFTR with actual human mutations. Examples include the Cambridge $\Delta F508$ mouse (Colledge et al, 1995) containing the commonest CF mutation and the G551D mouse (Delaney et al, 1996) containing a CF mutation associated with less severe gastrointestinal disease in humans.

All of the CF mouse models display disturbances in CFTR-dependent chloride channel conductance in secretory epithelia consistent with human CF. Furthermore, histopathological abnormalities similar to changes seen in human CF have been demonstrated in the lungs, colon and vas deferens of several mouse models (Dorin et al, 1992; Davidson et al, 1995; Colledge et al, 1995; Snouwaert et al, 1992). Pathological changes in the lungs are generally mild, with few mice showing overt

symptoms of respiratory disease when housed within laboratory conditions.

However, in an important study of the Edinburgh CF mouse, Davidson et al (1995) provided the first evidence to indicate that a CF mouse model displayed greater susceptibility to CF pathogens than non-CF mice. Following a single exposure to either *S. aureus* or *B. cepacia* by nebulisation, clearance was impaired in CF mice compared to non-CF littermates. Similarly, histopathological investigation indicated that repeated exposure to *S. aureus* was associated with a higher incidence of bronchiolitis, goblet cell hyperplasia or metaplasia, and mucus retention in CF mice than non-CF mice, whilst repeated exposure to *B. cepacia* was associated with a severe bronchopneumonia in some CF mice but none of their non-CF littermates.

The Edinburgh CF mouse model, which is bred onto an outbred murine strain (MF1), displays considerable variability in the degree of histopathological abnormalities, particularly in response to infection (Davidson et al, 1995; Dorin et al, 1992).

Reasons for such variability could include differences in the animals' environment, including exposure to pathogens, or variable degrees of 'leakiness' with respect to CFTR expression in CF mice. In addition, the presence of modifying genetic factors is likely to be of major importance in determining the phenotypic presentation of CF (Erickson, 1996). Sibling studies in human CF have demonstrated marked differences in severity between siblings presumably carrying the same mutations and living within similar environments (Sing et al, 1982). Recently a study of a CF mouse model outcrossed onto two different strains of normal mice, demonstrated milder phenotypic expression of CF in C57B6/J mice compared to DBA/2J mice (Rozmahel et al, 1996). The improved phenotype of C57B6/J mice may be related to

the higher expression of an alternative chloride channel to CFTR in these mice rather than DBA/2J mice. Further studies of different CF mouse models crossed onto inbred strains of mice will provide more information on the role of genetic modifiers in the pathogenesis of both mouse and human CF.

Gene therapy in CF

Perhaps the most exciting prospect for the successful treatment of CF is the development of somatic cell gene therapy. Since the discovery and cloning of the CF gene in 1989, CF research has been characterised by the speed with which gene therapy trials have been designed and initiated (Alton and Geddes, 1997). Already a number of phase I clinical trials have been completed, using both adenoviral and liposomal gene vectors to deliver the CF gene to nasal epithelia in adult CF patients, with successful transfection of the CF gene in a majority of recipients and at least partial correction of the electrophysiological abnormalities of CF in one third of patients. (Knowles et al, 1995; Zabner et al, 1993; Crystal et al, 1994; Caplen et al, 1995). Trials of adenovirus vectors have also included administration to the lung and have shown expression of CFTR in bronchial epithelium following CF gene transfection (Crystal et al, 1994); however no data is available on the functional correction of the CF defect due to the difficulty in assaying electrophysiological parameters in the lower respiratory tract. Several other trials have been completed or are in progress, including a large trial of liposomal delivery currently under way in Edinburgh.

Although early results from human gene therapy have provided much encouragement, problems remain with current gene therapy strategies. Firstly, even

if CFTR can be successfully replaced in the CF lung, it may be many years before clinical benefit can be shown in patients. At present, ethical considerations restrict trials to adult CF patients with established lung disease, but these patients are least likely to benefit from gene therapy. Furthermore, it is not clear how best to assess effectiveness of treatment, particularly as normalisation of electrophysiological measurements and CFTR expression in the lower respiratory tract may have little bearing on lung function, bacterial colonisation and patient well-being in the presence of pre-existing lung disease. Ultimately, trials will be needed in children prior to bacterial colonisation and the initiation of gross lung damage. Finally, the efficacy and toxicity of the various delivery systems will need careful monitoring. Adenoviral vectors have already been associated with dose-dependent toxicity in CF patients due to the generation of a cytotoxic T cell response and cytokine induction. Such an immune response may also be associated with a decrease in the effectiveness of gene delivery (Crystal et al, 1994). Thus, the number of successful applications of the adenoviral vector which can be made in any one patient is limited. Since CFTR expression is only maintained for approximately 1-3 weeks after transfection (Caplen et al, 1995; Crystal et al, 1994) repeated applications will be needed to induce any clinical benefit. Research is therefore under way to develop adenoviral vectors of low immunogenicity, allowing repeated administration of the CF gene to the respiratory epithelium. Alternatively, improved efficiency of non-toxic liposomal vectors may provide the sustainable delivery system needed for successful gene therapy (Alton and Geddes, 1997)

In view of the many problems outlined above, research in CF mouse models may be of crucial importance in expediting the development of efficient, safe and clinically effective gene delivery systems in CF patients. Already functional correction of the CF defect in the lower respiratory tract has been demonstrated in trials of gene therapy in CF mice (Hyde et al, 1993; Alton et al, 1993; Dorin et al, 1996). Significantly, in one study, correction of defective chloride transport was demonstrated after only partial correction of the genetic defect with CFTR mRNA levels of only 5% normal (Dorin et al, 1996). Trials of different delivery systems and regimes in CF mice may also allow greater targeting of delivery strategies in human CF. Recent trials of fetal gene therapy have also suggested that permanent clinical benefit can follow limited expression of CFTR by fetal mice, following administration of adenoviral vectors in utero (). Ultimately, studies in the CF mouse will indicate whether gene therapy can prevent the development of lung damage in response to bacterial pathogens in CF patients. The establishment of a CF mouse model of bacterial colonisation with common CF pathogens will greatly aid this research (Davidson et al, 1995; Anonymous, 1995).

1.2 INFLAMMATION IN CYSTIC FIBROSIS

1.2.1 CF as an inflammatory disease

Lung damage in CF is characterised by widespread destruction of the lung parenchyma giving rise to bronchiectasis. Bacterial colonisation alone is not likely to provoke this damage, particularly as the majority of pathogens in CF are associated with non-invasive disease. Instead, it would appear that chronic exposure to bacterial pathogens leads to a marked and prolonged pulmonary immune response, resulting in inflammatory damage to the lung parenchyma. Serum indicators of inflammation including C-reactive protein (CRP), tumour necrosis factor α (TNF α) and neutrophil elastase are consistently increased in CF patients and can be used as a marker for imminent or ongoing infective exacerbations (Rayner et al, 1991; Norman et al, 1991; Glass et al, 1988; Govan and Nelson, 1993). Patient response to antibiotic therapy is associated with a drop in CRP levels, but not necessarily in bacterial load, suggesting that sub-lethal doses of antibiotics suppress microbial virulence which results in a 'damping down' of the immune response (Glass et al, 1988; Govan and Nelson, 1993). Similarly the pronounced humoral response to colonisation with *P. aeruginosa* does not appear to be protective, but is associated with a poorer prognosis for patients with higher immunoglobulin levels (Pedersen et al, 1990).

These findings indicate a correlation between immune response and clinical condition in CF, but do not prove that the immune response *per se* is responsible for the damage seen in the CF lung. *P. aeruginosa*, for example, is known to produce a

range of toxins that may act directly on the lung, including elastase which may contribute to the destruction of the lung parenchyma (Suter, 1994). However studies using bronchoalveolar lavage (BAL) have provided evidence of an excessive local immune response within the CF lung. Concentrations of pro-inflammatory cytokines including IL-1, IL-6, IL-8 and TNF α are increased in BAL fluid from CF patients compared to healthy controls (Bonfield et al, 1995b). Konstan et al (1994) describe an increase in phagocytic cells and immunoglobulins in BAL fluid from CF adolescents or adults with clinically mild disease. Significantly, neutrophils accounted for 57% of inflammatory cells in CF BAL fluid, in contrast to the predominance of macrophages seen in healthy lungs. These findings were particularly interesting in view of the comparatively good clinical state of these patients, and imply that low-level inflammation is occurring continuously, even in CF patients with mild disease. Similarly Khan et al (1995) describe an augmented neutrophil load in infants with CF, in the absence of bacterial infection. Noah et al (1997) compared cytokine levels in BAL fluid from children with or without CF who were undergoing bronchoscopy. Infection in CF patients was associated with higher levels of the pro-inflammatory cytokine IL-8 in comparison to both infected and non-infected controls. As IL-8 is a potent chemotactic agent for neutrophils, these results imply that increased IL-8 production in CF may account for the excessive recruitment of neutrophils to the CF lung in response to infection. The source of increased IL-8 production could include alveolar macrophages, respiratory epithelial cells and neutrophils already recruited to the lung. A deficiency in the production of the anti-inflammatory cytokine IL-10 may also augment the release of pro-inflammatory cytokines (Bonfield et al, 1995a). Whether such a pro-inflammatory

state arises from the fundamental defect in CFTR function in CF remains to be determined. However, expression of CFTR in normal macrophages and the observation of elevated IL-8 levels in CF alveolar macrophages would indirectly support such an hypothesis (Clément, 1996; Khan et al, 1995).

Accumulated evidence indicates that neutrophils are the predominant inflammatory cell in the CF lung and are implicated in lung damage through the release of tissue damaging enzymes and other substances during activation. Thus, the following sections will provide a general overview of neutrophil activation processes and a critical discussion of current views on the role of neutrophil defence mechanisms in CF lung disease.

1.2.2 Neutrophils: an overview

Neutrophils are large granular polymorphonuclear leukocytes, constituting up to 70% of the white blood cell population in normal blood. Their primary function is the phagocytosis and killing of extracellular pathogens; thus neutrophils are equipped with an impressive array of microbial killing mechanisms, including proteases, acid hydrolases, iron-binding proteins, antimicrobial agents and enzyme systems to generate highly toxic reactive oxygen species (ROS). Neutrophil activation processes involve numerous stages, including upregulation of surface receptors, recruitment to inflammatory sites, binding and phagocytosis of non-opsonised and opsonised bacteria, degranulation into phagosomes and the extracellular environment and the initiation of the ROS-generating respiratory burst. Neutrophils, particularly if unactivated, are short-lived cells and normally undergo apoptotic cell death and

clearance by macrophages, thus preventing the inappropriate release of tissue-damaging enzymes and substances into the extracellular environment (Male et al, 1996).

The complex processes underlying neutrophil recruitment to inflammatory sites and subsequent activation are mediated by a diverse range of receptor molecules expressed at the neutrophil cell surface. Several classes of receptor have been described, including the 7-transmembrane domain chemokine receptors, adhesion mediating selectins and integrins, single domain immunoglobulin receptors and a variety of structurally unrelated receptors recognising neutrophil priming agents such as TNF α or GM-CSF. Individual neutrophil receptors will be discussed further in the relevant sections below.

Neutrophil granules and their constituents

Granules within the neutrophil cytoplasm act as an intracellular store for many of the receptors, inflammatory mediators and antimicrobial systems deployed by neutrophils during migration, phagocytosis and bacterial killing. At least 4 granule subtypes have been identified on the basis of size, contents, density and electrophoretic properties, but others may yet be described (Boxer and Smolen, 1988; Borregaard et al, 1993; Sengeløv et al, 1992). Degranulation, the process whereby granules discharge their contents by fusing with the plasma membrane or a developing phagosome, is under precise control, and can be initiated by a range of stimuli including chemoattractants, neutrophil priming agents, bacterial products such as lipopolysaccharide, and the phagocytosis of bound microorganisms. Granule fusion with the plasma membrane results in extracellular release of granule contents

(exocytosis), as well as an upregulation of specific cell surface receptors stored within the membrane of each granule subtype. Fusion with the phagosome results in the release of antimicrobial compounds into the immediate vicinity of an ingested microorganism, forming a phagolysosome.

Mobilisation of each granule subtype is regulated independently, reflecting the diverse functions of granular constituents and the potential for harm should toxic substances be released inappropriately (Boxer and Smolen, 1988; Borregaard et al 1993). Table 1.2 summarises the contents of the four main granule subtypes described to date. Azurophil (primary) granules are large granules formed early in neutrophil development. Many of the most toxic components of the neutrophil antimicrobial armoury are stored within azurophil granules, which are mobilised late in activation processes and fuse primarily with the phagosome to form the phagolysosome; hence azurophil granules can be regarded as the classic lysosome. Specific (secondary) granules are large granules which are formed later in neutrophil development and contain a range of substances involved in neutrophil recruitment (eg: integrins, FMLP receptors), the initiation of an inflammatory response (eg: complement activator, monocyte chemoattractant) and antimicrobial activity (eg lysozyme, cytochrome b_{558} , lactoferrin). Degranulation of specific granules occurs more readily than azurophilic granule mobilisation, and may be important in neutrophil chemotaxis, mobilisation of inflammatory mediators, and in preparing the neutrophil to mount an effective respiratory burst against ingested microorganisms (Boxer and Smolen, 1988; Borregaard et al, 1993). Tertiary granules are smaller

Table 1.2 Neutrophil granules and their constituents. (Adapted from Boxer and Smolen, 1988; Borregaard et al, 1993).

Granule	Membrane	Matrix
Azurophil (1 ⁰)	CD63, CD68	Myeloperoxidase Lysozyme Defensins Cationic peptides Elastase Cathepsin G Proteinase-3 N-acetyl-glucuronidase Cathepsin B, D β -glucuronidase α -mannosidase Kinin-generating enzyme C5a-inactivating factor
Specific (2 ⁰)	CD15, CD66 Cytochrome b ₅₅₈ , Rap-1, Rap-2 TNF-R FMLP-R CR3 Laminin-R Fibronectin-R Vitronectin-R	Lactoferrin Lysozyme Collagenase Complement activator Monocyte chemoattractant Plasminogen activator Protein kinase C inhibitor Histaminase Vitamin B12 binding protein
Tertiary	FMLP-R CR3 Diacylglycerol-deactivating enzyme	Gelatinase Acetyltransferase
Secretory Vesicles	Alkaline phosphatase Cytochrome b ₅₅₈ CR3 FMLP-R Uroplasminogen activator-R, CD10, CD13, CD45 (? Fc γ RIII, CR1, CD45)	Plasma proteins (incl. tetranectin)

readily mobilisable granules containing gelatinase as well as complement receptor 3 (CR3) and receptors for formylated peptides (FMLP-R). Finally, secretory vesicles are small endocytic vesicles characterised by latent alkaline phosphatase activity and containing CD66, CR3, FMLP receptors and cytochrome b₅₅₈. Mobilisation of secretory vesicles occurs early in response to low doses of chemoattractants and is important in neutrophil recruitment to inflammatory sites (Borregaard et al, 1987; 1993; Sengeløv et al, 1996).

Neutrophil recruitment

Neutrophil migration from the circulation to an inflammatory site depends upon the co-ordinated expression of several adhesion molecules on neutrophil and endothelial cell surfaces. Initial adherence of neutrophils to the endothelium is mediated by selectins which facilitate a transient 'rolling' adhesion of neutrophils along the endothelial surface (Condliffe et al, 1996). Neutrophil arrest and firm adherence, however, is promoted by the neutrophil β_2 integrin molecules, particularly complement receptor 3 (CR3, CD11b/CD18) and lymphocyte function associated antigen 1 (LFA-1: CD11a/CD18), which bind to members of the intercellular adhesion molecule family (ICAM-1, ICAM-2) expressed on endothelial cells. Adherence is followed by diapedesis between endothelial cells and migration to the inflammatory focus.

The process of neutrophil chemotaxis is initiated by the release of chemoattractants (chemokines) at the site of inflammation. These chemotactic factors diffuse into the local circulation and act on both circulating neutrophils and endothelial cells to promote neutrophil adherence. Neutrophil chemokines include small bacterially-

derived formylated peptides, platelet activating factor (PAF), complement component C5a and the cytokine, IL-8; all of which bind to specific receptors belonging to the 7-transmembrane-domain G-protein-linked family (Hallett and Lloyds, 1995). Receptor occupancy and G-protein activation ultimately trigger a rise in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), which in turn initiates actin polymerisation and cytoskeletal rearrangements (Bokoch, 1995; Chen et al, 1996). Alterations in endothelial cells are manifest as an increase in the expression of selectins and ICAMs, whilst neutrophil exocytosis of non-azurophilic granules results in an increased expression of several cell surface markers, including CR3. Once adherent, neutrophil forward motion through the endothelium is facilitated by actin polymerisation and CR3 ligand-binding primarily at the leading edge of the neutrophil (lamellipodium), thus 'pulling' the neutrophil through the endothelium and intercellular spaces along a chemokine concentration gradient. Simultaneously, release of ligand-bound CR3 at the trailing edge of the neutrophil (uropod), frees the neutrophil to move further forward. Thus, CR3 molecules undergo a cycle of attachment at the lamellipodium and release at the uropod during neutrophil migration. The mechanisms underlying this phenomenon are unclear but may include conformational changes in CR3 affecting binding affinity (Diamond and Springer, 1994). An additional explanation has been provided by the demonstration of competitive inhibition of CR3 binding sites by the endogenous ligand, neutrophil elastase (Cai and Wright, 1996), raising the hypothesis that increased neutrophil cell surface neutrophil elastase following limited azurophilic degranulation triggers the release of bound CR3 and permits the neutrophil to move forward. Released

proteolytic enzymes, such as neutrophil elastase, may also aid neutrophil passage between cells by digesting intercellular fibres.

Several lines of evidence suggest that upregulation of CR3 effector functions is associated with changes in the conformational state of CR3 as well as an increase in CR3 cell surface expression. For example, ligand-binding and non-binding forms of CR3 can be isolated from neutrophils and treatment of the inactive form with cellular lipids or with the monoclonal antibody KIM-127 restores ligand-binding activity (Cai and Wright 1995; 1996; Hermanowski-Vosatka et al, 1992). Furthermore, in purified neutrophils, stimulation with a range of priming agents increases CR3-mediated binding of albumin-coated latex beads in a manner that cannot be accounted for on the basis of increased CR3 expression alone (Condliffe et al, 1996). Conformational changes in CR3 associated with increased activity could be induced by derepression of a repressor protein, stimulation of an associated regulatory protein, or through covalent modifications such as phosphorylation (Diamond and Springer, 1994). Interestingly, a recent study has identified differential phosphorylation of CR3 in plasma and granule membranes when stimulated with the neutrophil agonist phorbol myristate acetate (PMA), suggesting that phosphorylation of the CD18 component of CR3 may be the basis of CR3 activation at the neutrophil cell surface (Buyon et al, 1997).

Neutrophil phagocytosis and activation

The primary function of neutrophils at an inflammatory site is to ingest and kill extracellular pathogens and, to this end, neutrophils express a number of receptors involved in bacterial recognition and binding. Ingestion may be triggered by either

non-opsonised or opsonised bacteria, although opsonisation greatly enhances phagocytosis and the subsequent deployment of killing mechanisms by the neutrophil. Non-opsonic phagocytosis, however, represents an important defence mechanism in the early stages of an infection or in inflammatory sites where levels of opsonins are low or opsonisation is inhibited by proteolytic cleavage of opsonins and opsonin receptors (Ofek et al, 1992; Speert, 1993; Döring 1994). Mechanisms underlying non-opsonic phagocytosis include recognition of lectins on the bacterial surface (lectinophagocytosis), and protein-protein interactions via an Arg-Gly-Asp (RGD) peptide sequence (Ofek et al, 1992). Neutrophil lectinophagocytosis of a range of bacterial species has been described, including type 1- and type P-fimbriated *E. coli*, *Neisseria gonorrhoeae*, *Actinomyces naeslundii*, and *Staphylococcus saprophyticus*. Interestingly, type-1 fimbriated *E. coli* have been shown to bind to components of the β_2 integrin family, including CR3, demonstrating that neutrophil receptors may play a dual role by facilitating both opsono- and lectinophagocytosis. Indeed CR3 has also been implicated in the RGD amino acid sequence-mediated uptake of *Bordetella pertussis* and *Leishmania donovani* by macrophages. Ligand-binding to CR3 alone induces little or no respiratory burst activity (Ofek et al, 1992; Berton et al, 1992; Gadd et al, 1994), a phenomenon exploited by both these intracellular pathogens, which gain entry to macrophages via CR3 without triggering ROS generation and hence microbial killing (Falkow et al, 1992). It is therefore possible that other pathogens may similarly evade neutrophil-mediated killing by binding to CR3 alone, triggering phagocytosis and exocytosis but not respiratory burst activity.

Oponisation of microorganisms with antibody and complement increases both the efficiency of phagocytosis and the initiation of the appropriate killing mechanisms. Neutrophil opsonin receptors include Fc receptors for the Fc region of immunoglobulin G molecules, and complement receptors recognising various activated forms of the complement component C3. Both medium affinity Fc γ RII (CD32) and low affinity Fc γ RIII (CD16) are found on neutrophils, although levels of Fc γ RII (10,000 - 20,000 per neutrophil) are much lower than levels of Fc γ RIII (100,000 - 300,000 per neutrophil) (Cohen, 1994). High affinity Fc γ RI may also be expressed by neutrophils following incubation with the pro-inflammatory cytokine interferon γ (IFN γ) (Gadd et al, 1996). Complement receptors include CR1 (CD35) which recognises the C3b form of complement component C3 and CR3 which recognises particles coated with the iC3b form of C3. An opsonised bacterium binding to Fc γ R and complement receptors on the surface of the neutrophil is engulfed by a 'zippering' motion, as progressive receptor binding pulls the plasma membrane around the bacterium until a phagosome is formed. Simultaneous degranulation of azurophil and specific granules in the vicinity of the phagosome results in phagolysosome formation, ROS generation, and increased cell surface expression of complement receptors, thus improving the efficiency of bacterial engulfment and initiating bacterial killing (Cohen, 1994).

The relative contributions of the various Fc γ R and complement receptors to phagocytosis and respiratory burst induction remains unclear. Cross-linking of CR3 alone induces an increase in [Ca²⁺]_i, triggering a variety of neutrophil responses, including phagocytosis, but not a substantial respiratory burst (Petersen et al, 1993;

Berton et al, 1992; Gadd et al, 1994). However FcγR-induced respiratory burst responses are enhanced by the presence of activated complement. Studies have shown the moderate affinity FcγRII to be a more effective trigger for phagocytosis and respiratory burst induction than the low affinity FcγRIII (Cohen, 1994; Edwards, 1995), and the latter was traditionally regarded as playing a subsidiary role in enhancing FcγRII functions and ingesting large immune complexes. However, cross-linking of either FcγRII and FcγRIII can independently lead to tyrosine phosphorylation and an elevation in $[Ca^{2+}]_i$, both of which are features of neutrophil activation processes (Edwards, 1995). Interestingly, interactions of FcγRIII with β_2 integrins appear to be particularly important in FcγRIII-mediated neutrophil activation (Edwards, 1995; Petty and Todd, 1996). The form of FcγRIII expressed in neutrophils (FcγRIIIB) lacks a transmembrane domain but is anchored to glycosylphosphatidylinositol (GPI) within the plasma membrane (Edwards, 1995; Petty and Todd, 1996). Consequently another molecule must be involved in signalling from activated FcγRIIIB molecules. Co-capping studies have demonstrated the close proximity of FcγRIIIB and CR3 in neutrophil plasma membranes and transfection studies in non-haematopoietic cells have indicated that CR3 is necessary for FcγRIIIB-mediated phagocytosis to occur. Coupled with studies of CR3 interactions with other GPI-linked neutrophil markers, these results suggest that the transmembrane CR3 molecule may function as a relay for signals generated by GPI-linked receptors (Petty and Todd, 1996).

Respiratory burst activity and ROS generation

Respiratory burst activity in phagocytic cells, manifest as an increase in oxygen consumption which is independent of mitochondrial activity, is the initiating event in the generation of ROS, which are necessary for the effective killing of many bacterial species. At the molecular level, ROS formation depends upon the transfer of electrons from molecular oxygen to H₂O via a series of intermediaries of varying toxicity (Fig 1.1, also reviewed in Miller and Britigan, 1997; Karnovsky, 1994). Respiratory burst activity is initiated by the assembly and activation of the enzyme NADPH oxidase, which catalyses the transfer of a single electron to molecular oxygen to form the superoxide anion, O₂⁻. Individuals with an inherited defect in any one of several of the components of the NADPH oxidase enzyme complex are unable to mount an appropriate respiratory burst in response to phagocytic cell activation, and consequently suffer from recurrent life-threatening infections with catalase positive bacteria, including *S. aureus* and *B. cepacia*. The failure of neutrophils to clear the infection promotes macrophage recruitment and granuloma formation; hence the syndrome is termed chronic granulomatous disease (CGD) (Dinauer, 1992).

The central component of the NADPH oxidase system is a flavicytochrome complex entitled cytochrome b₅₅₈, b₅₅₉ or b₋₂₄₅ and consisting of a 91 kDa glycoprotein, gp91-phox, and a 22 kDa protein, p21-phox (Deleo and Quinn, 1996; Wientjes et al, 1997). In resting cells, cytochrome b₅₅₈ is found predominantly within the membrane of specific granules, although approximately 10% is expressed within the plasma membrane. A further component, the GTP-binding protein, Rap1A, is also found

within the plasma and specific granule membranes in association with cytochrome b_{558} . Cytosolic components of NADPH oxidase include a further GTP-binding protein, Rac, and a complex of three proteins, p47-phox, p67-phox and p40-phox. On phagocytic cell activation, specific granules are translocated to the plasma membrane, leading to an increase in cytochrome b_{558} at the cell surface. Cytosolic components are also translocated to the plasma membrane and associate with the cytochrome b_{558} component to form the completed enzyme. In particular, phosphorylation of the p47 component induces conformational changes in the p47/p67/p40 complex which in turn permit binding to the cytochrome b_{558} component, and hence NADPH oxidase assembly. GTP-binding proteins appear to play a regulatory role, particularly in enzyme activation where GDP-GTP exchange may function as an on-off switch. Activated NADPH oxidase catalyses the transformation of NADPH to NADP and H^+ with the release of a single electron and hence the generation of O_2^- from O_2 .

Once formed O_2^- is rapidly dismutated to H_2O_2 either spontaneously or through the action of superoxide dismutase (SOD) (Fig 1.1; Miller and Britigan, 1996). Neutrophils, unlike monocytes/macrophages, contain a further enzyme, myeloperoxidase (MPO), which converts H_2O_2 to hypochlorous ion (OCl^-), a potent anti-bacterial agent. A variety of reactions between O_2^- , H_2O_2 and OCl^- lead to the formation of the highly reactive oxygen species, singlet oxygen ($O_2(^1\Delta_g)$) and hydroxyl radical ($\cdot OH$), both of which are short lived and highly potent oxidising agents. Cytotoxic chloramines and tyrosyl radicals are generated via an MPO-dependent pathway. In addition, O_2^- may react with extracellular nitric oxide ($NO\cdot$),

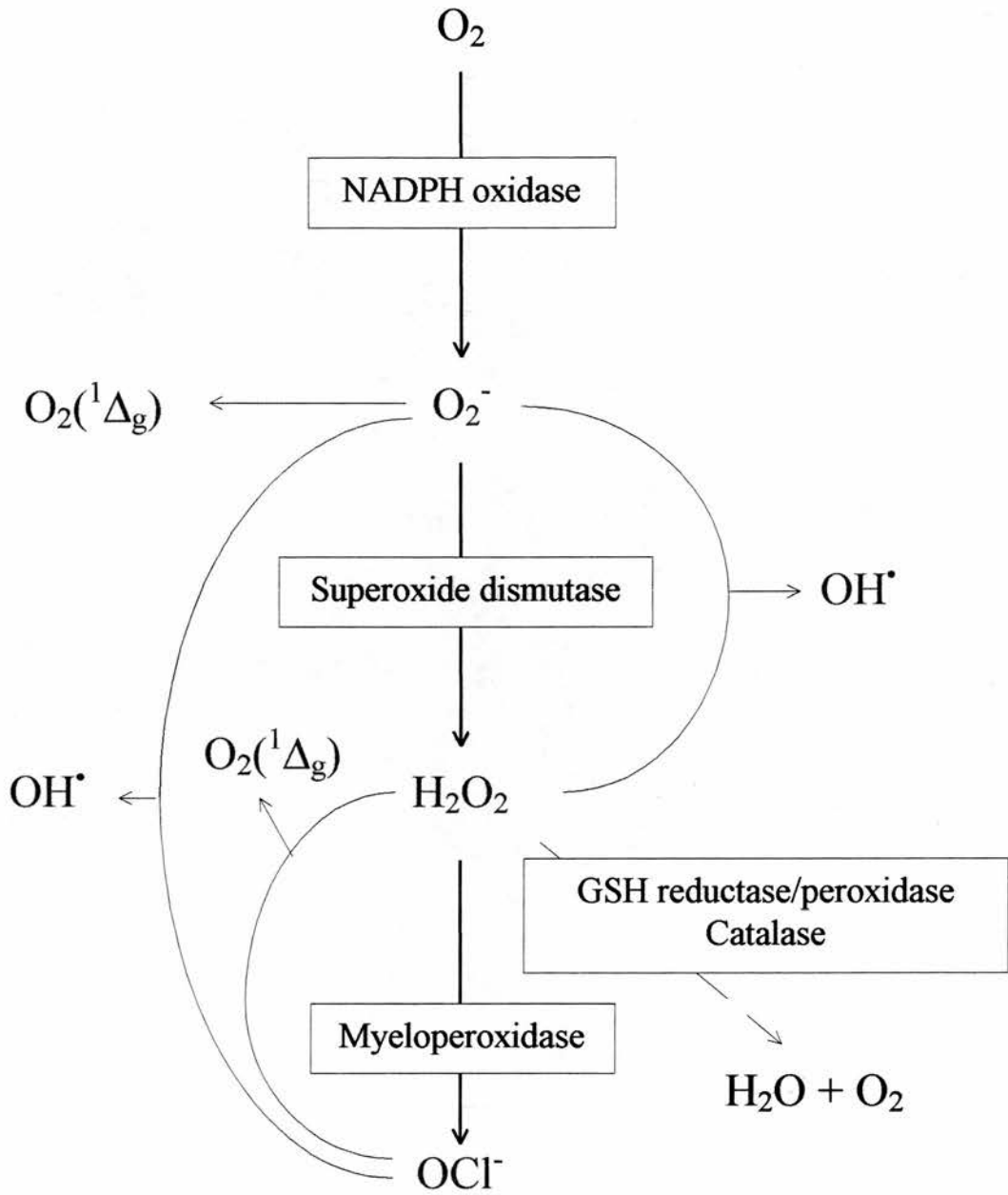


Fig 1.1 Respiratory burst induction and the production of reactive oxygen species in neutrophils. Solid lines: pro-oxidant reactions; dashed lines: antioxidant reactions. ROS shown in red.

released by macrophages or epithelial cells, to produce toxic peroxynitrites. In view of the toxicity of the many oxygen species generated during a respiratory burst, several regulatory mechanisms exist to prevent damage to host tissues, whilst maximising the effectiveness of bacterial killing. NADPH oxidase is stored primarily in specific granules and assembled at the plasma membrane which is then incorporated into the phagosome during phagocytosis. By contrast MPO is contained within azurophil granules and is released into the phagosome during phagolysosome formation via the fusion of azurophil granules. Thus highly active ROS are primarily generated within the immediate environment of the ingested microorganisms. However some leakage of ROS to the extracellular environment is inevitable and may be considerable in instances of frustrated phagocytosis or if neutrophils are killed and undergo lysis. Since H_2O_2 formation is pivotal in the formation of most ROS, neutrophils also contain mechanisms to remove excess H_2O_2 , including the enzyme catalase which converts H_2O_2 to water and oxygen, and the enzyme system glutathione reductase / peroxidase which converts H_2O_2 to water. Extracellular anti-oxidant molecules such as ascorbic acid (vitamin C), α -tocopherol (vitamin E), β -carotene and sulphhydryls also play a role in protection from oxidative damage via released ROS (Brown and Kelly, 1994b).

Neutrophil priming

In vitro, neutrophil responses to any given stimulus can be modulated by prior exposure to a wide range of inflammatory mediators. Such agents do not trigger a respiratory burst in themselves but 'prime' the neutrophil so that a subsequent respiratory burst is of a greater magnitude. Many substances have been demonstrated

to prime neutrophils, including cytokines such as IL-8, platelet activating factor (PAF), insulin-like growth factor 1 (IGF-1), granulocyte/macrophage-colony stimulating factor (GM-CSF) and TNF α (Hallet and Lloyds, 1995; Condliffe et al, 1996; Bjercknes and Aarskog, 1995; Wozniak et al, 1993; Daniels et al, 1994) or bacterially derived substances, such as LPS (Böhmer et al, 1992; Condliffe et al, 1996). Although the role of priming *in vivo* is less clear, the observation that many priming agents are also potent chemoattractants suggests that neutrophils are primed for more effective bacterial killing as they move along a chemokine gradient towards the inflammatory focus. Priming is also associated with degranulation of non-azurophilic granules and subsequent increased expression of complement receptors at the cell surface (Bjercknes and Aarskog, 1995; Condliffe et al, 1996), again preparing the neutrophil for more effective bacterial phagocytosis and killing.

LPS preparations from a range of bacterial species (Aida et al, 1995; Heiman et al, 1990; Karlsson et al, 1995; Kharazmi et al, 1991) have been shown to prime neutrophil respiratory burst responses *in vitro*. LPS is avidly bound by the serum protein, LPS-binding protein (LBP). Thus, in the presence of serum, LPS interacts with phagocytic cells as LPS-LBP complexes, which bind to a GPI-linked protein, CD14, expressed predominantly on monocyte/macrophages but also at lower levels on neutrophils. Along with other GPI-linked receptors, CD14 probably requires cross-linking with CR3 or other receptors to induce intracellular signalling and neutrophil priming (Petty and Todd, 1996). LPS can also bind to neutrophils and induce priming in the absence of serum and hence LBP, although the concentrations of LPS required to induce a level of priming equivalent to that induced in the

presence of serum, are generally much greater (Luchi and Munford, 1993; Lynn et al, 1991; I Dransfield, personal communication). In the absence of LBP, putative LPS receptors include a 70-80 kDa molecule (Halling et al, 1992; Jacques, 1996) and CR3 (Wright et al, 1989), but not CD14 (Luchi and Munford, 1993). Surprisingly, however, expression of functional CD14 does appear to be necessary for neutrophil priming to occur under these circumstances (Lynn et al, 1991).

Cellular mechanisms controlling neutrophil priming and activation

The potential toxicity of neutrophil defence mechanisms is matched by several regulatory systems controlling the many neutrophil effector functions. The complexity of such regulatory networks is reflected in the fact that one agonist can have many different effects upon neutrophil function depending on both the concentration of the stimulus and on the activation state of the neutrophil. For example at low concentrations, the formylated peptide, fMet-Leu-Phe (FMLP), is a potent stimulator of neutrophil chemotaxis and primes neutrophils for later respiratory burst responses. However at high concentrations, FMLP itself will induce a respiratory burst in unprimed neutrophils (Hallett and Lloyds, 1995; Jaconi et al, 1993). Neutrophil activation state is also important, since primed neutrophils will produce a respiratory burst in response to concentrations of FMLP that do not trigger respiratory burst induction in unprimed neutrophils (Condliffe et al, 1996).

Many investigators have attempted to unravel the complex regulatory webs controlling neutrophil functions, and considerable progress has been made. Diverse neutrophil effector functions, including adhesion, chemotaxis, priming, degranulation, and respiratory burst induction are dependent upon an increase in

$[Ca^{2+}]_i$ transients (Jaconi et al, 1993; Hallett and Lloyds, 1995). Different patterns of $[Ca^{2+}]_i$ release may dictate the nature of the neutrophil responses induced. For example, low concentrations of FMLP, which are associated with chemotaxis, induce a sustained increase in $[Ca^{2+}]_i$ transients, whilst high concentrations of FMLP, which are associated with the arrest of cell movement and the activation of degranulation, induce a single elevation of $[Ca^{2+}]_i$ which rapidly returns to baseline levels (Jaconi et al, 1993). Additional mechanisms involved in neutrophil regulatory pathways include both serine/threonine and tyrosine phosphorylation of intracellular proteins (Bokoch, 1995).

The biochemical pathways underlying changes in $[Ca^{2+}]_i$ and protein phosphorylation following receptor activation have been studied most fully in relation to the 7-transmembrane G-protein linked family of chemokine receptors (Bokoch, 1995). G proteins, which are heterotrimeric GTP-binding proteins comprising α , β and γ subunits, dissociate on activation, releasing $G\alpha$ and $G\beta\gamma$ components. In the classical model of intracellular signalling (illustrated in Fig 1.2), $G\beta\gamma$ activates phospholipase C which breaks down phosphatidylinositol 4,5-bisphosphate (PIP_2) to form the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilises intracellular Ca^{2+} stores and hence induces an increase in $[Ca^{2+}]_i$ which, along with DAG, activates protein kinase C (PKC) and initiates intracellular phosphorylation reactions (Bokoch, 1995; Worthen et al, 1994). Activation of phospholipases D (PLD) and A_2 (PLA_2) also occur as downstream events of G-protein activation, triggering the formation of phosphatidic acid (PA) and arachidonic acid (AA) respectively. PA is further degraded to DAG, whilst AA is metabolised to

produce a range of eicosanoid inflammatory mediators. In addition, both PA and AA have been linked to NADPH oxidase activation (Edwards, 1995; Jones et al, 1996) and hence respiratory burst induction.

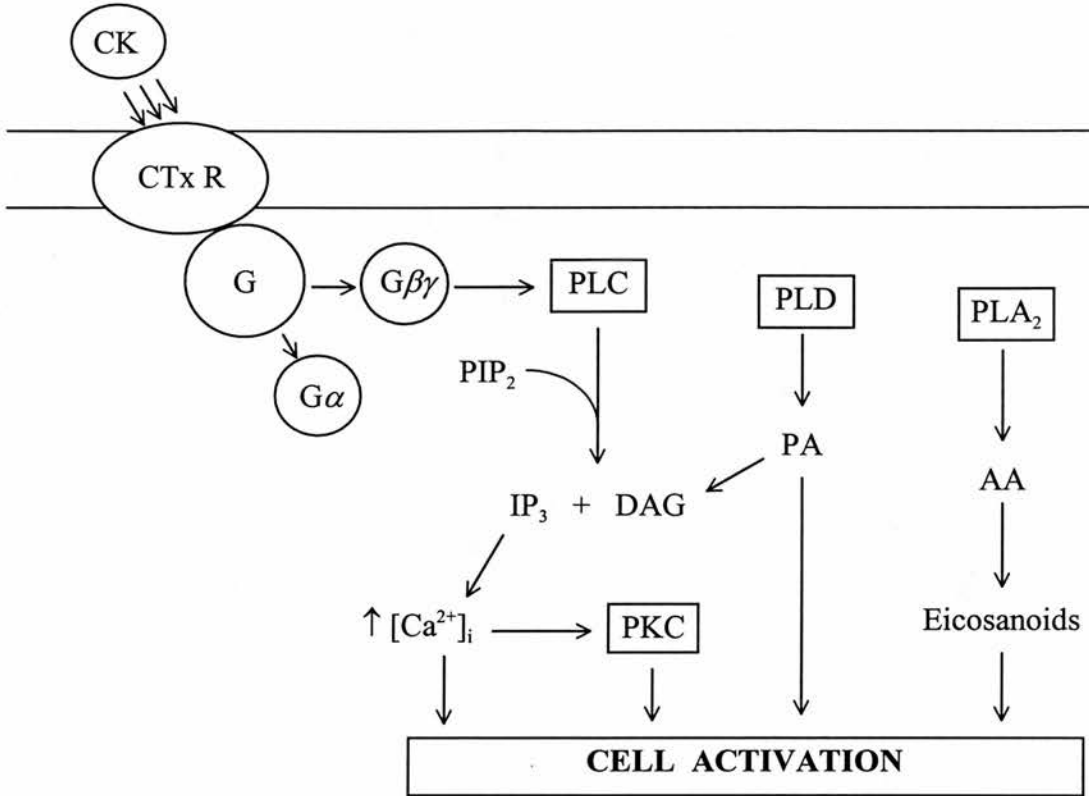


Fig 1.2. Classical pathway of chemokine receptor signalling in neutrophils. Binding of chemokine (CK) to a specific 7-transmembrane domain linked receptor (CTxR) triggers G protein dissociation into $G\alpha$ and $G\beta\gamma$ components and a cascade of events leading to cell activation. PLC, phospholipase C; PLD, phospholipase D; PLA₂, phospholipase A₂; PIP₂, phosphatidyl inositol-4,5-biphosphate; IP₃, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C; AA, arachidonic acid; PA, phosphatidic acid. (Adapted from Bokoch, 1995)

Recently, several alternative regulatory networks have been described involving a number of tyrosine kinases, mainly of the Src family, and low molecular weight GTPases of the Ras family (reviewed in Bokoch, 1995). Tyrosine kinase activation triggers GDP/GTP exchange on Ras, which in turn activates the Ras-dependent kinases, Raf and MAP/ERK kinase kinase (MEKK-1; Fig 1.3). Both Raf and MEKK-1 activate MAP/ERK kinase (MEK-1 and MEK-2), which subsequently activates a serine/threonine kinase termed mitogen activated protein kinase (p42/p44 MAPK), triggering a range of cellular events including activation of phospholipase A₂, cytoskeletal rearrangements, and activation of other serine/threonine kinases (Bokoch, 1995; Nick et al, 1997). Src tyrosine kinases also function in association with Ras to activate phosphatidylinositol 3-kinase (PI3K) which converts PIP₂ to phosphatidylinositol triphosphate (PIP₃). PIP₃ formation is associated with cytoskeletal rearrangements, whilst inhibition of PI3K blocks chemoattractant stimulation of NADPH oxidase activity, suggesting that PIP₃ may have a role in both of these processes (Bokoch, 1995).

Other less well-characterised signalling pathways have been described in neutrophils, including a novel form of PI3K activated by Gβγ (Bokoch, 1995) and a 38 kD MAP kinase (p38 MAPK) activated by a distinct pathway from the p42/p44 MAPK (Nick et al, 1997). Stimulation of FMLP and PAF receptors induces differential activation of p38 MAPK and p42/p44 MAPK, which in turn produces distinct effects on neutrophil activation processes (Nick et al, 1997). Similarly, the two IL-8 receptors expressed by neutrophils, IL-8R1 and IL-8R2, induce different effects, with both triggering an increase in [Ca²⁺]_i and exocytosis, whilst only IL-8R1 triggers

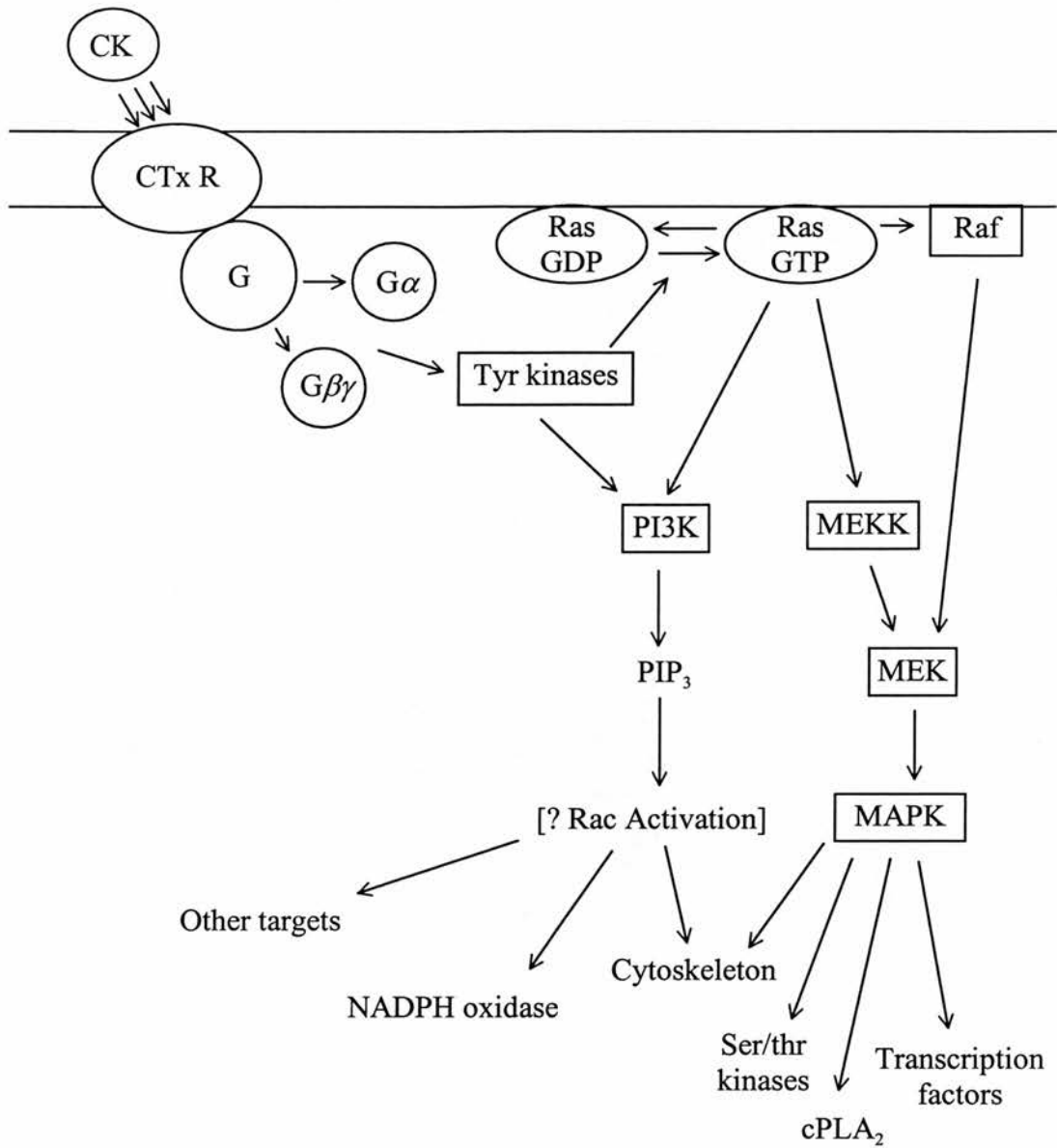


Fig 1.3. Chemokine receptor Ras-dependent signalling pathways. Nonreceptor tyrosine kinases of the Src family (Tyr kinases), activated by G protein components, trigger activation of the small GTPase, Ras. Subsequent stimulation of the serine/threonine kinase Raf, kinases of the MAPK cascade, and PI3K lead to various effects upon neutrophil activation processes. MAPK, mitogen-activated protein kinase (ERK); MEK, MAPK/ERK kinase; MEKK, MEK kinase; PI3K, phosphatidylinositol 3-kinase; PIP₃, phosphatidylinositol triphosphate. (Adapted from Bokoch, 1995; Nick et al, 1997)

respiratory burst activity and PLD activation (Jones et al, 1996). Thus neutrophil responses to an individual chemokine may be 'fine-tuned' by the selective upregulation of distinct intracellular signalling pathways, perhaps mediated by the coupling of G proteins with distinct α subunits to individual chemokine receptors (Gierschik et al, 1989).

The role of kinase activation and changes in $[Ca^{2+}]_i$ in neutrophil priming remains unclear. However, a recent review has suggested that tyrosine phosphorylation may be the major mechanism underlying priming activity (Hallett and Lloyds, 1995). Evidence in support of this hypothesis includes a number of observations: 1. changes in $[Ca^{2+}]_i$ following neutrophil stimulation are identical in unprimed and primed neutrophils, despite greater O_2^- production in the latter, suggesting that priming does not function by facilitating greater release of intracellular Ca^{2+} stores (Lloyds and Hallett, 1994); 2. receptors for agents such as $TNF\alpha$ or GM-CSF, which prime but do not activate neutrophils, do not trigger a rise in intracellular $[Ca^{2+}]_i$ but do increase intracellular tyrosine phosphorylation (Hallett and Lloyds, 1995); 3. tyrosine kinase activation occurs in response to primers and agonists at concentrations too low to trigger a rise in $[Ca^{2+}]_i$ (Lloyds et al, 1995; McColl et al, 1991); 4. neutrophil priming can be manipulated experimentally by inhibition or activation of tyrosine kinases and phosphatases (Lloyds and Hallett, 1994). The kinetics of tyrosine phosphorylation on neutrophil stimulation are much slower than those of an increase in $[Ca^{2+}]_i$, providing a model to explain the dose-dependent effects of several neutrophil agonists upon neutrophil priming and activation (Hallett and Lloyds, 1995). In this model, high agonist concentrations induce a rapid rise in $[Ca^{2+}]_i$ and NADPH oxidase

activation initiating a modest respiratory burst. Low agonist concentrations, however, fail to induce a rise in $[Ca^{2+}]_i$, but do initiate tyrosine phosphorylation, which in turn primes any subsequent activation of NADPH oxidase for a greater respiratory burst.

1.2.3 Neutrophil-mediated damage in the CF lung

As the predominant immune cell within the CF lung, neutrophils are regarded as major contributors to inflammatory damage in CF patients. Given the increased levels of pro-inflammatory cytokines and bacterial products such as LPS in the CF lung, conditions would seem ideal for priming and activation of neutrophil responses. Furthermore, exposure to colonising bacteria provides a constant stimulus for neutrophil activation, as demonstrated by the 'frustrated phagocytosis' model of neutrophil/mucoid *P. aeruginosa* (MPA) interactions (Govan and Glass, 1990; Govan and Deretic, 1996). In this model, antibodies to alginate and other pseudomonal antigens trigger neutrophil attempts to engulf large microcolonies of MPA within alginate biofilms. In the absence of effective phagosome formation, neutrophil granule exocytosis releases tissue-damaging substances into the extracellular environment, thus promoting further lung damage and continuing neutrophil activation.

Interest in neutrophil-mediated lung damage in CF has focused on two main areas; first, the generation of toxic ROS, and second, the release of proteolytic enzymes such as neutrophil elastase. Both will be discussed in the following sections.

ROS-mediated damage

The antimicrobial action of ROS are essential to the control of many bacterial pathogens, but, if present in excess, can provoke considerable damage to host tissues. Damage may be induced directly via cytotoxic reactions involving oxidation and peroxidation of proteins or lipids, or indirectly by the upregulation of pro-inflammatory cytokines and the inactivation of anti-inflammatory molecules such as the serine protease inhibitor, α_1 -antitrypsin (reviewed in Miller and Britigan, 1996). In CF, the observation of elevated plasma concentrations of oxidised lipids, proteins and DNA suggests the presence of an oxidant/antioxidant imbalance favouring the presence of excess ROS at inflammatory sites. Significantly, increased plasma levels of oxidation products correlate with reduced lung function, implying that oxidative tissue damage contributes to CF lung disease (Brown and Kelly, 1994a; Brown et al, 1995). Although it has been suggested that deficiencies in antioxidant vitamins such as α -tocopherol and ascorbic acid may contribute to oxidative stress, studies have indicated that antioxidant levels in CF children fall within normal ranges, reflecting the good nutritional management now provided in CF clinics (Brown and Kelly, 1994b; Vaisman et al, 1994). However a deficiency of the antioxidant glutathione has been demonstrated both systemically and in the airways lining fluid of CF patients (Roum et al, 1993).

Increased production of oxidant molecules by activated phagocytic cells may also contribute to an oxidant/antioxidant imbalance in CF. For example sputum concentrations of extracellular MPO are elevated in CF patients and correlate with the severity of lung disease (Regelmann et al, 1995). Furthermore, studies measuring

neutrophil luminol-enhanced chemiluminescence (LCL), which is myeloperoxidase-dependent, have shown increased neutrophil LCL activity in CF patients compared to healthy controls (Vaisman et al, 1994; Roberts and Stiehm, 1989). These findings were confirmed in a recent publication directly demonstrating increased myeloperoxidase activity and chloramine release, but not increased production of O_2^- or H_2O_2 in neutrophils from CF patients (Witko-Sarsat et al, 1996). Interestingly, asymptomatic CF heterozygotes also showed increased intracellular MPO activity, and, for both groups, MPO activity could be returned to control levels by amiloride-dependent inhibition of sodium/proton antiport activity. These results imply that increased MPO activity in CF is not solely a consequence of neutrophil priming or activation, but reflects a fundamental defect in the regulation of intracellular pH and/or ionic concentrations.

The proposed oxidant/antioxidant imbalance in CF has prompted calls to introduce antioxidant therapy in CF patients, including the use of recombinant SOD (Koyama et al, 1992), selenium (Sies, 1987), the glutathione precursor N-acetylcysteine (Leff et al, 1993) and catalase (Thibeault et al, 1991). However, it is still unclear how much tissue damage in CF can be attributed to oxidative processes. For example, levels of the oxidant species NO and H_2O_2 in exhaled air are increased in asthmatic children but not in CF patients or healthy controls (Dötsch et al, 1996; Worlitzsch et al, 1998). Both sputum, which largely consists of DNA, and pseudomonas alginate are powerful scavengers of oxygen radicals and may therefore remove ROS from the respiratory epithelium before damage can occur. In addition, the sequestration of the highly cationic enzyme MPO by negatively charged DNA within sputum may favour

the generation of ROS away from the epithelial cell surface, thus providing further protection against oxidative damage.

Proteolytic enzymes

Neutrophils contain both metalloproteinases and serine proteases which may be involved in inflammatory damage in CF patients. Most interest has focused on the role of serine proteinases, and particularly neutrophil elastase, which is capable of digesting elastin and fibronectin within the lung parenchyma, inducing a loss of lung elasticity and thus promoting the development of bronchiectasis (Döring and Bellon, 1996). Within the normal lung, free elastase activity is inhibited by the presence of α_1 -proteinase inhibitor (α_1 -PI) which forms an inactive complex with neutrophil elastase (NEAP). An imbalance of elastase/antiproteinase inhibitor is believed to be present within the CF lung, mediated by the inactivation of α_1 -PI by a range of substances, including neutrophil elastase itself (Döring, 1994), *P. aeruginosa* elastase (Suter, 1994) and MPO-derived oxidants (Miller and Britigan, 1996). Free neutrophil elastase may have a number of potentially detrimental effects in CF lung disease, including epithelial cell toxicity, degradation of the lung extracellular matrix, promotion of mucus hypersecretion and inhibition of mucociliary transport, induction of IL-8 and other neutrophil chemotactic factors, and interference with phagocytosis by degradation of immunoglobulin and CR1 (Vender 1996; Döring 1994). In addition, neutrophil elastase may impair surfactant function by degrading surfactant proteins (Liau et al, 1996). Consequently, antiproteinase supplementation has been proposed as a potential therapy in CF patients (Vender, 1996; Döring and Bellon, 1996). Although some trials of aerosolised α_1 -PI in CF patients have

demonstrated decreased concentrations of free neutrophil elastase in both sputum and airway lining fluid, not all have been successful and further work is required to improve delivery systems for α_1 -PI before a definite therapeutic benefit can be demonstrated (Döring and Bellon, 1996). As a caveat, disruption of phagocytosis and the cleavage of T lymphocyte receptors such as CD2, CD4, and CD8 by the serine proteinases neutrophil elastase and cathepsin G, may downregulate immune responses, and so protect lung tissue from further inflammatory damage (Döring et al, 1995). Thus a cycle of acute inflammation followed by serine protease-mediated immune downregulation may contribute to the typical pattern of intermittent infective exacerbations on a background of chronic inflammation seen in CF patients.

1.3 *BURKHOLDERIA CEPACIA*

1.3.1 Background

Burkholderia cepacia was first described as the cause of sour skin in onions in 1950 by Burkholder, who termed the organism *Pseudomonas cepacia* (Latin: *cepia* = onion) (Burkholder, 1950). Subsequently, *B. cepacia* was cultured from both clinical and environmental sources and appeared in the literature under various names, including eugonic oxidiser 1 (EO-1), *P. kingii* and *P. multivorans*. The latter name reflects the characteristic nutritional versatility of *B. cepacia*, which can metabolise a

wide variety of substrates and is even capable of utilising penicillin G as a sole carbon source (Beckman and Lessie, 1979).

As an environmental, non-fermentative, Gram-negative bacillus of high G-C content, *B. cepacia* was originally included in the genus *Pseudomonas*. The genomic heterogeneity of the phenotypically-diverse pseudomonads was confirmed by RNA-DNA hybridisation studies with five major groupings identified; RNA Group I included the type strain, *P. aeruginosa*, whilst RNA Group II included *P. cepacia* (Palleroni, 1984). In the early 1990's, the RNA group II pseudomonads were transferred to a new genus, *Burkholderia*, with *B. cepacia* as the type strain (Yabuuchi et al, 1992). Other members of the *Burkholderia* genus include *B. pseudomallei*, *B. mallei*, *B. gladioli*, *B. caryophilli*, (Yabuuchi et al, 1992), *B. plantarii*, *B. glumae*, *B. vandii*, (Urakami et al, 1994), *B. cocovenenans* (Zhao et al, 1995) and *B. vietnamiensis* (Gillis et al, 1995). Although members of the *Burkholderia* genus are typically environmental organisms and phytopathogens, several species have been associated with human disease. Most notably, *B. pseudomallei* is the causative agent of melioidosis, an often lethal infection which may present as septicaemia with multi-system abscess formation or as a chronic pneumonic illness resembling tuberculosis. *B. mallei* is associated with glanders, a predominantly respiratory disease of horses and occasionally man. Since the early 1980's, *B. cepacia* has been increasingly recognised as a human pathogen, particularly amongst CF patients.

1.3.2 Clinical impact of *B. cepacia*

Reports of *B. cepacia* infection in non-CF patients have typically been associated with immunocompromised individuals treated within intensive therapy units. In many instances, outbreaks of *B. cepacia* infection have been traced to contaminated aqueous or antiseptic solutions (Craven et al, 1981; Sobel et al, 1982; Rapkin et al, 1976) or to contaminated equipment (Takigawa et al, 1993; Poe et al, 1977; Berkelman et al, 1982; Gelbart, 1976), reflecting the organisms innate resistance to many commonly used disinfectants. Sites of infection have included the lungs (Maningo and Watanakunakorn, 1995; Takigawa et al, 1993; Poe et al, 1977) urinary tract (Levey et al, 1981; Sobel et al, 1982), and soft tissues (Basset, 1970), as well as bacteraemia (Henderson et al, 1988; Berkelman et al, 1981; Craven et al, 1981) and septic shock (Brauner et al, 1985; Thong and Tay, 1975; Phillips and Eyken, 1971). Lung infection has been associated with contaminated nebulisers (Poe et al, 1977), including an outbreak affecting 36 patients in a Japanese hospital (Takigawa et al, 1993). In contrast to these opportunistic infections, *B. cepacia* has been described as a pathogen in apparently immunocompetent individuals, including a report of macerated hyperkeratotic foot lesions or 'foot rot' amongst troops training in swamps in northern Florida (Taplin et al 1971), two reports of bacteraemic pneumonia in previously healthy children (Wong et al, 1991; Pujol et al, 1992) and a recent report of otitis media and cerebellar abscess formation in a UK offshore oil worker (Hobson et al, 1995). Serious infections with *B. cepacia*, particularly affecting the lower respiratory tract, are also a feature of CGD (Lacy et al, 1993; O'Neil et al, 1986). However, the greatest impact of *B. cepacia* has been as a major pathogen within the

CF community, where the increasing prevalence of *B. cepacia* within CF clinics, associated with the emergence of transmissible ‘epidemic’ lineages, has led to the introduction of segregation for colonised patients in both European and North American clinics. The role of *B. cepacia* in CF will be discussed in detail in section 1.4.

1.3.3 Characteristics and identification of *B. cepacia*

B. cepacia is a motile non-spore-forming, aerobic bacillus, with a G-C content of 66-67% and typically catalase- and oxidase-positive. Various non-fluorescent pigments may be produced and poly- β -hydroxyalkanoates can be accumulated as reserve materials. Optimal growth occurs at 30°C (Palleroni, 1984). The characteristic nutritional versatility and adaptability of *B. cepacia* has been attributed to its unusual genetic structure. *B. cepacia* possesses an exceptionally large genome with approximately 8 megabase pairs, contained within multiple replicons (Cheng and Lessie, 1994; Rodley et al, 1995). Using multilocus linkage disequilibrium analysis, Wise et al (1995) demonstrated a high degree of variability in a clonally-derived natural population of *B. cepacia*, which suggested an unusually high rate of recombination of *B. cepacia* relative to binary fission. Furthermore, *B. cepacia* has been shown to contain multiple insertion sequences (IS) (Rodley et al, 1995; Scordilis et al, 1987). Indeed migration of IS within the *B. cepacia* genome has been proposed as a mechanism to account for the phenotypic variability seen in *B. cepacia* infections, particularly in terms of changing antibiotic sensitivity patterns during

treatment (Hobson et al, 1995; Pitt et al, 1996; Larsen et al, 1993; Scordilis et al, 1987).

In the clinical setting, isolation and identification of *B. cepacia* has been greatly improved by the introduction of selective media and the use of commercial multitest systems (Gilligan, 1991; Pitt and Govan, 1993). However, accurate identification of *B. cepacia* is still problematic, particularly as other bacteria may grow on selective media and commercial test systems do not accurately identify all *B. cepacia* isolates (Kiska et al, 1996; Leff et al, 1995). Recent studies have identified further difficulties, by demonstrating the extreme diversity of bacterial isolates currently identified as *B. cepacia* by standard laboratory tests. Analysis of whole cell fatty acids as fatty acid methyl esters (FAME) by gas chromatography led Simpson et al (1994) to speculate that epidemic strains of *B. cepacia*, associated with outbreaks amongst CF patients, may actually be hybrids of *B. cepacia* and other members of the *Burkholderia* genus, including the phytopathogen *B. gladioli* and the potent intracellular pathogen *B. pseudomallei*. Taxonomic studies have also shown that strains currently identified as *B. cepacia* on the basis of standard laboratory tests, actually consist of at least 5 distinct species or 'genomovars' (Govan et al, 1996; Vandamme, 1995). Isolates were grouped according to polyacrylamide gel electrophoresis of whole cell proteins and DNA-DNA hybridisation studies. By convention, new species identified in such a manner are referred to as 'genomovars' until reliable phenotypic markers are found for species identification (Ursing et al, 1995). Thus, for example, genomovar V isolates have been identified as the new species, *B. vietnamiensis* on the basis of nitrogen-fixing ability (Gillis et al, 1995),

whilst genomovar II isolates have recently been proposed as the new species, *B. multivorans* (Vandamme et al, 1997).

1.3.4 Biotechnological exploitation of *B. cepacia*

Although originally identified as a phytopathogen, *B. cepacia* is currently being developed as an agent of biocontrol and bioremediation for agricultural use. The nutritional adaptability of *B. cepacia* makes it an attractive prospect for the 'neutralisation' of land contaminated by industrial wastes or herbicides (Bhat et al, 1994; Havel and Reineke, 1993; Folsom et al, 1990; Krumme et al, 1993). For example, *B. cepacia* has been shown to degrade the defoliant 2,4,5-trichlorophenoxyacetic acid (2,4,5-T; 'agent orange') at a rate up to 20,000 times greater than other degradative bacteria (Kilbane et al, 1982; Folsom et al, 1990). Furthermore, like many soil organisms, *B. cepacia* produces a range of antimicrobial compounds which enable it to compete for limited nutrients within the soil micro-environment. Agricultural microbiologists are investigating the use of *B. cepacia* in the control of a wide range of plant pathogens, including *Rhizoctonia solani*, *Fusarium monoliforme*, *Sarocladium oryzae*, *Pyricularia oryzae*, *Verticillium dahliae*, *Sclerotium rolfii*, *Pythium ultimum* and *Pseudomonas solanacearum* (Homma et al, 1989; Aoki et al 1991; Fridlender et al, 1993; Rosales et al, 1995).

The commercial release of *B. cepacia* in biocontrol programmes, involving either crop spraying or seed treatment, remains controversial, particularly as the mechanisms underlying *B. cepacia* pathogenicity are unclear. Bevivino et al (1994) compared two clinical and two environmental strains for a range of characteristics

associated with environmental organisms or human pathogens. Although the clinical strains were found to bind more effectively to urinary epithelial cells than the environmental strains, the limited number of strains involved and choice of 'virulence determinants' restricts the usefulness of this study in determining the safety of environmental *B. cepacia* isolates. In an earlier study, Gonzalez and Vidaver (1979) were able to differentiate clinical and environmental isolates on the basis of phytopathogenicity tests but found no difference in mouse lethality between the two groups. Gessner and Mortensen (1990) compared a larger panel of CF, non-CF clinical and environmental *B. cepacia* strains for a range of potential virulence factors but found few obvious differences between the two groups. Recent taxonomic analyses have indicated that most environmental isolates, including the *B. cepacia* type strain ATCC25416, are genomovar I isolates. The observation that isolates of genomovars I and IV are less frequently found in CF than genomovar II and III isolates is perhaps relevant to the choice of *B. cepacia* strains for development in biocontrol programmes. However pro-inflammatory activity induced by a genomovar I strain has been demonstrated in a study of monocyte TNF- α induction by bacterial lipopolysaccharide (Shaw et al, 1995a). Thus, controversy still surrounds the human hazards associated with the commercial use of *B. cepacia*; indeed, a recent application to release an environmental *B. cepacia* strain in a large scale agricultural programme in the USA has been vigorously opposed by clinicians and medical microbiologists involved in the care of CF patients. Further understanding of *B. cepacia* acquisition and pathogenicity is necessary to enable an adequate assessment of the 'safety' of any *B. cepacia* strain proposed for release.



1.4 *BURKHOLDERIA CEPACIA* IN CYSTIC FIBROSIS

1.4.1 Background

Culture of *B. cepacia* from a CF patient was first reported in the early 1970's as part of a large survey of *B. cepacia* in US hospitals (Ederer and Matsen, 1972). Several years later, Rosenstein and Hall (1980) reported a severe case of *B. cepacia* infection in a 17-year old female CF patient. Infection was characterised by acute pneumonia and septicaemia, a pattern of disease not seen with other CF pathogens. In the mid 1980's, an increase in *B. cepacia* prevalence within North American CF clinics was reported; significantly, a number of patients developed septicaemia as a consequence of *B. cepacia* infection. (Isles et al, 1984; Thomassen et al, 1985). In the UK, rapid pulmonary deterioration and fatal *B. cepacia* septicaemia in a CF patient was first reported in 1986 (Glass and Govan, 1986). However, by the early 1990's, an increase in *B. cepacia* prevalence equivalent to that seen in North American CF centres was observed in UK clinics (Govan et al, 1993; Smith et al, 1993).

B. cepacia colonisation in both North American and European CF centres has provoked concern for several reasons (Govan et al, 1996). The organism is innately resistant to available antibiotics and hence extremely difficult to treat. Colonisation is also associated with a poorer clinical outcome for most patients who acquire the organism, particularly those who succumb to the characteristic pneumonia and septicaemia described in early reports; the so-called 'cepacia syndrome'. Finally, there is compelling evidence that the increasing incidence of colonisation in some centres results from direct patient-to-patient transmission of *B. cepacia*. As fears of

B. cepacia acquisition have grown, segregation of colonised and non-colonised CF patients has been introduced in many centres worldwide. National CF organisations also introduced measures to prevent *B. cepacia* acquisition, including hygiene guidelines for hospitals, patients and carers, and a ban on summer camps and similar social events for CF patients (ACFA, 1993; Bingen et al, 1993; Pegues et al, 1994). The psychological, social and financial implications of these infection control measures for patients and carers have been considerable, particularly as patients may feel they have become social outcasts as a consequence of colonisation (Govan and Deretic, 1996). Reinfection of transplanted lungs with *B. cepacia*, and the spread of *B. cepacia* in transplant centres has even led to the inclusion of *B. cepacia* infection as a contra-indication to transplantation in some centres (Noyes et al, 1994; Snell et al, 1993).

1.4.2 *B. cepacia* and transmissibility

During the 1980's, the emergence of *B. cepacia* in CF was characterised by dramatically different prevalence figures at individual clinics. Possible explanations for such regional variation included difficulties in the isolation of *B. cepacia* from sputum specimens (Tablan et al, 1987). However, with the widespread introduction of improved cultural techniques (Gilligan, 1991), laboratory proficiency alone could not account for the observed differences in prevalence. Alternative explanations were the improved life expectancy of CF patients, with corresponding changes in the pattern of respiratory disease, or selective pressure caused by increased antibiotic use, in particular the introduction of nebulised colimycin for early treatment of

P. aeruginosa colonisation. Early studies attributed the increased *B. cepacia* prevalence to nosocomial acquisition, particularly from contaminated equipment (Isles et al, 1984; Thomassen et al, 1985). However growing suspicion that direct or indirect patient-to-patient transmission was responsible for new cases increased pressure to introduce cohorting of colonised patients. With the advent of molecular typing in the early 1990's, several studies indicated the existence of distinct *B. cepacia* lineages within CF centres (LiPuma et al, 1988; 1990; Govan et al, 1993; Smith et al, 1993). In addition, collection of detailed epidemiological data provided compelling evidence of direct patient-to-patient transmission of *B. cepacia* and validated the unpopular segregation policies introduced by CF centres and organisations. Table 1.2, which summarises studies of *B. cepacia* epidemiology, demonstrates the weight of evidence supporting the epidemic spread of *B. cepacia* within at least some CF centres.

Highly transmissible B. cepacia lineages

Epidemiological data from numerous studies indicated that some strains of *B. cepacia* were associated with epidemic spread within CF clinics whilst others remained restricted to individual patients. In a large outbreak of *B. cepacia* infection in Edinburgh and Manchester CF centres, genomic fingerprinting by pulsed field gel electrophoresis (PFGE) and extensive epidemiological investigation of patient contacts, demonstrated the spread of at least two epidemic strains of *B. cepacia* between patients (Govan et al, 1993). In both centres, transmission occurred mainly through social contact both within and outwith the hospital setting. Similarly, inter-regional spread of the so-called Edinburgh epidemic strain followed contacts at

Table 1.2a. Publications supporting person-to-person transmission of *B. cepacia*.

Publication	Comments
Isles et al 1984	Seminal paper: noted rising incidence of <i>B. cepacia</i> and cepacia syndrome in Toronto
Thomassen et al 1985	Rising <i>B. cepacia</i> incidence in US clinics
Thomassen et al 1986	Fall in incidence post segregation
Lipuma et al 1988	Prevalence of one ribotype in individual clinics
Lipuma et al 1990	Ribotyping demonstrates person-to person spread between two patients at a CF camp
Anderson et al 1991	Nosocomial outbreak
Millar-Jones et al 1992	UK nosocomial outbreak
Govan et al 1993	PFGE: person-to-person spread through social contact in and between two UK CF centres
Smith et al 1993	Further UK outbreak with transmission in clinical and social settings
Bingen et al 1993	International consensus confirming <i>B. cepacia</i> transmissibility
Corkill et al 1994	Highlights transmission particularly at UK CF events
Pegues et al 1994	Demonstration of transmission at US CF camps
Johnson et al 1994	Intercontinental spread of Edinburgh/Toronto strain ET12
Lipuma et al 1994	? Inapparent transmission from culture negative patient
Ryley et al 1995	Further UK outbreak
Sun et al 1995	Cable pili demonstrated on intercontinental strain (ET12)
Reverts and Lauwers 1995	Prevalent strain in Belgian clinic
Whiteford et al 1995	Outbreak in UK Paediatric clinic
Pitt et al 1996	Strain ET12 prevalent in UK clinics: accounting for 38% cases
Cazzola et al 1996	Variable transmission in an Italian clinic

Table 1.2b. Publications with no evidence of person-to-person transmission of *B. cepacia*.

Publication	Comment
Glass and Govan 1986	No transmission of pathogenic strain between siblings
Hardy et al 1986	No transmission to uncolonised patients during hospitalisation
Taylor 1994	No transmission in UK unit prior to segregation
Steinbach et al 1995	No transmission in large CF unit despite no segregation of hospitalised patients

national CF events. Further epidemiological studies using the PFGE typing method have now demonstrated the spread of this strain throughout many centres in the UK (Pitt et al, 1996) whilst a large study involving multilocus enzyme electrophoresis and ribotyping has demonstrated the presence of this *B. cepacia* lineage, termed electrophoretic type 12 (ET12), in both UK and North American CF centres (Johnson et al, 1994). Indeed it is now believed that the ET12 lineage originated in Toronto, Canada before spread to the UK at international CF camps during the mid 1980's. Throughout the rest of this thesis, the Edinburgh/Toronto epidemic strain will be referred to as the ET12 lineage.

A number of studies have provided additional evidence that not all *B. cepacia* strains are equally transmissible. For example, in the Edinburgh outbreak, one patient who was colonised with two *B. cepacia* strains, only transmitted one, the ET12 lineage, to his CF girlfriend (Govan et al, 1993). Furthermore evidence from studies of CF siblings indicates that not all strains will spread from sibling to sibling despite the close proximity of these patients (Glass and Govan, 1986; Cazzola et al, 1996). The occurrence of sporadic new cases of colonisation with genetically distinct *B. cepacia* in clinics where strict segregation measures have been applied, suggests that

environmental reservoirs may also be an important source of infection (Govan and Deretic, 1996). For patients acquiring such strains, segregation is particularly controversial as transmissibility has not been demonstrated. However to classify such strains as non-transmissible on the basis of current knowledge is difficult. Notably, a recent study has identified transmission of a *B. cepacia* lineage to 5 patients, 6 years after the index case was originally colonised (Mahenthiralingam et al, 1997). Consequently research has focused on identifying bacterial markers that might enable the identification of highly transmissible strains before transmission occurs. A greater understanding of such factors will not only allow the development of more rational segregation policies but may provide a basis for the development of an anti-*B. cepacia* vaccine.

B. cepacia transmissibility markers

Studies of *B. cepacia* ET12 demonstrated its enhanced adhesion to respiratory mucin mediated by a 22kDa protein (Sajjan et al, 1992; Sajjan and Forstner 1992; Sajjan and Forstner, 1993)) present on giant intertwined fibres expressed at the bacterial cell surface, the so-called 'cable pili' (Sajjan et al 1995). Sajjan et al (1995) identified the gene encoding cable pili, termed *cblA*, in isolates of the ET12 lineage, but extensive analyses failed to find *cblA* in representatives of other epidemic *B. cepacia* lineages, suggesting that other transmissibility factors exist (Sun et al, 1996).

However one 'non-transmissible' isolate from a CF patient in Mississippi was found to contain a variant of the *cblA* gene. Interestingly, this strain has now been implicated in a large outbreak involving over 200 non-CF patients in an intensive therapy unit, possibly through transmission from the original CF host (R. Goldstein,

personal communication). Further studies have identified enhanced adhesion of variants of the ET12 strain deficient in cable pili, to the cell surface glycolipid, globotriosylceramide (Sylvester et al, 1996). The authors speculate that increased expression of this glycolipid in response to pro-inflammatory cytokines may promote adhesion of *B. cepacia* to the CF lung. Using randomly amplified PCR, Mahenthiralingam et al (1997) have identified a novel genomic marker termed '*Burkholderia cepacia* epidemic strain marker' (BCESM). Significantly, BCESM was identified in all representatives of genetically distinct epidemic lineages tested, including the ET12 clone. The absence of BCESM in apparently non-transmissible clones suggests that it may provide a useful marker for transmissibility. Further studies are ongoing to identify the role of the putative EsmR protein encoded on the 1.4 kb BCESM DNA.

1.4.3 Is *B. cepacia* a pathogen in CF?

Whilst the existence of highly transmissible *B. cepacia* lineages has now been widely accepted, the pathogenicity of *B. cepacia* is still controversial, particularly in CF centres where *B. cepacia* colonisation has not been associated with poor clinical outcome. Some clinicians believe *B. cepacia* to be merely a marker of pre-existing lung disease, or indeed, CF itself, rather than a pathogen in its own right, and thus question the need to maintain current segregation protocols (Revetts and Lauwers, 1995; Conway et al, 1995). However other centres have experienced the rapid deterioration of previously well patients on acquisition of *B. cepacia*, consistent with a major pathogenic role for this organism (Govan et al, 1993). Furthermore, in

several case-controlled studies, *B. cepacia* colonisation has been associated with accelerated respiratory decline and increased mortality (Whiteford et al, 1995; Muhdi et al, 1996; Tablan et al, 1985; Lewin et al, 1990; Brown et al, 1993). In one study (Muhdi et al, 1996), retrospective analysis found no difference between colonised and non-colonised patients in lung function or hospital visits in the 24 months prior to *B. cepacia* acquisition, providing strong evidence that *B. cepacia* acquisition was not merely a marker of existing lung disease.

Further evidence for the pathogenic potential of *B. cepacia* in CF comes from studies in the Edinburgh CF mouse (Davidson et al, 1995). Following repeated exposure of mice to *B. cepacia* J2315, an ET12 isolate, by nebulisation, histological examination of lungs revealed more severe histopathological changes in CF mice than in their non-CF littermates. Pathological changes were consistent with a severe bronchopneumonia in the CF mice and were analogous to the clinical outcome of *B. cepacia* infection in some CF patients.

Although there is an inclination to link *B. cepacia* transmissibility with pathogenicity, this view is unfounded on the basis of the available evidence. On the one hand, apparently non-transmissible strains have caused cepacia syndrome (Glass and Govan, 1986) whilst on the other, epidemic lineages have not been universally associated with a poor clinical outcome (Revets and Lauwers, 1995; Whiteford et al, 1995). Even within patients colonised with the same epidemic strain, clinical outcomes can be widely variable, ranging from asymptomatic colonisation to fulminant cepacia syndrome (Isles et al, 1984; Govan et al, 1993). Possible explanations for the variable outcome of *B. cepacia* colonisation include differences

in strain virulence (Govan et al, 1996), the underlying severity of CF disease (Tablan et al, 1985; Lewin et al, 1990; Brown et al, 1993) host immune response (Shaw et al, 1995a), gender (Thomassen et al, 1985; Govan et al, 1993), age at colonisation (Whiteford et al, 1995) and the effect of co-colonising respiratory pathogens (Shaw et al, 1995b).

Genomovar status and pathogenicity

Recently, attempts have been made to link *B. cepacia* genomovar status with both transmissibility and pathogenicity in CF. Comparison of genomovar data for a panel of *B. cepacia* isolates found examples of genomovars I to IV and *B. vietnamiensis* amongst CF isolates (Govan et al, 1996). However, genomovars II and III were associated more frequently with CF than other genomovars and included examples of known epidemic lineages, including the ET12 lineage which belongs to genomovar III. Strains associated with severe disease and cepacia syndrome in CF belonged predominantly to genomovar III. One patient who was colonised with a genomovar I strain subsequently died, but it was not clear whether *B. cepacia* had contributed to his death. A survey of Belgian CF patients identified genomovars II, III and IV amongst 12 *B. cepacia*-colonised patients but no genomovar I isolates (Revets et al, 1996). PFGE patterns revealed transmission of one genomovar III strain between 4 CF patients within a clinic but not of two other genomovar III strains. Other isolates were unique, apart from one genomovar IV strain detected in two patients from another clinic. It may be relevant to note that of the 12 patients, only one individual who was colonised with the transmissible genomovar III strain, displayed a deterioration on acquisition of *B. cepacia* and died 5 years after first *B. cepacia*

culture. In conclusion, present knowledge suggests that genomovar III strains are associated with pathogenicity in CF. However further microbiological and epidemiological studies are required to validate this important association.

1.4.4 *B. cepacia* cell surface structures

The outer surface of Gram-negative microbes is characterised by two lipid bilayers, the cytoplasmic and outer membranes, located on either side of a thin peptidoglycan wall. LPS is expressed on the outer membrane and consists of a hydrophobic anchor region termed lipid A, embedded in the outer membrane and linked by core oligosaccharide to the hydrophilic O antigen region made up of repeating oligosaccharide units, extending into the surrounding environment of the bacterium. LPS may be complete, i.e. expressing O antigen, in which case it is referred to as 'smooth', or lacking in O antigen, in which case it is referred to as 'rough'. Outer membrane proteins (OMPs) are also expressed at the cell surface and include transmembrane structures involved in signalling, nutrient uptake and secretion processes, or proteins expressed only on the inner or outer surface of the membrane involved in bacterial signalling and adhesion. Production of extracellular polysaccharide may give rise to capsule formation around the organism. Other appendages, in particular pili and flagella may be present on the surface of the bacterium.

Several researchers have investigated various aspects of the *B. cepacia* outer surface including LPS structure, OMP profiles, extracellular polysaccharide production and expression of pili or flagella. The potential role of *B. cepacia* pili in adhesion and

transmissibility has already been discussed in section 1.4.2. Exopolysaccharide production, OMP expression and LPS structure will be discussed below.

Exopolysaccharide production

Given the importance of alginate production in the pathogenesis of *P. aeruginosa* infection in CF (Govan and Deretic, 1996), attempts have been made to identify alginate production in *B. cepacia* strains. To date, production of an alginate-like polysaccharide, containing 72% guluronic acid, has been reported in a single strain only (Straus et al, 1989). In another study, a pivotal gene in the alginate biosynthesis pathway termed *algD*, was not found in any of 10 *B. cepacia* isolates (Nelson et al, 1994). Other studies have identified non-alginate heteropolysaccharide production in some *B. cepacia* strains but no correlation has been found with colonisation or virulence in CF (Allison and Goldsborough, 1994; Sage et al, 1990; Nelson et al, 1994). Furthermore, unlike *P. aeruginosa*, isolation of mucoid *B. cepacia* variants amongst CF patients is rare and has not been associated with severe disease (Govan and Deretic, 1996).

Outer membrane protein expression

Researchers have identified various OMP profiles in *B. cepacia* strains. Aronoff and Stern (1988) identified 5 major OMP comprising OMP A (56kDa), OMP B (38kDa), OMP C (37kDa), OMP D (28kDa) and OMP E (21kDa). Anwar et al (1983) described major OMPs at 40kDa, 36kDa, 24.5kDa, 17kDa and 14.5kDa. An 81kDa OMP has also been described (Parr et al, 1987; Gotoh et al, 1994), which dissociates into 36kDa and 27kDa components, probably corresponding to the C and D proteins of Aronoff and Stern and the 36kDa and 24.5kDa proteins of Anwar et al. Nutrient

depletion has been associated with changes in OMP profiles, including the induction of a 66kDa OMP under conditions of iron depletion (Anwar et al, 1983).

Considerable phenotypic variability in the OMP expression of clonally related isolates may also occur. For example, in the study of Larsen et al (1993), up to 5 OMP profiles were observed from clonal isolates derived from one colonised CF patient.

Several studies have reported an association between OMP expression and antibiotic resistance in *B. cepacia* infection (Parr et al, 1987; Moore and Hancock, 1986; Aronoff, 1988). Many hydrophilic antibiotics, including β -lactams, depend on the presence of suitable transmembrane protein channels termed 'porins', to diffuse across the bacterial outer membrane. Parr et al (1987) demonstrated that the 81kDa complex referred to earlier forms an unusually small porin channel in lipid bilayers, through which β -lactam antibiotics would be unable to pass. Antibodies to both the 36kDa (OMP C) and the 27kDa (OMP D) OMPs of this complex have been demonstrated in the serum of *B. cepacia*-colonised patients, demonstrating that both molecules are expressed *in vivo* and may be involved in antibiotic resistance (Aronoff and Stern, 1988). Changes in OMP profiles may also be important in the development of resistance to other antimicrobial compounds, particularly *in vivo* where dramatic variation in antibiotic resistance patterns can be observed even within clonal isolates from individual patients (Corkill et al, 1994a; Larsen et al, 1993; Hobson et al, 1995; Pitt et al, 1996)

Lipopolysaccharide

Bacterial lipopolysaccharide is a potent inflammatory molecule and thus a major virulence factor in many infectious diseases. As biological activity is highly dependent on LPS structure, several researchers have investigated the nature of *B. cepacia* LPS. Strains of *B. cepacia* may produce rough or smooth LPS, although a predominance of strains producing rough LPS have been noted in CF patients, particularly amongst highly transmissible lineages (Nelson et al, 1994; Butler et al, 1994b; Simpson et al, 1994). Unlike *P. aeruginosa*, there is no evidence to suggest that *B. cepacia* initially colonises CF lungs in a smooth form before changing to a rough form (Nelson et al, 1994). However antibody to *B. cepacia* O-antigen has been demonstrated in serum from patients colonised with apparently rough *B. cepacia* strains (Lacy et al; 1995; Shaw, 1995). These observations suggest that *B. cepacia* may undergo phenotypic variation *in vivo*, expressing O-antigen under certain situations not yet reproduced in the laboratory. Butler et al (1994) demonstrated serum sensitivity of rough *B. cepacia* isolates despite the ability of the strains involved to cause septicaemia in CF patients. It is tempting to speculate that this observation may be due to transient expression of smooth LPS in bacteraemic *B. cepacia*. However comparison of blood culture and sputum isolates of *B. cepacia* shows no difference in LPS expression.

Chemical analyses and studies of antibody responses have indicated that the structure of *B. cepacia* core LPS is markedly different from that of *P. aeruginosa* LPS (Nelson et al, 1993; Lacy et al, 1995; Manniello et al, 1979). In an earlier study LPS from several strains of *B. cepacia* was found to contain less phosphorus but more heptose

than *P. aeruginosa* LPS (Manniello et al, 1979). The presence of 3-deoxy-D-manno-2-octulosonic acid (KDO) has been linked to LPS toxicity, but several studies have reported little or no detectable KDO in *B. cepacia* LPS (Manniello et al, 1979; Shaw et al, 1995a). Straus et al (1990) demonstrated the presence of KDO in only 1 out of 6 *B. cepacia* strains using standard assay conditions but were able to detect KDO in all strains using a modified assay procedure. They concluded that KDO in *B. cepacia* LPS may be altered by substitution at a carbon position, thus rendering the molecule undetectable under normal assay conditions. Simultaneous studies of mouse lethality, however, demonstrated no difference in toxicity, suggesting that the form of KDO present had no impact on LPS biological activity.

Although LPS is a major bacterial virulence determinant, studies of the biological activity of *B. cepacia* LPS are limited. The toxicity of an extracellular compound of LPS, carbohydrate and protein in a mouse model appeared to be related to the LPS content of the compound. (Straus et al, 1989). As discussed in section 1.4.7, Shaw et al (1995a) have shown LPS from a range of *B. cepacia* strains to be efficient inducers of the pro-inflammatory cytokine TNF- α from human monocytes. In this study, neither LPS O-antigen expression nor content of phosphorus and KDO correlated with cytokine-inducing activity.

1.4.5 Production of extracellular virulence factors by *B. cepacia*

B. cepacia produce a range of extracellular compounds including protease, lipase, gelatinase, haemolysin, catalase and siderophores (Nelson et al, 1994; Govan and Deretic, 1996) . No definitive role has been found for any of these substances in CF,

although the production of catalase is linked to the ability of *B. cepacia* to cause infection in CGD.

Protease, lipase and haemolysin production

A 34kDa proteinase sharing antigenic similarities with *P. aeruginosa* elastase and cleaving gelatin and collagen has been isolated from *B. cepacia* (McKevitt et al, 1989). Intratracheal instillation of the purified protease into rat lungs was associated with the development of bronchopneumonia. Despite these findings, the observation that many CF strains of *B. cepacia* do not produce any proteolytic activity, would suggest that protease production is not a major virulence determinant in *B. cepacia* infection of the CF lung (Govan and Deretic, 1996).

Lipase and haemolysin production by *B. cepacia* is also highly strain dependent. Haemolytic activity is found in a minority of clinical strains (McKevitt and Woods, 1984; Nakazawa et al, 1987; Gessner and Mortensen, 1990), although in one study, investigation of activity on a panel of erythrocytes from various species found 40% of strains to be haemolytic against at least one form of erythrocyte (Vasil et al, 1990). By contrast, lipase activity, including the production of phospholipase C (PLC), is found in over 60% of *B. cepacia* isolates (McKevitt and Woods, 1984; Gessner and Mortensen, 1990). Purified *B. cepacia* lipase (MW 25,000) demonstrated no toxicity for either mice or HeLa cells (Lonon et al, 1988) but in a later study this same lipase was found to disrupt rat alveolar macrophage phagocytosis in a dose-dependent manner, suggesting a possible role for lipase in human infection (Straus et al, 1992). However, as many CF isolates of *B. cepacia* do not possess lipolytic activity,

production of lipase may play only a minor role in *B. cepacia* infection (Govan and Deretic, 1996).

Unlike *P. aeruginosa*, PLC activity in *B. cepacia* does not correlate with haemolytic activity, although a heat-labile haemolysin with PLC and sphingomyelinase activities has been described (Vasil et al, 1990). Thus although haemolytic strains of *B. cepacia* display PLC activity, many strains positive for PLC do not express haemolytic activity (Nelson et al, 1994). Studies of PLC have revealed the complex nature of PLC regulation and expression in *B. cepacia*. In particular, a gene probe directed to the *B. cepacia* PLC gene was found to bind to restricted DNA in a highly variable manner (Vasil et al, 1990). One possible explanation for this finding is migration of insertion sequences (IS) within the *B. cepacia* genome. Multiple IS have been found within the *B. cepacia* genome and are believed to be involved in the activation or inactivation of gene expression (Scordilis et al, 1987). The variability in the expression of haemolytic and PLC activity and the observation of conversion of a haemolysin-positive strain to a haemolysin-negative mutant following DNA rearrangements (Vasil et al, 1990) would support this hypothesis.

Abe and Nakazawa (1993) described the production of a novel compound displaying haemolytic and antifungal activity, by a clinical isolate of *B. cepacia*. This substance, termed cepalycin, was a small non-proteinaceous heat-resistant compound which was demonstrated to form pores within erythrocyte membranes as determined by electronmicroscopy. Haemolytic activity was inhibited by sterols. Recently Hutchison et al (1998) have described a haemolysin extracted from a representative isolate of *B. cepacia* ET12 displaying many properties similar to cepalycin.

Biochemically the haemolysin behaves as a classical lipopeptide. Haemolytic activity follows pore formation in the erythrocyte membrane and is inhibitable by sterols. Biological assays have indicated a possible clinical role for this substance which induces neutrophil apoptosis at low concentrations and neutrophil degranulation at higher concentrations. As the haemolysin is only expressed in conditions of high oxygen tension, the authors speculate that the most likely role for this molecule is in the initial establishment of infection in the CF lung.

Gessner and Mortensen (1990) compared 98 CF and 21 control (environmental and non-CF clinical) isolates of *B. cepacia* for a wide range of potential virulence factors. Significant differences included greater catalase production, ornithine decarboxylase activity and α haemolytic activity amongst CF strains, whilst control strains were more likely to display nitrate reduction, urea hydrolysis, xanthine hydrolysis, valine aminopeptidase activity, C14 lipase activity, β haemolysis and alginase and trypsin activity. However, the role of any of these factors in pathogenesis remains unclear.

Siderophore production

The production of iron-binding siderophores enables bacteria to compete with host proteins for limited iron resources and is thus associated with virulence. *B. cepacia* produces at least three iron-binding siderophores, termed cepabactin, azurechelin and pyochelin, the latter being chemically unrelated to the *P. aeruginosa* siderophore of the same name (Sokol, 1986; Bukovits et al, 1982; Sokol et al, 1992; Meyer et al, 1989). To date, a definitive role for any of the *B. cepacia* siderophores in CF has not been demonstrated. Sokol (1986) reported increased morbidity and mortality in CF patients colonised with pyochelin-producing strains but half of all CF isolates

investigated were pyochelin-negative. Administration of exogenous pyochelin enhanced the virulence of non-pyochelin producing *B. cepacia* in a rat model of chronic pulmonary *B. cepacia* infection (Sokol and Woods, 1988), suggesting that pyochelin may aid *B. cepacia* dissemination through the lungs. A role for azurechelin in pathogenesis has also been suggested following the observation of azurechelin production in 88% of clinical isolates of *B. cepacia* (Sokol et al, 1992).

1.4.6 *B. cepacia* and intracellularity

Several puzzling clinical and scientific observations have led to speculation that *B. cepacia* may survive and grow within pulmonary phagocytes or respiratory epithelial cells: first, clinical resistance to antimicrobial therapy despite demonstration of in vitro susceptibility; second, culture of serum sensitive isolates in bacteraemic infection (Butler et al, 1994a); third, chronic pulmonary colonisation despite a pronounced antibody response (Nelson et al, 1993); fourth, the close taxonomic relationship between *B. cepacia* and the highly virulent intracellular pathogen, *Burkholderia pseudomallei* (Palleroni, 1984; Pruksachartvuthi et al, 1991). To date, however, the scientific evidence for intracellular survival or growth of *B. cepacia* is not convincing. Studies of intracellularity in bacterial pathogens can be difficult and in the case of *B. cepacia* are further complicated by the organism's innate resistance to antibiotics, including aminoglycosides, which are commonly used in intracellular assays to kill extracellular organisms. In a short abstract, Burns (1992) reported the observation of *B. cepacia* within CF post-mortem respiratory epithelial cells by electronmicroscopy. No further evidence was presented until 1996

when the same authors reported that *B. cepacia* ET12 invaded cultured respiratory epithelial cells to a greater extent than an apparently non-transmissible CF strain of *B. cepacia* (Burns et al, 1996). Other researchers have also demonstrated low level in vitro invasion of respiratory epithelial cells by the ET12 lineage (Tipper et al, 1995) but the role of epithelial invasion in vivo remains unclear. As a caveat, the demonstration of the intracellular survival and growth of *B. cepacia* within amoeba raises the possibility that free-living protozoa may act as an environmental reservoir from which CF patients could acquire the organism (Landers et al, 1995).

1.4.7 The immune response to *B. cepacia* infection in CF

Humoral immune responses

In CF patients, colonisation with *B. cepacia* is associated with a pronounced and specific humoral response, including raised serum IgG and IgA and sputum IgA titres against *B. cepacia* lipopolysaccharide (LPS) and outer membrane components (OMP) (Nelson et al, 1993; Aronoff et al, 1991). Anti-*B. cepacia* antibodies have also been detected in non-colonised CF patients, and particularly in patients colonised with *P. aeruginosa* (Nelson et al, 1993; Aronoff and Stern, 1988). Studies using pre-absorbed sera have failed to demonstrate an appreciable degree of cross-reactivity between the two species, either for OMP or LPS components (Nelson et al, 1993; Lacy et al, 1995) suggesting that the response to *P. aeruginosa* is not the source of pre-colonisation anti-*B. cepacia* antibody. Generally, levels of anti-*B. cepacia* immunoglobulin in non-colonised patients are low but the demonstration of substantially raised titres in a subset of patients may reflect prior exposure to

B. cepacia where an appropriate antibody response has prevented the occurrence of chronic colonisation. The role of antibody once *B. cepacia* is established in the CF lung is unclear; for example, clinical outcome is independent of the magnitude of anti-*B. cepacia* responses (Nelson et al, 1993). Measurement of humoral responses to *B. cepacia* core LPS amongst *B. cepacia*-colonised patients has demonstrated variable IgG subclass expression with a predominance of IgG2 and IgG3, leading the investigators to speculate that the poor clinical outcome in some colonised patients may be due to an inappropriate IgG subclass response (Butler et al, 1994b). A recent study by Burnie et al (1995), using immunoblotting techniques, suggested that IgG antibodies against a 30 kDa OMP, presumptively identified as the major immunodominant porin OMP D (Aronoff and Stern, 1988, Parr et al, 1987), are associated with a better prognosis in colonised patients. If these results are confirmed, it raises the possibility of using this OMP as a target for immunotherapy.

The association of *B. cepacia* with CGD (O'Neil et al, 1986; Lacy et al, 1993) and the role of neutrophils as the predominant immune effector cell in the CF lung (Konstan et al, 1994) have led to speculation that the interaction between *B. cepacia* and neutrophils may be important in the evasion of host defences by this organism. Speert et al (1994) demonstrated that unlike *P. aeruginosa*, *B. cepacia* is resistant to non-oxidative neutrophil killing mechanisms; hence the role of *B. cepacia* in CGD. Evasion of the normal neutrophil oxidative burst, through the avoidance of opsonisation with specific immunoglobulin, would aid the survival of *B. cepacia* in the presence of a pronounced immune response. Such a situation can be postulated within the CF lung, where normal opsonisation processes are disrupted by proteolytic

cleavage of complement receptors and immunoglobulin molecules (Döring, 1994; Suter, 1994), neutralising the humoral immune response to *B. cepacia* and enabling the organism to persist despite the high neutrophil presence. This hypothesis, however, does not explain the ability of rough, O antigen-deficient, serum-sensitive *B. cepacia* to cause invasive pneumonitis and septicaemia in patients with elevated anti-*B. cepacia* immunoglobulin titres (Butler et al, 1994a).

Inflammatory damage

Recently, increasing evidence has indicated that host immune responses are important in the pathogenesis of *B. cepacia* infection. A UK multicentre study has shown that levels of the inflammatory markers, C-reactive protein and neutrophil elastase α 1-antiproteinase complex, are significantly higher during *B. cepacia*-associated exacerbations than in exacerbations due to *P. aeruginosa*. Aggressive antibiotic treatment with the most active agents available did not eliminate *B. cepacia* but in most cases was associated with a decline in inflammatory markers to pre-exacerbation levels (Elborn et al, 1994). In addition, anecdotal evidence indicates that patients who exhibit rapid pulmonary decline and pronounced inflammatory symptoms, but do not respond to antibiotic therapy, nevertheless respond to treatment with commercial preparations of immunoglobulin (Govan et al, 1996). The relative absence of *B. cepacia* antibodies in healthy humans (Nelson et al, 1993), from whom these immunoglobulins are obtained, suggests that such preparations contain potentially useful anti-inflammatory activity.

Shaw et al (1995a) have demonstrated that LPS from clinical and environmental isolates of *B. cepacia* induces pro-inflammatory cytokines, including the major

cytokine TNF α , to a level ten times that induced by *P. aeruginosa* LPS and matching the inflammatory power of *Escherichia coli* endotoxin (Shaw, 1995; Shaw et al, 1995a). The mechanism involved in *B. cepacia* cytokine stimulation is unclear but is independent of CD14 receptors. Of interest, induction of TNF α by *B. cepacia* LPS was reduced in the presence of *P. aeruginosa* LPS which suggests that the diversity of clinical outcomes in patients colonised with *B. cepacia* may in part be influenced by the presence or absence of *P. aeruginosa* and other CF pathogens (Shaw et al, 1995a; b).

The pro-inflammatory effect of *B. cepacia* has also been shown in studies by Palfreyman et al (1997) who demonstrated the induction of the cytokine IL-8 in a respiratory epithelial cell line by supernatants from *B. cepacia* culture. Since purified LPS induced little or no IL-8 production by epithelial cells, activity did not appear to be related to the presence of LPS in the culture supernatants. As IL-8 is a potent chemotactic agent for neutrophils, these results suggest that *B. cepacia* may increase neutrophil recruitment, with a resulting increase in the inflammatory burden within the CF lung.

CHAPTER 2 METHODS

2.1 MATERIALS AND EQUIPMENT

2.1.1 Bacterial strains

Strains used in this thesis are from the collection of Dr John Govan (CFL, Dept of Medical Microbiology, Edinburgh University) and are described in Table 2.1. Information regarding pulsed field gel electrophoresis patterns and genomovar status was provided by Mrs C Doherty (CFL) and Dr P Vandamme (University of Gent, Belgium) respectively. Phenol water extracts of LPS from strains *P. aeruginosa* PAO1 and C1250, and *B. cepacia* J2540, J2505 and C1504, were provided by Dr D Shaw and from *E. coli* O18K- by Dr D Delahooke, (MPRL, Dept. of Medical Microbiology, Edinburgh University). *B. cepacia* haemolysin was provided by Dr M Hutchison (CFL).

2.1.2 Serum and antibodies

Serum from CF patients was provided by the adult CF clinic at the Western General Hospital, Edinburgh. Serum was extracted from clotted blood samples after overnight storage at 4°C. RBC were removed by centrifugation and samples stored at -20°C before use. Heat-inactivated sera were prepared by incubation of serum at 56°C for 1 hour before use. Pooled human serum and pooled human AB+ serum were provided by the Scottish Blood Transfusion Service, (SEBTS, Royal Infirmary Edinburgh).

Table 2.1a *Burkholderia cepacia* strains used in this thesis

Strains	Comment	Genomovar
CF strains		
A599/C1964	non-epidemic	I
C1394	Manchester epidemic strain	III
C1444	Manchester epidemic strain	III
C1504	non-epidemic	
C1524	non-epidemic	II
C1555	non-epidemic	
C1576	Glasgow epidemic strain	II
C1579	Glasgow epidemic strain	II
C1632	Newcastle epidemic strain	
C1873	non-epidemic	
C1985	non-epidemic	
C1987	non-epidemic	
C1989	non-epidemic	
C2040	non-epidemic	
J2315	ET12 lineage (Edinburgh, index case)	III
J415	non-epidemic	III
Non-CF clinical		
C1962	Cerebellar abscess	II
J2352	Neonatal infection	III
J2503	ATCC 25608, Wound infection	I, III, IV
J2505	ATCC 17762, UTI	I, III, IV
J2660	ITU infection	III
J2684	Pneumonia	
Environmental		
J2395	Soil (pot plant, respiratory unit)	II
J2504	ATCC 29424, Soil	
J2511	ATCC 17616, Soil	
J2540	RBGE, Soil	I
J2552	RBGE, Soil	I

Table 2.1b Other strains used in this thesis

<i>Pseudomonas aeruginosa</i>	
A15	non-mucoid CF strain
A593	non-mucoid CF strain
A603	non-mucoid CF strain
A605	non-mucoid CF strain
A607	non-mucoid CF strain
A609	non-mucoid CF strain
C1249	non-mucoid CF strain
C1250	mucoid CF strain (isogenic C1249)
H228	non-mucoid CF strain
H230	non-mucoid CF strain
J1385	non-mucoid CF strain
PAO1	non-CF clinical: classic laboratory strain
<i>Escherichia coli</i>	
O18K-	non-capsulate
<i>Listeria monocytogenes</i>	
J2563	haemolytic clinical isolate

For flow cytometry, murine monoclonal antibodies conjugated to FITC were provided by Serotec Ltd (Kidlington, Oxford, UK) including control IgG1 and anti-human CD11b.

2.1.3 Mice

Wild-type and CF mice were provided by Prof D. Porteous, MRC HGU, WGH Edinburgh. Mice were housed either in standard conditions or in a specific pathogen free environment as stated in the results section. Background colonisation of the

respiratory tract was investigated in a range of murine strains, including MF1, C57/Bl6, C129 and the Edinburgh transgenic CF mouse (Dorin et al, 1992). Clearance of *B. cepacia* J2315 was initially investigated in Swiss mice. In all other experiments, mice used were from breeding programmes involving heterozygotes of the Edinburgh transgenic CF mouse ($CF_{HGU}/+ : h/+$; Dorin et al, 1992) and the UNC knockout mouse ($CF_{UNC}/+ : u/+$; Snouwaert et al, 1992). Non-CF mice were therefore wildtype (+/+) or heterozygotes (h/+ or u/+), whilst CF mice were compound heterozygotes (h/u) or homozygotes (h/h or u/u).

2.1.4 Chemicals and media

Solutions

Unless otherwise stated, all solutions were sterilised by autoclaving at 121°C/15psi for 15 min. Saline solutions were prepared at both 0.85% and 0.9% (w/v) in distilled water. Phosphate buffered saline (PBS) was prepared using one PBS tablets (Oxoid, Basingstoke, Hants) per 100 ml distilled water. Pyrogen-free water (pH₂O) was obtained using a Milli-Q Reagent Grade Water system (Millipore Corporation, Molshiem, France). Pre-sterilised Hanks balanced salt solution with Ca²⁺ and Mg²⁺ but without phenol red (HBSS) was obtained from Sigma (Sigma-Aldrich Coy. Ltd., Poole, Dorset). For neutrophil incubations, HBSS was modified by addition of 0.1% (w/v) glucose from a filter-sterilised stock solution of 50% (w/v) glucose (mHBSS).

Bacteriological media

Unless otherwise stated media were provided by Oxoid. All media were sterilised by autoclaving at 121°C/15psi for 15 min except skimmed milk which was autoclaved

for only 5 min and Malka medium which was prepared from sterile stock solutions.

Nutrient agar: Columbia agar base, 39 g/L.

Tryptone soya agar (TSA): Tryptone soya agar, 40 g/L.

Blood agar: Columbia agar base, 39 g/L, with 5% sterile horse blood

(E-O Laboratories, Bonnybridge, Falkirk) added after autoclaving, when the agar has cooled to 50°C.

Cepacia agar (CEP): Cepacia agar (MAST Diagnostics Ltd., Bootle, UK), 32.5 g/L.

Add one Selectatab (MAST) per 100 ml agar after autoclaving, when the agar has cooled to 50°C. This gives a final antibiotic concentration of 300 U/ml polymixin and 100 µg/ml ticarcillin.

Pseudomonas isolation agar (PIA): Pseudomonas isolation agar (Difco

Laboratories, Michigan, USA) 45 g/L with 2% (v/v) glycerol (BDH, Merck Ltd., Dorset, UK), added prior to autoclaving.

Skimmed milk: Skimmed milk powder, 10% (w/v).

Nutrient broth (NYB): Oxoid broth No 2, 25 g/L, with 0.5% (w/v) yeast extract (Difco) added prior to autoclaving.

Malka medium stock solutions: All stock solutions were prepared with sterile pH₂O in clean sterile glassware (Table 2.2). All chemicals were Analar grade, supplied by BDH (Merck Ltd, Dorset, UK).

Table 2.2 Malka Medium stock solutions

Solution	Recipe
A	Na ₂ HPO ₄ (7.34 g/100 ml) and KH ₂ PO ₄ (3.24 g/100 ml). pH adjusted to 7.2. Stored over 3-5 ml chloroform.
B	MgSO ₄ .7H ₂ O (20.5 g/100 ml). Stored over 3-5 ml chloroform.
C	50% (w/v) glucose. Filter sterilised.
D	FeSO ₄ .7H ₂ O (0.183 g/100 ml). Add 1 drop of concentrated H ₂ SO ₄ or HCl.
E	(NH ₄) ₂ SO ₄ (5 g/100 ml). Stored over chloroform.

Malka medium (MMFe⁺): Add 20 ml A, 20 ml B, 5 ml C, 1 ml D, and 20 ml E to 934 ml sterile pH₂O in a sterile clean glass container. For iron-deficient Malka medium (MMFe⁻) omit solution D.

Chemicals

All chemicals were Analar grade, provided by BDH unless otherwise stated.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) and lucigenin (bis-N-methylacridinium nitrate; Sigma) were stored as 0.1 M stock solutions in dimethyl sulphoxide (DMSO) at -20°C. Other stock solutions included fMet-Leu-Phe (FMLP; Sigma), stored as a 10⁻³ M solution in DMSO at -20°C, and phorbol 12-myristate 13-acetate (PMA, Sigma), stored as a 1 mg/ml solution in distilled water at -20°C.

Solutions for flow cytometry, including FACS Lysing Solution and CellFix, were supplied by Becton-Dickinson (Cowley, Oxford). Dihydrorhodamine (DHR) was supplied by Cambridge Biosciences, Cambridge, UK and was stored as a 10 mg/ml stock solution in DMSO at -20°C in the dark.

2.1.5 Equipment

Chemiluminescence experiments were carried out using a LabSystems Luminometer (Labsystems, Life Sciences International UK Ltd, Basingstoke, Hants), in the laboratory of Dr GR Barclay, SEBTS. Flow cytometric analyses were conducted using a Becton Dickinson FACSort, also in the laboratory of Dr Barclay. Other items of equipment are described within the relevant section.

2.2 GENERAL BACTERIOLOGICAL METHODS

2.2.1 Storage, recovery and growth of bacteria

Bacteria were stored in skimmed milk at -70°C and were recovered by streaking onto nutrient agar or TSA. Bacterial cultures were maintained on the bench by weekly passage on CEP for *B. cepacia* or PIA for *P. aeruginosa*, but were replenished from frozen stores at 1-2 monthly intervals. For the majority of experiments bacteria were grown overnight in NYB at 37°C with orbital incubation at 140 rpm. Unless otherwise stated, bacterial cultures were standardised spectrophotometrically to a concentration of 1×10^9 cfu/ml using an OD_{590} of 1.0.

2.2.2 Extracellular products of *B. cepacia* isolates

Lecithinase test agar was prepared using standard TSA with 0.11% (w/v) CaCl_2 and 5% (w/v) egg yolk powder (Oxoid). Protease test agar was prepared as follows. 9.25 g of Brain, Heart Infusion powder (Oxoid) was dissolved in 25 ml of distilled

water and dialysed against 500 ml distilled water for 18h at 4°C. The resultant dialysate was combined with 3% bacteriological agar and 3% skimmed milk before sterilisation. Elastase test agar was prepared similarly except that skimmed milk was omitted and 1% UV-sterilised Elastin Congo Red stain was added to the medium at 50°C after autoclaving. Haemolysis was tested using blood agar with 5% horse or human blood.

For all media, bacterial strains were tested as follows. Bacteria were grown overnight in NYB and diluted 1 in 10 before use. Plates were inoculated using a multipoint inoculator (A400 Multipoint Inoculator, Denley, Billingshurst, Surrey) and incubated at 30°C and 37°C. Plates were assessed after 24h and 48h, with a zone of clearing around bacterial growth being a positive result.

Catalase testing was carried out in a bacteriological safety cabinet using 30% H₂O₂ solution. Bacteria were grown overnight on either nutrient or cepacia medium. Using sterile wooden sticks, a small sample of bacterial growth was smeared onto a glass slide and 1 to 2 drops of H₂O₂ solution added using a glass Pasteur pipette, before covering with a glass coverslip. Catalase activity was determined by bubbling of the bacterial culture. Activity was either brisk on the basis of immediate bubbling or slow on the basis of obvious bubbling only occurring after the addition of the glass coverslip.

2.2.3 Growth in iron-rich and iron-deficient media

Iron-rich and iron-deficient Malka media (MMFe⁺ and MMFe⁻ respectively) were prepared as described as in section 2.1.4. For selected experiments apotransferrin

(Sigma; stock solution: 10 mg/ml in pH₂O) was added to MMFe⁺ and MMFe⁻ to give a final concentration of 200 µg/ml (MMFe⁺TF and MMFe⁻TF). Bacteria were grown overnight in NYB at 37°C with orbital incubation at 140 rpm. After centrifugation at 2500 x g for 10 min, bacteria were resuspended in PBS and standardised spectrophotometrically to give a concentration of 5 x 10⁸ cfu/ml using an OD₅₂₅ of 0.43. Bacteria were further diluted in PBS to 5 x 10⁶ cfu/ml. 100 µl amounts of the 5 x 10⁸ cfu/ml suspension or the 5 x 10⁶ cfu/ml suspension were added to 10 ml aliquots of the appropriate Malka medium in sterile glass universals, giving inoculum sizes of 5 x 10⁶ cfu/ml and 5 x 10⁴ cfu/ml respectively. Cultures were grown for up to 5 days at 37°C, with orbital incubation, and aliquots removed at intervals for serial dilution and counting of viable bacteria.

2.2.4 Extraction of outer membrane proteins

Bacteria were grown up in at least a 500 ml volume in NYB over a 48h period, in either shaking culture in air or static culture in air + 5% CO₂. Cells were washed twice in PBS at 10,000 x g (Sorvall, RC-5B centrifuge, Dupont UK Ltd., Stevenage, Herts.) before resuspension in 10-20 ml PBS in a glass universal. Samples were sonicated at an amplitude of 10 µm for 6 x 30s pulses (Microson, Ultrasonic Cell Disrupter, Heat-Systems-Ultrasonic Inc., NY, USA). For each 10 ml of sample, 1 ml 7% Sarkosyl was added and the cell debris spun out at 10,000 x g for 15 min. The supernatant was removed and spun again to ensure complete removal of cell debris. Outer membrane proteins were precipitated by ultracentrifugation at 50,000 x g for 1 hour (Sorvall ultracentrifuge-OTD65B, Dupont UK Ltd.). After resuspension in

pfH₂O a further ultracentrifugation step was carried out. Finally the samples were resuspended in 2 ml pfH₂O and stored in 200 µl aliquots at -20°C. Protein concentration was determined using the method of Lowry et al (1951), with bovine serum albumin (BSA) as the protein standard.

2.2.5 Phenol water extraction of lipopolysaccharide

LPS extracts were prepared according to a modified aqueous phenol method of Westphal and Luderitz (1954) as described in Hancock and Poxton (1988). Bacteria were grown for 48 hours in a orbital incubator (140 rpm) at 37°C in NYB and washed twice in PBS at 10,000 x g (Sorvall RC-5B), before resuspension in PBS and lyophilisation over 48 hours (Edwards Modylo freeze dryer, Edwards High Vacuum Ltd., Surrey, UK). Dry cells were resuspended (2% w/v) in pfH₂O and softened for 1 hour at 60°C, before addition of an equal volume of 90% phenol, pre-warmed to 60°C. The aqueous phenol mixture was incubated at 60°C for 15 min, with shaking, before cooling on ice. Extracts were transferred to centrifuge tubes and spun at 13,000 x g for 20 min to ensure phase separation. The LPS rich upper aqueous layer was removed and dialysed overnight against running tap water, before 4 hours further dialysis against pfH₂O, with 3-4 changes of water. The resulting dialysate was lyophilised and resuspended in pfH₂O at a concentration of 2 mg/ml. Samples were sonicated at an amplitude of 10 µm for three to eight 30s pulses to ensure adequate solubilisation. All samples were stored at -20°C.

LPS purity was confirmed using the method of Bradford (1976) to determine protein content. Briefly, Bradford's reagent was prepared by dissolving 100 mg Coomassie

brilliant blue R-250 (Biorad, Hemel Hempstead, Herts.) in 50 ml 95% (v/v) ethanol. To this was added 100 ml of 85% (w/v) phosphoric acid and the resulting mixture made up to a volume of 1L in pH_2O . Standard solutions of BSA in pH_2O were prepared as a series of 1 in 10 dilutions from 2 mg/ml to 2 ng/ml. One part standard or LPS solution was diluted with 4 parts Bradfords reagent and kept at room temperature for at least 2 min before reading of the OD_{595} . Protein content of LPS samples was derived using a standard curve of OD_{595} against protein content prepared from results for the BSA standards.

2.2.6 SDS-PAGE gel analysis of OMP and LPS extractions

Solutions

OMP and LPS preparations were separated on polyacrylamide gels using the buffer system of Laemmli (1970). The following solutions were used:

Double strength sample buffer: 0.125M Tris-HCl, 4% (w/v) sodium dodecylsulphate (SDS), 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue; pH 6.8.

Double strength separating buffer: 0.75M Tris-HCl, 0.2% (w/v) SDS; pH 8.8.

Double strength stacking buffer: 0.25M Tris-HCl, 0.2% (w/v) SDS; pH 6.8.

Acrylamide: 40% (w/v) acrylamide consisting of 100g acrylamide and 2.7g methylene bis acrylamide.

Electrode buffer: 0.025M Tris, 0.192M glycine, 0.1% (w/v) SDS; pH 8.3.

LPS preparations

LPS samples (1 mg/ml) were prepared for polyacrylamide gel electrophoresis (PAGE) as follows: 100 µl LPS was mixed with 100 µl double strength sample buffer and 50 µl proteinase K solution (Sigma, 2.5 mg/ml in pH₂O). Samples were incubated for 2 hours at 37°C before storage at -20°C. Immediately prior to loading samples were thawed at room temperature and boiled for 10 min.

OMP preparations

200 µl of each OMP sample was mixed with 200 µl double strength sample buffer. Standards were prepared from a stock solution of 2 mg/ml protein standards (Sigma) in single strength sample buffer, by diluting one part standard solution to three parts single strength sample buffer. Both OMP preparations and standards were boiled for 2-5 min prior to loading onto the gel.

SDS Polyacrylamide gel electrophoresis

LPS preparations were run on a 14% separating gel, whilst OMP preparations were run on a 12% separating gel. Table 2.3 summarises the preparation of stacking and separating gels. All gels were degassed prior to addition of TEMED and APS.

Table 2.3 Preparation of polyacrylamide gels

Reagent	Stacking gel	Separating gel	
	4%	12%	14%
pfH ₂ O	3.5 ml	5.2 ml	3.45 ml
Separating buffer	-	17.5 ml	17.5 ml
Stacking buffer	5.0 ml	-	-
40% Acrylamide	1.0 ml	10.5 ml	12.25 ml
TEMED (NNN'N'-tetramethyl-1,2-diaminoethane)	20 µl	50 µl	50 µl
Ammonium persulphate (APS; 15 mg/ml)	0.5 ml	1.75 ml	1.75 ml

The separating gel was poured and overlaid with water-saturated butan-2-ol until set. After removal of the butan-2-ol, the stacking gel was poured on top and a 20 well comb inserted. Once set, the comb was removed and the gel apparatus placed into an electrophoresis tank. Wells were loaded with 40 µl OMP preparations or 20 µl LPS preparations, and any empty wells loaded with an equivalent volume of single strength sample buffer. Samples were electrophoresed through the stacking gel at 90V and through the separating gel at 150V. Once the dye front had run a sufficient distance, gels were removed and stained appropriately.

Silver staining for LPS

LPS was visualised using the method of Tsai and Frasch (1982) as described in Hancock and Poxton (1988). Solutions used were as follows:

Fixative solution: 7% (v/v) acetic acid, 25% (v/v) propan-2-ol.

Oxidising solution: 1.05 g periodic acid in 150 ml propan-2-ol with 4 ml fixative.

Ammoniacal silver nitrate solution: 1.4 ml ammonia solution and 21 ml 0.36%

(w/v) sodium hydroxide to which 4 ml of 19.4% (w/v) silver nitrate was slowly added with mixing. The solution was then made up to 100 ml in pyrogen free H₂O.

Developing solution: 150 ml formaldehyde solution with 10 mg citric acid, prewarmed to 22°C.

LPS gels were soaked in fixative solution overnight before oxidation for 10-15 min in freshly prepared oxidising solution. Gels were then washed in at least four changes of pffH₂O over a three hour period. The gel was silver-stained by the addition of freshly-prepared silver nitrate solution for 15 min before washing in at least 4 changes of pffH₂O over a 40 min period. The gel was developed by addition of fresh developing solution at 22°C, which was left on the gel until a sufficient intensity of staining had been reached. Finally the gel was washed thoroughly in at least four changes of pffH₂O and kept in water until photographed. All steps, except developing, were carried out on a shaking platform.

Coomassie blue staining for protein

Gels were stained for protein using the method described in Hancock and Poxton (1988). Solutions were made up in pffH₂O as follows:

Solution 1: 25% (v/v) propan-2-ol, 10% (v/v) acetic acid, 0.05% (w/v) Coomassie brilliant blue R-250 (Bio-Rad, Hemel Hempstead, Herts.).

Solution 2: 10% (v/v) propan-2-ol, 10% (v/v) acetic acid, 0.005% (w/v) Coomassie blue.

Solution 3: 10% (v/v) acetic acid, 0.025% (w/v) Coomassie blue.

Solution 4. 40% (v/v) methanol, 10% (v/v) acetic acid.

Solution 5: 10% acetic acid.

All steps were carried out on a shaking platform. Protein gels were soaked overnight in solution 1. Thereafter gels were placed sequentially in solutions 2 – 5 for one hour at a time. The stained gel was kept in solution 5 until photography.

2.2.7 Bacterial pre-opsonisation

Overnight bacterial cultures in NYB were pelleted by gentle centrifugation at 1500 x g for 20 min without braking. After at least one wash in either PBS or HBSS, bacteria were resuspended in PBS or mHBSS and adjusted spectrophotometrically to a concentration of 1×10^9 cfu/ml. Pre-opsonisation was carried out in 5 ml glass tubes. Aliquots of bacterial suspensions were mixed with sufficient serum to give a final concentration of 10% serum and incubated at 37°C for 30 min with gentle shaking. After further centrifugation at 2500 x g for 15 min, the bacterial pellets were resuspended in a volume of mHBSS equivalent to the initial aliquot used. Bacteria were diluted in PBS or mHBSS to the required concentration before use.

In some chemiluminescence experiments (see section 2.4.4), bacteria were also pre-opsonised with guinea pig complement (GPC, VH Bio Ltd., Gosforth, Newcastle upon Tyne). GPC was reconstituted at a concentration of 5 mg/ml in distilled water from lyophilised preparations stored at -20°C. The resulting stock solution was aliquoted and stored at -70°C until use. Aliquots were thawed on ice immediately prior to pre-opsonisation, which was carried out as for serum. Initial LCL experiments indicated that opsonisation with 4% (v/v) GPC solution gave optimum

complement activity, and further experiments were carried out using 4% GPC as standard.

2.3 EXTRACTION AND PREPARATION OF PHAGOCYtic CELLS

2.3.1 Preparation of monocytes

For assays of intracellular survival, monocytes were extracted from buffy coat preparations provided by SEBTS. Buffy coats were diluted 1 in 4 with PBS and layered onto Histopaque-1077 (Sigma). Gradients were spun at 400 x g for 25-30 min and cells harvested from the interface between the two layers. Cells were washed in RPMI 1640 (Gibco BRL, Life Technologies Ltd., Paisley, Renfrewshire) and pelleted by centrifugation at 150 x g for 5 min. After a further wash and pelleting at 150 x g for 5 min, monocytes were resuspended in a small volume of RPMI 1640. A 50 μ l aliquot was diluted in 450 μ l Trypan blue, and the concentration of monocytes determined by counting in a haemocytometer at a magnification of x 25. Viability, as determined by the exclusion of the Trypan blue dye, was greater than 95%. The monocyte suspension was adjusted to give a final concentration of 5×10^6 viable cells/ml.

For assays of intracellular uptake of bacteria by monocytes, cells were prepared from fresh blood donated by healthy volunteers. Blood was diluted 1 in 4 in PBS and monocytes extracted as above. Finally monocytes were resuspended in Eagles

medium (1% w/v Eagles salts (Gibco), 1% (v/v) HEPES (Sigma) and 1-2% (w/v) NaHCO_3) at a concentration of 5×10^6 /ml.

In some experiments, the mouse monocyte cell line J774.2 was used rather than human monocytes. Briefly J774.2 was cultured in Dulbecco's minimal essential medium (DMEM; Gibco) with 10% FCS (Sigma) and with 1% stock penicillin/streptomycin solution (Sigma) in a 5% CO_2 incubator at 37°C. Cells were harvested by gentle scraping and washed twice in PBS before resuspension at 1×10^7 cells/ml in DMEM with FCS but without antibiotics.

2.3.2 Neutrophil preparation using the plasma-Percoll method

For killing assays and chemiluminescence experiments neutrophils were extracted from fresh blood according to the plasma-Percoll method described by Haslett et al (1985) with minor modifications. Using 3.8% sodium citrate as an anticoagulant (1 ml citrate per 10 ml blood), fresh whole blood was collected from healthy volunteers and centrifuged at $300 \times g$ for 20 min. The platelet rich plasma (PRP) was removed and the cell pellet gently mixed with a solution of 6% (w/v) dextran (MW: 500,000; BDH) in 0.9% NaCl, prewarmed to 37°C, in a ratio of one part dextran solution to four parts cell pellet. After making up to double the volume with NaCl at 37°C, the blood was allowed to sediment at room temperature for 25-40 min. Platelet poor plasma (PPP) was prepared by centrifugation of PRP at $2500 \times g$ for 10 min. Percoll solutions were made up from a stock Percoll solution of 9 parts Percoll to 1 part 0.9% NaCl, as described below:

51%:	1.02 ml Percoll solution	0.98 ml PPP
42%	0.84 ml Percoll solution	1.16 ml PPP

The leucocyte rich upper layer was collected from the dextran sedimentation, spun at 300 x g for 6 min and resuspended in 2 ml PPP. The Percoll density gradient was overlaid (51% followed by 42% followed by the leukocyte layer) and spun at 275 x g for 10 min. Neutrophils were collected from the interface between the 51% and 42% layers, extending into 51% layer. After washing in PPP and then in PBS, RBC were lysed using 9 parts distilled water for a few seconds, before addition of 1 part x10 PBS. Neutrophils were washed for a final time in PBS, resuspended in mHBSS and adjusted to the desired concentration after counting in a haemocytometer. Cell viability was greater than 95% as confirmed by the trypan blue exclusion test. Neutrophil purity was confirmed by May-Grunwald/Giemsa staining. Briefly, a smear of the neutrophil suspension on a glass slide was dried in air before immediate fixing with methanol for at least 10 min. May-Grunwald stain (Sigma) was added to the neutrophil smear for 3 min, followed by Giemsa (Sigma) stain for a further 3 min. Smears were viewed under the light microscope at x 25 and were found to contain >95% polymorphonuclear cells of which >95% were neutrophils.

2.3.3 Neutrophil preparation using the Histopaque method

In superoxide and flow cytometric assays, neutrophils were prepared using the Histopaque method. Briefly, one part fresh heparinised blood was mixed with 4 parts 6% dextran in 0.9% NaCl and sedimented for 25-40 min at room temperature. The neutrophil-rich upper layer was collected and layered unto cold Histopaque

before centrifugation at 150 x g for 30 min. After discarding the upper two layers the neutrophil cell pellet was washed in HBSS at 150 x g for 5 min. RBC were lysed by the addition of 9 parts ice cold distilled water for a few seconds, followed by 1 part x10 PBS. After a further wash in HBSS, neutrophils were counted in a haemocytometer and resuspended in mHBSS to give the desired concentration.

2.3.4 Preparation of washed blood

In the majority of flow cytometry experiments, plasma was removed from whole blood to prevent any opsonic neutrophil responses to bacteria or LPS. Fresh heparinised blood from healthy volunteers was transferred to Eppendorf tubes and spun at 6500 rpm for 30 sec using a Micro Centaur microfuge (MSE, Loughborough, UK). Following the removal of plasma the cell pellet was washed 4 times in HBSS. Finally the cell pellet was resuspended in a volume of mHBSS equivalent to the initial volume of plasma removed. The resultant 'washed' blood was kept on ice until use.

2.4 STUDIES OF BACTERIA:PHAGOCYTE INTERACTIONS

2.4.1 Washing assay for intracellular survival in monocytes

Intracellular survival of bacteria in monocytes was estimated using an adaptation of the method utilised by Pruksachartvuthi et al (1990) to demonstrate intracellular survival of *B. pseudomallei* in phagocytic cells. Monocytes were prepared according

to the method outlined in section 2.3.1 above and were resuspended at a concentration of 5×10^6 cells/ml in RPMI 1640. Overnight cultures of bacteria in NYB were resuspended in RPMI 1640 at a concentration of 5×10^8 cfu/ml. Reaction mixtures were prepared in Eppendorf tubes and consisted of 0.5 ml monocyte suspension, 0.1 ml bacterial suspension, 0.1 ml FCS and 0.3 ml RPMI 1640. After 20 min incubation in a waterbath at 37°C, phagocytosis was stopped by centrifugation at 6500 rpm in an MSE Microcentaur for 2 min and the resuspension of the cell pellet in 1 ml ice cold RPMI. Monocytes were washed 5 times in ice cold RPMI to remove extracellular bacteria before resuspension in 1 ml RPMI + 10% FCS and incubation at 37°C with gentle agitation for 24 hours. Bacterial viability in RPMI + 10% FCS was assessed concurrently. At intervals samples were removed and 0.1 ml aliquots mixed with 0.9 ml ice cold distilled water. After vigorous vortexing to disrupt monocytes, viable bacteria were counted by serial dilution of the monocyte lysate. Of the remaining sample, 0.5 ml was removed and fixed with 0.5 ml 2.5% glutaraldehyde (v/v) for electronmicroscopy. After 1 hour, fixed preparations were washed twice in sodium cacodylate buffer and refrigerated overnight before electronmicroscopy.

2.4.2 Uptake of bacteria by monocytes using acridine orange/crystal violet staining

J774.2 cells or human monocytes from fresh whole blood were prepared using the methods outlined in section 2.3.1 above and resuspended in DMEM or Eagles medium respectively. Overnight cultures of bacteria in NYB were washed once and resuspended in PBS at a concentration of 5×10^8 cfu/ml, as determined by the OD₅₂₅.

Bacterial concentrations were adjusted to give a final concentration approximately 100 times the concentration of J774.2 / monocyte suspensions. In a 1.5 ml Eppendorf tube, 50 μ l pooled human serum, 150 μ l cell suspension, 150 μ l bacterial suspension and 50 μ l Acridine Orange solution (AO; Pro-Lab Diagnostics, Bromborough, Merseyside) were mixed and incubated at 37°C for 20 min with gentle shaking. A 50 μ l aliquot was removed from each sample and added to 90 μ l cold 0.2% EDTA in PBS to prevent further phagocytosis. After addition of 10 μ l saturated crystal violet solution (CV) for 2 min at room temperature, each sample was washed once in PBS and, after brief centrifugation at 6500 rpm (MSE Microcentaur), the supernatant discarded. The resulting cell deposits were transferred to a glass slide by cytospin and stained for a further 30s with AO at room temperature before washing in PBS. Finally the stained deposit was covered with a glass coverslip and sealed with clear nail varnish. Samples were viewed under incident light fluorescent microscopy and the number of organisms per monocyte counted for at least 100 cells. Final results were calculated as the average number of bacteria per monocyte.

To investigate the effect of bacterial viability on the colour of AO staining, studies were conducted on live and killed bacteria in the absence of monocytes. Bacteria were prepared as above and resuspended spectrophotometrically at a concentration of 5×10^8 cfu/ml. Aliquots were killed either by boiling at 100°C for 5 min or by exposure to short-wave UV irradiation for 2.5 min at close range. For each condition, 350 μ l bacterial suspension was incubated with 50 μ l AO at room temperature for 2 min before washing in PBS and centrifugation at 6500 rpm (MSE

Microcentaur) to pellet the bacterial cells. Deposits were cytospun onto glass slides and covered with glass coverslips before sealing with clear nail varnish and viewing under incident light fluorescence microscopy.

2.4.3 Killing of bacteria by neutrophils

Killing assays were carried out using neutrophils prepared from fresh whole blood according to the plasma-Percoll method described in section 2.3.2 above.

Neutrophils were resuspended in PBS at a concentration of 1×10^7 cells/ml.

Bacteria were grown overnight in NYB at 37°C, washed in PBS at 2500 x g for 15 min and finally resuspended in PBS at a concentration of 2.5×10^7 cfu/ml. Assays were carried out in triplicate in 96 well microtitre plates. To each well, 100 µl neutrophil suspension, 100 µl bacterial suspension, 5 µl 50 mM CaCl₂ solution and 5 µl 50 mM MgSO₄ solution were added. Control wells were identical except that the neutrophil suspension was replaced with PBS alone. Plates were incubated at 37°C with shaking. 20 µl aliquots were removed from each well at times 0, 15, 30, 60 and 120 min and added to 1980 µl ice cold distilled water. Samples were kept on ice for 10 min before vigorous vortexing to ensure adequate disruption of neutrophils. Bacterial survival was assessed by viable counting.

In initial experiments, killing of non-opsonised bacteria was compared with killing of bacteria opsonised with PHS or with autologous serum from the neutrophil preparation. Pre-opsonisation was carried out as described in section 2.2.7. Pre-opsonisation with PHS gave the greatest degree and consistency of bacterial killing and was used as standard in further killing assays unless otherwise stated.

2.4.4 Chemiluminescence assay of neutrophil respiratory burst activity

For chemiluminescence experiments neutrophils were prepared according to the plasma-Percoll method described in section 2.3.2. Cells were resuspended in mHBSS prior to use. Unless otherwise stated, bacteria were grown overnight in NYB at 37°C in an orbital incubator. After centrifugation at 1500 x g for 20 min, without braking, cultures were resuspended in mHBSS and adjusted spectrophotometrically to a final concentration of 5×10^8 cfu/ml before use. Where required, bacteria were pre-opsonised with 10% heat-inactivated serum or 4% GPC as described in section 2.2.7. Non-opsonised and opsonised zymosan were used as positive controls for chemiluminescence production. Initial studies indicated that a concentration of 6 mg/ml zymosan gave adequate results. Zymosan was opsonised by incubation with pooled human serum for 15 min at 37°C before washing in HBSS and resuspension in mHBSS at the appropriate concentration.

Studies were carried out in triplicate in 8 or 12 well luminostrips (LabSystems). Luminol, stored as a 0.1 M solution in DMSO at -20°C, was diluted to a 1 mM solution in mHBSS prior to use. Luminostrips were loaded in triplicate with 100 µl neutrophil suspension, 100 µl bacterial suspension and 100 µl luminol solution per well. Neutrophils were added last, immediately prior to incubation of luminostrips in the LabSystems luminometer, prewarmed to 37°C. Temperature was maintained at 37°C and light production measured at 5 min intervals for at least 90 min. Initial experiments indicated that a neutrophil concentration of 2.5×10^6 cells/ml (i.e. 2.5×10^5 cells/well) gave optimum light production. Bacteria alone produced no chemiluminescence response.

Studies using lucigenin rather than luminol chemiluminescence were similar except that lucigenin was diluted from a 0.1 M stock solution in DMSO to a 30 mM solution in mHBSS prior to use. In some experiments the inhibitors superoxide dismutase (SOD) and sodium azide (NaN_3) were used. SOD was resuspended in distilled water at a concentration of 1100 u/ml and stored at -20°C in 100 μl aliquots. NaN_3 was prepared as a 30 mM solution and stored at room temperature. In all experiments involving inhibitors, 10 μl SOD or 10 μl NaN_3 were added to luminostrips just prior to addition of neutrophil suspensions and incubation in the luminometer.

2.4.5 Cytochrome c reduction assay for superoxide production

Neutrophils were prepared using the Histopaque method and resuspended in PBS with 0.1% glucose (mPBS) at a concentration of 5×10^6 cells/ml. A solution of cytochrome c in mPBS at 10 mg/ml was prepared immediately prior to use. Experiments were carried out in duplicate in Eppendorf tubes, using an initial aliquot of 0.2 mls neutrophil suspension to which was added 80 μl cytochrome c and the required volume of stimulant in mPBS. For positive controls, 80 μl zymosan, previously opsonised with 30% AB+ serum for 15 min at 37°C and resuspended at a concentration of 10 mg/ml in mPBS, was used as the stimulant. For negative controls, 80 μl SOD (2000 u/ml) was added to the reaction mixture in addition to the zymosan suspension. The final volume for both samples and controls was made up to 0.8 mls with mPBS and the resulting reaction mixture incubated at 37°C with shaking for the desired time period. Reactions were stopped by transfer onto ice,

followed immediately by centrifugation at 5°C for 15 min at 400 x g to pellet the neutrophils. Supernatants were carefully removed and the OD₅₅₀ read spectrophotometrically. Extracellular O₂⁻ concentration was calculated using the following equation:

$$\text{O}_2^- \text{ nmol} / 10^6 \text{ cells} / t \text{ min} = (\text{Sample OD}_{550} - \text{Negative control OD}_{550}) \times 68$$

where t is the time point at which the reaction was stopped.

Stimulants used to assess O₂⁻ release included PMA (final concentration: 200 ng/ml) or bacteria. In the majority of experiments bacteria were resuspended at a concentration of 5 x 10⁸ cfu/ml and added to the reaction mixture to give a bacteria:neutrophil ratio of 100:1. However in some experiments varying amounts of bacterial suspensions were added to the reaction mixtures to assess the effect of bacterial concentration on respiratory burst induction. Where required, bacteria were pre-opsonised with heat-inactivated serum using the method outlined in section 2.2.7.

An attempt was made to develop a microtitre plate assay for cytochrome c reduction by extracellular O₂⁻. Briefly the assay conditions described above were reproduced in 200 µl volumes in triplicate within microtitre plates. After incubation at 37°C in a plate warmer for the desired period of time, reactions were stopped by centrifugation at 5°C for 15 min at 400 x g and 100 µl volumes of supernatant carefully transferred into ELISA plates for analysis. Optical density was read using a microtitre plate reader (Tmax Plate Reader, Molecular Devices) , and calculated automatically as the OD₅₅₀ - OD₆₅₀, to correct for any defects within the ELISA plate itself. In additional

experiments, reaction mixtures were prepared as for the standard cytochrome c method, but after the centrifugation step, supernatants were transferred to ELISA plates, in triplicate, for reading of the $OD_{550} - OD_{650}$.

2.4.6 Flow cytometric analysis of neutrophil surface marker expression

Flow cytometric analysis of neutrophil CR3 (CD18/CD11b) expression was carried out on purified neutrophils or on neutrophils within whole or washed blood as stated in the results section. For experiments involving purified cells, neutrophils were resuspended in mHBSS at a concentration of 5×10^6 cells/ml prior to use. In time course experiments, neutrophils / blood samples were kept on ice until use. In experiments involving whole bacteria, overnight cultures in NYB were spun down at $1500 \times g$ without braking, washed once in HBSS and resuspended at a concentration 1×10^9 cfu/ml, using the OD_{590} , before dilution to the desired concentration in mHBSS.

All experiments were carried out using 100 μ l aliquots of blood or neutrophils in 5 ml polystyrene tubes (Greiner, Stonehouse, Glouc., UK), to which was added an equal volume of mHBSS or neutrophil stimulant such as whole bacteria, LPS or haemolysin diluted in mHBSS. The tubes were incubated for the desired time period at 37°C with gentle shaking. Reactions were stopped by transferring the sample tubes onto ice. After the addition of anti-CD11b:FITC (5 μ l) or mouse isotype control antibody (5 μ l) to each sample, the tubes were mixed and incubated for 20 min on ice. Then red blood cells (RBC) were lysed by the addition of 2 ml Lysing Solution for 30 min. Neutrophils were pelleted by centrifugation at $400 \times g$, washed

once in PBS and resuspended in 500 μ l CellFIX. Samples were stored at 4°C until flow cytometric analysis within 24 hours. Neutrophils were gated on forward and side scatter characteristics using CellQuest 1.2.2 software. CD11b expression was determined as the mean channel fluorescence (MCF) of the neutrophil population using the green channel (FL1). As considerable inter-donor variation in the magnitude of neutrophil responses was noted, MCF values were standardised by expression as a percentage of the MCF of neutrophils treated with buffer alone.

2.4.7 Intracellular H₂O₂ production by neutrophils

Neutrophils, bacteria and LPS were prepared as for assessment of CD11b expression. Intracellular H₂O₂ production was assessed using the fluorescent dye, dihydrorhodamine (DHR), diluted from the stock solution 1 in 50 times in a 20% solution of DMSO in mHBSS, before addition to reaction tubes to give a final concentration of 30 mM DHR (10 μ l per 200 μ l reaction mixture). Tubes were incubated at 37°C with gentle shaking for the desired period of time. All experiments were carried out on an aliquot of 100 μ l blood, to which was added an equal volume of stimulant. Reactions were stopped by transferring the sample tubes onto ice, RBC were lysed by the addition of 2 ml Lysing Solution for 30 min and the remaining neutrophils processed as above. As H₂O₂ oxidises DHR to fluorescent rhodamine, intracellular H₂O₂ production was determined as the MCF of the neutrophil population using the red channel (FL2). Results were analysed using CellQuest and, where required, standardised by expression as a percentage of MCF of neutrophils stimulated with buffer alone.

In experiments investigating the priming of neutrophil respiratory burst responses by LPS or *B. cepacia* haemolysin, 100 μ l samples of washed blood were pre-incubated with 100 μ l priming agent at the required concentration for the desired period of time. In the majority of experiments, priming was assessed using the neutrophil stimulant fMet-Leu-Phe (FMLP). A 10^{-4} M stock solution of FMLP in HBSS was prepared from a 10^{-3} M solution in DMSO, and was stored at -20°C . Immediately before use FMLP was further diluted in mHBSS to give a 10^{-6} M solution. 200 μ l of FMLP or mHBSS were added to each reaction tube along with 30 mM DHR for a further 15 min at 37°C . Neutrophil activation was arrested by transfer of the tubes onto ice and lysis of RBC carried out using 2.4 ml FACS Lysing solution per tube before proceeding as above.

In experiments measuring priming induced by whole bacteria a similar procedure was followed except that 100 μ l washed blood was incubated with 100 μ l bacterial suspension in the presence of 30 mM DHR before the addition of 10 μ l FMLP in mHBSS at a concentration of 10^{-5} M to give a final concentration of 5×10^{-7} M. In experiments measuring the priming of responses to whole bacteria by LPS, bacteria were resuspended in mHBSS at a concentration of 1×10^8 M and 200 μ l added to each tube, along with 30 mM DHR, after pre-incubation with LPS or mHBSS. After 30 min at 37°C with gentle shaking, reactions were stopped and samples prepared as above.

2.5 STUDIES IN THE EDINBURGH CF MOUSE MODEL

Experiments involving Swiss mice were carried out in collaboration with Dr G McLachlan, MRC HGU, Western General Hospital, Edinburgh. Otherwise all studies were carried out in collaboration with Dr DJ Davidson, also from the MRC HGU.

2.5.1 Bacterial inoculation of mice

Bacteria for inoculation were cultured overnight in NYB, spun down at 2500 x g for 15 min and resuspended in PBS or normal saline before use. Mice were anaesthetised by intra-peritoneal (i.p.) injection of 0.6 ml 'Avertin', prepared by dissolving 2.5g tri-bromo-ethanol in 5 ml 2-methyl-2-butanol (tertiary amyl alcohol) and adding 200 ml distilled water. In the majority of experiments, intubation was carried out non-surgically using a modified Terumo spinal needle (25g x 3.5").

Mice were inoculated with a 25 µl volume of bacterial suspension or, in initial experiments, with 25 µl of methylene blue dye which allowed direct visualisation of administration to the lungs. In studies of the delivery method, mice were killed after 5 min and before recovery from anaesthesia by i.p. injection of 0.5 ml Sagatal (Rhone-Merieux, Harlow, Essex). In studies of bacterial clearance mice were returned to their cages and allowed to recover from anaesthesia before transfer to isolators within the animal facility.

In some studies, intubation was carried out surgically as follows. A mid-line incision was placed over the trachea of an anaesthetised mouse and the muscles of

the throat gently parted to expose the underlying trachea. A specially adapted needle was passed into the trachea and 25 μ l of bacterial suspension inoculated as above. In all such studies mice were killed by i.p. injection of Sagatal after 5 min and before recovery from anaesthesia.

2.5.2. Preparation of mice lungs

Mice were killed by i.p. injection of 0.5 ml Sagatal. Death was confirmed by opening of the peritoneal cavity and dissection of the aorta. A mid line incision was extended from the peritoneum to the throat, exposing the rib cage and muscles of the throat. Next the sternum was dissected and the throat muscles parted to reveal the underlying trachea. A sterile cord was passed under the trachea but above the oesophagus and tied in a knot around the trachea to prevent contamination with upper respiratory tract organisms. The trachea was cut above the knot and the lungs removed by gently pulling the trachea up and forward whilst cutting any connections of the lung to the underlying rib cage. In the majority of cases this procedure resulted in the clean removal of the whole lungs.

In some experiments the mouse oesophagus and stomach were also removed after the removal of the lungs. Briefly the oesophagus was cut at its upper end and gently peeled from the mediastinal tissue. The stomach was then lifted at its junction with the oesophagus and removed by applying gentle pressure while cutting any soft tissue connections.

Lung lavage was performed prior to lung removal in some experiments. The trachea was exposed and tied off as above and pierced with a Terumo Surflo i.v. catheter. 1 ml of PBS was infused using a 1 ml sterile syringe, withdrawn and infused again, and after a few seconds withdrawn again using the same syringe. Recovery of 0.6-0.8 ml lavage per 1 ml infused was generally obtained. Removal of lungs proceeded as normal after the lavage process.

Lungs and stomach were placed in sterile plastic bijoux along with 1 ml of PBS, and specimens kept at 4°C before processing within a 2-3 hour period. Specimens were homogenised using a hand-held homogeniser (OMNI TH Homogeniser, Camlab, Cambridge) and bacterial counts determined by serial dilution in saline and plating onto cepacia medium. Lavage specimens were processed identically except that no homogenisation step was required. Plates were incubated at 37°C for 48 hours before counting.

2.6 STATISTICAL ANALYSIS

Statistical analysis was carried out with the aid of Excel v5.0 (Microsoft). The Students t test was used for the comparison of the means of two samples, where a combined variance for the two samples was derived according to the following formula:

$$t = \frac{\sum (x_1 - \bar{x}_1)^2 + \sum (x_2 - \bar{x}_2)^2}{(n_1 - 1) + (n_2 - 1)}$$

Fisher's exact test, the χ squared test (with Yates correction), and rank correlation were carried out as described in Swinscow (1987). For analysis of variance (ANOVA) raw data were first transformed by conversion to Log_{10} . Mean values (\bar{x}) for each experimental conditions were used to calculate the variance of the means (SD^2) and t calculated according to the equation:

$$t = \frac{Z}{\sqrt{SD^2 \left(\frac{l_1^2}{n_1} + \frac{l_2^2}{n_2} + \dots + \frac{l_n^2}{n_n} \right)}}$$

where n is the number of observations per test condition, and Z is the contrast, derived from the equation:

$$Z = l_1 \bar{x}_1 + l_2 \bar{x}_2 + \dots + l_n \bar{x}_n$$

Values for $\{l_1, l_2, \dots, l_n\}$ were chosen according to the desired comparison to be made by the test. For example, in flow cytometric analyses of neutrophil priming by *B. cepacia*, *P. aeruginosa* and *E. coli* LPS, three *P. aeruginosa* strains were compared with 5 *B. cepacia* strains. For each *P. aeruginosa* strain, $l = -\frac{1}{3}$, for each *B. cepacia* strain, $l = \frac{1}{5}$ and for *E. coli* O18K- and untreated neutrophils, $l = 0$.

CHAPTER 3 CHARACTERISTICS OF CLINICAL AND ENVIRONMENTAL *B. CEPACIA*

3.1 PRODUCTION OF EXTRACELLULAR TOXINS

A representative panel of clinical and environmental strains of *B. cepacia* was assessed for protease, elastase and lipase activity. Clinical isolates consisted of 4 CF and 2 non-CF strains and included a representative of the ET12 epidemic lineage. Environmental isolates included 11 strains cultured from various sites within the Royal Botanic Gardens, Edinburgh (Butler et al, 1995) and representative strains from the CF Laboratory collection. No *B. cepacia* strain produced elastase activity at either 30°C or 37°C. Protease and lipase activity were observed more frequently at 30°C (Table 3.1). Protease activity was detectable in more clinical than environmental strains but this difference was not statistically significant at either 30°C or 37°C. Lipase activity was more frequent among environmental strains, particularly at 30°C ($p < 0.02$, Fisher's exact test).

The same panel of strains was tested for haemolytic activity on horse blood agar (Table 3.2). Haemolytic environmental strains produced either α or β haemolysis whilst all haemolytic clinical strains produced α haemolysis (Data not shown). Statistical analysis indicated that significantly more environmental strains displayed haemolytic activity at 30°C than at 37°C ($p < 0.05$, Fisher's exact test). Furthermore, fewer clinical strains than environmental produced activity at 30°C ($p < 0.02$, Fisher's exact test).

Table 3.1 Protease and lipase activity of environmental and clinical *B. cepacia*¹.

	Protease		Lipase	
	30°C	37°C	30°C ²	37°C
Environmental	63	56	100	81
Clinical	83	67	50	50

¹Results are expressed as the percentage of positive strains out of 15 environmental and 6 clinical strains

²Difference between clinical and environmental strains significant, ($p < 0.02$, Fisher's exact probability test).

Table 3.2 Haemolytic activity of clinical and environmental *B. cepacia* strains on horse blood and human blood agar¹.

	Environmental		Clinical	
	30°C	37°C	30°C	37°C
Blood				
Horse ²	81	44	17	32
Human	15	0	5	9

¹Results are the percentage of positive strains from 15 environmental and 6 clinical strains for horse blood, and from 13 environmental and 22 clinical strains for human blood

² Environmental strains at 30°C significantly different from the same strains at 37°C ($p < 0.05$, Fisher's exact test) and from clinical strains at 30°C ($p < 0.02$, Fisher's exact test)

Further studies were undertaken using human blood agar (Table 3.2). For both environmental and clinical strains levels of haemolytic activity against human blood were less than against horse blood, although the same overall trends could be observed. In general, clinical strains showed greater activity at 37°C whilst environmental strains showed greater activity at 30°C. All haemolytic environmental strains and one non-CF clinical strain showed β haemolysis; however the single

haemolytic CF strain, the Edinburgh epidemic strain, J2315, only demonstrated α haemolysis after at least 48 hours of incubation.

Catalase activity was assessed in a panel of 66 environmental and clinical strains grown at 37°C on cepacia medium. Only one environmental strain displayed no activity (Table 3.3). For the remaining strains results were classified on the basis of slow or brisk activity. CF strains demonstrated the greatest degree of catalase activity, environmental strains demonstrated intermediate levels and non-CF clinical strains demonstrated the least activity. Differences between environmental strains and all clinical strains, or CF or non-CF strains alone were not significant. However comparison of non-CF and CF strains found a significant difference between the two clinical groups ($p < 0.05$, Fisher's exact test).

Table 3.3 Catalase activity of environmental and clinical *B. cepacia*¹

	None	Slow	Brisk
Environmental	3	52	45
Non-CF clinical ²	0	73	27
CF ²	0	35	65

¹Results are the percentage of positive strains from 31 environmental strains, 15 non-CF clinical strains and 20 CF strains

²Significantly different, $p < 0.05$ (Fisher's exact test)

3. 2 EFFECT OF IRON ON GROWTH OF *B. CEPACIA*

Four *B. cepacia* strains, comprising the environmental strains J2511 and J2552, and the CF strains J2315 and C1524, were grown in iron-deficient (Fe-) or iron-rich (Fe+) minimal media, with or without transferrin (TF). Fig. 1.1 shows representative growth curves. Strains C1524, J2511 and J2552 displayed vigorous growth reaching steady state levels by 24 hours for C1524 and 48 hours for the two environmental strains. However in both Fe+ and Fe- conditions, J2552 and J2511 displayed an initial drop in viable counts, recovering rapidly between 12 and 24 hours. For C1524, growth was unaffected by iron concentration whilst for J2552 and J2511 growth in Fe- media was associated with a slight decrease in steady state viable counts. Growth of J2315 was associated with a long lag phase of approximately 36 hours followed by a slow rise in counts to reach a steady state by 84 hours. Iron-deficient conditions supported slightly faster growth than Fe+ conditions but final viable counts were similar in both media.

Addition of 200 µg/ml TF had no effect on the growth of C1524 in either media. For both environmental strains, growth in the presence of TF prevented the initial fall in bacterial numbers observed in TF-free media. However, the advantage gained in the presence of TF did not persist beyond 48 hours for Fe+ media and 24 hours for Fe- media. Indeed, ultimately the presence of TF was associated with a slight decrease in steady state bacterial counts. For J2315, the inclusion of TF in either Fe+ or Fe- media led to a decrease in bacterial growth. Thus for Fe+ media, bacterial levels after 132 hours were 59% of the levels in TF-free media and for Fe- media, only 3% of the levels obtained in TF-free media.

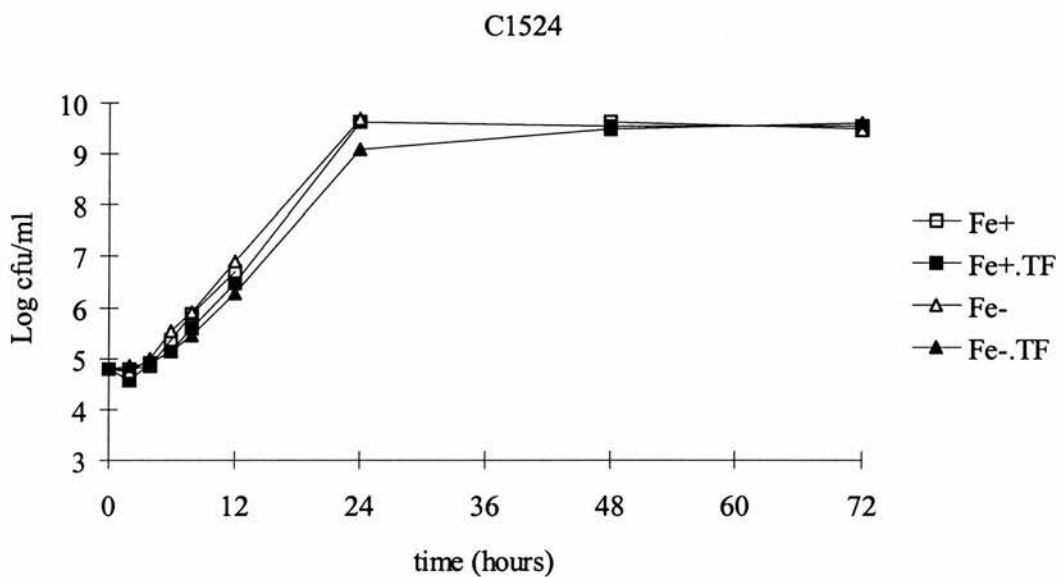
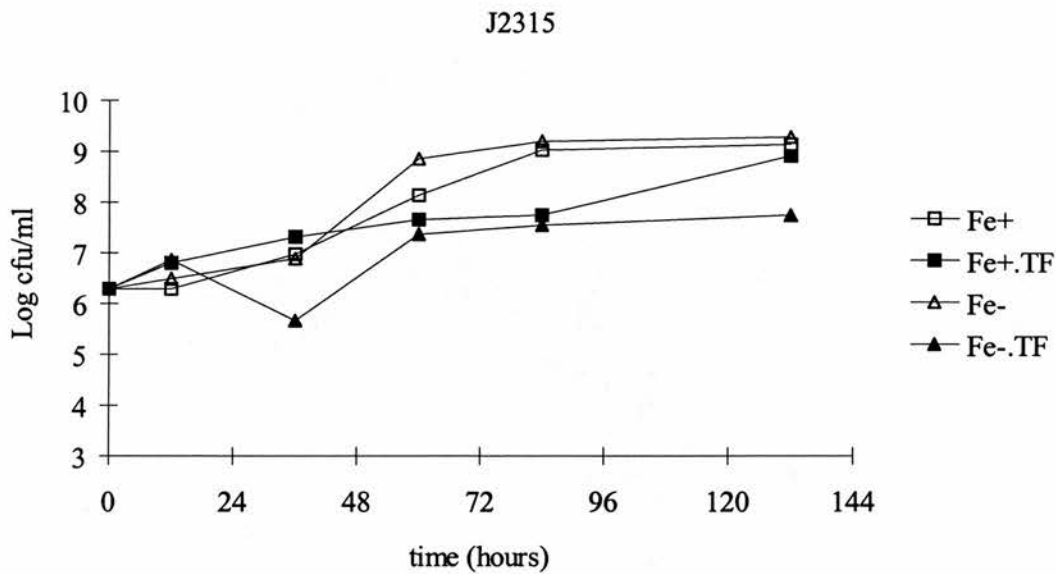


Fig 1.1a Growth of *B. cepacia* J2315 and C1524 in iron rich (Fe+) and iron poor (Fe-) minimal media with or without 200 µg/ml transferrin (TF).

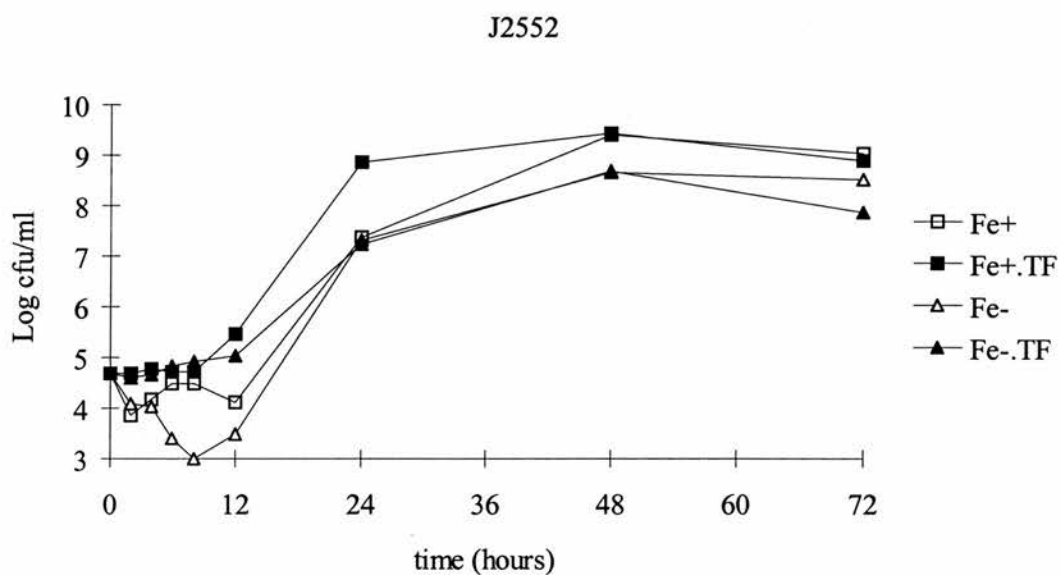
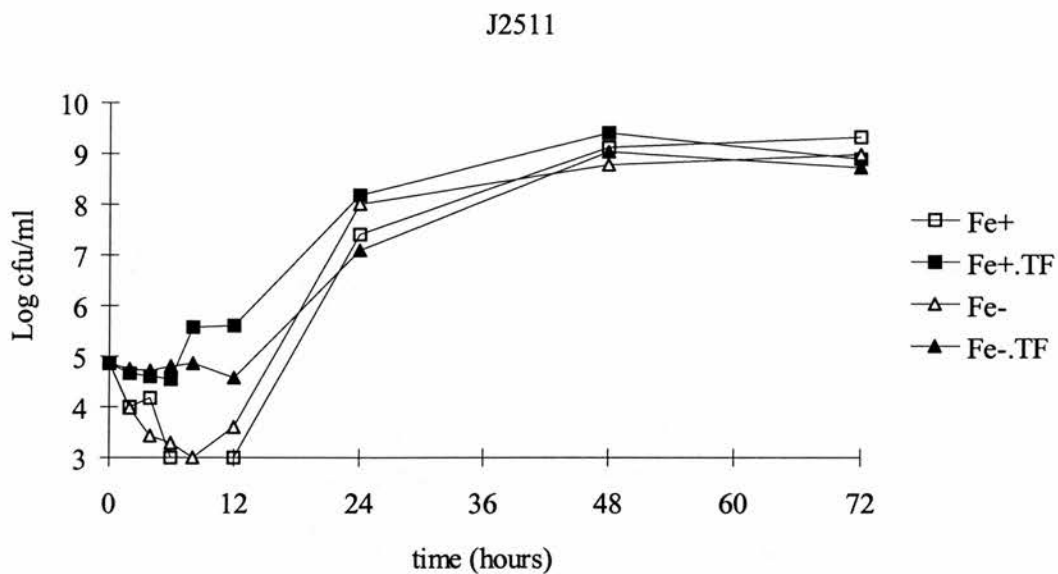


Fig 1.1b Growth of *B. cepacia* J2511 and J2552 in iron rich (Fe+) and iron poor (Fe-) minimal media with or without 200 µg/ml transferrin (TF).

CHAPTER 4 INTRACELLULARITY OF *B. CEPACIA*

4.1 UPTAKE BY HUMAN MONOCYTES

Intracellular uptake of environmental and clinical *B. cepacia* strains by human monocytes was compared using *P. aeruginosa* and *Listeria monocytogenes* as negative and positive control organisms respectively. Uptake was determined using acridine orange (AO) to stain intra- and extracellular bacteria before quenching extracellular fluorescence with crystal violet (CV) solution. AO is believed to produce green fluorescence in live bacteria but red fluorescence in dead bacteria; thus it was hoped that the AO/CV technique would allow estimation not only of uptake but also killing of bacteria by monocytes (West, 1969). In preliminary experiments, live bacteria were observed to stain a bright green colour. However after heat- or UV-killing, the majority of bacteria still maintained a bright green coloration. Furthermore, those bacteria which showed colour changes in response to heat or UV killing displayed an orange-green coloration rather than bright red.

Uptake assays were performed using either the monocyte/macrophage murine cell line J774.2 or purified human monocytes. Bacteria and cells in a ratio of 100 to 1 were co-incubated for 20 min in the presence of AO, before further AO staining and counter-staining with CV. Stained preparations were viewed under indirect fluorescence microscopy and bacterial numbers counted for at least 100 monocytes per slide. Uptake of both *L. monocytogenes* and *B. cepacia* could be demonstrated within J774.2 cells and human monocytes. However within any one experiment, most cells contained few if any organisms whilst a minority of cells were observed to

take up a large number of bacteria. Overall uptake was greater using human monocytes; further studies of *B. cepacia* and *P. aeruginosa* were therefore carried out using human monocytes only. Table 4.1 summarises results from three experiments. In all instances uptake of *L. monocytogenes* was greater than uptake of *P. aeruginosa* PAO1 or *B. cepacia* J2315 and J2552. In general uptake of *P. aeruginosa* appeared to be greater than uptake of both J2315 and J2552 but this difference was not significant. Virtually all intracellular *P. aeruginosa* and *B. cepacia* appeared orange/green. By contrast, the majority of *L. monocytogenes* were bright red, indicating bacterial cell death, although a few green organisms were also observed suggesting survival of a minority of ingested *L. monocytogenes*.

Table 4.1 Intracellular uptake of bacteria by human monocytes, including number of bacteria per monocyte and colour changes on AO staining.

Strain	N° bacteria per monocyte			Colour
	Exp. 1	Exp. 2	Exp. 3	
PAO1	6.4	3.01	13.1	orange/green
J2315	1	3.18	5.1	orange/green
J2552	3.9	1.84	4.6	orange/green
<i>L. monocytogenes</i>	18.3	11.87	>50	red, occasional green

4.2 SURVIVAL OF INTRACELLULAR BACTERIA

Monocytes pre-incubated with bacteria for 20 min at 37°C were repeatedly washed to remove extracellular bacteria before further incubation at 37°C. Intracellular bacterial survival was assessed at intervals by lysis of monocyte preparations and counting of surviving bacteria. Additional samples were simultaneously fixed for electronmicroscopy. Results from three experiments are summarised in Table 4.2.

Table 4.2 Intracellular survival of *L. monocytogenes*, *B. cepacia* J2315 and *P. aeruginosa* PAO1 in monocytes.

Time (hours)	Log % of colony forming units at time t=0 ¹			
	1 ²	6	19	24
<i>L. monocytogenes</i>	2.14		4.72 ± 0.39	3.44
<i>P. aeruginosa</i>	0.72 ± 0.16	1.51 ± 1.02	4.08 ± 0.08	3.32 ± 0.13
<i>B. cepacia</i>	1.79 ± 0.24	1.97 ± 0.45	3.10 ± 0.11	3.09 ± 0.11

¹Results are expressed as mean of three experiments ± SEM

²Difference between *P. aeruginosa* and *B. cepacia* significant; p<0.05 (Students t test)

The intracellular pathogen *L. monocytogenes* was included as a positive control and reached maximal levels after 19 hours of incubation when at least a 500-fold increase in viable counts was observed (Table 4.2). Unlike *L. monocytogenes*, both *P. aeruginosa* and *B. cepacia* showed an initial decrease in viable counts at time t=1. Although this decrease was significantly greater for *P. aeruginosa* than *B. cepacia* (p<0.05, Students t test), *P. aeruginosa* recovered to higher levels than *B. cepacia* by

19 hours incubation. By 24 hours incubation viable counts for both *P. aeruginosa* and *L. monocytogenes* began to decrease, whilst levels for *B. cepacia* continued to rise slowly. Monocyte viability was assessed at various time points using the trypan blue exclusion test. For all experiments >95% of monocytes were viable after one hour of incubation. For both *P. aeruginosa* and *B. cepacia*, total monocyte counts had dropped by 50% after 24 hours. Remaining monocytes were >95% and 72% viable for *P. aeruginosa*- and *B. cepacia*-treated preparations respectively. By contrast, *L. monocytogenes* was associated with only 6% monocyte viability after 19 hours and no monocyte survival after 24 hours.

Electronmicroscopy of monocyte preparations pre-incubated with *B. cepacia* or *P. aeruginosa* failed to show any evidence of intracellular bacterial growth as bacteria could not be identified in these preparations.

CHAPTER 5 *B. CEPACIA* AND NEUTROPHILS: BACTERIAL KILLING AND EFFECTS ON THE NEUTROPHIL CELL SURFACE

5.1 BACTERIAL KILLING BY NEUTROPHILS

Four *B. cepacia* strains were investigated in neutrophil killing assays. Bacteria were pre-incubated in 10% pooled human serum (PHS) prior to addition to neutrophils in a ratio of approximately 2:1. Control bacteria were pre-incubated in 10% PHS before addition to buffer alone. Samples were removed at intervals from neutrophil and control preparations and bacterial survival analysed by neutrophil lysis and viable counts (Fig 5.1). For strains C1524, J2511 and J2552, a drop in viable counts of 20%, 44% and 15% respectively was observed in controls over a two hour incubation period. For the environmental strains J2511 and J2552, little or no difference was observed between neutrophil and control preparations, suggesting that no neutrophil-mediated killing was taking place. By contrast, the CF strain C1524 showed a marked increase in viable counts in the presence of neutrophils, rising to 182% initial levels after 120 min. Although results for the Edinburgh epidemic strain, J2315, varied from experiment to experiment, a third distinct pattern could be observed. Control counts rose initially to an average of 138% starting levels by 15 min, followed by a drop to 100% by 1 hour and 93% by 2 hours. The initial rapid rise in levels suggested dissociation of clumped bacteria, rather than bacterial cell growth *per se*. J2315 incubated with neutrophils were observed to decrease in number, reaching an average of 73% of initial counts by 30 min before a gradual increase in numbers towards an average count of 83% at 120 min.

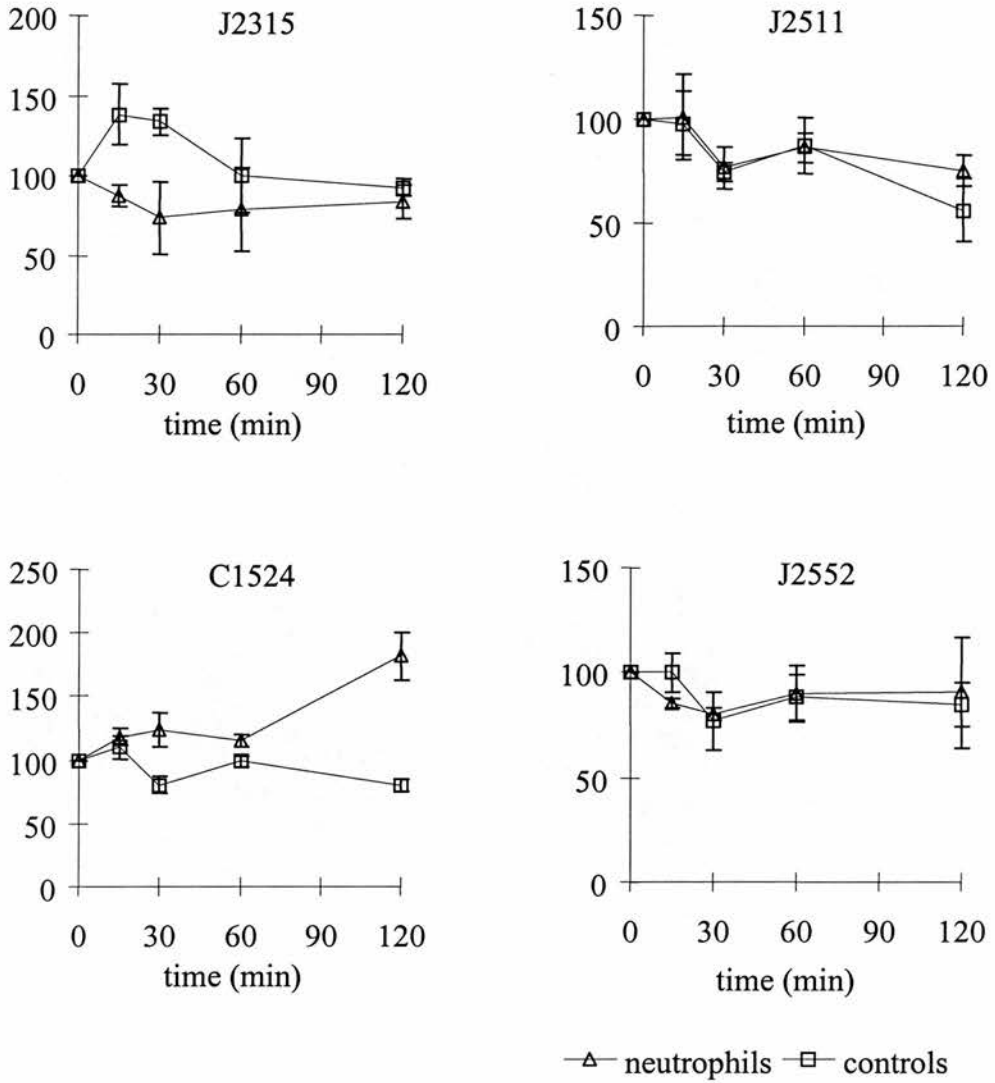


Fig 5.1 Survival of 4 strains of *B. cepacia* in the presence of neutrophils. Results are expressed as a percentage of cfu/ml at time $t=0$ and are the mean of at least three experiments \pm SEM. Controls are bacteria incubated in buffer alone.

Further experiments focused on J2315, and investigated the impact of opsonisation with immune serum (IS) from *B. cepacia*+ CF patients on neutrophil-mediated bacterial killing. In all studies, opsonisation of J2315 with IS resulted in a greater decrease in bacterial cfu/ml on incubation with neutrophils than no opsonisation or opsonisation with non-immune serum. However in neutrophil-free control samples, IS was associated with a greater drop in cfu/ml than in neutrophil-containing samples (Data not shown). As this effect was observed using both heat-inactivated and normal serum samples, it was concluded that the drop in counts represented bacterial clumping rather than actual killing of bacteria and further studies were discontinued.

5.2 FLOW CYTOMETRIC ANALYSES OF NEUTROPHIL: BACTERIA INTERACTIONS

5.2.1 Comparison of neutrophil preparation techniques on neutrophil:bacteria interactions

Initial studies were carried out to assess the impact of neutrophil preparation techniques on size, granularity and expression of the cell surface marker complement receptor 3 (CR3, CD18/CD11b). Neutrophils were gated according to forward scatter (FSC) and side scatter (SSC) characteristics, corresponding to size and granularity respectively (Fig 5.2). Both neutrophil purification methods used; namely, Histopaque density gradient separation and plasma-Percoll separation; were associated with an increase in FSC and a decrease in SSC for the neutrophil population, although these changes were most marked for the plasma-Percoll method. By contrast, a whole blood cell preparation from which plasma had been removed by repeated washing ('washed blood') demonstrated identical FSC and SSC characteristics as untreated whole blood.

CR3 expression was determined using mouse anti-human CD11b monoclonal antibodies conjugated to FITC (Anti-CD11b:FITC), with CR3 levels expressed as the mean channel fluorescence (MCF) of the neutrophil population. Comparison of MCF values showed that both neutrophil purification techniques were associated with a drop in CR3 expression compared to untreated blood, a finding which was surprising given that slight degrees of neutrophil activation during separation procedures are normally associated with increases in CR3 expression. It is possible, however, that the results reflect an increase in CR3 expression on neutrophils in

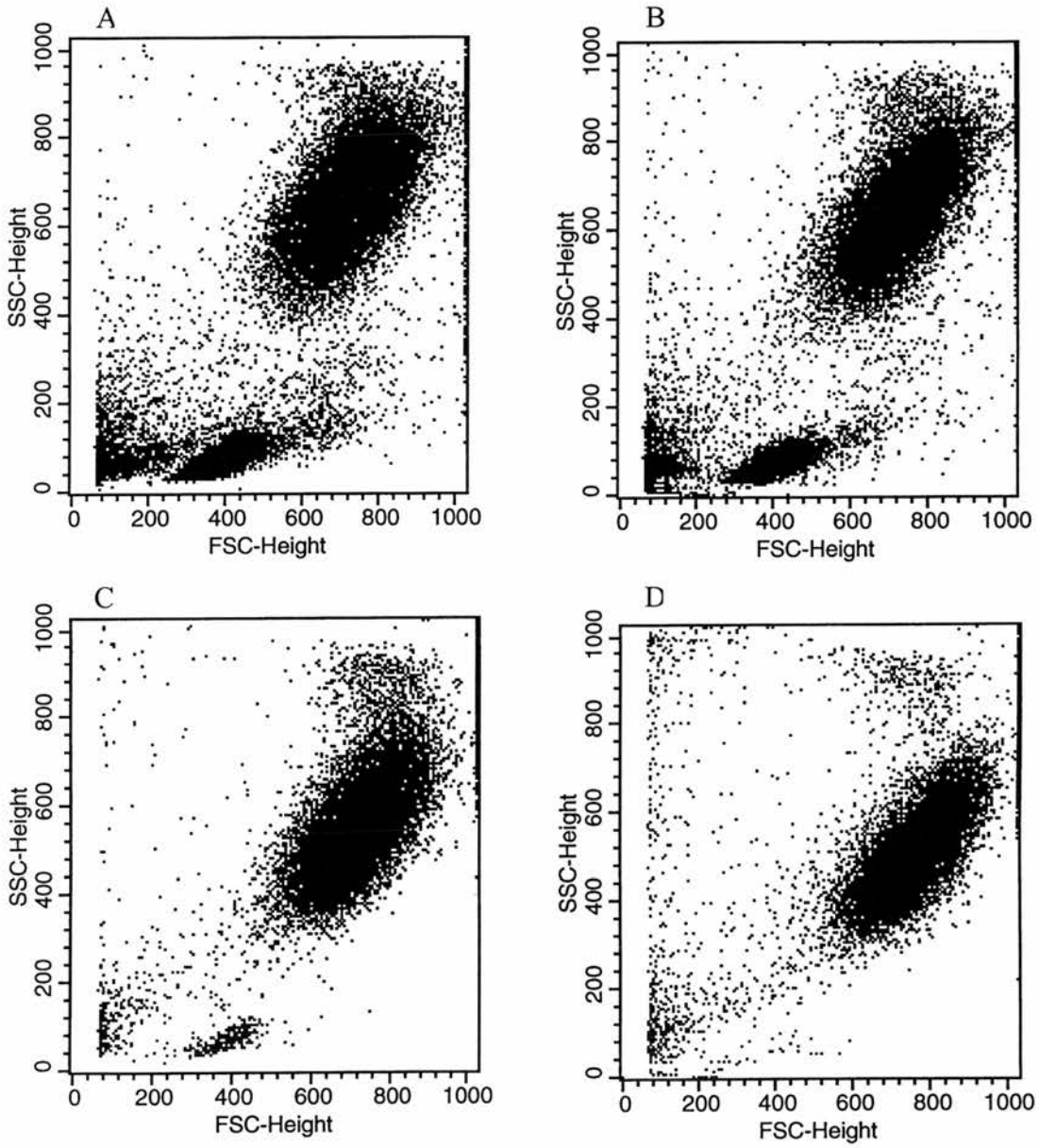


Fig 5.2 Comparison of neutrophils from A. whole blood, B. washed blood, C. Histopaque density gradient separation and D. plasma-Percoll separation. Charts show dot plots of SSC vs FSC following flow cytometric analysis.

whole blood kept at room temperature for 2-3 hours whilst purification of neutrophils using both the Histopaque and Percoll techniques was in progress, rather than a loss of CR3 from purified neutrophils. CR3 levels on neutrophils from the washed blood preparation were similar to levels on neutrophils from whole blood, indicating that the washing procedure was not associated with significant neutrophil activation.

Neutrophil responses to the stimulant PMA and to *P. aeruginosa* J1385 were compared using neutrophils prepared by each of the above methods (Fig 5.3). With both stimulants greater levels of CR3 were induced in neutrophils from whole or washed blood than in purified neutrophils. As expected, PMA induced an increase in CR3 expression on neutrophils from all of the preparation methods, although this effect was least marked for the washed blood preparation. *P. aeruginosa* J1385 was associated with increases in CR3 at bacterial concentrations of 2.5×10^7 and 2.5×10^8 cfu/ml. For washed blood and for purified neutrophils the increase in CR3 expression was considerably greater at a concentration of 2.5×10^8 cfu/ml, whilst in untreated blood, a smaller increase was seen at 2.5×10^8 cfu/ml than at 2.5×10^7 cfu/ml. In a larger study of *B. cepacia* strains and neutrophils in whole blood, a similar dose-dependent phenomenon was observed, with 4 clinical strains J2315, J415, C1576 and C1962 and one environmental strain J2540 inducing an increase in CR3 expression at a concentration of 2.5×10^7 cfu/ml, but provoking a decrease in expression at 2.5×10^8 cfu/ml. One strain, C1964, induced little change in CR3 at all concentrations tested whilst another strain, J2552, induced little response at a concentration of 2.5×10^7 cfu/ml, but a significant response at 2.5×10^8 cfu/ml.

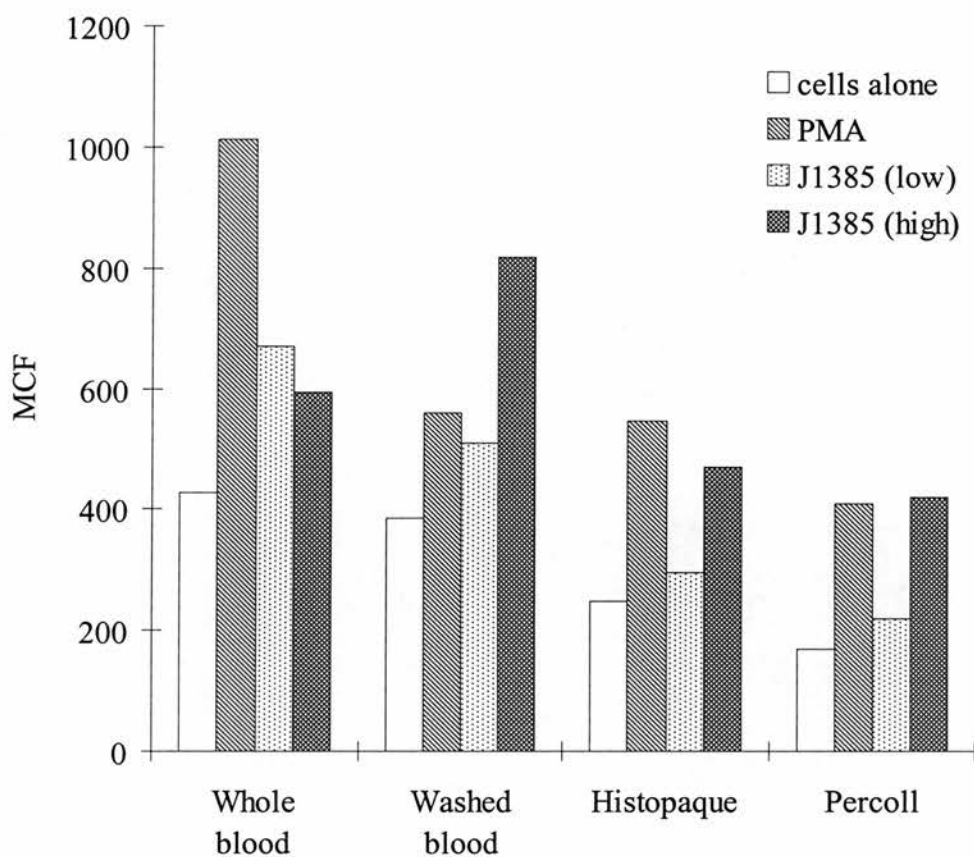


Fig 5.3 Effect of PMA and non-opsonised *P. aeruginosa* J1385 on expression of CR3 on neutrophils prepared by 4 different techniques. *P. aeruginosa* were at a concentration of either 2.5×10^7 cfu/ml (low) or 2.5×10^8 cfu/ml (high). Results are expressed as the mean channel fluorescence (MCF) of neutrophils treated with MAb anti-CD11b:FITC.

Further studies attempted to explain these observations by comparing CR3 expression in neutrophils from whole or washed blood incubated with bacterial strains at a wider range of concentrations (Fig 5.4). In the absence of plasma, CR3 expression on neutrophils increased proportionally with a logarithmic increase in bacterial concentration for both *P. aeruginosa* J1385 and *B. cepacia* J2315. However, incubation of *B. cepacia* J2315 with neutrophils in untreated blood was associated with a peak in CR3 expression at a concentration of 5×10^7 cfu/ml, whilst at higher concentrations a decrease in CR3 expression was seen. By contrast, incubation of the environmental *B. cepacia* strain J2552 with neutrophils in whole blood demonstrated a linear relationship between bacterial concentration and CR3 expression up to a concentration of 1×10^9 cfu/ml. In conclusion, the differences observed between washed and whole blood preparations implied a role for opsonisation, either with immunoglobulin or complement, in bacteria/neutrophil interactions within whole blood. Thus strains which are effectively opsonised induced increased CR3 expression at lower concentrations but were associated with a decrease in CR3 at higher concentrations, either through receptor down-regulation or simply through masking of anti-CD11b binding sites by bacteria. It may be relevant to note that both strains showing little opsonisation effect, C1964 and J2552, belong to *B. cepacia* genomovar I. However another genomovar I strain, J2540, appeared to be effectively opsonised within whole blood and, in this respect, seemed more akin to the genomovar III strains J2315 and J415, and the genomovar II strains C1576 and C1962. Overall, in view of the strain-specific effects observed above, further studies were carried out using washed blood as standard.

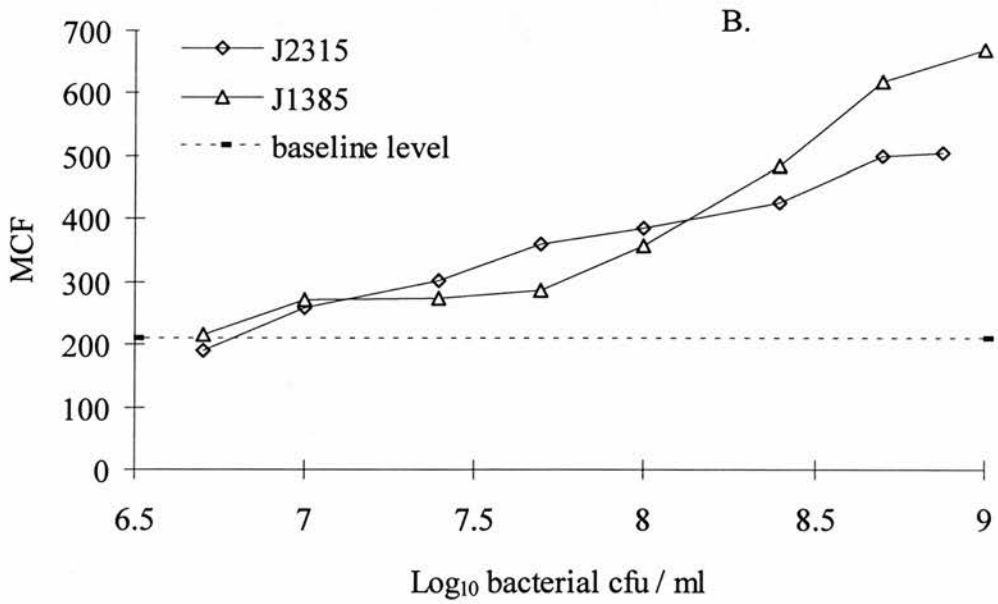
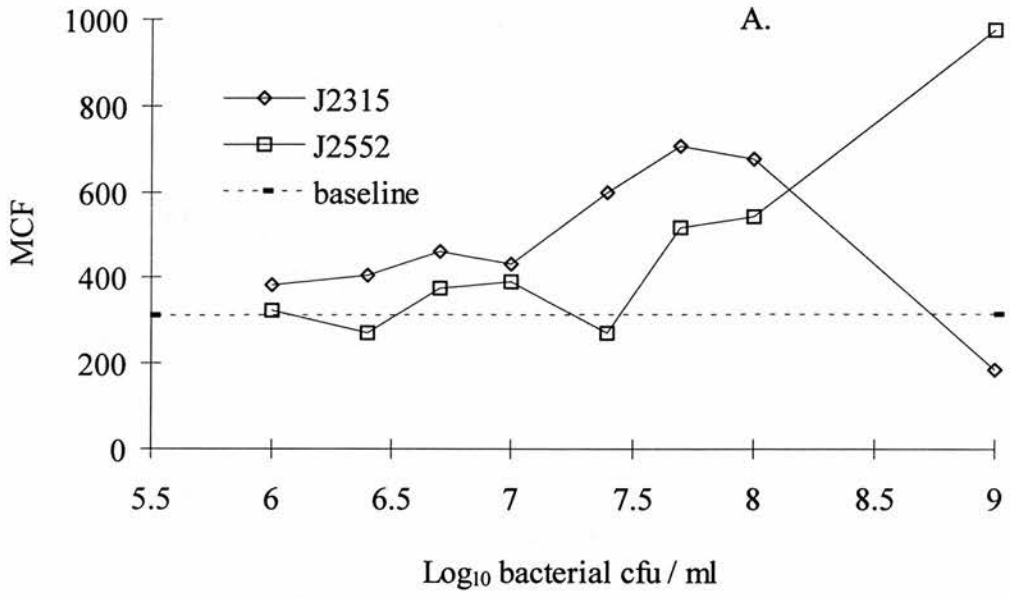


Fig 5.4 Expression of CR3 by neutrophils stimulated with increasing concentrations of non-opsonised bacteria for 60 min. Results are the MCF of neutrophils stained with anti-CD11b:FITC. A. whole blood. B. washed blood.

5.2.2 Induction of CR3 by non-opsonised bacteria on neutrophils in washed blood

A panel of CF isolates of *B. cepacia* and *P. aeruginosa* were compared for induction of CR3 on neutrophils in washed blood. Samples were incubated with bacteria at a concentration of 5×10^8 cfu/ml for 60 min at 37°C before staining with anti-CD11b:FITC. CR3 expression was determined as the MCF of the neutrophil population. All *B. cepacia* strains induced CR3 levels at least twice those of neutrophils exposed to buffer alone; by comparison, only 3 out of 5 *P. aeruginosa* strains induced similar levels. Mean CR3 expression induced by *B. cepacia* strains was 260% of levels on control neutrophils, compared to a mean of 212% control levels, induced by *P. aeruginosa* strains. However, this difference was not significant.

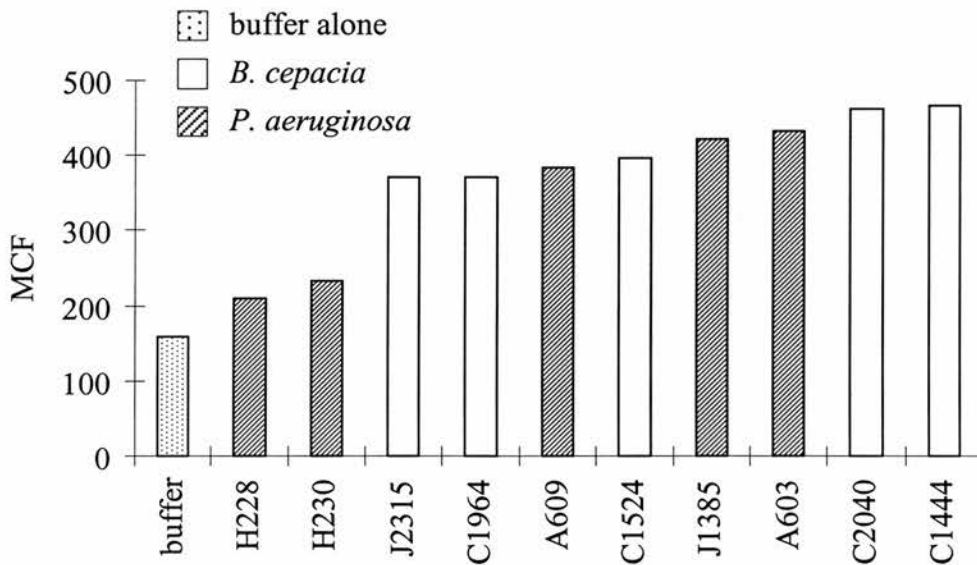


Fig 5.5 Expression of CR3 by neutrophils exposed to buffer alone, or *B. cepacia* or *P. aeruginosa* at a concentration of 5×10^8 cfu/ml for 60 min at 37°C. CR3 is determined as the MCF of neutrophils treated with anti-CD11b:FITC.

CHAPTER 6 NEUTROPHIL RESPIRATORY BURST RESPONSES TO *B. CEPACIA*

6.1 CHEMILUMINESCENCE RESPONSES TO NON-OPSONISED *B. CEPACIA* AND *P. AERUGINOSA*

6.1.1 Luminol chemiluminescence responses to non-opsonised bacteria

Bacteria were incubated with plasma-Percoll purified neutrophils in the presence of luminol over a 90 min period at 37°C and respiratory burst activity assessed by the production of light from activated luminol. Zymosan opsonised with normal serum (OZ) was used as a positive control and induced an early sharp peak in neutrophil responses, occasionally followed by a second smaller peak (Fig. 6.1). Bacteria alone produced no LCL responses, but did stimulate a variety of strain-dependent responses when co-incubated with neutrophils. Responses varied in terms of initial rates, peak LCL production and the sustained nature of the response. In general, bacterially-induced responses were slower than responses to zymosan and of a lesser magnitude. Wide inter-donor variations in the magnitude and speed of responses were observed, but patterns for individual bacterial species were consistent. Thus, for example, the CF *P. aeruginosa* strain J1385 was associated with an early and sustained rise in LCL, whilst the *B. cepacia* strain, C2040, showed a later response, decreasing more rapidly than that for J1385. The Edinburgh epidemic strain, J2315, was associated with either no response, or a late moderate rise in LCL after 1 hour of incubation. In the majority of experiments, results were analysed using both peak LCL up to 90 min to assess magnitude and LCL at 10 min (LCL₁₀) to assess initial

rate of responses. Preliminary studies using bacteria at a range of concentrations indicated that responses increased up to a ratio of 125 bacteria per neutrophil before declining. Further experiments were carried out using 100 bacteria per neutrophil as standard.

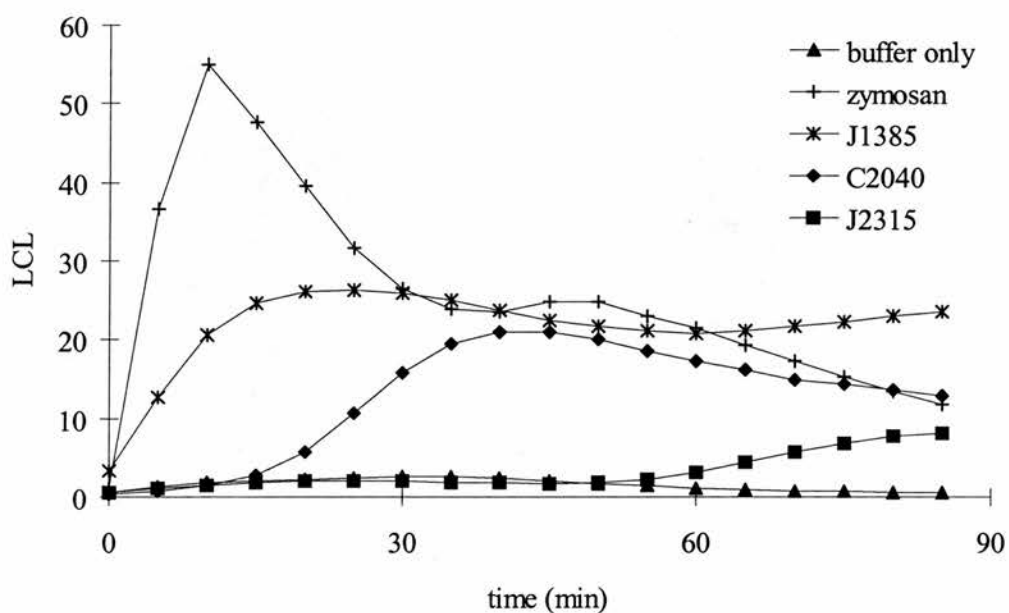


Fig 6.1 Examples of LCL induction in neutrophils exposed to zymosan, *P. aeruginosa* J1385 and *B. cepacia* J2315 and C2040.

6.1.2 Comparison of clinical and environmental *B. cepacia*

In a series of experiments, non-opsonised *B. cepacia* from clinical and environmental sources were compared. Results from 2-6 experiments per strain were standardised as the percentage of both peak LCL and LCL₁₀ in response to OZ (Fig 6.2).

Statistical analysis was carried out using analysis of variance. CF strains demonstrated a variety of responses, from virtually no activity to the moderate activity shown by J2315, with a mean peak LCL equal to 27.3% OZ levels and mean LCL₁₀ equal to 4.1% OZ levels. Environmental strains showed overall similar levels of activity (mean peak LCL = 32% OZ, mean LCL₁₀ = 8.2% OZ), although one strain, J2511, induced a much larger and faster response than all other environmental and CF strains tested. For non-CF clinical strains, results for both peak LCL (mean = 92.9% OZ) and LCL₁₀ (mean = 16.8% OZ) were significantly greater than levels induced by environmental and CF strains. (ANOVA, $p < 0.001$).

An attempt was made to confirm these results using a larger panel of strains with at least 9 isolates in each group. However, comparison of results from an individual experiment using the Wilcoxon rank sum test demonstrated no significant difference between any of the groups tested.

6.1.3 Comparison of *B. cepacia* and *P. aeruginosa* isolated from CF patients

It was noted in several experiments that the *P. aeruginosa* strain PAO1 induced neutrophil LCL responses greater than any of the *B. cepacia* strains tested. In one experiment, a panel of *B. cepacia* and non-mucoid *P. aeruginosa* strains isolated

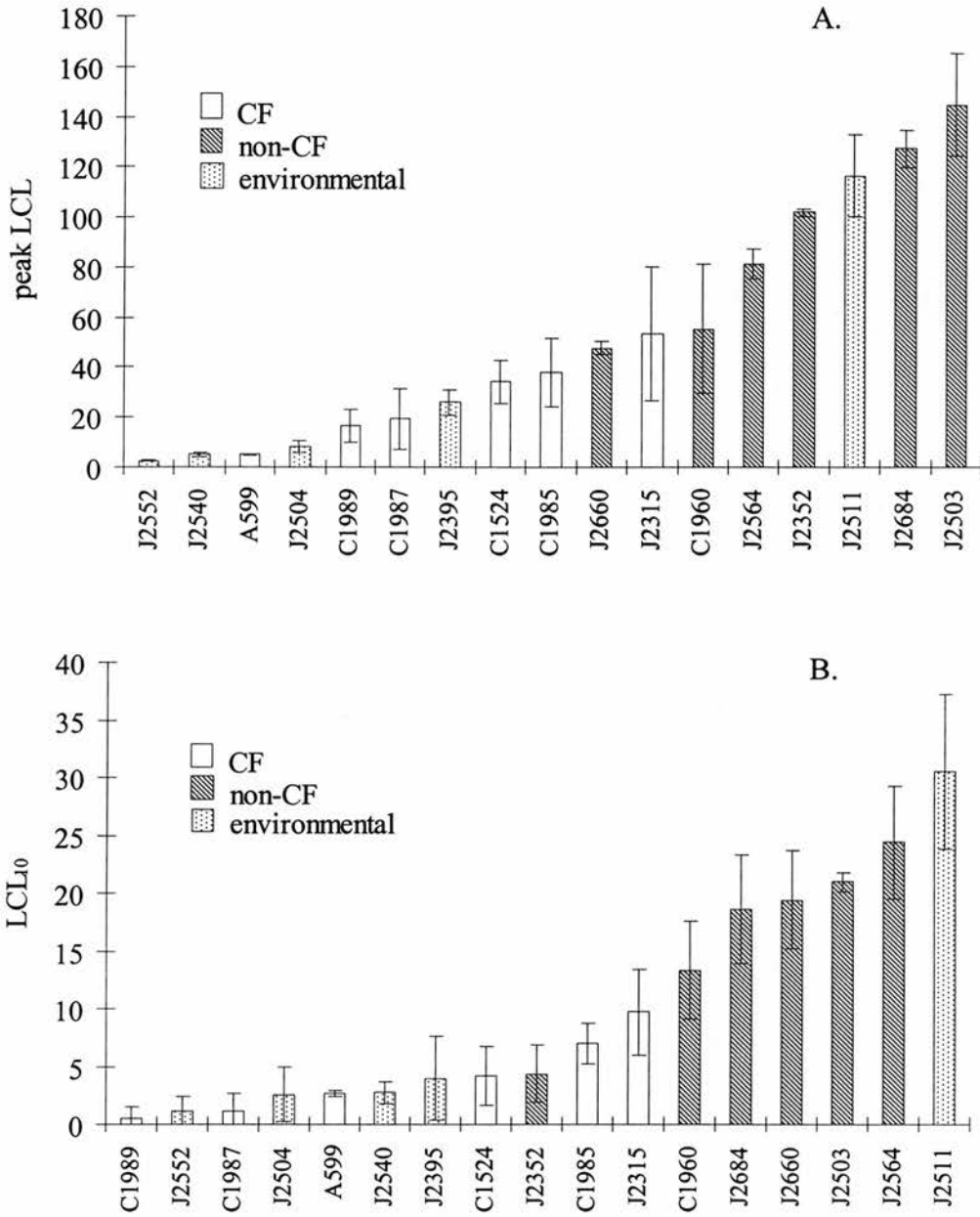


Fig 6.2 LCL activity in neutrophils stimulated with clinical and environmental *B. cepacia*. Results are expressed as the percentage of A. peak LCL and B. LCL₁₀ induced by opsonised zymosan in each experiment and are the mean of 2-6 experiments per strain \pm SEM

from CF patients were compared (Fig 6.3). Mean peak LCL and LCL₁₀ for *B. cepacia* strains were 8.0 and 1.8 respectively and for *P. aeruginosa* strains were 15.1 and 9.1 respectively. However considerable inter-strain variation in responses was noted, particularly for *P. aeruginosa* strains, where a subpopulation of 4 strains (A603, A607, J1385 and A15) induced early large peaks in LCL responses whilst a fifth strain, A605, was associated with a later marked peak in LCL. By contrast all *B. cepacia* were slow inducers of LCL responses and only one strain, C2040, induced a late peak equivalent to peak values for the highly active *P. aeruginosa* subpopulation. Although most strains of both species induced at least a slight LCL response, four strains; *B. cepacia* C1987 and A599, and *P. aeruginosa* H230 and A609; demonstrated no activity, i.e. both peak LCL and LCL₁₀ values were lower than values for neutrophils alone.

In view of the skewed distribution of responses to *P. aeruginosa* strains, statistical analysis was carried out by applying the Wilcoxon rank sum test. Differences between *B. cepacia* and *P. aeruginosa* strains failed to reach significance for peak LCL and were just short of significance at a 5% level for LCL₁₀. However, if the four strains inducing no response were excluded from analysis, results for LCL₁₀ reached significance at $p < 0.01$. Thus active *B. cepacia* strains were found to induce a slower response than active *P. aeruginosa* strains

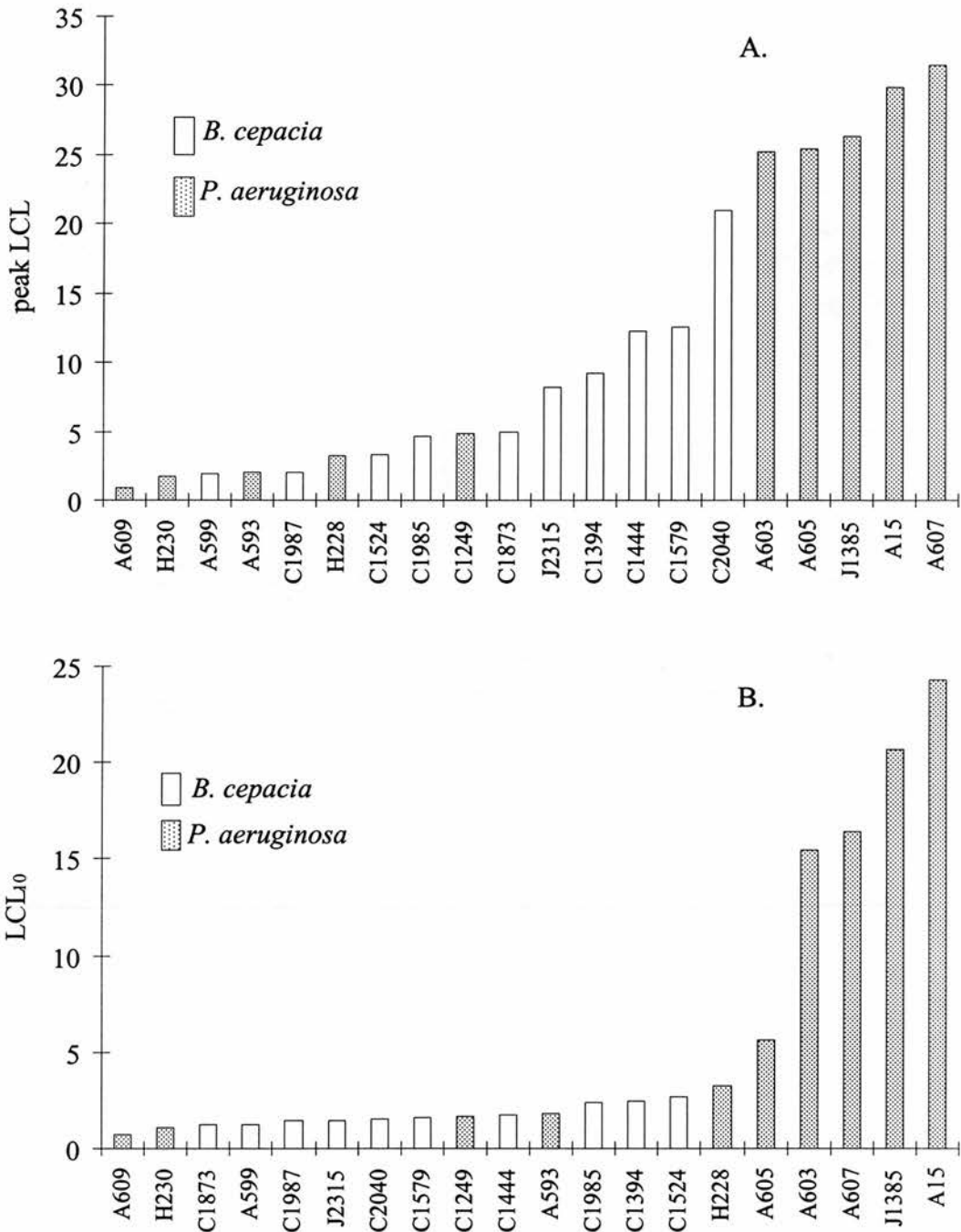


Fig 6.3 A. Peak LCL and B. LCL₁₀ induced in neutrophils by non-mucoid CF isolates of *B. cepacia* and *P. aeruginosa*.

6.1.4 Effect of growth conditions on LCL induction

A panel of *B. cepacia* were grown at 37°C in either shaken or static culture, in order to test the hypothesis that growth in conditions favouring hydrophobicity, ie static culture, would increase bacteria:neutrophil interactions and hence LCL induction. Changes in LCL induction following static growth varied in a strain-dependent but reproducible manner. Fig. 6.4 shows results from a representative experiment. For the *P. aeruginosa* strain PAO1 and two *B. cepacia* non-CF clinical strains, J2660 and J2684, static growth was associated with a decrease in peak LCL although initial rates of LCL induction remained largely unchanged. By contrast, results for two CF *B. cepacia*; C1987 and J2315; displayed an increase in both initial rates of LCL induction and peak LCL following static growth, although overall levels were still much lower than those for PAO1, J2660 or J2684. Of two environmental strains, J2511 and J2552, static growth conditions were associated with a slight increase in responses.

6.1.5 Comparison of luminol and lucigenin chemiluminescence responses

Whilst LCL detects ROS production secondary to myeloperoxidase (MPO) activity, lucigenin-dependent chemiluminescence (LuCL) detects the production of intracellular superoxide anions (O_2^-) and thus gives a direct measure of NADPH oxidase activity and hence respiratory burst initiation. LCL and LuCL responses were compared for a number of *B. cepacia* and *P. aeruginosa* strains (Fig 6.5). In all

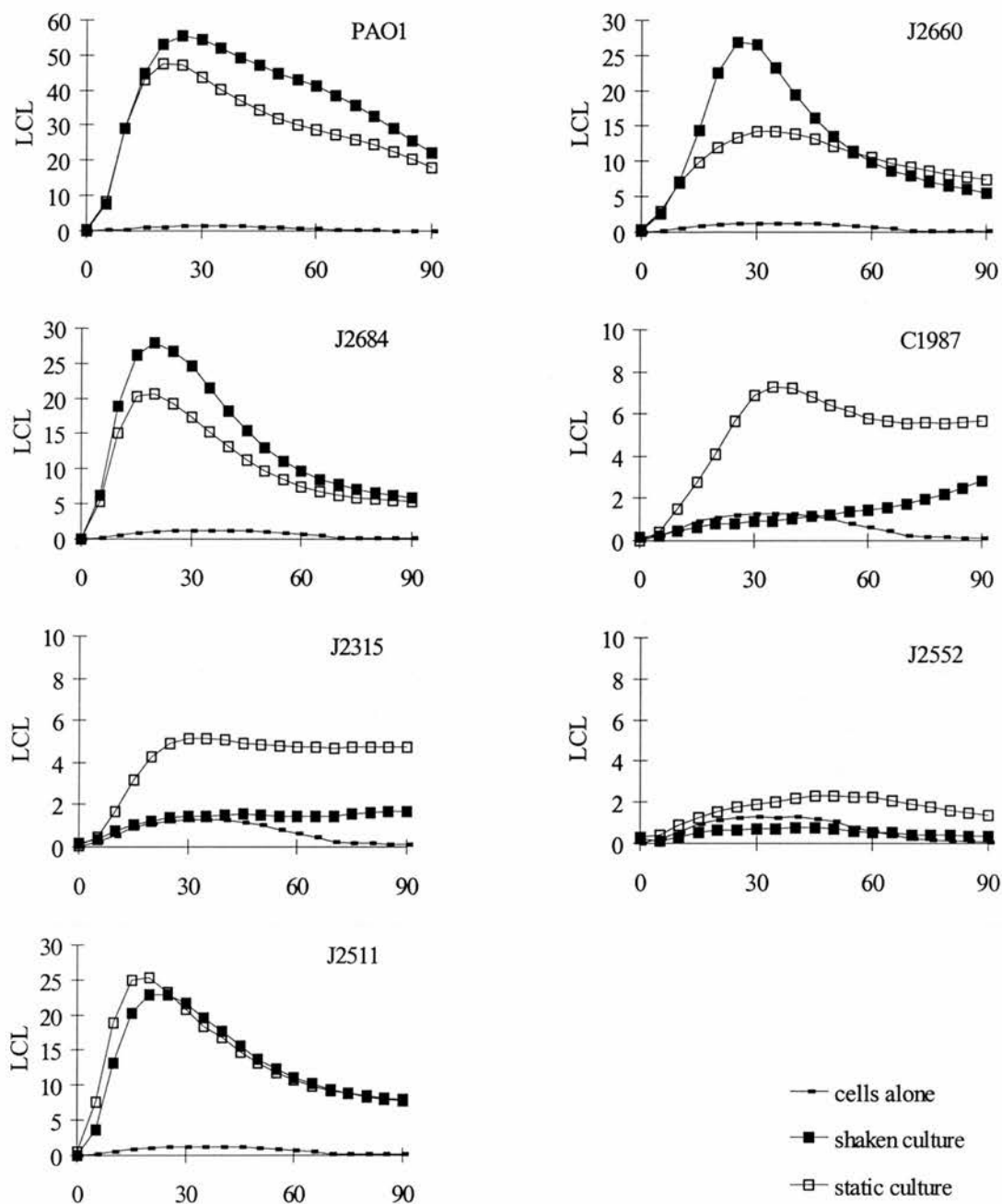


Fig 6.4 Effects of growth in an orbital incubator (shaken culture) or in static culture on LCL induction by *P. aeruginosa* PAO1 and representative strains of *B. cepacia*. Note that the scale on the LCL axis is not identical for all strains.

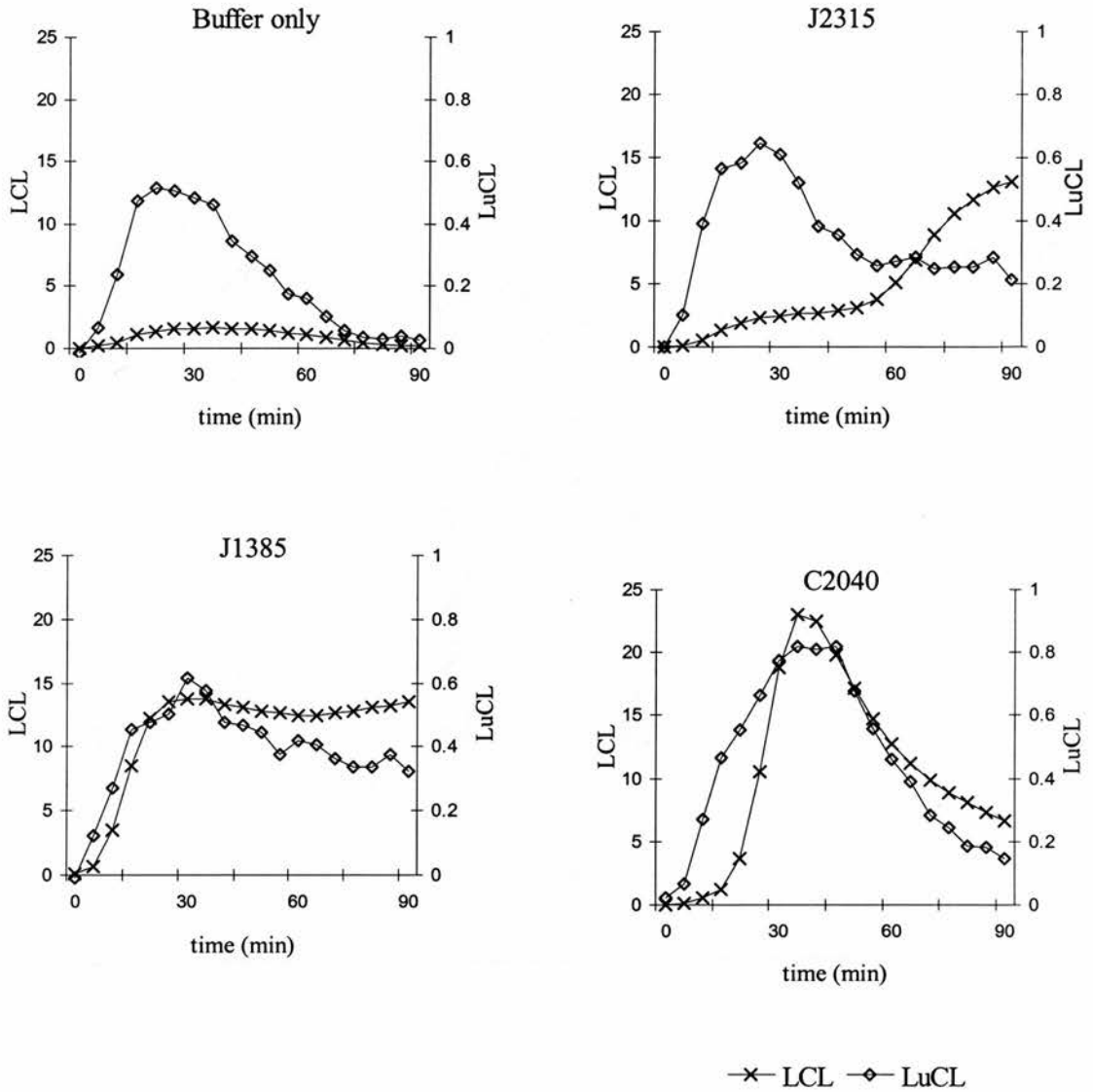


Fig 6.5 Comparison of LCL and LuCL induced in neutrophils incubated with buffer alone and three representative bacterial strains.

instances, including neutrophils incubated with buffer alone, a rise in LuCL was seen prior to or concurrent with any rise in LCL. However peak LuCL levels showed no consistent correlation with peak LCL levels induced by non-opsonised bacteria. Furthermore patterns of LuCL induction for individual bacterial strains did not correlate with patterns of LCL induction.

The observation of little or no correlation between LuCL and LCL activity was surprising, as it was supposed that both lucigenin and luminol detected different points within the same respiratory burst pathway. Thus experiments were conducted using superoxide dismutase (SOD) to selectively inhibit LuCL responses and sodium azide (NaN_3) to inhibit LCL responses. In neutrophils stimulated with non-opsonised zymosan or with non-opsonised J2315 (Fig 6.6), the use of NaN_3 was associated with the complete abolition of LCL responses but a marked increase in LuCL activity, consistent with the build up of O_2^- within the neutrophil. By contrast, SOD was associated with the complete abolition of LuCL responses and a marked reduction in LCL responses, suggesting that LCL activity is at least partly dependent on O_2^- production within the neutrophil.

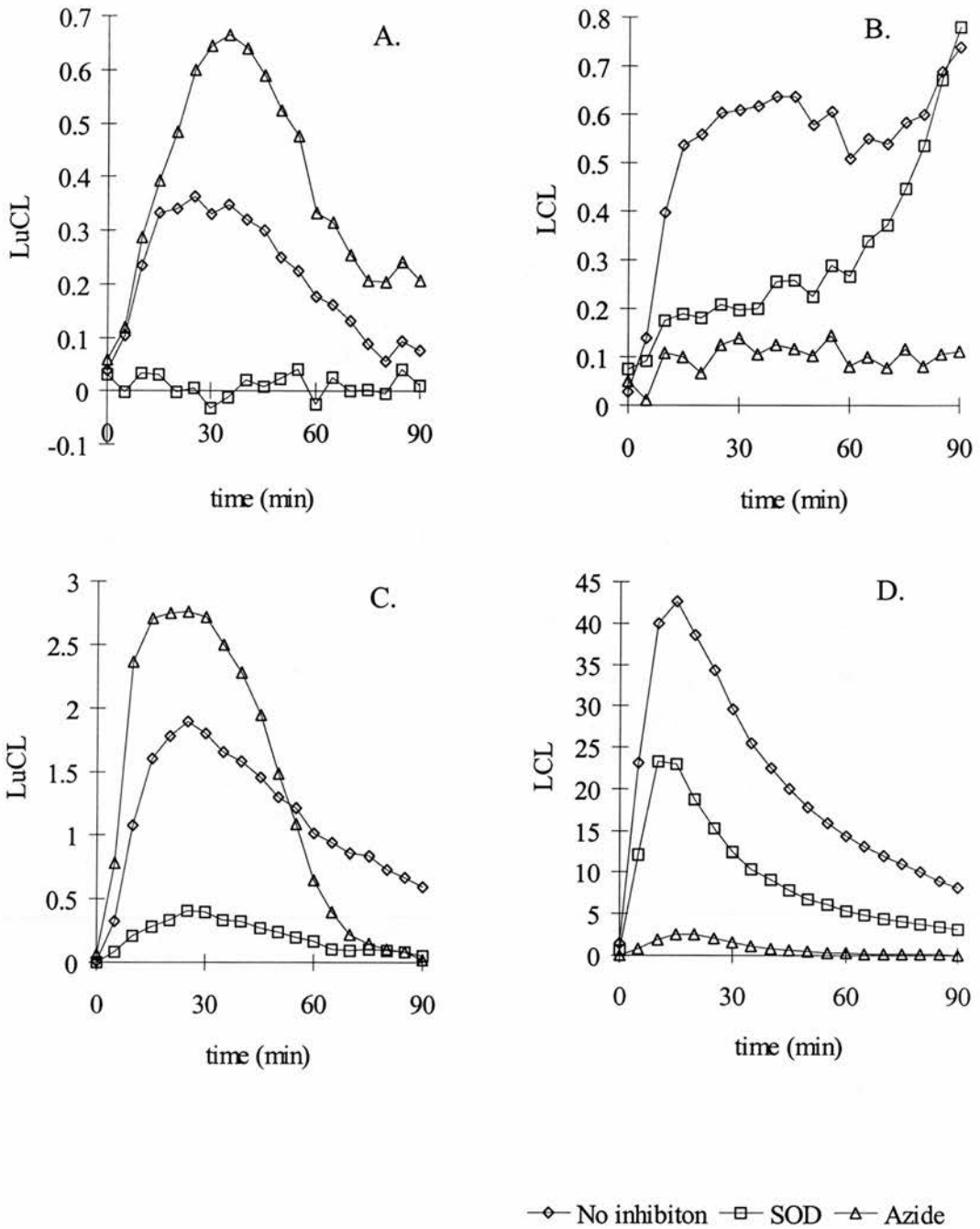


Fig 6.6 Effect of SOD and sodium azide on the induction of LCL and LuCL by non-opsonised *B. cepacia* J2315 and zymosan. Charts show LuCL for A. J2315 and C. zymosan, and LCL for B. J2315 and D. zymosan. Note the differences in scale for both LCL and LuCL between J2315 and zymosan.

6.2 EFFECT OF OPSONISATION ON CHEMILUMINESCENCE RESPONSES

6.2.1 Effect of complement and serum on LCL and LuCL responses to the Edinburgh epidemic strain of *B. cepacia*

Neutrophil LCL and LuCL activities were analysed in response to *B. cepacia* J2315 pre-incubated with 10% heat-inactivated serum from *B. cepacia*+ patients with or without 4% guinea pig complement (GPC). Pre-opsonisation was associated with reproducible changes in chemiluminescence induction. Results from a representative experiment are shown in Fig 6.7. Non-opsonised J2315 induced little or no LCL or LuCL activity above control neutrophils incubated with buffer alone. Bacterial pre-opsonisation with complement alone was associated with an initial slight reduction in LCL activity, before a gradual increase in levels after one hour of incubation. By contrast, any LuCL activity was completely abolished. Pre-opsonisation by serum alone led to an early peak in both LCL and LuCL induction. Similar LuCL levels were induced by bacteria opsonised with serum alone or with complement plus serum. However addition of complement to opsonising serum was associated with an augmentation of LCL activity with, in some instances, an increase in peak LCL or, as in the example shown, a more sustained LCL response.

Although direct correlation of LuCL and LCL levels could not be demonstrated, investigation of responses using the inhibitors NaN_3 and SOD confirmed the relationship between them (Fig 6.8). Thus, for bacteria pre-opsonised with immune serum, SOD led to an almost complete abolition in LuCL activity and a significant reduction in LCL levels. The presence of NaN_3 , however, was associated with an

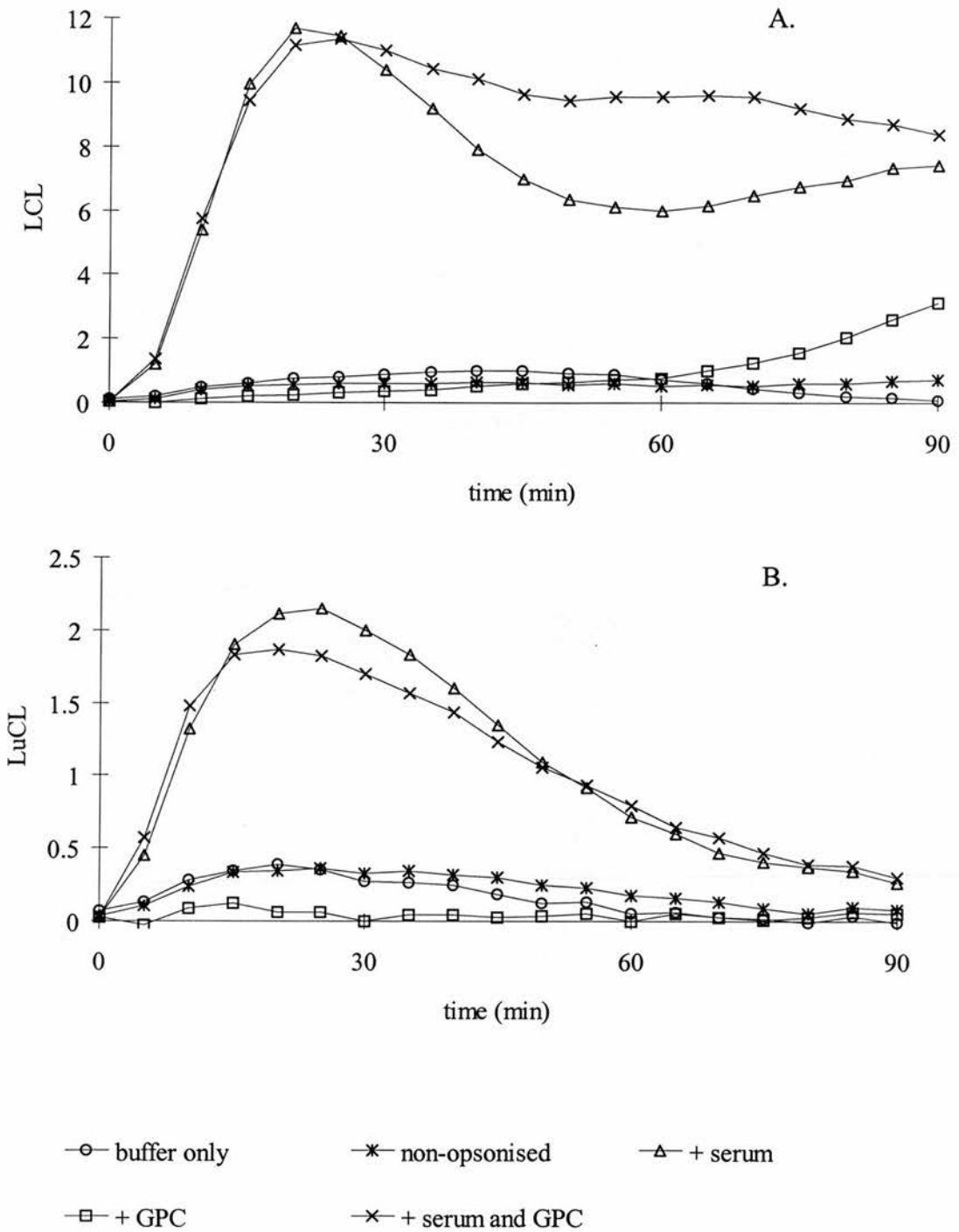


Fig 6.7 Effect of pre-opsonisation on A. LCL and B. LuCL induced by *B. cepacia* J2315. Bacteria were either non-opsonised or pre-opsonised with 10% heat-inactivated immune serum from a *B. cepacia*+ CF patient and/or 4% GPC

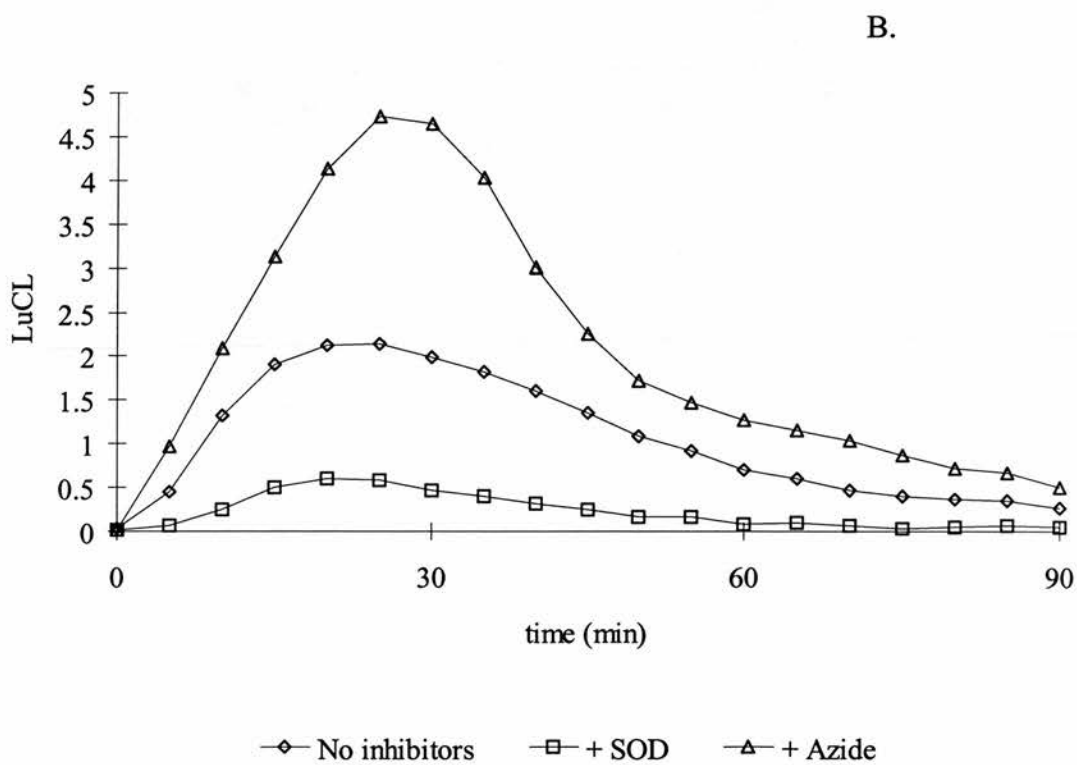
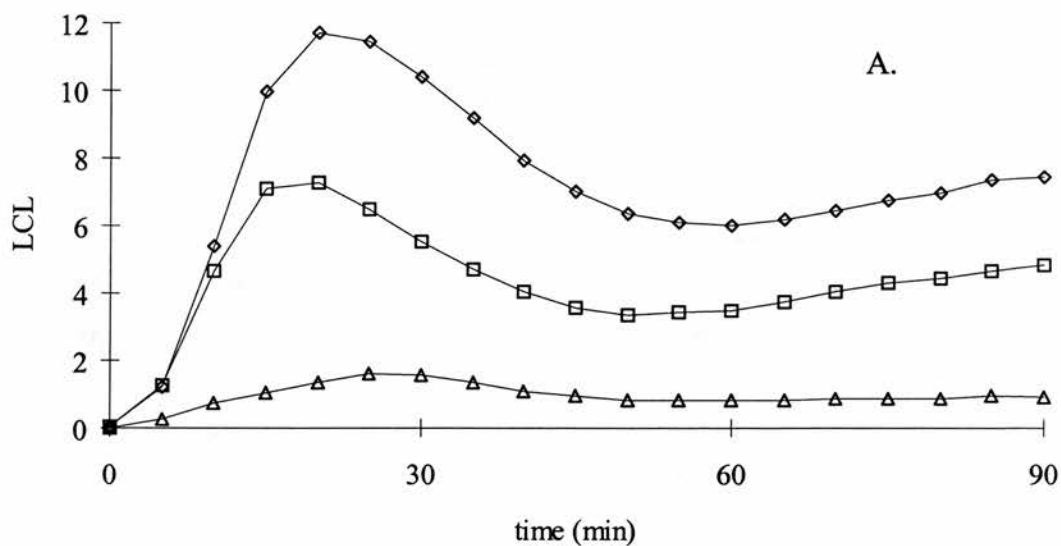


Fig 6.8 Effect of the inhibitors, SOD and sodium azide, on A. LCL and B. LuCL induction by *B. cepacia* J2315 pre-opsonised with 10% immune serum.

almost complete reduction in LCL levels but marked augmentation of LuCL activity consistent with O_2^- build up within the neutrophil. Similar changes in both LCL and LuCL induction were observed for bacteria opsonised with GPC alone or with immune serum and GPC (Data not shown).

6.2.2 Chemiluminescence induction following pre-opsonisation of a range of *B. cepacia* strains

Given the diversity of bacterial isolates presently identified as *B. cepacia*, the effects of pre-opsonisation of a panel of strains with immune serum were investigated. In all instances, pre-opsonisation was carried out using heat-inactivated serum from patients colonised with the Edinburgh epidemic strain of *B. cepacia*. Representative results are shown in Fig 6.9. Effects of opsonisation on LCL induction were strain dependent with the CF strains J2315, J415, C1632, C1576, C1444 and C1524, and one environmental strain, J2540, showing a rapid early rise in LCL levels followed by a sustained response. By contrast, the CF strains C1964 and C1962, and the environmental strain J2552, showed less augmentation of responses. Pre-opsonisation of the CF strain C2040 was associated with an earlier but diminished peak in LCL response in comparison to non-opsonised bacteria.

In a number of these studies, LuCL levels were measured simultaneously with LCL activity. Opsonisation with serum was associated with a marked increase in LuCL activity, generally occurring prior to or concurrently with any rise in LCL activity. However, as for non-opsonised bacteria, no direct correlation could be found between the magnitude or pattern of LuCL and LCL responses (Data not shown).

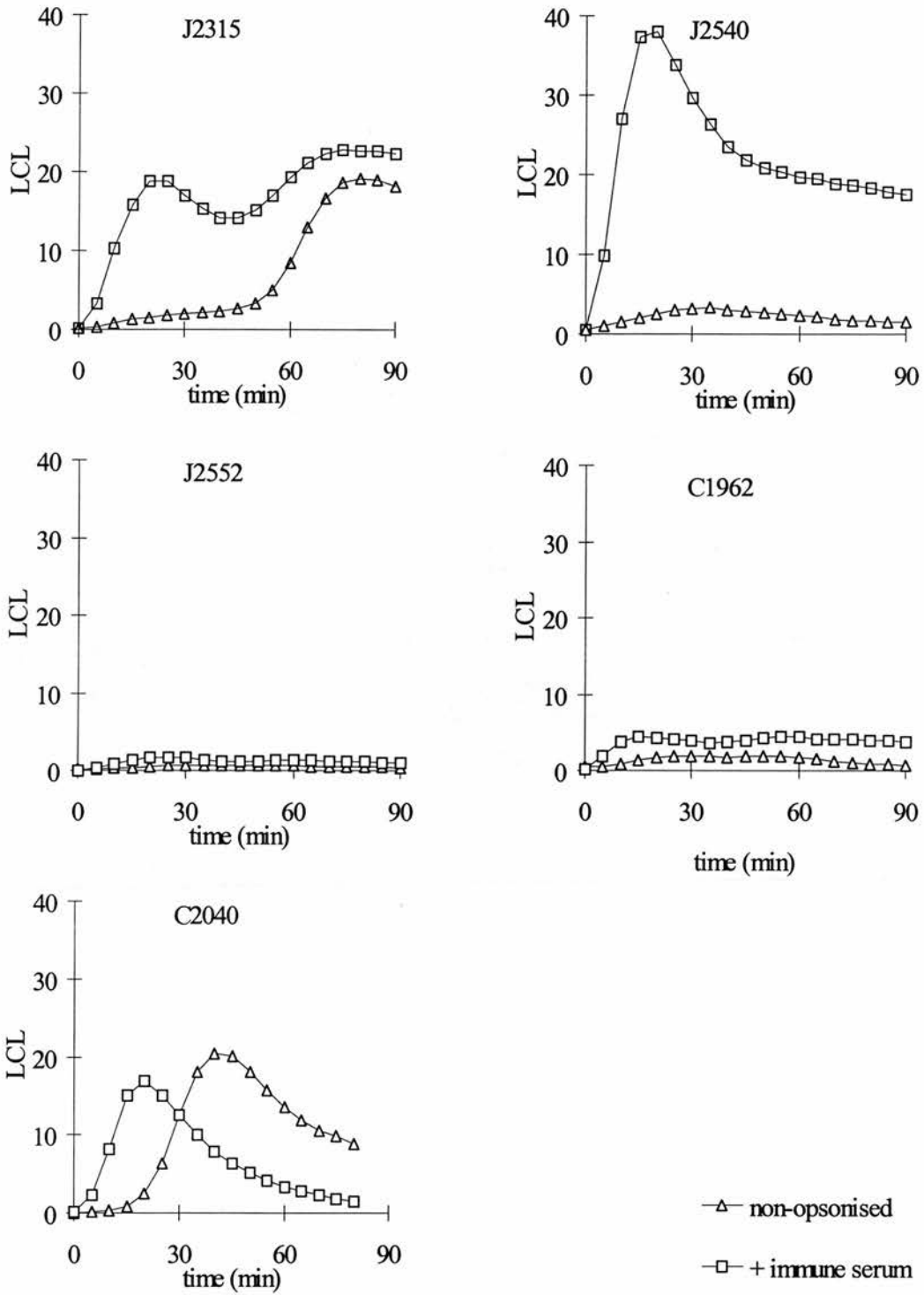


Fig 6.9 LCL in neutrophils stimulated with representative strains of *B. cepacia* pre-opsonised with immune serum from a *B. cepacia*+ CF patient.

6.2.3 Comparison of sera from *B. cepacia*+ and *B. cepacia*- CF patients

Preliminary studies using pooled human serum from healthy volunteers and pooled immune serum from CF patients colonised with the Edinburgh epidemic strain of *B. cepacia* indicated that the magnitude of peak responses to J2315 were much greater if bacteria were pre-opsonised with immune serum. Further studies were carried out to investigate the opsonising activity of sera from *B. cepacia*+ and *B. cepacia*- CF patients. Results from two experiments using serum samples from 6 *B. cepacia*+ and at least 5 *B. cepacia*- patients, are summarised in Table 6.1. Fig 6.10 also shows patterns of LCL induction in experiment 1.

Table 6.1 Peak LCL and LCL₁₀ induced by J2315 pre-opsonised with sera from *B. cepacia*+ and *B. cepacia*- CF patients¹.

	Exp. 1		Exp. 2	
	Peak LCL ²	LCL ₁₀ ³	Peak LCL ²	LCL ₁₀ ³
No opsonisation	3.7	2.1	5.1	0.5
<i>B. cepacia</i> +	24.4 ± 1.1	14.1 ± 1.0	12.7 ± 1.4	7.1 ± 1.4
<i>B. cepacia</i> -	18.8 ± 1.3	5.2 ± 2.0	8.5 ± 0.6	1.6 ± 0.7

¹ Results are mean ± SEM

² Difference between *B. cepacia*+ and *B. cepacia*- sera significant: p<0.05 (Students t test)

³ Difference between *B. cepacia*+ and *B. cepacia*- sera significant: p<0.01 (Students t test)

Non-opsonised J2315 induced little or no LCL response. In general pre-opsonisation with *B. cepacia*+ serum was associated with a greater rate and magnitude of LCL induction than pre-opsonisation with *B. cepacia*- serum. Both peak LCL and LCL₁₀

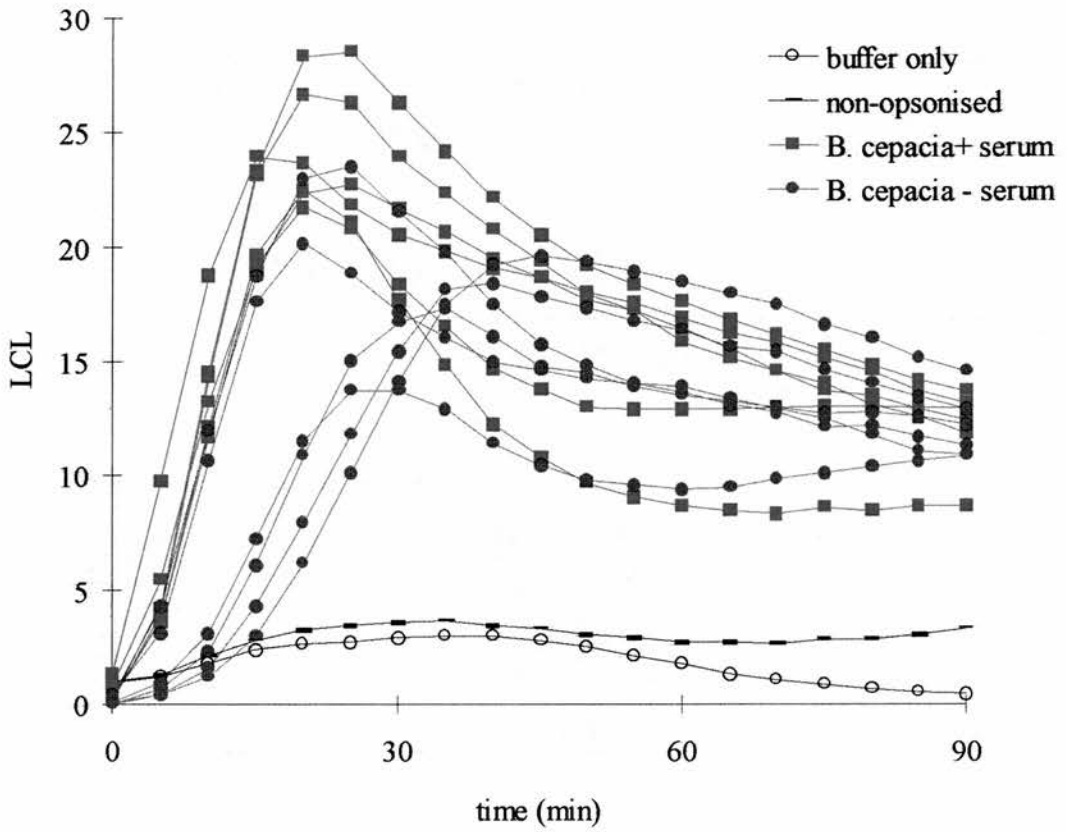


Fig 6.10 Comparison of serum samples from *B. cepacia*⁺ and *B. cepacia*⁻ CF patients. Results show LCL in neutrophils stimulated with non-opsonised *B. cepacia* J2315 or with *B. cepacia* J2315 pre-opsonised with serum from 6 *B. cepacia*⁺ patients (red lines) and 6 *B. cepacia*⁻ patients (blue lines).

were considerably greater following pre-opsonisation with *B. cepacia*+ sera than with *B. cepacia*- sera and these differences were statistically significant at a 5% level for peak LCL and a 1% level for LCL₁₀.

For two patients, G and S, stored serum samples were used to analyse changes in opsonising activity both before and after colonisation with *B. cepacia* (Fig 6.11). For both patients LCL₁₀ values displayed greater reproducibility than peak LCL values. The variability in peak LCL values made interpretation difficult; however a rising trend was detected for patient G before and after colonisation, whilst for patient S, peak values were observed to decline in the two year period prior to colonisation before a slight rise in the few months after colonisation. For patient G, LCL₁₀ also increased before and after colonisation whilst for patient S, LCL₁₀ values were comparatively constant until a few months before the first detection of *B. cepacia*, after which a slight rise in values was detected.

To confirm that the above results indicated a specific effect of serum from *B. cepacia*+ patients, further studies were carried out using immune sera absorbed against a panel of *B. cepacia* strains and a representative CF strain of *P. aeruginosa*, J1385 (Fig 6.12). Neutrophils were stimulated with *B. cepacia* J2315 pre-opsonised with absorbed sera and LCL recorded over a 90 min period. Sera absorbed against *P. aeruginosa* J1385 or *B. cepacia* C1444, C1524 or J2511 opsonised J2315 as effectively as non-absorbed serum. By contrast, pre-opsonisation with serum absorbed against J2315 was associated with a marked drop in LCL responses with peak LCL declining to 11.8 from 24.2 for non-absorbed serum. To confirm the specificity of this effect, neutrophils were also stimulated with pre-opsonised

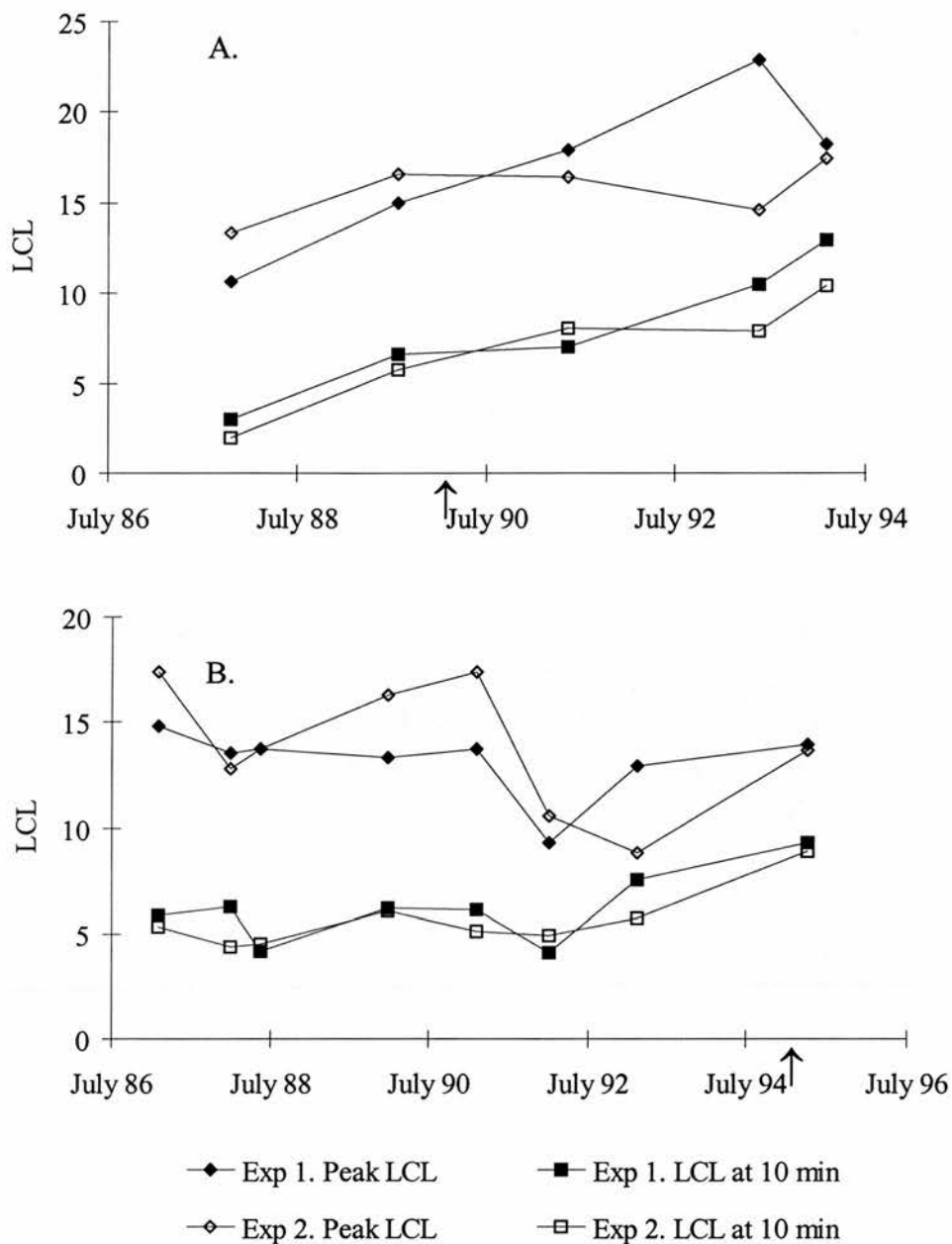


Fig 6.11 Peak LCL and LCL₁₀ induced by J2315 pre-opsonised with heat-inactivated serum collected over a 7-8 year period from A. patient G and B. patient S, both eventually colonised by the Edinburgh epidemic strain of *B. cepacia*. Results are shown from two separate experiments per patient. Arrows indicate date of first sputum sample positive for *B. cepacia*.

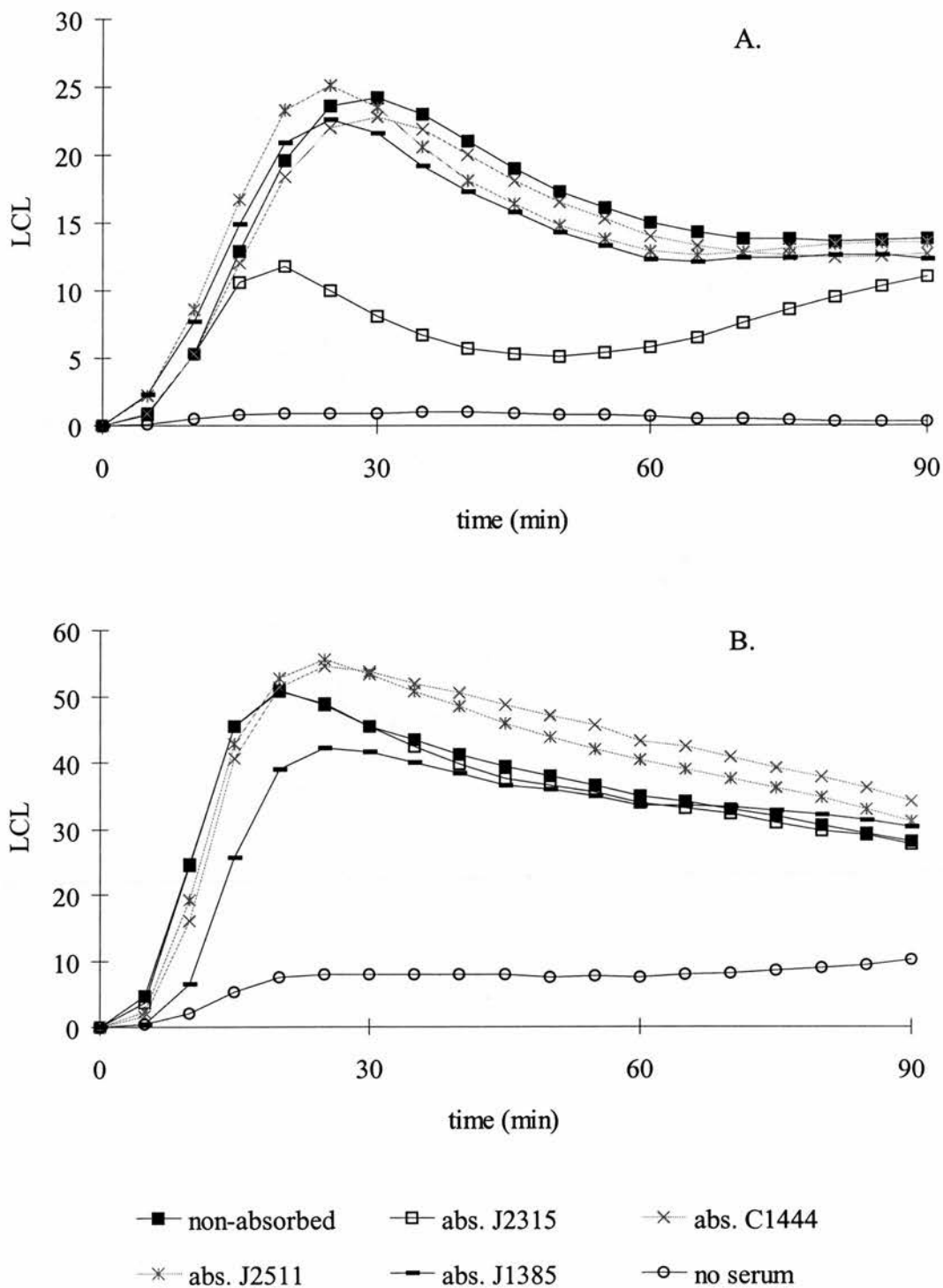


Fig 6.12 Effect of pre-opsonisation with absorbed sera on LCL induction by *A. B. cepacia* J2315 and *B. P. aeruginosa* J1385.

P. aeruginosa J1385. In this instance, serum absorbed against J2315 opsonised J1385 as effectively as non-absorbed serum whilst serum absorbed against J1385 was associated with a drop in peak LCL from 50.9 for non-absorbed serum to 42.2.

In the above experiment, LuCL was measured concurrently with LCL levels. As expected, pre-opsonisation of J2315 or J1385 with non-absorbed sera was associated with an increase in both the magnitude and initial rate of LuCL responses. Unlike LCL, however, pre-absorption of opsonising sera with J2315 or J1385 did not diminish the effects of opsonisation on LuCL induction by J2315 or J1385 respectively (Fig 6.13). Furthermore, comparison of peak LuCL and peak LCL for each test condition showed no correlation between values. Indeed, although opsonised J1385 induced LCL to levels twice those induced by opsonised J2315, induced LuCL levels were much lower for J1385 than for J2315 (Fig 6.13). A similar relationship between LCL and LuCL values was observed using non-opsonised J2315 and J1385 although overall levels were much lower.

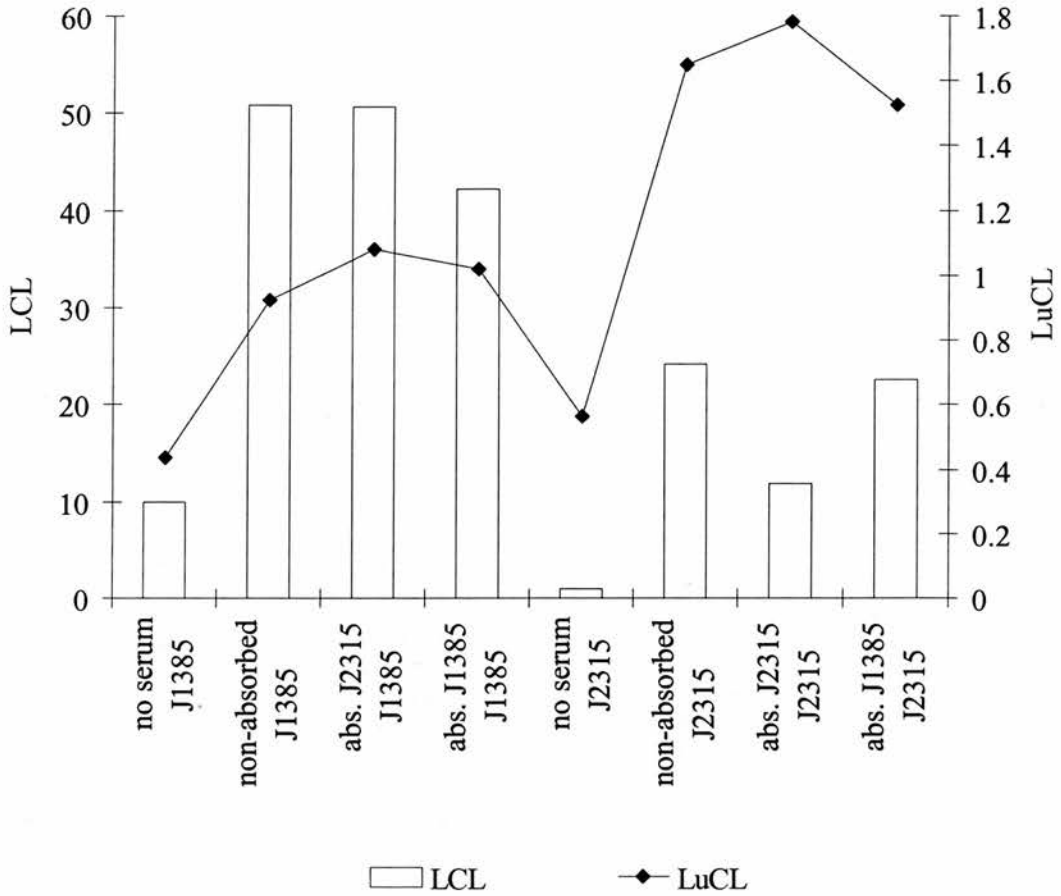


Fig 6.13 Comparison of peak LCL and LuCL values in neutrophils stimulated with *P. aeruginosa* J1385 or *B. cepacia* J2315. Bacteria were either non-opsonised or pre-opsonised with serum from a *P. aeruginosa*+, *B. cepacia*+ CF patient. Serum was non-absorbed or absorbed against *B. cepacia* J2315 (abs. J2315) or *P. aeruginosa* J1385 (abs. J1385).

6.3 PRODUCTION OF SUPEROXIDE ANIONS BY NEUTROPHILS

LCL results suggested that strains of *P. aeruginosa* isolated from CF patients were greater inducers of a respiratory burst than CF strains of *B. cepacia*. However, as LCL is a non-specific assay, and as interpretation of results is difficult due to the variety of responses obtained, an attempt was made to confirm LCL data using the reduction of cytochrome c following the extracellular release of O_2^- to detect respiratory burst initiation. These studies were performed by the candidate in the laboratory of Professor G. Döring, University of Tübingen, Germany. Neutrophils were prepared using Dextran sedimentation and Histopaque density gradient separation. Unstimulated neutrophils produced little O_2^- but could be induced to produce significant amounts by the chemical stimulus, PMA, or by opsonised zymosan.

All non-opsonised bacteria tested, including *P. aeruginosa* J1385, a non-mucoid CF isolate, and the *B. cepacia* strains J2315, C2040, C1524, C1555 and C1960, gave low levels of superoxide production after one hour of incubation with neutrophils. For strains J1385, J2315 and C1524, a further small increase in O_2^- production was detected after 90 min (Table 6.2). *P. aeruginosa* strain, J1385, consistently gave higher results than the *B. cepacia* strains used. However, for all isolates, absolute levels of O_2^- production were too low to signify a meaningful respiratory burst.

Table 6.2 Production of extracellular O₂⁻ anions by neutrophils stimulated with zymosan or non-opsonised bacteria.

Stimulant	Superoxide production (nmol/10 ⁶ cells)	
	60 min	90 min
Zymosan	23.26	22.71
J1385	4.42	6.05
J2315	1.16	2.65
C2040	1.22	0.75
C1524	1.97	2.86
C1444	0.82	0.82
C1960	2.11	2.04

The effect of bacterial numbers on O₂⁻ production was investigated in a further experiment using varying concentrations of non-opsonised *P. aeruginosa* J1385 and *B. cepacia* J2315 to stimulate neutrophils over a 60 min period (Fig 6.14). At all bacterial concentrations, extracellular release of O₂⁻ was markedly lower than that induced by opsonised zymosan (15.3 nmol / 10⁶ neutrophils after 15 min), and correlated in a linear fashion with the bacteria to neutrophil ratio (J1385: $r^2 = 0.989$, J2315: $r^2 = 0.945$). At all concentrations above 10 bacteria per neutrophil, J1385 induced greater O₂⁻ production than J2315. However, as bacterial cultures cannot be standardised with sufficient accuracy using optical density techniques, it is possible that the observed strain differences resulted from relatively small variations in the

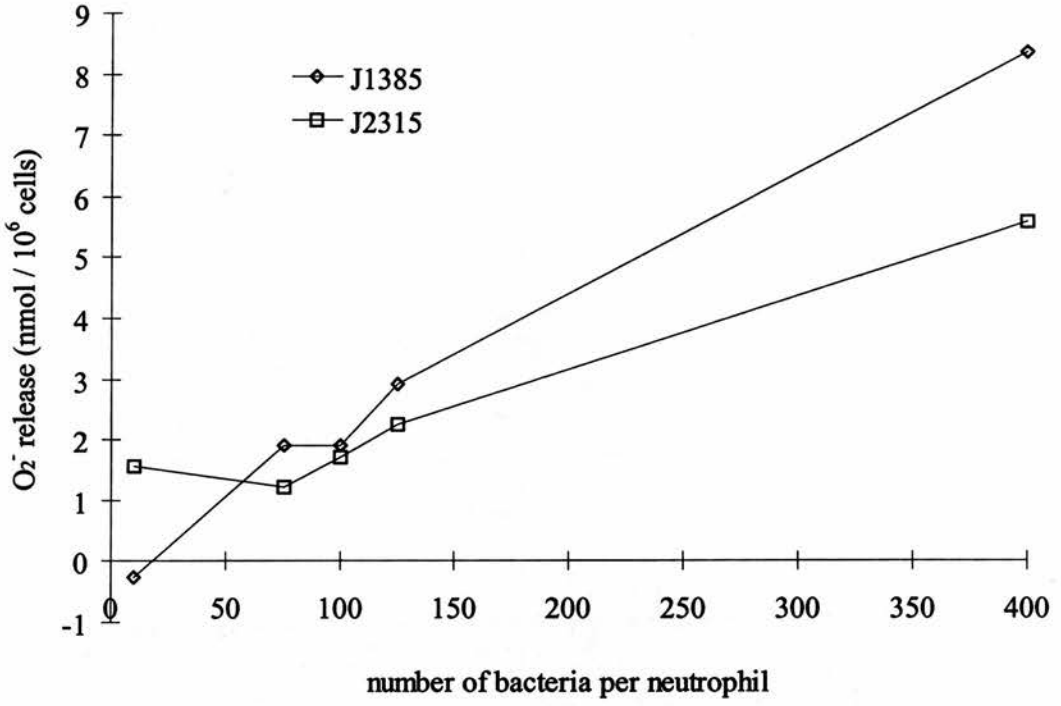


Fig 6.14 Production of extracellular O_2^- by neutrophils stimulated with non-opsonised *P. aeruginosa* J1385 and *B. cepacia* J2315 for 60 min at 37°C.

concentration of bacterial preparations. Overall, these results suggest that the interaction between neutrophils and non-opsonised bacteria is non-specific, depending only on the contact time between bacteria and neutrophils: hence increased responses occur over longer incubation periods and with higher concentrations of bacteria.

Pre-opsonisation of both *B. cepacia* J2315 and *P. aeruginosa* J1385 with immune serum was associated with a marked increase in O_2^- induction in neutrophils to levels greater than that induced by opsonised zymosan (Fig 6.15). For *B. cepacia* J2315, pre-absorption of sera with J2315 was associated with a 52% drop in O_2^- induction from 27.1 nmol/10⁶ cells to 13.1 nmol/10⁶ cells. By contrast, pre-absorption of sera with J1385 was associated with only a 13% drop to 23.7 nmol/10⁶ cells. Further investigation using serum samples from 4 CF patients colonised with *B. cepacia* and/or *P. aeruginosa* or with neither, indicated that only sera from patients colonised with the appropriate species were opsonic in this assay, since sera from two *B. cepacia*- patients did not opsonise *B. cepacia* J2315; nor did serum from one *P. aeruginosa*- patient opsonise *P. aeruginosa* J1385 (Fig 6.16). In the above assay, O_2^- production was measured at both 15 and 30 min; levels were raised after 15 min and no further increases were observed at 30 min (data not shown). Thus, the opsonic effects of serum appeared to be mediated by highly specific and non-crossreactive immunoglobulin interacting rapidly with Fc receptors on the surface of the neutrophil.

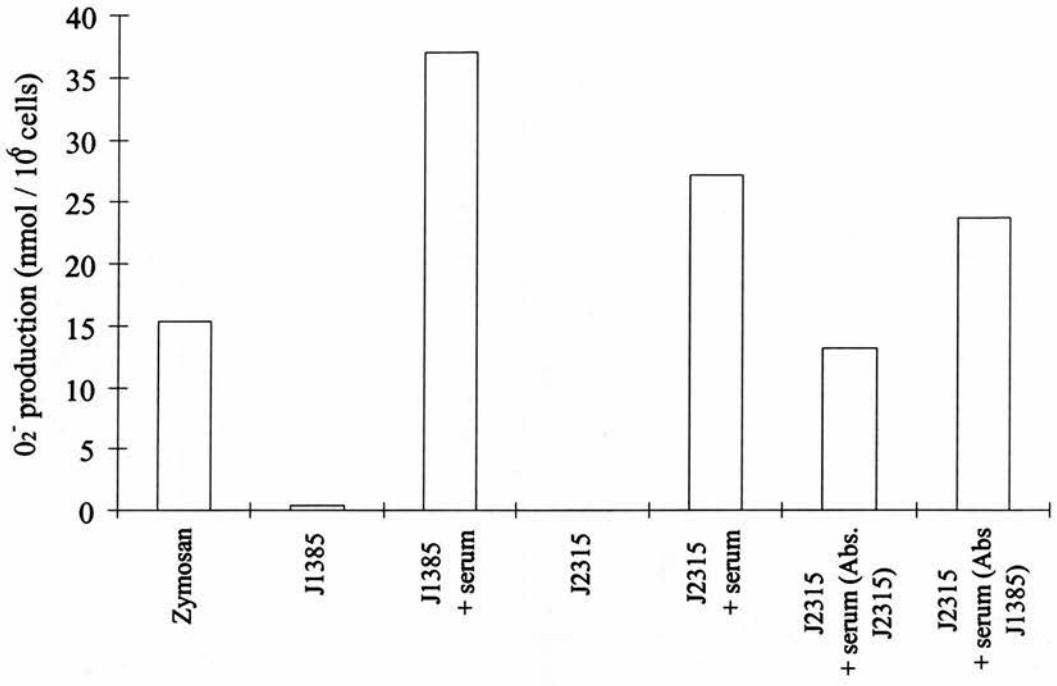


Fig 6.15 Effect of opsonisation on bacterial induction of extracellular O_2^- release by neutrophils incubated with bacteria for 30 min.

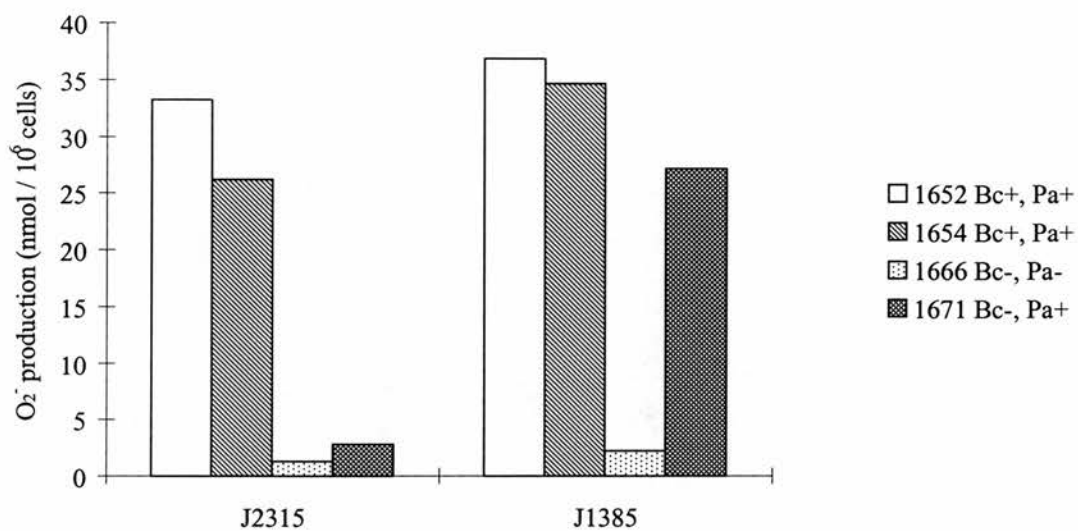


Fig 6.16 Extracellular release of O_2^- by neutrophils stimulated with *B. cepacia* J2315 or *P. aeruginosa* J1385 pre-opsonised with serum from 4 CF patients. Results are shown after 15 min of incubation. Legend key shows serum samples with colonisation status of each patient; Bc: *B. cepacia*; Pa: *P. aeruginosa*.

In a subsequent experiment, the effects of growth condition on bacterial opsonisation were investigated using *B. cepacia* J2315 and *P. aeruginosa* J1385 grown in air with shaking or in 5% CO₂ without shaking. Results are shown in Table 6.3. In all cases, the response provoked by the sera followed the same pattern as previous experiments. However for *P. aeruginosa*, growth in CO₂ was associated with an increase in opsonisation and superoxide induction whilst for *B. cepacia*, growth in CO₂ resulted in a diminution in superoxide induction.

Table 6.3 Effect of growth conditions on opsonisation of bacteria by immune sera.

Bacteria + serum ^a	O ₂ ⁻ production (nmol / 10 ⁶ neutrophils /15 min)	
	Air	CO ₂
J2315 + 1652	21.76	12.512
J2315 + 1654	18.224	9.792
J2315 + 1666	0	0.68
J2315 + 1671	0.204	0.68
J1385 + 1652	32.64	33.73
J1385 + 1654	17.54	25.77
J1385 + 1666	0.47	1.22
J1385 + 1671	13.26	20.06

^aSerum samples from 4 CF patients: 1652, *B. cepacia*+, *P. aeruginosa*+; 1654, *B. cepacia*+, *P. aeruginosa*+; 1666, *B. cepacia*-, *P. aeruginosa*-; 1671, *B. cepacia*-, *P. aeruginosa*+.

As these results may reflect changes in the bacterial outer surface, outer membrane protein (OMP) extracts were prepared for both J2315 and J1385 grown in air or in 5% CO₂. Polyacrylamide gel electrophoresis and Coomassie blue staining (Fig 6.17)

indicated a number of differences in the OMP profile of both J2315 and J1385 grown in different atmospheric conditions; notably the modification of a band at 36 kDa, the loss of bands at 63, 28 and 18 kDa and the appearance of bands at 46 and 26.5 kDa for J2315 grown in CO₂, and the loss of bands at 22 and 23.5 kDa for J1385 grown in CO₂. Surprisingly the potentially virulence-associated band at 30 kDa previously described for *B. cepacia* (Burnie et al, 1995; Shaw, 1995) was not observed in the profile of J2315, a representative of the ET12 intercontinental epidemic lineage; the nearest band being measured at 28 kDa. Repeat OMP extraction and PAGE confirmed the absence of the 30 kDa band for this strain of *B. cepacia*.

These studies of O₂⁻ production by neutrophils depended upon the spectrophotometric measurement of changes in the OD₅₅₀ of cytochrome C using a standard spectrophotometer. An attempt was made to adapt this method for use in a microtitre plate assay using an Rmax plate reader. Initial studies using neutrophil stimulants such as PMA or zymosan were encouraging as reproducible and marked changes in OD₅₅₀ could be detected. However, in subsequent experiments using opsonised bacteria the dramatic changes in OD₅₅₀ observed in our original studies could not be reproduced, and, although bacterial opsonisation was associated with slight increases in OD₅₅₀, absolute levels were too low to allow meaningful interpretation of the results. The basis for this discrepancy remains unclear but may reflect the different modes of determining optical density used by standard spectrophotometers and microtitre plate readers.

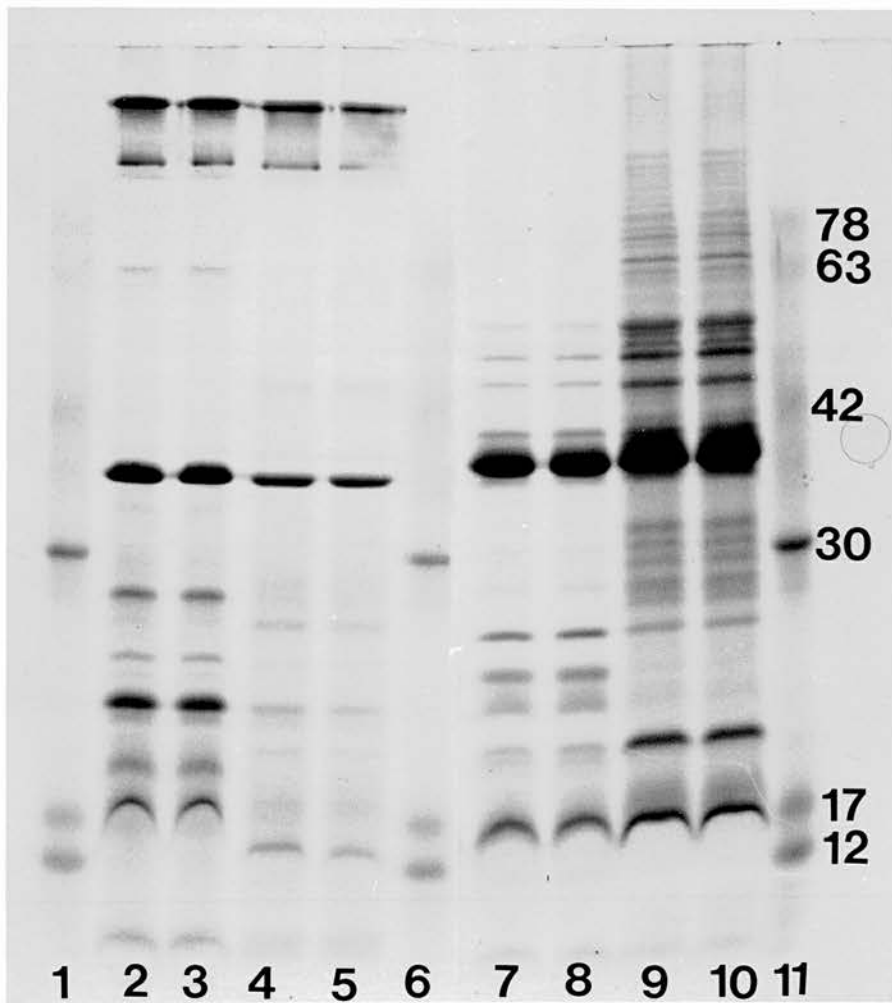


Fig 6.17 Coomassie blue-stained polyacrylamide gel showing outer membrane protein profiles of *B. cepacia* J2315 and *P. aeruginosa* J1385. Tracks are 1, 6, 11: Protein standards; 2, 3: J2315 grown in air; 4, 5: J2315 grown in air with 5% CO₂; 7, 8: J1385 grown in air; 9, 10: J1385 grown in air with 5% CO₂.

6.4 PRODUCTION OF INTRACELLULAR H₂O₂ BY NEUTROPHILS STIMULATED WITH *B. CEPACIA* AND *P. AERUGINOSA*

Dihydrorhodamine (DHR) is rapidly converted to fluorescent rhodamine in the presence of H₂O₂ and thus provides a rapid and simple means of detecting neutrophil respiratory burst production in a flow cytometric assay. As the use of flow cytometry permits the investigation of individual cell types within mixed populations, studies were carried out using whole blood cell preparations with plasma removed.

In initial studies, dose response curves to two strains of *B. cepacia*, J2315 and C2040, and two strains of *P. aeruginosa*, J1385 and H228 were generated (Fig 6.18A). Bacteria were incubated with neutrophils within washed blood at concentrations ranging from 2.5×10^6 cfu/ml to 5×10^8 cfu/ml. For all strains, neutrophil responses increased with increasing concentrations of bacteria, with little response being generated at the lowest concentration of bacteria. Considerable H₂O₂ production was detected following incubation of neutrophil with strains J1385, H228 and C2040. By contrast, responses to the ET12 strain, J2315 were limited, even at a concentration of 5×10^8 cfu/ml. To determine whether DHR responses were due to whole bacteria or released products, neutrophils were also incubated with filtrate from bacterial preparations at the highest concentration used. For strains J1385, J2315 and C2040, responses to bacterial filtrate was less than 10% responses to whole bacteria. For strain H228 however, responses to bacterial filtrate accounted for approximately 25% of the response to whole bacteria at the highest concentration (Data not shown)

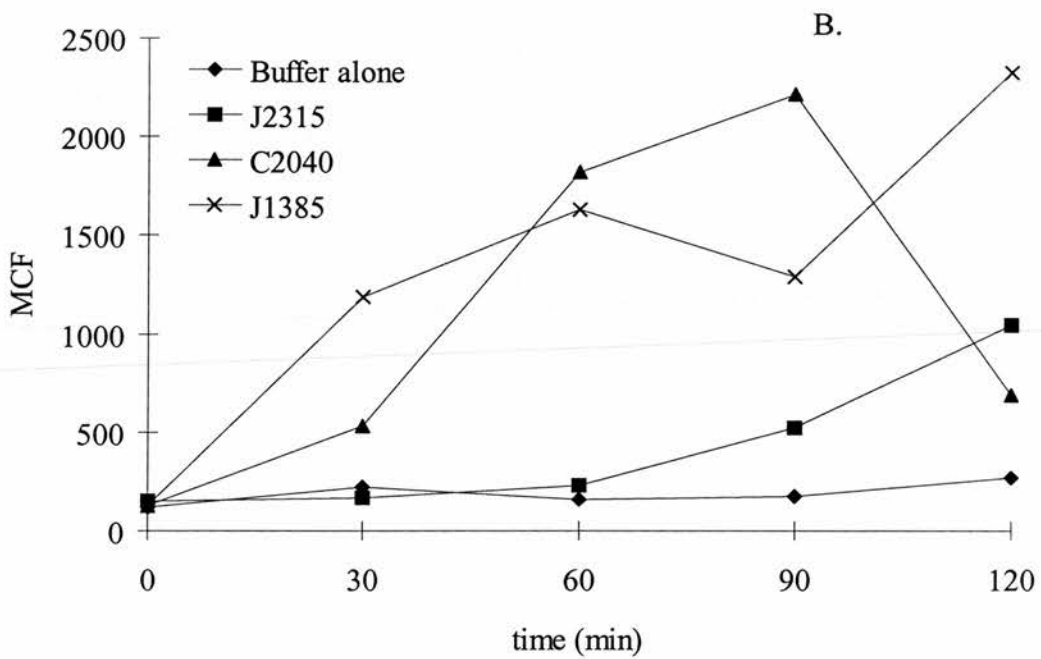
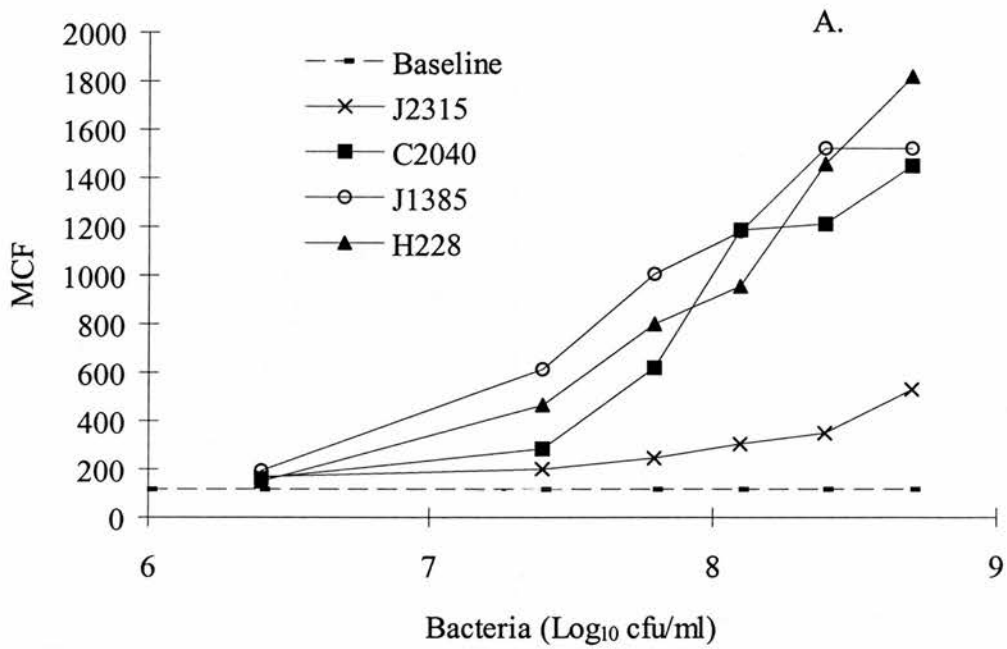


Fig 6.18 H₂O₂ production in neutrophils exposed to *B. cepacia* J2315 and C2040 and *P. aeruginosa* J1385 and H228. A. Dose response following 60 min incubation. B. Time course following incubation with 5 x 10⁸ cfu/ml bacteria. H₂O₂ production is determined as the MCF of neutrophil populations in the presence of DHR.

In time course experiments measuring the DHR response to bacteria incubated with washed blood at a concentration of 5×10^8 cfu/ml, *P. aeruginosa* J1385 was found to provoke the fastest neutrophil response with a considerable increase in intracellular H_2O_2 after 30 min incubation, whilst *B. cepacia* J2315 only induced slight H_2O_2 production after 90 min of incubation. Responses to C2040 rose after one hour of incubation, before decreasing to near baseline levels at two hours. Thus neutrophil H_2O_2 production in response to each strain followed a similar pattern to LCL induction in response to the same strains (Fig 6.1).

The above differences in *B. cepacia* J2315 and *P. aeruginosa* J1385 were confirmed in studies of LPS priming (see section 7.3, Fig 7.12), where J1385 were found to induce significantly more H_2O_2 production than *B. cepacia* J2315 following 30 min incubation with unprimed neutrophils in washed blood. In view of this finding and previous results from studies of LCL responses, a panel of CF isolates of *B. cepacia* and *P. aeruginosa* were compared for the stimulation of H_2O_2 production by neutrophils. Bacteria were incubated with washed blood for 60 min at a concentration of 5×10^8 cfu/ml. Results are shown in Fig 6.19. As for LCL results, considerable intraspecies variability in respiratory burst induction were detected. For most strains, results correlated well with LCL data, however two *P. aeruginosa* strains, H228 and C1249, which induced little LCL response were observed to induce a sizeable DHR response. Overall, *P. aeruginosa* strains appeared to induce a greater respiratory burst response than *B. cepacia* strains, although this trend failed to reach significance.

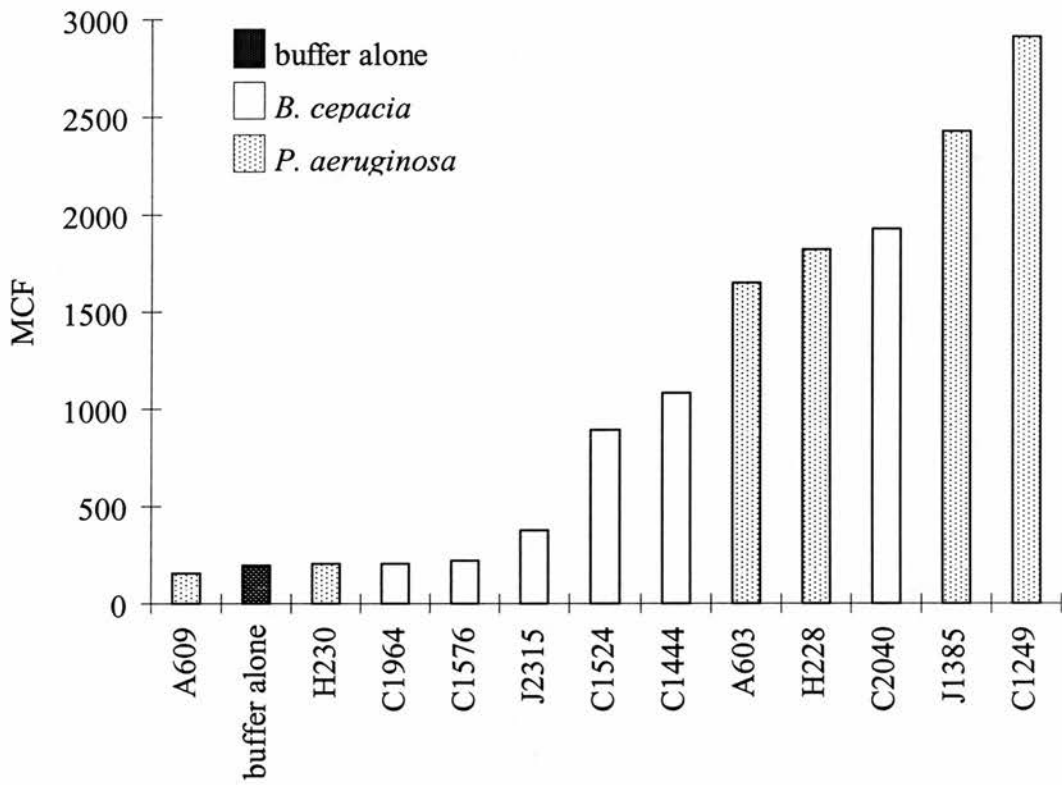


Fig 6.19 H₂O₂ production by neutrophils exposed to *B. cepacia* and *P. aeruginosa* strains at a concentration of 5×10^8 cfu/ml for a period of 60 min at 37°C. H₂O₂ production is determined as the MCF of the neutrophil population in the presence of DHR.

In the above experiment, individual bacterial strains were also compared for ability to prime neutrophil respiratory burst responses. Priming was assessed by addition of 5×10^{-7} M FMLP, 15 min before the end of the bacteria:neutrophil incubation period, and expressed as the difference between MCF in neutrophils treated with bacteria alone and MCF in neutrophils treated with bacteria followed by FMLP. Within both *P. aeruginosa* and *B. cepacia* populations considerable variation in neutrophil priming was observed, although all strains were observed to have at least a slight priming effect (Table 6.4). Overall results suggested that *B. cepacia* strains primed to a greater degree than *P. aeruginosa* strains (mean priming effect: *B. cepacia*, 717.27; *P. aeruginosa* 406.03) but this difference failed to reach statistical significance. Indeed, it is possible that the lesser priming effect seen for *P. aeruginosa* is merely a reflection of the greater respiratory burst activity induced by some *P. aeruginosa* strains, particularly if the respiratory burst induced by an individual strain approaches the maximum capacity of a neutrophil to generate H_2O_2 . However the spread of results was such that this effect did not appear to play a major role in determining the degree of priming for any individual strain.

Table 6.4 Priming of neutrophils by whole bacteria

Strain	MCF		Priming effect ^c
	Bacteria alone ^a	Bacteria + FMLP ^b	
<i>P. aeruginosa</i>			
C1249	2910.78	3035.57	124.79
H230	204.64	333.74	129.1
J1385	2430.09	2633.29	203.2
H228	1817.49	2132.88	315.39
A609	157.9	688.72	530.82
A603	1645.34	2778.23	1132.89
<i>B. cepacia</i>			
J2315	377.7	656.96	279.26
C1964	206.02	598.38	392.36
C1524	896.65	1301.55	404.9
C1444	1081.65	1847.96	766.31
C2040	1928.97	2986.91	1057.94
C1576	218.35	1621.2	1402.85

^a Washed blood incubated with bacteria at a concentration of 5×10^8 cfu/ml bacteria for 60 min in the presence of DHR.

^b Washed blood incubated with bacteria (5×10^8 cfu/ml) in the presence of DHR for 45 min, followed by the addition of 5×10^{-7} M FMLP for 15 min.

^c Determined as the difference between the MCF of neutrophils exposed to bacteria alone and the MCF of neutrophils exposed to bacteria followed by FMLP

CHAPTER 7 EFFECT OF *B. CEPACIA* PRODUCTS ON NEUTROPHILS

7.1 EFFECT OF LIPOPOLYSACCHARIDE ON NEUTROPHIL CR3 EXPRESSION

7.1.1 Lipopolysaccharide extracts

Lipopolysaccharide (LPS) was extracted from *B. cepacia* J2315 and J2552 and *P. aeruginosa* J1385 using the aqueous phenol method. Fig 7.1 shows polyacrylamide gel electrophoresis and silver staining of the above extracts and extracts of other *B. cepacia* and *P. aeruginosa* provided by Dr D Shaw and of a representative *E. coli* strain provided by Dr D Delahooke, MPRL, Medical Microbiology. LPS preparations from *B. cepacia* strains J2315 and C1504 and *P. aeruginosa* strains J1385 and C1250 showed profiles consistent with rough LPS. By contrast, clear ladder patterns could be distinguished for *E. coli* O18K- and *B. cepacia* J2540 and J2552. Less distinct ladder patterns were also visible for *P. aeruginosa* PAO1 and *B. cepacia* J2505.

7.1.2 Induction of CR3 on neutrophils stimulated with LPS from representative strains.

The effect of bacterial LPS on neutrophil CR3 expression was initially compared in washed and whole blood samples. Neutrophil cell surface CR3 was detected using anti-CD11b: FITC and results were expressed as the MCF of the neutrophil population gated on FSC and SSC characteristics. Although increased CR3 expression could be demonstrated in whole blood using lower LPS concentrations

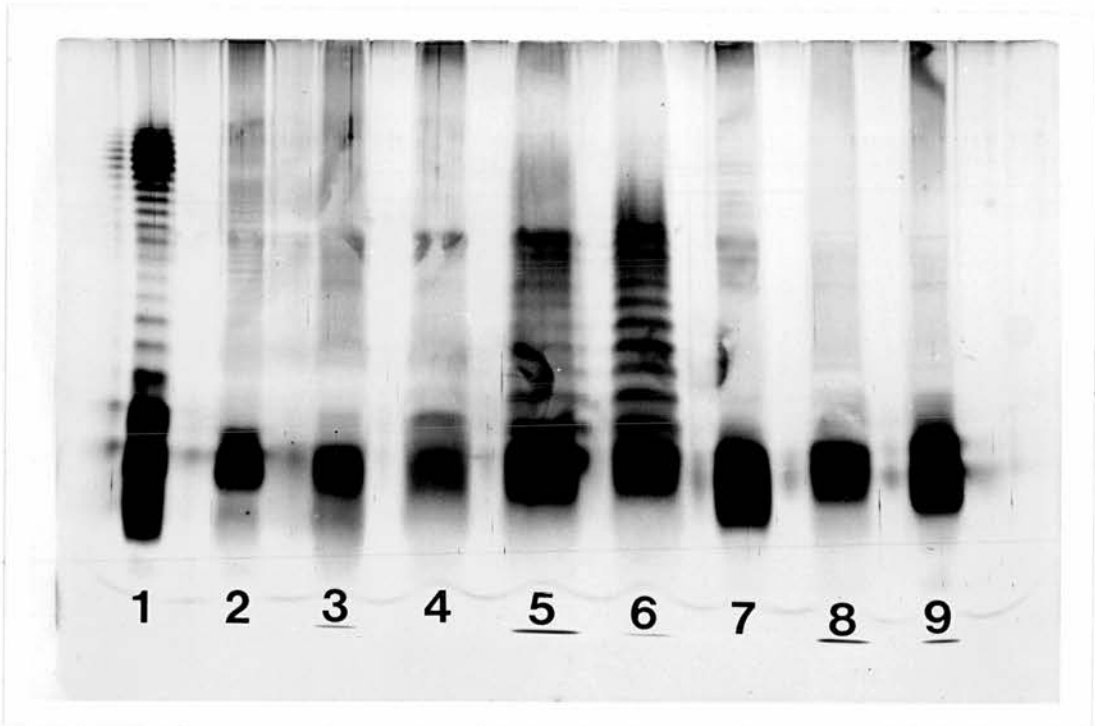


Fig 7.1 Polyacrylamide gel electrophoresis and silver staining of LPS from *B. cepacia* and *P. aeruginosa* strains. Lanes are aqueous phenol extracts of **1**: *E. coli* O18K-, **2-4**: *P. aeruginosa* PAO1, J1385 and C1250, **5-9**: *B. cepacia* J2540, J2552, J2505, J2315, and C1504.

than in washed blood, levels induced by LPS concentrations of 100 ng/ml and above were much greater in washed rather than whole blood. Further studies were therefore conducted in washed blood standard. CR3 expression amongst the washed blood neutrophil population was unimodal in distribution with MCF generally lying in the 100 - 200 region for unstimulated neutrophils (Fig 7.2). Up-regulation of CR3 following exposure to LPS was also unimodal in distribution, indicating that changes in CR3 expression were largely homogenous within the neutrophil population.

Neutrophils were exposed to LPS at a range of concentrations from 1 ng/ml to 1 mg/ml for a 30 min period. It was noted that both *B. cepacia* J2552 and J2315 LPS extracts induced a slight increase in CR3 expression at a concentration of 10 µg/ml and marked increases to at least 300% baseline levels at concentrations of 100 µg/ml and above. By contrast, LPS from *P. aeruginosa* J1385 only induced an increase in CR3 expression at a concentration of 1 mg/ml when MCF rose to 175% baseline. However as these LPS concentrations were too high to be considered physiological, further studies were carried out using lower concentrations over longer time periods. Fig 7.3 shows the effect of increasing concentrations of LPS from *B. cepacia* J2315, *P. aeruginosa* J1385 and *E. coli* O18K- on CR3 expression after two hours of incubation at 37°C. Both *E. coli* and *B. cepacia* LPS increased CR3 expression on neutrophils, although responses to *E. coli* LPS could be demonstrated at a lower concentration (1 ng/ml) than *B. cepacia* LPS (10 ng/ml). The greatest effect was seen using 100 ng/ml *E. coli* LPS where CR3 expression reached 2.2 times levels on neutrophils incubated with buffer alone.

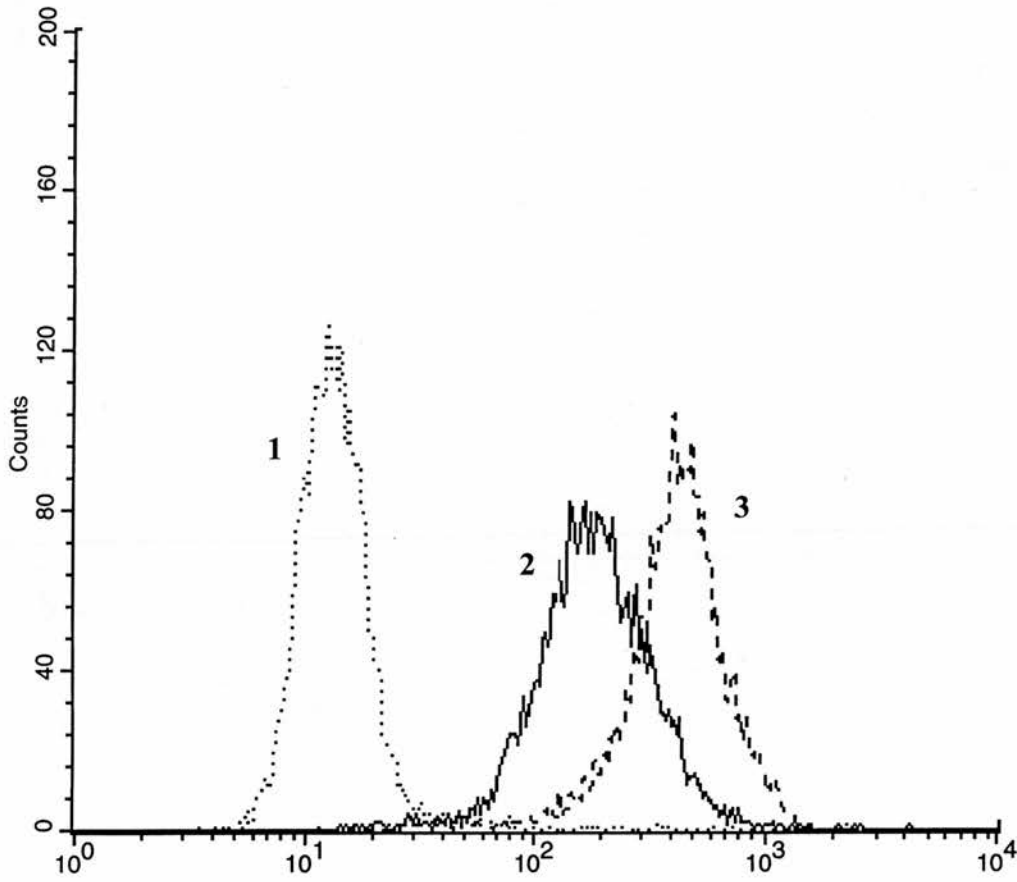


Fig 7.2 Histogram showing distribution of CR3 expression on neutrophils within a washed blood preparation. Curves are **1**: unstimulated neutrophils treated with control antibody, **2**: unstimulated neutrophils treated with anti-CD11b monoclonal antibody, and **3**: neutrophils pre-incubated with 100 ng/ml *E. coli* LPS before addition of anti-CD11b monoclonal antibody.

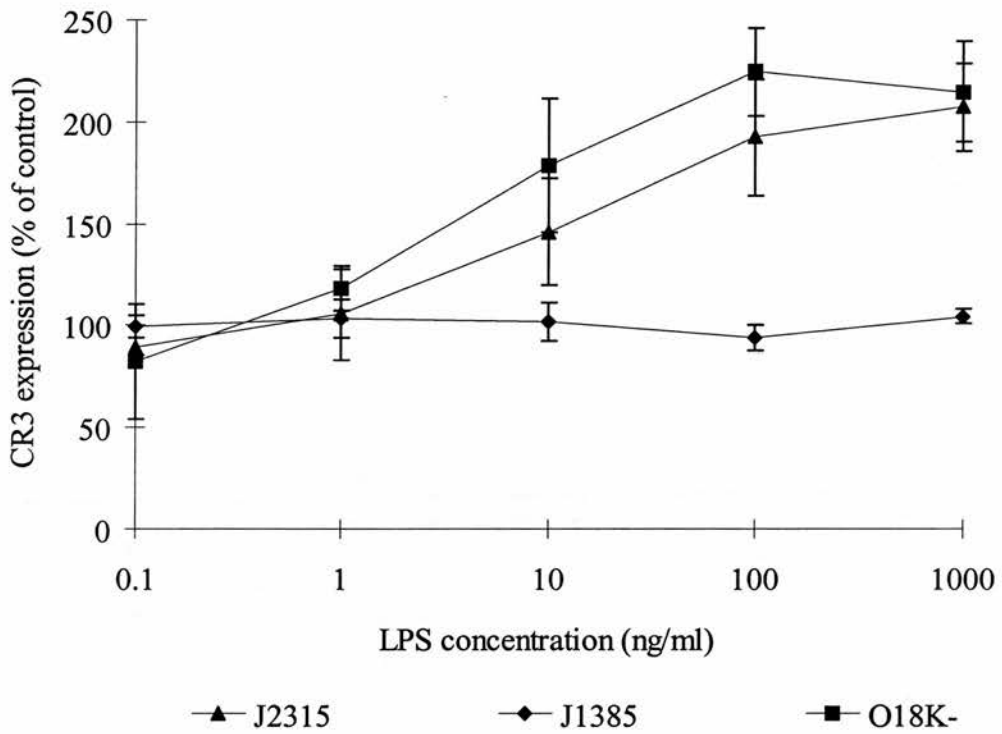


Fig 7.3 Induction of CR3 on neutrophils following stimulation with varying concentrations of LPS from *E. coli* O18K-, *B. cepacia* J2315 and *P. aeruginosa* J1385. Results are expressed as a percentage of the MCF of neutrophils treated with buffer alone for 2 hours (mean of 4 experiments \pm SEM).

At all LPS concentrations, CR3 levels induced by *E. coli* were greater than those induced by *B. cepacia*. However for both organisms, raised CR3 levels were significantly above baseline only at concentrations of 100 ng/ml and above ($p < 0.02$, Student's t test). LPS from the CF strain of *P. aeruginosa*, J1385, had no effect on neutrophil CR3 expression at all concentrations tested.

Preliminary time course experiments indicated that at low concentrations (1 ng/ml) *B. cepacia* LPS was associated with a late rise (after 3 hours) in neutrophil CR3 expression (Fig 7.4). By contrast, higher LPS concentrations induced a rise in CR3 expression to equivalent levels in under 2 hours. Subsequent experiments comparing LPS from *B. cepacia*, *P. aeruginosa* and *E. coli* were carried out using a concentration of 100 ng/ml LPS (Fig 7.5). Incubation with buffer alone was associated with a slight elevation in CR3 expression between 60 and 120 min, returning to baseline levels by 150 min. *E. coli* LPS was found to induce statistically significant levels of CR3 by 60 min ($p < 0.01$), peaking at 90 min ($p < 0.001$) with levels 2.3 times greater than those on neutrophils treated with buffer alone. Responses to *B. cepacia* LPS were slower to develop, rising to 1.8 times the levels on neutrophils treated with buffer alone by 90 min and reaching statistical significance after 120 min ($p < 0.01$). Incubation with *P. aeruginosa* LPS had little effect on CR3 expression over the entire incubation period.

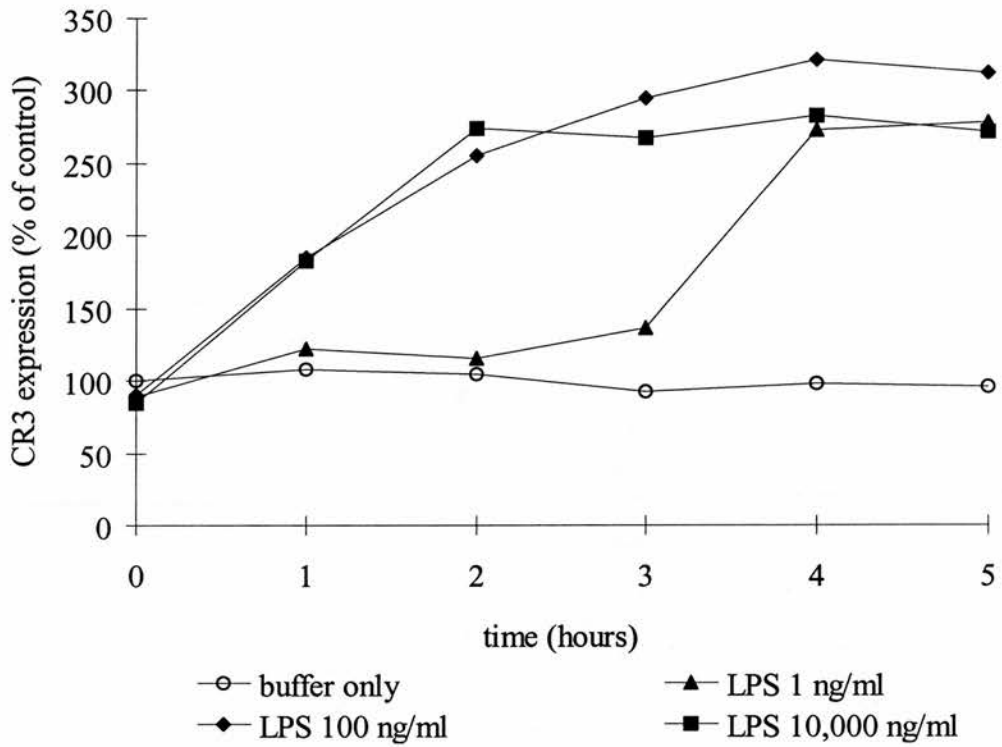


Fig 7.4 Representative time course of CR3 induction on neutrophils exposed to buffer alone, or LPS from *B. cepacia* J2315 at a range of concentrations. Results are expressed as a percentage of the MCF of neutrophils treated with buffer alone at time $t=0$.

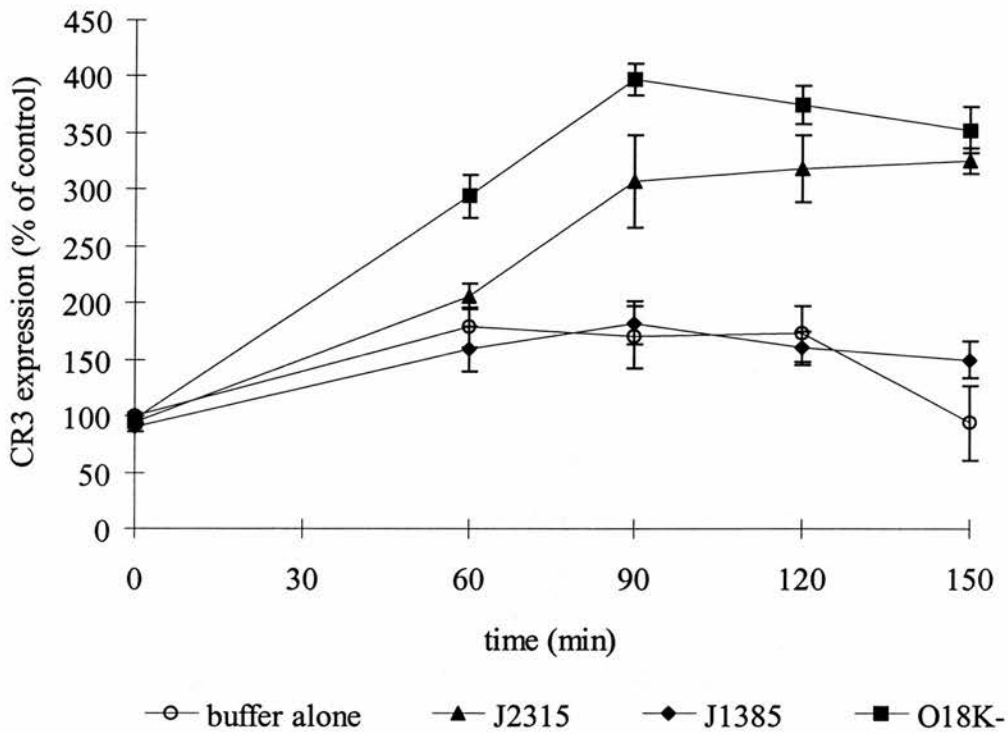


Fig 7.5 Time course of induction of CR3 on neutrophils following stimulation with buffer alone or 100 ng/ml LPS from *E. coli* O18K-, *B. cepacia* J2315 and *P. aeruginosa* J1385 at 37°C. Results are expressed as a percentage of the MCF of neutrophil treated with buffer alone at time t=0 (mean of 4 experiments \pm SEM).

7.1.3 Neutrophil CR3 induction by LPS from a range of bacterial strains

LPS samples from a wider range of *B. cepacia* and *P. aeruginosa* strains were investigated for their ability to induce CR3. Neutrophils were incubated with 100 ng/ml LPS for 90 min at 37°C before staining for CD11b. LPS from *E. coli* O18K- was included as a positive control and induced maximal CR3 expression at 270% baseline (Fig 7.6). LPS from all *B. cepacia* strains, except the environmental strain J2540, induced levels of CR3 expression comparable with those induced by *E. coli* LPS. Furthermore LPS from all *B. cepacia* strains, including J2540, induced CR3 levels which were higher than those induced by all *P. aeruginosa* LPS preparations. Of the *P. aeruginosa* strains tested, only LPS from strain PAO1, the classic laboratory strain, increased CR3 expression above that of unprimed neutrophils, whilst LPS from both a non-mucoid (J1385) and a mucoid CF strain (C1250) had no effect. Direct comparison of individual *P. aeruginosa* with *B. cepacia* strains using the Students' t test found a significant difference ($p < 0.05$) for all combinations except PAO1 and J2540. Finally, analysis of variance between mean results for *P. aeruginosa* and *B. cepacia* strains indicated a significant difference between these populations for CR3 induction ($p < 0.001$)

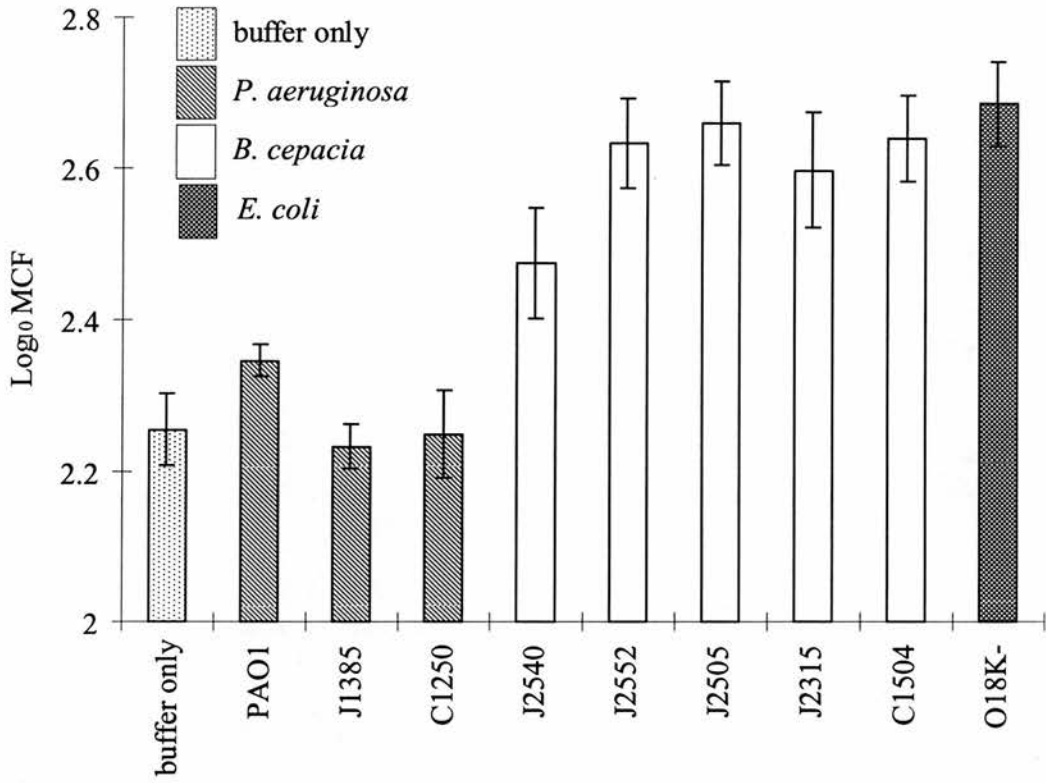


Fig 7.6 Induction of CR3 on neutrophils following 90 min incubation at 37°C with buffer alone, or 100 ng/ml LPS from strains of *P. aeruginosa*, *B. cepacia*, and *E. coli* (mean of 6 experiments \pm SEM).

7.2 PRIMING OF FMLP-INDUCED RESPIRATORY BURST ACTIVITY BY BACTERIAL PRODUCTS

7.2.1 Priming by LPS from *B. cepacia* J2315 and *E. coli* O18K-

Dihydrorhodamine was used in a flow cytometric assay to detect the presence of intracellular H_2O_2 as a measure of neutrophil respiratory burst activity. Neutrophils were primed with buffer alone or with LPS before the addition of the neutrophil stimulant FMLP. Initial studies indicated that FMLP induced little or no respiratory burst activity in unprimed neutrophils but that priming with *E. coli* LPS was associated with a marked increase in intracellular H_2O_2 production on exposure of neutrophils to FMLP, thus providing an effective assay of LPS priming activity.

Comparison of unprimed and primed neutrophils in their responses to FMLP revealed the presence of a highly responsive sub-population within the primed neutrophil population (Fig 7.7). Unstimulated neutrophils and unprimed neutrophils stimulated with FMLP were gated within the R1 region (Fig 7.7A) However, stimulation of LPS-primed neutrophils with FMLP led to the appearance of a second population of neutrophils with greater FSC but lower SSC values than the original population (Fig 7.7B, C; R2). Neither LPS-priming alone nor stimulation of unprimed neutrophils with FMLP were associated with a shift in neutrophils from the R1 to the R2 population (Fig 7.7A). In all instances, MCF for R2 populations was greater than MCF for R1 populations (Fig 7.7D-F), indicating greater H_2O_2 production within R2 cells. However, the patterns of change in MCF within each individual experiment were virtually identical for both R1 and R2 neutrophils and in

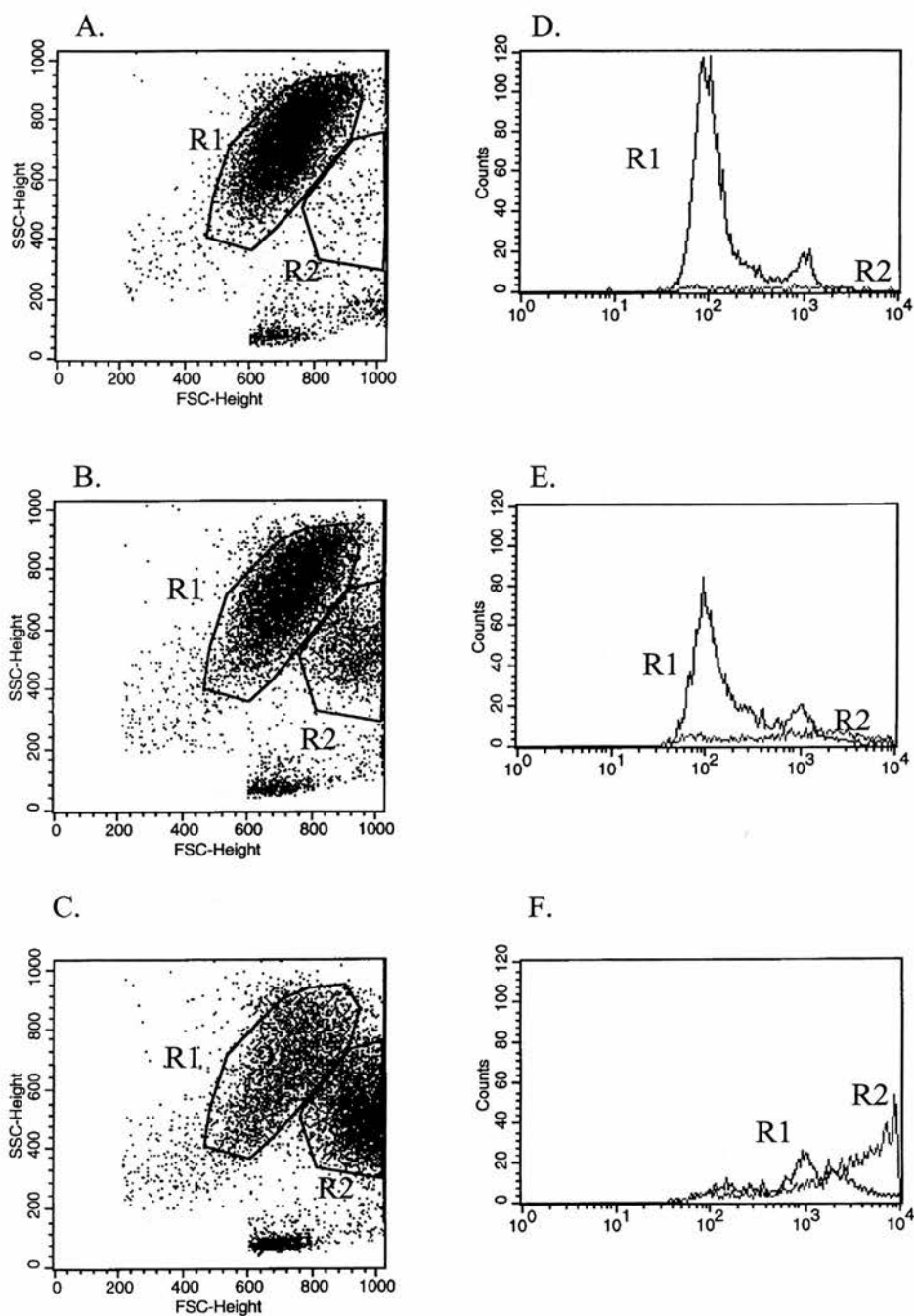


Fig 7.7 Size, granularity and intracellular H₂O₂ production of neutrophils stimulated with FMLP following pre-incubation with *E. coli* LPS. A-C: Dot plots of SSC versus FSC. Neutrophils are gated as two populations, R1 and R2. D-F: Histograms showing fluorescence of neutrophils in populations R1 and R2 in the presence of DHR. Neutrophils were pre-incubated with A, D: buffer alone; B, E: 1 ng/ml LPS; C, F: 100 ng/ml LPS.

subsequent experiments, respiratory burst activity was generally expressed as the MCF of the total neutrophil population (R1 + R2).

Both *E. coli* O18K- and *B. cepacia* J2315 LPS primed for responses to FMLP in a dose dependent manner. By contrast, *P. aeruginosa* J1385 LPS had no priming effect on neutrophils at all concentrations tested. Priming effects using *E. coli* LPS could be detected at lower concentrations (1 ng/ml) than *B. cepacia* LPS (100 ng/ml) and were greater in magnitude at all concentrations tested (Data not shown). Subsequent time course experiments were carried out using 100 ng/ml LPS as standard. Neutrophils from individual donors varied considerably in both the magnitude and timing of priming responses (Fig 7.8), but several general trends could be observed. None of the LPS preparations induced a respiratory burst in the absence of stimulation with FMLP (Data not shown). Pre-incubation with buffer alone was associated with little or no priming of responses to FMLP. However, both *E. coli* LPS and *B. cepacia* LPS were potent priming agents of a respiratory burst response, with priming occurring after as little as 30 min incubation. Responses to FMLP following stimulation with *E. coli* LPS generally peaked earlier and were of a greater overall magnitude than responses to *B. cepacia* LPS.

7.2.2 Priming of neutrophil respiratory burst responses by a panel of representative strains

In a series of experiments, priming of neutrophil responses to FMLP by LPS from representative *B. cepacia* and *P. aeruginosa* were assessed. Similar trends were observed for priming effects as for induction of CR3 expression (Fig 7.9). LPS from

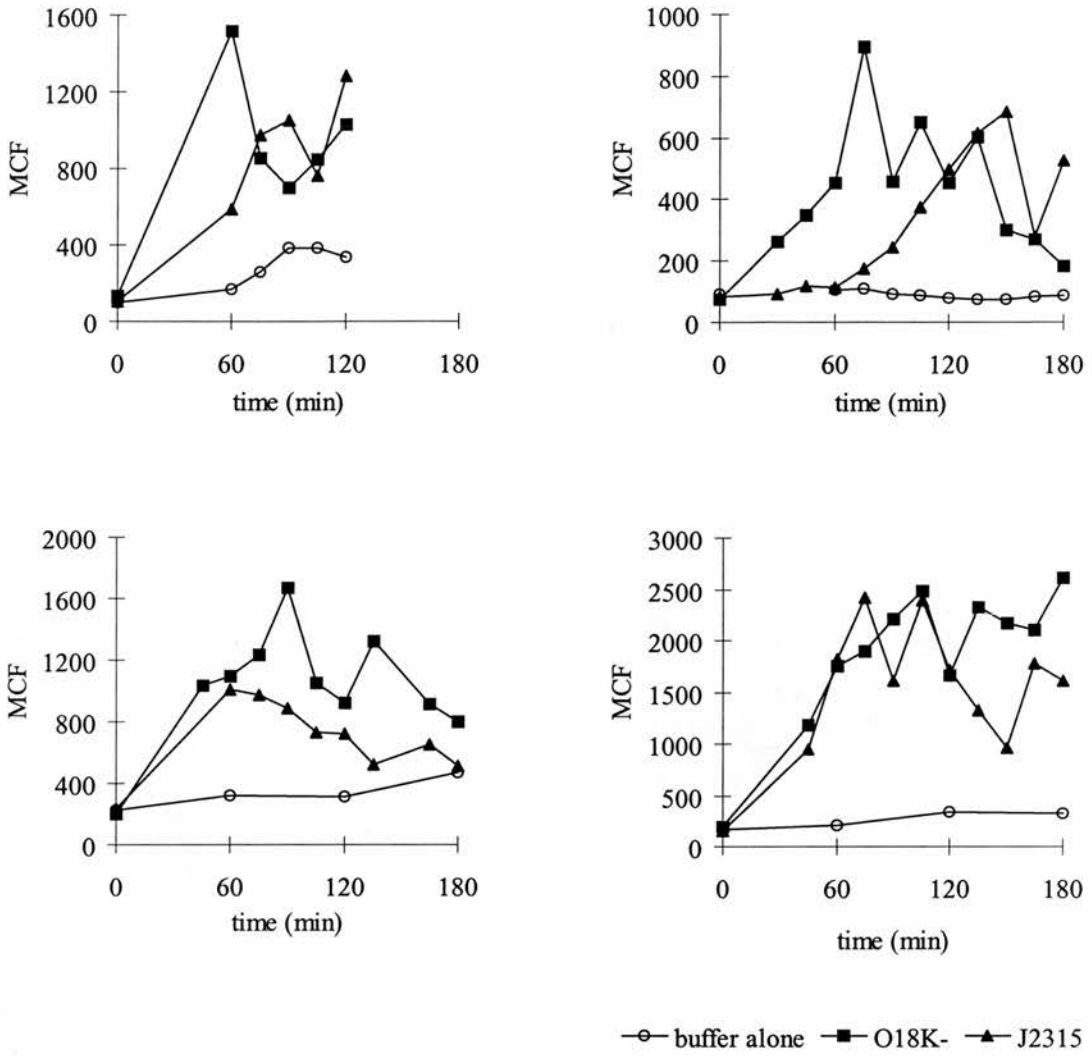


Fig 7.8 Priming of FMLP-induced neutrophil respiratory burst responses by pre-incubation with buffer alone or 100 ng/ml LPS from *E. coli* O18K- or *B. cepacia* J2315. Charts show results from studies on 4 individual donors. Results are the MCF of the total neutrophil population stimulated with FMLP in the presence of DHR.

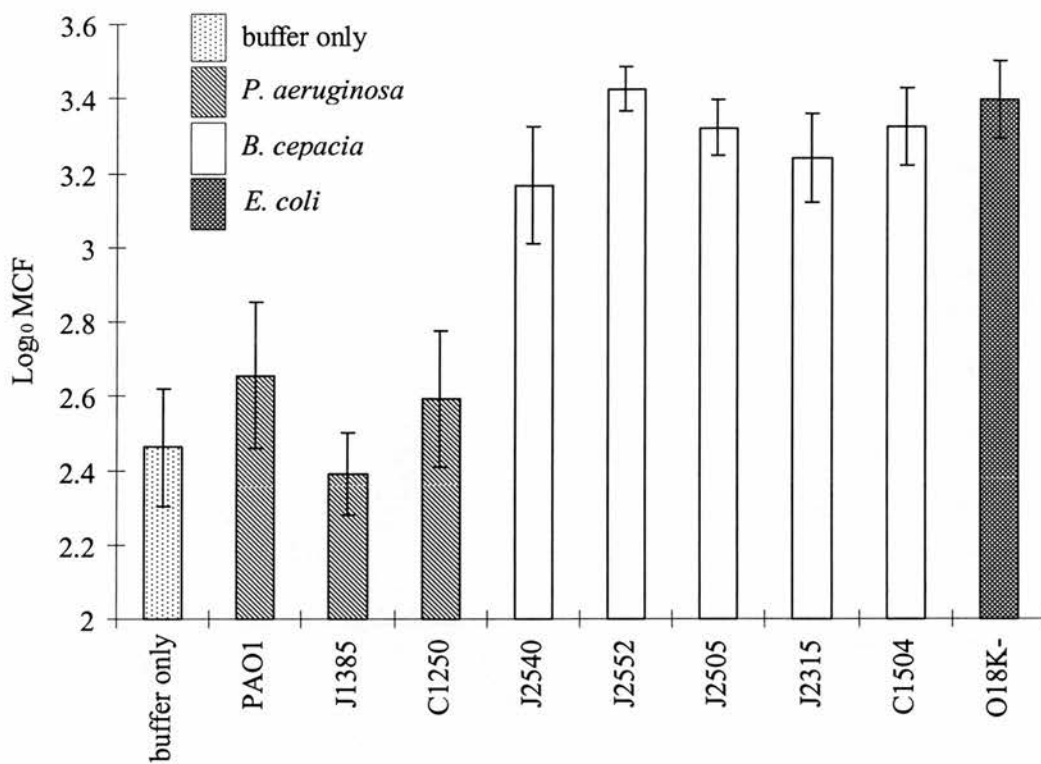


Fig 7.9 Priming of FMLP-induced intracellular H₂O₂ production in neutrophils following 90 min incubation at 37°C with buffer alone or 100 ng/ml LPS from strains of *P. aeruginosa*, *B. cepacia*, and *E. coli* (mean of 5 experiments ± SEM).

B. cepacia J2552 and *E. coli* O18K- primed FMLP responses to the greatest degree (1100% and 1041% baseline respectively). LPS from all *B. cepacia* strains primed responses to levels greater than all *P. aeruginosa* strains tested and these observations were significant for all combinations of strains except PAO1 and J2540 (Students' t test, $p < 0.05$). Although slight neutrophil priming effects were observed for LPS preparations from both *P. aeruginosa* PAO1 and *P. aeruginosa* C1250 these results were not statistically significant. LPS from *P. aeruginosa* J1385 had almost no effect on FMLP-induced respiratory burst activity compared to buffer controls. Again, analysis of variance confirmed a significant difference between LPS from the *P. aeruginosa* and *B. cepacia* populations tested ($p < 0.001$).

7.2.3 Analysis of neutrophil subpopulations

In selected experiments, R1 and R2 populations were analysed individually with respect both to MCF and the number of neutrophils in each population. In all instances, MCF activity was greatest within the R2 population, although considerable overlap in results for individual neutrophils occurred (Fig 7.7D-F). Priming with both *E. coli* O18K- and *B. cepacia* J2315 LPS increased FMLP-induced respiratory burst responses in both R1 and R2 populations in a dose dependent manner. Furthermore, LPS was associated with a shift of neutrophils from the R1 to the R2 population on stimulation with FMLP (Fig 7.10A). Movement from the R1 to R2 population was dose-dependent for both *E. coli* and *B. cepacia* LPS but at each concentration was greater for *E. coli* than *B. cepacia*. Furthermore, for priming with both *E. coli* and *B. cepacia* LPS, the increase in MCF within R2 correlated in a linear

fashion with an increase in the percentage of total neutrophils within the R2 population ($r^2 = 0.94$, $p < 0.01$) whilst the increase of MCF within R1 correlated with a decrease in the percentage of neutrophils within the R1 population ($r^2 = 0.94$, $p < 0.01$) (Fig 7.10B). Thus, LPS priming appeared to have a dual effect by firstly increasing the magnitude of responses in both R1 and R2 neutrophils and secondly by shifting more neutrophils into the highly responsive R2 population.

The nature of the phenotypic change underlying the shift from R1 to R2 populations by neutrophils was unclear from the above experiments. The observation of an increase in MCF in both R1 and R2 populations, and the overlap in MCF values for neutrophils within both populations suggested that the R2 phenotype was not merely a result of respiratory burst induction *per se*. Comparison of mean CR3 expression and mean intracellular H_2O_2 induction in total neutrophil populations stimulated with LPS from a range of strains (Fig 7.6, 7.9) revealed a close correlation between both parameters ($R^2 = 0.96$, $p < 0.01$). However, the skewed distribution of fluorescence values in experiments using DHR was in sharp contrast to the unimodal distribution of increased CR3 expression on neutrophils incubated with LPS (Fig 7.2, 7.7), suggesting that a simple increase in generalised receptor expression was not responsible for the R2 phenotype. An attempt was made to develop the DHR technique for two-colour fluorescence experiments to allow comparison of CR3 expression and H_2O_2 production in individual neutrophils. Unfortunately, however, the overlap in the emission spectra between DHR and FITC was too great to allow meaningful data to be collected in this way.

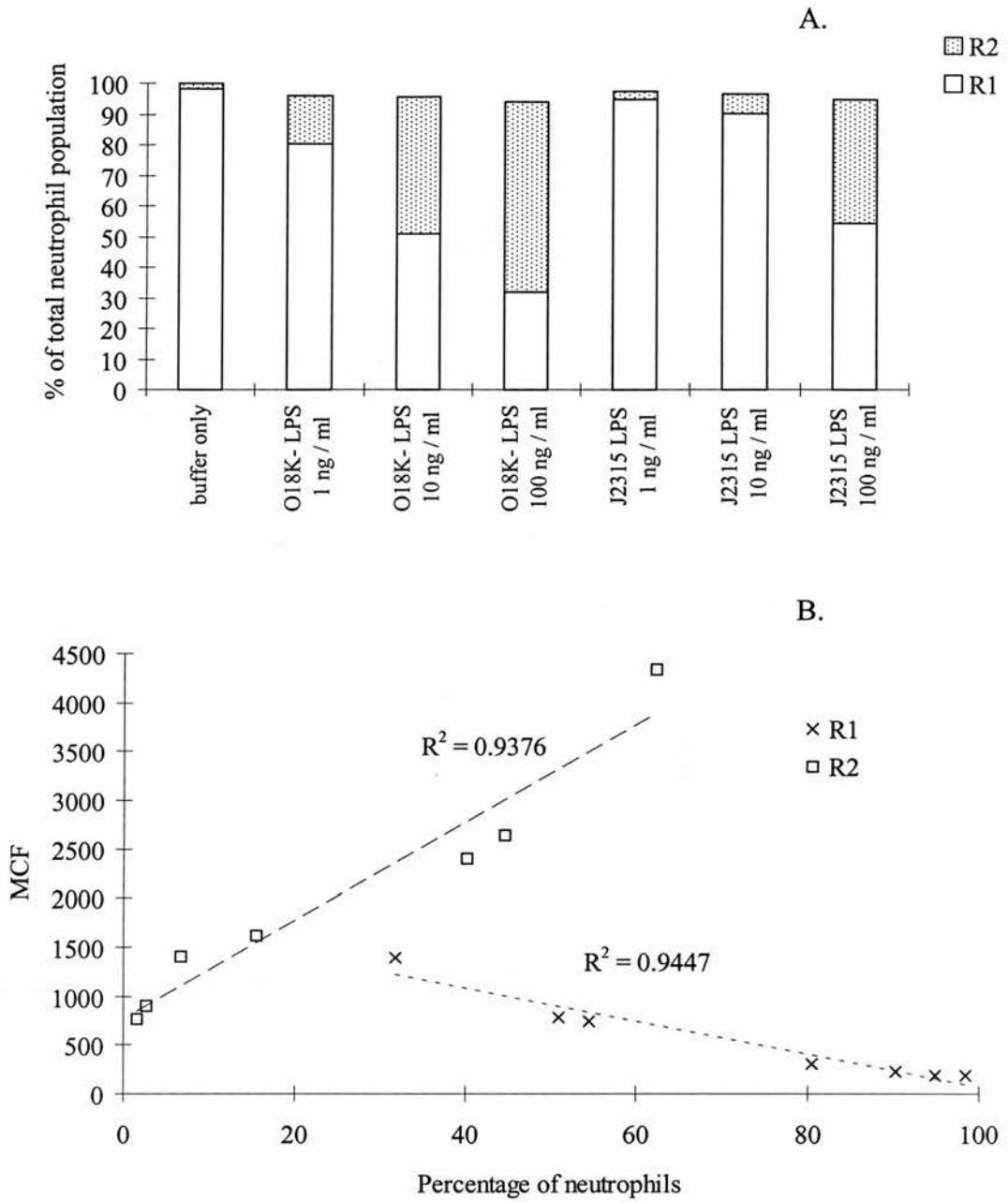


Fig 7.10 Effect of LPS priming on the production of H_2O_2 by neutrophils following exposure to FMLP. A. Percentage of neutrophils falling within the R1 and R2 populations following 15 min incubation with FMLP. B. Relationship between percentage of neutrophils within the R1 and R2 populations and H_2O_2 production, expressed as the MCF in the presence of DHR.

7.2.4 Effect of *B. cepacia* J2315 haemolysin on neutrophil respiratory burst activity

Neutrophils were pre-incubated with *B. cepacia* J2315 haemolysin at concentrations of 50-500 ng/ml for 90 min before addition of either buffer alone or 5×10^{-7} M FMLP in the presence of DHR for 15 min. Haemolysin alone induced no respiratory burst activity but did, however, prime responses to FMLP in a dose-dependent manner (Fig 7.11). At all concentrations from 50 ng/ml to 500 ng/ml, haemolysin primed neutrophil activity to levels greater than pre-incubation with 100 ng/ml *B. cepacia* J2315 LPS.

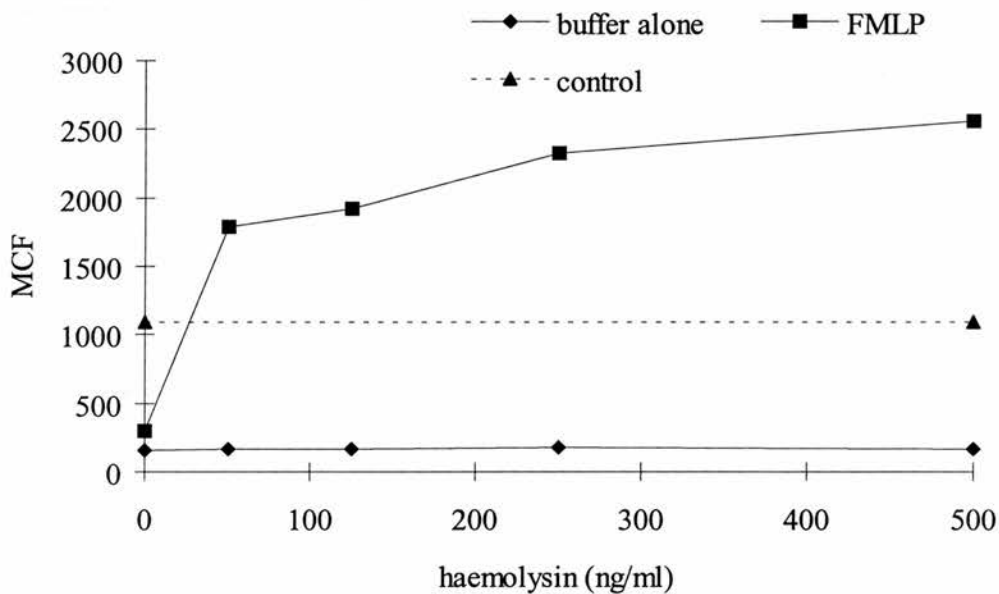


Fig 7.11 Neutrophil respiratory burst activity following pre-incubation with *B. cepacia* J2315 haemolysin. Neutrophils were pre-incubated with haemolysin for 90 min at 37°C before addition of either buffer alone or 5×10^{-7} M FMLP. Control neutrophils were pre-incubated with 100 ng/ml *B. cepacia* J2315 LPS before addition of FMLP. Intracellular H_2O_2 production was detected as increased MCF in the presence of DHR.

7.3 PRIMING OF NEUTROPHIL RESPIRATORY BURST RESPONSES TO WHOLE BACTERIA

In a series of experiments, the effect of LPS on neutrophil respiratory burst responses to whole bacteria were compared. Neutrophils within washed blood were pre-incubated with buffer or with 100 ng/ml LPS for 90 min at 37°C before addition of DHR and either buffer alone, bacteria to a final concentration of 2.5×10^7 /ml, supernatant from filtered bacterial suspensions or FMLP. Results expressed as MCF of neutrophil populations were transformed by conversion to Log_{10} . Only *P. aeruginosa* J1385 induced a statistically significant respiratory burst in non-primed neutrophils ($p < 0.01$, Student's t test) with an average MCF of 425.1 against an average baseline MCF of 143.9 (Fig 7.12). Neither *B. cepacia* J2315, nor supernatants from *B. cepacia* and *P. aeruginosa* suspensions, nor FMLP induced a respiratory burst in unprimed neutrophils. However, as previously, significant respiratory burst activity was seen in response to FMLP in neutrophils primed with *E. coli* O18K- and *B. cepacia* J2315 LPS but not *P. aeruginosa* J1385 LPS. Responses to *P. aeruginosa* were significantly greater in neutrophils primed with LPS from both *B. cepacia* and *E. coli* than in unprimed neutrophils, with average MCF values of 1039.9 ($P < 0.05$) and 1374.5 ($p < 0.05$) respectively. By contrast, LPS priming induced only slight increases in responses to *B. cepacia* J2315. Furthermore, the observation of a similar small increase in responses to *B. cepacia* filtrate indicated that any respiratory burst activity induced by *B. cepacia* was due to extracellular factors rather than the bacteria themselves. Pre-incubation of neutrophils with LPS from *P. aeruginosa* J1385 had no effect on either FMLP- or *P. aeruginosa*-induced respiratory burst activity.

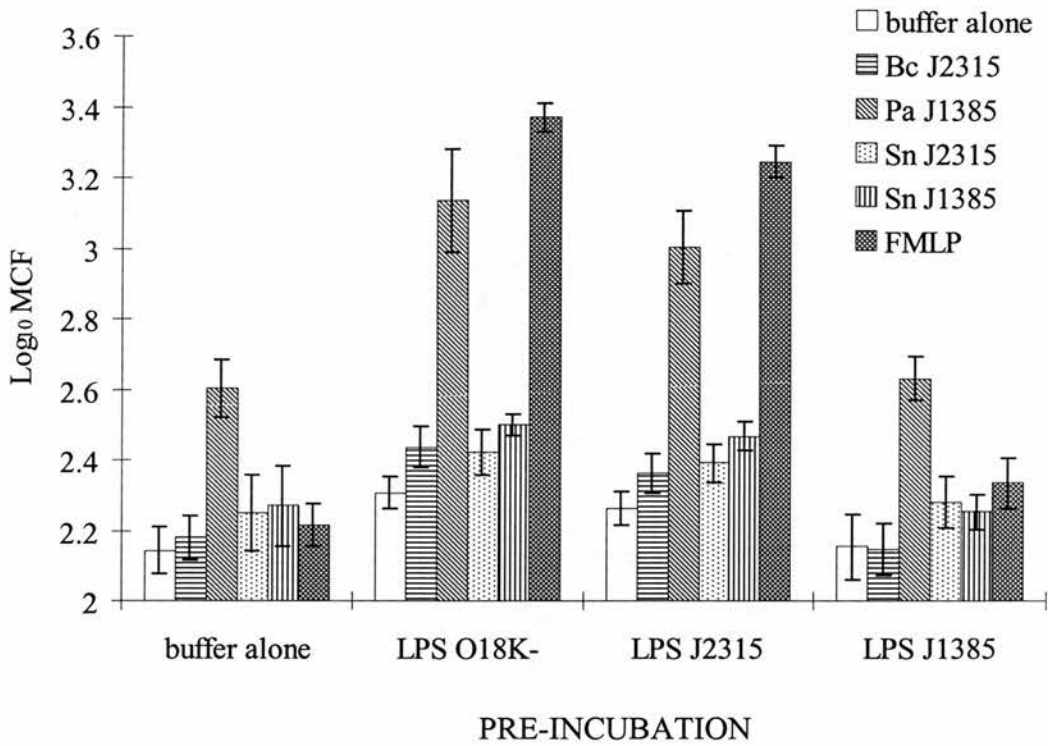


Fig 7.12 Effect of LPS-priming on neutrophil respiratory burst responses to whole *B. cepacia* J2315 and *P. aeruginosa* J1385. Neutrophils were pre-incubated with LPS or buffer alone for 90 min prior to addition of buffer alone, whole bacteria, supernatant from bacterial suspensions or FMLP. Data was transformed by conversion to Log_{10} . Results are the mean of three experiments \pm SEM. Legend key shows stimuli for primed neutrophils: Bc, *B. cepacia*; Pa, *P. aeruginosa*; Sn, bacterial supernatant.

CHAPTER 8 STUDIES OF *B. CEPACIA* IN A MOUSE MODEL

8.1 BACKGROUND FLORA AND DELIVERY OF BACTERIA

8.1.1 Background flora

Studies carried out by previous investigators indicated considerable variability in the background respiratory flora of mice housed in standard conditions. Thus, in an early study, respiratory flora of three strains of mice were compared with mice of the CF_{HGU} lineage by plating out of lung homogenates (Table 6.1). For each strain of mouse considerable background flora was apparent. Although the variety and number of bacteria varied considerably from mouse to mouse, strain-specific patterns of colonisation could be detected. Both inbred murine strains, C129 and C57/B1, generally had heavy contamination of the respiratory tract, particularly with Gram-negative organisms, including coliforms. Mice of strain C129 differed from C57/B1 mice with heavy colonisation by non-coliform Gram-negative organisms.

Furthermore those organisms identified as coliforms for both C129 and C57 mice were distinct for each strain. Unlike inbred strains, an outbred mouse strain, MF1, was predominantly colonised by streptococcal organisms with only one mouse showing slight growth of coliform organisms. CF_{HGU} mice showed a mixed pattern of growth with high numbers of both streptococcal and coliform organisms in three mice whilst a fourth mouse showed only an occasional staphylococcal organism. For all strains little staphylococcal colonisation was apparent.

Table 8.1 Background flora of 4 strains of mice

		<i>Staphylococci</i>	<i>Streptococci</i>	Coliforms ^a	Other ^b
C129	1				++++ GNB (Mac ⁻) ++++ GNC
	2	+ ^c	++	+	++ GNC
	3	+	++		++ GNC
	4		++	++	
C57	1	+			
	2	++		+++	
	3	++	+++	++++	
	4	++	++++	+++	
MF1	1	+	++++		
	2	+	++++		
	3	+	+++	++	
	4	+			
CF _{HGU}	1		++++	++++	
	2		++++	++++	
	3		++++	+++	
	4	+			

^a Defined as Gram-negative bacilli which grow on MacConkey's agar.

^b GNB: Gram-negative bacilli; GNC: Gram-negative cocci; Mac⁻: No growth on MacConkey's agar.

^c + < 100 cfu / lung, ++ 101-1000 cfu / lung, +++ 1001-10,000 cfu / lung,
++++ > 10,000 cfu / lung.

In view of the variety and degree of colonisation of the respiratory tract in mice within standard housing conditions, a further experiment was carried out to compare mice from standard housing and from the SPF housing within the transgenic animal unit (TGU). Results are shown in Table 8.2. For both standard and TGU mice, overall levels of background flora were much lower than those observed in the previous study, with little evidence of coliform (GNB) colonisation and only small numbers of staphylococcal and streptococcal organisms. This difference may reflect changes in practices within the animal house or in the source of the mice used; significantly all of the mice used were bred in-house rather than imported from other animal units.

Although overall colonisation levels were low, two unusual organisms; filamentous bacteria and yeast-like organisms; were detected in three mice from the TGU and one mouse from standard housing. Growth of both organisms was much heavier in the mouse from standard housing conditions. Furthermore in a comparison of nasal lavage, tracheal homogenates and lung homogenates for TGU mouse 1, yeast-like organisms were only cultivated from nasal lavage and tracheal samples but not lung homogenates, suggesting that the low numbers of organisms in other TGU mice may arise from contamination of lung homogenates with intra-tracheal bacteria rather than lower respiratory tract colonisation *per se*. However for mouse 5 from standard housing, high counts of yeast-like and filamentous organisms were present in tracheal and lung homogenates, indicating genuine lower respiratory tract colonisation.

Table 8.2 Comparison of mice in standard animal house and transgenic unit (TGU).

Housing	Mice ^a	Staphylococci	Streptococci	GNB ^b	Other
TGU	1 N	+		++	+ ? yeast
	1 T	+			+ ? yeast
	1 L				
	2	+			+ ? yeast + filamentous
	3	+			+ ? yeast
	4	+			
Standard	5 N	+	++	+	
	5 T	++	+	+	+++ ? yeast +++ filamentous
	5 L	+		+	+++ ? yeast +++ filamentous
	6 N	+			
	6 T	+		++	
	6 L	+			
	7	+		+	
	8	+			

^a Mice strains were heterozygote CF_{HGU} and CF_{UNC}. For mice 1, 5 and 6, samples include N: nasal lavage, T: tracheal homogenate and L: lung homogenate. For other mice, samples are homogenates of lungs plus trachea.

^b Gram-negative bacilli

8.1.2 Intratracheal inoculation of bacteria: delivery and bacterial recovery

Surgical and non-surgical techniques were compared in order to identify the most effective and reproducible method of intratracheal inoculation of bacteria with minimum harm to the mouse. Mice were inoculated with *B. cepacia* J2315 and delivery assessed by removal of both lungs and stomach 5 - 10 minutes after inoculation. In initial studies surgical inoculation was more reliable than non-surgical inoculation, with no delivery failures (defined as recovery from lungs < 1% total bacteria) for surgical techniques but 3 out of 8 for non-surgical techniques. On exclusion of delivery failures, surgical inoculation was associated with higher delivery to the lungs but lower delivery to the stomach (Lungs: mean 21.8%, range 9.1% - 37.1%; Stomach: mean 0.2%, range <0.1% - 1.2%) than non-surgical inoculation (Lungs: mean 14.5%, range 5.6% - 35.4 %, Stomach: mean 3.1%, range <0.1 - 6.7%). However it was noted that non-surgical delivery improved both in terms of greater bacterial recovery from the lung and fewer delivery failures with the experience of the inoculator and that there was considerable overlap between the ranges of percentage recovery for surgical and non-surgical techniques. Reliability was further improved by the development of a frame to hold anaesthetised mice in an upright position whilst illuminating the trachea via a cold light source. Given that non-surgical intubation involved much less trauma to the mouse, further experiments were conducted using non-surgical intratracheal inoculation as standard.

8.2 CLEARANCE OF *B. CEPACIA* J2315 IN CF AND NON-CF MICE

Clearance of *B. cepacia* J2315 after intratracheal inoculation in non-CF mice was determined in Swiss mice. Mice were inoculated with a high dose of *B. cepacia* (1.25×10^7 bacteria) and kept in isolated conditions until culling. *B. cepacia* were recovered from lung homogenates in 1 ml PBS onto cepacia medium. Results are shown in Fig 8.1. Rates of bacterial recovery immediately post-inoculation were low but rose by over 100-fold by 1 day post inoculation. Subsequently levels dropped until 5 days post-inoculation when no mice remained colonised.

A time point of 48 hours was chosen for further clearance experiments comparing CF and non-CF mice from the standard animal house. Mice were a combination of CF_{HGU} and CF_{UNC} wild-type, heterozygote, compound heterozygote, and homozygote mice. Following intratracheal inoculation with 2.5×10^7 bacteria, mice were transferred to isolators for 48 hours before culling and plating out of lung homogenates. Results from three experiments are summarised in Fig 6.2 and in Table 8.3.

Recovery of *B. cepacia* J2315 varied considerably both between and within individual experiments. In experiments 1 and 3, average bacterial recovery was lower from non-CF than CF mice. By contrast, in experiment 2, two CF mice compared to only one non-CF mouse had completely cleared *B. cepacia* by 48 hours post-inoculation, resulting in a higher average recovery for non-CF than CF mice. Attempts at statistical analysis were confounded by the high degree of variability from experiment to experiment, particularly as numbers of mice within individual

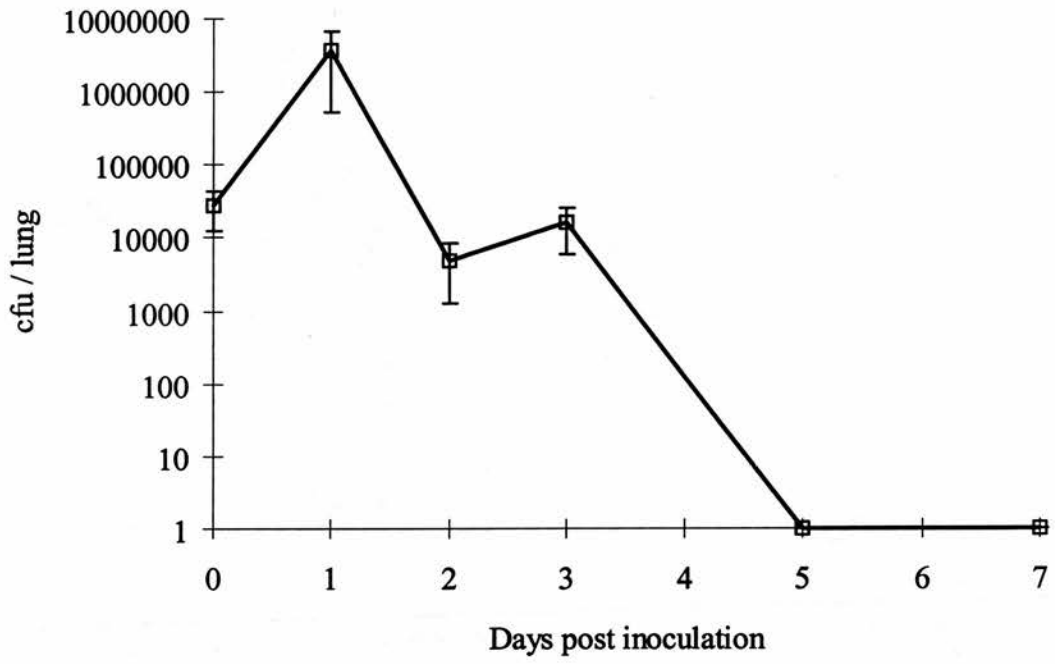


Fig 8.1 Recovery of *B. cepacia* J2315 from lung homogenates of Swiss mice inoculated with 1.25×10^7 bacteria at time $t=0$.

experiments were low. However the overall trend of results was towards a higher bacterial recovery and hence poorer clearance in CF than non-CF mice. It may be relevant to note that in each experiment, mice demonstrating the poorest clearance were always CF rather than non-CF mice.

Table 8.3. Recovery of *B. cepacia* J2315 from the lungs of mice inoculated intratracheally with 2.5×10^7 bacteria, 48 hours previously.

Experiment	CF		Non-CF	
	N ^o of mice	Mean cfu/lung (range)	N ^o of mice	Mean cfu/lung (range)
1	4	8.7×10^3 ($5.4 \times 10^3 - 1.2 \times 10^4$)	4	1.3×10^3 ($0 - 5.1 \times 10^3$)
2	3	3.7×10^3 ($0 - 1.1 \times 10^4$)	3	6.7×10^3 ($0 - 1.0 \times 10^4$)
3	3	6.7×10^6 ($3 \times 10^3 - 1 \times 10^7$)	4	9.6×10^5 ($5.1 \times 10^3 - 3.5 \times 10^6$)

The variability between individual mice in the above experiments may reflect differences in the background flora of animals reared within the standard animal house. Thus a further clearance experiment was undertaken using CF mice and non-CF littermates from the TGU. Results are shown in Fig 8.3. As for previous experiments a wide range of values was obtained within both populations, with the highest bacterial recovery occurring in two CF mice. Although the trend was again towards poorer clearance by CF mice, results failed to reach statistical significance (CF: mean 2.6×10^4 , range $5.6 \times 10^3 - 6.3 \times 10^4$; non-CF: mean 1.1×10^4 , range $3.2 \times 10^3 - 1.6 \times 10^4$).

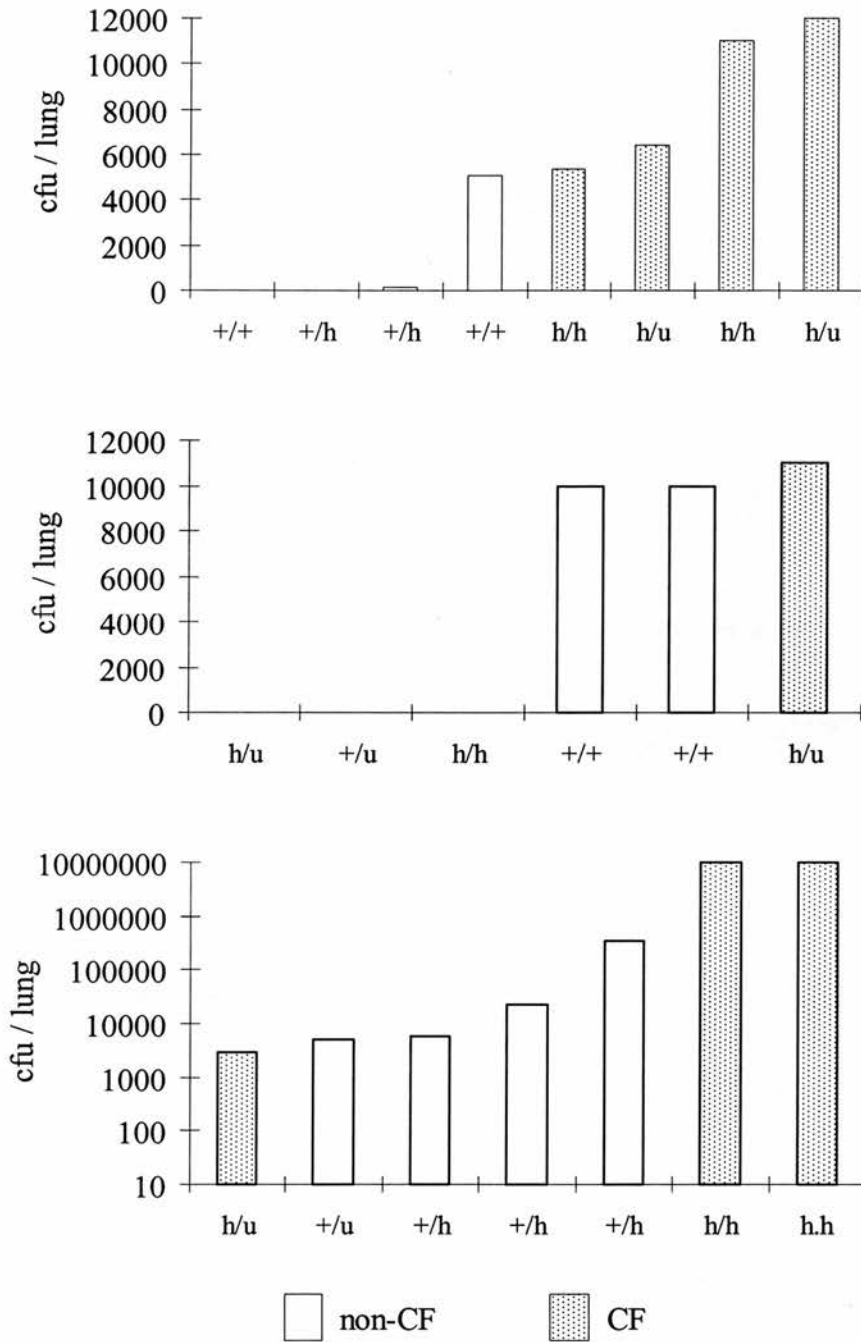


Fig 8.2 Clearance of *B. cepacia* J2315 by CF and non-CF mice from the standard animal house. Results are from three experiments and show bacterial recovery from mice lungs, 48 hours after intratracheal delivery of 2.5×10^7 bacteria. Note the differences in scale for cfu/lung. Genotypes: + - wildtype, h - CF_{HGU}, u - CF_{UNC}.

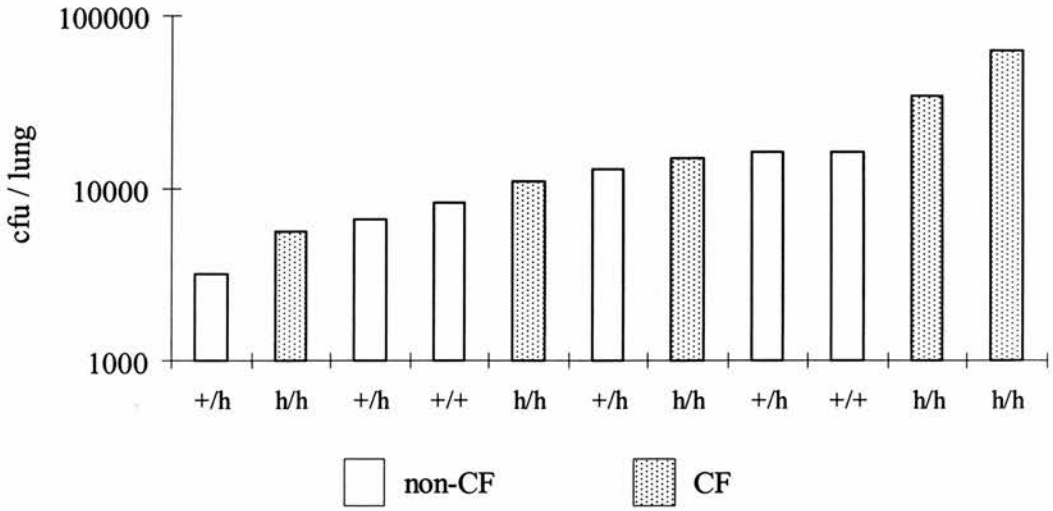


Fig 8.3 Clearance of *B. cepacia* J2315 by CF and non-CF mice from the TGU. Results show recovery of bacteria from mice lungs 48 hour after intratracheal delivery of 2.5×10^7 bacteria. Genotypes: + - wildtype, h - CF_{HGU}, u - CF_{UNC}.

8.3 CLEARANCE OF ENVIRONMENTAL AND CLINICAL *B. CEPACIA*

Clearance of *B. cepacia* J2315 and the environmental *B. cepacia* J2552 were compared in wild-type and non-CF heterozygote mice from the TGU CF breeding programme. Mice were inoculated intratracheally with 2.5×10^7 bacteria per mouse. Clearance was assessed after 48 hours by plating out of lung homogenates. Results of two experiments involving a total of 24 mice are summarised in Fig 8.4. Mean recovery of *B. cepacia* J2552 was 2.4×10^6 cfu/lung (range 8.4×10^4 - 5.9×10^6) whilst mean recovery of *B. cepacia* J2315 was 2.9×10^4 cfu/lung (range 3.2×10^3 - 9.2×10^5). Thus, recovery of *B. cepacia* J2552 was on average 80 times that of *B. cepacia* J2315 and this difference was significant at a 1% level (Students t test, $p < 0.01$).

In a further experiment, clearance of J2552 and J2315 were compared in CF TGU mice. Mean recovery of J2552 from three mice was 6.2×10^4 (range 5.2×10^4 - 8.1×10^4) whilst mean recovery of J2315 from 4 mice was 6.2×10^3 (range 1.9×10^3 - 1.5×10^4). Despite the small numbers of mice involved, the 10 fold difference in recovery of J2552 from recovery of J2315 was statistically significant (Students t test, $p < 0.01$). Surprisingly, whilst clearance of J2315 was similar in experiments involving non-CF or CF mice, clearance of J2552 appeared to be greater in CF than non-CF mice. However direct comparison of J2552 clearance in CF and non-CF mice in a single experiment would be necessary to confirm this apparent difference.

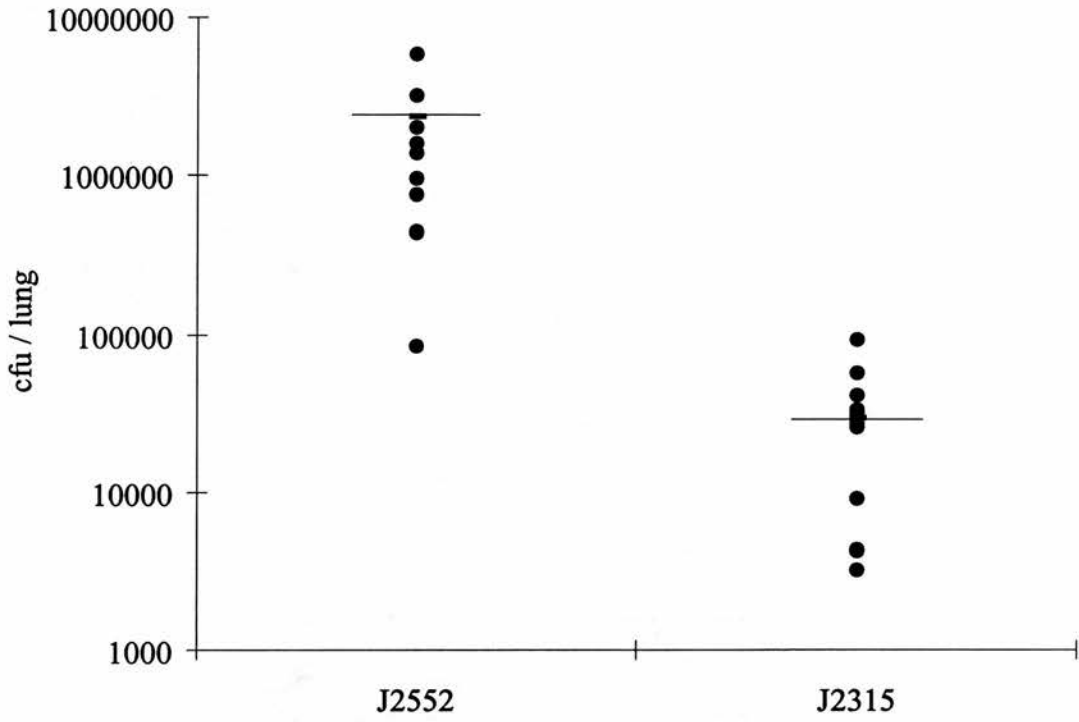


Fig 8.4 Recovery of *B. cepacia* J2552 and *B. cepacia* J2315 from non-CF mice 48 hours post intratracheal inoculation of 2.5×10^7 bacteria. Bars show mean cfu/lung for each bacterial species

CHAPTER 9 DISCUSSION

9.1 THE PATHOGENICITY OF *B. CEPACIA*

The impact of *B. cepacia* on the CF community cannot be overemphasised. First, colonisation is associated with deteriorating lung function and decreased life expectancy. Second, current antibiotic regimes are ineffective against this multiresistant pathogen. Third, fears of epidemic spread of *B. cepacia* within and between CF centres have led to the introduction of draconian and indiscriminate segregation policies for colonised patients. Although our understanding of factors responsible for patient-to-patient spread of epidemic *B. cepacia* lineages is increasing, knowledge of the pathogenic mechanisms underlying disease in colonised patients remains elusive. Several key questions need to be addressed if effective and novel strategies are to be developed to face the challenge of *B. cepacia*. For example, how do *B. cepacia* establish colonisation in the hostile environment of the CF lung, particularly in the presence of mucoid *P. aeruginosa* and other CF pathogens? Furthermore, what are the mechanisms underlying lung damage in the *B. cepacia*-colonised lung and what role do host factors play in these processes, particularly in the development of cepacia syndrome in a subset of colonised patients. The aims of this project were therefore to identify and investigate potential pathogenic mechanisms employed by *B. cepacia* in CF lung disease.

Several hypotheses of *B. cepacia* pathogenesis have been proposed. Initial comparisons of *B. cepacia* with *P. aeruginosa* found no evidence of classic *P. aeruginosa* virulence factors, including exotoxin A, elastase, pyocyanin and

alginate, amongst CF isolates of *B. cepacia* (Nelson et al, 1994). Comparative studies of clinical and environmental isolates of *B. cepacia* for other putative virulence factors including proteases, lipases, haemolysins and siderophores, have also failed to conclusively identify virulence-related determinants. The close relationship of *B. cepacia* with *B. pseudomallei*, the lack of obvious virulence factors and the persistence of *B. cepacia* in the presence of a pronounced humoral response in CF patients led to suggestions that *B. cepacia* may survive intracellularly within either epithelial or phagocytic cells. Finally, increasing evidence has suggested that *B. cepacia* may exert a marked pro-inflammatory effect within the CF lung, thus triggering further damage to the lung parenchyma and consequent respiratory deterioration (Shaw et al, 1995a; Palfreyman et al, 1997). In the following sections, these various hypotheses will be discussed in relation to investigations described in this thesis.

9.2 *B. CEPACIA* EXOPRODUCTS

Historically there has been little evidence to associate *B. cepacia* exoproducts with virulence. Nevertheless, in view of the availability of a well-defined panel of *B. cepacia* isolates, clinical and environmental strains were compared for production of a range of potential exotoxins, including protease, elastase, lipase (lecithinase / phospholipase C), haemolysin and catalase. Results were largely in agreement with those of other investigators; for example virtually all strains produced catalase whilst only a few produced haemolytic activity and none produced elastase (Gessner and

Mortensen, 1990). Protease activity was found in the majority of isolates, although more frequently in clinical than environmental strains. By contrast, lecithinase activity was found more frequently in environmental strains, particularly when tested at 30°C. Overall, the observation of non-proteolytic, non-lipolytic strains amongst clinical isolates suggests that production of these enzymes may not be important for colonisation and pathogenesis in CF patients.

Bacterial haemolytic activity is generally mediated by small pore-forming molecules, which insert into lipid bilayers, giving rise to abnormal ionic movement across cell membranes and subsequent erythrocyte lysis (Hutchison et al, 1997, 1998).

However, the pathogenic significance of bacterial haemolysins is usually due to their effects on cell types other than erythrocytes. For example, the ability of *L. monocytogenes* to survive intracellularly within macrophages is dependent on the haemolysin-mediated escape of ingested organisms from the phagosome into the cytoplasm prior to formation of a phagolysosome (Falkow et al, 1992; Brunt et al, 1990). Haemolysins may also interfere with phagocytic cell activity by inducing apoptosis, resulting in cell death and loss of an important constituent of antimicrobial defences, or by triggering degranulation, with release of harmful substances into the extracellular environment (Jonas et al, 1994). The observation of haemolytic activity amongst environmental *B. cepacia* strains is not surprising, given the role these agents may also play in survival within the soil ecosystem and in phytopathogenicity, particularly in facilitating invasive infections of plant material.

Previous investigators have identified haemolytic activity in some CF strains of *B. cepacia*, but in all studies, the majority of CF isolates appear to be non-haemolytic

(Gessner and Mortensen, 1990; McKevitt and Woods, 1984). Indeed, in the present study, activity against human erythrocytes was virtually absent amongst clinical *B. cepacia* isolates with only two non-CF isolates producing β haemolysis at 30°C, and one CF isolate, a representative of the ET12 lineage, producing a small degree of α haemolysis after prolonged incubation at 37°C. This relatively simple screening study implies that haemolytic activity is not important in the pathogenesis of *B. cepacia* respiratory disease, particularly in CF. However, a recent in depth analysis (Hutchison et al, 1998) has demonstrated the production of a powerful and multifunctional haemolysin by a representative strain of the ET12 lineage. Significantly, activity was only produced when bacterial cells were grown under highly-oxygenated conditions and not under conditions of colonial growth on agar at 37°C. Similar environmental regulation of haemolysin production in vivo may be important in the early stages of *B. cepacia* colonisation, when interference with neutrophil functions, including the induction of apoptosis by low concentrations of haemolysin, may enable *B. cepacia* to establish a foothold in the CF lung.

Catalase is produced by many bacterial species and functions primarily to degrade endogenously-produced H_2O_2 , although the protective effect of catalase against host H_2O_2 may also be important in infectious diseases. Our investigation of a large panel of strains confirmed the general description of *B. cepacia* as a catalase-positive species, with only one strain showing no catalase activity. However, in a semi-quantitative assay, CF strains demonstrated significantly greater catalase activity than non-CF strains. *B. cepacia* are sensitive to oxidative killing mechanisms employed by neutrophils (Speert et al, 1994) and must therefore have evolved strategies to

evade ROS generation within the context of the CF lung. One such strategy may be the degradation of H₂O₂ by catalase to minimise the burden of ROS, thus enabling bacterial survival. It seems probable that *B. cepacia* strains with high catalase activity would be better adapted to survival within the oxidant-rich environment of the CF lung. By contrast, many non-CF *B. cepacia* infections occur in situations where oxidative defences are reduced; for example in individuals with CGD, in neutropenic patients within intensive therapy units, or, in patients with adequate numbers of functional neutrophils, at sites where phagocytic cell numbers are generally low.

An ability to sequester iron in competition with natural iron chelators is a prerequisite for bacterial growth in both clinical and environmental situations. Bacteria have evolved a number of strategies to compete for reduced iron supplies; most notably, the production of small iron-binding compounds, termed siderophores, which compete for iron with host iron-storage proteins and bind to specific receptors expressed at the bacterial outer membrane. In addition, some bacterial species, such as pathogenic *Neisseriae* produce receptors for the human iron storage compounds transferrin and lactoferrin, thus allowing these organisms to directly utilise host iron for bacterial growth. In many instances, the potential pathogenicity of an individual bacterial strain has been linked to an ability to compete effectively for iron in iron-limited conditions. However, to date, a direct link between *B. cepacia* siderophore production and human pathogenicity has not been demonstrated.

To investigate the role of iron limitation in the colonisation of the CF lung by *B. cepacia*, four *B. cepacia* strains were screened for ability to grow in iron-rich and

iron-deficient conditions, with or without the human iron-binding protein, transferrin. Surprisingly, the representative ET12 strain, J2315, demonstrated slower growth than all other strains tested, even when using a 100-fold greater inoculum size. In addition, inclusion of transferrin in the growth medium was associated with a decrease in the growth rate for J2315 and, particularly in iron-deficient conditions, a lower steady state viable count. By contrast, growth of the CF strain C1524 was unaffected by iron-deficiency or the presence of transferrin, whilst inclusion of transferrin appeared to enhance the initial survival and growth of both environmental strains, but had little effect upon steady state levels. These results support previous studies which failed to categorise clinical and environmental strains on the basis of siderophore production (Nelson et al, 1994) and suggest that, at least for the ET12 lineage, adaptation to conditions of iron-limitation are not a major feature of pathogenicity in the CF lung. Studies of the effects of iron-limitation on *B. cepacia* were therefore not pursued.

In conclusion, attempts to separate clinical and environmental *B. cepacia* on the basis of exotoxin production were largely unsuccessful, and, with the possible exception of catalase activity amongst CF *B. cepacia* strains, no extracellular virulence factors were identified in comparative studies of *B. cepacia* populations. Further studies therefore focused on two areas; firstly the proposed intracellular model of *B. cepacia* infection, and secondly the interaction of *B. cepacia* with neutrophils, the predominant immune cell in the CF lung.

9.3 *B. CEPACIA* AND INTRACELLULARITY

B. cepacia infection in CF shares several features with known infections caused by intracellular pathogens; for example, persistent lung colonisation in the presence of a pronounced, specific and damaging immune response bears some similarity to infection with *B. pseudomallei* and *Mycobacterium tuberculosis*, both of which survive intracellularly within phagocytic cells (Falkow et al, 1992). Many invasive gastrointestinal pathogens, such as enteroinvasive *E. coli*, *Shigella* or *Salmonella* spp, penetrate and survive within epithelial cells (Falkow et al, 1992; Korth et al, 1994), suggesting that invasive respiratory pathogens may utilise a similar mechanism to spread beyond the respiratory tract. Published studies of *B. cepacia* intracellularity are few and have dealt solely with *B. cepacia* invasion of epithelial cells; for example, a recent publication demonstrated enhanced invasion of a respiratory cell line by a representative strain of the ET12 lineage, in comparison to a non-epidemic *B. cepacia* strain (Burns et al, 1996). However, the significance of in vitro invasion of epithelial cell lines within tissue culture systems remains unclear, particularly in relation to pathogenesis in respiratory tract infections (Miller, 1995; Pier et al, 1996). Indeed, a recent publication has even suggested that enhanced uptake of CF pathogens by epithelial cells expressing normal CFTR, followed by epithelial desquamation, may be an important host defence mechanism rather than a bacterial virulence determinant (Pier et al, 1996).

B. cepacia is closely related to the intracellular pathogen *B. pseudomallei*, which survives within phagocytic cells; thus the current project focused on the uptake and survival of *B. cepacia* in monocytes, using *L. monocytogenes* and *P. aeruginosa* as

positive and negative control organisms respectively. Conventional techniques for investigating intracellular bacteria have depended on the use of non-penetrating antibiotics to kill extracellular organisms. However, the intrinsic antibiotic resistance of *B. cepacia* severely restricts the use of this approach. Similar problems have plagued the investigation of *B. pseudomallei* as an intracellular pathogen and it was not until 1990 that definitive evidence of *B. pseudomallei* intracellularity was presented by Pruksachartvuthi et al who used a washing technique coupled with EM to demonstrate that *B. pseudomallei* can survive and multiply within human leukocytes. However, our attempts to repeat this experiment using *B. cepacia* were largely unsuccessful; neither *B. cepacia* nor *P. aeruginosa* were demonstrated intracellularly by EM implying that the increasing bacterial counts for both of these organisms reflected growth of extracellular but adherent bacteria, rather than intracellular organisms. Both *B. cepacia* and *P. aeruginosa* showed a decrease in viable counts after one hour of incubation, suggesting killing of ingested organisms. Interestingly, however, this decrease was significantly greater for *P. aeruginosa* than *B. cepacia*, implying that *B. cepacia* may display greater resistance to monocyte killing mechanisms than *P. aeruginosa*.

An alternative approach to the study of intracellularity is the utilisation of a differential staining technique, in this case, the acridine orange (AO) / crystal violet (CV) method. AO is a fluorescent dye which binds to DNA in a bimodal fashion, whereby binding to intact DNA is characterised by green fluorescence whilst binding to denatured DNA is associated with cross-linking of the AO molecules and the emission of red fluorescence (West, 1969). Theoretically, AO can therefore

distinguish viable and non-viable bacteria on the basis of DNA denaturation.

Furthermore, AO penetrates mammalian cells whilst CV, which quenches the fluorescence of AO, remains in the extracellular environment. Hence addition of CV to AO-stained monocytes will mask the fluorescence of any extracellular bacteria adherent to the monocyte cell surface.

AO/CV staining was used to assess uptake and initial survival of *B. cepacia* within monocytes, with *L. monocytogenes* and *P. aeruginosa* as positive and negative controls respectively. Results indicated no increased uptake of the representative ET12 strain, *B. cepacia* J2315, in relation to an environmental *B. cepacia* strain, J2540, or a representative *P. aeruginosa* strain, PAO1. By contrast, uptake of *L. monocytogenes* was at least three times greater than that of the other strains tested. Interpretation of colour changes with respect to bacterial viability proved difficult, particularly as earlier studies of heat-killed or UV-irradiated bacteria suggested that neither *B. cepacia* nor *P. aeruginosa* displayed the characteristic green to red colour change associated with bacterial cell death. Nevertheless, the majority of ingested organisms for both *P. aeruginosa* and *B. cepacia* were orange/green in colour implying some degree of DNA damage, and suggesting that some bacterial killing was taking place. By contrast, the majority of ingested *L. monocytogenes* appeared bright red in colour, indicating rapid killing of ingested bacteria. This latter observation seems paradoxical, given the proven intracellularity of *L. monocytogenes*. However, previous investigators have also demonstrated that the majority of ingested *L. monocytogenes* are killed by phagocytic cells with only a minority escaping the phagolysosome to grow within the cytoplasm (de Chastellier

and Berche, 1994). Furthermore, in the assays of bacterial intracellular survival over a 24 hour period, the observation of killing of all monocytic cells after 24 hours incubation with *L. monocytogenes* supports a model of *L. monocytogenes* intracellular survival where a small number of ingested organisms multiply within the cytoplasm until monocyte death and release of the intracellular organisms occurs.

In conclusion, no evidence was found to support the hypothesis that *B. cepacia* persistently colonises the CF lung by intracellular growth within monocytic cells. As a caveat, however, comparison of results for *B. cepacia* and *P. aeruginosa* in assays of intracellular bacterial survival, suggested that the evasion of phagocytic cell killing mechanisms may be important in the persistence of *B. cepacia* in the CF lung.

9.4 INTERACTION OF *B. CEPACIA* WITH NEUTROPHILS

Since neutrophils are the predominant immune cell in the CF lung, survival of *B. cepacia* depends upon either evasion of or resistance to neutrophil-mediated killing mechanisms. Further studies therefore focused on the interaction of *B. cepacia* with neutrophils, particularly in relation to bacterial killing and neutrophil respiratory burst induction.

Killing of B. cepacia by neutrophils

Initial assays of neutrophil-mediated bacterial killing compared killing of non-opsonised *B. cepacia*, with killing of bacteria opsonised in 10% pooled human serum (PHS) and indicated that serum factors were required for bacterial killing. However,

in further studies of a panel of *B. cepacia* strains pre-opsonised with PHS, no evidence of significant killing of clinical or environmental strains could be demonstrated. For example, levels of the environmental strains, J2511 and J2552, were similar following incubation with neutrophils or buffer alone, whilst one CF isolate, C1524, actually increased in number to almost double initial counts when incubated with neutrophils for a two hour period. Results for the ET12 strain, J2315, were even more intriguing; bacteria incubated with buffer alone displayed a marked increase in numbers, rising to 138% initial levels after 15 min incubation followed by a steady decline towards initial levels, whilst bacteria incubated with neutrophils displayed a slight decrease in numbers. The rapid and early rise in counts observed in control bacteria is consistent with dissociation of bacterial aggregates rather than bacterial growth *per se*; it is possible, therefore, that the extent of bacterial killing in the presence of neutrophils has been masked by the dissociation of existing bacterial clumps. Conversely the tendency of *B. cepacia* J2315 to form aggregates could lead to an underestimation of bacterial viability in the presence of neutrophils. Thus any data generated from assays of neutrophil-mediated killing of *B. cepacia*, particularly of the ET12 lineage, must be interpreted with caution.

The confounding effect of bacterial aggregation was further demonstrated in comparative studies of neutrophil-mediated killing following opsonisation with immune and non-immune sera. Initial studies suggested that heat-inactivated immune serum was associated with greater bacterial killing than similarly treated non-immune serum. However, the observation of a similar or even greater decline amongst pre-opsonised bacteria in the absence of neutrophils, indicated that the

apparent killing of *B. cepacia* by neutrophils was in fact due either to serum sensitivity or bacterial aggregation. Serum sensitivity depends upon the presence of active complement; thus the observation of a similar pattern of viable counts following pre-opsonisation with either heat-inactivated or untreated serum suggests that antibody-mediated bacterial aggregation is the basis of any decline in bacterial counts. Given this phenomenon, further killing assays using pre-opsonised bacteria were discontinued.

Respiratory burst activity

In view of the difficulties encountered with neutrophil-mediated killing assays, an alternative approach to the assessment of neutrophil defences against *B. cepacia* was required. Speert et al (1994) have demonstrated that *B. cepacia* are resistant to neutrophil non-oxidative killing mechanisms; the generation of an effective respiratory burst is therefore necessary for the clearance of these organisms. Thus, the induction of neutrophil respiratory burst activity in response to *B. cepacia* was investigated.

Respiratory burst activity can be assessed using a variety of methodologies, all of which measure different products in the sequence of events leading to ROS generation. Techniques used in this thesis included luminol- and lucigenin-enhanced chemiluminescence (LCL and LuCL respectively), flow cytometric analysis using the fluorescent dye dihydrorhodamine (DHR) and spectrophotometric measurement of extracellular cytochrome c reduction. Both LuCL and the cytochrome c assay detect O_2^- , the first product of the NADPH oxidase pathway; notably, however, LuCL detects intracellular O_2^- whilst the cytochrome C assay only detects O_2^- released

extracellularly. DHR is used to detect intracellular production of H_2O_2 whilst LCL detects the generation of MPO-dependent ROS, although the exact nature of the oxidant species involved is not clear.

Initial studies focused on the induction of LCL by non-opsonised *B. cepacia* and *P. aeruginosa*. Interpretation of the results generated by this technique was difficult. For example, considerable inter-donor variation was observed, with wide variations in the magnitude and timing of responses from donor to donor. However, this was partly corrected by using the LCL response to opsonised zymosan (OZ) as a standard, and calculating activity of all other agents as a percentage of OZ activity. Perhaps a more difficult problem was how best to analyse the wide variety of patterns of activity obtained, with individual bacterial isolates inducing peaks of activity at differing time points, or a sustained response over the duration of the assay period. Data could be interpreted in a number of ways, including the magnitude and timing of peak responses, the initial rate of responses or the area under the LCL curve. Comparison of various parameters indicated that analysis of peak LCL and LCL at 10 min (LCL_{10}), gave the most consistent results and further data were interpreted on the basis of these two parameters. Since previous investigators have demonstrated that the timing of LCL responses correlates with neutrophil phagocytosis and bacterial killing to a greater degree than the magnitude of peak responses (Speert et al; 1986), LCL_{10} data may be particularly important in terms of bacterial sensitivity to neutrophil-mediated killing.

Comparison of a panel of environmental, non-CF clinical and CF *B. cepacia* strains indicated that LCL induction varied considerably from strain to strain. Nevertheless,

CF strains, including representative epidemic strains, did appear to induce less respiratory burst activity than non-CF strains, whilst environmental strains displayed a variety of responses (Fig 6.2). This observation supports a hypothesis of *B. cepacia* infection in CF where bacterial survival depends upon avoidance of a meaningful neutrophil respiratory burst; thus those strains of *B. cepacia* less likely to induce activity have an advantage in terms of respiratory colonisation and survival. However, given the diversity of bacterial strains currently identified as *B. cepacia*, a greater number of isolates would need investigation to confirm these findings. Perhaps more significantly, CF strains of *B. cepacia* were observed to display less respiratory burst activity than CF strains of non-mucoid *P. aeruginosa*. In particular, the range of activities induced by *P. aeruginosa* was much greater than that induced by *B. cepacia* strains, suggesting that the evasion of a neutrophil respiratory burst is less relevant to survival of *P. aeruginosa* than *B. cepacia* (Fig 6.3). Indeed, as *P. aeruginosa* is sensitive to non-oxidative neutrophil defence mechanisms (Speert et al, 1994), killing of *P. aeruginosa* will occur whether or not an effective respiratory burst is mounted. Survival of *P. aeruginosa* within the CF lung depends upon the conversion of non-mucoid *P. aeruginosa* (NMPA) to a mucoid phenotype (MPA), which enables the organism to evade host defence mechanisms by sheltering within a protective layer of alginate. A model of *P. aeruginosa* infection can be hypothesised where MPA within the alginate biofilm, periodically revert to non-mucoid forms, resulting in the presence of both MPA and NMPA in sputum. Thus, the persistence of NMPA within the CF lung depends upon the continued presence of a pool of mucoid strains which sporadically give rise to non-mucoid forms, rather than the direct avoidance of neutrophil-killing mechanisms.

The interaction of non-opsonised bacteria with neutrophils is generally believed to be mediated or enhanced by hydrophobic interactions between the bacterial and neutrophil cell surfaces. The characteristics of bacterial cell surfaces are in part determined by cultural conditions; for example, bacterial growth in static conditions favours bacterial aggregation and enhanced hydrophobicity, whilst bacteria grown in shaken culture will tend to be less hydrophobic. Therefore the effects of growth in either static or shaken culture on LCL induction were compared for a panel of CF, non-CF clinical and environmental *B. cepacia* strains (Fig 6.4). Both CF strains, J2315 and C1987, and the environmental strain, J2552, induced little LCL activity when grown in shaking condition; static growth, however, was associated with an increase in reactivity, implying that, for these strains, bacteria/neutrophil interactions were dependent on non-specific hydrophobic bacterial binding. By contrast, when grown under shaken conditions, two non-CF clinical strains, J2660 and J2684, induced a sizeable respiratory burst, which was not enhanced by growth under static conditions, suggesting that receptor-mediated rather than non-specific hydrophobic reactions were responsible for LCL induction by these bacteria. Again, these results support the hypothesis that evasion of respiratory burst induction by CF strains of *B. cepacia* is important for the survival of *B. cepacia* within the CF lung; strains which are more likely to trigger a respiratory burst are less likely to be implicated in CF respiratory disease.

As LCL is a relatively non-specific technique for the detection of respiratory burst activity, attempts were made to confirm the above results using lucigenin to detect the generation of O_2^- . Theoretically, LCL detects oxidant species produced in a

MPO-dependent manner, suggesting that activity requires the presence of H_2O_2 , generated from superoxide anions. Measurement of LuCL and LCL levels in simultaneous experiments allowed direct comparison of NADPH oxidase activation and O_2^- production with LCL generation. Neutrophils treated with buffer alone demonstrated a small peak in LuCL activity at 30 min, which declined to baseline levels after one hour of incubation, whilst LCL activity remained unchanged. Incubation of neutrophils with bacteria had varying effects on LuCL activity, either inducing a greater peak than untreated neutrophils (eg *B. cepacia* C2040), or a more sustained LuCL response (eg *B. cepacia* J2315, *P. aeruginosa* J1385). Surprisingly, little consistent correlation was seen between LCL and LuCL with respect to both peak activity and the initial rate of responses (Fig 6.5). However, the presence of a sustained LuCL response did appear to correlate with either a sustained LCL response (eg *P. aeruginosa* J1385) or a late LCL response (eg *B. cepacia* J2315). Furthermore, studies using two inhibitors of respiratory burst activity provided strong evidence to support the proposed relationship between LuCL and LCL activity (Fig 6.6). Treatment with NaN_3 virtually abolished LCL activity whilst augmenting LuCL activity, suggesting that at least a component of LCL activity is a downstream event of O_2^- production. Simultaneous experiments using SOD confirmed this relationship by demonstrating a complete abolition of LuCL activity associated with a reduction in LCL activity.

Further studies of neutrophil respiratory burst induction were carried out using alternative methodological approaches, including the detection of extracellular O_2^- by reduction of ferricytochrome c, and the detection of intracellular H_2O_2 by oxidation

of dihydrorhodamine (DHR). Virtually no extracellular O_2^- could be detected following exposure of neutrophils to a range of *B. cepacia* strains, whilst only a slight response was induced by a representative *P. aeruginosa* strain, under similar assay conditions to those utilised in LCL and LuCL experiments (Table 6.2). By contrast, flow cytometric analysis of neutrophils treated with DHR demonstrated the generation of intracellular H_2O_2 in response to a range of bacterial strains (Fig 6.19). However, as for LCL assays, a greater range of activities was detected in response to representative CF isolates of *P. aeruginosa* than *B. cepacia*. Furthermore, for the majority of bacterial isolates, considerable correspondence in the timing and level of DHR and LCL responses was observed. In particular, the pattern of DHR activity over time seen in response to the strains *P. aeruginosa* J1385, *B. cepacia* C2040 and *B. cepacia* J2315 correlated well with the respective early, medium and late rises seen in LCL responses to these bacteria (Fig 6.1, Fig 6.18). In conclusion, results from studies of respiratory burst activity using a variety of methodologies supported our hypothesis that non-opsonised CF isolates of *B. cepacia* induce little respiratory burst activity in human neutrophils.

9.5 BACTERIAL OPSONISATION AND RESPIRATORY BURST INDUCTION

Colonisation with *B. cepacia* in CF is associated with a marked and specific humoral immune response. Unlike *P. aeruginosa*, elevated titres of anti-*B. cepacia* antibodies do not correlate with a decline in lung function but nor do they appear to confer a protective effect upon the patient (Nelson et al, 1993) leading some observers to

speculate that the IgG response seen in colonised patients may be largely non-opsonic, and thus have little impact on killing of *B. cepacia* by phagocytic cells (Butler et al, 1994b). ELISA techniques to detect immunoglobulin give little information regarding the opsonic activity of serum antibody; by contrast, measurement of neutrophil respiratory burst induction by bacteria following pre-opsonisation with non-immune and immune serum provides a functional assay for the effectiveness of an immunoglobulin response. We thus investigated the effects of pre-opsonisation in response to a representative of the ET12 lineage, J2315, using serum from the adult CF clinic based at the Western General Hospital, Edinburgh, where several patients are colonised by this strain.

Pre-opsonisation of J2315 with heat-inactivated immune serum was associated with an increase in LCL induction; this response was enhanced still further if pre-opsonisation was carried out with serum plus active complement, primarily through the induction of a more sustained LCL response (Fig 6.7). Pre-opsonisation with complement alone, however, was associated with the induction of little or no LCL activity. LuCL activity induced by J2315 was also increased if bacteria were pre-opsonised with immune serum; however, opsonisation with complement and serum was not associated with any further increase in activity and opsonisation with complement alone was associated with the complete abolition of LuCL responses. Although this latter result agrees with the observation that complement receptors alone do not provoke a respiratory burst, the abolition of even the slight LuCL response observed in neutrophils treated with buffer alone suggests that under these circumstances, complement may actually down-regulate respiratory burst activity.

Furthermore, the enhancement of LCL but not LuCL induction by pre-opsonisation of bacteria with both complement and serum suggests that complement receptor activation may have downstream effects during neutrophil respiratory burst activation processes. For example, complement-mediated enhancement of azurophil degranulation would increase the availability of MPO in phagolysosomes, thus promoting the generation of the MPO-dependent ROS detected by the LCL assay.

The role of opsonic activity in disease progression

Since the inclusion of complement with serum had comparatively little effect on peak LCL or LCL₁₀, further studies of immune and non-immune serum from colonised patients were conducted using serum alone, rather than serum plus complement. Heat-inactivated serum from a panel of CF patients, of whom all the *B. cepacia*-colonised patients harboured the ET12 lineage of *B. cepacia*, were compared for opsonic activity against *B. cepacia* J2315. Although bacterial pre-opsonisation with serum from both colonised and non-colonised patients increased LCL responses dramatically, significantly greater activity in terms of both peak LCL and LCL₁₀ were induced by serum samples from colonised patients (Table 6.1; Fig 6.10). Absorption studies using a CF strain of *P. aeruginosa*, and a range of other *B. cepacia* strains indicated that this effect was specific to *B. cepacia* J2315 and was not due to cross-reacting antibodies against *P. aeruginosa* (Fig 6.12). In further studies, the cytochrome c assay was used to assess the effects of opsonisation on induction of extracellular O₂⁻ release. Results confirmed those of the LCL assay, by indicating the species-specific effect of pre-opsonisation of both *B. cepacia* and *P. aeruginosa* with patient serum (Table 6.3, Fig 6.16). For example, serum from a patient colonised

with *P. aeruginosa* alone had no opsonic activity against *B. cepacia*, whilst patients colonised with both organisms possessed opsonic activity against both *B. cepacia* and *P. aeruginosa*. Again, the use of absorbed serum confirmed the lack of cross-reactivity between the *B. cepacia* and *P. aeruginosa* responses (Fig 6.15).

Of the *B. cepacia*-positive patients investigated, no correlation could be observed between serum opsonic activity and clinical status; thus a specific role for anti-*B. cepacia* opsonic antibody in the pathogenesis of *B. cepacia* lung disease could not be demonstrated. Nelson et al (1993) had previously demonstrated high anti-*B. cepacia* LPS immunoglobulin titres in both patients with stable clinically mild disease and patients who subsequently succumbed to severe lung disease. Notably, the development of septicaemia in patients with cepacia syndrome was not prevented by the presence of high immunoglobulin titres, suggesting that an inappropriate non-opsonic IgG subclass response may be made to *B. cepacia*, preventing effective complement- and phagocytic cell-mediated killing of this organism (Butler et al, 1994b). However in the present study, we have demonstrated the presence of specific and opsonic anti-*B. cepacia* immunoglobulin, suggesting that an appropriate IgG response is mounted. The persistence of *B. cepacia* in the face of a pronounced humoral response may therefore reflect the disruption of opsonic processes within the CF lung by proteolytic cleavage of complement receptors and immunoglobulin, rather than a defect in the immunoglobulin response. Nevertheless, such a hypothesis does not explain the ability of serum-sensitive *B. cepacia* to cause septicaemia in a subset of colonised patients.

Effect of atmospheric conditions

An interesting observation from the above studies was the demonstration of differing opsonic activity of serum against *B. cepacia* and *P. aeruginosa* grown in different atmospheric conditions. As expected, growth in conditions of air enriched with 5% CO₂, which resemble the in vivo situation, was associated with increased opsonisation of a CF strain of *P. aeruginosa*. By contrast, however, opsonisation of the ET12 lineage of *B. cepacia* was decreased when the bacteria were grown under CO₂-enriched conditions (Table 6.3). Analysis of OMP expression for both organisms suggested that changes in OMP profile may be responsible for the differing opsonisation patterns seen. Burnie et al (1995) have hypothesised that an improved prognosis in *B. cepacia*-colonised patients can be correlated with the production of antibodies against a 30 kDa protein, presumptively identified as the 28 kDa OMP D porin (Aronoff and Stern, 1988), which is believed to associate with the 37 kDa OMP C porin to form an 81 kDa band (Gotoh et al, 1994). In the present study, analysis of the OMP profile of *B. cepacia* ET12 grown in air revealed no obvious band at 81 kDa, but major bands were apparent at 36 kDa and 28 kDa (Fig 6.17). Interestingly, growth in CO₂-enriched conditions was associated with the loss of the 28 kDa band and the appearance of a new band at 26.5 kDa. Furthermore, a slight shift was observed in the 36 kDa band, suggesting some modification of this OMP. Thus, phenotypic modification of *B. cepacia* within the CF lung may alter the organisms antigenic profile, enabling bacterial persistence in the presence of a specific immunoglobulin response. In addition, growth in a CO₂-enriched environment has been implicated in increased resistance of *B. cepacia* to antimicrobial agents, possibly through altered OMP expression (Corkill et al, 1994a);

the changes observed in the present study may contribute to the phenomenon whereby *B. cepacia* which display antibiotic sensitivity in vitro, are found to be resistant to antibiotic therapy in vivo.

Opsonic immunoglobulin and B. cepacia colonisation

An attempt was made to assess the relationship of opsonic immunoglobulin production to *B. cepacia* colonisation by conducting longitudinal studies in two patients, using serum samples taken before and after the first positive sputum for *B. cepacia* (Fig 6.11). Both patients became persistently colonised with the ET12 lineage of *B. cepacia*. For patient G, a steady increase in opsonic activity, as determined by both peak LCL and LCL₁₀, was observed after colonisation with *B. cepacia*, although rising activity was also detected prior to colonisation. Results for patient S were more intriguing, with the demonstration of considerable pre-colonisation opsonic activity for several years before a fall in activity in the two year period prior to the isolation of *B. cepacia*. Previous investigators have demonstrated the presence of high titres of anti-*B. cepacia* LPS antibodies in a small number of apparently *B. cepacia*-negative patients (Nelson et al, 1993), leading to speculation that, in some patients, exposure to *B. cepacia* may be followed by a protective immune response, preventing the establishment of colonisation. Such a hypothesis would be consistent with our observations regarding patient S; colonisation with *B. cepacia* only occurred after a demonstrable drop in anti-*B. cepacia* opsonic activity. Alternatively, the presence of an apparently pre-colonisation antibody response to *B. cepacia* may represent the presence of low numbers of *B. cepacia* within the CF lung, which are not detected by standard cultural techniques. Indeed

the possibility of culture-negative *B. cepacia* colonisation in CF patients has been suggested by a number of investigators. For example, Nelson et al (1993) described a rise in patient anti-*B. cepacia* titres in the months immediately before the first positive sputum for *B. cepacia*, implying that *B. cepacia* was not detected by sputum culture until a certain level of colonisation was reached. LiPuma et al (1994) have also speculated that *B. cepacia* sputum-negative patients may actually harbour the organism, following the apparent transmission of a *B. cepacia* strain between two patients prior to the first positive sputum culture for either patient. Most recently, PCR of sputum from apparently non-colonised patients using *B. cepacia*-specific primers, has demonstrated positive results in a proportion of patients, again suggesting that colonisation may be undetected in some CF individuals (S Elborn, personal communication). Thus the rise in titres seen in patients S and G in the months immediately prior to positive sputum culture may reflect early undetected *B. cepacia* colonisation. However, epidemiological data for patient S has identified only one definite episode of exposure to *B. cepacia*, occurring a few weeks before positive sputum culture; thus a prolonged period of inapparent *B. cepacia* colonisation seems unlikely for this patient.

Relationship to other B. cepacia strains

Immune sera from CF patients colonised with the ET12 lineage of *B. cepacia* were used to assess the relationship between *B. cepacia* ET12 and other *B. cepacia* strains. Considerable variation was seen in the opsonic activity of individual serum samples in response to a panel of clinical and environmental strains. However, in all instances, genomovar III strains, of which the ET12 lineage is a member, were most

likely to be opsonised by serum from ET12-colonised patients, as determined by the earlier induction of an LCL response (Fig 6.9). Results for representative strains of other genomovars were less clear cut. For example, the genomovar I strains, C1964 (CF) and J2552 (environmental), were not opsonised by serum from an ET12-colonised patient, whilst another environmental genomovar I strain, J2540, induced a dramatically enhanced LCL response following pre-opsonisation. It may be relevant to note that the OMP profile of *B. cepacia* J2540 appears to be closely related to that of the ET12 lineage (Dr Kathy Taylor, personal communication); by contrast, studies of TNF- α induction by *B. cepacia* LPS have indicated that LPS from J2540 is less active than that of a representative ET12 strain (C1359), or other clinical *B. cepacia* strains (Shaw et al, 1995a). Thus the exact nature of the relationship between *B. cepacia* J2540 and the ET12 lineage remains unclear. Genomovar II strains were also opsonised to differing degrees by anti-ET12 serum, with no opsonic activity demonstrated against a non-CF clinical strain (C1962) whilst considerable activity was demonstrated against a CF isolate, C1576. This latter isolate is a representative of the Glasgow epidemic strain; thus a common transmissibility factor may be present in both the Glasgow and ET12 epidemic lineages. Indeed, both of these lineages are known to contain the *B. cepacia* epidemic strain marker described by Mahenthiralingam et al (1997), although the role of this marker in antigen expression by *B. cepacia* is as yet unknown.

9.6 *B. CEPACIA* AND THE RESPIRATORY BURST PATHWAY

Interpretation of data regarding the induction of a neutrophil respiratory burst by non-opsonised and opsonised bacteria was complicated by the apparently contradictory results obtained using a range of techniques to determine respiratory burst activity. Thus, for example, the bacterial strains *P. aeruginosa* J1385 and *B. cepacia* C2040, which both induced marked LCL and DHR responses, induced little detectable extracellular O_2^- , as determined by the cytochrome c assay (Fig 6.1; 6.3; Table 6.2). Similarly, little correlation was observed between LCL and LuCL activity, despite the demonstration of a relationship between these two parameters when respiratory burst induction was analysed in the presence of specific inhibitors. Opsonisation of bacteria with immunoglobulin enhanced both O_2^- production and the generation of MPO-dependent ROS, but complement appeared to enhance only the latter stages of the respiratory burst pathway and not the initial production of O_2^- . One explanation for these anomalies may be the generation of MPO-dependent ROS from bacterially-derived rather than neutrophil-derived H_2O_2 . Alternatively, neutrophil stimulation may upregulate later processes in the respiratory burst pathway as well as O_2^- generation. Indeed, recent studies have indicated that MPO activity can be increased independently of NADPH oxidase activation. For example, Witko-Sarsat et al (1996) demonstrated a constitutive increase in MPO activity in neutrophils from CF patients and heterozygote carriers. Other investigators have demonstrated an increase in MPO activity, independent of an increase in NADPH oxidase activity, in neutrophils from healthy individuals following in vivo cytokine treatment (Allen et al, 1997; Zipfel et al, 1997). It is possible that an MPO-

dependent increase in H_2O_2 utilisation and hence O_2^- dismutation may have removed O_2^- before detection in the various assays used in the present study, thus masking the true extent of O_2^- generation. One criticism of this hypothesis arises from the observation of dramatically increased levels of O_2^- detected by cytochrome c, following neutrophil stimulation with pre-opsonised rather than non-opsonised bacteria. However, since the cytochrome c method detects only extracellular O_2^- , this phenomenon may represent increased granule exocytosis in addition to the induction of respiratory burst activity. Evidently, where possible, respiratory burst activity should be analysed by more than one technique, if a complete picture of neutrophil-bacterial interactions is to be obtained.

In any investigation of neutrophil-bacterial interactions, consideration must be given to bacterial products which may modulate respiratory burst responses. A possible role for catalase production in the down-regulation of ROS responses by *B. cepacia* has already been discussed, but other bacterial substances may provide the organism with protection against neutrophil oxidative killing mechanisms. In the present study, comparison of LuCL and LCL induction by a pre-opsonised non-mucoid *P. aeruginosa* strain and a pre-opsonised *B. cepacia* of the ET12 lineage revealed marked differences in the patterns of activity induced. In particular, LCL responses were greater for the *P. aeruginosa* strain, whilst the *B. cepacia* strain induced greater LuCL activity (Fig 6.13). This intriguing observation suggests that *B. cepacia* ET12 may actually inhibit the generation of MPO-dependent ROS, even in the presence of NADPH activation and O_2^- production. Production of carotenoid pigments which quench singlet oxygen, one of the main ROS detected by the LCL assay, has been

proposed as a bacterial defence mechanism employed to evade oxidative killing (Krinsky, 1974). It would be tempting to speculate that the ET12 lineage of *B. cepacia*, which, unlike most other *B. cepacia*, is characterised by the production of a brown diffusible pigment, may utilise a similar mechanism to evade oxidative killing mechanisms. However, other investigators have failed to detect carotenoid pigments in this strain of *B. cepacia* (Dr Kathy Taylor, personal communication) and preliminary experiments towards the present study failed to demonstrate any inhibition of LCL activity by supernatant from *B. cepacia* cultures. Nevertheless, the findings presented above are consistent with the hypothesis that inhibition of ROS generation by *B. cepacia* ET12 may play a role in the persistent colonisation of the CF lung.

9.7 THE PRO-INFLAMMATORY EFFECT OF *B. CEPACIA*

Accumulated evidence indicates that *B. cepacia* colonisation in CF is associated with an upregulation in respiratory inflammation, promoting further lung damage in patients who acquire this pathogen. Given that neutrophil products, including ROS and proteolytic enzymes, are widely believed to be the pathogenic mediators of this inflammatory damage; it seems reasonable to investigate the ability of *B. cepacia* to promote neutrophil activation and degranulation. Although we have already demonstrated that CF strains of *B. cepacia* tend to be poor inducers of a respiratory burst, these strains may augment neutrophil activation in other ways, either through enhancing neutrophil recruitment to the lung or by increasing the magnitude of

activation events. Respiratory burst induction and degranulation of azurophil granules are end-stage events in neutrophil activation. By contrast, degranulation of other granule subtypes and priming of respiratory burst activity, whereby responses to any subsequent stimulus are increased, occur earlier in neutrophil activation processes. Neutrophil degranulation can be assayed through the measurement of released enzyme activity in neutrophil supernatants or increased expression of cell surface receptors originally stored within the granule membrane. In the present study, flow cytometry was used to assess CR3 expression as a measurement of non-azurophil degranulation in response to *B. cepacia* and *P. aeruginosa*. Since many agents induce neutrophil priming at the same time as non-azurophil degranulation, priming of neutrophil responses to the stimulant FMLP was also measured, using DHR to detect intracellular H₂O₂ production.

CR3 is a pivotal receptor in such diverse neutrophil functions as adhesion, transmigration, phagocytosis and degranulation; therefore an increase in CR3 expression is a key event in neutrophil activation processes. Analysis of a panel of *B. cepacia* strains indicated that these bacteria induced increased CR3 expression both in the presence and absence of serum. However, for some strains, including the ET12 lineage and other representatives of genomovar III, CR3 responses to high concentrations of bacteria were down-regulated in the presence of serum. By contrast, responses in the absence of serum correlated logarithmically with the concentration of bacteria present (Fig 5.4). The nature of this strain-specific effect remains unclear, but, given the low anti-*B. cepacia* titres generally found in healthy individuals, is unlikely to involve opsonisation with immunoglobulin. As fresh

blood samples were used, the effect of serum may be linked to the binding and activation of complement by some *B. cepacia* strains. Decreased detection of CR3 expression at high bacterial concentrations would thus represent either masking of antibody binding sites on the CR3 molecule by bound bacteria or the internalisation of bound bacteria with consequent down-regulation of CR3 expression. Binding of activated complement is severely disrupted within the CF lung by the proteolytic cleavage of complement receptors; thus the majority of further studies were conducted on neutrophils in the absence of serum.

Pro-inflammatory effects of whole bacteria

Neutrophil responses to a panel of *B. cepacia* and *P. aeruginosa* strains isolated from CF patients were compared for both induction of non-azurophil degranulation and priming of respiratory burst activity. All *B. cepacia* strains were found to induce a marked increase in CR3 expression, whilst several strains primed respiratory burst responses to FMLP. (Fig 5.5; Fig 6.19; Table 6.4). Little CR3-inducing activity or priming activity was present in filtered supernatants from bacterial suspensions; thus activity appeared to depend on the presence of whole bacteria, rather than on bacterial products. However, as incubation times of up to one hour were used, it is possible that bacterial products, released during the course of the experiment, were responsible for these effects. Comparison of *B. cepacia* and *P. aeruginosa* strains, supported our hypothesis that *B. cepacia* are less likely to induce a respiratory burst response but do trigger other pro-inflammatory neutrophil responses. Thus, *P. aeruginosa* triggered a greater average respiratory burst response, while *B. cepacia* induced greater average CR3 expression. Priming activity also tended to be greater

for *B. cepacia* than *P. aeruginosa* strains. However, overall differences between *P. aeruginosa* and *B. cepacia* failed to reach statistical significance, and further studies using a greater number of strains for each bacterial species are required to confirm these observations.

The nature of the *B. cepacia*-neutrophil interaction inducing increased CR3 expression and priming remains to be determined. *B. cepacia* may bind to specific receptors or may bind non-specifically to the neutrophil surface by hydrophobic interactions. Results from LCL studies of bacterial growth conditions, and the observation by previous investigators of high surface hydrophobicity in CF strains of *B. cepacia*, including the ET12 lineage (Shaw, 1995), suggest that the latter mechanism is probably the basis of *B. cepacia*-neutrophil interactions. However, receptor-mediated binding may also be enhanced by hydrophobic interactions, and seems more likely to trigger the neutrophil degranulation and priming seen in response to *B. cepacia* strains. One intriguing possibility is that *B. cepacia* may actually bind to the CR3 molecule itself, thus triggering neutrophil degranulation and phagocytosis without inducing a respiratory burst. Ideally, studies of binding of *B. cepacia* to neutrophils utilising specific monoclonal antibodies raised against *B. cepacia*, would be required to establish the exact nature of *B. cepacia*-neutrophil interactions. It is hoped that the current development of anti-*B. cepacia* monoclonal antibodies will allow rapid progress in this field of research.

B. cepacia products in neutrophil activation processes

Bacterial products may also interact with neutrophils to induce priming and degranulation. For example, LPS from a number of bacterial species are potent

neutrophil priming agents. *B. cepacia*, including the ET12 lineage, is known to produce LPS which is active in bioassays measuring induction of the pro-inflammatory cytokine, TNF α from monocytes (Shaw et al, 1995a). Thus the interaction of LPS molecules extracted from strains of *B. cepacia* and *P. aeruginosa* with neutrophils was investigated, using *E. coli* LPS as a positive control.

In initial studies, induction of CR3 by LPS from *B. cepacia* J2315 was compared in washed and whole blood. High levels of CR3 were induced on neutrophils within washed blood by concentrations of LPS of 100 ng/ml or greater. LPS activity was demonstrated at lower concentrations when incubated with neutrophils in whole blood, but no CR3 induction was seen at higher concentrations. Furthermore, the overall level of activity was much lower for LPS in whole blood than washed blood. One explanation for this observation may be the presence of monocytes within the blood samples used; monocytes express much greater levels of CD14 than resting neutrophils and may therefore bind LPS-LBP complexes before any interaction with neutrophils can occur. Thus in the absence of serum and hence LBP, more LPS would be available for binding to neutrophils in a non-CD14 dependent manner.

While these results suggest that circulating *B. cepacia* LPS may have little impact on neutrophils within the bloodstream, the observation of enhanced CR3 expression in neutrophils treated with LPS in the absence of serum factors is particularly relevant to inflammation in the CF lung, where concentrations of serum factors are likely to be low. As both *P. aeruginosa* and *B. cepacia* are typically found in CF sputum at high concentrations of approximately 10^8 - 10^9 cfu/ml, it is likely that concentrations of shed LPS from both viable and non-viable bacteria will also be elevated. Thus,

further studies were conducted in serum-free conditions using a concentration of 100 ng/ml LPS as standard. Extracts of LPS from a panel of *B. cepacia* and *P. aeruginosa* isolates were compared for both induction of increased CR3 expression and priming of neutrophil responses to FMLP. LPS from both clinical and environmental strains of *B. cepacia* induced a marked increase in neutrophil CR3 expression (Fig 7.6). By contrast, LPS from three representative *P. aeruginosa* strains had little effect on CR3 expression. These results are consistent with those of Shaw et al (1995a) who first reported the surprising finding that *B. cepacia* LPS induced a TNF α response from circulating blood monocytes, which was 9-fold greater than that induced by *P. aeruginosa* LPS. In these studies, TNF α was detectable between 2.5 - 4.5 hours after the addition of *B. cepacia* LPS, and levels peaked at approximately 3.5 hours. As the assays in the present study were carried out on a whole blood cell population, it is possible that the increase in CR3 expression was due to secondary stimulation of neutrophils following the release of cytokines from the monocyte population. Indeed at low LPS concentrations an increase in neutrophil CR3 expression occurred after 3 hours, possibly through stimulation by monocyte-derived TNF α (Fig 7.4). However the earlier timing of responses to higher concentrations of LPS (< 2 hours) suggests a direct effect of LPS on neutrophils.

E. coli LPS has been demonstrated to prime FMLP-induced respiratory burst responses in neutrophils (Karlsson et al, 1995). Comparison of *B. cepacia* and *P. aeruginosa* LPS with *E. coli* LPS in the current study indicated that whilst *B. cepacia* LPS molecules were potent neutrophil priming agents, little priming was

induced by *P. aeruginosa* LPS (Fig 7.9). Of the *B. cepacia* strains tested only J2540, an environmental strain, failed to induce priming significantly above levels of all *P. aeruginosa* strains tested. In time course experiments using LPS from *B. cepacia* J2315 and *E. coli* O18K-, priming was observed after as little as 30 min and 45 min respectively, suggesting that neutrophil priming responses were not secondary to LPS-induced cytokine release from circulating monocytes. LPS from two *P. aeruginosa* strains: PAO1, a well-characterised laboratory strain and C1250, a mucoid strain isolated from a CF patient, primed neutrophils to a low degree. However LPS from the non-mucoid CF strain, J1385, had no priming effect. Interestingly in a study of LPS extracted from 5 CF strains of *P. aeruginosa*, Kharazmi et al (1991) observed similar variation in neutrophil priming, with LPS from two mucoid strains showing the greatest overall activity.

Our observation of a high degree of correlation between mean CR3 surface expression and mean FMLP-induced respiratory burst activity agrees with published studies (Yee and Christou, 1993). Unlike increased CR3 expression, however, priming responses were not uniform throughout the neutrophil population and a subpopulation of highly responsive neutrophils were identified by changes in size (forward scatter) and granularity (side scatter) on stimulation with FMLP (Fig 7.7). These results agree with those of Yee and Christou (1993) who observed that the FMLP-induced elevation of intracellular Ca^{2+} levels in neutrophils primed with LPS was due to raised levels in a subpopulation of responsive cells. Similarly, Daniels et al (1994) demonstrated the recruitment of previously non-responsive neutrophils into a responsive population following treatment with physiological priming agents.

In the present study, highly responsive cells were virtually absent in unprimed populations but appeared in samples primed with LPS from both *B. cepacia* and *E. coli*. The observed changes in forward and side scatter for R2 neutrophils (Fig 7.7) may simply reflect the activation of these cells following FMLP stimulation. However, since respiratory burst responses also increased amongst R1 neutrophils, and since a considerable overlap in individual responses for neutrophils in R1 and R2 populations was observed, it seems more probable that LPS is associated with a significant phenotypic change in R2 neutrophils prior to stimulation with FMLP. The nature of such a phenotypic change remains unclear but could involve changes in receptor expression. However comparison of histogram distributions of CR3 expression and FMLP-induced intracellular H₂O₂ production indicated that the highly responsive subpopulation of neutrophils could not be identified on the basis of increased CR3 expression. FMLP receptors were not measured in the present study; however, previous investigators have found that both CR3 and FMLP receptors are upregulated on LPS-treated neutrophils in a unimodal fashion, with no evidence of neutrophil subpopulations (Yee and Christou, 1993), suggesting that increased receptor expression is not the mechanism underlying the highly responsive phenotype.

Additional studies assessed the impact of neutrophil exposure to LPS on respiratory burst induction by whole bacteria and provided an insight into the potential role of LPS in the colonisation of the CF lung by *B. cepacia*. The majority of *B. cepacia*-colonised CF individuals harbour large numbers of *P. aeruginosa* within their sputum prior to acquisition of *B. cepacia*. In our studies of representative *B. cepacia*

and *P. aeruginosa* strains, priming with LPS from *B. cepacia* J2315 was associated with a significant increase in respiratory burst induction by whole *P. aeruginosa* J1385. By contrast, whole *B. cepacia* J2315 induced little or no respiratory burst in both primed and unprimed neutrophils. Thus, *B. cepacia* LPS appeared to selectively upregulate responses to non-mucoid *P. aeruginosa*, whilst having little effect upon neutrophil interactions with *B. cepacia* itself. It seems reasonable to speculate that the induction of an increased anti-pseudomonal neutrophil response may enable *B. cepacia* to establish itself within the bacteriologically-crowded CF lung.

The interaction of *B. cepacia* haemolysin with neutrophils has recently been described by Hutchison et al (1998). This lipopeptide induces neutrophil azurophil degranulation, as determined by increased expression of surface neutrophil elastase, when incubated with neutrophils at concentrations of 100 ng/ml and above. In the present study, concentrations of haemolysin from 50 ng/ml to 500 ng/ml induced no respiratory burst response, but primed FMLP-induced responses to a level at least twice that induced by *E. coli* LPS. These results indicate a further mechanism by which *B. cepacia* strains of the ET12 lineage may potentiate neutrophil activation within the CF lung, thus increasing the inflammatory damage seen in colonised patients.

9.8 STUDIES IN THE CF MOUSE

CF lung disease is characterised by complex physiological and pathological defects, associated with respiratory colonisation by a diverse spectrum of microbial

pathogens. Consequently, many aspects of pathogenesis in CF can only be definitively investigated within a suitable animal model. CF has not been identified within any naturally-occurring animal population; however, CF mice have recently been developed by genetic manipulation of the gene coding for the murine homolog of CFTR. Many of these mutant mice display physiological and pathological defects similar to those seen in human CF, particularly in relation to gastrointestinal disease and the development of intestinal obstruction. However, less information is available on the development of respiratory disease and susceptibility to CF microbial pathogens in these animals. Indeed, microbiological data is only available for one murine model, the Edinburgh HGU/HGU CF mouse, which is more susceptible to the development of lung pathology in response to the CF pathogens, *S. aureus* and *B. cepacia*, than non-CF siblings (Davidson et al, 1995). As part of this thesis, further studies of the interaction of *B. cepacia* with the Edinburgh CF mouse were conducted.

Results from the present investigations have highlighted several potential problems in the analysis of data from bacteriological studies in CF mouse models. For example, considerable variability was observed in results within both CF and non-CF groupings in experiments assaying the clearance of *B. cepacia* J2315. Such variability was also noted in previous studies (Davidson et al, 1995) and may be due to several factors, including the effects of the variable genetic background of the outbred murine strain on the severity of the CF phenotype, the variable expression of residual CFTR due to the 'leaky' mutation in this strain, or differential exposure of mice within standard animal housing to environmental factors such as potential

respiratory pathogens. The extent of variability in the present study was such that a significant difference in terms of clearance of *B. cepacia* could not be demonstrated between CF mice and their non-CF siblings. Nevertheless, trends were clearly towards poorer clearance in CF than non-CF mice, suggesting that CF mice may be more susceptible to colonisation with *B. cepacia* than non-CF mice. In particular, the observation that individual CF mice demonstrated the poorest clearance in all experiments implies that the CF phenotype may only be fully expressed in a subset of these mice, reflecting the genetic diversity of the outbred murine strains used. Development of inbred strains may aid investigators in attempts to establish more fully the CF mouse model for bacteriological studies; however, as inbred strains displaying a less CF-like phenotype are more likely to breed successfully, development of such strains will require careful monitoring to ensure that the CF phenotype is not 'bred out' despite the presence of a genetic CF defect. In the meantime, studies in the Edinburgh CF mouse are continuing in an effort to establish more fully this model of microbiological disease in CF.

9.9 GENOMOVAR STATUS AND BACTERIAL ORIGIN: ARE ENVIRONMENTAL STRAINS OF *B. CEPACIA* PATHOGENIC?

Concern regarding the development of *B. cepacia* as a biopesticide has reached a new level with a recent application to the US Environmental Protection Agency for permission to conduct large scale field trials involving the administration of several tons of *B. cepacia* annually across the USA. Thus, an urgent need has arisen to define the features of *B. cepacia* which are linked to pathogenicity and hence prevent

the commercial development of strains which may present a human hazard. Whilst the increased incidence of *B. cepacia* infection in CF can be attributed to a small number of epidemic strains which may be only distantly related to environmental strains, the continuing acquisition of apparently non-epidemic strains of *B. cepacia* by CF patients, particularly within segregated clinics, suggests that environmental strains may pose a threat to patients. In addition, *B. cepacia* in chronic granulomatous disease and, indeed, in the rare cases of infection in apparently immunocompetent individuals, is presumably acquired from an environmental rather than clinical source. Furthermore, in view of the genetic complexity and inherent variability of *B. cepacia* (Wise et al, 1995), it is feasible that the emergence of epidemic strains represents the in vivo adaptation of environmental organisms to the CF lung, possibly through genetic recombination with other *Burkholderia* spp (Simpson et al, 1995). Such a possibility must be taken into consideration in any programmes developing environmental *B. cepacia* for large-scale release.

An understanding of the bacterial factors underlying *B. cepacia* colonisation and pathogenesis in CF would greatly assist in attempts to select 'safe' strains for commercial exploitation. Shaw et al (1995a) produced evidence suggesting that LPS from environmental strains is less inflammatory than LPS from CF and other clinical isolates; however, the inflammatory potential of environmental *B. cepacia* LPS was still significantly greater than that of representative strains of *P. aeruginosa*. In the present study we have demonstrated the induction of both neutrophil priming and non-azurophil degranulation by LPS from CF, non-CF clinical and environmental strains, with one environmental strain, J2552, showing the greatest activity. By

comparison, *P. aeruginosa* LPS has little or no activity. Thus it would seem that all *B. cepacia* strains possess LPS capable of producing a marked and damaging inflammatory response in colonised individuals. Furthermore, comparison of an ET12 strain, J2315, with the environmental strain, J2552, in studies of clearance from mouse lungs following intratracheal bacterial instillation suggested that the environmental strain had the greater colonising ability. As this finding was apparent in both CF and non-CF mice, a respiratory predilection for J2552-like *B. cepacia* in healthy as well as CF individuals cannot be ruled out, despite the apparently innocuous origin of this strain.

The identification of individual *B. cepacia* genomovars within panels of clinical and environmental strains may provide a means by which pathogenic and non-pathogenic strains can be separated. To date, the majority of environmental isolates tested have belonged to genomovars I or IV, whilst genomovars II and III have predominated amongst CF patients, and are associated both with transmissibility and, particularly for genomovar III, with rapid pulmonary deterioration (Govan et al, 1996; Revets et al, 1996; Dr P Vandamme, personal communication). However, such data has been gathered on a relatively small number of strains, and further work is required to confirm these findings. In the meantime, efforts to develop *B. cepacia* for biocontrol have focused on environmental isolates, particularly the genomovar V AMMD strain which has recently been proposed for large scale release in North American crop spraying trials. Little data is available, however, on the potential pathogenicity of this strain, particularly in relation to CF and other respiratory diseases.

As work towards this thesis has reached its final stages, information regarding the genomovar status of some of the strains investigated has become available. Initially it was hoped that separation of strains into individual genomovars would help explain some of the variability found between isolates, particularly in chemiluminescence experiments. However, little correlation was observed between genomovar status and neutrophil respiratory burst induction. In studies of opsonisation, all genomovar III strains were well opsonised by immune serum from patients colonised with the ET12 lineage. Results from isolates belonging to genomovars I and II were less clear-cut, with opsonic activity against some strains but not others, suggesting the existence of some cross-reactive epitopes on the surface of genomovar I, II and III isolates. In further studies of LPS activity in neutrophil degranulation and priming assays, both the genomovar I strains, J2540 and J2552, and the ET12 genomovar III strain, J2315, showed similar levels of pro-inflammatory activity to *E. coli* LPS, suggesting that both genomovars have the potential to initiate inflammatory damage within the CF lung. A further strain, J2505, which shares characteristics of genomovars I, III and IV was also highly pro-inflammatory in these assays. Finally, the results of clearance studies in non-CF and CF mice, suggested that genomovar I strains such as J2552 may actually demonstrate an equivalent or greater colonising ability than genomovar III strains such as J2315. As current proposals to release the environmental *B. cepacia* strain, AMMD, recently identified as a genomovar V strain (i.e. *B. vietnamiensis*), consist of the repeated administration of several ounces of organisms per acre to fields in several States of the USA, individuals may be exposed to abnormally high inoculae of this organism. The risks of such exposure for both healthy individuals and individuals with CF seem impossible to predict, particularly

in the absence of data regarding the inflammatory potential of the AMMD strain. Thus, further studies should be urgently conducted on this strain before permission for environmental release is granted.

CHAPTER 10 CONCLUSIONS

Increasing evidence suggests that the pathogenesis of lung disease in CF patients colonised with *B. cepacia* is mediated by a marked inflammatory response to this unusual but potentially devastating respiratory pathogen. Research carried out in pursuit of this thesis has provided further evidence to support this hypothesis, by indicating the potent pro-inflammatory potential of *B. cepacia* and *B. cepacia* products, particularly LPS. Thus, induction of non-azurophil degranulation, associated with the priming of respiratory burst responses, may promote both neutrophil recruitment to the respiratory tract, followed by an enhanced response on exposure to a suitable stimulus within the lung. It is tempting to speculate that cepacia syndrome develops whenever the burden of activated neutrophils within the lung becomes too great for over-stretched regulatory mechanisms, increasing the rate of inflammatory damage and permitting the bacteraemic spread of *B. cepacia* beyond the lung parenchyma. *B. cepacia* itself seems to induce little respiratory burst activity and may thus evade neutrophil killing mechanisms, particularly if neutrophil-bacteria interactions occur in the absence of effective opsonisation. Indeed, the development of rapid lung deterioration and cepacia syndrome may be a consequence of activation of *B. cepacia* LPS-primed neutrophils by a secondary stimulus such as other co-colonising bacteria, including *P. aeruginosa*, or exposure to a respiratory viral infection. Such a model of *B. cepacia* pathogenesis may go some way to explaining the great variety of clinical outcomes seen in colonised patients, with the prognosis for individual patients linked not only to the response to *B. cepacia* but also to other co-colonising organisms.

The development of an inflammatory model of *B. cepacia* pathogenesis provides several potential avenues for investigation. In particular, the effects of co-colonisation with *B. cepacia* and *P. aeruginosa* requires further study. Ideally, confirmation of this hypothesis will come with the refinement of current animal models of CF, enabling in vivo studies of bacterial co-colonisation on pathogenesis. Considerable work will be required, however, if mouse models are to reach this stage of development, particularly in the establishment of chronic *P. aeruginosa* infection within the mouse lung. An alternative approach could include the in vitro comparison of the inflammatory potential of combinations of paired *B. cepacia* and *P. aeruginosa* strains from individual patients with clinical data regarding patient well-being and lung function.

Further investigations of the nature of *B. cepacia* LPS-neutrophil interactions are anticipated in an attempt to identify the main neutrophil receptors utilised by *B. cepacia* LPS, particularly in the absence of serum, and to analyse the nature of signal transduction events leading to the induction of non-azurophil degranulation and priming. At present, there is an urgent need for a novel therapeutic approach to the treatment of *B. cepacia* infection, particularly as there is little prospect of the development of an effective anti-*B. cepacia* antimicrobial agent at present. It is therefore hoped that an understanding of the molecular processes by which *B. cepacia* LPS upregulates neutrophil activation will provide new target molecules for the development of anti-inflammatory therapies.

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Burkholderia cepacia and Cystic Fibrosis: Do Natural Environments Present a Potential Hazard?

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Received 30 September 1994/Returned for modification 11 November 1994/Accepted 6 January 1995

An environmental survey of 55 sites yielded only 12 *Burkholderia cepacia* isolates, none of which displayed the phenotypic properties of a multiresistant epidemic strain associated with pulmonary colonization in patients with cystic fibrosis. Although the environment probably poses a low risk for patients with cystic fibrosis as a source of *B. cepacia*, the pathogenic potential of individual environmental strains remains unclear. We advise caution in the development of *B. cepacia* as a biocontrol agent.

First described in 1950 by Burkholder (5) as the cause of soft rot of onions, *Burkholderia cepacia* (basonym *Pseudomonas cepacia*) is now of wider and increasing interest in agriculture, biotechnology, and medicine. Reasons for this interest include the organism's abilities to promote plant growth by antagonizing soilborne plant pathogens (15, 16, 25), to degrade hydrocarbons and thus assist in the bioremediation of contaminated soil and water (11), and to cause opportunistic human infections, particularly in patients with chronic granulomatous disease (22, 30) and cystic fibrosis (CF) (1, 14, 19, 36). In patients with CF, *B. cepacia* is now a major pathogen because of the following: first, its association with cepacia syndrome, a rapidly fatal necrotizing pneumonia, sometimes accompanied by septicemia (19); second, its innate multiresistance to antibiotics (23, 34); third, person-to-person transmission through social contact (14, 24, 38); and fourth, the risk of nosocomial acquisition (40). In the last decade, there have been increased attempts to reduce the risk of *B. cepacia* transmission by segregation of *B. cepacia*-colonized patients and publication of guidelines concerned with hygiene and social behavior (1, 29, 42). The social effects of segregation on patients with CF and organizations for people with CF are psychologically devastating, and the policy has not been universally welcomed, particularly in centers for patients with CF in which *B. cepacia* colonization or transmission has remained low (1, 33, 39). The introduction of hygiene guidelines and segregation has undoubtedly reduced acquisition of *B. cepacia* in patients with CF (14, 38, 42), but acquisition has not been eliminated. The source of *B. cepacia* responsible for new acquisitions is unclear, and there is increasing concern about the risk that the environment may present as a source of this opportunistic saprophyte (10, 20). At present, rational judgments on this issue are frustrated by the following uncertainties: first, the distribution of *B. cepacia* in natural environments; second, the pathogenic potential of environmental isolates; and third, the potential hazards associated with the development and use of *B. cepacia* as a biological control agent.

B. cepacia has been cultured from a range of environmental sources, including soil, water, and vegetation (2, 15, 26), and from clinical material (17). There are few reports, however, of prospective studies of the organism's natural habitats, and there is little documented evidence to support the use of the

term ubiquitous to describe the organism's environmental distribution. LiPuma et al. (24) cited previous failures to recover *B. cepacia* from environmental surfaces to support person-to-person transmission as the primary mode of *B. cepacia* acquisition in patients with CF. In a subsequent study, *B. cepacia* was cultured from only 1% of samples obtained from homes and 4.5% of samples obtained from salad bars and food stores (10). The use of the term environmental isolates also can be misleading since in epidemiological studies of nosocomial *B. cepacia* infections, it is not always possible to identify whether isolates cultured from environmental surfaces have contaminated patients or vice versa. The aims of our study were to investigate prospectively the distribution of *B. cepacia* in other natural environments and to obtain authentic environmental isolates for future comparative studies with clinical isolates to investigate *B. cepacia* virulence factors and to determine the pathogenic potential of environmental strains.

A large botanical complex was chosen for this survey to provide an extensive range of soils, aquatic sites, and vegetation located in a natural temperate climate and tropical, subtropical, and arid microclimates within greenhouses. Fifty-five samples of soil, soil rhizosphere, water, and vegetation were collected and cultured for *B. cepacia* by using selective media and screening procedures adapted from two previous surveillance studies (10, 28). Prior to sampling, swabs were first moistened in a selective broth that consisted of Malka minimal broth (35) supplemented with polymyxin (300 U/ml) and plated directly onto selective agar that consisted of 300 U of polymyxin per ml and 100 µg of ticarcillin per ml (Mast cepacia agar; Mast Diagnostics, Ltd., Bootle, United Kingdom). Water samples (100 ml) were filtered (pore size, 0.2 µm; Millipore). The same swabs, filter membranes, soils, and vegetation were transferred to Malka minimal broth supplemented with polymyxin, incubated at 30°C for 5 days, and subcultured to the selective agar described. After 48 h at 30°C, bacterial colonies were subcultured to MacConkey agar and fluorescent pigment-enhancing King's B medium (21). After a further 48 h at 30°C, nonfluorescent, non-lactose fermenters were screened on arginine-glucose medium, *B. cepacia* isolates were presumptively identified by biochemical reactions (13), and identification was confirmed by the API 20 NE system (bioMérieux, Basingstoke, United Kingdom). Individual *B. cepacia* isolates were then investigated for catalase production (32), lipopolysaccharide (LPS) content by polyacrylamide gel electrophoresis following proteinase K extraction as previously described (6), and antimicrobial susceptibility by an agar dilution method on Mueller-Hinton agar (23), with sensitivity defined in terms of the British

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TABLE 1. Antibiotic resistance of 48 clinical and 25 environmental isolates of *B. cepacia* with sensitivity defined in terms of breakpoints

Antibiotic	Breakpoint (mg/liter) ^a	No. of resistant isolates (%)	
		Clinical	Environmental ^b
Piperacillin	64	20 (42)	1 (4)
Ceftazidime	2	39 (81)	4 (16)
Meropenem	4	25 (52)	2 (8)
Chloramphenicol	8	44 (92)	18 (72)
Ciprofloxacin	4	18 (38)	2 (8)
PD 127391	4	10 (21)	0 (0)
PD 131628	4	14 (29)	0 (0)

^a Defined by the British Society for Antimicrobial Chemotherapy Working Party (4), apart from PD 127391 and PD 131628, which have been defined by Lewin et al. (23).

^b Includes 10 isolates in this study and 15 isolates previously held in our collection.

Society for Antimicrobial Chemotherapy Working Party breakpoints (4) (Table 1). Pulsed-field gel electrophoresis (PFGE) was performed by the technique of Vasil et al. with some modifications (43). Bacteria were grown in nutrient broth no. 2 (Oxoid, Basingstoke, United Kingdom) that contained 0.5% yeast extract (Difco, Surrey, United Kingdom). Cells were washed with 75 mM NaCl–25 mM EDTA, standardized to an optical density at 590 nm of 1.0, and mixed with an equal volume of molten 1% low-melting-point preparative-grade agarose (Bio-Rad, Hemel Hempstead, United Kingdom). DNA restricted with *Xba*I (TCTAGA) and *Spe*I (ACTAGT) (Gibco BRL, Paisley, United Kingdom) was separated by PFGE by using the CHEF DRII system (Bio-Rad) with pulses of 2.9 to 35.4 s at 200 V for 22 h at 14°C. Gels were stained with ethidium bromide at a concentration of 1 µg/ml for 15 min and then destained in distilled water for 30 min.

B. cepacia was cultured from 12 (21.8%) of 55 samples and sites (Table 2). This recovery rate exceeds that reported by Fisher and colleagues (10) for homes (1%) and food stores (4.5%); however, both studies indicate that it is an exaggeration to describe *B. cepacia* as ubiquitous or widely distributed in nature. The data also emphasize the biological puzzle posed by Palleroni (31), namely, if *B. cepacia* is so nutritionally versatile, why is this species not more readily cultured from natural habitats? In our study, *B. cepacia* isolates were not recov-

TABLE 2. Environmental sites in botanical gardens from which *B. cepacia* strains were cultured

Isolate	Site	Area
J2534	Rotting bark	Orchid and cycad house
J2535	Rotting bark	Orchid and cycad house
J2536	Soil	Gardens
J2537	Soil	Rhododendron house
J2538	Soil	Rhododendron house
J2539 ^a	Rhizosphere of <i>Hohenbergia stalata</i>	Temperate aquatic house
J2540	Rhizosphere of banana plant	Tropical aquatic house
J2541	Rhizosphere of <i>Epidendrum obrieniamum</i>	Orchid and cycad house
J2542	Rhizosphere of <i>Nautilocalyx lynchii</i>	Orchid and cycad house
J2543 ^a	Pond water	Tropical aquatic house
J2552	Rhizosphere of <i>Carludouca palmata</i>	Tropical aquatic house
J2553	Sansevieria leaf	Tropical palm house

^a Identical PFGE profiles.

ered from healthy or diseased cacti or from soil within the cactus greenhouse; the majority of recovery sites were in moist soil or soil rhizosphere, which suggests that these environments are natural habitats for this microbial saprophyte.

Previous studies (6,14) have shown that *B. cepacia* J2315, an epidemic strain associated with morbidity and mortality in patients with CF and epidemic outbreaks in regional clinics for those with CF in the United Kingdom, has unusual phenotypic characteristics, including a dry rough colonial morphotype, dark melanin-like pigmentation, a sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile indicative of rough LPS, and multiresistance to co-trimoxazole and all of the antibiotics listed in Table 1. These characteristics were not observed for any of the 12 environmental isolates of *B. cepacia*. The results of antibiotic sensitivity testing are shown in Table 1; in general, environmental isolates, including 15 previously held in our collection, showed greater sensitivity than did CF isolates of *B. cepacia*. Interestingly, however, epidemic strain J2315 and 10 of 12 environmental isolates were catalase positive, a characteristic of opportunistic pathogens responsible for recurrent life-threatening infections in patients with chronic granulomatous disease (22).

Genomic analysis of DNA from environmental isolates of *B. cepacia* by PFGE following endonuclease digestion with *Spe*I and *Xba*I illustrated the genomic diversity of this species. With one exception, each of these 12 isolates produced a distinct endonuclease restriction profile which also differed from the PFGE profiles of epidemic strain J2315 and 10 other clinical isolates of *B. cepacia* cultured from patients with CF.

Accumulated evidence from this study and previous reports (10, 18) suggests that compared with the heavily colonized respiratory secretions of *B. cepacia*-colonized patients with CF, the environment is a relatively low-risk source of *B. cepacia* for patients with CF. Honicky et al. recovered *B. cepacia* from only 4 (3 from lake water and 1 from ice water from a picnic jug) of 58 environmental samples in a study of *B. cepacia* acquisition in patients with CF who attended summer camps (18). Furthermore, genomic fingerprinting of *B. cepacia* cultures from the 16 (6.6%) of 244 campers whose acquisition of *B. cepacia* was associated with camp attendance showed that the majority of new colonizations involved strains shared by other camp attenders and that no patient with CF had acquired a strain which shared a genotype found in environmental isolates.

An important caveat to the conclusion that the environment is not a major source of *B. cepacia* infection in humans concerns the development and release of *B. cepacia* as a biological agent to control plant disease and soil decontamination. In a recent report, Bevivino et al. (3) concluded that environmental isolates probably pose little threat in human disease since they lack the abilities to produce gelatinase and a hydroxamate siderophore and to adhere to human uroepithelial cells. This conclusion needs to be treated with considerable caution. Only two environmental isolates and two clinical isolates were investigated; furthermore, there is no evidence that the putative virulence determinants investigated in this study play a role in human infections, including colonization and pathogenesis in patients with CF (27).

Equally relevant to the issue of the use of *B. cepacia* in biological control are the questions of whether clinical and environmental *B. cepacia* organisms represent two distinct groups and, in particular, whether environmental strains cause human infections, either de novo or through adaptation. In respect to the latter, in longitudinal studies of *B. cepacia*-colonized patients with CF, there is no phenotypic evidence of adaptation in vivo, which is so characteristic of mucoid alginate-producing *Pseudomonas aeruginosa* (27). Previous re-

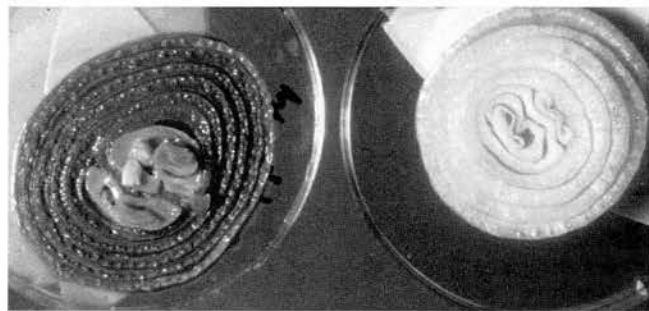


FIG. 1. Soft rot of onion segments inoculated with *B. cepacia* clinical isolate J2315 (left) and an uninoculated, undamaged control (right), both incubated at 30°C for 72 h.

ports (12, 44) concluded that the two groups of *B. cepacia* are distinct and that clinical isolates do not have the ability to act as phytopathogens; however, this distinction is unfounded since most clinical isolates, including epidemic strain J2315, readily cause soft rot of onions (Fig. 1). In turn, evidence that environmental isolates have the ability to cause human infection is provided by the macerated, hyperkeratotic foot lesions encountered by troops during swamp training, known as swamp rot (41). The distinctive phenotypic properties of multiresistant epidemic *B. cepacia* isolates, including J2315 (14, 37), suggest that a subpopulation of *B. cepacia* may have a predilection for the lungs of patients with CF. Recent evidence (37) that such strains possess characteristics of *B. cepacia* and the closely related phytopathogen *Burkholderia gladioli* and that the genomic constitution of *B. cepacia* may facilitate genetic rearrangements (7) supports speculation that the transition of environmental *B. cepacia* isolates from phytopathogen to pulmonary colonization in patients with CF is enhanced by intraspecies adaptation or the emergence of bacterial hybrids of the two species.

At present, unequivocal statements on the potential of environmental *B. cepacia* isolates to cause infections in patients with CF and in other immunocompromised hosts are frustrated by our scanty knowledge of the virulence factors and mechanisms of pathogenesis involved (27). The recent development of mutant mice with CF (9) and the demonstration that *B. cepacia* J2315 causes pneumonia in mice with CF but not in control animals (8) provide a useful model to investigate the pathogenic potential of environmental *B. cepacia* isolates for patients with CF.

We acknowledge the interest and hospitality of the curator and staff of the Royal Botanic Gardens, Edinburgh, Scotland, in the collection of environmental samples for this study.

The financial support of the Cystic Fibrosis Trust in the form of a CF Studentship held by S.L.B. is also acknowledged.

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REVIEW ARTICLE

***Burkholderia cepacia*: medical, taxonomic and ecological issues**

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The increasing challenge posed by multiresistant saprophytes in medical microbiology is strikingly demonstrated by the emergence of *Burkholderia* (formerly *Pseudomonas*) *cepacia* as an opportunist pathogen in immunocompromised patients, particularly individuals with chronic granulomatous disease and cystic fibrosis (CF). Best known previously as a phytopathogen and the cause of soft rot of onions, *B. cepacia* presents three major problems for the CF community: innate multiresistance to antimicrobial agents; person-to-person transmission of epidemic strains through nosocomial or social contacts; and 'cepacia syndrome', a fulminating fatal pneumonia, sometimes associated with septicaemia, that occurs in approximately 20% of colonised patients, including those with previously mild disease. Accumulated evidence to dispel earlier suggestions that the organism is avirulent and merely a marker of existing lung disease includes: case-controlled studies in CF patients; reports of serious infections in non-CF patients; in-vitro and in-vivo evidence that *B. cepacia* induces production of pro-inflammatory markers, including the major cytokine TNF α ; and histopathological evidence that exposure of transgenic CF mice to *B. cepacia* results in pneumonia. By the early 1990s, the use of selective culture media and DNA-based bacterial fingerprinting confirmed suspicions of epidemic person-to-person spread of *B. cepacia*. This evidence provided scientific justification for draconian and controversial measures for infection control, in particular, segregation of *B. cepacia*-colonised patients during treatment at CF centres and their exclusion from social gatherings and national conferences. Recently, molecular analyses of type strains and clinical isolates have revealed that isolates identified previously as *B. cepacia* belong to at least three distinct species and have increased concern regarding the reliability of current laboratory detection and identification systems. Clarification of the taxonomy of *B. cepacia*-like organisms and the pathogenic potential of environmental isolates remains a high priority, particularly when the organism's antifungal and degradative properties have created interest in its potential use as a biological control agent to improve crop yields and its use for the bioremediation of contaminated soils.

Introduction

'The development of multiresistance in major microbial pathogens is well-recognised; in contrast, little attention has been paid to the pathogenic potential of naturally resistant environmental saprophytes'.

Known originally as a phytopathogen, *Burkholderia cepacia* (previously *Pseudomonas cepacia*, *P. multi-*

vorans, *P. kingii*, 'Eugonic oxidiser 1') exhibits impressive nutritional versatility. Some microbes have an inherent or acquired ability to degrade antibiotics, but few have the ability to use penicillin as a sole carbon source [1] or to reduce onions to a macerated pulp! The earlier name, *P. multivorans*, reflected the organism's omnivorous appetite, but it was not until 1950 that its pathogenic potential was recognised when Burkholder identified the organism as the cause of soft rot of onions—particularly 'compromised' onions damaged during harvesting—and provided an appropriate species epithet (Latin: *cepia* = onion) [2]. In the early 1990s, following taxonomic re-appraisal, the RNA group II pseudomonads were recognised as

the new genus *Burkholderia*, with *B. cepacia* as the type species [3]. At present, the genus *Burkholderia* comprises *B. cepacia*, *B. gladioli*, *B. mallei*, *B. pseudomallei*, *B. caryophylli*, and recently added to the group, *B. plantarii*, *B. glumae*, *B. vandii* [4], *B. cocovenenans* [5] and *B. vietnamiensis* [6].

The general characteristics of *B. cepacia* include the following: gram-negative, non-spore-forming, aerobic bacillus; motile with a respiratory metabolism and typically catalase- and oxidase-positive; various non-fluorescent pigments may be produced and poly- β -hydroxyalkanoates can be accumulated as reserve materials; the optimal temperature for growth is 30–35°C [7]. Recently, elegant molecular analyses have provided scientific evidence that may account for the organism's impressive versatility, including multilocus linkage disequilibrium analysis of environmental populations [8]—which suggested an extraordinarily high rate of recombination in *B. cepacia* relative to binary fission—and demonstration of multiple replicons and insertion sequences in type strains [9, 10].

The natural habitats of *B. cepacia* have been described as soil, water and vegetation [11]. However, it is a common but erroneous belief that *B. cepacia* is a ubiquitous saprophyte sharing similar environmental habitats with *Pseudomonas aeruginosa* and other pseudomonads. Extensive surveillance studies have shown that culture of *B. cepacia* from natural sources, including soil, water and plants, or from hospitals, foodstores, restaurant salad bars and patients' homes is surprisingly difficult, with detection rates of only 1–16% [12–16].

In agricultural microbiology, ecological awareness and an increasing incidence of pesticide-resistant pathogens have led to interest in *B. cepacia* as a potential agent for biological control and soil decontamination. *B. cepacia* produces several antimicrobial agents, including pyrrolnitrins, altericidins, cepalycins and bacteriocin-like agents [17–20], that inhibit bacterial and fungal phytopathogens and suppress tobacco wilt and other plant diseases [21]. *B. cepacia* is also capable of degrading industrial waste and herbicides, including 2,4,5-trichlorophenoxyacetic acid (2,4,5-T),

the principal ingredient of the highly potent 'agent orange' [22]. Indeed, *B. cepacia* has been shown to degrade 2,4,5-T in heavily contaminated soils at a rate up to 20 000-fold greater than other known degradative bacteria [23].

In contrast to its potential agricultural benefits, *B. cepacia* has also emerged as a multiresistant opportunist human pathogen, leading to concern about the relationship between environmental and clinical isolates [14, 24–26] and the potential hazards of releasing *B. cepacia* as a biological control agent [14, 24]. This review will provide an update on microbes currently described as *B. cepacia*, with particular focus on clinical, taxonomic and ecological issues (Table 1) associated with pulmonary infection in patients with cystic fibrosis (CF).

The emergence of *B. cepacia* as a human pathogen

Before the early 1980s, reports of human infections caused by *B. cepacia* were sporadic and generally restricted to hospitalised patients exposed to contaminated disinfectant and anaesthetic solutions in which this nutritionally adaptable saprophyte survives for long periods. Infections included those of soft tissues and the respiratory and urinary tracts, but bacteraemia also occurred, sometimes associated with endocarditis and septic shock [27–31]. A rising incidence of *B. cepacia* infection was noted during the early 1980s and, although in some cases culture of *B. cepacia* was thought to reflect mere colonisation or contamination rather than infection [11, 32], extensive analyses of USA databases of nosocomial infections confirmed a significant increase in clinically significant *B. cepacia* infections [33, 34]. The apparent propensity of *B. cepacia* to cause fatal pulmonary infections, as suggested by these analyses, is emphasised in patients with chronic granulomatous disease (CGD)—in whom *B. cepacia* pneumonia and septicaemia are life-threatening [35, 36]—and in its emergence as a major pathogen in patients with CF [37–39]. By the 1990s, disturbing reports also emerged of fatal cases of *B. cepacia* pneumonia and septicaemia in previously

Table 1. Major issues associated with *B. cepacia* and cystic fibrosis (CF)

- Is there convincing evidence to confirm that *B. cepacia* has pathogenic potential and is not merely a marker of pulmonary disease?
- Based on the success, but unpopularity, of segregation and advances in clarifying the taxonomy of the genus *Burkholderia*, should all *B. cepacia* be treated as equal? Can phenotypic or genomic markers be found which would identify highly transmissible or virulent clones?
- To what degree do natural environments represent a reservoir for *B. cepacia* and a hazard for CF patients? What hazards are associated with the development and use of *B. cepacia* as a biological control agent?
- Could an improved understanding of the host immune response, including enhanced cytokine induction by bacterial surface components, clarify the immunopathology of *B. cepacia* and lead to innovative forms of immunotherapy?
- At present, it is not possible to forecast the clinical outcome of *B. cepacia* colonisation. Can host and bacterial factors responsible for initial colonisation and poor clinical outcome be identified?
- Recently, it has been demonstrated that CF airway epithelia contain bactericidal activity that is inhibited reversibly by high NaCl concentrations. Does this killing potential include *B. cepacia* and is it host or strain specific?
- Ultimately, the identification of bacterial and host factors associated with transmission and virulence would assist greatly in the rational design of an effective *B. cepacia* vaccine.

healthy individuals [40, 41]. Community-acquired *B. cepacia* infections are uncommon, but the organism's pathogenic potential and the financial implications of antimicrobial therapy were recently strikingly demonstrated when an offshore oil worker developed multiple brain abscesses secondary to suppurative otitis media. Therapy involved four neurosurgical operations, an extensive period of hospitalisation and an antibiotic bill of £10K [42].

The above case also demonstrated an interesting and unexplained variability in antibiotic susceptibility profiles that has been observed in serial *B. cepacia* isolates from single patients and during epidemic outbreaks [43–46]. The mechanism responsible for variable susceptibility is unclear, but may be associated with the observation that migration of insertion sequences within the *B. cepacia* genome can affect the expression of genes that modulate antibiotic resistance [47].

B. cepacia and cystic fibrosis

During the last decade, the major clinical interest in *B. cepacia* has focused on its addition to the relatively narrow spectrum of microbial pathogens responsible for debilitating and ultimately fatal pulmonary infections in patients with CF [26, 39, 48, 49]. In the late 1980s, surveillance studies in the UK indicated a maximum prevalence of 7% [39, 50–52]; however, in some CF centres this later increased to approach the prevalence of 40% described in contemporary North American studies [53]. The three major issues concerning *B. cepacia* can be summarised as follows: 1, the clinical risk of rapid and fatal pulmonary decline, even in patients with previously mild disease; 2, patient-to-patient spread of epidemic strains within and between regional CF centres and between centres in the UK and North America; and 3, the innate multiresistance of most *B. cepacia* isolates to available antibiotics—which deprives patients of effective antimicrobial therapy [46, 54]—combined with the failure to reduce the bacterial population in sputum and a relatively poor clinical response even when the colonising strain exhibits in-vitro susceptibility.

The clinical significance of *B. cepacia* in CF patients was first described in 1984 in a seminal report by Isles *et al.* [37]. In addition to noting the increased prevalence of *B. cepacia* colonisation in patients attending Toronto clinics, Isles *et al.* described a rapid and unexpected clinical decline, including necrotising pneumonia and bacteraemia, that occurred in *c.* 20% of colonised patients. This acute clinical decline is sometimes referred to as 'cepacia syndrome' [37]. It is important to note that acute clinical deterioration and bacterial spread to sites other than the lung is not observed with the other major CF pathogens, *Staphylococcus aureus*, *Haemophilus influenzae* and *P. aeruginosa*.

The second major issue relating to *B. cepacia* arose in the mid 1980s as an increasing—but scientifically unproven—conviction held by some CF carers that the clustering of cases in some large North American clinics had arisen from cross-infection. At that time, an alternative explanation for clustering was the difficulty in culturing this relatively new pathogen from CF sputa [48]. As evidence, in a controlled study involving 115 North American CF centres, only 36 (31%) cultured the organism successfully from a seeded sputum specimen [55]. However, by the early 1990s, the availability of selective culture media [48] and awareness of the organism's cultural idiosyncrasies [56] indicated that regional variation in the prevalence of *B. cepacia* colonisation could not be explained simply by laboratory methodology. Furthermore, the development and use of bacterial fingerprinting techniques—including multilocus enzyme electrophoresis (MLEE), pyrolysis mass spectroscopy, PCR-ribotyping and pulsed-field gel electrophoresis (PFGE)—provided compelling evidence for person-to-person spread of *B. cepacia* through nosocomial and social contacts (Table 2) [25, 37, 46, 57–75] and, occasionally, in the absence of proven sputum colonisation [67]. Epidemiological data also provided scientific justification for the introduction of guidelines by national CF organisations to improve personal and hospital hygiene and, more controversially, for the implementation of segregation policies to limit contact between colonised and non-colonised individuals [76]. Surveillance studies show that segregation undoubtedly reduces the incidence of *B. cepacia* cross-infection [38, 62, 71, 77], but the strategy has not eliminated acquisition. Furthermore, the logistic and social consequences of draconian infection control measures reminiscent of mediaeval approaches to leprosy have not been accepted universally. In particular, the need for such measures has been questioned fiercely by patients and care-givers in CF centres where intensive surveillance has not revealed a high incidence or prevalence of *B. cepacia* colonisation.

A pathogen or a marker of lung disease?

In the 1970s, some microbiologists and clinicians considered *S. aureus* to be the only true microbial pathogen in CF patients and viewed *P. aeruginosa* as merely a marker of disease. A similar doubt has accompanied the emergence of *B. cepacia* and has exacerbated the controversy surrounding segregation of colonised individuals. In discussions of any potential opportunist pathogen, it is easy to find evidence of asymptomatic carriage; even *Salmonella typhi* and *Vibrio cholerae* do not invariably exhibit pathogenicity!

Clarification of the clinical relevance of *B. cepacia* is also thwarted by the fact that the available scientific evidence requires particularly careful analysis. There is an inclination to link bacterial transmissibility and virulence, and to categorise individual *B. cepacia*

Table 2. Evidence for and against person-to-person transmission of *B. cepacia*

Reference	Comments
A. Evidence in favour of person-to-person transmission	
Isles <i>et al.</i> [37]	Seminal paper: noted rising incidence of <i>B. cepacia</i> and cepacia syndrome in Canadian clinics
Thomassen <i>et al.</i> [57]	Fall in incidence after segregation
LiPuma <i>et al.</i> [58]	Prevalence of one ribotype in individual clinics
LiPuma <i>et al.</i> [59]	Ribotyping demonstrates person-to-person spread between two patients at a CF camp
Anderson <i>et al.</i> [60]	Nosocomial outbreak
Millar-Jones <i>et al.</i> [61]	UK nosocomial outbreak
Govan <i>et al.</i> [62]	Genotypic fingerprinting and extensive epidemiological data provides compelling evidence of person-to-person spread through social contact in and between two UK CF centres
Smith <i>et al.</i> [63]	Further UK outbreak with transmission in clinical and social settings
Bingen <i>et al.</i> [64]	International consensus confirming <i>B. cepacia</i> transmissibility
Corkill <i>et al.</i> [65]	Highlights transmission particularly at UK CF events
Pegues <i>et al.</i> [66]	Demonstration of transmission at USA CF camps
Johnson <i>et al.</i> [25]	Intercontinental spread of Edinburgh/Toronto strain ET12
LiPuma <i>et al.</i> [67]	Inapparent transmission from culture-negative patient (?)
Ryley <i>et al.</i> [68]	Further UK outbreak
Sun <i>et al.</i> [69]	Cable pili demonstrated on intercontinental strain (ET12)
Reverts <i>et al.</i> [70]	Prevalent strain in Belgian clinic
Whiteford <i>et al.</i> [71]	Outbreak in UK paediatric clinic
Pitt <i>et al.</i> [46]	Strain ET12 prevalent in UK clinics: accounting for 38% of cases
B. Cases with no evidence of person-to-person transmission	
Glass and Govan [72]	No transmission of pathogenic strain between siblings
Hardy <i>et al.</i> [73]	No transmission to uncolonised patients during hospitalisation
Taylor <i>et al.</i> [74]	No transmission in UK unit before segregation
Steinbach <i>et al.</i> [75]	No transmission in large CF unit despite no segregation of hospitalised patients

strains as either transmissible and virulent, or non-transmissible and avirulent. There is no scientific justification for this view. In epidemic outbreaks in which patients are colonised by the same strain, some patients may remain asymptomatic whilst other individuals succumb to rapid and unexpected fatal deterioration [37, 62]. In the case of transmission, epidemiological evidence has clearly identified lineages with enhanced transmissibility [25, 46, 62, 69]; however, based on present knowledge, it cannot be stated with confidence that a strain inherently lacks the ability for epidemic spread. Furthermore, apparently 'non-transmissible' strains that have not spread even to a patient's CF sibling have been responsible for fatal infection [72]. Finally, it could be argued that transmission is not strain-dependent, but is associated with nosocomial or social opportunities for transmission. This hypothesis is certainly not supported by the behaviour of the particular *B. cepacia* lineage with a notorious ability to spread in CF centres in the UK [46, 62] and North America [25, 69], referred to as the Edinburgh/Toronto lineage [69] or ET12 intercontinental clone (multilocus enzyme electrophoresis type 12) [25]. For convenience, this particular *B. cepacia* lineage will be referred to as the ET12 lineage in the remainder of this review.

Some CF carers who have experienced transmission of *B. cepacia* amongst small numbers of their patients have argued against segregation on the grounds that no significant clinical deterioration was observed and that implementation of such draconian measures stigmatises patients and prevents valuable social contacts with other CF patients [70]. However, the hypothesis that *B. cepacia* is transmissible but merely a marker of pulmonary deterioration can be chal-

lenged. A recent retrospective study of the clinical status of *B. cepacia*-colonised adults in the 24-month period before colonisation found no difference in their lung function, number of days in hospital or outpatient visits [77]. Furthermore, in numerous case-controlled studies involving large numbers of patients, *B. cepacia* colonisation has been associated in some but not all patients with an accelerated decline in pulmonary function and a poor prognosis [71, 77–81]. Most studies have reported that the risk of clinical deterioration on acquisition of *B. cepacia* is increased in adult patients with severe disease [78–80]. This contrasts with an epidemic outbreak of *B. cepacia* among children, in whom the dominant impact on respiratory function was greater in patients with better levels of respiratory function [71]. Explanations for the range of clinical responses associated with *B. cepacia* colonisation and inability to predict the clinical outcome in individual patients could include: 1, differences in strain virulence; 2, the relatively low 20% 'strike rate' of cepacia syndrome; 3, the influence of co-colonisation by other pathogens; 4, the age at which colonisation occurs; 5, individual host immune responses; and 6, the severity of underlying CF disease.

The hypothesis that *B. cepacia* colonisation is merely a marker of severe lung disease is also undermined by the fact that fatalities have occurred in CF adults with mild CF disease, including individuals not already harbouring *P. aeruginosa* [62]. Finally, one of the most striking results from the first microbiological studies in transgenic CF mice showed that 70% of CF mice exposed to *B. cepacia* succumbed to more severe broncho-pulmonary infection than control animals [82].

The Edinburgh/Toronto/ET12 epidemic lineage

In reviewing the emergence of *B. cepacia* in CF populations in Europe and North America, it is necessary to emphasise the influence of epidemic lineages on the incidence and prevalence of *B. cepacia* within CF centres. Evidence shows that the incidence in a centre can be influenced greatly by the epidemic spread of a single lineage, and that if such spread is discounted then the prevalence of *B. cepacia* in most CF centres remains relatively low at 5–10%. Transient colonisation by *B. cepacia* also influences prevalence and occurs in *c.* 5% of CF patients; however, transient colonisation is observed very rarely with the ET12 lineage (authors' unpublished observations), perhaps reflecting the high colonisation potential of this clone. From a clinical, epidemiological and evolutionary viewpoint, the influence of this single clone on the CF community is considerable. In the UK alone, it has been isolated in eight (50%) of 16 CF centres and from 68 (38%) of 178 *B. cepacia*-colonised patients [46]. Attempts to identify its origins have been frustrated by a lack of stored isolates; however, investigation of available isolates allows several conclusions to be reached. Based on evidence from MLEE and ribotyping [25] and PFGE [46, 62], the first known isolates of this epidemic lineage were cultured from Ontario paediatric patients in the latter half of the 1980s [25]. In the UK, the first recorded isolate of the same lineage was in August 1989 [62] from a patient who had never been out of the UK nor shown any evidence of *B. cepacia* colonisation during previous bacteriological investigations. The patient had previous contacts with other UK patients colonised by *B. cepacia*, but the isolates from these patients were not available.

From the available evidence, it appears that the Edinburgh/Toronto/ET12 lineage was established in Canada before its appearance in the UK, and that at some stage in the late 1980s, intercontinental spread occurred between UK and Canadian patients whilst attending summer camps in Ontario, followed by inter-regional spread in the UK during social contacts at meetings [25, 62]. It is tempting to conclude that this highly transmissible strain is clonally related to the isolates cultured during the first documented outbreak of *B. cepacia* in CF patients in Ontario, reported in 1984 [37].

Potential pathogenic mechanisms of *B. cepacia*

Although *B. cepacia* produces several putative virulence determinants—including haemolysins, proteases, lipases, siderophores and catalase—a major clinical role for these factors has not been demonstrated convincingly in CF [83, 84]. However, catalase is associated with the organism's ability to resist killing by professional phagocytes and to produce serious infection in patients with CGD [85].

Intracellular survival

Several puzzling clinical and scientific observations have led to speculation that *B. cepacia* can survive and grow within pulmonary phagocytes or respiratory epithelial cells. First, clinical resistance to antimicrobial therapy despite demonstration of an isolate's susceptibility *in vitro*; second, isolation of serum-sensitive isolates in bacteraemic infection [86]; third, chronic pulmonary colonisation despite a pronounced antibody response [87]; and fourth, the close taxonomic relationship between *B. cepacia* and the intracellular pathogen, *B. pseudomallei*. However, to date, the scientific evidence for intracellular survival or growth of *B. cepacia* is not convincing. Studies of intracellularly in bacterial pathogens can be difficult and, in the case of *B. cepacia*, are complicated further by the organism's innate resistance to antibiotics, including aminoglycosides, which are used commonly in intracellular assays to kill extracellular organisms. As it is known that *B. pseudomallei* survives and multiplies within professional phagocytes [88], studies within our group have focused on monocytes, with *Listeria monocytogenes* and *P. aeruginosa* as positive and negative controls, respectively. However, it was not possible to demonstrate either enhanced uptake or survival of *B. cepacia* in monocytes. Previously, Burns [89] reported the observation of *B. cepacia* within CF post-mortem respiratory epithelial cells by electron microscopy, but no further data have been published to validate this important finding. Low-level invasion *in vitro* of a respiratory epithelial cell line by the epidemic ET12 lineage has been demonstrated [90], but the significance of limited epithelial invasion by bacteria remains unclear [91]. A recent and potentially seminal publication has even suggested that enhanced uptake of CF pathogens by epithelial cells expressing surface cystic fibrosis transmembrane conductance regulator (CFTR), followed by epithelial desquamation, may be an important host defence mechanism rather than a bacterial virulence determinant [92].

Overall, the role of intracellularly in the pathogenesis of *B. cepacia* infection in CF patients is still in doubt. As a caveat, the demonstration of its intracellular survival and growth within amoebae, raises the possibility that these free-living protozoa may act as an environmental reservoir from which CF patients could acquire the organism [93].

B. cepacia and host immune responses

Colonisation with *B. cepacia* is associated with a pronounced and specific humoral response, including raised serum IgG and IgA and sputum IgA titres against *B. cepacia* lipopolysaccharide (LPS) and outer-membrane protein (OMP) components [87, 94]. Anti-*B. cepacia* antibodies have also been detected in non-colonised CF patients, and particularly in patients colonised with *P. aeruginosa* [87, 95]. Studies with pre-absorbed sera have failed to demonstrate an appreciable

degree of cross-reactivity between the two species, either for OMP or LPS components [87, 96], suggesting that the response to *P. aeruginosa* is not the source of pre-colonisation anti-*B. cepacia* antibody. Generally, levels of anti-*B. cepacia* immunoglobulin in non-colonised patients are low, but the demonstration of substantially raised titres in a subset of patients may reflect previous exposure to *B. cepacia* where an appropriate antibody response has prevented the occurrence of colonisation. On the other hand, the demonstration of antibody in stored pre-colonisation sera from patients who subsequently became colonised, indicates that antibody does not always play a preventative role. Similarly, the role of antibody in patients once they are colonised is unclear; for example, clinical outcome is independent of the magnitude of anti-*B. cepacia* responses [87]. A recent study [97] with immunoblotting techniques has suggested that IgG antibodies against a 30-kDa OMP, identified presumptively as the major immunodominant porin, OMP D [95, 98], are associated with a better prognosis in colonised patients. If these results are confirmed, it raises the possibility of using this OMP as a target for immunotherapy.

The association of *B. cepacia* with CGD, an inherited defect in neutrophil oxidative killing pathways, and the role of neutrophils as the predominant immune effector cell in the CF lung [99], have led to speculation that the interaction between *B. cepacia* and neutrophils may be important in the evasion of host defences by this organism. Speert *et al.* [85] demonstrated that, unlike *P. aeruginosa*, *B. cepacia* is resistant to non-oxidative neutrophil killing mechanisms; hence the role of *B. cepacia* in CGD. Evasion of the normal neutrophil oxidative burst would aid the survival of *B. cepacia* in the presence of a pronounced immune response. Within the CF lung, normal opsonisation processes are compromised severely through the disruption of immune effector molecules by bacterial and host proteases [100, 101]. In particular, cleavage of complement receptors and immunoglobulin molecules within the respiratory tract may neutralise the humoral immune response to *B. cepacia* and enable the organism to persist in the lungs of colonised patients. However, this observation does not explain the ability of rough, LPS-deficient, serum-sensitive *B. cepacia* to cause invasive pneumonitis and septicaemia in patients with elevated anti-*B. cepacia* immunoglobulin titres [86].

Inflammatory damage

Increasing evidence has emerged to suggest that host immune responses are important in the pathogenesis of *B. cepacia* infection. A UK multicentre study has shown that levels of the inflammatory markers, C-reactive protein and neutrophil elastase α 1-antiproteinase complex, are significantly higher during *B. cepacia*-associated exacerbations than in exacerbations

caused by *P. aeruginosa* alone. Aggressive antibiotic treatment with the most active agents available did not eliminate *B. cepacia*, but in most cases was associated with a decline in inflammatory markers to pre-exacerbation levels [102]. In addition, anecdotal evidence indicates that patients who exhibit rapid pulmonary decline and pronounced inflammatory symptoms, but who do not respond to antibiotic therapy, nevertheless respond to treatment with commercial preparations of immunoglobulin. The relative absence of *B. cepacia* antibodies in healthy human donors [87], from whom these immunoglobulins are obtained, suggests that such preparations contain potentially useful anti-inflammatory activity.

An unexpected but informative result from our own studies has demonstrated that LPS from clinical and environmental isolates of *B. cepacia* induces pro-inflammatory cytokines, including the major cytokine tumour necrosis factor α (TNF α), to a level 10-fold that induced by *P. aeruginosa* LPS and matching the inflammatory power of *Escherichia coli* endotoxin [103, 104]. The mechanism involved in *B. cepacia* cytokine stimulation is unclear, but is independent of CD14 receptors. Of interest, induction of TNF α by *B. cepacia* LPS is reduced in the presence of *P. aeruginosa* LPS, suggesting that the diversity of clinical outcomes in patients colonised with *B. cepacia* may be influenced in part by the presence or absence of *P. aeruginosa* and other CF pathogens [105].

What is a true *B. cepacia*?

Further research to establish a gold standard for laboratory identification of *B. cepacia* has assumed high priority. Reliable identification is important not only in attempts to clarify the organism's pathogenic potential, but also because of the clinical, social, psychological and potentially litigious consequences for patients, carers and diagnostic laboratories associated with the organism's acquisition and transmission. Selective media and laboratory protocols for culture and presumptive identification of *B. cepacia* from clinical or environmental sources have been described and their value in microbiological surveillance established [14, 48, 56, 106]. However, existing selective media also support the growth of other gram-negative non-fermenting bacilli [46, 48, 56] and unequivocal identification of *B. cepacia* by multitest commercial systems can present difficulties [44, 56, 106, 107].

There is increasing evidence that organisms presently identified as *B. cepacia* by standard laboratory procedures exhibit such diverse genotypic and phenotypic properties that attempts to generalise on virulence, transmission and antibiotic susceptibility are ill-founded. Simpson *et al.* [44] speculated that epidemic strains may represent a *B. cepacia* sub-

population, arising as bacterial hybrids or through horizontal transfer of virulence genes from the closely related pseudomonads *B. gladioli* and the highly dangerous intracellular pathogen *B. pseudomallei*. Recently, isolates identified as *B. cepacia* were characterised further by analysis of cellular proteins and fatty acid components and clustered by means of computer-assisted numerical comparison of the profiles. Representative isolates from individual clusters were selected to determine genotypic relatedness within and between clusters by means of DNA–DNA and DNA–rRNA hybridisation assays. These molecular phylogenetic studies revealed that organisms identified by conventional tests as *B. cepacia* comprised several new *Burkholderia* spp. [108].

According to taxonomic conventions, new species names should not be given to bacteria that cannot be identified reliably by phenotypic characteristics; instead, such groups can be described by the terms genomovar I, II, etc. [109]. Following this convention, isolates identified as *B. cepacia* by conventional multitest systems such as the API 20NE system (API-bioMerieux, Marcy l'Etoile, France) constitute at least four different genomovars of *B. cepacia*; other presumed *B. cepacia* strains are identified as the nitrogen-fixing organism *B. vietnamiensis*. Preliminary studies on a small number of isolates have indicated that the majority of CF isolates from Belgium and the UK tend to cluster in genomovar III [70, 108]. Subsequent ongoing analyses of a larger collection of environmental, phytopathogenic and clinical isolates in our laboratories have confirmed the potential importance of genomovar identification. For example, the isolate responsible for the first UK report of cepacia syndrome [72], and three individual epidemic clones including the highly transmissible ET12 lineage [25, 44, 62, 69] each belong to genomovar III. It should be stressed that *B. vietnamiensis* and the remaining *B. cepacia* genomovars were also identified amongst isolates from CF patients, and that genomovar III status is not synonymous with high transmissibility [72]. Of the 150 '*B. cepacia*' isolates studied to date, most environmental isolates (including the phytopathogenic type strain ATCC 25416) belong to genomovar I; in contrast, isolates associated with acute clinical decline in CF patients are restricted to genomovar III. These results confirm the complex taxonomic heterogeneity within the genus *Burkholderia* and have important diagnostic implications for infection control in the CF community.

Unique bacterial clones and *B. cepacia* transmission factors

Epidemiological data and genomic fingerprinting suggest that the variable incidence of *B. cepacia*—in particular, the lack of cross-infection in some centres

[75, 81], and the contrasting epidemic spread in others—reflects the behaviour of a relatively small number of highly transmissible clones [46, 69, 110–112].

It seems reasonable to speculate that *B. cepacia* strains responsible for epidemic spread may harbour a common colonising factor whose identification could be exploited for diagnostic and therapeutic purposes. At present, the most significant of these factors is adhesion to respiratory mucin [53, 113–115], associated with giant intertwined fibres referred to as cable pili [53, 114]. The gene responsible for cable pili, *cbl*, has been detected in the highly transmissible ET12 lineage, represented by the Edinburgh isolate CF5610 (J2315) [16, 25, 62, 69, 115], and responsible for *B. cepacia* colonisation in 38% of UK patients [46]. In a slightly different form, *cbl* has also been associated with epidemic transfer of *B. cepacia* from CF to non-CF patients in a Mississippi outbreak [16, 69, 115]. However, studies with a *cbl* DNA probe indicated that *cbl* is not present in all epidemic clones, suggesting that other bacterial and host factors need to be identified [69]. Interestingly, a recent study [116] has described enhanced binding of the ET12 lineage to lipid receptors, particularly the galactolipid globotriosphylceramide (GB₃), and led to speculation that upregulation of GB₃, mediated through the infection process and TNF stimulation within the lung, may provide an alternative receptor for isolates in which cable pili are poorly expressed and a second receptor system for the epithelial attachment of bacteria that have migrated through the mucosal blanket.

Experimental proof of direct or indirect transmission of epidemic *B. cepacia* is not feasible and can be judged only by circumstantial evidence. However, epidemiological data has strikingly demonstrated such potential. Colonisation with more than one strain of *B. cepacia* is unusual and has been reported in <10% of patients [46]. During the Edinburgh outbreak, PFGE fingerprinting showed that one patient harboured two *B. cepacia* strains in his respiratory secretions, including the ET12 clone; however, only the epidemic strain was transmitted subsequently to his girlfriend [62].

Modes of transmission and the risks of acquisition

The potential risks of *B. cepacia* transmission, either directly by person-to-person spread or indirectly from contaminated fomites, continue to be a major concern to the CF community. Table 2 summarises the extensive documented evidence for direct transmission of *B. cepacia* between CF patients during close contacts within hospitals [61, 63, 65], at educational or summer camps [59, 66] and through other social contacts [62, 63]; in contrast, several reliable studies have found no evidence of cross-infection [72–75]. In

their initial report, LiPuma *et al.* [59] cited previous failures to culture *B. cepacia* from respiratory equipment and environmental surfaces as circumstantial evidence that direct person-to-person spread might be the primary means of transmission. However, a subsequent prospective study [117] with selective culture and DNA-based typing of isolates showed that colonised patients can contaminate their environment; thus indirect transmission might occur *via* contaminated surfaces. The intrinsic resistance of *B. cepacia* to many antibiotics also raised justifiable concern that the use of contaminated home-use nebulisers might present a special hazard for *B. cepacia* acquisition. Currently, evidence for nebuliser-associated transmission is scanty and equivocal. A case-controlled retrospective study of five CF patients undergoing treatment in a CF centre [118] showed a significant association between outpatient nebuliser use and *B. cepacia* colonisation. *B. cepacia* was also cultured from nebulisers used by colonised patients. Unfortunately, no bacterial typing was performed to confirm the clonal relationships of the human and nebuliser isolates. Recently, in a prospective study [119], *B. cepacia* was cultured from three of 35 home-use nebulisers. DNA macrorestriction analysis by PFGE revealed that one of two strains of *B. cepacia* recovered from the nebuliser of one patient was also present in the patient's sputum. However, sputum cultures from the two other patients whose nebulisers harboured *B. cepacia* did not yield the organism, suggesting an environmental origin for the *B. cepacia* strain isolated from the nebuliser. Other studies of nosocomial acquisition of *B. cepacia* in non-CF patients have suggested that respiratory infection probably occurred by indirect transmission following use of contaminated nebuliser devices [31, 120]. Airborne dissemination may also present a small risk of *B. cepacia* acquisition. In a prospective study, *B. cepacia* was recovered from the room air during occupation by five of six patients, but to only a limited extent, with the number of bacteria ranging from 1 to 158 cfu/m³ [121]. Maximum yields were associated with episodes of coughing and, after a patient left the room, the organism persisted in room air for up to 45 min.

To conclude, ethical considerations prevent experi-

ments that could provide scientific data to assess the risks of *B. cepacia* acquisition, including clarification of the frequency of contact and the infectious dose required. Based on accumulated evidence (Tables 2 and 3), skin contact, respiratory aerosols, sharing food, contaminated equipment, co-habitation or undergoing physiotherapy in the same room as a *B. cepacia*-positive individual present reasonable risks of acquisition. However, epidemiological evidence [38, 62], including the high numbers (typically >10⁸ cfu/ml) of *B. cepacia* present in the saliva of colonised patients, suggests that the close and frequent social contact that occurs between siblings, the direct exchange of respiratory secretions associated with kissing, and the involvement of a highly transmissible *B. cepacia* lineage arguably present the greatest risks of acquisition.

Environmental release of *B. cepacia* as a biological control agent

Whilst the CF community debates the clinical issues of *B. cepacia* colonisation and transmission, agricultural microbiologists continue to develop the organism as a biological control agent to exploit its antifungal activity (Fig. 1) for the enhancement of crop yields [122, 123] and its nutritional adaptability in the bioremediation of landfill sites, contaminated soils and ground water aquifers [124–126]. Deliberate environmental distribution of *B. cepacia* as field inoculants raises the issue of the phylogenetic relationship between *B. cepacia* of environmental and clinical origin and the potential hazard for human infection. The debate on this relationship has revealed the gulf that exists between different areas of interest and microbiological expertise and, as stated recently in an editorial comment on another contentious issue, bovine spongiform encephalopathy, 'underscores the weakness of separating agricultural and medical science' [127].

We have stated previously that the scientific evidence that environmental strains of *B. cepacia* present little hazard to man is weak [14] and is based on examination of only a few bacterial isolates and

Table 3. Factors that may influence acquisition of *B. cepacia*

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- In colonised individuals, *B. cepacia* saliva counts can exceed 10⁸ cfu/ml, suggesting that the highest risk of patient-to-patient spread is transmission of respiratory secretions during kissing or through sharing of eating or drinking utensils.
 - Spirometer mouthpieces become heavily contaminated during lung function tests. Risk avoided by use of disposable mouthpieces. Recovery from the surface of lung function equipment is low.
 - Recovery from antibiotic reservoirs of nebulisers has been reported, but incidence is low and the degree of risk appears secondary to the preceding factors.
 - Aerosol recovery is low, suggesting low risk of aerosol transmission.
 - Hands become contaminated after coughing and the organism can be transmitted by handshake. Survival on hands reduced to 10% after 30 min; this varies in different individuals and may account for variable recovery in surveillance studies.
 - Gastrointestinal carriage has not been demonstrated, even in colonised individuals, suggesting that the risk of faecal-oral spread is minimal.
 - After surface contamination with *B. cepacia*-containing sputum, viable bacteria can be recovered for several weeks.
 - Surface contamination by *B. cepacia* sputum is eliminated by treatment with UV irradiation and with common hospital disinfectants, including Milton, Dettol, alcohol 70%, phenols, iodine and cetrimide. Careful drying is important after washing or disinfection.
 - Recovery of *B. cepacia* from soil, plants, drains, lakes and surface waters is low, suggesting that natural environments present a possible but low risk for acquisition.
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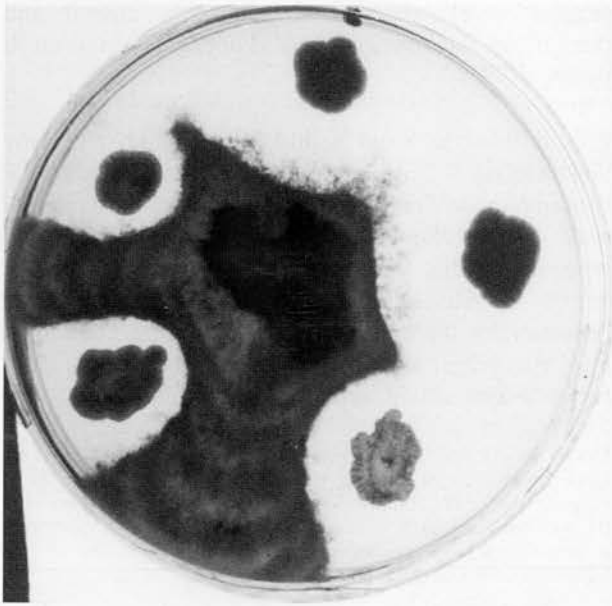


Fig. 1. Inhibition of the phytopathogenic fungus *Rhizoctonia solani* by five isolates of *B. cepacia*. The fungus was inoculated in the centre of the plate and bacteria around the perimeter. Cultures were photographed after incubation for 14 days at room temperature.

inappropriate bacterial properties [24]. Although some studies have indicated that environmental and clinical isolates are distinct, no reliable phenotypic markers have been identified [25, 45, 108]. The suggestion that clinical isolates can be distinguished from soil isolates based on the former's lack of plant pathogenicity [45] is discounted by the fact that CF isolates of *B. cepacia* will readily macerate onion tissue (Fig. 2) [14]. In addition, the invasive *B. cepacia* foot lesions

known as swamp foot [128], acquired by military personnel during jungle training, confirm the pathogenic potential of environmental strains of *B. cepacia* for man.

The potential hazard that some or all environmental *B. cepacia* strains present to the CF community is unclear and requires investigation. The fact that new cases of *B. cepacia* colonisation continue to occur with strains that show no genotypic relationship to other isolates within the same CF centre, points to the environment as a potential source. However, the extent of this risk is difficult to assess. Extensive microbiological safaris into supermarkets and domestic homes [15], and a range of botanical soils and cultivars [14], indicate that *B. cepacia* can be cultured from up to 20% of warm moist environmental sites, particularly soils, but that it is not as ubiquitous as other pseudomonads. Interestingly, in our studies to date, none of the environmental isolates of *B. cepacia* have been identified as belonging to genomovar III.

Conclusions and future prospects

B. cepacia is a striking example of a multiresistant soil saprophyte and phytopathogen that has emerged as an important threat to susceptible human hosts. In the CF community, the degree to which infection control measures should be implemented continues to arouse strong scientific and social debate. The validity of strict control is supported by circumstantial, but nevertheless compelling, evidence for direct person-to-person transmission of epidemic strains through nosocomial and social contact. In contrast, although the risk of indirect iatrogenic spread from contaminated fomites remains

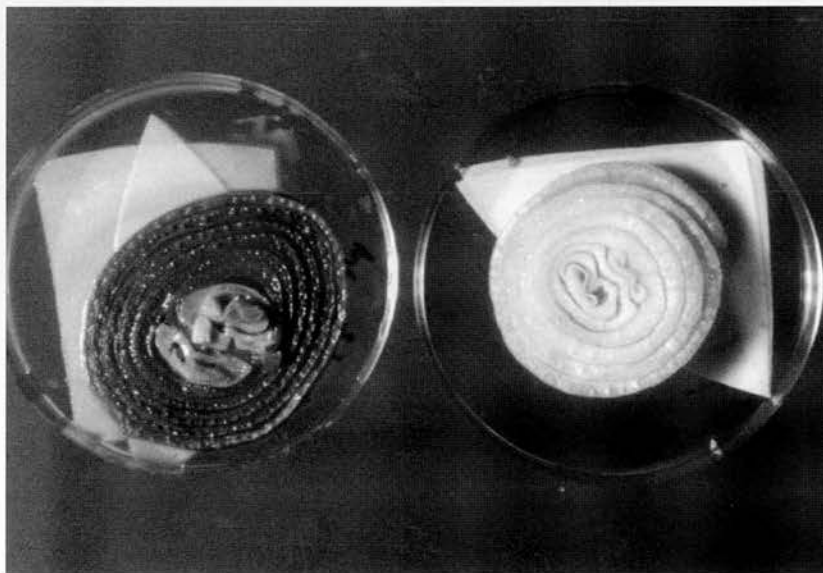


Fig. 2. Soft rot of a segmented 'compromised' onion inoculated with a clinical *B. cepacia* isolate of the epidemic ET12 lineage (left) and an uninoculated control (right), both incubated at 30°C for 72 h. Reproduced with permission from Butler *et al.* [14].

unclear, available evidence suggests that this route is less important than direct transfer. An important caveat in attempts to generalise on *B. cepacia* transmission is evidence that the major epidemics of *B. cepacia* involve a subpopulation of highly epidemic lineages which might be re-allocated ultimately to new species; '*Burkholderia cfei*' might be an appropriate but probably controversial choice! Ongoing microbiological surveillance in CF centres indicates that sporadic acquisition of epidemic lineages continues to occur when there is a failure to comply with infection control measures. For example, a striking demonstration of the continued potential for transmission of the ET12 lineage was its recent acquisition by an Edinburgh CF adult; extensive inquiries suggested that this patient had social contact for only 10 min whilst visiting another CF male who was hospitalised during an episode of *B. cepacia* septicaemia. Even when infection control appears effective in preventing spread of epidemic lineages, new cases of *B. cepacia* colonisation continue to occur with isolates that exhibit unique PCR ribotyping or PFGE profiles. Such sporadic acquisitions raise a fundamental question concerning the source and colonising potential of individual *B. cepacia* strains. For example, does the environment contain a subpopulation of *B. cepacia* clones that are innately primed for human colonisation, or does colonisation and virulence in man require in-vivo adaptation? Future improvements in laboratory identification of *B. cepacia* subpopulations associated with CF disease and identification of transmission factors, in addition to cable pili, may provide scientific justification for relaxation of segregation in the absence of known epidemic and potentially virulent lineages. Turning our attention to CF patients, we need to clarify why colonisation by the same strain of *B. cepacia* leads to variable clinical responses, ranging from asymptomatic colonisation to rapid fatal pulmonary deterioration. It could be argued that this particular problem is not unique to *B. cepacia*, and that applying Koch's postulates in an attempt to distinguish between sycophancy and pathogenic potential is difficult when dealing with any opportunist pathogen. Certainly, host factors cannot be ignored in attempts to understand the pathogenic processes involved in CF lung infections.

During the final preparation of this review, a deceptively simple and elegant study has illustrated how CFTR-associated defective Cl⁻ transport across airway epithelia might lead to bacterial colonisation in CF patients. Smith *et al.* [129] showed that the normal human apical epithelial surface is bactericidal for *P. aeruginosa* and *S. aureus*; in contrast, the bactericidal activity was inhibited reversibly in CF epithelia because of a high NaCl concentration. If this phenomenon varies in individual CF patients—or if individual *B. cepacia* strains differ in susceptibility to the defensin-like bactericidal agent—it might explain some of the host- and pathogen-specific anomalies associated with *B. cepacia* pulmonary infection and

suggest novel strategies for infection control and therapy of this unusual and challenging opportunist pathogen.

It is difficult to avoid a final comment on the irony that whilst *B. cepacia* continues to hold the CF community to ransom, agricultural microbiologists seek to develop the commercial and beneficial potential of this microbial Jekyll and Hyde in their search for biological control agents. This situation demonstrates the diversity of microbiology, but should also encourage attempts to reduce the present gulf between agricultural and medical science.

Studies by J.R.W.G. and J.E.H. were supported by grants from the Cystic Fibrosis Trust and the Medical Research Council. J.E.H. is supported by a MRC Clinical Training Fellowship. P.V. is indebted to the National Fund for Scientific Research (Belgium) for a position as a postdoctoral research fellow. Special thanks are due to our laboratory colleagues past and present.

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Note added in proof

Following submission of this review, the results of an epidemiological study of *B. cepacia* in a large series of CF patients attending the CF centre in Verona were published. Cazzola *et al.* concluded that their results are difficult to interpret. Nevertheless, data are essential if progress is to be made in unravelling *B. cepacia* epidemiology, and the results of this study are particularly relevant to the major issues discussed in our review.

Between Nov. 1991 and Dec. 1994, *B. cepacia* was cultured from 85 (11.0%) of 769 CF patients attending the Verona centre. Based on genomic fingerprinting, 32 (53.3%) patients were colonised by individual *B. cepacia* strains; the remaining 28 (46.7%) patients were divided into 10 subgroups, each colonised by a distinct strain. As previously encountered with the ET12 lineage, the outcome of *B. cepacia* colonisation in the Verona study varied from rapidly fatal septicaemia to maintenance of reasonably stable respiratory function, even in patients colonised by the same strain. Cazzola *et al.* provide further evidence for hypotheses discussed in our review that some *B. cepacia* strains exhibit and low transmissibility that the environment is a likely source of sporadic new cases: e.g., transmission was observed in only three of eight pairs of CF siblings; in unrelated patients, direct person-to-person transmission was evident in only 10 cases (16.7%); despite a strict segregation policy, whether as in- or out-patients, 15 new colonised patients were identified during 1993. Considering social implications and the paucity of previous data, it was particularly interesting to note that transmission was demonstrated between two unrelated CF schoolmates.

Cazzola G, Amalfitano G, Tonelli E, Perazzoli C, Piacentini I, Mastella G. *Burkholderia (Pseudomonas) cepacia* epidemiology in a cystic fibrosis population: a genome finger-printing study. *Acta Paediatr* 1996; **85**: 554–557.

BACTERIAL CHARACTERISATION

Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*

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The biochemical characteristics of 213 isolates of *Burkholderia pseudomallei* from patients with melioidosis and 140 isolates from the soil in central and northeastern Thailand were compared. Whereas the biochemical profiles of all the clinical isolates were similar, all soil isolates from the central area and 25% of isolates from northeastern Thailand comprised a different phenotype. This was characterised by the ability to assimilate L-arabinose (100%), adonitol (100%), 5-keto-gluconate (90%) and D-xylose (84%), but failure to assimilate dulcitol (0%), erythritol (0%) and trehalose (10%). Compared with clinical isolates, these organisms had similar antibiotic susceptibility profiles and were also recognised by a specific polyclonal antibody against *B. pseudomallei*. As melioidosis is rare in central Thailand, but common in the northeast, this raises the possibility that this biochemical phenotype may be less virulent, or may even represent a different species.

Introduction

Burkholderia (formerly *Pseudomonas*) *pseudomallei* is an environmental saprophyte capable of causing devastating systemic infections in many species of mammals and birds. Although *B. pseudomallei* has been isolated from environmental samples across the tropical world, the disease in man is confined to the endemic areas of eastern Asia and northern Australia [1]. In northeastern Thailand *B. pseudomallei* is a major cause of morbidity and mortality, causing one-fifth of all community-acquired septicaemias [2]. However, *B. pseudomallei* is also isolated readily from soil samples in the southern and central regions of Thailand, where clinical disease is rare [3, 4]. To investigate this discrepancy between the distribution of disease and the distribution of the organism in the environment, this study compared the biochemical and antigenic features, and antibiotic susceptibilities, of clinical and environmental isolates of *B. pseudomallei*.

Materials and methods

Bacterial isolates

Clinical and laboratory studies on melioidosis have been conducted in Ubon Ratchatani, northeast Thailand, since 1986. More than 1100 patients with culture-proven melioidosis have been seen during this time. Single isolates of *B. pseudomallei* from 213 of these patients were used for this study.

The soil isolates of *B. pseudomallei* were selected from those collected in a survey of rice paddies in northeastern Thailand [5] and in a quantitative comparison of the isolation of the organism from soil in central and northeastern Thailand [4]. A maximum of two isolates was selected from each site sampled, giving a total of 140 isolates, 115 from the northeast and 25 from the central region.

Organism identification

All isolates were identified initially by their characteristic colonial morphology on a differential agar medium [6], positive oxidase reaction, and resistance to colistin and gentamicin [7].

Priming of Neutrophil Respiratory Burst Activity by Lipopolysaccharide from *Burkholderia cepacia*

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Received 18 April 1997/Returned for modification 27 May 1997/Accepted 2 July 1997

Neutrophil activation may play an important role in the pathogenesis of respiratory disease in *Burkholderia cepacia*-colonized cystic fibrosis (CF) patients. As bacterial lipopolysaccharides (LPS) are potent immunostimulatory molecules, we investigated the role of *B. cepacia* LPS in neutrophil activation processes. LPS extracted from a highly transmissible and virulent strain of *B. cepacia* (J2315) was found to increase neutrophil surface expression of the β_2 integrin, complement receptor 3, and to prime neutrophil respiratory burst responses to the neutrophil-activating agent fMet-Leu-Phe. By contrast, LPS extracted from a nonmucoid *Pseudomonas aeruginosa* strain isolated from a patient with CF showed little or no priming activity. As *B. cepacia* is currently being developed as a biocontrol agent for large-scale agricultural release, we compared LPS molecules from a range of bacterial strains for their proinflammatory ability. Priming activity was demonstrated in LPS extracts from all *B. cepacia* strains tested, with one environmental strain, J2552, showing the highest activity. These findings indicate (i) that *B. cepacia* LPS may contribute to the inflammatory nature of *B. cepacia* infection in CF patients, both by promoting increased neutrophil recruitment and by priming neutrophil respiratory burst responses, and (ii) that environmental strains of *B. cepacia* may have considerable inflammatory potential in susceptible individuals.

During the last 15 years, *Burkholderia cepacia* has emerged as a serious respiratory pathogen in cystic fibrosis (CF) (16). Epidemic spread of *B. cepacia* between CF patients has been associated with a subset of highly transmissible strains, including the Edinburgh/Toronto or electrophoretic type ET12 strain, which has been implicated in numerous outbreaks in both North American and United Kingdom CF centers and has been isolated from up to 40% of *B. cepacia*-colonized patients in the United Kingdom (23, 34). Recently, investigators have identified putative transmissibility markers, including adhesion to mucin and respiratory epithelium mediated via type 4 pili (39); enhanced adhesion to the cell surface glycolipid, globotriosylceramide (40); and a genomic marker termed the *Burkholderia cepacia* epidemic strain marker (30). In contrast, little is known of the pathogenic mechanisms responsible for severe *B. cepacia* infection. For example, it is not clear why some patients appear unaffected by *B. cepacia* colonization while others succumb to rapidly fatal necrotizing pneumonia and septicemia, the so-called cepacia syndrome.

There is increasing evidence that acute pulmonary deterioration associated with *B. cepacia* infection is due to a marked inflammatory response induced by this organism. In a case-controlled study, raised serum levels of the inflammatory markers C-reactive protein and neutrophil elastase were found during exacerbations with *B. cepacia* (11). In addition, anecdotal evidence has suggested that severe *B. cepacia* infection can be treated with anti-inflammatory therapy, in particular the use of hypergammaglobulin preparations (16). Shaw et al. (37) described the in vitro induction of the proinflammatory cytokine tumor necrosis factor alpha (TNF- α) from monocytes stimulated by lipopolysaccharides (LPS) extracted from clinical

strains of *B. cepacia*, including a representative of the ET12 lineage (C1359). Interestingly, levels of TNF- α induced were over nine times greater than levels induced by LPS from *Pseudomonas aeruginosa*, the major respiratory pathogen in CF. Recently Palfreyman et al. (33) have described the induction of biologically active interleukin-8 from lung epithelial cells and human monocytes by a *B. cepacia* extracellular factor distinct from LPS.

Neutrophils have been implicated in immunologically mediated lung damage in CF through the release of reactive oxygen species (ROS) and proteolytic enzymes during activation processes (6, 9, 26). Chronic colonization with mucoid *P. aeruginosa* is believed to exacerbate neutrophil-mediated damage through "frustrated phagocytosis," as neutrophils attempt to engulf *P. aeruginosa* microcolonies embedded in a protective layer of alginate and release their granule contents in the process (14, 15). Similarly, bacterial extracellular products from *B. cepacia* or *P. aeruginosa* may also act directly on neutrophils either by inducing end stage activation with the release of granule contents or by priming responses to other immunostimulatory agents. Colonization with both *P. aeruginosa* and *B. cepacia* is associated with high bacterial counts in sputum, typically 10^9 CFU/ml, suggesting that high concentrations of LPS shed from both viable and nonviable bacteria will also be present. As LPS molecules from a number of bacterial species have been recognized as neutrophil-priming agents (1, 21, 24, 25, 32), we investigated the interaction of *B. cepacia* LPS with neutrophils. Our results indicate the potent priming activity of *B. cepacia* LPS and confirm the proinflammatory capabilities of this unusual and challenging pathogen.

MATERIALS AND METHODS

Materials and equipment. Bacterial strains used in this study are from the collection held in the Cystic Fibrosis Laboratory, Department of Medical Microbiology, Edinburgh University, and are described in Table 1. Strains were stored at -70°C in 5% skim milk (Oxoid, Basingstoke, United Kingdom) and were recovered onto nutrient agar (Oxoid) before use. Fluorescence-activated cell sorter lysing solution and CellFIX were supplied by Becton Dickinson UK

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TABLE 1. Bacterial strains used in this study

Strain	Comment	Reference(s)
<i>B. cepacia</i>		
J2315 ^a	CF, genomovar III ^b	13, ^c 33
C1504	CF	37
J2540	Environment, genomovar I	7, 37
J2552	Environment, genomovar I	7, 33
J2505	Non-CF clinical, ATCC 17762	37
<i>P. aeruginosa</i>		
PAO1	Non-CF clinical isolate	29, 37
J1385	Nonmucoid, CF	17, ^d 41
C1250	Mucoid, CF	37
<i>E. coli</i> O18K-		
		31

^a Representative strain of the transcontinental ET12 lineage (23).

^b Based on polyacrylamide gel electrophoresis of whole-cell proteins and DNA-DNA hybridization of selected *B. cepacia* strains.

^c Identified as CF5610 in this reference.

^d Pyocin type 1/b (4, 8, b) s.

Ltd., Oxford, United Kingdom. Monoclonal antibodies (mouse anti-human CD11b and mouse isotype control antibody) conjugated with fluorescein isothiocyanate were obtained from Serotec, Oxford, United Kingdom. Dihydrorhodamine (DHR; Cambridge Biosciences, Cambridge, United Kingdom) was stored at -20°C as a 10-mg/ml stock solution in dimethyl sulfoxide and was diluted to a 200-µg/ml solution in 20% (vol/vol) dimethyl sulfoxide in Hanks balanced salt solution (HBSS) with 0.1% (wt/vol) glucose (mHBSS) immediately prior to use. All other reagents were provided by Sigma Chemical Corporation (Poole, United Kingdom). Flow cytometry was carried out on a Becton Dickinson FACSort, and results were analyzed by using CellQuest 1.2.2 (Becton Dickinson).

LPS extraction. Bacteria were grown for 48 h in an orbital incubator (140 rpm) at 37°C in nutrient broth no. 2 (Oxoid) containing 0.5% yeast extract (Difco Laboratories, Detroit, Mich.). LPS was extracted by a modification of the aqueous phenol method of Westphal and Luderitz (44) as described by Hancock and Poxton (19). LPS samples were resuspended in pyrogen-free water at a concentration of 2 mg/ml. Samples were sonicated at an amplitude of 10 µm for three to eight 30-s pulses to ensure adequate solubilization. Protein concentration was determined by using the Coomassie brilliant blue protein assay of Bradford (5), with bovine serum albumin as the standard. For all LPS samples, protein content was less than 0.5% of the total dry weight of LPS. LPS characterization was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the buffer system of Laemmli (28), and silver staining was done by a modification of the method of Tsai and Frasch (42) as described by Hancock and Poxton (19). Samples from *Escherichia coli* O18K-, *P. aeruginosa* PAO1, and *B. cepacia* J2540, J2552, and J2505 were found to be smooth in character, while samples from *P. aeruginosa* J1385 and C1250 and *B. cepacia* J2315 and C1504 were rough in character. All samples were stored at -20°C.

Preparation of plasma-free whole blood cells. Fresh heparinized blood was collected from healthy volunteers. As we have observed low levels of anti-*B. cepacia* LPS antibodies in healthy individuals (22a), plasma was removed from blood samples to prevent any opsonic neutrophil responses. Blood was transferred to Eppendorf tubes and spun at 6,500 rpm for 30 s in a Micro Centaur microcentrifuge (MSE, Loughborough, United Kingdom). Following the removal of plasma, the cell pellet was washed four times in HBSS. Finally, the cell pellet was resuspended in a volume of mHBSS equivalent to the initial volume of plasma removed. The resultant washed blood was kept on ice until use. All experiments were initiated within 1 h of blood collection. Polymorphonuclear cell viability was determined by trypan blue exclusion and was found to be greater than 95%. Comparison of washed whole blood neutrophils with untreated blood showed no differences in size, granularity, or baseline CD11b expression, indicating that little neutrophil activation had occurred during plasma removal.

Detection of surface CD11b following neutrophil exposure to LPS. All experiments were carried out with 100-µl aliquots of washed blood in 5-ml polystyrene tubes (Greiner, Stonehouse, Gloucestershire, United Kingdom), to which was added an equal volume of mHBSS or LPS diluted in mHBSS. The tubes were incubated for the desired time period at 37°C with gentle shaking. Reactions were stopped by transferring the sample tubes onto ice. After the addition of anti-CD11b-fluorescein isothiocyanate (5 µl) or control antibody (5 µl) to each sample, the tubes were mixed and incubated for 20 min on ice. Then erythrocytes were lysed by the addition of 2 ml of lysing solution for 30 min. Neutrophils were pelleted by centrifugation at 500 × g, washed once in phosphate-buffered saline and resuspended in 500 µl of CellFIX. Samples were stored at 4°C until flow cytometric analysis on the same day as the experiment. Neutrophils were gated on forward and side scatter characteristics by using CellQuest 1.2.2 software. CD11b expression was determined as the mean channel fluorescence (MCF) of

the neutrophil population, using the green channel (FL1). As considerable interdonor variation in the magnitude of neutrophil responses was noted, MCF values were standardized by expression as a percentage of the MCF of neutrophils treated with buffer alone. Each complete experiment was conducted at least four times, using blood from a different donor on each occasion.

Intracellular H₂O₂ production following neutrophil priming and stimulation. All experiments were carried out with an initial aliquot of 100 µl of washed blood. Where required, samples were preincubated with mHBSS or LPS at the desired concentration at 37°C. Samples were then stimulated with mHBSS or fMet-Leu-Phe (FMLP; 5 × 10⁻⁷ M) for 15 min at 37°C in the presence of 30 mM DHR (4, 43). Reactions were stopped by transferring the sample tubes onto ice. Erythrocytes were lysed by the addition of 2 ml of lysing solution for 30 min, and the remaining neutrophils were processed as described above. As H₂O₂ oxidized DHR to fluorescent rhodamine, intracellular H₂O₂ production was determined as the MCF of the neutrophil population, using the red channel (FL2) (43). Results were analyzed by using CellQuest and expressed as the MCF of the total neutrophil population. Each complete experiment was conducted at least four times, using blood from a different donor on each occasion.

Statistical analysis. Statistical analysis was carried out by using Student's *t* test to compare the means of two populations. For comparison of *B. cepacia* and *P. aeruginosa* populations, data were transformed by conversion to logarithms, and analysis of variance was performed.

RESULTS

Induction of CD11b on neutrophils stimulated with LPS from representative strains. Neutrophils were exposed to increasing concentrations of LPS from representative strains of *B. cepacia*, *P. aeruginosa*, and *E. coli* for a 2-h period at 37°C. Both *E. coli* and *B. cepacia* LPS increased CD11b expression on neutrophils, although responses to *E. coli* LPS could be demonstrated at a lower concentration (1 ng/ml) than for *B. cepacia* LPS (10 ng/ml). The greatest effect was seen with *E. coli* LPS at 100 ng/ml, where CD11b expression reached 2.5 times levels on neutrophils incubated with buffer alone (Fig. 1). At all LPS concentrations, CD11b levels induced by *E. coli* tended to be greater than those induced by *B. cepacia*. However, for both organisms, raised CD11b levels above baseline became statistically significant only at concentrations of 10 ng/ml and above ($P < 0.02$). LPS from a nonmucoid CF strain of *P. aeruginosa*, J1385, had no effect on neutrophil CD11b expression at all concentrations tested.

Preliminary time course experiments indicated that at a low concentration (1 ng/ml), *B. cepacia* LPS was associated with a late rise in neutrophil CD11b expression, occurring after 3 h of incubation. By contrast, higher LPS concentrations induced a rise in CD11b expression to maximal levels in less than 2 h of incubation (data not shown). Subsequent experiments compared

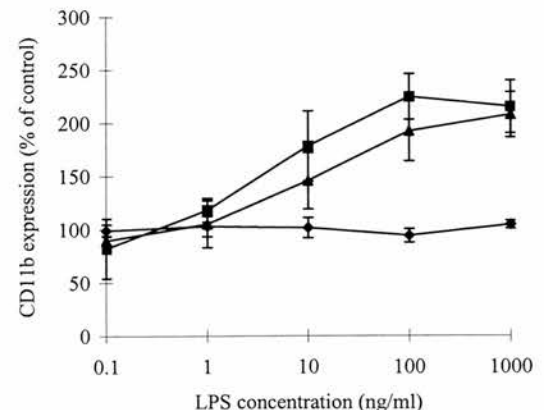


FIG. 1. Induction of CD11b on neutrophils following stimulation with various concentrations of LPS from *E. coli* O18K- (■), *B. cepacia* J2315 (▲), and *P. aeruginosa* J1385 (◆). Results are expressed as a percentage of the MCF of neutrophils treated with buffer alone for 2 h (mean of four experiments ± standard error of the mean).

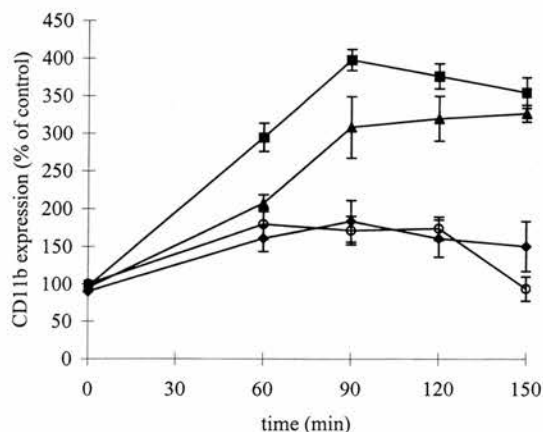


FIG. 2. Time course of induction of CD11b on neutrophils following stimulation with buffer alone (○) or LPS (100 ng/ml) from *E. coli* O18K- (■), *B. cepacia* J2315 (▲), and *P. aeruginosa* J1385 (◆) at 37°C. Results are expressed as a percentage of the MCF of neutrophils treated with buffer alone at time zero (mean of four experiments \pm standard error of the mean).

ng LPS from *B. cepacia*, *P. aeruginosa*, and *E. coli* were carried out with an LPS concentration of 100 ng/ml (Fig. 2). Incubation with buffer alone was associated with a slight elevation in CD11b expression between 60 and 120 min, returning to baseline levels by 150 min. *E. coli* LPS was found to induce statistically significant levels of CD11b by 60 min ($P < 0.01$), peaking at 90 min ($P < 0.001$) with levels 2.3 times greater than those on neutrophils treated with buffer alone. Responses to *B. cepacia* LPS were slower to develop, rising to 1.8 times the levels on neutrophils treated with buffer alone by 90 min and reaching statistical significance after 120 min ($P < 0.01$). Incubation with *P. aeruginosa* LPS had little effect on CD11b expression over the entire incubation period.

Priming of FMLP-induced respiratory burst activity by LPS from *B. cepacia* and *E. coli*. DHR was used to detect the presence of intracellular H_2O_2 as a measure of neutrophil respiratory burst activity. Unstimulated neutrophils and unprimed neutrophils stimulated with FMLP fell within the R1 region (Fig. 3A) on the basis of size and granularity. Stimulation of LPS-primed neutrophils with FMLP was associated with changes in the forward and side scatter characteristics of a subpopulation of neutrophils (Fig. 3B and C, R2). By contrast, either LPS priming alone nor stimulation of unprimed neutrophils with FMLP was associated with a shift in neutrophils from the R1 to the R2 population (Fig. 3A). In all instances, MCF for R2 populations was greater than MCF for R1 populations (Fig. 3D to F). However, as the patterns of change in MCF within each individual experiment were identical for both R1 and R2 neutrophils, respiratory burst activity was generally expressed as the MCF of the total neutrophil population (R1 plus R2).

Both *E. coli* O18K- and *B. cepacia* J2315 LPS primed for responses to FMLP in a dose-dependent manner. By contrast, *P. aeruginosa* J1385 LPS had no priming effect on neutrophils. Priming effects of *E. coli* LPS could be detected at lower concentrations (1 ng/ml) than for *B. cepacia* LPS (10 ng/ml) and were greater in magnitude at all concentrations tested (data not shown). Subsequent time course experiments were carried out with 100 ng of LPS per ml as standard. Neutrophils from individual donors varied considerably in both the magnitude and timing of priming responses (Fig. 4), but several general trends could be observed. None of the LPS preparations induced a respiratory burst in the absence of stimulation

with FMLP (data not shown). Preincubation with buffer alone was associated with little or no priming of responses to FMLP. However, both *E. coli* LPS and *B. cepacia* LPS were potent priming agents of a respiratory burst response, with priming occurring after as little as 30 min of incubation. In assays using neutrophils from three of four donors, priming responses occurred more rapidly and displayed greater peak activity following stimulation with *E. coli* LPS rather than *B. cepacia* LPS (Fig. 4A to C). However, for one individual, little difference was observed between *E. coli* and *B. cepacia* LPS in both the speed and magnitude of priming responses (Fig. 4D).

In selected experiments, R1 and R2 populations were analyzed individually with respect to both MCF and the number of neutrophils in each population. In all instances, MCF activity was greatest within the R2 population, although considerable overlap in results for individual neutrophils occurred (Fig. 3D to F). Priming with both *E. coli* and *B. cepacia* LPS increased FMLP-induced respiratory burst responses in both R1 and R2 populations in a dose-dependent manner. Furthermore, LPS was associated with a shift of neutrophils from the R1 to the R2 population on stimulation with FMLP, so that the increase in MCF within R2 correlated in a linear fashion with an increase in the percentage of total neutrophils within the R2 population ($r^2 = 0.94$), while an increase of MCF within R1 correlated with a decrease in the percentage of neutrophils within the R1 population ($r^2 = 0.94$). Thus, LPS priming appeared to have a dual effect by first increasing the magnitude of responses in both R1 and R2 neutrophils and second shifting neutrophils into the highly responsive R2 population.

Neutrophil activation by LPS from a range of *B. cepacia* and *P. aeruginosa* strains. LPS from representative strains of *B. cepacia* and *P. aeruginosa* (Table 1) were investigated for the ability to induce CD11b and to prime FMLP-induced respiratory burst responses. LPS from *E. coli* O18K- was included as a positive control and induced maximal CD11b expression at 2.7 times the levels induced by buffer alone (Fig. 5). LPS from all *B. cepacia* strains, except the environmental strain J2540, induced levels of CD11b expression comparable to those induced by *E. coli* LPS. Furthermore, LPS from all *B. cepacia* strains, including J2540, induced CD11b levels which were higher than those induced by all *P. aeruginosa* LPS preparations. Of the *P. aeruginosa* strains tested, only LPS from strain PAO1, the classic laboratory strain, increased CD11b expression above that of unprimed neutrophils, while LPS from both a nonmucoid (J1385) and a mucoid (C1250) CF strain had no effect. Direct comparison of individual *P. aeruginosa* with *B. cepacia* strains by Student's *t* test found a significant difference ($P < 0.05$) for all combinations except PAO1 and J2540. Finally, analysis of variance between mean results for *P. aeruginosa* and *B. cepacia* strains indicated a significant difference between these populations for CD11b induction ($P < 0.001$).

Similar trends were observed for the priming effect of LPS on an FMLP-induced respiratory burst (Fig. 6). LPS from *B. cepacia* J2552 and *E. coli* O18K- primed intracellular H_2O_2 production to the greatest degree, with an approximately 10-fold increase in responses to FMLP following incubation with both LPS compared to neutrophils stimulated with buffer alone. LPS from all *B. cepacia* strains primed responses to levels greater than for all *P. aeruginosa* strains tested, and these observations were significant for all combinations of strains except PAO1 and J2540 (Student's *t* test, $P < 0.05$). Although slight neutrophil-priming effects were observed for LPS preparations from both *P. aeruginosa* PAO1 and *P. aeruginosa* C1250, these results were not statistically significant. LPS from *P. aeruginosa* J1385 had almost no effect on FMLP-induced respiratory burst activity compared to buffer controls. Again,

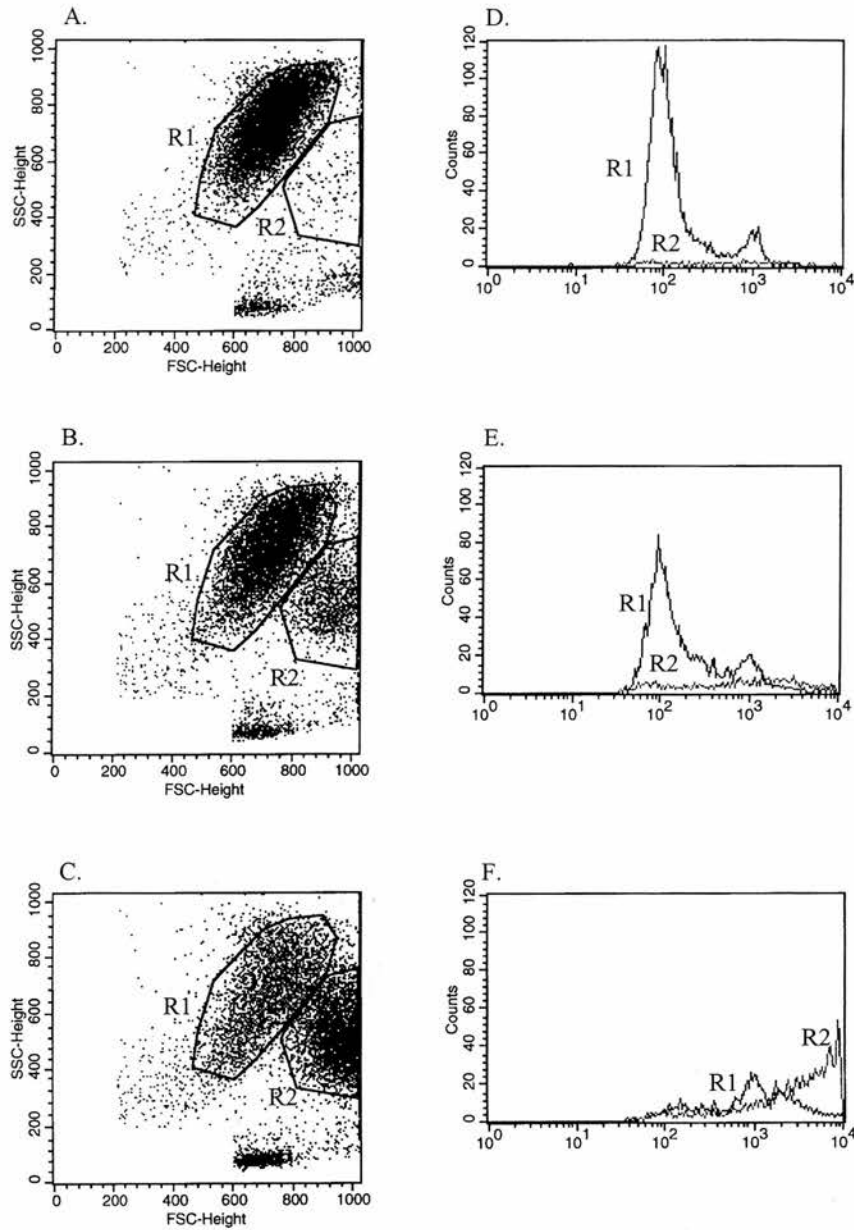


FIG. 3. Size, granularity, and intracellular H_2O_2 production of neutrophils stimulated with FMLP following preincubation with *E. coli* LPS. (A to C) In the dot plots, neutrophils are gated as two populations, R1 and R2. (D to F) Histograms showing fluorescence of neutrophils in populations R1 and R2 in the presence of DHR. Neutrophils were preincubated for 90 min with buffer alone (A and D) or LPS at 1 (B and E) or 100 (C and F) ng/ml.

analysis of variance confirmed a significant difference between LPS from the *P. aeruginosa* and *B. cepacia* populations tested ($P < 0.001$).

DISCUSSION

Neutrophil activation in response to bacterial colonization has been implicated in the pathogenesis of CF lung disease by initiating and sustaining a cycle of increasing lung damage and bacterial colonization (9, 26). In this study, we investigated the role of *B. cepacia* LPS in two aspects of neutrophil activation: first, the up-regulation of surface CD11b; and second, the priming of neutrophil respiratory burst responses. We used flow cytometry to analyze neutrophils within a whole blood cell population from which plasma had been removed. Comparison

of neutrophil size, granularity, and CD11b expression indicated that this procedure did not activate neutrophils to any significant degree.

CD11b associates with CD18 to form the β_2 integrin complement receptor 3 (CR3), which is expressed on the cell surface of granulocytes and macrophages/monocytes and is involved in numerous neutrophil functions, including adhesion, transmigration, phagocytosis, and activation (10). Increased surface expression of CR3 is a prerequisite for neutrophil transmigration from the pulmonary circulation to the alveolar spaces; thus, up-regulation is seen early in neutrophil activation processes (2, 38). In the present study, we have demonstrated that LPS from both clinical and environmental strains of *B. cepacia* induced a marked increase in neutrophil CR3 expression. By contrast, LPS from three representative

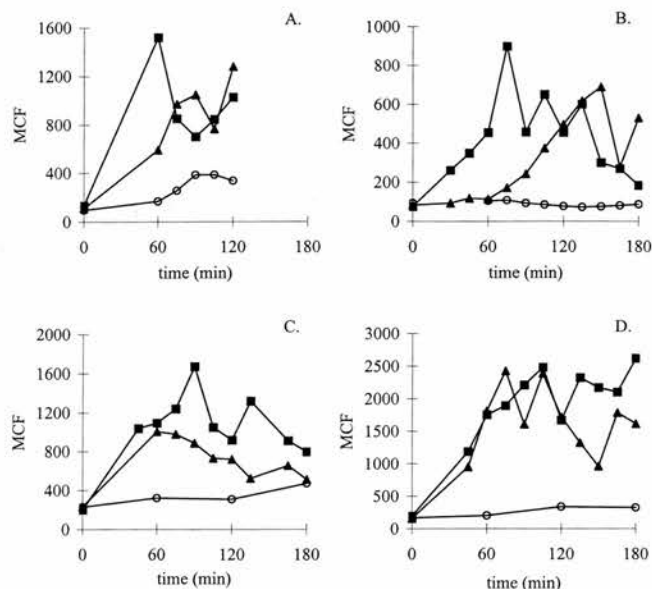


FIG. 4. Priming of FMLP-induced neutrophil respiratory burst responses by preincubation with buffer alone (○) or LPS (100 ng/ml) from *E. coli* O18K- (■) or *B. cepacia* J2315 (▲) for up to 3 h. Charts show results from studies on four individual donors. Intracellular H₂O₂ production is expressed as the MCF of the total neutrophil population stimulated with FMLP in the presence of DHR.

aeruginosa strains had little effect on CR3 expression. These results are consistent with those of Shaw et al. (37), who first reported that *B. cepacia* LPS induced a TNF- α response from circulating blood monocytes which was ninefold greater than that induced by *P. aeruginosa* LPS. TNF- α was detectable between 2.5 and 4.5 h after the addition of *B. cepacia* LPS, and levels peaked at approximately 3.5 h. As the assays in the present study were carried out on a whole blood cell population, it is possible that the increase in CR3 expression was due to secondary stimulation of neutrophils following the release of cytokines from the monocyte population. Indeed, at low LPS concentrations, an increase in neutrophil CD11b expression occurred after 3 h, possibly through stimulation by monocyte-derived TNF- α . However, the earlier timing of responses to

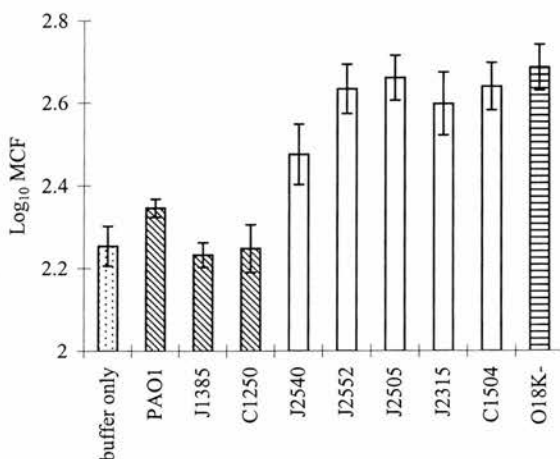


FIG. 5. Induction of CD11b on neutrophils following 90 min of incubation at 37°C with buffer alone (□) or LPS (100 ng/ml) from strains of *P. aeruginosa* (▨), *B. cepacia* (□), and *E. coli* (▩). Results are shown as the log₁₀ MCF of the neutrophil population (mean of six experiments \pm standard error of the mean).

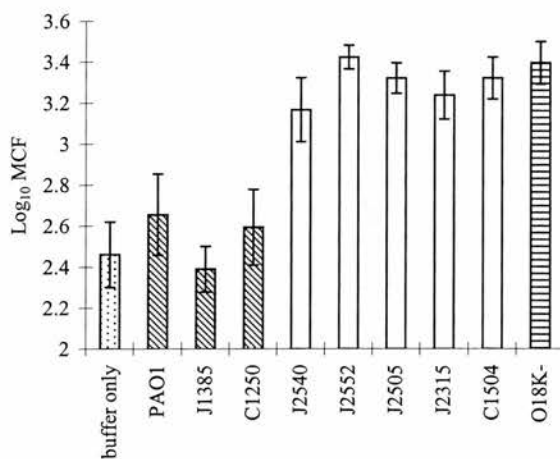


FIG. 6. Priming of FMLP-induced intracellular H₂O₂ production in neutrophils following 90 min of incubation at 37°C with buffer alone (□) or LPS (100 ng/ml) from strains of *P. aeruginosa* (▨), *B. cepacia* (□), and *E. coli* (▩). Results are shown as the log₁₀ MCF of the total neutrophil population in the presence of DHR (mean of five experiments \pm standard error of the mean).

higher concentrations of LPS (<2 h) suggests a direct effect of LPS on neutrophils.

Respiratory burst activity occurs late in neutrophil activation processes and may be increased by prior exposure of neutrophils to priming agents, including LPS (1, 18, 21, 24, 25, 32). The bacterially derived peptide FMLP triggers respiratory burst activity in primed neutrophils. In the present study, little or no respiratory burst response was observed in unprimed neutrophils stimulated with FMLP. This is in agreement with the observations of previous investigators (8, 45) and confirms the low activation state of neutrophils in the whole blood cell preparations used. *E. coli* LPS has been demonstrated to prime FMLP-induced respiratory burst responses in neutrophils (24). Comparison of *B. cepacia* and *P. aeruginosa* LPS with *E. coli* LPS in the present study indicates that while *B. cepacia* LPS molecules were potent neutrophil-priming agents, little priming activity was induced by *P. aeruginosa* LPS. Of the *B. cepacia* strains tested, only J2540, an environmental strain, failed to induce priming significantly above the levels of all of the *P. aeruginosa* strains tested. In time course experiments using LPS from *B. cepacia* J2315 and *E. coli* O18K-, priming was observed after as little as 30 and 45 min, respectively, suggesting that neutrophil-priming responses were not secondary to LPS-induced cytokine release from circulating monocytes. LPS from two *P. aeruginosa* strains, PAO1, a well-characterized laboratory strain, and C1250, a mucoid strain isolated from a CF patient, primed neutrophils to a low degree. However, LPS from a nonmucoid CF strain, J1385, had no priming effect. Interestingly in a study of LPS extracted from five CF strains of *P. aeruginosa*, Kharazmi et al. (25) observed similar variation in neutrophil priming, with LPS from two mucoid strains showing the greatest overall activity.

Our observation of a high degree of correlation between mean CR3 surface expression and mean FMLP-induced respiratory burst activity agrees with recently published studies (8). Unlike increased CR3 expression, however, priming responses were not uniform throughout the neutrophil population, and a subpopulation of highly responsive neutrophils was identified by changes in size (forward scatter) and granularity (side scatter) on stimulation with FMLP (Fig. 3, R2). These results correspond to those of Yee and Christou (45), who observed that the FMLP-induced elevation of intracellular Ca²⁺ levels

in neutrophils primed with LPS was due to raised levels in a subpopulation of responsive cells. In the present study, highly responsive cells were virtually absent in unprimed populations but appeared in samples primed with LPS from both *B. cepacia* and *E. coli*. The observed changes in forward and side scatter for R2 neutrophils may simply reflect the activation of these cells following FMLP stimulation. However, since respiratory burst responses also increased among R1 neutrophils, and since a considerable overlap in individual responses for neutrophils in R1 and R2 populations was observed, it seems more probable that LPS is associated with a significant phenotypic change in R2 neutrophils prior to stimulation with FMLP. The nature of such a phenotypic change remains unclear but could involve changes in receptor expression. However, comparison of histogram distributions of CD11b expression and FMLP-induced intracellular H₂O₂ production indicated that the highly responsive subpopulation of neutrophils could not be identified on the basis of increased CR3 expression. FMLP receptors were not measured in the present study; however, previous investigators have found that both CR3 and FMLP receptors are up-regulated on LPS-treated neutrophils in a unimodal fashion, with no evidence of neutrophil subpopulations (45), suggesting that increased receptor expression is not the mechanism underlying the highly responsive phenotype.

The data presented here indicate that *B. cepacia* LPS may be an important virulence determinant in the development of inflammation in response to *B. cepacia* infection. The demonstration of neutrophil-priming activity even in the absence of plasma is particularly relevant to the CF lung, where concentrations of plasma factors are likely to be low, due both to poor diffusion into bronchiectatic airways and to the presence of high concentrations of proteolytic enzymes (9). The necrotizing pneumonitis and bacteremia which are characteristic of cepacia syndrome are unique to *B. cepacia* infection and have never been described in CF infection caused by *P. aeruginosa*. The up-regulation of CR3 expression in neutrophils exposed to *B. cepacia* LPS may be involved in the increased recruitment of neutrophils to the lung. At the same time, *B. cepacia* LPS may prime neutrophil responses to increase the release of tissue-damaging enzymes and reactive oxygen species from activated neutrophils. It is tempting to speculate that cepacia syndrome develops whenever the burden of activated neutrophils within the lung becomes too great for overstretched regulatory mechanisms, increasing the rate of inflammatory damage and permitting the bacteremic spread of *B. cepacia* beyond the lung parenchyma.

Environmental *B. cepacia* strains are currently being developed as biological control agents, both in the control of fungal plant pathogens and in the bioremediation of contaminated landfill sites (3, 12, 20, 22, 27, 36). Identification of strains which may represent a human hazard is therefore of paramount importance if *B. cepacia* is to be released on a large scale, particularly in agricultural programs. Recently, taxonomic analyses have revealed that bacterial strains currently identified as *B. cepacia* consist of at least four distinct species or "genomovars" (16, 35). Although numbers were small, investigation of a panel of CF, non-CF clinical, and non-CF environmental isolates suggested that strains belonging to genomovars II and III were associated with colonization and, in some cases, transmissibility in CF, while strains belonging to genomovar III were associated with severe disease and cepacia syndrome in CF patients (16). By contrast, most environmental isolates, including the *B. cepacia* phytopathogenic type strain ATCC 25416, belonged to genomovar I, which is less common in CF patients. A survey of Belgian CF patients identified genomovars II, III, and IV among *B. cepacia*-colonized pa-

tients but no genomovar I isolates (35). Comparison of a genomovar III strain (J2315) with two genomovar I strains (J2540 and J2552) in the present study has shown, however, the inflammatory potential of LPS from strains of both genomovars. Thus, the rarity of genomovar I isolates in CF may reflect the poor colonizing ability of these strains rather than any intrinsic lack of pathogenicity. As it is currently impossible to predict the effect of increased exposure on the risk of a CF patient acquiring a genomovar I strain, we believe that the use of environmental *B. cepacia* strains in large-scale agricultural release programs must be carefully reviewed and monitored.

ACKNOWLEDGMENTS

This study was supported by an MRC clinical training fellowship held by J.E.H.

We thank C. Taylor for assistance with LPS extraction procedures. We also thank D. Shaw and D. Delahooke, Department of Medical Microbiology, University of Edinburgh, for the provision of LPS samples from strains PAO1, C1250, J2505, J2540, C1504, and O18K-.

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