TUBERCULOSIS: PROSPECTS FOR AN ORAL VACCINE USING NOVEL ANTIGENS AND ADJUVANTS

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I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Abstract

In spite of vaccine and treatment strategies, *Mycobacterium tuberculosis* kills more than 3 people per minute. The emergence of drug-resistant strains makes treating the disease complicated and expensive for government health departments, and unpleasant and laborious for patients. The current vaccine, parenterally administered BCG, is only 50% effective. Oral vaccination has the advantage of targeting the mucosal immune system, which acts at the direct site of initial exposure to the infecting airborne pathogen. In addition, oral vaccines are cheaper and safer to administer than parenteral vaccines. This dissertation provides a conceptual framework for the prevention of the disease by means of oral vaccination and outlines methods that were developed for the production of concentrated purified somatic and extracellular antigens. Immune responses to somatic antigens were also examined in conjunction with established and novel adjuvants. The role of *Propionibacterium jensenii* 702 as a suitable mucosal adjuvant was supported by the results obtained.

Abbreviations

”	inch
%	percent
α	alpha
Ag	antigen
AIDS	Acquired Immunodeficiency Syndrome
A-SA	apical-subapical
ATCC	American Type Culture Collection
β	beta
BCG	Bacille Calmette-Guérin
BSA	bovine serum albumin
ºC	degrees Celsius
CD	cluster of differentiation
CDC	Centers for Diseases Control and Prevention
CFP	culture filtrate protein
cfu	colony-forming unit
cm	centimetres
CMI	cell-mediated immunity
Con A	Concanavalin A
cpm	counts per minute
CSF	cerebrospinal fluid
CT	cholera toxin
δ	delta
Da	Daltons
DDA	Dimethyl dioctadecyl ammonium bromide
dd-mm-yy	date, in the format day-month-year, each with two digits
dH_20	distilled water
DN	double negative
DNA	deoxyribonucleic acid
DOTS	Directly Observed Therapy – Short-course

DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
ESAT	early secretory antigen target
et al.	and others
FCA	Freund's Complete Adjuvant
FDCs	fixed dose combinations
g	gram
γ	gamma
HCl	hydrochloric acid
HIV	human immunodeficiency virus
IFA	Incomplete Freund's Adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Ipr1	Intracellular pathogen resistance 1
ISCOMS	immunostimulant complexes
IV	intravenous
kDa	kilo Daltons
L	litre
LabVISE	Laboratory Virology and Serology Reporting Scheme
LJ	Lowenstein-Jensen (medium)
LT	heat-labile enterotoxin
M	molar
M.	*Mycobacterium*
mA	milli Amperes
MDR	multi-drug-resistant
mg	milligram
MHC	major histocompatibility complex
min	minutes
ml	millilitres

mm	millimetres
MPB	*Mycobacterium bovis* protein
MPL	Monophosphoryl lipid A
MPT	*Mycobacterium tuberculosis* protein
MSM	Modified Sauton's Medium
MWCO	Molecular Weight Cut-off
N	no
N/A	not applicable
NaOH	sodium hydroxide
nm	nanometres
NNDSS	National Notifiable Disease Surveillance System
NSW	New South Wales, Australia
OD	optical density
P.	*Propionibacterium*
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picograms
PI	Protease Inhibitor solution
PJ702	*Propionibacterium jensenii* 702
PLG	polylactide-coglycolides
PPD	purified protein derivative
rpm	revolutions per minute
RNA	ribonucleic acid
SAF	Syntex Adjuvant Formulation
SDS	sodium dodecylsulfate
sec	seconds
STCF	short-term culture filtrate
TB	tuberculosis
Tc	cytotoxic T-lymphocyte
Th	helper T-lymphocyte

TLR	Toll-like receptor
TNF	Tumour necrosis factor
TST	Tuberculin skin test
µCi	micro Curie
µg	micrograms
µL	microliters
µm	micrometres
US	United States
UV	ultraviolet
V	Volts
VIC	Victoria, Australia
WA	Fraction A from whole *Mycobacterium tuberculosis* Sonicate
WCAB	Wilkins-Chalgren Anaerobe Broth
WE	Fraction E from whole *Mycobacterium tuberculosis* Sonicate
WHO	World Health Organization
WTB	Whole *Mycobacterium tuberculosis* Sonicate
x g	times gravity
Y	yes

Chapter 1: Introduction

Tuberculosis is a widespread respiratory infectious disease caused by the acid-fast organism, *Mycobacterium tuberculosis*. Between 2000 and 2020 it is expected that TB will infect nearly one billion people worldwide, 200 million of whom will develop active disease, and 35 million will die (World Health Organization 2000). Over the course of recorded history, tuberculosis has experienced numerous rises and falls in prevalence. The most recent epidemic occurred in the mid-1980s alongside the emergence of the Acquired Immunodeficiency Syndrome (AIDS) (Dove 1998). Multi-drug-resistant strains have made treatments complicated and expensive (CDC Fact Sheet #250112; TB "Hot Zones" Emerging 1998). Today, the disease is most prevalent in developing countries, where diagnosis and treatment are difficult to monitor, although drug-resistant strains have resulted in increased rates worldwide, including developed countries (Tuberculosis Elimination Revisited 1999; Cantwell et al. 1994).

In 1993, the World Health Organisation focused researchers' attention on combating the disease by declaring tuberculosis to be a Global Health Emergency (Raviglione et al 1995) and put procedures in place for increasing the effectiveness of disease treatment. Since that time, considerable research has occurred in the field of tuberculosis prevention. The current vaccine against tuberculosis is the Bacille Calmette-Guérin (BCG), which was first administered in 1921 (Harboe et al 1996a). Although it has an overall efficacy of only 50% (Colditz et al 1994), a

better alternative has not yet been developed. Many studies are examining novel vaccines based on subunit proteins of *M. tuberculosis*, DNA vaccines, recombinant vaccines and auxotrophs, some of which are progressing to clinical trials.

The majority of vaccines being developed are designed for parenteral administration. One of the major obvious disadvantages of this delivery method is the risk of needle-stick injuries to health care workers, particularly in high prevalence areas, such as developing countries, where other diseases including HIV are also prevalent. Mucosal delivery systems, including oral administration, overcome this problem. In addition, they are able to deliver the vaccine directly to the mucosal immune system, which is required to protect the host at the point of infection, that is, the lung mucosa.

One of the major problems associated with oral vaccination is oral tolerance. Oral tolerance is the suppression of potentially harmful immune responses to something the body considers to be a “safe” antigen. Oral administration of a vaccine can result in the permanent switching of the immune system from responsiveness to tolerance of that antigen (Czerkinsky & Holmgren 1995). The tactic employed to prevent oral tolerance to a vaccine is co-administration of an adjuvant. Until recently, the available mucosal adjuvants were all toxic to humans and could only be used in animal experimentation. Many resources have been poured into reducing the toxicity of available adjuvants. A better alternative, however, may be the use of a probiotic adjuvant, such as *Propionibacterium jensenii* 702 (Lean

2002; Adams et. al. 2005). Thus, this dissertation further examines the use of this probiotic as an adjuvant.

An additional issue surrounding oral vaccination is the requirement to administer greater amounts of antigen than the quantities required for parenteral vaccination. Current available literature provides information on the extraction of *M. tuberculosis* antigens at low concentrations, which are suitable for parenteral vaccination or lymphocyte culture stimulation. Thus, a considerable portion of this study was dedicated to the preparation and purification of antigens at sufficiently high concentrations to be useful for oral vaccination.

Finally, the purified antigens were tested with the novel adjuvant in a murine model. Unfortunately, unforseen problems interrupted the progress of experimentation and limited the scope of the study. These problems included air-conditioning failure, theft, laboratory closure and numerous equipment malfunctions. The experimental results that were obtained were carefully examined and some conclusions were able to be drawn.

In summary, the purpose of the research outlined in this dissertation was to show that subunit antigens could be purified from cellular extracts of *Mycobacterium tuberculosis* and culture media. These antigens were to be obtained in sufficiently high concentrations to be suitable for oral vaccination of mice. It was expected that vaccination with these antigens would lead to effective induction of protective immune responses.

Chapter 2: Literature Review

2.1 *Mycobacterium* Genus

2.1.1 Structure

Mycobacteria are a unique genus of organisms, due to the mycolic acid, which comprises approximately 60% of the cell wall. In structure, mycobacteria are similar to Gram negative bacteria, with a more complex cell wall structure than Gram positive bacteria. However, they are not considered to be a type of Gram negative bacteria because the lipopolysaccharides of Gram negative bacteria are replaced by mycolic acids in mycobacteria. The mycolic acids give each organism a water resistant waxy coating, which is responsible for many unusual characteristics related not only to physical properties, but also to metabolism and pathogenicity (Tortora et al. 1998a; Tortora et al. 1998b).

Mycobacteria grow in aerobic conditions and do not form spores (Tortora et al. 1998b). They are slender rod shaped organisms and grow either curved or straight. In size they are 0.2-0.6 x 1.0-10 μm (Murray et al. 2003). Mycobacteria usually occur singly or in clusters, but occasionally they may exhibit filamentous growth, particularly on the surface of liquid media. It is this characteristic that has provided the genus name ("myco" meaning fungus) (Tortora et al. 1998c).

2.1.2 Species

2.1.2.1 *M. tuberculosis*

Mycobacterium tuberculosis, the organism responsible for tuberculosis (TB), is one of the more notorious species in the *Mycobacterium* genus. The optimal growth temperature for this organism is 37°C (Prescott et al. 1999a) and it grows readily on simple substrates using glycerol as a carbon source and ammonia or amino acids as nitrogen sources (Murray et al. 2003). Some types of media currently used for culturing *M. tuberculosis* include Lowenstein Jensen Agar (bioMérieux), Mycobacteria 7H11 Agar with OADC enrichment (Difco), and modified Sauton's medium (Figure 2.1).

Even on an appropriate medium, the growth of *M. tuberculosis* is very slow. The waxy cell wall decreases the entry of nutrients into the cell, and thus reduces the metabolic rate of the organism (Tortora et al. 1998b). It takes 24 hours for a cell to divide and can take 2-3 weeks for visible cultures to appear on a solid medium (Young & Garbe 1991).

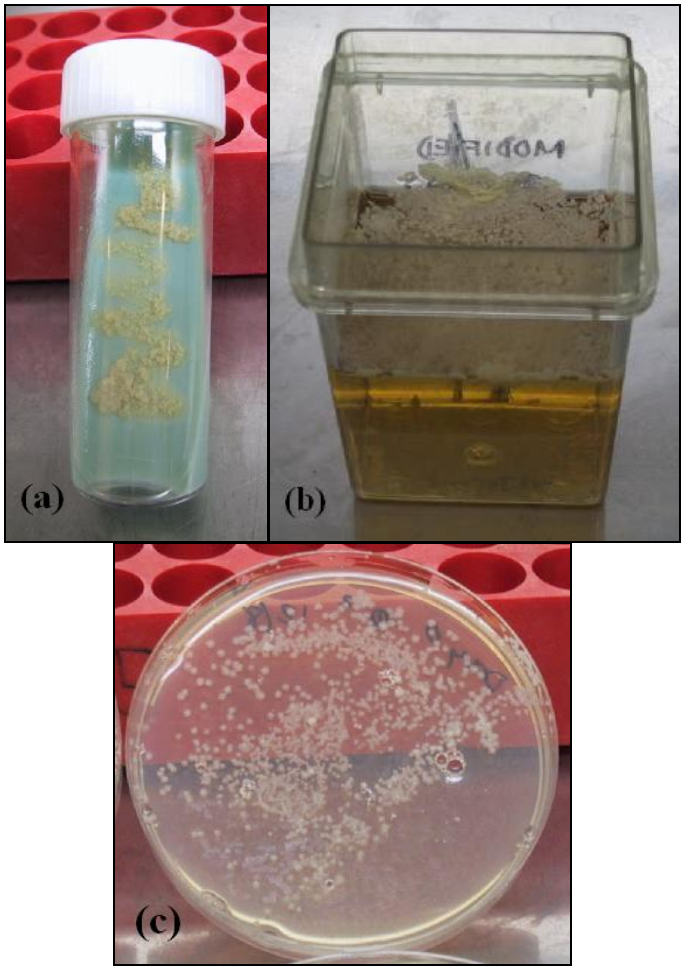

Figure 2.1. Growth of *M. tuberculosis* on (a) Lowenstein Jensen agar; (b) modified Sauton's medium; and, (c) Mycobacteria 7H11 Agar with OADC enrichment.

M. tuberculosis was first isolated by Robert Koch in 1882 using his newly reported postulates (Prescott et al. 1993b, Daniel 1997a). In spite of this, however, *M. tuberculosis* is one of the few species that is an exception to Koch's postulates. The same organism is responsible for causing a disease that can be manifest in a number of different organs (for example, lungs, skin, bones, and other internal organs), which, to an untrained eye, may appear to be a variety of completely unrelated diseases (Tortora et al. 1998d).

2.1.2.2 *M. bovis*

Mycobacterium bovis, as its name suggests, is an organism that causes tuberculosis in cattle, although it can also infect dogs, cats, pigs, parrots, badgers primates and humans (Murray et al. 2003). The disease can be transmitted from cattle to humans via contaminated milk or food. This was a common occurrence before the days of pasteurized milk and screening of cattle for the disease. In more recent years, *M. bovis* accounts for less than 1% of human tuberculosis cases in the United States (Tortora et al. 1998c).

2.1.2.3 *M. avium*

M. avium causes tuberculosis in birds. Its optimal growth temperature is 41-45°C and its pathogenicity in other animals is mild to non-existent (Pelczar & Reid 1972). It is very uncommon for this organism to infect humans, except in the late stages of HIV infection (Tortora et al. 1998c).

2.1.2.4 *M. leprae*

M. leprae is another well known species of the *Mycobacterium* genus. It is the organism that causes the disease most commonly known as leprosy, although the disease is also referred to as Hansen's disease, to avoid the fear associated with the word "leprosy". Gerhard A. Hansen was the first scientist to isolate and identify the organism in 1870 in Norway. The fear of leprosy is attributed to the biblical and historical references to the disease and the descriptions of its sufferers as being outcasts due to their infectivity. They

were expelled from cities and sometimes wore bells so as passers by would be aware of their disease and give them a wide berth (Tortora et al. 1998e; The Holy Bible 1986). In fact, leprosy is not a very contagious organism, and most transmission occurs by the secretions of an infected person contacting the nasal mucosa of an uninfected individual. Intimate contact over a considerable period of time is usually required for transmission to occur. Isolation is no longer required because the sufferer can be treated with sulfone drugs, such as dapsone, which make them non-infectious within a few days (Tortora et al. 1998e).

Once a person contracts leprosy, it may take a number of years for symptoms to appear, although the incubation time for children is considerably less. There are two stages to the disease. The first, called the “tuberculoid (neural) form”, is characterized by depigmented areas of skin, which have no sensation, and each surrounded by a border of nodules. Spontaneous recovery has been known to happen with respect to this form of the disease, however in patients with poorly effective cell-mediated immune responses, the disease progresses into the “lepromatous (progressive) form”. At this stage, disfiguring nodules may occur all over the body, particularly the cooler extremities. A “lion-faced” appearance is associated with infection of the mucous membranes of the nose (Tortora et al. 1998e).

Diagnosis of leprosy is by the detection of acid fast bacilli in body fluids drained from cool sites on the body, such as the ear lobe. The lepromin test

can also be used, which involves an intradermal injection of a lepromatous tissue extract. A visible skin reaction indicates an immune response to *M. leprae*; however the test is negative in the lepromatous form of the disease (Tortora et al. 1998e).

Although it is now known that the fear associated with leprosy is unfounded, the disease rate continues to increase and resistance to dapsone is becoming a major problem. It is now administered along with rifampin and clofazimine to reduce the risk of creating other resistant forms of the disease. Research is continuing into creating an effective vaccine. This has been encouraged by the fact that the BCG vaccinated individuals have experience some protection from leprosy (Tortora et al. 1998e).

M. leprae has an optimum growth temperature of 30°C, which explains its preference for the extremities of the body. Its generation time has been estimated at a very long 12 days. It has never been cultivated in laboratory conditions and is, therefore, one of the few organisms that is does not follow Koch's postulates. South American armadillos, which have a low body temperature of 30-35°C, are often infected with *M. leprae* in the wild and have been used for laboratory studies into examining the pathogenesis of the disease and treatment methods (Tortora et al. 1998e).

(The remainder of this thesis discusses *M. tuberculosis*, and more specifically, the pulmonary disease caused by *M. tuberculosis*, unless stated otherwise.)

2.2 History of *Mycobacterium tuberculosis*

2.2.1 Ancient history

Although *M. tuberculosis* was not isolated by Robert Koch until 1882, tuberculosis has been a recognized disease for thousands of years. Tuberculosis has been identified from images on ancient Egyptian art works and stone statues from around 3000 BC. Ancient texts from around 2000 BC in India and approximately the third century BC in China also describe the condition we now know to be tuberculosis (Daniel 1997a). It is mentioned in the Bible (Deut. 28:22, Lev. 26:16) where it is referred to as “consumption” (Tortora et al. 1998c). In Australia there is no evidence of tuberculosis among the indigenous Australians prior to the arrival of European explorers (Plant et al. 1995).

Throughout recorded history, the prevalence of tuberculosis has risen and fallen following the “herd immunity” concept, whereby those who become infected either die, or develop immunity. Thus the disease runs rampant until it exhausts the host supply for some time, only to rise again at a later date (Daniel 1997b).

For many years, tuberculosis was thought to be a hereditary disease until Benjamin Marten suggested, in 1720, that “animacula” (microorganisms) were responsible for the disease. Quarantine laws were introduced, although not strictly

enforced until the 1780s in Italy, and as late as the 1820s in England. The following years saw significant advances in science and technology that improved the quality of life and raised awareness of the infectious nature of diseases, including tuberculosis. In 1892 the first voluntary health agency, a tuberculosis association, was organised in Philadelphia (Daniel 1997a).

2.2.2 Modern history

The concept that a bacterium was responsible for tuberculosis was presented by Koch to a gathering of scientists in 1882 (Prescott et al. 1999b). This news spread quickly throughout the civilized world, sparking a fever of scientific research into the disease.

Four years after Koch's discovery that tuberculosis was a bacterial disease, two German chemists, V.H. and F. Soxhlet adapted the pasteurization techniques to the preservation of milk (Prescott et al. 1999c). This resulted in the reduction of milk-transmissible diseases, including tuberculosis (*M.* bovis). Pasteurization of milk was introduced in the United States in 1889 (Prescott et al. 1999c). By 1907, the mortality rate from tuberculosis had declined significantly in all developed countries. The high public awareness of measures to avoid TB infection lead to expectations that the disease would be completely wiped out within a short time (Bröker 1999). Developed countries established sanatoria to care for the disease stricken consumptives and to reduce the spread of the disease (Pearn 1987).

Scientific research aimed at reducing the burden of tuberculosis and focussed on prevention and treatment. A vaccine against tuberculosis, called BCG, was developed by Albert Calmette and Camille Guérin and was first administered in 1921 (Harboe et al. 1996a). BCG vaccination is effective in reducing tuberculosis infection and only slight changes have been made to this vaccine since its development (Pelczar & Reid 1972; Colditz et al 1994).

The first effective antibiotic for TB treatment was streptomycin (Tortora et al. 1998c). The development of this antibiotic was announced by Selman Waksman in 1944 (Prescott et al. 1999d). Other effective drugs for treating TB were also discovered in the 20 years that followed. These treatments further reduced incidence and mortality. It was believed that TB was in the process of eradication in developed countries and funding for TB research diminished. Sanatoria were closed down and public health systems that related to TB were dismantled, with the focus on a greatly improved community care service (Brown 1993).

By the 1970s, the incidence of tuberculosis was so low that funding for the community care services was eliminated. Patients who did not complete therapy were not followed up and it was not long before drug-resistant TB mutants began to emerge. Global economic recession, military unrest, poverty, malnutrition and human migration, IV drug use, homelessness, and overcrowding contributed to an increased spread of tuberculosis (Mori 2000; Bloom & Murray 1992; Brown 1993; Yang et al. 1998). In the 1980s, the human immunodeficiency virus (HIV) began to emerge (Brown 1993). HIV and TB form an unusual partnership (which will be

discussed in following sections), with each disease speeding the progress of the other (Ginsberg 2000; World Health Organization 2000).

The declining rate of incidence of TB, which had been 10% per year since the 1950s, became completely reversed in the 1980s. Between 1985 and 1992 the number of TB cases increased 18% in the United States (Bloom & Murray 1992). In April 1993 the World Health Organization (WHO) showed its concern about the neglected state of tuberculosis control and declared tuberculosis to be a global emergency (World Health Organization 2000).

Since 1993 many strategies have been established to reduce the incidence of tuberculosis. As a result, in the United States TB morbidity has steadily declined, reaching a record low in 1998. In addition, TB incidence has largely been reduced to a level where its occurrence is largely in well-defined risk groups (Tuberculosis Elimination Revisited 1999).

Between 1992 and 1997 in the United States, the number of TB cases decreased among U.S.-born individuals by 38%, but the incidence among foreign-born persons increased 6% (Tuberculosis Elimination Revisited 1999). In Eastern Europe, tuberculosis incidence rates peaked around 2001 and, with the exception of the African region, 2003 statistics indicate that worldwide incidence rate of tuberculosis are falling or stable (WHO Report 2005).

In spite of the encouraging statistics, the battle against TB is far from over, and complacency with respect to this disease should be feared lest the situation realised in the 1970s and 1980s returns.

2.3 Current Incidence

2.3.1 Worldwide

Tuberculosis is responsible for 5% of all worldwide deaths (Ginsberg 2000). 9.6% of adults between the ages of 15 and 59 die from tuberculosis, making it the foremost cause of death from a single infectious agent (Raviglione et al. 1995; Ginsberg 2000). Approximately one third of the world's population harbours the disease and is at risk of developing tuberculosis (Orme 1999a). In 2003, 8.81 million new cases were diagnosed, of which approximately 85% were pulmonary disease. 1.75 million people, that is more than 3 people per minute, died as a result of TB infection. The prevalence of tuberculosis in India alone accounts for almost one quarter of the world's total TB infections (WHO Report 2005). Between 2000 and 2020 it is expected that TB will infect nearly one billion people, 200 million of whom will develop active disease, and 35 million will die (World Health Organization 2000).

Of the 15 countries with the highest TB incidence rates per capita, 12 are from the African region, which also experience the highest rates of HIV/TB co-infection. The highest incidence of TB infection is in Swaziland where almost 1100 per 100,000 population were diagnosed in 2003 (WHO Report 2005). In spite of the high

incidence in several African countries, the South-East Asia Region accounts for 33% of global incidence (World Health Organization 2005).

2.3.2 Australia

There was no evidence of tuberculosis among indigenous Australians before the discovery of our country by European explorers (Plant et al. 1995). Like the rest of the world, Australia experienced a high rate of tuberculosis cases early last century, followed by a decline in TB rates since the 1950s (Dwyer 1996). In Australia, this decline is attributed to participation in the National TB Campaign (Gilroy, 1999). Unlike the global TB rates, however, the Australian statistics fail to reflect the increase in incidence seen in the late 1980s (Li et al. 2004). The rates of TB notification in Australia have remained relatively consistent since 1991 (Thomson et al. 1999; Gilroy 1999).

Australia continues to report one of the lowest rates of tuberculosis in the World with incidence rates of 4.9 new cases per 100,000 population in 2003 (Gilroy, 1999; Li et al. 2005). As such, TB may not be considered to be a problem in Australia. To the contrary, however, tuberculosis ranked tenth highest (out of 40) among Australia's notifiable diseases in 1998 and ranked 14th (out of 59) in 2003 (Thomson et al. 1999; Miller et al. 2005). Pulmonary tuberculosis accounted for 57.3% of all new and relapsed TB cases reported in 2003 (Li et al. 2005).

New South Wales (NSW) and Victoria (VIC), the two most populous states, have continually reported rates of between 5 and 8 per 100,000 over the last 10 years,

with 2003 rates in NSW and VIC being 5.4 and 6.5 per 100,000 population respectively. Lower notification rates have been reported in all other states and territories with the exception of the Northern Territory. This region consistently reports significantly higher rates per capita than any other state or territory, with 13.1 new cases per 100,000 population in 2003 (Antic et al. 2001; Li et al. 2004).

The high rate of tuberculosis in the Northern Territory is unusual for such a sparsely populated region and is attributed to a larger percentage of Indigenous persons occupying the area. Indigenous Australians represent about 25% of the population of the Northern Territory (Australian Bureau of Statistics 2005), and this subgroup of the Australian population has continually experienced almost 10-fold higher TB incidence rates than their non-Indigenous counterparts (Li et al. 2005). Indigenous Australians suffer from a social disadvantage that leads to poverty, underweight, homelessness, overcrowding and poor nutrition. These factors are all responsible for increasing the risk of contracting tuberculosis. In addition to these social factors, Indigenous Australians typically have a higher prevalence of diabetes mellitus, renal failure, alcohol abuse and smoking, all of which are risk factors for tuberculosis (Plant et al. 1995).

It is well recognised that Australians born overseas also experience higher tuberculosis notification rates. In 2003, the notification rate of tuberculosis among overseas-born Australians was 19.9 per 100,000 population. The equivalent rate for Australian-born persons was only 0.9 per 100,000 population (Li et al. 2004).

2.3.3 Prisons

The term “prison” incorporates a wide range of detention centres, from illegal immigrants and pre-trial holding, through to maximum security. At any given time, the world’s prisons may hold 8-10 million people and, due to the high turnover in this population, approximately 4-6 times this number pass through the prison systems each year (World Health Organization 2000).

Prisoners are considered to be a high-incidence population for tuberculosis infection, and may experience TB rates up to 100 times higher than the civilian population (World Health Organization 2000). There are numerous reasons for this high infection and disease rate. They include late diagnosis, inadequate treatment, overcrowding, poor ventilation, malnutrition, substance abuse and higher rates of HIV and other diseases (World Health Organization 2000). In addition to these factors, the screening of prisoners for TB can become very complicated for simple logistic reasons. Prisoners may remain in detention for short periods of time; they may be moved frequently or may spend considerable time in court (Jones et al. 1999). As a result, they may enter the prison system unscreened, thus introducing the infection to not only their fellow inmates, but also to the staff and visitors, and then on into the civilian community (World Health Organization 2000).

2.4 Pathogenesis

2.4.1 Risk factors

Many social and health factors contribute to an increased risk of not only becoming infected with tuberculosis, but also subsequently developing the active disease. These factors include increased population mobility due to military unrest and political instability, conflict, natural disasters and human migration. Risks are also increased among defined population subgroups, such as prisons, ethnic minorities, immigrants, indigenous persons and the elderly. In addition, economic recession leads to overcrowding, poor ventilation, poverty, homelessness and malnutrition, all of which are factors that provide optimum conditions for the spread of tuberculosis. The risk of developing tuberculosis is also increased among those with pathological diseases, such as diabetes mellitus, renal failure, HIV and immunosuppression; and among drug users, including IV drug use, alcohol abuse and smoking (Mori 2000; Bloom & Murray 1992; Brown 1993; Yang et al. 1998; Plant et al. 1995; World Health Organization 2000; Frieden et al. 1993; Prescott et al. 1999b; Fischer 1999; Antic et al. 2001).

All these risk factors contribute to disease risks in different ways. Population mobility not only increases the number of people that TB can be spread to, but it also poses significant problems in tracing contacts, which can result in delayed diagnosis of new cases, and in ensuring that treatments are completed. Military or political unrest is capable of destroying infrastructure that is put in place for the control of TB. Overcrowding, and defined population subgroups living in close

proximity have increased risks simply through higher levels of contact with an infected individual. The risk of transmission increases as the amount of time spent with an infectious person increases (Braden 1995). Malnutrition, substance abuse and pathology increase the risk of developing active disease once infected.

2.4.2 Natural resistance

In spite of the risk factors mentioned, studies have shown that humans have a natural resistance to acquiring TB (Schurr et al. 1991). In mice, the gene that confers natural resistance is called the "intracellular pathogen resistance 1 (*Ipr1*) gene" in the *sst1* (super-susceptibility to tuberculosis 1) region of chromosome 1. The product encoded by *Ipr1* may be responsible for integrating macrophage mechanisms with signals produced by intracellular pathogens, such as *M. tuberculosis* (Pan et al. 2005). It remains to be seen whether natural resistance in humans is controlled by a gene equivalent to that identified in the murine genome.

2.4.3 Methods of transmission

2.4.3.1 Respiratory

Respiratory mechanisms are by far the most common cause of TB transmission. Bacteria are expelled in tiny droplets from people with active tuberculosis either by sneezing, singing, talking, or coughing (Bloom & Murray 1992; Loudon & Roberts 1968; Loudon & Roberts 1967). These droplets can vary considerably in size and number. On average, one cough produces as many particles as about five minutes of loud continuous talking (Loudon & Roberts 1967). The smaller droplets fall to the ground much slower than

larger droplets. After 30 minutes, 40% of particles produced by coughing remain suspended in the air, compared with 6% of those produced during talking (Loudon & Roberts 1967). As the smaller droplets spend much longer in the air, they are more exposed to evaporation, leaving a residue that contains the bacteria. This residue is called a “droplet nucleus”, and is so light that it may drift in air currents until it is either inhaled, vented or destroyed (Wells et al. 1948).

Due to the waxy coating around *M. tuberculosis* cells, the bacteria are resistant to drying out and remain virulent, floating in the air currents, with the potential to cause infection (Tortora et al. 1998c). They are however, sensitive to UV light, which will eventually kill the bacteria (Peccia & Hernandez 2004).

2.4.3.2 Other means of transmission

Extrapulmonary tuberculosis is rarely considered contagious; however some cases have been reported. In 1992, a case was reported of a *M. tuberculosis* infection in an ulcer on the upper thigh of a patient. 18% of the persons present during dressing changes of that wound became newly infected with *M. tuberculosis* (Frampton 1992). A 1997 report traced the infection of a patient back to the use of a bronchoscope used by a tuberculous patient 6 months earlier. This case led to the review of endoscopic cleaning techniques (Michele et al. 1997). A tuberculous finger lesion has also been reported to have occurred as a result of a needle-stick injury obtained during incision and

draining of a patient's tuberculous thigh abscess (Hutton et al. 1990). Oral transmission is also possible, but is far less effective than respiratory transmission of the organism (Bloom & Murray 1992).

2.4.3.3 Inhalation of bacteria by a new host

A single droplet nucleus contains one to three *M. tuberculosis* cells. When inhaled, this is sufficient to begin the infection process (Wiegeshaus et al. 1989). For this infection to occur, the droplet nucleus usually needs to reach the apical-subapical (A-SA) area of the lungs, a region where bacilli can survive in low numbers even after the development of an immune response. This zone is relatively poorly ventilated and multiple exposures to *M. tuberculosis* are usually required before a primary infection can develop (Smith & Wiegeshaus 1989).

2.4.4 Disease progression

2.4.4.1 Macrophage involvement

In the lungs, the infecting bacteria are engulfed by alveolar macrophages, which recognize the bacteria merely as foreign matter. At this point, the destruction of the bacteria depends upon the microbicidal power of the macrophage, which reflects the interaction between the genetics of the host and the virulence of the bacterial strain. If the macrophage is able to destroy the bacteria, then the infection is arrested. If, however, the macrophage fails to destroy the bacteria, the infection progresses to the next stage (Dannenberg 1991).

2.4.4.2 Tubercle formation

The macrophages that are struggling to control the bacterial infection release pro-inflammatory cytokines and chemokines to recruit monocytes and lymphocytes to assist with control of the infection. The monocytes mature into multi-nucleated giant cells surrounded by T lymphocytes, producing a granulomatous lesion called a tubercle, which walls off the infection. These microscopic tubercles form approximately two to three weeks after inhalation of the organism. The logarithmic growth of the bacteria ceases due to the cell death at the caseous centre of the tubercle and the equilibrium formed by the marginal cell division and destruction within the macrophages on the periphery of the lesion (Dannenberg 1991; Andersen 1997; Tortora et al. 1998c; Young & Robertson 1998).

This stationary phase lasts approximately 8 weeks, by which time the host's immune system has switched from the cell-mediated immunity (CMI), which failed to stop intracellular growth of the bacteria, to delayed-type hypersensitivity (DTH) response, which sacrifices the host's own tissues (Dannenberg 1991). At this time, the tubercles may begin to regress and become calcified, thus showing up clearly on radiological testing (Tortora et al. 1998c). In spite of the calcification on the lesions, the bacteria can survive for many years in a dormant state in the centre of the tubercle, with potential for reactivation of the disease (Andersen 1997).

In some hosts, the macrophages fail to effectively wall off the lesion and the necrotic centre of the tubercle, including dead macrophages and bacilli, continues to enlarge and becomes macroscopically visible within four to five weeks (Dannenberg 1991). The host may then manifest the infection as symptomatic active disease.

2.4.4.3 Reactivation

M. tuberculosis bacteria held in the caseous tubercles in the lungs of an infected individual may exist in static form for many years without the individual experiencing any symptoms or, in fact, even being aware that they have been infected. At some stage, a triggering factor, such as immunosuppression, causes reactivation of the tubercle and the progression to active disease (Andersen 1997; Ginsberg 1998). It is possible for some lesions to progress to an active disease state, whilst other tubercles in the same lung may actually regress (Dannenberg 1991). Progression to active disease over the lifetime of an individual only occurs in 10% of those infected with *M. tuberculosis* (Ginsberg 2000).

2.4.4.4 Active disease

Active disease may follow on from infection, either immediately or at a later time following reactivation. Hydrolytic enzymes, produced by the activated macrophages, cause liquefaction of the tubercle (Harboe et al. 1996a), providing a liquid environment that enables the bacteria to proliferate outside of the host macrophages for the first time (Dannenberg 1991). The fast

growing bacteria produce toxins that attack the host tissues and cause necrosis and rupture of nearby bronchi resulting in cavity formation. The cavity provides an oxygen rich environment that further favours proliferation of the bacilli (Dannenberg 1991; Tortora et al. 1998c).

Liquid from the tuberculous lesions, containing large numbers of bacteria, provide a perfect vehicle for aerosol formation when air is expelled from the host lung. In this manner, the organisms can be transported to other regions of the lung and also to other individuals (Dannenberg 1991; Tortora et al. 1998c). The more progressed the disease, the greater the number of bacteria expressed into the air and the greater the chance of infecting another individual (Prescott et al. 1999b).

Cavity formation in the host lung also provides a direct method of entry for the bacilli into the circulatory and lymphatic systems of the infected individual (Tortora et al. 1998c). Thus, secondary infections may occur throughout the body, a disease state referred to as extrapulmonary or miliary tuberculosis (Ginsberg 1998; Prescott et al. 1999b).

The clinical symptoms of active pulmonary tuberculosis include chest pain, coughing, shortness of breath, fever, night sweats, fatigue and weight loss (Murray et al. 2003). The prognosis for treated tuberculosis sufferers is reasonably positive with 73% cure rate of registered TB sufferers worldwide (WHO Report 2005).

2.5 Diagnosis

2.5.1 Microscopy

Smears can be made from cultures or directly from samples and then stained for microscopic examination. Mycobacteria stain positively by Gram stain, making them difficult to distinguish by this traditional method. Because of their waxy coating, mycobacteria can, however, be stained for microscopy by using an acid-fast staining technique. In this technique, carbol fuchsin is applied to a fixed smear, and heated to enhance cell wall penetration and retention of the dye. Acid alcohol is used as a decolouriser. The carbol fuchsin is retained by mycobacteria because it is more soluble in the waxy cell wall than in the acid alcohol. Methylene blue is used as a counterstain (Tortora et al. 1998f). Under the microscope, mycobacteria stained by this method appear as pink rods on a blue background (Figure 2.2).

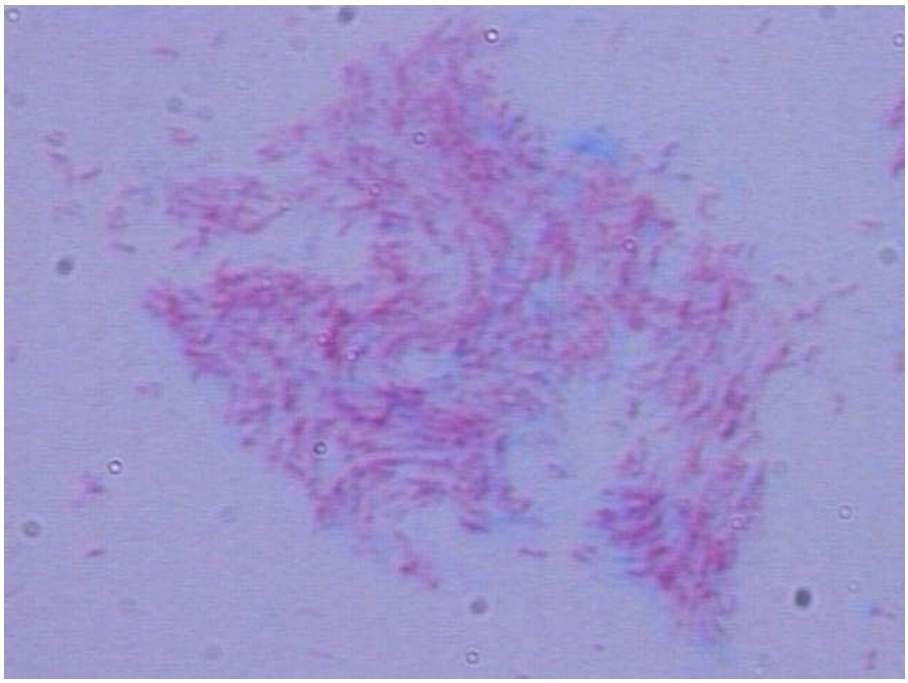

Figure 2.2. Ziehl Nielsen strain of *Mycobacterium tuberculosis* showing the typical "clumping" of the bacteria. The acid fast organisms appear pink and the background staining is blue.

Another microscopic technique for examining mycobacteria is fluorescence microscopy. The fluorochrome, auramine O, is strongly absorbed by *M. tuberculosis* and, when examined under a microscope using ultra-violet light, it appears bright yellow against a dark background (Tortora et al. 1998g).

Smears are used as a diagnostic tool as they quickly provide results with a relatively high specificity. The disadvantage of relying on smears for diagnosis is that the bacteria appear in a smear only when the disease has progressed to a point where the patient is highly infectious. In addition, this method of diagnosis

requires well-trained staff and sufficiently maintained laboratory equipment (O'Brien 1993; Zumla & Grange 1998).

2.5.2 Culture

Tubercle bacilli can be isolated from sputum, bronchial aspirates, gastric contents, spinal fluid, urine and tissue samples of persons infected with the microorganism (Murray et al. 2003). The bacteria can be grown on solid or liquid media, however diagnosis from culture alone is very slow. It takes 24 hours for a cell to divide and can take 2-3 weeks for visible cultures to appear on a solid medium (Young & Garbe 1991). In spite of the time taken to culture *M. tuberculosis*, culture is considered the "gold standard", to which all other identification methods are compared (Gillespie et al. 1997).

Automated culture systems are available, such as the bioMérieux BacT/ALERT® system, which employs colorimetric technology (bioMérieux 2005), and the Becton Dickensen BACTEC™ MGIT™ 960 system, which uses fluorometric technology, for rapid detection of bacterial growth (Pfyfer et al. 1997). Automated systems, however, are more costly than basic culture methods in terms of requiring skilled training and operation. As a result of the difficulty in culture and identification of *Mycobacterium* isolates, centralised laboratories that specialize in identification of these organisms are beneficial (Foulds & O'Brien 1998; Zumla & Grange 1998).

2.5.3 Tuberculin skin test

The tuberculin skin test, called a “Mantoux test”, is the traditional method of diagnosing *M. tuberculosis* infected individuals. It was a technique initially used by an Austrian physician, Clemens Freherr Baron von Pirquet in 1907, and refined by Mantoux in 1908. The technique used “tuberculin”, which was described by Robert Koch in 1890 (Daniel 1997a). Tuberculin, which is actually a purified protein derivative (PPD) of *M. tuberculosis* cultures, is intradermally injected into the forearm. A delayed type hypersensitivity response (DTH) results in an inflamed area within 2-3 days, the size of which is relative to the levels of the immune response that the body has against the PPD (Figure 2.3) (World Health Organization 2004).

This method of diagnosis, however, is only effective in countries where tuberculosis is not endemic and where few people have received BCG vaccinations (Ginsberg 1998). Exposure to TB infected individuals may result in seroconversion to one or more antigens in PPD. Also, BCG vaccines can expose individuals to antigens that are present in PPD. In both of these circumstances, a positive Mantoux test does not necessarily indicate infection by *M. tuberculosis*. In addition, 20-30% of patients with active tuberculosis infections are negative for the tuberculin skin test (Bonay et al. 1999).

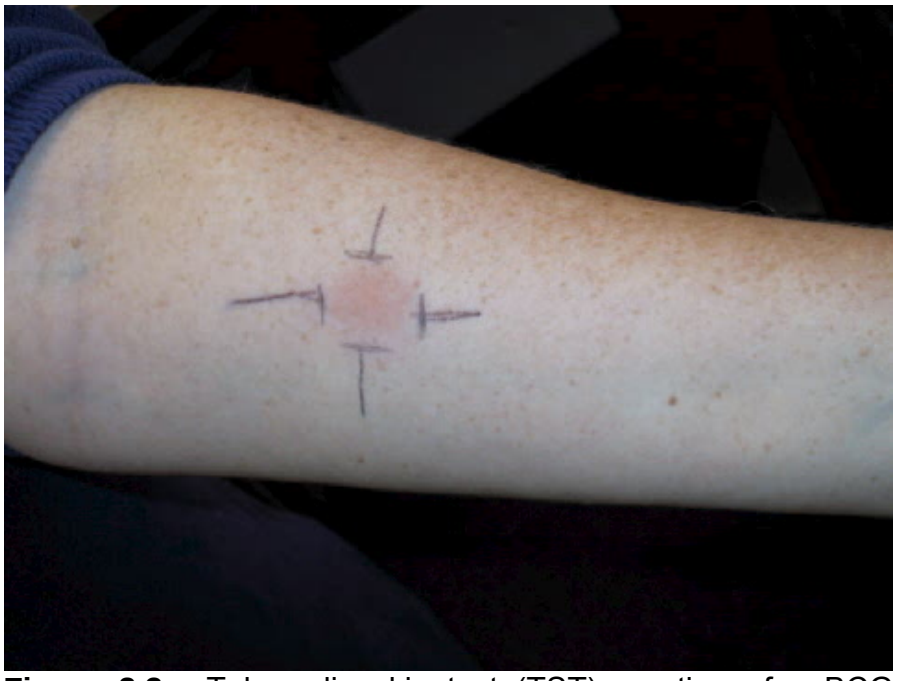

Figure 2.3. Tuberculin skin test (TST) reaction of a BCG vaccinated individual following and exposure to *Mycobacterium tuberculosis*. Pen marks on the arm indicate the extent of swelling associated with the tuberculin skin test. This reaction was found to be 24 mm in diameter.

2.5.4 Use of secreted proteins

Proteins that are secreted from actively growing *M. tuberculosis* have been examined, with variable success, for diagnostic purposes with the hope of developing an “easy-to-use” method of detecting infection by the organism due to the immunological response of the host (Ginsberg 1998; Lagrange et al. 1999; Saunders 1999).

ESAT-6, a 9.9 kDa protein, and culture filtrate protein 10 (CFP10), are both found in culture filtrates of infectious strains of *M. tuberculosis* and *M. bovis* but not in *M. bovis* BCG (Harboe et al. 1996b). These two antigens have been incorporated into

two commercially available diagnostic tests used for detecting tuberculosis infection.

The T-SPOT™ TB assay (Oxford Immunotec), an enzyme-linked immunospot (ELISPOT) test, was released in August 2004. It works by detection of interferon-γ (IFN-γ) levels induced by incubation of patient mononuclear cells from a blood sample with recombinant ESAT-6 or CFP10 (Oxford Immunotec 2005; Ewer et al. 2003; Richeldi et al 2004). This assay has been shown to be more sensitive and specific than TST for the detection of TB (Lalvani et al. 2001).

Another assay that has been developed using similar technology is the QuantiFERON®-TB Gold assay (Cellestis). This assay measures plasma IFN-γ levels using an enzyme-linked immunosorbent assay (ELISA) following centrifugation of whole blood that has been incubated in tubes coated with ESAT-6 or CFP10 (Cellestis 2005; Brock et al. 2004). This method is simpler and less labour intensive than the ELISPOT method and is also more accurate than the TST method for the diagnosis of tuberculosis (Ferrara et al. 2004; Brock et al. 2004).

2.5.5 Radiological methods

Chest X-Rays are the traditional diagnostic tool for tuberculosis. They provide immediate results without having to wait for several weeks for culture results. There are, however, numerous disadvantages with relying on X-Rays for the diagnosis of tuberculosis. Firstly, the technique has very poor specificity, with many other chest conditions resulting in similar radiographic abnormalities (Foulds

& O'Brien 1998). This can lead to over-diagnosis of tuberculosis and unnecessary prescription of antibiotics against tuberculosis (O'Brien 1993). Sensitivity is also poor, with the early stages of infection failing to appear on X-Rays (O'Brien 1993; Kline et al. 1995). Finally, X-Rays cannot be used to determine whether the patient is part of an outbreak, or simply suffering reactivation of an old infection (Kline et al. 1995). For these reasons, X-Rays should only be used as a diagnostic tool for tuberculosis in conjunction with microscopy and cultures.

2.5.6 Molecular biology methods

In the early 1990s, molecular biology techniques used in research began to be applied as a laboratory diagnostic tools (Kulaga et al. 1999). Initially, Southern blotting was used for detection of *M. tuberculosis* DNA restriction fragments. PCR techniques were developed that detected a fragment of IS*6110*, an insertion sequence specific to *M. tuberculosis*. Other insertion sequences specific to other mycobacterial species have also been identified (Kent et al. 1995; Hellyer et al. 1996; Gillespie et al. 1997; Saunders 1999)

The current role of PCR is limited and its sensitivity and specificity is low. PCR techniques are expensive and complicated and require specialised equipment and dedicated space. Although this technique may play a small role in current diagnostic methods, the recent developments of new simpler methods, such as those using secreted proteins, have attracted more attention (Lodha & Kabra 2004).

2.5.7 Susceptibility testing

The Becton Dickensen BACTEC™ MGIT™ 960 system, an automated mycobacterial culture system, can also be used for susceptibility testing of *M. tuberculosis*. This system provides relatively rapid results to the common antibiotics used for treatment of tuberculosis, including streptomycin, isoniazid, rifampin, ethambutol and pyrazinamide (Becton, Dickinson and Company 2005).

The manual technique employed for susceptibility testing of mycobacterial isolates is the agar proportion method. In this technique, a specific quantity of antibiotic is included into the agar before inoculation and an isolate is classified as sensitive if the number of colonies on that plate is <1% of the colony count on a control plate (Yajko et al. 1995). Whilst this method is the cheaper option, it is labour intensive and slow, taking up to 2 months to achieve results (Franzblau et al. 1998).

Due to financial and technical restraints, developing countries have difficulty in obtaining susceptibility patterns of *M. tuberculosis* isolates. The agar proportion method is the logical choice from the financial perspective, but from a clinical perspective it is almost worthless due to the time taken to achieve results. Commercially available systems are able to provide results in real time, but are too expensive to install and operate in developing countries. Thus, assumptions are made with regard to treatment of infected individuals, which can lead to treatment failure and the development of resistant isolates (Franzblau et al. 1998).

2.5.8 Epidemiology studies

In Australia, *M. tuberculosis* infections are notified to either the National Notifiable Disease Surveillance System (NNDSS) by means of notifications to local public health units by the health professionals who diagnosed the disease, or to the Laboratory Virology and Serology Reporting Scheme (LabVISE). Communicable Diseases Australia, a government agency, combines the data from these two surveillance schemes and publishes quarterly reports (Department of Health and Aging 2005). This information assists in epidemiological studies and the rapid detection and subsequent isolation of outbreaks.

Follow-up of individual cases by public health departments is essential in containing infections and reassuring contacts of infected individuals. Tuberculin skin testing (TST) is performed on close contacts and flexibility is required in broadening or reducing the sphere of testing depending on results obtained, particularly if the infected individual is a health care worker (Askew et al. 1997).

M. tuberculosis strains can be genetically analysed for similarities to assist in epidemiology studies (Michele et al. 1997; Yang et al. 1998; Jones et al. 1999; Kline et al. 1995; Porco & Blower 1998). Genetic analysis can also identify emerging resistance patterns and follow the progression of the strain (Small et al. 1993). Thus genetic tools can be used in epidemiological studies of tuberculosis to help prevent further spread of the disease.

2.6 Treatment

2.6.1 History

From the time they were first prescribed by Galen around 100 BC, and for many hundreds of years following, fresh milk, open air, sea voyages and dry elevated places were the treatments of choice for the disease now known to be tuberculosis. The coronation ceremony of Clovis of France in 496 AD, introduced the “royal touch” as a cure for scrofula, a disease also known as “The King’s Evil” in which tuberculosis infects the lymph glands of the neck. The “royal touch” became a ritual which involved prayers, a blessing, and the hanging of a gold piece around the patient’s neck. This treatment peaked in popularity between 1662 and 1682 during which time King Charles II touched an average of almost 90 people per week (Daniel 1997c).

After the infectious nature of tuberculosis was demonstrated in 1865 by French scientist, Jean-Antoine Villemin, the treatment of tuberculosis sufferers was largely focused on prevention of the spread of the disease. Sanatoriums and hospitals were built to specifically house and confine the patients. It was not until the 1940s and 1950s that antibiotics were introduced and refined into disease-fighting drugs (Daniel 1997a).

2.6.2 Alternative treatments

Resectional surgery has been used for the treatment of tuberculosis with relatively successful results (Iseman 1993). Surgery, however, introduces another whole

field of complications and is a severe form of treatment when chemotherapy is an available option. Immunotherapy involving administration of recombinant interleukin-2 has also been attempted with some success although some side effects, including fever and appetite loss, were noted (Tsuyuguchi 1996).

2.6.3 Antibiotics

Current treatment of tuberculosis involves the use of a cocktail of chemotherapeutic agents. The preferred treatment regimen is an initial 8 weeks of daily doses of isoniazid, rifampin, pyrazinamide and ethambutol. This is followed up by either daily or twice-weekly doses of isoniazid and rifampin for a further 18 weeks, giving a total treatment time of approximately 6 months as a minimum (CDC Fact Sheet #250111, 2003).

Antibiotics are not only used for the treatment of tuberculosis, but are also prescribed as preventative therapy for latent tuberculosis infections. Isoniazid is the drug of choice for prophylaxis and is given daily or twice weekly for 6-9 months. Alternatively, rifampin may be given daily for 4 months. Previously, a combination of rifampin and pyrazinamide has also been available for prophylaxis. This combination, however, is now strongly discouraged due to the risks of severe liver injury and death as a result of the treatment (Valway et al. 1998; CDC Fact Sheet #250110, 2004).

2.6.3.1 Streptomycin

The discovery of the first antibiotic therapy, streptomycin, was announced by Selman Waksman in 1944 (Prescott et al. 1999d; Tortora et al. 1998c). It is an aminoglycoside antibiotic synthesised by *Streptomyces*. Streptomycin attacks a broad spectrum of bacteria, including Gram positive, Gram negative and mycobacteria. It kills bacterial cells by binding with the 30S subunit of the ribosome, thus inhibiting protein synthesis and causing misreading of mRNA. The side effects associated with use of streptomycin include allergic responses, nausea, loss of hearing and renal damage (Prescott et al. 1999e).

2.6.3.2 Isoniazid

Isoniazid is a drug that specifically targets mycobacteria. It is a metabolic antagonist that prevents mycolic acid synthesis, thus either halting cellular activity or causing cell death. Side effects related to isoniazid use include allergic reactions, gastrointestinal upset, peripheral neuropathy and liver damage (Prescott et al. 1999e; MIMS Annual, 2000).

2.6.3.3 Rifampin

Rifampin halts cellular activity of Gram positive bacteria and mycobacteria by binding to RNA polymerases, thus inhibiting nucleic acid synthesis. This antibiotic is structurally related to macrolides, although it is more effective at penetrating tissues, reaching therapeutic levels in CSF and abscesses. Side effects include allergic reactions, liver damage, nausea, mental confusion, muscular weakness and visual disturbances. An additional unique

idiosyncrasy of rifampin is its ability to stain urine, faeces, saliva, sputum, sweat and even tears a bright orange-red colour (Prescott et al. 1999e; Tortora et al. 1998h; MIMS Annual, 2000).

2.6.3.4 Ethambutol

The activity of ethambutol is specific to mycobacteria, although its efficacy is comparatively weak and thus it is used only as a secondary drug for the prevention of drug resistance. Its action is to inhibit the incorporation of mycolic acids into the cell walls of the bacilli (Tortora et al. 1998h). Adverse reactions include optic neuritis, allergic reactions, gastrointestinal upset and mental confusion (MIMS Annual, 2000).

2.6.3.5 Pyrazinamide

Pyrazinamide is only used in patients with active *M. tuberculosis* infections and has no action against atypical mycobacteria. The most common side effect is liver damage, however gout, anaemia, nausea, arthralgia, malaise and fever have also been reported. (MIMS Annual, 2000).

2.6.3.6 Other chemotherapeutic agents

Other antibiotics less commonly used for the treatment of tuberculosis include dapsone, a metabolic antagonist; capreomycin, which must be given parenterally; cycloserine, a broad spectrum antibiotic; and, mycobutin, which is closely related to rifampin (MIMS Annual, 2000).

2.6.4 Compliance

Due to prolonged treatment and the side effects experienced, compliance with chemotherapeutic agents is one of the greatest hurdles in the treatment of tuberculosis. Whilst relatively short lapses in therapy have no substantial effect on the rate of recovery, patients who default on greater than 2 months of treatment experience a 10-fold increased risk of poor outcomes of therapy (Burman et al. 1997).

2.6.5 Multi-drug-resistance

A multi-drug-resistant (MDR) strain of *M. tuberculosis* is one that is resistant to isoniazid and rifampin. It is not necessarily resistant to additional drugs. Spontaneous chromosomal mutations within the cell that cause MDR occur in approximately 1 in 10^{16} replications of the bacteria. Ignoring spontaneous mutations, infection of a multi-drug-resistant (MDR) form of tuberculosis can occur in two ways. Firstly, the strain can be contracted from another individual who is infected with that MDR strain of *M. tuberculosis*. Alternatively, a patient with tuberculosis can develop drug resistance due to treatment failure (Prescott et al. 1999b; Iseman 1993).

Treatment failure can be due to a number of factors. Inadequate treatment includes prescription of antibiotics for a shortened time period, prescription of a single antibiotic in a case of active tuberculosis, or addition of a single drug to a failing regimen. Another factor that can lead to drug resistance is the prescription of antibiotics prior to susceptibility results being examined or acted upon. Finally,

treatment of tuberculosis can fail if the patient is non-compliant with the drug regime (Mahmoudi & Iseman 1993; Prescott et al. 1999b).

Treatment of MDR strains of tuberculosis require a number of chemotherapeutic agents to be taken for up to 2 years depending on the resistance patterns (CDC Fact Sheet #250112). Drug regimens required for these treatments cost up to $250,000 per patient, thus making MDR-TB an untreatable disease in developing countries (TB “Hot Zones” Emerging 1998).

2.6.6 Directly Observed Therapy – Short-course (DOTS)

Directly Observed Therapy – Short-course, or DOTS, is the World Health Organization (WHO) initiative to prevent drug-resistance due to non-compliance of patients with their treatments and to improve the recovery rates of tuberculosis sufferers. As its name suggests, the strategy involves supervised treatment of tuberculosis. Moreover, DOTS encompasses five key components essential for combating the progress of tuberculosis (World Health Organization 2000).

The first component of DOTS is sustained political commitment. It deals with the perception of tuberculosis as a preventable disease and the development of organised strategies at all levels of government to combat TB and alleviate the poverty and social issues that are associated with high TB incidence. Governments need to provide financial and human resources for making control of TB a part of their national health strategy (World Health Organization 2000).

The second component of DOTS discusses case detection by sputum smear microscopy. Access to quality health care and rapid detection of symptomatic patients is a priority. Particular attention must be paid to high-risk categories, such as HIV sufferers and institutionalised persons (World Health Organization 2000).

Standardised treatment and proper case management are the third component of DOTS. Ensuring that standardised treatments are administered involves educating the health care providers regarding the standard drug regimes. Provision of support and care of the patient is essential for motivation and adherence to treatments. This may require establishing community- or workplace-based direct observation of therapy. WHO recommends direct observation of therapy whenever rifampin is administered for the control of tuberculosis (World Health Organization 2000).

Uninterrupted supply of tuberculosis treatments, as the fourth component of DOTS, covers the reliability and quality of available chemotherapeutic agents and their distribution to required destinations. It is also suggested that fixed dose combinations (FDCs) of the numerous drugs required during initial TB therapy be provided in a single tablet to assist patients with the complicated nature of treatment, thus reducing the risks of development of multi-drug-resistant strains (World Health Organization 2000).

Finally, DOTS incorporates the establishment of a recording and reporting system of detection, treatment and outcomes of each tuberculosis patient. This enables

surveillance and monitoring of national programmes and encourages communication and support on all levels from local health care to international strategies (World Health Organization 2000).

In 2003, 182 countries, covering 77% of the worldwide population, were implementing the DOTS strategy. Between 1995 and 2003, DOTS programmes treated 17.1 million TB patients and 8.6 million smear positive patients. DOTS programmes resulted in a case detection rate of 45% in 2003, which was considerably higher than previous years. The 5% decrease in prevalence since 1990 has also been attributed to DOTS (WHO Report 2005).

2.6.7 Recovery rates

The treatment success rate of patients registered in DOTS programmes in 2003 was 82% (data for non-DOTS areas was incomplete). Morbidity rates for treated tuberculosis patients were the worst in the African Region, which has a higher HIV rate, where 7% of patients died. A 6% mortality rate was noted among treated patients in the European Region, where drug-resistant strains are more common (WHO Report 2005).

2.7 Effect of HIV

2.7.1 Disease process

Tuberculosis and the human immunodeficiency virus (HIV) are both sinister diseases that develop an unusual partnership. As a result of the poor immune

response of HIV patients, they are less capable of fighting new and/or latent TB infections, which progress rapidly to active disease with mortalities occurring in as little as 4 to 16 weeks (Alito et al. 1999). In addition, the tuberculosis infection activates T-cells and macrophages that may harbour latent HIV, which can exacerbate the HIV infection and progress it to AIDS (acquired immunodeficiency syndrome) (Silveira et al. 1997; Barnes & Modlin 1996). HIV sufferers are 8.3 times more likely to contract TB infections than the HIV-negative population (WHO Report 2005).

2.7.2 Diagnosis

Tuberculosis, unlike most diseases that affect HIV sufferers, usually develops earlier in the course of HIV infection. Due to the low CD4 levels of the HIV patients, tuberculosis is very difficult to diagnose. Chest X-rays may appear normal and the tuberculin skin test (TST) may be negative (Hanson & Harkness 2001). A TST as small as 5 mm in diameter is sufficient to indicate TB infection in a HIV patient (CDC Fact Sheet #250120, 2005).

2.7.3 Vaccination

Vaccination with the current BCG vaccine is not recommended in any immunocompromised persons, including those infected with HIV (CDC Fact Sheet #250120, 2005). Although BCG is an avirulent strain of *Mycobacteria*, it is still a live organism and the diminished immune system of HIV patients can be incapable of containing it (Elhay & Andersen 1997).

2.7.4 Treatment and recovery (Prognosis)

Treatment of tuberculosis is also complicated as a result of HIV infection and it is a fine line between treating the TB and exacerbating the HIV (Kirschner 1999). DOTS should always be used to avoid the added complication of drug-resistant organisms. In addition, rifampin has been known to interact with antiretroviral agents and an alternative to this chemotherapeutic drug may be required (CDC Fact Sheet #250113, 2003).

2.8 Immunological Manifestation

2.8.1 Antigen presentation

When *M. tuberculosis* bacteria are ingested by the macrophage they excrete metabolites that prevent the fusion of the phagosome and the lysosomes, thus preventing contact with destructive lysosomal contents (Prescott et al. 1999b). The bacteria multiply within the host cell and the macrophage processes and presents bacterial antigens as MHC (major histocompatibility complex) class II antigens (Andersen et al. 1991a; Anderson et al. 1995), which focus on the presentation of exogenous or vacuolar antigens (Harding 1996).

MHC class II antigens have been associated with CD4 (Cluster of Differentiation surface markers type 4) T-lymphocyte activation (Kaufmann & Hess 1999). It is now known that some MHC class I presentation and CD8 T-cell activity may also associated with TB infections (Kaufmann & Hess 1999; Feng & Britton 2000). In

addition, a small group of cells, called γδ T-cells, are capable of recognising antigens in the absence of traditional presentation molecules (Chien et al. 1996).

Infected macrophages also secrete interleukin-12 (IL-12), a cytokine that activates natural killer (NK) cells to produce interferon-γ (IFN-γ). The IFN-γ in turn reactivates the macrophage to kill the phagocytosed organisms (Tsuyuguchi 1996).

2.8.2 T-lymphocyte types

2.8.2.1 CD4 cells and immunological memory

CD4 cells express αβ T-cell receptors on their surface. They are often referred to as T-helper (Th) cells and recognise antigens associated with MHC class II molecules (Roitt 1991; Roitt et al. 1993). They are activated by antigen presenting cells when only small numbers of T-cells are active, thus leading to clonal expansion. The influx of Th-cells secretes large numbers of cytokines that assist in granuloma formation and inhibition of bacterial growth (Orme et al. 1993a).

CD4 cells can further be divided into Th-1 and Th-2 cell types according to the cytokines they produce (Mosmann et al. 1986). These specific cytokines inhibit activity by the other Th-cell-type and direct certain pathways of immune responses (Barnes & Modlin 1996), such as activating macrophages to control or eliminate intracellular organisms using interferon-γ (IFN-γ) (Flynn 2004).

Th-1 cells secrete IFN-γ and interleukin-2 (IL-2), and are responsible for primary antibody responses and strong cellular immunity against intracellular pathogens (Mosmann et al. 1986; Orme et al. 1993b; Ferrick et al. 1995). It is this cell subset that develops into a long-lived "memory" phenotype once direct exposure to the antigen ceases (Orme et al. 1998b; Andersen et al. 1995).

Very high levels of IFN-γ have been measured in memory-immune mice within the first 24 hours following infection with *M. tuberculosis*, suggesting that they are rapidly recruited from the circulating pool to the site of infection. Although CD8 cells are also initially recruited to the site, it is the CD4 cells that continue to proliferate (Andersen et al. 1995).

Th-2 cells produce IL-4 and IL-5, which lead to antibody production in B cells and the development of humoral responses (Mosmann et al. 1986; Ferrick et al. 1995; Barnes & Modlin 1996). These cells play very little role in fighting tuberculosis infections and are present simply as regulatory cells (Orme et al. 1993b; Tsuyuguchi 1996).

2.8.2.2 CD8 cells and cytolytic response

For many years it was thought that CD8 cells did not play an important role in controlling TB infections. Now, however, it appears that this category of cells does, in fact, assist in controlling this disease within the host to some degree (Feng & Britton 2000; Flynn 2004). Like CD4 cells, CD8 cells fall into the

category of cells expressing αβ T-cell receptors on their surface. CD8 cells are often referred to as cytotoxic T-cells (Tc) and recognise antigens associated with MHC class I molecules (Roitt 1991; Roitt et al. 1993). It has been suggested that this type of antigen presentation may be due to the ingested bacilli having access to the macrophage cytoplasm via pores in the vacuole membrane (Mazzaccaro et al. 1996; Teitelbaum et al. 1999).

Cytokines secreted by CD8 cells include IFN-γ and tumour-necrosis-factor-α (TNFα), which enables them to activate macrophages (Flynn 2004). These cells are associated with the formation of granulomas successful in restricting cellular infiltrates are responsible for macrophage lysis within the granuloma (Andersen 1997; Ladel et al. 1995b). CD8 T-cells in humans can contribute to bacterial death within macrophages by releasing granulysin, which is capable of entering the macrophage and is directly toxic to *M. tuberculosis* (Stenger et al. 1998).

2.8.2.3 DN αβ T-cells

Cells that express αβ T-cell receptors, but are negative for CD4 and CD8 surface markers are referred to as double negative (DN) αβ T-cells (Roitt 1991; Roitt et al. 1993). These cells are present in very low numbers and have been shown to recognise *M. tuberculosis* mycolic acids in association with CD1 molecules rather than MHC (Andersen 1997; Beckman et al. 1994).

2.8.2.4 DN γδ T-cells

T-cells that express γδ, instead of αβ T-cell receptors, are usually CD4 and CD8 negative and are often simply referred to as γδ T-cells. They constitute 5% of T-cells in lymphoid organs and 1-5% of circulating T-lymphocytes (Orme et al. 1993a). Studies in mice show that the DN population in the spleens of infected mice is predominantly γδ T-cells, whereas the pulmonary DN population is largely αβ T-cells (Phyu et al. 1999). γδ T-cells are capable of recognising protein antigens directly, without the assistance of any antigen processing molecules. It is therefore possible that the types of proteins stimulating these cells are completely different from those involved in αβ T-cell activation (Chien et al. 1996).

The exact role of this cell type, however, is unclear. It has been shown that γδ T-cell lines experience rapid expansion within 4 days following stimulation and that the immune response is directed towards a Th-1 type response, suggesting contribution toward cell-mediated immunity (Garcia et al. 1997). In addition, when systemic immune responses fail, mucosal γδ T-cells regulate the maintenance of IgA responses (Fujihashi et al. 1996), and show Th-2-like activity (Wen et al. 1998). It has been suggested that γδ T-cells are able to mediate a protective immune response in the absence of αβ cells (Chien et al. 1996). Further studies, however, have shown that rather than being protective, the γδ cells control the migration of macrophages to the site of infection (Orme 1999a).

2.8.3 Cytokines

Many cytokines are involved in the immune response to *M. tuberculosis* infection. Interleukins, such as IL-1, IL-6 and IL-4, transforming growth factor (TGF-β) and various chemokines recruit monocytes and lymphocytes to the infected area and regulate the extent of the immune response (Mosmann & Coffman 1989; Andersen 1997; Strober et al. 1998). Other cytokines that play a more direct role in combating TB infections are discussed below in greater detail.

2.8.3.1 Interferon-γ (IFN- γ)

IFN-γ is secreted by CD4, CD8 and γδ T-cells and levels of IFN-γ peak around 2 weeks following infection (Flynn 2004; Ferrick et al. 1995; Ohga et al. 1990; Andersen et al. 1995). γδ T-cells are the most efficient at producing IFN-γ and CD8 T-cells are the least effective, being unable to produce the cytokine in the absence of CD4 cells (Ladel et al. 1995b; Tsukaguchi et al. 1999). Production of IFN-γ is dependent on antigen presentation and co-stimulators provided by monocytes (Tsukaguchi et al. 1999).

Mice with a genetic disruption disabling them from producing IFN-γ suffer severe caseous necroses, large abscesses and widespread dissemination of *M. tuberculosis* following infection, resulting in rapid death. These mice are inadequately able to activate both infected macrophages and arriving monocytes (Flynn et al. 1993; Cooper et al. 1993). IFN-γ is essential for control of tuberculosis infection by retaining the integrity of the granuloma that is formed (Cooper et al. 1993). Due to the ability of IFN-γ to control infection,

this cytokine can be used to monitor emergence of protective T-cells (Orme et al. 1992).

2.8.3.2 Interleukin-12 (IL-12)

IL-12 is an important cytokine produced by macrophages and B cells that stimulates IFN-γ production and enhances the cytolytic activity of CD4, CD8 and natural killer cells (Flynn & Bloom 1996). The presence of IL-12 improves the systemic and mucosal Th-1 cytokine responses and the similar cytokine responses of γδ T-cells to nonpeptide antigens (García et al. 1997; Williams et al. 1999; Arulanandam & Metzger 1999). This presence can be artificially created by oral or intra-nasal administration of IL-12 (Marinaro et al. 1997; Arulanandam & Metzger 1999). These anti-tuberculous effects, however, have not been observed in the absence of IFN-γ (Flynn et al. 1995).

2.8.3.3 Tumour necrosis factor (TNF-α)

TNF-α is produced by macrophages, CD4 T-cells, γδ T-cells, and monocytes (Tsukaguchi et al. 1999; Flynn 2004). It contributes to both pathogenesis and protection against tuberculosis infection. TNF-α can cause severe tissue necrosis that aid progression of the disease by releasing otherwise trapped bacilli (Barnes & Modlin 1996). Alternatively, this cytokine aids protection against tuberculosis by activating macrophages and influencing cell migration to infected regions. It affects chemokines and their receptors and adhesion molecules, thereby contributing to the formation of granulomas (Flynn 2004; Cooper et al. 1993).

2.9 Mucosal Immunity and Tuberculosis

The study of mucosal immunity is important in the understanding of tuberculosis, as tuberculosis infections usually initiate in the lungs. The mucosal immune system protects the mucous membranes that line the respiratory system, digestive system, urogenital tracts, eye conjunctiva, inner ear and the ducts of exocrine glands (Mowat & Viney 1997; Holmgren & Czerkinsky 2005). The main functions of this system include protection of mucous membranes from pathogen infection and colonisation and, conversely, to prevent antigen uptake and potentially harmful immune responses to proteins derived from ingested food, airborne particles and commensal microorganisms (Holmgren & Czerkinsky 2005).

The mucosal immune system is completely separate from the systemic immune system. Varying mechanisms and functions of these two immune systems are based on the fact that the systemic immune system operates in a normally sterile environment and responds vigorously to any foreign material, whereas the mucosal immune system protects organs that are routinely exposed to “safe” foreign particles. This core difference results in slightly different subsets of lymphoid cells regulating the responses in each system (Fujihashi et al. 1996; Holmgren & Czerkinsky 2005).

2.9.1 Mechanisms

Protective immune responses are initiated in the mucosal immune system in lymphoid microcompartments, which include Peyer's patches, mesenteric lymph nodes and lymphoid cells in the lamina propria and intestinal epithelium (Mowat & Viney 1997). Antigens are taken up into these sites from the mucosa via M cells (membranous cells). Dendritic cells process the antigens and present them to the T-cells, which stimulates T-cell differentiation into effector T-cells that either result in immunisation or oral tolerance (Strober et al. 1998; Holmgren & Czerkinsky 2005).

In addition, the T-cells interact with B-cells to produce secretory IgA. Both types of lymphocytes then leave the initial site through the lymphoid system and migrate to mucosal sites, usually the mucosa where the antigen was initially detected, and differentiate into memory or effector cells (Strober et al. 1998; Holmgren & Czerkinsky 2005). An increase in secretory IgA levels on a mucosal surface can neutralise bacterial toxins and prevent attachment (and hence infection) of the bacteria at the site (Freytag & Clements 2005).

Thus, it was found that there exists in humans a "common mucosal immune system", in which immunity conferred at one site can be effective in another mucosal location (Czerkinsky & Holmgren 1995). This basic common system is now known to be a little more restricted that previously expected, with some degree of compartmentalisation. Strongest immune responses take place at the antigen-exposed mucosa, for example, the respiratory mucosa, and secondary

responses occur at specific effector sites, for example, the genital mucosa (Holmgren & Czerkinsky 2005).

The systemic immune system can be activated if the antigens follow an alternate pathway involving direct entry into the circulation and dissemination to systemic lymphoid sites (Strober et al. 1998).

2.9.2 Oral tolerance

Suppression of potentially harmful immune responses to “safe” antigens employs a tactic of the mucosal immune system known as oral tolerance (Strobel 1995). The type of T-cell response initiated by the dendritic cells determines whether tolerance or immunisation ensues. In immunisation responses, the T-cells stimulated are cytotoxic (CD8) T-lymphocytes, Th1-like cells (Holmgren & Czerkinsky 2005). Alternatively, the T-cells stimulated in tolerance responses include CD4 and CD8 T-cells that secrete TGF-β (transforming growth factor β); Th2-like cells that produce IL-4 and IL-10 and down regulate Th1-like cells; and T_{reg} cells (regulatory T-cells) that suppress proliferation (Holmgren & Czerkinsky 2005; Thornton & Shevach 1998; Groux et al. 1997; Chen et al. 1994).

Oral tolerance is largely determined by T-cell differentiation and the only effect on B-cells is that in tolerance situations, the lack of T-cells results in a reduced B-cell stimulation. The inhibited B-cell response leads to decreased levels of IgA, which can be measured to determine whether the immune response is of tolerance or immunisation (Strober et al. 1998; Elson & Ealding 1984).

A small number of substances fail to induce oral tolerance responses in the mucosal immune system. These include polysaccharide antigens, which bypass T-cell responses and interact directly with B-cells; bacterial toxins that serve as adjuvants, which bind to cells and alter their function; and, proteins that bind to epithelial cells (Strober et al. 1998).

Oral tolerance can be used beneficially for controlling conditions such as food sensitivities, inflammatory bowel disease and autoimmune disease (Strober et al. 1998). In mucosal vaccine design, however, oral tolerance poses serious dangers. If a vaccine that is administered using a mucosal route results in the development of oral tolerance, that tolerance is passed on to the systemic immune system and even parenteral administration of that antigen fails to induce immune responses (Czerkinsky & Holmgren 1995). Dose size and frequency can be altered to prevent tolerance to administered antigens. Single low dose (>0.1 mg/g body weight) oral antigen administration can lead to tolerance of that antigen. Alternatively, a high dose of the antigen (>1 mg/g body weight) is more likely to lead to immunisation. Multiple doses of each antigen load simply polarise the type of response, be it tolerance or immunisation (Strober et al. 1998; Strobel 1995).

2.10 Bacille Calmette-Guérin (BCG) – the Current Vaccine

2.10.1 History

The current vaccine used for protection against tuberculosis is the bacille Calmette-Guérin (BCG) vaccine. The BCG vaccine was developed by Albert Calmette and Camille Guérin by isolation of *M. bovis* from a cow with mastitis and more than 230 serial passages in potato-glycerin medium over a period of 13 years. In 1908, these scientists noted a change in colony morphology and found that the strain had become attenuated (Harboe et al. 1996a; Hess & Kaufmann 1999; Fine 1998). It was first given as a vaccine in 1921 to a baby who was heavily exposed to TB and followed up by vaccine trials mainly in children (Harboe et al. 1996a).

The initial BCG vaccine preparations were stable for approximately 2 weeks and had to be refrigerated. In many cases, laboratory testing for vaccine potency and safety exceeded this 2 week time-frame making it impossible to use the vaccine in many places around the world. In 1957 a heat-resistant freeze-dried BCG vaccine was developed, which could withstand temperatures up to 37ºC for a number of weeks (Pelczar & Reid 1972). Once reconstituted, the vaccine must be kept cool, protected from light and discarded at the end of the day (World Health Organization 2001). The BCG vaccine is cheap to produce, costing only a few cents per dose (Orme 1999a).

Disadvantages of BCG vaccination include the localised skin reactions experienced, adverse outcomes in immunocompromised individuals, complication of TST use for diagnosis and the difficulty of administration (Bellet & Prose 2005; World Health Organization 2001). BCG is administered intradermally, which is more difficult than most other vaccines that are given into the deeper layers of the skin (World Health Organization 2001).

M. bovis and *M. tuberculosis* have numerous similarities in the primary structures of their constituent proteins (Nagai et al. 1991), which accounts for the inter-strain immunity. The BCG vaccine has also been shown to have some protective effect against *M. leprae* (Gormus et al. 2002). It prevents the spread of disease rather than prevention of infection and has been shown to exert a protective effect for 20 to 40 years (Boesen et al. 1995; Pal & Horwitz 1992).

Due to the difficulty of diagnosing TB using the TST in individuals who have been vaccination with BCG, many countries do not recommend this vaccine for general use (Iseman 1993, Bröker 1999). The CDC (Centers for Diseases Control and Prevention) in the USA recommends vaccinating children with a negative TST who are continually exposed to inadequately treated adults or adults with MDR-TB. Health care workers should only be vaccinated if they are dealing with a high percentage of patients infected with drug-resistant TB strains, or if infection control procedures have been unsuccessful (CDC Fact Sheet #250120 2005).

2.10.2 Efficacy

Worldwide, BCG vaccinations have been administered to 3000 million people. 172 countries are involved in BCG vaccination programmes and 85% of people worldwide are vaccinated within the first year of their lives (Guérin 1997). In spite of the BCG immunisation coverage being the highest of all vaccines of the Expanded Programme on Immunisation (EPI), almost 9 million people die from tuberculosis each year (WHO Report 2005), strongly highlighting the inadequacy of the current vaccine.

Overall, BCG vaccination reduces the risk of TB infection by 50%, which can be broken up into a 78% protective effect against disseminated TB; a 64% protective effect against tuberculous meningitis; and, a 71% reduction in death rate (Colditz et al 1994). Alternatively, vaccination localises the infection and there is an increased incidence of mediastinal adenopathy (Ortona & De Luca 1998). Clinical trials show that the efficacy of BCG ranges from zero protection in Southern USA and India to 77% in the UK (World Health Organization 2001, Bröker 1999).

Variations in efficacy of BCG vaccination have been attributed to a number of causes, not the least of which is age and gender variation. It is generally accepted that BCG vaccination has a definite protective effect in children with immune protection lasting up to 10 years (Sterne et al. 1998), but for unknown reasons is much less effective in adults (Orme 1999a). There are also gender differences with a recent study showing a 70% efficacy for women, but only 29% protection against TB for men (Aronson et al. 2004).

The *M. bovis* BCG strain has been maintained on various different culture media, which can induce different expression of proteins in the organisms and may be an attributing factor to the variable efficacy of BCG vaccination (Fine 1998). There is also evidence that the number of serial cultures performed is indirectly proportional to the efficacy of that strain's protective effect (Behr & Small 1997; Ginsberg 1998). These strain variations have been confirmed by genetic studies that reveal an absence of the *M. tuberculosis* ESAT-6 protein in all *M. bovis* BCG strains, but the MPB64 (or MPT-64) protein is present in some but not all BCG strains. It appears that there was a significant deviation from the original BCG Pasteur strain some time between 1925 and 1934 (Weldingh & Andersen 1999; Mahairas et al. 1996). In spite of these discoveries, the impact of strain variation on the efficacy of BCG vaccination remains to be seen (Melles & Gunilla 2005).

Exposure to environmental organisms has also been blamed for variation in the efficacy of the BCG vaccine. Environmental mycobacteria are common in some countries and it is thought that exposure to these organisms may sensitise the immune system of the individual. Thus, when vaccination occurs, the immune system attacks the BCG strain very quickly, preventing persistence of the vaccine strain for a sufficient length of time to create memory-immune T-cells against *M. tuberculosis* (Brandt et al. 2002). In addition, environmental mycobacteria and helminth infections have been implicated in shifting the immune response towards a Th-2 type and reduce the efficacy of BCG vaccination (Rook et al. 2005).

Finally, variations in the efficacy of the BCG vaccine may be due to worldwide variations in dosage and immunisation schedules (World Health Organization 2001). The BCG vaccine may also be more effective if it were delivered directly to the mucosal immune system, rather than being given parenterally (Haile & Gunilla 2005).

2.10.3 Contraindications

Although the EPI recommends BCG vaccination at or soon after birth in countries with a high incidence of TB, if the mother develops TB around the time of delivery it is recommended that the newborn be given prophylactic treatment for 6 months and then vaccinated (World Health Organization 2001). In addition, BCG vaccination should never be given to pregnant women as there is insufficient data of the effects on the foetus (CDC Fact Sheet #250120 2005). Immunosuppressed individuals, such as HIV sufferers and recipients for organ donation, should not be vaccinated with BCG due to the risks of infection in spite of the attenuation of the strain (CDC Fact Sheet #250120 2005).

2.10.4 Side effects

Side effects related to BCG vaccination in otherwise healthy individuals are negligible. Following administration the individual experiences self-limiting bacterial multiplication resulting in a minor lesion (Hess & Kaufmann 1999). If the vaccine is administered too deeply, local reactions, ulcers and regional lymphadenitis may occur (World Health Organization 2000).

2.11 Vaccine Design

2.11.1 The ideal vaccine

The purpose of vaccination against tuberculosis is to boost the CMI response, thus preventing the immune progression to a DTH response, which sacrifices host tissues in its attempt to combat disease. In tuberculosis the protective immune responses are the same as those involved in the pathology underlying the disease and stimulating immune protection without worsening the disease is a fine line (Doherty & Andersen 2005). The understanding of the immune responses to infection is paramount to discoveries leading to successful vaccine development.

The characteristics of an ideal vaccine against tuberculosis include the following: the vaccine should be safe enough to administer to immunocompromised patients and have minimal side effects; it should be effective in spite of malnutrition of the recipient; it should have a demonstrated protective effect in animal models; it should provide protection in naïve individuals as well as those previously exposed to mycobacterium; it should be a single dose, which is not administered by injection; it should elicit a long-lasting immune response; it should not interfere with the TST method of diagnosis; it should be able to be integrated into current immunisation schedules; it should be cheap to produce; and, it should be able to withstand heat and long storage times (Brennan 2005; Ginsberg 2000; Hess & Kaufmann 1999; McMurray et al. 1985).

The above list outlines the “ideal”, and vaccine candidates that fulfil most of these criteria could be adequate. For criteria that a vaccine does not fulfil, alternatives may be considered. For example, an antigen used for vaccination that interferes with TST methods of diagnosis could still be considered ideal if an alternative diagnostic method and/or surrogate marker of protective immunity were developed in parallel (Root-Bernstein 2005; Ginsberg 2000; Harboe et al. 1996a).

The search for an ideal antigen for use as a vaccine has three basic prerequisites for the expression of protection. Firstly, it must result in proliferation of T-cells; secondly, those T-cells must be the appropriate phenotype; and thirdly, the chosen antigen must be adequately expressed on the surface of infected macrophages (Silva et al. 1996).

The ultimate goal of all tuberculosis vaccination studies is to prevent the spread of the disease and work towards complete eradication of tuberculosis. Effective vaccination of individuals with latent TB infections could reduce disease rates by greater than 70% within two decades (Dye 2000).

2.11.2 Testing in animal models

The purpose of using animal models for testing vaccine candidates is to assess the safety and predict the efficacy of the vaccine before it is administered to humans. The majority of animal vaccine trials have been in murine models and following success in mice, some have also been tested in guinea pigs. Rabbits and rats have also been used occasionally and the fact that these animals are more

expensive, difficult to house and exhibit similar responses to the mouse model has resulted in very limited interest in this field of research. Larger animals such as primates and cattle have been suggested as an additional step between vaccine testing in guinea pigs and human clinical trials. Each animal model reacts slightly differently to vaccination against and infection with *M. tuberculosis*. Although no particular model is ideal, much information can be obtained from each and be related back to human disease (Pal & Horwitz 1992; Orme 2005b).

2.11.2.1 Mice

The mouse model has been used extensively in the field of tuberculosis research, not only for vaccine testing, but also to examine the immune responses to TB infection. A considerable quantity of discoveries have lead to increased knowledge regarding differentiation of T-cell subsets, cytokines, the function of macrophages and antigen presentation (Orme 2005b). Many of the discoveries relating to immunity that have been studied in mouse models have been as a result of gene disruption in the animals (Harboe et al. 1996a; Chien et al. 1996; Flynn et al. 1995; Flynn et al. 1993). In fact, more is known about immunity to tuberculosis in mice than in humans (Malin & Young 1996)!

Mice are the cheapest animals to house, readily available and many reagents are available to test immunological responses (Malin & Young 1996; McMurray 2000). In addition, mice are easy to handle and the information available relating to their immune systems makes them an attractive animal

model (Harboe et al. 1996a). The main disadvantage of using mice in tuberculosis studies is that the pathology of the disease in this model does not correlate well with that in humans. Similarly, mice are not as susceptible to disease, nor do they develop DTH skin test responses following vaccination (McMurray 2000).

The two strains of mice that have been used in most studies examining tuberculosis and immunity are C57BL/6 and BALB/c. It has been found that C57BL/6 mice have much higher levels of Th1 type responses and less readily succumb to infection (Wakeham et al. 2000; Flynn & Bloom 1996; Flynn et al. 1995). Thus, in recent years, BALB/c mice have rarely been used for tuberculosis vaccine research.

2.11.2.2 Guinea pigs

Compared to the mouse model, guinea pigs exhibit disease pathology that more closely compares to tuberculosis in humans (Elhay & Andersen 1997). They are quite susceptible to infection following inhalation of the organism and develop the necrotic lesions seen in untreated humans (Harboe et al. 1996a; Orme 1999a). Additionally, they exhibit strong DTH skin test responses (McMurray 2000) and remarkable success in vaccine-initiated protection has been observed in these animals (Brandt et al. 2004). Guinea pigs, however, are very expensive to keep under level III biosafety condition (Orme 1999a) and specific immunologic reagents are rare (McMurray 2000). In spite of these disadvantages, the guinea pig is considered the gold

standard for testing vaccine candidates against tuberculosis (Orme et al. 2001).

2.11.2.3 Cattle and primates

Following success in guinea pigs, assessment of vaccine candidates in larger animals prior to human clinical trials has been advocated. Some believe the cow to be the best model of human tuberculosis (Hewinson et al. 2003). Equally, the primate model has also been promoted (Langermans et al. 2005; Reed et al. 2003). Primate models of TB vaccine trials have included Rhesus monkeys and, more frequently, the cynomolgus macaque (Orme 2005b).

The obvious advantages with using cattle and primate models are the similarities of disease progression and immune manifestations when compared to the human model. Drawbacks pertaining to the use of these animals include availability, housing costs, and public acceptance (McMurray 2000; Orme et al. 2001; Orme 2005b).

An alternative to using large animals for examination of vaccine candidates prior to clinical trials is a new method of in vitro testing using BCG *lux* (Kampmann et al. 2004). This new method of in vitro testing uses a BCG strain that carries *luxA* and *luxB* genes from *Vibrio harveyi*, which enables luminescent detection that correlates with mycobacterial growth.

2.11.2.4 Human clinical trials

In the human model particularly, there are defined steps and regulations for evaluating vaccine candidates to ensure that the vaccine administered is pure, sterile, non-toxic and effective (Ginsberg 2000; Brennan 2005). Other issues that must be addressed prior to clinical trials are: the trial site (certain populations experience higher latent infection and active TB rates); logistics (including resources and trained staff); standardisation of protocols (including vaccine administration and measurement of immune response); ethics approval; definition of endpoints; medical care and counselling; and data management (Ginsberg 2000; Melles & Gunilla 2005).

There are three phases of clinical trials, each of which must experience success before progression to the next phase. Phase I, involving between 10 and 60 volunteers, establishes the safety of the vaccine in humans and determines the maximal dose that can be tolerated. Phase II is a larger study involving 200-300 participants. In addition to observing any adverse effects, it characterises the immune responses to the vaccine and examines variable doses and schedules (Ginsberg 2000).

The scope of testing is broadened in Phase III trials with 7000 to 300,000 subjects participating, and a trial period of 10 to 30 years may be required. Phase III trials can include three different designs. Pre-infection vaccine trials can be performed in children or young adults who have not previously been infected with mycobacteria. Complete prevention of infection is the obvious

advantageous outcome; however these trials may be required to continue for 20-30 years for adequate evaluation. Alternatively, post-infection vaccine trials can provide indications of efficacy within 5-10 years and examines vaccination of individuals with latent TB infection. The advantages of both methods can be gleaned by total-population vaccine trials in areas of high incidence of infection. Establishment of infectivity by performing TSTs at the start of the trial can lead to preliminary results within 5-10 years and a long-term follow up of naïve subjects (Ginsberg 2000).

2.12 Vaccine Types Undergoing Research

Since tuberculosis was declared a global health emergency in 1993 (World Health Organization 2000), funding has been made available for vaccine research and considerable efforts have been expended in this field over the last 15 years (McCarthy 2004; Hampton 2004). Different groups of researches have examined various types of antigens as prospective vaccines and these will be outlined below. Immune responses to proposed antigens have largely been measured by bacterial loads following aerosol challenge, lymphocyte proliferation and IFN-γ production (Orme 2005b).

2.12.1 Subunit vaccines

In the 1980s it was learned that individual proteins constituting *M. tuberculosis* cells or their contents were able to invoke immune responses (Chaparas et al. 1980; Nagai et al. 1981; Chawla et al. 1986; Abou-Zeid et al. 1988; F. Collins et al. 1988;

Wallis et al. 1989; Verbon et al. 1990). Proteins identified were referred to by their molecular weight in kDa and their derivation, that is, MPT (from *M. tuberculosis*) and MPB (from *M. bovis*) (Andersen & Doherty 2005).

Due to the intracellular nature of *M. tuberculosis*, secreted proteins are required for rapid responses against infection with the organism as long as the bacteria are surviving and replicating within the macrophages (Kaufmann & Hess 1999). Short-term culture filtrate (STCF) was defined by Andersen and colleagues (1991a) and contains approximately 200 proteins, which vary depending on when the culture is harvested (Nagai et al. 1991; Pal & Horwitz 1992). In 1992 it was found that secreted, or extracellular, proteins from the bacteria are able to protect guinea pigs from aerosol challenge with live *M. tuberculosis* (Pal & Horwitz 1992).

A number of these extracellular antigens have now been defined, although the exact functions of many of them are unknown (Andersen & Doherty 2005). Two molecular mass fractions (<14 kDa and 26-34 kDa) have been shown to contain particularly strong immunoreactive antigens (Andersen et al. 1992; Boesen et al. 1995) and the proteins responsible for the antigenicity of these fractions are described below. Individual culture filtrate proteins have given variable results when tested as vaccine candidates and more success has been experienced with protein mixtures or “cocktails” (Orme 2005a).

Somatic antigens include cell wall and cytoplasmic proteins, and many of the culture filtrate proteins can also be found within somatic antigen preparations

(Wiker 2001). These antigens stimulate protective T-cell responses (Andersen & Doherty 2005), although they induce DTH, rather than CMI and memory immunity. For this reason, it was thought for many years that efforts for a subunit vaccine should only concentrate on extracellular proteins (Orme 1988a; Andersen & Heron 1993b; Andersen 1994). Somatic antigens, however, are experiencing a somewhat renewed interest in the context of CD8 T-cell involvement in the control of active disease (Feng & Britton 2000; Flynn 2004) and also in the suggestion that somatic antigens may sustain a protective immune response that has previously been initiated (Kaufmann & Hess 1999).

Complex antigen preparations induce stronger proliferative responses than single purified antigens (Mustafa et al. 1998), suggesting that responses to a number of different antigens within the bacilli may be required for successful vaccination (Sable et al. 2005).

2.12.1.1 ESAT-6

The name “ESAT-6” is used to describe a 6 kDa early secretory antigen target (Andersen et al. 1995) that was isolated from culture filtrates after only 7 days growth of *M. tuberculosis* (Andersen & Heron 1993b; Andersen et al. 1995; Boesen et al. 1995; Sørensen et al. 1995). The 6 kDa size of this protein was determined by Western blot, however using gel filtration and nondenaturing PAGE the molecular mass of ESAT-6 was found to be 24 kDa. The reason for the difference in size was thought to be due the presence of polymers.

Laser desorption mass spectrometry revealed the mass of the protein to be 9.9 kDa (Harboe et al. 1996b; Sørensen et al. 1995).

ESAT-6 is located in the RD1 gene region of mycobacteria (Andersen & Doherty 2005), a region that is absent in attenuated strains of *M. tuberculosis* and *M. bovis* (Harboe et al. 1996b). This antigen is only present in clinical strains of *M. tuberculosis* and *M. bovis* (Harboe et al. 1996b; Mustafa et al. 1998) and is responsible for the immunogenicity of the STCF fraction of low molecular weight proteins (Weldingh & Andersen 1999; Elhay & Andersen 1997). As a result, ESAT-6 has attracted much interest as both a potential vaccine candidate and as a diagnostic reagent (Orme 1999a). It has been shown to induce both a Th1-type immune response as well as CD8 T-cell responses (Lalvani et al. 1998). In spite of all the attention this molecule has drawn, the function of ESAT-6 proteins remains unknown (Andersen & Doherty 2005).

2.12.1.2 Antigen 85 complex

The antigen 85 complex, made up of Ag85a, Ag85B and Ag85C, is believed to be responsible for the immunogenicity of the mid-range fraction of STCF (Andersen et al. 1992; Boesen et al. 1995; Weldingh & Andersen 1999). Ag85A is a 35.7 kDa fibronectin-binding surface protein and is sometimes referred to as MPT44. Ag85B, or MPT59, is 34.6 kDa in size and is a mycosyl-transferase surface protein. Ag85C, which is also part of this complex is sometimes referred to as MPT45, and is not thought to be an

immunodominant antigen. All components of the Ag85 complex are present in culture filtrates of *M. tuberculosis* (Andersen & Doherty 2005; Nagai et al. 1991; Abou-Zeid et al. 1988).

2.12.1.3 Mtb72F

Mtb72F is a new 72 kDa fusion protein derived from two known antigens, namely Mtb32, a serine pronase that cannot be expressed individually, and Mtb39 (Skeiky et al. 2004). The resultant fusion protein has been combined with the adjuvant AS02A and administered to mice and guinea pigs. The results show that this antigen increases the efficacy of BCG vaccination when it is co-administered or used as a booster (Skeiky et al. 2004; Brandt et al. 2004).

2.12.2 DNA vaccines

DNA vaccination involves the production and purification of the DNA that encodes the antigen, in an inexpensive bacterial host, such as *Escherichia coli*. The resultant DNA can then be injected into either the skin or the muscle and is expressed by the cells of the recipient (Ginsberg 2000). There are numerous advantages with this method of antigen delivery, including cheap, simple production; no infectious agents are involved in the administration of the antigen; the ability to rapidly test different combinations of antigens; and, modification of antigen expression to tailor the type of immune response (Ulmer et al. 1993; Lowrie et al. 1997b; Donnelly et al. 1994). The only disadvantage with this method

of vaccination, and one that is extremely significant, is that to date DNA vaccines have performed very poorly in humans (Orme 2005).

DNA vaccination has been investigated as a delivery method for several different antigens including heat shock proteins (Lowrie et al. 1997a; Silva et al. 1999), Ag85A (Lozes et al. 1997; Kamath et al. 1999; Kirman et al. 2003), MPT 64 (Kamath et al. 1999) and Mtb72F (Brandt et al. 2004). These vaccines have been tested in mice and guinea pigs with promising results, eliciting CD8 T-cell responses in addition to Th1 responses, with antigen expression continuing for a considerable amount of time (Lowrie et al. 1997a; Lozes et al. 1997). Single DNA vaccinations, however, have failed to protect mice following aerosol challenge with *M. tuberculosis* (Kamath et al. 1999), although greater protective efficacy has been achieved using DNA booster doses (Kirman et al. 2003).

2.12.3 Recombinant vaccines

Recombinant vaccines deliver organisms in which the DNA has been engineered to overexpress a certain antigen or they have an additional gene sequence spliced into their own genome. Most efforts in developing recombinant vaccines for tuberculosis have focused on BCG as the vector, however other vectors examined have included *M. smegmatis* (Garbe et al. 1993; Laqueyrerie et al. 1995), *M. vaccae* (Abou-Zeid et al. 1995), *M. aurum* (Hermans& de Bont 1993), vaccinia virus (Lyons et al. 1990; Baumgart et al. 1996) and mixed results have been achieved with these vectors.

The results, however, are more promising with recombinant BCG, and in most situations the genetic insertions do not damage the recombinant organism. Studies have included alteration of the genome to overexpress immunodominant antigens, such as Ag85, or to sway the immune response to improve CD8 T-cell recognition (Orme 2005a; Horwitz & Harth 2003). The advantage of using BCG as the recombinant vector is that it mimics *M. tuberculosis* infections by its intracellular replication and it is capable of stimulating immune responses (Ginsberg 1998). Recombinant BCG is, however, a live organism and faces the same problems associated with the current BCG vaccine in that it cannot be administered to immunocompromised individuals. In addition, there is a potential for inhibition as a result of exposure to environmental mycobacteria (CDC Fact Sheet #250120 2005; Brandt et al. 2002).

In many situations, antibiotic resistance genes have been used as markers for the detection and selection of recombinants. It has been suggested, however, that this method may confer drug-resistance to the infecting organism and thus an alternative selection method, such as mercury resistance, should be employed (Baulard et al. 1996).

2.12.4 Auxotrophs

Auxotrophs are the opposite of recombinant vaccines, with gene sequences being switched off or deleted. These organisms rely on the addition of the absent growth factor for their survival, enabling external control of the degree of proliferation of the vaccine (Guleria et al. 1996; Ginsberg 1998; Orme 1999a). Many studies of

auxotrophic vaccines have involved manipulation of *M. bovis* BCG (McAdam et al. 1995; Reyrat et al. 1995; Guleria et al. 1996); however when *M. tuberculosis* is used as an auxotrophic vaccine more than one essential gene should be deleted to ensure the organism does not revert back to its virulent form (Guleria et al. 1996).

Gene deletions have been used to prevent production of leucine, methionine, mycolic acids, and to disrupt iron starvation responses and vitamin B metabolic pathways. Variable vaccination success has been achieved with these auxotrophs (Orme 2005a; Guleria et al. 1996; McAdam et al. 1995).

2.12.5 Mucosal delivery systems

Initial studies of BCG in 1921 were performed using oral vaccination due to the ease of administration by this method (Lagranderie et al. 2000). Subjects receiving a high oral dose of BCG experienced severe side effects (Lagranderie et al 2000). It was found that oral, intragastric, and subcutaneous administration of BCG provided similar levels of protection. As a result, subcutaneous vaccine injection, which resulted in reduced side effects, became the standard method of administration. It is, however, thought that encapsulation of orally administered BCG would prevent the described side effects (Lagranderie et al. 2000; Buddle et al. 1997).

The increased antigen concentrations required for oral vaccination as compared to parenteral vaccines (Rappuoli et al. 1999) may pose production difficulties and increased costs depending on the type of antigen used. In addition, efforts to

prevent oral tolerance must be incorporated into vaccine design and may include studying different available adjuvants. Delivery systems must be established that transport the vaccine directly to the intestine for presentation without disruption by the acidic conditions of the stomach and avoiding the side effects noted in early BCG oral vaccination. The advantages of oral vaccination include: ease of administration; safety (that is, negate the risk of needle stick injuries in HIV endemic areas); and the immediate stimulation of mucosal immune responses (Buddle et al. 1997). Nasal delivery systems may provide a method of mucosal vaccination that overcomes some of the difficulties associated with oral administration.

Several mucosal immunisation strategies have been examined. Two such studies using oral administration of Ag85-ESAT-6 fusion protein (Doherty et al. 2002) and nasal administration of heat-killed BCG or arabinomannan (Haile et al. 2005) have failed to induce primary immune responses, but achieved some success as booster vaccines following BCG vaccination.

To date, the only successful oral vaccine in naïve mice has used crude *M. tuberculosis* sonicate (Hosken 1999) or a crude preparation of STCF (Adams et al. 2005), both of which were delivered intragastrically. Both preparations resulted in significant immune responses as measured by T-cell proliferation, with the latter producing a Th1-type response (the type of T-cell response was not determined in the earlier study). Successful vaccination has also been achieved using an intranasal delivery of a recombinant adenoviral Ag85 vaccine (Wang et al. 2004).

Reasons for the variable results obtained in these studies may include the type of adjuvant used, which was different in every study, or the antigen dose administered. Subunit antigens that resulted in priming immune responses were administered at a dose of 200 µg per mouse. Alternatively, only 1-10 µg of antigen was administered to each mouse in the studies that were able only to induce boosting responses (Hosken 1999; Doherty et al. 2002; Haile et al. 2005; Adams et al. 2005; Rappuoli et al. 1999).

Due to limited availability of mucosal adjuvants for the prevention of oral tolerance, very few vaccines have been examined for mucosal delivery and considerable work remains to be done in this area (Freytag & Clements 2005).

2.12.6 Current clinical trials

One of the first vaccines to progress to phase I clinical trials since BCG was introduced was a recombinant vaccinia virus Ankara, expressing Ag85A, administered intradermally. This clinical trial found that the vaccine produced good immune responses in naïve participants and even greater responses when used as a booster following BCG. No CD8 T-cells responses were detected (McShane et al. 2004).

Another candidate for clinical trials has been inactivated *Mycobacterium vaccae*. The organism was heat inactivated and multiple doses were administered intradermally to HIV positive patients. The results showed that the vaccine was

well tolerated by all the patients. The type of immune responses obtained were dependant upon the dosage size, with smaller doses resulting in Th1 responses and larger doses also introducing some Th2 responses. It was also recognised in this clinical trial that inactivated *M. vaccae* is more effective at boosting immune responses initiated by prior BCG vaccination than inducing a primary immune response (Waddell et al. 2004; Vuola et al. 2003).

The most recent phase I clinical trial to be undertaken was announced in August 2004 (Bosch 2004; von Reyn 2005). This African trial uses rBCG30 (recombinant BCG overexpressing Ag85B) as the vaccine candidate and the scientific community is optimistically awaiting results due to the outstanding success of this vaccine in the guinea pig model (Bosch 2004; Horwitz 2005; Horwitz & Harth 2003).

Other clinical trials in primate models have involved the administration of fusion proteins to cynomolgous monkeys. The two fusion proteins that have been examined in this model as vaccine candidates are Ag85B-ESAT-6 (Langermans et al. 2005) and Mtb72F (Reed et al. 2003). Both studies have demonstrated increased protection against infection as a result of vaccination and have been recommended for further clinical evaluation.

2.13 Adjuvants

An adjuvant, derived from the Latin “adjuvare” meaning to help, is any product that, when incorporated into a vaccine formulation, acts to accelerate, modulate, prolong or enhance the quality of the immune response against an antigen (Vogel 2000; Lima et al. 2004). All effective vaccines must contain three important factors: (i) one or more antigens to which the immune response is directed; (ii) a method of activating antigen presenting cells; and (iii) signals or cytokines for modulating the immune response between Th1- and Th2-types. Many tuberculosis vaccines currently under review are either synthetically derived or are purified derivatives of whole organisms. As such, adjuvants are required for fulfilment of all three factors required for effective vaccination and recent trends have been to combine different adjuvants and delivery systems for optimal results (Lima et al. 2004).

In addition to tailoring immune responses to an antigen, adjuvants reduce the dose and frequency of immunisations required for protective immunity (Vogel 2000). This property of adjuvants makes them ideal for mucosal immunisation and the prevention of oral tolerance.

For vaccines against tuberculosis and other intracellular bacterial infections, adjuvants should be selected that promote Th1 and CD8 T-cells (Harboe et al. 1996a; Elhay & Andersen 1997; Lima 2004). Numerous different adjuvants have been studied and the properties of some of the more common ones are outlined below.

2.13.1 Aluminium hydroxide

Aluminium hydroxide is often simply referred to as alum and is the only parenteral adjuvant currently licensed for use in humans. Unfortunately, this adjuvant promotes a dominant Th2 response with the production of IL-4 and IgG1, which leads to exacerbation of TB infections in mice (Andersen 1997; Elhay & Andersen 1997; Vogel 2000).

2.13.2 Freund's adjuvant

Freund's Complete Adjuvant (FCA), which was developed in the 1930s, contains killed mycobacteria in a water-in-mineral-oil emulsion with an additional immunomodulator. It is one of the most effective parenteral adjuvants known and induces strong immune reactions, including granulomatous lesions at the site of injection. This, along with other toxic effects has resulted in FCA not being licensed for use in humans (Vogel 2000; Gupta et al. 1993; Sinha & Khuller 1997).

In an effort to reduce the side effects of FCA, the mycobacteria were removed from the adjuvant to create Incomplete Freund's Adjuvant (IFA), which induces effective Th1 responses and cell-mediated immunity (Andersen 1997; Elhay & Andersen 1997). IFA has been used in several experimental HIV vaccines and a licensed influenza vaccine in the United Kingdom (Vogel 2000).

2.13.3 DDA

Dimethyl dioctadecyl ammonium bromide (DDA) is a parenteral adjuvant that has been shown to promote strong cell-mediated immune responses to mycobacterial

antigens with production of high levels of IFN-γ. Side effects such as local ulceration and toxicity, which are noted in other adjuvants, are not experienced in administration of DDA (Andersen 1994; Andersen 1997).

2.13.4 ADP-ribosylating Enterotoxins

Cholera toxin (CT), which is produced by various strains of *Vibrio cholera*, and heat-labile enterotoxin (LT), produced by *Escherichia coli*, are both soluble proteins that are also potent mucosal adjuvants (Williams et al. 1999). These two toxins are similar in structure and mechanism.

At the amino acid level, they are 82% homologous (Williams et al. 1999), and both consist of A and B subunits. The A subunit, which is made up of A1 and A2 chains joined together by a disulfide loop, is linked to the pentameric B molecule by an α-helix. Both toxins are responsible for profuse watery diarrhoea in humans. The mechanisms leading to such a reaction include a complex series of chemical reactions within host cells, beginning with binding of the B subunit to GM1-ganglioside receptors followed by unfolding and disassembly of the A subunit in the endoplasmic reticulum (Rappuoli et al. 1999; Freytag & Clements 2005).

Although there is considerable knowledge regarding the mechanisms of these toxins, very little is known regarding the mechanisms of their function as adjuvants. It has been hypothesised that they undergo complex and dynamic interactions with a variety of different cell types and change the context in which antigens are processed and presented (Freytag & Clements 2005).

The obvious disadvantage of using CT and LT as adjuvants is the toxic responses experienced by human hosts. As little as 5 μg of CT and 2.5 μg of LT are sufficient to induce significant diarrhoea. Mutations of the active sites and protease sites in these two toxins have been studied in an attempt to retain adjuvant properties, but negate or reduce their toxicity (Freytag & Clements 2005).

LTK63 and CTK63, in which serine 63 is substituted for lysine; CTS106, in which proline 106 is replaced with serine; and LTR72, in which alanine 72 is replaced with arginine in the A subunits; are mutant forms of LT and CT that have significantly reduced toxicity. Good results have been achieved with LT mutants and CTS106, but CTK63 appears to have reduced adjuvanticity (Freytag & Clements 2005; Vajdy et al. 2004; Rappuoli et al. 1999; Pizza et al. 1994; Partidos et al. 1996; Fiuliani et al. 1998; Douce et al. 1997). Another successful LT mutant LTG192 has an altered cleavage site of its two A-chains (Dickinson & Clements 1995). This prevents its destruction in the endoplasmic reticulum and the resultant chemical cascade (Tsai et al. 2001). The attenuated LTG192 has equal adjuvant activity to native LT (Freytag & Clements 2005).

The major difference between the two families of ADP-ribosylating enterotoxins is the type of immune responses they induce. CT is associated with Th2 responses, characterized by the CD4 T-cell secretion of IL-4, IL-5, IL-6 and IL-10, and the production by B-cells of secretory IgA and serum IgG1, IgA and IgE. In contrast, LT induces both Th1 and Th2 responses in addition to B-cell secretion of secretory

IgA and serum IgG1, IgG2a and IgA (Freytag & Clements 2005; Doherty et al. 2002).

2.13.5 Adjuvants that use Toll-like receptors (TLR)

The signal pathway for this type of mucosal adjuvant begins with engagement of intramembrane receptors, called Toll-like receptors (TLR), on various cells of the immune system (Kopp & Medzhitov 1999; Freytag & Clements 2005). CpG ODN is a synthetic oligonucleotide that uses TLR9 to stimulate monocytes, macrophages and dendritic cells, which in turn activate T-cells in a typical Th1-like response (Freytag & Clements 2005). Monophosphoryl lipid A (MPL), another adjuvant in this category, is isolated from the lipopolysaccharide of *Salmonella minnesota* R595. It uses TLR2 and TLR4 to activate monocytes and also promotes Th1-type immune responses (Vajdy et al. 2004; Puggioni et al. 2005; Freytag & Clements 2005).

2.13.6 Propionibacterium

Propionibacterium acnes (EqStim®) is a recognised commercially available parenteral adjuvant used in the equine industry (Flaminio et al. 1998) and results in the production of high levels of IFN-γ (Megid et al. 1999). Recently, a probiotic strain of *P. jensenii* (PJ702) was also shown to induce a Th1-type response in vaccination against tuberculosis and shows promise as a mucosal adjuvant (Adams et al. 2005).

2.13.7 Quil A

Quil A is extracted from the bark of *Quillaja saponaria*, a tree native to Chile (Vajdy et al. 2004). QS21, or AS02A, is a highly purified saponin from Quil A that produces strong Th1-type immune responses (Brandt et al. 348). Quil A is haemolytic, but QS21 is suitable for either parenteral or mucosal immunisation (Vajdy et al. 2004).

ISCOMS are immunostimulant complexes, in which Quil A is incorporated into particles that also contain cholesterol, phospholipids and cell membrane antigens (Kamstrup et al. 2000). These complexes enable a reduction in the amount of the haemolytic Quil A to be administered, however the inclusion of antigens in ISCOMS can be complicated (Vajdy et al. 2004).

2.13.8 Particulate adjuvants/Delivery systems

Particulate adjuvants are considerably larger than other adjuvants described. They provide delivery systems that can limit adverse effects of the antigen or other co-administered adjuvants in addition to contributing adjuvant properties of their own (Vajdy et al. 2004; Lima et al. 2004).

Microparticles are a type of particulate mucosal adjuvant and are capable of presenting multiple copies of an antigen to the immune system. Common microparticles tested in vaccine models are polylactide-coglycolides (PLG) and have been shown to elicit strong humoral immune responses both by

microencapsulation of the antigen and by adsorption of the antigen onto the surface of the particle (Vajdy et al. 2004; Heritage et al. 1998).

Another particulate adjuvant is MF59, a squalene oil-in-water adjuvant. This potent parenteral adjuvant was designed to replace the toxic Syntex Adjuvant Formulation (SAF) and Freund's Complete Adjuvant (FCA), both of which are also oil-in-water adjuvants (Vajdy et al. 2004; Vogel 2000; Byars & Allison 1987; Byars et al. 1990).

Liposomes are phospholipid adjuvants that have also been examined as delivery systems for both hydrophilic and hydrophobic antigens with promising results. The activity attributed to these particles, however, is often difficult to determine as they are frequently co-administered with MPL (Vajdy et al. 2004; Sinha & Khuller 1997; Andersen 1994).

2.13.9 Cytokines

As an alternative to using adjuvants to sway immune responses toward the production of certain cytokines, the cytokines can be administered directly with the antigen to modulate the response (Vajdy et al. 2004). Cytokines that have been evaluated in tuberculosis vaccination include IL-12, IL-18, IL-15 and IFN-γ, and good immune responses have been achieved although some cytokines boost the activity of another adjuvant rather than acting independently (Hovav et al. 2005; Arulanandam & Metzger 1999; Cooper et al. 1995; Baek et al. 2003; Umemura et al. 2003). Some cytokines have been evaluated as mucosal adjuvants in addition to parenteral administration; however cytokines tend to exhibit dose-related

toxicity, are expensive to produce and require stringent storage conditions for relatively short periods of time (Vajdy et al. 2004).

Chapter 3: General Methods, Cultures and Tools for Protein Analysis and Purification

3.1. Introduction

This chapter explains the general laboratory methods and cultures used as a basis of all experiments in this dissertation. In the 1980s it was learned that individual proteins constituting *M. tuberculosis* cells or their contents were able to invoke immune responses (Chaparas et al. 1980; Nagai et al. 1981; Chawla et al. 1986; Abou-Zeid et al. 1988; F. Collins et al. 1988; Wallis et al. 1989; Verbon et al. 1990). As these subunit proteins are the focus of this dissertation, this chapter outlines the basic methods used for purification and analysis of proteins.

3.2. Methods

3.2.1. General equipment

Unless stated otherwise, all pipetting was performed using Gilson automatic pipettes and Bonnet Equipment pipette tips. All reagents mentioned in this section that were not acquired ready-made are described in Appendix A of this thesis. A Biological Safety Cabinet Class II (Gelman Sciences) was used whenever work was performed using live bacteria.

3.2.2. Bacterial culture methods

3.2.2.1. *Mycobacterium tuberculosis* strain used

Mycobacterium tuberculosis H37Rv (ATCC 27294) was used in all bacterial experiments described in this thesis. This strain was obtained in lyophilised form from American Type Culture Collection (ATCC) in the United States, and was reconstituted with 0.4 ml PBS (Appendix A 1.1).

3.2.2.2. Culture on Lowenstein-Jensen (LJ) media

Two drops of reconstituted *M. tuberculosis* (ATCC 27294) were placed on each Lowenstein-Jensen (LJ) slope (bioMérieux) using a transfer pipette (Biolab Scientific). The cultures were incubated (Thermoline) at 37°C for approximately 3 weeks until sufficient culture was obtained. A portion of this culture was stored (Section 3.2.2.4.) and the remainder was subcultured onto LJ slopes. Subcultures were continued onto LJ slopes approximately every 2-3 weeks to keep the bacteria actively growing. Approximately every 3 months a fresh culture was begun using one of the stored aliquots (Section 3.2.2.4.).

3.2.2.3. Culture on Modified Sauton's Medium (MSM)

M. tuberculosis bacteria grow optimally on the surface of Modified Sauton's Medium due to their preference for highly aerobic conditions and they grow best if they are sub-cultured on a weekly basis (H. G. Wiker, personal communication). Autoclavable plant culture vessels (Sigma, Magenta vessels) were obtained with lids that have a filter set into them, allowing fresh

air to enter the vessel, without allowing the escape of particulate aerosols. A surface culture was initiated by smearing *M. tuberculosis* cells from cultures on LJ medium (section 3.2.2.2) onto a mixed cellulose ester membrane (MFS, 47 mm, 0.45 μm pore size) using a sterile swab (Livingstone). The membrane was lifted, using two pairs of sterile forceps, and floated onto the surface of 200 mL of Modified Sauton's Medium (Appendix A 2.2). Following inoculation, the vessel was incubated (unshaken) at 37°C for approximately 10 days, by which time the culture had grown enough to sub-culture onto fresh MSM without the flotation aid provided by the membrane. The culture was separated from the membrane, using a sterile teaspoon, of which the handle had been bent to form a ladle, and transferred to 200 ml fresh Sauton's medium. Subcultures were performed on a weekly basis by the scooping up of cells using the teaspoon ladle.

3.2.2.4. Storage of bacterial strain

Following culture, the bacteria were suspended in Nutrient Broth with 10% glycerol (Appendix A 2.1). 1.5 ml aliquots of bacteria were placed into 2.0 ml cryogenic vials (Corning). The cultures were "snap-frozen" by placing in a Pyrex beaker containing dry ice (BOC gases) and ethanol (Fronine). Frozen cultures were stored at -80°C (GS Laboratory Equipment).

3.2.3. Protein assay methods

A Bio-Rad Protein Assay kit was used. Initially, the standard method was performed, and then the microtitre plate method was instigated upon purchase of the Bio-Rad Microplate Reader (Model 550). For samples of adequate volume that were expected to contain very small amounts of protein, the Microassay Microtitre Plate Method was used.

3.2.3.1. Standard protein assay method

The standard protein assay was performed as per the manufacturer's instructions (Bio-Rad). In brief, triplicates of 100 µL of blank (distilled water), standard (Appendix A 3.4) or sample were mixed with 5.0 mL of dye reagent (Appendix A 3.1) in a clean dry test tube and vortexed (Ratek Instruments). Following 5 minutes incubation at room temperature the absorbances were measured (Novaspec II spectrophotometer, Pharmacia Biotech) at 595 nm on a distilled water blank within one hour. A standard curve, such as that shown in Figure 3.1, with a linear range of 0.2 to 0.9 mg/mL was prepared for the determination of protein concentrations in the samples.

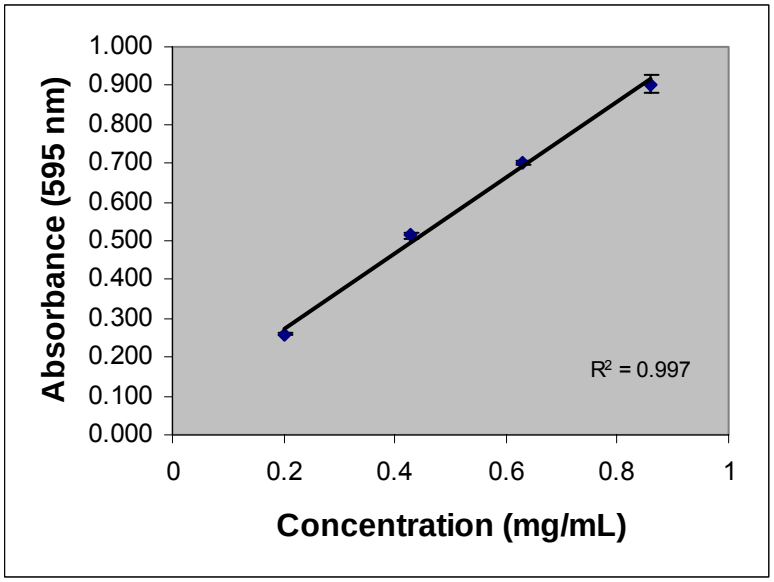

Figure 3.1. A typical standard curve obtained using the Bio-Rad Standard Protein Assay. Error bars indicate one standard deviation and the R^2 value for the line of best fit is displayed on the graph.

3.2.3.2. Microtitre plate method

The microtitre plate method of protein analysis was used in preference to the standard method following the purchase of the microtitre plate reader and was performed as per the manufacturer's instructions (Bio-Rad). In brief, 10 µL of blank (distilled water), standard (Appendix A 3.5) or sample were pipetted into microtitre wells in triplicate. 100 µL of diluted dye reagent (Appendix A 3.2) was added to each well and mixed using the pipette. Following 5 minutes incubation at room temperature, the absorbances were measured at 595 nm using a microplate reader (Bio-Rad Model 550). Bio-Rad Microplate Manager (Version 5.0.1) was used for the construction of a standard curve, such as that shown in Figure 3.2, with a linear range of 0.05 mg/mL to approximately 0.5 mg/mL and subsequent calculation of protein concentration in samples.

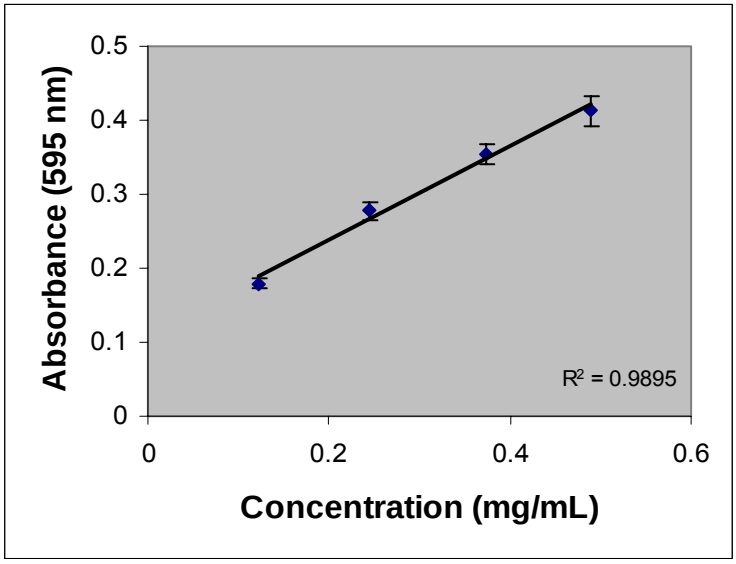

Figure 3.2. A typical standard curve obtained using the Bio-Rad Microtitre Plate Protein Assay. Error bars indicate one standard deviation and the R^2 value for the line of best fit is displayed on the graph.

3.2.3.3. Microassay microtitre plate method

The microassay microtitre plate method was used for samples with very low protein levels, as the linear range of this assay was 8.0 μg/mL to approximately 80 μg/mL. 160 μL of blank (distilled water), standard (Appendix A 3.7) or sample were pipetted into microtitre plate wells. Blanks and standards were always assayed in triplicate. Samples were assayed in duplicate or triplicate, depending on available volume. 40 μL of dye reagent concentrate (Coomassie® Brilliant Blue G-250, Bio-Rad) was added to each well and mixed using the pipette. Following 5 minutes incubation at room temperature, the absorbances were measured at 595 nm using a microplate

reader (Bio-Rad Model 550). Bio-Rad Microplate Manager (Version 5.0.1) was used for the construction of a standard curve, such as that shown in Figure 3.3, and the calculation of protein concentration in the samples.

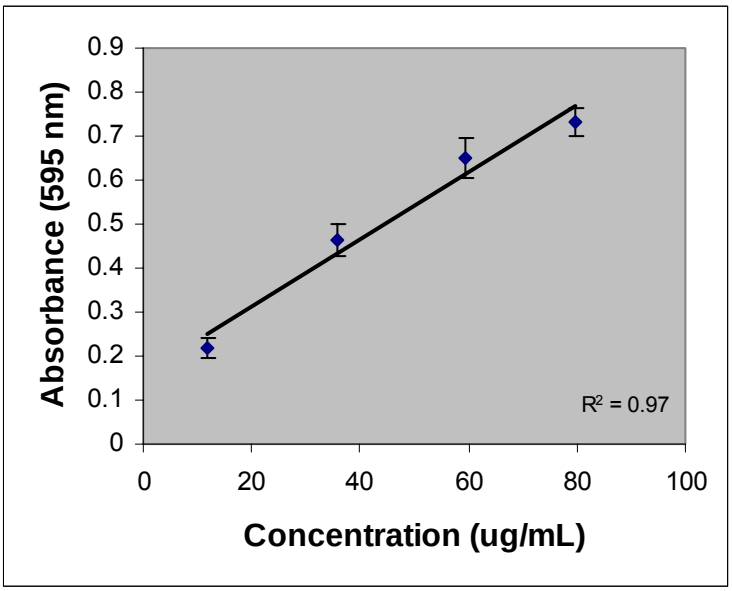

Figure 3.3. A typical standard curve obtained using the Bio-Rad Microassay Microtitre Plate Protein Assay. Error bars indicate one standard deviation and the R^2 value for the line of best fit is displayed on the graph.

3.2.4. SDS-PAGE Methods

3.2.4.1. Equipment and assembly of gel

The Bio-Rad Protean II xi Cell electrophoresis system was used for SDS-PAGE analysis. This method is based on the methods described by Laemmli (Laemmli, 1970). A 12 % separating gel (Appendix A 4.6), overlaid with a 4% stacking gel (Appendix A 4.7), was poured according to the manufacturer's instructions. The gel thickness was 0.75 mm, the length was 16 cm and a 15-

well comb was used to make the wells in the stacking gel. The gels and running buffer (Appendix A 4.11) were assembled in the electrophoresis cell according to the manufacturer's instructions.

3.2.4.2. Molecular weight standards used

Two types of molecular weight standards were used in SDS-PAGE. Gels that were to be used for electro-elution were loaded with 25 µL of neat pre-stained low range SDS-PAGE standard (Bio-Rad) in lanes 1 and 15. This standard comprised 6 different proteins ranging in size from approximately 20-110 kDa (exact molecular weights were supplied with each lot number). The purpose of using the pre-stained standard was to provide markers for division of protein fractions in the adjacent lanes according to their size. The eluted unstained proteins, extracted from the lanes adjacent to the standards, would be suitable for use in cell cultures and vaccinations.

Alternatively, an unstained low range SDS-PAGE molecular weight standard (Bio-Rad) was applied to the gel on occasions when the completed gel was to be stained for visualization of protein bands. This provided a form of positive control for the staining process and also indicated the distances that certain proteins had travelled according to their molecular weights. The unstained low range SDS-PAGE standard comprised 6 different proteins, which are listed with their molecular weights in Table 3.1. This standard was diluted 1 in 10 with sample buffer in a microfuge tube (Sarstedt) and a hole was pierced in the lid using a pin. The tube was heated at 95°C for 4 minutes and cooled.

10 μL of treated sample was added to the appropriate well/s of the prepared SDS-PAGE gel.

Table 3.1. Unstained low range SDS-PAGE standard constituents and their molecular weights.

Protein	Source	MW (kDa)
Phosphorylase B	Rabbit muscle	97.4
Bovine serum albumin	Bovine plasma	66.2
Ovalbumin	Chicken egg white	45
Carbonic anhydrase	Bovine erythrocytes	31
Soybean trypsin inhibitor	Soybean	21.5
Lysozyme	Chicken egg white	14.4

3.2.4.3. Sample preparation

Samples were diluted 1 in 3 with sample buffer (Appendix A 4.9) in microfuge tubes (Sarstedt). A hole was pierced in the lid of each tube using a pin and the tubes were heated at 95°C for 4 minutes and cooled. 60 μL of treated sample was added to the designated wells of a prepared SDS-PAGE gel prior to electrophoresis.

3.2.4.4. Running Conditions

SDS-PAGE was performed at a constant current. Optimum running conditions were found to be 25 mA for approximately 45 minutes (or until the dye front passed through the stacking gel), and then the current was reduced to 20 mA for approximately 12 hours (or until the dye front was approximately 1 cm from the bottom of the gel). These conditions were found to produce

clear separation of proteins without the appearance of “drag lines” at the edges of each lane.

3.2.5. Gel staining

All staining was performed in a glass dish of proportions 21 x 21 x 5 cm. During staining, the dish was covered and placed on a Ratek rocker to ensure even diffusion of staining solutions.

3.2.5.1. Coomassie Blue

Following SDS-PAGE, the gel was placed in Coomassie Blue Stain (Bio-Rad) for 30 minutes. The gel was then washed with 3 changes of Fixative I (Appendix A 5.1) over a period of 90 minutes and rinsed for 5 minutes in distilled water.

3.2.5.2. Silver Stain

The Bio-Rad Silver Stain Kit was used to stain the gels following SDS-PAGE as per the manufacturer’s instructions. Briefly, the gel was placed in 400 mL of Fixative I (Appendix A 5.1) for a minimum of 30 minutes followed by Fixative II (Appendix A 5.2) for two 15 minute intervals. 200 mL of Oxidizer Reagent (Appendix A 5.3) was applied to the gel for 15 minutes and then washed three times by soaking in distilled water for 5 minutes per wash. The gel was placed in a bath containing 200 mL Silver Reagent (Appendix A 5.4) for 20 minutes and given a 1-minute distilled water rinse before developing. Developing the gel involved placing the gel in 200 mL Developer (Appendix A

5.5) for approximately 30 seconds until a brown “smoky” precipitate appeared. Immediately the gel was removed and placed into a 200 mL of fresh developer for 5 minutes. One final soak in developer for 5 minutes was performed and the reaction was stopped using 400 mL Stop Solution for 5 minutes.

3.2.5.3. Gel drying

Prior to drying, stained gels were soaked in Gel Drying Solution (Bio-Rad) for 30 minutes. The Gel Drying Assembly (Bio-Rad) was constructed according to the manufacturer’s instructions. The gel was placed in the GelAir Dryer (Bio-Rad) over night, with the fan running for the first 3 hours. Photography of gels was performed using the Bio-Rad Gel Doc 2000 system and Bio-Rad Quantity One (Version 4.1.1).

3.2.6. Method for fractioning crude proteins according to their molecular weights

3.2.6.1. Electro-elution method

The electro-elution method used was based on the methods reported by Andersen and Heron (1993a). Following electrophoresis (Section 3.2.4.4.) using pre-stained standards and molecular markers, the gel was soaked in three changes of Phosphate Buffer (Appendix A 6.1) on a Ratek rocker for a total of 20 minutes. This allowed the gel to swell prior to electro-elution, and also removed excess SDS. The gel was then removed from the buffer and placed on a clean flat surface. The position of the molecular weight markers

was recorded prior to trimming, and the gel was trimmed to the size of the electroeluter. A Bio-Rad Whole Gel Eluter was used and the equipment was assembled according to the manufacturer's instructions. The electro-elution was performed at 40 V for 20 minutes. At the end of the elution period, the current was reversed for 15 seconds to dislodge proteins bound to the cellophane membrane.

3.2.6.2. Harvesting

Following elution, the electrodes were removed from the Whole Gel Eluter (Bio-Rad) and the harvesting box was attached. The harvester was connected to suction and the thirty fractions were harvested into small test tubes. The harvesting box was then removed and a Pasteur pipette was used to ensure that the harvest was complete.

3.2.7. Ultra-filtration

Ultra-filtration was used to concentrate protein solutions. A 50 mL stirred cell apparatus (Advantec MFS, UHP-43) was assembled according to the manufacturer's instructions, using a regenerated cellulose ultrafiltration membrane (Amicon) with a 3,000 Da molecular weight cut-off. The stirred cell was connected to a nitrogen gas cylinder (BOC Gases) and the flow rate of the nitrogen gas was gradually increased until no bubbles appeared in the waste line. Distilled water was run through the stirred cell for a minimum of 5 minutes to rinse the membrane. Any remaining water was tipped out before the sample was placed into the stirred cell. Ultra-filtration was used to reduce the sample volume to approximately 2 mL.

Membranes were stored in 10% ethanol (Appendix A 1.2) at 4°C and reused several times.

3.2.8. Statistical analysis

All graphical and statistical analysis was performed using Microsoft® Excel 2002. The statistical analysis involved multiple two-sample two-tailed t-tests, assuming equal variances.

Chapter 4: Production of Antigens From Sonicated *Mycobacterium tuberculosis*

4.1. Introduction

Somatic (or sonicate) antigens, including cell wall and cytoplasmic proteins (Wiker 2001), have generally not received much attention among those searching for an improved vaccine against tuberculosis due to the fact that they do not evoke long-term specific immunity (Orme 1988a; Andersen & Heron 1993b; Andersen 1994). In recent years, however, somatic antigens have received a somewhat renewed interest as a result of the realization of CD8 T-cell involvement in the control of active disease (Feng & Britton 2000; Flynn 2004) and also in the suggestion that they may sustain previously initiated protective immune responses (Kaufmann & Hess 1999). It is also conceivable that the sonicate antigens of *Mycobacterium tuberculosis* may act as an adjuvant if given in conjunction with extracellular proteins, which would result in lower doses of extracellular proteins required for the desired long-term specific immunity. Thus, it was decided to examine the effect of oral vaccination with somatic antigens.

Several factors had to be considered in extracting cellular proteins from *Mycobacterium tuberculosis* for use as oral antigens. The time at room temperature had to be kept to an absolute minimum in order to reduce degradation of proteins. Likewise, the refrigeration time was kept to not more than a few days and samples were stored at -80°C as soon as possible. Methods of sonicate

production were developed with the aim of increasing protein concentration as much as possible, thus decreasing the number of repetitions of the developed method that were required to obtained a sufficient quantity of antigen. Fewer repetitions lead to reduced storage time prior to vaccination of laboratory animals and use in follow-up testing of immune responses, thus decreasing the chances of protein degradation.

In addition, methods had to be designed for the production of sonicate containing high protein concentrations in order to reduce the volume of the antigen feed to a level that maintained the comfort of laboratory animals. From previous experiments (Hosken 1999; Lean 2002) it was decided to set the antigen dose at 200 μg. For the comfort of the mouse, the volume injected into the stomach by a gavage needle was kept at not more than 100 μL. As a result, the quantitative aim of the experiments outlined in this chapter was the production of 1.1 mL (per group of 10 mice) of sonicate antigen solution containing approximately 4 mg/mL protein.

Fraction antigens, as well as the whole sonicate antigens, were required for use in stimulating lymphocytes extracted from the spleens and lymph nodes of the mice, so testing the immune response to the ingested whole antigens (Chapter 6). From examination of the literature (Andersen et al. 1992), it was expected that the optimal protein concentration of fractions for lymphocyte stimulation would be between approximately 1 μg/mL and 30 μg/mL. For the ease of doubling dilutions, the maximum concentration was set at 32 μg/mL. Thus, a minimum of 600 μL of a

64 μg/mL solution of each fraction antigen and whole sonicate solution was required for one dose curve experiment.

4.2. Methods

4.2.1. Cell preparation

M. tuberculosis cells were recovered from Modified Sauton's Medium (MSM) or Lowenstein-Jensen (LJ) slopes (Chapter 3). A Biological Safety Cabinet Class II (Gelman Sciences) was used for safety purposes whenever work was performed using live bacteria. The cells were suspended in 2% glutaraldehyde (Appendix A 1.4) and killed by soaking overnight at 4°C. The cells were then transferred to 10 mL screw cap sterile centrifuge tubes (Sarstedt) and centrifuged for 20 minutes at 4000 rpm (1,000 x g) using a Hettich EBA 8S centrifuge. Following centrifugation, the supernatant was discarded and the cells were washed 4 times in sterile distilled water (dH_2O) or Protease Inhibitor (PI) Solution (Appendix A 7.4) by centrifugation using the previous mentioned parameters.

Killed washed cells were added in various quantities to a 5 mL Pyrex centrifuge tube for sonication. Additional dH_2O or PI was added in sufficient quantity to just loosen the cells. In some circumstances, 1.0 mL glass beads (Fortuna, 0.25-0.5 mm) were also added. The test tubes containing the samples were kept cool on crushed ice.

4.2.2. Basic sonication method – Pilot study

A Branson Digital Sonifier 250 was purchased and a Microtip Tapered ⅛" horn was used for all sonication in this project. All test tubes containing killed *M. tuberculosis* samples were placed in a 200 mL Pyrex beaker containing crushed ice to ensure that the sample was kept cool during sonication. Samples were sonicated at amplitudes between 35% and 50% for time intervals of 2-5 minutes. 1-8 repetitions were performed, resulting in total sonication times of 2-40 minutes. Larger debris was removed from most samples using a sterile Sartorius Minisart filter (25 mm, 0.2 μm pore size) and the protein content of the filtrate was determined using the standard Bio-Rad protein assay (Chapter 3). All samples were assayed in duplicate and average values were calculated.

4.2.3. Sonication method – Revision 1

After consulting journal articles referring to the sonication of *M. tuberculosis*, it was found that most authors sonicated their cell suspensions for at least 20 minutes (Daniel 1975; Closs 1980). Following sonication, they used ultracentrifugation (20,000 x g for 30 minutes) to clarify the samples and remove hydrophobic proteins. It was also decided that cell volumes could be increased if the vessel was changed to incorporate the larger volume.

The 5 mL Pyrex centrifuge tube was replaced with a 10 mL round bottom test tube. Two cell volumes were experimented with. Experiment 4.2.3.1 constituted 2.0 mL of glass beads, 2.0 mL of washed packed *M. tuberculosis* cells and 2.5 mL sterile

distilled water combined in the test tube. The cell suspension was sonicated in a beaker of crushed ice for 7 repetitions of 3-minute sonications at 35% amplitude, giving a total sonication time of 21 minutes. In experiment 4.3.2.2, 3.0 mL of cells from LJ slopes were added to 2.0 mL glass beads, with just enough sterile distilled water to make a runny paste (approximately 1.0 mL). Due to the thickness of the paste, the amplification of the sonicator was increased to 40%.

Following sonication, the sample was transferred without the glass beads, to a 30 mL plastic ultracentrifuge tube (Nalgene) using a transfer pipette (Biolab Scientific). The sample was centrifuged (Beckman, J2-MC centrifuge) at 20,000 x g for 30 minutes at 4°C. Following ultracentrifugation, the sample was sterile filtered using a sterile Sartorius Minisart filter (25 mm, 0.2 μm pore size) and the protein concentration was determined using the microtitre plate method (Bio-Rad) (Chapter 3).

4.2.4. Sonication method – Revision 2 (Temperature probe method)

It was suspected that the poor protein yields obtained using the sonication methods previously described may be due to denaturation of proteins at high temperatures. As a result, a temperature probe was obtained for the Branson Digital Sonifier.

For this experiment, the water washes and supernatant (Section 4.2.1) were replaced with Phosphate Buffered Saline (PBS) (Appendix A 1.1) so as to be more biologically conducive to antigen administration.

The temperature probe sonication method was based on the method described in Section 4.2.3, but with the following changes. Due to the fact that two probes now needed to be placed into the cell suspension, test tubes with a larger brim were required. 50 mL round bottom Pyrex test tubes were obtained (Crown Scientific), which had a diameter at the brim of 25 mm.

With the increase in vessel size, the sample size also had to be increased. It was decided to use the same ratios that had been used in successful prior experiments (Section 4.2.3.1), and the volume was tripled (that is, 6.0 mL of washed packed cells, 6.0 mL glass beads, 7.5 mL PBS). It was found that a greater volume of cells could be obtained by culture on MSM than by culture on LJ slopes, and so from this point forward, all cells were obtained from MSM.

At this point, it was also decided to add ethanol to the ice-filled beaker that the sample rested in during sonication, in an effort to cool the sample more effectively. Five experiments (designated 4.2.4.1, 4.2.4.2, 4.2.4.3, 4.2.4.4 and 4.2.4.5) were prepared with the only variable being the quantities of ethanol added to the crushed ice. After the beaker was filled with crushed ice, ethanol was added to the desired graduations on the beaker (Table 4.1). The temperature limit was set at

45°C and the sonication time was set to 20 minutes. Temperature measurements were recorded throughout the sonication.

Table 4.1. The variable ethanol quantities defining the five experiments used for the temperature probe sonication experiment.

Experiment Number	Beaker graduation (mL) to which ethanol was added
4.2.4.1	20
4.2.4.2	25
4.2.4.3	50
4.2.4.4	50
4.2.4.5	100

The samples were ultra-centrifuged and sterile filtered following sonication as per the method described previously (Section 4.2.3) and the protein concentrations were measured using the microtitre plate method (Bio-Rad) (Chapter 3).

The sonicates obtained in Experiments 4.2.4.1 and 4.2.4.2 were then combined and concentrated by ultra-filtration (Chapter 3) in an attempt to further increase protein concentration in the sample. Likewise, experiments 4.2.4.3, 4.2.4.4 and 4.2.4.5 were also combined and concentrated by ultra-filtration. The average protein production for the experiments included in each batch were not significantly different ($P>0.1$), thus only the quality of proteins had to be considered in the combinations. It was assumed that similar sonication temperatures would result in similar protein production and the experiments were divided into the two different batches for concentration according to their temperature curve similarities (Section 4.3.3.2, Figure 4.1).

4.2.5. TES buffer lysis

Prior to the purchase of the temperature probe for the Sonifier, an alternative method was sought that would prevent the samples becoming hot whilst protein was extracted from the cells, and also one that would produce a higher protein concentration in the sample. Previous laboratory experience had demonstrated greater success using a TES buffer lysis method than using sonication and so this method (Fessler, et. al. 1999; Huang 2003) was attempted on *M. tuberculosis* cells.

4.2.5.1. TES buffer lysis method

A 1.5 mL microfuge tube (Sarstedt) was used to contain the sample. 300 μL of glass beads (Fortuna, 0.25-0.5 mm) that were wet with TES buffer (Appendix A 7.5) were placed in the tube. 200 μl TES buffer and approximately 100 μL killed washed (Section 4.2.1) *M. tuberculosis* cells (a generous loop full) were added to the tube. Each microfuge tube was vortexed (Ratek Instruments) for 1 minute and then placed on crushed ice for 1 minute. This process was repeated until the desired total vortex time had elapsed. Total vortex times attempted were 5, 10 and 20 minutes. The sample was then centrifuged for 10 minutes using a Beckman Microfuge 11. Seven centrifuge speeds were tested between 3000 rpm (1,400 x g) and 10,000 rpm (15,000 x g). Samples were then filtered using a Gelman GHP Acrodisc syringe filter (13 mm, 0.45 μm pore size) and the protein

concentration was measured using the microtitre plate method (Bio-Rad) (Chapter 3).

4.2.5.2. Modified TES buffer lysis method

A 10 mL Pyrex round bottom test tube was used to contain the sample. 2 mL of glass beads (Fortuna, 0.25-0.5 mm) that were wet with TES buffer (Appendix A 7.5) were placed in the tube. 2 mL TES buffer and approximately 1.5 mL killed washed (Section 4.2.1) *M. tuberculosis* cells were added to the tube. The test tube was vortexed (Ratek Instruments) for 1 minute and then placed on crushed ice for 1 minute until a total vortex time of 20 minutes had elapsed. The sample was divided into 1.5 mL microfuge tubes (Sarstedt) and centrifuged for 10 minutes using a Beckman Microfuge 11. Six centrifuge speeds were tested between 3000 rpm (1,400 x g) and 13,000 rpm (25,000 x g). Samples were then filtered using a Gelman GHP Acrodisc syringe filter (13 mm, 0.45 μm pore size) and the protein concentration was measured using the microtitre plate method (Bio-Rad) (Chapter 3).

4.2.6. Fraction production

Protein fractions are small portions of the entire protein spectrum in a sample. Use of protein fractions can help to narrow down immune responses to certain antigens, the ultimate goal being to isolate a single antigen (from the entire spectrum) to which the immune response is mounted against. This would be

advantageous because the antigen used for immunization could be different from that used for detection of infection. Also, a single antigen can be synthetically produced, or genetically engineered into a vector.

The proteins in the whole sonicate (WTB) produced using the method developed in this chapter were separated by electrophoresis and removed from the gel by electro-elution (Chapter 3). The eluted proteins were labelled and frozen at -80°C until a considerable number of elutions had been performed. Like fractions were thawed and combined before the proteins were concentrated by ultra-filtration (Chapter 3). The protein concentration of each fraction was determined by the microassay microtitre plate method (Bio-Rad) (Chapter 3).

4.2.7. Sample storage

Following protein concentration determination, WTB samples and fractions were aliquoted, labelled and stored at -80°C. Aliquots of fractions taken from WTB were labelled WA through to WE, according to their fraction, followed by the date in the format dd-mm-yy.

4.3. Results and Discussion

4.3.1. Results of sonication pilot study

In many cases, the protein yield in initial sonication experiments was below the linear range for the standard protein assay. At this early stage in the project, the Bio-Rad microplate reader had not been purchased, and hence the microassay

microtitre plate method for the assay of small amounts of protein had not been developed. As a result, when the protein concentration was <0.200 mg/mL, broad comparisons were performed by examining the raw absorbance data.

4.3.1.1. The effect of time

Initially, samples were sonicated for 5-minute intervals; however at the end of this time it was found that the sample was extremely hot. To avoid denaturation of proteins as a result of excessive heat, decreased time intervals were tested (Table 4.2).

The 3-minute time interval using water as a supernatant resulted in some detectable protein. The amount of protein was less than that obtained by using the 5-minute interval; however the difference in cell volume must be taken into consideration. A 2-minute time interval was also attempted with the use of glass beads, but compared to the equivalent 3-minute intervals the protein yield either decreased or did not change. A 3-minute time interval was used for subsequent sonication.

Table 4.2. Sonication conditions and protein yield for *M. tuberculosis* samples.

Cell Origin	Packed Cell Volume (mL)	Supernatant	Glass Beads	Time (minutes)	Average Absorbance (595 nm)	Protein Concentration (mg/mL)
MSM	1.0	PI	N	5	0.193	<0.200
LJ	0.5	dH_2O	Y	3	0.120	<0.200
LJ	0.5	dH_2O	Y	2	0.094	<0.200
LJ	0.5	PI	Y	3	0.048	<0.200
LJ	0.5	PI	Y	2	0.046	<0.200

All samples were sonicated at 35% amplitude. All experiments were performed with only one repetition of the stated time interval and all samples were filtered prior to protein assay. (MSM = Modified Sauton's Medium, LJ = Lowenstein Jensen Media, PI = protease inhibitor solution, dH_2O = sterile distilled water, N = no, Y = yes.)

4.3.1.2. The effect of repetitions

It was suspected that several repetitions of a 3-minute sonication time might result in a greater protein yield than a single 5-minute sonication. This assumption was based on the concept of protein denaturation at high temperatures. It was expected that if the sample was allowed to cool after each 3-minute burst of sonication, the sample might be kept cool enough to reduce denaturation of the proteins. Numerous experiments were performed and the results are shown in Table 4.3.

In all but two cases, increasing the number of repetitions resulted in an increase in the protein yield. The first exception resulted in identical absorbance measurements (0.063). The protein content of each sample involved in this experiment was determined on separate occasions using slightly different standard curves. Extrapolating the absorbances into the non-linear region of the curve resulted in the identification of a higher protein yield in the sample that had 3 sonication repetitions.

The second exception was the repetitions using 5-minute intervals. In this situation, the similar protein yields of all three experiments was most likely due to protein precipitation of all susceptible proteins during the first 5-minute interval, which resulted in a constant filtrate protein concentration over the times tested.

Of the times and intervals tested, 5 repetitions of 3 minutes (total sonication time of 15 minutes) produced the greatest protein yield.

4.3.1.3. The effect of amplitude

In most cases, the Pyrex centrifuge tubes were unable to withstand amplitudes greater than 35%. Thus, all further experiments were performed at an amplitude of 35%.

4.3.1.4. The effect of glass beads

Glass beads were added to samples to increase shear forces during sonication. Equivalent samples were sonicated without glass beads and the results are compared in Table 4.4. In most cases, the addition of glass beads assists sonication as detected by an increase in protein yield. In two of the experiments, addition of glass beads resulted in negligible change in the protein yield. In each of these experiments, however, the other conditions were less than optimal and the protein yield was very low. Therefore, under optimal conditions, glass beads improve the protein yield.

Table 4.3. The effect of repetitions of the same sonication time on the protein yield.

Cell Origin	Packed Cell Volume (mL)	Supernatant	Glass Beads	Time (min)	Repetitions	Total Sonication Time (min)	Filtered	Average Absorbance (595 nm)	Protein Concentration (mg/mL)
MSM	0.5	dH_2O	Y	3	3	9	N	0.437*	3.816
MSM	0.5	dH_2O	Y	3	5	15	N	0.509 *	4.583
MSM	0.5	dH_2O	Y	3	3	9	Y	0.171	<0.200
MSM	0.5	dH_2O	Y	3	5	15	Y	0.207	<0.200
MSM	0.5	PI	Y	3	3	9	N	0.339*	2.772
MSM	0.5	PI	Y	3	5	15	N	0.394*	3.356
LJ	0.5	dH_2O	N	3	1	3	Y	0.063	<0.200
LJ	0.5	dH_2O	N	3	3	9	Y	0.063	<0.200
LJ	0.5	dH_2O	Y	3	1	3	Y	0.120	<0.200
LJ	0.5	dH_2O	Y	3	2	6	Y	0.166	<0.200
LJ	0.5	dH_2O	Y	3	3	9	Y	0.189	<0.200
LJ	0.5	PI	N	3	1	3	Y	0.026	<0.200
LJ	0.5	PI	N	3	3	9	Y	0.061	<0.200
LJ	0.5	PI	Y	3	1	3	Y	0.048	<0.200
LJ	0.5	PI	Y	3	2	6	Y	0.053	<0.200
LJ	0.5	PI	Y	3	3	9	Y	0.059	<0.200
MSM	1.0	PI	N	5	1	5	Y	0.193	<0.200
MSM	1.0	PI	N	5	4	20	Y	0.191	<0.200
MSM	1.0	PI	N	5	8	40	Y	0.196	<0.200

The experiments were all performed in singlicate at 35% amplitude. Horizontal lines are used to divide comparable experiments. (* = The absorbance recorded refers to that measured on a 1/10 dilution of the sample; MSM = Modified Sauton's Medium, LJ = Lowenstein Jensen Media, PI = protease inhibitor solution, dH_2O = sterile distilled water, N = no, Y = yes.)

Table 4.4. The effect that addition of glass beads has on the protein yield from sonicated samples.

Cell Origin	Supernatant	Repetitions	Total Sonication Time (min)	Filtered	Protein Assay Results			
					With Glass Beads		Without Glass Beads	
					Average Absorbance (595 nm)	Protein Concentration (mg/mL)	Average Absorbance (595 nm)	Protein Concentration (mg/mL)
MSM	dH_2O	3	9	N	0.437*	3.816	0.389*	3.305**
MSM	PI	3	9	N	0.339*	2.772	0.343	0.366**
MSM	PI	3	9	Y	0.097	<0.200	0.099	<0.200
LJ	dH_2O	1	3	Y	0.120	<0.200	0.063	<0.200
LJ	dH_2O	3	9	Y	0.189	<0.200	0.063	<0.200
LJ	PI	1	3	Y	0.048	<0.200	0.026	<0.200
LJ	PI	3	9	Y	0.059	<0.200	0.061	<0.200

The experiments were performed in singlicate and all used a packed cell volume of 0.5 mL and all samples were sonicated at 35% amplitude with each repetition extending 3 minutes. (* = The absorbance recorded refers to that measured on a 1/10 dilution of the sample; ** = although not filtered, the sample was centrifuged prior to protein analysis due to the very large amount of visible precipitate in the sample; MSM = Modified Sauton's Medium, LJ = Lowenstein Jensen Media, PI = protease inhibitor solution, dH_2O = sterile distilled water, N = no, Y = yes.)

4.3.1.5. The effect of supernatant

Protease Inhibitor Solution (PI) had been used as a supernatant during sonication to prevent denaturation of the proteins during storage following sonication (S. Harris, personal communication). Experiments were performed to determine the effect of the PI solution on the protein yield and the results are shown in Table 4.5.

In all but one case, the addition of protease inhibitors prior to sonication resulted in a decreased protein yield. In the one exception, the difference in the protein yield between the two experiments was negligible. Therefore, sterile distilled water was considered the superior supernatant.

Table 4.5. A comparison of dH_2O and PI as supernatants for sonication and their effect on protein yield.

Cell Origin	Glass Beads	Time (min) x Repetitions	Total Sonication Time (min)	Filtered	Protein Assay Results			
					Supernatant: dH_2O		Supernatant: PI	
					Absorbance (595 nm)	Protein Concentration (mg/mL)	Absorbance (595 nm)	Protein Concentration (mg/mL)
MSM	Y	3 x 5	15	N	0.509*	4.583	0.394*	3.356
MSM	Y	3 x 3	9	N	0.437*	3.816	0.339*	2.772
MSM	N	3 x 3	9	N	0.389*	3.305**	0.343	0.366**
MSM	Y	3 x 3	9	Y	0.171	<0.200	0.097	<0.200
LJ	N	3 x 1	3	Y	0.063	<0.200	0.026	<0.200
LJ	N	3 x 3	9	Y	0.063	<0.200	0.061	<0.200
LJ	Y	2 x 1	2	Y	0.094	<0.200	0.046	<0.200
LJ	Y	3 x 1	3	Y	0.120	<0.200	0.048	<0.200
LJ	Y	3 x 2	6	Y	0.166	<0.200	0.053	<0.200
LJ	Y	3 x 3	9	Y	0.189	<0.200	0.059	<0.200

The experiments all used a packed cell volume of 0.5 mL and all samples were sonicated at 35% amplitude. (* = The absorbance recorded refers to that measured on a 1/10 dilution of the sample; ** = although not filtered, the sample was centrifuged prior to protein analysis due to the very large amount of visible precipitate in the sample; MSM = Modified Sauton's Medium, LJ = Lowenstein Jensen Media, PI = protease inhibitor solution, dH_2O = sterile distilled water, N = no, Y = yes.)

4.3.1.6. The effect of cell volume and origin

For each experiment, the washed packed *M. tuberculosis* cells were loosened in the small amount of remaining supernatant before a volume was measured and transferred to the Pyrex centrifuge tube for sonication. 0.5 mL of cells from LJ slopes was measured using a transfer pipette. When cells were measured from the Modified Sauton's Medium, measurements were far less exact due to the considerably greater clumping of cells, making it impossible to use a transfer pipette. This suggests that cells from MSM were more densely packed than the cells from LJ medium. Thus, 0.5 mL washed LJ cells actually contains less cells than 0.5 mL washed MSM cells. As a result, the protein yield for 0.5 mL of cells from MSM would be expected to be higher than the equivalent experiment using cells from LJ medium. Table 4.6 shows this to be true with only one exception. In general, however, the preliminary data show that increasing the number of cells results in an increased protein yield.

Table 4.6. The effect that cell volume has on the protein yield of sonicated samples.

Cell Origin	Packed Cell Volume (mL)	Supernatant	Glass Beads	Average Absorbance (595 nm)	Protein Concentration (mg/mL)
MSM	1.0	PI	N	0.371	0.305
LJ	0.5	dH_2O	Y	0.189	<0.200
MSM	0.5	dH_2O	Y	0.166	<0.200
MSM	0.5	PI	N	0.099	<0.200
MSM	0.5	PI	Y	0.097	<0.200
LJ	0.5	PI	N	0.061	<0.200
LJ	0.5	PI	Y	0.059	<0.200

All samples were sonicated 3 times for 3 minutes each (total time of 9 minutes) at an amplitude of 35%. All samples were filtered prior to protein quantification. (MSM = Modified Sauton's Medium, LJ = Lowenstein Jensen Media, PI = protease inhibitor solution, dH_2O = sterile distilled water, N = no, Y = yes.)

Table 4.7. Sonication experiments performed to confirm observations made during pilot study.

Cell Origin	Packed Cell Volume (mL)	Glass Beads (mL)	dH_2O Added (mL)	Time (min) x Repetitions	Total Sonication Time (min)	Absorbance (595 nm)	Protein Concentration (mg/mL)
LJ	1.0	1.0	1.5	3 x 3	9	0.148	<0.200
LJ	1.0	1.0	1.5	3 x 5	15	0.116	<0.200

(LJ = Lowenstein Jensen Media, PI = protease inhibitor solution, dH_2O = sterile distilled water.)

4.3.1.7. Further observations from sonication pilot study

Two further experiments were performed to confirm the observations made in the pilot study (Table 4.7). Although the protein yields were superior to many obtained during the pilot study, they were well below levels required for use as an oral vaccine in murine studies.

In order to increase the protein yield, an attempt was made at increasing the packed cell volume to 2.0 mL. Following sonication of this sample, there was so much solid material that it was impossible to sterile filter the suspension to obtain any hydrophilic soluble proteins. The insoluble material may have been either whole cells that were not broken up by the sonication (due to the increased cell volume), precipitated proteins, hydrophobic proteins, cell wall carbohydrates or, most likely, a combination of the above.

4.3.2. Sonication results – Revision 1

Sonication Experiment 4.2.3.1 (Section 4.2.3) resulted in a protein yield of 0.962 mg/mL protein, the highest protein concentration obtained so far for a sterile filtered sample. Following this success, an attempt was made at increasing the cell volume and the volumes used are described as Experiment 4.2.3.2 (Section 4.2.3). This experiment resulted in a protein yield of 0.544 mg/mL, which was much lower than expected, in light of the increased concentration and amplification. Thus, it was decided to continue with the volumes used in Experiment 4.2.3.1. These conditions were repeated three times to confirm the

method, and the results shown in Table 4.8 are consistent with the original higher protein yield.

Table 4.8. Sonication results using the method described in Section 4.2.3 – Experiment 4.2.3.1.

Cell Origin	Packed Cell Volume (mL)	Glass Beads (mL)	dH_2O Added (mL)	Time (min) x Repetitions	Total Sonication Time (min)	Protein Concentration (mg/mL)
LJ	2.0	2.0	2.5	3 x 7	21	2.353
LJ	2.0	2.0	2.5	3 x 7	21	1.466
LJ	2.7	2.0	2.5	3 x 7	21	0.862

Although this method for sonication of *M. tuberculosis* yielded the highest protein concentrations, it remained lower than the required 4 mg/mL concentration and the final volumes obtained were less than 2 mL. In addition, there was still some concern over the heat generated in the sample during sonication. After a 3-minute sonication, the sample was almost too hot to handle, raising suspicions over the quality of sample.

4.3.3. Sonication results – Revision 2 (Temperature probe method)

4.3.3.1. Quantity of protein produced

The protein concentrations achieved using the temperature probe method (Table 4.9) were comparable to optimal protein yields obtained prior to use of the temperature probe. Interestingly, the amount of ethanol added, which influenced the maximum temperature during sonication (Figure 4.1), did not affect the quantity of protein produced. It can be assumed, however, that keeping the protein solution at lower temperatures preserves the quality, if not the quantity, of the proteins.

Table 4.9. The protein concentrations of sonicated clarified samples after sonication using the temperature probe and the addition of ethanol to the ice bath.

Experiment Number	Ethanol added to x mL	Protein Concentration (mg/mL)
4.2.4.1	20	0.974
4.2.4.2	25	1.150
4.2.4.3	50	1.046
4.2.4.4	50	1.333
4.2.4.5	100	1.063

4.3.3.2. The effect of an ethanol bath on sonication

Figure 4.1 shows that as the amount of ethanol added to the ice bath increases, the temperature of the sample during sonication decreases. When ethanol was added to the 20 mL mark on the beaker, the sample reached the maximum temperature of 45ºC in just over 7 minutes. The zigzag pattern shown for this curve on the graph is a result of the sonicator cutting out until the sample temperature decreased to 42ºC. The peak temperatures for Experiments 4.2.4.3 and 4.2.4.4 (each containing ethanol to 50 mL in the ice bath) were 16.9°C and 17.5°C, respectively. When ethanol was added to 100 mL (Experiment 4.2.4.5), the temperature reached a maximum of 11.9°C.

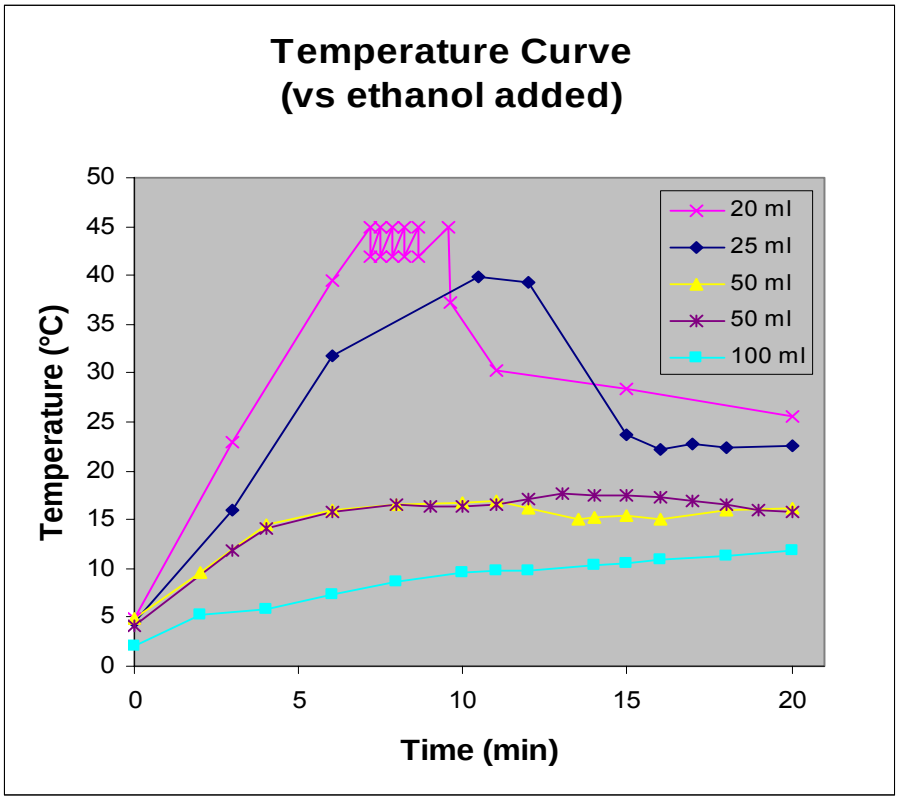

Figure 4.1. The temperature curve obtained during sonication of *M. tuberculosis* cells. The legend refers to the mark on the ice-bath beaker to which ethanol was added following the addition of crushed ice. The ice bath was topped up with crushed ice at the highest temperatures for both the 20 mL and the 25 mL temperature curves.

4.3.3.3. Combination and concentration of sonicates

Approximately 7.5 mL of sterile filtered whole sonicate (WTB) was obtained for each experiment using the temperature probe method of sonication. The combined and concentrated WTB from Experiments 4.2.4.1 and 4.2.4.2 yielded a protein concentration of 3.975 mg/mL. Experiments 4.2.4.3, 4.2.4.4 and 4.2.4.5 were also combined and concentrated by ultra-filtration, resulting in a final protein concentration of 12.769 mg/mL, well above the required 4 mg/mL protein level.

4.3.3.4. Quantity of insoluble proteins in sonicate

As a point of interest, the protein was measured in Experiments 4.2.4.1 and 4.2.4.2 of both the crude sonicate, and the centrifuged sterile-filtered sonicate. The results showed that the protein contained in the clarified sonicate solution, that is, the water-soluble proteins, comprised 10.64% and 11.20% of the total protein produced by sonication in Experiments 4.2.4.1 and 4.2.4.2 respectively. These results are not surprising considering the waxy nature of mycobacterial cell walls (Tortora et al. 1998b).

4.3.3.5. Visualisation of WTB proteins

The concentrated proteins produced from each experiment using the temperature probe method (Section 4.2.4) were examined using SDS-PAGE (Section 3.2.4) following dilution with sample buffer (Appendix A 4.9). The gel was stained using the Silver Stain method (Section 3.2.5.2). The sonication process involved in the extraction of WTB broke up existing proteins into smaller fragments. As a result, SDS-PAGE did not reveal distinct bands of proteins. Instead a continuous band containing proteins of all sizes was seen (Figure 4.2) as compared to excreted STCF proteins, which were not exposed to sonication.

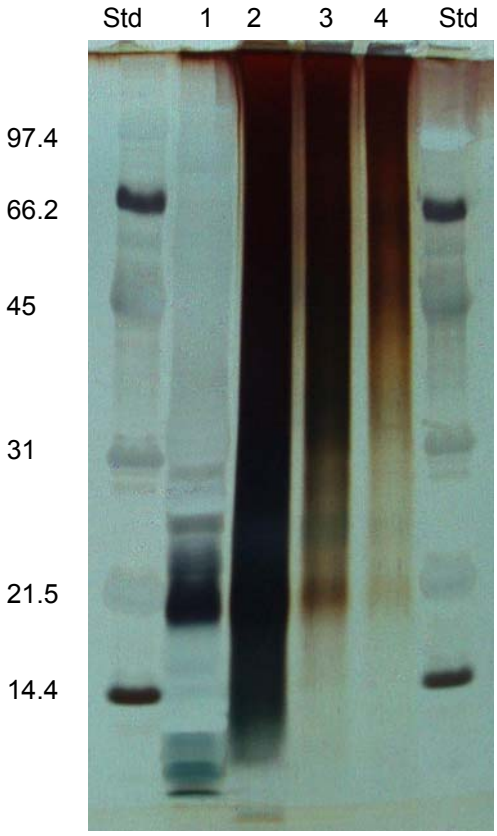

Figure 4.2. Silver Stain following SDS-PAGE of standards (Std) and *M. tuberculosis* proteins. Lane 1 contains STCF proteins (developed in Chapter 5). Lane 2 contains neat WTB. Lanes 3 and 4 contain 1/5 and 1/10 dilutions of WTB respectively. The molecular weights (kDa) of the standard proteins are indicated.

4.3.4. TES buffer lysis method results

The TES Buffer Lysis Method (Section 4.2.5.1) was attempted using various parameters on three separate occasions. On each of these occasions, no protein was detected in any of the samples. It was suspected that the packed cell volume of *M. tuberculosis* might be too low to produce a sufficient protein yield, and so the Modified TES Buffer Lysis Method (Section 4.2.5.2) was developed. This method also failed to produce any detectable protein.

Although the TES Buffer Method may have produced sufficient amounts of protein from other strains of bacteria (Fessler, et. al. 1999; Huang 2003), this method failed to produce any significant amounts of protein from *M. tuberculosis* cells. This suggests that the *M. tuberculosis* cells were not broken up by the shear forces of the glass beads during vortexing, a phenomenon which may be due to the hard waxy coating around the bacterium (Tortora et al. 1998b). As a result, the TES Buffer Method was discarded for the remainder of this project and it was decided to focus on further refining the sonication methods.

4.3.5. Fraction production results

Following electrophoresis of WTB, the molecular weight measurements taken from the prestained standard lane were used to create a standard curve (Figure 4.2). This curve was used to determine the migration through the gel of the following molecular weights: 20 kDa, 25 kDa, 35 kDa, 65 kDa. The wells corresponding to these critical points were determined and thus the thirty fractions aspirated from the Eluter were combined into five fractions (Table 4.10).

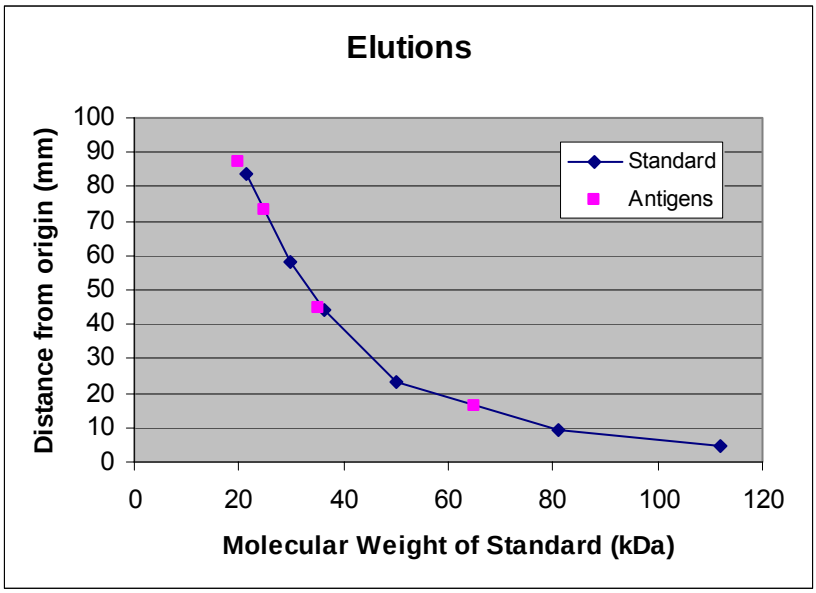

Figure 4.2. A typical standard curve used to determine the distance from the origin of the critical points for the fraction cut-offs.

Table 4.10. The final molecular weight range of each fraction.

Fraction	Molecular Weight Range (kDa)
A	3-20*
B	20-25
C	25-35
D	35-65
E	>65

(*The low molecular weight cut-off value is 3 kDa due to the pore sizes of the membranes used.)

Following combination and concentration of like fractions, no protein was detected in fractions B, C or D. Fraction A produced a protein yield of 9.274 µg/mL and the protein concentration in Fraction E was 29.141 µg/mL. Time limits prevented further experimentation in fraction production and thus only

Fractions A (WA) and E (WE) were used for animal experiments in vaccination with extracted tuberculosis antigens (Chapter 6).

4.4. Discussion

The sonication pilot study not only allowed familiarisation with the new Sonifier purchased, but also revealed some important observations. It was found that the protein yield could be maximised by increasing the cell volume and by adding glass beads to the sample prior to sonication. Optimal amplitude for the Branson Digital Sonifier 250 using a Microtip Tapered ⅛" horn was found to be 35%. It was also noted that samples should be syringe filtered prior to protein assay to remove insoluble/precipitated proteins and other materials.

One of the most important observations from the pilot study was the recognition of heat generated during the sonication process. This observation lead to the purchase of the temperature probe for the Sonifier and experiments were performed in an attempt to keep the sample at or below 45ºC. It was found that placing the sample into a 200 mL beaker of crushed ice and adding ethanol to the 50 mL graduation provided suitable conditions for keeping the sample below room temperature during a 20-minute sonication.

The method developed for production of whole sonicate focused on quality and quantity of the proteins recovered. The success of the quality of the proteins was largely placed upon the fact that the solution was kept cool and thus protein denaturation due to elevated temperatures was avoided.

It was subsequently discovered that the glutaraldehyde used to kill *M. tuberculosis* cells can cause alteration in protein structure (Bollag 1996). This alteration was initially noticed due to inconsistencies in staining SDS-PAGE gels. It was found that the molecular weight standards (Bio-Rad, Low Range) and STCF antigens (Chapter 5) were staining only by the Coomassie Blue method (Section 3.2.5.1.). It was also noted that only WTB antigens (Chapter 4) were appearing on gels stained using the Silver Stain method (Section 3.2.5.2.).

Some acidic proteins are able to escape detection by Coomassie Blue staining and basic proteins stain poorly by Silver Stains (Bollag 1996). Soaking the *M. tuberculosis* cells in glutaraldehyde overnight appeared to change some amino acids and acidify the proteins that were extracted by sonication. This alteration to the protein structure was evidenced by the staining patterns of the sonicate on SDS-PAGE gels (data not shown). Alteration of protein structure is a significant problem in vaccine development. It is important that proteins constituting a vaccine mirror the protein structure of the target antigen. This enables rapid detection upon antigen exposure. Therefore another method of killing the cells must be sought (outside the scope of this study) if an effective method of vaccination is to be based on these proteins.

4.5. Conclusion

The process outlined in this chapter provided sufficient quantities of whole sonicate containing hydrophilic intracellular and cell wall proteins, which were required for oral vaccination of mice (Chapter 6).

Chapter 5: Short-Term Culture Filtrate (STCF) Production

5.1. Introduction

Although somatic proteins are believed to play a role in immunization against *M. tuberculosis* (Feng & Britton 2000; Flynn 2004; Kaufmann & Hess 1999), it is the extracellular proteins that are responsible for specific long-term immunity (Andersen et. al. 1991b). Short-term culture filtrate (STCF), which contains the excreted (or extracellular) proteins, has been used by many researchers in searching for better vaccinations as well as diagnostic markers (Weldingh 1999; Moran 1999; Andersen 1997; Elhay 1997).

As with production of sonicate antigens (Chapter 4), denaturation of proteins due to extended periods of time at elevated temperatures was of paramount concern in the production of STCF antigens. Thus, similarly to sonicate antigens, the refrigeration time of STCF antigens was kept to not more than a few days and samples were stored at -80°C as soon as possible. Once again, antigen production methods were developed with the aim of producing concentrated solutions in order to decrease the number of repetitions necessary for obtaining sufficient antigen. This would enable reduced storage times prior to vaccination of laboratory animals and follow-up testing.

The STCF antigens were purified specifically for use in oral vaccination experiments with laboratory mice and antigen solutions were required to contain

high protein concentrations in order to reduce the volume of the antigen feed to a level that maintained the comfort of laboratory animals. If an antigen dose of 200 μg was to be used, as indicated from previous experiments (Hosken 1999), and a volume of not more than 100 μL was to be administered to each mouse, the STCF antigen solution would have to contain approximately 4 mg/mL protein. Thus, the quantitative aim of the experiments outlined in this chapter was the production of 1.1 mL (per group of 10 mice) of 4 mg/mL STCF antigen in phosphate buffered saline.

5.2. Methods

5.2.1. Obtaining crude STCF

An active *M. tuberculosis* culture was cultivated on the surface of Modified Sauton's Medium (MSM) (Chapter 3). A Biological Safety Cabinet Class II (Gelman Sciences) was used for safety purposes whenever work was performed using live bacteria. The cells were sub-cultured onto fresh MSM and incubated at 37°C for 4 days, thus providing the "short-term" component of STCF. Following culture, the cells were scooped off the surface of the media, and the MSM was filtered using a 60 ml syringe (Terumo) and a Sartorius Minisart filter (25 mm, 0.2 μm pore size) to remove any suspended bacterial cells. Approximately 90 mL of crude STCF was obtained from each culture vessel.

5.2.2. Concentration of STCF by ultrafiltration

Two ultrafiltration experiments were performed in order to optimise conditions for STCF protein purification. In Experiment 5.2.2.1, 90 mL of crude STCF was

concentrated using standard ultrafiltration as previously described (Chapter 3). This method took approximately 15 hours at room temperature. The protein in the concentrated solution was assayed using the Bio-Rad microtitre plate method (Chapter 3) and samples were aliquoted and frozen at -80°C.

Experiment 5.2.2.2 involved a two-step method of ultra-filtration that made it possible to concentrate approximately 90 mL of STCF in approximately 7 hours. In this revised method, a regenerated cellulose ultrafiltration membrane (Amicon) with a 10,000 Da molecular weight cut-off was used to filter the sample until its volume was approximately 2 mL. The sample was then removed and placed into a 5 mL sterile screw-cap tube (Sarstedt) and refrigerated. The waste, which contained all proteins having a molecular weight less than 10 kDa, was then re-filtered using a regenerated cellulose ultrafiltration membrane (Amicon) with a 3,000 Da molecular weight cut-off. When the sample volume was reduced to approximately 2 ml, the initial filtrate (containing proteins greater than 10 kDa) was added to the stirred cell apparatus. When the volume was once again reduced, 15 mL sterile phosphate buffered saline (Appendix A 1.1) was added to the stirred cell and filtered until the volume was reduced to approximately 2 ml. An additional 15 mL sterile phosphate buffered saline was added and the volume reduced 3 times, effectively providing a “wash” and removing remaining MSM from the protein solution. The protein in the concentrated solution was assayed using the Bio-Rad microtitre plate method (Chapter 3) and samples were aliquoted and frozen at -80°C.

5.2.3. Concentration of STCF by freeze-drying

Freeze-drying was expected to provide the opportunity to significantly reduce the volume of crude STCF without exposing the proteins to temperatures above freezing point, thus retaining their integrity. Two methods of freeze-drying were attempted, namely, Experiment 5.2.3.1 and Experiment 5.2.3.2. For each experiment, crude STCF from several culture vessels was pooled and 90 mL of the pooled STCF was concentrated by ultrafiltration (Experiment 5.2.2.2, Section 5.2.2) in parallel to the freeze-drying experiment.

In Experiment 5.2.3.1, approximately 150 mL of crude STCF was placed into a 250 mL Magenta vessel (Sigma) and frozen to -80°C. Two vessels each containing approximately 150 mL of frozen crude STCF were placed in the freeze-dryer (Dynavac Engineering) for 24 hours. A small amount of sterile distilled water had to be used to remove all the concentrated STCF from the Magenta vessels. The freeze-dried STCF was then further concentrated using ultrafiltration (Chapter 3), and the protein concentration was determined using the Bio-Rad microtitre plate method (Chapter 3). Samples were aliquoted and frozen at -80°C.

In Experiment 5.2.3.2, the freeze-drying method was repeated using three magenta vessels each containing 60 mL of frozen (-80°C) crude STCF in Magenta vessels. Freeze-drying was continued for 7 days until the STCF formed a sticky sludge in the bottom of each container. Two STCF samples were reconstituted using 10 mL sterile distilled water and poured into the third sample. The two empty vessels were rinsed with an additional 5 mL sterile

distilled water, which was added to the STCF mixture. Therefore, the total volume was approximately 30 mL. This sample was then concentrated using ultrafiltration (Experiment 5.2.2.2, Section 5.2.2), and the protein concentration was determined using the Bio-Rad microtitre plate method (Chapter 3). Samples were aliquoted and frozen at -80°C.

5.2.4. Concentration of STCF by ammonium sulfate precipitation and dialysis purification

This traditional method of protein concentration was based on methods supplied by H.G. Wiker and S. Harris (personal communication).

The quantity of crude STCF was measured and poured into a beaker. The appropriate amount of ammonium sulfate crystals (Sigma) based on solubility (The Merck Index) was weighed out to allow for 70 g ammonium sulfate per 100 mL crude STCF. The ammonium sulfate was gradually added to the sample during stirring, allowing time to dissolve before addition of more ammonium sulfate. The solution was then poured into 250 mL plastic ultracentrifuge tubes (Nalgene) and ultracentrifuged at 20,000 x g (14,000 rpm) using the Beckman J2-MC centrifuge for 30 minutes at 4°C.

Following ultracentrifugation, the samples were carefully removed and the supernatants were pipetted off using a transfer pipette (Biolab Scientific). A small amount of sterile distilled water was added to the precipitate and it was transferred to washed cellulose ester dialysis tubing (Sigma, 10 kDa cut-off) using a transfer pipette. The ends of the dialysis tubing were clamped and the

tubing was suspended in PBS (Appendix A 1.1) in a 2 L beaker. A magnetic stir bar was added and the sample was dialysed for 48 hours at 4°C on a Chiltern Scientific stirrer, with 4 changes of PBS during that time.

Following dialysis, the sample was removed from the dialysis tubing and concentrated by ultrafiltration (Experiment 5.2.2.2, Section 5.2.2). The protein concentration was determined using the Bio-Rad microtitre plate method (Chapter 3) and samples were aliquoted and frozen at -80°C. Once again a parallel sample was concentrated solely by ultrafiltration (Experiment 5.2.2.2, Section 5.2.2).

The STCF proteins produced using ammonium sulfate precipitation and dialysis purification were examined using SDS-PAGE (Section 3.2.4). The gel was stained using the Coomassie Blue method (Section 3.2.5.1).

5.3. Results/Discussion

5.3.1. Concentration of STCF by ultrafiltration

Protein concentrations obtained using the method described as Experiment 5.2.2.1 in ultrafiltration ranged from approximately 0.6 to 1 mg/mL. Experiment 2 increased the protein yield to approximately 1-2 mg/mL. In addition, Experiment 5.2.2.2 significantly reduced the amount of time at room temperature, thus decreasing the risk of protein degradation. In spite of the improvements noted in Experiment 5.2.2.2, the protein yield remained at less than half the required concentration.

5.3.2. Concentration of STCF by freeze-drying

In Experiment 5.2.3.1, the freeze-drying process resulted in copious amount of bubbles being generated in the STCF, causing it to overflow from the container. It was estimated that 1-2 mL concentrated STCF was lost. The protein produced using this method was equivalent to 87.0% of the protein produced in a parallel sample that was concentrated solely by ultra-filtration. It was suspected that a significant amount of antigen had been lost due to the "boiling-over" of the sample in the freeze-drier.

The bubbling that occurred in the sample during freeze-drying may have been due to a number of factors including: insufficient freezing of the sample prior to freeze-drying; thawing of the sample during freeze-drying as a result of insufficient surface area in relation to the depth of the sample volume; or an air leak in the freeze-dryer (R. W. Hosken, personal communication). As the samples had been frozen at -80ºC for a number of weeks prior to the freeze-drying process, it was suspected that the surface area of the samples needed to be increased in relation to their depth and reduced volumes were used in Experiment 5.2.3.2.

Some overflow was still noted with two of the samples in Experiment 5.2.3.2. The amount of protein produced using this method was 88.7% of the protein produced in an equivalent sample that was concentrated solely by ultra-filtration. Once again, it was suspected that a significant amount of sample had been lost due to the "boiling-over" of the sample in the freeze-drier. To further increase the surface area of sample, the volume of STCF able to be processed

in the freeze-dryer for any one run had to be reduced. This reduction in sample volume made freeze-drying impractical as a method of protein concentration, particularly for larger scale production of STCF proteins if the scope of vaccine testing were to be broadened in the future. Thus, freeze-drying as a method of concentrating crude STCF was abandoned.

5.3.3. Concentration of STCF by ammonium sulfate precipitation and dialysis purification

Using the ammonium sulfate precipitation and dialysis purification method for the concentration of STCF, 2.3 times more protein was produced than using concentration solely by ultrafiltration. 2.27 mL of concentrated STCF was produced containing 7.090 mg/mL of protein and the experiment was repeated an additional time with similar results. SDS-PAGE revealed distinct bands of proteins (Figure 5.1).

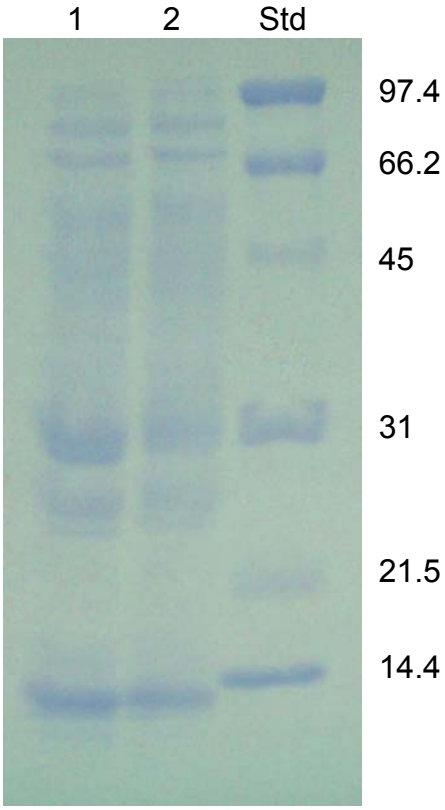

Figure 5.1. Coomassie Blue stain following SDS-PAGE of standards (Std) and STCF proteins. Lanes 1 and 2 contain STCF proteins from separate extractions using the Ammonium Sulfate Precipitation and Dialysis Purification method. The molecular weights (kDa) of the standard proteins are indicated.

Despite the success of this method of concentration, it must be noted that the dialysis tubing used had a 10 kDa pore size, which would have allowed considerable loss of low molecular weight antigens. Some of these antigens, particularly ESAT-6, are significant immunostimulants in the field of tuberculosis vaccine research (Weldingh & Andersen 1999; Orme 1999a; Lalvani et al. 1998; Elhay & Andersen 1997). Further use of this method of protein extraction should investigate using dialysis tubing with a lower molecular weight cut-off.

A very important characteristic of this method of protein concentration and purification is that it could be scaled-up for the production of larger volumes of concentrated STCF without greatly increasing the time taken for concentration. This method is also advantageous because it minimizes the amount of time that the sample is exposed to temperatures above 4°C.

5.4. Discussion

Three methods were attempted in order to concentrate the crude STCF. Ultrafiltration produced an STCF protein solution with an insufficient concentration for the immunization of mice. Freeze-drying was investigated in order to concentrate a large volume of crude STCF into a volume small enough to follow up with ultra-filtration. This experiment also failed to produce the required results and the traditional technique of ammonium sulfate precipitation of proteins was examined. The precipitated proteins were purified using dialysis techniques and concentrated using ultrafiltration. Sufficient quantities of protein were extracted from culture filtrate using this method.

In addition, this technique required the STCF to be at room temperature for approximately 2 hours for protein precipitation, followed by refrigeration for 2 days during dialysis, and another short period at room temperature for ultracentrifugation prior to freezing. These considerations given to temperature would have decreased the likelihood of protein degradation.

The STCF antigen purification techniques established in this dissertation (Section 5.2.4) were used for vaccination of mice and immune response

analysis by a collaborating student and the subsequent results were published (Adams et al. 2005).

5.5. Conclusion

The quantitative aim of producing 1.1 mL of 4 mg/mL STCF antigen in phosphate buffered saline was achieved.

Chapter 6: Vaccine Trials of Whole Sonicate from *Mycobacterium tuberculosis* in the Murine Model

6.1. Introduction

Oral tolerance is a well known issue that impedes upon the simplicity of oral vaccination (Strobel 1995, Strober 1998). Adjuvants are given along with the antigens in an oral vaccine to boost the immune response to the vaccine, thus making it possible to reduce the quantities of antigen given and hence reduce the likelihood of tolerance to the antigen. Cholera toxin has been used widely in murine vaccination models (representative examples include Elson and Ealdin, 1984; Douce et al. 1997; Marinaro et al. 1997; Arulanandam and Metzger 1999) as mice are resistant to its toxic effects. To enable transfer of any murine model used to a human system, an alternative adjuvant is required.

Studies in the equine industry have shown that *Propionibacterium acnes* (commercially available inactivated strain, Eqstim®) can generate a non-specific immune response when given intravenously to horses (Flaminio et. al. 1998). Most notably, *P. acnes* has been shown to induce a cellular response that involves the release of high levels of interferon-γ (IFN-γ) (Flaminio et. al. 1998, Megid et. al. 1999). *Propionibacterium jensenii* 702 (PJ702), a strain of dairy propionibacteria first reported by Adams in 2002, was found to adhere to the intestinal epithelium (Huang & Adams 2003). A study run in parallel to those described in this chapter used oral administration of STCF antigens and PJ702 as an adjuvant (Lean 2002; Adams et. al. 2005). It concluded that oral

vaccination with the culture filtrate proteins of *M. tuberculosis* can induce a Th1-type immune response and, in addition, that PJ702 boosted that immune response.

It has previously been shown that extracellular proteins of *M. tuberculosis* are required for long-term immunity following parenteral vaccination (Orme 1988a; Andersen et. al. 1991b). It is also believed that somatic antigens may play a role in vaccination against tuberculosis (Feng & Britton 2000; Flynn 2004; Kaufmann & Hess 1999). Previous studies in this laboratory (Hosken 1999) have shown that cellular proteins given orally can induce an immune response, but the type of response was not specified. Thus, it was necessary to perform studies examining the immune responses to somatic antigens in an oral vaccination model, particularly in light of the recent progress in the field of oral adjuvants.

Experiments involving the use of live animals were necessary in examining potential vaccines and adjuvants. A fully intact immune system was required to examine mucosal and systemic responses to antigens administered. Thus, mice were used for immunological experiments in this dissertation.

Mycobacterium tuberculosis proteins isolated in chapters 4 and 5 of this dissertation were designed for use in oral vaccination. This chapter focuses on trialling sonicate antigens (WTB) (Chapter 4) and examines adjuvants that can be given to boost the immune responses to the vaccines. It was expected that an immune response would be detected following oral vaccination with WTB in

conjunction with cholera toxin. This response would then be compared to that obtained using the novel adjuvant, PJ702.

Some experiments described in this chapter were unable to be completed due to unrelated and unforeseen misfortunes. In spite of these situations, interesting conclusions were able to be drawn regarding the nature of the adjuvants studied and the prospects for oral vaccination against tuberculosis.

6.2. Methods

6.2.1. General methods

6.2.1.1. Mice used in experiments

Ethics approval was obtained for all experiments involving laboratory animals and ethics numbers are stated under the methods of each experiment. Female C57Bl/6 mice were used in all experiments and 4-5 mice were housed in each standard cage and received the standard food and water provided by animal house staff. On each occasion, mice were given one week to rest following arrival at the David Maddison Building Animal Facility, University of Newcastle. The mice were weighed weekly by placing a single mouse into a tared cage on a Snowrex Electronic Balance. Weights were recorded and monitored over the experimental period as an indicator of well-being.

6.2.1.2. PJ702 plate counts

Plate counts were performed using a standard method. In brief, the stock suspension was diluted 1 in 10 with Wilkins-Chalgren Anaerobe Broth

(WCAB) (Appendix A 9.1). 10-fold serial dilutions in WCAB were performed and 100 µL of each dilution was inoculated onto SLA plates (Appendix A 9.2) in duplicate and spread using a sterile glass spreader. Plates were incubated anaerobically at 30ºC for 5 days, at which time colony counts were performed.

6.2.1.3. Production of live PJ702

An aliquot of *Propionibacterium jensenii* (PJ702), which had previously been stored at -80ºC, was subcultured onto SLA plates (Appendix A 9.2). A stock bacterial suspension was made by suspending PJ702 from fresh cultures in sterile PBS (Appendix A 1.1) in a 10 mL sterile centrifuge tube (Sarstedt). The suspension was centrifuged (Hettich EBA 8S) at 4000 rpm (1,000 x g) for 5 minutes and the supernatant was removed using a sterile transfer pipette (Samco). The cells were resuspended in approximately 6 mL sterile PBS. This wash procedure was repeated 3 times and plate counts were performed as previously described (Section 6.2.1.2)

6.2.1.4. Production of heat-killed PJ702

A 10^{10} cfu/mL suspension of live PJ702 was made as described in Section 6.2.1.3. 5 mL aliquots of this suspension were each heat treated for 3 hours, 4 hours or 5 hours in triplicate. 100 µL of each aliquot was plated in duplicate onto SLA plates and incubated anaerobically at 30ºC for 5 days at which time colony counts were recorded. The samples that had been heat treated for 3 hours were used for vaccinations that required killed PJ702 as the adjuvant. Plate counts were also performed, as previously

described (Section 6.2.1.2), on the unheated suspension and the volumes required for vaccination were adjusted accordingly.

6.2.1.5. Whole sonicated *Mycobacterium tuberculosis* (WTB) vaccine

Whole sonicated *Mycobacterium tuberculosis* (WTB) was prepared as described previously (Chapter 4). A stock aliquot of WTB containing 21.02 mg/mL of protein was used. To obtain 200 µg of WTB protein in the 90 µL per mouse volume that was not taken up by the adjuvant, a WTB protein concentration of 2.22 mg/mL was required. Thus, for each week that vaccines were required, 422 µL of stock WTB protein was made up to 4 mL with sterile PBS and divided appropriately amongst the 4 vaccines of which this protein was a component.

6.2.1.6. Antigen administration

Mice were anaesthetised by inhalation (Advanced Anaesthesia Vaporiser) using isofluorane (Laser) at a rate of 4 L/min. Vaccines were given as antigen feeds by use of a 21 gauge lavage needle inserted into the stomach of each mouse under anaesthetic. On each occasion, antigen feeds were 100 µL in size.

6.2.1.7. Saphenous blood collection for IgG analysis

Blood samples were collected from the saphenous vein of each mouse. Mice were restrained using a blackened 50 mL Falcon centrifuge tube (Becton Dickinson) with four holes drilled in the end for ventilation. The rear leg was cleaned using a gauze swab (Smith+Nephew) soaked in

Hibiclens (ICI Pharmaceuticals) and shaved using a No. 11 scalpel blade (Swann-Morton). Vaseline was applied to the area and the vein was punctured using a 23 gauge 1½ inch needle (Terumo). Blood was collected into capillary tubes (Hirschmann Laborgaräte) and transferred into micro test tubes (Eppendorf). Samples were centrifuged at the highest speed setting (unknown rpm) for 10 minutes at 4ºC (Eppendorf Centrifuge 5415C). The serum was then separated into a clean micro test tube (Eppendorf) and stored at -80ºC.

6.2.1.8. Blood collection by cardiac puncture

Mice were anaesthetised using isofluorane (Laser), and the procedure was performed and completed within 20 seconds. The chest and abdominal area were sterilised with 70% ethanol (Appendix A 1.3) and the chest cavity was opened using sterile scissors and forceps. A 1 mL syringe (Terumo) and 23 gauge 1½ inch needle (Terumo) were used to collect the total blood volume directly from the heart. Mice were not revived and the heart was removed to ensure the animal was deceased.

Blood was transferred into micro test tubes (Eppendorf) and centrifuged at the highest speed setting (unknown rpm) for 10 minutes at 4ºC (Eppendorf Centrifuge 5415C). The serum was then separated into a clean micro test tube (Eppendorf) and stored at -80ºC until IgG analysis could be performed.

6.2.1.9. Faeces collection for IgA analysis

While mice were in separate cages for weighing, a faecal pellet was collected from each one and placed into a micro test tube (Eppendorf) containing 0.5 mL Faecal Protease Inhibitor (Appendix A 8.2). The test tubes were vortexed for 15 minutes at room temperature to suspend the pellet, then centrifuged (Eppendorf Centrifuge 5415C) at full speed for 15 minutes at 4ºC. 400 µL of supernatant was transferred to another micro test tube containing 100 µL glycerol and 10 µL Faecal PMSF Solution (Appendix A 8.3). The tubes were vortexed briefly to mix and stored at -80ºC.

6.2.1.10. Faeces collection for PJ702 culture

One pellet of faeces was collected from each mouse while the mouse was in isolation for weighing. Each pellet was placed into preweighed sterile 5 mL screw-cap vials (Sarstedt) containing 3 mL of 0.05 M sodium phosphate buffer (Appendix A 8.1) and re-weighed. Initially, samples were stored at 4ºC for 12-16 hours before testing, but following laboratory closure, the samples were frozen at -80ºC until access was available (approximately 4 months).

Samples were vortexed at maximum setting for 10 seconds to suspend the faecal pellet. 50 µL of the faecal suspension was inoculated onto SLA plates (Appendix A 9.2) and streaked out to produce isolated colonies. Plates were set up in duplicate and incubated anaerobically at 30ºC for 5 days. Cultures were examined for the presence of dairy propionibacteria,

which appear as creamy white colonies approximately 2 mm in size. Gram stains were performed on any suspect colonies.

6.2.1.11. Saliva collection for IgA analysis

The abdominal region of each mouse was cleansed with 70% ethanol (Appendix A 1.3). 100 µL of dilute Isoptocarpine (Appendix A 8.4) was injected into the peritoneum of each mouse using a 1 mL syringe (Terumo) and a 30 gauge ½ inch needle (Terumo). When the mice began to salivate approximately 12 minutes later, they were anaesthetised (Advanced Anaesthesia) by inhalation of isofluorane (Laser) at a rate of 4 L/min. The mice were held with the head slightly lower than the rest of the body and the saliva was collected into a capillary tube (Hirschmann Laborgaräte). The saliva was transferred to micro test tubes (Eppendorf) for storage at -80ºC until IgA analysis could be performed.

6.2.1.12. Collection of spleen and Peyer's Patches lymphocytes

Mice were sacrificed by carbon dioxide inhalation for 30 seconds. The abdomen was washed with 70% ethanol (Appendix A 1.3) and the abdominal cavity of the mouse was opened using sterile scissors and forceps. The area was washed again using 70% ethanol and spleens from each treatment group were removed, trimming off excess. The spleens were gently diced using the dissection scissors and pooled in 70 mL sterile containers (Sarstedt) containing 40 mL of cold sterile PBS (Appendix A 1.1). Small intestines were removed from the mice and spread out on a piece of sterile gauze (Kimberly-Clark). Peyer's patches were removed

from the intestines using the dissection scissors and pooled in 70 mL sterile containers containing 40 mL of cold sterile PBS. All specimens were stored on crushed ice for up to 1 hour before processing.

From this point onwards, spleens and Peyer's patches were processed in a sterile Clemco Laminar flow hood. The flow hood had been subjected to UV light overnight and all items entering the sterile environment were sprayed with 70% ethanol (Appendix A 1.3).

Single cell suspensions from the spleens and Peyer's patches were obtained using the sieve method. The pooled tissues were macerated through a sterile sieve using the flat end of a plunger from a 60 mL syringe (Terumo). Cells were collected into a sterile Petri dish (Becton Dickinson) and the sieve was flushed with the remaining PBS from the pooled tissues. The cell suspension in PBS was transferred to a 50 mL Falcon centrifuge tube (Becton Dickinson) and stored on crushed ice for several minutes.

6.2.1.13. Preparation of spleen lymphocytes

Large pieces of debris were removed using a sterile transfer pipette (Samco) before the specimen was centrifuged (Heraeus Megafuge 1.0R) at 1500 rpm (450 x g) for 5 minutes at 4ºC. The supernatant was removed using a sterile transfer pipette and the deposit was resuspended in 5 mL of Red Blood Cell Lysis Buffer (Appendix A 10.2) for 5 minutes. 20 mL of RPMI complete medium (Appendix A 10.1) was added and the suspension was centrifuged using the same conditions as previously described. The

spleen cell pellet was washed 3 times with 20 mL of RPMI complete medium and finally resuspended in 10 mL RPMI complete medium. Cell counts were performed by diluting the suspension 1 in 5 with trypan blue (Appendix A 10.3) and loading into a disposable plastic counting chamber (Vetri-plast). Cells were stored on ice until required.

6.2.1.14. Preparation of Peyer's Patches lymphocytes

Peyer's patches lymphocytes were prepared in the same way as spleen lymphocytes (section 4.5.2.9), but omitting the step involving red cell lysis buffer. Cell counts were performed by diluting the suspension 1 in 2 with trypan blue (Appendix A 10.3) and loading into a disposable plastic counting chamber (Vetri-plast). Cells were stored on ice until required.

6.2.1.15. Lymphocyte proliferation cultures

Nunclon Surface tissue culture plates were used and samples and controls were set up in quadruplicate. Spleen and Peyer's patches cells were used at a concentration of 2 x 10^6 cells/mL and 100 µL was added to each appropriate well.

100 µL of each antigen was added to the appropriate wells containing 100 µL of cells, thus halving the concentrations of the antigens added. Positive controls were performed by using 100 µL Lectin Concanavalin A (Con A) (Boehringer Mannheim) as the stimulating antigen. Negative controls were performed by the addition of 100 µL RPMI Complete Medium to the wells

instead of antigen. Culture plates were incubated in 5% carbon dioxide at 37ºC for 2 days for control plates and 3 days for test plates.

6.2.1.16. Harvesting and reading lymphocyte cultures

Following incubation, 10 µL of working thymidine (Appendix A 10.4) was added to each well (0.5 µCi/well). The plates were returned to the incubator for 6 hours then harvested (Packard Filtermate 196, Perkin Elmer) onto Packard glass fibre filters (Perkin Elmer). The filters were dried at room temperature overnight then a scintillator sheet (Wallac) was melted onto each filter using a hot plate (CHILTERN Scientific). The filter was cooled and placed in a sample bag (Wallac), which was sealed (Wallac 1295-012 Heat Sealer) and trimmed for reading on a Liquid Scintillation and Luminescence Counter (TRILUX 1450 MicroBeta, Perkin Elmer).

6.2.1.17. Lymphocyte cultures for cytokine analysis

Multiwell tissue culture plates (Becton Dickinson) were used and samples and controls were set up in duplicate. Spleen and Peyer's patches lymphocytes were used at a concentration of 2×10^6 cells/mL and 0.5 mL was added to each well. 0.5 mL of antigen was added to each appropriate well and 0.5 mL RPMI complete medium was added instead of antigen to the negative control wells. All cultures were performed in duplicate.

Tissue culture plates were incubated in 5% carbon dioxide at 37º C for 6 days. The supernatants were then removed using a sterile transfer pipette

(Samco) and frozen at -80ºC in micro test tubes (Eppendorf) until IFN-γ and IL-4 assays could be performed.

6.2.1.18. Cytokine ELISA assays

OptEIA™ Mouse IFN-γ and OptEIA™ Mouse IL-4 ELISA assay kits (BD Bioscience Pharmingen) were used to assay the thawed lymphocyte culture supernatants (section 4.5.2.17) according to the manufacturer's instructions outlined in the kit inserts. Nunc Immunosorbent multi-well plates were used for the ELISA assays.

In brief, 100 µL of diluted Capture Antibody (Appendix A 12.3 and 12.6) was added to each well and the ELISA plates were incubated overnight at 4ºC. Wells were aspirated and washed 5 times in PBST (Appendix A 11.1). The plates were blocked using 200 µL of assay diluent (Appendix A 12.2) and incubated for 1 hour at room temperature. Wells were aspirated and washed 5 times in PBST. 100 µL of standard (Pharmingen) or sample was added to the appropriate wells and the plates were incubated for 2 hours at room temperature. Wells were again aspirated and washed 5 times in PBST. 100 µL of Working Detector (Appendix A 12.5 and 12.8) was added to each well and plates were incubated for 1 hour at room temperature. For the final wash, wells were aspirated and washed 10 times in PBST with 30-60 second soaks. Colour development was performed by incubating 100 µL of TMB Substrate Solution (Appendix A 11.2) in each well for 30 minutes at room temperature in the dark. 50 µL of Stop Solution (Appendix A 11.3) was added to each well and absorbances

were measured at 450 nm within 30 minutes with a wavelength correction of 570 nm (BioRad Microplate reader, Model 450).

6.2.1.19. Immunoglobulin ELISA assays

IgA analysis was performed on faeces and saliva samples collected from the mice (Sections 6.2.1.9 and 6.2.1.11) and IgG analysis was performed on the blood samples (Sections 6.2.1.7 and 6.2.1.8). The IgG and IgA ELISA methods used followed the same protocol, but used slightly different reagents as outlined in the Appendix A.

In brief, the wells of Nunc Immunosorbent multi-well plate were coated with 100 µL of 25 µg/mL WTB in bicarbonate buffer (Appendix A 13.1). Plates were covered with foil and incubated overnight at 4ºC. Wells were aspirated and washed 3 times in PBST (Appendix A 11.1). Each well was blocked using 100 µL of 5% foetal calf serum (Appendix A 13.2) and incubated for 1 hour at 37ºC. Wells were again aspirated and washed 3 times in PBST. 100 µL of diluted sample (in PBS) or control sera was added to the appropriate wells and plates were incubated for 1 hour at 37ºC. Wells were aspirated and washed 5 times in PBST. 100 µL of appropriate biotinylated conjugate (Appendix A 13.3 and 13.4) was added to each well and incubated for 1 hour at 37ºC. Wells were again aspirated and washed 5 times in PBST. 100 µL of Streptavidin Horseradish Peroxidase (SA-HRP) (Appendix A 13.5) was added to each well and incubated 1 hour at 37ºC. PBS (Appendix A 1.1), instead of PBST, was used for the final wash, and wash repetitions were increased to 7 times.

Colour development was performed by incubating 100 µL of TMB Substrate Solution (Appendix A 11.2) in each well for 10 minutes at room temperature. 50 µL of stop solution (Appendix A 11.3) was added to each well and absorbances were measured at 450 nm (BioRad Microplate reader, Model 450).

6.2.2. Experiment using whole sonicate (WTB) and cholera toxin (CT)

This experiment was designed to be the first in a large series. Each experiment was to follow the same protocol, with just the vaccine (antigen feed) varying from group to group. A negative control group was to be run at some time over the course of experiments as time allowed. The groups were to be staggered so as to allow production of the vaccines and lymphocyte stimulants; and also to reduce the time delay in processing samples, which can reduce the viability of the lymphocyte cultures.

The purpose of the series of experiments was to assess a number of different vaccines, both cellular and extracellular, to determine the optimum concentration for administration. It was also taken into consideration that various concentrations of antigens, including fractions of the original vaccine, would need to be assessed for optimal lymphocyte stimulation in cell cultures.

The first in the series of experiments is outlined in this thesis.

6.2.2.1. Animals and ethics

Ethics approval was obtained for this experiment (number 712 0901). The animals used for this experiment were C57Bl/6 female mice. Ten 10-week-old mice were obtained for the experiment and all mice were classified as a treatment group, with 5 mice per cage.

6.2.2.2. Antigen administration

Each mouse was given an antigen dose of 200 µg of sonicated whole *Mycobacterium tuberculosis* (WTB) (Chapter 4) and 10 µg of cholera toxin (Sigma) in 0.1 mL sterile PBS (Appendix A 1.1). An antigen feed was given to each mouse on days 7, 14 and 21.

6.2.2.3. Blood and faeces collection

Blood samples for IgG analysis were collected from each mouse on day 7 and day 24 (Section 6.2.1.7) and faecal pellets were collected from each mouse (Section 6.2.1.9) on day 7 and day 24 of the experiment.

6.2.2.4. Lymphocyte cultures

Mice were sacrificed on day 24 and spleens and Peyer's patches were removed (Sections 6.2.1.12-6.2.1.14). Lymphocyte cultures were prepared (Section 6.2.1.15) using whole sonicated *Mycobacterium tuberculosis* (WTB) and fractions A (WA) and E (WE) of WTB (Chapter 4) as the stimulating antigens. WTB was added to the wells at concentrations of 16, 8, 4, 2, and 1 µg/mL; WA at concentrations of 4, 2 and 1 µg/mL; and, WE at concentrations of 16, 8, 4, 2, and 1 µg/mL. Con

A was used at a concentration of 10 µg/mL and 100 µL was added to each appropriate well giving a final concentration in each well of 5 µg/mL. Cultures were pulsed and harvested as previously described (Section 6.2.1.16).

6.2.3. Concanavalin A optimisation experiment

The Concanavalin A optimisation experiment was performed as a follow-up to the initial experiment outlined in Section 6.2.2. Its purpose was to confirm the stability of the Con A and working thymidine used and to determine the best concentration of Con A for following experiments.

6.2.3.1. Animals and ethics

This experiment was performed under the ethics approval granted for the previous experiment (Section 6.2.2, ethics number 712 0901). Two 13-week-old mice were obtained and immediately upon arrival at the David Maddison Building Animal Facility the mice were sacrificed by carbon dioxide inhalation for 30 seconds.

6.2.3.2. Lymphocyte cultures

Splenic lymphocytes were collected and prepared for culture as previously described (Sections 6.2.1.12-6.2.1.13). For the lymphocyte cultures (Section 6.2.1.15), Concanavalin A (Con A) was used as the stimulating antigen and the Con A used in the previous experiment (made 16/4/1998, referred to as “Con A 16/4/98”) was tested alongside freshly made Con A (made 20/3/2001, referred to as “Con A 20/3/01”). 100 µL was added to

the relevant wells to achieve final concentrations of 20, 10, 5, 2.5, 1.25 and 0.625 µg/mL. 8 replicates were set up for each concentration for both Con A batches. Con A 16/4/98 was not run at 0.625 µg/mL to allow room on the culture plate for a blank to be run. 100 µL of RPMI complete medium was added to four wells as a blank.

Lymphocytes were harvested and scintillation counts were performed as previously described (Section 6.2.1.16) with one exception. The working thymidine used in the previous experiment (Section 6.2.2) (referred to as "old" in this dissertation, as the date it had been made was not recorded on the label), was compared with freshly made working thymidine (referred to as "new" in this dissertation), by pulsing 4 of the 8 replicates with each different batch of working thymidine.

6.2.4. PJ 702 adjuvant experiment

Previous studies (Adams et. al. 2005) have shown that live PJ702 can increase the immune response towards culture filtrate (excreted) antigens of *Mycobacterium tuberculosis*. In this experiment, the antigens investigated were obtained from sonicated *Mycobacterium tuberculosis* and included cellular and intracellular proteins. This experiment was performed to investigate the adjuvant capacity of *Propionibacterium jensenii* 702 (PJ702), both living and killed bacteria, and to compare the results obtained with cholera toxin, a well known murine adjuvant.

6.2.4.1. Animals and ethics

40 mice were obtained for this experiment under ethics approval number 856 0904. All mice were between 4-6 weeks old upon arrival at the David Maddison Building Animal Facility. The mice were divided into 5 treatment groups, each consisting of 8 mice, and caged in standard cages with 4 mice of the same treatment group in each cage. Following division into the different cages, mice were given one week of rest prior to any additional handling.

6.2.4.2. Antigen administration

In addition to the vaccine administration, the intestinal tracts of the mice in Group 1 were primed with live PJ702 prior to vaccination. It was decided to administer the organisms in the same way as an oral vaccine so that the number of organisms ingested could be most accurately controlled. These mice received 10^8 cfu in 0.1 mL sterile PBS (Appendix A 1.1) on days 7, 14 and 21. The first suspension for administration (used on day 7), was made by diluting 66 µL of stock suspension (Section 6.2.1.3) in 5 mL sterile PBS. This volume was adjusted to 6 µL of stock in 5 mL of sterile PBS for administration of days 14 and 21.

For vaccine administration, 10^8 cfu of PJ702 was required to be reduced to a volume of 10 µL, that is, 10^{10} cfu/mL. This allowed the remaining 90 µL volume available for administration to each mouse to be taken up by the relevant antigen that the PJ702 was combined with. Thus, 66 µL of stock

PJ702 suspension (Section 6.2.1.3) was made up to 5 mL with sterile distilled water.

Plate counts were performed, using the standard method, on an aliquot of each live PJ702 vaccine administered. Thus the concentration of bacteria administered to each mouse could be retrospectively determined.

The vaccines given to each treatment group are outlined in Table 6.1. 1.0 mL of each vaccine was freshly made in sterile PBS (Appendix A 1.1) immediately prior to administration. Each mouse was given 0.1 mL of the relevant vaccine. Vaccines were administered on days 28, 35 and 42.

Table 6.1. Treatment groups and the relevant quantities of antigen and adjuvant per mouse per vaccine administration.

Group Number	Antigen	Adjuvant
1	200 µg WTB	10^8 cfu PJ702, live (section 4.5.2.3.2)
2	200 µg WTB	10^8 cfu PJ702, killed (section 4.5.2.3.3)
3	200 µg WTB	10 µg cholera toxin (Sigma)
4	200 µg WTB	NIL
5	NIL	NIL

6.2.4.3. Blood, faeces and saliva collection and analysis

Blood samples for IgG analysis (Section 6.2.1.19) were collected from each mouse on day 28 from the saphenous vein and by cardiac puncture on day 45 using the method described previously (Sections 6.2.1.7 and

6.2.1.8). A faeces sample was collected from each mouse for PJ702 culture (Section 6.2.1.10) on days 14, 21, 28, 35, and 42. Additional faecal pellets for IgA analysis (Section 6.2.1.19) were collected as previously described (Section 6.2.1.9) on days 28 and 45. Saliva samples were collected on day 45 (Section 6.2.1.11) and were also stored until IgA analysis could be performed (Section 6.2.1.19).

6.2.4.4. Lymphocyte proliferation cultures

Following cardiac puncture, spleen and Peyer's patches lymphocytes were collected and prepared as previously described (Sections 6.2.1.12-6.2.1.14). Lymphocyte cultures were performed (section 6.2.1.15) using Whole Sonicated *Mycobacterium tuberculosis* (WTB) at a concentration of 5 µg/ mL as the stimulating antigen. The same stock lot that was used for vaccination was used for cell cultures. Positive controls were performed by using Lectin Concanavalin A (Con A) (Boehringer Mannheim) at a concentration of 5 µg/mL. Following incubation, lymphocyte cultures were harvested and prepared for reading as described previously (Section 6.2.1.16).

6.2.4.5. Cytokine analysis

Lymphocyte cultures using increased volumes were performed as previously described (Section 6.2.1.17). The same stock lot of Whole Sonicated *Mycobacterium tuberculosis* (WTB) that was used previously in this experiment was used as the stimulating antigen. WTB was used at a concentration of 5 µg/mL ad 0.5 mL was added to each appropriate well.

Following incubation, supernatants were stored for approximately 10 months until cytokine analysis could be performed (Section 6.2.1.18).

6.2.4.6. Storage of samples

As previously stated, the laboratory was closed on day 22 of this experiment. As the animal trial had started, all samples that were collected after the closure were stored at -80ºC until processing could be undertaken.

6.3. Results

6.3.1. Experiment using whole sonicate (WTB) and cholera toxin (CT)

Positive control wells in both spleen lymphocyte cultures and Peyer's Patches cultures were not significantly different from the negative control wells ($P>0.5$), thus, failing to validate the assay. In addition, test results (Table 6.2) were negligible compared to previous experiments (Hosken 1999). The large standard deviations emphasise the negligible levels of the mean scintillation counts.

Failure of mice to express an immune response to all antigens, including Concanavalin A (Con A) in the previous experiment raised two possibilities. The first possibility was that an air-conditioning failure and hot weather had resulted in the immune systems of the mice becoming compromised. During the third week of this experiment, in the mid-January heat, the air-conditioning in the animal house failed. Several other students experienced similar unusual results at this time (K. Beagley, personal communication), and it was suspected

that the extreme weather may have contributed to the poor immune responses of the mice. Increasing air temperatures to 27ºC has previously been shown to reduce immunogenicity (Stark 1970).

Table 6.2. Scintillation Counts per minute of lymphocyte cultures for various concentrations of different antigens.

Antigen	Concentration (µg/mL per well)	Average Count (min^{-1}) ± SD	
		Spleen	Peyer's Patches
Nil (RPMI)	N/A	193 ± 79	141 ± 41
Con A	5	183 ± 108	126 ± 23
WTB	0.5	156 ± 96	167 ± 102
	1	118 ± 25	137 ± 54
	2	122 ± 58	227 ± 67
	4	84 ± 31	135 ± 53
	8	133 ± 15	181 ± 68
WA	0.5	111 ± 33	135 ± 75
	1	158 ± 85	114 ± 17
	2	159 ± 87	158 ± 79
WE	0.5	94 ± 29	183 ± 31
	1	154 ± 51	222 ± 103
	2	118 ± 61	230 ± 74
	4	140 ± 9	369 ± 70
	8	131 ± 32	251 ± 93

(RPMI = RPMI complete medium; N/A = not applicable; Con A = Concanavalin A; WTB = whole sonicated *Mycobacterium tuberculosis*; WA = fraction A of WTB; WE = fraction E of WTB. Note: fractions are described in section 3.5.)

The second possibility was that the Con A or working thymidine was defective and failed to stimulate the lymphocytes, in addition to the test vaccines not stimulating an immune response. Previous results (Hosken 1999) indicated that at least some of the antigens should have induced a detectable immune response.

Further experiments in this series were postponed until it could be determined whether the reagents were the cause of the problem, or whether environmental conditions had played a major part in the invalid results. The latter, and more likely, cause for the invalid results obtained from the lymphocyte cultures would have influenced all immune responses so serum and faeces samples were not assayed.

6.3.2. Concanavalin A optimisation experiment

The results for the four different combinations of batches of Con A and working thymidine (shown in Figure 6.1), indicate that for optimal results Con A should be used at a final concentration of 2.5 µg/mL. Thus, the results for this concentration for each combination of reagents were compared to all other concentrations of the same reagents. It was found that for all reagent combinations, the results obtained for 2.5 µg/mL of Con A were significantly higher ($P<0.5$) than all other concentrations tested except for 0.625 µg/mL. The results for the 2.5 µg/mL and 0.625 µg/mL concentrations of Con A were found to be statistically similar ($P>0.1$).

Following the discovery that 2.5 µg/mL is the optimal Con A concentration, results for this concentration were compared among the different reagent concentrations. For Con A concentrations at this level, no significant difference ($P>0.1$) existed between the different reagent combinations. Thus, it appeared that there was no difference in lymphocyte proliferations when Con A 16/4/98 or Con A 23/3/01 was used. It was interesting to note that although Con A 16/4/98 was made almost 3 years earlier, it produced proliferative responses

equivalent to fresh Con A. In addition, it made no difference to the level of proliferation when old working thymidine or new working thymidine is used.

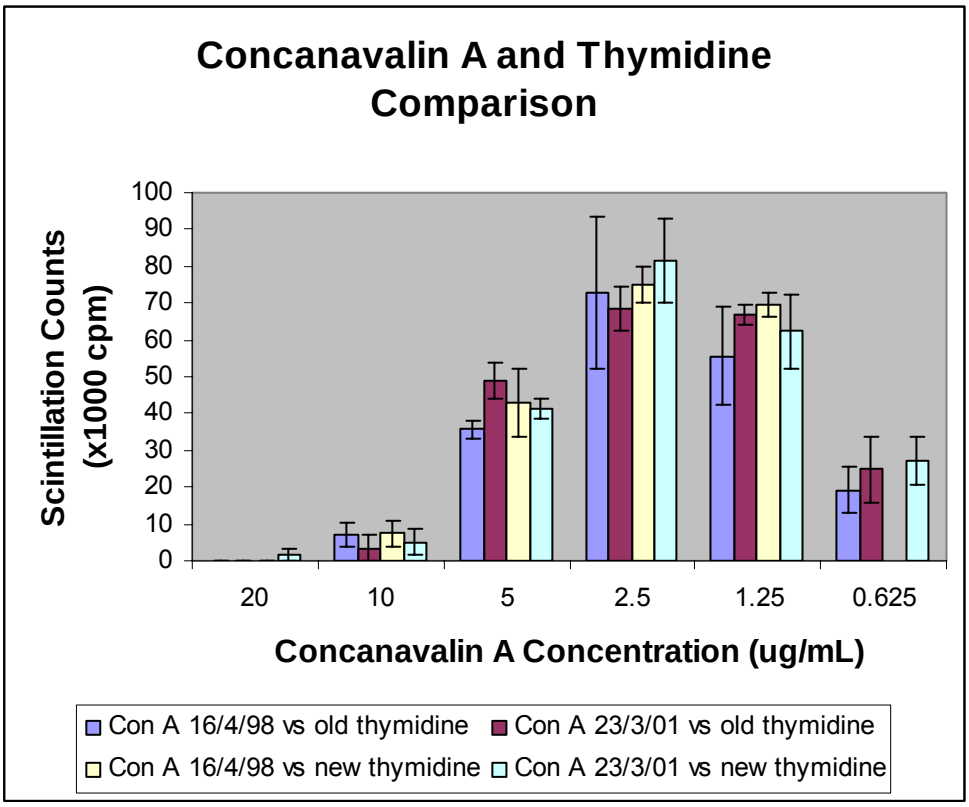

Figure 6.1. Comparison of different thymidine and Concanavalin A (Con A) batches. (cpm = counts per minute).

It is also important to note that for the Con A 16/4/98 and old working thymidine reagent, the average scintillation counts per minute were 35632, with a standard deviation of 2343. These levels are significantly higher ($P<0.001$) than those achieved on the previous experiment (Table 6.2).

The reagents that were used in the previous experiment (Section 6.2.2) were found to be working to the same level as fresh reagents in spite of the age difference of the reagents. In addition, the same reagents that were used in the previous experiment produced significantly higher results when repeated under

ideal conditions. Thus, it can only be concluded that the failure of mice to produce immune responses in the previous experiment was due to environmental conditions, possibly temperature variations, and not reagent failure.

6.3.3. PJ 702 adjuvant experiment

6.3.3.1. Mouse weights

The weights of the mice were found to slightly increase over the course of the experiment (Figure 6.2). The weights in each group did not significantly differ from Group 4, the group receiving WTB only (P>0.1).

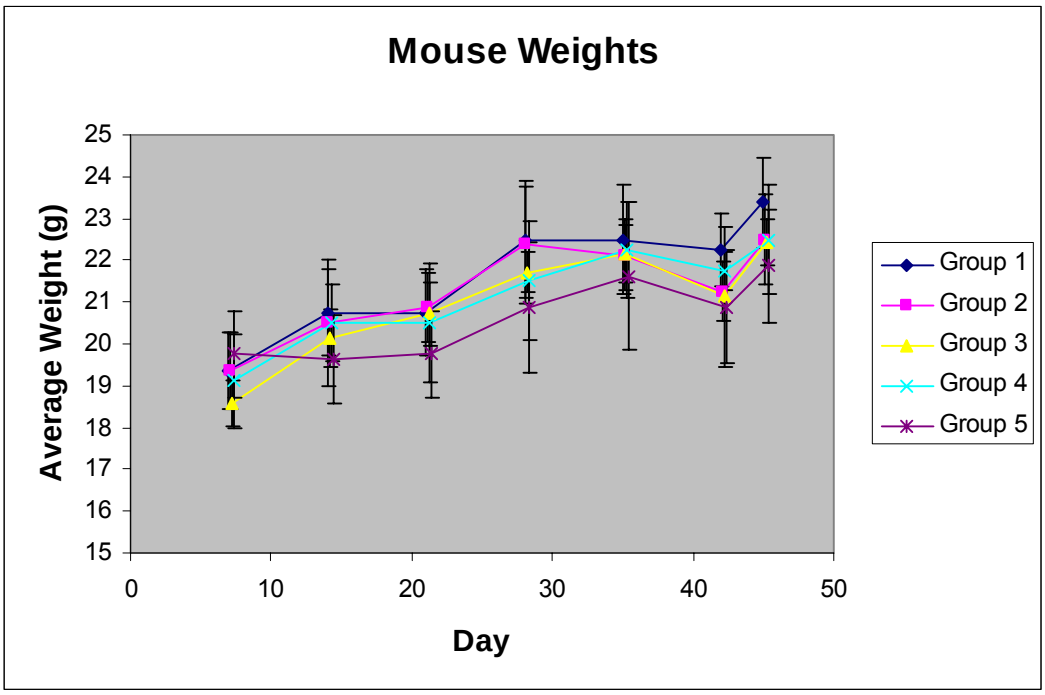

Figure 6.2. Average weights of the mice over the course of the experiment. Error bars show one standard deviation. Note: graphical data excludes one mouse from group 3 that had congenital hydrocephaly and was considerably underweight.

One mouse from group 3 was excluded from the graphical and statistical analysis. Its weight was 14 g on day 7 and failed to increase, weighing in at 13 g on day 14. It was also observed on day 14 that the mouse had decreased activity levels and a hunched posture. The mouse was immediately excluded from the experiment and placed in a separate cage under a heat lamp. Half an hour later, the activity of the mouse had slightly increased, however its posture remained hunched. The mouse was kept in a separate cage and monitored closely over the next 3 days until it died on day 17 and was sent to The University of Newcastle Veterinary Officer for autopsy. The autopsy revealed that the mouse suffered from congenital hydrocephaly and had no cerebellum. Thus, its death was unrelated to the experimental conditions it experienced.

With the exception of the one mouse that died of unrelated causes, all mice in this experiment experienced good health and an expected marginal increase in weight. This experiment confirms previous studies (Adams et. al. 2005, Hosken 1999) in showing that neither oral administration of live *Propionibacterium jensenii 702* (PJ702) nor whole sonicated *Mycobacterium tuberculosis* (WTB) appears to affect the well-being of the mice. In addition, it shows that heat-killed PJ702 did not detrimentally affect the health of the mice.

6.3.3.2. Vaccines – PJ702 cell counts

6.3.3.2.1. Heat-killed PJ702 vaccine

The unheated suspension of PJ702 had a colony count of 7.4 x 10^8 cfu/mL. When this suspension was heat treated at 90ºC for 5 hours, one replicate from one sample grew 1 colony; and a single replicate from another aliquot grew 6 colonies. A 4-hour heat treatment resulted in one replicate from one aliquot growing 5 colonies. No colonies were isolated from any replicates of any tubes heated for 3 hours. Thus, the samples that received a 3-hour heat treatment were used for the vaccinations.

It can only be speculated why sterility was not achieved at the higher exposure times. It may be simply that the cells were insufficiently resuspended in some tubes, and hence the packing of the cells provided some thermal protection. The results did demonstrate that PJ702 does have high thermal tolerance, a useful trait for industrial bacteria and one not commonly observed for this genus of bacteria.

6.3.3.2.2. Live PJ702 vaccine

Plate counts revealed that the live suspension of PJ702 given to the mice in group 1 on day 7 was 1.135 x 10^{10} cfu/mL. Therefore, each mouse in that group was given approximately 1.135 x 10^9 cfu of PJ702. Dilutions made from the heavy stock suspension were adjusted accordingly for the second week's administration and a 10-fold weaker dilution of the stock bacterial suspension was given to the mice. This dilution was found to contain 1.32 x 10^9 cfu/mL of PJ702. Therefore, each mouse in that group

was given 1.32 x 10^8 cfu of PJ702. These calculations confirmed the adjusted dilution to be correct thus the amended quantities were used on subsequent weeks.

The third week, however, access to the laboratory was denied and retrospective analysis to confirm the amount of PJ702 given to the mice each week could not be performed in real time. As dilutions of the stock suspension were made fresh weekly, it was assumed that this stock suspension did not deteriorate over the course of the experiment. Weight was added to this assumption by numerous studies using the stock suspension (data not shown) leading up to the experiment showing no change in the concentration of the organism in that suspension.

The vaccine samples from the remaining weeks of this experiment, which had been frozen for 4 months prior to processing, were found to contain PJ702 levels from <1000 cfu/mL in the oldest samples to 7.5 x 10^5 cfu/mL in the more recent samples. This 1000-fold decrease in organisms over 4 months stored at -80ºC suggests considerable cell death as a result of freezing and storage of the more dilute bacterial suspensions. This result is expected at it has previously been demonstrated that freezing of PJ702 without a cryoprotectant does lead to considerable cell death (Kotula and Adams 2004). If this organism were to be used as a live adjuvant at some time in the future, consideration would need to be given to use of a cryoprotectant to prevent deterioration of the organism.

6.3.3.3. Faeces cultures

The faeces cultures performed on all mice in all groups from days 7 and 14 showed that the mice had no PJ702 in their intestinal tract. These cultures were performed in real-time. The frozen faeces samples from subsequent weeks that were stored for 4 months prior to analysis resulted in one colony of Propionibacterium being isolated from a singe mouse in group 1. All other specimens contained no Propionibacterium.

It was expected that considerable amounts of PJ702 would be isolated from faeces samples collected from the mice in Group 1 and that no PJ702 would be isolated from faeces samples of any other group. Previously it had been demonstrated that PJ702 did survive the gastrointestinal tract of mice, and have been recovered in mice faeces previously when administered in similar conditions (Adams et al. 2005). In this experiment it was anticipated that quantities of PJ702 would be isolated from group 1, albeit not as concentrated as the vaccine given, from approximately day 28 onwards.

There are two possibilities for the lack of PJ702 recovered from the faeces. Firstly, the prolonged storage time of the faeces prior to culture may have considerably reduced bacteria counts below detectable levels, as evidenced in section 6.3.3.2.2. Importantly, the one colony of PJ702 that was isolated came from a Group 1 faeces sample from the latest collection date. These results suggest that if the faeces were able to be cultured

more promptly, greater numbers of Propionibacteria may have been isolated from the samples obtained from mice in group 1.

Additionally, the poor recovery rate of PJ702 from the faeces may be related to the dosage rate of the organism. It is a reasonable assumption that most of the PJ702 given in the vaccine was excreted as live bacteria in faeces, however the amount of PJ702 given to the mouse at each dose, diluted throughout the gastrointestinal tract, would not leave many bacteria in each faeces pellet, which has an average weight of 67.9 mg. In previous work with mice and other species, animals were given PJ702 daily (Adams et al. 2005; Adams et al. 2002), and this may be important to establish colonisation of PJ702 in their gastrointestinal system.

Previous studies have demonstrated dosage and colonisation are important for probiotics to exert clinical efficacy (J. Collins et al. 1998; Gopal et al. 2001). The adjuvant application of PJ702 in this experiment may have been limited by the colonisation rate. Application of PJ702 as an adjuvant will require further substantial studies on its action as an immune stimulant before the true potential can be identified.

6.3.3.4. Lymphocyte cultures

As analysis of lymphocytes required the use of radiation, this part of the study was required to be undertaken in an approved laboratory, which was kindly provided by the Department of Immunology, Faculty of Health, University of Newcastle. Unfortunately though, after the first two plates

had been harvested, that department's harvester malfunctioned and further analysis could not be performed. In addition, it was learned at that time, that the beta-counter was experiencing technical difficulties. Thus, no reliable results were obtainable from the lymphocyte cultures due to unforseen equipment failure part-way through the experiment.

Due to the absence of lymphocyte proliferation, an assumption had to be made for the purposes of analysing additional data. Based on previous experiments in the same laboratory using the same antigen (Hosken 1999), and the cytokine results obtained (Section 6.3.3.5), it was expected that if lymphocyte proliferation data had been available, it would reveal an increased immune response among spleen lymphocyte cultures but not in Peyer's patches lymphocyte cultures (due to no significant difference in cytokine levels for the Peyer's patches culture supernatants).

6.3.3.5. Lymphocyte culture supernatant cytokine results

The cytokine analysis on the lymphocyte culture supernatants was performed for the purpose of determining the type of T-cell response to the antigens. Increased levels of IFN-γ are indicative of a Th1 response (Kaufmann et. al. 1998), which is the desired response for protection against tuberculosis infection (Elhay & Anderson 1997). Alternatively, Th2 responses are associated with increased levels of IL-4 (Mosmann & Coffman 1989).

6.3.3.5.1. Interferon-γ results

None of the Peyer's patches lymphocytes that were stimulated with WTB were found to induce IFN-γ levels that were significantly higher than those that were not stimulated (P>0.1) (data not shown). From the spleen results (Figure 6.3), it was found that only group 2 had significantly higher levels (P<0.05) in the stimulated lymphocyte cultures when compared with the equivalent unstimulated cultures.

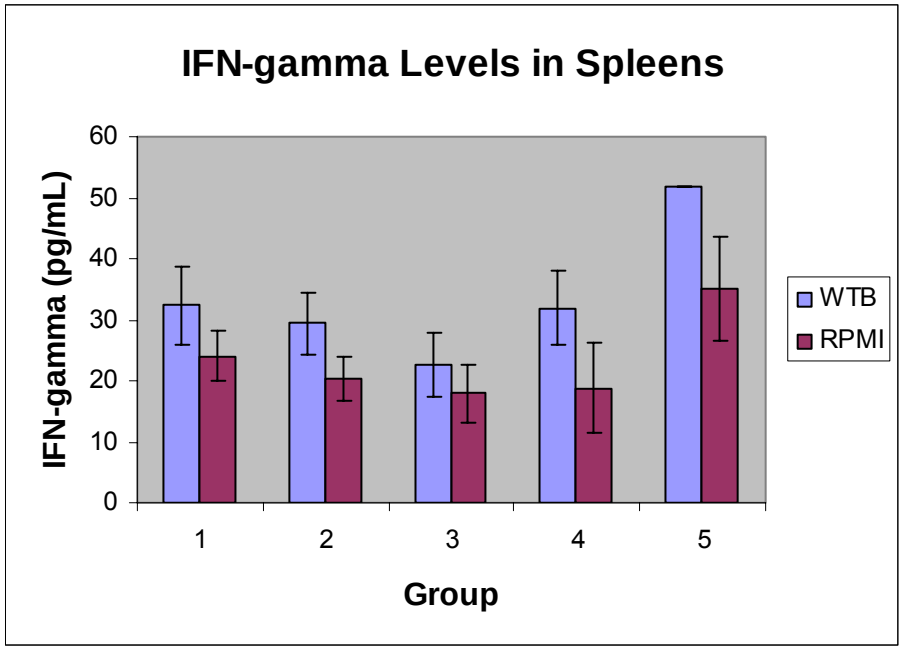

Figure 6.3. Interferon-γ (IFN-γ) levels in murine spleen lymphocyte culture supernatants from cultures stimulated with whole sonicated *Mycobacterium tuberculosis* antigens (WTB), or RPMI complete culture medium (unstimulated). Error bars indicate the standard deviation (Note: standard deviation for group 5 WTB was 0, thus no error bar appears for that group).

Group 2, which used killed PJ702 as the adjuvant, was the only group to experience a significant increase in IFN-γ levels. This Th1 response, however, was not observed in Group 1, which used live PJ702 as the

adjuvant. This is discrepant with previous studies (Adams et al. 2005) in which Th1 responses were observed in vaccinated animals where live PJ702 was used as an adjuvant.

Inadequate quantities of PJ702 bacteria administered may be responsible for this discrepancy. When live bacteria are given to animals, they must compete with indigenous flora to survive and/or colonise the gastrointestinal tract. PJ702 is known to be able to do both (Huang 2003; Huang & Adams 2003). Each time a dose of live bacteria is given to an animal, only a small percentage will be able to compete effectively, and therefore the more doses, the faster the colonisation rate. Once colonised, the bacteria will grow, multiply and eventually die, effectively establishing a new culture environment. Over a period of time a continuous culture can be established, the stability of which may be influenced by many things, including continual reinoculation of the strain. PJ702 is a slow growing organism, taking at least three to five days to produce visible colonies on laboratory media (personal observation).

With this in mind, it is speculated that when Group 1 were given live PJ702 a small percentage of bacteria colonised the mouse, but a combination of dosage rate, experimental duration and relatively slow growth rate of the bacteria, resulted in insufficient time to establish a microenvironmental niche of PJ702 in their gastrointestinal tract (Section 6.3.3.3). With only a small bacterial population, the amount of intracellular bacterial material released would have been low. Importantly, however, the killed bacterial

suspensions given to group 2 would contain intracellular as well as extracellular components of PJ702. Therefore, groups 1 and 2 were given equal quantities of extracellular PJ702 components, but only group 2 received intracellular components of the bacteria. No other group received any PJ702 compounds. Although many studies identify the principle non-specific immunostimulating property of bacteria to be related to cell wall constituents (Harboe et al. 1996a; Orme 1988a), this study may suggest a role for intracellular components.

6.3.3.5.2. IL-4 results

None of the WTB-stimulated Peyer's patches lymphocyte cultures were found to have significantly higher levels ($P>0.1$) than their equivalent unstimulated cultures (data not shown). Among the spleen cell lymphocyte culture results, groups 1 ($P<0.01$), 2 ($P<0.01$) and 4 ($P<0.05$) were found to have significantly higher IL-4 levels in the stimulated cultures than the unstimulated cultures (Figure 6.4).

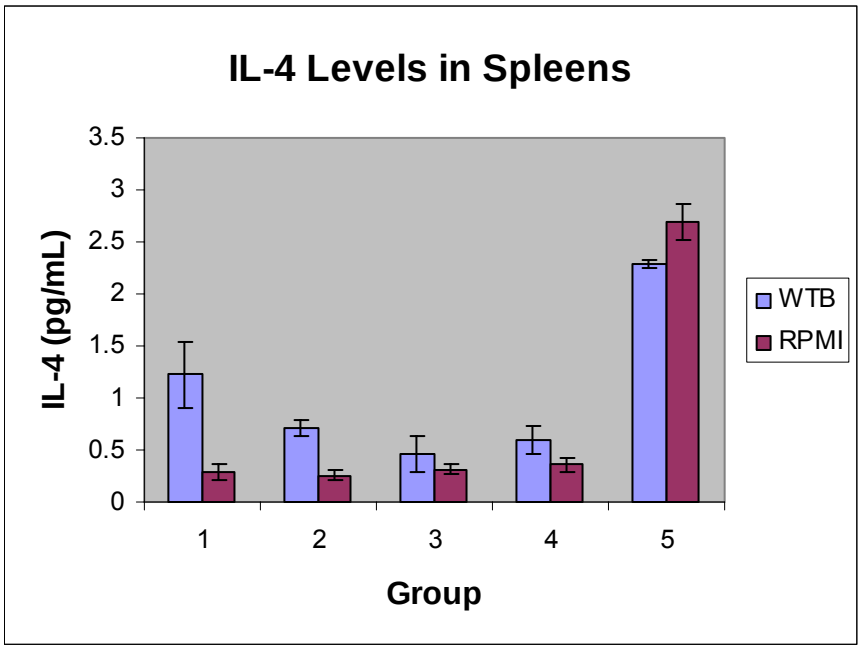

Figure 6.4. Interleukin-4 (IL-4) levels in murine spleen lymphocyte culture supernatants from cultures stimulated with whole sonicated *Mycobacterium tuberculosis* antigens (WTB), or RPMI complete culture medium (unstimulated). Error bars indicate the standard deviation.

Group 4, which was given WTB without adjuvant, experienced a significant increase in IL-4 levels in the culture supernatant. Tuberculosis proteins are known for their adjuvant properties and they are the major component of Freund's Complete Adjuvant (FCA) (Sigma). FCA usually sways the response towards a Th1 type, although Th2 cytokines may also be noted (Eisenberg et al. 2003). This experiment shows that some immune response of these orally vaccinated mice is attributable to the somatic tuberculosis proteins administered. In accordance with previous findings (Andersen and Doherty 2005; Harboe et al. 1996a, Skinner 1997), the somatic antigens are responsible for delayed-type hypersensitivity associated with Th2 stimulation.

This Th2 stimulation caused by the WTB vaccine, as evidenced by increased IL-4 levels, was also observed in groups 1 and 2, which were administered live PJ702 and killed PJ702, respectively, along with the WTB. Group 4 experienced a 1.7-fold increase in IL-4 levels, which was boosted by the presence of PJ702 to a 2.8-fold increase for Group 2 and a 4.3-fold increase for Group 1. These results indicate that low levels of the extracellular components of PJ702, which were given to groups 1 and 2, are sufficient to boost Th2 responses. The results are also in accordance with the IFN-γ results, which indicated that immune responses to intracellular components, such as those given to group 2 only, favour Th1 type responses.

It is interesting to note that use of cholera toxin, a well known adjuvant, did not result in any significant changes to cytokine levels in group 3 mice. Without lymphocyte proliferation results, it is impossible to make assumptions regarding the significance of this observation. It is possible, however, that as both the WTB and cholera toxin are known to act as adjuvants, there may have been an interference in their modes of action by using both together, although this has not been observed from lymphocyte proliferation data gained from previous experiments (Hosken 1999).

It must also be noted, when considering the cytokine results, that the test results obtained from the standard curve required extrapolation of the curve below the level achieved by the standards. Curvature of the line created by the standards may mask the true values of the test samples,

resulting in slightly lower levels and greater differences between results. Further experiments in this area may require use of standards of lower cytokine levels to create a more accurate standard curve.

6.3.3.6. Faeces IgA levels

The initial and final faeces IgA levels were tested on separate occasions, and as no standards or controls were available the results from the separate test runs cannot be accurately compared. Thus, the initial results were compared to each other, as were the final results.

The initial results showed some variation of IgA levels (Figure 6.5). Average levels and significant differences ($P<0.05$) were determined and are indicated in Table 6.3. The results show that initial faecal IgA levels were significantly lower in group 2 than any other group. Groups 1, 3 and 4 were not statistically different from group 5. As mice were randomly placed in groups, and no statistical difference was observed in weights of mice in any group (with the exception of the single mouse from group 3 that was excluded from the experiment), it is not possible to identify the reason for the lower IgA response for Group 2.

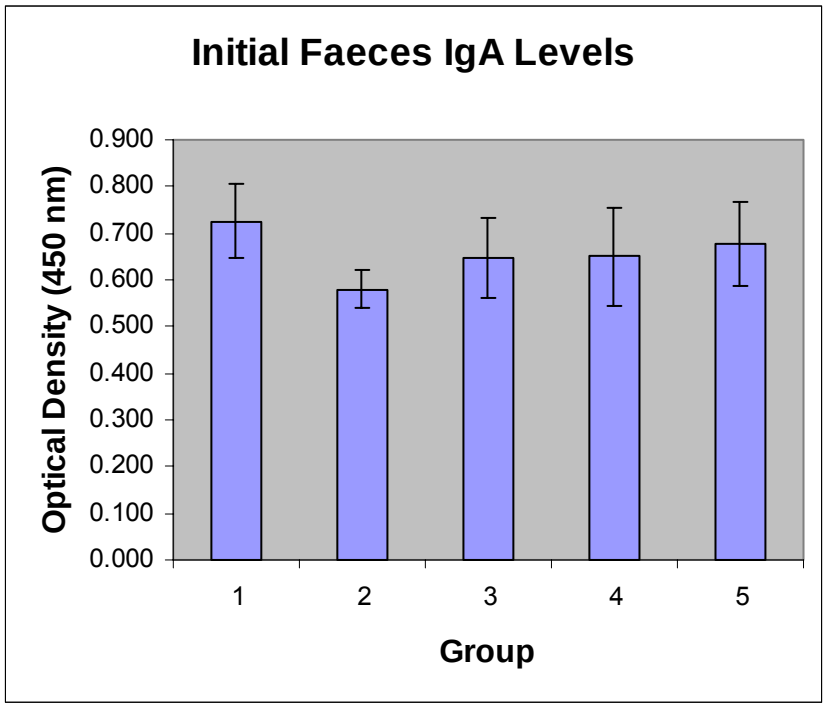

Figure 6.5. IgA levels in faeces collected on day 7. The column for each group represents the average optical density (n=14) and error bars indicate the standard deviation.

Table 6.3. Average initial optical densities (OD) measured in faeces by IgA ELISA.

Group	Average OD
1	[a]0.726
2	[c]0.579
3	[b]0.647
4	[b]0.650
5	[ab]0.676

Significantly similar results (P>0.05) are indicated by the same letter prefixing the OD result.

The final faeces results again showed some variation in IgA levels (Figure 6.6). Average levels and significant differences (P<0.05) were determined and are indicated in Table 6.4. The results show a gradual consecutive

decrease from group to group, with group 1 having the highest levels and group 5 (negative control) having the lowest levels. It is important to note that in the final results, groups 1 and 2 do not have significantly different IgA levels, whereas in the initial results, group 2 had significantly lower levels. All groups, except group 4, are significantly higher than group 5. Groups 3 and 4 did not significantly different final IgA levels.

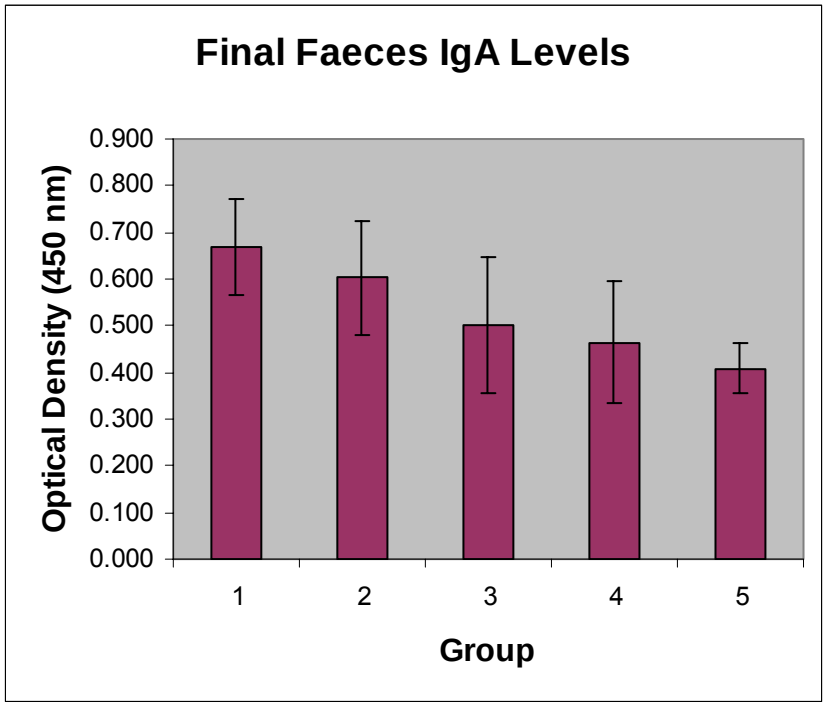

Figure 6.6. IgA levels in faeces collected on day 45. The column for each group represents the average optical density (n=14) and error bars indicate the standard deviation.

Table 6.4. Average final optical densities (OD) measured in faeces by IgA ELISA.

Group	Average OD
1	[a]0.667
2	[ab]0.604
3	[bc]0.502
4	[cd]0.464
5	[d]0.409

Significantly similar results ($P>0.05$) are indicated by the same letter prefixing the OD result.

Initial faecal IgA analysis showed that baselines for all groups were similar with the exception of group 2, which were significantly lower. Throughout the course of the experiment, the faecal IgA levels in group 2 increased to surpass groups 3, 4 and 5 (although, not being statistically higher than group 3, or statistically lower than group 1). Group 1, although not having significantly higher final faecal IgA levels than group 2, had higher levels than any other group.

The high faecal IgA level obtained by group 1, combined with the large change in levels from group 2, suggest that PJ702 is a very effective mucosal stimulant, with live organisms being slightly, but not more significantly, more effective than killed bacteria. This emphasises the importance of intestinal colonisation, and a combination of extracellular and intracellular components of PJ702 for optimal immune responses.

The faecal IgA results are in accordance with the conclusions made earlier with respect to serum IL-4 levels and reiterates the participation of

extracellular components in the stimulation of humoral immune responses (Harboe et al. 1996a; Orme 1988a). The amount of extracellular material given to the mice was equivalent for both the killed and live PJ702 groups.

Cholera toxin was only marginally better than no adjuvant at eliciting a mucosal immune response, once again leading to the possibility of a negative interference between WTB and cholera toxin, although this is contradictory to lymphocyte proliferation results from previous studies (Hosken 1999).

6.3.3.7. Saliva IgA levels

The saliva IgA levels from day 45 are shown in figure 6.7. The results showed no statistical significant difference ($P>0.5$) between any of the groups.

The indications regarding mucosal immunity, made by examining the faecal IgA results, could not be confirmed using the salivary IgA results. Isoptocarpine, which induces salivation, can also increase intestinal passage (Vivino et al. 1999; Answers Corporation 2005) and has antimicrobial properties against PJ702 (Rajvanshi 2004). Thus, no initial saliva samples were collected due to the fact that it would compromise the PJ702 habitation of the intestines required for group 1 of this experiment. The final salivary IgA results did not differ significantly between any groups. Without initial salivary IgA levels however, any changes similar to that experienced by group 2 in the faecal IgA results could not be noted.

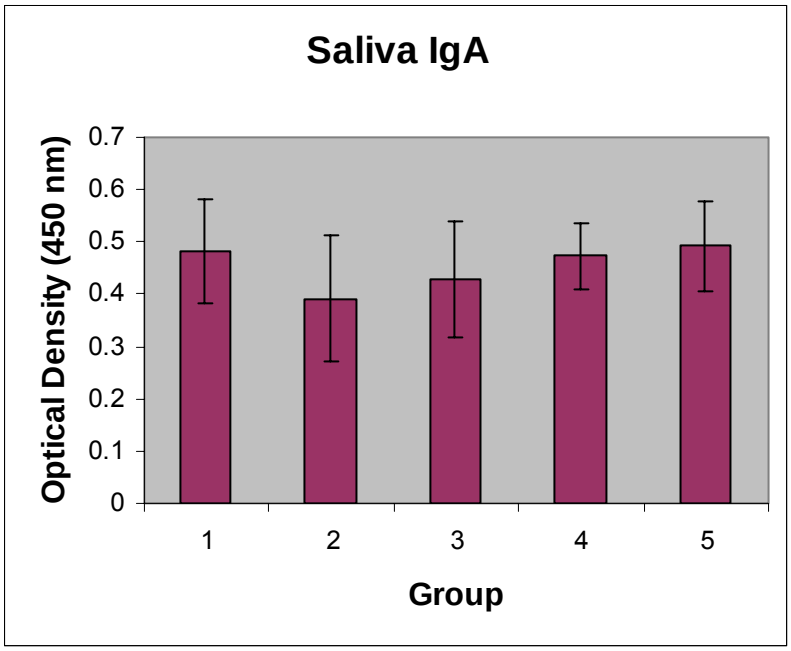

Figure 6.7. IgA levels in saliva from mice on day 45. Columns indicate average of duplicate assays on pooled samples from each group. Error bars indicate the standard deviation.

It is known that most rapid and strongest immune responses to a mucosal antigen occur at the site of antigen administration. Slightly weaker secondary responses can also be observed at adjacent mucosa or specifically inter-connected mucosal systems (Holmgren and Czerkinsky 2005). Thus, had the study duration been longer, a salivary IgA response would be anticipated.

6.3.3.8. Serum IgG levels

Serum samples from each group were pooled and initial and final serum samples were assayed on the same ELISA run, enabling comparisons to

be made between initial and final IgG levels for each group (results shown in Figure 6.8).

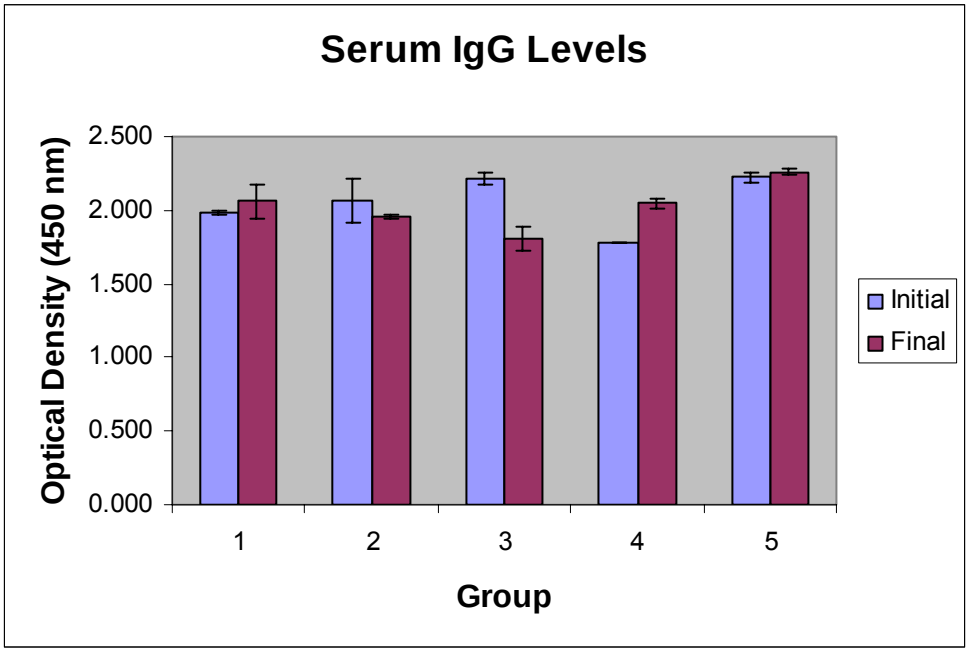

Figure 6.8. Serum IgG levels for pooled serum samples from day 7 (initial) and day 45 (final). Columns indicate average of 2-4 replicates and error bars indicate the standard deviation.

There was no significant change ($P>0.1$) in IgG levels over the course of the experiment for groups 1, 2 or 5. Group 3 experienced a significant ($P<0.05$) drop in IgG levels and group 4 experience a significant ($P<0.01$) increase in serum IgG levels.

The mucosal immune responses observed in the faecal IgA results did not transfer to systemic immunity as evidenced by the serum IgG results. Test groups 1 and 2 (using PJ702 as the adjuvant) experienced no change in serum IgG levels. The success of this organism as a mucosal adjuvant, suggests that a longer period of time or increased numbers of doses, may

be required for the immunity to transfer from the mucosal to the systemic immune system.

There was a significant decrease in serum IgG levels in mice that were given cholera toxin as an adjuvant. This is an unexpected result due to the widespread use of cholera toxin as a murine adjuvant in the field of experimental immunology. This lack of response, however, correlates with the negligible changes to cytokine levels in this group of mice (Section 6.3.3.5) and may indicate a problem with the lymphocyte cultures for this group. Without actual figures for lymphocyte proliferation cultures it is impossible to draw conclusions as to the significance of the results for this group.

Another point of interest with regard to serum IgG levels was the significant increase noted in the group that was given WTB as the antigen with no adjuvant. Once again, this is most likely due to the known adjuvant capacities of *Mycobacterium tuberculosis* proteins previously mentioned.

6.4. Discussion

The animal experiments outlined in this dissertation only examined immune responses to somatic antigens obtained by sonication of *M. tuberculosis*. The initial experiment using Whole Sonicate (WTB) and Cholera Toxin (CT), which obtained unexpectedly poor results, lead to an investigation of reagent stability. Concanavalin A (Con A) and [methyl-^{3}H] thymidine, the reagents used in these experiments, were found to be stable over a considerable period of time.

Three-year-old Con A induced lymphocyte proliferations equal to that of fresh Con A, with both having an optimal concentration of 2.5 µg/mL per well for lymphocyte cultures. Thus, the failure of the initial experiment was attributed to ambient temperature instability following air-conditioning failure in hot weather. Temperature instability has previously been reported to affect immune responses (Stark 1970).

The experiment using WTB antigens described in this dissertation, in conjunction with PJ702 as an adjuvant, confirmed previous findings that dairy propionibacteria are not normal colonisers of murine intestinal tracts (Adams et. al. 2005). As opposed to the previous study, however, dairy propionibacteria were not recoverable from the intestinal tract following introduction of PJ702 to the diet by means of oral vaccine administration. The deterioration of live PJ702 in weak suspensions that had been frozen over a number of months, as observed in the frozen vaccine samples, is a possible cause of poor results in the cultures of the faecal samples from the mice. It is also hypothesised that the reason for the lack of recovery of PJ702 from the faeces is the lower dosage given and the shorter duration of the study, resulting in insufficient colonisation.

The findings for the PJ702 adjuvant experiment in this chapter confirmed the effectiveness of this organism as an adjuvant. In addition, these results appear to indicate that various components of the Propionibacterium may stimulate different cell lines. The production of IFN-γ in lymphocytes exposed to somatic *M. tuberculosis* antigens only occurred in the group of mice that were exposed to intracellular components of PJ702. Extracellular components of PJ702,

however, boosted Th2 responses to WTB. All responses were due to mucosal immunity as evidenced by increased faecal IgA levels. These results indicate the importance of intestinal colonisation by PJ702 for effective stimulation of Th1 immune responses to a co-administered antigen.

Unrelated and unforeseen misfortunes prevented complete analysis of all objectives on many occasions throughout the experiments performed on the animal models outlined in this chapter. Thus, interpretation of results required assumptions to be made and further experiments in this field would shed more light on the significance of some of the data obtained. Investigations regarding PJ702 as an adjuvant should involve higher adjuvant doses, with extended follow-up intestinal examination of subjects.

6.5. Conclusions Drawn from Animal Experiments

In spite of technical difficulties, the results of this chapter clearly indicate that *Propionibacterium jensenii* 702 is an effective and safe oral adjuvant for inducing mucosal immunity in mice. In addition, the Th2-stimulating nature of somatic *M. tuberculosis* antigens has been shown to occur following oral vaccination.

Chapter 7: Conclusion

This dissertation describes the development of purification methods of both somatic and extracellular antigens from *Mycobacterium tuberculosis* cultures. Consideration was given to obtaining high protein concentrations, with both methods producing greater than 7 mg/mL protein. Quality was also considered, with minimal purification processes requiring the sample to be at room temperature, and refrigeration kept to less than 5 days.

The somatic antigen purification process involved harvesting *M. tuberculosis* cells from Modified Sauton's Medium and killing them by an overnight soaking in 2% glutaraldehyde. The cells were then washed in phosphate buffered saline and sonicated in an ethanol-ice bath. Following sonication, the insoluble material was removed by ultracentrifugation and the hydrophilic proteins in the supernatant were concentrated by ultrafiltration.

Extracellular antigens were purified from the liquid Modified Sauton's Medium following short-term culture on the surface of the media. The optimal method for purifying the antigens involved addition of ammonium sulfate to the filtered media, which resulted in precipitation of the hydrophilic proteins. This enabled the media volume to be rapidly reduced by ultracentrifugation. The precipitated proteins in the deposit were dissolved in phosphate buffered saline and purified by dialysis. Proteins produced using this method have been shown to induce a Th1 response, which is boosted by the use of *Propionibacterium jensenii* 702

(PJ702) as an adjuvant (Lean 2002; Huang and Adams 2003; Adams et. al. 2005).

Several conclusions can be drawn from animal experimentation in this dissertation. Firstly, the importance of the comfort of the animals for effective immune functions has been emphasised by absent responses following air-conditioning failure in the animal house during summer. Secondly, the results confirmed the “safe” administration of PJ702 and the ability of the organism to stimulate mucosal immune responses.

The most significant findings of the experiments performed in this dissertation relate to the nature of the responses to live and killed PJ702. It appears that extracellular components, present in live and killed preparations of the organism, are capable of boosting immune responses to an antigen. In this case the antigen (WTB) is known to induce Th2 responses and the PJ702 was able to increase that response. Alternatively, intracellular components of PJ702, present in killed preparations and actively growing (or established) cultures, appear to be capable of inducing Th1 responses to WTB antigens.

Finally, the experiments performed indicated that a longer duration of PJ702 administration may be required. The IgA responses measured in the faecal samples were not observed in the secondary location examined (saliva). Prolonged ingestion of the probiotic adjuvant may also assist in the intestinal colonisation of the organism and provide clarity with regard to the type of immune responses the organism instigates.

Appendix A - Reagents

Unless stated otherwise, pH adjustments were performed using a Microprocessor Bench pH Meter (Hanna) with the addition of concentrated HCl (Ajax) or concentrated NaOH (Ajax).

1. General Reagents

1.1. Phosphate Buffered Saline (PBS), pH 7.3

Reagent	Quantity	Supplier
Sodium chloride	8.0 g	Sigma
Potassium chloride	0.2 g	Sigma
Disodium hydrogen phosphate	1.15 g	Sigma
Potassium dihydrogen phosphate	0.2 g	BDH
Dissolve all reagents in 1.0 L distilled water. Adjust to pH 7.3. Autoclave 15 minutes at 121°C if desired. Store at 4°C.		

1.2. 10% ethanol

Reagent	Quantity	Supplier
Ethanol	5 ml	Fronine
Distilled water	45 ml	
Mix well.		

1.3. 70% ethanol

Reagent	Quantity	Supplier
Ethanol	700 mL	Fronine
Distilled water	300 mL	
Mix well.		

2. Mycobacterial Culture Media

2.1. Nutrient Broth with 10% glycerol

Reagent	Quantity	Supplier
Nutrient Broth powder	1.3 g	Amyl Media
Distilled water	90 mL	
Glycerol	10 mL	Sigma
Mix together. Apply gentle heat if necessary to dissolve media powder. Dispense into desired containers. Autoclave at 121°C for 15 minutes. Store at 4°C.		

2.2. Modified Sauton's Medium (MSM)

Reagent	Quantity (for 1 L MSM)	Quantity (for 2 L MSM)	Supplier
Asparagine	4 g	8 g	Sigma
Tap water	250 mL	500 mL	
Dissolve asparagine in water (warm to 80°C in water bath if necessary)			
Magnesium sulfate heptahydrate ($MgSO_4.7H_20$)	0.5 g	1.0 g	Sigma
Citric Acid	1.83 g	3.66 g	Sigma
Potassium orthophosphate (K_2HPO_4)	0.5 g	1.0 g	Fronine
Ferric Ammonium Citrate	0.05 g	0.10 g	Sigma
D-Glucose	4.38 g	8.76 g	Sigma
Sodium Pyruvate	4.82 g	9.64 g	Sigma
Glycerol	53 mL	106 mL	Sigma
Tap water	710 mL	1420 mL	
Add to dissolved asparagine. Adjust to pH 6.8 with concentrated ammonia (25-32%) (Ajax). Dispense into desired containers. Autoclave at 127°C for 20 minutes. Allow to cool in autoclave for at least 2 hours. Store at 4°C for up to 6 weeks. (CAUTION: EXPLOSIVE!)			

2.3. Mycobacteria 7H11 Agar

Reagent	Quantity	Supplier
Mycobacteria 7H11 Agar Powder	21 g	Difco
Distilled water	895 mL	
Glycerol	5 mL	Sigma
Boil to dissolve. Sterilize at 121°C for 15 minutes. Cool to 50-55°C.		
Middlebrook OADC Enrichment	100 mL	Bacto
Aseptically add OADC Enrichment to sterile media. Mix well. Pour into Petri dishes (Sarstedt). Cool to set. Store at 4°C.		

3. Protein Assay Reagents

3.1. Dye Reagent (for standard procedure)

Reagent	Quantity	Supplier
Dye Reagent Concentrate (Coomassie® Brilliant Blue G-250)	20 mL	Bio-Rad
Distilled Water	80 mL	
Mix well and filter through Whatman #1 filter. Store at room temperature for 2 weeks.		

3.2. Dye Reagent (for microtitre plate method)

Reagent	Quantity	Supplier
Dye Reagent Concentrate (Coomassie® Brilliant Blue G-250) Distilled Water	4 mL 16 mL	Bio-Rad
Mix well and filter through Whatman #1 filter. Store at room temperature for 2 weeks.		

3.3. Stock Standard (1.43 mg/ml protein)

Reagent	Quantity	Supplier
Lyophilized Bovine Serum Albumin (BSA) Distilled Water	1 bottle 20 mL	Bio-Rad
Aliquot 50 μL quantities and store at -80°C.		

3.4. Working Standards (standard method)

Standard	Stock Standard Volume (μL)	Distilled Water Volume (μL)	Protein Concentration (mg/mL)
1	70	430	0.200
2	150	350	0.429
3	220	280	0.629
4	300	200	0.858
Prepare the standards according to the above dilutions. Use immediately.			

3.5. Working Standards (microtitre plate method)

Standard	Protein Concentration (mg/mL)	Dilutions
Standard 1 (S1)	0.050	50 μL S2 + 50 μL dH_20
Standard 2 (S2)	0.100	50 μL S3 + 50 μL dH_20
Standard 3 (S3)	0.200	50 μL S4 + 50 μL dH_20
Standard 4 (S4)	0.400	28 μL Stock standard + 72 μL dH_20
Prepare the standards according to the above dilutions. Use immediately.		

3.6. Secondary Standard Preparation

Reagent	Quantity	Supplier
Stock Standard Distilled water	100 μL 900 μL	Bio-Rad
Mix well and use immediately.		

3.7. Working Standards (microassay microtitre plate method)

Standard	Secondary Standard Volume (μL)	Distilled Water Volume (μL)	Protein Concentration (μg/mL)
1	50	550	11.92
2	125	475	29.79
3	200	400	47.67
4	250	350	59.58
5	300	300	71.50
Prepare the standards according to the above dilutions. Use immediately.			

4. SDS-PAGE Reagents

4.1. Acryliamide/Bis

Reagent	Quantity	Supplier
Acrylamide	146.0 g	Bio-Rad
N,N'-Methylene-bis Acrylamide	4.0 g	Bio-Rad
Add distilled water to 500 mL. Filter and store at 4°C in the dark for up to 30 days.		

4.2. 1.5 M Tris-HCl, pH 8.8

Reagent	Quantity	Supplier
Tris base	54.45 g	Sigma
Distilled water	150 mL	
Mix to dissolve. Adjust to pH 8.8. Add distilled water to 300 mL. Store at 4°C.		

4.3. 0.5 M Tris-HCl, pH 6.8

Reagent	Quantity	Supplier
Tris-HCl	7.88 g	Sigma
Distilled water	60 mL	
Mix to dissolve. Adjust to pH 6.8. Add distilled water to 100 mL. Store at 4°C.		

4.4. 10% (w/v) SDS

Reagent	Quantity	Supplier
SDS	10 g	Bio-Rad
Distilled water	60 ml	
Dissolve with gentle stirring. Add distilled water to 100 mL.		

4.5. 10% (w/v) Ammonium Persulfate

Reagent	Quantity	Supplier
Ammonium Persulfate	100 mg	Bio-Rad
Distilled water	1.0 mL	
Dissolve and use immediately.		

4.6. Separating Gel (12%, pH 8.8)

Reagent	Quantity	Supplier
Acrylamide/bis	40.0 mL	Appendix 4.1
Distilled water	33.5 mL	
1.5 M Tris-HCl, pH 8.8	25.0 mL	Appendix 4.2
10% (w/v) SDS	1.0 mL	Appendix 4.4
10% (w/v) ammonium persulfate	500 μL	Appendix 4.5
TEMED	50 μL	Bio-Rad
Combine all reagents without frothing, except ammonium persulfate and TEMED. Allow to stand 15 minutes. Add the two catalysts immediately prior to casting the gel.		

4.7. Stacking Gel (4%, pH 6.8)

Reagent	Quantity	Supplier
Acrylamide/bis	1.3 mL	Appendix 4.1
Distilled water	6.1 mL	
0.5 M Tris-HCl, pH 6.8	2.5 mL	Appendix 4.3
10% (w/v) SDS	100 μL	Appendix 4.4
10% (w/v) ammonium persulfate	50 μL	Appendix 4.5
TEMED	10 μL	Bio-Rad
Combine all reagents without frothing, except ammonium persulfate and TEMED. Allow to stand 15 minutes. Add the two catalysts immediately prior to casting the gel.		

4.8. 0.5% (w/v) Bromophenol Blue

Reagent	Quantity	Supplier
Bromophenol Blue	0.05 g	Bio-Rad
Distilled water	10.0 mL	
Mix and store in a dark place.		

4.9. Sample Buffer, pH 6.8

Reagent	Quantity	Supplier
Distilled water	3.0 mL	
0.5 M Tris-HCl, pH 6.8	1.0 mL	Appendix 4.3
Glycerol	1.6 mL	Sigma
10% SDS	1.6 mL	Appendix 4.4
2-mercaptoethanol	0.4 mL	Bio-Rad
0.5% (w/v) bromophenol blue	0.4 mL	Appendix 4.8
Mix well. Store 4°C.		

4.10. 5X Electrode Buffer

Reagent	Quantity	Supplier
Tris base	45.0 g	Sigma
Glycine	216.0 g	Sigma
SDS	15.0 g	Bio-Rad
Add distilled water to 3.0 L. Do not adjust the pH. Store at 4°C. Warm to 37°C before use if precipitation occurs.		

4.11. Running Buffer

Reagent	Quantity	Supplier
5x Electrode buffer	0.5 L	Appendix 4.10
Distilled water	2.0 L	
Running buffer may be reused several times. Store 4°C.		

5. Gel Staining Reagents

5.1. Fixative I

Reagent	Quantity	Supplier
Methanol	800 mL	Fronine
Acetic Acid	200 mL	BDH
Distilled water	1.0 L	
Mix well. Tightly cover and store at room temperature.		

5.2. Fixative II

Reagent	Quantity	Supplier
Ethanol	100 mL	Fronine
Acetic Acid	50 mL	BDH
Distilled water	850 mL	
Mix well. Tightly cover and store at room temperature.		

5.3. Oxidizer Reagent

Reagent	Quantity	Supplier
Oxidizer Reagent Concentrate	20 mL	Bio-Rad
Distilled water	180 mL	
Mix well. Use immediately.		

5.4. Silver Reagent

Reagent	Quantity	Supplier
Silver Reagent Concentrate	20 mL	Bio-Rad
Distilled water	180 mL	
Mix well. Use immediately.		

5.5. Developer

Reagent	Quantity	Supplier
Developer powder	32 g	Bio-Rad
Distilled water	1.0 L	
Stir to dissolve. Tightly cover and store at room temperature for up to 1 month.		

5.6. Stop Solution

Reagent	Quantity	Supplier
Acetic Acid	50 mL	BDH
Distilled water	950 mL	
Mix well. Tightly cover and store at room temperature.		

6. Electroelution Reagents

6.1. Phosphate Buffer (2 mM, pH 6.8)

Reagent	Quantity	Supplier
Sodium orthophosphate (NaH_2PO_4) Distilled water	0.48 g 1.8 L	BDH
Dissolve and adjust to pH 6.79. Make up to 2.0 L with distilled water and store at 4°C.		

7. Somatic Antigen Production Reagents

7.1. 2% glutaraldehyde

Reagent	Quantity	Supplier
Glutaraldehyde (25%) Distilled water	4 mL 46 mL	Sigma
Mix together in a fume hood.		

7.2. Sodium Orthovanadate (0.14 M)

Reagent	Quantity	Supplier
Sodium Orthovanadate Distilled water	0.100 g 4.0 mL	Sigma
Adjust to pH 10 and heat to boiling. Cool.		

7.3. Concentrated PMSF (50X)

Reagent	Quantity	Supplier
PMSF Methanol	0.174 g 20 mL	Sigma Fronine
Dissolve. Freeze 200 µL aliquots at -70ºC.		

7.4. Protease Inhibitor Solution

Reagent	Quantity	Supplier
Na_2EDTA Distilled water	0.2922 g to 978 mL	Sigma
Dissolve Na_2EDTA before adding additional reagents.		
Sodium Orthovanadate (0.14 M) Leupeptin Aprotinin Pepstatin A	7.36 µL 85.5 µL 1.3 mL 275 µL	Appendix 7.2 Sigma Sigma Sigma
Aliquot 9.8 mL volumes and freeze at -70ºC. Just prior to use, add 200 µL concentrated PMSF (50X) (Appendix 7.3).		

7.5. TES Buffer

Reagent	Quantity	Supplier
Tris-HCl	0.315 g	Sigma
$Na_2EDTA.2H_2O$	0.074 g	Bio-Rad
Sodium Chloride	1.169 g	Sigma
Distilled Water	180 mL	
Mix well to dissolve. Adjust to pH 6.8. Make up to 200 mL with distilled water and store at 4°C.		

8. Reagents for Animal Experiments

8.1. Sodium Phosphate Buffer (0.05 M, pH 7.0)

Reagent	Quantity	Supplier
0.5 M Na_2HPO_4	500 mL	Chem Supply
0.5 M NaH_2PO_4	500 mL	Chem Supply
Mix and store at 4ºC.		

8.2. Faecal Protease Inhibitor Solution

Reagent	Quantity	Supplier
Soybean Trypsin Inhibitor (100 mg/mL)	3.0 mg	Sigma
PBS	300 mL	Appendix 1.1
Autoclave 15 minutes at 121ºC. Store at 4ºC.		

8.3. Faecal PMSF Solution

Reagent	Quantity	Supplier
PMSF	20 mg	Sigma
Absolute ethanol	1 mL	Fronine
Mix to dissolve. Store at 4ºC.		

8.4. Dilute Isoptocarpine

Reagent	Quantity	Supplier
Pilopt 1%	250 µL	Allergan
Sterile PBS	4. 75 mL	Appendix 1.1
Invert to mix and use immediately.		

9. Propionibacterium Culture Medium

9.1. Wilkins-Chalgren Anaerobe Broth (WCAB)

Reagent	Quantity	Supplier
WCAB	16.5 g	Oxoid
Distilled water	1 L	
Dissolve and autoclave at 121ºC for 15 minutes. Store at 4ºC.		

9.2. Sodium Lactate Agar (SLA)

Reagent	Quantity	Supplier
Tryptone	10 g	Oxoid
Yeast Extract	10 g	Oxoid
Sodium lactate (60%)	16.5 mL	Sigma
Di-potassium hydrogen phosphate	0.25 g	Ajax
Manganese sulfate	0.05 g	BDH
Bacteriological agar	15 g	Oxoid
Distilled water	1 L	
Heat using a magnetic stirrer to dissolve. Adjust to pH 7.0. Autoclave at 121ºC for 15 minutes. Pour 20 mL aliquots into sterile Petri dishes (Becton Dickinson) in a sterile manner. Leave flat to set. Store at 4ºC.		

10. Lymphocyte Culture Reagents

10.1. RPMI Complete Medium

Reagent	Quantity	Supplier
DMEM/RPMI	500 mL	Thermo Trace
10,000 U/mL Penicillin, 10 mg/mL Streptamycin	5 mL	Thermo Trace
L-Glutamine (200 mM)	5 mL	Thermo Trace
2-Mercaptoethanol (5 mM)	5 mL	Thermo Trace
HEPES buffer (1 M)	10 mL	Thermo Trace
Foetal Calf Serum (heat inactivated*)	50 mL	Thermo Trace
Combine reagents under sterile conditions. Store at 4ºC. (*To inactivate Foetal Calf Serum: place in 56ºC water bath for 45 minutes.)		

10.2. Red Blood Cell Lysis Buffer

Reagent	Quantity	Supplier
NH_4Cl	4.15 g	BDH
$NaHCO_3$	0.5 g	BDH
EDTA di-sodium salt	0.0185 g	Ajax
Dissolve in approximately 400 mL sterile distilled water. Adjust the pH to 7.35 and make up to 500 mL. Filter sterilize and store at 4ºC.		

10.3. Trypan Blue (0.1%)

Reagent	Quantity	Supplier
Trypan Blue	0.05 g	Sigma
PBS	50 mL	Appendix 1.1
Store at room temperature.		

10.4. Working Thymidine

Reagent	Quantity	Supplier
[methyl-^{3}H] thymidine (1 mCi/mL)	100 µL	Amersham
RPMI complete medium	1.9 mL	Appendix 10.1
Store at 4ºC.		

11. General ELISA Reagents

11.1. PBST (PBS + 0.05% Tween 20)

Reagent	Quantity	Supplier
PBS	20 L	Appendix 1.1
Tween 20	10 mL	Sigma
Store at 2-8ºC for up to 7 days.		

11.2. TMB Substrate Solution

Reagent	Quantity	Supplier
Substrate A	15.0 mL	Pharmingen
Substrate B	15.0 mL	Pharmingen
Mix well. Use immediately.		

11.3. Stop Solution (1 M H_2SO_4)

Reagent	Quantity	Supplier
Sulphuric Acid (18 M)	55.5 mL	BDH
Distilled water	to 1.0 L	
Store at room temperature.		

12. Cytokine ELISA Reagents

12.1. Coating Buffer (0.1 M Carbonate Buffer, pH 9.5)

Reagent	Quantity	Supplier
$NaHCO_3$	8.40 g	BDH
Na_2CO_3	3.56 g	BDH
Distilled water	1.0 L	
Mix to dissolve. Adjust pH to 9.5. Store at 4ºC for up to 30 days.		

12.2. Assay Diluent

Reagent	Quantity	Supplier
PBS	90 mL	Appendix 1.1
Foetal Calf Serum (heat inactivated*)	10 mL	Thermo Trace
Store at 4ºC for up to 3 days. (*To inactivate Foetal Calf Serum: place in 56ºC water bath for 45 minutes.)		

12.3. Capture Antibody (for IL-4 assay)

Reagent	Quantity	Supplier
Capture Antibody	50 µL	Pharmingen
Coating Buffer	11.95 mL	Appendix 12.1
Make up fresh. This is sufficient quantities for one ELISA plate.		

12.4. Detection Antibody (for IL-4 assay)

Reagent	Quantity	Supplier
Biotinylated anti-mouse IL-4	100 µL	Pharmingen
Assay diluent	24.9 mL	Appendix 12.2
Invert to mix.		

12.5. Working Detector (for IL-4 assay)

Reagent	Quantity	Supplier
Enzyme Reagent	200 µL	Pharmingen
Detection Antibody (for IL-4 assay)	24.8 mL	Appendix 12.4
Mix well. Use within 15 minutes.		

12.6. Capture Antibody (for IFN-γ assay)

Reagent	Quantity	Supplier
Capture Antibody	10 µL	Pharmingen
Coating Buffer	20.0 mL	Appendix 12.1
Mix well.		

12.7. Detection Antibody (for IFN-γ assay)

Reagent	Quantity	Supplier
Biotinylated anti-mouse IFN-γ	100 µL	Pharmingen
Assay diluent	24.9 mL	Appendix 12.2
Invert to mix.		

12.8. Working Detector (for IFN-γ assay)

Reagent	Quantity	Supplier
Enzyme Reagent	100 µL	Pharmingen
Detection Antibody (for IFN-γ assay)	11.95 mL	Appendix 12.7
Mix well. Use within 15 minutes.		

13. Immunoglobulin ELISA Reagents

13.1. Bicarbonate buffer

Reagent	Quantity	Supplier
Na_2CO_3	1.192 g	BDH
$NaHCO_3$	2.205 g	BDH
Distilled water	650 mL	
Mix to dissolve. Adjust to pH 9.8 and make up to 750 mL with distilled water. Autoclave 121ºC for 15 minutes. Store at 4ºC.		

13.2. Foetal Calf Serum (5%)

Reagent	Quantity	Supplier
PBS	95 mL	Appendix 1.1
Foetal Calf Serum (heat inactivated*)	5 mL	Thermo Trace
Store at 4ºC for up to 3 days. (*To inactivate Foetal Calf Serum: place in 56ºC water bath for 45 minutes.)		

13.3. Biotinylated IgG Conjugate

Reagent	Quantity	Supplier
Anti-mouse IgG	20 µL	Amersham Pharmacia
PBST	20.0 mL	Appendix 11.1
Use immediately.		

13.4. Biotinylated IgA Conjugate

Reagent	Quantity	Supplier
Anti-mouse IgA	10 µL	Amersham Pharmacia
PBST	20.0 mL	Appendix 11.1
Use immediately.		

13.5. Streptavidin Horseradish Peroxidase (SA-HRP)

Reagent	Quantity	Supplier
SA-HRP	10 µL	Amersham Pharmacia
PBST	9.9 mL	Appendix 11.1
Use immediately.		

Appendix B – Manufacturer Details

Advanced Anaesthesia Specialists
Unit 13
46-48 Buffalo Road
Gladesville NSW 2111
Ph. (02) 9808 1844
Fax. (02) 9808 1866

Advantec MFS, Inc.
6691 Owens Drive
Pleasanton CA 94588-3335
United States of America
Phone (925) 225-0349
Fax (925) 225-0353

Ajax Finechem
8 Abbott Rd
Seven Hills NSW 2147
Phone: (02) 9839-4000
Fax: (02) 9674-6225

Allergan Australia
77 Ridge St
Gordon NSW 2072

Amicon
72 Cherry Hill Drive
Beverly, MA 01915
United States of America
Ph. (508) 777 3622
Fax. (508) 777 6204

Amyl Media
39 Healey Road
Dandenong, Victoria 3175
Ph. (03) 9706 5666

American Type Culture Collection (ATCC)
PO Box 1549
Manassas VA 20108
United States of America
Ph. (703) 365 2700

Amersham Pharmacia (now GE Healthcare Life Sciences)
Unit 1
22 Hudson Avenue
Castle Hill NSW 2154
Ph. (02) 9899 0999 or 1800 150 522
Fax. (02) 9899 7511

Bacto Laboratories
310-312 Elizabeth Drive
Liverpool NSW 2170
Ph. (02) 9602 5499 or 1800 819994
Fax. (02) 9601 8293

BD Biosciences Pharmingen
2350 Qume Drive
San Jose CA 95131
United States of America
Ph. (877) 232 8995

BDH (Merck)
Frankfurter Str. 250
64293 Darmstadt
Germany
Ph. +49 6151 72-0
Fax. +49 6151 72-2000

Beckman Coulter
Unit D
24 College Street
Gladesville NSW 2111
Ph. (02) 9844 6000
Fax. (02) 9844 6098

Becton, Dickinson and Company
1 Becton Drive
Franklin Lakes NJ 07417
United States of America
Ph. (201) 847 6800

Bio-Rad
Unit 1, Block Y
Regents Park Industrial Estate
391 Park Road
Regents Park NSW 2143
Ph. (02) 9914 2800 or 1800 224 354
Fax. (02) 9914 2889

Biolab Scientific
2 Clayton Rd
Clayton VIC 3167
Ph. (03) 9263 4300 or 1300 735 292
Fax. (03) 9562 9840

bioMérieux
Unit 25
Parkview Business Centre
1 Maitland Place
Baulkham Hills NSW 2153
Ph. (02) 8852 4700
Fax. (02) 8852 4777

BOC Gases
258 Manns Rd
West Gosford NSW 2250
Ph. (02) 4324 5444

Boehringer Ingelheim
Ellesfield Avenue
Bracknell
Berkshire
RG12 8YS
United Kingdom
Tel. +44 (0) 1344 424600
Fax. +44 (0) 1344 741444

Branson Ultrasonics Corporation
41 Eagle Road
Danbury CT 06313-1961
United States of America

Cellestis
1046A Dandenong Rd
Carnegie VIC 3163
Ph. (03) 9571 3500
Fax. (03) 9571 3544

Chem Supply
38-50 Bedford St
Gilman SA 5013
Ph. (08) 8440 2000
Fax. (08) 8440 2001

CHILTERN Scientific
171 Bath Road
Slough
Berkshire, SL1 4AA
United Kingdom
Ph. +44 (0) 1753 512000
Fax. +44 (0) 1753 511116

Copeland
Ashville, North Carolina
United States of America

Corning Costar Corporation
Cambridge, MA 02140
United States of America
Ph. (800) 492-1110

Crown Scientific
1 Culverston Rd
Minto NSW 2566
Ph. (02) 9933 4000
Fax. (02) 9603 5155

Difco
(see Becton, Dickinson and Company)

Dynavac Engineering (now Rietschle Thomas)
30 Bearing Rd
Seven Hills NSW 2147
Ph. 1800 822 886

Eppendorf
Unit 4
112 Talavera Rd
North Ryde NSW 2113
Ph. (02) 9889 5000
Fax. (02) 9889 5111

Fortuna Technologies
1270A Lawrence Station Rd
Sunnyvale CA 94089
United States of America
Ph. (408) 541 0200
Fax. (408) 541 0300

Fronine
144 Hamilton St
Riverstone NSW 2765
Ph. (02) 9627 3600
Fax. (02) 9627 2052

Gilson
3000 Parmenter St
Middleton WI 53562
United States of America
Ph. (608) 836 1551
Fax. (608) 831 4451

Gelman Sciences (now Pall Corporation)
Unit 2
11-13 Orion Road
Lane Cove NSW 2066
Ph. (02) 9424 3000
Fax. (02) 9420 3388

GS Laboratory Equipment
(see Copeland)

Hanna Instruments Pty Ltd
1/11 Lindaway Place
Tullamarine VIC 3043
Australia
Phone (03) 9335 2418
Fax (03) 9338 5136

Hirschmann Laborgaräte
Hauptstr. 7-15
D-74246 Eberstadt
Germany
Ph. +49 7134 5110
Fax. +49 7134 51190

Hettich Zentrifugen
Föhrenstr.12
D-78532 Tuttlingen
Denmark
Ph. 0049 7461 705 0
Fax. 0049 7461 705 125

Kimberly-Clark
52 Alfred Street
Milsons Point NSW 2061
Ph. (02) 9963 8888
Fax. (02) 9957 5687

Laser Animal Health
3/70 Raynham St
Salisbury QLD 4107

Livingstone
106-116 Epsom Rd
Rosebery NSW 2018
Ph. 1300 780 780
Fax. 1300 780 008

Nalgene (Nalge Nunc International)
75 Panorama Creek Drive
Rochester NY 14625
United States of America
Ph. 585 899 7198
Fax. 585 899 7195

Oxoid Australia
20 Dalgleish St
Thebarton SA 5031
Ph. 1800 331 163
Fax. 1800 007 054

Perkin Elmer Life and Analytical Sciences
45 William Street
Wellesley MA 02481
United States of America
Ph. 203 925 4602
Fax. 203 944 4904

ICI Pharmaceuticals
20 Manchester Square
London W1U 3AN
United Kingdom
Ph. +44 20 7009 5000
Fax. +44 20 7009 5001

Ratek Instruments
1/3 Wadhurst Drive
Boronia VIC 3155
Phone (03) 9887 2161
Fax (03) 9887 2163

Samco Scientific Corporation
1050 Amoyo Ave
San Fernando CA 91340
United States of America

Sarstedt Australia
16 Park Way
Technology Park SA 5095
Ph. (08) 8349 6555
Fax. (08) 8349 6882

Sartorius
Unit 17
104 Ferntree Gully Road
Waverley Business Park
East Oakleigh VIC 3166
Ph. (03) 9590 8800
Fax. (03) 9590 8828

Sigma-Aldrich
PO Box 970
Castle Hill NSW 1765
Ph. 1800 800 097
Fax. 1800 800 096

Smith+Nephew
315 Ferntree Gully Rd
Mount Waverley VIC 3149
Ph. (03) 8540 6777
Fax. (03) 9544 5086

Swann-Morton
Owlerton Green
Sheffield S6 2BJ
United Kingdom
Ph. +44 (0)114 234 4231
Fax. +44 (0)114 231 4966

Spectrum Laboratories, Inc.
18617 Broadwick Street
Rancho Dominquez, CA 90220-6435
United States of America
Ph. (310) 885-4600, (800) 634-3300
Fax. (310) 885-4666

Terumo
Level 4, Building B
11 Talavera Road
Macquarie Park NSW 2113
Ph. (02) 9878 5122
Fax. (02) 9878 5085

Thermoline Scientific Equipment Pty Ltd
40 Blackstone Street
Wetherill Park NSW 2164
Phone (02) 9604 3911

Thermo Trace
189-199 Browns Rd
Noble Park VIC 3174
Ph. (03) 9790 4100

Vetri-plast
(distributed by Fronine, see Fronine)

Wallac
(now Perkin Elmer, see Perkin Elmer)

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