Murine mechanisms of resistance to *Streptococcus pneumoniae*

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By

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Finally, I would like to dedicate this thesis to the loving memory of Moya Adamson (Grin-Gran).
Statement of originality

The accompanying thesis submitted for the degree of Ph.D. entitled "Murine mechanisms of resistance to Streptococcus pneumoniae" is based on work conducted by the author in the Department of Microbiology and Immunology of the University of Leicester mainly during the period between July 1995 and November 1998.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed: Neil Zig الك ار Date: 20/1/00
MURINE MECHANISMS OF RESISTANCE TO *STREPTOCOCCUS PNEUMONIAE*.

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The aim of this thesis was to investigate the basis of genetic resistance or susceptibility to infection with *S. pneumoniae* using a mouse model of bronchopneumonia. To this end, a panel of strains of inbred mice was intranasally infected with *S. pneumoniae* type 2 strain (D39), and type 3 (GB05). BALB/c mice were resistant and CBA/Ca were susceptible to infection, particularly with D39 pneumococci. To analyse the genetic inheritance, filial generations (F1 mice and F2 mice) were produced from intercross breeding these two strains and subsequently challenged with D39 pneumococci.

Further investigation of the parental strains using D39 pneumococci revealed that BALB/c mice, unlike CBA/Ca mice, were able to prevent proliferation of pneumococci in lungs and blood. Rapidly increasing numbers of bacteria in the blood was a feature of CBA/Ca, in contrast to BALB/c.

Analysis of the cellular response to infection provided data to show that BALB/c mice were able to recruit more leukocytes into the lungs than CBA/Ca mice, following infection. In the lungs, BALB/c mice recruited significantly more neutrophils than CBA/Ca mice at 12 and 24 hours post-infection. Histological studies reflected the cellular recruitment data. Inflammatory lesions in BALB/c mice were visible much earlier than in CBA/Ca mice and there was a greater cellular infiltration into the lung tissue of BALB/c mice, at the earlier time points.

Data presented in this thesis suggest that resistance or susceptibility to intranasal pneumococci may have an association with recruitment and/or function of neutrophils. Ongoing genetic analysis using tissue samples from F2 generation mice obtained during this project has revealed linkage to one murine chromosome. Continuing analysis will reveal possible additional loci and further define the genes involved in the susceptibility or resistance to pneumococcal disease.
**Abbreviations:**

Acronym | Definition
---|---
Akp-1 | Alkaline phosphatase 1
BAB | Blood agar base
BAL | Bronchoalveolar lavage
BALF | Bronchoalveolar lavage fluid
bg | Biege mutation
BHI | Brain heart infusion
BSA | Bovine serum albumin
Btk | Bruton agammaglobulinemia tyrosine kinase
CFU | Colony forming units
CR | Complement receptor
CRP | C-reactive protein
CSF | Cerebrospinal fluid
DNA | Deoxyribonucleic acid
ED | Effective dose
EDTA | Ethylenediaminetetraacetic acid
ELISA | Enzyme-linked immuno-sorbent assay
F1 | First filial generation
F2 | Second filial generation
FACS | Fluorescent activated cell sorter
FBS | Foetal Bovine Serum
FITC | Fluorescein isothiocyanate
GRO | Growth related oncogen
H₂O₂ | Hydrogen peroxide
HBSS | Hanks Balanced Salt Solution
HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP | Horseradish peroxidase
HTAB | Hexadecyl-trimethyl ammonium bromide
ICAM-1 | Intercellular adhesion molecule-1
IFNγ | Interferon gamma
Ig | Immunoglobulin
IL - Interleukin
LPS - Lipopolysaccharide
LytA - N-acetylMuramyl-L-alanine amidase
MBL - Mannan binding lectin
µg - Microgram
mg - Milligram
MHC - Major histocompatibility complex
MIP - Macrophage inflammatory protein
µl - Microlitre
ml - Millilitre
mM - Millimolar
MPO - Myeloperoxidase
NanA - Neuraminidase A
NanB - Neuraminidase B
nm - Nanometre
Nrampl - Natural resistance macrophage protein 1
O.D. - Optical density
PAF - Platelet activating factor
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
PECAM-1 - Platelet endothelial cell adhesion molecule
PLN-A - Pneumolysin negative type 2 pneumococcal strain
PMN - Polymorphonuclear leukocyte
POHPAA - p-hydroxyphenyl acetic acid
PsaA - Pneumococcal surface adhesion A
PspA - Pneumococcal surface protein A
QTL - Quantitative trait loci
R-PE - R form of phycoerythrin
SOD - Superoxide dismutase
SP-A - Surfactant protein A
SP-D - Surfactant protein D
TMB - Tetramethylbenzidine
TNF-α - Tumour necrosis factor alpha
<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen-4</td>
</tr>
<tr>
<td>Xid</td>
<td>X-linked immunodeficiency</td>
</tr>
<tr>
<td>XLA</td>
<td>X-Linked Agammaglobulinemia</td>
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Chapter 1: Introduction

1.1 *Streptococcus pneumoniae* and pneumococcal disease

*Streptococcus pneumoniae* or the pneumococcus as it is frequently referred to, has a characteristic ovoid or lancet shaped appearance. These Gram-positive cocci are commonly associated in pairs (diplococci) but can form chains particularly in culture. This non-motile, aerobic/facultatively anaerobic *Streptococcus* species is an extracellular pathogen of important clinical significance.

Since the discovery of this organism in 1881 by Pasteur and Sternberg, the pneumococcus has been found to cause a variety of medical manifestations, including pneumonia, bacteraemia, meningitis and otitis media. Globally the pneumococcus causes many fatalities, particularly in the developing world were annually over 1 million children and infants succumb to respiratory infections caused by this bacterium (Greenwood 1999, Mulholland 1999). In the 1999 World Health Organisation (WHO) report it states that “by 2025 there will still be 5 million deaths among children under five – 97% of them in the developing world and most of them due to infectious diseases such as pneumonia and diarrhoea, combined with malnutrition”.

In the United States the number of cases, annually reach between 150,000 to 570,000 for pneumonia, 16,000 to 55,000 for bacteraemia and 3,000 to 6,000 for meningitis (Centers for Disease Control 2000). This accounts for 36% of adult community acquired pneumonia and 50% of hospital acquired pneumonia, with 25% to 30% of pneumococcal pneumonia patients further developing bacteraemia (Centers for Disease Control 2000). Cases of pneumococcal meningitis in the United States account for 13% to 19% of the overall cases of bacterial meningitis, and within the patient group of children under five, *S. pneumoniae* is the leading cause of meningitis (Centers for Disease Control 2000). Mortality varies particular in elderly people but statistics associate pneumococcal pneumonia with 5% to 7%, bacteraemia with 20% and meningitis with 30% overall case mortality rate (Centers for Disease Control 2000). In the United Kingdom, the pneumococcus accounts for 30-50% of community acquired and 8% of nosocomial pneumonia (Meyer and Finch 1992). In England and Wales, pneumococcal isolates have been shown to account for a mean annual incidence of 6.7
per 100,000 episodes of bacteraemia and 0.44 per 100,000 of meningitis, with an overall case mortality of 20% and 22% respectively (Laurichesse et al., 1998).

This bacterium also accounts for 30% of all cases of otitis media (Luotonen et al., 1981). In the United States, pneumococci cause 7 to 10 million cases of this disease each year (Reichler et al., 1992). This raises the issue of not only the consequence of invasive disease and mortality rates by the pneumococcus but the overall cost and reduction in the efficiency of medical services by this pathogen.

Predisposition to pneumococcal disease in patients encompasses a number of factors ranging from the age of the patient to the clinical state of the host. Pneumococcal invasive disease is prevalent at the extremes of age, in the very young and the very old (Musher 1992). Individuals with underlying medical conditions are also at higher risk from invasive pneumococcal infections. These include: patients with HIV infection, other viral infections, other pulmonary diseases, asplenic patients, alcoholism and liver disease, patients with sickle cell disease, with malignancies, and with defects of phagocyte function and of the humoral system (Johnston 1981, 1991, Taussig 1984, Musher 1992, Bruyn et al., 1992, Kemper and Deresinski 1994, Obaro et al., 1996, Centers for Disease Control 2000, Catterall 1999).

The pneumococcus is asymptomatically carried by healthy people in the nasopharynx and depending on the age of the host, environment of the host and the presence of underlying respiratory infections, carriage can vary from 5% to 70% (Centers for Disease Control 2000).

For pneumococci, over 90 capsular serotypes have been characterised (Henrichssen 1995, Kalin 1998). The distribution of these serotypes changes temporally and geographically, together with the carrier age groups (Henrichssen 1995, Kalin 1998). Carriage can occur within hours of birth and most children up to 2 years old, are or have been colonised at some point by pneumococci (Gray and Dillon 1989). Colonisation by pneumococci in individuals is not always confined to the presence of one pneumococcal serotype; at the same time, a number of serotypes can reside in the nasopharynx (Austrian 1986). Invasive pneumococcal infection usually follows the acquisition of a new serotype rather from the prolonged carriage of a resident serotype (Gray and Dillon 1989). Pneumococci can be transmitted via droplet dispersal, dust
from dried secretions and contact with contaminated articles (Taussig 1984). Over crowding and poor ventilation can contribute to the spread of organisms (Musher 1992). Like other pathogens, the social and economic conditions in the population can have an effect on the epidemiology of infection.

Through the advent of antibiotics and the polysaccharide vaccine the overall incidence of the bacteria has decreased. However, mortality has remained high for all invasive pneumococcal infections, despite antibiotic treatment. For example, after successful antibiotic treatment the host can be free of invasive pneumococci but death can still occur (Austrian and Gold 1964). In addition, non-fatal sequelae following successful antibiotic therapy occurs in some instances; in pneumococcal meningitis over 20% of patients suffer from loss of hearing (Kennedy et al., 1991).

Administration of antibiotics such as penicillin is the most commonly used therapeutic approach in the treatment of pneumococcal infections (Musher 1992, Obaro 1996). However, since the emergence of antibiotic resistant phenotypes first discovered in the 1960's, there has been an increase globally in the level of antibiotic resistance identified among pneumococcal clinical isolates (Tomasz 1997). The level of resistance varies between geographical regions. For instance in England and Wales, a study between 1989 and 1995 showed an increase in both penicillin (0.3% to 2.9%) and erythromycin (3.3% to 10.9%) (Laurichesse et al., 1998). In other parts of the world like Spain, Eastern Europe, South Africa, South America, New Guinea and Korea the level of penicillin resistance in pneumococcal isolates has been reported to be between 30% to 50% (Appelbaum 1996). An alarming study has found the first incidence of tolerance to vancomycin (Novak et al., 1999). This antibiotic is one of the few remaining in the arsenal of compounds to fight multi-resistant invasive bacterial isolates including pneumococci (Tomasz 1997). Due to the emergence of this phenotype, vancomycin tolerant pneumococci can now more easily make the transformation into high-level resistance against this antibiotic (Novak et al., 1999).

Of a number of factors that may influence antibiotic resistance levels and frequency, one factor that is likely to be involved is the usage of antibiotics themselves (Dowson et al., 1997). Evidence suggests that antibiotic resistant isolates are more prevalent in areas of high antibiotic prescription. For instance, in Hungary a high level
of penicillin-resistance in pneumococcal isolates can be correlated with an over-use of penicillin (Marton et al., 1991). Not only are further anti-microbials needed but also a carefully directed use of future and present compounds.

Vaccination using polysaccharide antigens from the capsule of pneumococci has been practised for many years and shown to be effective in the protection against pneumococcal infection (MacLeod et al., 1945). The present-day 23-valent polysaccharide vaccine although effective in adults is underused (Shapiro et al., 1991), and inefficient at extremes of age and amongst immuno-compromised individuals (White 1988, Musher et al., 1990a, Musher 1992). These patient groups are the most at risk of invasive pneumococcal disease, and often show an insufficient antibody response to the vaccine (White 1988, Musher et al., 1990a, Musher 1992, Rodriguez-Barradas et al., 1992). Impairment of immunoglobulin response and polymorphonuclear leukocyte (PMN) function, such as chemotaxis and phagocytosis, is a consequence of a compromised immune response in elderly patients (Simons and Reynolds 1990).

The inefficiency in response to the 23-valent polysaccharide vaccine is in part due to the fact that the normal B and T lymphocyte interactions (to elicit an antibody response) are bypassed by pneumococcal capsular polysaccharide, a type 2 thymus-independent antigen (Davies et al., 1970, Howard et al., 1971, Manning et al., 1972). This subject is discussed further in relation to host immunity in section 1.2.1.3.

The polysaccharide vaccine also has one other important failing: the protection elicited from the antibodies generated after vaccination is serotype specific. As discussed earlier, serotype distributions vary geographically. Therefore, although the 23-valent vaccine developed in the United States encompasses serotypes relevant to disease in North America and Europe, in regions of Asia the efficiency of this vaccine drops below 63% (Lee et al., 1991). To deal with both serotype specificity and the insufficient antibody production to solely polysaccharide vaccines, new approaches are being adopted. New conjugate vaccines have been developed, and are presently tested in clinical trials (Käyhty and Eskola 1996). In these conjugate vaccines, polysaccharides are linked with a protein carrier thus eliciting a thymus-dependent antigenic response (Käyhty and Eskola 1996). The protein carrier can be presented in the normal way with major histocompatibility complex class II molecules (MHC II) thus stimulating T-helper
cells and allowing the development of immunological memory (Kähhty and Eskola 1996). Antibody response is therefore more efficient when the host is faced with a pneumococcal vaccine serotype which is colonising or invading; not relying on the overall existing concentration of antibody resulting from a type-2 thymus-independent response (Kähhty and Eskola 1996).

Resistance to antibiotic treatment coupled with the inefficacy of protection provided by present polysaccharide vaccines has prompted further research into the pneumococcus to establish new therapeutic approaches and vaccine development. Thus further investigation towards the understanding of the mechanisms involved in the pathogenesis of pneumococcal disease, host-bacterial interactions and host defense mechanisms is ongoing.

1.2 Pathogenesis of pneumococcal disease: Part I

1.2.1 Host immunity

To understand the relationship between the host and the pneumococcus it is important to look at the mechanisms that have evolved in host immunity to prevent or combat infectious disease.

1.2.1.1 Innate non-immune mechanisms

Aerodynamic barriers provide the first protective barrier (Canto et al., 1994) against respiratory pathogens like S. pneumoniae. Filtration of particles occurs in the nasal passages (Canto et al., 1994). Furthermore, the airways are designed to allow turbulent flow of air upon inhalation and this has the consequence of deposition of particulates on the mucociliary blanket (Canto et al., 1994). Lower in the tract sedimentation occurs until the smallest particulates reaching the alveoli are distributed by Brownian motion and are unaffected by inertia and gravitational forces and thus are removed rapidly by exhalation (Canto et al., 1994). Airway reflexes (such as sneezing, coughing and bronchoconstriction) help to clear the airways from trapped particles, prevent much of the oropharyngeal secretions passing down the trachea and restricts particulate entry to the distal tracheobronchial tree (Canto et al., 1994).

In conjunction with these mechanisms, removal of particulate matter deposited
in the airways involves mucociliary clearance (Canto et al., 1994). Goblet cells, together with mucous secretory cells and glands, produce the mucus blanket involved in this process (Canto et al., 1994). Ciliated epithelial cells provide the motor for moving this mucus blanket by rapid cilia beating; on average about 600 beats per minute (Rutland et al., 1982). Trapped particles such as bacteria are transported from the airways into the hypopharynx where they are usually swallowed; in humans this process occurs in mucus volumes of between 10ml to 100ml per day (Toremalm 1960, Reid 1965). Incorporated with mucus, the host has other defensive molecules, such as secretory immunoglobulin A (sIgA), lysozyme, lactoferrin and peroxidases (Nicod 1999). Lysozyme is effective towards pneumococcal infiltration, as it causes the breakdown of the peptidoglycan cell wall (Coonrod et al., 1991).

In addition to the mucociliary clearance, a mechanical barrier is provided by the respiratory epithelium joined by apical tight junctions (Hogg et al., 1979). Interaction with these cells and the integrity of this barrier is an important event in the colonisation and invasion of the pneumococcus as will be discussed later in section 1.3. However, the host regulates the adherence of some microbes; nasooropharynx epithelial cells allow the preferential binding of these microorganisms (Canto et al., 1994). A number of species can be isolated from the nasooropharynx of a normal immunocompetent host (Mackowiak 1982). The symbiosis that can occur with the normal flora can prevent adherence by pathogens. These resident microbes can also actively produce antimicrobials that can also inhibit the establishment of pathogens. Under normal conditions, the population of microbes in the nasooropharynx do not usually cause infection in an immunocompetent host (Canto et al., 1994). However, if the immunological state of the host changes, which allows an increase in the number of adherent Gram-negative bacteria at the epithelial surface (Johanson et al., 1980), an overshooting colonization may lead to a predisposition to infection (Canto et al., 1994).

1.2.1.2 Humoral systems

Once the pneumococcus bypasses the general host defense mechanisms (discussed in the previous section), specific immunity to the bacterium requires the interaction of both innate and acquired mechanisms in both humoral and cellular immunity.
Phagocytosis has been shown to occur in the lungs in the absence of complement activation and specific antibody responses (Rehm and Coonrod 1982). This process may involve the phenomenon of surface phagocytosis; the close proximity with the alveolar walls allowing contact between phagocyte and bacterium (Wood 1960). The effect of alveoli lining material may also have a role and has been shown with some bacterial sp. to have an effect on viability (Juers et al., 1976, LaForce et al., 1973). In the case of the pneumococcus, it has been shown by Coonrod et al., (1984) that this may be an effect of surfactant-derived free fatty acids. These act by increasing the permeability of the cell membrane thus increasing the bacterial killing by alveolar macrophages (Coonrod et al., 1984). However, the effectiveness of these mechanisms in pneumococcal disease has not been fully demonstrated.

Another mechanism is the interaction between the invading pathogen and surfactant proteins (SP-A and SP-D) in the lung. These proteins are members of the collectin family, which can bind carbohydrate moieties common to a number of bacterial species and have now been considered to provide an innate humoral defense mechanism in the lung (Holmskov et al., 1994). Clearance of bacteria by alveolar macrophages with the involvement of surfactant collectins, in the absence of effective complement and specific immunoglobulins, may be the first line of defense to delay bacterial spreading in the lungs. Reports on the interaction with SP-A and the uptake of pneumococci by macrophages have so far provided conflicting evidence (McNeely and Coonrod 1993, Tino and Wright 1996) and may reflect the differences in the specificities of interactions with different pneumococcal serotypes or strains. However, enhancement of the uptake of pneumococci by neutrophils has been observed with both SP-A and SP-D (Hartshorn et al., 1998). This enhanced uptake, associated with these surfactant collectins, was found to occur by both aggregation of the bacteria and direct interaction with neutrophils (Hartshorn et al., 1998). Therefore, these surfactant collectins may enhance the removal of bacteria from the lungs by neutrophils and/or mucociliary-mediated clearance (Hartshorn et al., 1998). Additionally, these surfactant proteins have also been demonstrated to have an effect on leukocyte chemotactic responses; SP-D effecting macrophages and neutrophils (Crouch et al., 1995) and SP-A affecting macrophages (Wright and Youmans 1993).

However, due to the antiphagocytic properties of the capsule (Lee et al., 1991),
immunoglobulins directed at the capsule are the main opsonins required for efficient phagocytosis of pneumococci, as demonstrated by the effective protection to infection shown by immunisation with type-specific capsular polysaccharides (MacLeod et al., 1945). Efficient bacterial clearance from the host is dependent on opsonisation; once opsonised, pneumococci can be effectively removed from the lung by resident and infiltrating phagocytes and from the blood by the reticuloendothelial system (Bruyn et al., 1992). The requirement for immunoglobulins is demonstrated in patients with hypogammaglobulinemia who have an increase in the susceptibility to pneumococcal disease (Rosen and Janeway 1966).

Demonstrated by passive immunisation animal studies, IgM and IgG are the most important immunoglobulins in the humoral defence against the pneumococcus (Musher et al., 1990b). While IgG can directly act as an opsonin on phagocytes regardless of their activation state, IgM mediated enhancement of phagocytosis is only observed on activated phagocytes (Janeway and Travers 1996). However, for complement activation IgM is more efficient than IgG (Janeway and Travers 1996, Austyn and Wood 1993).

A study of leukemic patients undergoing allogeneic bone marrow transplantation, demonstrated that post-transplant a deficiency in IgG2 and IgG4 isotypes was associated with susceptibility to pneumococcal infection (Sheridan et al., 1990). From studies after capsular polysaccharide vaccination, antibodies of the IgG2 subclass predominate in adults (Bruyn et al., 1992), whereas in children it is antibodies of the IgG1 subclass (Freijd et al., 1984).

High serum and secretory IgA levels following vaccination have been shown in another study (Cummins et al., 1988). Both IgA and IgG are produced by plasma cells in the lung lamina propria and respiratory mucosal sites have both IgA and IgG in the mucus (Canto et al., 1994). Compared to IgG, complement activation by IgA is weak and it is inefficient as an opsonin (Janeway and Travers 1996). The role of IgA in pneumococcal immunity remains unclear at present. In one study, patients with hypogammaglobulinemia with selective IgA deficiency did not appear to have an increase susceptibility to pneumococcal disease (Ammann and Hong 1971). However, the fact that the pneumococcus produces IgA1 protease to cleave IgA1 is suggestive of a role in the pathogenesis of this bacterium (Male 1979).
Interactions with the complement system are also important in pneumococcal disease. Both the classical and alternative pathways are involved and it is thought that these two pathways cooperate to increase the rapidity of pneumococcal opsonisation (Kemper and Deresinski 1994). Complement may also be activated by a third route by mannann binding lectin (MBL) mediated activation (the lectin pathway) (Hoppe and Reid 1994). However, the role of this pathway in pneumococcal disease remains unclear. MBL binds with a low affinity to pneumococci as compared to other microorganisms (Neth et al., 2000).

Activation of the classical complement pathway is initiated by C1q binding to surface bound IgG and IgM (Janeway and Travers 1996). Studies have shown this pathway can also be activated directly in the absence of specific antibody, by the pneumococcal toxin pneumolysin (Paton et al., 1984, Mitchell et al., 1991) and the binding of the acute phase protein, C-reactive protein (CRP) (Kaplan and Volanakis 1974). The alternative pathway does not utilise C1q and in this process, C3b molecules generated by spontaneous cleavage of C3 can bind to the bacterial surface and activate the alternative complement cascade (Janeway and Travers 1996). The pneumococcal capsule does not activate the alternative pathway, as Factor B is inefficient in binding to capsule bound C3b (Brown et al., 1983). Winkelstein and Tomasz (1978) have found that in vitro, teichoic acid a component of pneumococcal cell wall can activate the alternative pathway. It is important to note that molecules bound to the pneumococcal cell surface are covered by the capsule, therefore C3b, iC3b, and the Fc fragment of fixed IgG, below the capsule are masked from receptors on phagocytic cells (Osmond et al., 1975, Winkelstein et al., 1976, Holzer et al., 1984, Musher 1992).

All three complement activation pathways lead to the production of C3 convertases, which cleave C3 and deposits C3 components on the bacterial surface (Goldstein 1992). On the surface of phagocytes, C3b binds to complement receptor CR1, and iC3b is recognised by complement receptors CR3 and CR4 (Frank and Fries 1991). These complement receptors bind weakly and numerous ligand-receptor interactions are required, which can under some circumstances lead to the stimulation of oxidative burst, degranulation and upregulation of Fc receptor-mediated phagocytic activity (Frank and Fries 1991).
However, stimulation of phagocytes by C3b and iC3b differs, with iC3b triggering phagocytosis, the release of superoxide, myeloperoxidase and lactoferrin more efficiently than C3b (Verhoef and Visser 1993). The way complement components are bound to the pneumococcal surface also affects phagocytosis; where amide linked binding is more effective than thiolester-reactive site binding (Verhoef and Visser 1993). The observation that encapsulated pneumococci usually bind C3 complement components by thiolester-reactive site binding whereas amide linked binding occurs with unencapsulated pneumococci, indicates a possible antiphagocytic property (Verhoef and Visser 1993).

The importance of complement can be demonstrated with decomplementation studies. In a study with decomplemented mice, a deficiency in C3 was suggested to be associated with an increase in the susceptibility to pulmonary pneumococcal infection (Nakajima et al., 1990). In their study, Nakajima et al., (1990) suggested that the lack of pneumococcal clearance in decomplemented mice, was a deficiency of opsonisation and not of chemotaxis, as a large number of PMNs were observed in the lungs of these mice.

Further evidence, has been gained from studies with patients deficient in complement components. C3 complement deficiency in humans has been documented to increase the susceptibility to lethal pneumococcal infection (Alper et al., 1972). Deficiencies in components such as C2 deficiency (Newman et al., 1978) and C3b inactivator deficiency are also indicative of their importance for resistance to pneumococcal disease (Alper et al., 1970, Thompson et al., 1977).

Although pneumococci have shown to activate both the alternative and classical complement pathways, the generation of the membrane attack complex (C5-C9) is ineffective against Gram-positive organisms, such as the pneumococcus (Kemper and Deresinski 1994, Cooper 1991). However, C5 components generated in this cascade, together with C3 components, provide other important functions besides opsonisation. Both complement molecules are mediators of host inflammation (Frank and Fries 1991). Complement fragments C4a, C3a and C5a are known as anaphylatoxins due to their potent response when injected into animals (Frank and Fries 1991).

C5 components can increase the adhesiveness of neutrophils, induce their
aggregation, and evoke chemotaxis (Frank and Fries 1991). This can be demonstrated in a rabbit model of pneumococcal meningitis; C5 derived peptides were found to recruit PMNs into the cerebrospinal fluid (Ernst et al., 1984). The C5 derived peptide, C5a, can also influence the behaviour of phagocytes by stimulating neutrophil oxidative metabolism, the production of toxic oxygen species (respiratory burst), and release of lysosomal enzyme from a number phagocytic cells (Goldstein 1988). Pro-inflammatory peptides like C5a upregulate the number of complement receptors and/or effect the activation state of these receptors for phagocytosis (Unkeless and Wright 1988). C5a can stimulate the release of the pro-inflammatory cytokine interleukin 1 (IL-1) from macrophages (Frank and Fries 1991). Fixed C3 fragments iC3b and C3b can also induce the production of IL-1 (Couturier et al., 1990, Bacle et al., 1990). C5a and other complement components elicit the release of PAF and which influences arachidonate metabolism, both of which are mediators of inflammation (Vogt 1986, Pinckard et al., 1988).

As discussed previously in this section, C3 deficiency, C2 deficiency and C3b inactivator deficiency increase the susceptibility to lethal pneumococcal infection. However, to the knowledge of this author, a raised susceptibility to pneumococcal disease has not been specifically reported in C5 deficient patients. This may be due to the rarity of this disease; a total of 27 cases were reported up until 1991 (Morgan and Walport 1991).

Another component of the humoral system involved in pneumococcal infection is the acute phase protein C-reactive protein (CRP). CRP is produced in the liver with synthesis induced by the cytokine interleukin-6, as demonstrated following the intravenous challenge of pigs with a pneumococcal isolate (Ziegler-Heitbrock et al., 1992). CRP binds to phosphorylcholine on the pneumococcus to act as an opsonin (Bruyn et al., 1992) and has been demonstrated to provide protection against pneumococcal infection in a mouse model of sepsis (Mold et al., 1981). A study with CRP-transgenic mice showed that protection afforded from pneumococcal challenge was primarily mediated by complement (Szalai et al., 1996), as CRP can directly activate the classical complement pathway (Kaplan and Volanakis 1974). However, without complement, CRP can partially protect against invasive pneumococcal challenge, indicating that alone this acute phase molecule can directly act as an opsonin
for opsonophagocytosis (Nakayama et al., 1983, Szalai et al., 1996). This is made possible by the fact that monocytes have been shown to bind CRP via a CRP receptor (Tebo and Mortensen 1990) and the high affinity Fc receptor FcγRI (Marnell et al., 1995). Additionally, neutrophils can bind CRP via FcγRI (Marnell et al., 1995). Interestingly, it has been observed that streptococcal infection induces the elevation of the expression of FcγRI on neutrophils and monocytes (Guyre et al., 1990). However, the specific role of these receptors in the phagocytosis of CRP bound pneumococci remains to be investigated (Szalai et al., 1996).

It is postulated that CRP binding to the cell wall associated phosphorylcholine blocks the inflammation caused by cell wall lipoteichoic acid and teichoic acid (Mold et al., 1981). CRP has been shown to bind more avidly to killed rather than live pneumococci and it has been postulated that the role of this protein is in clearance of killed bacteria, bacterial and cellular debris (De Beaufort et al., 1997).

1.2.1.3 Main immune effector cells

Following infection in the lungs the first immune cell encountered by invading bacteria is the macrophage (Kemper and Deresinski 1994). These mononuclear cells are found in the airways, alveoli, pulmonary intravascular component, connective tissue and the pleura in the lungs (Canto et al., 1994). The role of lung associated macrophages in pneumococcal disease remains to be elucidated. However, alveolar macrophages resident in the lungs have an important function as the first line of immunological defence (Canto et al., 1994). These cells are attached to epithelial cells and migrate in a layer of surfactant (Lipscomb et al., 1995), acting both as effective phagocytes and/or mediators of immunity by the release a number of mediator molecules to regulate the immune response (Speert 1992).

Phagocytosis and activation of these cells is regulated by receptor ligand interactions. Although there are differences between the type and location of macrophage (Speert 1992, Lipscomb et al., 1995), macrophages generally possess Fcγ receptors, complement receptors, C1q receptor, lipopolysaccharide (LPS) receptor (CD14), mannosyl/fucosyl receptor and adhesion promoting receptors (CD18) (Speert 1992). Following receptor recognition, stimulation evokes phagocytosis and the activation of the phagocyte to release immune mediators discussed later in section
1.2.1.4. Activation of these cells and/or phagocytosis also evokes the release and production of an array of antimicrobial agents both in the phagocytic vacuole and externally (Sibille and Reynolds 1990).

Macrophages undergo a respiratory burst following activation (Austyn and Wood 1993). This system, gives rise to reactive oxygen intermediates such as superoxide ions, hydroxyl radicals and the production of hydrogen peroxide, all of which are potent oxidative antibacterial agents (Austyn and Wood 1993). Macrophages also produce nitric oxide by the mechanism of inducible nitric oxide synthase (Moncada et al. 1991). This system generates a number of reactive nitrogen intermediates, resulting in another source of potent oxidative antibacterial agents (Austyn and Wood 1993). However, it is important to note that there is a difference in stimuli needed to induce the production of nitric oxide between human and mouse cells (Spitsin et al., 1997).

Macrophages also produce a variety of enzymes such as lysosomal acid hydrolases and neutral proteases; with the hydrolytic enzyme lysozyme contributing to 25% of all protein released from the macrophage (Gordon 1980). Lysozyme is effective against pneumococci causing the breakdown of cell wall (Coonrod et al., 1991).

The digestive, degradative and oxidative power of all these cell-derived components gives these phagocytes a powerful antibacterial activity. Although pneumococci inhibit phagocytosis, they are easily killed once engulfed in the phagocytic vacuole (Johnston 1981, 1991). This process has been postulated to occur, even in the absence of phagocyte derived hydrogen peroxide, due to the presence of hydrogen peroxide generated intrinsically by these bacteria, which are incapable of degradation of this molecule because they lack catalase (Johnston 1981, 1991).

The role of macrophages extends to disseminated pneumococcal disease. In intravascular clearance, neutrophils do not play a major role in removal of pneumococci during bacteraemia (Bruyn et al., 1992). Tissue associated macrophages of the reticuloendothelial system serve as the main pneumococcal blood clearance mechanism, via binding of opsonised bacteria by Fc, CR1 (C3b receptor) and CR3 (iC3b) receptors (Bruyn et al., 1992). Both the reticuloendothelial organs, the spleen and the liver, are important in this process as demonstrated during clinical conditions affecting their functionality (Bruyn et al., 1992). It is widely known that patients who are asplenic or
where the function of the spleen is disrupted, such as sickle cell anaemia, are susceptible to pneumococcal disease. Cirrhosis of the liver also predisposes individuals to invasive pneumococcal disease (Bruyn et al., 1992). These organs work in combination as splenic removal of pneumococci can occur without the need for complement (Brown et al., 1981). Thus splenic clearance allows the removal of pneumococci that have not been removed by the liver due to a reduction in complement or the virulence of the invading organism (Brown et al., 1981).

Although alveolar macrophages and tissue associated macrophages involved in the reticuloendothelial system have a function in pneumococcal disease, neutrophils are the main leukocyte involved in pneumococcal clearance and host inflammation in the lungs. Under normal circumstances, neutrophils only comprise of 2% of the cell population from human bronchoalveolar lavage (Reynolds and Newball 1974) but they are rapidly recruited following infection, as demonstrated by several studies of animal models of pneumococcal disease (Gunn and Nungester 1936, Robertson et al., 1933a, b, Terrell et al., 1933, Loosli and Baker 1962, Doerschuk et al., 1994, Garvy and Harmsen 1996, Bergeron et al., 1998, Kadioglu et al., 2000).

Neutrophils are predominately found at other sites of infection. In experimental pneumococcal meningitis, numerous neutrophils are isolated in the cerebrospinal fluid (CSF) (Ernst et al., 1984). Their effect is both beneficial and detrimental; the presence of these cells has been found to be associated with the possible containment or impedance of pneumococci to prevent dissemination from the CSF (Ernst et al., 1983). Again, with pneumococcal otitis media, neutrophils are recruited in abundance into the middle ear fluid in both human patients and animal models (Nonomura et al., 1991). The detrimental consequences for the host in this cellular recruitment to these sites, together with the adhesion molecules and interactions to bring about migration and the chemotaxis of these cells will be discussed later in this and subsequent sections.

Neutrophils have a variety of cell surface receptors that recognise humoral system ligands associated with pneumococci (discussed in section 1.2.1.2). These cells bind the ligands iC3b and C3b by complement receptors CR3 and CR1 respectively (Verhoef and Visser 1993). Neutrophils are also found to bind IgG by FcyRII and FcyRIII (Rosales and Brown 1993, Verhoef and Visser 1993); receptors for other
immunoglobulin classes appear to be absent (Canto et al., 1994). This may form the reason why IgG is the most important immunoglobulin class in pneumococcal disease. Binding to Fcy receptors on leukocytes elicits a number of functions including, the clearance of immune complexes, antibody-dependent cell-mediated cytotoxicity, triggering of respiratory burst, secretion of inflammatory mediators, and phagocytosis (Rosales and Brown 1993).

Neutrophils undergo a respiratory burst, as described for macrophages, generating potent reactive oxidant species from the NADPH oxidase system as part of its antimicrobial system (Sibille and Reynolds 1990). In addition, the antimicrobial system is also enhanced by the enzyme myeloperoxidase. This enzyme is stored in the primary granules in the cytoplasm of neutrophils (Klebanoff 1980), and catalyses the production of hypohalous acids from hydrogen peroxide in the presence of halide ions (Klebanoff 1988). Neutrophils also have other potent antimicrobial constituents in their granules, and these are released upon stimulation of the cells. Table 1.1 shows a list of the numerous components found in the three populations of granules present in mature neutrophils (Bainton 1992).

Both oxidative metabolites and granule constituents are effective antimicrobial agents. The interaction of the different antimicrobial mechanisms and constituents provide numerous ways to inhibit or kill microorganisms and thus there is no one exclusive mechanism. Interestingly, although the pneumococcus has antiphagocytic properties (Lee et al., 1991), once engulfed, as described for macrophages, pneumococci are readily killed within the confines of the phagocytic vacuole (Johnston 1981, 1991). That there is no one exclusive killing mechanism, and pneumococci are easily killed in the phagocytic vacuole, is demonstrated in patients with abnormal neutrophils. Patients with a defective phagocyte respiratory burst, such as chronic granulomatous disease or patients with an abnormality of neutrophil granules, do not appear to have an increased susceptibility to pneumococcal disease (Strauss et al., 1974, Johnston and Newman 1977, Komiyama et al., 1979).
<table>
<thead>
<tr>
<th>Azurophil granules</th>
<th>Specific granules</th>
<th>Other intracellular compartments</th>
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<tr>
<td><strong>Microbicidal enzymes</strong></td>
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<tr>
<td>Myeloperoxidase, Lysozyme, Bacterial permeability increasing protein (BPI) or CAP 57, Defensins, Serprocidins, Elastase, Cathepsin G, Proteinase 3, Azurocidin or CAP 37</td>
<td>Lysozyme</td>
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<td></td>
<td>Collagenase, Gelatinase</td>
<td>Alkaline phosphatase, Tetranection</td>
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<td><strong>Acid hydrolases</strong></td>
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<td>β-Glycerophosphatase, β-Glucuronidase, N-Acetyl-β-glucosaminidase, α-Mannosidase, Cathepsin B, Cathepsin D</td>
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<td>Lactoferrin, Vitamin B₁₂-binding proteins, Plasminogen activator, Histamine, Cytochrome b, β₂-microglobulin</td>
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<td><strong>Receptors</strong></td>
<td><strong>Receptors</strong></td>
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<tr>
<td>FMLP (N-Formylmethionyl-leucyl-phenylalanine), CR3 (C₃bi), Laminin, CD11b/CD18, Vitronectin</td>
<td>Fc RIII, CR1, CD11b/CD18</td>
<td></td>
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</table>

**Table 1.1 Human neutrophil granule constituents (Taken from Bainton 1992).**
It is important to note that the anti-microbial components of both neutrophils and macrophages can cause and augment detrimental inflammation in the host. Indeed, inflammation and toxaemia generated by the host is important in pneumococcal disease, as demonstrated in the fact that even when tissues are found to be sterile, death can still occur (Austrian and Gold 1964).

Under normal circumstances, host cells have mechanisms to overcome the toxicity of the oxidative burst. These include cellular derived catalases, superoxide dismutases and peroxidases (Del Maestro 1980). To alleviate the toxicity of enzymes produced by degranulation, antiproteases found in the serum and produced by macrophages, can prevent tissue injury by irreversibly inhibiting proteases (Travis and Salvesen 1983, Sibille and Reynolds 1990). However, during pneumococcal infection, the level of cellular recruitment and inflammation would out-weight the antagonistic effect of these mechanisms.

Oxidants play a role in inactivating protease inhibitors, thus oxygen metabolites derived from the NADPH oxidase system interact together with granule constituents such as proteolytic enzymes to cause host cell and tissue damage (Weiss 1989). Proteases have the ability to attack key components of the extracellular matrix (Hay 1981). In particular, the major neutral serine protease produced from neutrophils, elastase (Campbell 1986) has the potential to detach, lyse or cause the derangement of endothelial cell barrier properties (Ricevuti 1997). Free radicals themselves are injurious via their reaction with proteins, nucleic acids and lipids (Ricevuti et al., 1993, Piani et al., 1994). Oxidants can directly injure lung tissue, as demonstrated in rats (Johnson et al., 1981, Johnson and Ward 1981), and can affect fibroblasts, erythrocytes, endothelial cells, lung parenchymal cells and hyaluronic acid (McCord 1974, Sacks et al., 1978, Weiss 1980, Weiss et al., 1981, Simon et al., 1981, Martin et al., 1981).

Although alveoli contain approximately 10% lymphocytes (Canto et al., 1994, Nicod 1999), the role of these cells in pneumococcal disease is considered to be of a lesser extent to that described of macrophages and neutrophils. This is demonstrated in patients with congenital thymic hypoplasia who have a lack of T lymphocytes but are not overly susceptible to pneumococcal disease (Di George 1968). Moreover, activation of macrophages by T lymphocytes does not also appear to be necessary, because of the
relative ease of destruction of pneumococci once phagocytosed, as discussed previously in this section (Johnston 1981).

Pneumococcal capsular polysaccharide is also a type 2 thymus-independent antigen and thus bypasses the normal B and T lymphocyte interactions to produce antibody (Davies et al., 1970, Howard et al., 1971, Manning et al., 1972). B cell activation may occur by repetitive determinants on the polysaccharide molecule cross-linking with surface receptors (Austyn and Wood 1993), eliciting an IgM response, which is devoid of memory or booster function and isotype switching (Beuvery et al., 1982). A subset of B cells known as CD5 B cells, are thought to be involved in this type 2 thymus-independent response (Janeway and Travers 1996).

Stimulation of B cells by type 2 thymus-independent antigens is dependent on the maturity of the B cells (Käähty and Eskola 1996, Janeway and Travers 1996). It is thought that young children who have a poor antibody response and are more susceptible to pneumococcal disease than adults, have immature B cells (Rijkers et al., 1987). Although not needed for the initiation, subsets of T lymphocytes in mice, T suppressor and T amplifier cells, do however modulate the magnitude of the antibody response (Baker et al., 1970, 1974, 1981, Taylor and Bright 1989).

A further direct role for lymphocytes in pneumococcal disease may yet be uncovered as T and B lymphocytes in a mouse model of bronchopneumonia were found to accumulate at sites of inflammation (Kadioglu et al., 2000). CD4 cells may play a part in pneumococcal disease by the cytokines they produce. Both Th1 and Th2 response-derived cytokines have been implicated in pneumococcal disease. These components of immunity will be discussed further in the next section. The reader is referred to Lipscomb et al., (1995), for further details of the general role of lymphocytes in pulmonary immunity.

1.2.1.4 Cytokines, chemokines and other effector molecules

Leukocytes, epithelial cells, endothelial cells and other cell types can produce soluble mediators of inflammation that effect the function, recruitment of phagocytes and the overall outcome of the disease. These cytokines, chemokines and other mediators of inflammation, have an important role in pneumococcal disease, interacting
to modulate adhesion, function, the recruitment and chemotaxis of phagocytes and host inflammation.

Cytokines and chemokines are a family of potent polypeptide molecules, which have a number of important immunological functions. In clinical studies, an increase in the cytokines tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), interleukin 1 beta (IL-1β) and the chemokine interleukin 8 (IL-8) has been observed in the lungs during pneumococcal community acquired pneumonia (Boutten et al., 1996, Dehoux et al., 1994). In the blood, this was confined to IL-6 with the absence of detectable levels of the other molecules (Boutten et al., 1996, Dehoux et al., 1994). Experimental models have also supported these findings with an increase in TNF-α, IL-1 and IL-6 in the lungs following pneumococcal infection (van der Poll et al., 1996, 1997, Bergeron et al., 1998). Additionally, in experimental models IL-10, and interferon gamma (IFN-γ) levels in the lungs have been reported to be elevated following pneumococcal pneumonia (van der Poll et al., 1996, 1997). Experimental models have also shown the release of TNF-α, IL-1, IL-6 and IFN-γ in the blood following pneumococcal infection (Benton et al., 1995, 1998, Takashima et al., 1997, van der Poll et al., 1997, Rubins and Pomeroy 1997, Bergeron et al., 1998).

In vitro experiments have shown the release of a number of mediators upon stimulation of mammalian cells with pneumococci or pneumococcal components. Pneumolysin, the pneumococcal toxin discussed in section 1.3.2, at sub-lytic levels stimulated IL-1β and TNF-α release from both a cultured cell line and isolated human monocytes (Houldsworth et al., 1994). Production of IL-1 was associated with the stimulation of monocytes by the pneumococcal cell wall, however, the production of TNF-α was not observed (Reisenfeld-Orn et al., 1989). In an endothelial cell model, heat killed pneumococci or purified cell wall caused effects, such as cell separation and destruction of cell monolayer, attributable to the induction of cytokines; IL-1 and TNF-α (Geelen et al., 1993). Indeed, it is proposed that the cytokine activation of epithelium and endothelium cells, which allows pneumococci to invade the host; discussed in detail in section 1.3.

In another in vitro study, human peripheral blood mononuclear cells were stimulated by intact killed pneumococci isolated from different clinical sources (Arvå
and Andersson 1999). This resulted in the observation of an induction of a proliferative response in these cells, together with the production of a number of mediators of inflammation and phagocyte function (Arvå and Andersson 1999). These were pro-inflammatory, anti-inflammatory and immunomodulatory: IL-1β, IL-6, TNF-α, IL-8, IL-10, IL-12, IFN-γ and granulocyte macrophage colony stimulating factor (GM-CSF) (Arvå and Andersson 1999). Interestingly, from this list the cytokine profiles between different isolates varied but all isolates produced more IL-12 than IL-10. Thus in this model, pneumococci promote more of a T-helper type 1 (Th-1) response (Arvå and Andersson 1999).

*In vivo* experiments have probed the function of these molecules on the outcome of pneumococcal disease using transgenic mice and passive immunisation. Takashima *et al.* (1997) demonstrated that modulation of TNF-α, by administration of specific anti-TNF-α immunoglobulin during pneumococcal pneumonia in mice, lessened survival time and increased bacterial counts in the blood. Neutrophils were also found to decrease in immunised animals and thus TNF-α was postulated to be involved in the prevention of bacteraemia in this model by increasing neutrophils in the blood (Takashima *et al.*, 1997). Further experiments have provided evidence that TNF-α receptor 1 (p55) which is one of the two receptors for the signalling of TNF-α is important in pneumococcal susceptibility (O’Brien *et al.*, 1999). O’Brien *et al.*, (1999) with their intraperitoneal model of infection, also indicated that p55-deficient mice did not lack recruited neutrophils in the peritoneum. TNF-α thus may act more by the activation of neutrophils than by the recruitment of these cells (O’Brien *et al.*, 1999). Benton *et al.*, (1998) showed that survival and bacterial counts in mice infected with pneumolysin-deficient pneumococci, was reliant on TNF-α. Past the initial phase of infection, IL-1β was found to be important in survival (Benton *et al.*, 1998). In these experiments, CBA/CaHN-XID/J (CBA/N mice were shown to have elevated levels of TNF-α, IL-1β and IFN-γ when pneumococcal numbers in the blood reached high levels (Benton *et al.*, 1998).

In another study the sustained production of the anti-inflammatory cytokine IL-10 locally in the lungs was found to occur after experimental infection of mice (van der Poll *et al.*, 1996). van der Poll *et al.*, (1996) also showed that a certain level of
constitutive expression of this cytokine occurs in murine lungs. Following the addition of IL-10 at the same time as the inoculum, the antagonist properties of this cytokine were associated with a decrease in lung levels of TNF-α and IFN-γ with a concomitant increase in pneumococcal numbers in the blood and lungs and a shorter survival time (van der Poll et al., 1996). This scenario was reversed when anti-IL-10 immunoglobulin was administered, thus IL-10 may, in this model, attenuate immunological defence to pneumococci (van der Poll et al., 1996).

The importance of the cytokine IFN-γ has been identified experimentally in normal and knockout mice (Rubins and Pomeroy 1997). Post-infection levels of IFN-γ did not significantly change in the lungs but levels of this cytokine were found to increase in the serum (Rubins and Pomeroy 1997). Lethality and bacterial numbers were also found to increase in animals deficient in interferon-γ (Rubins and Pomeroy 1997).

Another important mediator of both pro-inflammatory and anti-inflammatory elements of the inflammatory network is the cytokine IL-6. Present during pneumococcal infections as described earlier in this section, this cytokine has been shown to be important in experimental pneumococcal pneumonia in mice (van der Pol et al., 1997). van der Pol et al., (1997) observed that endogenous levels of serum and lung IL-6 increased post-infection in normal immuno-competent mice. IL-6 deficient mice had a greater pneumococcal growth in the lungs and a higher mortality than was observed after pneumococcal infection in their wild-type counterparts (van der Pol et al., 1997). In addition, these mice were shown to have significantly higher lung levels of TNF-α, IL-1β, IFN-γ, IL-10 and myeloperoxidase, as a determinant of neutrophil numbers, than the normal sufficient IL-6-murine strain (van der Pol et al., 1997). This was also accompanied by a higher level of soluble TNF-α receptors in IL-6 deficient mice and a lower level of the acute phase proteins, serum amyloid P and C3 (van der Pol et al., 1997). Induction of acute phase proteins is an important function of IL-6 and in another study, by Ziegler-Heitbrock et al., (1992) is associated with the production of CRP in pigs following pneumococcal infection.

IL-8 has been shown to be present in the lungs during infection, discussed earlier in this section. This chemokine has been found to be produced by cells including neutrophils, which have been directly stimulated in vitro to produce IL-8 by incubation
with pneumococci (Hachicha et al., 1998). IL-8 is a member of the CXC chemokine family, one of four groups of related molecules with a conserved group of cysteine amino acids in the primary sequence; C, CC, CXC and CX3C (X designates a variable amino acid residue) (Strieter and Kunkel, 1997, Rollins 1997, Luster 1998.). Importantly, no mouse homologue of IL-8 has been found but human IL-8 binds to an IL-8 receptor homologue in mice as does mouse macrophage inflammatory protein-2 (MIP-2), mouse KC (IL-8r homologue) (Cacalano et al., 1994, Lee et al., 1995). In mice, the homologue for the human chemokines growth related oncogen-β and growth related oncogen-γ (GRO-β/γ) is MIP-2 (Lecture 1999). In vivo experiments with MIP-2 have shown the importance of this molecule in murine models. MIP-2 mimics part of the function of IL-8 in neutrophil recruitment in a model of urinary tract infection by Escherichia coli (Hang et al., 1999). In a model of Klebsiella pneumoniae pneumonia, MIP-2 was found to be important for appropriate pulmonary cellular recruitment and the clearance of bacterial numbers (Greenberger et al., 1996).

It is likely though, that other chemokines apart from IL-8 interact in humans, for instance due to the fact that cell types such as epithelial cells, can produce other chemokine molecules such as GRO-α, GRO-γ and monocyte chemotactic protein-1 (MCP-1) (Nicod 1999). The production of CXC molecules by pulmonary fibroblasts and epithelial cells also is induced by IL-1 and TNF-α (Lecture 1999), with these cytokines already shown to be involved in pneumococcal disease. Due to the production of CXC chemokines by both immune and non-immune cells, it is thought that these molecules orchestrate the overall cellular recruitment in the lungs during inflammation (Lecture 1999). Chemokine functions can overlap with a number of molecules, thus other chemokine family member can also play a role. For instance, MIP-1α, a chemokine from the CC family of chemokines, is also produced by neutrophils in response to stimulation with pneumococci in vitro (Hachicha et al., 1998). Neutrophils can therefore add to the orchestration of their own recruitment and that of mononuclear leukocytes, to sites of infection (Hachicha et al., 1998).

It is important to note that the orchestration of leukocyte recruitment by mediators such as chemokines is tightly controlled. Although a large pool of marginated neutrophils exists in the vascular bed in the lungs, neutrophils are inhibited from crossing into the alveolar spaces by soluble components in the serum and macrophage
derived polymorphonuclear inhibitors (Goetzl 1975, Sibille and Reynolds 1990). This process is observed during pneumococcal pneumonia as recruitment occurs in infected regions of the lungs, whereas in normal uninfected regions, marginated neutrophils are not seen to transmigrate (Doerschuk et al., 1994).

Research continues into the roles of cytokines and chemokines in pneumococcal disease, particularly in view of conflicting reports that show different correlations between the levels of cytokines such as TNF-α, IL-6, IL-10 and patient outcome during pneumonia (Chollet-Martin et al., 1993, Marik et al., 1993, Moussa et al., 1994, Puren et al., 1995, Glynn et al., 1997). Further studies are also needed to test data obtained in animal in vivo models, particularly in the light of the possible use of cytokines as therapeutic agents or adjuvants in some clinical conditions (van der Poll et al., 1996, 1997, Rubins and Pomeroy 1997, O'Brien et al., 1999).

Together, these in vitro, in vivo and clinical studies indicate the intricacy in the modulation of inflammation and host defence by mediator molecules. The focus of research has been on these aforementioned mediators, in particular cytokines and chemokines, however, other molecules have been implicated in pneumococcal disease.

Another class of molecules, the lipid mediators, contribute to inflammation in the host. During pneumococcal pneumonia, neutrophils have been documented to be recruited by the products of the arachidonic acid pathway (Tuomanen et al., 1987). Pneumococcal cell wall components have been implicated in the activation of this pathway, connected with lung inflammation in pneumococcal pneumonia (Tuomanen et al., 1987).

In vitro it has been found that pneumolysin can activate phospholipase A (Rubins et al., 1994). Phospholipases metabolise membrane phospholipids to arachidonic acid (Austyn and Wood 1993). Products from the modification of arachidonic acid by the oxygenase and lipooxygenase pathways are prostaglandins, prostacyclin, thromboxanes, leukotrienes and hydro(pero)xyeicosatetraenoate derivatives (H(P)ETEs), which are important in inflammation in the host (Austyn and Wood 1993). In particular, leukotriene B₄ has been shown to be elevated in the lungs, in an experimental pneumonia model (Bergeron et al., 1998). In addition to arachidonic acid and the oxygenase and lipooxygenase pathway products, free fatty acids and
lysophosphatides produced from the action of phospholipases are found to be cytotoxic, and may contribute to inflammation in the host (Rubins et al., 1994).

Another lipid mediator found to be important in pneumococcal disease is platelet-activating factor (PAF). This molecule is produced in response to injury by macrophages, neutrophils, platelets and endothelial cells (Wissner et al., 1986, Issekutz and Szpejda 1986). PAF is a mediator of inflammation causing effects such as cytokine activation, bronchoconstriction, increased vascular permeability connected with protein exudation, platelet aggregation and accumulation of leukocytes (Cabellos et al., 1992, Issekutz and Szpejda 1986). PAF has been found to play a role in inflammation during pneumococcal disease, and is associated with a CD18 dependent inflammatory cellular recruitment in the central nervous system and a CD18 independent inflammatory cellular recruitment in the lungs (Cabellos et al., 1992). Clinically, PAF has been shown to be important, with the levels of this lipid mediator found to correlate with clinical outcome in children with pneumococcal meningitis (Arditi et al., 1990). The receptor for PAF is also involved in pneumococcal pneumonia, and is an important component of the pathogenesis of pneumococci discussed in more detail in section 1.3.1.

The list of molecules found to interact or play a role in pneumococcal disease, will undoubtingly increase. For example, other mediators that are released during inflammation, such as serotonin and histamine from mast cells (Janeway and Travers 1996) will play a role in causing and augmenting inflammation in the host.

1.2.1.5 Adhesion molecules

Migration of leukocytes to sites of infection, by the process of extravasation and diapedesis is dependent upon the interactions of adhesion molecules, leukocytes, epithelial and endothelial cells. Four stages are involved in the transmigration of leukocytes from the vasculature to the infected tissue (Janeway and Travers 1996). Firstly, an attachment and rolling stage involves the adhesion molecules of the selectin family (L-selectin, E-selectin and P-selectin) (Hogg and Doerschuk 1995). P-selectin is carried in pre-formed granules known as Weibel-palade bodies, does not require protein synthesis and is released in response to histamine, thrombin and oxidants (Hogg and Doerschuk 1995). These molecules are the first inflammatory induced adhesion molecules to appear on the endothelial surface, appearing within minutes after contact
with inflammatory stimuli (Janeway and Travers 1996). Within hours after inflammation of the endothelium, E-selectin is then upregulated on the cell surface (Janeway and Travers 1996). Low affinity interactions between these selectins together with L-selectin and other ligands on the leukocyte surface, cause these cells to roll along the activated blood vessel wall (Janeway and Travers 1996). This has the effect of slowing the travel of these cells in the blood flow to allow the interaction of other adhesion molecules upregulated at a later stage (Hogg and Doershuk 1995, Janeway and Travers 1996).

In the next stage, stronger affinity occurs between the leukocyte and the endothelial surface after integrin-ligand binding. Integrins are expressed by a number of cells and are important adhesion molecules found on leukocytes (Pigott and Power 1993). The main integrins associated with leukocyte endothelial interactions are the β2 integrins; CD11/CD18 molecules and VLA-4 (very late antigen-4) (Hogg and Doershuk 1995). Their respective ligands are from the immunoglobulin superfamily; intercellular adhesion molecule (ICAM-1) for CD11/CD18 molecules and vascular cell adhesion molecule (VCAM-1) for VLA-4 (Pigott and Power 1993). However, it must be noted that dependent on the site of leukocyte migration, other integrin-ligand pairs may be involved (Kadioglu 1996).

During inflammation, mediators such as IL-8, PAF and complement activation products induces shedding of L-selectin and the upregulation and activation of CD11/CD18 molecules (Hogg and Doershuk 1995). With lymphocytes, there is a different interaction involving VLA-4 on the leukocyte binding to VCAM-1 on endothelial cell (Hogg and Doershuk 1995). However, although the upregulation of these molecules occurs, it is a change in the conformational state of the integrin that affects the leukocyte’s ability to adhere more firmly to endothelial cells (Hogg and Doershuk 1995, Janeway and Travers 1996).

Together with the effect of pro-inflammatory stimuli on leukocytes, during inflammation, ICAM-1 and E-selectin are upregulated on the endothelial surface by TNF-α and IL-1 (Hogg and Doershuk 1995). The net effect of these interactions is the firm adhesion of leukocytes to the endothelial surface to allow transendothelial migration (Hogg and Doershuk 1995).
It is suggested that transendothelial migration involves the adhesion molecule PECAM-1 (platelet endothelial cell adhesion molecule), together with a CD11/CD18 mechanism (Vaporciyan et al., 1993, Hogg and Doerschuk 1995). Interactions with PECAM-1 on the surface of leukocytes, involves binding to analogous PECAM-1 molecules on the endothelial cell junctions, and other unknown ligands (Vaporciyan et al., 1993). Diapedesis occurs when the leukocyte migrates between the tight junctions of the endothelium to cross the vascular endothelial wall (Janeway and Travers 1996). Leukocytes then migrate to the site of infection by following a gradient of chemotactic molecules produced at the site of infection (Janeway and Travers 1996). For a more detailed explanation of leukocyte transendothelial migration, the reader is referred to Bianchi et al., (1997).

This model is a generalised overview of leukocyte emigration and the complexity of adhesion interactions occurring in systemic blood vessels. These events can be ascribed to leukocyte migration during disseminated pneumococcal infection, which has been implicated with experiments on mice deficient in adhesion molecules (E-selection, P-selectin, ICAM-1, ICAM-1/CD18) (Tan et al., 1995a, b, Bullard et al., 1996, Munoz et al., 1997). However, migration has been postulated to differ within the pulmonary capillaries in context of inflammation in the lungs (Hogg and Doerschuk 1995). Due to the size of these vessels, the rolling of leukocytes cannot take place (Doerschuk et al., 1993). Normally cytoskeletal deformability occurs in the leukocytes, allowing the formation of a flexible elongated shape, which allows these cells to bypass these vessels (Hogg and Doerschuk 1995). During inflammation, inflammatory mediators activate a change in this cytoskeletal deformability, which prevents this flexible elongated shape from occurring causing leukocytes to sequester in these pulmonary capillaries (Hogg 1987, Downey and Worthen 1988, Worthen et al., 1989, Selby et al., 1991, Inano et al., 1992). This increases the contact time in these vessels and thus it has been proposed replaces normal L-selectin adhesion (Hogg and Doerschuk 1995). Studies on mice with selectin-deficiency have also shown that pneumococcal pneumonia induces a neutrophil mechanism which bypasses normal selectin mediated margination or migration (Bullard et al., 1995, Mizgerd et al., 1996, Doyle et al., 1997).

During pulmonary infection with the pneumococcus, as previously discussed,
the main cell type involved and found in the greatest quantity is the neutrophil. These leukocytes also have another important mechanism in pulmonary leukocyte emigration, which is different to that found in systemic circulation. It has been shown that *S. pneumoniae* can elicit an integrin (CD11/CD18) independent emigration of neutrophils in pulmonary capillaries (Doerschuk *et al.*, 1990, Harlan *et al.*, 1992, Hellewell *et al.*, 1994, Mizgerd *et al.*, 1999).

Endothelial levels of the CD11/CD18 ligand ICAM-1 are not found to be upregulated in pneumococcal pneumonia (Burns *et al.*, 1994). It has therefore been suggested, that it is the ability of different stimuli (such as those induced by different microorganisms) to stimulate the expression of this adhesion molecule during neutrophil migration, which accounts for the use of either CD18-dependent/independent mechanism of neutrophil emigration (Burns *et al.*, 1994). Interestingly, although CD18 independent adhesion and emigration occurs, CD18 expression on the neutrophil increases following pneumococcal infection in the lung, with a down-regulation of L-selectin (Burns and Doerschuk 1994). This CD18 expression, which is greatest in airspace neutrophils within the pneumatic site, is thought to be involved in phagocytosis (Burns and Doerschuk 1994) by binding to iC3b (Wright *et al.*, 1990).

1.3 **Pathogenesis of pneumococcal disease: Part II**

In the nasopharynx, pneumococci colonise the epithelium by adhering to glycoconjugate receptors. This process of adherence allows “quiet” carriage, which does not cause host inflammation but evokes anti-capsular serotype immunity (Tuomanen 1997, Tuomanen and Masure 1997). However, in conditions resulting in an invasive process, pneumococci can migrate from the nasopharynx up the eustachian tube to the middle ear to cause otitis media (Cundell *et al.*, 1995a). From here, pneumococci can spread to the subarachnoid space to cause meningitis (Taussig 1984).

Alternatively, from the initial site of colonisation in the nasopharynx, pneumococci can travel/migrate to the lower airways by aspiration/aerosol dispersion (Rake 1936, Cundell *et al.*, 1995a, Tuomanen *et al.*, 1995, Musher 1992) or by means of adherence to bronchial epithelia as a second colonisation site (Adamou *et al.*, 1998). In the mucosa of the alveolar space, low levels of pneumococci can exist without inducing clinical symptoms, observed in an experimental model of canine pneumonia.
(Hamburger and Robertson 1940). However, establishment of pneumococci in the lower airways can cause lobar and bronchopneumonia. Lobar pneumonia is characterised by alveoli consolidated with inflammatory exudate, to an extent that the whole lobe is involved and this is thought to be caused by infection with an acquired virulent serotype (Taussig 1984). On the other hand, bronchopneumonia is characterised by discrete foci of inflammatory lesions around terminal and respiratory bronchioles and is thought to develop from an opportunistic infection from resident pneumococci (Taussig 1984).

Pneumococci in the lower respiratory airways can also provide a route to systemic infection. Invasion of the lower respiratory epithelium provides migrating pneumococci access to the pulmonary circulation and lymphatic system (Rake 1936, Cundell et al., 1995a). Pneumococci entering the blood from the alveoli can lead to septicaemia, which in certain instances can have the additional effect of causing meningitis (Cundell et al., 1995b).

The condition in which pneumococcal carriage changes to invasion is not completely understood. As discussed in section 1.1, between 5% to 70% of individuals in the normal population carry the pneumococcus asymptomatically (Centers for Disease Control 2000). However, pre-proposing factors such as defects in host immunity, as discussed in section 1.1, may contribute to the shift from carriage to disease. In particular, it has been postulated that pneumococci can exploit a change in the host. This is indicated by in vitro viral or cytokine enhancement of pneumococcal adherence (Plotowski et al., 1986, Håkansson et al., 1994, Cundell et al., 1995c).

1.3.1 Phase variance

Pneumococci can exhibit a changing interaction with the host. This is the phenomenon of phase variance that occurs within a single strain of pneumococcus (Weiser et al., 1994). It involves spontaneous phenotype switching observed as a transparent, semi-transparent and opaque colonial morphology (Weiser et al., 1994). These phenotypes are derived from the ability of pneumococci to vary the amount of choline binding proteins, cell wall–associated teichoic acid and capsular polysaccharide (Weiser et al., 1996, Kim and Weiser 1998, Rosenow et al., 1997, Kim et al., 1999, Weiser and Kapoor 1999). The transparent phenotype is associated with an increase in cell wall-associated teichoic acid and a difference in choline binding proteins, such as
the increased expression of autolysin (LytA) and choline binding protein A (Cbp A) compared with the opaque form (Weiser et al., 1996, Rosenow et al., 1997, Kim and Weiser 1998). However, the opaque phenotype is associated with an increase in capsular polysaccharide over that of the transparent form (Kim and Weiser 1998, Kim et al., 1999). Therefore, this phase variance has important consequences \textit{in vivo}, with both opaque and transparent forms important at different stages in the pathogenesis of pneumococcal disease. The transparent phenotype is better adapted for colonisation in the nasopharynx, as it adheres more efficiently than the opaque form to cell receptors in the nasopharynx (Weiser et al., 1994, Cundell et al., 1995c). However, in the lower respiratory tract both variants adhered to normal resting respiratory epithelium and endothelium equally (Cundell et al., 1995c).

Cundell et al., (1995c) also showed that transparent variants adhered to cytokine activated respiratory epithelium and endothelium far more than opaque variants. Upon further investigation, transparent pneumococci were found to adhere to the platelet-activating factor (PAF) receptor by cell wall-associated phosphorylcholine, which is up-regulated on activated cells (Cundell et al., 1995d).

Pneumococci bound to the PAF receptor do not interfere with or induce signalling from the receptor (Cundell et al., 1995d). However, the PAF receptor undergoes receptor-mediated endocytosis (Gerard and Gerard 1994) and when pneumococci are adherent to the receptor, this provides a mechanism of internalisation into the cell (Cundell et al., 1995a, d). Studies by both Cundell et al., (1995d) and Geelen et al., (1993) have shown pneumococci inside activated endothelial cells. Another report has also shown that pneumococci can enter respiratory epithelial cells and are found in the cytoplasm either freely in damaged cells or enclosed in vacuoles in intact cells (Talbot et al., 1996). PAF receptor internalisation thus may give transparent variant pneumococci the means to transmigrate from the lungs to bloodstream (Cundell et al., 1995c, d). The mechanism by which pneumococci can exit once internalised has not yet been determined. However, studies have indicated that they do exit, such as demonstrated with an \textit{in vivo} intratracheal model of rabbit pneumonia (Cundell et al., 1995d). Cundell et al., (1995d) observed that when animals were treated with a PAF receptor antagonist, thus blocking PAF receptor dependent adherence and transmigration, an attenuation of bacteraemia occurred.
Most of the discussion of these phase variants has centred on the transparent variant form of pneumococci, however after adherence and translocation from the lungs, the opaque phenotype becomes advantageous to the pneumococcus. The opaque phase form, with a greater amount of capsule, inhibits opsonophagocytosis more than the transparent phase form (Kim et al., 1999). This has a ramification for invasive disease, as demonstrated in an intraperitoneal model of sepsis in which animals infected with opaque variants were shown to have a greater mortality than transparent variants (Kim and Weiser 1998). Thus, there is a relationship between phase shift and colonisation or invasive disease, giving pneumococci the ability to adapt to different host niches.

Although a hypothesis on colonisation and invasion has been based around these phase variants (Cundell et al., 1995a, Tuomanen 1997, Tuomanen and Masure 1997), this hypothesis does not address the interaction of other pneumococcal factors with the host, in the pathogenesis of pneumococcal disease. Pneumolysin and other pneumococcal proteins, for instance, have a role that is discussed further in section 1.3.2. Together, in combination with phase variance, these virulence factors provide the pneumococcus with the mechanism for pathogenesis in the host. However, a role or a defined series of events remains to be confirmed for a number of these factors.

1.3.2 Virulence factors

The pneumococcus produces a number of factors, which are a pre-requisite for causing disease in the host.

1.3.2.1 Capsule

Pneumococci isolated on agar can appear smooth (encapsulated) or rough (unencapsulated). This phenomenon is due to a surface layer of polysaccharide. This polysaccharide capsule enclosing the bacteria is fundamental but not exclusive to the virulence of this organism, as demonstrated by its removal by Avery and Dubos (1931) and by immunisation with type-specific capsular polysaccharides, which provided effective protection from pneumococcal infection (MacLeod et al., 1945). There are over 90 capsular serotypes (Henrichssen 1995, Kalin 1998), and the virulence of pneumococci has been found to be dependent on capsular serotype. This has been demonstrated in vivo; with mortality and the duration before fatal illness in mice, shown
to be directly attributed to differences in capsular type (Briles et al., 1992). It has been suggested that the differences between serotypes in virulence, reflects differences in the composition, more than the size of the capsule (Knecht et al., 1970). The mechanism by which virulence is attributed to the capsule is suggested to lie in the anti-phagocytic protection it confers to the bacteria (Lee et al., 1991). Unlike other virulence factors, the polysaccharide capsule has no inflammatory or toxic properties in the host (Tuomanen et al., 1987). It also does not appear to be involved in adherence to respiratory epithelium, as Talbot et al., (1996) found that the presence of a capsule was associated with a reduction in pneumococcal adherence and penetration in an in vitro assay using a respiratory epithelial cell line.

1.3.2.2 Cell wall

The cell wall of the pneumococcus is an important mediator of inflammation in pneumococcal disease. Cell wall alone can mimic clinical features of pneumococcal infection in animal models of meningitis, pneumonia, otitis media (Tuomanen et al., 1985, 1987, Ripley-Petzoldt et al., 1988). It has been found that cell wall components, teichoic and lipoteichoic acids can activate the alternative complement pathway, acute phase protein C reactive protein (CRP), and procoagulant activity on epithelium cell surfaces, induce cytokines and PAF upon binding to cells (epithelial, endothelial and macrophages) and effect the recruitment of leukocytes (Winkelstein and Tomasz 1978, Tomasz and Saukkonen 1989, Tuomanen and Sande 1989, Reisenfeld-Orn et al., 1989, Geelen et al., 1992, 1993, Cabellos et al., 1992, Heumann et al., 1994). Glycopeptides, the major building block of pneumococcal cell wall peptidoglycan can affect the host by increasing blood-brain barrier permeability (Spellerberg et al., 1995).

The cytotoxic effects of cell wall components can occur not just from their release after autolysis but also from the attachment of intact pneumococci (Geelen et al., 1993). These cytotoxic effects, on an endothelial cell monolayer, were observed as a progression, from cell separation through to the destruction of the cells (Geelen et al., 1993). This was found to be attributable to the induction of the cytokines IL-1 and TNF-α (Geelen et al., 1993). Apart from modulation of host immunity and inflammation, cell wall and cell wall associated components have an important role in pneumococcal adherence to host cells (Geelen et al., 1993). Phosphorylcholine in particular has an
important role in binding pneumococci to cellular ligands. As discussed previously in section 1.3.1, cell wall-associated phosphorylcholine attaches to the PAF receptor for mediation of adherence and internalisation into epithelial cells (Cundell et al., 1995a, d).

1.3.2.3 Adhesins and surface proteins

A number of adhesins and surface proteins have been implicated in the attachment of pneumococci and the pathogenesis of pneumococcal disease. One such protein, pneumococcal surface protein A (PspA) has been identified in all pneumococci, although it is antigenically very variable (Crain et al., 1990). The exact function of this protein has not been elucidated but the involvement of PspA in pneumococcal virulence has been shown: PspA negative pneumococci were less virulent than wildtype pneumococci (Briles et al., 1988). This observation was supported further by studies indicating that both passive immunisation with anti-PspA monoclonal immunoglobulin, and vaccination with the N-terminal half of PspA, provided protection against pneumococcal challenge (Briles et al., 1989, Talkington et al., 1991).

Another surface protein identified in the pneumococcus is pneumococcal surface adhesion A (PsaA). PsaA is homologous to cell surface adhesion proteins of other Streptococcus species (Sampson et al., 1994). However, this protein has not been found to function as an adhesin (Talbot et al., 1996). In their study, Talbot et al., (1996) used a pneumococcal strain lacking PsaA in an in vitro assay with a respiratory epithelial cell line. The results indicated that PsaA was not involved in adherence to or penetration of the respiratory epithelial cell line (Talbot et al., 1996). However, in contradiction to these results, a report by Berry and Paton (1996) indicated a role of PsaA in pneumococcal adhesion by the reduction of adherence to type II pneumocytes of PsaA negative pneumococci.

Further investigation of PsaA has revealed that the gene for this protein is part of the Psa locus, which encodes a manganese transporter (ABC-type Mn permease complex) (Dintilhac et al., 1997). The hypothesis of the function of this complex is that it indirectly affects pneumococcal adhesion, lysis, transformation and virulence, as part of a signalling pathway (Novak et al., 1998). However, there are conflicting data on some of the pleiotropic effects of experimentally mutated psa genes, such as the effects on autolysis and penicillin tolerance (Claverys et al., 1999). The role of this protein in
the pathogenesis of pneumococcal disease has not been clarified. However, some studies have indicated the potential importance of PsaA. Talkington et al., (1996) showed that following vaccination with PsaA, mice were protected from challenge with pneumococci by intravenous challenge. Another study by Berry and Paton (1996) has shown that PsaA negative pneumococci exhibit a reduction in virulence when inoculated into mice by either the intraperitoneal or intranasal route.

Besides the aforementioned proteins, the surface of the pneumococcus is a complex landscape of molecules, interacting with host ligands, that may give rise to colonisation and invasion. In the host, one of the primary ligands on resting nasopharyngeal epithelial for pneumococcal adherence is the carbohydrate N-acetyl-D-glucosamine 81-3 galactose (Andersson et al., 1983). This has been suggested as the ligand for colonisation of the nasopharynx, however the mechanism of adherence to carbohydrate receptors in the upper respiratory tract is still not fully clear. In a report by Barthelson et al., (1998), sialylated oligosaccharides were shown to interfere with pneumococcal binding to primary bronchial epithelial cells and Chang conjunctival cells (Barthelson et al., 1998).

The ligands identified as important in resting cells in the lower respiratory tract include N-acetyl-D-galactosamine 81-4 galactose and N-acetyl-D-galactosamine 81-3 galactose (present on vascular endothelial cells and type II pneumocytes) (Krivan et al., 1988, Cundell and Tuomanen 1994). As discussed in section 1.3.1 the PAF receptor is an important ligand in activated cells for adherence and transmigration of virulent pneumococci. With activated cells, the sugars N-acetylgalactosamine or lacto-N-neotetraose, and PAF receptor antagonists have been found to inhibit adherence of pneumococci (Cundell et al., 1995d, Tuomanen and Masure 1997). Cundell et al., (1995d) showed that phosphorylcholine a component of pneumococcal cell wall acts as an adhesive ligand by attaching to the PAF receptor to mediate adherence of pneumococci to activated cells. Therefore, the proposed mechanisms of pneumococcal adherence to the PAF receptor are via sharing the site to which PAF itself binds or via binding to a glycosylated site on the receptor or a co-receptor bound to the PAF receptor (Tuomanen and Masure 1997).
1.3.2.4 Pneumolysin

Pneumolysin is a cytosolic protein released when pneumococci autolyse. The function of this protein outside the bacteria is that of a membrane-inserting toxin. Pneumolysin belongs to a group of pore forming toxins, known as thiol-activated cytolysins, which share similar characteristics (Alouf and Geoffroy 1991). Oligomerisation of monomeric protein units forms pores in cholesterol-containing membranes and dependent on concentration, this can cause lysis of affected cells (Bhakdi and Tranum-Jensen 1988). Although some sequence variation occurs (Mitchell et al., 1990, Lock et al., 1996), the pneumolysin gene remains very conserved between isolates of pneumococci. Pneumolysin is also produced from mostly all clinical isolates (Kanclerski and Möllby 1987). The important of this toxin as a virulence factor has been shown by vaccination experiments. In these experiments, mice that had been immunised with either native or altered versions of pneumolysin made by in vitro mutagenesis, were significantly protected following pneumococcal infection (Paton et al., 1983, Lock et al., 1992, Alexander et al., 1994).

Evidence continues to accrue on the effect of pneumolysin on host cells and in the virulence of the pneumococcus. The toxin has been found to be directly injurious to nasal epithelia and tracheobronchial ciliated epithelia (Steinfort et al., 1989, Feldman et al., 1990). At lower, sub-lytic concentrations, a depression/stasis of ciliary beat frequency was found to occur in ciliated respiratory epithelium (Feldman et al., 1990). This effect may provide an important mechanism to over-ride mucociliary clearance in the host, for the purposes of pneumococcal colonisation and invasion.

In an organ culture of respiratory mucosa infected with isogenic pneumococci, deficient and sufficient in pneumolysin, a study by Rayner et al., (1995) showed that the toxin is involved in the separation of epithelial tight junctions. Pneumococci were observed to adhere in the tight junctions and thus may invade the host by this toxin dependant process (Rayner et al., 1995). However, the role of pneumolysin in this process has not been fully defined, whereas PAF receptor mediated invasion, as discussed in section 1.3.1 has been clearly demonstrated. Lower down the respiratory tract in the host, the toxin also has a cytolytic effect on pulmonary alveolar epithelia and pulmonary endothelia (Rubins et al., 1992, 1993). By injuring these cells, this allows
haemorrhaging and a permeation of serum into the alveoli, and presumably promotes growth and spread of pneumococci (Rubins et al., 1993). In addition, following membrane damage by pneumolysin, phospholipase A is activated in pulmonary artery endothelial cells (Rubins et al., 1994). This enzyme produces inflammatory lipid mediators, thus promoting further cellular damage and the induction of inflammatory responses (Rubins et al., 1994). The effects of pneumolysin in the lungs as a whole, has been demonstrated; Feldman et al., (1991) showed that administration of the toxin into the lungs of rats generated pathological lesions comparable to that of pneumococcal pneumonia.

The toxicity of pneumolysin to cells is not confined to the respiratory tract. Cytotoxic effects of this toxin on guinea pig cochlea have been demonstrated, indicating that deafness following pneumococcal otitis media and meningitis may be contributed, in part, by pneumolysin (Comis et al., 1993). Subsequent study has defined this damage by pneumolysin as due to stimulation of production of nitric oxide (Amaee et al., 1995).

Another important effect of this toxin on host cells is the interaction with cellular and humoral immunity. Pneumolysin directly affects leukocytes at sub-lytic concentrations of toxin. Anti-microbial systems are affected in phagocytes, with respiratory burst and chemotaxis decreased in toxin treated neutrophils (Paton and Ferrante 1983). Pneumolysin was also found to affect the function of monocytes with a depression of respiratory burst and lysosomal enzyme release (Nandoskar et al., 1986). In addition to interfering with phagocytes, pneumolysin has been found to be inhibitory to lymphocytes with regard to proliferative responses to mitogens, immunoglobulin and lymphokine production (Ferrante et al., 1984). This pneumococcal toxin not only affects the function of leukocytes but also enhances inflammation via the stimulation of the production of pro-inflammatory cytokines. Interleukin-1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) were produced from monocytes following treatment with low concentrations of pneumolysin (Houldsworth et al., 1994), indicating that pneumolysin has a direct role in cytokine-mediated inflammation. In addition, in other studies it has been shown that pneumolysin can stimulate macrophages to produce nitric oxide and mediators such as interleukin-6, cyclooxygenase-2 (COX-2) as well as TNF-α (Braun et al., 1999).
Studies have shown that pneumolysin can activate the classical pathway of complement activation (Paton et al., 1984, Mitchell et al., 1991). This pneumococcal protein has been shown to have regions of amino acid sequence homology to C reactive protein (CRP) (Mitchell et al., 1991) an acute phase protein shown to bind to pneumococcal C-polysaccharide and to activate the classical complement pathway (Kaplan and Volanakis 1974). However, unlike CRP, pneumolysin requires the presence of immunoglobulin G (IgG) for complement activation to occur (Mitchell et al., 1991). An Fc-binding site in the pneumolysin protein binds to IgG and this antibody-toxin complex initiates the classical complement pathway cascade (Mitchell et al., 1991). Evidence for the role of complement activation in the pathogenesis of pneumococcal disease, comes from experiments using a mutated form of pneumolysin without complement activating potential, achieved by in vitro mutagenesis (Feldman et al., 1991). Feldman et al. (1991) showed that inflammation caused by this complement activating deficient pneumolysin was observed to be less compared to that induced by wild-type toxin, when administered into the lungs of rats.

The use of pneumococcal strains, in which the gene for pneumolysin has been mutated or knocked out, has extended knowledge of the importance of pneumolysin as a virulence factor. Canvin et al., (1995) reported that a pneumolysin negative type 2 pneumococcal strain (PLN-A) caused a less severe lung inflammation, had slower growth in the lungs and a delay in bacteraemia, compared to that of wild-type pneumococci in a model of bronchopneumonia. Infection with PLN-A was also associated with less virulence as compared to wild-type pneumococci after intranasal, intraperitoneal and intravenous administration of inoculum (Berry et al., 1989a, Berry et al., 1992, Benton et al., 1995). Other experiments used a model of pneumococcal pneumonia following endotracheal administration, to compare pneumolysin-deficient and sufficient-strains (Rubins et al., 1995). These experiments showed both the cytotoxicity and complement activating ability of the toxin independently play a role on the intraalveolar replication of pneumococci, and the invasion and dissemination of bacteria into lung tissue and blood (Rubins et al., 1995).

The route and the site of infection are an important factors in the role of pneumolysin in the pathogenesis of pneumococcal disease. Studies have suggested that the roles for cytolytic activity and complement binding differed in the route of infection.
(Berry et al., 1995, Rubins et al., 1996). Friedman et al., (1995) have also shown in a model of meningitis in rabbits, that pneumolysin does not contribute to host inflammation at this site.

1.3.2.5 **Autolysin**

*N*-acetylmuramyl- L-alanine amidase (Lyt A) is an autolysin found on the pneumococcal cell envelope (Diaz et al., 1989). Here LytA is bound to choline moieties on lipoteichoic acid, which is important in the regulation of the autolysin (Briese and Hakenbeck 1985). Under normal conditions, the enzyme is inactive but during stationary phase when nutrients are low, daughter cell separation or after binding of penicillin, Lyt A is activated (Tomasz 1979, Horne et al., 1977, Ronda et al., 1987). Activated Lyt A degrades cell wall by catabolism of the amide bonds in peptidoglycan (Dowson et al., 1997).

Studies showed that vaccination of mice with autolysin provided partial protection against subsequent pneumococcal challenge (Lock et al., 1992, Berry et al., 1989b). In their experiments, Lock et al., (1992) ascribed protection to the role of autolysin mediating the release of pneumolysin. In accordance with these results, pneumococci without autolysin were also shown to have reduced virulence in mice (Berry et al., 1989b). In time course experiments, Canvin et al., (1995) found that mice did not become bacteraemic after intranasal administration of autolysin-negative mutant pneumococci, and were shown to clear bacteria from the lungs, in contrast to infection with wildtype pneumococci. Furthermore, no inflammation was observed in autolysin-negative mutant-infected lungs, in contrast with the inflammation seen with wildtype pneumococci (Canvin et al., 1995). It has been suggested that the role in virulence of autolysin is the release of pneumolysin, hydrolytic enzymes and inflammatory cell wall components (Paton et al., 1993).

1.3.2.6 **IgA1 Protease**

Pneumococci have been shown to possess the enzyme Immunoglobulin A1 protease (Male 1979). This enzyme is proposed to be important in the virulence of IgA1 protease secreting bacteria, by cleavage of mucosal IgA1 (Plaut et al., 1974). Neisseria meningitidis, Haemophilus influenzae and the pneumococcus have this enzyme, and the
suggested involvement of IgA1 protease in pneumococcal disease comes from the fact that related non-pathogenic bacteria, do not produce this enzyme (Mitchell et al., 1997). However, as discussed in section 1.2.1.2, patients who have an IgA deficiency are not found to be at a higher risk from infection with S. pneumoniae (Ammann and Hong 1971).

The role of IgA1 protease in pneumococcal disease has not been fully characterised, and because of its species specificity, current animal models of infection are inadequate (Mitchell et al., 1997, Paton et al., 1993). To demonstrate the role of IgA protease in pneumococcal infection, investigators would have to use transgenic animals possessing human IgA1 (Paton et al., 1993).

1.3.2.7 Hyaluronidase

The hydrolytic enzyme hyaluronidase is produced by the pneumococcus (Humphrey 1944), and has been proposed to play a part in pneumococcal virulence. The mode of action of this enzyme is thought to be the hydrolysis of hyaluronic acid in connective tissue allowing the spread and invasion of pneumococci in the host (Berry et al., 1994, Mitchell et al., 1997). However, these reports do not mention the role of the PAF receptor and the use of other virulence factors such as pneumolysin, which must be borne in mind when discussing the spread and/or invasion of pneumococci in the host.

Support for the role of this enzyme in pneumococcal disease is limited. In an unpublished observation, mice that were intranasally infected with a mutant pneumococcus deficient in hyaluronidase survived longer than after infection with wildtype pneumococci (T. Mitchell, University of Glasgow, UK, personal communication). The growth of these mutant organisms in lungs and blood was also found to be less than wildtype pneumococci (T. Mitchell, University of Glasgow, UK, personal communication). In a study of invasive strains causing meningitis, Kostyukova et al., (1995) demonstrated that the majority of meningitis isolates produced hyaluronidase compared with carriage strains; these strains were capable of infecting the brain after intranasal introduction into mice whereas infection with strains deficient in this enzyme, did not infect the brain.
1.3.2.8 Neuraminidase

Two studies have indicated that the enzyme neuraminidase is produced by all pneumococcal clinical isolates (Kelly et al., 1967, O'Toole et al., 1971). This enzyme catabolises the cleavage of terminal sialic acid residues from mucin, glycoproteins, glycolipids and gangliosides (Kelly et al., 1967, Scanlon et al., 1989) and has lead to the proposition that this enzyme could expose cell surface receptors and could help to penetrate the mucin layer to allow colonisation in the lungs (Andersson et al., 1983, Scanlon et al., 1989). Neuraminidase may also contribute directly to lesions in the host as circumstantial evidence has shown that the prognosis in pneumococcal meningitis is correlated with the levels of N-acetylneuraminic acid in the cerebrospinal fluid (O'Toole et al., 1971). However, the detection of the activity of pneumococcal neuraminidase may just be an indicator of pneumococcal numbers. Nevertheless, neuraminidase is further supported as a pneumococcal virulence factor by protection studies. Lock et al., (1988) found that mice were partially protected from invasive pneumococcal infection following vaccination with formaldehyde-treated neuraminidase. The toxicity of the native enzyme to animals also provided evidence for neuraminidase as a virulence factor in the pathogenesis of the pneumococcus (Lock et al., 1988).

Two genetically unrelated neuraminidases have been found, to date, to be produced by the pneumococcus (Cámara et al., 1991). These are designated NanA (Cámara et al., 1991) and NanB (Berry et al., 1988, Berry et al., 1996). Most studies have centred upon NanA, which is found to be associated on the cell surface of pneumococci (Cámara et al., 1994). In vivo experiments, with a mouse model of pneumonia have shown an attenuated virulence in pneumococci with an insertion-duplication mutation of nanA and provided evidence that this neuraminidase is important in the growth and survival of pneumococci in the lung (T. Mitchell, University of Glasgow, UK, personal communication). In further studies, vaccinating mice with genetically altered versions of NanA have shown a partial protection against intranasal challenge with virulent pneumococci (S. Soma, University of Leicester, UK, personal communication). Although, most studies to date have been centred on NanA, studies with NanB are in progress.

However, like pneumolysin, neuraminidase may only be an important virulence
factor at particular host sites. Winter et al. (1997) found that neuraminidase did not play a pathogenic role in causing deafness and cochlear damage, in a model of meningitis, as compared to the significant importance of pneumolysin in this model. Support for this conclusion comes from the fact that toxicity after administration of the enzyme alone, is affected by the site of introduction. Kelly and Greiff (1970) found that intracerebral administration of neuraminidase in mice caused toxicity, whereas by the intrathecal route O'Toole and Stahl (1975) found that neuraminidase induced no effect in the dog. However, these differences could be a reflection of animal variation.

1.3.2.9 Other factors which play a role in virulence

Virulence and host-pathogen interactions are complex and a number of additional factors may play a role in pneumococcal disease. Ongoing studies will uncover as yet unknown factors and further enhance knowledge of existing factors. Some examples are given below.

Further work in the field of pneumolysin research identified another toxin produced by the pneumococcus responsible for beta haemolysis on blood agar plates incubated anaerobically (Canvin et al., 1997). The part this haemolysin plays in virulence of the pneumococci is unknown at present.

Hydrogen peroxide generated from pneumococci has been found to be a source of pneumococcal toxicity to alveolar epithelia, additional to that of pneumolysin (Duane et al., 1993). Also, in addition to pneumolysin, the production of hydrogen peroxide has also been shown to be responsible for the reduction of ciliary beating on rat brain ependyma by pneumococci (Hirst et al., 2000).

Recently a study has shown the importance of superoxide dismutase (SOD) in pneumococcal virulence (Yesilkaya et al., 2000). A SOD inactivated mutant was found to have a lower virulence compared to wild-type pneumococci when mice were intranasally infected (Yesilkaya et al., 2000). Growth of this SOD mutant in the lungs of infected mice, as well as the appearance of these pneumococci in the blood, was found to be delayed compared to that of wild-type infection (Yesilkaya et al., 2000). However, after intravenous administration of these mutant pneumococci virulence was matched with that of wild-type bacteria (Yesilkaya et al., 2000).
In a study by Spellerberg et al., (1996), pyruvate oxidase was found to be a virulence factor in the pneumococcus. In models of nasopharyngeal colonization, pneumonia and sepsis a reduction in virulence was associated with infection of a pyruvate oxidase-deficient strain of pneumococci (Spellerberg et al., 1996). The conjecture formed for the reduction in virulence is that the pyruvate oxidase-deficient strain with a mutation in the spxB locus, down-regulates multiple adhesive properties of pneumococcus (Spellerberg et al., 1996). Interestingly, these mutants produce much less hydrogen peroxide than wild-type pneumococci under aerobic conditions, thus Spellerberg et al., (1996) have suggested that this maybe another mechanism that could account for the observed reduction in virulence.

Methods such as in vivo expression technology and signature-tagged mutagenesis have identified previously confirmed virulence factors and a number of new genes in the pathogenesis of pneumococcal disease (Polissi et al., 1998). New choline binding proteins, other than autolysin and PspA have been discovered (Rosenow et al., 1997, Hammerschmidt et al., 1997, Polissi et al., 1998, Paton 1998). These less defined choline-binding proteins may provide addition colonisation and virulence factors (Rosenow et al., 1997, Hammerschmidt et al., 1997, Polissi et al., 1998, Paton 1998). In addition to these technologies, analysis of the genome sequence of the pneumococcus (www.tigr.org), and DNA microarray or protein analysis will reveal new genes.

As this section has described, further research will continue to expand the number of virulence factors involved in pneumococcal disease. These may involve a number of interactions that effect or regulate previously characterised virulence factors. Further work may even uncover presently unknown novel interactions with the host in the pathogenesis of pneumococcal disease.

1.4 Genetic resistance and susceptibility to microbial disease

In the study of disease caused by the pneumococcus and that of a number of other pathogens, primarily work has examined virulence factors and the pathogenesis of the organism. The effect of the genetic background of the host has been largely unexplored. Although there is an understanding of basic immunology in a number of diseases, genetic analysis of the host could provide a number of novel elements of the innate and
acquired immunity. The benefits of exploring this would be to study the genes involved and their products for possible diagnostic, therapeutic and vaccine development; thus susceptible individuals could be identified as being at risk, and possible approaches to bypass genetic defects could be investigated (Malo and Skamene 1994).

However, the dissection of susceptibility to infectious disease has a number of problems. Firstly, a number of traits are complex and may not simply be the result of the Mendelian inheritance of a single gene (monogenic). Departure from simple Mendelian inheritance encompasses genetic traits involving a number of genes (polygenic), although this does not exclusively define some complex traits (Lander and Schork 1994). A number of external factors may come into play, such as environment, sex, age and other genes (Lander and Schork 1994). These factors can affect the genotype, such that the trait even though inherited is not expressed; this is known as incomplete penetrance (Lander and Schork 1994). The cause of the disease maybe due to random or environmental factors other than the inheritance of the disease-causing trait and therefore may complicate the genetic association with disease; this is known as phenocopy (Lander and Schork 1994). A further complication can be that of genetic heterogeneity, whereby a mutation occurring in a number of different genes can result in the same disease phenotype (Lander and Schork 1994). This is illustrated in the degenerative disease of the retina, retinitis pigmentosa, where if any of 14 different loci have a mutation, disease results (Bleeker-Wagemakers et al., 1992, Kumar-Singh et al., 1993). Another difficulty in examining genetic traits, both monogenic and polygenic, is a problem of high frequency of the disease-causing allele in the population interfering with normal linkage analysis of the trait (Lander and Schork 1994).

Although these problems hamper genetic analysis, in a number of infectious diseases, studies in both humans and mice have suggested that genetic factors strongly influence the host's response to infection. In the next section, the methodology behind the genetic analysis of infectious disease is discussed.

1.4.1 Comparative genetic analysis of infectious disease

Together with the underlying complicating genetic factors, study of the host can be problematic. The human host experimentally is difficult to both control and collect sufficiently large groups for study (Qureshi et al., 1999). The long generation times and
the large genome, together with the lack of the ability of experimental manipulation, add to these difficulties (Qureshi et al., 1999). Animal models (in particular mouse models) provide an important tool for dissecting genetic associations in disease and studying the function of these genetic elements. In contrast to humans, advantages of using mice include ease of controlled breeding, availability of inbred strains, high density of genetic markers, generation times and litter size (Malo and Skamene 1994). By comparative mapping, a resistant locus or loci identified in the mouse genome can be found in the human genome, due to the genetic homology between mouse and human conserved linkage groups (DeBry and Seldin 1996, Qureshi et al., 1999, Mcleod et al., 1995).

Identified gene(s) can then be used in mutation analysis in the mouse to further elucidate their functionality and effect in the host (Qureshi et al., 1999). Together with the development of experimental models of the infectious disease, the host response during the pathogenesis of the disease under investigation can be studied further. This methodology can be adopted in a reciprocal fashion so when disease loci are identified in humans, animal models can be created to study the disease further (Primose 1995). Additionally, a number of mouse mutants have been identified which mimic disease in humans, where as yet no gene(s) have been cloned (Primrose 1995). In this case or when a mouse model for a disease is unavailable, mapping the gene(s) involved is easier to do in the mouse and then, by using comparative mapping, to predict its location in humans (Primrose 1995).

1.4.1.1 Genetic mapping of resistance or susceptibility traits

The approach to find genetic associations using animal models involves a number of steps (Figure 1.2). Firstly, a describable phenotype must be observed, such as survival time or pathogen density within target organs (Malo and Skamene 1994). Following observations on a number of inbred strains the basis for the genetic susceptibility or resistance to the infectious disease studied can be further analysed. The pattern of distribution of phenotypes among strains, gives an indication of the possible inheritance; continuous variation with susceptible, resistant and intermediate phenotypes is indicative of a complex trait whereas discontinuous variation with clearly susceptible and resistant phenotypes indicates the possibility of a monogenic trait (Malo and Skamene 1994).
To further examine the inheritance involved, 'classical' breeding crosses are performed; familial intercross generations F1 and F2 and backcrosses with parental strains (Festing 1979). Phenotypic data are then compared to genotype data, generated from genetic marker typing. Using DNA from these familial generations and backcross progeny, analysis by genetic marker typing is achieved by amplification, by the polymerase chain reaction (PCR), of polymorphic markers, such as microsatellite regions (simple DNA tandem repeats such as [CA]n), which span the genome (Love et al., 1990).
Figure 1.2 A representation of the approach to identifying genetic loci involved resistance and susceptibility to infectious disease (taken from Malo and Skamene 1994).
Approximately 17,000 markers with intervals of 200Kb have been mapped in the mouse genome, of these markers 5,000 are genes and >10,000 are mostly microsatellite markers (Qureshi et al., 1999). Linkage analysis can then be used to identify chromosomal regions involved in the genetic trait (Qureshi et al., 1999). This process is relatively simple in dissecting monogenic traits, however if the genetic trait is a complex polygenic trait other methods have to be adopted.

Polygenic inheritance can be either a discrete trait (for example either dead/alive or responder/non-responder) or quantitative trait, such as a continuous variable, for instance, blood pressure (Festing 1979, Lander and Schork 1994). The complex inheritance of resistance or susceptibility to infectious disease is more often a quantitative trait. The genetic determinants of this variation are referred to as quantitative trait loci (QTLs) (Primose 1995, Fincham 1994). These QTLs can be located using genetic maps already obtained for the mouse, combined with markers that span the genome. In the mapping of QTLs, the segregation of markers is compared with the quantitative trait examined, with the simplest approach involving statistical comparison of each marker in turn to the quantitative trait, to identify whether there is correlation between them (Primose 1995, Fincham 1994). However, the main approach to investigating QTLs involved in the trait is by the method of interval mapping (Lander and Botstein 1989). Interval mapping (adapted from LOD score analysis in humans (Morton 1955) provides a value of the odds ratio that a QTL will or will not be present along the length of the chromosome examined (Primose 1995). This odds ratio is expressed as the logarithm to the base 10 known as a LOD score (Primose 1995). LOD scores over the threshold value (or significance) set by the size of the genome and marker number indicate the probable existence of a QTL in the region studied (Primrose 1995).

Once QTLs have been located or chromosomal regions found to contain a locus or loci of interest, identification of the gene(s) involved requires further steps. Further resolution using more markers around the chromosomal region of interest can continue, before moving to other methods. A method of isolating the relevant chromosomal region for further investigation is by congenics (Snell 1948). The gene donor strain is serially backcrossed to the gene recipient strain, such that the genome of the resultant progeny is serially diluted of the donor strain genome (Wakeland et al., 1997). The
resultant congenic strain has 99% of its genome derived from the recipient strain, together with the region of interest from the donor strain (Wakeland et al., 1997). Traditionally 12 backcrosses are done with a final F1 intercross stage to select for homozygous alleles; progeny produced from this process are selected for the gene(s) of interest to ensure transfer to subsequent generations (Wakeland et al., 1997). An adaptation of this method is speed congenics (Wakeland et al., 1997). In this process, progeny to be used subsequently as breeding pairs are selected by analysis of polymorphic markers along the genome, for gene(s) or chromosomal region of interest together with the lowest amount of donor genome (Wakeland et al., 1997). This process can cut the number of generations and thus the time taken in producing a congenic strain, resulting in a congenic strain line with less contaminating donor genome than the traditional congenic method (Wakeland et al., 1997).

If gene products, such as proteins, define the phenotype, a panel of genetic probes can be made to identify the coding sequence (Fincham 1994). This candidate gene approach to identification of the gene(s) involved in the examined trait can also involve studying other genes within the chromosomal region of interest, for which both their function and genetic map position are known (Ballabio 1993, Primose 1995). A source for these data comes from genomic sequencing projects or expressed sequence tags/cDNA fingerprints (Primose 1995). This analysis would uncover whether the genetic trait is associated or linked with the candidate gene(s) studied. Although the candidate gene approach is useful, the method of positional cloning provides the main way to isolate unknown gene(s) (Fincham 1994). Positional cloning involves finding the gene(s) of interest by direct cloning of the DNA within the chromosomal region of interest, which has been demarcated previously by genetic markers (Fincham 1994, Primose 1995). Cloning can be achieved by chromosomal walking (Bender et al., 1983). From the starting marker, a clone of known sequence is used as a probe to find the next overlapping clone in a chromosomal fragment library; this process is continued until a number of overlapping clones stretch across the region of interest (Bender et al., 1983). Isolation of the gene(s) is then found by using a number of approaches such as sequencing the region or analysing sequence data already obtained for that region (Primose 1995); other methods include exon amplification (Buckler et al., 1991) and cDNA selection (Lovett et al., 1991, Parimoo et al., 1991).
A problem in chromosomal walking is that repetitive sequences in the chromosome can inhibit the procedure but this can be overcome by the method of chromosomal jumping (Poustka et al., 1987, Rommens et al., 1989). Chromosomal jumping can also reduce the distance between the nearest marker and the gene(s) of interest, if the distance for chromosomal walking is too large (Primose 1995). In brief, this involves taking large genomic fragments and circularising them, where the ends of these circularised fragments meet, these sites are cloned, thus bringing distantly separated sequences together (Primose 1995). A number of these clones constitute a 'jumping library' (Primose 1995). One such strategy for this process uses the restriction enzyme Not I, which has infrequent sites in mammalian DNA and is illustrated in Figure 1.3.

Figure 1.3 An illustration of the construction of a jumping library (modified from Poustka et al., 1987).
Clones of these fragments are then used as subsequent probes bypassing (jumping) the repetitive region to find the next overlapping clone. Once a putative sequence has been identified, gene addition (knock-in) or knock-out studies with transgenic mice can reveal whether the gene(s) identified effect the phenotype associated with the disease (Lander and Schork 1994).

To examine the corresponding human disease association, comparative mapping can lead to location of the homologous locus (or loci) in the human genome. At least 1,416 loci have been mapped between humans and mice, relating to 181 conserved linkage groups contributing to approximately 90% of the mouse genome (DeBry and Seldin 1996). Comparative genome analysis between mice and humans has already uncovered novel genes hypothesised to be involved in innate immunity, some of which are discussed below.

Nrampl

Natural resistance to infection with intracellular parasites *Leishmania donovani*, *Mycobacterium* sp. and *Salmonella typhimurium* has been found to be controlled by a dominant genetic locus on mouse chromosome 1 referred to as Lsh, Bcg or Ity (Bradley 1977, Gros et al., 1981, Skamene et al., 1982, Plant et al., 1979, 1982). Nrampl is the candidate gene for this trait and it encodes natural resistance macrophage protein 1 (Nrampl) which affects the macrophage’s ability to destroy ingested intracellular parasites early in the infectious process (Vidal et al., 1993). In this early non-immune phase, rapid growth of bacterial pathogens occurs in the organs of the reticuloendothelial system of susceptible mice (Gros et al., 1981).

Nrampl is located on the membrane of late endosome/lysosome compartments in resting macrophages and is recruited to the phagosomal membrane during maturation of the phagolysosome (Gruenhied et al., 1997). Nrampl has structural similarity to ion channels and transporter proteins (Gruenhied et al., 1997), and has been shown to be involved as a divalent cation transporter affecting the production of antimicrobial hydroxyl radicals, endosomal metalloprotease activity and phagolysosome fusion (Blackwell et al., 2000).

In humans, Nrampl has been found to map to the telomeric end of chromosome
2q35 (Schurr et al., 1990). The human orthologue of the gene represents a conservation of 85% identity and 92% similarity as compared to the gene in the mouse (Cellier et al., 1994). However, human polymorphisms of *Nramp1* lack the point mutation found in the *Nramp1* gene of susceptible mouse strains (Liu et al., 1995, Blackwell et al., 1995). Disease associations have been reported in humans, such as the work of Bellamy et al., (1998) showing susceptibility to pulmonary tuberculosis in West Africans linked with variants of *Nramp1*. However, in contrast to the striking susceptibility shown in the mouse, in humans, reports of association with susceptibility and linkage to *Nramp1* are confined to certain ethnic population groups (Shaw et al., 1993, Levee et al., 1994). *Nramp1* may confer an advantage within these ethnic population groups to an as yet undefined disease or it may have another functional role.

**Bg (biege mutation)**

The biege (*bg*) mutation in mice has been found to mimic the disease of Chediak-Higashi syndrome (Kelley 1957). The phenotypic effects of this mutation include a lack of cathepsin G and elastase in neutrophil granules, impairment of neutrophil functions of chemotaxis and intracellular killing (Gallin et al., 1974, Ganz et al., 1988), defective natural killer (NK) cell function and abnormal cytotoxic T-cell function (Saxena et al., 1982, Haliotis et al., 1980), pigment dilution, large melanin granules, large lysosomal granules (Lutzner et al., 1967) and an increase susceptibility to infections (Shellam et al., 1981, Kirkpatrick and Farrell 1982, Elin et al., 1974). This increase in susceptibility to infections results in the main characteristic of the disease of high patient mortality during the first two decades of life (Qureshi et al., 1999). The beige mutation in mice causes an increase in the susceptibility to infection by *C. neoformans*, *C. albicans*, *E. coli*, *K. pneumoniae*, *Staph. aureus*, *S. pneumoniae* and cytomegalovirus (Shellam et al., 1981, Kirkpatrick and Farrell 1982, Elin et al., 1974).

In mice, the *bg* locus was located on chromosome 13 (Kingsmore et al., 1996). Comparative mapping indicated that the human orthologue of *bg* was located on the distal end of chromosome 1q (Qureshi et al., 1999). The mouse and human candidate *bg* gene, *Lst* (referring to lysosomal trafficking regulator) (Perou et al., 1996, Barbosa et al., 1996) has been found to have significant nucleotide sequence homology (77.2%) and amino acid homology (87.9%) (Qureshi et al., 1999). *Lst* has been found to be
homologous to Vps15, a yeast serine/threonine kinase protein kinase thought to be involved in the regulation of intracellular protein trafficking, via a membrane-associated signal transduction complex (Klionsky and Emr 1990). Although a full account of the function of the Chediak-Higashi gene, however, as yet has not been reported (Qureshi et al., 1999).

**xid/btk**

X-Linked Agammaglobulinemia (XLA), a recessive humoral immunodeficiency disease in humans and the mutation X-linked immunodeficiency (xid) in the mouse strain CBA/N were described separately. In humans, XLA is a deficiency of two stages of B cell maturation (Ochs and Edvard Smith 1996), resulting in a decrease in the numbers of these cells (both peripherally and within the lymph nodes), together with low levels of the three main immunoglobulin isotypes (Qureshi et al., 1999). The consequence for the human host is an increase in the susceptibility to infectious disease with pathogens such as *S. pneumoniae*, *H. influenzae*, *Staph. aureus*, *Pseudomonas* sp., *Salmonella* sp., *Campylobacter* sp., *Mycoplasma* sp., *Chlamydia* sp. and enteroviruses (Qureshi et al., 1999). X-linked inheritance of the xid mutation in mice is manifested by the inability to mount a humoral response to a group of thymus-independent (T1-2) carbohydrate antigens (Amsbaugh et al., 1972, Scher 1982, Slack et al., 1980, Briles et al., 1980, 1982). In these mice, the numbers of peripheral B-cells are decreased, coupled with an abnormal surface marker phenotype (Qureshi et al., 1999). Xid mice also have an impairment of the activation of B-cell proliferation via surface immunoglobulin M or surface immunoglobulin cross linking, together with the loss of response to mitogens such as lipopolysaccharide, IL-5 and IL-10, CD38 receptors and CD40 ligands (Qureshi et al., 1999). Resulting immunoglobulin responses have a reduction of serum IgM and IgG3 (Qureshi et al., 1999). Like XLA, the xid mutation confers susceptibility to infectious pathogens, such as *S. pneumoniae* (Briles et al., 1980).

A candidate gene for causing XLA in humans is the gene *btk* (Bruton agammaglobulinemia tyrosine kinase) (Vetrie et al., 1993, Tsukada et al., 1993). Btk is an src-like cytoplasmic tyrosine kinase involved in haematopoietic cell signal transduction (Vetrie et al., 1993). Comparative mapping and sequence analysis between human and mouse, showed that the *btk* gene in humans was linked with a locus on the
distal X chromosome of the mouse, corresponding to \textit{xid} (Rawlings \textit{et al.}, 1993).

Severity of disease is found to be greater in humans than in mice. It has been postulated that this is due to different point mutations and deletions in the human \textit{btk} gene than the single point mutation in the mouse, resulting in a greater effect on the expression of \textit{btk}, and subsequent protein production and kinase activity (Vetrie \textit{et al.}, 1993, Tsukada \textit{et al.}, 1993). In the mouse, an additional mechanism interacts with the early the B cell maturation process to compensate for the severe early B-lymphocyte developmental arrest, which occurs in humans (Qureshi \textit{et al.}, 1999). The full function of the \textit{btk} gene, particularly its role in normal B-cells, is still unclear at present (Qureshi \textit{et al.}, 1999).

1.4.2 Genetic resistance and susceptibility to \textit{S. pneumoniae}

Webster (1933a), using the pathogen \textit{S. enteritidis}, developed resistant and susceptible lines of mice by selective breeding. To investigate whether susceptibility or resistant of these mice occurred with other pathogens, Webster included the pneumococcus, as well as other bacterial and viral pathogens, in her experiments (Webster 1933b). In her study, mice susceptible to \textit{S. enteritidis} were also found to be susceptible to two type 3 pneumococcal isolates from cases of human pneumonia, another type 3 pneumococcal isolate from a healthy carrier and a type 8 isolate from another case of human pneumonia. However, this observation was only seen when mice were challenged by intranasal administration. After either intraperitoneal or intravenous infection, no differences in susceptible and resistant lines of mice were observed, even when the dose was modified (Webster 1933b). This variance in the virulence depending on the route of infection was observed by Webster and Clow (1933). For example, a pneumococcal strain showing virulence when given intraperitoneally was avirulent intranasally. Further to this, Rake (1936) found that lesions in the lungs during pneumococcal induced pneumonia varied with the mouse strain used. Rake (1936) when analysing the pathology post-infection, also observed that lesions varied when different pneumococcal isolates were administered into the lungs of a single murine strain.

The concept that the genetic makeup of the pathogen as well as the host genetic background is important in the susceptibility or resistance to pneumococcal infection
was further addressed (Briles et al., 1983). Briles et al., (1983) reported that when two pneumococcal strains (a type 1 and a type 3 isolate) were given intravenously to inbred mice, susceptibilities to infection varied not only with the mouse strain but also according to the type of pneumococcus administered.

Investigators revisited the earlier report of a link between susceptibility to *S. pneumoniae* and to *S. enteritidis* (Webster 1933b). Murine susceptibility to *Salmonella* sp. was found to be associated with the *Ity* locus (Plant et al., 1979, 1982), and as discussed previously, this is now designated *Nramp1* (Vidal et al., 1993). Upon investigation, mice with the susceptible genotype of *Nramp1* were found to be no different in the susceptibility or resistance to *S. pneumoniae* administered intravenously, than mice with a resistant *Nramp1* genotype (Briles et al., 1986). Thus, the genes for the resistance and susceptibility to *Salmonella* sp. appear unrelated to pneumococcal infection in mice.

Briles et al., (1986) attempted to further identify associations with individual genes involved in murine resistance to pneumococcal infection. Recombinant inbred mice were generated by crossing resistant C57BL/6J mice with susceptible C3H/HeJ mice (designated B x H), and resistant C57L/J mice with susceptible AKR/J mice (designated AK x L) (Briles et al., 1986). These mice were then challenged with a type 1 strain and a type 3 strain of *S. pneumoniae* (Briles et al., 1986). Following analysis of the phenotypic traits of these mice and the known distribution of parental alleles, resistance to *S. pneumoniae* was statistically linked to the *Akp-1* locus in AK x L mice infected with the type 3 strain WU2 (Briles et al., 1986). *Akp-1* is found on murine chromosome 1 (Wilcox et al., 1978) and encodes alkaline phosphatase 1. However, no other study has found or confirmed this association.

Susceptibility has also been connected with the *xid* locus described in the previous section, 1.4.1.1. The *xid* locus found in the mouse strain CBA/N gives rise to a defect that makes these mice incapable of producing anti-polysaccharide antibodies and therefore being highly susceptible to pneumococcal infection (Briles et al., 1980). Susceptibility in these mice is a consequence of the lack of antibodies generated against phosphorylcholine (Briles et al., 1981a), which is found in the cell wall of pneumococci and the F antigen (Tomasz 1967, Brundish and Baddiley 1968, Briles and Tomasz...
1973). These anti-phosphorylcholine antibodies are presumably generated in response to normal flora colonisation (Briles et al., 1986). In vivo infection models have shown phosphorylcholine antibodies to be protective against invasive pneumococcal disease (Briles et al., 1981a, Szu et al., 1983, McDaniel et al., 1984). Intravenous infection studies showed that anti-phosphorylcholine antibodies were protective against a number of pneumococcal samples, although there were exceptions such as isolates of type 3, 4, 5 and 6A serotype (Yother et al., 1982, Szu et al., 1983, Briles et al., 1986 unpublished). Other studies showed that when phosphorylcholine was conjugated to a protein carrier (keyhole limpet haemocyanin), it stimulated an antibody response similar to an anti-capsular antibody response (Wallick et al., 1983). Another report indicated that encapsulated pneumococci bound phosphorylcholine antibodies, therefore the presence of capsule does not completely or effectively, inhibit or shield antibody binding to pneumococcal cell wall phosphorylcholine residues (Yother et al., 1982).

The main isotypes of immunoglobulin found to be protective were IgM and IgG, as IgA was shown to be not protective (Briles et al., 1981b). It was concluded that this was likely due to the fact that IgA does not activate complement (Szu et al., 1983). Evidence that the protective affect of these phosphorylcholine antibodies involves the interaction of complement comes from a study showing that C3 deficiency removes the protective effect of these antibodies (Briles et al., 1985).

Reports that antibodies to phosphorylcholine exist, and are found to be effective in mouse models, indicate that they may play an important role as part of a pre-existing immune response to the pneumococcus. However, a number of authors have indicated that the general genetic background of CBA mice is susceptible to pneumococcal infection and that this is not just accounted for by the xid locus but the abrogating effect of other unknown genes (Briles et al., 1986, 1992, Takashima et al., 1996). For instance, CBA/J mice without the xid genotype are as susceptible to pneumococcal infection as the CBA/N strain carrying the trait (Briles et al., 1986). Furthermore, in pneumococcal challenges with offspring generated from intercrosses between CBA/N mice and BALB/c or DBA/2 mice, the dominance of an undefined resistance trait from the parents BALB/c and DBA/2 masked any susceptibility due to the xid locus (Briles et al., 1986). In another interesting observation, the effect of the pneumococcal toxin pneumolysin during bacteraemia has been shown to be dependent on the genetic background of the mice used, outside the background xid (Benton et al., 1997a). In their
study, Benton et al., (1997a) found that growth of wildtype pneumococci and pneumococci deficient in pneumolysin were comparable in CBA/N, C3H/HeJ and C3H/HeOuJ mice. However, in 129/SvJ and C57BL/6J mice, the growth patterns of both pneumococcal strains differed in comparison to CBA/N, C3H/HeJ and C3H/HeOuJ mice.

1.5 Aims of the project

1.5.1 General aims inclusive of collaborations

In this study the susceptibility and resistance to S. pneumoniae was revisited. Both humans and mice were studied in collaboration with AstraZeneca plc in Alderley Edge together with the MRC Mouse Genome Centre in Harwell and the Wellcome Trust Centre for Human Genetics in Oxford. This was for the purposes of finding novel genes for the resistance or susceptibility to invasive infectious diseases, using S. pneumoniae as the model microorganism.

1.5.1.1 Murine studies

Specific aims of this thesis

As discussed in section 1.4, the mouse provides an important model to study and search for genes related to the susceptibility or resistance to disease. In this context, this project used a mouse model of bronchopneumonia and bacteraemia to study the genetic resistance and susceptibility to S. pneumoniae. Phenotype data would be combined with genotype data using a strategy that would allow detailed genetic mapping and subsequently identification of any resistance loci. The first stage was to identify resistant and susceptible mouse strains. A mouse model of pneumococcal pneumonia was used to examine the susceptibility of a number of inbred mouse strains to infection with a type 2 and a type 3 pneumococcus. In previous studies, no attempt had been made to define resistance by means other than survival time. Therefore, to enhance the data collected, further phenotypic observations were taken during examination of the development of invasive pneumococcal disease in these mice.

This thesis reports the identification of murine strains that are resistant and susceptible to invasive pneumococcal disease. Furthermore, in an attempt to identify at
what point in the course of pneumococcal infection the genes involved in susceptibility
or resistance exert their effect, a number of parameters in the pathogenesis of
pneumococcal disease in these mice were analysed. A further objective of this thesis
was the characterisation of F1 and F2 generations of mice, generated by crossing
resistant and susceptible parental inbred strains for further genetic study. To this end, a
number of progeny were bred and further phenotypic study was performed on these
mice using the type 2 pneumococcus. During this process, collection of tissue samples
was achieved for DNA analysis and genome scanning.

**Murine genome scanning**

In tandem with this thesis, and continuing thereafter, the DNA collected from
progeny produced from the mating of resistant and susceptible murine inbred strains
was to be analysed by the MRC Mouse Genome Centre in Harwell. The objective of
this study was the genetic mapping and identification of a locus or loci, which
corresponds to the genetic resistance or susceptibility to *S. pneumoniae* in mice. The
ultimate aim would be to use these data to identify a locus or loci in humans.

To achieve the objective of this study, polymorphic markers were selected to
span the mouse genome in the parental strains (the susceptible and resistance mice).
DNA from F2 generation mice was analysed by amplification of polymorphic markers
by PCR. Products from these reactions were then analysed by gel electrophoresis. To
examine the segregation of markers for possible association of QTLs, Mapmaker – QTL
software package was utilised. The objective of this study has not yet been concluded.
Continuing analysis and mapping to finer resolution, to the point where sequencing and
cloning of the locus or loci is achievable, is in progress.

1.5.1.2 Human studies

Although as discussed in section 1.4, analysis of susceptibility to infectious
disease in humans is more difficult than in mice, a candidate gene approach was
adopted. The objective of this study was to run in tandem with the mouse genome
scanning project, to provide case studies for pneumococcal patients and controls and
generate a possible locus or loci from known candidates. To this end, sequencing and
polymorphism analysis of candidate loci was undertaken with an initial list of target
candidates given in Table 1.2. These candidates were chosen on the basis of known immunological importance. DNA samples were obtained from the blood of patients with pneumococcal disease in the Oxfordshire area, together with control blood samples from plasma donors (S. Roy, Wellcome Trust Centre, Oxford University, UK, personal communication). In some analyses, other population groups from Gambia and Calcutta were also used (S. Roy, Wellcome Trust Centre, Oxford University, UK, personal communication).

<table>
<thead>
<tr>
<th>Candidate loci</th>
<th>Candidate loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannose binding protein</td>
<td>HLA</td>
</tr>
<tr>
<td>Inducible NO synthase</td>
<td>IL-12</td>
</tr>
<tr>
<td>Natural resistance macrophage protein</td>
<td>IL-1 receptor</td>
</tr>
<tr>
<td>Tumour necrosis factor α and β</td>
<td>Fce receptor II</td>
</tr>
<tr>
<td>Fc gamma receptor II</td>
<td>Duffy blood group</td>
</tr>
<tr>
<td>Km allotypes</td>
<td>IgH allotypes</td>
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<tr>
<td>T cell receptor variants</td>
<td>Fc gamma receptor III</td>
</tr>
<tr>
<td>Interferon gamma receptor</td>
<td>Tumour necrosis receptor-p55</td>
</tr>
<tr>
<td>Interleukin-4</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 Initial choices of candidate loci for sequence and polymorphism analysis for association to the susceptibility or resistance to pneumococcal disease in humans (S. Roy, Wellcome Trust Centre, Oxford University, UK, personal communication).
Chapter 2: Materials and Methods

2.1 Materials

Sigma and BDH supplied all chemicals unless otherwise stated. Oxoid supplied all bacteriological growth media unless otherwise stated. Gibco Technologies supplied tissue culture media and solutions unless otherwise stated.

2.2 Mouse Strains

Inbred mice: BALB/c, C57BL/6, DBA/2, AKR, CBA/Ca, C3H/HeN, FVB/n, NIH, SJL CCBA F1, CBAC F1 and outbred MF1 mice were obtained from Harlan Olac. CCBA F2 and CBAC F2 mice were bred in the Biomedical Services Department of Leicester University (section 2.2.1). All mice were kept under standard housing conditions, with free access to food and water. Mice were obtained from Harlan Olac at approximately 7 weeks old and were used between ≥ 9 weeks and ≤ 30 weeks old. All mice were kept a minimum of 2 weeks in Biomedical Services under standard conditions, before experimental use, to acclimatise. Following challenge with virulent pneumococci, infected mice were housed for the duration of the experiment in an isolator (MDH). A selection of mice from the strains used was screened by Dr Janet Alexander (Department of Microbiology and Immunology, Leicester University) to check for pre-existing anti-pneumococcal capsular antibodies. Screening for these antibodies was performed using ELISA. This screening test indicated that this type of antibody was not present before infection in these mice.

2.2.1 Production of CBAC F2 and CCBA F2 mice

F2 mice were bred from a staggered breeding protocol, using a breeding panel of 8 males to 16 females. Briefly, once male and female F1 mice that were obtained from Harlan Olac arrived at Biomedical Services, mice were caged separately and acclimatised; one male caged between two females. Staggered breeding commenced each week with 2 males mating 4 females (1 male to two females in each cage). This method was repeated for 4 weeks to generate 4 mating groups. During this period, each female from each group was observed daily for signs of a vaginal plug to indicate pregnancy. Once confirmed pregnant, females were separated into individual cages. Following birth, progeny were weaned at 20 days and male and female littermates were
then caged separately. 4 groups generated approximately 100 progeny, staggered in age. Subsequent phenotyping challenges could then be easily managed. This breeding protocol was repeated twice for each F1 reciprocal cross (males and females from both CBA/Ca mice and BALB/c mice). Therefore both F1 crosses, designated CBAC F1 and CCBA F1 were used to generate approximately 200 CBAC F2 mice and CCBA F2 mice.

2.3 Pneumococcal Strains

Strains used were D39 (type 2) derived from the National Collection of Type Cultures (N.C.T.C strain No. 7466), and GB05 (type 3), a clinical isolate obtained from the Leicester Royal Infirmary.

2.3.1 Pneumococcal Strain validation

Each strain was streaked to single colonies from strain collection stocks on blood agar base (BAB) with 5% v/v aerated horse blood (TCS Microbiology). Confirmation of optochin (Ethylhydrocupreine hydrochloride) sensitivity was achieved by placing an optochin antibiotic disc (Dibco) on the plates at the first site of the streak. This was done as a test to identify *S. pneumoniae* against any other contaminating alpha-hemolytic streptococci. Plates were then inverted and incubated in a candle jar overnight at 37°C. After incubation, a sweep of colonies were taken from these plates and used to inoculate 10 ml brain heart infusion (BHI) broths. These broths were then incubated overnight statically at 37°C. Viability was checked by performing a colony count on these cultures (section 2.3.3). The quellung reaction (section 2.3.2) was used to verify each strain’s serotype.

Another sweep of colonies from the plate was used to inoculate another 10ml BHI broth. Following overnight static incubation at 37°C, the culture was centrifuged at 18,000g (Heraeus Christ centrifuge) for 15 minutes. The pellet was resuspended in 1ml of BHI broth with 20% v/v irradiated heat-inactivated foetal bovine serum (fresh serum broth). This foetal bovine serum (FBS), which had been filter-sterilised through 0.2μm filters, was stored routinely at -20°C in 5 to 10ml aliquots from the original 500ml batch. Once the cell pellet was resuspended, 200μl of this suspension was added to 3ml of fresh serum broth to give an optical density (OD$_{500nm}$) of approximately 0.7 when
measured in a Ultrospec 3000 spectrophotometer (Pharmacia Biotech). The inoculated serum broth was subsequently left to incubate at 37°C for 5 hours to reach an optical density (OD$_{500nm}$) of approximately 1.6. After incubation 0.5ml aliquots were pipetted into sterile cryotubes (Sarstedt), frozen and stored at -70°C. Following 24 hours at -70°C, one vial from the collection of vials was streaked out to single colonies on BAB with 5% V/V aerated horse blood to check for contamination. Optochin sensitivity, viability and a quellung reaction were again performed to check each strain.

2.3.2 Quellung reaction

This reaction was to check for the presence of type-specific polysaccharide capsule (Austrian 1976). A loop of overnight broth culture was smeared onto a slide and left to dry in air. On a coverslip, 10µl of type specific capsular antiserum (Statens Seruminstitut) was mixed with 10µl of 1% W/V methylene blue in water. This was done before placing the fluid side down onto the prepared culture smear. The slide was then examined with a light microscope under x1000 oil immersion and compared to a control slide prepared with non-immune serum (heat inactivated foetal bovine serum). If the bacteria were positive, the capsule was seen clearly around the cells, which stain blue. If the reaction was not immediately seen, the slide was re-examined after leaving in a Petri dish on a damp tissue for 1 hour.

2.3.3 Viable counting

Serial dilutions of 20µl samples were done in sterile 96 well microtitre plates (Nunc A/S) containing 180µl of sterile nanopure water per well. Serial dilutions to $10^{-7}$ were made with each sample. Dried BAB with 5% V/V aerated horse blood plates were marked into six sectors and 3 x 20µl of each dilution were plated into each sector: this procedure was repeated in duplicate. The spots on each sector were allowed to dry into the agar for 10 minutes. Once dry, plates were placed inverted into a candle jar and incubated at 37°C overnight. Plates were observed and colonies were counted in sectors containing a measurable number (approximately <300). The following equation was then used to calculate the number of colony forming units from each sample:

$$\text{CFU per ml} = \frac{\text{Total number of colonies in sector}}{3 \times 50 \times \text{Dilution factor}}$$
2.4 Infection studies

2.4.1 Animal passage of pneumococci

Pneumococci were streaked to single colonies on BAB with 5% \( v/v \) aerated horse blood plates from frozen stock cultures. Plates were incubated overnight in a candle jar at 37°C. From the streak plates, a sweep of colonies were inoculated into BHI broth and incubated overnight statically at 37°C. Following incubation the culture was centrifuged at 18,000g for 15 minutes. The supernatant was then discarded, the pellet retained and resuspended in 10ml of sterile 1x Phosphate buffered saline (PBS) before being placed on ice until required. Using a 1ml insulin syringe (Becton Dickinson), 200μl from the 10ml cell suspension was injected intraperitoneally into each of two MF1 mice.

After 24 hours the animals were anaesthetised with 5% \( v/v \) fluothane (Zeneca Pharmaceuticals) with 1 Litre \( O_2 \)/min using a Fluotec 3 calibrated vaporiser (Cyprane). Pinching joints and observing no reflex reaction from the animal confirmed effective anaesthesia had been achieved. Once fully anaesthetised, exsanguination by cardiac puncture was performed using a 2ml syringe with a 23G needle (Terumo). Immediately after removal, blood was placed into cryotubes and put on ice. Once the procedure was completed, each animal was culled by dislocation of the neck without allowing recovery from anaesthesia. 50μl of blood from each mouse was placed into separate universal tubes with 10ml of BHI broth and incubated overnight statically at 37°C.

2.4.2 Preparation of standard inoculum

Following an overnight culture of animal-passaged pneumococci in BHI broth, prepared as in section 2.4.1, pneumococci were centrifuged at 18,000g for 15 minutes. The pellet was retained and the supernatant discarded before resuspension of the cells in 1ml of freshly made BHI broth with 20% \( v/v \) irradiated heat inactivated foetal bovine serum. Once resuspended in serum broth mixture, 400μl of this solution was added to 6ml of fresh serum broth to give an OD\(_{500nm}\) of approximately 0.7. The serum broth culture was then incubated statically at 37°C for 5 hours. Following 5 hours of incubation a sample of the culture’s optical density was measured. Samples at approximately an OD\(_{500nm}\) of 1.6 were removed from the incubator, and cultures with a
lower OD_{500nm} were allowed further incubation time with 30-minute optical density measurements. If after approximately 8 hours incubation the appropriate OD_{500nm} of 1.6 was not reached, the samples were discarded.

After the appropriate incubation time, the culture was vortexed before 0.5ml aliquots were placed into sterile cryotubes and stored at -70°C. 24 hours after storage at -70°C an aliquot was checked for viability, confirmation of optochin sensitivity and a quellung reaction performed. Viability was confirmed approximately at monthly intervals and the standard inocula were not used after approximately 4 months storage.

2.4.3 Procedure for intranasal challenge

Standard inoculum samples to be tested were thawed at room temperature. Bacteria were pelleted in a microfuge at 13000rpm for 10min, then resuspended in 0.5ml sterile PBS. From the viable count data taken from samples of frozen standard inocula (section 2.4.2), the number of CFU per ml were known for each sample. Further dilutions were then made in 1x PBS to achieve a CFU count in 50μl of 1x PBS equal to ED_{100} of the pneumococci to be used in the experiment. Once the dilution was completed, the cell suspension was kept on ice and used as soon, as was possible.

Mice of the appropriate strain to be used in the experiment were anaesthetised with 2.5 to 5% v/v fluothane, 1 litre O_2/min. Confirmation of effective anaesthesia was done by observation of no pinch reflex reaction. The mice following anaesthesia were then scruffed at the back of the neck and held upright. Using a Gilson pipette the pneumococcal inoculum of 50μl was placed in the nostrils in small drops. Once this was performed, the animals were placed in their cages on their backs to recover. After intranasal challenge, the viable count of the inoculum was determined (section 2.3.3). The infected animals were then kept in an isolator and monitored for symptoms of disease. Symptoms were scored in severity from hunched, starry coat, lethargic through to moribund or found dead. Each score was marked as one or two, dependant on the severity of each symptom. The condition of the animals was recorded up to 7 to 14 days post infection (dependent on the experiment) or until they became moribund. The time that the animals became moribund was recorded and the animals were then culled by dislocation of the neck. Death was confirmed by the absence of eye and joint reflexes. Carcasses and bedding were then removed and disposed by the appropriate local
procedure by Biomedical Services.

2.4.4 Determination of bacterial numbers in lung tissue

After the required period of infection, animals were culled by dislocation of the neck. When each mouse was confirmed dead, the fur was drenched with ethanol or hibitane (Zeneca Pharmaceuticals)/ethanol mixture. Using scissors and forceps sterilised in ethanol, the fur was removed from the chest and abdomen and care was taken not to penetrate the chest cavity or peritoneum. After re-sterilising the instruments in ethanol, the chest was opened with care taken to avoid the internal organs and an incision was made in the rib cage. The lungs were then aseptically dissected from the chest cavity, placed into a pre-weighed universal tube containing 10ml of sterile water and kept on ice.

After this procedure, each tube containing lung tissue was weighed to determine the weight of tissue. As soon as was practical but within 1 hour following removal, the tissue was homogenised. This was done by aseptically transferring all of the contents of a universal tube into a sterile Stomacher bag (Seward Medical) and homogenising for 90 seconds in a Stomacher Lab Blender 80 (Seward Medical). Following homogenisation of the lung tissue, the contents from the Stomacher bags were transferred back to the appropriate universal tube and the number of CFU/ml in each homogenate sample determined using the section 2.3.3.

2.4.5 Determination of bacterial numbers in peripheral blood

Mice were warmed in their cages under an infrared heat lamp for approximately 10 to 30 minutes. Each mouse was then placed in a 50ml syringe tube with padding and air holes bored along the width of the tube. Mice were monitored for signs of distress throughout the procedure and removed from the container if any distress occurred. Mice were orientated in such a way as to expose their tails through the hole in the lid of the tube. By means of a 1ml insulin syringe, blood was withdrawn from the tail vein. Immediately after removal of ≥ 100µl, blood was placed into sterile eppendorf tubes. As soon as was possible the blood was then taken and diluted in sterile nanopure water to determine viable numbers of bacteria in the blood (section 2.3.3).
2.4.6 Tissue for DNA analysis

Immediately after death, the liver and tail were removed from the animal. Tissue samples were placed into separate sterile cryotubes, and stored in air at -70°C. Each sample was labelled with mouse identification, date, mouse and pneumococcal strain. Each mouse sample had a corresponding symptom monitoring sheet and a record sheet of date, identification number, challenge details, strain and issue information.

2.5 Bronchoalveolar lavage

Mice were sacrificed by cervical dislocation and experimental procedures started following confirmation of death. The fur of each mouse was soaked in alcohol or hibitane/alcohol mixture and an incision was made in the abdomen using sterile scissors and forceps. Fur was then removed from the chest and neck area. The chest was opened and the sternum removed from each animal to expose the lungs. A further incision was made at the base of the neck to expose the trachea from the surrounding tissue. Once exposed, the top of the trachea (nearest the throat) was raised using a pair of scissors. A 16 gauge intravenous catheter (Becton Dickinson) was then carefully inserted into the trachea below the raised section. Once the catheter was inserted into the trachea the needle was removed and a three-way tap (B. Braun) attached to the top of the tubing. A 10ml syringe containing 10ml of sterile lavage buffer was attached to the end of the first tap, and an empty 10ml syringe to the second.

**Lavage buffer (per 50ml):**
Hank's balanced salt solution (HBSS)
250 units of heparin (CP Pharmaceuticals)
0.6mM EDTA
10mM HEPES (from a 1M stock solution of pH7.2 to 7.5)

0.5ml volumes of solution were slowly injected into the lungs. Once the lungs were fully extended, the fluid was drawn back into the empty syringe after switching the tap position. The lavage fluid collected, was placed into 5ml sterile tubes (Sarstedt) and put on ice. This process was repeated for 6 times for each mouse, thus making up a volume of approximately 2 to 3ml recovered fluid. After each lavage the catheter unit was discarded and the three-way tap washed through with lavage buffer.
2.6 **Total leukocyte count and differential analysis on bronchoalveolar lavage fluid (BALF)**

BALF was spun at 271g at 4°C for 15 minutes and resuspended in 1640 RPMI medium with 10% v/v foetal bovine serum (FBS) and 17mM HEPES. A cell count was performed with a 1/1 ratio of 1% w/v trypan blue stain in a haemocytometer (Improved Neubauer, Weber Scientific International Limited). Samples were then adjusted to give a concentration of approximately $1 \times 10^5$ total cells in a volume of 50µl. Cells were then spun onto cytospin slides (Shandon) using a cytocentrifuge (Cytospin 2, Shandon) at 108g for 3 minutes. Slides were air-dried briefly following centrifugation, then fixed in 100% methanol for 10 minutes. Differential staining was then achieved by flooding the slides with 1 part in 8 Giemsa’s stain to tap water for 10 minutes before rinsing in tap water. Slides were examined using a light microscope under 400x and mononuclear leucocytes, lymphocytes and polymorphonuclear leukocytes identified. At least 200 cells were counted on each slide. For each of the types of cells identified, a percentage of the total number of cells counted was calculated. These percentages were used with the total leukocyte counts in each lung sample, to calculate the total numbers of each type of leukocyte.

2.7 **Measurement of total protein concentration in BALF**

Protein concentration was measured using the Biorad microtitre microassay for protein determination that is based on the Bradford protein assay (Bradford et al., 1976). Standards were made between 8 and 80µg/ml in the diluent used for collection of the protein. The standard protein used was bovine serum albumin (BSA) (Sigma). 160µl of each standard solution and samples were placed into 96-well microtitre plates. After the addition of 40µl of the dye assay reagent (Biorad), the plate was left for 5 minutes before absorbance at 595nm was measured in an ELISA plate reader (Dynatech).

2.8 **Myeloperoxidase assay**

This method was modified from the methods of Jackett et al., (1981), Bradley et al., (1982), Shayevitz et al., (1995) and Metcalf et al., (1986). In this method, the enzyme concentration was used as the rate-limiting factor in the oxidation of the substrate, p-hydroxyphenyl acetic acid (POHPAA) to a fluorescent product by $\text{H}_2\text{O}_2$. 
Due to the expense of myeloperoxidase an alternative enzyme, horseradish peroxidase (HRP) with analogous activity was used in all standard curves.

2.8.1 Standard curve and sample preparation

HRP concentrations were made up from 100 unit stock batches (stored in frozen aliquots at -20°C) to give amounts of between 0.01 and 0.025 units in 50μl of diluent. 50μl of each of the enzyme standards and experimental samples were then added to duplicate tubes containing 950μl of reaction mixture. The reaction mixture in each tube was made up with the following components: 50μl of substrate POHPAA at a concentration of 7.4mg/ml, H_2O_2 at a concentration of 0.85mM in the final 1ml reaction and 50mM phosphate buffer (pH 6.0) with 0.5mM EDTA. The total reaction mixture was 1ml after addition of the enzyme solutions.

Tubes containing the reaction mixtures were left in a 37°C water bath to equilibrate before addition of the enzyme. 10 minutes after the addition of standards and samples, 1ml of ice-cold 0.2M borate buffer (pH 10.4) was added to the tubes to terminate the reaction. After reactions were allowed to come to room temperature, fluorescence was measured in a spectrofluorophotometer (Shimadzu) with wavelengths set at 313nm for excitation and 414nm for analysis. Peroxidase concentrations were calculated from a standard curve of fluorescence versus the concentration of enzyme. A new standard curve was determined for each batch of samples.

2.8.2 Myeloperoxidase extraction from tissue samples

This method was modified from the methods of Bradley et al., (1982), Shayevitz et al., (1995), and Metcalf et al., (1986). Lung tissue samples to be assayed for myeloperoxidase were homogenised using a glass Dounce homogeniser (F.T. Scientific Instruments Limited). Tissue was homogenised in a buffer consisting of 50mM phosphate buffer (pH 6.0), 0.5% w/v hexadecyl-trimethyl ammonium bromide (HTAB) and 5mM EDTA. Once tissue was sufficiently homogenised (i.e. no solid material remained visible) samples were sonicated for 3x10 seconds at a setting of 5 amplitude microns with a Soniprep 150 sonicator (Sanyo). Following sonication, samples were spun in centrifuge (Sorvall) at 3000g for 30minutes. The supernatant was retained and the pellet discarded after centrifugation. Lung homogenate samples at various
concentrations were then added to reaction mixtures set up as previously described (section 2.8.1).

2.9 Lung homogenate cell preparation

2.9.1 Lung homogenisation and enzyme digestion

This method was modified from the methods of Curtis et al., (1994) and Huffnagle et al., (1995). Before removal of the lungs from sacrificed animals, vascular perfusion was performed by injection of 1ml of HBSS into the left ventricle of the heart. The tissue was then removed and placed into 10ml of HBSS. The lungs were then cut into small pieces and homogenised in 5ml of digestion buffer through a tea strainer a total of three times. The digestion buffer was comprised of 5% v/v FBS in RPMI 1640 medium with 0.5mg (207 collagen digestion units)/ml collagenase (Sigma) and 30μg (87 units)/ml Dnase I from bovine pancreas (Sigma). After homogenising, lung samples were placed into 15ml tubes and incubated at 37°C for 30 minutes. Following incubation, digested tissue samples were pipetted up and down to break up tissue fragments before passing cells through a column of non-absorbent cotton wool to remove large pieces of debris. This column was made with approximately 1cm of non-absorbent cotton wool in a glass Pasteur pipette. Cells were collected from the bottom of the column in Falcon 2052 tubes (Becton Dickinson) and spun at 322g for 5 minutes at 4°C. The supernatant was removed and the cells were resuspended in 1ml of 1x lysing solution (Pharmingen). Lysis of red blood cells was achieved by incubating the sample for 5 minutes at room temperature. After this period the cells were brought to isotonicity by adding an excess of ice cold 1x PBS (approximately 10ml). Again the cells were pelleted at 322g for 5 minutes at 4°C. Once the supernatant was removed, the cells were washed with 1ml of 1x PBS before centrifugation as in the previous step. The method of resuspension of the pellet was determined by the method to be used subsequently.

2.9.2 Lung homogenate cytocarin

Lung cells, prepared as described in method 2.9.1, were resuspended in 1ml of 5% v/v FBS in RPMI 1640 medium. The number of cells then enumerated with a haemocytometer using trypan blue in 1x PBS at a 1:1 ratio of stain to cells. The sample was diluted to give a total of 7x10^4 to 1x10^5 cells per 50μl (optimum concentration of
7x10⁴ gave a good distribution of leukocytes on the slide for ease of identification and counting. Cytocentrifugation of these cells was performed as previously stated (section 2.6).

2.10 Flow cytometric analysis of leukocytes

2.10.1 Preparation of lung homogenate cells

The flow cytometric analysis method was modified from the methods of Curtis et al., (1994) and Huffman et al., (1995). Lung cells, prepared as previously stated (section 2.9.1), were resuspended in 1ml of 1x PBS. The number of cells was then enumerated using a haemocytometer as described in section 2.9.2 to determine the total number of leukocytes. Lung samples were then diluted to give a total of 1x10⁵ cells per 50μl. When staining the cells, samples of 50μl were incubated with appropriate concentrations of fluorescein isothiocyanate (FITC) and the R form of phycoerythrin (R-PE) labelled antibodies in 2052 Falcon tubes (Table 2.1). Cells were then incubated on ice for a total of 60 minutes, covered from daylight. Antibody concentrations were used as given in the supplier's instructions to label 5x10⁵ cells and to give an excess of antibody to the sample size of 1x10⁵ cells.

Once the incubation step was complete, 1ml of 1x PBS with 0.5% v/v BSA was added to each tube of labelled cells. Cells were pelleted by centrifugation at 322g for 5 minutes at 4°C. The cells were washed with approximately 1ml of 1xPBS, before a final resuspension in 1ml of 1% w/v paraformaldehyde to fix the cells. Samples were kept overnight protected from light, at 4°C, before flow cytometric analysis.
<table>
<thead>
<tr>
<th>Antibody:</th>
<th>Target cell:</th>
<th>Supplier:</th>
<th>Amount added to 1x10⁵ cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse CD45 (R-PE)</td>
<td>All leukocytes (Watt et al., 1987)</td>
<td>Serotec</td>
<td>5µl</td>
</tr>
<tr>
<td>Anti-mouse CD3e (FITC)</td>
<td>Pan T-cell marker (Portoles et al., 1989)</td>
<td>Cambridge Bioscience</td>
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<tr>
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<td>Pan B-cell marker (Krop et al., 1996)</td>
<td>Cambridge Bioscience</td>
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<tr>
<td>Anti-mouse F4/80 (FITC)</td>
<td>Mature tissue macrophage marker (Austyn et al., 1981)</td>
<td>Serotec</td>
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<td>Anti-mouse Ly6G (Gr-1) (FITC)</td>
<td>Neutrophil marker (Fleming et al., 1993)</td>
<td>Pharmingen</td>
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<tr>
<td>Anti-mouse IgG2B (FITC) isotype control</td>
<td>isotype control</td>
<td>Cambridge Bioscience</td>
<td>5µl</td>
</tr>
<tr>
<td>Anti-mouse IgG2B (R-PE) isotype control</td>
<td>isotype control</td>
<td>Serotec</td>
<td>5µl</td>
</tr>
</tbody>
</table>

**Table 2.1** A list of antibodies, used in FACS analysis experiments with their suppliers.
*(Amount used from suppliers stock solution).*
2.10.2 Preparation of murine blood leukocytes for flow cytometry

50μl of heparin was added to each syringe before removal of blood via cardiac puncture as described previously (section 2.4.1). Blood from each animal was placed as 100μl samples into 2052 Falcon tubes. 1ml of lysing solution (Pharminen) was added to each 100μl blood sample and incubated for 5 minutes at room temperature to lyse red blood cells. Blood leukocytes were brought to isotonicity after this step by adding an excess of ice cold 1x PBS (approximately 10ml). Cells were pelleted by centrifugation at 322g for 5 minutes at 4°C and the supernatant removed before a repeat of the lysis step. Following the second lysis stage, cells were pelleted and washed in 1x PBS as before. After another centrifugation step, cells were resuspended in 50μl of 1xPBS, then labelled with appropriate antibodies as described in the previous section (2.10.1). Cells were then fixed in 1% w/v paraformaldehyde and kept overnight protected from light at 4°C before flow cytometric analysis.

2.10.3 Flow cytometric analysis procedure

Fixed blood and lung leukocyte samples, were analysed with a FACScan (Becton Dickinson) using the manufacturer's standard protocols. The number of events counted in each sample was set to 10,000. Acquisition and analysis of FACS data was done on Becton Dickinson LYSIS software. On dot plots and fluorescence histograms, gates and marker regions were set using blood samples and the same settings then used to analyse lung sample cells.

2.11 Histology

2.11.1 Preparation of formaldehyde fixed tissue

Gross pathology was examined by preparation of wax sections. Preparation of wax embedded sections was performed by immersing samples in neutral buffered formaldehyde (NBF) before embedding in paraffin wax. Embedding and sectioning was done by Dr Huw B. Jones (AstraZeneca plc, Alderley Edge, UK).

Neutral buffered formaldehyde: 100ml of 40% formaldehyde
900ml of distilled water
4g of sodium dihydrogen phosphate monohydrate
6.5g disodium hydrogen phosphate anhydrous

2.11.2 Preparation of frozen tissue

The embedding compound Tissue Tek OCT (Sakura) was placed in aluminium foil containers made by moulding the foil around 7ml Bijou tubes. With the embedding compound at a depth to cover the bottom of the foil container (approximately 0.5cm), tissue for frozen cryostat sectioning was added. The tissue was then completely immersed in embedding compound. Once completed the container was placed into a beaker of n-pentane, which was suspended, in liquid nitrogen. Tissue samples were then gradually frozen before storage at -70°C.

2.11.3 Cryostat sectioning

Before use, samples were removed from storage at -70°C and placed at -20°C overnight before sectioning. Immediately before sectioning, tissue was fixed on a cryostat mount with Tissue Tek OCT embedding compound and allowed to acclimatised to between -18°C and -25°C, the working temperature of the cryostat (Bright Instrument Company Limited). Once the tissue was acclimatised, sections were cut between 10 to 25 microns in thickness using a re-usable microtome blade (Bright Instrument Company Limited). After sectioning, samples were allowed to dry at room temperature for 20 minutes. Embedding compound surrounding the sections was removed and the samples were then ready for staining. Sections to be stored were either wrapped in aluminium foil at -20°C or placed in a container with silica gel at 4°C.

2.11.4 Haematoxylin and Eosin stained tissue sections

Slides containing tissue sections were first placed into pre-filtered Meyers Haematoxylin stain (BDH) (filtered through tissue paper). After 30 seconds in the solution, slides were washed under tap water. Once washed, slides were then immersed in a solution of Eosin stain (BDH) and again left for 30 seconds. A second wash step was performed under tap water. Slides were dehydrated through a series of alcohol washes (70% V/V, 90% V/V and 100% V/V solutions) for a period of 30 seconds in each solution, then placed into xylene. Finally, sections were left to dry, mounted in DPX solution (BDH) and covered with a coverslip.
2.12 Analysis of data

All data were expressed as either median values with maximum and minimum values or the mean +/- standard error of the mean (SEM) unless otherwise stated in the text. Statistical analysis was by the Mann-Whitney U test, unpaired t-test, analysis of variance (ANOVA) with tukey post test, as appropriate. Significance was considered as p values < 0.05.
Chapter 3: Results

3.1 Distribution of phenotypes in infection studies using a panel of nine murine inbred strains

Nine inbred mouse strains were chosen to look at the genetic resistance and susceptibility to invasive pneumococcal infection. Strains were selected on the criteria of genetic diversity, ease of handling, breeding vigour and availability. From previous studies in Leicester (Canvin et al., 1995), the outbred murine strain MF1 was shown to have a reproducible phenotype in survival time and lung and blood bacterial counts following intranasal inoculation of pneumococci. The required dose for D39 (type 2) and GB05 (type 3) had been established in the outbred strain MF1 mouse: $1 \times 10^6$ cfu in 50μl for D39 and $5 \times 10^5$ cfu in 50μl for GB05. This was calculated as the effective dose to render a group of MF1 mice moribund within three days (ED100). Prior to the challenges of inbred strains, this dose was confirmed by virulence studies using MF1 mice. To look for phenotypic differences within the panel of inbred mice selected for analysis, the ED100 of D39 (type 2) and GB05 (type 3) for outbred MF1 mice was used. Outbred MF1 mice were used as a control group for comparison with all the inbred strains of mice. If these mice were found to behave differently from expected, the challenge data for the inbred mice were discarded. During both initial pilot studies and full-scale challenges, female outbred and inbred murine strains were used in the experiments for ease of handling.

3.1.1 Pilot study

A pilot study was performed with groups of five to six mice, primarily to check the efficacy of the dose selected for both strains of pneumococci. This was also done to investigate whether the dose selected would give a sufficient spread of phenotypes to give a measurable indication of susceptibility or resistance to pneumococcal infection. Nine inbred strains as listed previously (section 2.2) were infected with D39 and GB05 pneumococci. Animals were intranasally infected with previously prepared, passaged and virulence-tested pneumococci. Following infection, the animals were monitored, until the experiment was terminated. Both the pilot study and the subsequent full-scale study ran from between >90 to ≥168 hours depending upon logistical constraints. To
compare these studies more easily, the median survival times for surviving mice in both studies is shown as 168 hours.

Symptoms of sickness were recorded during the experiment for all mice infected. Any animals found moribund were culled and the survival time noted. To monitor the invasiveness of the infection, blood samples were taken from each mouse 24 hours post-infection and checked for viable pneumococci. The median survival time and mean log CFU/ml of pneumococci in the blood 24 hours post-infection for mice infected with D39 is shown in Figure 3.1 and Figure 3.2 respectively.

The resultant spread of phenotypes from infection with D39 pneumococci gave a distribution of susceptible, intermediate and resistant mice. This was in respect to both median survival times and the blood count 24 hours post-infection. BALB/c mice showed the greatest resistance to this strain of pneumococcus with 4 out of 5 mice surviving the challenge and showing a low CFU count in the blood following infection (1.12 mean Log CFU/ml 24 hours post-infection). This number was the consequence of bacterial colonies recovered from only one of the five mice challenged.

Two strains were shown to have a susceptible phenotype to D39 pneumococcus. CBA/Ca and SJL mice had low median survival times: 28 hours (CBA/Ca mice) and 26 hours (SJL mice). Coupled with low survival times, an increase in the number of pneumococci isolated from the peripheral blood was observed. CBA/Ca had the highest blood count of all the inbred strains tested (7.27 mean Log CFU/ml 24 hour post-infection) with SJL following second (6.30 mean Log CFU/ml 24 hours post-infection).

A group of inbred mice constituted an intermediate phenotype following infection with D39. Among these strains were DBA/2 and FVB/n mice, which were both found to have a median survival time of 69 hours. However, DBA/2 mice were found to have a marginally higher mean Log CFU/ml blood count 24 hours post-infection of 4.86 in contrast to FVB/n mice, showing a mean Log CFU/ml blood count 24 hours post-infection of 4.68. NIH and AKR mice were also found to have an intermediate susceptibility comparable with DBA/2 and FVB/n mice. NIH mice showed a median survival time of 69 hours and AKR mice were found to have a median survival time of 70 hours. However, AKR mice with a higher median survival time than NIH, had greater numbers of pneumococci isolated from the blood at 24 hours post-
infection. AKR had a mean Log CFU/ml blood count 24 hours post-infection of 3.76 in comparison with a mean Log CFU/ml blood count 24 hours post-infection of 3.49 found for NIH mice.

At the lower end of the intermediate phenotype group, C3H/He and C57BL/6 mice were shown to be sensitive to D39 with median survival times of 45 and 47 hours respectively. No blood samples were taken from the group of C57BL/6 mice and therefore no colony counts were recorded for this strain. Colony counts from C3H/He showed a higher number (5.72 mean Log CFU/ml) with relation to the other intermediate mice, reflecting a reduction in survival time.

Outbred mice (MF1) were included in all experiments as a control. They were shown to behave as expected after infection with D39, having a survival time of (52 hours) and a mean log CFU/ml 24 hours post-infection of 3.25.

Examination of the symptoms of disease scored as described in the methods section 2.4.3, was consistent with the survival and CFU/ml data. Symptom data for the pilot study shown in Figure 3.5, illustrate the difference in susceptible and resistant strains. After D39 infection, BALB/c mice showed little or no symptoms (median symptom score of 1) between 0 to 24 hours or the period between 24 to 48 hours post-infection. In contrast to BALB/c mice, CBA/Ca and SJL mice developed symptoms earlier, with greater severity between 0 to 24 hours. During this time, symptom data indicated that CBA/Ca mice (median symptom score 7) developed more severe symptoms than SJL (median symptom score 4). However, both strains developed severe symptoms (median symptom scores of 9) between 24 to 48 hours reflecting the low median survival times for these strains. These data therefore reflect the observations during these experiments that SJL mice succumbed to the disease rapidly after the first signs of illness, compared to CBA/Ca mice which developed symptoms earlier but remained symptomatic longer before reaching moribund state.
Figure 3.1 Median survival time from the pilot study of murine strains (n=5) intranasally challenged with D39 (type 2).

Figures in parentheses are the range above and below the median survival time (hours).
Figure 3.2 Number of pneumococci in the blood at 24 hours post-infection, from the pilot study of murine strains (n=5) intranasally challenged with D39 (type 2).

Error bars are the standard error of the mean Log CFU/ml.
For mice infected with GB05 pneumococcus, the results are shown in Figures 3.3 and 3.4. With this strain, the distribution pattern of phenotypes was not so clearly demarcated into groups. Of the murine strains challenged with GB05, BALB/c showed the greatest resistance to infection. However, these mice were more susceptible to infection with GB05 than with D39. After infection with GB05, BALB/c mice had a high bacterial load at 24 hours post-infection (mean Log CFU/ml blood count of 5.65) and a survival time of 81 hours. Grouped closely with BALB/c mice, were the inbred strains C57BL/6 and NIH with survival times of 69 and 71 respectively. Interestingly, the blood counts 24 hours post-infection for both these mice were much lower than BALB/c mice. C57BL/6 mice were found to have a mean Log CFU/ml blood count 24 hours post-infection of 1.16 and NIH mice a mean Log CFU/ml blood count 24 hours post-infection of 0.69. Closely related to the phenotypes of C57BL/6 and NIH mice, DBA/2 were found to have a median survival time of 61.5 hours and a blood count 24 hours post-infection of 1.95.

The mice most susceptible to GB05 infection were the inbred strains C3H/He, CBA/Ca and FVB/n, with median survival times of 46, 46, and 49 hours respectively. Pneumococcal numbers cultured from blood samples 24 hours post-infection were found to be a mean Log CFU/ml of 3.48 for C3H/He mice, a mean Log CFU/ml of 5.37 for CBA/Ca mice and a mean Log CFU/ml of 4.14 for FVB/n mice. AKR and SJL mice were unobtainable from the supplier for this experiment and thus no data were generated for these mice.

As with the previous experiment with D39, MF1 outbred mice used as a control, were found to behave as expected following infection with GB05. These mice were found to have a median survival time of 69 hours and a mean Log CFU/ml blood count 24 hours post-infection of 1.59.

Following observation of symptoms following infection (Figure 3.5), the picture found after GB05 infection was different to that after D39 infection in both susceptible and resistant mice. Both BALB/c and CBA/Ca mice showed no symptoms (median score of 1) between 0 to 24 hours post-infection. Between 24 to 48 hours BALB/c mice had a median symptom score of 4; higher than after D39 infection. This was consistent with the lower median survival time observed after infection with GB05. However,
these mice were found to have less severe symptoms than CBA/Ca (median symptom score of 7.5) at this time.
Figure 3.3 Median survival time from the pilot study of murine strains (n=5 or 6) intranasally challenged with GB05 (type 3).

Figures in parentheses are the range above and below the median survival time (hours).
Figure 3.4 Numbers of pneumococci in the blood at 24 hours post-infection, from the pilot study of murine strains (n=5 or 6) intranasally challenged with GB05 (Type 3).

Error bars are the standard error of the mean Log CFU/ml.
Figure 3.5 Median symptom scores for periods between 0 to 24 hours and 24 to 48 hours following infection in a pilot study of inbred strains infected with D39 (A) and GB05 (B) pneumococci.
3.1.2 Full scale studies

Following the results of the pilot study, a full-scale study using a group size of twenty mice was undertaken. This was to check the reproducibility of the pilot study challenges and to improve statistical analysis of the comparison of phenotypes between inbred strains. Challenges were done under the same conditions as the pilot study. The median survival times and mean Log CFU/ml pneumococcal blood counts 24 hours post-infection are given in Figures 3.6 and 3.7 for infection with D39 (type2) pneumococci. Data from infection with GB05 (type3) pneumococci are shown in Figures 3.8 and 3.9.

As shown in Figure 3.6, after infection with D39 pneumococci, the full-scale study reflected the data generated in the pilot study, as inbred mouse strains again could be divided into three phenotype groups; resistant, intermediate, and susceptible. BALB/c mice were again more resistant to this infection than all the other strains. Although one mouse succumbed to the infection (at 93 hours), the remaining nineteen mice survived to seven days post challenge at which time the experiment was ended (median survival time of >168 hours). Consistent with this median survival time, no pneumococci were isolated from the blood 24 hours post-infection from any of the mice challenged (Figure 3.7). BALB/c mice were found to be statistically different to all the other inbred strains in both median survival time (p<0.01) and blood counts 24 hours post-infection (p<0.001).

The majority of the strains tested (C3H/He, DBA/2, C57BL/6, AKR, NIH and FVB/n) constituted an intermediate phenotype group for susceptibility to infection with D39 (Figures 3.6 and 3.7, Table 3.1). This was in both phenotypic parameters: blood counts 24 hours post-infection and median survival time.

As observed in the pilot study, CBA/Ca and SJL mice were shown to be susceptible to D39 (type 2) infection in the full-scale study. These mice were found to be similar to each other in blood counts 24 hours post-infection (p>0.05), although different in median survival time (p<0.05). CBA/Ca were very sensitive to type 2 pneumococcal infection, quickly developing severe disease (median survival time of 27 hours). Bacteraemia also developed quickly with high numbers (mean Log CFU/ml of 7.8) detected in the blood 24 hours post-infection (Figure 3.7). SJL mice also showed
severe bacteraemia (6.1 mean Log CFU/ml) with a short survival time (median survival time of 28 hours). Both mice were statistically different in survival time (SJL p<0.001; CBA/Ca p<0.0001) to the other inbred mice. CBA/Ca mice, with the highest number of pneumococcal in the blood counts, were significantly different (p<0.01) in blood counts 24 hours post-infection to the other inbred mice. However, SJL mice, although having the second highest blood count 24 hours post-infection, were comparable to the strains C3H/He, FVB/n and NIH (p>0.05).

The symptoms of invasive disease in the full-scale study (Figure 3.10), again correlated with the data obtained for the median survival times and mean Log CFU/ml blood counts 24 hours post-infection. After D39 infection, BALB/c mice again showed little or no symptoms (median symptom score of 1) between 0 to 24 hours or the period between 24 to 48 hours. Of the susceptible strains, CBA/Ca mice showed no symptoms between 0 to 24 hours post-infection in contrast to SJL mice, which had a median symptom score of 3. For symptom scores calculated between 24 to 48 hours, both of these strains were shown to be symptomatic with severe median symptom scores of 8, reflecting the median survival times being at this time. Contrary to the pilot study, symptom data in the full-scale study indicated that SJL mice developed symptoms greater than CBA/Ca in the first period post-infection (0 to 24 hours). However, on closer examination of the data, SJL mice rapidly reached moribund state from the onset of symptoms within a few hours, whereas CBA/Ca mice were observed to be symptomatic at 12 hours but reached moribund state 15 hours later at 27 hours.

In agreement with the pilot study, less variation in resistance to GB05 pneumococcal infection was observed, compared with the observations following infection with D39. The narrower range of survival times after infection with this pneumococcal strain was also reflected in the narrower range of numbers of pneumococci in the blood 24 hours post-infection (Figures 3.8 and 3.9). Inbred mouse strains could not be clearly divided into the groups defined for resistance or susceptibility to D39 infection (Table 3.2).

BALB/c mice, previously shown to be resistant to D39, were shown to be more sensitive to the GB05 strain of pneumococci. The full-scale study indicated that BALB/c mice statistically were no more resistant to GB05 infection (p>0.05) in median
survival time than C3H/He, DBA/2 and NIH mice (Table 3.2). The bacterial blood
count data also indicated that BALB/c mice were not phenotypically distinct from most
of the murine strains infected with GB05. However, these mice were found to be
significantly more resistant to GB05 (p<0.05) than C57BL/6 and AKR mice in median
survival times. Additionally, BALB/c mice had a statistically higher (p<0.05) median
survival time of 52 hours, compared to CBA/Ca mice with a median survival time of 46
hours. The numbers of pneumococci in the blood 24 hours post-infection (3.4 mean Log
CFU/ml) compared to CBA/Ca mice (5.1 mean Log CFU/ml) was also statistically
lower (p<0.01). Together with support from the pilot study data, these results suggest
that BALB/c mice are more resistant to both pneumococcal strains investigated
compared to CBA/Ca mice.

CBA/Ca mice were more susceptible to GB05 than C3H/He, DBA/2 and NIH
with a statistically lower (p<0.01) median survival time. In blood counts 24 hours post-
infection CBA/Ca were found to have statistically higher (p<0.01) numbers of
pneumococci in the peripheral blood than BALB/c, C3H/He, DBA/2 and NIH. In
further comparisons, these mice did not define a susceptibility group to GB05.
Compared to AKR, and C57BL/6, CBA/Ca mice were not found to differ statistically
(p>0.05) in median survival time. In blood counts 24 hours post-infection, these mice
were also not significantly different (p>0.05) to that of AKR, C57BL/6 and NIH mice.
Unfortunately, no data were generated for SJL and FVB/n in this experiment.

CBA/Ca mice were less susceptible to infection with GB05 than to D39 (Figures
3.8 and 3.9). In the GB05 study, a significantly (p< 0.001) longer median survival time
(46 hours) together with significantly lower (p<0.0001) blood counts (mean Log
CFU/ml 24 hours post-infection of 5.1) was observed compared to data from D39
infection (median survival time 27 hours, mean Log CFU/ml 24 hours post-infection of
7.8).

Symptoms following GB05 infection (Figure 3.10), confirmed data shown in the
pilot study. Both susceptible and resistant strains did not show any signs of illness with
a median symptom score of 1 between 0 to 24 hours. During the full scale study
BALB/c developed symptoms at 24 to 48 hours post infection. Complementary to the
pilot study, these symptoms were higher than observed during infection with D39, with
a median symptom score of 4.5. However, symptoms observed with BALB/c mice between 24 to 48 hours post infection were less severe than CBA/Ca mice, with a median symptom score of 6.
Figure 3.6 Scatter graph of survival times in the full-scale study of murine strains (n=20*) intranasally challenged with D39 (type 2).

Figures in parentheses are the median survival time (hours). Each datum point is from one mouse. * except MF1 were n=40 and SJL were n=15.
Figure 3.7 Numbers of pneumococci in the blood 24 hours post-infection, in the full-scale study of murine strains (n=20*) intranasally challenged with D39 (type 2).

Error bars are the standard error of the mean. * except MF1 were n=40 and SJL were n=15.
Figure 3.8 Scatter graph of survival times in the full-scale study of murine strains (n=20*) intranasally challenged with GB05 (type 3).

Figures in parentheses are the median survival time (hours). Each datum point is from one mouse. * except MF1 were n=34.
Figure 3.9 Numbers of pneumococci in the blood 24 hours post-infection, in the full-scale study of murine strains (n=20*) intranasally challenged with GB05 (Type 3).

Error bars are the standard error of the mean. * except MF1 were n=34.
### Table 3.1 Statistical analysis of (A) median survival time and (B) mean blood counts 24 hours post infection of inbred strains challenged with D39 (type 2).

Figures represent approximate calculated P value. NS= not significant.
Table 3.2 Statistical analysis of (A) median survival time and (B) mean blood counts 24 hours post infection in inbred strains challenged with GB05 (type 3).

Figures represent approximate calculated P value. NS= not significant, NA= not analysed in this experiment.
Figure 3.10 Median symptom scores for periods 0 to 24 hours and 24 to 48 hours following infection in a full-scale study of inbred strains infected with D39 (A) and GB05 (B) pneumococci.
3.2 Progeny phenotype distributions from crosses of susceptible and resistant parents

From the results indicated in the pilot study and the full-scale studies, BALB/c mice were selected as resistant and CBA/Ca mice were selected as a susceptible strain. These parental strains were then used for further genetic and phenotypic analysis. As the phenotype distribution from infection with D39 (type 2) showed the greatest variation in susceptibility or resistance, this strain was chosen over GB05 (type 3) to continue further infection studies.

To follow the inheritance pattern of resistance or susceptibility genetic loci, progeny from crossing parental BALB/c mice and CBA/Ca mice (F1 generation) were challenged with D39. For genome scanning to further analyse the segregation of genes of resistance or susceptibility, F2 generation mice were produced from an intercross of F1 parents and these progeny were tested for phenotypic differences and tissue kept for DNA analysis. In both familial generations, the reciprocal parental crosses were also done to look for genetic imprinting from the parental genotype (i.e. both males and females were used in crosses from both CBA/Ca and BALB/c genetic backgrounds). F1 and F2 generation mice were both produced by a staggered breeding protocol described in section 2.2. The nomenclature for progeny generated in the F1 and F2 generations is indicated in Figure 3.11.
Figure 3.11 Strain nomenclature diagram to illustrate parental crosses through to the production of F2 generation mice.
3.2.1 Results of the F1 generation infection study

Once 9 to 30 weeks old, groups of 20 F1 generation mice were intranasally challenged with virulent D39 pneumococci. This was done for both CCBA F1 and CBAC F1 mice. Challenges were done on several separate occasions for both F1 progeny crosses. These challenges for CBAC F1 were denoted A through to C, and for CCBA F1 A through to F. Results from the infection study of CCBA F1 mice are represented in Figures 3.12-3.15. Results from CBAC F1 mice are represented in Figures 3.16-3.19.

CBAC F1 mice showed an intermediate value in both bacterial blood counts and survival time after infection with D39 in both male and female mice. The analysis of the results for challenges A and B (Figure 3.12) showed that both male and female groups were not statistically different (p>0.05) in median survival time. In both males and females from challenge C (Figure 3.12), again no statistical difference (p>0.05) was found in median survival time. However, when comparing between challenges, female mice from the challenge group C were found to be statistically different in median survival time (p<0.05) to the male and female progeny in groups A and B.

When analysing bacterial blood counts in all the challenges done (Figure 3.13), males and females from challenges A and B were shown not to be statistically different (p>0.05). In challenge C, both males and females were phenotypically paired as the blood count data were found not to be statistically different (p>0.05). However, comparing challenges A and B with challenge C, revealed that there was a significant difference (p<0.01) in blood count data between the male and female mice in these groups.

The scatter graphs of the phenotypic data (Figures 3.14 and 3.15) show the distribution of phenotypes in the separate challenges. The significant difference between challenges can be seen by the shift in phenotypes between the challenges. Figure 3.14 shows that in challenge C (both male and female mice) there are fewer surviving mice than in challenges A and B. The blood counts 24 hours post-infection correlate with this observation, with higher numbers for males and females in challenge C compared to challenges A and B (Figure 3.15).
This variation between different challenges was reflected much more with CCBA F1 mice, therefore, for this reason further challenges were done (challenges d, e, and f) in addition to challenges a to c. Due to the distribution of mean Log CFU/ml data in all of these challenges, blood counts were analysed by a non-parametric method (two tailed Mann-Whitney U test). The results for these challenges segregated into two statistically different groups; mice surviving the experiment and mice susceptible to infection. Challenges a, c and f (males and females) were found not to be statistically different (p>0.05) in median survival time. In addition, these mice were not significantly different in blood counts 24 hours post-infection (p>0.05), with the exception of challenge a which was significantly different (p<0.05) to challenge f (male). CCBA F1 mice that showed a resistant phenotype in challenges b, d and e were found not to be statistically different (p>0.05) in median survival time. These mice were also not significantly different in blood counts 24 hours post-infection (p>0.05), apart from challenge b which was found to differ statistically (p<0.05) from challenge d.

In comparison, challenge b, d and e were found to be significantly different to challenges a, c and f (males and females) in median survival time (p<0.01). In addition, these mice were also significantly different in blood counts 24 hours post-infection (p<0.05), except challenge c, which had comparable blood counts to d and e challenges. In the scatter graphs showing the distribution of phenotypes (Figures 3.18 and 3.19), the number of surviving mice was higher in challenges b, d and e correlating with a lower number of blood counts. In challenges a, c and f (males and females), few mice survived the challenge, and there was a higher pattern in the distribution of blood counts for each mouse.

As outlined above, several additional challenges (d to f) were done using CCBA F1 female mice because of the variation in the phenotypes seen. In particular, female mice were shown to have been sensitive to the factors that may have varied between challenges, regardless of mouse batch differences. Factors explaining this variation could have been the variation of dose or virulence differences within different batches of passaged pneumococci. However, each passaged batch of inoculum was routinely tested on MF1 outbred mice for virulence. In addition, if any dose variation was present that could have accounted for challenge variation, it was not observed in the phenotype of MF1 mice used as a control in both CBAC F1 and CCBA F1 challenges (Figures
MF1 mice were not found to be statistically different in median survival time throughout all the challenges (p>0.05). However, in the blood count data 24 hours post-infection, MF1 mice were found to differ between CBAC F1 challenges A and C (p<0.01), and in CCBA F1 challenges a and b, b and c, and c and e (p<0.05).

To summarise both F1 crosses, the data showed a general intermediate phenotype with both males and females. The results from the F1 data showed a continuous variation within the observed phenotypic traits to infection with D39 (type 2). This observation implies that the genetic trait is not a simple Mendelian inheritance but a polygenic effect. Possible sex linkage effects and any genetic imprinting that may occur between parental alleles from reciprocal crosses may have been masked by the variation in phenotype between challenges.
Figure 3.12 Median survival time from the study of CBAC F1 mice (n=20 for each group*) intranasally challenged with D39 (type 2).

Figures in parentheses illustrate the range above and below the median survival time (MF1 control mice n=5 for each group except challenge group A were n=4). Challenges denoted (A), (B) and (C) were done on separate occasions. *except challenge group C (males and females) were n=11.
Figure 3.13 Number of pneumococci in the blood 24 hours post-infection, from the study of CBAC F1 mice (n=20 for each group*) intranasally challenged with D39 (Type 2).

Error bars illustrate the standard error of the mean. (MF1 control mice n=5 for each group except challenge group A were n=4). Challenges denoted (A), (B) and (C) were done on separate occasions. *except challenge group C (males and females) were n=11.
**Figure 3.14** Scatter graph of survival times from the study of CBAC F1 mice (n=20 for each group*) intranasally challenged with D39 (type 2).

Challenges denoted (A), (B) and (C) were done on separate occasions. Each datum point is from one mouse. *except challenge group C (males and females) were n=11.
Figure 3.15 Scatter graph of the number of pneumococci in the blood 24 hours post-infection, from the study of CBAC F1 mice (n=20 for each group) intranasally challenged with D39 (type 2).

Challenges denoted (A), (B) and (C) were done on separate occasions. Each datum point is from one mouse. *except challenge group C (males and females) were n=11.
Figure 3.16 Median survival time from the study of CCBA F1 mice (n=20 for each group*) intranasally challenged with D39 (type 2).

Figures in parentheses illustrate the range above and below the median survival time (MF1 control mice n=5 for each group except challenge c were n=4). Challenges denoted (a), (b), (c), (d), (e) and (f) were done on separate occasions. *except challenge c were n=4, challenge e (female) were n=11, challenge e (male) were n=9, challenge f (female) were n=18, and challenge f (male) were n=15.
Figure 3.17 Number of pneumococci in the blood 24 hours post-infection, from the study of CCBA F1 mice (n=20 for each group*) intranasally challenged with D39 (type 2).

Error bars illustrate the standard error of the mean. (MF1 control mice n=5 for each group except challenge c were n=4). Challenges denoted (a), (b), (c), (d), (e) and (f) were done on separate occasions. *except challenge c were n=4, challenge e (female) were n=11, challenge e (male) were n=9, challenge f (female) were n=18, and challenge f (male) were n=15.
Figure 3.18 Scatter graph of survival times from the study of CCBA F1 mice (n=20 for each group) intranasally challenged with D39 (type 2).

Challenges denoted (a), (b), (c), (d), (e) and (f) were done on separate occasions. Each datum point is from one mouse. * except challenge c were n=4, challenge e (female) were n=11, challenge e (male) were n=9, challenge f (female) were n=18, and challenge f (male) were n=15.
Figure 3.19 Scatter graph of the number of pneumococci in the blood 24 hours post-infection, from the study of CCBA F1 mice (n=20 for each group) intranasally challenged with D39 (type 2).

Challenges denoted (a), (b), (c), (d), (e) and (f) were done on separate occasions. Each datum point is from one mouse. *except challenge c were n=4, challenge e (female) were n=11, challenge e (male) were n=9, challenge f (female) were n=18, and challenge f (male) were n=15.
3.2.2 Intercross F2 generation: results of infection studies

F1 offspring were intercrossed to produce F2 progeny for genome scanning. Both CCBA F2 mice (n=251) and CBAC F2 mice (n=223) were challenged with D39 pneumococci. Infection data and tissue samples for DNA analysis were collected to determine linkage between genotype and phenotype characteristics. Following infection studies, tissue samples were sent to the MRC Mouse Genome Centre in Harwell. Genome scanning from these F2 tissues is currently underway.

Figures 3.20 and 3.21 show the distribution of phenotypes in both F2 crosses with regard to median survival time and bacterial counts in the blood 24 hours post-infection. As can be seen in Figure 3.20, F2 mice showed an intermediate median survival time with a spread of phenotypes from susceptible to resistant (median survival time for CCBA F2 was 72 hours and CBAC F2 70 hours). This phenotype was reflected in the number of pneumococci in the blood seen 24 hours post-infection. An intermediate level of bacteraemia (CCBA F2 mean Log CFU/ml of 3.5 and CBAC F2 mean Log CFU/ml of 2.8) was observed. Consistent with the survival data, the blood counts showed a spread from low (resistant phenotype) to high (susceptible phenotype). However, within the F2 reciprocal crosses statistically differences (p<0.0001) were found to each other in both median survival time and mean Log CFU/ml counts 24 hours post-infection. Due to the significant difference in standard deviations between mean Log CFU/ml data, blood counts were analysed by a non-parametric method (two tailed Mann-Whitney U test).

Median survival times of the two groups of control MF1 mice, used alongside the F2 challenge groups, were found to be statistically similar (p>0.05). This analysis of control MF1 mice suggests that the phenotypic difference between both F2 groups, may be a genotypic trait and not due to external differences between challenges. However, MF1 control mice from the CBAC F2 challenge were found to have a significantly (p<0.005) lower numbers of pneumococci in the blood 24 hours post-infection than the control mice from the CCBA F2 challenge.

The scatter graph representation of the data (Figures 3.22 and 3.23) indicates the variation within each F2 cross and between CCBA F2 mice compared to CBAC F2 mice. Plotting the distribution of survival times against percentage survival (Figure
3.24), shows that between 27 and 52 hours less CCBA F2 mice survived. Blood count data, represented by ranking each mouse according to Log CFU/ml counts 24 hours post-infection (Figure 3.25), showed that the CCBA F2 group had mice with numbers of pneumocci in the blood higher than any of the CBAC F2 mice tested. Both these phenotypic differences in survival times and blood count data may have accounted for the calculated statistical difference between CCBA F2 mice and CBAC F2 mice.

The spread of phenotypes seen in both F2 groups again is suggestive of an effect of multiple loci. Due to the statistical difference in phenotypes from both F2 crosses, genetic imprinting from either parental background was possible. However, the results may not reflect this genetic phenomenon, due to the possible variation in challenges as implied by the difference in MF1 blood data, together with data from the previous F1 challenges (section 3.2.1). Ongoing genotyping of F2 tissue samples, from these experiments, will define loci responsible for the susceptibility or resistance to invasive pneumococcal disease in this mouse model. In addition, genotyping will elucidate whether there is any association to sex linkage or genetic imprinting with this genetic trait.
Figure 3.20 Median survival time from the study of CBAC F2 mice (n=223) and CCBA F2 mice (n=251) intranasally challenged with D39 (type 2).

Figures in parentheses illustrate the range above and below the median survival time (MF1 control mice n=44 for the CBAC F2 challenges and n=50 for the CCBA F2 challenges).
Figure 3.21 Numbers of pneumococci in the blood 24 hours post-infection, from the study of CBAC F2 mice (n=223) and CCBA F2 mice (n=251) intranasally challenged with D39 (type 2).

Error bars illustrate the standard error of the mean. (MF1 control mice n=44 for the CBAC F2 challenges and n=50 for the CCBA F2 challenges).
Figure 3.22 Scatter graph of the median survival time from the study of CBAC F2 mice (n=223) and CCBA F2 mice (n=251) intranasally challenged with D39 (type 2).

Each data point refers to one mouse.
Figure 3.23 Scatter graph of the numbers of pneumococci in the blood 24 hours post-infection, from the study of CBAC F2 mice (n=223) and CCBA F2 mice (n=251) intranasally challenged with D39 (type 2).

Each data point refers to one mouse.
Figure 3.24 Distribution of survival time from the study of CBAC F2 mice (n=223) and CCBA F2 mice (n=251) intranasally challenged with D39 (type 2).

To illustrate the distribution, overlapping time points in which both F2 mice strains cross are plotted against the percentage of the total number of mice surviving. Key: (→) CCBA F2 mice and (←) CBAC F2 mice.
Figure 3.25 Distribution of the number of pneumococci in the blood 24 hours post-infection, from the study of CBAC F2 mice (n=223) and CCBA F2 mice (n=251), intranasally challenged with D39 (type 2).

Values represent mice ranked in order of ascending value against Log CFU/ml. Key: (→) CCBA F2 mice and (←) CBAC F2 mice.
3.3 Time course investigations of the number of viable bacteria in the blood and lungs of both CBA/Ca mice and BALB/c mice, following intranasal infection with D39 pneumococci

Further analysis was performed on the parental strains (BALB/c mice selected as resistant and CBA/Ca mice selected as susceptible), to compare bacterial numbers in vivo over time. The type 2 strain D39 was selected for further studies due to a greater separation of BALB/c and CBA/Ca phenotypes than was seen with infection with the type 3 strain GB05. Pre-selected groups of five mice were allocated to each planned post-infection time point. At these times, blood samples were taken and the lungs removed (section 2.5.4 and 2.5.5). For each mouse, a viable count was performed on blood and lung tissue (section 2.4.3).

Initial time course experiments were done using a method previously developed in Leicester University (Canvin et al., 1995). However, this procedure, which involved the removal of blood via cardiac puncture under terminal anaesthesia, was found to be prone to operational error. Blood samples at 0 hours following infection that were taken using this method, were found to contain viable pneumococci which was considered to be due to contamination of the blood. This contamination was thought to be the result of puncturing the lung whilst removing blood from the heart. The method for removal of blood during a time course experiment was subsequently changed. Blood was removed by tail bleeding (section 2.5.5) at each time point, before sacrificing each animal to remove the lungs.

A time course of infection of CBA/Ca mice and BALB/c mice was done using the refined method and the results are illustrated in Figures 3.26 and 3.27. In BALB/c mice, pneumococci were shown to have a different pattern of growth compared to CBA/Ca mice. Following infection, BALB/c mice had an initial level of bacteria in the lungs of 3.46 mean Log CFU/mg post-infection (Figure 3.26). There was no significant change in pneumococcal numbers in the lungs of BALB/c mice over the first 24 hours post-infection. The numbers of pneumococci were found to be 2.79 mean Log CFU/mg at 6 hours post-infection, 2.56 mean Log CFU/mg at 12 post-infection and 3.13 mean Log CFU/mg at 24 hours. After this time numbers declined by 48 hours post-infection to a mean Log CFU/mg count of 1.25. In comparison to 0 hours the numbers of
pneumococci in the lungs at 48 hours were significantly (p<0.001) lower. After this point, bacterial numbers remained significantly lower compared to 0 hours (p<0.001) at both 96 hours (a mean Log CFU/mg count of 0.87) and 168 hours (a mean Log CFU/mg count of 0.45) until 336 hours post-infection. At this time, all five mice examined were found to have no detectable pneumococci in the lungs.

As can be seen in Figure 3.26, in CBA/Ca mice the profile of infection in the lungs was different from BALB/c mice. From an initial level of 3.42 Log CFU/mg following infection, numbers did not change significantly (p>0.05 as compared to 0 hours) by 6 hours (3.40 mean Log CFU/mg) or 12 hours (3.58 mean Log CFU/mg) post-infection. However, by 24 hours a significant increase (p<0.05 compared to 0 hours) in pneumococci was evident. By this time, numbers of viable pneumococci in the lungs of CBA/Ca mice had reached 4.20 mean Log CFU/mg. At 24 hours post-infection, the experiment with CBA/Ca mice was ended. This was because mice were not expected to survive beyond this time given that the median survival time had been determined as 27 hours (section 3.1.1 and 3.1.2).

Post-infection pneumococcal numbers in the peripheral blood for both mice were also found to differ in each murine strain. Following infection, BALB/c mice were found to have no detectable pneumococci in the blood until 12 hours post-infection (mean Log CFU/ml of 0.36) (Figure 3.27). This 12 hour count was due to bacteria being isolated from one of the ten mice tested. Pneumococci were also detected at 24 hours post-infection (mean Log CFU/ml of 0.42). As previously, this viable count was the result of pneumococci in the blood of one out of ten mice. The highest level of pneumococcal numbers in the blood of BALB/c mice was found to occur at 48 hours (mean Log CFU/ml of 2.06), with pneumococci isolated from four out of the ten mice challenged. At 96 hours post-infection pneumococci in the lungs declined to reach a level of a mean Log CFU/ml of 1.40, which was the result of pneumococci in the blood of one out of five mice. After this time point, at both 168 hours and 336 hours post-infection, no pneumococci were isolated from any of the five mice tested.

In contrast to BALB/c mice, following infection CBA/Ca mice were observed to have viable pneumococci in the peripheral blood (p<0.01 as compared to 0 hours), as early as 6 hours post-infection (mean Log CFU/ml of 2.11) (Figure 3.27). After this
time point, numbers of bacteria increased (p<0.001 as compared to 0 hours) to 12 hours post-infection (5.47 mean Log CFU/ml). Pneumococci thereafter, continued to rapidly rise in number, reaching a blood count level of 6.98 mean Log CFU/ml at 24 hours after infection. This increase in pneumococci was found to be significant (p<0.001), compared to 0 hours. After 24 hours, the experiment was not continued with CBA/Ca mice.
Figure 3.26 Time course of the growth of *S. pneumoniae* D39 in the lungs of BALB/c (n=10 except 96 hrs, 168 hrs and 336hrs in which n=5) and CBA/Ca mice (n=5).

Data are the mean of 5 or 10 mice per point with error bars showing the standard error of the mean. *p<0.05, †p<0.001 as compared to time zero. Key: (▲) CBA/Ca mice and (■) BALB/c mice.
Figure 3.27 Time course of the growth of *S. pneumoniae* D39 in the blood of BALB/c (n=10 except 96 hrs, 168 hrs and 336 hrs in which n=5) and CBA/Ca mice (n=5).

Data are the mean of 5 or 10 mice per point with error bars showing the standard error of the mean. *p<0.01, †p<0.001 as compared to time zero. Key: (▲) CBA/Ca mice and (■) BALB/c mice.
3.4 Development of methods to investigate immunological differences in the lungs of parental strains CBA/Ca and BALB/c mice

A number of different methods to investigate the immunological differences between CBA/Ca and BALB/c mice following infection with D39 were studied (sections 2.5 through to 2.10). MF1 mice were used to set up these methods as these mice were found to be reliability reproducible in phenotype from previous experiments (Canvin et al., 1995). Furthermore, they were relatively inexpensive and readily available.

It is important to note that although in this section, a number of these methods (BAL analysis, MPO assay and FACS analysis) were unsuccessful; they provided the necessary steps towards the final method adopted. This final method of analysis of cellular recruitment by homogenisation and enzymic digestion of whole lungs, proved to be successful in the investigation of immunological differences between BALB/c and CBA/Ca mice.

3.4.1 Bronchoalveolar lavage (BAL) to investigate bacterial numbers and inflammatory response following infection with D39 pneumococci in MF1 mice

The first investigation was to look at the effect of D39 infection on the inflammatory response in the lungs using the technique of BAL (section 2.5). This technique was used to set up as an assay to quantify inflammation in the lung; to investigate the number and type of infiltrating leukocytes and to measure bacterial numbers and protein levels in the alveolar spaces.

Groups of five mice were challenged with D39 and at specific time points following infection, mice were sacrificed and BAL performed (section 2.5). Total leukocyte numbers were then enumerated and differential analysis performed using the BAL fluid (section 2.6). Protein measurements (section 2.7) were also made from this lavage fluid to look at the leakage of serum albumin, to be used as an indicator of inflammation in the lung.

Figure 3.28 shows the total numbers of leukocytes calculated per ml of lavage fluid. Recovery from 3 ml of added fluid ranged from approximately 1.4 ml to 2.6 ml. Leukocyte numbers were not found to statistically differ throughout the time course
(p>0.05). Although the numbers of leukocytes in the alveolar spaces were not found to differ, differential analysis shown in Figure 3.29 indicates that the populations of cell types did alter over time. However, due to the small sample size of two mice at each time point statistical analysis could not be achieved. The dominant cell type found at 0 hours was identified as mononuclear leukocytes (alveolar macrophages), with low numbers of both lymphocytes and PMNs; exclusively made up of neutrophils. At further time points, between 0 hours and 12 hours, the populations changed with an increase in PMNs and a decrease in macrophages. At 24 hours to 48 hours both of these cell populations reached a plateau. From the differential analysis, lymphocytes were not found to feature in the population changes during the time course.

Figure 3.30 shows the results generated from the measurement of protein concentration in lavage fluid. Although a trend can be seen in the Figure, with an unexpected decrease in protein concentration, statistical analysis revealed that there was no significant difference (p>0.05) between all the time points from 0 hours through to 48 hours.

![Figure 3.28](image) 

**Figure 3.28** Time course of the total leukocyte count in BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice per point with error bars showing the standard error of the mean.
Figure 3.29 Differential analysis of leukocytes within BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 2 mice per point with error bars showing the standard error of the mean. Key: macrophage (■), lymphocyte (▲) and polymorphonuclear leukocyte (▲).  

Figure 3.30 Time course of the total protein concentration in BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice per point with error bars showing the standard error of the mean.
This experiment was repeated to see whether these data obtained were the true representation of the immunological picture during infection in these mice. A second group of mice was infected and lavage fluid collected. During this experiment, a collection of between 1.9 ml and 2.7 ml, from the added 3ml wash was achieved. Figure 3.31 shows the total number of leukocytes counted per ml of lavage fluid. Total numbers of leukocytes in the lungs of these mice following infection were found not to differ in the earlier time points as was found in the previous experiment; 0 hours to 12 hours were not statistically different (p>0.05). However, the later time points differed between the experiments as the drop in total leukocyte numbers occurring after 12 hours at 24 hours in this experiment, was found to be significantly different (<0.01) compared to 0 hours. No comparison could be made at 48 hours as the mice in this group succumbed to the infection before 48 hours.

Differential analysis of the leukocyte population (Figure 3.32) revealed that primarily cells in the alveolar spaces were mononuclear leukocytes (alveolar macrophages). Following intranasal infection, these leukocytes were found to dramatically decrease from an initial level of a mean number of $1.08 \times 10^5$ macrophages/ml to reach a level of $3.38 \times 10^4$ macrophages/ml by 6 hours (p<0.001). This decrease was also evident at 12 hours (p<0.001 compared to 0 hours). By 24 hours the numbers of cells reached $2.20 \times 10^3$ macrophages/ml (p<0.001 compared to 0 hours). As in the previous experiment, PMNs were found at low numbers at 0 hours (42 PMNs/ml). However, these leukocytes were found to rise in numbers to a peak of recruitment at 12 hours. At this time point the numbers of PMNs in the BAL fluid reached $5.66 \times 10^4$ PMNs/ml (p<0.01 compared to 0 hours). After 12 hours, PMN numbers declined until the final time point at 24 hours (a mean number of $1.18 \times 10^4$ PMNs/ml), when the numbers were the same as at 0 hours (p>0.05). Lymphocytes did not feature in the population fluctuations as was found in the previous experiment. The numbers of lymphocytes at each time point were not found to be significantly different to the numbers at 0 hours (a mean number of $1.05 \times 10^3$ lymphocytes/ml) (p>0.05).

Figure 3.33 shows the protein concentration determined from the lavage fluid at the time points selected. In contrast to the previous experiment, the concentration of protein in the BAL fluid increased post-infection during this experiment and by 12 hours the concentration of protein had risen to a mean value of 121 mg/ml. This was
significantly different (p<0.01) to the mean value of 63 mg/ml at 0 hours. By 24 hours, protein concentration in the BAL fluid (a mean value of 144.20 mg/ml) reached a plateau and was not significantly different to the concentration of protein at 12 hours.

Figure 3.31 Time course of the total leukocyte count in BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice per point with error bars showing the standard error of the mean. *p<0.01 for leukocyte levels as compared to 0 hours.
Figure 3.32 Differential analysis of leukocytes within BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice per point with error bars showing the standard error of the mean. Key: macrophage (■), lymphocyte (▲) and polymorphonuclear leukocyte (▼). *p<0.001 for macrophage levels as compared to 0 hours, †p<0.01 for PMN levels as compared to 0 hours.

Figure 3.33 Time course of the total protein concentration in BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice per point with error bars showing the standard error of the mean. *p<0.01, †p<0.001 for protein concentration as compared to 0 hours.
A further group of mice were infected with D39 to look at the growth of bacteria in the alveolar spaces compared to the lung tissue and to monitor the recovery of pneumococci from BAL fluid. This was undertaken, as recovery differences of pneumococci between the BAL fluid and lung homogenates taken from the same animals would indicate whether the infection was best monitored in the lung tissue and not in the alveolar spaces by using BAL. In addition, analysis of total leukocyte counts and protein concentrations were again performed on BAL fluid, to compare this experiment to the previous experiments carried out.

Figure 3.34 shows the results for the number of pneumococci in the lavage fluid (mean Log CFU/ml) and the lung homogenate (mean Log CFU/mg), together with the total lung CFU count (lavage + homogenate). At time 0 hours, the number of bacteria isolated by lavage was 96.1 +/- 0.4% of the pneumococci in the lungs (mean log CFU/ml of 4.67). Although growth of pneumococci in the lungs was observed (Figure 3.32), statistical analysis indicated that bacterial numbers in the lavage fluid did not change significantly over 48 hours (p>0.05 compared to 0 hours). In contrast to the numbers of pneumococci post-infection in BAL fluid, pneumococcal numbers recovered from the lung homogenate samples increased between 0 hours and 48 hours. Up to 24 hours post-infection numbers of bacteria in the lung homogenate samples were not significantly different to the numbers of pneumococci found at 0 hours (p>0.05). At 48 hours following infection, bacterial numbers had risen from a mean log CFU/ml count of 3.27 at 0 hours to a mean log CFU/ml count of 6.47; a significant increase (p<0.05). On analysis of the total bacterial load in the lungs (BAL + homogenate) the mean number of pneumococci at all the time points measured, was not found to be significantly different (p>0.05) compared to 0 hours. These data indicate that the picture of infection differs between lavage and homogenate. At the start of infection, most of the pneumococci are in the bronchoalveolar air spaces as demonstrated in the BAL fluid counts. Later in the infection, bacteria enter and proliferate in the tissue shown by the homogenate counts. Therefore observing events in the bronchoalveolar air spaces may not give an entire representation of the events occurring in the lungs post-infection.

Figure 3.35 shows the total numbers of leukocytes enumerated in the BAL fluid in this experiment. Statistical analysis revealed that the number of leukocytes in the
BAL fluid did not significantly increase over the time points selected (p>0.05 as compared to 0 hours). This result was seen in the original experiment in this series, although the numbers of leukocytes at the start of this experiment were less than the original experiment (p<0.01). However, leukocyte numbers at 24 hours and 48 hours in this experiment were not lower than the original experiment (p>0.05).

Protein concentration in the lavage fluid in this experiment (Figure 3.36) did not significantly increase (p>0.05) from the start of the experiment at 0 hours (mean value of 62 mg/ml) to 24 hours (mean value of 69 mg/ml). However, after 24 hours the concentration of protein in the BAL fluid samples reached a mean value of 125 mg/ml at 48 hours. This was a statistically significance increase (p<0.05) compared to 0 hours. These data reflect the observations from the previous experiment where an increase in the protein concentration was found to occur following intranasal challenge with D39.

Although BAL provided a means of observing events in the lung and had indicated an influx of neutrophils into the alveolar spaces (figures 3.29 and 3.32), it was not able to show the full immunological picture in the lungs. This was indicated by the discrepancy between all BAL experiments and the recovery of pneumococci from the lungs and BAL fluid. Therefore, further studies were performed to look at neutrophils in the lung tissue as a whole.
Figure 3.34 Time course of the growth of D39 in BAL fluid and lung homogenate samples of MF1 mice.

Data are the mean of 5 mice per point (except 0 and 48 hours n=4) with error bars showing the standard error of the mean. Key: homogenate samples; mean Log CFU counts per mg of tissue (▼), BAL samples; mean Log CFU per ml of fluid (▲) and total (Homogenate + BAL) (■). *p<0.05 for homogenate mean Log CFU counts as compared to 0 hours. Both BAL samples and total (Homogenate + BAL) time points were not significantly different.
Figure 3.35 Time course of the total leukocyte count in BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice (except 0 and 48 hours n=4) per point with error bars showing the standard error of the mean. The means at all time points showed no significant difference to 0 hours.

Figure 3.36 Time course of the total protein concentration in BAL fluid samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice (except 0 and 48 hours n=4) per point with error bars showing the standard error of the mean. *p<0.05 for protein concentration as compared to 0 hours.
3.4.2 Development of a myeloperoxidase assay to investigate neutrophil recruitment

Following the observation in the BAL experiments (section 3.4.1), that neutrophils were recruited into the lungs, the phenomenon was investigated further. Experiments were designed to quantify the neutrophil response in the whole lung tissue during a time course of infection with D39. Initially, experiments were set up in the MF1 mouse model of infection for reasons described previously (section 3.4).

Myeloperoxidase (MPO) is an abundant enzyme found in the granules of neutrophils (Bretz et al., 1974) which has been used before to quantify of neutrophils in different tissues (Bradley et al., 1982). In these experiments, an adaptation of a fluorescent assay (section 2.8) for a similar enzyme, horseradish peroxidase (HRP) (Jackett et al., 1981) was used. This assay quantifies the amount of enzyme via oxidation of the substrate p-hydroxyphenyl acetic acid (POHAA) by H$_2$O$_2$ and measuring the fluorescence of the end product. Myeloperoxidase was extracted from lung tissue (section 2.8.2) by a modification of the methods of Shayevitz et al., (1995) and Bradley et al., (1982). Extracts of lung homogenate were analysed in parallel with standard curves generated with HRP.

A standard curve of HRP concentration against fluorescence was generated for concentrations of 0 to 0.025 units/ml of HRP. These values were chosen as an initial study (not shown) indicated that a concentration above 0.025 units/ml of HRP did not give a linear relationship in this assay. Using this standard curve, the amount of myeloperoxidase from a frozen lung sample obtained from an MF1 mouse intranasally infected with D39 was calculated. This was done to observe whether the concentration of myeloperoxidase from an infected mouse (which would have high numbers of neutrophils in the lungs) lay within the standard curve and if it would be necessary to adjust the standard concentration ranges. Figure 3.37 shows the standard curve from this experiment with the concentration of a duplicate sample of infected tissue.

The amount of enzyme extracted from the infected tissue sample was found to lie within the enzyme concentration range where the assay was linear (0.0046 units/ml). The standard range from 0 to 0.025 units/ml of HRP was set for further experiments.
Figure 3.37 A standard curve of fluorescence versus HRP concentration.

Lines on the axes indicate where the fluorescence of an unknown quantity of MPO, from an MF1 mouse lung tissue sample, intercepts with the standard curve. Unknown sample enzyme units were calculated by linear regression. Each point represents the average of two fluorescence readings.

From the above experiment, the amount of MPO was found to lie on the lower values of the standard curve. To increase the amount of MPO detected and therefore move the fluorescence values into the centre of the standard curve, an additional experiment was done to ascertain the effect of increasing the volume of homogenisation buffer and the amount of homogenate added to the reaction mixture. In this experiment, the homogenisation was performed in 10ml of buffer (compared to 6ml previously), and the sample volume added to the reaction mixture was increased to 900μl (compared to the previous experiment of 50μl of homogenate sample). To compensate for this increase in sample volume, standard HRP samples were analysed with the same buffer concentrations as the homogenate samples tested (50μl of appropriate enzyme concentration added with 850μl of buffer). Duplicate lung samples from two infected MF1 mice were compared to a standard curve generated with the same concentrations of HRP used in the previous experiment (0 to 0.025 units/ml).

Both lung homogenate samples were found to be below the fluorescence for 0 units of HRP from the standard curve (Sample 1: 0.91, sample 2: 1.9 compared to 3.8
for 0 units of HRP). It was concluded that the homogenate might be quenching fluorescence.

To investigate this phenomenon, the effect on fluorescence of varying the amount of homogenate was examined. The amount of homogenate added was varied between 50μl and 700μl, with all other components and conditions remaining the same. In addition, a comparison between MF1 lung tissue from an uninfected mouse and an infected mouse was also made, to see whether this quenching effect differed when using uninfected versus infected lung tissue.

The data shown in Table 3.3 of results confirm this quenching effect. Increasing the amount of homogenate inversely decreases the fluorescence of the samples. This was found to be more pronounced with amounts above 100μl of homogenate. From this experiment, it was observed that homogenate from infected lungs had a greater amount of MPO than homogenate from uninfected lungs as judged by a greater fluorescence value. However, both uninfected and infected homogenate samples were found to be equally susceptible to the quenching of fluorescence, when the amount of homogenate added was increased.
### Table 3.3
The effect of varying the amount of homogenate on fluorescence using both homogenate lung samples from an uninfected and infected MF1 mouse.

<table>
<thead>
<tr>
<th>Samples and amounts:</th>
<th>Average Fluorescence value</th>
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</thead>
<tbody>
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<td>Standards – 0 units/ml</td>
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<tr>
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<tr>
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<tr>
<td>Infected lung sample 100μl homogenate</td>
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<td>Uninfected lung sample 700μl homogenate</td>
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</tbody>
</table>

Results are expressed as average fluorescence values from duplicate samples.
Further experiments were done, to define the cause of this fluorescence quenching. Firstly, an experiment to determine whether the concentration of detergent in the reaction mixture (used to solublise MPO from the lung homogenate tissue) may play a role in interfering with the fluorescence generated was performed. Conditions were as in the previous experiments, apart from the detergent and its concentration. Triton X100, used in previous reports to extract MPO (Metcalf et al., 1986) was compared to hexadecyl-trimethyl ammonium bromide (HTAB) used previously in MPO experiments done here. To the reaction mixtures containing 0.025 units/ml of HRP, amounts of both detergents were added to give final concentrations for comparison for Triton X100 of 0.05% v/v and 0.5% v/v, and for HTAB of 0.5% w/v and 5% w/v.

Table 3.4 shows the results from this investigation. A slight quenching of fluorescence can be observed with an increase in the concentration of each detergent but the fluorescence values were actually higher with both types of detergents than the control with no detergent added. The effect on the fluorescence of either detergent did not match with the previous experiments with homogenate added. It therefore was concluded that there was no interference with either detergents and that the quenching observed when homogenate is added to the reaction mixture is independent of the effect of detergent.
Table 3.4 Analysis of the effect of the amount and type of detergent used to solubilise MPO from lung tissue on fluorescence measurement in the assay to determine the amount MPO in lung tissue samples.

Results are the average fluorescence values from duplicate samples.
To try to overcome the quenching effect of the homogenate, an experiment was performed to test the effect of increasing the reaction time from 10 minutes to 30 minutes. This was done to see whether a longer incubation might increase the fluorescence signal in the reaction over the interference produced by the homogenate. Experimental conditions were kept the same apart from the homogenisation volume and H$_2$O$_2$ concentration (adjusted to 0.85mM in the reaction mixture as was indicated in Metcalf et al., 1986). To observe what effect the concentration of homogenate may play in the reaction, homogenising of lung tissue was performed in a Dounce homogeniser (F.T. Scientific Instruments Limited) in 1ml and 6ml volumes (6ml was found to be the maximum volume allowable). This homogeniser was used because it was found to be more efficient in homogenisation the lung tissue in these volumes than a Stomacher Lab Blender 80 (Seward Medical). Additionally, infected and uninfected lung samples were also compared in this experiment.

Table 3.5 shows the results for this investigation. The effect of increasing the reaction time shows an increase in the fluorescence of the HRP standards. Although an increase in fluorescence was observed with infected over uninfected lung tissue, increasing the reaction time did not increase the level of fluorescence in both samples. The 1ml samples compared to the 6ml samples showed a greater fluorescence as expected from previous experiments. However, no increase was seen over a reaction time of 30 minutes in either 1ml or 6ml samples with both uninfected and infected homogenate lung samples. This indicated that MPO was possibly not active or was not detectable in any of the homogenate samples tested beyond 10 minutes incubation time.
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<th>Sample</th>
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</tbody>
</table>

**Table 3.5** Analysis of the effect of increasing the reaction time and the amount of homogenate on fluorescent measurement in the assay to determine the amount MPO in lung tissue samples.

Results are expressed as average fluorescence values from duplicate samples.
From these data, it was decided to examine the possibility of an inhibition effect from the homogenate on the activity of the enzyme MPO. To investigate this, HRP was added to homogenate samples to monitor the interference of enzyme activity by the homogenate. In addition, the effect on the reaction of the exclusion of some of the assay components was also done. Two HRP concentrations were selected for this experiment; 0.005 units/ml and 0.025 units/ml. Addition of HRP to 50μl homogenate samples was used to monitor the interference of enzyme activity by the homogenate. Controls were included with or without enzyme, substrate or hydrogen peroxide. All other experimental conditions remained the same as in previous experiments.

Table 3.6 shows the results for this experiment. Interestingly, the addition of HRP to the reaction mixture increased the level of fluorescence at both HRP concentrations. Therefore, the quenching phenomenon seen with the addition of lung homogenate was not due to an inhibition effect on enzymic activity. In addition, fluorescence was only seen in the presence of homogenate as in previous experiments, with both substrate and H₂O₂. These results indicate that the homogenate solution itself does not interfere with the enzyme activity in this assay. Possible problems with the pre-treatment of the tissue, which renders the enzyme inactive or other external factors may account for the lack of activity based upon MPO extracted from infiltrating lung neutrophils in this assay.
Table 3.6 Observations on the addition of HRP to homogenate samples and exclusion of assay components on fluorescent measurement in the assay to determine the amount MPO in lung tissue samples.

Results are expressed as average fluorescence values from duplicate samples.

<table>
<thead>
<tr>
<th>Samples:</th>
<th>Average Fluorescence value after 10 minutes incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No HRP + substrate + H₂O₂</td>
<td>3</td>
</tr>
<tr>
<td>No HRP + 50μl homogenate + no substrate + no H₂O₂</td>
<td>1</td>
</tr>
<tr>
<td>No HRP + 50μl homogenate + substrate + no H₂O₂</td>
<td>1</td>
</tr>
<tr>
<td>No HRP + 50μl homogenate + substrate + H₂O₂</td>
<td>39</td>
</tr>
<tr>
<td>HRP (0.005 units/ml) + 50μl homogenate + substrate + H₂O₂</td>
<td>42</td>
</tr>
<tr>
<td>HRP (0.025 units/ml) + 50μl homogenate + substrate + H₂O₂</td>
<td>86</td>
</tr>
<tr>
<td>HRP (0.025 units/ml) + 50μl buffer/detergent + substrate + H₂O₂</td>
<td>97</td>
</tr>
</tbody>
</table>
The final test of the MPO assay was to assess the affect of storage of the tissue on enzyme activity. The results for this experiment are shown in Table 3.7. No difference was found between freshly prepared lung tissue and frozen stored tissue. As observed previously, the activity of the homogenate did not change between the two reaction times tested (10 and 30 minutes). In comparison, it can be seen that increasing reaction time in the HRP control samples increases the overall fluorescence detected.

From these series of experiments, a reliable fluorescence MPO assay was not achieved. Intrinsic chemical activity of the homogenate may have accounted for the observed fluorescence and not enzyme activity. Further investigation continued into finding reliable methods that could be used to observe cellular recruitment into the lungs.

<table>
<thead>
<tr>
<th>Samples:</th>
<th>Average Fluorescence value after 10 minutes incubation</th>
<th>Average Fluorescence value after 30 minutes incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no HRP)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Control (0.025 units/ml of HRP)</td>
<td>364</td>
<td>555</td>
</tr>
<tr>
<td>Frozen lung sample</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Fresh lung sample</td>
<td>28</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 3.7 Analysis of the effect of fresh versus frozen homogenate samples on fluorescent measurement in the assay to determine the amount MPO in lung tissue samples.

Results are expressed as average fluorescence values from duplicate samples.
3.4.3 Development of flow cytometry to investigate cellular influx in both lung and blood

Following the attempts to set up a reliable MPO enzyme assay, it was decided to look at alternative methods to further examine neutrophil recruitment into the lungs. Flow cytometry (FACS analysis) of lung homogenates was investigated because of the high through-put and the versatility that this technique has over more conventional methods.

Initially the analysis of murine blood was done for leukocytes to set up the conditions for FACS analysis. Blood was analysed rather than homogenate samples because these homogenate samples would be contaminated with other cell types. Leukocytes in the blood following the lysis of erythrocytes would be clearly segregated into distinct populations, that could be easily identified via fluorescence from labelled marker antibody and, from size and granularity of the cells.

A blood sample was removed by cardiac puncture (section 2.4.1) of an MF1 mouse. Blood cells for FACS analysis were prepared and analysed via a FACScan by standard protocols (section 2.10). Blood cell samples were separately labelled with CD45 and GR-1 conjugated monoclonal antibodies (section 2.10). Once in the acquisition mode of the FACScan during real time operation, parameter settings were adjusted so that all leukocytes could be seen in both forward scatter and side scatter axes. These settings were then subsequently used for all FACS experiments, including lung homogenate cell suspensions.

Once settings and conditions were set for blood samples, an experiment was set up to compare flow cytometry of blood samples with that of lung samples. A blood sample from an uninfected MF1 mouse was prepared as previously described. Vascular perfusion of the lungs was also compared with removal of lungs without this treatment (section 2.9.1). Lung cell suspensions were made by homogenisation and enzyme digestion of lung tissue (section 2.9.1). With both blood and lung samples, cell samples were separately labelled (section 2.10) with monoclonal antibodies to CD45, CD45 + F4/80 and CD45 + Gr-1.

Following acquisition of the cell data from the blood sample, analysis showed a
segregation pattern of the three leukocyte cell populations (Figure 3.37). Using the gating functions available in the FACS analysis software, a gate was set around all the leukocytes. Fluorescence in this region (R1) was analysed via histograms drawn of fluorescence (channels FL-1 and FL-2) against cell counts, for each marker. From Figure 3.37 it can be seen that most of the cells in this region were confirmed as leukocytes (74.9% CD45 positive calculated from the histogram in Figure 3.37 entitled CD45 (FL-2)). Gr-1 positive cells constituted 21.1% (calculated from the histogram in Figure 3.37 entitled Double stained CD45 + Gr-1 (FL-1)) of the total cell population in this region, thus indicating the percentage of neutrophils in the blood of an uninfected MF1 mouse.

F4/80 monoclonal antibody was included as it binds to an antigen on the surface of mouse macrophages (Austyn and Gordon 1981). Although detectable amounts of F4/80 antigen have been shown to be found in the blood of mice, it is not of a measurable quantity (Lee et al., 1985). In this experiment, some cells were shown to be F4/80 positive (3.4%) calculated from the histogram in Figure 3.37 entitled Double stained CD45 + F4/80 (FL-1). This may indicate the possibility of a background level of staining.

Further examination of the FACS data revealed the location of these cells within three regions visible from dot plot analysis (Figure 3.38). A gate was set around each of these regions shown in the dot plot representing side scatter plotted with forward scatter, labelled as R2, R3 and R4 (Figure 3.38). Dot plots of each of these regions were then plotted with the fluorescence of the CD45-PE marker (FL-2) against the FITC markers F4/80 or Gr-1 (FL-1) (Figure 3.38). Comparison of the dot plots in Figure 3.38 entitled Blood cell control and Double stained CD45 + Gr-1 reveals a shift in the distribution of cells along the fluorescence measurement axes, when positive with either CD45 or both CD45 + Gr-1 antibodies. When comparing each of the dot plots from each of the regions (R2, R3 and R4), the area indicated as region 2 (R2) contains the majority of Gr-1 stained cells (represented by the dot plot entitled Double stained CD45 + Gr-1 (R2) in Figure 3.38). However, some Gr-1 positive cells were also located in region 3 (R3) (represented by the dot plot entitled Double stained CD45 + Gr-1 (R3) in Figure 3.38), though in smaller numbers than in region 2 (R2).
Comparison of the dot plots in Figure 3.38 entitled Blood cell control and Double stained CD45 + F4/80 reveals a shift in the distribution of cells along the fluorescence measurement axes, when positive with either CD45 or both CD45 + F4/80 antibodies. Examination of the dot plots from each of the regions (R2, R3 and R4) indicated that the F4/80 positive cells detected in this experiment were found to be located in gated region (R2) (represented by the dot plot entitled Double stained CD45 + F4/80 (R2) in Figure 3.38).

For the purposes of comparison, the gated regions set for blood samples were used in the analysis of prefused and non-prefused lung samples. In the lung homogenates, as seen in Figure 3.39, the cells were not found in the identifiable pattern previously observed for blood leukocytes. However, analysis of the fluorescence of these non-perfused lung cells revealed that within the region set to contain leukocytes, by the parameters set by blood samples, a high percentage (67.5%) of CD45 positive cells are found (calculated from the histogram in Figure 3.39 entitled CD45 (FL-2)). Analysis of Gr-1 and F4/80 positively stained cells indicates that within this region 20.1% of neutrophils are present with 14.8% of mature macrophages (calculated from the histograms in Figure 3.39 entitled Double stained CD45 + Gr-1 and Double stained CD45 + F4/80). However, the result for Gr-1 showed an unexpected high percentage of positively stained cells (20.1%), as the majority leukocyte found in uninfected lung tissue would be expected to be the macrophage (F4/80 positive cells).

Dot plots showing fluorescence in both channels (FL-1 and FL-2) using the regions indicated for blood leukocytes (regions 2, 3 and 4), were used to locate Gr-1 and F4/80 positive cells (Figure 3.40). Dot plots in Figure 3.40 entitled Blood cell control, Double stained CD45 + Gr-1 and Double stained CD45 + F4/80 reveal a shift in the distribution of cells along the fluorescence measurement axes, when positive with CD45/CD45 + F4/80 or CD45 +Gr-1 antibodies. Examination of the dot plots from each of the regions (R2, R3 and R4) indicated that both F4/80 and Gr-1 positive cells detected in this experiment were found to be located in gated region (R2) (represented by the dot plots entitled Double stained CD45 + F4/80 (R2) and Double stained CD45 + F4/80 in Figure 3.40). This was similar to the analysis of blood leukocytes (Figure 3.38). Therefore, for analysis of all subsequent FACS analysis gating was made using blood samples to identify the region enclosing all of the leukocytes (R1).
Vascular perfusion was preformed so that leukocytes identified would be from within the lung tissue without interference from peripheral blood leukocytes. When comparing perfused to non-perfused lungs a small difference between these samples was observed (Figure 3.39; 67.5% of cells where CD45 positive in the non-perfused sample compared to 59.2% of cells in the perfused sample). These percentages were calculated from the histograms in Figure 3.39 entitled CD45 (FL-2) and CD45 (FL-2) (prefused lung sample). For further experiments, it was decided to continue to perfuse the lungs with buffer prior to extraction, to elevate any effect that leukocytes from the pulmonary circulation may have on homogenised whole lung samples.
Figure 3.37 Dot plot and histogram data from the flow cytometry of an MF1 mouse blood sample.

R1 represents gated region 1. M1=marker set for threshold level of fluorescence. M2=marker set for sample fluorescence detection. FSC=forward scatter, SSC=side scatter, FL1=FITC detected fluorescence, FL2=PE detected fluorescence.
**Figure 3.38** Dot plot data showing stained leukocytes in different regions from the flow cytometry of an MF1 mouse blood sample.

R1, R2, R3, R4 represent gated regions 1, 2, 3 and 4. FSC=forward scatter, SSC=side scatter, FL1=FITC detected fluorescence, FL2=PE detected fluorescence.
Figure 3.39 Dot plot and histogram data from the flow cytometry of MF1 mice lung samples, comparing both non-vascular prefused and vascular prefused lungs.

R1 represents gated region 1. M1=marker set for threshold level of fluorescence. M2=marker set for sample fluorescence detection. FSC=forward scatter, SSC=side scatter, FL1=FITC detected fluorescence, FL2=PE detected fluorescence.
Figure 3.40 Dot plot data showing stained leukocytes in different regions from the flow cytometry of a non-vascular prefused MF1 mouse lung sample.

R1, R2, R3, R4 represent gated regions 1, 2, 3 and 4. FSC=forward scatter, SSC=side scatter, FL1=FITC detected fluorescence, FL2=PE detected fluorescence.
Following the results of the previous experiment, flow cytometry of lung cells from MF1 mice was further investigated. Cell samples were generated from lung tissue (section 2.9.1) taken from an animal that was infected with D39 pneumococci and one that was uninfected. Analysis was performed on these lung cell samples, together with blood cell samples from an infected MF1 mouse.

In this experiment a pan B-cell and pan T-cell marker was included (CD19 and CD3), along with isotype controls IgG2B (FITC) and IgG2B (PE), together with the previous markers Gr-1, F4/80 and CD45. The results from this experiment are shown in Table 3.10.

Comparing infected versus uninfected lung samples it can be seen from Table 3.8 that the cell populations shift in proportion. Both samples show a high level of CD45 positive cells, with an increase in the levels of both B cells and neutrophils in the infected sample. Macrophages and T cells were however, found to fall in percentage compared to the uninfected sample. Noteworthy is the low level of F4/80 positive cells in uninfected lung tissue.

The effect of gating can be seen in both infected and uninfected lung samples (Table 3.8). In the ungated analysis, a lower percentage of cells were stained positively for CD45 and the other cell markers, compared to analysis using a gate. This was expected due to the fact that not using gates would include more non-leukocyte lung cells in the analysis.

The gate used for analysis of both infected and uninfected lung samples was set with a sample of blood cells. With this infected blood sample, neutrophils make up a higher percentage (55.3%) of cells than seen with a sample from uninfected animal (21.1%) (Figure 3.37). However, no other comparisons were made between infected and uninfected blood as these samples were primarily used for setting the gating region for lung samples. Interestingly, B cells in the infected blood sample were found only to be slightly higher than the percentage proportion of T cells, in contrast to the major differences found in the infected lung sample.

Isotype matched controls of both PE and FITC labelled antibody were included in both blood and lung samples to show the level of background staining (Table 3.8). In
all of the samples, a higher level of background staining was observed with isotype control FITC labelled antibody. In addition, background staining with either isotype control (PE or FITC labelled antibody) was greatest in the uninfected lung sample.

Cytocentrifugation was also performed on homogenate lung cell samples isolated for FACS analysis. This was done to compare the data from the two techniques. Table 3.9 shows the percentages of the cell populations in infected and uninfected lung samples. These data show that the majority of leukocytes found in the lungs of uninfected MF1 mice, are macrophages. Lymphocytes were the next most abundant cell type, then PMNs (which were mostly neutrophils). In contrast, in the infected sample, the PMN population was found to dominate, with both macrophage and lymphocyte populations found at a similar percentage. Although FACS analysis did show population shifts in neutrophils, observation of cytocentrifuged slides indicated that the numbers of macrophages were possibly misrepresented in the FACS experiments.
Table 3.8 Comparison of the flow cytometry of infected and uninfected lung leukocytes showing the effect of using the gating function selected around blood leukocytes to analyse lung leukocytes. ND = not done.
<table>
<thead>
<tr>
<th>Cells:</th>
<th>Lung leukocytes (infected) % of total cell population</th>
<th>Lung leukocytes (uninfected) % of total cell population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>24.9</td>
<td>53.3</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>22.3</td>
<td>34.4</td>
</tr>
<tr>
<td>Polymorphonuclear leukocytes (mostly neutrophils)</td>
<td>52.8</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Table 3.9 Differential leukocyte analysis from cytocentrifuged cell samples prepared for FACS analysis.

Cells were Giemsa-stained and observed under 400x magnification. A total of at least 200 cells was counted on each slide.

A limited experiment was done because of the expense of reagents, to alter the digestion conditions, to reduce the amount of collagenase and RPMI tissue culture medium used in the homogenisation. Two lungs from MF1 mice were homogenised in a digestion buffer of: 1mg/ml collagenase with 30 μg/ml Dnase in a total volume of 15ml of 5% v/v FBS in RPMI 1640 medium. Another two MF1 mice lungs were homogenised in a digestion buffer of: 0.5mg/ml of collagenase with 30 μg/ml of Dnase in a total volume of 5ml of 5% v/v FBS in RPMI 1640 medium. From Figure 3.10 it can be seen that the effect on total cell number was negligible. From this point onwards the digestion conditions were changed to a reaction volume of 5ml and collagenase concentration of 0.5mg/ml.
Table 3.10 The effect of changing the digestion conditions on the recovery of lung leukocytes from MF1 mice.

Following the previous experiments, it was decided to investigate for one last time, the differential analysis by FACS analysis of the lung samples generated by the homogenisation and enzyme digestion method. In addition, the influx of leukocytes in the lung samples was measured by a total cell count.

Following the experience of the previous experiments, gates and histogram markers were set using blood samples. Groups of five mice were intranasally infected with D39 pneumococci. At selected time points (0, 6, 12, 24, and 48 hours) mice were humanely sacrificed, blood samples taken and lungs perfused before removal; cell samples were then generated. Total cell numbers were enumerated and cell samples were analysed by flow cytometry using fluorescent conjugated cell markers: F4/80, Gr-1, CD3, CD19, CD45 with isotype controls IgG2B (FITC), IgG2B (PE). The results from this experiment are shown in Figures 3.41 to 3.44. They show the total cell numbers throughout the time course of infection in the MF1 mice and the analysis of the

<table>
<thead>
<tr>
<th>Digestion conditions:</th>
<th>Total cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Total reaction volume 15ml with 1mg/ml Collagenase+ 30μg/ml Dnase</td>
<td>1.54 x 10^6</td>
</tr>
<tr>
<td>2. Total reaction volume 15ml with 1mg/ml Collagenase+ 30μg/ml Dnase</td>
<td>1.38 x 10^6</td>
</tr>
<tr>
<td>3. Total reaction volume 5ml with 0.5mg/ml Collagenase+ 30μg/ml Dnase</td>
<td>1.22 x 10^6</td>
</tr>
<tr>
<td>4. Total reaction volume 5ml with 0.5mg/ml Collagenase+ 30μg/ml Dnase</td>
<td>1.82 x 10^6</td>
</tr>
</tbody>
</table>
leukocyte populations by FACS analysis.

From the Figure 3.41 it can be seen that from the start of the infection there is an identifiable increase in leukocyte numbers until termination of the experiment at 48 hours. Total leukocytes were found to significantly rise to a mean of $1.47 \times 10^6$ leukocytes per ml at 48 hours post-infection ($p<0.05$ as compared to 0 hours; mean number of $9.06 \times 10^5$ leukocytes/ml).

The control blood samples run in parallel with lung homogenate samples, for the purposes of gating lung leukocytes, showed an increase in neutrophils post-infection. From Figure 3.42 it can be seen that within the total population of cells a high percentage of these were CD45 positive. Background staining with both isotype controls was low, although FITC labelled IgG2B showed a higher percentage of positive cells than PE labelled IgG2B. In contrast to blood, lung cells incubated with the FITC labelled IgG2B showed a high percentage of staining (Figure 3.43). This indicates a high level of background staining, although contrary to this, lung cells incubated with PE labelled IgG2B showed a less staining (Figure 3.43). Lung cells were found to have a high percentage of CD45 positive cells (Figure 3.43).

On examination of the leukocyte populations post-infection in MF1 lung tissue by FACS analysis, all types of leukocytes were found to decrease over time (Figure 3.44). Neutrophils (Gr-1 positive cells) at 0 hours were the lowest percentage leukocyte type. Although the overall level of these cells dropped by 48 hours, they reached a higher percentage of the total than macrophages (F4/80 positive cells) and T cells (CD19 positive cells) at 48 hours. At both 0 hours and 48 hours, the highest proportion of cells above all the other leukocytes was found to be B cells (CD 3 positive cells).

Overall, lung cell FACS analysis experiments suffered from two inaccuracies: background staining, which varied between experiments (Table 3.8, Figure 3.43) and an inheritant difficulty in precisely setting a marker for the threshold level of fluorescence and sample fluorescence detection (Figure 3.39). Therefore, although these data indicate that a high proportion of cells were found to be leukocytes by CD45 staining (Figure 3.39, Table 3.8, Figure 3.43), differential analysis of the leukocyte populations by FACS analysis in these experiments, may not have reliably measured the change in cell populations post-infection.
**Figure 3.41** The total leukocyte count in lungs from MF1 mice following infection with D39 pneumococci.

Data are the mean of five mice per time point. Error bars illustrate the standard error of the mean.
Figure 3.42 Flow cytometry of MF1 blood leukocytes taken over time following infection with D39 pneumococci.

Data are the mean of five mice per time point. Error bars illustrate the standard error of the mean. Key: IgG2B-PE isotype control (▼), IgG2B-FITC isotype control (■), GR-1 (▲) and CD45 (●).
Figure 3.43 Flow cytometry of MF1 lung cells taken over time following infection with D39 pneumococci.

Data are the mean of five mice per time point. Error bars illustrate the standard error of the mean. Key: IgG2B-PE isotype control (▼), IgG2B-FITC isotype control (■), and CD45 (◆).
Figure 3.44 Flow cytometry of MF1 lung cells taken over time following infection with D39 pneumococci.

Data are the mean of five mice per time point. Error bars illustrate the standard error of the mean. Key: Gr-1 (■), F4/80 (▲), CD3 (▼) and CD19 (●).
3.4.4 Development of lung homogenisation and enzyme digestion to investigate immunological cellular responses

Due to the problems experienced with FACS analysis of lung homogenate cells it was decided to explore other avenues to examine the immunological response in the lungs following infection with the pneumococcal strain D39. From the FACS analysis experiments, it was shown that a sufficient number of cells were generated from homogenisation and enzyme digestion of lung tissue samples for analysis (in contrast to BAL). These cells also were found to be mostly CD45 positive and post-infection were found to increase in numbers (section 3.4.3). Hence, the approach of using the enzyme digestion and homogenisation procedure developed in the FACS analysis was adopted. This methodology was also combined with counting the total number of cells by haemocytometer and performing differential analysis by counting stained cytocentrifuged cell samples.

Initially the above methodology was tried with a time course experiment using MF1 mice, to develop upon the results generated from section 3.4.3 (Figure 3.41) and to set up the methodology before analysing the resistant and susceptible inbred mice. This experiment was done in collaboration with Kate Grattan and Dr Aras Kadioglu (Kadioglu et al., 2000). Groups of five mice were infected with D39 pneumococci and at the selected time points of 0, 6, 12, 24 and 48 hours mice were humanely sacrificed. Lungs were removed following perfusion and cells were obtained as described in section 2.9. The total number of cells from each lung sample was enumerated and differential analysis was performed on cytocentrifuged cell samples (section 2.9.2).

The results for this experiment are shown in Figures 3.45 and 3.46 for the total leukocyte count and differential cell count respectively. Observation of the recruitment of leukocytes shows that following intranasal infection in the outbred murine strain MF1, total leukocyte numbers increased. From a mean number of $1.55 \times 10^6$ leukocytes per ml at 0 hours, pulmonary inflammatory cells remained relatively unchanged by 6 hours post-infection (a mean number of $1.54 \times 10^6$ leukocytes per ml). However, after this time, the number of leukocytes in the lungs increased and by 24 hours had reached a mean number of $2.53 \times 10^6$ leukocytes per ml. At 48 hours, the mean number of inflammatory leukocytes in the lungs was $2.64 \times 10^6$ per ml. Statistical analysis using
ANOVA showed that overall, the mean number of leukocytes at all of the time points were significantly different (p<0.05). However, it must be noted that the tukey post test revealed no statistical difference between the mean number of leukocytes at individual time points (6, 12, 24 and 48 hours), compared to the mean number of leukocytes at 0 hours.

Differential analysis showed no difference in the influx of macrophages during the time course of infection (Figure 3.46). These leukocytes were found not to significantly change from the base level at 0 hours of a mean number of 6.82x10⁵ macrophages per ml. The macrophage was the majority cell type in the lungs at this time point accounting for 46.7 +/- 7.6% of the total leukocyte cell population. Lymphocytes also were found not to rise significantly above the level at 0 hours (a mean number of 5.64x10⁵ lymphocytes per ml) accounting for 35.0 +/- 4.7% of the total leukocyte cell population. However, analysis of the level of infiltrating polymorphonuclear leukocytes (PMNs) showed that numbers of these cells increased significantly during the time course of infection. At 24 hours, the mean number of PMNs rose from 3.04x10⁵ per ml at 0 hours, accounting for 18.2 +/- 3.3% of the total leukocyte cell population, to a peak of 1.41x10⁶ per ml (p<0.001 compared to 0 hours). By 48 hours, the numbers of PMNs had dropped in the lungs of MF1 mice, reaching a level of a mean number of 1.34x10⁶ PMNs per ml. At this time point the numbers of PMNs were still significantly higher than compared to 0 hours (p<0.01).
**Figure 3.45** The total leukocyte count in digested lung samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of five mice per time point, except 48 hours, which is from three mice. Error bars are the standard error of the mean.
Figure 3.46 Differential analysis of leukocytes within digested lung samples from MF1 mice following infection with D39 pneumococci.

Data are the mean of 5 mice (except 48 hours which is the mean of 3 mice) per point. Error bars are the standard error of the mean. Key: macrophage (■—), lymphocyte (▲—) and neutrophil (▼—).
3.5 Cellular recruitment into the lungs following infection of BALB/c mice and CBA/Ca mice with *S. pneumoniae* strain D39 (type 2)

Following the results generated using MF1 outbred mice (section 3.4.4) the same methodology was used to examine cellular recruitment into the lungs in both susceptible and resistant inbred murine strains. Groups of five BALB/c mice and CBA/Ca mice were taken at each time point following intranasal infection. Lungs were removed and the total number and differential analysis of leukocytes evaluated (section 2.9).

3.5.1 Analysis of the total number of leukocytes from homogenised lungs of BALB/c mice and CBA/Ca mice post-infection

Figure 3.47 shows the results for the total number of leukocytes enumerated in lungs of both murine strains following infection with D39 pneumococci. Susceptible CBA/Ca mice were analysed only until 24 hours post-infection due to this murine strain's calculated median survival time of 27 hours (section 3.1.2). BALB/c were analysed until 336 hours post-infection as this strain was found to have a median survival time of >168 hours (section 3.1.2).

Following infection with the type 2 pneumococcus, cellular recruitment was found to increase in the CBA/Ca mice. From a mean base level of $8.80 \times 10^5$ leukocytes per ml at 0 hours, CBA/Ca mice recruited significantly more pulmonary inflammatory leukocytes following infection. At 12 hours post-infection, the level of leukocytes in the lungs of CBA/Ca mice reached a mean number of $1.39 \times 10^6$ per ml. These cells continued to rise after 12 hours, reaching a level of statistical significance at 24 hours ($p<0.01$ compared to 0 hours) with a mean number of $1.71 \times 10^6$ leukocytes per ml, shortly before the animals became moribund.

With BALB/c mice following intranasal infection with the type 2 pneumococcus, the pattern of recruitment also was found to change post-infection. At 6 hours (a mean number of $1.62 \times 10^6$ leukocytes per ml) the number of leukocytes in the lungs was not significantly different to the level at 0 hours (a mean number of $1.32 \times 10^6$ leukocytes per ml). However, at 12 hours (a mean number of $2.37 \times 10^6$ leukocytes per ml) the number of leukocytes infiltrating into the lungs was significantly different ($p<0.001$) to 0 hours. This recruitment continued with an increase in cell numbers to a
mean number of $2.76 \times 10^6$ leukocytes per ml at 24 hours post-infection ($p<0.001$ compared to 0 hours). At 48 hours, the influx of pulmonary inflammatory leukocytes reached a peak, with a level of a mean number of $3.96 \times 10^6$ leukocytes per ml ($p<0.001$ compared to 0 hours). After this time point the cellular response in the lungs of BALB/c mice changed, with a decrease in the numbers of total leukocytes present in the lungs (a mean number of $2.65 \times 10^6$ leukocytes per ml at 168 hours). By 336 hours this decrease had continued, as the level of cells had dropped to a mean number of $2.34 \times 10^6$ leukocytes per ml. Interestingly, the number of the leukocytes was observed to be still significantly greater than that found at 0 hours ($p<0.001$).

BALB/c mice had a significantly different pattern of cellular recruitment compared to CBA/Ca mice. Statistical analysis revealed that BALB/c mice had significantly greater numbers of leukocytes than CBA/Ca mice at 0 hours ($p<0.005$), 12 hours ($p<0.005$) and 24 hours ($p<0.01$) post-infection. Analysis of the rate of recruitment of leukocytes, calculated from the total leukocyte data and the time points taken, showed that BALB/c mice recruited leukocytes at a faster rate (a mean number of $8.75 \times 10^4$ leukocytes per ml per hour) than CBA/Ca mice (a mean number of $4.25 \times 10^4$ leukocytes per ml per hour) between 0 hours and 12 hours. Between 12 hours and 24 hours, BALB/c mice and CBA/Ca mice were found to recruit leukocytes at a similar rate (a mean number of $3.25 \times 10^4$ leukocytes per ml per hour in BALB/c mice versus a mean number of $2.67 \times 10^4$ leukocytes per ml per hour in CBA/Ca mice). Both strains of mice were found to recruit cells faster between 0 and 12 hours than 12 to 24 hours.
Figure 3.47 Comparison of the total leukocyte count (in digested lung samples) from CBA/Ca (■) and BALB/c (▲) mice following infection with D39 (type 2) pneumococci.

Data are the mean of five mice per time point except 168 hours, which is from three mice. Error bars show the standard error of the mean. *p<0.005, †p<0.01 for BALB/c leukocyte levels as compared to CBA/Ca.
3.5.2 Comparison of the differential analysis of BALB/c and CBA/Ca lung cell homogenates

Figure 3.48 shows the comparison of the different leukocyte populations from samples generated from the total leukocyte counts, from (A) CBA/Ca mouse and (B) BALB/c mouse lungs, following infection with *S. pneumoniae* strain D39.

Examination of the leukocyte population in CBA/Ca lungs at 0 hours, indicated the major type of leukocyte present was the macrophage, which accounted for 53.1 +/- 1.9% of the total leukocyte cell population. The number of cells were unaltered from a basal level of a mean number of 4.69x10^5 macrophages per ml at 0 hours during the course of infection; there was no significant rise or decline at both 12 and 24 hours. However lymphocytes, which constituted 34.5 +/- 1.6% of the total leukocyte cell population at 0 hours, were found to differ in numbers post-infection. At 12 hours after infection, an observable decline was evident (p<0.05 compared to 0 hours); from a mean number of 3.06x10^5 lymphocytes per ml at 0 hours to 1.52x10^5 lymphocytes per ml at 12 hours. Pulmonary lymphocytes were found to continue to significantly decrease in number after this time point to reach a level of a mean number of 1.43x10^5 lymphocytes per ml at 24 hours (p<0.05 compared to 0 hours).

Polymorphonuclear leukocytes constituted the main population of infiltrating inflammatory leukocytes. Post-infection the PMN population, apparently exclusively made up of neutrophils, was found to increase in number. These leukocytes were lower than both macrophage and lymphocyte populations in the lungs at 0 hours making up 12.4 +/- 1.9% of the total leukocyte cell population. From an initial level of a mean number of 1.06x10^5 PMNs per ml, the number of cells recruited into the lungs of CBA/Ca mice was found to increase significantly by 12 hours post-infection, reaching 8.82x10^5 PMNs per ml (p<0.001 compared to 0 hours). By 24 hours this influx of PMNs reached a mean of 1.18x10^6 PMNs per ml; significantly different (p<0.001) compared to 0 hours.

Analysis of the pulmonary leukocytes in BALB/c mice revealed that, not unlike CBA/Ca mice, the macrophage was the most dominant cell type at 0 hours (47.3 +/- 4.7% of the total leukocyte cell population). However, in contrast to CBA/Ca mice post-infection, macrophages in BALB/c mice fell in numbers. At 12 hours after intranasal
infection the numbers of cells had significantly reduced, from a mean number of 6.20\times10^5 macrophages per ml at 0 hours to 3.09\times10^5 macrophages per ml (p<0.01 as compared to 0 hours). This trend was maintained after this time point, as the numbers of macrophages continued to fall between 12 hours to 24 hours post-infection. 24 hours after infection, the concentration of macrophages was a mean number of 2.91\times10^5 per ml (p<0.01 compared to 0 hours).

Lymphocytes were found to form 37.8 +/- 2.8% of the total leukocyte cell population analysed at 0 hours in the lungs of BALB/c mice. Following pneumococcal infection, these leukocytes declined, as was seen with CBA/Ca mice and by 12 hours post-infection the numbers of cells was a mean number of 2.17\times10^5 lymphocytes per ml; this was found to be a significant decrease (p<0.05) compared with 4.99\times10^5 lymphocytes per ml at 0 hours. In contrast to CBA/Ca mice, lymphocyte numbers at 24 hours in BALB/c mice were not found to change from the numbers seen at 0 hours post-infection. The number of cells at this time point, 2.67\times10^4 mean number of lymphocytes per ml, was not significantly different to that at 0 hours (p>0.05).

Like the situation in CBA/Ca mice, after infection, the influx of inflammatory cells into the lungs of BALB/c mice was due to recruited PMNs (exclusively neutrophils). At 0 hours, the number of PMNs in the lungs of BALB/c mice was a mean number of 1.97\times10^5 per ml which constituted 14.9 +/- 2.8% of the total leukocyte cell population. The level of PMNs was the same at 0 hours between both murine strains (p>0.05 difference between strains). At 6 hours post-infection, PMNs were found to rise to a level of a mean number of 5.58\times10^5 per ml. This recruitment of PMNs into the lungs of BALB/c mice reached a level of significant difference by 12 hours, rising to a level of a mean number of 1.85\times10^6 PMNs per ml (p<0.001 compared to 0 hours). Compared to CBA/Ca at 12 hours, BALB/c mice recruited significantly more PMNs (p<0.001). Recruitment of these leukocytes continued in the lungs of BALB/c mice after 12 hours with a further increase in PMNs by 24 hours post-infection. At this time point the level of these cells reached a mean number of 2.07\times10^6 PMNs per ml; significantly different (p<0.001) compared to the number of cells at 0 hours. Statistical analysis between both inbred murine strains at 24 hours showed that BALB/c mice recruited significantly more PMNs than CBA/Ca mice (p<0.01).
Further analysis of the rate of recruitment of PMNs, revealed that BALB/c mice recruited PMNs faster than CBA/Ca mice between 0 hours to 12 hours. BALB/c mice were found to have a rate of recruitment of a mean number of $1.38 \times 10^5$ PMNs per ml per hour whereas CBA/Ca mice recruited $6.47 \times 10^4$ PMNs per ml per hour. Both murine strains were found to have similar rates of recruitment between 12 hours through to 24 hours post-infection. Between these time points, BALB/c mice recruited a mean number of $1.83 \times 10^4$ PMNs per ml per hour compared to the recruitment of $2.48 \times 10^4$ PMNs per ml per hour by CBA/Ca mice.
Figure 3.48 Differential analysis of leukocytes within digested lung samples from CBA/Ca (A) and BALB/c (B) mice following infection with D39 (type 2) pneumococci.

Data are the mean of 5 mice per point with error bars showing the standard error of the mean. Key: macrophage (■—■), lymphocyte (▲—▲) and polymorphonuclear leukocyte (▼—▼). *p<0.001, †p<0.01 for BALB/c PMN levels as compared to CBA/Ca.
3.6 Histological studies with BALB/c mice and CBA/Ca mice following infection with *S. pneumoniae* strain D39 (type 2)

After the analysis of the cellular response to intranasal pneumococcal infection in both susceptible CBA/Ca mice and resistant BALB/c mice, histological studies in both murine strains were undertaken to further define events in the lungs. Histological examination (section 2.11) of the tissue in BALB/c and CBA/Ca was done on several mice taken at selected points after intranasal infection with D39. In one time course, tissue was embedded in paraffin wax (section 2.11.1). Following sectioning and haematoxylin and eosin staining, each section was examined for gross pathology. In a second time course, tissue samples were frozen (section 2.11.2). Following cryostat sectioning and haematoxylin and eosin staining, each section was examined blind for analysis of specific indications of inflammation based on pre-selected criteria to give a numerical comparison between the murine strains.

3.6.1 Paraffin wax sections

Done in collaboration with Dr Huw Jones of AstraZeneca, histopathological assessment was performed on lung tissue samples taken from at least two mice sacrificed at each of various times following intranasal infection. These time points were between 0 hours to 24 hours for CBA/Ca mice and 0 hours to 336 hours for BALB/c mice. Figures 3.49 through to 3.55 are photographs that show CBA/Ca and BALB/c lung sections at each time point taken.

Post-infection, CBA/Ca mice showed no obvious lesions at 6 hours (Figure 3.50). At 12 hours following pneumococcal intranasal infection (Figure 3.50), the lungs of one of the CBA/Ca mice examined showed mild, multifocal mixed inflammatory infiltration. One mouse showed a mild, multifocal acute peribronchial infiltration. In this animal, extension of lesions into other areas of the lung was evident, with a minimal diffuse, acute/sub-acute interstitial alveolitis. At 24 hours post-infection (Figure 3.51), lesions in the lungs of both mice examined were generally mild in severity and were more diffuse in distribution, involving peribronchial and perivascular regions through to interstitial alveolitis, in which the inflammatory infiltrate was acute/sub-acute in nature. The infection in these mice was also shown to extend to inflammation of the pleural
membrane with a multifocally distributed, acute pleuritis (moderate severity grading).

Compared with CBA/Ca mice, BALB/c mice showed inflammatory lesions at a much earlier time post-infection. Although minimal in severity, a multifocal, peribronchial and perivascular acute, inflammatory cellular infiltration was seen at 6 hours following intranasal pneumococcal infection in one of the two mice examined (Figure 3.52). By 12 hours post-infection (Figure 3.53), these lesions were more severe (minimal/mild severity grading). In one of the mice examined, there was evidence of involvement of the lung parenchyma, observed as a minimal, acute multifocal interstitial alveolitis. At 24 hours (Figure 3.53), BALB/c mice showed peribronchial and perivascular, inflammatory cellular infiltration that was greater in severity (mild severity grading) and more diffuse than observed at 12 hours post-infection. At this time point interstitial alveolitis was mild/moderate in severity grading with an acute/sub-acute inflammatory infiltrate. The distribution of this lesion at 24 hours was observed to be no different than at 12 hours (multifocal). In addition to the above lesions, at this time point signs of an acute mild bronchitis was evident in one of three mice examined. It was notable that CBA/Ca mice showed no signs of this type of lesion at this time point.

At 48 hours post-infection (Figure 3.54), peribronchial and perivascular, inflammatory cellular infiltration in the lungs of BALB/c mice was of the same severity as that seen at 24 hours post-infection (mild severity grading). However, at this time point, these lesions were more multifocal in distribution and the inflammatory infiltrate was acute/sub-acute in nature. Interstitial alveolitis was observed to be less severe than at 24 hours post-infection (mild severity grading). The bronchitis, again from one of three mice examined, was also less marked (minimal severity grading) and more multifocal, together with a more acute/sub-acute peribronchial and perivascular inflammatory cellular infiltrate. By 72 hours following intranasal pneumococcal infection (Figure 3.54), BALB/c mice showed an increase in the severity of perivascular infiltration (mild/moderate severity grading) and interstitial alveolitis (minimal/moderate severity grading). Both peribronchial and perivascular inflammatory cellular infiltrate were sub-acute in nature at this time point, but the perivascular lesions were more diffuse in distribution. At this stage of the infection, inflammation had also extended to the pleural membrane, as a minimal multifocal pleuritis in one out of three mice. One week after initial infection (168 hours), one BALB/c mouse showed signs of
a chronic, multifocal interstitial alveolitis (mild severity grading). In the lungs of the second mouse examined, there was evidence of a marked multifocal lymphoid hyperplasia (moderate severity grading) (Figure 3.55).

Figure 3.49 Photograph from the paraffin wax sections of CBA/Ca mice at 0 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: b = bronchiole, v = venule.
Figure 3.50 Photographs from paraffin wax sections of CBA/Ca mice, (A) 6 hours and (B) 12 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: ia = interstitial alveolitis, pv = perivascular and pb = peribronchial cellular infiltration.
Figure 3.51 Photographs from paraffin wax sections of CBA/Ca mice, 24 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: ia = interstitial alveolitis, pv = perivascular and pb = peribronchial cellular infiltration.
Figure 3.52 Photographs from paraffin wax sections of BALB/c mice, (A) 0 hours and (B) 6 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: pv = perivascular and pb = peribronchial cellular infiltration.
**Figure 3.53** Photographs from paraffin wax sections of BALB/c mice, (A) 12 hours and (B) 24 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: ia = interstitialalveolitis, pv = perivascular and pb = peribronchial cellular infiltration.
Figure 3.54 Photograph from paraffin wax sections of BALB/c mice, (A) 48 hours and (B) 72 hours post-infection with S. pneumoniae strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: ia = interstitial alveolitis.
Figure 3.55 Photograph from paraffin wax sections of BALB/c mice, 168 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: lh = lymphoid hyperplasia.
3.6.2 Frozen sections

Cryostat-cut frozen sections (10 to 25 microns thick) were scored blind to look for a difference between strains in the inflammatory response in the lungs. Sections were given an inflammatory lesion score for a numerical comparison, as described in methods section 2.11.3. Figure 3.56 shows the numerical comparison of inflammatory lesions in both strains of mice. Figures 3.57 and 3.58 are photographs of CBA/Ca and BALB/c lung sections at 0 hours and 12 hours post-infection.

BALB/c mice and CBA/Ca mice at 0 hours (median lesion scores of 7.5 and 6 respectively), were found to be significantly different to each other in median lesion score (p<0.05). At 6 hours post-infection, a statistical difference between both strains (p<0.05) was still evident, with a median lesion score of 9.5 in BALB/c mice and 8 in CBA/Ca mice. Again, at 12 hours post-infection, BALB/c mice were statistically different in median lesion score than CBA/Ca mice (p<0.0005). A difference in the cellular infiltration throughout the tissue at these times was obvious between the two strains. BALB/c mice were found to have a peak median lesion score at 12 hours of 12, the highest throughout the time course. This score reflected the high level of cellular recruitment throughout the tissue and in and around the bronchioles, and a greater thickening (hyperplasia) of bronchiole walls, compared to that of CBA/Ca mice (median lesion score at 12 hours of 7) (Figures 3.57 and 3.58).

At 24 hours, BALB/c mice were again found to have a significantly higher (p<0.05) median lesion score compared to CBA/Ca mice (median lesion scores of 9 and 8 respectively). The time course of infection for CBA/Ca mice was terminated after 24 hours due to the median survival time being around that time. BALB/c mice were analysed until 336 hours post-infection. Lung sections in BALB/c mice were found to have a median lesion score of 10 at 48 hours, a median lesion score of 8 at 168 hours, and a median lesion score of 8.5 at 336 hours.

In summary, BALB/c had a greater cellular infiltration than CBA/Ca at 12 hours post-infection. These mice were also shown to have a marked hyperplasia of the bronchiole walls with an increased level of exudate and cellular infiltration in the bronchioles compared to the situation in CBA/Ca mice.
Figure 3.56 Median inflammatory lesion score of histological sections from BALB/c (→) and CBA/Ca (↔) lungs (n=2) during a time course infection with D39 (type 2) pneumococci.

Median lesion score were generated from anatomical criteria for inflammatory lesions from a total of 4 sections per mouse at each time point. Bars are the minimum and maximum lesion score values.
Figure 3.57 Photographs from frozen cut sections of CBA/Ca mice, (A) 0 hours and (B) 12 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: b = bronchiole, v = venule, mci = mild cellular infiltration.
Figure 3.58 Photographs from frozen cut sections of BALB/c mice, (A) 0 hours and (B) 12 hours post-infection with *S. pneumoniae* strain D39 (type 2).

Haematoxylin and eosin stained sections at 328x magnification. Key: sci = substantial cellular infiltration.
Chapter 4: Discussion

The host genetic factors that control innate resistance to pneumococcal disease are not well understood. To begin to define host genetic factors that may play a role in susceptibility to invasive pneumococcal disease this study has used a murine model of pneumococcal disease. Murine models provide an important tool in examining both familial inheritance and the dissection of the biology behind genetic traits. The long-term objective of this study is to identify and map candidate genes of the mouse genome. This thesis describes the results of the first stage of this process. This was to select phenotypically distinct resistant and susceptible parental strains for further genetic analysis and examine the inheritance of phenotypes in subsequent generations (F1 and F2 progeny) produced from crossing these selected parental strains. In addition, the objective was to further characterize the nature of disease progression in these identified resistant and susceptible parental strains.

4.1 Discussion of the distribution of phenotypes in infection studies using a panel of nine murine inbred strains

Following pneumococcal infection, the inbred mouse strains examined, differed markedly in susceptibility as represented by survival time and numbers of pneumococci in the blood (section 3.1). The greatest phenotypic variation was seen with D39 (type 2) pneumococci compared to GB05 (type 3), with a clear indication of a susceptible phenotype (CBA/Ca and SJL mice) and a resistant phenotype (BALB/c mice). These results were seen both in a pilot study undertaken with five mice in each group and a larger full-scale study with twenty mice in each group (section 3.1.1 and section 3.1.2).

By virtue of the variation in the distribution of phenotypes seen between all 9 inbred strains following infection with D39, the data suggest that resistance to pneumococcal infection is a complex trait. Continuous variation of phenotypes from susceptible, intermediate through to resistant in both blood counts and survival data was observed. However, the results do not rule out the possibility of a single gene effect with the different phenotypes observed generated from allelic variation in the inbred strains.

Both the pilot study and full-scale study showed that although SJL and CBA/Ca
mice were similar in median survival times, a difference in symptoms was observed post-infection (section 3.1.1 and section 3.1.2). These data indicate the possibility of additional differences in the genetic susceptibility to pneumococcal infection between SJL mice and CBA/Ca mice.

Although BALB/c mice were significantly more resistant than CBA/Ca mice to infection with GB05, the pattern of phenotypic variation in the panel of inbred mice was less defined than for D39. In these data, neither BALB/c mice nor CBA/Ca mice constituted a distinct resistant or susceptible group to infection with GB05 compared to the other inbred mice (section 3.1.1 and section 3.1.2). The highly virulent nature of GB05 pneumococci may have masked the clear distribution of a genetic susceptible and resistant phenotype to this strain. However, any difference in virulence between D39 and GB05 was compensated by the dose of intranasally administered pneumococci given to the mice.

Data in this study may not rule out the possibility that the same genetic determinants control susceptibility to both D39 and GB05 but they indicate a possible difference in the genetic susceptibility that is dependent on the identity of the infecting pneumococci. That the interaction between the pneumococcal and murine genotypes is important in pneumococcal pathogenesis has been reported previously (Briles et al., 1983, 1986). Pneumococci have a number of virulence factors important in the pathogenesis of the bacteria, as discussed in section 1.3.2. Therefore, the genetic heterogeneity between pneumococcal strains, particularly in their virulence factors, may account for different susceptibilities within the same host genetic background.

One such virulence factor that varies between pneumococcal strains is the polysaccharide capsule (section 1.3.2.1). Virulence is known to be associated with capsular serotype of the pneumococcal isolate (Briles et al., 1992). Additionally, the kinetics of pneumococcal growth in the blood following intravenous inoculation was found to be different dependent on the strain of pneumococcus, with an apparent association to serotype (Benton et al., 1997b).

The capsule is only one of many virulence factors interacting with the host to cause disease (section 1.3.2). Therefore, the possible numerous gene interactions involved with different strains of pneumococci and different host backgrounds may
hamper the dissection of specific genes involved in the susceptibility or resistance to this bacterium.

For the purposes of further study, BALB/c was chosen as a resistant strain and CBA/Ca a susceptible strain. CBA/Ca was chosen over SJL due to availability. Available resources and time restricted analysis to one resistant and one susceptible parental inbred strain. In addition, infection with D39 pneumococci was chosen over further analysis with GB05, due to the greater phenotypic variation seen in the panel of inbred mice infected with D39.

4.2 Progeny phenotype distributions from susceptible and resistant parental crosses: discussion of F1 and F2 generations

Following selection of parental strains for analysis of the genetic inheritance of resistance or susceptibility to pneumococcal disease, filial generations (F1 mice and then subsequently F2 mice) were bred for further study. To observe whether genetic imprinting occurred from either parent, reciprocal crosses were made, crossing CBA/Ca males and females with BALB/c males and females. To achieve all possible phenotypes for linkage to genotypes, F2 generation mice were produced from intercross breeding from F1 progeny.

At first data for both CCBA F1 and CBAC F1 mice was suggestive of a codominant trait, as phenotypes in both survival and blood counts were intermediate compared to the susceptible parent strain (CBA/Ca) and the resistant parent strain (BALB/c). Taken together, the survival times and bacterial counts 24 hours post-infection of both F1 and F2 mice were indicative of quantitative variation. Greater variation of phenotypes was observed in the F2 progeny because of a greater number of genotypes present in these mice. Although with this variation of phenotypes, the overall distribution was observed as skewed, as compared to the expected normal bell-shaped distribution in a quantitative trait (Fincham 1994). In both F2 crosses, the number of mice surviving the experiment and having no pneumococci in the blood was disproportionate to the numbers of mice most susceptible to infection and with the highest numbers of pneumococci in the blood (section 3.2.2). This skewed distribution is suggestive of a major dominant locus (S. Brown, MRC Mouse Genome Centre, Harwell, UK, personal communication) within the loci responsible for the trait.
Epistatic interactions between this major dominant locus and additional loci may occur and these data do not rule out the possibility of genetic heterogeneity.

In both F1 and F2 generation mice, differences were seen between CCBA and CBAC mice, suggestive of an effect of genetic imprinting from parental or maternal inheritance of CBA/Ca or BALB/c alleles (section 3.2). In addition, differences were seen in some experiments, suggestive of an X-linked effect, although the pattern of differences between males and females was not consistent between challenges (section 3.2.1). Thus, the possibility of genetic imprinting and X-linked effects seen in challenges may have been accounted for by environmental factors.

A proportion of quantitative variation is not associated with genetic inheritance but is attributed to environmental fluctuation (Fincham 1994). In this study, these environmental fluctuations could have been virulence differences between batches of pneumococci, variation in the dose given, differences in the mice, for example the immunological state of the mice pre-infection. However, during these experiments the dose was checked by viable counting, and the virulence of pneumococcal batches was monitored using MF1 outbred mice as a marker. In both F1 and F2 challenges, MF1 mice were also included as a control for other unknown challenge variables, and during these experiments, these mice were not found to differ greatly in phenotype (section 3.2). Additionally, all mice were housed under the same conditions, location and were checked frequently for any health problems.

Despite the variation found, this study was successful in breeding and phenotyping both filial generations (F1 mice and then subsequently F2 mice). F1 generation mice were challenged to monitor the phenotypic inheritance pattern and to give an idea of the number of segregating genes. These F1 mice were not intended to be used for the genetic analysis of susceptibility by genome scanning. Although variation was also found in the F2 challenges, the large number of F2 mice used in this study, together with genome scanning of these mice, should overcome possible environmental interactions.

Genome scanning of the F2 mice tissue samples is currently in progress. The results generated so far are discussed in section 4.8. This analysis will identify possible QTLs associated with the resistance or susceptibility to the pneumococcus, and may
lead to the eventually uncovering of a major dominant gene involved and/or other genes, such as modifier or enhancer genes, that interact to produce the phenotypes observed. Additionally, further genetic analysis may reveal whether genetic imprinting or X-linked effects are involved in the phenotypic differences in resistant and susceptible mice.

4.3 **Time course investigations into the number of viable bacteria in the blood and lungs of both CBA/Ca mice and BALB/c mice, following intranasal infection with D39 (type 2) pneumococci**

From the study of the panel of inbred mice, results section 3.1, resistant BALB/c and susceptible CBA/Ca mice were chosen for further study, on the basis of continuing studies to identify the genes involved in host resistance. With a view to complementing future data from gene identification studies, these mouse strains were examined to investigate the biology behind their susceptibility/resistance phenotypes. Time course investigations into pneumococci in the blood and lungs were performed, to determine how the different phenotypic characteristics of the murine strains were related to the changes in pneumococcal numbers.

Consideration of the bacterial growth curves suggests that the difference between murine strains in pneumococcal growth in the lungs is critical to explain subsequent events. In CBA/Ca mice, pneumococci increased in the lungs over 24 hours whereas in BALB/c mice the numbers were unchanged during this time. By 48 hours, numbers of bacteria had declined in BALB/c whereas by 27 hours CBA/Ca were moribund and obviously could not enter this phase of bacterial decline. The events in the blood mirrored those seen in the lungs. CBA/Ca mice developed an early sepsis, extending to an uncontrolled bacterial increase whereas in BALB/c mice only small numbers of bacteria were recovered from the blood, and then only occasionally. Maintenance of bacteraemia appears to be dependent on the occurrence of high numbers of pulmonary bacteria and BALB/c mice are able to manage the infection in the lungs. In other experiments conducted in this laboratory (A. Kadioglu, University of Leicester, UK, personal communication), this type of infection profile pattern has been confirmed in the lungs and blood of BALB/c mice, although the timing of these events has been found to fluctuate.
The rapid increase in bacterial numbers in the blood of CBA/Ca mice may not
be entirely the consequence of pneumococci disseminating from the lungs. Rapid
growth in the blood may have contributed to, or be the sole cause of, the large numbers
isolated, and the growth seen in the lungs at 24 hours may have been due to increased
numbers of pneumococci in the vascular bed. However, dissemination from the lungs
may have occurred to reach a level to overcome the appropriate immune defences and to
contribute to an uncontrolled bacteraemia. Interestingly, this event must have occurred
very early post-infection, as by 6 hours, bacteria were observed in the blood. Arguably,
pneumococci may have translocated from the nasopharynx or other areas of the upper
respiratory tract. However, this seems unlikely, due to the fact that the main bolus of
bacteria would have been distributed in the lower airways. Data from this study
provides evidence for this statement, as pneumococcal numbers recovered from the
lungs at the start of the infection (0 hours) in BALB/c and CBA/Ca mice, constituted
approximately 73% of the inoculum given. Although it is speculation, in addition to the
distribution of inoculum, the pulmonary vascular system in the lower respiratory tract
could allow easier access for pneumococci to disseminate into the blood.

To investigate if the blood is not the critical site to explain the observed
differences between the strains, both BALB/c and CBA/Ca mice would have to be
compared following intravenous infection. However, studies in this thesis were limited
to an examination of the genetic susceptibility to invasive pneumococcal disease in a
model of bronchopneumonia. Alternative routes of infection and therefore different
models of infection in these mouse strains may enhance the genetic associations to
particular genetic loci or uncover additional or different genetic determinants of
resistance or susceptibility to pneumococcal infection.

4.4 Development of methods to investigate immunological differences in the lungs
between parental strains CBA/Ca and BALB/c mice

Methods were investigated to look for ways to examine the immunology behind
the phenotypes seen in susceptible CBA/Ca mice and resistant BALB/c mice. Due to the
expense, availability issues and ease of handling, the outbred strain MF1 was used in
these experiments. These mice were also used because of their reproducible phenotype
following infection with D39 in this study (section 3.1 and 3.2) and previous studies in
Leicester (Canvin et al., 1995).

The first method chosen for investigation was BAL, to analyse a difference in inflammation by assessment of the numbers and type of leukocytes in the bronchoalveolar air spaces. Measurement of total protein concentration in BAL fluid as further indication of inflammation was also used, following the hypothesis that alveoli would leak serum proteins during inflammation in the lungs (Rubins et al., 1995). In the initial experiment, a lack of leukocyte recruitment coupled with no change in BAL fluid protein concentration indicated that inflammation within the bronchoalveolar air spaces was very low (section 3.4.1). The only indication of any change in the immunological response post-infection was a shift in the population of leukocytes, with an increase in the number of PMNs and a decrease in macrophages. The lack of a difference in leukocyte numbers may have been due a net loss of macrophages, balanced with the net gain of PMNs. However, these data were not truly representative of events occurring in MF1 lungs. In the second time course, although the initial leukocyte levels in the BAL fluid at 0, 6 and 12 hours post-infection were similar to the previous time course, at 24 hours post-infection, a significant decrease in leukocytes was observed which was not seen in the previous BAL experiment (section 3.4.1). Coupled with this difference, an elevation was seen in the protein concentration, reaching a plateau between 12 and 24 hours post-infection. Although in both time courses, the number of PMNs increased post-infection with a decline in the numbers of macrophages, in this second time course levels of PMNs rose above the level of macrophages at 12 hours post-infection. Interestingly, in contrast again with the first time course, there was a decrease in the total number of leukocytes at 48 hours, reflected in the differential count by lower numbers of both macrophages and PMNs.

The apparent difference in the time courses may have been accounted by differences in infection in the mice tested. In the second time course, the group designated for 48 hours succumbed to infection before sampling could occur, indicating a more aggressive progression of disease in these animals. This may have affected leukocyte numbers due to high levels of pneumococci possibility present, overwhelming and indeed reducing the numbers of leukocytes. However, a more probable explanation for the decline in numbers of leukocytes at 24 hours coupled with a decrease in both macrophage and PMNs numbers at this time, is the possibility of operational error.
Differences between the time courses could have been due to sampling error when recovering BAL fluid from the lungs.

The results from the third and final BAL experiment that examined differences in pneumococcal numbers in the lungs, showed that in the later stages post-infection, most pneumococci were recovered from homogenate samples and not BAL fluid samples (section 3.4.1). At 0 hours, pneumococci were mostly in the BAL fluid and had not yet invaded into the surrounding lung tissue. In homogenate samples, an increase of pneumococcal numbers was observed, indicating a spread and growth of these bacteria in the lung tissue and not in the BAL fluid. Interestingly, the level of lavage CFU counts did not decline post-infection. This may indicate that sufficient growth of pneumococcal numbers was occurring to supplement pneumococci leaving the air spaces and invading the lungs.

The final BAL experiment was similar in total leukocytes to the first BAL time course performed, with no change found at any time point (section 3.4.1). This observation in two of the three experiments done suggests that the numbers of leukocytes do not change post-infection in the bronchoalveolar air spaces even though pneumococci are present at high numbers throughout.

In this final BAL experiment, protein levels were elevated post-infection, which also occurred in the second time course done (section 3.4.1). Therefore, two out of the three BAL experiments performed indicated a change in the permeability of alveoli allowing leakage of serum proteins. Although leukocytes levels remained unchanged, the influx of PMNs and the effect of intact pneumococci and components of lysed bacteria (particularly pneumolysin and cell wall constituents), would contribute to this effect as discussed in section 1.2 and 1.3.

Comparing all BAL experiments, the lack of consistency between them may reflect subtle differences in the severity of infection in the experimental groups, together with the inherent problems in the use of BAL. Why the numbers of leukocytes and protein concentrations varied in the BAL experiments could reflect the methodology employed and operational error. For instance, the variation in the amount of recovered lavage fluid reflected the degree of difficulty in performing this procedure during a time course investigation (section 3.4.1). This variation in recovery could have had a
profound effect on the concentration of both leukocytes and protein in the BAL fluid. In these BAL experiments, six washes with 0.5ml volumes of buffer were used (section 2.5), therefore efficiency between washes may have varied and thus effected the end concentration of the total recovered lavage fluid. Another factor that may have reduced the efficiency of BAL during these time courses could be the fact that inflammation and pathology in the lungs may have restricted the recovery of lavage fluid.

BAL, by virtue of the technique, only recovers a proportion of the leukocyte population in the lung; those in the air spaces. Moreover, BAL experiments also only reflect the inflammation and events occurring in the bronchoalveolar spaces and therefore not representative of the lung as a whole. This may be illustrated from the CFU data in the third experiment (section 3.4.1), when numbers remained unchanged in the lavage fluid but increased in the homogenate.

Evidence for the problems of using BAL to examine immunological events in the lungs comes from a study by Ishimine et al., (1995). In a study of mice infected with *Pneumocystis carinii*, analysis of the cellular response and IFN-γ synthesis in the lungs was compared in tissue homogenate and BAL fluid samples (Ishimine et al., 1995). Post-infection in these mice it was found that leukocytes did not significantly increase in number in the BAL samples whereas in homogenates a significant increase was observed (Ishimine et al., 1995). In addition, a significant level of IFN-γ was detected in homogenate samples compared to that found in BAL fluid (Ishimine et al., 1995).

Other studies have used BAL to monitor cellular infiltration, cytokine levels and other factors in the airways following infection. One such study using BAL, obtained a significant chronological picture of infection and inflammation, following intranasal inoculation of pneumococci in mice (Bergenon et al., 1998). However, this study used a type 3 pneumococcal isolate, in CD1 Swiss mice with a high challenge dose of 10⁷ pneumococci (Bergenon et al., 1998). The median survival time in these mice was greater than MF1 mice infected with D39 and time points continued in CD1 Swiss mice until 96 hours post-infection (Bergenon et al., 1998). These differences make difficult a direct comparison between both the study by Bergenon et al., (1998) and the BAL experiments described in this report.

Due to the results observed in the BAL experiments in this thesis (section 3.4.1),
further experimentation was directed towards the use of total lung tissue samples to examine the infection profile following intranasal challenge with pneumococcal strain D39. To follow the influx of PMNs (which were made up of exclusively neutrophils in the BAL experiments), an MPO assay was examined to quantify these cells in the lungs of MF1 mice post-infection.

The assay commonly used for MPO utilises the substrate o-dianisidine dihydrochloride (Worthington 1993). However, due to the carcinogenic properties of o-dianisidine dihydrochloride, and the availability of other resources in the laboratory, a modification of a fluorescent assay for \( \text{H}_2\text{O}_2 \) to assay for MPO was undertaken (modification of Jackett et al., 1981). Modifications initially were made to the amount of both \( \text{H}_2\text{O}_2 \) and substrate, so these components were not rate limiting in the reaction. The initial experiment with this assay showed that HRP worked in this system, and a standard curve was obtained from a range of 0 to 0.025 units/ml of HRP.

When lung homogenates were examined in this assay, fluorescence was observed in the presence of both \( \text{H}_2\text{O}_2 \) and substrate (section 3.4.2), and therefore oxidation of the substrate had occurred. However, experiments indicated that the addition of homogenate itself caused a quenching of fluorescence (section 3.4.2). Changing the amount of homogenate in the reaction mixture had an effect on fluorescence obtained, although this did not fully alleviate the problems of analysis of lung homogenates. Extension of the incubation time showed that MPO activity had ceased or was undetectable after 10 minutes. Therefore, the phenomenon of fluorescence quenching was not found to be purely an affect of particulate matter or the opacity of the solution contributing to a reduction in detected fluorescence.

Examination of the detergent for possible quenching effects within this system indicated that neither the type of detergent nor its concentration effected fluorescence (section 3.4.2). This was in keeping with reports that have used HTAB and Triton X-100 to successfully solubilise MPO (Bradley et al., 1982, Metcalf et al., 1986, Shayevitz et al., 1995).

Lack of activity because of potential competitive or non-competitive inhibition, due to components in the homogenate or temperature or pH effects seemed unlikely because of the fact that when HRP was added to the reaction (in addition to
homogenate), activity was observed following incubation (section 3.4.2). Other factors 
such as storage of lung tissue could have affected enzyme activity. However, no 
difference was seen in activity between fresh and frozen tissue (section 3.4.2). This is in 
keeping with Bradley et al., (1982) and Shayevitz et al., (1995) who have used freeze-
thawing of tissue samples in the process of MPO extraction and detection.

No firm conclusions can be drawn from the experiments with this MPO assay, particularly in view of the control HRP standard curves being reproducible during the experiments. A number of questions remain unresolved, but because of the time and resources, this method was abandoned and other immunological tests sought for the analysis and quantification of inflammation in the lungs. If this assay had been continued, the next step would have been to possibly clean up the homogenate samples by fractionation on a sucrose gradient.

Neutrophils extracted from peripheral blood could have been used as a control for homogenate samples. To monitor the recovery and activity of MPO following solubilisation in buffer and detergent, the spike of activity of MPO extracted from neutrophils in the blood could be used to compare to the activity of MPO from lung homogenate samples. Moreover, to indicate whether appropriate solubilisation and extraction of MPO from lung samples was being achieved, MPO activity from pellets following the centrifugation the homogenised lung tissue could have been examined. Both these suggestions are based from a study of tissue neutrophil content by Bradley et al., (1982).

Finally, other methods using different substrates for the analysis of MPO activity could have been investigated, such as an adaptation of the method of Root et al., (1975) using scopoletin as a substrate, which decreases in fluorescence upon oxidation. In this method, the high starting fluorescence may minimise the impact of background fluorescence that may mask a level of enzyme activity. Alternatively, methods not reliant on the measurement of fluorescence could have been used such as the use of tetramethylbenzidine (TMB) as a substrate (Andrews and Krinsky 1981). TMB is a non-carcinogenic compound, which has been used successfully for the measurement of MPO (Andrews and Krinsky 1981).

Following from the MPO assay experiments, FACS analysis was evaluated as a
method to investigate leukocytes in the lungs. This technique was chosen as a method of rapid differential counting, which could be used to further differentiate lymphocytes into T-cells and B-cells.

Experiments were first set up using blood samples, which showed the different populations of leukocytes, (granulocytes, monocytes and lymphocytes) based on their granularity and size (section 3.4.3). Gating these regions and determining the level of positively stained cells was easier than for lung samples. During the course of the FACS experiments, the analysis of blood samples was reproducible (section 3.4.3). Moreover, in blood samples a difference between mice infected with D39 pneumococci and uninfected mice was correlated with increase in the population of neutrophils (section 3.4.3). This is in keeping with a previous study were Canvin et al., (1995) noted an increase of neutrophils following the differential analysis of blood smears post-infection from D39 infected MF1 mice.

Throughout the FACS experiments with lung samples, blood samples were used for the purposes of deciding gating regions because of the ease of distinguishing populations of cells by granularity and size (section 3.4.3). This was because of the heterogeneous nature of the cell population in the lungs made distinguishing the regions containing leukocytes difficult. Nonetheless, lung samples were found to contain a high proportion of CD45 positive cells, indicating that the majority of the cells were leukocytes (section 3.4.3).

During the analysis of lung samples, in some of the experiments performed, Gr-1 positive cells or neutrophils, were shown to increase in infected lungs as compared to uninfected lungs (section 3.4.3). This is in agreement with a number of studies that have observed that neutrophils are recruited into the lungs after pneumococcal infection (Gunn and Nungester 1936, Robertson et al., 1933a, b, Terrell et al., 1933, Loosli and Baker 1962, Doerschuk et al., 1994, Garvy and Harmsen 1996, Bergeron et al., 1998, Kadioglu et al., 2000). However, during these experiments, F4/80 positive cells (indicating macrophage and monocyte cell populations) were low in both uninfected and infected lung samples (section 3.4.3). Although these cells were found to decrease in infected lung samples, not dissimilar to the change in macrophage population found in the BAL experiments, F4/80 positive cells were generally low throughout the
experiments. One would expect a higher percentage of macrophages than neutrophils in uninfected lungs. Neutrophils, as observed in tissue sections, are recruited post-infection in the lungs and are associated with inflammatory lesions in the lung tissue in MF1 mice (Canvin et al., 1995, Kadioglu et al., 2000).

Interestingly, in one experiment B cells increased in proportion to T cells in infected lung samples compared to uninfected lung samples (section 3.4.3). In this FACS experiment (section 3.4.3) the proportion of B lymphocytes were observed to be lower than T lymphocytes at the start of the infection, and T lymphocytes were found to decline in proportion to B lymphocytes post-infection. In a study by Kadioglu et al., (2000) with MF1 mice infected with D39 pneumococci, the total numbers of lymphocytes in the lungs were found to remain the same post-infection. However, following infection the distribution of T and B lymphocytes changed (Kadioglu et al., 2000). By analysis of cells in tissue sections by immunohistochemistry, Kadioglu et al., (2000) demonstrated that following infection, the distribution of B lymphocytes increased in inflamed areas of the lungs. However, in contrast to the FACS experiment, Kadioglu et al., (2000) found that the proportion of B lymphocytes were not lower than T lymphocytes at the start of the infection. In addition, they found that the proportion T lymphocytes were also found to increase in inflamed areas of the lungs post-infection (Kadioglu et al., 2000).

A definite increase in total leukocytes was seen during the FACS time course experiment in MF1 mice (section 3.4.3). This was in contrast to the total leukocyte levels seen in the BAL experiments (section 3.4.1), furthering the belief that events post-infection occur primarily in the lung tissue and not the bronchoalveolar airspaces. However, unlike the BAL experiments, no firm data on the differential analysis were given by the FACS analysis. Furthermore, during the time course experiment, all cell populations analysed were found to decline (section 3.4.3). This suggests that although inflammatory leukocytes were observed to be recruited into the lungs post-infection, with some experiments recognising this to be an influx of neutrophils, the FACS analysis experiments were misrepresenting the true cell populations in the lungs.

One possible explanation to account for inconsistencies in the FACS experiments could be that throughout all the FACS experiments, high background
staining was observed, particularly during the time course experiments (section 3.4.3). Background fluorescence of different cell populations was a possible problem, and can be seen as two peaks of fluorescence observed in the histograms for the lung homogenate samples (section 3.4.3, Figure 3.39). Therefore, setting histogram markers in the control unstained lung homogenate cell samples, to select between negatively stained and positively stained cells, was difficult due to this background fluorescence (section 3.4.3).

Dead cells are a factor in non-specific staining in FACS analysis (Crockard and Scott 1995). By nature of the tissue disruption in the homogenisation and enzyme digestion procedure, a number of dead cells would be contained in the cell population. These cells would contribute to the level of background staining.

Together with the inconsistencies of differential cell data provided by FACS analysis, data observed from counting stained cytocentrifuged lung cells indicated the expected representation of the different leukocyte cell populations in the lungs (section 3.4.3). These data demonstrated a higher level of macrophages than other leukocyte types in uninfected lungs with an increase in PMNs (which were identified as exclusively neutrophils) in infected lungs. This suggested that simply counting stained cytocentrifuged lung cells would give a clearer representation of the cell populations in the lungs of mice than the previously tried FACS method.

In further pursuit of a FACS analysis method to examine the different leukocyte populations in lung samples, exploration of the cell population with two or three different fluorescent labelled antibodies against other cell antigens could have been done. For instance, the double staining of CD11b (Mac-1) with Gr-1 or F4/80 may have further separated the neutrophils and macrophage cell populations from the lungs. However, this may not overcome the problem of background fluorescence observed during the FACS experiments. In addition, although running samples in the flow cytometer was easy and quick, the time taken for preparation of samples and data acquisition was greater than anticipated. Data from counting stained cytocentrifuged lung cells indicated a simpler, less costly (in comparison to the high cost of monoclonal antibodies for FACS analysis) and more effective method of differential cell analysis. Thus, it was decided to incorporate the homogenisation and enzyme digestion method.
developed during these experiments, and analyse the cellular response in the lungs by total cell counts and differential cell analysis by counting stained cytocentrifuged lung cells.

Using the whole lung homogenisation and enzyme digestion method, leukocyte counting and differential analysis of the cell population by cytocentrifugation was done in a further time course of D39 infection in MF1 mice (section 3.4.4). These data were included in the publication Kadioglu et al., 2000, in which this technique was used to compare the difference in cellular responses between a pneumococcal strain of D39 lacking the gene for pneumolysin (designated PLN-A) and wildtype D39 pneumococci. The results showed a difference in leukocyte levels post-infection in the lungs, with an overall increase in the number of recruited cells (section 3.4.4). These recruited cells were found to be predominantly PMNs, which were exclusively made up of neutrophils, thus reiterating the importance of these cells during pneumococcal infection in the lungs.

This method of whole lung homogenisation and enzyme digestion, with examination of total leukocyte levels and differential analysis of the cell population by cytocentrifugation was able to distinguish differences in the leukocyte response to two different pneumococcal strains, indicating the effect of pneumolysin on the cellular recruitment into the lungs (Kadioglu et al., 2000). Therefore, these data suggested the feasibility of this method to look for differences between CBA/Ca and BALB/c mice in context of the susceptibility to pneumococcal infection.

In hindsight, the simple approach of the analysis by homogenisation and enzyme digestion of whole lung samples should have been adopted at the start of the experiments into the development of methods to investigate immunological differences in the lungs. Additionally, the appeal of methods, such as FACS analysis, that could investigate the cellular response involving a high through-put, reproducible non-counting methodology, out-weighted the simplistic approach of counting stained cytocentrifuged lung cells that was finally adopted. However, although unsuccessful, these methods (BAL analysis, MPO assay and FACS analysis) provided the necessary steps towards the final enzymic digestion and homogenisation procedures used.
4.5 Cellular recruitment and pathology in the lungs of BALB/c mice and CBA/Ca mice following infection with *S. pneumoniae* strain D39

Following the methodology for homogenisation and enzyme digestion of whole lung samples developed and tested in MF1 mice (section 3.4.4), BALB/c and CBA/Ca mice were examined to explore cellular recruitment and pathology following intranasal infection with D39 pneumococci.

The bacterial time courses suggested that the site of the difference between BALB/c and CBA/Ca mice was the lung. Supportive evidence for this conclusion came from the analysis of the cellular response in the lungs of CBA/Ca and BALB/c mice. The data imply that genes associated with the susceptibility or resistance to invasive pneumococcal disease are connected to the recruitment of PMNs. Importantly, this leukocyte population was exclusively neutrophils and therefore these data implies a difference in neutrophil recruitment.

In BALB/c mice, a greater inflammatory response post-infection was elicited than in CBA/Ca mice. The influx of neutrophils in BALB/c mice was greater, particularly at 12 hours post-infection, than that in CBA/Ca mice. This difference in the recruitment of neutrophils may account for the susceptibility in CBA/Ca mice and the resistance in BALB/c mice to invasive pneumococcal lung infection. This could be explained by comparing the pattern of cellular recruitment into the lungs with the numbers of viable bacteria in the lungs and blood. Thus, neutrophils infiltrate into the lungs in BALB/c mice, either at a sufficient rate and/or number, to limit the number of pneumococci. These leukocytes presumably prevent the dissemination of pneumococci into the systemic circulation. At the peak of leukocyte recruitment, at 48 hours, the numbers of pneumococci had declined, consistent with an important role for these inflammatory leukocytes in the clearance of pneumococci and recovery from infection. Although it must be stated that cause and effect has not been proven.

In contrast to BALB/c mice, the influx of neutrophils into the lungs of CBA/Ca mice was both slower in rate between 0 to 12 hours post-infection and with less total numbers of cells (at all time points taken post-infection). This situation could explain why pneumococcal levels rise unchecked. Presumably, levels of these bacteria reached a point where the growth and spread of pneumococci outstripped the influence of the
influx of neutrophils. Although the influx of neutrophils continued into lungs (at 24 hours post-infection just before these mice were moribund), systemic spread of pneumococci had occurred by this stage, giving rise to a lethal septicaemia.

That the ability to recruit inflammatory cells is fundamental to the phenotypic differences between the two strains was supported by histopathological evidence (section 3.6). In the wax sections examined, inflammatory lesions in BALB/c lungs were visible much earlier than those in CBA/Ca lungs (section 3.6.1). At 6 hours post-infection, no lesions in the lungs of CBA/Ca mice were observed, even though pneumococci had spread at this point, from the airways to the systemic circulation (section 3.3). This illustrates the inability of CBA/Ca mice to mount an effective immune response in the lungs to prevent the spread of pneumococci.

That an effective immune response was associated with the recruitment of neutrophils was indicated in BALB/c mice. At 12 hours post-infection, BALB/c mice were observed to have perivascular infiltration, not observed in CBA/Ca mice at this time point, and additionally, a more severe peribronchial cellular infiltration (section 3.6.1). A greater influx of neutrophils had occurred in BALB/c lungs at 12 hours post-infection, compared to CBA/Ca lungs (section 3.5.2). Therefore, in the lungs of BALB/c, an influx of neutrophils around both bronchioles and venules may have prevented pneumococcal dissemination.

In CBA/Ca mice, pneumococcal levels significantly increased by 24 hours post-infection accompanied by a rise in the numbers of neutrophils (section 3.3 and 3.5.2) and a greater severity of inflammatory lesions observed than at the previous times post-infection. At this time, these inflammatory lesions in CBA/Ca were as severe as those found in BALB/c mice (section 3.6.1). This may indicate that CBA/Ca mice may have succumbed to an acute sepsis by large numbers of bacterial cells in the peripheral blood circulation rather than host inflammatory response in classic pneumococcal pneumonia. However, this is speculative as cause and effect is not proven.

Application of a numerical score to grade the response seen in frozen sections, gave an opportunity for statistical analysis. These data indicated that lesions in the lungs in BALB/c mice were significantly different to CBA/Ca mice. The difference in 'inflammatory score' being accounted for primarily by the greater perivascular,
peribronchial, intrabronchial cellular infiltration and bronchiole hyperplasia in BALB/c mice compared to CBA/Ca mice. That the sections were significantly different at 12 hours post-infection, reflects the observation on the total leukocyte population and the recruitment of neutrophils (section 3.5.1 and 3.5.2). In addition, it compliments observations in the paraffin wax sections, that the inflammatory lesions were greater in BALB/c mice than CBA/Ca mice as a consequence of the influx of neutrophils.

Whether the marked hyperplasia of the bronchioles of BALB/c mice is an important difference between both strains of mice remains in question, because this type of lesion was observed in the frozen sections, but was not in the paraffin wax sections (section 3.6.1 and 3.6.2). This may demonstrate a difference between both methods of histological preparation. Although paraffin wax sections give a clearer resolution of tissue the process exerts different pressures on the tissue compared to frozen sections, which may stretch the tissue thereby masking some detail (H. Jones, AstraZeneca plc, Alderley Edge, UK, personal communication).

Interestingly, both BALB/c mice and CBA/Ca mice differed in the histological median score at 0 hours (section 3.6.2). Additionally, the level of total leukocytes in the lungs of CBA/Ca mice at 0 hours were lower than BALB/c mice (section 3.5.1). These data may suggest a deficiency in the baseline numbers of leukocytes in the lungs of CBA/Ca mice, as the cause of susceptibility to pneumococcal infection. Supportive evidence for this hypothesis comes from a study by Russell et al., (1951) who compared inbred strains for leukocyte counts in the blood and found CBA/J to have a low baseline level. However, these data by Russell et al., (1951) did not compare lung leukocyte levels. Importantly, a clear difference in neutrophil recruitment was seen between BALB/c and CBA/Ca strains in the experiments in this thesis. In addition to this, even though total leukocyte levels in the lungs of BALB/c mice were statistically higher than in CBA/Ca mice at 0 hours, the numbers of neutrophils in the lungs were no different between both strains at this time point. These reasons lead to the conclusion that purely a deficiency in the numbers of leukocytes may not be the factor attributable to the susceptibility or resistance to pneumococcal infection in these mice.

There is evidence that a difference in the recruitment of neutrophils is the basis of innate susceptibility to other infections. For example, the differences in response of
resistant BALB/c-H-2\textsuperscript{k} and susceptible CBA/CaH mice to \textit{Candida albicans} was suggested as representing differences in differentiation and recruitment of PMNs between the strains (Ashman and Papadimitriou 1992). In an endobronchial model of infection with \textit{Pseudomonas aeruginosa}, resistant BALB/c mice showed greater recruitment of PMNs into the bronchoalveolar spaces, than susceptible DBA/2 mice (Morissette \textit{et al}., 1995). Morissette \textit{et al}., (1995) also suggested that the function of PMNs may be a factor in the genetic resistance to \textit{P. aeruginosa}, together with chemotactic factors and proinflammatory cytokines. Interestingly, DBA/2 mice are deficient in the complement component C5, and this will be discussed in context of the susceptibility of inbred mice to pneumococci later in this section.

The recruitment of neutrophils into the peritoneal cavity of mice has been reported to be under the genetic influence of the H-2 haplotype, in conjunction with genes unconnected with the major histocompatibility complex (MHC), to enhance the response (Marley \textit{et al}., 1994). Thus, it is a polygenic effect (Marley \textit{et al}., 1994). In their study, Marley \textit{et al}., (1994) showed that strains also differed in neutrophil response to different stimuli (sterile irritant, killed Gram positive bacteria and Gram negative bacteria). C57BL/10 had a high responder phenotype (greatest neutrophil response to sterile irritant) than BALB/c mice (Marley \textit{et al}., 1994). Baseline numbers of neutrophils did not account for the phenotypes observed because C57BL/10 mice had lower numbers of resting neutrophils in blood, bone marrow and peritoneal cavity compared to the other strains in the study. Interestingly, BALB/c mice recruited neutrophils in greater numbers in response to Gram positive \textit{Staphylococcus aureus} than C57BL/10 (Marley \textit{et al}., 1994).

Importantly, Marley \textit{et al}., (1994) found that genes not associated with MHC were also involved in the neutrophil response in their study. Evidence suggests that in the experiments in this thesis, genes unconnected to H-2 haplotype are involved in the resistance and susceptibility to the pneumococcus. CBA/Ca with H-2\textsuperscript{k} haplotype were significantly more susceptible to pneumococcal infection than the H-2\textsuperscript{k} AKR (section 3.1). BALB/c with H-2\textsuperscript{d} haplotype was significantly more resistant to pneumococcal infection than DBA/2, which is also H-2\textsuperscript{d} (section 3.1). Although we are not yet in a position to identify the genes that underpin resistance, the continuing genotyping of F2 CBA/Ca x BALB/c crosses, indicates that an association with H-2 has been excluded.
Discussion in this section so far has centred upon the recruitment of neutrophils as the basis of innate susceptibility or resistance to pneumococcal infection. It is important to discuss the recruitment of neutrophils in a wider context, as this process is a complex interaction of a number of factors. Chemokines, cytokines and other mediators of inflammation have a profound effect on the recruitment and function of neutrophils. In addition, these molecules modulate inflammation and affect the overall outcome of the infection.

Discussed in section 1.2.1.4, the main cytokines so far found to be involved in experimental models of pneumococcal disease have been TNF-α, IL-1, IL-6, IL-10 and IFN-γ (Benton et al., 1995, 1998, Takashima et al., 1997, van der Poll et al., 1996, 1997, Rubins and Pomeroy 1997, Bergeron et al., 1998). One could speculate that a difference in the balance of anti-inflammatory mediators and pro-inflammatory mediators would affect the recruitment and function of neutrophils. Reports have shown the effects of modulating anti-inflammatory and pro-inflammatory cytokines in mice and the outcome of disease (Takashima et al., 1997, van der Poll et al., 1996, 1997, Rubins and Pomeroy 1997).

Takashima et al., (1997) suggested that TNF-α was important in preventing bacteremia in a mouse model of pneumococcal pneumonia after intranasal infection, by increasing the numbers of neutrophils in the blood. Therefore, a potential difference between CBA/Ca mice and BALB/c mice may be due to differences in the levels or effects of TNF-α, particularly in view of the fact that CBA/Ca mice had a lower number of neutrophils in the lungs post-infection and developed a high bacteremia. Interestingly, following the results with the experiments in this thesis, both CBA/Ca mice and BALB/c mice have been examined for cytokine differences in some of the important mediators of inflammation (A. Kerr, University of Glasgow, UK, personal communication). At 6 hours and 12 hours post-infection, levels of TNF-α in BALB/c lungs exceeded those found in CBA/Ca mice. This could indicate that this cytokine is involved in the genetic susceptibility to pneumococcal infection in this model. However, this difference probably reflects the numbers of neutrophils present, as an association with TNF-α in our study has been excluded (P. Denney, MRC Mouse Genome Centre, Harwell, UK, personal communication).
Genome Centre, Harwell, UK, personal communication). Although it is interesting to note that *in vitro* BALB/c mouse cells recovered from the airways have been found to produce more TNF-α after stimulation, than CBA/Ca mouse cells (A. Kerr, University of Glasgow, UK, personal communication).

The antagonist to TNF-α, the anti-inflammatory cytokine IL-10, has also been suggested to be important in the outcome of pneumococcal disease in a mouse model of infection. van der Poll *et al.*, (1996) showed that addition of IL-10 antibodies had an effect of reducing the level of TNF-α and IFN-γ in the lungs, increasing pneumococcal levels in the lungs and blood, and shortening survival time. Therefore, a difference in the level or function of IL-10 between CBA/Ca mice and BALB/c mice may account for the difference in susceptibility to pneumococcal infection. Interestingly, in the lungs of mice, constitutive expression of IL-10 has been observed (van der Poll *et al.*, 1996). This constitutive expression is under genetic control and could therefore, be effected by genetic variation within strains. Thus, CBA/Ca mice may have a higher constitutive expression of IL-10 or differ in the production of this cytokine post-infection compared with BALB/c mice.

Studies with IL-6 knockout mice, IFN-γ knockout mice have suggested the importance of these molecules in determining higher mortality and pneumococcal numbers (van der Poll *et al.*, 1997, Rubins and Pomeroy 1997). These studies indicate that these molecules may be involved in the genetic susceptibility to pneumococcal infection.

In spite of evidence in favour of certain cytokines, the overall role of cytokines in pneumococcal disease and the susceptibility to pneumococcal disease needs to be further addressed. Interestingly, the studies examining the role of cytokines in animal models of pneumococcal disease have used C57BL/6 mice and strains with a C57BL/6 background, CBA/J, CBA/N, and CD1 Swiss mice (Benton *et al.*, 1995, 1998, Takashima *et al.*, 1997, van der Poll *et al.*, 1996, 1997, Rubins and Pomeroy 1997, Bergeron *et al.*, 1998). These strains of mice all have differing susceptibilities to pneumococcal infection, which is dependent on the genetic background of the mouse and the pneumococcal strain used for infection. Therefore, further work within this area should focus upon possible differences between the genetic background of murine
strains and the importance of different cytokines in the outcome of disease.

Chemokines (discussed in section 1.2.1.4) are important chemotactic proteins. The role of chemokines in mouse models of pneumococcal disease has not been fully elucidated. However, it is important to mention these molecules in context to this study, as differences in chemokines are likely to have a profound effect on the recruitment of neutrophils. Chemokines such as MIP-2 have been shown to be important in neutrophil recruitment in models of *Escherichia coli* urinary tract infection and *Klebsiella pneumoniae* pneumonia (Hang et al., 1999, Greenberger et al., 1996). MIP-1α has been shown to be produced by neutrophils following *in vitro* stimulation with pneumococci (Hachicha et al., 1998). Therefore, these and other chemokines are candidates to account for the difference between the neutrophil recruitment in BALB/c and CBA/Ca.

A difference in the recruitment of neutrophils in the murine strains could even extend to differences in other mediators of inflammation such as the lipid mediators, and in particular PAF (discussed in section 1.2.1.4). However, at present there is a lack of a full understanding of the interaction of these mediators in pneumococcal disease. Whether a difference between these lipid mediators in CBA/Ca and BALB/c mice would account for the difference in susceptibility to pneumococcal disease remains to be seen.

Although cytokines, chemokines and other mediators can effect the recruitment of neutrophils, in order for these leukocytes to reach their target sites an interaction with adhesion molecules usually occurs. Therefore, a difference in the function or production of these molecules could effect neutrophil recruitment. This may not relate to a difference of adhesion molecules on neutrophils themselves, but differences in these molecules on endothelial and epithelial cells.

Adhesion molecules have been discussed in section 1.2.1.5. Interestingly, neutrophil recruitment into the lungs from pulmonary capillaries following pneumococcal infection is both independent of integrins (CD11/CD18) (Doerschuk et al., 1990, Harlan et al., 1992, Hellewell et al., 1994, Mizgerd et al., 1999), and selectins (Bullard et al., 1995, Mizgerd et al., 1996, Doyle et al., 1997). However, this area of pneumococcal research needs to be addressed further. The mechanism of integrin (CD11/CD18) independent emigration of neutrophils is not fully elucidated but implies
involvement of other adhesion molecules such as integrin α4β7, VLA-4, with their ligands MAdCAM-1 (mucosal addressin cell adhesion molecule), VCAM-1 is a possibility (A. Kadioglu, University of Leicester, UK, personal communication).

Some of the experiments to examine the interaction with adhesion molecules were done using inbred mice, particularly on a C57BL/6 genetic background (found to be intermediate in susceptibility to the pneumococcus), which may have affected the results. In one study looking at ICAM-1 expression in the lungs of mice, BALB/c mice were used and were given an extremely high dose of $10^9$ pneumococci/ml intratracheally (Burns et al., 1994).

Although this study suggests the recruitment of neutrophils is involved in the resistance and susceptibility of pneumococcal infection, from the data one cannot determine if, additionally there is a difference in the anti-microbial activity of the two sets of neutrophils. The functionality of these cells may be as, or more important than the rate of influx or total numbers of neutrophils.

As discussed in section 1.2.1.3, once engulfed by phagocytes pneumococci are easily killed inside the phagocytic vacuole, which in part may be a consequence of its own $\text{H}_2\text{O}_2$ production (Johnston 1981, 1991). Human patients with impairment of the production of neutrophil-derived oxidants or abnormality of neutrophil granules do not appear to have an increased susceptibility to pneumococcal disease (Strauss et al., 1974, Johnston and Newman 1977, Komiyama et al., 1979). With the array of bactericidal components available (discussed in section 1.2.1.3), there appears to be no one exclusive anti-microbial mechanism. Therefore, a deficiency in one component may be compensated by another. Thus it is more likely that differences in anti-microbial may be due to a difference in the phagocytosis of pneumococci. However, a major difference of phagocytic function between these mice has (to the knowledge of this author) not been reported. Within the time frame and resources available during this project, further experiments to pursue questions of this nature were not achievable. Further work, such as phagocytosis, chemotaxis and bacterial killing assays involving D39 pneumococci and CBA/Ca and BALB/c neutrophils would address this issue.
4.6 Other possible mechanisms involved in the resistance or susceptibility to *S. pneumoniae*

Interestingly, during the time course investigations in CBA/Ca and BALB/c mice, pneumococci were detected in the blood of CBA/Ca mice early in infection (section 3.3). This was before a significant recruitment of neutrophils into the lungs of CBA/Ca mice had begun (section 3.5.2). This suggests a role for other components of the immune system other than or in addition to neutrophils.

Evidence in support of this comes from the examination of outbred MF1 mice. Kadioglu *et al.*, (2000) compared the difference in cellular responses following infection with a mutant of D39 lacking the gene for pneumolysin (designated PLN-A) and D39. After intranasal infection, the presence of pneumolysin had an effect on inflammation, increasing both the timing and extent of neutrophil recruitment into the lungs (Kadioglu *et al.*, 2000). However, in both wildtype and PLN-A pneumococcal infected MF1 mice, in the early time points post-infection, bacterial numbers declined before an elevation of leukocyte levels in the lungs (Kadioglu *et al.*, 2000). This suggested that early in infection there was an interaction with other components of the immune system. Possibilities are surfactants, complement and/or resident macrophages (Kadioglu *et al.*, 2000).

Macrophages are the first line of defense in the lungs (Kemper and Deresinski 1994). These cells are effective phagocytes and/or mediators of immunity by the release of a number of mediator molecules to regulate the immune response (Speert 1992). Macrophages could control bacterial dissemination in the lungs and recruit leukocytes to the sites of infection; therefore, a genetic difference in the function of these cells could affect the susceptibility to disease.

Susceptibility to some infectious diseases, as discussed in section 1.4, is controlled by a dominant locus on mouse chromosome 1, called *Lsr1*, *Ity* or *Bcg* (Bradley 1977, Gros *et al.*, 1981, Skamene *et al.*, 1982, Plant *et al.*, 1979, 1982). *Nramp1* is the candidate gene for this trait (Vidal *et al.*, 1993). Nramp1 has been shown to be a divalent cation transporter affecting the production of antimicrobial hydroxyl radicals, endosomal metalloprotease activity and phagolysosome fusion (Blackwell *et al.*, 2000). However, although the *Nramp1* genotype has an effect on the innate
resistance to some intracellular pathogens, no reports have shown any association to extracellular pathogens such as *S. pneumoniae*. Furthermore, Briles *et al.*, (1986) have suggested that inheritance of either a susceptible or resistant *Nramp1* genotype is unrelated to the susceptibility of mice to pneumococcal infection. Studies in this thesis indicate that neither the susceptibility or the resistance phenotype of *Nramp1* plays a role in pneumococcal infection; CBA/Ca mice were more susceptible than AKR mice, and BALB/c mice were more resistant than C57BL/6 mice (section 3.1). AKR/J mice and CBA/J mice have the resistant *Nramp1* genotype whereas BALB/cJ and C57BL/6J have the susceptible *Nramp1* genotype (Skamene *et al.*, 1982).

Although *Nramp1* may not be associated, this does not preclude a genetic association to an involvement of macrophages in the genetic susceptibility to pneumococcal disease. Indeed, one report showed that the response of macrophages to stimuli differed between different inbred strains (Sluiter *et al.*, 1985). Marley *et al.*, (1994) suggested that these responses in macrophages might be correlated in function, with responses in neutrophils. However, the involvement of macrophages in pneumococcal disease is poorly understood.

The humoral immune system is an important host defense (discussed in section 1.2.1.2), which could be involved in the susceptibility or resistance to the pneumococcus. There is evidence, for example in the study of Amsbaugh *et al.*, (1972), that different mice strains have different anti-capsular antibody responses. From a panel of different mouse strains, BALB/c mice where found to have the highest response to polysaccharide antigen (Amsbaugh *et al.*, 1972). This response was not associated with H-2 haplotype (Amsbaugh *et al.*, 1972). Another study by Smith *et al.*, (1976), showed that BALB/c mice had a greater antibody response to lipopolysaccharide and type 3 capsular polysaccharide both at a young age and later in life than that of SJL/J mice.

However, immunoglobulins are usually produced on the 5th or 6th day following pneumococcal infection (Austrian 1980). The peak of antibody responses, as assessed by plaque-forming cells to type 3 pneumococcal polysaccharide, occurs at 5 days post-immunisation in mice (Baker *et al.*, 1970). Therefore, acquired immunoglobulin differences between resistant and susceptible mice would appear unlikely, as the phenotypic difference between BALB/c and CBA/Ca mice occurs early in infection. In
addition, a selection of mice used in the experiments in this thesis were tested for pre-existing antibody to the capsular type 2, and were found to have none. Furthermore, several studies have suggested that pneumococcal susceptibility in mice with a CBA background is not exclusively associated the *xid* locus but involves other unknown genes (Briles *et al.*, 1986, 1992, Takashima *et al.*, 1996). The *xid* locus confers in CBA/N mice an inability to produce anti-polysaccharide including anti-phosphorylcholine antibodies (Briles *et al.*, 1980, 1981a).

Although susceptibility to pneumococcal disease associated with a difference in humoral response seems unlikely, a difference in the anti-phosphorylcholine antibody response between susceptible and resistant mice cannot be ruled out. This innate immune mechanism has been discussed in section 1.4.2. A number of reports have indicated that anti-phosphorylcholine antibodies are protective in infection models of pneumococcal disease (Briles *et al.*, 1981, Szu *et al.*, 1983, McDaniel *et al.*, 1984). Protection with phosphorylcholine antibodies appears to be mediated via the activation of complement (Briles *et al.*, 1985, Briles *et al.*, 1989). Therefore, in the model of infection in this thesis, a difference in anti-phosphorylcholine antibodies between CBA/Ca and BALB/c may account for the difference in the level of neutrophil recruitment and susceptibility to infection.

An anti-phosphorylcholine antibody response in mice generates different idiotype groups (Perlmutter *et al.*, 1984). Of these groups, the T15 idiotype has been found to be the most protective in mice (Briles *et al.*, 1981a). Interestingly, expression of the T15 idiotype is dominant in BALB/c mice in over 90% of primary anti-phosphorylcholine plaque-forming cells, whereas in other mouse strains such as CBA, less than 20% of anti-phosphorylcholine plaque-forming cells are positive for the T15 idiotype (Sher and Cohn 1972, Cosenza and Hohler 1972, Stall *et al.*, 1986).

It has been found that phosphorylcholine antibodies give no protection against some strains of pneumococci (Yother *et al.*, 1982, Szu *et al.*, 1983, Briles *et al.*, 1986 unpublished), one of which was a type 3 serotype. In this thesis, in addition to type 2 D39 pneumococci, a type 3 strain GB05, was used in the initial challenges on a panel of inbred strains (section 3.1). The results with this strain still indicated that CBA/Ca mice were susceptible to pneumococcal infection whereas BALB/c mice were resistant.
As previously mentioned, an assay was used to test a selection of mice used in the experiments in this thesis for pre-existing antibody to the capsular type 2. In this ELISA assay, the 23-valent polysaccharide vaccine was used to coat microtitre plates. Cell wall polysaccharide is found in pneumococcal capsular polysaccharide preparations (Soininen et al., 2000). Therefore, the detection of anti-polysaccharide antibodies would include detection of a level of phosphorylcholine antibodies. A difference between the levels of antibodies against the 23 valent polysaccharide vaccine in the serum of these mice was not found. However, on reflection it may have been useful to analyse the levels of T15 idiotype antibody in these mice.

Another factor in the humoral system, discussed in section 1.2.1, is the complement system and in particular, C3 and C5 derived components. Human case studies and animal models have show the importance of complement pathway deficiencies in pneumococcal disease. Therefore, it could be postulated that differences between susceptible mice and resistant mice may be attributed to a genetic factors giving rise to deficiency in either the amount or function of complement. These differences would then affect opsonisation, chemotaxis or stimulation of phagocytes.

Mice generally have lower complement levels than other mammals (Rice 1950). Haemolytic complement activities are found to vary in inbred mice (Ong and Mattes 1989). However, even though in one study BALB/c mice were found to have a higher haemolytic complement activity that CBA/J mice (Rosenberg and Tachibana 1969), BALB/c mice were found to be similar to SJL/J in another study (Ong and Mattes 1989). These results indicate that the susceptibility difference between BALB/c and SJL mice in this thesis is probably not due to haemolytic complement differences. Furthermore, the membrane attack complex of complement does not lead to lysis of pneumococci (Kemper and Deresinski 1994, Cooper 1991), therefore it may be a difference in the opsonic action and chemotactic properties of the early complement components that could potentially differ between susceptible and resistant phenotypes.

Evidence from the results in this thesis suggests that C5 complement component is not associated with susceptibility in this model of pneumococcal disease. In this study DBA/2 mice, which are C5 deficient, were more resistant to infection than CBA/Ca mice to D39 pneumococci and not statistically different from BALB/c mice in
susceptibility to infection with GB05 pneumococci (section 3.1.2).

Interestingly, in a study by Shin et al., (1969) with B10.D2 mice deficient in C5, the absence of C5 did have a detrimental effect on mortality rate post-infection. This suggests that C5 has an effect, but is dependent on the mouse genetic background. This phenomenon has been demonstrated in other models of infection. Cerquetti et al., (1986) have shown that in an endobronchial model of infection with *Pseudomonas aeruginosa*, bacterial clearance was modulated by C5 but was affected by the genetic background of the C5 deficient strain. C5 deficient B10.D2 mice were observed to have a difference in pulmonary bacterial clearance and PMN recruitment compared to C5 deficient DBA/2 mice (Cerquetti et al., 1986).

Studies in the literature discussed in section 1.2.1 suggest the role of C3 in pneumococcal disease. However, to the knowledge of this author no studies have specifically looked at the difference in C3 between BALB/c and CBA/Ca mice. Early complement components such as C3 could play a role in the genetic susceptibility to pneumococcal disease.

The continuing genome analysis of the BALB/c and CBA/Ca F2 generation tissue samples will allow any association to be seen with genes connected with areas of the immune system discussed in this section.

4.7 Progress on the murine genome scanning project

The murine genome scanning for QTLs involved in the susceptibility or resistance to pneumococcal infection, started during this study, has not yet been completed. As mentioned in section 4.6 no link has been found to genes associated with H-2 or the TNF-α gene. The statistical link discovered by Briles et al., (1986) with susceptibility to pneumococcal infection and the *Akp-1* locus on chromosome 1, has also been excluded. However, an association between markers on one of the murine chromosomes has suggested a location for a QTL (P. Denney, MRC Mouse Genome Centre, Harwell, UK, personal communication). For confidentiality reasons, details on these results cannot be given in this thesis. Further fine mapping with more markers in this area, together with congenics and positional cloning, will elucidate the exact location and sequence of this identified QTL.
In experiments in this thesis, SJL mice were found to be susceptible to pneumococcal infection (section 3.1). In addition, these mice were observed to have a difference in symptoms post-infection compared to CBA/Ca mice (section 3.1). This may suggest a possible genotypic difference between both these susceptible strains of mice. Although it cannot be ruled out that the susceptibility gene or genes, could be the same, but behave differently dependant on the genetic background of the mouse strain. However, until further investigation with this strain of mouse, genes associated with H-2, the TNF-α gene, or the Akp-1 locus on chromosome 1 cannot be excluded.

That there is a difference between the genetic association to pneumococcal disease obtained by Briles et al., (1986) and this study may also signify the effect of both the genetic background of the host and the pathogen. Therefore, different genes maybe associated with susceptibility to other pneumococcal strains. Additionally, genetic susceptibility may be dependant on the site of infection. Briles et al., (1986) used an intravenous route of infection whereas in this study mice were challenged intranasally, thus a different combination of genes may occur in the genetic susceptibility or resistance to pneumococcal pneumonia, meningitis, and bacteremia.

4.8 Human studies

From the list of genes of known immunological importance (Figure 1.4), a possible association with mannose binding lectin (MBL) was found in the human studies that have been done (S. Roy, Wellcome Trust Centre, Oxford University, UK, personal communication). However, these data were generated from a small limited human case study. Therefore, further case studies are needed from different population groups, to determine how important MBL is in the susceptibility to pneumococcal disease.

Point mutations in MBL are associated with codons 52, 54 and 57 (Lipscombe et al., 1992, Madsen et al., 1994, Sumiya et al., 1991), and are connected with a low level of MBL (Holmskov et al., 1994), which is associated with susceptibility to disease particularly in childhood (Turner 1998). However, the fact that these mutations are relatively common suggests a selective advantage of MBL deficiency (Holmskov et al., 1994, Turner 1998). The significance of this observation is undetermined at present (Babovic-Vuksanovic 1999).
Interestingly, it has been shown that pneumococci (10 strains of serotype 1, 3 to 6, 9V, 14, 19F, 18, and 23) in vitro do not bind MBL efficiently, compared to other microorganisms (Neth et al., 2000). Therefore, complement activation by MBL (Hoppe and Reid 1994) maybe a more important mechanism associated with the susceptibility or resistance to pneumococcal disease, than opsonophagocytosis via MBL. MBL activates complement (Hoppe and Reid 1994), by the formation of a complex with MBL associated serine protease 1 (MASP1) (Matsushita and Fujita 1992), and MBL associated serine protease 2 (MASP2) (Thiel et al., 1997). Another component has been recognised in this complex (Thiel et al., 1997), although the role of MBL associated protein (Map19) has not been clarified (Stover et al., 1999). The presence of these components of the MBL-mediated complement activation pathway raises the question that polymorphisms may occur in the genes encoding them. These genetic differences could be associated with the susceptibility to pneumococcal disease by affecting the MBL-mediated complement activation pathway. This could give a hereditary disadvantage/advantage in innate complement activation, which could therefore affect complement-mediated immunity to the pneumococcus. However, this is speculative and further study is needed to clarify the role of MBL the susceptibility to pneumococcal disease.

4.9 Summary

In summary, the results of this study demonstrate that susceptibilities in inbred mice to pneumococcal isolates varies in both the genetic background of the mouse strain and the capsular serotype. BALB/c mice are genetically resistant to intranasal pneumococcal infection compared to CBA/Ca and SJL mice. In both BALB/c and CBA/Ca mice, the cellular response observed during infection was different and these early cellular responses may explain the genetic resistance or susceptible phenotype observed. These data suggest that BALB/c mice have a greater neutrophil response to pneumococcal infection than CBA/Ca. The process of neutrophil recruitment is complex and an active area of research in pneumococcal pathogenesis. Mediators of inflammation, such as cytokines and chemokines have been previously implicated in pneumococcal disease discussed in section 1.2.1.4. Cytokines and chemokines that influence the recruitment and function of neutrophils warrant further investigation. However, other components related to innate immunity and the inflammatory process
should not be overlooked, such as the role of adhesion molecules, complement, collectins, and resident macrophages.

Studies with both BALB/c and CBA/Ca mouse strains will continue to elucidate the key components involved in the behaviour of neutrophils in pneumococcal disease. Data from this study also emphasises the need for further understanding of the effect on the immune system of the genetic background of the host. An understanding of the host immunity at the genetic level may uncover potential target sites in the host for therapeutic and prophylactic treatments. In addition, may also identify individuals who may require special treatment or observation, for example genetically susceptible patients attending hospital.
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