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Identification and characterization of an 8.4 kDa protein antigen of *Mycobacterium bovis*.

A thesis presented in partial fulfilment of the requirements for the degree of

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ABSTRACT.

The culture filtrate (CF) derived from a *M. smegmatis* subclone transformed with the mycobacteria/*E. coli* plasmid shuttle vector pSU4511 containing a 4.3 kb fragment of *M. bovis* DNA (*M. smegmatis* pSU151.43), was observed to stimulate PBMC from a steer vaccinated with *M. bovis* BCG to proliferate and produce IFN-γ. To identify the source of immunoreactivity, the proteins in CF derived from *M. smegmatis* pSU151.43 were separated by fast protein liquid chromotography (FPLC) and the fractions were screened in whole blood IFN-γ assays. A stimulatory protein was purified that had a molecular mass of 8335 Da and the N-terminal amino acid sequence: DPVDAVINTT. Polyclonal antisera were raised against the purified recombinant antigen in rabbits and used for Western blotting.

The nucleotide sequence of the 4.3 kb insert of *M. bovis* DNA was determined, and the open reading frame (ORF) coding for the 8.4 kDa protein was identified. Computer analysis of the deduced amino acid sequence with the programme PSORT predicted that the nascent protein consisted of a 28 amino acid export signal sequence followed by an 82 amino acid mature protein. It was also found that *M. avium* possesses a nucleotide sequence that potentially codes for a protein with a high degree of homology to the 8.4 kDa antigen of *M. bovis.*

A segment of the 4.3 kb insert of *M. bovis* DNA adjacent to the gene coding for the 8.4 kDa antigen was found to be polymorphic between the strain of *M. bovis* from which the cosmid library was constructed and the published sequence of *M. tuberculosis* H37Rv (Cole *et al.* 1998). The *M. bovis* sequence contained 1.7 copies of a 62 bp exact tandem repeat and the *M. tuberculosis* sequence contained 2.7 copies. The species distribution of the 62 bp exact tandem repeat (ETR) locus was characterized by polymerase chain reaction (PCR) and Southern blotting. The 62 bp ETR was found to occur only in *M. tuberculosis* complex species and may be a useful genetic marker for differentiating between *M. bovis* and *M. tuberculosis.*

Lymphocyte proliferation and IFN-γ assays were used to measure the responses of ten BCG vaccinated and ten unvaccinated calves the 8.4 kDa antigen, PPD-B and PPD-A tuberculins, both before and after intratracheal challenge infection with virulent *M. bovis.*

The results provided evidence that vaccination of cattle with *M. bovis* BCG but not infection with *M. bovis* appeared to elicit an immune response to the 8.4 kDa antigen of *M. bovis.*

To obtain greater quantities of recombinant 8.4 kDa antigen, the gene that codes for the protein was cloned into *E. coli* and *M. smegmatis* expression plasmids. The 8.4 kDa antigen was overexpressed and secreted with an N-terminal 6 x Histidine tag by *M. smegmatis.* Approximately 500 μg of 6 x Histidine tagged 8.4 kDa Ag were purified / litre of CF in one step by metal chelate affinity chromatography. The recombinant protein was shown to elicit specific IFN-γ responses *in vitro.*

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ABBREVIATIONS.

2D-PAGE	two dimensional polyacrylamide gel electrophoresis.
2-ME	2-mercaptoethanol.
2xSLB	2 x sample loading buffer.
6 x His	6 x Histidine.
A_{280}	absorbance at 280 nm.
Ag	antigen.
AHB	Animal Health Board.
APC	antigen presenting cell.
APS	ammonium persulfate.
ATCC	American Type Culture Collection.
BCG	*M. bovis* bacillus Calmette-Guérin.
BLAST	basic local alignment search tool.
bp	nucleotide base pairs
BSA	bovine serum albumin.
CCT	comparative cervical test.
CD	cluster of differentiation.
CF	culture filtrate.
Cos151	*M. smegmatis* cosmid library clone 151.
ConA	concanavalin A.
dH_2O	double distilled water.
c.p.m	counts per minute.
Δ c.p.m	difference in counts per minute.
$\Delta\ OD_{450}$	difference in optical density at 450 nm.
DAB	3, 3'-Diaminobenzidine.
DIG-dUTP	Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate.
DMSO	dimethyl sulfoxide.
DNA	deoxyribonucleic acid.
dsDNA	double stranded DNA.
dNTP	deoxynucleoside triphosphate.
DR	direct repeat.
DTH	delayed type hypersensitivity.
DTT	dithiothreitol.

EIA	enzyme immunoassay.
ELISA	enzyme linked immunosorbent assay.
ERMA	Environmental Risk Management Authority.
ETR	exact tandem repeat.
FAO	Food and Agriculture Organization of the United Nations.
FCS	foetal calf serum.
FPLC	fast protein liquid chromatography.
g	gravity (a force of ~ 10 N).
GST	glutathione-S-transferase.
HRP	horesradish peroxidase.
ICAM	intercellular adhesion molecule.
IFA	Incomplete Freund's Adjuvant.
IFN	interferon.
IFN-γ	interferon gamma.
IL	interleukin.
IPTG	isopropylthio-β-galactoside.
IS	insertion sequence.
IU	international units.
IUATLD	International Union Against Tuberculosis and Lung Disease.
IUPAC	International Union of Pure and Applied Chemists.
IWGMT	International Working Group on Mycobacterial Taxonomy.
kb	kilobase pairs.
LB	Lauria-Bertani.
LøP	lymphocyte proliferation.
MHC	major histocompatability complex.
MIRU	mycobacterial interspersed repetitive unit.
MPTR	major polymorphic tandem repeat.
MW	molecular weight.
MWCO	molecular weight cut-off.
NCBI	National Center for Biotechnology Information.
NK	natural killer T-lymphocyte.
NVL	no visible lesions.
OD	optical density.
OIE	Office International des Epizooties.
ORF	open reading frame.

PBMC	peripheral blood mononuclear cells.
PBS	phosphate buffered saline.
PCR	polymerase chain reaction.
PGRS	polymorphic GC rich repetitive sequence.
PMSF	phenylmethylsulfonyl flouride.
PO_4SB	phosphate start buffer.
PO_4SB	phosphate wash buffer.
PPD	purified protein deriviative.
PPD-A	PPD derived from *M. avium.*
PPD-B	PPD derived from *M. bovis.*
RFLP	restriction fragment length polymorphism.
RNA	ribosomal nucleic acid.
r.p.m	revolutions per minute.
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide electrophoresis.
SIT	single intradermal test.
TAE	Tris-acetate.
TB complex	*Mycobacterium tuberculosis* complex.
TBE	Tris-borate.
TIGR	The Institute for Genomic Research.
TEMED	N, N, N', N'-tetramethylethylenediamine.
Th 1/Th 2	T-helper cell phenotype Type 1/Type 2.
TNF	tumour necrosis factor.
TTBS	Tween Tris-buffered saline.
U	units.
UV	ultraviolet.
V	volts.
VNTR	variable number of tandem repeats.
WHO	World Health Organization.
w/v	weight for volume.
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside.

INTRODUCTION.

In New Zealand, cattle continue to become infected with *Mycobacterium bovis* transmitted from feral reservoir hosts such as possums (*Trichosurus vulpecula*), ferrets (*Mustela putorius furo*), and deer (*Cervus elaphus*) (Cooke 2000). At 30 June 2000, 568 of the approximately 32,000 cattle herds in New Zealand remained under movement control restrictions, and 33% of the country was designated as Vector Risk Areas (AHB 2000, Statistics N.Z. 2000).

Continued *M. bovis* infection of domestic livestock could potentially have significant economic implications, if importing countries were to apply more stringent phytosanitary conditions upon agricultural products exported from New Zealand. In 1999/2000, control of bovine tuberculosis cost a total of approximately $NZ 52 million. The test and slaughter programme and meatworks surveillance were funded by producer levies of $NZ 25 million. In addition, central and local government spent a further $NZ 26 million on vector control (AHB 2000).

In 1992, a report to the Minister of Research Science and Technology concluded that because feral vectors act as maintenance hosts for *M. bovis* and contribute to continued infection of cattle, it was unlikely that bovine tuberculosis could be eradicated by the currently applied control measures. Therefore, it was recommended that further coordinated research into alternative tuberculosis control techniques was required (Allison 1992).

The studies described herein are the continuation of a project conducted at Massey University within the framework for research coordinated by the Possum and Bovine Tuberculosis Control National Science Strategy Committee. The research was funded by the Agricultural Marketing and Development Trust (AGMARDT) and the objective was to identify protein antigens of *M. bovis* that potentially have application as components of more specific diagnostic reagents and/or vaccines against tuberculosis.

A novel method was employed to identify the segments of the *M. bovis* genome that code for proteins against which infected cattle mount a cellular immune response. A cosmid library of *M.bovis* genomic DNA was transformed into the non-pathogenic fast growing mycobacterial species *M. smegmatis*. Filtered culture medium (CF) derived from 356 individual library clones was screened in lymphocyte proliferation and interferon-gamma

production assays, for the presence of recombinant *M. bovis* antigens recognized by T-lymphocytes from cattle that had been vaccinated with *M. bovis* BCG. The CF from one cosmid clone (Cos151) stimulated responses from the PBMC of seven out of eight *M. bovis* BCG vaccinated cattle in lymphocyte proliferation and interferon gamma assays (Carpenter *et al.* 1995, Gormley *et al.* 1999).

This study describes the purification and characterization of a recombinant 8.4 kDa *M. bovis* protein that was the immunologically active component of CF derived from a subclone of *M. smegmatis* Cos151, and the identification of its gene.

Approval for the use of experimental animals was granted by the animal ethics committees of Massey University and AgResearch Wallaceville. The development in containment of the genetically modified *E. coli* and *M. smegmatis* clones described herein was approved by the Massey University Genetic Technology Committee under Section 40 of the Hazardous Substances and New Organisms Act 1996 (GTC application GM099/MU/68: 'Identification, production and immunological characterization of protein antigens from *Mycobacterium bovis/Mycobacterium tuberculosis*, which are potential components of improved preparations for diagnosis and/or vaccination.').

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1.1 HISTORICAL OVERVIEW.

Archeological evidence indicates the disease tuberculosis has had an intimate association with human civilization since man first kept domesticated livestock (Ryan 1993). From around 2000 B.C, Hindu writings described a disease with clinical manifestations resembling tuberculosis in man and elephants (Kleeburg 1975), and the Old Testament describes a gradual progressive emaciation as a *consumption.*

The Lord shall smite thee with a consumption, and with a fever, and with an inflammation...and they shall pursue thee until thou perish.

Deuteronomy 28 : 22

Hippocrates was the first to describe the progressive wasting associated with lung disease as *phthisis*, and Columella described *consumption* of cattle in 14 A.D (Kleeburg 1975). Much later in 1679, Franciscus Sylvius was the first to describe the lung nodules characteristically associated with *phthisis* as "tubercula" (Daniel *et al.* 1994).

Until the late nineteenth century tuberculosis was described in terms of its clinical and pathological manifestations. For example, Gaspard-Laurent Bayle (1774 - 1816) advanced the concept that *phthisis* was progressive and that each stage might have different manifestations. He offered the analogy of an oak tree. “The mature oak would represent the florid disease. But the seedling oak, while very different from the full grown tree, is none the less an oak”. Similarly, René-Théophile-Hyacinthe Laennec (1781 - 1826) recognized that although *phthisis* might take on more than one form, he considered “tuberculous phthisis” to be a specific destructive disease of the lung uniquely associated with the tubercle (King 1982).

In 1868, Jean-Antoinne Villeman published the book *Études sur la tuberculose* in which he contended that the manifestations of tuberculosis were the end result of processes initiated by an inciting aetiologic agent. Villeman conducted a series of transmission experiments, and successfully reproduced the disease in animals by injecting caseous material taken from human and bovine cases of tuberculosis.

In 1882, Robert Koch revolutionized the understanding of infectious diseases, and tuberculosis in particular. He isolated bacilli from human tubercles and grew them in pure

culture on solid media. He also stained the tubercle bacilli and described their microscopic appearance. Finally, he injected the cultured bacteria into guinea-pigs and recreated the disease.

Koch also made the first experimental observations of immunological interactions between host species and tubercle bacilli (Koch 1891 cited by Rook 1983). He found that naïve guinea-pigs developed a tubercle at the site of a subcutaneous injection of live bacilli. The lesion ulcerated and persisted without healing until the guinea-pig died of disseminated disease. However, if guinea-pigs were given a second subcutaneous injection of live bacilli, the injection site became necrotic, sloughed and healed. Koch also used the supernatant from actively growing 8 week old glycerol broth cultures of tubercle bacilli ("tuberculin") as the second inoculation. A small dose induced a swelling at the injection site 24 to 72 hours after inoculation. A large dose induced necrosis at the site of the second injection, and very large doses resulted in necrosis at both sites. "Tuberculin" had no effect on uninfected guinea-pigs. Consequently, Koch proposed using subcutaneous injections of "tuberculin" for the diagnosis and treatment of tuberculosis (reviewed by Brown 1983, Rook 1983, Stanford 1983, Dungworth 1993, Andersen and Brennan 1994, Rook and Bloom 1994, Rook 1994).

In 1907, von Pirquet reported that when infected guinea-pigs were injected intradermally with small doses of tuberculin, an oedematous indurated swelling occured at the site 24 to 96 hours later. The phenomenon he observed is now known as cutaneous delayed-type hypersensitivity (cutaneous DTH). The following year, Moussu and Mantoux evaluated the responses elicited by injecting tuberculin into the caudal fold of tuberculous cattle, and since 1910 the intradermal tuberculin test has been the standard ante-mortem diagnostic test for tuberculosis in both man and domestic livestock (Francis 1958).

1.1.1 Control measures implemented in the twentieth century.

In developed countries, human tuberculosis was brought under control by a combination of diagnosis using the intradermal test and drug treatment, with or without BCG vaccination. From the early 1920s, attenuated strains of bovine tubercle bacilli (*M. bovis* BCG) have been used to vaccinate children considered to be at risk of contracting the disease. However, in the 1950s the advent of combined antibacterial and antibiotic treatment revolutionized tuberculosis control in humans, and since the late 1960s few developed countries continued to practice widespread BCG vaccination (Ryan 1993).

As an adjunct to control of human tuberculosis, developed countries introduced measures to prevent transmission of the disease from domestic livestock. Bovine tuberculosis was eradicated or reduced to a very low prevalence by slaughter of test-positive animals, and milk pasteurisation became almost universal. Consequently, transmission of disease from cattle to humans virtually ceased, and tuberculosis came to be considered a disease of the past. Nevertheless, despite implementing standard test and slaughter control programmes, some developed nations have failed to prevent continued infection of domestic livestock. In those countries, although few animals develop clinical tuberculosis and the public health significance is negligible, the implications for trade in animal products imposes significant economic costs (Livingstone 2000 cited by AHB 2000).

The intradermal diagnostic test is not particularly accurate and BCG vaccination is not fully protective (Colditz *et al.* 1994, Monaghan *et al.* 1994), therefore tuberculosis control programmes have been expensive and protracted (Brennan 1997). Recent advances in the fields molecular biology and immunology have already resulted in improvements in both laboratory and field diagnosis. However, there remains considerable scope for more accurate diagnostic methods, and for vaccines that stimulate immune responses that successfully prevent tubercle bacilli establishing infection (Andersen 2001).

1.2 THE BACTERIUM.

1.2.1 Taxonomy.

Tubercle bacilli are mycobacteria. They belong to the genus *Mycobacterium*, which is the only genus in the family *Mycobacteriaceae*, within the order *Actinomycetales.* Mycobacteria are classified by phenotypic and genotypic methods under the oversight of the International Working Group on Mycobacterial Taxonomy (IWGMT). In 1998, the genus *Mycobacterium* consisted of 74 recognized species of saprophytes, facultative or obligate pathogens (Goodfellow and Magee 1998).

Mycobacteria are described as acid-fast. Arylmethane dyes in phenol-water such as carbol fuchsin, carbol crystal violet, or carbol auramine O, form acid-stable complexes with mycolic acids in the mycobacterial cell envelope and are not decolourized by one to two per cent hydrochloric acid in ethanol (Barksdale and Kim 1977).

Members of the genus *Mycobacterium* are broadly categorized as either slow or fast growing species. Slow growing species take longer than seven days to appear as visible colonies on solid media (Levy-Frebault and Portaels 1992). There are 40 recognized fast growing species (9 pathogenic, 31 non-pathogenic), 33 slow growing species (26 pathogenic, 7 non-pathogenic) and one uncultivable pathogenic species (*M. leprae*) (Goodfellow and Magee 1998).

The mycobacterial species that cause tuberculosis in man and domestic animals are *M. tuberculosis*, *M. bovis* and *M. africanum*. They are slow growing and microbiological diagnosis is protracted, whilst it can take up to three months to identify them by the traditional means of culture and biochemical tests (Goodfellow and Magee 1998). The procedures currently recommended for identifying slow growing pathogenic mycobacteria are detailed in the World Health Organisation publication 'WHO / Guidelines for speciation within the *M. tuberculosis* complex' (Grange and Yates 1994), and the necropsy and culture techniques for post-mortem microbiological diagnosis of tuberculosis in livestock have been descibed in detail (Corner 1993, Corner 1994).

1.2.2 Mycobacterium tuberculosis complex.

The *Mycobacterium tuberculosis* (TB) complex consists of the slow growing mycobacterial species: *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti* (Wayne and Kubica 1986, Levy-Frebault and Portaels 1992). The mycobacterium that causes tuberculosis in seals (Cousins *et al.* 1993, Romano *et al.* 1995), and *M. canetti* (van Soolingen 1997) are also closely related to the TB complex species. Isolates from tuberculous goats in Spain were found to be intermediate between *M. tuberculosis* and *M. bovis*, and it has been proposed that they be classified as a new member of the TB complex named *M. tuberculosis* subsp. caprae subsp. nov. (Aranaz *et al.* 1999). The common feature of all TB complex species is that they are obligate pathogens of mammals (Goodfellow and Magee 1998).

Although humans are the definitive host for *M. tuberculosis* and *M. africanum*, other animals can become infected (Aranaz 1996). Mice, rabbits and guinea-pigs can be infected with *M. tuberculosis* experimentally, and develop progressive disease (McMurray *et al.* 1996). Cattle can be infected with *M. tuberculosis* and become transiently intradermal tuberculin test positive, but infection has not been found to persist (Francis 1958).

M. bovis has the widest host range of any member of the TB complex, and causes disease in many species of herbivores, carnivores, and omnivores (O'Reilly and Daborn 1995, Cooke 2000). *M. bovis* can be transmitted from animals to humans, and tuberculosis in humans caused by *M. tuberculosis*, *M. bovis* and *M. africanum* is clinically indistinguishable (Cosivi *et al.* 1998).

The *M. bovis* BCG vaccine (Bacillus Calmette Guérin) was derived from a French isolate of bovine origin, and passaged on potato medium supplemented with ox bile from 1908 to 1921 (Calmette 1928). Subsequently numerous daughter strains were distributed to various laboratories in many countries (Oettinger *et al.* 1999, Grange 2000).

Members of the TB complex share at least 99.9% identity at the DNA level (Imaeda 1985, Rogall 1990), which was demonstrated by comparison of 16S RNA (Böddinghaus 1990, Kirschner 1993), 16S and 23S ribosomal RNA intergenic spacer sequences (Frothingham *et al.* 1994), and structural genes (Kapur *et al.* 1994, Sreevatsan *et al.* 1997a). Based on the assumption that *M. tuberculosis* accumulated synonymous mutations (silent changes in the third base position) at a constant rate similar to other bacteria, it was estimated that divergence within the TB complex has occured over 15,300 to 20,400 years (Kapur *et al.* 1994). Hence, it has been speculated that *M. tuberculosis* arose from *M. bovis*, since cattle and goats were domesticated more than 10,000 years ago (Kapur *et al.* 1994, Espinosa de los Monteros *et al.* 1998, Taylor *et al.* 1999).

1.2.3 Genotypic typing of mycobacterial isolates.

In the last decade, numerous researchers have investigated a variety of methods for genotypic typing of mycobacterial isolates. Genotypic typing provides more information then phenotyping, thus facilitates molecular epidemiological traceback studies (Kremer *et al.* 1999). In New Zealand, a restriction fragment analysis technique has been used to identify the source of *M. bovis* during outbreaks affecting domestic livestock, but it is technically demanding (Collins and de Lisle 1984, Collins and de Lisle 1985, Collins *et al.* 1993, Collins *et al.* 1994). The Tuberculosis in Animals Subsection of the International Union Against Tuberculosis and Lung Disease (IUATLD) recently identified a need to ensure the methods used for genotypic typing of *M. bovis* isolates were comparable, and recommended a standardized approach to be used for DNA fingerprinting (Cousins *et al.* 1998b).

A number of PCR based techniques have been investigated for genotypic typing of *M. bovis* (Cousins *et al.* 1998a, Aranaz *et al.* 1998, Zumárraga *et al.* 1999a, Costello *et al.* 1999). The Australian bovine tuberculosis eradication programme applied a multiplex PCR technique for rapid presumptive bacteriological diagnosis of lesions found in cattle at slaughter. The technique distinguished between TB complex, *M. avium* complex and *M. intracellulare* infection (Wilton and Cousins 1992).

Recently, a number of newly described repetitive and non-repetitive polymorphic genetic loci have been used as markers for genotypic typing of mycobacterial isolates (Mahairas *et al.* 1996, Philipp *et al.* 1996a, Philipp *et al.* 1996b, Brosch *et al.* 1998, Gordon *et al.* 1999a). The polymorphic genetic loci that have been found to differentiate between *M. tuberculosis* and *M. bovis* are described in sections 1.2.3.1 and 1.2.3.2.

1.2.3.1 Non-repetitive polymorphic loci:

A number of genes have been identified that are unique to the TB complex species. The gene *mpb-70* occurs in all members of the TB complex, but is invariant (Terasaka *et al.* 1989, Cousins *et al.* 1992). The gene *mpt-40* occurs in most but not all isolates of *M. tuberculosis* and *M. africanum*, some *M. microti*, but not in *M. bovis*, *M. bovis* BCG or non TB complex mycobacteria (Del Portillo *et al.* 1991, Parra *et al.* 1991, Liébana *et al.* 1996, Weil *et al.* 1996). There is a single nucleotide difference in the *oxy R* gene between *M. tuberculosis* and *M. bovis* (Sreevatsan *et al.* 1996). Similarly, the *pnc A* gene of *M. bovis* has a single nucleotide change that results in an inactive pyrazinamidase enzyme (Scorpio and Zhang 1996, Sreevatsan *et al.* 1997b).

Some strains of *M. bovis* BCG lack the TB complex specific RD2 region that contains the gene *mpb-64* (Li *et al.* 1993), and a polymorphism downstream from the gene *mpb-64* enabled *M. tuberculosis*, *M. bovis*, and the seal mycobacterium to be differentiated (Fisanotti *et al.* 1997). The *M. tuberculosis* region designated alternatively as RD9 (Gordon *et al.* 1999a), or RD12 (Behr *et al.* 1999), and a 12.7 kb fragment downstream from the RD2 region (Zumárraga *et al.* 1999b) are absent from *M. bovis*. Whereas, a fragment specific to *M. bovis* by PCR has differentiated *M. tuberculosis* from *M. bovis* following probe hybridization RFLP (Rodriguez *et al.* 1995). A 2405 base pair (bp) fragment adjacent to the P_{AN} promoter occurs in *M. tuberculosis*, *M. bovis* BCG and some *M. bovis* isolates of French origin. However, the fragment appears to be absent from *M.*

bovis isolates from other geographical locations and non-TB complex mycobacteria (Rauzier *et al.* 1999, A. Murray *pers comm*).

1.2.3.2 Repetitive polymorphic loci.

There are a number of repetitive DNA sequences in TB complex species (Gordon *et al.* 1999b). At some loci the repetitive elements are polymorphic not only between TB complex species, but also between isolates of the same species. Therefore, genotypic typing based on polymorphic repetitive loci has found particular application in molecular epidemiological studies. Isolates of *M. tuberculosis* and *M. bovis* have been differentiated by polymorphisms in the number of copies of repeat loci, sequence differences between repeat elements within loci, or the number of tandem repeats occuring at particular loci (Fisanotti *et al.* 1998, Kremer *et al.* 1999). The features of the most widely studied polmorphic loci are described below.

Insertion sequences.

IS6110 (also known as IS986 in *M. tuberculosis*, or IS987 in *M. bovis* BCG) was the first repetitive DNA sequence reported that was thought to be exclusive to the TB complex (Eisenach *et al.* 1988, Zainuddin and Dale 1989, Eisenach *et al.* 1990, Hermans *et al.* 1990, Thierey *et al.* 1990). It is an insertion sequence of 1355 bp, and is related to the IS*3* family of insertion sequences found in *Enterobacteriacae* (Zainuddin and Dale 1989, McAdam *et al.* 1990). The genomes of *M. tuberculosis* and *M. africanum* generally contain six to 25 copies, *M. bovis* has one to five copies, whereas *M. bovis* BCG has a single copy at an invariable locus (Eisenach *et al.* 1990).

The polymorphic chromosomal distribution of copies of IS6110 forms the basis of a widely used RFLP fingerprint analysis technique (Hermans *et al.* 1990, van Soolingen *et al.* 1991, van Soolingen *et al.* 1993, van Embden *et al.* 1993, Poulet and Cole 1995a, Liébana *et al.* 1997, Yang *et al.* 1998, Kremer *et al.* 1999). For *M. bovis*, the discriminitive power of IS6110 based typing is poor because 80 to 90% of isolates have only one copy of IS6110, and some strains have no copies (Cousins *et al.* 1998a).

Sequences homologous to IS6110 have been found in non-TB complex species of mycobacteria (Liébana *et al.* 1996, Hellyer *et al.* 1998), *Arthrobacter* species and *Pseudomonas aeruginosa* (Kato *et al.* 1994). Therefore, target sequences for PCR amplification of IS6110 must be chosen with care (van Soolingen *et al.* 1993, Yuen *et al*

1993, Kent *et al.* 1995, Hellyer *et al.* 1996). A number of PCR based typing methods directed at IS6110 have been developed, but it is reported that a mixed-linker PCR technique provides the most information for epidemiological investigations (Kremer *et al.* 1999).

Another repetitive insertion sequence specific for the TB complex is IS1081 (Collins and Stephens 1991). It is 1324 bp long and occurs five or six times per genome, however its chromosomal distribution is relatively invariant. Therefore, although IS1081 fingerprinting distinguishes *M. bovis* BCG from other members of TB complex (van Soolingen *et al.* 1992), for RFLP hybridization typing of *M. bovis* IS1081 has been used in combination with IS6110 (Collins *et al.* 1993).

The Direct Repeat locus.

The DR locus contains multiple copies of conserved 36 bp direct repeats (DRs) interspersed with nonrepetitive 34 to 41 bp spacer sequences. A direct variable repeat (DVR) is comprised of one repeat unit plus the adjacent spacer sequence, and the number of DVRs varies between isolates (Hermans *et al.* 1991). The direct repeat (DR) locus is present only in TB complex bacteria, and usually contains a copy of IS6110 (Groenen *et al.* 1993, Fang *et al.* 1998).

A method of simultaneous detection and strain differentiation known as spacer oligotyping (spoligotyping) is based on the hybridization patterns of PCR amplified DVRs with a panel of 43 spacer oligonucleotides. Spoligotyping not only differentiates strains within TB complex species, but also between *M. tuberculosis* and *M. bovis* (Kamerbeek *et al.* 1997). The spoligotyping technique has been extensively characterized (Aranaz *et al.* 1998, Cousins *et al.* 1998a, Fisanotti *et al.* 1998, Costello *et al.* 1999, Kremer *et al.* 1999, Zumárraga *et al.* 1999a, van Emben *et al.* 2000), and is one of the techniques recommended by IUATLD for DNA fingerprinting *M. bovis* isolates (Cousins *et al.* 1998b).

Polymorphic GC rich repetitive sequences.

Polymorphic GC Rich Repetitive Sequences (PGRS) are the most abundant repetitive elements in mycobacteria, and display significant polymorphism (De Wit *et al.* 1990, Ross *et al.* 1992, Doran *et al.* 1993, van Soolingen *et al.* 1993). PGRS have a GC content of approximately 80% and are derived from a 9 bp repeat of $^{5'}$CGGCGGCAA$^{3'}$ arranged in tandem copies. PGRS clusters vary in size from 0.7 to 1.3 kb and share about 70%

sequence identity (Poulet and Cole 1995b). A comparison of techniques for DNA fingerprinting *M. bovis* isolates found that PGRS are the most powerful marker for RFLP analysis (Cousins *et al.* 1998a), but because PGRS elements exist in many non-TB complex mycobacteria their usefulness for primary identification is limited (Poulet and Cole 1995b).

Variable numbers of tandem repeats loci.

The genomes of TB complex species contain numerous loci that consist of tandemly repeated DNA sequences. Some loci are polymorphic with respect to the number of copies of the repeated units and are described as variable numbers of tandem repeats (VNTR) loci. Polymorphisms in the number of tandem repeats at each VNTR locus can be easily determined by analysis of PCR product sizes following agarose gel electrophoresis (Hermans *et al.* 1992, Frothingham 1995).

There are two types of tandem repeat loci, Major Polymorphic Tandem Repeats (MPTR) and Exact Tandem Repeats (ETR). MPTR consist of 15 bp repeats of a single consensus sequence, but with substantial sequence variation between repeats. Of the five TB complex MPTR loci that have been analysed, one (MPTR-A) is a VNTR locus (Frothingham 1995).

Exact tandem repeat (ETR) loci consist of large (40 to 110 bp) tandem repeats without spacer DNA. There is minimal sequence variation between repeat units, although the outermost repeat units may be truncated (Frothingham 1995). A study which investigated six ETR and MPTR-A by VNTR analysis identified 22 allele profiles in 25 TB complex isolates, and the allele profiles of each TB complex species were distinctly clustered. The same study reported that computer analysis of the sequences of *M. tuberculosis* H37Rv available in October 1997 identified 34 ETR loci, of which at least 13 were found to be informative VNTR loci (Frothingham and Meeker-O'Connell 1998).

Two of the VNTR loci (ETR-D and ETR-E) analysed by Frothingham and Meeker-O'Connell (1998) had been identified as Mycobacterial Interspersed Repetitive Units (MIRU) (Supply *et al.* 1997). The MIRUs are a subset of ETR composed of homologous 40 to 100 bp DNA elements. Recently, analysis of the complete *M. tuberculosis* H37Rv genome has identified 41 MIRU loci, of which 12 are VNTR loci that are polymorphic between isolates of TB complex species. The MIRU locus 21 does not occur in *M. bovis*,

as it is located in the region known alternatively as RD9 (Gordon *et al.* 1999a) or RD12 (Behr *et al.* 1999) that is absent from *M. bovis* (Supply *et al.* 2000).

1.3 PATHOGENESIS OF TUBERCULOSIS IN CATTLE.

1.3.1 Route of infection.

Cattle are the definitive maintenance host and vector of *M. bovis* and primarily contract infection by the aerogenous route (Francis 1958, O'Reilly and Daborn 1995). Infected cattle with respiratory disease disseminate bacilli when they exhale droplets containing a few bacilli, which partially evaporate to become droplet nuclei and stay airborne for extended periods. Inhaled droplet nuclei are carried deep into the lung on inspired air and infection establishes in the alveoli (Langmuir 1961). Although the alimentary tract can be affected, large infective doses must be ingested to establish infection (reviewed by Francis 1958, Lepper and Pearson 1973, Collins and Grange 1983, Pritchard 1988, Morris *et al.* 1994).

1.3.2 Development of tuberculous granulomas.

M. bovis bacilli that are phagocytosed by the resident tissue macrophages persist as facultative intracellular parasites and incite a distinctive form of chronic inflamation known as granulomatous inflammation (Rhoades and Ullrich 2000). A granuloma forms when an organized infiltrate of monocytes and lymphocytes accumulates around a focus of infected macrophages (reviewed by Cotran, Kumar and Robbins 1992, Dungworth 1993, Neill *et al.* 1994a, Cassidy *et al.* 1998).

The distinctive granuloma of established tuberculosis is the tubercle, which ranges in size from 2 to 500 mm in diameter. Tubercles are comprised of three distinct concentric layers. The centre consists of caseous nectoric debris that may be calcified. The middle cellular layer is composed of organized concentric sheets of differentiated epithelioid macrophages and multinucleate giant cells, interspersed with rafts of T-lymphocytes and some plasma cells. An outer fibrous capsule circumscribes the lesion, but varies from being virtually non-existent to constituting the majority of the lesion (reviewed by Francis 1958, Stamp 1959, Pallaske 1971, Gracey 1986, Dungworth 1993, Neill *et al.* 1994a).

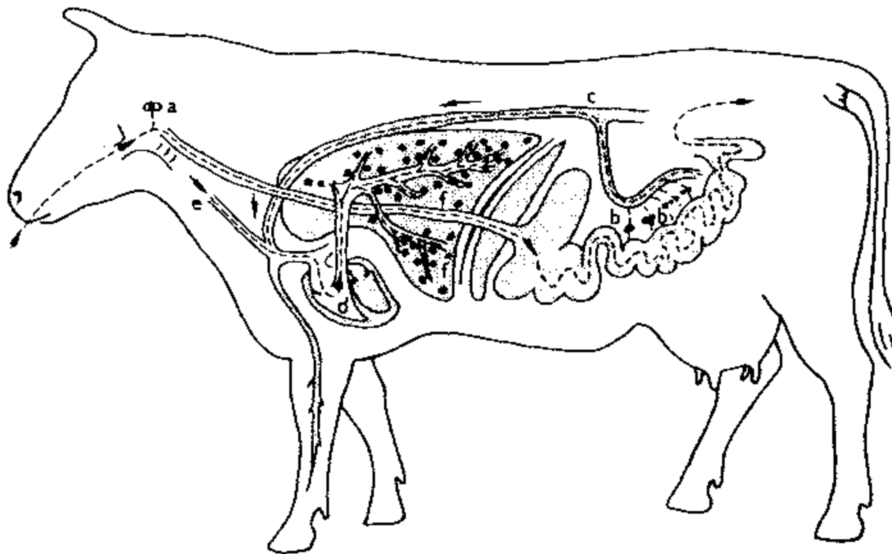

Figure 1.1 Spread of bovine tuberculosis by the lympho-haematogenous route. Tubercle bacilli enter the body by inhalation or ingestion, and may infect the retro-pharangeal lymph nodes (a). Ingestion of bacilli leads to infection of the mesenteric lymph nodes (b), with eventual passage of bacilli into the thoracic duct (c), and thence to the right ventricle (d). Breakdown of tuberculous foci in the lymph nodes of the head may allow bacilli to enter the tracheal lymph duct (e). Entry of bacilli into the pulmonary artery results in haematogenous infection of the lungs (f), and if infection is massive is manifested as numerous miliary tubercles scattered uniformly throughout both lungs. The development of generalized tuberculosis is considered to occur more frequently from lesions in the lung than from the alimentary tract (adapted from Gracey 1986).

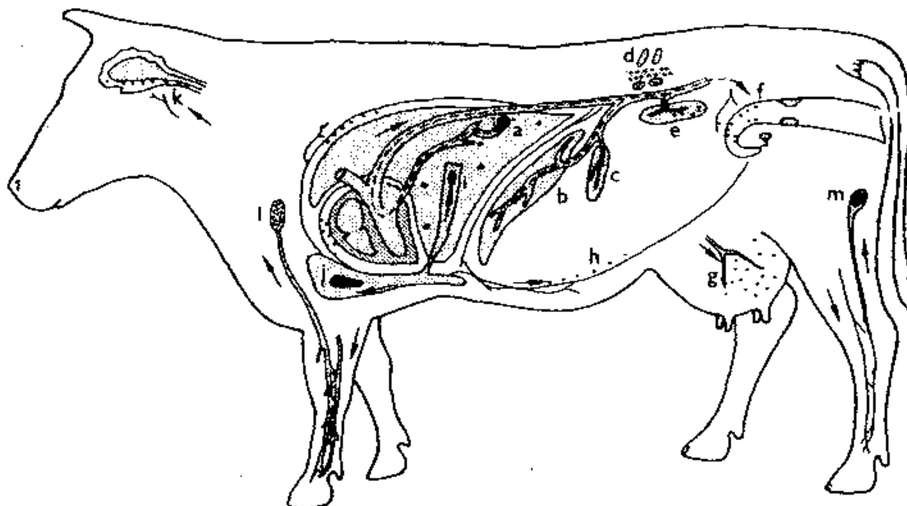

Figure 1.2 Generalization of tuberculosis from a lung lesion (a). If such a lesion erodes into a branch of the pulmonary vein, bacilli are carried to the left ventricle and enter the systemic circulation to involve the liver (b), spleen (c), bodies of the vertebrae (d) or other bones, kidneys (e), uterus (f), udder (g), ribs (i), and sternum (j). If bacilli enter the arteries of the legs, the result may be infection of the carcase lymph nodes e.g. prescapular (l) and popliteal (m). Bacilli entering the aorta may give rise to lesions in the brain or cranium, eventually producing a tuberculous meningitis (k). The peritoneum (h) may also be affected following haematogenous spread (adapted from Gracey 1986).

The tubercle is a typical high turnover immune-mediated granuloma, and the few bacilli within the lesion reside within a small percentage of the epithelioid macrophages. The tuberculous granuloma is a dynamic structure and cell recruitment and differentiation is dependent on a continued T-lymphocyte mediated immune response directed against *M. bovis* antigens presented by the infected macrophages (Dungworth 1993).

1.3.3 Progression of tuberculous disease.

After *M. bovis* bacilli become lodged in the repiratory or alimentary tracts, one or more small foci of infection are established. Later, bacilli travel via the draining lymphatics and lodge in the regional lymph nodes where macroscopic tubercles develop. The combination of a lesion at the site of primary infection and in the regional lymph node is called the "primary complex of Ranke" (see Figure 1.1) (Francis 1958, Gracey 1986).

Although tuberculosis of cattle is invariably progressive, under usual husbandry conditions cattle are slaughtered before the tuberculous process has progressed beyond the primary complex, and before clinical signs of disease are apparent (Francis 1958). Slaughter surveys conducted in Northern Ireland found that tuberculin test positive animals had a mean of 1.2 macroscopic lesions, of which 85% were associated with the respiratory tract (Neill *et al.* 1994a). Similarly, in New Zealand the mean number of tuberculous lesions found at slaughter was 1.2 per animal. Most lesions were less than 20 mm in diameter, and 65% were pharangeal or thoracic whilst 16% were abdominal (Crews 1991).

If infected animals are not slaughtered, lesions expand by direct extension and satellite tubercles form around bacilli that escape the initial focus of infection. Later, as peripheral fibroplasia and central necrosis develop concomitantly, the encapsulating fibroplasia may become greater in extent than the central cellular and necrotic parts of the lesion. Eventually the lesions may coalesce to form large multi-loculated tubercles.

When the immune response of the host is compromised, containment of the tuberculous process fails and post-primary dissemination occurs. The bacilli spread locally via the airways and lymphatics. If bacilli gain entry to the systemic circulation, either via the efferent lymphatics or directly through eroded veins, "generalization" occurs. When small numbers of bacilli enter the blood stream intermittently, generalization is protracted. Typically, well encapsulated tubercles with inspissated, calcified, caseous centres form in

many organs resulting in any combination of caseous pneumonia, caseous metritis, caseous mastitis, caseous pleuritis, caseous lymphadenitis or caseous nephritis (see Figure 1.2).

If large numbers of bacilli enter the circulation in a single episode, generalization is said to be massive. The disease becomes fulminant, and clinically the animal becomes cachectic. Numerous small miliary tubercles form in the lung, lymph nodes, skeleton and serous membranes. However, metastatic tubercles rarely affect the salivary glands, pancreas, spleen, liver, brain, myocardium or skeletal muscles (reviewed by Francis 1958, Stamp 1959, Pallaske 1971, Gracey 1986, Dungworth 1993, Neill *et al.* 1994a).

1.4 THE IMMUNE RESPONSE TO INFECTION WITH *M. bovis*.

Conceptual models to explain the mechanisms of the immune responses to mycobacteria have been developed from studies of *M. tuberculosis* and *M. bovis* BCG infection in laboratory animals. In recent years, studies with gene knock-out mice have enabled the function of various components of the immune response to be dissected. Consequently, there have been great advances in the conceptual understanding of the interdependent pathways that contribute to the development of the tubercle and effect protective immunity (reviewed by Stenger *et al.* 1997, Kaufmann and Andersen 1998, Ehlers 1999, Kerksiek and Pamer 1999, Silva *et al.* 1999, Orme 2000, Saunders and Cooper 2000). Recent detailed immunological studies of *M. bovis* infection in cattle, have shown that the murine models appear to reflect the immune responses of cattle remarkably well (Pollock *et al.* 1996, Ng *et al.* 1997, Lyashchenko *et al.* 1998b, Liébana *et al.* 1999, Wedlock *et al.* 1999, Hope *et al.* 2000, Rhodes *et al.* 2000c).

According to the current models, tubercle bacilli that lodge in the host's lung are phagocytosed by alveolar macrophages, that respond by flattening against the alveolar wall. The bacilli gain access to the interstitium of the lung where they and their products are phagocytosed by resident tissue macrophages, or are endocytosed by immature dendritic cells after binding to receptor molecules on the cell surface including those for complement and mannose (Schlesinger 1993, Mosser 1995), or to Toll-like receptors (Aderem and Ulevitch 2000).

Mycobacterial components directly induce dendritic cells to mature (Henderson *et al.* 1997). The maturing dendritic cells migrate out of the lung, travel via the draining

lymphatics and lodge in the T-cell areas of the regional lymph node. The mature infected dendritic cells have upregulated expression of the costimulatory molecules B7-1 and B7-2, the intercellular adhesion molecule (ICAM-1), the signalling molecule CD40, and the major histocompatability complex (MHC) molecules that present antigen to T-lymphocytes. At this time, the activated dendritic cells have lost the ability to capture antigen, but have aquired the ability to activate naïve T-cells, initiate their clonal expansion and modulate the immune response by producing soluble mediators. They secrete the regulatory cytokines interleukin-12 (IL-12), IL-1●, interferon alpha (IFN-α) and IFN-β. Interleukin-12 bridges the innate and aquired immune responses, whilst it activates naïve natural killer (NK) and other T-cells, and influences their phenotypic differentiation (reviewed by Reis e Sousa *et al.* 1999, Busch *et al.* 2000, Demangel and Britton 2000, Bodnar *et al.* 2001).

Infected macrophages at the site of infection and dendritic cells in the lymph nodes also produce the pro-inflammatory cytokines IL-1, IL-6 and tumour necrosis factor alpha (TNF-α). The pro-inflammatory cytokines act locally to upregulate the expression of various surface molecules on vascular endothelial cells, which facilitates recruitment of leukocytes from the circulation (Bodnar *et al.* 2001, Gonzalez-Juarrero and Orme 2001).

Monocytes and lymphocytes accumulate around the infected cells to form a granuloma, which by definition consists of an organized cellular infiltrate. The infiltrating $\gamma\delta$ T-lymphocytes have an innate capacity to respond to mycobacterial antigens without prior activation, and coordinate the aggregation of infiltrating cells. The granuloma constitutes a physical barrier that prevents bacilli from disseminating. It also enables the closely apposed macrophages and T-cells to interact by direct contact, and communicate via cytokines and chemokines to coordinate their functional differentiation (reviewed by Saunders and Cooper 2000).

Both live and dead bacilli at the centre of the developing granulomas are phagocytosed by macrophages. Phagosomes containing live bacilli do not undergo the normal sequential stepwise maturation process of fusion with early endosomes, late endosomes and lysosomes. Instead, infected phagosomes interact with the early endocytic recycling system, but do not acquire the protein Rab-7 which is a component of the vesicle fusion machinery. Therefore, the infected phagosomes cannot fuse with late endosomes and lysosomes. The consequence of the failure of phago-lysosome fusion is that live bacilli

avoid the bacteriostatic mechanisms of the host macrophage, and thrive in intracellular vesicles that are not acidified (reviewed by Rhoades and Ullrich 2000).

In contrast, phagosomes that contain dead bacilli traffic normally and become mature phago-lysosomes. Current models of mycobacterial pathogenesis suggest that live bacilli secrete virulence factors that integrate into the phagosomal membrane and prevent fusion. Alternatively, it is suggested that virulence factors escaping into the cytosol could interfere with the host cell's cytoskeletal system and prevent phagosomes from trafficking along the endocytic pathway (Wei *et al.* 2000).

Macrophages are described as professional phagocytes. They initiate and perpetuate the acquired immune response to *M. bovis* by presenting processed antigen on their surface to various T-lymphocyte subsets (Ramachandra *et al.* 1999, Underhill *et al.* 1999, Aderem and Ulevitch 2000). Particulate antigens are degraded in phago-lysosomes and transit through the cytosol to be presented in association with MHC class II molecules to $CD4^+$ $\alpha\beta$ T-cells (Schaible and Kaufmann 2000). Recently, *in vitro* studies have also shown that when bone marrow derived macrophages have phagocytosed live *M. bovis* BCG, proteins secreted by the bacilli are released from the BCG containing phagosomes into the cytosol where they intersect with vesicular compartments containing MHC class II molecules (Beatty and Russell 2000).

Phagocytosed particulate antigens are also processed along an alternative antigen processing pathway and presented in association with MHC class I molecules to $CD8^+$ $\alpha\beta$ T-cells (Lewinsohn *et al.* 1998, Canaday *et al.* 1999). Additionally, it has been shown that antigens produced by live bacilli that appear in the cytosol can be processed along the transporter associated with antigen processing (TAP) pathway and are presented in association with MHC class I to $CD8^+$ $\alpha\beta$ T-cells (Mazzaccaro *et al.* 1996, Smith and Dockrell 2000). Mycobacterial glycolipid antigens are presented by cluster of differentiation (CD1) molecules to double negative $CD4^-$ $CD8^-$ $\alpha\beta$ T-cells (DN T-cells) (reviewed by Porcelli *et al.* 1998, Park and Bendelac 2000).

Activated T-cells that bind presented antigen, proliferate and differentiate. Their functional differentiation is influenced by the relative amounts of cytokines within the granuloma. In general, macrophages infected with live bacilli produce more IL-12 than

IL-6, whereas macrophages that have phagocytosed particulate material or dead bacilli produce more IL-6 (Sinigaglia *et al.* 1999). The effect of IL-12 is to influence $CD4^+$ and $CD8^+$ T-cells to develop a Type 1 phenotype and produce IL-2, IFN-γ and TNF-α. In contrast, IL-6 promotes the development of a Type 2 phenotype, and $CD4^+$ and $CD8^+$ T-cells produce IL-4, IL-5, IL-10, IL-13 and TNF-α (reviewed by Andersen and Smedegaard 2000, Smith and Dockrell 2000).

The division of T-cell responses into Type 1 and 2 phenotypes is not absolute, but is a functional description of the two opposite poles of a spectrum of responses. Within the granuloma, cellular interactions are dynamic and the phenotype that predominates is largely dependent on the relative amounts of IFN-γ or IL-4 present. The IFN-γ produced by Type 1 T-cells inhibits the proliferation of Type 2 T-cells. Whereas, IL-4 produced by Type 2 T-cells skews differentiation towards the Type 2 phenotype, and IL-10 inhibits IFN-γ production (reviewed by Romagnani 1997, Lanzavecchia and Sallusto 2000).

The IFN-γ and TNF-α produced by Type 1 $CD4^+$ and $CD8^+$ T-cells act in concert to activate macrophages infiltrating the granuloma. Activated epithelioid macrophages are the effector cells of the immune response, and phagocytosed live bacilli are unable to block maturation of their phagosomes. A phago-lysosome forms and the oxygen dependent and oxygen independent bacteriostatic mechanisms of the macrophages are potentiated (reviewed by Andersen 1997, Orme and Cooper 1999, Stenger and Modlin 1999, Liébana *et al.* 2000a, Rhoades and Ullrich 2000).

Macrophages that support bacilliary multiplication are lysed by activated T-cells (Liébana *et al.* 2000b). A subset of $CD4^+$ T-cells and cytotoxic $CD8^+$ T-cells induce apoptosis of infected macrophages by releasing granules that contain perforin and granzyme proteases. The cytotoxic granules of $CD8^+$ T-cells also contain the recently discovered protein granulysin, which has direct bacteriocidal activity in conjunction with perforin (reviewed by Stenger *et al.* 1999, Seder and Hill 2000, Smith and Dockrell 2000). Alternatively, apoptosis is induced when the Fas ligand (CD95L) on the surface of the T-cells engages the Fas molecule (CD95) on the surface of the macrophage (Liébana *et al.* 2000a). The Fas-Fas ligand pathway is mainly employed by CD1 restricted DN T-cells to induce apoptosis of macrophages that present mycobacterial glycolipid antigens (reviewed by Porcelli *et al.* 1998, Park and Bendelac 2000).

The granuloma is a dynamic structure, in which there is continuous cell turnover. Studies with gene knock-out mice have shown that both TNF-α and IFN-γ are essential mediators for the formation and maintenance of an effective granuloma, and that $CD4^+$, $CD8^+$ and $\gamma\delta$ T-cells are all required (Roach *et al.* 1999). Following *M. bovis* infection the Type 1 immune response fails to eliminate all the bacilli, and the infection ultimately progresses to disease. At this stage, the granuloma breaks down, neutrophils infiltrate and there is liquefactive necrosis of cells. A Type 2 response predominates and antibodies are produced (Pedrosa *et al.* 2000, Saunders and Cooper 2000). In cattle, the apperance of antibodies in the serum is considered to be coincidental to disease progression (Ritacco *et al.* 1991, Hanna *et al.* 1992, Neill *et al.* 1994b), however that contention is currently being re-evaluated (Glatman-Feedman and Casadevall 1998, Teitelbaum *et al.* 1998).

In cattle, there are dynamic changes in the T-cell subpopulations circulating in the peripheral blood as the disease progresses. In ruminants, approximately 40% of circulating lymphocytes are $\gamma\delta$ T-cells (Hein and Mackay 1991, Wyatt *et al.* 1994). Soon after infection, the lymphocytes responsive to mycobacterial antigens are primarily $CD4^+$ $\alpha\beta$ and $WC1^+$ $\gamma\delta$ T-cells (Pollock *et al.* 1996). Later, reactive $CD8^+$ $\alpha\beta$ T-cells predominate (Liébana *et al.* 1999). A recent study has demonstrated that the peripheral blood lymphocyte subsets responsive to mycobacterial antigens appear to be representative of the T-cell subsets orchestrating the immune response within the tubercle (Rhodes *et al.* 2000b).

Peripheral blood mononuclear cells (PBMC) of experimentally infected calves produce both IFN-γ and IL-2 when stimulated *in vitro* with mycobacterial antigens (PPD-B tuberculin), but the cytokine response is not always correlated with cutaneous DTH. Hence, it has been speculated that different T-cell subsets mediate different arms of the immune response (Ng *et al.* 1997). Subsequently, it has been reported that following experimental infection of cattle with *M. bovis*, the IFN-γ response began approximately two weeks post-infection and was maintained, whereas the IL-4 response was confined to a peak of activity from six to twelve weeks post-infection (Rhodes *et al.* 2000c).

Another study compared the cellular and humoral immune responses of cattle infected intratracheally with virulent *M. bovis*, three attenuated strains of *M. bovis* and *M. bovis* BCG. Infection with virulent *M. bovis* induced persistently high IFN-γ and IL-2 responses and antibodies specific for PPD tuberculin. The attenuated strains induced low (1 strain) or no (2 strains and BCG) antibody responses, and the IFN-γ and IL-2 responses peaked at 2 to 4 weeks then rapidly declined. The author concluded that continued active infection is required to induce and maintain strong IFN-γ responses in cattle. However, since there was no correlation between the virulence of the *M. bovis* strain with which the cattle were infected and their cutaneous DTH responses, it was suggested that the IFN-γ and cutaneous DTH responses are dissociated (Wedlock *et al.* 1999).

Recently, it was shown that BCG vaccinated cattle posessed $CD4^+$ and $CD8^+$, but not $WC1^+$ γδ memory T-lymphocytes that proliferate and produce IFN-γ in response to mycobacterial antigens. Moreover, whilst the the $CD4^+$ T-cells demonstrated greater proliferative responses, the $CD8^+$ cells produced more IFN-γ (Hope *et al.* 2000).

1.5 CONTROL AND ERADICATION OF BOVINE TUBERCULOSIS.

Early in the twentieth century it was established that bovine tuberculosis is a zoonotic disease that can be transmitted from domestic livestock to humans. Humans usually became infected with *M. bovis* by the alimentary route, and suffered from extrapulmonary tuberculosis (Ostertag 1934). It was shown that although meat taken aseptically from infected animals was not infectious, it became contaminated from workers' equipment during dressing, and cows with tuberculous mastitis excreted *M. bovis* in their milk (reviewed by Ostertag 1934, Francis 1958, Kleeburg 1975, Pritchard 1988). Measures to control bovine tuberculosis were instituted primarily to prevent *M. bovis* being transmitted to humans. Milk was pasteurised, meat inspection ensured hygenic processing of carcasses, and programmes were implemented to eradicate tuberculosis from domestic cattle (reviewed by Francis 1958, Kleeburg 1975, Pritchard 1988).

1.5.1 Principles of bovine tuberculosis control and eradication.

The principles of infectious disease control have been known for over 100 years. To prevent infection it is necessary to cut the chain of transmission. Therefore, infected animals are identified by diagnostic tests and removed, and/or vaccination renders the population capable of resisting infection. Initially, both techniques were applied to the control of bovine tuberculosis. With experience, it was found that although the intradermal tuberculin test did not identify all infected animals it could identify infected herds, and it became the mainstay of bovine tuberculosis test and slaughter control programmes. In contrast, vaccination of cattle was discontinued because it was not efficacious, and interfered with the intradermal test (Francis 1958, Ritchie 1959, Tweddle and Livingstone 1994).

1.5.1.1 Bovine tuberculosis was successfully eradicated from some countries.

Notwithstanding the limitations imposed by the deficiencies of the intradermal test, many developed countries, including Finland, Denmark and the Netherlands had eradicated bovine tuberculosis by the middle of the twentieth century (Stenins 1949, Plum 1955, Van den Born 1956 cited by Ritchie 1959). The USA was essentially free by 1941 (Meyers and Steele 1969), and in 1997 Australia was declared a tuberculosis "Free Area" in terms of the definition of the Office International des Epizooties 'International Animal Health Code' (Cousins and Corner 1998).

In all cases, control was implemented on an area basis. Infected herds were identified both on farm by intradermal tuberculin testing, and by slaughterhouse surveillance supported by diagnostic laboratory services. To ensure that the chain of transmission was cut, test positive animals were slaughtered immediately and stock movement from infected herds to uninfected herds was prohibited (reviewed by Francis 1958, Ritchie 1959, Kleeburg 1975, Cousins and Corner 1998).

1.5.1.2 Other countries have failed to eradicate bovine tuberculosis.

Despite implementing standard control measures, some countries have failed to achieve eradication of bovine tuberculosis. Reservoirs of *M. bovis* are maintained in wild and feral animal hosts, and are a continued source of infection for domestic livestock. In New Zealand, the maintenance hosts and disease vectors are possums (*Trichosurus vulpecula*),

ferrets (*Mustela putorius furo*), and deer (*Cervus elaphus*). In Britian and Ireland, the principal feral animal reservoir host is the badger (*Meles meles*) (Morris and Pfeiffer 1991, Willesmith 1991, Morris and Pfeiffer 1995, O'Reilly and Daborn 1995, Davidson 1998, Cooke 2000).

1.5.2 Control of bovine tuberculosis in New Zealand.

The bovine tuberculosis control programme was instituted as an adjunct to the control of tuberculosis in the human population. Consequently, few infected cattle now develop disease, and they are no longer an epidemiologically significant source of human infection. However, *M. bovis* infection has become primarily an issue that affects trade in agricultural products (reviewed by Tweddle and Livingstone 1994, Davidson 1998).

In the early 1950s, the national prevalence of bovine tuberculosis was estimated to be eight to ten per cent. Compulsory testing of dairy cattle began on a regional basis in 1961 and all herds were being tested regularly by 1970. At that time, it was recognized that possums were both a maintenance host and vector for *M. bovis*, consequently possum control measures began in 1972 (Hickling 1991). Compulsory testing of beef herds began in 1970 and all herds were under surveillance by 1977. Between 1977 and 1981 the number of cattle herds under movement control restrictions declined from approximately 1500 to 511, and in 1978 the government reduced funding for the bovine tuberculosis control programme. During the 1980s, the number of herds on movement control increased steadily (reviewed by Livingstone 1991).

The Possum and Bovine Tuberculosis Control National Science Strategy Committee was established in 1991 to coordinate research on alternative methods of bovine tuberculosis control. A report prepared for the Minister of Research, Science and Technology outlined how the bovine tuberculosis control programme was failing, and recommended research into improved control techniques including diagnostic tests and vaccines against *M. bovis* infection (Allison 1992).

At present, the primary basis of the New Zealand bovine tuberculosis control programme remains test and slaughter, supplemented by population control of feral vectors. In the 1999/2000 year, 4.84 million primary intradermal tuberculin tests were conducted on 2.79 million dairy and 2.05 million beef cattle. There were 2356 test reactors and 1377 tuberculous animals were identified at slaughter. At 30 June 2000, there were 568 cattle

herds still on movement control. Despite the success of the traditional control measures, it is recognized that as the programme moves from the control phase to eradication, a prerequisite for success will be more rapid and accurate ante-mortem and post-mortem diagnosis of individual tuberculous animals (AHB 2000).

1.6 DIAGNOSIS OF INFECTION IN CATTLE.

The immune responses mounted by cattle against intracellular mycobacteria have been considered to be primarily cell mediated (Parish 1972, Pollock *et al.* 1996, Tan *et al.* 1997, Bonecini-Almeida *et al.* 1998, Hope *et al.* 2000). Therefore, to make a presumptive ante-mortem diagnosis of tuberculosis in cattle, the presence or absence of activated T-cells with specificity for *M. bovis* is determined indirectly. The principle of the tests is that animals which have previously encountered *M. bovis* possess activated T-cells with specificity for mycobacterial antigens, that respond by proliferating and producing cytokines when exposed to *M. bovis* antigens either *in vivo* or *in vitro* (Lepper and Corner 1983, Rothel *et al.* 1993, Wood and Rothel 1994, Monaghan *et al.* 1994, Cousins and Corner 1998, Hope *et al.* 2000, Buddle *et al.* 2001).

The preparations of mycobacterial antigens most commonly used for diagnostic testing are purified protein deriviative (PPD) tuberculins. In 1934, Siebert and Munday derived PPD tuberculins from liquid cultures of various mycobacterial species, and although they are described as "pure", PPD tuberculins are complex mixtures of proteins, lipids, sugars and nucleic acids. The specificity of diagnostic tests using PPD tuberculins can be compromised because many of the antigens in PPD tuberculins derived from *M. bovis* cultures are common to other members of the TB complex and to opportunistic slow growing species such as *M. avium* (Daniel and Janicki 1978, Stanford 1983, Pritchard 1988).

The processes for manufacturing and standardizing PPD tuberculins have been refined, and the currently recommended international standards for PPD tuberculin manufacture are published in the 'Office International des Epizooties, Manual of Standards for Diagnostic Tests and Vaccines' (1996). In summary, PPD-B tuberculin is produced from *M. bovis* strains AN5 or Vallee, and PPD-A from *M. avium* strains D4ER or TB56. The organisms are grown in a synthetic medium for up to three months, then killed by flowing steam and filtered. The protein in the filtrate is precipitated with ammonium sulphate or trichloro-acetic acid, washed and resuspended in a physiological buffer at a pH of 6.6 to 6.7. The

biological potency of PPDs varies between batches and cannot be predicted from the protein concentration. Therefore, batches are standardized in bioassays with homologously sensitized guinea-pigs. The cutaneous reactions elicited by serial dilutions of the test PPD tuberculin are compared to a reference tuberculin (Haagsma 1986). Subsequently, PPD-B tuberculin is diluted to a final concentration of approximately 1.0 mg / ml, and PPD-A tuberculin to approximately 0.5 mg / ml, which equates to 2000 (± 25%) International Units (IU) per 0.1 ml dose. Preparations destined for use in *in vivo* testing may have 0.5% w/v phenol added as a preservative.

1.6.1 The intradermal test.

The internationally prescribed standard method for making a presumptive ante-mortem diagnosis of *M. bovis* infection in cattle is the intradermal tuberculin test (●IE Manual of Standards for Diagnostic tests and Vaccines 1996). The most common primary test is the single intradermal test (SIT). A dose of at least 0.1 mg PPD-B tuberculin, equivalent to a minimum of 2000 IU in a volume not exceeding 0.2 ml, is injected intradermally into the side of the neck or the caudal fold. The animal is considered to be a positive reactor if a delayed type hypersensitivity reaction is visibly or palpably detectable as an indurated swelling at the injection site 72 hours later (reviewed by Monaghan *et al.* 1994).

The mechanism of the cutaneous DTH reaction is that the injected PPD tuberculin antigens are taken up by APCs in the dermis and presented in association with MHC molecules. If the animal has circulating activated or memory T-cells with specificity for the presented mycobacterial antigens a cellular infiltrate accumulates at the injection site. Histologically, the infiltrate initially consists of neutrophils and mononuclear cells, but once the swelling is grossly obvious mononuclear cells predominate (Monaghan *et al.* 1994). Skin biopsies taken from the tuberculin reactions of cattle have shown that DTH response was associated with expression of mRNA for IFN-γ, IL-2, IL-4, IL-10 and TNF-α by the infiltrating cells (Ng *et al.* 1995).

The caudal fold test was found to have a sensitivity of 82% and a specificity of 96% in the United Kingdom (Francis *et al.* 1973, Francis *et al.* 1978), a sensitivity of 80% and a specificity of 89% in Australia (Lepper *et al.* 1977), and a sensitivity of 66% and a specificity of 95% in New Zealand (Ryan *et al.* 1991). Therefore, because the accuracy of

the intradermal tuberculin test is limited it can be used for surveillance to identify infected herds but not to define the disease status of individual animals (Monaghan *et al.* 1994).

The sensitivity of the intradermal test can be increased by injecting greater amounts of PPD-B tuberculin but the specificity is decreased, especially in environments where cattle become sensitized by transient infection with non-TB complex mycobacteria such as *M. avium* (Lepper *et al.* 1979a, Pollock *et al.* 2000, Ryan *et al.* 2000). Australia was fortunate to have little problem with sensitization by non-TB complex mycobacteria causing false positive reactions (Lepper *et al.* 1977, Lepper *et al.* 1979b). Therefore, the Australian bovine tuberculosis eradication campaign adopted a dose of 0.3 mg or 15,000 IU of PPD-B tuberculin for intradermal testing, which gave a sensitivity of 88% without compromising specificity (Rothel *et al.* 1993, Cousins and Corner 1998). In New Zealand, where sensitization with *M. avium* causes test interpretation problems in some herds, the dose of PPD-B injected into the caudal fold is currently 5000 IU in 0.1 ml (Ryan *et al.* 2000).

1.6.1.1 The comparative cervical test.

Where it is suspected that positive reactions to a primary SIT are false due to sensitization by non-TB complex bacteria such as *M. avium*, a comparative test can be applied. In Europe, where the comparative test is applied as a primary test, no less than 2500 IU of PPD-B and PPD-A tuberculins in 0.1 ml are injected intradermally in separate sites 12 to 15 cm apart on the side of the neck, and the skin-fold thickness is remeasured 72 hours later. The test is interpreted according to the presence or absence of reactions, and the difference between any increase in skin-fold thickness between the two injection sites. The interpretation varies in stringency between countries, but the animal is considered to have been sensitized by the mycobacterial species that elicits the largest reaction if any, and are described as bovine or avian reactors accordingly. However, the comparative cervical test (CCT) has a lower sensitivity than the single intradermal test (reviewed by Francis 1958, Patterson 1959, Ritchie 1959, Lepper and Corner 1983, Crews 1991, Rothel *et al.* 1993, Monaghan *et al.* 1994, OIE 1996, Cousins and Corner 1998).

In New Zealand, the CCT has been used as an ancillary test applied in series to confirm sensitization by *M. avium*, but not to exclude *M. bovis* infection. The CCT is conducted a minimum of 60 days after the SIT because cutaneous reactivity is diminished for 60 to 90 days following any intradermal tuberculin test (Radunz and Lepper 1985, Monaghan *et al.*

1994). The dose of PPD-B tuberculin is 5000 IU in 0.1 ml, and the dose of PPD-A tuberculin is 2500 IU in 0.1 ml. An animal is considered to be a bovine reactor if the increase in skin thickness at the PPD-B tuberculin injection site is more than 4 mm greater than the PPD-A injection site. If all the animals in a group subjected to a serial CCT are either avian reactors or do not react to PPD-A or PPD-B tuberculin, it is strong evidence that the previous positive reactions were due to sensitization by non-TB complex mycobacteria. The lack of a reaction to either PPD-A or PPD-B tuberculins upon retest is a common occurrence, and is consistent with elimination of a transient infection of non-pathogenic mycobacteria. Conversely, if there are bovine reactors no conclusions can be drawn regarding the *M. bovis* infection status of the avian reactors, and they must be considered to be potentially tuberculous (McLaughlin 1991, Ryan *et al.* 2000).

At the outset of bovine tuberculosis control programmes when the prevalence of infection is high, the majority of test positive animals are tuberculous and the intradermal test has a high positive predictive value. As the prevalence of infection is reduced, fewer test positive animals are diseased and proportionally more animals with no visible lesions (NVL) are slaughtered. NVL reactors can be interpreted either as tuberculous animals in the early stage of disease, false positives due to sensitization by non-TB complex mycobacteria, or false positives due to inaccuracy of the intradermal tuberculin test (Monaghan *et al.* 1994, Cousins and Corner 1998).

For bovine tuberculosis control campaigns that are entering the eradication phase, it is important to have tests of high sensitivity to detect the remaining infected animals. It is equally important to have tests with high specificity to minimize the number of NVL reactors, which are a problem because cattle producers perceive the intradermal test to be inaccurate and lose confidence in the control programme (Cousins and Corner 1998). In New Zealand, unnecessary slaughter of NVL reactors was estimated to cost the industry $900,000 per annum (Ryan 1994). In 1999/2000, 55% of the 2356 reactor animals slaughtered did not have visible lesions (AHB 2000).

1.6.2 *In vitro* diagnostic assays.

To maintain producer confidence in the efficacy of bovine tuberculosis control programmes as they moved into the eradication phase, *in vitro* diagnostic assays have been developed. The principle of the *in vitro* tests of a cellular immune response is that blood lymphocytes are cultured with PPD-B and PPD-A tuberculins, and either proliferation or

cytokine production is measured. The perceived advantage is that the T-lymphocytes can interact with the antigens in standardized controlled conditions, thus the tests should be both more sensitive and specific. The practical advantages are that animals only need to be mustered once, and if repeat testing is required there is no need to wait 60 to 90 days. The practical disadvantages of *in vitro* assays are that considerable laboratory resources are required and the tests are costly (reviewed by Wood and Rothel 1994).

1.6.2.1 Lymphocyte transformation.

The usual format is that peripheral blood mononuclear cells (PBMC) are separated from whole blood, and replicate cultures are stimulated with bovine and avian tuberculins for up to five days. To measure the proliferative response, the cultures are pulsed with radioactive nucleoside towards the end of the incubation period, and the amount of radioactivity incorporated by the stimulated cultures is compared with unstimulated control cultures (reviewed by Lepper and Corner 1983). The lymphocyte transformation assays provide semi-quantitative estimates of the T-cell proliferative response to mycobacterial antigens. Although useful for research purposes, lymphocyte proliferation assays are technically demanding, time consuming and expensive. Therefore, they were not considered to be a practical alternative to the intradermal test (Wood and Rothel 1998, Cousins and Corner 1998).

1.6.2.2 Interferon-gamma assay.

The IFN-γ assay was developed in Australia in the closing stages of their bovine tuberculosis eradication programme, and provides an indirect measure of the acquired cellular immune response to *M. bovis* (Wood *et al.* 1991). To use the commercially available BOVIGAM™ Bovine Interferon Test Kit (CSL Veterinary, Australia), aliquots of whole blood are incubated for 16 to 24 hours with PPB-B or PPD-A tuberculin and a nil antigen control. The relative amounts of IFN-γ produced are determined in an enzyme immunoassay (EIA). The test is interpreted according to the differences in optical density (OD) between the three aliquots (Rothel *et al.* 1993, Buddle *et al.* 2001).

The cut-off values defining a positive response can be altered according to the purpose for which the test is applied. When the IFN-γ assay is used as a primary or parallel test it can be interpreted with low cut-off values to maximize sensitivity. In Australian field

trials, the IFN-γ assay had a sensitivity of 93.6% and a specificity of 96.3%. In the same trial, the sensitivity of the single intradermal test was 65.6%, but when the tests were interpreted in parallel the overall sensitivity was 95.2%. Although the IFN-γ assay detected infected cattle that were negative to the intradermal test, there were also infected cattle that were intradermal test positive and negative to the IFN-γ assay (Wood *et al.* 1991).

Subsequently, field trials have been conducted in several countries including Ireland, Northern Ireland, USA, Italy, Spain, New Zealand and Romania. When the IFN-γ assay was used as a parallel test its performance largely reflected the Australian findings. The sensitivity of the IFN-γ assay and the intradermal tests were not significantly different, but the two tests detected different overlapping populations of infected animals (Neill *et al.* 1994b, Wood and Rothel 1998, CSL quarterly bulletins).

However, in some instances the performance of the IFN-γ assay has been inadequate. In Ireland, the specificity of the test was unacceptable unless PPD tuberculins of local manufacture were used (Wood and Rothel 1998). Whilst in South Africa, when the IFN-γ assay was used as a primary test in buffalo, cross-reactivity caused by *M. fortuitum* infection resulted in a high incidence of false positive results (Michel and Jones 1998). The specificity was improved by stimulating a third aliqout of blood with 'tuberculin' derived from *M. fortuitum*, and interpreting the assay as a triple comparative test (CSL quarterly bulletins).

Although the IFN-γ assay is inherently a comparative test because the responses to PPD-B and PPD-A tuberculins are compared, when the IFN-γ assay is applied as a serial ancillary test following a positive intradermal test, interpretation is not straightfoward. The constraints on interpretation that apply to the CCT also apply to the IFN-γ assay. Moreover, the animals are known to be sensitized to mycobacterial antigens and the cut-off thresholds need to be set higher (Rothel *et al.* 1993, Ryan 1994, Monaghan *et al.* 1997).

Following extensive trials in New Zealand, the BOVIGAM™ test was introduced in March 1997 as an ancillary serial test, applied 10 to 30 days following a positive caudal fold test result (Ryan *et al.* 1991, Ryan 1994, Ryan *et al.* 2000). Initially, the cut-off was set at twice the level recommended by the manufacturer, and the interpretation of a positive bovine reactor was a PPD-B response ≥ 100 units above the nil antigen control, and ≥ 100 units above the PPD-A response. In 1999, the interpretation schedule was altered, and additional cut-off levels were defined. In some circumstances, the results are now interpreted only according to the difference between the PPD-B and PPD-A responses, and one of two cut-offs are employed (40 units or 70 units) depending on the results of the group being tested (CSL quarterly bulletins, Buddle *et al.* 2001).

1.6.2.3 Serological assays.

To diagnose *M. bovis* infection in cattle, current control programmes have relied on assays of the acquired T-lymphocyte response rather than the humoral response (Rothel *et al.* 1993). Although serological assays have been extensively investigated, it was found that using the available antigen preparations, assays of the humoral response to *M. bovis* suffered from a lack of specificity (Fifis *et al.* 1994b). Furthermore, serum antibodies usually do not appear until the cell mediated immune response wanes (Plackett *et al.* 1989, Ritacco *et al.* 1991). Consequent to annual intradermal testing, few infected animals remain in the herd long enough to develop advanced disease, therefore serological tests were found to lack sensitivity (reviewed by Wood *et al.* 1992, Wood and Rothel 1994, Cousins and Corner 1998).

Nevertheless, if suitable antigen preparations were available, serological tests could be applied in parallel with the intradermal test to detect the few animals with advanced disease that are anergic in the assays of the cellular response to *M. bovis* (Fifis *et al.* 1994b, Buddle *et al.* 1994, Buddle *et al.* 1995a, Buddle *et al.* 1995b, Costello *et al.* 1997, Ng *et al.* 1997, Lightbody *et al.* 1998b, Lyashchenko *et al.* 1998b, Lightbody *et al.* 2000).

1.6.3 Application of parallel *in vitro* diagnostic tests to bovine tuberculosis control in New Zealand.

Parallel *in vitro* diagnostic tests were included in the New Zealand bovine tuberculosis control programme for the first time in 1999/2000. Ancillary parallel IFN-γ and lymphocyte transformation assays (BOVIGAM™ and 'Leukocyte Screen') were

performed on 21,349 skin test negative cattle in infected herds. One percent of the animals recorded positive results, of which 22% had macroscopic tuberculous lesions at slaughter (AHB 2000).

1.6.4 Investigation of alternatives to PPD tuberculins.

Sensitization by antigens common to slow-growing mycobacterial species can complicate interpretation of diagnostic tests based on the response to PPD tuberculins. Initial investigations of the cellular and humoral responses of tuberculous cattle to partially purified *M. bovis* antigens found that many were cross-reactive. Furthermore, individual antigens were not recognized consistently by a high enough proportion of infected cattle to enable diagnostic tests with acceptable sensitivity to be developed using single antigens (Fifis *et al.* 1994a, Fifis *et al.* 1994b).

Recently, antigens with restricted species distribution such as ESAT-6 have been identified, and it has been suggested that they could be used for specific diagnosis of tuberculosis (Pollock and Andersen 1997a, Pollock and Andersen 1997b, Buddle *et al.* 2001). Experiments with guinea-pigs sensitized with either *M. bovis* BCG or *M. avium*, investigated the intradermal responses elicited by preparations of recombinant mycobacterial antigens MPT63, MPT64, MCT28, MPT32, MPT51 and the 38kDa antigen purified from *E. coli.* Four of the antigens were TB complex specific (MPT63, MPT64, MTC28 and MPT70). As expected, only *M. bovis* BCG sensitized animals responded to a 'cocktail' of the TB complex specific antigens, whereas both ***M. avium*** and ***M.bovis*** BCG sensitized animals responded to a 'cocktail' of cross-reactive antigens (Lyashchenko *et al.* 1998a).

A number of studies have also been conducted in naturally and experimentally infected cattle to investigate both cellular and humoral responses to individual mycobacterial antigens. Investigations of the cellular responses using either lymphocyte proliferation or cytokine production assays, found that fewer animals had positive responses to single antigens than to PPD-B, and that the responses of individuals fluctuated over time. However, whilst the sensitivity of testing with the individual TB complex specific antigens MPB70, MPB64, ESAT-6 and CFP10 was lower than testing with PPD-B, the specificity approached 100%. Moreover, it was found that when antigens were combined as 'cocktails' or the responses to each antigen were interpreted in parallel, the sensitivity was

increased substantially (Lightbody *et al.* 1998a, Pollock *et al.* 2000, Rhodes *et al.* 2000a, van Pinxteren *et al.* 2000).

Similar studies have investigated the feasibility of discriminating between cattle vaccinated with *M. bovis* BCG or infected with *M. bovis* by using antigens that are either not produced by *M. bovis* BCG Pasteur (ESAT-6, MPB64), or are only produced at low levels (MPB70, MPB83). The assays were discriminatory but lacked sensitivity, since only 60 to 70% of PPD-B positive infected animals had positive responses to the individual antigens (Buddle *et al.* 1999, Vordermeier *et al.* 1999).

Studies of the humoral responses of tuberculous cattle found that the major seroreactive antigens were ESAT-6, 14 kDa, MPT63, MPT70, MPT51 and MPT32, but the appearance of serum antibodies was delayed until five months to one year post-infection, and there was marked heterogeneity in antigen recognition between animals over time (Lyashchenko *et al.* 1998b). Other studies reported that the serum antibodies that became detectable from 400 to 600 days post-infection were predominantly against MPB70, and to a lesser extent MPB64 and MPB59 (Lightbody *et al.* 1998b). Interestingly, it was found that cattle with macroscopic tuberculous lesions had enhanced IgG responses from seven to 16 days after intradermal tuberculin injection (Lightbody *et al.* 2000).

Those studies have demonstrated that it is feasible to enhance the specificity of tuberculosis diagnosis by using cocktails of *M. bovis* specific antigens in place of PPD-B, but a greater number of antigens need to be included if sensitivity is to be maintained.

1.7 VACCINATION OF CATTLE AGAINST TUBERCULOSIS.

1.7.1 Origins of BCG and its use in humans.

If cattle were vaccinated against *M. bovis*, the aim would be to stimulate acquisition of a cell-mediated immune response that would prevent establishment of infection (Livingstone 2000 cited by AHB 2000, Vordermeier *et al.* 2001). The most widely trialed vaccine against tuberculosis is *M. bovis* BCG (Bacillus Calmette Guérin). It is a variant of *M. bovis* that was developed from tubercle bacilli isolated from a case of tuberculous mastitis in a heifer by Nocard in 1901. The isolate was passaged 230 times on potato-glycerin media with added ox bile, for 13 years from 1908 to 1921. Calmette and Guérin noted that the colony morphology had changed and determined that the strain was no longer virulent

(Calmette 1928). Subsequently, many daughter strains were distributed worldwide (Oettinger *et al.* 1999). Genetic differences have been described between *M. bovis* BCG substrains and *M. bovis*, but the differences that account for attenuation have not been determined (Mahairas *et al.* 1996, Philipp *et al.* 1996b).

There is conflicting evidence regarding the efficacy of BCG vaccination. The consensus of various trials, in both humans and laboratory animals, is that BCG vaccination in the absence of progressive disease elicits a protective cell-mediated immune response that prevents uncontrolled replication of infecting tubercle bacilli and dissemination from the primary focus. Thus, although BCG vaccination effectively protects against extrapulmonary disease it has not been found to protect against the establishment of primary aerogenous infection and pulmonary disease (reviewed by Bloom and Fine 1994, Colditz *et al.* 1994, Hart and Parry 1996, Huebner 1996, Oettinger *et al.* 1999, McMurray 1999, Grange 2000).

1.7.2 Vaccination of cattle with BCG.

BCG vaccination of cattle was trialed in Britian during the 1950s. It was found that *M. bovis* BCG injected subcutaneously into calves did not persist, but could produce lesions depending on the dose. Field trials of the protective efficacy of BCG vaccination were not encouraging. When natural challenge was high, vaccination of young stock had little effect on the incidence of tuberculosis, and vaccinated animals were sensitized to tuberculin for at least one year. Therefore, it was concluded that BCG vaccination would not be useful in those countries with control programmes based on intradermal testing and slaughter (reviewed by Glover and Ritchie 1953, Ritchie 1953 cited by Stamp 1959, Doyle and Stuart 1958 cited by Lepper and Corner 1983, Ritchie 1959, Daborn and Grange 1993, Newell and Hewinson 1995).

Nevertheless, it was thought that vaccination might be beneficial in countries with a high prevalence of infection, where test and slaughter were not economically feasible. Subsequently, trials conducted in Africa during the 1970s found that BCG vaccination had little effect on the incidence of infection, and since that time BCG vaccination of cattle has not been used as part of any bovine tuberculosis control programmes (Ellwood 1972, Waddington and Ellwood 1972, Cheneau and Blancou 1975, Berggren 1977).

Since the early 1990s, there has been renewed interest in vaccinating cattle in the UK, Ireland and New Zealand, where domestic livestock continue to contract tuberculosis from feral animal maintenance hosts and vectors of *M. bovis* (NSSC 1993, Krebs *et al.* 1997, Cooke 2000). Furthermore, it has been suggested that vaccination of cattle against tuberculosis could have a positive impact on both public health and the animal industries in developing nations, where the social and economic costs of test and slaughter programmes are prohibitive and there is continued intra-herd transmission of *M. bovis* (Daborn and Grange 1993, WHO 1993, WHO/FAO/OIE 1994).

It has been shown that low doses of subcutaneous BCG can significantly protect experimentally infected calves from disease (Buddle *et al.* 1994, Buddle *et al.* 1995a, Buddle *et al.* 1995b), and low doses but not high doses of BCG have also protected deer (Griffin *et al.* 1999). Nevertheless, in New Zealand there has been considerable controversy regarding the potential place of vaccination in the national bovine tuberculosis control programme. At present there is limited within herd transmission of *M. bovis*, and it has been questioned how vaccination might be expected to reduce reactor numbers (Livingstone and Davidson 1993, NSSC 1993).

The main objections to vaccinating cattle with BCG are the potential ramifications for the export beef trade. Test positive infected animals without gross lesions at slaughter would fall outside the framework of current trade protocols. Further, it has been questioned whether markets would accept beef from animals vaccinated with live bacteria. It is considered that countries that have successfully eradicated bovine tuberculosis by test and slaughter are unlikely to alter their import standards to accomodate the trading aspirations of those countries with an intractable problem (reviewed by Livingstone and Davidson 1993, NSSC 1995, Morris *et al.* 1994, Morris and Pfeiffer 1995, de Lisle *et al.* 1998).

In contrast, in the UK the potential trade ramifications of cattle vaccination are limited because most of the beef produced is consumed domestically. Badgers are the feral reservoir host of *M. bovis*, but are protected. Therefore, since depopulation of badgers is not a politically feasible solution and groups of badgers remain static, it has been suggested that vaccination of cattle could prevent tuberculosis from spreading outwards through the cattle population from a geographical focus (reviewed by Newell and Hewinson 1995, Krebs *et al.* 1997, Livingstone 2000 cited by AHB 2000).

1.7.3 Alternatives to BCG.

The recognized difficulties encountered using *M. bovis* BCG as a vaccine have prompted a search for alternatives. Ideally, such vaccines would stimulate a memory immune response that would eliminate infection before it became established, protect against the establishment of pulmonary infection, and not compromise diagnostic tests (reviewed by Doherty and Andersen 2000, Seder and Hill 2000, Andersen 2001, Orme *et al.* 2001).

To achieve improved vaccine efficacy, live attenuated strains and auxotrophic mutants of *M. tuberculosis* and *M. bovis* other than *M. bovis* BCG have been trialed in laboratory animals (Guleria *et al.* 1996, de Lisle *et al.* 1999, Jackson *et al.* 1999, Smith *et al.* 2001). An alternative approach has been to genetically engineer BCG to express genes for cytokines such as IL-12 and IL-2 that drive a Type 1 immune response (O'Donnell *et al.* 1994, Murray *et al.* 1996, Slobbe *et al.* 1999).

DNA vaccination is in its infancy, but is showing promise. The principle of DNA vaccination is that genes coding for antigens are carried on a plasmid downstream from a strong promoter. The naked DNA is injected intramuscularly and transfects the muscle cells. The antigens are expressed within the cell and presented in association with MHC class I. It is thought that antigens released from lysed transfected cells are phagocytosed by macrophages and presented in association with MHC class II. The result is both a cell mediated and a humoral response to the antigen (Denis *et al.* 1998, Huygen 1998, Lowrie *et al.* 1999, Chambers *et al.* 2000a, Lefévre *et al.* 2000, McShane *et al.* 2001)

A number of studies have shown that mice or guinea-pigs vaccinated with plasmids encoding hsp65, 36 kDaAg, 38 kDaAg, hsp70, Ag85A, Ag85B, MPT63, MPT83, ESAT-6 and a multivalent combination of ESAT-6, MPT64, MPT63 acquired a measurable specific immune response to and/or a degree of protection from *M. tuberculosis* or *M. bovis* BCG challenge infection (Huygen *et al.* 1996, Tascon *et al.* 1996, Lowrie *et al.* 1997, Zhu *et al.* 1997, Baldwin *et al.* 1998, Kamath *et al.* 1999, Li *et al.* 1999, Morris *et al.* 2000).

To date, there is only one reported study of the immunological responses of cattle following vaccination with DNA encoding mycobacterial antigens. Calves were vaccinated with plasmids encoding either MPB83, MPB70 or Ag85A. All calves vaccinated with MPB83 DNA acquired an antigen specific cellular response characterized by $CD4^+$ T-cells

that produced IFN-γ, and a predominantly IgG_1 biased humoral response. Only half the animals responded to MPB70, and none responded to Ag85A. Moreover, no vaccinated calves were positive to the intradermal tuberculin test (Chambers *et al.* 2000b, Vordermeier *et al.* 2000). The ability of the vaccines to induce protection against challenge infection was not reported.

Antigens secreted by tubercle bacilli multiplying in liquid culture can also stimulate significant protective immunity, in some cases without inciting cutaneous reactivity to tuberculin. A number of studies with experimental animals, in which preparations of 'culture filtrate' (CF) were given alone and in combination with various adjuvants have demonstrated slowed progression of disease (Hubbard *et al.* 1992, Pal and Horwitz 1992, Andersen 1994, Horwitz *et al.* 1995, Roberts *et al.* 1995, Baldwin *et al.* 1998, Bosio and Orme 1998, Venkataprasad *et al.* 1999, Brandt *et al.* 2000, Olsen *et al.* 2000).

However, CF antigens differ in their ability to prime effective protection. For example, in mice that had been vaccinated with either the 19 kDa lipoprotein or hsp60, the course of disease was more fulminant following challenge with *M. tuberculosis* (Turner *et al.* 2000, Yeremeev *et al.* 2000). Other groups have demonstrated that vaccination with heat shock proteins induced Type I immune responses, had adjuvant activity and mice were protected from challenge infection with virulent tubercle bacilli (reviewed by Silva 1999). Other investigators have identified epitopes of *M. tuberculosis* antigens that are MHC class I or MHC class II restricted in murine models, and suggested that antigens used for vaccination could be chosen on the basis of their ability to induce a particular type of immune response (Lalvani *et al.* 1998, Mohagheghpour *et al.* 1998).

To date, only one study has tested the ability of CF proteins to induce protective immunity in cattle. Groups of calves were vaccinated with different combinations of *M. bovis* culture filtrate proteins (CFP) and bovine IL-2 in a monophosphoryl lipid A adjuvant. The protective efficacy of CFP and BCG vaccination was compared following intratracheal challenge with virulent *M. bovis*. Vaccination with BCG gave the greatest overall level of protection. However, whilst CFP+IL-2 vaccination reduced lung lesion scores and did not induce cutaneous reactivity to intradermal PPD tuberculin, there was greater extrathoracic spread of infection than in either BCG vaccinated or unvaccinated control animals (Wedlock *et al.* 2000).

1.8 IDENTIFICATION OF MYCOBACTERIAL PROTEIN ANTIGENS.

The genome of *M. tuberculosis* codes for more than 3900 proteins, many of which are antigens recognized by infected hosts (Cole *et al.* 1998). Numerous methods have been employed to identify the immunologically active components of tubercle bacilli, and to characterize the immune responses mounted against them. Initially, crossed immunoelectrophoresis with hyperimmune antisera showed that *M. bovis* BCG and *M. tuberculosis* have as many as 50 antigens (Harboe *et al.* 1992). Later, technical advances in liquid chromatography enabled a number of mycobacterial proteins to be purified to homogeneity, identified with monoclonal antibodies and N-terminal sequenced (reviewed by Fifis 1991). However, there were long delays between purifying antigens and identifying their genes (Radford *et al.* 1988, Terasaka *et al.* 1989).

An alternative strategy for identifying mycobacterial antigens was to construct libraries of the *M. tuberculosis* and *M. bovis* BCG genomes in the phage vectors λgt11 and λEMBL3, and screen them with hyperimmune antisera. Although only seven antigens were identified by the available monoclonal antibodies, the coding DNA could be easily propogated in *E. coli* and restriction mapped. Some of the antigens expressed in *E.coli* were different sizes than the native mycobacterial antigens, and it was suggested that differences in the transcription and translation apparatus of *E. coli* could account for altered or inefficient expression of mycobacterial proteins. Furthermore, many mycobacterial antigens exist in multiple forms due to post-translational modification. The immunological consequence is that recombinant proteins expressed in *E. coli* may not be recognized to the same degree as native antigens (Thole *et al.* 1985, Young *et al.* 1987, Schinnick *et al.* 1987, Andersen *et al.* 1988).

By 1986, 33 mycobacterial antigens had been identified by monoclonal antibodies (Anon. 1986). However, it was recognized that monoclonal antibodies detected antigens that were probable targets of the humoral immune response against mycobacteria, but did not necessarily detect antigens that were targets of the cellular immune responses of natural hosts (Lamb and Young 1987, Abou-Zeid *et al.* 1987). A 1992 compilation listed 52 antigens from various mycobacterial species, and many of the antigens from *M. tuberculosis*, *M. bovis* and *M. bovis* BCG were identical (Young *et al.* 1992).

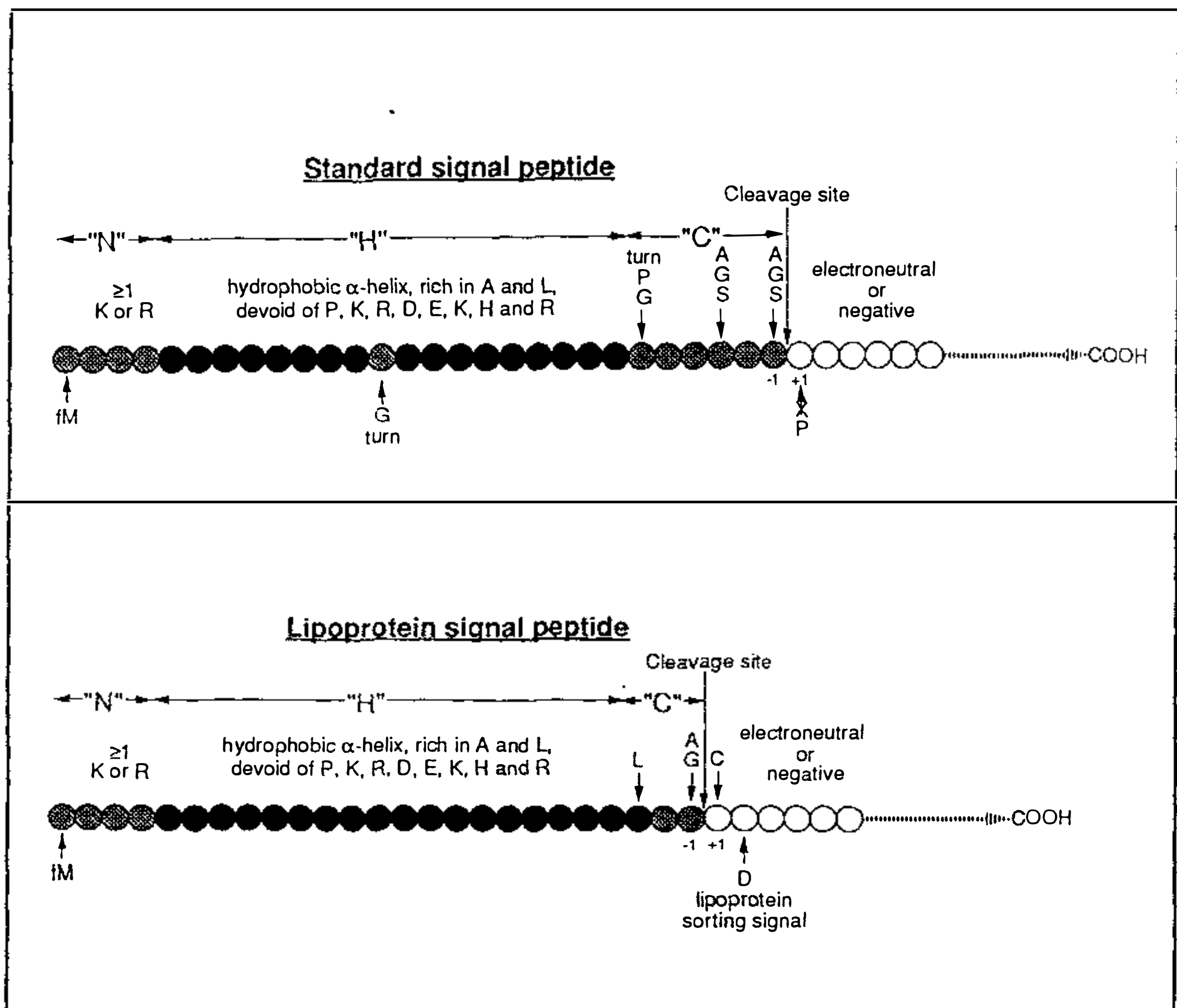

Figure 1.3 Schematic representation of the domain structure of signal sequences, at the N-terminus of precursor proteins exported by the *sec*- dependent general secretory pathway (Type II secretion system). The amino acid residues that constitute the signal peptides are indicated by solid or shaded circles. Open circles represent residues of the mature cleaved protein. The amino acids are indicated by IUPAC code letter.

Signal peptides have three domains (N, H, and C). The N domain (2 to >15 residues) is polar and carries a net positive charge. The H domain (>8 residues) is composed of predominantly hydrophobic residues and alanine, and lacks strongly polar or charged residues. The high leucine content causes signal peptides to adopt an α-helical configuration in apolar environments. The C domain is usually less hydrophobic and contains the sequences recognized by LepB or LspA signal peptidases.

Lipoprotein signal peptides are usually shorter and have more hydrophobic amino acids in the H region. The turn residue upstream from the cleavage site is also less readily identifiable than in standard signal peptides. The amino terminal cysteine residue of these polypeptides must be modified to glycerylcysteine for cleavage to occur and is usually acetylated.

The standard signal peptide is cleaved by Type I Lep with cleavage site recognition constraints on the -1 and -3 amino acids. Lipoprotein signal peptides are cleaved by Type II Lep with cleavage site recognition dependent on the -3 and +1 residues (adapted from Pugsley 1993, Fekkes and Driessen 1999).

1.8.1 Somatic and secreted mycobacterial antigens.

Mycobacterial antigens have been conceptually separated into two groups, somatic and secreted. Somatic antigens are the constituents of the cell wall, the cytosolic components and intracellular metabolic enzymes. Secreted antigens are exported from live mycobacterial cells (Andersen *et al.* 1991a, Wiker *et al.* 1991, reviewed by Andersen 1997, Kaufmann and Andersen 1998).

Many of the somatic antigens are constituents of the mycobacterial envelope. Models of the proposed structure of the mycobacterial cell envelope describe four layers. The innermost is a typical bacterial plasma membrane. The cell wall skeleton is a giant peptidoglycan macromolecule that entirely surrounds the bacterial cell. About half the weight of the cell envelope is lipid covalently bound to the outer surface of the peptidoglycan and non-covalently bound associated lipids. The outermost layer is a capsule visible as an electron transparent zone on electron micrographs (Daffé and Draper 1998).

The capsule is thought to consist of protein and polysaccharide exported across the cell wall, and it has been hypothesized that capsule components may modify the host cell's metabolism. When liquid cultures of mycobacteria are grown with shaking and detergents to prevent clumping, the hydrophilic polysaccharide and protein molecules are shed into the medium and can be recovered by harvesting the CF (reviewed by Barksdale and Kim 1977, Rastogi 1991, Brennan and Draper 1994, Brennan and Nikaido 1995, Ortalo-Magné *et al.* 1995, Lee *et al.* 1996, Daffé and Draper 1998, Draper 1998).

1.8.2 Protein export by mycobacteria.

Five non-homologous protein export systems have been identified in bacteria. The Sec (secretion) system, also called the Type II secretion system, is essential for bacterial viability (Economou 1999). Proteins with characteristic N-terminal signal peptides are translocated across the cytoplasmic membrane of Gram-negative and Gram-positive bacteria (see Figure 1.3) (Pugsley 1993, Salmond and Reeves 1993). *M. tuberculosis* has homologues of *secB, secC, secD, secE, secF secY,* and two copies of *secA* (Chubb *et al.* 1998, Cole *et al.* 1998).

The general secretory pathway has three distinct sequential interdependent stages, and the signal sequence of exported proteins functions as both the targeting and recognition signal

(see Figure 1.3). Following translation, pre-proteins are guided to exit sites in the plasma membrane by secretion specific chaperones such as signal recognition peptide (SRP), SecB, or in some circumstances housekeeping chaperones such as GroEL or DnaK. The exiting chain crosses the lipid bilayer of the plasma membrane through a protein channel known as the translocon, which catalyses transport of the exported protein across the cell membrane. The translocon is a ring made up of SecY, SecE and SecG, and the motor device is SecA. Once outside the plasma membrane, the signal peptide is cleaved from the mature protein by a serine peptidase; Lep (leader peptidase) (Paetzel *et al.* 1998, Economou 1999, Fekkes and Dreissen 1999). Some extracellular proteins do not have identifiable signal peptides. Therefore, it has been suggested that the sequence that facilitates export is contained within the protein itself (Harth and Horwitz 1999).

1.8.3 The immunological implications of somatic and secreted antigens.

Studies with guinea-pigs found that inoculations of both live and dead, slow growing mycobacteria resulted in cutaneous sensitization to tuberculin (Stanford 1983). However, it was observed that differences between the cutaneous responses to tuberculins prepared from the various mycobacterial species, could be used to differentiate between sensitization by live but not dead mycobacteria of different species. It was concluded that many somatic antigens were common to all species of slow growing mycobacteria, whereas subsets of the antigens produced by live bacteria were unique to each species. In addition, it was noticed that guinea-pigs sensitized with live mycobacteria had necrotic DTH responses to homologous tuberculin, and it was suggested that the antigens secreted by live mycobacteria elicited a different kind of immune response (Stanford 1983).

Vaccination with live BCG or recovery from active infection confers both cutaneous DTH and protection from re-challenge, whereas vaccination with killed bacilli results in cutaneous DTH but not protection (Orme *et al.* 1993, Chambers *et al.* 1997, Kaufmann and Andersen 1998, Seder and Hill 2000). It has been suggested that the antigens secreted by live mycobacteria are the primary targets of a protective cell mediated immune response against infection with pathogenic mycobacteria (Andersen *et al.* 1991b, Romain *et al.* 1993, Amara and Satchidandam 1997). Therefore, since the early 1990s the search for immunologically important mycobacterial antigens has focused on the proteins secreted by live TB complex bacilli that are recognized by T-cells of vaccinated or infected hosts (reviewed by Andersen 2001).

1.8.4 Culture Filtrate as a source of secreted mycobacterial antigens.

When *M. tuberculosis* was grown in the presence of [^{35}S] Methionine a number of unidentified radioactively labelled proteins were observed to appear in the culture media during the early growth phase (Abou-Zeid *et al.* 1988). The metabolic enzyme isocitrate dehydrogenase was used as a marker of autolysis, and preparations of proteins released into CF prior to autolysis were defined as 'Short-term culture filtrate' (ST-CF) (Andersen *et al.* 1991a). Other studies sought to define the differential distribution of antigens in CF and cell sonicates, and the concept of a localization index to distinguish between intracellular and extracellular antigens was proposed (Wiker *et al.* 1991).

Immunological studies compared the kinetics of T-cell responses to CF and cell sonicates, and demonstrated that mycobacterial antigens became available to the immune system in a sequential manner. In the first phase of infection, T-cell responses were directed against CF antigens. Later, once the initial host response had killed some of the bacteria, T-cells also recognized bacilliary somatic antigens (reviewed by Kaufmann and Andersen 1998).

T-lymphocytes isolated from tuberculous laboratory animals and cattle infected with *M. bovis*, were primarily stimulated to produce IFN-γ by CF antigens with molecular weights < 10 kDa and between 25 to 38 kDa (Orme *et al.* 1992, Fifis *et al.* 1994a, Boesen *et al.* 1995, Hasløv *et al.* 1995). However, in bovine infection models the responses to particular antigens varied between individuals over the time course of infection (Fifis *et al.* 1994a, Pollock and Andersen 1997b, Lyashchenko *et al.* 1998b, Lightbody *et al.* 1998a, Buddle *et al.* 1999, Rhodes *et al.* 2000a).

The known antigens in the 26 to 38 kDa molecular mass range include MPT64, 27 kDa, MPT51, MPT59, 31/32 kDa, 32 kDa, PhoS, the Ag85 complex, and the 19 kDa lipoprotein (Hasløv 1995, Horwitz *et al.* 1995, Huygen *et al.* 1996, Rosenkrands *et al.* 1998). The antigen ESAT-6 is responsible for much of the activity in the <10 kDa fractions (Sørensen *et al.* 1995, Brandt *et al.* 1996). However, other low molecular weight members of the ESAT-6 family such as CFP10 have been identified recently (see Appendix I) (Berthet *et al.* 1998, Skjøt *et al.* 2000).

The ability of CF proteins to induce protective immune responses against tubercle bacilli was demonstrated in experimental animal infection models. Mice and guinea-pigs vaccinated with CF, or individual secreted proteins from TB complex species were substantially protected from subsequent challenge infection (reviewed by Orme *et al.* 2001).

1.8.5 Methods used to identify Culture Filtrate antigens.

Short-term culture filtrate consists of numerous proteins, but large scale isolation and purification of *M. bovis* protein antigens by physicochemical methods is complicated, because many mycobacterial proteins aggregate or exist in multiple forms (reviewed by Fifis *et al.* 1994b). Thirty three protein bands were visible when ST-CF was separated by SDS-PAGE (Andersen 1997). Following 2D-PAGE of ST-CF, 205 protein spots were visible and a number of novel proteins were identified by N-terminal amino acid sequence analysis and mass spectrometry (Sonnenberg and Belisle 1997). Recently, a 2D-PAGE reference map of *M. tuberculosis* CF was constructed, and seven novel proteins have been identified by N-terminal sequence (Weldingh *et al.* 1998, Weldingh *et al.* 2000).

A technique is described whereby *M. bovis* BCG CF was separated by 2D-PAGE and 400 fractions were eluted into physiological buffer for screening in so called 'T-cell Western blot' assays of lymphocyte proliferation and cytokine production. Despite marked individual variation in the T-lymphocyte responses of BCG vaccinated cattle, it was demonstrated that cattle immunized with viable BCG responded to CF proteins, whereas T-cells from cattle that had received killed BCG did not (Gulle *et al.* 1995).

1.8.6 Cosmid libraries in *M. smegmatis*.

In 1993, a novel approach to identifying mycobacterial antigens was reported (Averill *et al.* 1993). A cosmid library of *M. bovis* BCG genomic DNA had been constructed in a mycobacteria/*E. coli* shuttle vector and transformed into *M. smegmatis*. The CFs derived from individual *M. smegmatis* clones were screened using *in vitro* assays, and some CFs stimulated lymphocytes obtained from healthy tuberculin positive humans. The technique was proposed to have several theoretical advantages compared to the traditional methods employed to identify mycobacterial antigens. The large inserts in the cosmid vector (approximately 40 kb) potentially contained as many as 20 to 40 genes including operons coding for the synthesis of complex antigenic determinants. Therefore, the cosmid library

could represent 99% of the *M. bovis* BCG genome in as few as 300 clones, as opposed to several thousand clones for a λgt11 library. In a mycobacterial host such as *M. smegmatis* there is a greater likelihood that the promoter sequences of genes will be recognized and that full length proteins will be expressed with the appropriate post-translational modifications. Also, it may be expected that proteins with export signal sequences would be secreted into the culture media. The practical advantages were that because *M. smegmatis* is a fast growing non-pathogenic species, CF could be prepared quickly and safely. The shuttle vector also allowed the library plasmids to be readily propogated in *E. coli* facilitating identification of the genes encoded by the insert (Averill *et al.* 1993).

Subsequently, it has been demonstrated that recombinant TB complex species antigens derived from *M. smegmatis* clones elicited *in vitro* immune responses similar to those measured in response to preparations of native *M. tuberculosis* and *M. bovis* antigens (Laqueyrerie *et al.* 1995, Roche *et al.* 1996, Harth *et al.* 1997).

1.9 PREVIOUS STUDIES IN THIS LABORATORY.

A library of *M. bovis* genomic DNA that had been constructed in the mycobacteria/*E. coli* shuttle vector pYUB18 (Wilson *et al.* 1995) was transformed into *M. smegmatis* (Carpenter *et al.* 1995). The CFs derived from 356 recombinant *M. smegmatis* cosmid library clones were screened for stimulatory activity in lymphocyte proliferation and IFN-γ assays, using PBMC from cattle vaccinated with a low dose of *M. bovis* BCG. It was found that CFs from 24 *M. smegmatis* clones were stimulatory, and analysis of the *M. bovis* DNA inserts by PCR found that none contained the genes of 10 known extracellular antigens (Gormley *et al.* 1999).

The CF derived from one *M. smegmatis* cosmid clone (Cos151) stimulated *in vitro* lymphocyte proliferation and IFN-γ responses from seven out of eight BCG vaccinated cattle. The approximately 40 kb insert of *M. bovis* DNA from Cos151 was digested with restriction endonucleases, subcloned into the mycobacteria/*E. coli* shuttle vector pSU4511 and transformed into *M. smegmatis*. The CFs from the *M. smegmatis* subclones derived from Cos151 were screened in lymphocyte proliferation and IFN-γ assays using PBMC from the same group of BCG vaccinated cattle. It was found that the CF from a subclone

that contained a 4.3 kb insert of *M. bovis* DNA was stimulatory in lymphocyte proliferation and IFN-γ assays.

1.10 THE OBJECTIVES AND SCOPE OF THIS STUDY.

The objectives of this study were to identify and characterize the immunoreactive component of CF derived from the *M. smegmatis* subclone of Cos151 that contained a 4.3 kb insert of *M. bovis* DNA.

In order to meet the objectives the following strategy was adopted. The nucleotide sequence of the 4.3 kb fragment of *M. bovis* DNA was determined and compared with the published sequence of *M. tuberculosis* (Chapter 2). Concurrently, the CF from the *M. smegmatis* subclone containing the 4.3 kb insert of *M. bovis* DNA was fractionated by liquid chromatography. The immunoreactive protein was purified and specific polyclonal antisera were produced in rabbits and used to detect the antigen on Western blots. The molecular mass and N-terminal amino acid sequence of the immunoreactive protein were determined, and the corresponding nucleotide coding sequence was identified (Chapter 3). The species distribution of a nucleotide sequence within the 4.3 kb fragment of *M. bovis* that differed from the homologous *M. tuberculosis* sequence was investigated by polymerase chain reaction and Southern hybridization analyses (Chapter 4). A nucleotide sequence potentially coding for a protein with a high degree of homology to the *M. bovis* antigen was identified in *M. avium.* (Chapter 5). The antigen specific immune responses of cattle infected with virulent *M. bovis* were investigated, and compared with the immune responses of cattle vaccinated with *M. bovis* BCG (Chapter 6). Finally, preliminary studies were conducted to explore methods of overexpressing the antigen in both *E. coli* and *M. smegmatis* (Chapter 7).

Chapter 2. Nucleotide sequence analysis of the 4.3 kb *M. bovis* DNA insert.

2.1 INTRODUCTION. 42

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2.1 INTRODUCTION.

A library of *M. bovis* genomic DNA that had been constructed in the mycobacteria/*E. coli* cosmid shuttle vector pYUB18 (Wilson *et al.* 1995), was transformed into *M. smegmatis* (Carpenter *et al.* 1995). Culture filtrates (CF) derived from 356 *M. smegmatis* clones were screened for stimulatory activity in lymphocyte proliferation and interferon-gamma (IFN-γ) assays using peripheral blood mononuclear cells (PBMC) from cattle vaccinated with *M. bovis* BCG (Carpenter *et al.* 1995). The CF derived from a *M. smegmatis* clone (Cos151) with an approximately 40 kb *M. bovis* DNA insert stimulated PBMCs from seven out of eight animals to proliferate and produce IFN-γ (Gormley *et al.* 1999).

Restriction digestion fragments of the approximately 40 kb insert of *M. bovis* DNA in Cos151 were subcloned into the mycobacteria/*E. coli* shuttle vector pSU4511 and transformed into *M. smegmatis*. It was found that the CF from a *M. smegmatis* subclone that contained a 4.3 kb *Sph* I fragment (*M. smegmatis* pSU151.43) also stimulated lymphocyte proliferation and IFN-γ production from the PBMCs of the same group of BCG vaccinated cattle, and was selected for further study.

As part of the strategy adopted to identify the gene coding for the immunoreactive component of the CF derived from the subclone *M. smegmatis* pSU151.43, the 4.3 kb *Sph* I fragment was cloned into the plasmid vector pUC18, and its nucleotide sequence was determined. The sequence of the 4.3 kb insert was aligned against the published sequence of *M. tuberculosis* H37Rv and analysed for the presence of potential genes, or any differences between the sequences of *M. bovis* and *M. tuberculosis.*

2.2 MATERIALS AND METHODS.

2.2.1 Bacterial growth conditions.

Liquid cultures of *E. coli* hosting plasmids were grown in Luria Bertani (LB) broth (Sambrook *et al.* 1989), with 100 μg / ml ampicillin (Sigma-Aldrich, Sydney, Australia) or 20 μg / ml kanamycin (Life Technologies, Auckland, NZ) as appropriate to the

plasmid's selection marker. The cultures were incubated at 37°C in aerobic conditions for 12 to 16 hours, with shaking at approximately 150 rpm.

On solid media, *E. coli* were grown on plates of LB agar with ampicillin (100 µg / ml) or kanamycin (20 µg / ml) for 12 to 24 hours at 37°C in aerobic conditions. For medium term storage, plates were sealed with Parafilm®"M" (American National Can$_{TM}$, Greenwich, USA) and kept at 4°C. For long term storage, aliquots taken from 5 ml liquid cultures were kept in 20% Bacto glycerol (Difco Laboratories, Detroit, USA) at -70°C.

Table 2.1 The strain of *E. coli* used in this study.

Species	Genotype / Phenotype	Source
E. coli DH10B ELECTR●MAX DH10B™	F⁻ *mcr* A D(*mrr-hsd* RMS-*mcr* BC) ϕ80d*lac* Z●M15 D*lac* X74 *end* A1 *rec* A1 *deo* R D(*ara, leu*)7697 *ara* D139 *gal* U *gal* K *nup* G *rps* L Γ.	Life Technologies

2.2.2 Extraction of plasmid DNA from *E. coli.*

Plasmids were extracted from *E. coli* by a modification of the alkaline lysis/silica binding mini-prep method (Sambrook *et al.* 1989). Plasmid DNA was purified from liquid cultures of *E. coli* (5 ml) using the BRESAspin Plasmid Mini Kit (Bresatec Pty Ltd, Thebarton, Australia) according to the manufacturer's instructions. When required, larger amounts of plasmid DNA were extracted from 20 ml liquid cultures. The cells in aliquots (4 x 1ml) of liquid culture were pelleted in 4 x Eppendorf tubes (Life Technologies) by four cycles of loading and centrifugation at 12,600 x g for 1 minute. The cells were resuspended, lysed, and the cellular debris were precipitated. Following centrifugation at 12,600 x g for 10 minutes, the supernatants were double loaded onto four silica matrix binding spin columns as recommended by the manufacturer, and the plasmid DNA was eluted in dH_2O (50 µl / spin column).

2.2.3 Restriction endonuclease digestion of plasmid DNA.

Typically, plasmid DNA was digested with restriction endonuclease (1 to 2 units) in 20 to 30 μl of 1 x SuRE/Cut Buffer (Roche Diagnostics, Auckland, NZ) at 37°C for 1 to 6 hours.

2.2.4 Separation of DNA fragments by electrophoresis.

Aliquots of solutions containing DNA fragments (typically 5 to 20 μl) were mixed with 0.2 volumes of 6 x Loading Buffer and loaded onto horizontal agarose (1%) slab gels (Agarose Ultra Pure; Life Technologies), submerged in 1 x Tris-acetate buffer (TAE). The DNA fragments were separated by electrophoresis at 60 to 100 volts, in either a MINI SUB™ DNA CELL, or a WIDE MINI SUB™ DNA CELL apparatus (Bio-Rad Laboratories Pty Ltd, Auckland, NZ). The DNA and commercial molecular weight markers that were run in adjacent lanes were stained with the flourescent intercalating dye ethidium bromide and visualized by ultraviolet transillumination. Images of the separated DNA fragments were recorded photographically on Polaroid 667 film.

Table 2.2 Plasmids used in this study.

Plasmid	Description	Source / Reference
pSU151.43	pSU4511(a) containing a 4.3 kb *Sph* I fragment derived from the *M. bovis* DNA insert in the plasmid pYUBCos151(b). Selection marker, kanamycin resistance.	C. Dupont Massey University
pUC18	pBR322 origin of replication, *lac* Z blue/white selection of clones containing inserts, M13/pUC sequencing primer binding sites. Selection marker, ampicillin resistance.	Life Technologies (Yanisch-Perron *et al.* 1985)
pUC18/4.3#1 pUC18/4.3#2	pUC18 containing the 4.3 kb *Sph* I fragment of *M. bovis* DNA from pSU151.43.	This study

(a) pSU4511 is a mycobacteria/*E. coli* shuttle vector derived from plasmids pAL5000 and pACYC184 (Ainsa *et al.* 1996). The selection marker is kanamycin resistance and the multiple cloning site is from plasmid pSU40 (Bartolomé *et al.* 1991).

(b) pYUBCos151 is the plasmid contained by the cosmid library clone *M. smegmatis* (Cos151).

2.2.5 Extraction of DNA from agarose gels.

DNA was purified from excised slices of agarose gels using the GENECLEAN *II* ® Kit (Bio 101 Inc, La Jolla, USA) according to the manufacturer's instructions. The DNA was suspended in sodium iodide, and bound to a silica matrix. The impurities were removed by a series of washes with a high salt buffer, and the bound DNA was eluted in dH_2O. To concentrate the eluted DNA, it was precipitated by adding 0.1 volumes of 3 M sodium acetate (Sigma-Aldrich) and 2.5 volumes of 100% ethanol (AnalaR; BDH, Auckland, NZ) and chilled at -20°C for more than three hours. The DNA was pelleted by centrifuging at 15,800 x g for 30 minutes, washed with 70% ethanol, air dried, and resuspended in a smaller volume of dH_2O.

2.2.6 Phosphatase treatment of plasmid.

Digested plasmid, that had been extracted from agarose gels and precipitated, was dephosphorylated to prevent recircularization. Plasmid DNA was dialysed against dH_2O for 10 minutes on membrane filters (25 nm pore size; Millipore Corporation, Bedford, USA), and mixed with 1 x dephosphorylation buffer (Tris-HCl 50 mM, EDTA 0.1 mM, pH 8.5), and 1 unit of calf intestinal alkaline phosphatase (Roche). The reaction mixture (20 µl) was incubated at 37°C for 1 hour, and the phosphatase was inactivated by incubating at 75°C for 15 minutes.

2.2.7 Quantitation of DNA.

The concentration of DNA in solution was estimated spectrophotometrically by standard methods (Sambrook *et al.* 1989). Aliquots of solutions containing DNA were diluted 1/40 with dH_2O, and the optical density was measured at 260 nm (OD_{260}). The DNA concentration was calculated using the formula:

dsDNA concentration (ng / µl) = OD_{260} x 50 x 40 (dilution factor)

(OD_{260} = 1, corresponds to approximately 50 ng dsDNA / µl.)

2.2.8 Ligation of DNA fragments.

Digested insert and plasmid DNA with complementary ends were ligated with T4 DNA ligase. The DNA fragments were mixed in 1 x ligation buffer buffer (Tris-HCl 66 mM,

dithiothreitol (DTT) 1 mM, $MgCl_2$ 5 mM, adenosine triphosphate (ATP) 1 mM, pH 7.5), with 1 unit of T4 DNA ligase (Roche), and the ligation reactions (15 µl) were incubated at 15°C for 16 to 20 hours. Insert and vector DNA were combined at a molar ratio of ends of 5 : 1. The volumes of insert and vector DNA (µl) included in the reaction mixtures were calculated using the formula:

$$\text{Insert vol } (\mu l) = \text{Vector vol } (\mu l) \times \frac{\text{Vector concentration}}{\text{Insert concentration}} \times \frac{\text{Insert length (bp)}}{\text{Vector length (bp)}} \times 5$$

2.2.9 Transformation of *E. coli* by electroporation.

E. coli ELECTROMAX DH10B™ (Life Technologies) were transformed with ligated plasmids using a Gene Pulser™ apparatus with PULSE CONTROLLER (Bio-Rad) according to the manufacturer's instructions. Aliquots of ligation reactions (5 µl) were dialysed against dH_2O on 25 nm pore size membrane filters for 15 minutes then mixed with electrocompetent *E. coli* DH10B (10 µl), and incubated on ice for 2 minutes. The cells were pulsed in electroporation cuvettes (0.2 cm electrode gap), at settings of 2.5 kV, 25 µF capacitance and 200 ohms resistance. LB broth (1 ml) was added immediately and the electroporated cells were incubated at 37°C for one hour, to enable expression of the antibiotic resistance gene carried on the plasmid.

Aliquots of transformed cells (100 to 200 µl) were spread on LB agar that contained ampicillin (100 µg / ml), and had the surface coated with 40 µl of 5-bromo-4-chloro-3-indoyl-β-D-galactoside 20 mg / ml (X-Gal; Life Technologies), and 8 µl of isopropylthio-β-galactoside 0.1 M (IPTG; Life Technologies). After incubating at 37°C for 16 to 20 hours, individual colonies of white transformants were streaked onto new LB agar plates containing ampicillin (100 µg / ml). Plasmid DNA was extracted from liquid cultures (5 ml) derived from the streaked *E. coli* clones, and the presence of inserts in the plasmids was verified by analysis of restriction endonuclease digestion fragments.

2.2.10 DNA sequencing.

Nucleotide sequences were determined by automated dideoxy chain-termination reaction on an ABI PRISM™ Model 377-18 Version 3.0 DNA Analysis System with Big Dye Terminator (Applied Biosystems, Foster City, USA), at the Massey University DNA Analysis Service (MUSeq). Overlapping nucleotide sequences were assembled into a contiguous sequence using GCG Gel assemble software (Genetics Computer Group (GCG) Wisconson Package™, website: http//www.gcg.com).

2.2.11 Computer analysis of DNA sequences.

The EMBL and GeneBank non-redundant databases were searched using the BLAST algorithims, for sequences that had homology to submitted query sequences (Altschul *et al.* 1997). The databases were accessed via the NCBI-BLAST server version 2.0 (Altschul *et al.* 1997) through the National Centre for Biotechnology Information (NCBI; National Library of Medicine, Bethesda, MD, USA, website: http://www.ncbi.nlm.nih.gov). The BLAST programmes are a set of sequence comparison algorithms that search sequence databases for optimal local alignment to a query sequence. The programme BLASTN was used in the Advanced BLAST mode to perform gapped alignments of the query sequences against the nucleotide sequence databases.

Multiple sequence alignments were conducted online using the programme MAP (Smith *et al.* 1996), and then completed manually. The programme uses an alignment algorithim that is global progressive in linear space, and was accessed from the Human Genome Centre, Department of Molecular and Human Genomics, Baylor College of Medicine, TX, USA, website: http://www.hgsc.bcm.tmc.edu.

2.3 RESULTS.

2.3.1 Construction of the pUC18/4.3 plasmids.

The plasmid pSU151.43 that contains a 4.3 kb insert of *M. bovis* DNA was extracted from *E. coli* DH10B and digested with the restriction endonuclease *Sph* I. The 4.3 kb fragment was separated from the plasmid DNA by agarose gel electrophoresis, extracted from the gel, purified, and concentrated by precipitation. The plasmid pUC18 was tranformed into electrocompetent *E. coli* DH10B, amplified, extracted, digested with *Sph* I, and dephosphorylated. The digested 4.3 kb fragments and pUC18 were ligated and

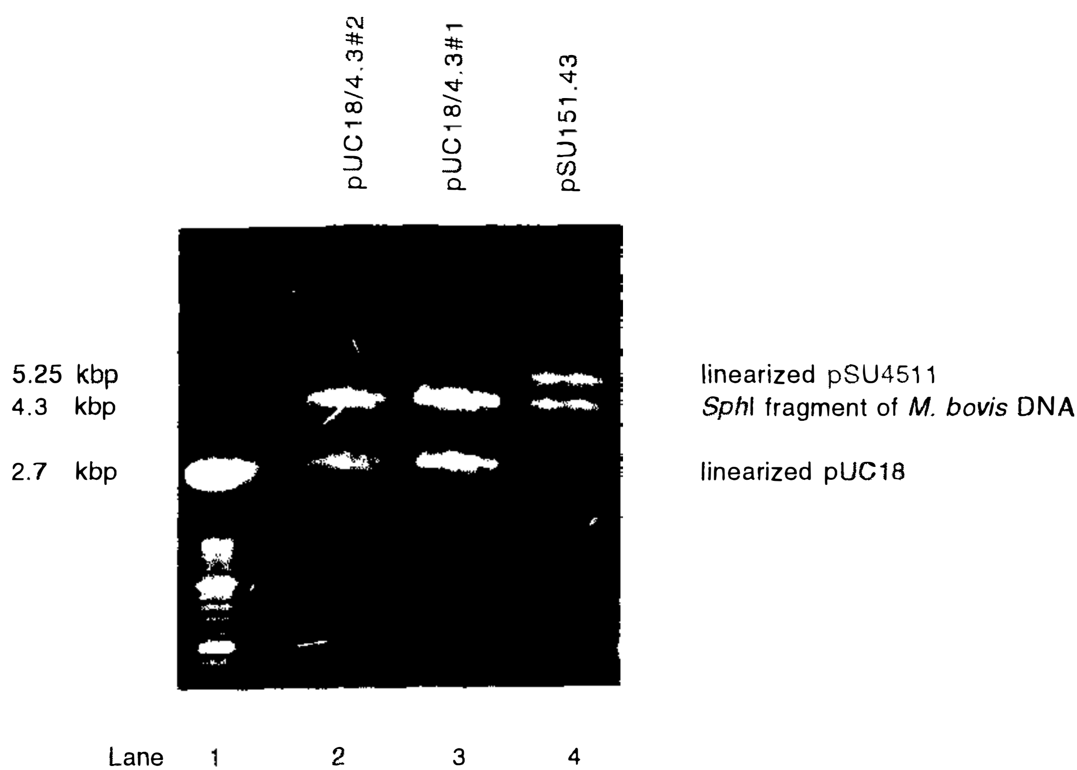

Figure 2.1 Agarose gel (1% TAE) showing restriction digestion fragments of plasmids pSU151.43, pUC18/4.3#1 and pUC18/4.3#2. The plasmids were extracted from *E. coli*.DH10B, digested with *Sph* I and separated by agarose gel electrophoresis. Lane 1, 100 bp ladder (Life Technologies); Lane 2, digested plasmid pUC18/4.3#2; Lane 3, digested plasmid pSU151.43#1; Lane 4, digested plasmid pSU151.43. The inserts from all the plasmids are 4.3 kbp. Linearized pSU4511 is 5.25 kbp, and linearized pUC18 is 2.7 kbp.

transformed into *E. coli* DH10B. Colonies of white transformants with the *lacZ* gene disrupted by the presence of insert were picked and streaked onto fresh plates of LB agar.

To verify that the 4.3 kb *Sph* I fragment had been cloned into pUC18, plasmids from two transformant clones were subjected to restriction digestion analysis. The plasmids pUC18/4.3#1, pUC18/4.3#2 and pSU151.43 were extracted from *E. coli* DH10B, digested with *Sph* I, and the DNA fragments were separated by agarose gel electrophoresis for comparison (see Figure 2.1). Analysis of the restriction digestion fragments showed that the plasmids pUC18/4.3#1 and pUC18/4.3#2 both contained a fragment the same size as the 4.3 kb insert in pSU151.43.

2.3.2 Nucleotide sequence of the 4.3 kb *M. bovis* DNA insert.

The plasmid pUC18/4.3#2 was extracted from an overnight liquid culture (20 ml) of *E. coli* DH10B, and 40 µg of plasmid DNA (0.2 mg / ml) was submitted for sequencing. The M13/pUC universal primers were used to generate the nucleotide sequence of approximately 500 bp at each end of the insert. Primers complementary to the 3' ends of the newly generated sequences were designed, and the primer walking technique was used to sequence further ~500 bp segments. The 10 overlapping sequence segments in the foward direction and 8 overlapping segments in the reverse direction were aligned into a contiguous 4267 bp long nucleotide sequence, that had a GC content of 64.5% (see Appendix II).

2.3.3 BLASTN search with the 4.3 kb *M. bovis* sequence.

The nucleotide sequence of the 4.3 kb insert of *M. bovis* DNA was submitted to the NCBI-BLAST server for a BLASTN alignment search. The 4.3 kb *M. bovis* sequence scored two discontinuous hits against the *M. tuberculosis* H37Rv sequence; segment 51/162, GenBank accession number: AL010186 (Cole *et al.* 1998, Parkhill 1998), and the *M. tuberculosis* MSGY348 sequence; clone Y348, GenBank accession number: AD000020 (L. Du, Genome Therapeutics Corp, MA, USA; Direct submission). The annotations to the homologous *M. tuberculosis* H37Rv sequence predict the segment contains three genes (see Figure 2.2). In addition, it was found that the 4.3 kb insert of *M. bovis* DNA was 62 base pairs shorter than the homologous *M. tuberculosis* sequence.

```
                 556            570 571            585 586            600 601            615 616            630
M. bovis         AGCTGGGTGAAACTT GGAGTGCGGCGCACC CAGGTGATGCTCGAA GGTGGCGCCAACGAC CTGGGCGGCACGCTG   630
M. tuberculosis  AGCTGGGTGAAACTT GGAGTGCGGCGCACC CAGGTGATGCTCGAA GGTGGCGCCAACGAC CTGGGCGGCACGCTG   630

631            645 646            660 661            675 676            690 691            705 706            720
ATGGAGGAGACCATC TCGCGGATGGCCGGT TCCGAACACGGATCG GCCAAGACCGTCGCT GAGCTGGTCGCGATC GCCGAAGGCATCGGC   720
ATGGAGGAGACCATC TCGCGGATGGCCGGT TCCGAACACGGATCG GCCAAGACCGTCGCT GAGCTGGTCGCGATC GCCGAAGGCATCGGC   720

721            735 736            750 751              765 766            780 781            795 796              810
CGCCCGGCGCGCCAG CGCACTACCACATAC GCCCTGCTTGC * GGCC TAGCCCCGGCGACGA TGCCGGGTCGCGGGA TG C GGCCCGTTGAGG   810
CGCCCGGCGCGCCAG CGCACTACCACATAC GCCCTGCTTGC * GGCC TAGCCCCGGCGACGA TGCCGGGTCGCGGGA TG C GGCCCGTTGAGG   810
                                                       "62 bp direct repeat copy 1"

811              825 826              840 841            855 856              870 871              885 886            900
AGCGGGCAATCT     ---------------- --------------- ------ ---------- ----------------   GGCCTAGCCCCGGCG   838
AGCGGGGCAATCT * GG CCTAGCCCCGGCGAC GATGCCGGGTCGCGG GATG C GGCCCGTTGA GGAGCGGGGCAATCT * GGCCTAGCCCCGGCG   900
                        "62 bp direct repeat copy 2"                                  "62 bp direct

901            915 916                 930 931              945 946            960 961            975 976            990
ACGATGCCGGGTCGC GGGATG G GGCCCG * CA TGGG CTTAATAGTTG TTGCAGGAGCCGGCA ACCGACTCGACAAGG CCGATGTACTGTGCC   928
ACGATGCCGGGTCGC GGGATG G GGCCCG * CA TGGG CTTAATAGTTG TTGCAGGAGCCGGCA ACCGACTCGACAAGG CCGATGTACTGTGCC   990
repeat partial copy (43/62)"           ==================================================================

991           1005 1006          1020 1021          1035 1036          1050 1051          1065 1066          1080
GCCCCCGGCACAGCT TGCAATTGCGCGGCC ATGGCAGCGCGCTGA GGTGGCGGTGCGGCG AGGAAATTGCGCAAA TAGGACTGCGCCACC   1018
GCCCCCGGCACAGCT TGCAATTGCGCGGCC ATGGCAGCGCGCTGA GGTGGCGGTGCGGCG AGGAAATTGCGCAAA TAGGACTGCGCCACC   1080
===============================================================================================

1081          1095 1096          1110 1111          1125 1126          1140 1141          1155 1156          1170
GGTGAGGCGTTGAAC TGTGCGGCAGCCCCC GGATCCGTCGCGTTG AGCGCAGCTACTACC TGCCCGTAATTGCAG GTGGTGTTAATGACC   1108
GGTGAGGCGTTGAAC TGTGCGGCAGCCCCC GGATCCGTCGCGTTG AGCGCAGCTACTACC TGCCCGTAATTGCAG GTGGTGTTAATGACC   1170
===============================================================================================

1171          1185 1186          1200 1201          1215 1216          1230 1231          1245 1246          1260
GCGTCCACGGGATCT GCGGAGGCGACCCCG GCCCCGACGGTCAAC GACATTGCCACGGCG CCTACACCGGCGCTC AATGC GGTCAACGAC   1198
GCGTCCACGGGATCT GCGGAGGCGACCCCG GCCCCGACGGTCAAC GACATTGCCACGGCG CCTACACCGGCGCTC AATGC GGTCAACGAC   1260
===============================================================================================

1261          1275 1276          1290 1291          1305 1306          1320 1321          1335 1336          1350
AGCCTCATTTATGGA CACCTTCCCCAAACT ATTGCACCGTCGTTA AGACGGCGACGACAT CTGCCCAGCGGTTGC CGTCTGCGGTCGAGG   1288
AGCCTCATTTATGGA CACCTTCCCCAAACT ATTGCACCGTCGTTA AGACGGCGACGACAT CTGCCCAGCGGTTGC CGTCTGCGGTCGAGG   1350
=========  ← Rv1174c
```

Figure 2.3 Alignment of part of the nucleotide sequence of the 4.3 kb *M. bovis* insert (above) against the corresponding sequence of *M. tuberculosis* H37Rv (below), showing the 62 bp ETR locus. The 62 bp direct repeat copies (italics) are marked at each end by astrisks (*). The annotations for the copies of the repeat units of *M. tuberculosis* (from Cole *et al.* 1998) are below the corresponding segments of the sequences. The repeat unit absent from *M. bovis* is indicated by a single broken line between the two asterisks above the *M. tuberculosis* "62 bp direct repeat copy 2". The predicted gene Rv1174c, that runs on the reverse strand, is indicated by double broken lines below the sequences. The nucleotide sequence of the "62 bp direct repeat partial copy" differs from the complete copies by one nucleotide at position 37 (bold).

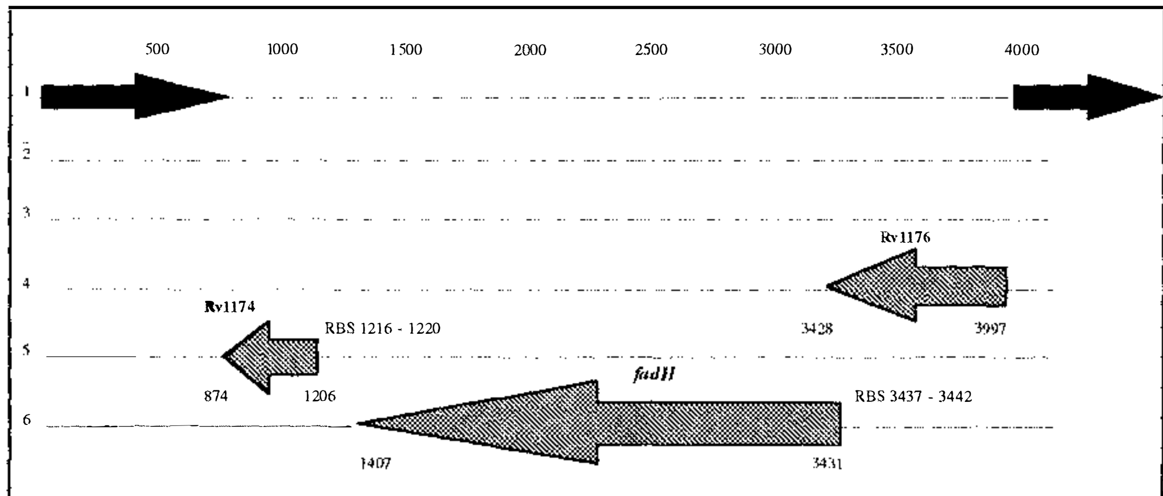

Figure 2.2 The predicted genes within the *M. tuberculosis* H37Rv sequence that is homologous to the 4.3 kb insert of *M. bovis* DNA. The open reading frames (ORFs) were predicted with a Hidden Markov Model of TB genes implemented in Tbparse. Where possible ATG, GTG or TTG start codons were chosen that are preceded by a ribosome binding site (RBS) 5 to 13 base pairs upstream. The corresponding location of the ORFs and RBSs on the 4.3 kb *M. bovis* contig are indicated by nucleotide number. The predicted genes are indicated by the designations used for the published sequence of *M. tuberculosis* H37Rv. The ORFs in frame 1 were truncated by the *Sph* I sites (Adapted from annotations to the *M. tuberculosis* H37Rv segment 51/162, Cole *et al.* 1998, Parkhill 1998).

2.3.4 Alignment of the 4.3 kb *M. bovis* DNA insert sequence against the homologous *M. tuberculosis* sequences.

The nucleotide sequence of the 4.3 kb *M. bovis* DNA insert was aligned against the published *M. tuberculosis* sequences. It was found that a 62 bp direct tandem repeat that occurs 2.7 times in *M. tuberculosis* only occured 1.7 times in the 4.3 kb *M. bovis* fragment (see Figure 2.3).

2.3.5 BLASTN search with the 62 bp repeat sequence.

The nucleotide sequence of the 62 bp tandem repeat unit from *M. bovis* was submitted for a BLASTN homology search. The sequence of the 62 bp element of *M. bovis* was identical to the two 62 bp direct repeat copies that are annotated to the published sequence of *M. tuberculosis* H37Rv; segment 51 / 162 (Cole *et al.* 1998). Likewise, the sequence of the direct repeat partial copy from *M. bovis* matched exactly the direct repeat partial copy adjacent to the 62 bp direct repeat copies, and is located immediately downstream from the predicted *M. tuberculosis* gene Rv1174c. The sequences of the 62 bp direct

repeat units did not have any significant matches to any other sequences on the EMBL and GenBank databases.

2.4 DISCUSSION.

The CF from a recombinant *M smegmatis* clone that contained the plasmid pSU151.43 stimulated the PBMCs of BCG vaccinated cattle to proliferate and produce IFN-γ. The 4.3 kb insert of *M. bovis* DNA was sequenced, and found to contain three complete open reading frames identical to predicted *M. tuberculosis* H37Rv genes, two of which have identifiable potential ribosomal binding sites. The largest predicted gene *fadH* codes for 2,4-Dienoyl-CoA Reductase, which is a metabolic enzyme involved in lipid catabolism. The gene Rv1176c is predicted to code for a hypothetical protein with an estimated molecular weight of >18 kDa, and the smallest predicted gene is Rv1174c, which codes for a hypothetical protein of approximately 10 kDa (Cole *et al.* 1998).

The 4.3 kb *Sph* I fragment of *M. bovis* is 62 bp shorter than the homologous *M. tuberculosis* sequence. The absence of one 62 bp direct tandem repeat unit results in a DNA polymorphism between the *M. bovis* isolate from which the cosmid library was constructed, and the *M. tuberculosis* H37Rv sequence. To date, very few genetic differences between *M. bovis* and *M. tuberculosis* have been reported (Behr *et al.* 1999, Gordon *et al.* 1999a), and the polymorphism found in this study has not been reported previously.

The repeat region appears to be an example of an exact tandem repeat (ETR) locus, but the repeat units share no sequence homology with the subset of ETR known as mycobacterial interspersed repetitive units (MIRU) (Supply *et al.* 1997, Frothingham and Meeker-O'Connell 1998, Supply *et al.* 2000). Six similar ETR have been reported that are polymorphic with respect to the number of tandem repeats, both within and between mycobacterial species belonging to the TB complex. The combined results of variable numbers of tandem repeats (VNTR) analyses of those six informative VNTR loci, generated allele profiles that distinguished between TB complex species (Magdalena *et al.* 1998, Frothingham and Meeker-O'Connell 1998). The distribution of the polymorphic 62 bp ETR locus in mycobacterial species is described in Chapter 4.

Chapter 3. Identification of an immunoreactive 8.4 kDa protein in culture filtrate from *M. smegmatis* pSU151.43

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3.1 INTRODUCTION.

The CF derived from the *M. smegmatis* subclone containing the plasmid pSU151.43 had been found to stimulate PBMC from BCG vaccinated cattle to proliferate and produce IFN-γ *in vitro*. To identify and characterize the nature of the immunoreactive component, the proteins in CF from *M. smegmatis* pSU151.43 were separated by fast protein liquid chromatography (FPLC), and the fractions were screened for stimulatory activity in whole blood IFN-γ assays. An 8.4 kDa protein that stimulated *in vitro* proliferation and IFN-γ production by PBMC from a BCG vaccinated animal was purified from *M. smegmatis* pSU151.43 CF and characterized in terms of its molecular mass and N-terminal amino acid sequence. The N-terminal amino acid sequence of the 8.4 kDa protein was found to correspond to the translated amino acid sequence of one of the predicted genes located on the 4.3 kb insert of *M. bovis* DNA in the plasmid pSU151.43.

3.2 MATERIALS AND METHODS.

3.2.1 Bacterial growth conditions.

Liquid cultures of recombinant *M. smegmatis* mc^2 155, a high transformation efficiency strain (Snapper *et al.* 1990), were grown in a modified version of Bacto Middlebrook 7H9 Broth (Difco Laboratories, Detroit, USA). The broth medium was prepared in dH_2O according to the manufacturer's directions, except neither Bacto Middlebrook ADC Enrichment nor Tween 80 were added. After autoclaving, the broth was supplemented with 0.2% D (+) glucose (AnalaR; BDH, Auckland, NZ) and 20 µg / ml kanamycin (Life Technologies, Auckland, NZ). Cultures were incubated at 37°C with shaking at approximately 150 r.p.m in aerobic conditions.

On solid media, recombinant *M. smegmatis* clones were grown on plates of Bacto Middlebrook 7H10 Agar supplemented with Bacto OADC Enrichment and kanamycin (20 µg / ml). The plates were incubated at 37°C in aerobic conditions for 3 to 4 days, until large colonies were visible. For medium term storage, plates were sealed with Parafilm® "M" and kept at 4°C. For long term storage, aliquots of liquid culture in 20% glycerol (Difco) were kept at -70°C.

Table 3.1 Plasmids used in this study.

Plasmid	Description	Source / Reference
pYUB18	Non-integrating mycobacteria/*E. coli* cosmid shuttle vector. Selection marker, kanamycin resistance	Belisle *et al.* 1991
pYUBCos151	pYUB18 containing a ~40 kb insert of *M. bovis* DNA	Wilson *et al.* 1995
pSU4511	mycobacteria/*E. coli* shuttle vector derived from pAL5000 and pACYC184, multiple cloning site from pSU40. Selection marker, kanamycin resistance	Ainsa *et al.* 1996
pSU151.43	pSU4511 containing a 4.3 kb *Sph* I fragment from the *M. bovis* DNA insert in pYUBCos151	C. Dupont Massey University

3.2.2 Preparation of culture filtrates for immunological assays.

Primary cultures of *M. smegmatis* recombinants were grown in supplemented 7H9 minimal medium (15 ml) for 72 hours. Aliquots (2 ml) of primary cultures were inoculated 1:40 into fresh medium (80 ml) and incubated at 37°C with shaking at 150 r.p.m. At intervals, the bacteria were uniformly suspended by shaking manually and samples (1 ml) were removed for optical density measurement at 580 nm (OD_{580}). When bacterial growth reached an OD_{580} of 0.8 to 0.9 (log phase), or after five days (stationary phase), the cultures were centrifuged at 5000 x g for 15 minutes and the supernatants were filtered through 0.22 μm pore size syringe filters (Millipore, Bedford, USA). The CFs were concentrated to a volume of approximately 1.5 ml, using CENTRIPLUS™ concentrators with a 3 kDa molecular weight cut-off (MWCO ≥ 3 kDa) according to the manufacturer's instructions (Amicon Inc, Beverly, USA).

3.2.3 Estimation of protein concentrations.

Protein concentrations were determined using a Micro BCA Protein Assay Kit (Labsupply Pierce, Auckland, NZ) according to the manufacturer's instructions. Briefly, aliquots of sample (10 μl) or Bovine Serum Albumin Protein Standard 2 mg / ml (8, 12, 16, 20, 24, 28 μg) were diluted in dH_2O (1 ml) and mixed with Working Reagent (1 ml),

in semi-micro 3.0 ml disposable polystyrene cuvettes (Labsupply Pierce). The reaction mixtures were incubated at 37°C for 30 to 60 minutes until the colour developed, and the OD_{562} was measured. The protein concentrations of the samples were estimated, by reference to the standard curve generated from the OD_{562} readings of the BSA protein standards.

3.2.4 Preparation of culture filtrate for biochemical fractionation.

Aliquots of primary culture (900 µl) were inoculated 1:1000 into fresh supplemented 7H9 minimal medium (5 x 900 ml) and incubated in 2 litre conical flasks at 37°C, with shaking at 150 r.p.m for approximately 60 hours until the late log growth phase was reached (OD_{580} 0.8 to 1.0). The cultures were centrifuged at 5000 x g for 10 minutes, and the supernatants were filtered through 0.22 µm GP Express membranes with prefilters using a Steritop vacuum filtration system (Millipore). Sodium azide (NaN_3; BDH) was added to a final concentration of 0.5%, and the culture filtrate was concentrated in 250 ml and 50 ml Amicon magnetic stir-cell ultrafiltration apparatuses fitted with YM3 membranes (MWCO ≥ 3 kDa).

3.2.5 Fractionation of the culture filtrate.

Culture filtrate proteins were separated by FPLC. The apparatus used was an LKB Bromma 2249 Gradient Pump linked to an LKB Bromma 2141 Variable Wavelength Monitor (Amersham Pharmacia Biotech, Auckland, NZ). To detect protein in the eluate the absorbance was measured at 280 nm (A_{280}) and recorded by PowerChrome v2.1 software (ADInstruments Pty Ltd, Castle Hill, Australia).

Aliquots of concentrated CF (1 ml) were exchanged into Tris-HCl 20 mM (pH 8.2), 0.5% NaN_3 by loading onto a Fast Desalting Column HR 10/10 (Pharmacia Biotech AB, Uppsala, Sweden) run at a flow rate of 2 ml / minute, and eluate fractions were collected every 1 minute. The first fractionation step was performed by anion exchange chromatography using an Uno™ Q-6 column (Bio-Rad Laboratories Pty Ltd, Auckland, NZ), equilibrated with Tris-HCl 20 mM, 0.5% NaN_3, (pH 8.2). All the CF was loaded and the bound proteins were eluted by applying a linear gradient of 0 to 1 M NaCl in equilibration buffer over 30 minutes at a flow rate of 2 ml / minute, and eluted fractions were collected every 2 minutes. The eluate fractions (4 ml) were exchanged 1:1000 into

phosphate buffered saline (PBS), and concentrated to approximately 1.5 ml by three dilution/concentration cycles in a CENTRIPLUS™ concentrator (MWCO ≥ 3 kDa).

The second fractionation step was performed either by size exclusion or reverse phase chromatography. For size exclusion chromatography, aliquots (0.2 ml) were separated through a Superose 6 HR 10/30 size exclusion column (Pharmacia Biotech AB) at a flow rate of 0.3 ml PBS / minute, and fractions were collected every 1 minute. Fractionation by reverse phase chromatography was performed with a ProRPC HR 5/2 column (Amersham Pharmacia Biotech) equilibrated with 0.1% trifluoroacetic acid (TFA; Sigma-Aldrich, Sydney, Australia) in dH_2O. Proteins were loaded in 0.1 or 0.15% TFA, and eluted by applying a linear gradient of 0 to 50% acetonitrile in water over 30 minutes at a flow rate of 0.7 ml / minute. To evaporate the TFA and acetonitrile, the eluted fractions (0.7 ml) were concentrated to near dryness in a SpeedVac SC 100 (Savant Instruments Inc, New York, USA), and the volume was adjusted to 100 μl with dH_2O.

3.2.6 Cattle used for this study.

Blood for use in immunoassays was taken from three, rising-4-year-old, cattle kept at the Massey University Large Animal Teaching Unit. A steer (No.5) had previously been given five subcutaneous inoculations of *M.bovis* BCG Pasteur (3×10^5 to 5×10^6 colony forming units) between the ages of 6 months and 2.5 years. Two cows (Nos.53 and 55) were unvaccinated control animals.

3.2.7 Lymphocyte proliferation assays.

Venous blood (20 to 30 ml) was collected into 10 ml Vacutainers (Becton-Dickinson, Franklin Lakes, USA) containing lithium heparin as anticoagulant. The blood was transferred into 50 ml polypropylene conical tubes (Falcon 2070 BLUE MAX™; Becton-Dickinson). The volume was adjusted to 40 ml with PBS (supplemented with $MgCl_2$ 0.25 mM to prevent the cells clumping), and the blood was centrifuged at 750 x g for 10 minutes. The buffy coats were aspirated, adjusted to 20 ml with PBS, layered onto 20 ml of Lymphoprep (density = 1.077 g / ml; Nycomed Pharma As, Oslo, Norway) and centrifuged at 750 x g for 20 minutes. The interface layers were collected and the PBMC were pelleted by centrifuging at 500 x g for 5 minutes, washed twice in PBS and resuspended in 5 ml of RPMI-1640 Culture Medium (Life Technologies), supplemented with Hepes Buffer 0.27 mM (HEPES Sodium Salt; Sigma-Aldrich), $NaHCO_3$ 0.24 mM

(Koch-Light Laboratories, Colnbrook, England), gentamicin 50 μg / ml (Sigma-Aldrich), 2-mercaptoethanol 0.05 mM (2-ME; Sigma-Aldrich), GLUTAMAX™-I 10 ml / litre (Life Technologies) and 5% heat-inactivated foetal calf serum (Qualified FCS; Life Technologies).

An aliquot (20 μl) of cells from each animal was diluted in 180 μl Trypan blue (Sigma-Aldrich) and the viable cells were counted in an Improved Neubauer haemocytometer. The cell concentrations were adjusted to 2 x 10^6 cells / ml with RPMI-1640 culture medium, and aliquots of suspended cells (2 x 10^5 PBMCs in 100 μl) were dispensed into 96-well flat-bottomed tissue culture plates (Nunc, Roskilde, Denmark).

Antigens were diluted in RPMI-1640 culture medium and aliquots (100 μl) were added to quadruplicate wells. The PBMC from each animal were stimulated with concanavalin A (ConA; Sigma-Aldrich) to check cell viability, PPD-B and PPD-A tuberculins (Sterile Preservative Free 0.3 mg / ml; CSL Veterinary (NZ) Ltd, Upper Hutt, NZ) to monitor responses to mycobacterial antigens, and CF proteins from recombinant *M. smegmatis* clones. RPMI-1640 culture medium without antigen (100 μl) was added to one quadruplicate of PBMC from each animal as an unstimulated control.

The cultures of PBMC (200 μl) were incubated at 37°C in a humidified atmosphere supplemented with 5% CO_2 for five days. On day five, aliquots (50 μl) of supernatant were harvested from each well, and the supernatants from quadruplicate wells were pooled and frozen at -20°C until assayed for IFN-γ. The cell cultures were pulsed for the final 16 hours with 0.5 μCi of [*methyl-* ^{3}H] Thymidine (Amersham Pharmacia Biotech) diluted in 10 μl of dH_20. The cells were harvested onto glass fibre mats (Printed Filtermat A; Wallac Oy, Turku, Finland) using an LKB Wallac 1295-001 Cell Harvester, and the incorporated radioactivity was measured as counts per minute (c.p.m) in an LKB Wallac 1205 BETAPLATE™ liquid scintillation counter. The results were expressed as the difference between the mean c.p.m of the stimulated and unstimulated quadruplicate wells (Δ c.p.m). A positive response was defined as the mean c.p.m of the stimulated quadruplicates being

greater than three times the mean c.p.m of the unstimulated wells, if Δ c.p.m was greater than 2000 (Buddle *et al.* 1995a, Rhodes *et al.* 2000a).

3.2.8 IFN-γ assays.

The relative IFN-γ concentrations in pooled supernatants from cultures of PBMC and whole blood were estimated using the BOVIGAM™ Interferon Test Kit (CSL). The kit detects bovine IFN-γ by enzyme immunoassay (EIA). An anti-bovine IFN-γ antibody adsorbed to the solid phase captures bovine IFN-γ present in the sample. A second anti-bovine IFN-γ antibody conjugated to horseradish peroxidase binds the captured IFN-γ. The rate at which the enzyme substrate 3',3',5',5'-tetramethylbenzidine (TMB) is converted is proportional to the amount of bound IFN-γ. The reaction is stopped with sulphuric acid after 30 minutes (Rothel *et al.* 1990) .

Colour development was estimated by measuring the OD_{450} in a 340 ATC ELISA Plate Reader (SLT Labinstruments GmbH, Grödig, Austria), and the readings were multiplied by 1000 to eliminate the decimal point. A positive response was defined as a difference in OD_{450} between stimulated and non-stimulated wells (ΔOD_{450}) of greater than or equal to 100.

Positive IFN-γ response = $\Delta OD_{450} \geq 100$ (Wood *et al.* 1991, Fifis *et al.* 1994a).

3.2.9 N-terminal sequencing.

The N-terminal amino acid sequences of proteins in chromatographic fractions, were determined by automated Edman degradation at Massey University Amino Acid and Protein Sequencing Services. The apparatus used was an Applied Biosystems Protein Sequencer, Model 476A, with data collection and analysis performed with a Model 610 Data Analysis Module (Applied Biosystems, Foster City, USA).

3.2.10 Mass spectrometry.

The molecular mass of proteins in eluates from the ProRPC 5/2 reverse phase column were determined using a Sciex API 300 Electrospray MS-Mass Spectrometer (MDS Sciex, Toronto, Canada) by Massey University MasSpec Services.

3.2.11 Computer aided sequence analysis.

The programmes BLASTN and BLASTP (Altschul *et al.* 1997) were accessed from the National Centre for Biotechnology Information website: http://www.ncbi.nlm.nih.gov, and used to search the EMBL and GenBank non-redundant databases for homologous sequences. Amino acid sequences were also analysed using programmes available through the ExPASy website: http://expasy.proteome.org.ch. The programme SignalP at: http://www.cbs.dtu.dk was used for signal peptide prediction. The programme PSORT at: http://psort.nibb.ac.jp was used to predict protein location with respect to the bacterial cell membrane. ProtParam tools was used to calculate the predicted molecular weight and theoretical pI from the submitted amino acid sequence.

3.2.12 Immunoblotting.

Culture filtrate proteins were separated by electrophoresis (Tris-tricine SDS-PAGE) by a modification of the method described by Schagger and von Jackow (1987). Ten lane 0.75 mm thick gels were poured in two layers using a Mini-PROTEAN II Cell and accessories (Bio-Rad). The resolving gel consisted of 15.5% Acrylamide/Bis 29:1 (Bio-Rad), Tris-HCl 1.08 M (Roche), HCl 0.36 M (Roche), 0.11% sodium dodecyl sulphate (SDS; Roche), 12% glycerol, 0.47% ammonium persulfate (APS; Sigma-Aldrich), 0.47% N,N,N',N'-tetramethylethylenediamine (TEMED; Bio-Rad). The stacking gel consisted of 3.9% Acrylamide/Bis 29:1, Tris-HCl 0.75 M, HCl 0.25 M, 0.075% SDS, 0.82% APS, 0.82% TEMED. The anode buffer was Tris-HCl 0.2 M (pH 8.9), and the cathode buffer was Tris-HCl 0.1 M, Tricine 0.1 M (AnalaR;BDH), 0.1% SDS (pH 8.25).

Aliquots (10 µl) of CF were mixed with equal volumes of non-reducing or reducing sample loading buffers (2xSLB; 15% glycerol, Tris-HCl 50 mM, 5% SDS, 0.003% bromophenol blue; with 1.0 M DTT or 2% 2-ME), heated to 100°C for 10 minutes, centrifuged briefly at 12,600 x g, and loaded onto the gels. A molecular weight marker was loaded either side of the samples. A voltage of 150 V was applied until the dye front reached the bottom of the gels after approximately 1.5 hours.

The proteins were transferred onto BioTrace™ PVDF membranes with a pore size of 0.45 µm (Pall Corporation, Ann Arbor, USA) in a TRANS-BLOT®SD SEMI-DRY TRANSFER CELL (Bio-Rad). The transfer buffer was Tris-HCl 48 mM, glycine 39 mM, 0.015% SDS, 20% methanol (pH >8.5). A voltage of 15 V was applied for 35 minutes at a maximum current of 0.2 mAmps per gel blotted.

The membranes were blocked overnight with 2% BSA (Sigma-Aldrich) or 2.5% non-fat skim milk dissolved in Tween Tris Buffered Saline (TTBS; Tris-HCl 25 mM, 0.9% NaCl, 0.1% Tween 20 (Bio-Rad), pH 7.5). The antibodies and enzyme-conjugate were also diluted in TTBS. Incubations were for one hour with shaking at room temperature, and between each step the membranes were washed three times in TTBS. The primary antibody was rabbit polyclonal antiserum, typically diluted 1/1000. The secondary antibody was goat anti-rabbit IgG biotin conjugate (Sigma Chemical Co, St Louis, USA) diluted 1/5000. The enzyme conjugate was Streptavidin-POD (Roche) diluted 1/5000. The conjugated horseradish peroxidase, immobilised on the membranes was detected by reaction with DAB peroxidase substrate (SIGMA FAST™ 3,3'-Diaminobenzidine Tablet Sets; Sigma-Aldrich).

3.2.13 Production of polyclonal antisera in rabbits.

Polyclonal antisera were raised against the 8.4 kDa antigen in two New Zealand white rabbits. The preparation of antigen injected into the rabbits consisted of pooled Superose 6 HR 10/30 and ProRPC HR 5/2 FPLC column eluate fractions derived from CF of *M. smegmatis* pSU151.43. Prior to being pooled the protein in the fractions was identified as the 8.4 kDa antigen by N-terminal amino acid sequence. Aliquots of the preparation of 8.4 kDa antigen (30 µg) were suspended in 1 ml of Incomplete Freund's Adjuvant (IFA, Life Technologies) and the rabbits were inoculated twice subcutaneously at an interval of three weeks.

One month after the second injection, blood samples were taken from an ear vein and the titres of the antisera were determined. To collect bulk polyclonal antiserum, the rabbits were anaesthetized with a proprietary combination of tiletamine hydrochloride and zolazepam hydrochloride (Zoletil 50; Virbac Laboratories (NZ) Ltd., Auckland, NZ) and exsanguinated by cardiac puncture. The blood was incubated at 37°C for one hour while the clot formed, stored at 4°C overnight to effect clot retraction, and the clotted blood was

centrifuged at ~1000 x g for 10 minutes. The serum was aspirated and aliquots (10 ml) were stored at -20°C.

3.3 RESULTS.

3.3.1 Growth curves of *M. smegmatis* recombinants.

Cultures (80 ml) of *M. smegmatis* clones containing the plasmids pSU4511, pSU151.43 and pYUBCos151 were grown as described. At intervals, OD_{580} measurements were taken and plotted against time to generate bacterial growth curves. The growth curves of *M. smegmatis* clones varied according to the plasmid each contained. Typically, *M. smegmatis* pSU4511 and pSU151.43 reached an OD_{580} of 0.9 after approximately 55 hours, whereas pYUBCos151 reached an OD_{580} of 0.8 after 80 hours (data not shown).

3.3.2 Stimulatory activity of *M. smegmatis* culture filtrates in lymphocyte proliferation and IFN-γ production assays.

Lymphocyte proliferation and IFN-γ assays were performed as described. PBMC were stimulated with CF from log and stationary phase cultures (80 ml) of recombinant *M. smegmatis* clones containing plasmids pYUB18, pYUBCos151, pSU4511 and pSU151.43 at concentrations of 0.25, 0.5, 2.5 and 10 μg protein / ml. The responses to stimulation at 2.5 μg protein / ml are shown in Figures 3.1 and 3.2..

In the lymphocyte proliferation assays, ConA stimulated strong proliferation responses from all the animals (Δ c.p.m > 34,000). PBMC from the BCG vaccinated animal (No.5) proliferated in response to PPD-B (Δ c.p.m 30,538), Cos151 log phase CF (Δ c.p.m 23,095), Cos151 stationary phase CF (Δ c.p.m 24,623), pSU151.43 log phase CF (Δ c.p.m 27,959), and pSU151.43 stationary phase CF (Δ c.p.m 12,057). The unstimulated wells recorded a mean c.p.m of 339. PBMC from control animal No.53 proliferated in response to PPD-A (Δ c.p.m 5564), but not PPD-B. The unstimulated control wells recorded a mean c.p.m of 162. PBMC from control animal No.55 proliferated in response

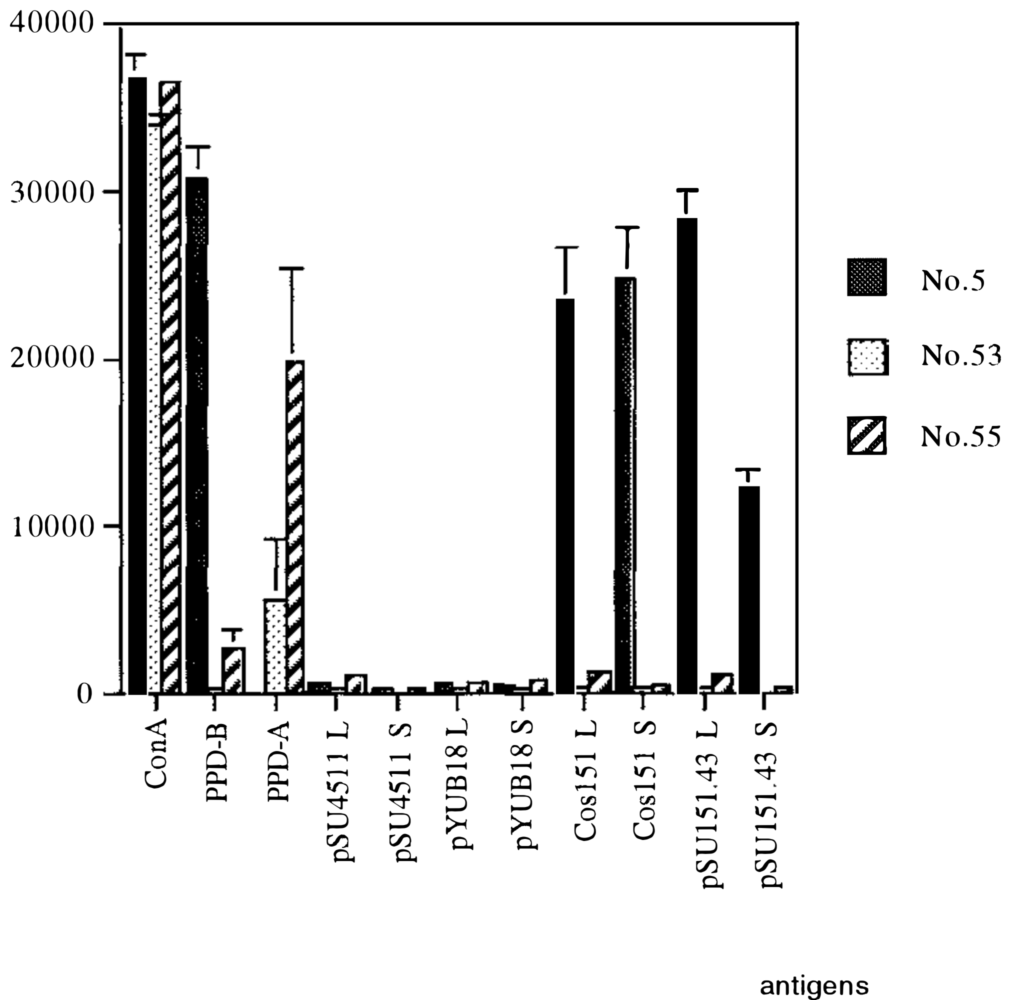

Figure 3.1 Lymphocyte proliferation responses (Δ c.p.m) of PBMC from the BCG vaccinated animal No.5 and the unvaccinated control animals Nos. 53 and 55, stimulated at 2.5 μg protein / ml. The stimulatory antigens were ConA, PPD-B, PPD-A and CFs from log (L) and stationary (S) phase cultures of the recombinant clones *M. smegmatis* pSU4511 and *M. smegmatis* pYUB18 that contained plasmids without inserts, or *M. smegmatis* Cos151 and *M. smegmatis* pSU151.43 that contained plasmids with inserts of *M. bovis* DNA. Standard errors of the mean are shown. The response of No.5 to PPD-A was not determined.

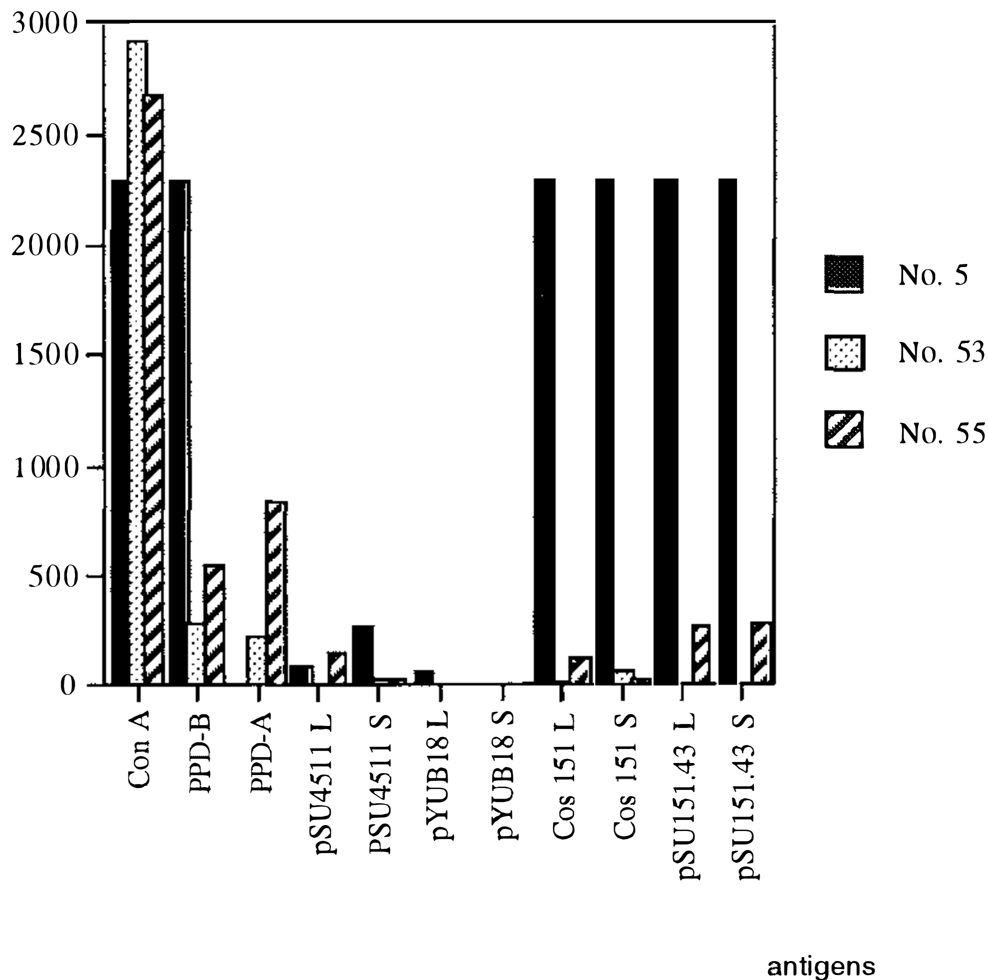

Figure 3.2 IFN-γ responses (Δ OD_{450}) of PBMC from the BCG vaccinated animal No.5 and the unvaccinated control animals Nos. 53 and 55, stimulated at 2.5 μg protein / ml. The stimulatory antigens were ConA, PPD-B, PPD-A and CFs from log (L) and stationary (S) phase cultures of the recombinant clones *M. smegmatis* pSU4511 and *M. smegmatis* pYUB18 that contained plasmids without inserts, or *M. smegmatis* pYUBCos151 and *M. smegmatis* pSU151.43 that contained plasmids with inserts of *M. bovis* DNA. The response of No.5 to PPD-A was not determined.

to PPD-A (Δ c.p.m 19,988) and PPD-B (Δ c.p.m 2326). The unstimulated control wells recorded a mean c.p.m of 445 (see Figure 3.1).

In the IFN-γ assays, the BCG vaccinated animal (No.5) had responses to ConA, PPD-B, Cos151 CFs and pSU151.43 CFs that exceeded the range of the ELISA plate reader (Δ $OD_{450} \geq 2288$), and a low level response to pSU4511 stationary phase CF (Δ OD_{450} 280). Control animal No.53 had low level responses to PPD-B (Δ OD_{450} 292) and PPD-A (Δ OD_{450} 225), and a saturated ConA response (Δ $OD_{450} \geq 2916$). Control animal No.55 had moderate responses to PPD-A (Δ OD_{450} 847) and PPD-B (Δ OD_{450} 547). Animal No.55 had low level responses to pSU4511 log phase CF, Cos151 log phase CF, pSU151.43 log phase CF, and pSU151.43 stationary phase CF (Δ OD_{450} 130 to 290). Animal No. 55 also had a saturated ConA response (Δ $OD_{450} \geq 2677$) (see Figure 3.2).

3.3.3 Fractionation of *M. smegmatis* pSU151.43 culture filtrate.

Cultures (5 x 900 ml) of *M. smegmatis* pSU151.43 were grown and harvested as described. Concentrated CF that contained approximately 20 mg of protein was separated by anion exchange FPLC. The 21 eluate fractions (A to U) were tested in whole blood IFN-γ assays at a protein concentration of 6.7 μg / ml. The fractions A, B, C, D, and E elicited IFN-γ production responses (Δ $OD_{450} > 300$) from the BCG vaccinated animal (No.5) only. The pooled fractions LM and NO elicited IFN-γ responses of similar or lower magnitude from both the BCG vaccinated animal and the two control animals (see Figures 3.3 and 3.4).

The Uno™Q-6 anion exchange column eluate fractions A, C, D and E were separated by size exclusion chromatography with a Superose 6 HR 10/30 column. Up to eight aliquots (0.2 ml) of each fraction were separated by consecutive runs. The corresponding eluates from each run were combined. The protein concentration could be quantified only in the combined eluate fractions that corresponded to A_{280} peaks of fractions A and C that eluted at 67 minutes (see Figure 3.5), the A_{280} peak of fraction D that eluted at 62 minutes and E

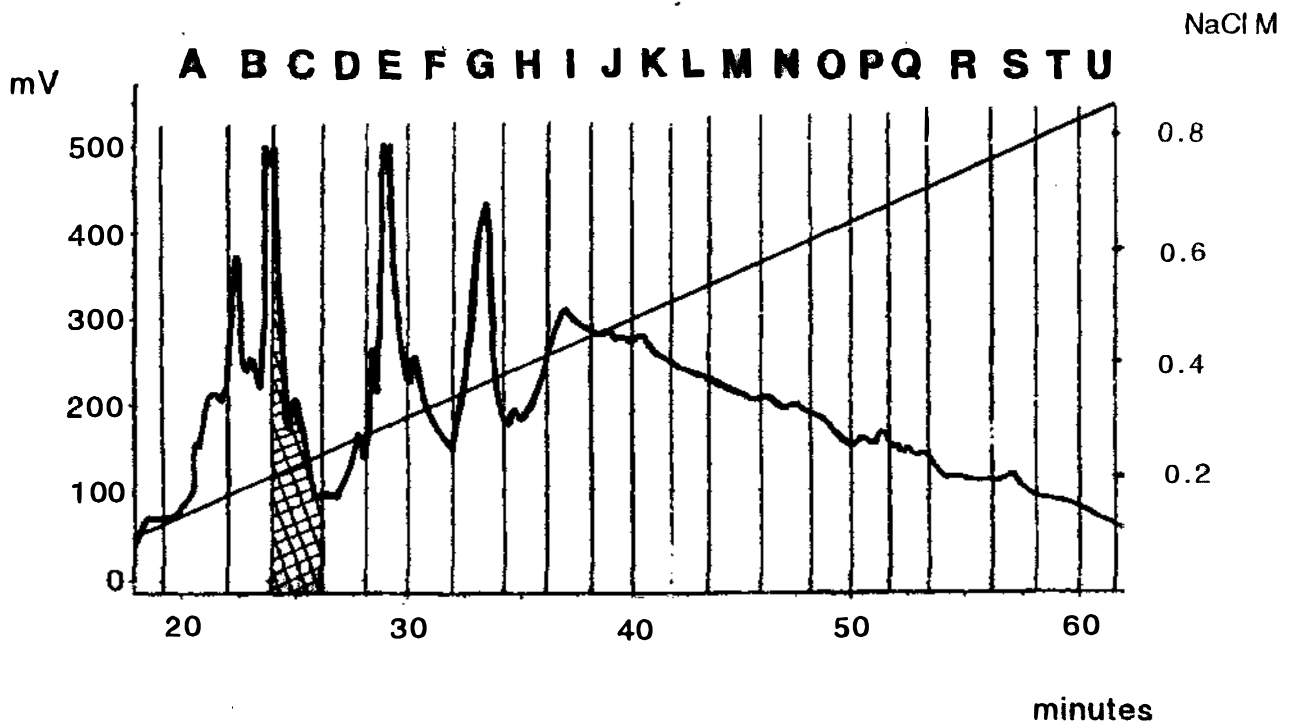

Figure 3.3 A section of a typical Uno™Q-6 anion exchange FPLC fractionation run. The trace indicates the A_{280} of the eluate. Concentrated CF from *M. smegmatis* pSU151.43 that contained 20 mg of protein was loaded onto the column in Tris-HCl 20 mM. The bound proteins were eluted by an NaCl concentration gradient (0 to 1 M) applied from 12 to 72 minutes. A to U represent the 4 ml eluate fractions that were collected between 18 and 64 minutes as the NaCl concentration increased from 0.1 M to 0.87 M. No A_{280} peaks were observed before 18 minutes. Fractions A to U were assayed for stimulatory activity in whole blood IFN-γ assays at 6.7 µg protein / ml (see Figure 3.4). Fraction C (shaded) was further fractionated by gel filtration (see Figure 3.5) or reverse phase FPLC (see Figure 3.6).

that eluted at 63 minutes. Fraction B was not separated by size exclusion chromatography because the column ceased to function.

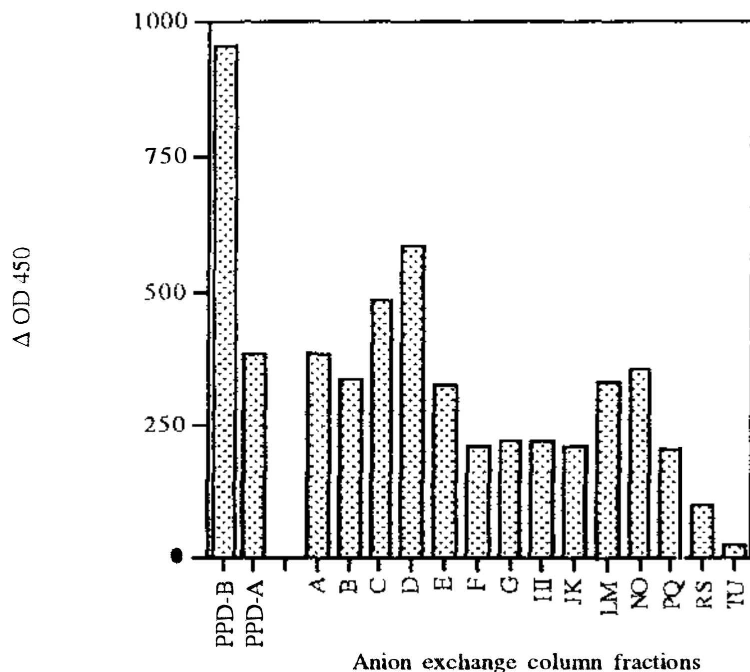

Figure 3.4 Representative whole blood IFN-γ assay responses (Δ OD_{450}) of the BCG vaccinated animal No.5 to Uno™Q-6 anion exchange column fractions. Culture filtrate from *M. smegmatis* pSU151.43 was fractionated by FPLC through an Uno™Q-6 anion exchange column (see Figure 3.3). The column eluate fractions (A to U) were assayed for stimulatory activity in whole blood IFN-γ assays at a concentration of 6.7 μg protein / ml blood. Fractions A to G were assayed individually and H to U were assayed as pooled pairs of fractions. The fractions A, B, C, D, and E elicited responses (Δ OD_{450} > 300) from the BCG vaccinated animal No.5 only. The responses to PPD-B and PPD-A (20 μg / ml) are shown for comparison.

The Superose 6 HR 10/30 eluate pools derived from each of A, C, D and E in which the protein concentration was quantified were tested for stimulatory activity in whole blood IFN-γ assays at 3.3 μg protein / ml. The one eluate pool derived from A and another from C elicited positive IFN-γ responses (Δ OD_{450} ~150) from the BCG vaccinated animal

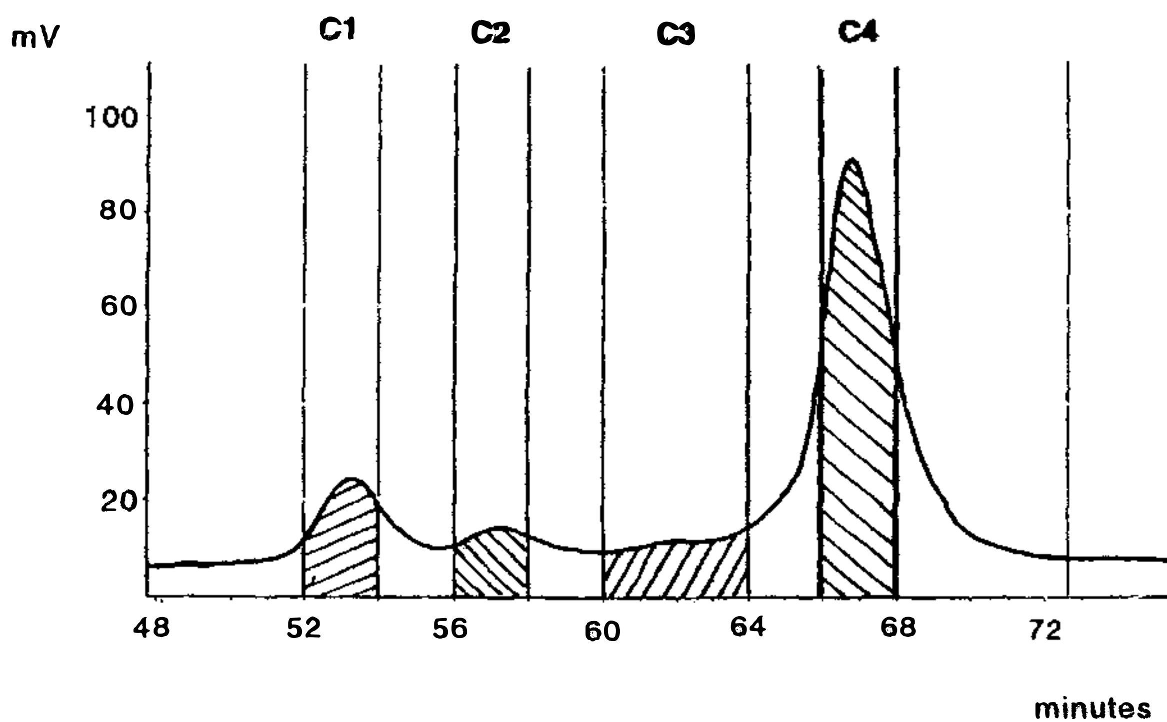

Figure 3.5 A section of a typical Superose 6 HR 10/30 gel filtration FPLC run showing fractionation of the Uno™Q-6 eluate fraction C (see Figure 3.3). Only the observed A_{280} peaks are shown. The sample was separated by consecutive runs of 0.2 ml aliquots and the A_{280} peaks of each run occurred at the same timepoints. The fractions corresponding to the peaks C_1, C_2, C_3 and C_4 (hatched) were combined and the protein concentrations were measured by MicroBCA assay. Only the pool of fractions corresponding to C_4 contained sufficient protein to be quantified.

(No.5). The one eluate pool derived from D which included the the fraction that eluted at 67 minutes elicited an IFN-γ response of Δ OD_{450} 120. The one eluate pool derived from E elicited an IFN-γ response of Δ OD_{450} 60.

To purify the protein from a further batch of *M. smegmatis* pSU151.43, the Uno™Q-6 eluate fraction corresponding to fraction C was separated by reverse phase chromatography. Initially fraction 'C' was loaded onto the ProRPC 5/2 column in 0.1% TFA. The eluates were tested for stimulatory activity in whole blood IFN-γ assays at 3.3 μg protein / ml.

The fractions that had eluted at 12, 14 and 25% acetonitrile elicited IFN-γ responses of Δ OD_{450} 270, 270 and 210 respectively from the BCG vaccinated animal (No.5). Those fractions were reloaded onto the ProRPC 5/2 column in 0.15% TFA. Each eluted as a single A_{280} peak at 31% acetonitrile. The fractions corresponding to the A_{280} peak of each sample were combined to give the pools 12a, 14a and 25a (see Figure 3.6). The pools were tested for stimulatory activity in lymphocyte proliferation and IFN-γ assays at 0.25 μg protein / ml (see Table 3.2).

Table 3.2 Lymphocyte proliferation and IFN-γ responses to ProRPC column eluates derived from CF of *M. smegmatis* pSU151.43.

	Lymphocyte proliferation (SI).			IFN-γ (Δ OD_{450})			
ProRPC #	5	53	55	5	53	55	Animal
12a	4.1	0.9	1.4	160	28	53	
14a	5.4	1.0	1.2	297	104	-27	
25a	1.7	1.1	1.2	64	-45	-7	

Positive responses were defined as Lymphocyte proliferation SI ≥ 3, IFN-γ assay Δ OD_{450} ≥ 100.

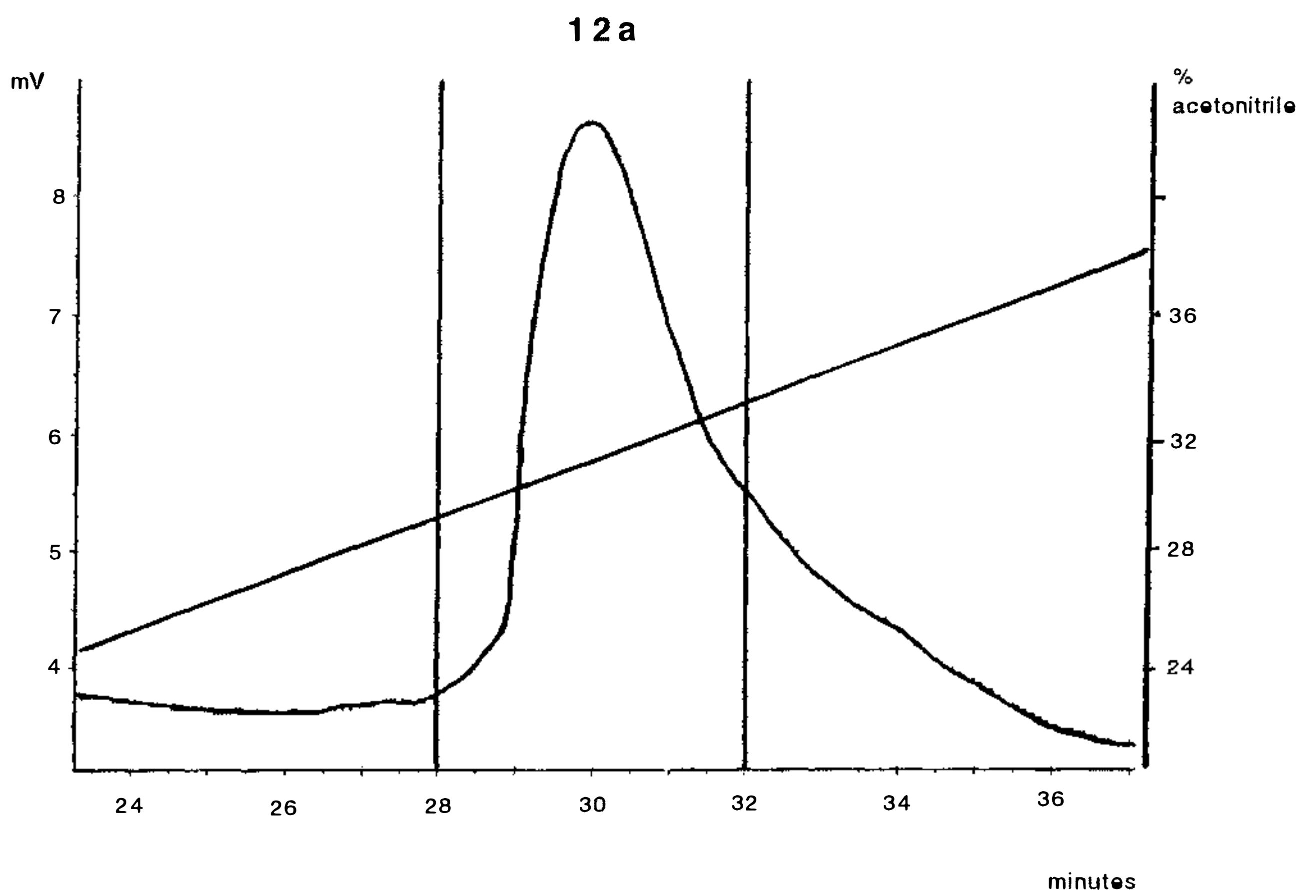

Figure 3.6 A section of a ProRPC 5/2 reverse phase FLPC run showing elution of the 8.4 kDa protein as a single peak at 31% acetonitrile. The ProRPC 5/2 fraction that eluted at 12% acetonitrile when loaded in 0.1% TFA was reloaded onto the ProRPC 5/2 column in 0.15% TFA. An elution gradient of 0 to 50% acetonitrile was applied. The A_{280} peak shown was the only elution peak detected. The eluate fractions collected between 28 and 32 minutes were combined to make pool 12a and tested for activity in lymphocyte proliferation and IFN-γ assays.

3.3.4 N-terminal sequencing.

An aliquot of fraction C_4 eluted from the Superose 6 HR 10/30 size exclusion column (see Figure 3.5) containing 30 μg of protein was submitted for N-terminal sequence analysis. The ten amino acids at the amino terminus of the purified protein were determined to be **D P V D A V I N T T**.

3.3.5 Computer analysis of the predicted amino acid sequence.

The experimentally determined N-terminal amino acid sequence was submitted for a BLASTP homology search and aligned with the *M. tuberculosis* H37Rv segment 51/162. The N-terminal 10 amino acids of the purified protein corresponded to amino acids 29 to 38 of the hypothetical *M. tuberculosis* H37Rv protein Rv1174c (Cole *et al.* 1998), which has 100% homology with the previously described 8.4 kDa proteins of *M. tuberculosis* and *M. bovis* BCG (Coler *et al.* 1998, Freer *et al.* 1998a).

The EMBL data base annotation for the *M. tuberculosis* H37Rv protein was:

DEFINITION hypothetical protein Rv1174c [Mycobacterium tuberculosis].
ACCESSION CAA15851
DBSOURCE embl locus MTV005, accession AL010186.1
FEATURES
Protein /product="hypothetical protein Rv1174c"
CDS /gene="Rv1174c"
/db_xref="SPTREMBL:050430"
/coded_by="complement (AL010186.1:9587..9919)"
/transl_table=11
/note="Rv1174c, (MTV005.10c). len: 110. Unknown."

The EMBL database annotation for the *M. tuberculosis* H37Rv gene was:

Accession: emb I AL010186.1 I MTV005 Mycobacterium tuberculosis H37Rv complete genome; segment 51/162
complement (9587-9919)
gene="Rv1174c"
product="hypothetical protein Rv1174c"
protein_id="CAA15851.1"

The 40 N-terminal amino acids of the 110 amino acid deduced protein sequence were submitted for SignalP analysis. The analysis predicted that the protein has an N-terminal signal region with a probable cleavage site between positions 28 and 29 (ASA/DP). The 110 amino acids of translated sequence was submitted for PSORT analyses, which

predicted a cleavable N-terminal signal sequence and that the mature protein would be located outside a gram-positive bacterial cell membrane. Analysis of the 82 amino acid sequence of the mature exported protein by ProtParam tools predicted that the cleaved protein would have a molecular weight of 8337.3Da and a theoretical pI of 4.23.

3.3.6 Mass Spectrometry.

The ProRPC 5/2 reverse phase column fraction that eluted at 31% acetonitrile was submitted for determination of the molecular mass and N-terminal sequence of the eluted protein. The actual molecular mass of the purified protein was determined to be **8397.0 Da** and the first four N-terminal amino acid residues were **DPVD**.

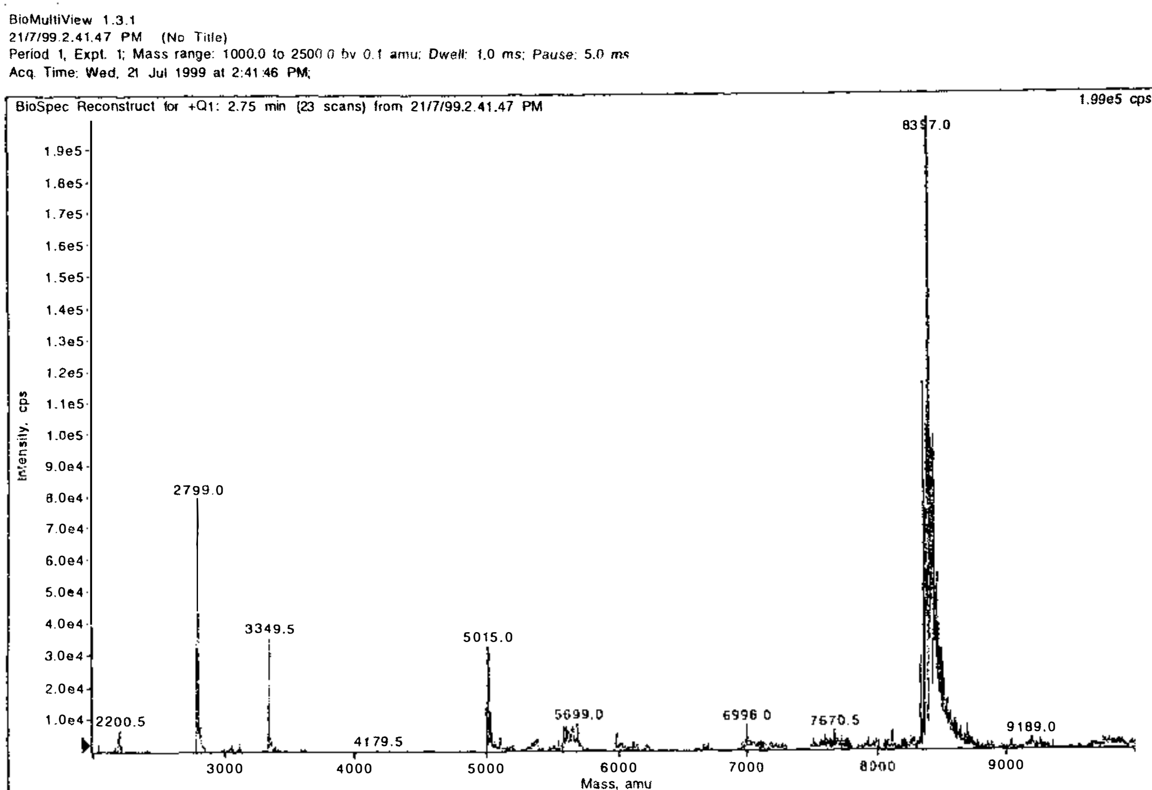

Figure 3.7 Mass spectrometric analysis of a preparation of the 8.4 antigen purified by reverse phase chromatography. The output of the Sciex API 300 Electrospray MS-Mass Spectrometer shows a strong signal at 8397.0 amu.

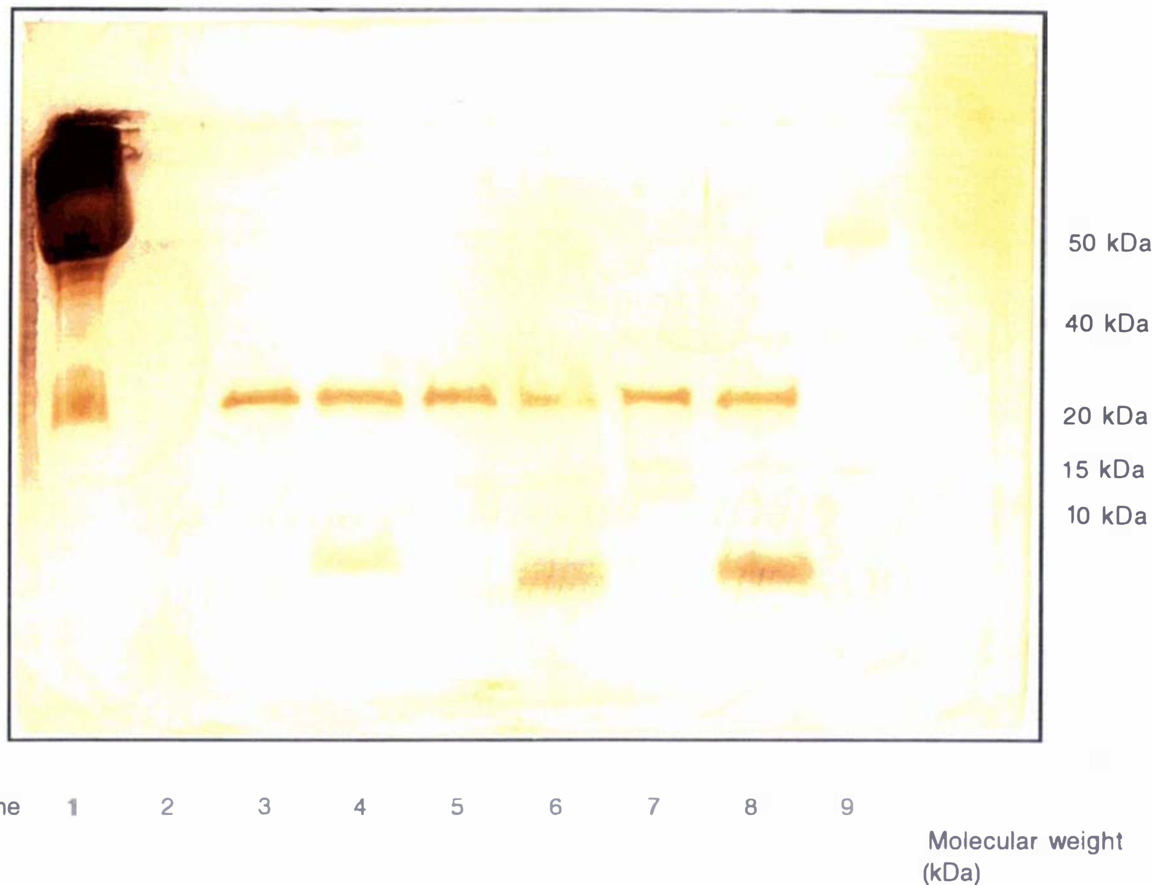

Figure 3.8 Western blot showing the 8.4 kDa Ag detected by specific polyclonal antiserum following separation of *M. smegmatis* pSU151.43 CF in reducing and non-reducing conditions. CFs from the *M. smegmatis* clone pSU4511 that contained plasmid without insert (control) and *M. smegmatis* pSU151.43. that contained a 4.3 kb insert of *M. bovis* DNA coding for the 8.4 kDa Ag were separated by Tris-tricine SDS-PAGE in non-reducing conditions (lanes 3 and 4), reduced with 2% 2-ME (lanes 5 and 6) or reduced with 0.5 M DTT (lanes 7 and 8). The Benchmark Prestained Protein Ladder (Life Technologies) was run in lanes 2 and 9. The proteins were transferred onto PVDF membrane and the 8.4 kDa Ag was detected with rabbit anti-8.4 kDa polyclonal serum (1/1000 dilution), goat anti-rabbit biotin conjugate (1/5000), Streptavidin-POD (1/5000) and DAB. The apparent molecular weights of the detected proteins were estimated from a relative electrophoretic mobility curve (MW (log_{10}) of the protein standards vs migration distance (mm)). Unreduced 8.4 kDa Ag was weakly detected and had an apparent molecular weight of 8 kDa (lane 4), whereas reduced 8.4 kDa Ag was strongly detected and had migrated further (lanes 6 and 8). Each band represents 1.2 µg of 8.4 kDa Ag. The identity of the band with an apparent molecular weight of 22 to 23 kDa is unknown, but it was also detected by sera from unvaccinated rabbits (not shown).

3.3.7 Immunoblotting.

Samples (10 µl) of concentrated CF from *M. smegmatis* pSU4511 and pSU151.43 were separated by Tris-tricine SDS-PAGE in reducing and non-reducing conditions. Western blotting was performed and the rabbit polyclonal antiserum detected a protein with an apparent molecular weight of approximately 8 kDa in the lanes corresponding to CF from *M. smegmatis* pSU151.43. The N-terminal sequence of the detected protein was DPVDA, which is identical to the sequence of the purified 8.4 kDa protein that was inoculated into the rabbits. The apparent molecular weights of the reduced and un-reduced forms of the 8.4 kDa were observed to differ by approximately 1 to 2 kDa (see Figure 3.8).

3.4 DISCUSSION.

Numerous *M. bovis* and *M. tuberculosis* extracellular protein antigens have been identified on the basis of their recognition by murine monoclonal antibodies (Young *et al.* 1992). However, it is technically difficult to identify the *M. bovis* and *M. tuberculosis* antigens that are targets of the T-cell mediated responses of natural hosts (Fifis *et al.* 1994b, Gulle *et al.* 1995, Sonnenberg and Belisle 1997, Rosenkrands *et al.* 1998, Weldingh *et al.* 1998). It had been proposed that CFs from cosmid libraries of *M. bovis* BCG and *M. bovis* constructed in *M. smegmatis* could be screened in lymphocyte proliferation and IFN-γ assays to identify the regions of the *M. bovis* genome coding for secreted recombinant *M. bovis* antigens recognized by the T-cells of homologously sensitized individuals (Averill *et al.* 1993).

In this laboratory a number of CFs from a cosmid library of *M. bovis* constructed in *M. smegmatis* had been found to stimulate PBMC from cattle vaccinated with *M. bovis* BCG (Gormley *et al.* 1999). However, it remained to be determined whether the source of immunoreactivity was indeed a secreted *M. bovis* antigen, or if the insert simply caused *M. smegmatis* to overproduce non-specific stimulatory substances such as heat shock proteins.

This study found that CFs from both log and stationary phase cultures of the *M. smegmatis* subclone containing a 4.3 kb *Sph* I fragment of *M. bovis* DNA stimulated high level lymphocyte proliferation and IFN-γ production responses from the PBMCs of a steer that had been vaccinated with *M. bovis* BCG. However, it was noted that CFs from some

of the subclones without inserts of *M. bovis* DNA stimulated low level IFN-γ responses from the PBMCs of the animal that had been vaccinated with *M. bovis* BCG and the control animal that was an avian reactor.

To identify the source of immunoreactivity, CF from the *M. smegmatis* subclone pSU151.43 was fractionated by FPLC. The only known feature of the protein was its immunoreactivity with PBMC from the steer that had been vaccinated with *M. bovis* BCG, therefore fractionation was monitored with lymphocyte proliferation and whole blood IFN-γ assays. Immunological assays are subject to the variability inherent in bioassays and a potential weakness of this study was that it relied on the specific responses of only one animal. Nevertheless, the immune responses of the BCG vaccinated animal enabled the eluate fractions containing the 8.4 kDa protein to be identified.

Approximately 120 µg of recombinant 8.4 kDa protein antigen was purified from each 4.5 litre batch of *M. smegmatis* pSU151.43 CF. The N-terminal amino acid sequence of the 8.4 kDa protein was determined and is consistent with being encoded by a predicted gene present within the 4.3 kb fragment of *M. bovis* DNA. The nucleotide sequence of the gene is identical to the predicted *M. tuberculosis* H37Rv gene Rv1174c, and the genes coding for the 8.4 kDa antigens previously identified in the CFs of *M. bovis* BCG and *M. tuberculosis* (Coler *et al.* 1998, Freer *et al.* 1998a) (see Appendix III). The gene codes for a 110 amino acid protein, which consists of a predicted 28 amino acid export signal sequence and an 82 amino acid exported protein. The N-terminal sequence determined in this study was identical to amino acids 29 to 38 of the deduced protein.

A previous study by Freer *et al.* (1998a) had determined by localization index analysis that the 8.4 kDa antigen of *M. bovis* BCG was a secreted protein, and also reported that the 8.4 kDa antigen was detected by the monoclonal antibody L8D8 on Western blots only after CF had been separated by SDS-PAGE in reducing conditions. In contrast, the study reported here found that the polyclonal antisera raised in rabbits detected the 8.4 kDa antigen on Western blots following separation by Tris-tricine SDS-PAGE in both reducing and non-reducing conditions. However, the electrophoretic mobility and antibody binding properties of the reduced and non-reduced forms of the protein were different.

The altered electrophoretic mobility of the reduced protein is consistent with reduction having broken a disulphide bond between the cysteine residues at positions 11 and 79 of

the exported protein. It has been reported that alterations to electrophoretic mobility following reduction of disulphide bonds are dependent on the net effect of more SDS bound to the unfolded polypeptide increasing the speed of migration, and greater frictional resistance slowing passage of the linearized protein through the polyacrylamide matrix (Dunker and Rueckert 1969, Allore and Barber 1984). It was also observed that the reduced protein appeared to be detected more strongly on Western blots than the unreduced protein. The apparent greater affinity of the polyclonal antisera for the reduced protein could have been because changes in the conformation of the molecule exposed more antibody binding sites.

The molecular mass of the exported 8.4 kDa protein was predicted from the deduced amino acid sequence to be 8337 Da. However, if the protein had an intramolecular disulphide bond the molecular mass was predicted to be 8335 Da, due to the loss of two hydrogen atoms. The measured molecular mass of the purified protein was 8397 Da. Analysis of the mass spectrographic readout at high resolution revealed shoulder peaks surrounding the 8397 peak that had molecular masses consistent with the adduction of Na^+ and K^+ ions to the protein. Adduction of Na^+ and K^+ ions to proteins is a common artifact encountered in mass spectrometry and occurs when proteins are purified in buffers containing Na^+, particularly if the eluates are stored in glass containers (Dr. Gill Norris, Massey University MasSpec Services - personal communication). The adduction of a Na^+ (23 Da) and a K^+ (39 Da) to the purified protein would have increased the molecular mass of the protein by 62 Da. Therefore, by subtraction the actual molecular mass of the protein was calculated to be 8335 Da. Mass spectrometry provided additional evidence that the gene identified within the 4.3 kb insert of *M. bovis* DNA codes for the 8.4 kDa antigen and that the protein has an intramolecular disulphide bond.

In conclusion, this study employed immunological and biochemical techniques to identify the gene coding for an 8.4 kDa secreted protein antigen of *M. bovis*. Moreover, this is the first study to report that PMBC from a bovine sensitized to *M. bovis* antigens by vaccination with *M. bovis* BCG proliferate and produce IFN-γ in response to stimulation with a recombinant 8.4 kDa antigen of *M. bovis* produced in *M. smegmatis*.

Chapter 4. Species distribution of the 62 base pair polymorphic tandem repeat locus.

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4.4 DISCUSSION..81

4.1 INTRODUCTION.

The mycobacterial species that constitute the TB complex are *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti* (Levy-Frebault and Portals 1992). The mycobacterium that causes tuberculosis in seals (Cousins *et al.* 1990) and *M. canetti* (van Soolingen 1997) are also closely related to the TB complex species. The classification of mycobacterial species by numeric taxonomy is based on a series of genotypic and phenotypic studies carried out under the auspices of the International Working Group on Mycobacterial Taxonomy (Goodfellow and Magee 1998). The procedures currently recommended for differentiating between TB complex isolates in diagnostic microbiology laboratories have been detailed in 'WHO / Guidelines for speciation within the *M. tuberculosis* complex' (Grange and Yates 1994).

Recently, the potential for typing TB complex isolates by genotypic methods has been investigated in detail (Kremer *et al.* 1999), and recommendations have been made to standardize typing protocols (Cousins *et al.* 1998b). To date, genetic characterization of mycobacterial isolates has been based on polymorphisms of the non repetitive *oxyR*, *pncA*, and *mpt-40* loci, or restriction fragment length polymorphisms (RFLP) of the insertion sequences IS6110 and IS1081, or spacer oligonucleotide typing of the direct repeat (DR) locus (spoligotyping), or variable number of tandem repeats (VNTR) typing (Frothingham *et al.* 1999, Vianca-Niero *et al.* 2001).

In Chapter 2 it was shown that the 4.3 kb insert of *M. bovis* DNA in plasmid pSU151.43 was 62 bp shorter than the homologous sequence of *M. tuberculosis* H37Rv. The *M. bovis* sequence derived from a New Zealand clinical isolate had 1.7 copies of a 62 bp tandem repeat, whereas *M. tuberculosis* H37Rv had 2.7 copies. The repeat region is a typical exact tandem repeat (ETR) locus, but it was not known whether the polymorphism observed was peculiar to those isolates or is a consistent difference between *M. bovis* and *M. tuberculosis*.

The objectives of this study were to determine the species distribution of the 62 bp ETR, and to ascertain whether it is an informative VNTR locus that can be used to differentiate *M. bovis* from *M. tuberculosis*. Isolates of a number of mycobacterial species were investigated by polymerase chain reaction (PCR) and Southern hybridization analysis.

Table 4.1 The bacterial species and strains analysed at Massey University.

Species	Details	Source / Reference
M. tuberculosis	5 NZ human isolates	Palmerston North Hospital
	2 NZ seal isolates	Hunter *et al.* 1998
	H37Ra (ATCC 25177)	ATCC, Rockville, USA
M. bovis	ATCC 19210, 35725, 35726, 35746	ATCC, Rockville, USA
	1 NZ bovine isolate (KML)	Massey University
	1 NZ canine isolate	Massey University
M. bovis BCG	Copenhagen (ATCC 27290)	ATCC, Rockville, USA
	Danish (ATCC 35733)	
	Glaxo (ATCC 35741)	
	Japan (ATCC 35737)	
	Montreal (ATCC 35735)	
	Pasteur (ATCC 35748)	
	Russian (ATCC 35740)	
M. avium	1 NZ veterinary isolate	Massey University
MAIS	2 NZ human isolates	Palmerston North Hospital
M. fortuitum	ATCC 6841	ATCC, Rockville, USA
M. gordonnae	ATCC 144470	ATCC, Rockville, USA
M. intracellulare	ATCC 35848	ATCC, Rockville, USA
M. kansasii	ATCC 12478	ATCC, Rockville, USA
M. marinum	ATCC 927	ATCC, Rockville, USA
M. scrofulaceum	ATCC 19981	ATCC, Rockville, USA
M. smegmatis	1 NZ veterinary isolate	Massey University
M. terrae	ATCC 15755	ATCC, Rockville, USA

4.2 MATERIALS AND METHODS.

4.2.1 Culture of mycobacteria.

Mycobacteria were inoculated onto slants of Bacto Lowenstein Medium Jensen, Bacto Middlebrook 7H10 Agar supplemented with Bacto Middlebrook OADC Enrichment, or Bacto Middlebrook 7H11 Agar supplemented with Bacto Middlebrook OADC Enrichment (Difco Laboratories, Detroit, USA), incubated at 37°C for 3 to 4 weeks until large colonies became visible, and were stored at 4°C.

4.2.2 Extraction of genomic DNA from mycobacteria for PCR.

Genomic DNA was extracted from mycobacteria by a modification of the method described by Shawar (1993). A small amount of bacterial culture was removed from the slant on a pipette tip and suspended in 100 µl of TE buffer, 1% Triton X-114 (Sigma Aldrich, Sydney, Australia), incubated at 95°C for 25 minutes, agitated briefly and centrifuged at 12,600 x g for 30 seconds. The aqueous top layer containing the extracted DNA was aspirated and stored at -20°C.

4.2.3 Design of PCR Primers.

A set of primers was designed to amplify the 62 bp ETR locus, and enable PCR product length polymorphisms between *M. bovis* and *M. tuberculosis* to be detected by agarose gel electrophoresis. The computer programme GeneWorks (IntelliGenetics Inc, Mountain View, USA) was used to identify primer pairs along the length of the 4.3 kb *M. bovis* sequence. The primer pair SM3/SM4 that hybridizes approximately 150 bp either side of the the 62 bp ETR locus was selected, because PCR products of between 300 and 400bp can be easily discriminated by agarose gel electrophoresis. The programme AMPLIFY version 1.2 (Engels 1992) was used to predict the sizes of the PCR products that would be amplified from the *M. bovis* and *M. tuberculosis* H37Rv sequences. The primer pair SM3/SM4 was predicted to generate PCR products of 315 bp from *M. bovis*, and 377 bp from *M. tuberculosis.*

A second set of primers was used to produce a labelled polynucleotide probe complementary to the 62 bp ETR locus. The primers SM21F and SM21R were designed with the assistance of the programmes MacVector™ 6.0 (Oxford Molecular Ltd, UK) and AMPLIFY version 1.2. The predicted PCR product amplified from *M. tuberculosis*

H37Rv is 213 bp long, and consists of 2.7 copies of the 62 bp repeat unit and 23 bp of flanking sequence at both ends.

The nucleotide sequences of the primers were submitted to the NCBI-BLAST server version 2.0 (Altschul *et al.* 1997) at the website: http://www.ncbi.nlm.nih.gov. The programme BLASTN was used in the Advanced BLAST mode to perform gapped alignments of the primer sequences against the nucleotide sequences in the EMBL and GeneBank non-redundant databases. Each primer aligned against only one segment of the *M. tuberculosis* H37Rv sequence.

The primers were manufactured by GIBCO BRL (USA) and supplied by Life Technologies, Auckland, NZ. Upon receipt, the primers were resuspended in dH_2O at a concentration of 100 mM and stored at -20°C.

Table 4.2 Primers used in this study.

Primer	Sequence	Source/Reference
SM3	5' GAG ACC ATC TCG CGG ATG G 3'	This study
SM4	5' CAA TTG CAA GCT GTG CCG G 3'	
Flank the 62 bp ETR. Amplify a 377 bp PCR product from *M. tuberculosis* and a 315 bp product from *M. bovis.*		
246	5' AGA GTT TGA TCC TGG CTC AG 3'	Böddinghaus *et al.* (1990)
264	5' TGC ACA CAG GCC ACA AGG GA 3'	
Hybridize specifically to the 16s rRNA genes of all mycobacterial species. Amplify a 1030 bp PCR product.		
SM21F	5' ACT ACC ACA TAC GCC CTG CTT G 3'	This study
SM21R	5' CTG CAA CAA CTA TTA AGC CCA TGC 3'	
Flank the 62 bp ETR, and used to make a polynucleotide probe complementary to the 62 bp repeat locus. Amplify a 213 bp product from *M. tuberculosis* , consisting of 2.7 copies of the repeat units and 23 bp of flanking sequence at both ends.		

4.2.4 PCR reaction conditions.

PCRs were performed in a total volume of 25 or 50 µl, in 0.2 ml thin walled PCR tubes (Life Technologies). The reaction mixture consisted of 1 x PCR Buffer (Tris-HCl 20 mM, KCl 50 mM, pH 8.4; Life Technologies), $MgCl_2$ 1.7 mM, dNTP 0.2 mM of each deoxynucleoside triphosphate (PCR Nucleotide Mix; Roche Diagnostics, Auckland, NZ), 1 unit of *Taq* DNA Polymerase (Promega Corporation, Madison, USA) and 0.4 mM of each primer. Reactions with primers SM3/SM4 also contained 10% dimethylsulfoxide (DMSO; Sigma-Aldrich, Sydney, Australia). Template DNA (10 µl) was added in a separate laboratory.

Control reactions were run in parallel. The positive control template DNA was previously extracted *M. tuberculosis* H37Ra and *M. bovis* genomic DNA. The negative control reactions contained an aliquot (10 µl) of TE buffer. The temperature cycling was performed in a Perkin Elmer GeneAmp® 9600 PCR System Thermocycler (Applied Biosystems, Foster City, USA), and the reaction conditions were; denaturation at 94°C for 5 minutes; followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds and extension at 70°C for 1 minute; followed by final extension at 70°C for 5 minutes.

4.2.5 Separation of PCR products by electrophoresis.

PCR products were separated by electrophoresis through agarose gels (2.5% TAE) submerged in 1 x TAE buffer. Aliquots of completed PCR reactions (10 to 15 µl) were mixed with 6 x Loading Buffer (5 µl) and separated by electrophoresis at 80 to 100 V. Commercial molecular weight markers were loaded in lanes flanking the samples. The DNA fragments were stained with ethidium bromide, visualized by UV transillumination and recorded photographically on Polaroid 667 film.

4.2.6 Production of a DIG labelled probe by PCR.

Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, alkali labile (DIG-dUTP; Roche) was incorporated by PCR into a 213 bp polynucleotide with sequence complementary to the 62 bp ETR of *M. tuberculosis*. The reaction mixture (25 µl), consisted of 1 x PCR Buffer

(Life Technologies), $MgCl_2$ 1.5 mM, dGTP, dCTP and dATP 0.2 mM, dTTP 0.12 mM, 1 x PCR_X Enhancer (Life Technologies), DIG-dUTP 0.02 mM, 1 unit of *Taq* DNA polymerase (Life Technologies) and 0.8 mM of each primer.

The reaction conditions were; denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 45 seconds, annealing at 62.5°C for 1 minute, extension at 72°C for 2 minutes; and final extension at 72°C for 10 minutes. A control reaction with 0.2 mM of each dNTP and without DIG-dUTP was run in parallel.

The template DNA from which the 213 bp probe was amplified was a PCR product consisting of the *M. tuberculosis* gene Rv1174c and its downstream intergenic region. The template had been amplified from *M. tuberculosis* H37Ra genomic DNA using the primer pair SM5f/SM5r and purified from agarose gels (1% TAE) using the CONCERT™ Rapid Gel Extraction System (Life Technologies) as described in Chapter 7.

4.2.7 Extraction of genomic DNA from mycobacteria for Southern blotting.

Mycobacteria were grown on slants and genomic DNA was extracted using the method described by Gwozdz (1999). Bacterial colonies were scraped from the slants (approximately 50 mg wet weight), suspended in 600 µl of Lysis Buffer (NaCl 100 mM, EDTA 25 mM, Tris-HCl 10 mM, 0.5% SDS, pH 8.0), mixed with Proteinase K 20 mg / ml (10 µl), RNAse A 10 mg / ml (10 µl) and incubated at 50°C for 12 to 18 hours.

Following incubation, NaCl 5.0 M (100 µl) and 6.7% cetyltrimethylamonium bromide (CTAB) in 0.5% NaCl (120 µl) were added and mixed by inversion. The DNA was extracted twice with 700 µl of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Ultra Pure; Life Technologies), once with one volume (700 µl) of chloroform (AnalaR; BDH, Auckland, NZ), then precipitated with one volume (700 µl) of chilled (-20°C) 100% isopropyl alcohol (AnalR; BDH), incubated at -20°C overnight, and centrifuged at 15,800 x g for 30 minutes. The pelleted DNA was washed with 70% ethanol and resuspended in

dH_2O (100 µl). The DNA concentration was estimated by measuring the OD_{260} as described in Chapter 2.

4.2.8 Southern Blotting; digestion and transfer of genomic DNA

Extracted genomic DNA (approximately 1 µg) was digested with the restriction enzyme *Eco* RI for > 16 hours, and the DNA fragments were separated by agarose gel (0.8% TAE) electrophoresis at 40 V. The gels were incubated in denaturing solution (NaOH 1.0 M, NaCl 0.5 M) for > 30 minutes, then in neutralizing solution (Tris-HCl 1.0 M, NaCl 3.0 M, pH 5.5) for > 30 minutes. The DNA was transferred from the gels onto either Biodyne B (Pall Corporation, Ann Arbor, USA) or Hybond™ N+ (Amersham Pharmacia Biotech, Auckland, NZ) nylon membranes by overnight capilliary transfer according to standard methods, with 20 x SSC (NaCl 3.0 M, Sodium citrate 0.3 M, pH 7.0) as the carrier (Sambrook *et al.* 1989). The transferred DNA was cross-linked to the membranes by irradiation on a UV transilluminator for 3 minutes.

4.2.9 Detection of blotted DNA with sequence complementary to the DIG-labelled probe.

The membranes were sealed in plastic bags and pre-hybridized in DIG Easy Hyb (Roche) at 42°C for ≥ 2 hours. DIG labelled probe was added to a final concentration of 20 ng / ml and incubated overnight at 42°C in a shaking water bath. The membranes were washed 2 x 15 minutes in 2 x SSC, 0.1% SDS at room temperature, then 2 x 15 minutes in 0.5 x SSC, 0.1% SDS at 68°C.

The hybridized probe was immunodetected using the DIG Chemiluminescent Detection System (Roche) according to the manufacturer's instructions. Briefly, the membranes were incubated in 1 x Blocking Solution diluted in maleic acid buffer (maleic acid 0.1 M, NaCl 0.15 M, pH 7.5) at room temperature with shaking for more than one hour. Anti-DIG-alkaline phosphatase conjugated Fab fragments were added to a final dilution of 1/1000 and incubated for ≥ 30 minutes with shaking.

The membranes were washed 2 x 5 minutes in maleic acid wash buffer (maleic acid buffer, 0.3% Tween 20), then equilibrated for one minute in detection buffer. CSPD® substrate (Disodium3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'chloro)tricyclo [$3.3.1^{3,7}$]

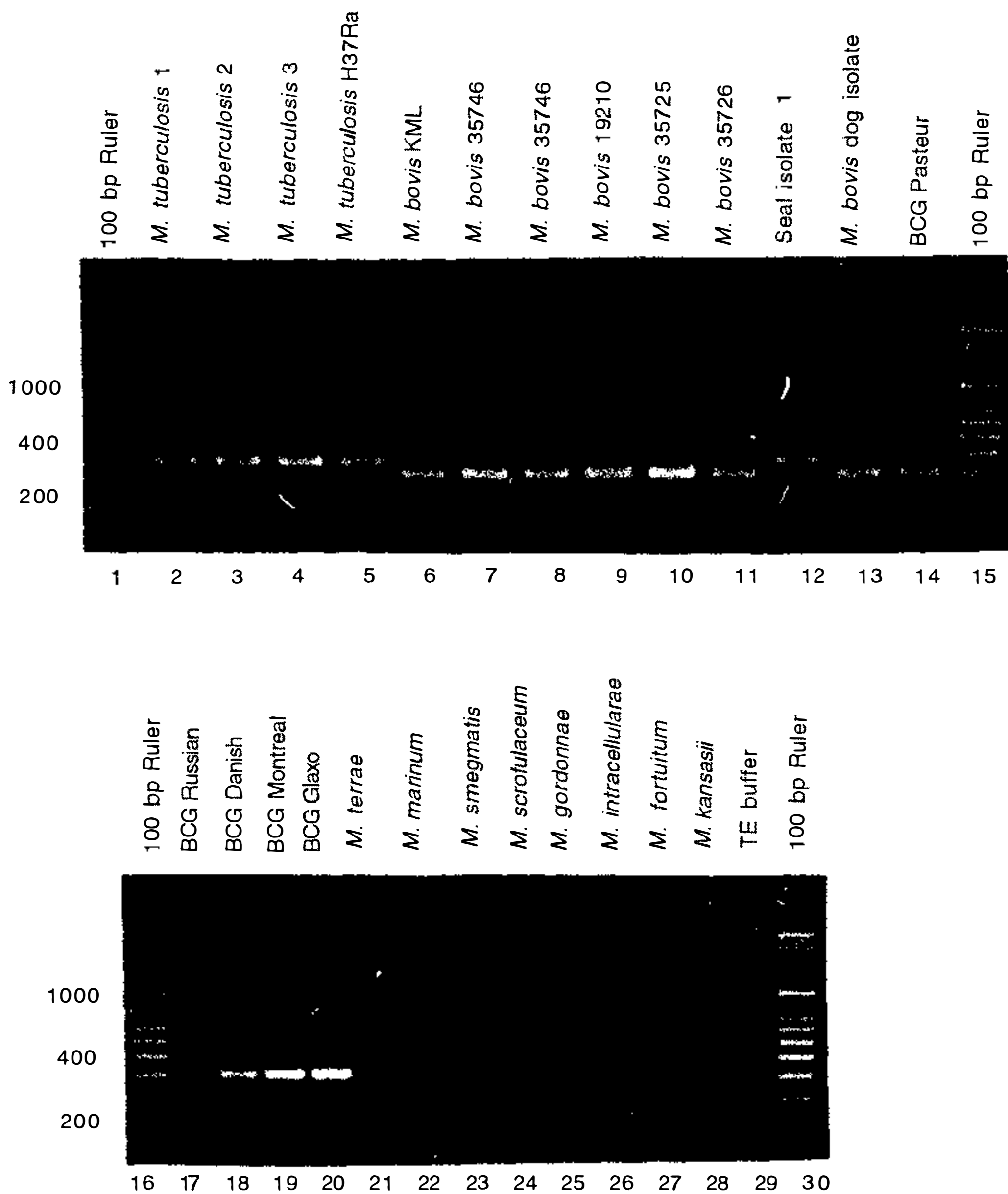

Figure 4.1 Representative agarose gel electrophoresis of the products of PCR with primers SM3 and SM4 directed against genomic DNA extracted from the species listed in Table 4.1. The reaction conditions were as described in section 4.2.4, and electrophoresis was through 2.5% TAE agarose gels. Lanes 1, 15, 16 and 30 are a 100 bp PCR Molecular Ruler (BioRad); lanes 2 to 5 were amplified from *M. tuberculosis*; lanes 6 to 11 were amplified from *M. bovis*; lane 12 was amplified from a seal isolate; lane 13 was amplified from a canine isolate of *M. bovis*; lanes 14, and 17 to 20 were amplified from *M. bovis* BCG; lanes 21 to 28 were amplified from non-TB complex mycobacteria; lane 29 was the TE-buffer control.

decan}-4-yl) phenyl phosphate) was diluted 1:99 in detection buffer (Tris-HCl 100 mM, NaCl 100mM, pH 9.5) and sprinkled onto the DNA side of the membranes, which were sealed in plastic bags and incubated at 37°C for 15 minutes. X-OMAT™ AR scientific imaging film (Eastman Kodak Co, Rochester, USA) was exposed to the membranes for 30 minutes in a film cassette equipped with a Kodak Lanex™ REGULAR intensifying screen then developed in an automatic processor

4.3 RESULTS.

4.3.1 PCR with mycobacterial DNA.

Genomic DNA was extracted from the mycobacterial species listed in Table 4.1. To assess DNA template integrity, PCRs were performed with the primer pair 246/264 that hybridizes specifically to the 16s RNA subunit gene of all mycobacterial species. Preparations of genomic DNA that gave a 1030 bp PCR product were used for further PCR analysis.

PCR products of 377 bp were amplified from five isolates of *M. tuberculosis* with the primer pair SM3/SM4. However, with template DNA from *M. bovis* and *M. bovis* BCG the amplified PCR products were 315 bp. The PCR product obtained from the mycobacteria isolated from seals was 377 bp. The isolate of *M. bovis* from a dog gave a PCR product of 315 bp. PCR products of 315 or 377 bp were not obtained when the primer pair SM3/SM4 was directed against template DNA extracted from non-TB complex mycobacterial isolates. Large PCR products >1000bp were weakly amplified from a few isolates of non-TB complex mycobacterial species (see Figure 4.1).

4.3.2 Southern hybridization analysis.

A 213 bp DIG-dUTP labelled DNA probe complementary to the 62 bp ETR was amplified by PCR with the primers SM21F and SM21R, using *M. tuberculosis* H37Ra DNA as the template. To verify DIG-dUTP labelling, the PCR products of the labelling and control reactions were separated by agarose gel (1% TAE) electrophoresis. The 213 bp PCR product of the DIG-dUTP incorporation reaction appeared to be approximately 50 bp larger than the product of the control reaction, which is consistent with incorporation of DIG-dUTP into the probe (not shown).

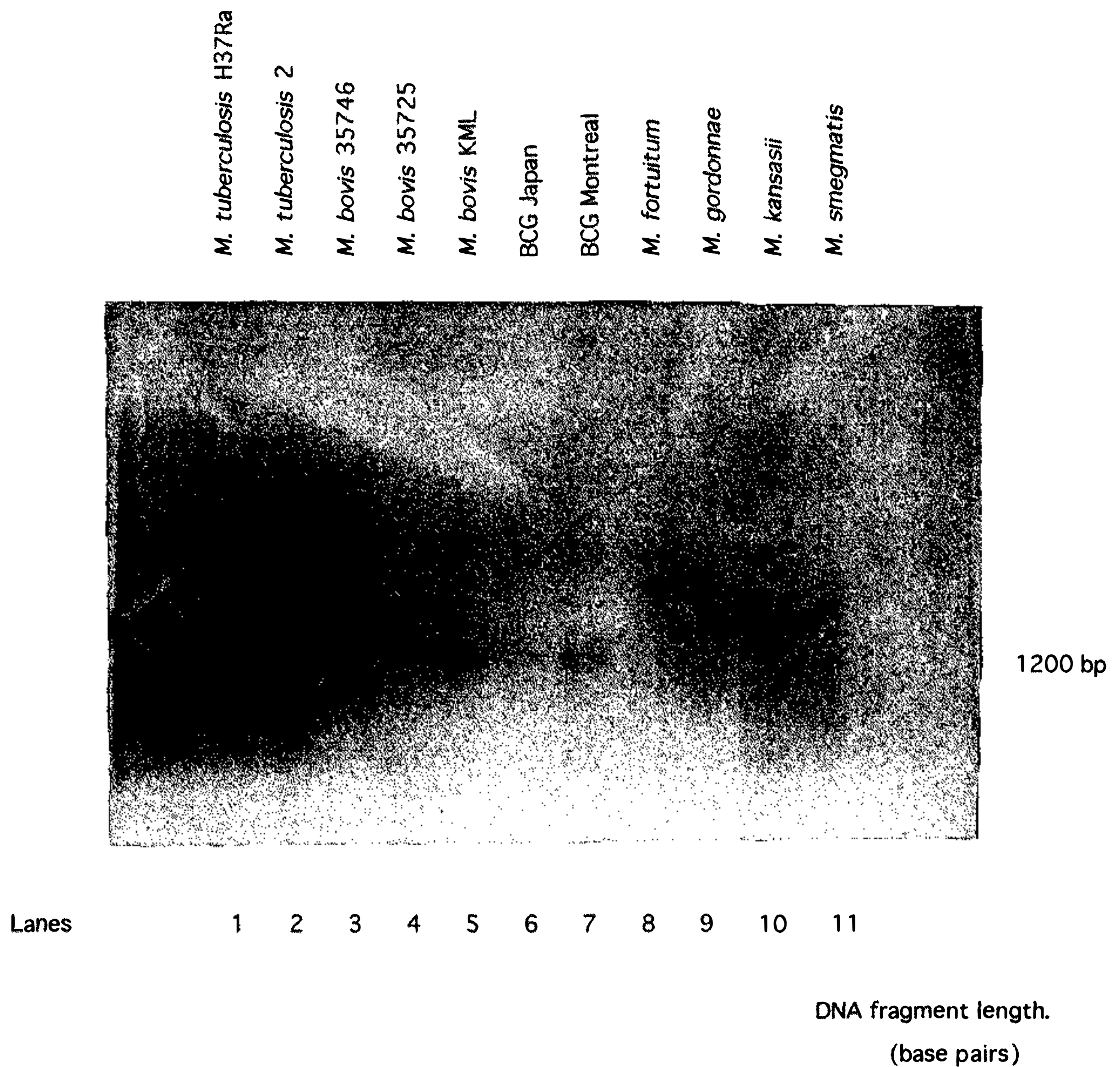

Figure 4.2 Southern blot showing the species distribution of the 62 bp ETR locus. Approximately 1.0 μg of genomic DNA from the mycobacterial species shown was digested with *Eco* RI and transferred onto Hybond™ N+ membrane. A DIG-dUTP labelled probe complementary to the 62 bp ETR of *M. tuberculosis* H37Ra hybridized to a 1200 bp *Eco* RI fragment of genomic DNA from the TB complex species *M. tuberculosis* (lanes 1 & 2), *M. bovis* (lanes 3 to 5) and *M. bovis* BCG (lanes 6 & 7), but not to DNA extracted from *M. fortuitum*, *M. gordonnae*, *M. kansasii* or *M. smegmatis* (lanes 8 to 11).

Blots of genomic DNA extracted from *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. fortuitum*, *M. gordonnae*, *M. kansasii*, *M. marinum* and *M. scrofulaceum* were probed with the 213 bp DIG-labelled DNA fragment. The probe hybridized to bands corresponding to a 1200 bp *Eco* RI fragment of *M. tuberculosis*, *M. bovis* and *M. bovis* BCG genomic DNA. Under the conditions employed the probe did not hybridize to DNA from *M. fortuitum*, *M. gordonnae*, *M. kansasii*, *M. marinum* or *M. scrofulaceum*. (see Figure 4.2). A Southern hybridization experiment conducted at the Pasteur Institute in Paris found that a 213 bp DNA fragment complementary to the 62 bp ETR hybridized to genomic DNA from *M. tuberculosis*, but not to DNA from *M. avium*, *M. chelonei*, *M. fortuitum*, *M. gordonnae*, *M. kansasii*, *M. marinum* or *M. scrofulaceum*.

4.3.3 Investigations conducted at the Pasteur Institute, Paris.

PCR with primers SM3 and SM4.

A collaborative study was conducted with Dr. Cristina Gutierrez at the Centre National de Référence des Mycobactéries, Institut Pasteur, Paris to investigate the occurrence of the polymorphic 62 bp ETR locus in a number of isolates of different mycobacterial species. The isolates had been typed as described by Viana-Niero *et al.* (2001), and PCR was performed using the primers SM3 and SM4.

Long 377 bp products were amplified from 16/20 isolates of *M. tuberculosis*, and 12/12 *M. africanum* isolates (see Tables 4.3 and 4.4). An additional 450 bp product was obtained from one *M. tuberculosis* isolate. A short 315 bp PCR product was obtained from 4/20 *M. tuberculosis* isolates. Two of the *M. tuberculosis* isolates from which a short 315 bp product was amplified and the isolate from which an additional 450 bp product was obtained also lacked the *mpt-40* locus. The *mpt-40* locus occurs in most but not all isolates of *M. tuberculosis* but not in *M. bovis* (Liébana *et al.* 1996). The results obtained from *M. canetti* isolates varied. A 377 bp product was amplified from one isolate of *M. canetti*, a 315 bp product from another isolate, and a product was not obtained from three isolates.

Short 315 bp PCR products were amplified from 30 different *M. bovis* isolates obtained from animal and human cases of tuberculosis. Shorter 250 bp products were obtained from the two isolates of *M. bovis* derived from macaque monkeys (see Tables 4.3, 4.5 and 4.6).

Species	Source	PCR product length
M. tuberculosis	9 European human isolates	7 x 377 bp
		1 x 377 bp & 450 bp
		1 x 315 bp
	10 African human isolates	7 x 377 bp
		3 x 315 bp
	1 Caribbean human isolate	1 x 377 bp
	1 Sea lion isolate	1 x 377 bp
M. africanum	12 human isolates	12 x 377 bp
M. bovis	11 French human isolates	11 x 315 bp
	3 North African human isolates	3 x 315 bp
	1 Asian human isolate	1 x 315 bp
	17 Veterinary isolates	15 x 315 bp
	(Macaque monkeys)	2 x ~ 250 bp
M. canetti	5 caprine isolates	1 x 377 bp
		1 x 315 bp
		3 x no PCR product
M. avium	3 human isolates	1 x a doublet at 1600 bp
		2 x no PCR product
M. chelonae	2 isolates	1 x ~ 2000 bp
		1 x ~ 600 bp
M. fortuitum	3 isolates	3 x no PCR product
M. intracellulare	3 isolates	3 x no PCR product
M. kansasii	3 isolates	1 x ~ 5000 bp
		1 x ~ 2000 bp
		1 x no PCR product
M. marinum	3 isolates	1 x ~ 2000 bp
		2 x no PCR product
M. scrofulaceum	3 isolates	3 x ~ 2000 bp
M. simiae	3 isolates	1 x ~ 4000 bp
		2 x no PCR product
M. xenopi	3 isolates	3 x ~ 2000 bp

Table 4.3 The species distribution of the 62 bp ETR locus. Genomic DNA extracted from the species listed was used as template for PCR reactions performed with primers SM3 and SM4 to amplify the region containing the 62 bp ETR locus. The sizes of the PCR products obtained are shown. A 315 bp PCR product corresponds to 1.7 copies of the 62 bp tandem repeat unit and a 377 bp PCR product corresponds to 2.7 copies.

PCR products of 315 bp or 377 bp were not obtained from any isolates of non-TB complex mycobacteria, however PCR products > 1600 bp were obtained from some isolates. Partial nucleotide sequences of the 2000 bp PCR products amplified from *M. scrofulaceum* and *M. xenopi* were determined. Approximately 500 bp of sequence adjacent to the primer binding site at each end of the PCR products was submitted for BLAST analyses. It was found that the sequences did not align to either the 62 bp ETR locus or to each other (data not shown).

Nationality	age/sex	loc	No.IS6110	*pncA*	*mpt-40*	62bpETR#
Zaire	29 / m	p	4			377
Spain	50 / m	p + e	7			377
Mali	49 / m	p	7	+	—	* 315
France	10 / m	e	8			377
Morocco	34 / m	p	8			377
Romania	30 / f	p	9			377
France	53 / m	p	10	+	+	* 315
Morocco	11 / f	p	10			377
France	50 / m	p	11			377
Martinique	39 / m	p	11			377
France	37 / m	p	12			377
Morocco	63 / m	p	12			377
Algeria	47 / m	p	13			377
France	47 / m	p	14			377
Morocco	21 / f	e	14			377
Senegal	37 / f	p + e	15	+	+	* 315
France	63 / f	p	16	+	—	377 + 450
Senegal	35 / m	p + e	16	+	—	* 315
Algeria	11 / m	p	18			377
France	49 / m	p + e	20			377

Table 4.4 Genotypic typing results obtained with *M. tuberculosis* isolates from human patients. The nationality, age and sex (m/f) of the patients, the location of the tuberculous process; pulmonary (p) or extrapulmonary (e), and the number of copies of IS6110 occuring in each isolate are shown. Genomic DNA from the isolates was used as template for PCR with primers SM3 and SM4 that are complementary to sequences either side of the 62 bp ETR locus. A long 377 PCR product was amplified from 15 isolates, and two products (377 and 450 bp) were obtained from one isolate. A 315 bp product was obtained from four isolates (*). Two of the isolates from which the 315 bp product was obtained and the isolate from which the additional 450 bp PCR product was amplified lacked the gene *mpt-40*. The four isolates from which the 315 bp product was amplified and the isolate that gave the 377 and 450 bp products all possessed an undisrupted gene for pyrazinamidase, *pncA* (+).

Nationality	age/sex	Location	IS6110	62bpETR#
France	86 / f	extra	1aG	315
France	52 / m	pulm	3a	315
France	39 / m	pulm	1dA	315
Algeria	31 / m	extra	1aE	315
France	73 / f	extra	1bB	315
France	35 / f	pulm	3b	315
France	75 / f	pulm	6a	315
France	24 / f	extra	2f	315
France	69 / m	pulm	2b	315
France	4 / m	extra	1aB	315
Morocco	23 / m	pulm	1aD	315
Morocco	31 / m	extra	1bA	315
France	44 / m		1dB	315
Cambodia	48 / m	extra	2d	315
France	70 / f	extra	2e	315

Table 4.5 Genotypic typing results obtained with ***M. bovis*** isolates from human patients. The nationality, age and sex (m/f) of the patients, and the location of the tuberculous process pulmonary (pulm) or extrapulmonary (extra) are shown if reported. Genomic DNA from the isolates was used as template for PCR with the primers SM3 and SM4 which are complementary to sequences either side of the 62 bp ETR locus. A 315 bp PCR product was obtained from all the isolates. The number of copies of IS6110 occurring in each isolate is shown. The letters refer to the chromosomal distribution of IS6110 and illustrates the range of genotypes investigated (Dr. C. Gutierrez personal communication).

Species	IS6110	62bpETR#
bovine	1aB	315
baboon	1aC	315
caprine	1aJ	315
cervine	2c	315
canine	2f	315
feline	3c	315
macaque	3d	* 250
ovine	4b	315
bovine	4c	315
porcine	5a	315
feline	5c	315
bovine	5d	315
porcine	6c	315
oryx	6d	315
bovine	9a	315
cervine	13a	315
macaque	13a	* 250

Table 4.6 Genotypic typing results obtained with *M. bovis* isolates from animals. Genomic DNA from the isolates was used as template for PCR with primers SM3 and SM4 that are complementary to sequences either side of the 62 bp ETR locus. A 315 bp product was obtained from all isolates except the two isolates from macaque monkeys (*), from which PCR products of ~250 bp were amplified. The number of copies of IS6110 occurring in each isolate is shown. The letters refer to the chromosomal distribution of IS6110 and illustrates the range of genotypes investigated (Dr. C. Gutierrez - personal communication).

4.4 DISCUSSION.

This study examined the species distribution of a 62 bp ETR locus that was found to be polymorphic when the nucleotide sequence from a New Zealand isolate of *M. bovis* (WAg201) was aligned against the corresponding *M. tuberculosis* H37Rv sequence (see Chapter 2). At Massey University, a small number of mycobacterial isolates were examined by both PCR and Southern hybridization analyses. The 62 bp ETR locus was found only in isolates of TB complex species. Short 315 bp PCR products corresponding to 1.7 copies of the repeat unit were amplified from all the *M. bovis* and *M. bovis* BCG isolates. Long 377 bp products corresponding to 2.7 copies of the repeat unit were amplified from all the *M. tuberculosis* isolates.

The initial findings were confirmed and extended by the collaborative study conducted with the Pasteur Institute, Paris. It was found that a 377 bp PCR product was only obtained from *M. tuberculosis*, whereas a 315 bp or shorter product was obtained from all isolates of *M. bovis* and some isolates of *M. tuberculosis.* A shorter approximately 250 bp product was obtained from the two isolates of *M. bovis* from macaque monkeys, and an additional 450 bp product was obtained from one isolate of *M. tuberculosis.* The nature of those products was not investigated because they were not made available for sequence analysis.

The isolates investigated had been assigned to the species *M. tuberculosis* and *M. bovis* on the basis of the aggregate results obtained from a number of phenotypic and genotypic typing techniques. However, as was observed with the isolates of *M. tuberculosis* that lacked the *mpt-40* locus, some isolates are not characteristic with respect to all features. The isolates analysed at the Pasteur Institute, Paris represented a wide variety of *M. tuberculosis* and *M. bovis* genotypes, including rare isolates. Therefore, the proportion of *M. tuberculosis* isolates from which a 315 bp product was amplified may not reflect the results that would be obtained in a routine diagnostic laboratory.

Genotypic typing of TB complex isolates by RFLP analysis of IS6110 and/or IS1081 polymorphisms is technically demanding and requires more DNA than PCR based methods (Collins *et al.* 1993). Spoligotyping requires small amounts of DNA because the direct variable repeats within the DR locus are amplified by PCR, but a hybridization step is required (Kamerbeek *et al.* 1997, Kremer *et al.* 1999). To differentiate between *M. bovis* and *M. tuberculosis* on the basis of the single nucleotide polymorphisims occurring

within the *oxyR* and *pncA* loci the PCR product must be sequenced (Sreevatsan *et al.* 1996, Sreevatsan *et al.* 1997b).

Although *M. tuberculosis* can be rapidly identified using PCR directed at the *mpt-40* locus, the absence of a product is not definitive and extra care must be taken to ensure that the amplification reactions are adequately controlled (Liébana *et al.* 1996). In contrast, PCR directed at variable numbers of tandem repeats (VNTR) loci, as described in this study, enables mycobacterial isolates to be differentiated on the basis of PCR product length polymorphisms observable following agarose gel electrophoresis. Moreover, the amplification reactions are effectively internally controlled because a product should always be obtained.

The VNTR typing technique is similar to the Short Tandem Repeat typing technique used for DNA fingerprinting mammals (Supply *et al.* 2000), but to date very few mycobacterial ETR loci have been investigated extensively. It has been reported that the polymorphic locus ETR-D is specific to TB complex species (Magdalena *et al.* 1998a, 1998b). Strains of *M. bovis* BCG contain 1, 2 or 3 copies of the repeat unit constituting ETR-D, whereas other TB complex species contain 3, 4, 6 or 7 copies of the repeat unit. In a study of six polymorphic ETR loci (including ETR-D), the combined results of VNTR analyses generated allele profiles that distinguished between TB complex species (Frothingham and Meeker-O'Connell 1998). It has also been reported that 12 mycobacterial interspersed repetitive units (MIRU) loci are polymorphic between isolates of TB complex species (Supply *et al.* 1997). Two recent studies have reported using VNTR typing for molecular epidemiological investigation of *M. africanum* isolates (Frothingham *et al.* 1999, Vianca-Niero *et al.* 2001).

This study is the first reported investigation of polymorphisms at the 62 bp ETR locus that occurs adjacent to the gene coding for the 8.4 kDa Ag of *M. bovis* and the *M. tuberculosis* gene Rv1174c. The 62 bp ETR locus appears to be restricted to TB complex species. It is also an informative VNTR locus whilst it can be used to differentiate between a high percentage of *M. bovis* and *M.tuberculosis* isolates. Therefore, in conjunction with other ETR loci the polymorphic 62 bp ETR locus may be useful for VNTR typing of mycobacterial isolates.

Chapter 5. Identification of a sequence in *M. avium* with homology to the gene coding for the 8.4 kDa Ag of *M. bovis.*

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5.1 INTRODUCTION.

The intradermal tuberculin test for diagnosis of *M. bovis* infection in cattle lacks specificity in environments where animals are sensitized to components of PPD-B tuberculin by exposure to *M. avium* (Ryan *et al.* 2000, Andersen *et al.* 2000). Recently, it was demonstrated that the specificity of diagnosis can be improved if antigens that are not produced by *M. avium* are used instead of PPD-B tuberculin, however test sensitivity is reduced (Buddle *et al.* 2001).

Consequently, it has been suggested that test sensitivity could be improved without sacrificing specificity, if a mixture of antigens with restricted species distribution were used as the stimulatory antigen preparation (Andersen *et al.* 2000). However, before individual *M. bovis* antigens can be considered for use as diagnostic reagents, it is necessary to determine whether *M. avium* has the potential to produce homologous proteins that could sensitize animals to the *M. bovis* antigens.

The gene coding for the 8.4 kDa antigen of *M. bovis* identified in Chapter 3 is identical to the genes coding for the 8.4 kDa antigens previously identified in *M. tuberculosis* and *M. bovis* BCG (Coler *et al.* 1998, Freer *et al.* 1998a). However, it was reported that a DNA probe complementary to the nucleotide sequence coding for the 8.4 kDa antigen hybridized to genomic DNA from *M. tuberculosis*, *M. bovis* BCG and *M. avium* (Coler *et al.* 1998).

In order to establish whether *M. avium* could potentially produce a protein that sensitizes animals to the 8.4 kDa antigen of *M. bovis*, this study used PCR analysis and BLAST searches to determine whether the *M. avium* genome posesses sequence that could code for a protein with homology to the 8.4 kDa Ag of *M. bovis.*

5.2 MATERIALS AND METHODS.

5.2.1 Polymerase chain reactions.

The primers SM5f and SM5r are complementary to each end of the nucleotide sequence coding for the mature secreted 8.4 kDa antigen of *M. bovis*. They were designed to amplify a 271 bp PCR product that incorporates restriction sites at each end for directional cloning of the gene (Chapter 7). The complementary sequences are longer than the usual

18 to 24 nucleotides to counteract any disruptive effect the non-complementary sequences at the 5' ends might have on annealing during PCR. The nucleotide sequences of both primers were submitted to the NCBI-BLAST 2.0 search engine (Altschul *et al.* 1997) for BLASTN homology searches as described in Chapter 4.

At the Pasteur Institute, Paris, Dr Alan Murray performed PCRs with the primer pair SM5f/SM5r and template DNA from isolates of *M. avium*, *M. fortuitum*, *M. gordonnae*, *M. kansasii*, *M. marinum* and *M. tuberculosis*. The reaction conditions were 94°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and extension at 72°C for 10 minutes.

Table 5.1 Primers used in this study.

Primer	Sequence
SM5f	5' CCG CGA ATT CAG ATC CCG TGG ACG CGG TCA TAA ACA CC 3'
SM5r	5' AAT GTC GAC TAA ATA GTT GTT GCA GGA GCC GGC 3'

5.2.2 Sequence homology searches.

The unpublished genome of *M. avium* was searched for sequences similar to the nucleotide and deduced amino acid sequences of the 8.4 kDa antigen of *M. bovis*. Preliminary sequence data was obtained from The Institute for Genomic Research (TIGR) website at http://www.tigr.org. The TIGR BLAST server runs the Washington University BLAST (WU-BLAST) version 2.0a19 software package, which makes available searches using BLASTN for nucleotide sequence queries, and TBLASTN for amino-acid sequence queries (Gish 1996 to 1999, http://blast.wustl.edu).

5.3 RESULTS.

5.3.1 Polymerase chain reactions.

PCRs were performed using the primers SM5f and SM5r as described. A strong 271 bp PCR product was amplified from *M. tuberculosis* DNA, and a weak product the same size

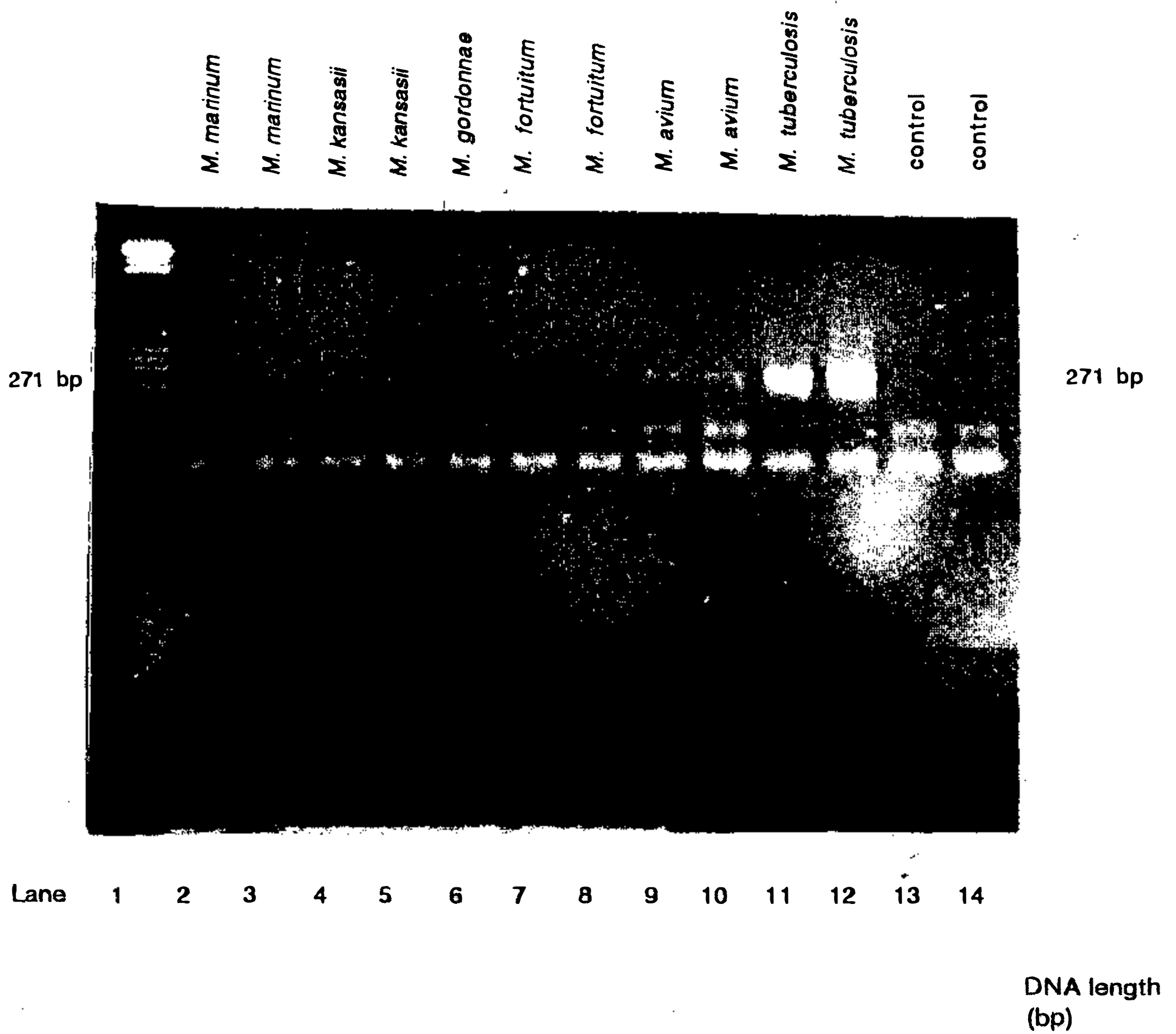

Figure 5.1 Agarose gel showing the results of PCR with the primers SM5f and SM5r directed against genomic DNA from isolates of *M. marinum, M. kansasii, M. gordonnae, M. fortuitum, M. avium*, and *M. tuberculosis*. At an annealing temperature of 60°C a PCR product was not amplified from *M. marinum* (lanes 2 and 3), *M. kansasii* (lanes 4 and 5), *M. gordonnae* (lane 6), *M. fortuitum* (lanes 7 and 8) or the controls without template (lanes 13 and 14). A 271 bp PCR product was weakly amplified from *M. avium* (lanes 9 and 10), and a 271 bp PCR product was strongly amplified from *M. tuberculosis* (lanes 11 and 12).

was obtained from *M. avium.* No products were amplified from the non-TB complex mycobacterial species (see Figure 5.1).

5.3.2 BLAST homology searches.

The nucleotide sequence of the gene coding for the 8.4 kDa antigen of *M. bovis* and its deduced amino acid sequence were submitted for alignment against the unpublished genome of *M. avium.* The BLAST algorithms identified three segments of the *M. avium* genome that produced high-scoring segment pairs. The translated amino acid sequences of the 8.4 kDa antigen and the *M. avium* sequence that produced the highest scoring segment pairs shared approximately 61% identity (see Figure 5.2).

Alignment of the translated sequence of the 8.4 kDa protein of *M. bovis* against the matching translated sequence of *M. avium* sequence 41

```
 Score = 363 (132.8 bits), Expect = 2.3e-33, P = 2.3e-33
 Identities = 68/110 (61%), Positives = 87/110 (79%), Frame = -2

                                       -6        +2
Query:      1 MRLSLTALSAGVGAVAMSLTVGAGVASA*DPVDAVINTTCNYGQVVAALNATDPGAAAQFN 60
              MRLSL+ L    VG+ A++LT  AGVASA*DP+DA+INTTCNYGQV+AALNA+DP AA Q N
Sbjct: 13103 MRLSLSKLGVAVGSAAVALTAAAGVASA*DPMDAIINTTCNYGQVIAALNASDPAAAQQLN 12924

Query:     61 ASPVAQSYLRNFLAAPPPQRAAMAAQLQAVPGAAQYIGLVESVAGSCNNY 110
              +SP+AQSY++ FLA+PP +R  MA Q+Q +P A QYI  +  VA +CNN+
Sbjct: 12923 SSPMAQSYIQRFLASPPAKRQQMAQQIQGMPAAQQYINDINQVAVTCNNF 12774
```

Figure 5.2 Gapped alignment of the translated amino acid sequence of the *M. bovis* 8.4 kDa protein antigen against the translated *M. avium* sequence that produced the maximal-scoring segment pairs. The sequence comparison was run at TIGR by WU-BLAST version 2.0a19, using the programme TBLASTN at the default settings. The submitted amino acid sequence of the 8.4 kDa Ag of *M. bovis* (query), shown by IUPAC code letter, is aligned against the translation of the corresponding *M. avium* sequence (subject). The position of the alignment on the *M. avium* sequence is indicated by amino acid number. The amino acid identities are shown between the query and subject sequences. Similar amino acids are designated positive (+). The number and percentage of identical (Identities) and similar (Positives) aligning amino acids is annotated above the aligned sequences. The scores for alignments (Score) are the sum of the pairwise scores that were calculated using the BLOSUM62 substitution matrix. The significance of alignment is expressed as an expectation (Expect) or a probability (P), which indicate how often the score would be expected to occur by chance. The reading frame of translated amino acid sequence relative to the *M. avium* nucleotide sequence is indicated (Frame). The predicted signal sequence cleavage site is shown (*).

The corresponding *M. bovis* and *M. avium* nucleotide sequences shared 73% identity. The *M. bovis* and *M. avium* nucleotide coding sequences were both 110 bp long and had corresponding start and stop codons (see Figure 5.3).

Alignment of the gene coding for the 8.4 kDa antigen of *M. bovis* against *M. avium* sequence 41

```
 Score = 811 (127.7 bits), Expect = 7.8e-32, P = 7.8e-32
 Identities = 243/332 (73%), Positives = 243/332 (73%), Strand = Minus / Plus

                       SM5r
Query:   328 AGTTGTTGCAGGAGCCGGCAACCGACTCGACAAGGCCGATGTACTG-TGCCGCCCCCGGC 270
             ||||||| |||| | | || |||   | ||    |  ||||||||| ||| ||  |||||
Sbjct: 12776 AGTTGTTACAGGTGACCGCGACCTGGTTGATGTCGTTGATGTACTGCTGC-GCGGCCGGC 12834

Query:   269 ACAGCTTGCAATTGCGCGGCCATG-GCAGCGCGCTGAGGTGGCGGTGCGGCGAGGAAATT 211
             |   | || |  |||  ||||||  || |  ||||  |  ||||| | ||| |||||
Sbjct: 12835 ATGCCCTGGATCTGCTGGGCCATCTGCTGA-CGCTTCGCCGGCGGGAGGCCAGGAACC- 12892

Query:   210 GC-GCAAATAGGACTGCGCCACCGGTGAGGCGTTGAACTG-TGCGGCAGCCCCCGGATCC 153
             || | |  ||||||||||||| ||| || | ||| | ||| || ||||||| |||| ||
Sbjct: 12893 GCTGGATGTAGGACTGCGCCATCGGCGACGAGTTCAGCTGCTG-GGCAGCCGCCGGGTCG 12951
                                                                  SM5f
Query:   152 GTCGCGTTGAGCGCAGCTACTACCTGCCCGTAATTGCAGGTGGTGTTAATGACCGCGTCC 93
             | |||||| ||||| || |  ||||||||||| |||||||||||||| |||| |||||||
Sbjct: 12952 GACGCGTTCAGCGCGGCGATCACCTGCCCGTAGTTGCAGGTGGTGTTGATGATCGCGTCC 13011

Query:    92 ACGGGATCTGCGGAGGCGACCCCGGCCCCGACGGTCAACGACATTGCCACGGCGCCTACA 33
             | ||| || ||||| ||||| |||||| || ||||||| | || |||| ||  ||| ||
Sbjct: 13012 ATGGGGTCGGCGGATGCGACACCGGCCGCGGCGGTCAATGCCACTGCCGCGCTGCCCACC 13071

Query:    32 CCGGCGCTCAATGCGGTCAACGACAGCCTCAT 1
              |  ||| ||||  | ||||||||||||||||
Sbjct: 13072 GCAACGCCCAATTTGCTCAACGACAGCCTCAT 13103
```

Figure 5.3 Gapped alignment of the nucleotide sequence coding for the 8.4 kDa protein antigen of *M. bovis* against the *M. avium* sequence that produced the maximal-scoring segment pairs. The sequence comparison was run at TIGR by WU-BLAST version 2.0a19, using the programme BLASTN at the default settings. The submitted nucleotide sequence of *M. bovis* (query), shown by IUPAC code letter, is shown aligned against the corresponding *M. avium* sequence (subject). The orientation (plus/minus) indicated that the coding sequence is on the reverse strand. The position of the alignment on the *M. avium* sequence is indicated by nucleotide number. The nucleotide identities are shown linked by a vertical bar. Gaps are indicated by a horizontal bar. The number and percentage of identical (Identities) and similar (Positives) aligning nucleotides is annotated above the aligned sequences. The scores for the alignments (Score) are the sum of the pairwise scores calculated using the +5, -4 unitary matrix. The significance of each alignment is expressed as expectations (Expect) or probabilities (P), which indicate how often the score would be expected to occur by chance. Start and stop codons are shown in bold type. Primer binding sites are underlined (SM5f and SM5r).

The nucleotide triplets coding for 22 of the 68 amino acids with identity had synonomous differences that did not alter the amino acid. The amino acid sequence homology was located at the beginning of the predicted signal peptide (amino acids 1 to 5), around the predicted cleavage site (amino acids -6 to +2 from the cleavage site between positions 28 and 29), and at the N-terminus of the mature protein (first 25 amino acids).

5.4 DISCUSSION.

This study investigated whether a nucleotide sequence with homology to the gene coding for the 8.4 kDa antigen of *M. bovis* occurs in *M. avium*. The primers used for PCR were designed to hybridize to the sequence coding for the secreted portion of the 8.4 kDa protein of *M. bovis*, because it has been reported that the amino acid sequences of the export leader peptides of homologous proteins can differ without affecting export of the mature protein (Economou 1999, Fekkes and Dreissen 1999). A PCR product of the expected size was obtained from *M. tuberculosis* DNA and the same sized product was weakly amplified from *M. avium* DNA. Thus, it appeared that a nucleotide sequence with some similarity to the gene for the 8.4 kDa antigen of *M. bovis* existed in *M. avium*.

Subsequent BLAST searches of the unfinished *M. avium* genome identified a nucleotide sequence that potentially codes for a protein with similarity to the 8.4 kDa antigen of *M. bovis*. The potential coding sequence from *M. avium* and the gene coding for the 8.4 kDa antigen of *M. bovis* were the same length. Furthermore, some segments of the deduced amino acid sequences were highly conserved, although 22/69 (32%) of the identical amino acids were coded for by nucleotide triplets with synonomous differences. Thus, it appears that *M. avium* could potentially produce a protein that sensitizes animals to the 8.4 kDa Ag of *M. bovis*.

If time had allowed, Western blot analyses would have been performed as described in Chapter 3 to ascertain whether *M. avium* expresses a protein with similarity to the 8.4 kDa antigen of *M. bovis*. If the rabbit anti-8.4 kDa polyclonal sera had detected a protein of the appropriate size in preparations derived from *M. avium*, it would have provided evidence that *M. avium* produces a protein with antigenic homology to the 8.4 kDa antigen of *M. bovis*. The polyclonal antisera could also have been used to investigate the report that a similar sized protein had been detected in the CFs of *M. tuberculosis*, *M. bovis* BCG, *M. marinum*, *M. kansasii* and *M. gordonnae* using a monoclonal antibody with specificity for the 8.4 kDa antigen of *M. bovis* BCG (Freer *et al.* 1998b).

Chapter 6. Immune responses to the 8.4 kDa antigen of BCG vaccinated and unvaccinated calves before and after challenge with *M. bovis.*

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6.1 INTRODUCTION.

There are genotypic and phenotypic differences between *M. bovis* BCG and virulent *M. bovis.* Following inoculation, *M. bovis* BCG does not cause disease in immunocompetent hosts, whereas virulent *M. bovis* persists and causes progressive disease (Orme *et al.* 2001). The genotypic differences that account for the attenuation of *M.bovis* BCG have not been determined (Behr *et al.* 1999, Gordon *et al.* 1999a), but *M. bovis* BCG lacks the genes coding for some antigens such as ESAT-6, CFP10 and MPB64 (some strains) that are the predominant targets of T-cell responses mounted by cattle infected with virulent *M. bovis* (Buddle *et al.* 1999, Vordermeier *et al.* 1999). It has also been shown that there is differential expression of some of the antigens that are common to both *M. bovis* BCG and *M. bovis* in liquid culture (Andersen 1997).

The recombinant 8.4 kDa antigen of *M. bovis* identified in the CF of *M. smegmatis* pSU151.43 stimulated PBMC from one steer vaccinated with *M. bovis* BCG to proliferate and produce IFN-γ (Chapter 3). However, there was no data to indicate whether the 8.4 kDa antigen is a target of the immune response mounted by cattle infected with virulent *M. bovis.* At AgResearch Wallaceville a trial was conducted in which calves vaccinated with BCG and unvaccinated calves were challenged intratracheally with virulent *M. bovis.* Dr. Bryce Buddle kindly supplied blood from the animals so that the immune responses of the two groups of cattle to the 8.4 kDa antigen could be investigated.

The objectives of this study were twofold. The first was to extend the observation made in Chapter 3 and investigate whether other BCG vaccinated cattle also mounted an immune response to the 8.4 kDa antigen. The second was to determine whether the 8.4 kDa antigen is a target of the immune responses mounted by cattle experimentally infected with virulent *M. bovis.* Lymphocyte proliferation and IFN-γ production assays were used to measure the responses of PBMC from ten unvaccinated and ten *M. bovis* BCG vaccinated calves to PPD-B, PPD-A and the 8.4 kDa antigen before and after intratracheal challenge with virulent *M. bovis.* The responses to the 8.4 kDa antigen were compared to the responses to PPD-B and PPD-A, both with respect to the number of animals exceeding cut-off response levels and the magnitude of the responses.

Table 6.1 Trial protocol.

Sampling time	Date	Procedure	Week
Sampling time 1 (*Pre-vaccination*)	(16/06/99)	Blood taken from all 20 animals 10 animals vaccinated with BCG	week 0
Sampling time 2 (*Post-vaccination*)	(07/07/99)	Blood taken from all 20 animals	week 3
	(29/07/99)	Revaccinated with BCG	week 6
Sampling time 3 (*Post-2nd vaccination*)	(18/08/99)	Blood taken from all 20 animals	week 9
Sampling time 4 (*Pre-challenge*)	(14/09/99)	Blood taken from all 20 animals. Challenged all 20 animals intratracheally with *M. bovis*.	week 13
Sampling time 5 (*Five weeks post-challenge*)	(20/10/99)	Blood taken from all 20 animals 5 weeks post challenge	week 18
Sampling time 6 (*Ten weeks post-challenge*)	(24/11/99)	Blood taken from all 20 animals 10 weeks post challenge intradermal tuberculin test	week 23
	(mid January)	Necropsied all 20 animals 17 weeks post challenge	week 30

6.2 MATERIALS AND METHODS.

6.2.1 Experimental animals.

Blood and sera were obtained from twenty rising one-year-old steers and heifers that were part of a larger experiment conducted at Wallaceville Animal Research Centre. Ten of the animals were vaccinated subcutaneously twice with 10^6 colony forming units (CFU) *M. bovis* BCG Pasteur 117P2. All twenty animals were challenged intratracheally with 5 x 10^3 CFU of virulent *M. bovis* Wag201 (see Table 6.1). At the conclusion of the experiment all the animals were killed and subjected to post-mortem examination as described by Wedlock *et al.* (2000).

6.2.2 Lymphocyte proliferation assays.

Venous blood was collected from each animal into 2 x 10 ml Vacutainers containing lithium heparin (Becton-Dickenson, Franklin Lakes, USA). The blood samples were transported to Massey University at ambient temperature and processed the same day in two batches of ten samples. To avoid bias, samples from unvaccinated and BCG vaccinated animals were processed alternately in ascending order of ear tag number. The PBMC were separated and quadruplicate cultures of 2 x 10^5 PBMC in RPMI-1640 culture medium were stimulated with antigen in 96 well plates as described in Chapter 3. Details of the stimulatory antigens and controls are listed in Table 6.2. The cell cultures were incubated in a humidified atmosphere of 5% CO_2 at 37°C. On the fifth day, 50 µl of supernatant was harvested from quadruplicate wells, pooled and frozen at -20°C until assayed for IFN-γ. The dividing cells were radioactively labelled by adding 0.5 µCi [*methyl*- ^{3}H] Thymidine in 10 µl dH_2O to each well. After 16 hours the incorporated radioactivity was measured in a liquid scintillation counter. The results of the lymphocyte proliferation assays were expressed as differences in counts per minute (Δ c.p.m) and as stimulation indices (SI).

Δ c.p.m = mean c.p.m stimulated wells — mean c.p.m unstimulated wells.

SI = mean c.p.m of stimulated wells / mean c.p.m of unstimulated wells.

A positive response was defined as: SI $\geq$ 3 (Vordermeier *et al.* 1999, Rhodes *et al.* 2000a).

Table 6.2 Antigens used in lymphocyte proliferation assays.

Antigen	Function	Stimulatory concentration	Sampling times.
RPMI-1640 medium	negative control		all
Con $A_{(a)}$	postive control for cell viability	0.25 µg/ml	all
PPD-$B_{(b)}$	control for sensitization by *M. bovis.*	0.05 µg/ml	all
		0.25	all
		1.25	4, 5 and 6
		6.25	4, 5 and 6
PPD-$A_{(b)}$	control for sensitization by *M. avium.*	0.05 µg/ml	all
		0.25	all
		1.25	4, 5 and 6
		6.25	4, 5 and 6
8.4 kDa $Ag_{(c)}$		0.05 µg/ml	all
		0.25	all
		1.25	4, 5 and 6
		6.25	4, 5 and 6

(a) Conavalin A (Sigma Aldrich, Sydney), stock solution 1 mg / ml in PBS.
(b) PPD-B and PPD-A (CSL NZ Ltd., Upper Hutt), Sterile Preservative Free 0.3 mg protein / ml.
(c) Recombinant *M. bovis* 8.4 kDa antigen FPLC purified from the CF of *M. smegmatis* pSU151.43 and N-terminal sequenced as described in Chapter 3. Stock: Superose 6 HR 10/30 column eluant fraction C_4 containing 0.4 mg protein / ml in PBS.

6.2.3 IFN-γ assays.

The relative IFN-γ concentrations in pooled supernatants from the lymphocyte proliferation assays were estimated using the BOVIGAM™ Interferon Test Kit as described in Chapter 3. The optical density (OD_{450}) readings were multiplied by 1000 to eliminate the decimal point and results were expressed as optical density differences (Δ OD_{450}).

$\Delta\ OD_{450}$ = mean OD_{450} stimulated wells — mean OD_{450} unstimulated wells.

A positive response was defined as: $\Delta\ OD_{450} \geq 100$ (Fifis *et al.* 1994a),

6.2.4 Immunoblotting.

PPD-B tuberculin and CF from *M. smegmatis* pSU151.43 were separated by Tris-tricine SDS-PAGE through 0.75 mm thick 15 lane gels and transferred onto PVDF membranes as described in Chapter 3. The antigen preparations were mixed with an equal volume of reducing sample loading buffer (2x SLB+DTT), heated to 100°C for ten minutes, centrifuged briefly and loaded in alternate lanes of Tris-tricine polyacrylamide gels (see Table 6.3). A voltage of 150 V was applied until the dye front reached the bottom of the gel after approximately 1 hour 30 minutes. The proteins were transferred onto BioTrace™ PVDF membranes with a pore size of 0.45 µm (Pall Gelman Sciences, Life Technologies) in a TRANS-BLOT®SD SEMI-DRY TRANSFER CELL (Bio-Rad Laboratories, Auckland, NZ). A voltage of 15 V was applied for 35 minutes at a maximum current of 0.2 mAmps per gel blotted.

Table 6.3 Details of antigens used in Western blots.

Antigen	Amount	Description
PPD-B	1.2 µg / lane	PPD-B, Sterile Preservative Free 0.3 mg / ml.
8.4 kDa Ag	0.12 µg / lane	*M. smegmatis* pSU151.43 CF buffer exchanged into Tris-HCl 20 mM.

The membranes were blocked overnight with with 2.5% non-fat skim milk in Tween Tris buffered saline (TTBS), washed three times in TTBS and cut into strips, each with lanes of blotted PPD-B, 8.4 kDa Ag and marker. The primary and secondary antibodies were diluted in TTBS and incubations were for one hour with shaking at room temperature. The membranes were washed 3 x 5 minutes in TTBS between each step.

The strips were incubated sequentially with individual cattle sera diluted 1/100, rabbit anti-bovine IgG Biotin conjugate (Sigma Chemical Co, St Louis, USA) diluted 1/5000, and Streptavidin-POD (Roche Diagnostics, Auckland, NZ) diluted 1/5000. The optimal concentrations of secondary antibody and Streptavidin-POD had been determined in preliminary titration trials. Horseradish peroxidase (HRP) immobilised on the membranes was detected as a brown precipitate formed following reaction with DAB peroxidase substrate (SIGMA FAST™ 3,3'-Diaminobenzidine Tablet Sets; Sigma-Aldrich, Sydney, Australia). As a positive control, one strip from each membrane was developed with rabbit anti-8.4 kDa Ag polyclonal serum diluted 1/1000 as the primary antibody, goat anti-rabbit biotin conjugate diluted 1/5000 as the secondary antibody, then streptavidin-POD and DAB as described above.

6.2.5 Statistical analyses.

The proportions of unvaccinated and BCG vaccinated animals with positive lymphocyte proliferation (SI $\geq$ 3) and IFN-γ (Δ OD_{450} $\geq$ 100) responses to each antigen were compared by Fisher's exact test ($P \leq 0.05$). Analysis of variance of lymphocyte proliferation (Δ c.p.m and SI) and IFN-γ (Δ OD_{450}) responses to each antigen were performed with log_{10} transformed data (Δ c.p.m ≤ 0 were designated a value of 10, and Δ $OD_{450} \leq 0$ were designated a value of 1.5). The mean responses of the BCG vaccinated and unvaccinated groups were compared using the least squares means test ($P \leq 0.05$).

6.3 RESULTS.

6.3.1 Lymphocyte proliferation and IFN-γ assay responses before and after challenge. Sampling times 4, 5 and 6.

The number of unvaccinated and BCG vaccinated animals with positive lymphocyte proliferation (LøP) and IFN-γ assay responses to PPD-B, PPD-A and the 8.4 kDa Ag at concentrations of 6.25, 1.25, 0.25 and 0.05 μg protein / ml before and after intratracheal challenge with virulent *M. bovis* (Sampling times 4, 5 and 6) are shown in Tables 6.4, 6.5 and 6.6 (see Appendix IV for raw data).

Table 6.4 Number of responses, Pre-challenge (Sampling 4).

	Unvaccinated		BCG Vaccinated	
Antigen	LøP (SI)	IFN-γ (ΔOD_{450})	LøP (SI)	IFN-γ (ΔOD_{450})
PPD-B				
6.25 μg/ml	**2** / 10	**3** / 10	**9** / 10	**6** / 10
1.25	1 / 10	1 / 10	8 / 10	4 / 10
0.25	1 / 10	1 / 10	6 / 10	3 / 10
0.05	1 / 10	1 / 10	1 / 10	1 / 10
PPD-A				
6.25 μg/ml	**9** / 10	**9** / 10	**10** / 10	**9** / 10
1.25	5 / 10	6 / 10	9 / 10	5 / 10
0.25	4 / 10	1 / 10	8 / 10	2 / 10
0.05	1 / 10	1 / 10	4 / 10	2 / 10
8.4 kDa Ag				
6.25 μg/ml	**1** / 10	**3** / 10	**7** / 10	**6** / 10
1.25	1 / 10	1 / 10	5 / 10	3 / 10
0.25	0 / 10	0 / 10	4 / 10	1 / 10
0.05	0 / 10	0 / 10	2 / 10	0 / 10

Number of unvaccinated and BCG vaccinated animals that responded to stimulation with different concentrations of PPD-B, PPD-A and the 8.4 kDa Ag in lymphocyte proliferation (LøP) and IFN-γ assays before intratracheal challenge with virulent *M. bovis*. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ assay, Δ OD_{450} ≥ 100.

Table 6.5 Number of responses, Five weeks post-challenge (Sampling 5).

	Unvaccinated		BCG Vaccinated	
Antigen	LøP (SI)	IFN-γ (ΔOD_{450})	LøP (SI)	IFN-γ (ΔOD_{450})
PPD-B				
6.25 μg/ml	**8** / 10	**6** / 10	**9** / 10	**7** / 10
1.25	8 / 10	5 / 10	9 / 10	4 / 10
0.25	7 / 10	3 / 10	6 / 10	2 / 10
0.05	4 / 10	2 / 10	4 / 10	2 / 10
PPD-A				
6.25 μg/ml	**8** / 10	**7** / 10	**10** / 10	**8** / 10
1.25	7 / 10	4 / 10	9 / 10	6 / 10
0.25	5 / 10	2 / 10	7 / 10	2 / 10
0.05	2 / 10	1 / 10	6 / 10	3 / 10
8.4 kDa Ag				
6.25 μg/ml	**3** / 10	**2** / 10	**7** / 10	**4** / 10
1.25	3 / 10	2 / 10	6 / 10	2 / 10
0.25	0 / 10	0 / 10	5 / 10	0 / 10
0.05	0 / 10	1 / 10	2 / 10	1 / 10

Number of unvaccinated and BCG vaccinated animals that responded to stimulation with different concentrations of PPD-B, PPD-A and the 8.4 kDa Ag in lymphocyte proliferation (LøP) and IFN-γ assays five weeks after intratracheal challenge with virulent *M. bovis*. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ assay, Δ OD_{450} ≥ 100.

A titration effect of antigen concentration is evident with more animals responding to 6.25 μg protein / ml than to the lower concentrations. Only the responses to stimulation with antigen at 6.25 μg / ml were subjected to statistical analysis (bold type). There were no significant differences between the LøP and IFN-γ assays with respect to the numbers of animals responding to each antigen ($P < 0.05$) (see Appendix Va).

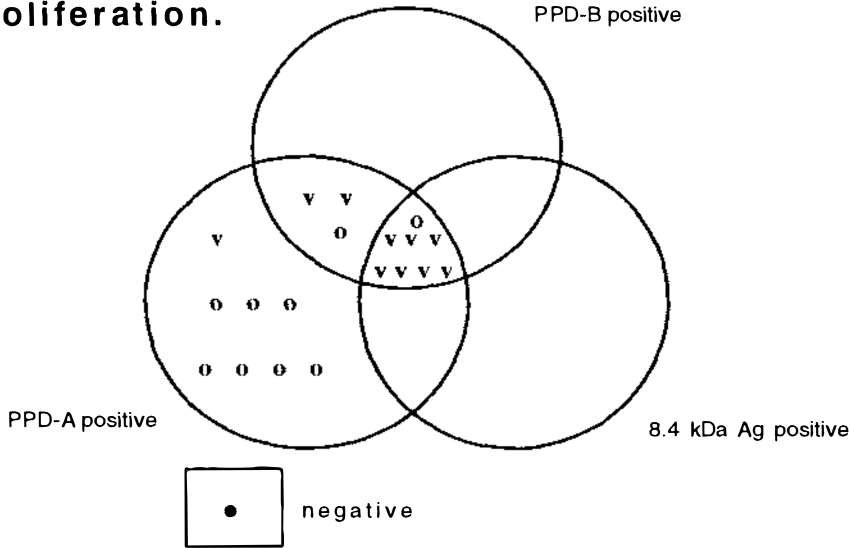
Table 6.6 Number of responses, Ten weeks post-challenge (Sampling 6).

	Unvaccinated		BCG Vaccinated	
Antigen	LøP (SI)	IFN-γ (ΔOD_{450})	LøP (SI)	IFN-γ (ΔOD_{450})
PPD-B				
6.25 μg/ml	**8** / 10	**9** / 10	**7** / 10	**8** / 10
1.25	6 / 10	7 / 10	7 / 10	5 / 10
0.25	5 / 10	8 / 10	5 / 10	5 / 10
0.05	3 / 10	5 / 10	3 / 10	1 / 10
PPD-A				
6.25 μg/ml	**7** / 10	**10** / 10	**7** / 10	**8** / 10
1.25	7 / 10	8 / 10	6 / 10	7 / 10
0.25	5 / 10	5 / 10	5 / 10	5 / 10
0.05	4 / 10	2 / 10	6 / 10	5 / 10
8.4 kDa Ag				
6.25 μg/ml	**2** / 10	**6** / 10	**5** / 10	**8** / 10
1.25	3 / 10	1 / 10	2 / 10	1 / 10
0.25	1 / 10	1 / 10	3 / 10	2 / 10
0.05	1 / 10	2 / 10	2 / 10	0 / 10

Number of unvaccinated and BCG vaccinated animals that responded to stimulation with different concentrations of PPD-B, PPD-A and the 8.4 kDa Ag in lymphocyte proliferation (LøP) and IFN-γ assays ten weeks after intratracheal challenge with virulent *M. bovis*. Positive responses were defined as; lymphocyte proliferation, $SI \geq 3$; IFN-γ assay, $\Delta OD_{450} \geq 100$.

The numbers of individual unvaccinated and BCG vaccinated animals that responded to each antigen at 6.25 μg protein / ml in LøP and IFN-γ assays, before and after challenge infection with *M. bovis* are illustrated schematically in Figures 6.1, 6.2 and 6.3. Prior to intratracheal challenge with virulent *M. bovis* (Pre-challenge, Sampling time 4) nine out of ten BCG vaccinated animals had positive LøP responses to PPD-B. Only two out of ten unvaccinated animals had positive LøP responses to PPD-B, and the difference between the BCG vaccinated group and the unvaccinated group was significant ($P = 0.003$) (see Appendix Vd).

Positive responses to PPD-B, PPD-A and the 8.4 kDa Ag; Pre-challenge (Sampling time 4).



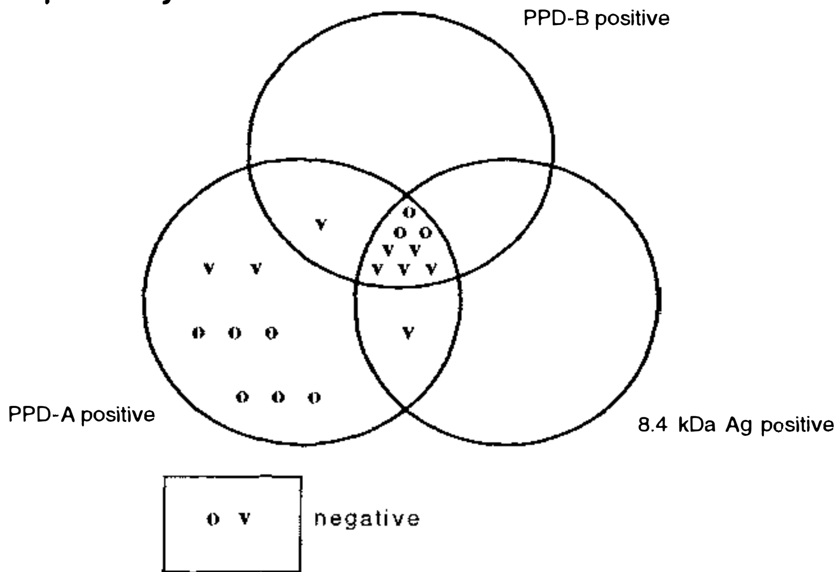
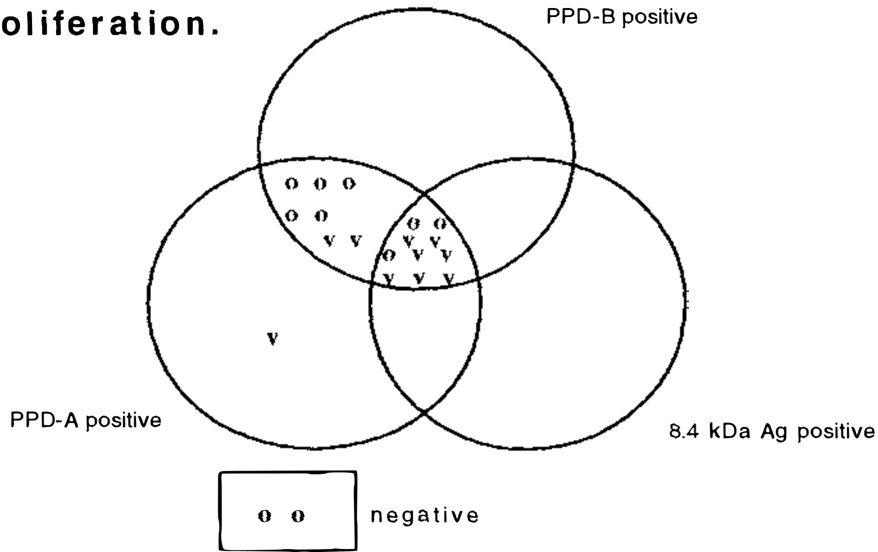


Figure 6.1 Lymphocyte proliferation and IFN-γ assay responses of ten unvaccinated (**o**) and ten BCG vaccinated (**v**) calves, before intratracheal challenge with virulent *M. bovis*. PBMC were stimulated with PPD-B, PPD-A and the 8.4 kDa antigen at 6.25 mg/ml. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ assay, Δ $OD_{450} \geq 100$, and are shown as **o** or **v** within the circle/s corresponding to the stimulatory antigen/s. Animals that did not respond to any antigens are shown in boxes outside the circles.

Positive responses to PPD-B, PPD-A and the 8.4 kDa Ag;
Five weeks post-challenge (Sampling time 5).



IFN-γ assay.

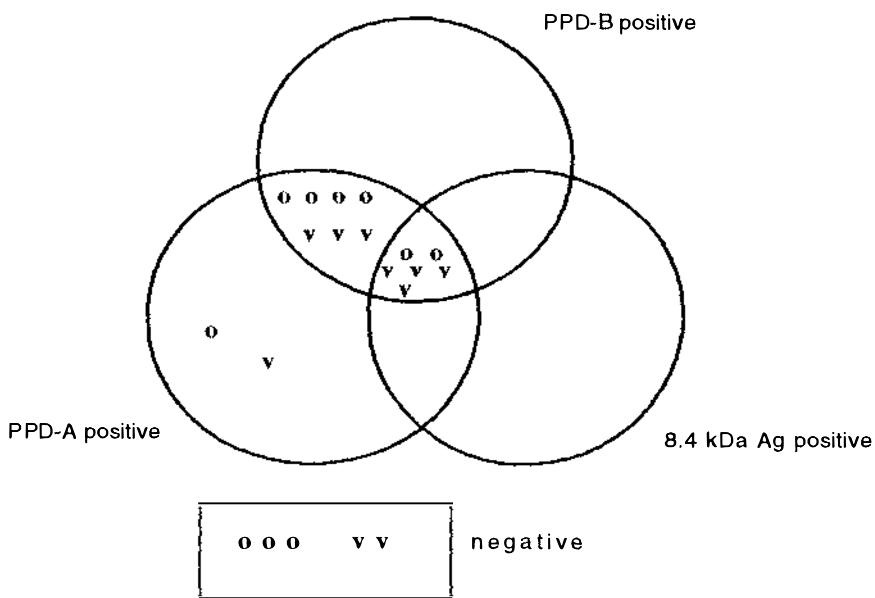
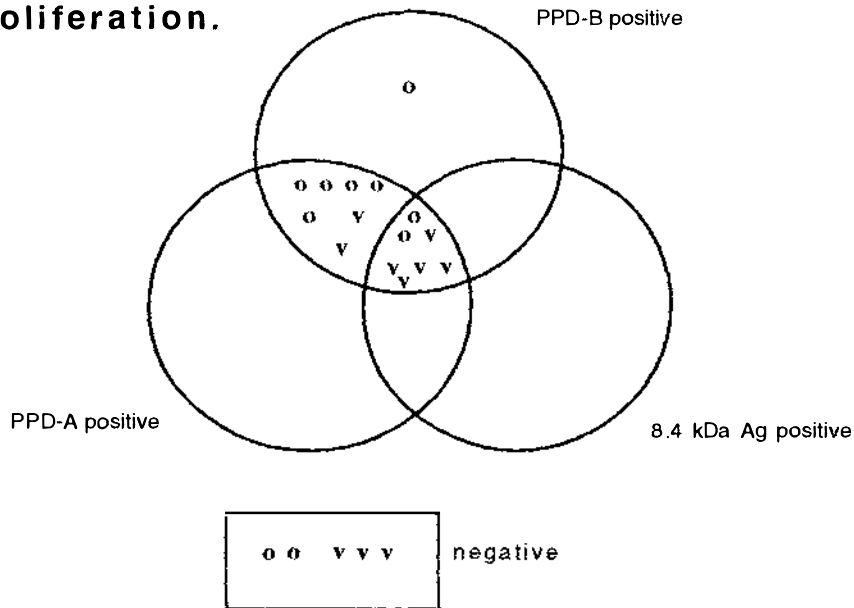


Figure 6.2 Lymphocyte proliferation and IFN-γ assay responses of ten unvaccinated (**o**) and ten BCG vaccinated (**v**) calves, five weeks after intratracheal challenge with virulent *M. bovis*. PBMC were stimulated with PPD-B, PPD-A and the 8.4 kDa antigen at 6.25 mg/ml. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ assay, Δ $OD_{450} \geq 100$, and are shown as **o** or **v** within the circle/s corresponding to the stimulatory antigen/s. Animals that did not respond to any antigens are shown in boxes outside the circles.

Positive responses to PPD-B, PPD-A and the 8.4 kDa Ag;
Ten weeks post-challenge (Sampling time 6).



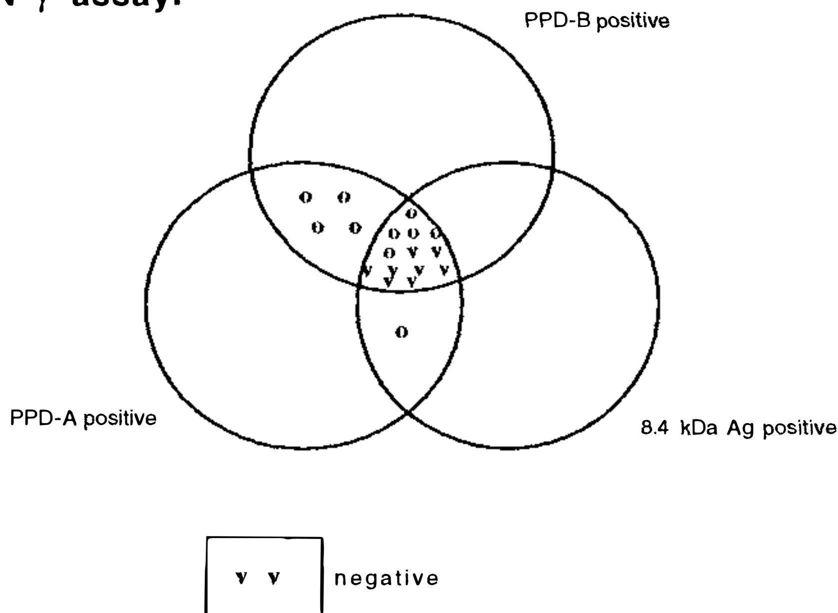


Figure 6.3 Lymphocyte proliferation and IFN-γ assay responses of ten unvaccinated (**o**) and ten BCG vaccinated (**v**) calves, ten weeks after intratracheal challenge with virulent *M. bovis*. PBMC were stimulated with PPD-B, PPD-A and the 8.4 kDa antigen at 6.25 mg/ml. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ assay, Δ OD_{450} ≥ 100, and are shown as **o** or **v** within the circle/s corresponding to the stimulatory antigen/s. Animals that did not respond to any antigens are shown in boxes outside the circles.

Of the nine BCG vaccinated animals with positive LøP responses to PPD-B, seven responsed to the 8.4 kDa Ag. In contrast, only one unvaccinated animal had a positive LøP response to the 8.4 kDa Ag (see Figure 6.1). The difference between the number of unvaccinated and BCG vaccinated animals responding to the 8.4 kDa Ag was significant ($P = 0.01$).

In IFN-γ assays (Sampling time 4), six out of ten BCG vaccinated animals had positive responses to PPD-B, of which five also responded to the 8.4 kDa Ag. Three unvaccinated animals had positive IFN-γ responses to both PPD-B and the 8.4 kDa Ag. However, there was no significant difference between the number of BCG vaccinated and unvaccinated animals responding to either PPD-B or the 8.4 kDa Ag in the IFN-γ assays (see Figure 6.1). The three unvaccinated animals that responded to the 8.4 kDa Ag were not a significantly greater number than would be expected if the assay had a specificity of 95%.

It was noted that nine out of ten unvaccinated animals responded to PPD-A in both LøP and IFN-γ assays, and was significantly greater than the number that responded to either PPD-B or the 8.4 kDa Ag ($P < 0.05$) (see Appendices Vbi and Vbii). The number of BCG vaccinated or unvaccinated animals responding to PPD-A in either LøP or IFN-γ assays was not significantly different and did not change significantly throughout the trial (see Appendices Va and Vc).

Following intratracheal challenge with virulent *M. bovis* (5 weeks post-challenge, Sampling time 5) the number of unvaccinated animals responding to PPD-B increased to eight out of ten in LøP assays and six out of ten in IFN-γ assays (see Figure 6.2). The increase was significant for the LøP assay ($P = 0.01$) but not the IFN-γ assay ($P = 0.15$). The number of unvaccinated animals with positive LøP and IFN-γ responses to the 8.4 kDa Ag did not change significantly following challenge. Significantly fewer unvaccinated animals had positive LøP responses to the 8.4 kDa Ag than to PPD-B ($P = 0.03$). However, in IFN-γ assays the difference between the number of unvaccinated animals responding to the 8.4 kDa Ag and PPD-B was not significant ($P = 0.08$) (see Appendix Vbii).

Although seven BCG vaccinated animals and three unvaccinated animals had positive LøP responses to the 8.4 kDa Ag, the difference between the two groups was not significant (P = 0.08). There was no significant difference between the number of BCG vaccinated and unvaccinated animals with positive IFN-γ responses to the 8.4 kDa Ag (see Appendix Vd).

At ten weeks post-challenge (Sampling time 6) the number of BCG vaccinated and unvaccinated animals responding to each antigen in LøP and IFN-γ assays had not changed significantly from five weeks post-challenge (Sampling time 5) (see Figure 6.3). However, the number of unvaccinated animals with positive IFN-γ responses to PPD-B had become significantly greater than the number responding prior to challenge (P = 0.01) (see Appendix Vc). At ten weeks post-challenge there was no significant difference between the number of BCG vaccinated and unvaccinated animals with positive responses to the 8.4 kDa Ag in either LøP or IFN-γ assays (see Appendix Vd).

There was a consistent pattern to the relationships between the positive responses to PPD-B, PPD-A and the 8.4 kDa Ag. Animals that had a positive response to PPD-B also had a positive response to PPD-A (one exception), but the converse was not true. Animals that had a positive response to the 8.4 kDa Ag also had a positive responses to PPD-B (two exceptions), but the converse was not true. Thus, animals that responded to the 8.4 kDa Ag responded to PPD-B (one exception) and also to PPD-A.

6.3.2 Magnitude of responses to PPD-B, PPD-A and the 8.4 kDa Ag.

The magnitude of individual LøP and IFN-γ responses of BCG vaccinated and unvaccinated animals to PPD-B, PPD-A and to the 8.4 kDa Ag (6.25 μg / ml) at Sampling times 4, 5 and 6 are shown in Figures 6.4, 6.5 and 6.6 respectively. The LøP (SI) data are shown on a $\log_{10}$ scale to illustrate the distribution of the proliferative responses. Prior to challenge (Sampling time 4) the LøP responses of the unvaccinated animals to PPD-B and the 8.4 kDa Ag were clustered below the positive response cut-off level. The LøP responses of the BCG vaccinated group are clustered above the cut-off (see Figures 6.4). The difference between the mean LøP responses of the BCG vaccinated and unvaccinated

Magnitude of responses to PPD-B, PPD-A and the 8.4 kDa Ag; Pre-challenge (Sampling time 4).

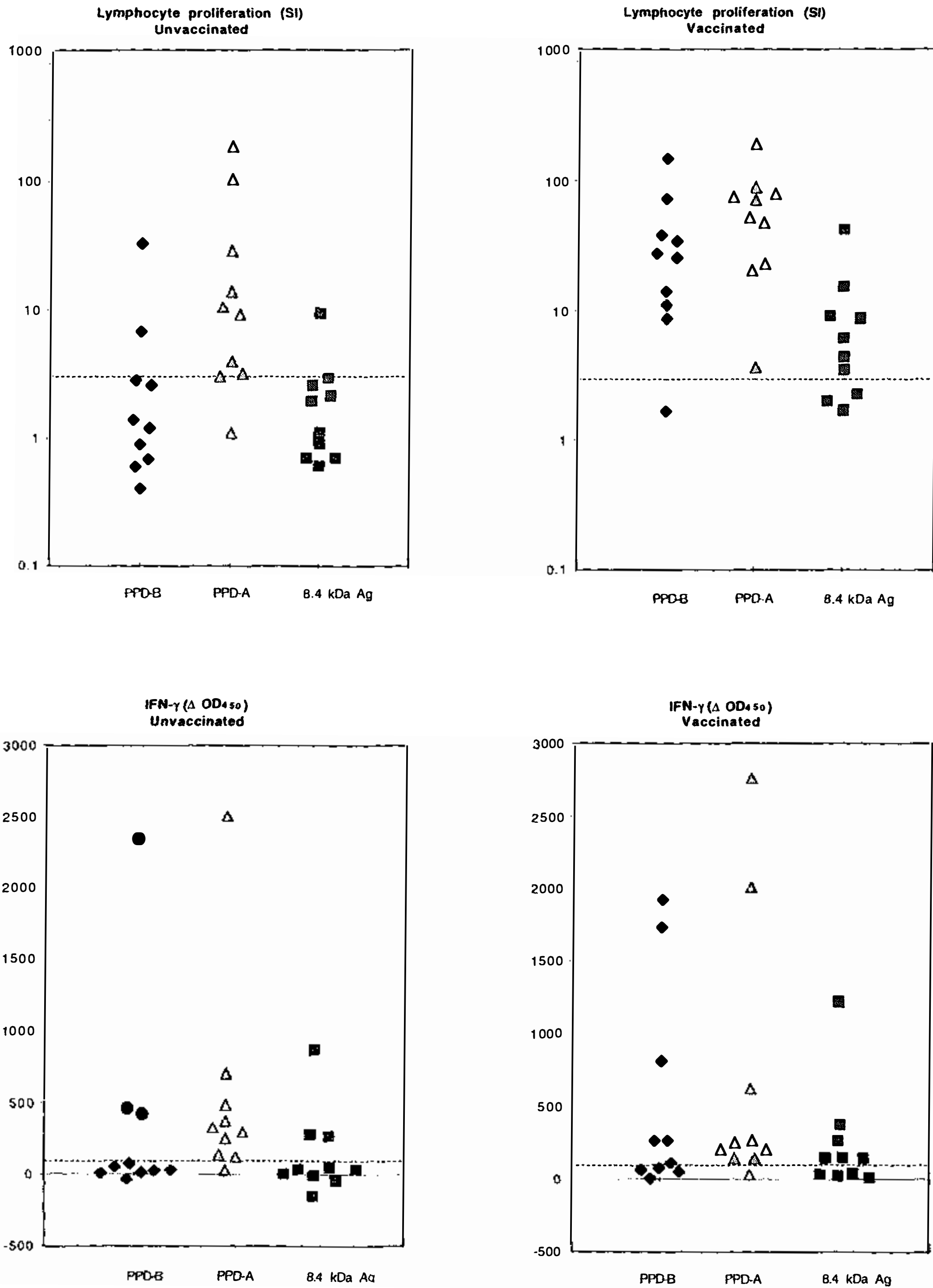


Figure 6.4 Lymphocyte proliferation (SI) and IFN-γ (Δ OD450) responses of ten unvaccinated and ten BCG vaccinated calves, to PPD-B, PPD-A and the 8.4 kDa Ag, before intratracheal challenge with virulent *M. bovis*. PBMC were stimulated with each antigen at 6.25 μg / ml. The data points represent the means of quadruplicates. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ, Δ OD450 ≥ 100. The cut-off levels for positive responses are indicated by broken lines.

Magnitude of responses to PPD-B, PPD-A and the 8.4 kDa Ag; Five weeks post-challenge (Sampling time 5).

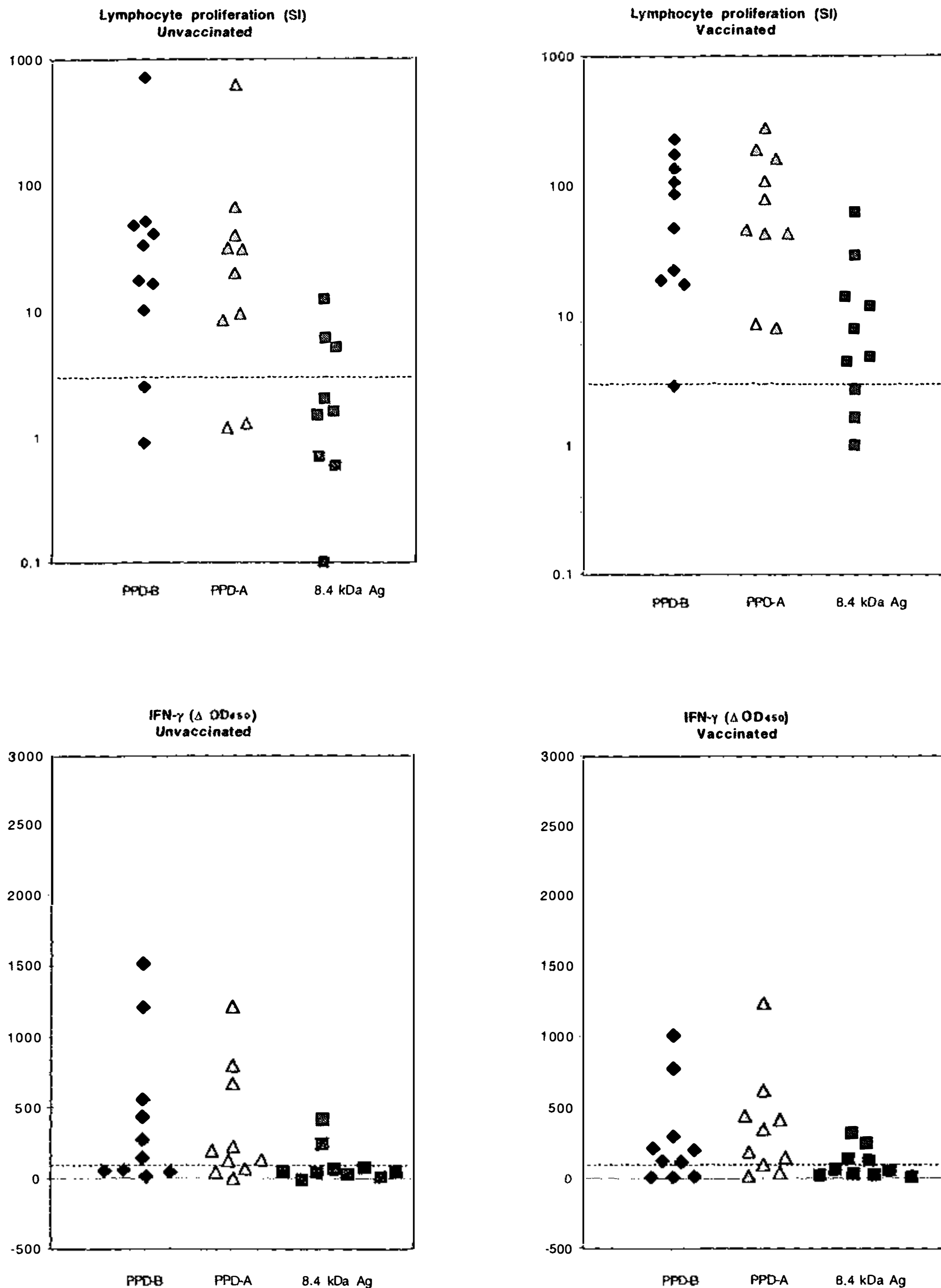


Figure 6.5 Lymphocyte proliferation (SI) and IFN-γ (Δ OD450) responses of ten unvaccinated and ten BCG vaccinated calves, to PPD-B, PPD-A and the 8.4 kDa Ag, five weeks after intratracheal challenge with virulent *M. bovis*. PBMC were stimulated with each antigen at 6.25 μg / ml. The data points represent the means of quadruplicates. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ, Δ OD450 ≥ 100. The cut-off levels for positive responses are indicated by broken lines.

Magnitude of responses to PPD-B, PPD-A and the 8.4 kDa Ag; Ten weeks post-challenge (Sampling time 6).

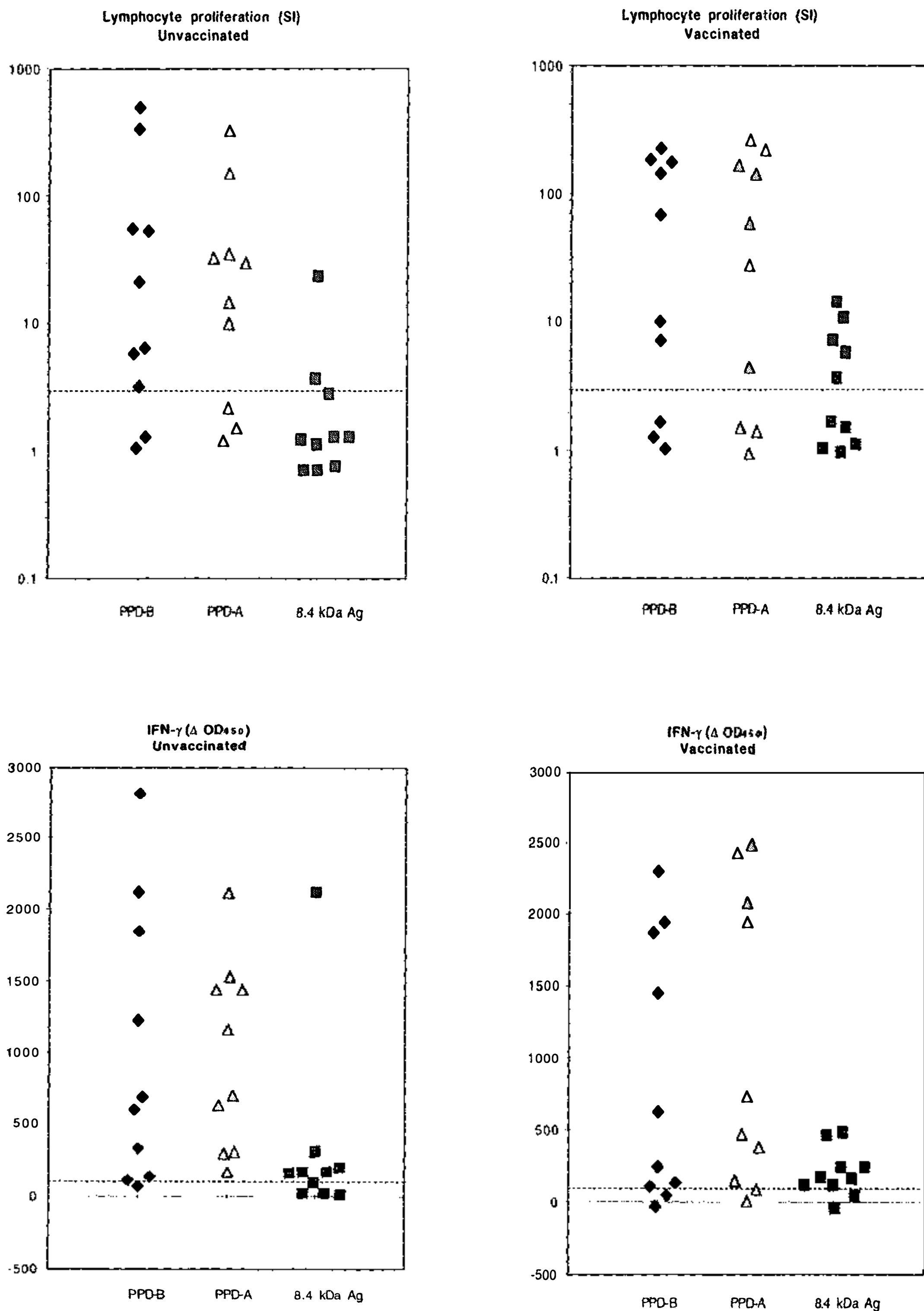


Figure 6.6 Lymphocyte proliferation (SI) and IFN-γ (Δ OD450) responses of ten unvaccinated and ten BCG vaccinated calves, to PPD-B, PPD-A and the 8.4 kDa Ag, ten weeks after intratracheal challenge with virulent *M. bovis*. PBMC were stimulated with each antigen at 6.25 μg / ml. The data points represent the means of quadruplicates. Positive responses were defined as; lymphocyte proliferation, SI ≥ 3; IFN-γ, Δ OD450 ≥ 100. The cut-off levels for positive responses are indicated by broken lines.

Mean lymphocyte proliferation and IFN-γ responses to PPD-B, PPD-A and the 8.4 kDa Ag. Pre-challenge, five and ten weeks post-challenge.

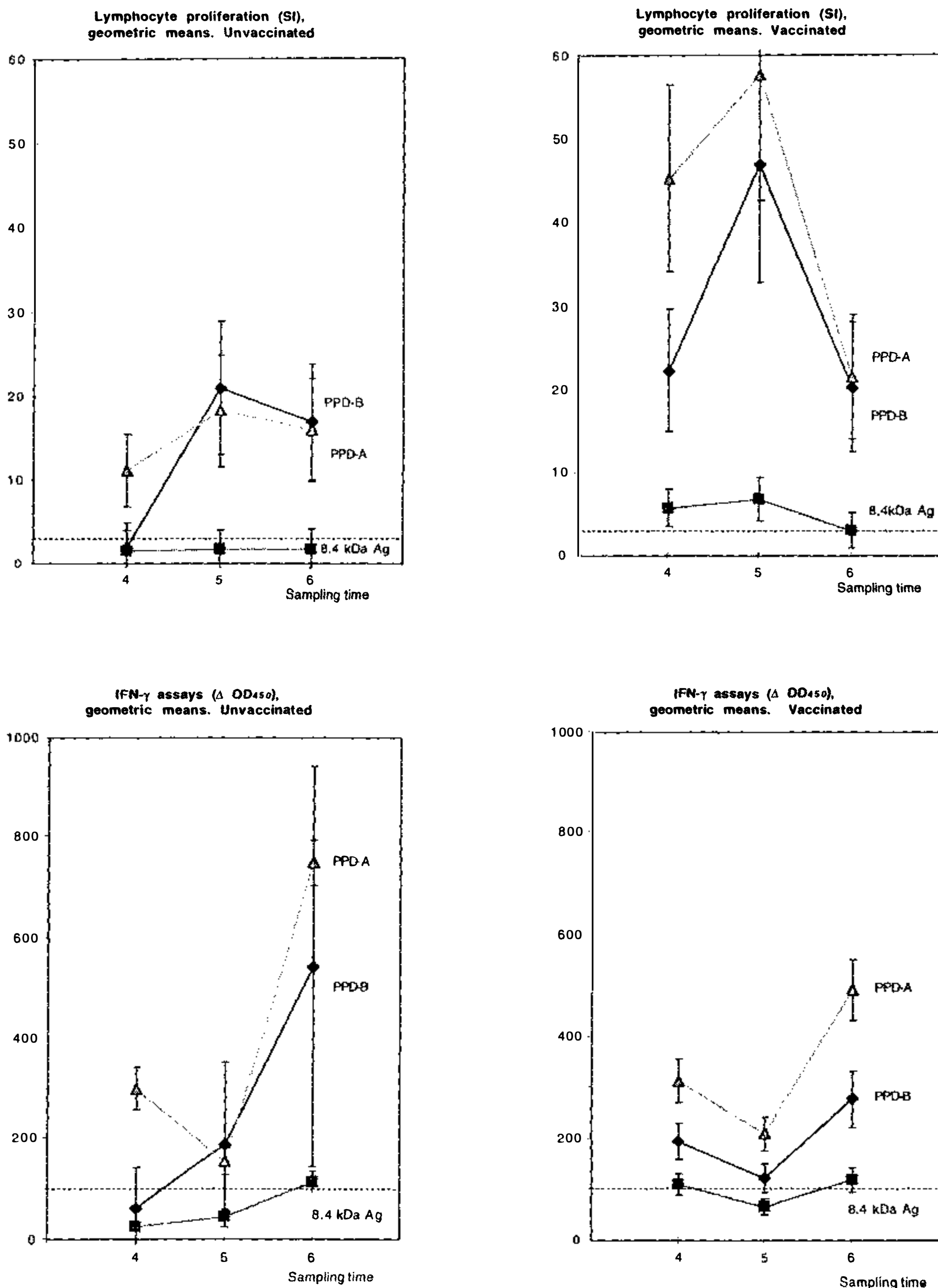


Figure 6.7 Comparison of the geometric mean responses of ten unvaccinated and ten BCG vaccinated calves to PPD-B (diamonds), PPD-A (triangles) and the 8.4 kDa Ag (squares), in lymphocyte proliferation (SI) and IFN-γ (Δ OD450) assays. PBMC were stimulated with antigen at 6.26 μg / ml, before and after intratracheal challenge with virulent *M. bovis* (Sampling time 4, pre-challenge; Sampling time 5, five weeks post-challenge; Sampling time 6, ten weeks post-challenge). Positive responses were defined as; IFN-γ, Δ OD450 ≥ 100. The cut-off levels for positive IFN-γ responses are indicated by broken lines. Vertical bars show 95% confidence intervals.

animals to PPD-B and the 8.4 kDa Ag was significant ($P < 0.01$) (see Figure 6.7 and Appendix Vf).

The IFN-γ data are shown on an arithmetic scale. The IFN-γ responses of both the unvaccinated and BCG vaccinated animals were clustered around the cut-off with a few outlying high level responses (see Figure 6.4). The mean IFN-γ responses of the BCG vaccinated animals to PPD-B or the 8.4 kDa Ag were not significantly higher than the mean responses of the unvaccinated animals (see Figure 6.7 and Appendix Vg). In both LøP and IFN-γ assays the BCG vaccinated and unvaccinated groups of animals had mean responses to PPD-A that were significantly higher than the mean responses to PPD-B and the 8.4 kDa Ag ($P < 0.05$) (see Figure 6.7).

Following challenge infection (Sampling time 5) the mean LøP response of the unvaccinated group to PPD-B increased significantly ($P = 0.0002$) and the responses were clustered above the cut-off (see Figure 6.5). In contrast, the responses of the unvaccinated animals to the 8.4 kDa Ag did not alter significantly following challenge. The difference between the mean LøP responses of the BCG vaccinated and unvaccinated groups to the 8.4 kDa Ag was significant ($P = 0.01$) (see Figure 6.7 and Appendix Vf).

The mean IFN-γ response of the unvaccinated animals to PPD-B and the 8.4 kDa Ag did not increase significantly by five weeks post-challenge, and there was no significant difference between the mean IFN-γ responses of the BCG vaccinated and unvaccinated groups of animals to either PPD-B or the 8.4 kDa Ag (see Figure 6.7 and Appendix Vg). The LøP responses to PPD-A had become more widely distributed, and in both LøP and IFN-γ assays there was no significant difference between the responses of BCG vaccinated or unvaccinated animals to PPD-A (see Figures 6.5 and 6.7).

At ten weeks post-challenge (Sampling time 6) the LøP responses of the unvaccinated animals had not changed significantly from five weeks post challenge (Sampling time 5), (see Figures 6.6 and 6.7). The IFN-γ assay responses of both the BCG vaccinated and unvaccinated group to all antigens and especially PPD-A had become more widely distributed. The mean IFN-γ response of the unvaccinated animals to PPD-B had become

significantly higher than prior to challenge (P = 0.002). The increase in the mean IFN-γ response of the unvaccinated animals to the 8.4 kDa Ag was not significant (see Appendix Vg).

It was noted that the unvaccinated animals had a low but detectable level of IFN-γ response to the 8.4 kDa Ag both before and after challenge infection. The mean IFN-γ level of the 8.4 kDa stimulated wells was 2.1, 2.0 and 2.7 times the 95% confidence interval above the level of the unstimulated wells at Sampling times 4, 5 and 6 respectively (see Figure 6.7).

6.3.3 Antibody responses.

Western blots were performed with sera taken ten weeks post-challenge (Sampling time 6). None of the antigens of PPD-B nor the 8.4 kDa Ag were detected by sera from any of the animals. A band of approximately 23 kDa in the CF of *M. smegmatis* pSU151.43 was weakly detected by sera from two animals. The 23 kDa band had also been detected by sera from unvaccinated control rabbits (not shown).

6.4 DISCUSSION.

In recent years, numerous studies have investigated the immune responses of cattle to individual *M. bovis* antigens following both BCG vaccination and *M. bovis* infection. It has been reported that up to 70% of animals that have positive *in vitro* responses to PPD tuberculin also record responses to any individual antigen. However, the magnitude of the responses to individual antigens is lower than the responses to PPD tuberculin. It has been concluded, that in an outbred population of animals each individual infected with *M. bovis* responds to a different repertoire of antigens, and that the response to each antigen is a fraction of the total response (Fifis *et al.* 1994a, Fifis *et al.* 1994b, Pollock and Andersen 1997, Lyashchenko *et al.* 1998b, Lightbody *et al.* 1998a, Lightbody *et al.* 1998b, Buddle *et al.* 1999, Vordermeier *et al.* 1999, van Pinxteren *et al.* 2000, Pollock *et al.* 2000, Rhodes *et al.* 2000a, Buddle *et al.* 2001).

In this study, the immune responses of ten BCG vaccinated and ten unvaccinated calves to recombinant 8.4 kDa antigen of *M. bovis* were compared with their responses to PPD-B and PPD-A before and after intratracheal challenge infection with virulent *M. bovis*. Ten

weeks after challenge with virulent *M. bovis*, the calves were subjected to an intradermal tuberculin test using PPD-B and all twenty calves had positive cutaneous DTH reactions. At post-mortem examination 17 weeks after challenge, all calves had macroscopic tuberculous lesions associated with the respiratory tract, but the pathologic changes associated with the lesions occurring in the BCG vaccinated calves were significantly less severe than in the unvaccinated calves (Wedlock *et al.* 2000).

Lymphocyte proliferation and IFN-γ assays were used to measure the cellular responses to PPD-B, PPD-A and the 8.4 kDa antigen. PBMC from the calves were stimulated with mycobacterial antigens at a range of concentrations. The concentrations of antigen used at Sampling times 1, 2 and 3 were selected on the basis of the response to the 8.4 kDa Ag of the BCG vaccinated animal at Massey University (Chapter 3). However, following BCG vaccination the proportion of animals with positive responses to PPD-B was less than the approximately 70% reported in similar studies (Lightbody *et al.* 1998a, Vordermeier *et al.* 1999, Hope *et al.* 2000, Rhodes *et al.* 2000a). Those studies indicated that the optimal concentrations of antigen for stimulating PBMC are between 4 and 10 μg protein / ml. Therefore, two five-fold higher concentrations of antigen were used at Sampling times 4, 5 and 6, but only the responses to 6.25 μg protein / ml were subjected to statistical analysis.

Western blotting was used to measure the humoral responses to PPD-B or the 8.4 kDa antigen. The animals had not developed a humoral response to either PPD-B or the 8.4 kDa Ag by 10 weeks post-challenge. It has been reported that humoral responses are indicative of disease progression (Fifis *et al.* 1994b, Lightbody *et al.* 2000). Therefore, sera taken prior to ten weeks post-challenge were not analysed for the presence of specific antibodies. The lack of humoral responses to PPD-B was consistent with the results of ELISA assays for serum antibodies conducted at AgResearch Wallaceville (Wedlock *et al.* 2000).

All but one of the unvaccinated animals were found to have positive responses to PPD-A before they were challenged with *M. bovis*. The responses to PPD-A indicate that environmental exposure to *M. avium* had sensitized the animals to antigens present in PPD-A. The distribution of the LøP responses of the unvaccinated animals to PPD-B and PPD-A overlapped. The greatest differentiation between responses to PPD-B and PPD-A

was achieved with a cut-off level of SI ≥ 3, which has also been used to define positive LøP responses in other recent studies (Vordermeier *et al.* 1999, Rhodes *et al.* 2000a).

A positive IFN-γ response was defined in terms of $\Delta\ OD_{450}$ in order to be consistent with the method used to interpret the BOVIGAM™ assay. The applicability of the interperetation schedules for whole blood IFN-γ assays used by other workers was investigated. If the definition of a positive responses as $\Delta\ OD_{450} > 50$ (Wood *et al.* 1992) was applied instead of $\Delta\ OD_{450} \geq 100$, a significantly greater proportion of animals had positive responses. For example, prior to challenge 3/10 unvaccinated animals had positive responses to PPD-B using the cut-off of $\Delta\ OD_{450} \geq 100$, which is not significant if the test has a specificity of 95%. However using a cut-off of $\Delta\ OD_{450} > 50$, it was found that 5/10 of the same animals had positive responses to PPD-B, which is not consistent with a test specificity of 95%.

If the definition of a positive IFN-γ response as $\Delta\ OD_{450} \geq 100$ and PPD-B OD_{450} — PPD-A $OD_{450} \geq 100$ (Ryan *et al.* 2000) was applied, there were only five positive IFN-γ responses in the whole trial. That finding could have been peculiar to this trial where there was a substantial level of IFN-γ response to PPD-A. Nevertheless, the findings of this study indicate that IFN-γ assays performed using supernatants from five day cultures of separated PBMC are not strictly comparable with whole blood IFN-γ assays. Therefore, caution must be exercised when extrapolating from one assay format to the other.

Consequently, a positive IFN-γ response was defined as $\Delta\ OD_{450} \geq 100$. The number of positive IFN-γ responses to each antigen were compared with the number of positive LøP responses. It was found that there were no significant differences between the two assays with respect to the number of animals responding to each of the antigens.

The first objective of this study was to determine whether the animals vaccinated with *M. bovis* BCG had developed an immune response to the 8.4 kDa Ag. The responses to PPD-B were used as a positive control for responses to *M. bovis* antigens, because it would be

expected that following BCG vaccination and/or challenge with *M. bovis* the animals would have positive responses to PPD-B.

If the BCG vaccinated animals responded to both PPD-B and the 8.4 kDa Ag and the unvaccinated animals responded to neither antigen it would support the conclusion that BCG vaccination had induced an immune response to the 8.4 kDa Ag. It was found that significantly more BCG vaccinated animals than unvaccinated animals responded to PPD-B and also to the 8.4 kDa Ag. In addition, the mean proliferative responses of the BCG vaccinated animals to PPD-B and to the 8.4 kDa Ag were greater than the mean responses of the unvaccinated animals. Thus, it appears that vaccination with BCG had induced the animals to develop immune responses against the 8.4 kDa Ag.

The second objective of this study was to determine whether animals experimentally infected with virulent *M. bovis* developed an immune response to the 8.4 kDa Ag. It was found that following challenge infection, the unvaccinated animals responded to PPD-B but not to the 8.4 kDa Ag in lymphocyte proliferation assays. In addition, the BCG vaccinated animals had greater proliferative responses to the 8.4 kDa Ag than the unvaccinated animals. Thus, it appears that respiratory infection with *M. bovis* did not elicit an immune response to the 8.4 kDa Ag.

The IFN-γ assay results were inconclusive. It was found that there were no significant differences between the responses of the BCG vaccinated and unvaccinated animals to either PPD-B or the 8.4 kDa Ag at any time during the trial. Furthermore, following challenge infection the IFN-γ responses of the unvaccinated animals to PPD-B did not increase significantly until ten weeks post-infection. The IFN-γ response data had greater variance than the LøP data and it is possible that real effects were not statistically apparent due to the small number of animals in each group.

However, prior to challenge infection the mean IFN-γ response level of the unvaccinated animals to the 8.4 kDa Ag was greater than the level of the unstimulated wells by twice the 95% confidence interval. The mean IFN-γ response of the unvaccinated animals to PPD-A was also high indicating that the animals had been exposed to *M. avium*. The higher than expected response of the unvaccinated animals to the 8.4 kDa Ag, and the lack of a

difference between the IFN-γ responses of the BCG vaccinated and unvaccinated animals to the 8.4 kDa Ag could be explained if exposure to *M. avium* had sensitized the animals to the 8.4 kDa Ag.

In Chapter 5 it was shown that the genome of *M. avium* contains a sequence that could potentially code for a protein with homology to the 8.4 kDa Ag of *M. bovis*. This study has provided some preliminary indirect evidence that the *M. avium* protein may be expressed *in vivo*. Thus, it appears that the 8.4 kDa Ag of *M. bovis* is not a candidate for specific diagnosis of *M. bovis* infection in cattle.

Chapter 7. Overexpression of the 8.4 kDa Ag by *E. coli* and *M. smegmatis.*

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7.1 INTRODUCTION.

Several mycobacterial antigens recognized by hosts infected with *M. bovis* have been identified and proposed as potential components of improved diagnostic tests or novel vaccines (Garbe *et al.* 1993, Matsumoto *et al.* 1995, Sørensen *et al.* 1995, Bigi *et al.* 1997, Harth *et al.* 1997, Berthet *et al.* 1998). However, a prerequisite for studies of the host immune responses to *M. bovis* antigens is the ready availability of pure immunologically active antigen (Colangeli *et al.* 1998). Production and purification of sufficient quantities of individual antigens from cultures of *M. bovis* is cumbersome due to the requirements for containment, slow growth, difficulties experienced with chromatographic purification, and the small amounts of antigen recovered (Nagai *et al.* 1991, Garbe *et al.* 1993, Fifis *et al.* 1994b, Colangli *et al.* 1998).

Several *M. bovis* antigens have been overexpressed as recombinant proteins in *E. coli*, and screened for immunological activity in assays of both cellular and humoral responses (Colangeli *et al.* 1998, Lightbody *et al.* 1998a, Lightbody *et al.* 1998b, Lyashchenko *et al.* 1998a, Lyashchenko *et al.* 1998b, Buddle *et al.* 1999, Vordermeier *et al.* 1999, van Pinxteren *et al.* 2000). However, production and purification of individual mycobacterial antigens in *E. coli* is not straightfoward since the proteins are not always expressed, or the expressed protein is insoluble (Harth *et al.* 1997, Colangeli *et al.* 1998).

Furthermore, it has been found that recombinant antigens expressed in *E. coli*, especially glutathione-S-transferase (GST) fusion proteins, sometimes do not stimulate lymphocyte proliferation, IFN-γ production or particularly cutaneous DTH to the same degree as native antigens (Hewinson *et al.* 1996, Roche *et al.* 1996, Triccas *et al.* 1998). It has been suggested that the reduced immunological activity could be due to differences in protein folding and/or lack of post-translational modification by *E. coli* (Garbe *et al.* 1993). However, the particular limitation of producing mycobacterial antigens in *E. coli* for use in cell-based immunological assays is the necessity for extensive purification to remove *E. coli* lipopolysaccharide, which is a potent non-specific activator of lymphoid cells (Colangeli *et al.* 1998).

In order to overcome the practical limitations of producing recombinant antigens in *E. coli*, it has been proposed that recombinant *M. bovis* antigens could be overproduced by *M. smegmatis*. Transcription and translation of *M. bovis* antigens by a mycobacterial host

should enable more accurate processing of the gene products. Thus, recombinant proteins should fold, be post-translationally modified, and if appropriate be exported in a manner similar to the native antigens. In support of that hypothesis, recombinant 19 kDa antigen expressed by *M. smegmatis* was observed to be glycosylated and more immunogenic than the *E. coli* recombinant (Garbe *et al.* 1993, Harth *et al.* 1997). Recently strong promoters have been used to upregulate expression of recombinant proteins of TB complex species by *M. smegmatis.* The recombinant antigens were purified from CF by antibody affinity chromatography, or alternatively antigen with a C-terminal polyhistidine tag was purified from cell sonicates by metal chelate affinity chromatography (Roche *et al.* 1996, Triccas *et al.* 1998).

In Chapter 3 it was demonstrated that the 8.4 kDa protein antigen of *M. bovis* was expressed by *M. smegmatis* under the control its own promoter, and its presence in unpurified CF could be detected in lymphocyte proliferation and IFN-γ assays. However, the *M. smegmatis* subclone containing the plasmid pSU151.43 expressed very small amounts of recombinant *M. bovis* 8.4 kDa antigen. It constituted approximately 0.6% of total CF protein, and only 25 µg was able to be purified from each liter of culture after two steps of FPLC.

The objective of the studies reported in this chapter was to explore strategies that could be employed to produce milligram amounts of recombinant 8.4 kDa antigen in a form that could be used in assays of the cellular immune response to *M. bovis* without the need for extensive purification. Initially, recombinant 8.4 kDa antigen was overexpressed in *E. coli* as a GST fusion protein. Subsequently, because proteins produced in *E. coli* require extensive purification to remove lipopolysaccharide prior to use in cell based assays, expression of the 8.4 kDa antigen by *M. smegmatis* was investigated. Immunologically active 8.4 kDa antigen with an N-terminal 6 x Histidine tag was overexpressed under the control of a strong promoter by *M. smegmatis* and purified from the CF in one step by metal chelate affinity chromatography.

Table 7.1 Plasmids used in this study.

Plasmid	Description	Source / Reference
pUC18/4.3#2	pUC18 containing the 4.3 kb *SphI* fragment of *M. bovis* DNA from pSU151.43 that contains the 8.4 kDa Ag gene. Selection marker, ampicillin resistance.	This study (Chapter 2)
pGEX-6P-3	Vector with pBR322 origin of replication. The *trc* promoter and *lac*I^q gene for IPTG inducible overexpression of GST fusion proteins in *E. coli*. Selection marker, ampicillin resistance.	Amersham Pharmacia
pGEX-6P-3:8.4	pGEX-6P-3 with an insert coding for the mature 8.4 kDa Ag.	This study
pGEX-6P-3:8.4-DS(B)	pGEX-6P-3 with an insert coding for the mature 8.4 kDa Ag and including the downstream intergenic sequence of *M. bovis*.	This study
pGEX-6P-3:8.4-DS(T)	pGEX-6P-3 with an insert coding for the mature 8.4 kDa Ag and including the downstream intergenic sequence of *M. tuberculosis*H37Ra.	This study
pPROEX HTa pPROEX HTb pPROEX HTc	Vectors with pBR322 origin of replication. The *trc* promoter and *lac*I^q gene for IPTG inducible overexpression of N-terminal 6xHistidine tagged proteins in *E. coli*. Selection marker, ampicillin resistance.	Life Technologies
pPROEX HT-CAT	pPROEX HTa containing the chloramphenicol acetyl transferase gene (CAT). Used as a positive control for induced expression.	Life Technologies
pPROEX HTc:8.4	pPROEX HTc with an insert coding for the mature 8.4 kDa Ag.	This study
pPROEX HTc:8.4-DS(B)	pPROEX HTc with an insert coding for the mature 8.4 kDa Ag and including the downstream intergenic sequence from *M. bovis*.	This study
pSU4511	*E. coli*- mycobacterial shuttle vector derived from pAL5000 and pACYC184. Multiple cloning site (MCS) from pSU40. Selection marker, kanamycin resistance.	Ainsa *et al.* 1996
pSUPSSHTb:8.4-DS(B)	pSU4511 with inserts for the *blaF** promoter from *M. fortuitum*, a signal sequence modified from the 8.4 kDa Ag gene, the 6xHis-MCS of pPROEX HTb, and the 8.4 kDa Ag including the downstream intergenic region from *M. bovis*.	This study

7.2 MATERIALS AND METHODS.

7.2.1 Plasmid propagation and extraction.

The plasmids were propagated in *E. coli* DH10B as described in Chapter 2. Liquid cultures were grown overnight in LB broth (20 ml) containing either ampicillin (100 µg / ml) or kanamycin (20 µg / ml) as appropriate. Plasmid DNA was extracted using either the BRESAspin Plasmid Mini Kit (Bresatec Pty Ltd, Thebarton, Australia) or the High Pure Plasmid Isolation Kit (Roche Diagnostics, Auckland, NZ) according to the manufacturers' double loading protocols, and eluted in dH_20 (50 µl / spin column) (see Tables 7.1 and 7.2).

Table 7.2 Bacteria used in this study.

Species	Genotype / Phenotype	Source / Reference
E. coli DH10B	F^- *mcr* A D(*mrr-hsd* RMS-*mcr* BC) φ80d*lac* ZDM15 D*lac* X74 *end* A1 *rec* A1 *deo* R D(*ara, leu*)7697 *ara* D139 *gal* U *gal* K *nup* G *rps* L l⁻.	Life Technologies
E. coli BL21	F^- *omp* T *hsd* S (r_B^-, m_B^-) *gal dcm.*	Amersham Pharmacia
M. smegmatis mc^2 155	high transformation efficiency strain	Snapper *et al.* 1990
M. fortuitum	ATCC 6841	ATCC, Rockville, USA
M. tuberculosis H37Ra	ATCC 25177	ATCC, Rockville, USA

7.2.2 Amplification of insert DNA by polymerase chain reaction.

The DNA inserts amplified by PCR with the primer pairs SM5f/SM5r, SM5f/SM12 and 14F/14R (see Table 7.3) were prepared in 12 x 0.2 ml thin walled PCR tubes (Life Technologies, Auckland, NZ). The reaction mixtures (50 µl) consisted of 1 x Pwo Amplification Buffer (Tris-HCl 10 mM, KCl 25 mM, $(NH_4)_2SO_4$ 50 mM, pH 8.85), dNTPs (0.2 mM each), primers (0.4 mM each), $MgSO_4$ (2 tubes of 1.5, 2.0, 2.5, 3.0 or 4.0 mM) and 2.4 units of Pwo DNA polymerase (Roche). Six tubes also contained 10% DMSO (Sigma-Aldrich, Sydney, Australia).

Table 7.3 Primers used in this study.

Primer	Sequence
	Eco RI
SM5f	5' CCGCG * AATTCAGATCCCGTGGACGCGGTCATTAACACC 3'
	Sal I
SM5r	5' AATG * TCGACTTAATAGTTGTTGCAGGAGCCGGC 3'
Used to amplify the coding region of the mature 8.4 kDa Ag from pUC18/4.3#2, with restriction enzyme recognition sites added at each end (* italics) for cloning into plasmids pGEX-6P-3 and pPROEX HTc.	
	Sal I
SM12	5' GTTAATG * TCGACGATGGAGGAGACCATCTCGCGGATGG 3'
Paired with SM5f used to amplify the coding sequence of the mature 8.4 kDa Ag and its downstream intergenic region from plasmid pUC18/4.3#2 or *M. tuberculosis* genomic DNA, with restriction enzyme recognition sites added at each end (* italics) for cloning into plasmids pGEX-6P-3 and pPROEX HTc.	
	Hind III
SM13F	5' TATGCTA * AGCTTGTTCCAACAGATTCGCGAGTCCCG 3'
	Sph I
SM13R	5' TGCGCGCATG * CTCAGCCTCATTGGACCCAG **T** GTAGCGGGACTGCCG 3'
Used to amplify the promoter of the β-lactamase gene from *M. fortuitum*, and introduce the *blaF** mutation **T**. Restriction enzyme recognition sites added at each end (* italics) for cloning into the MCS of plasmid pSU4511.	
	Sph I
SM14F	5' AGGCTGAGCATG * CGCGCATTGAGCGCCGGTGTAGGCG 3'
	Cla I
SM14R	5' TGATGAT * CGATGGGATCTGCGGAGGCGACCCCG 3'
Used to amplify the coding region of the signal sequence of the *M. tuberculosis* Rv1174c gene from pUC18/4.3#2, with restriction enzyme recognition sites added at each end (* italics) for cloning into the MCS of plasmid pSU4511.	
	Cla I
SM15F	5' ATCCCAT * CGATCATCACCATCACCATCACGATTACG 3'
	Kpn I
SM15R	5' CCAAAACAGCCAAGCTTGGTAC * CGCATGCC 3'
Used to amplify the coding sequence for the 6xHis tag, the spacer arm, Tobacco Etch Virus enzyme cleavage site and MCS from plasmid pPROEX HTb, with restriction enzyme recognition sites added at each end (* italics) for cloning into the MCS of plasmid pSU4511.	

Table 7.3 continued.

	Kpn I
SM16	5' GTTAAGGTAC * CGATGGAGGAGACCATCTCGCGGATGG 3'
	Xba I
SM17	5' TTGAAT * CTAGAGATCCCGTGGACGCGGTCATTAACACC 3'

Used to amplify the coding region of the mature 8.4 kDa Ag and its downstream intergenic region from plasmid pUC18/4.3#2, with restriction sites added at each end (* italics) for cloning into the pPROEX HTb MCS.

pGEX 3' Sequencing Primer	5' CCGGGAGCTGCATGTGTCAGAGG 3'	(Amersham Pharmacia)

The DNA inserts amplified with the primer pairs SM13F/SM13R, SM15F/SM15R, SM16/SM17 (see Table 7.3) were prepared in reaction mixtures (50 μl) that consisted of 1 x Expand High Fidelity PCR Amplification Buffer with $MgCl_2$ (Mg^{2+} 2.0 mM), dNTPs (0.2 mM each), primers (0.4 mM each) and 1 unit of Expand™ enzyme mix (Roche).

Template DNA was either plasmid pUC18/4.3#2 (see Table 7.1) or mycobacterial genomic DNA prepared as described in Chapter 4 (see Table 7.2). The reaction conditions were; 94°C for 5 minutes; 10 cycles of 94°C for 30 seconds, 65°C for 45 seconds, 72°C for 1 minute; 20 cycles of 94°C for 30 seconds, 65°C for 45 seconds, 72°C for 1 minute plus 20 seconds each cycle; followed by 72°C for 5 minutes. For amplification with the primer pair SM13F/SM13R the reaction conditions were; 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, 70°C for 1 minute; followed by 70°C for 5 minutes.

After cycling in a Perkin Elmer GeneAmp 9600 Thermocycler, the PCR mixtures were separated by agarose gel electrophoresis (1% TAE) and the amplified insert DNA was extracted using the Concert Rapid Gel Purification System (Life Technologies) according to the manufacturer's instructions.

7.2.3 Restriction endonuclease digestion of DNA.

Purified insert and vector DNA was either double digested by two enzymes simultaneously, or following incubation with one enzyme the reaction mixtures were dialysed against dH_2O and the second enzyme and buffer were added. After up to 16 hours of incubation at 37°C, the digested DNA was purified with the Concert Rapid PCR Purification System (Life Technologies).

7.2.4 Ligation of DNA and transformation of *E. coli.*

Insert and vector DNA was ligated by T4 DNA ligase (Roche) in a volume of 15 µl as described in Chapter 2. $CaCl_2$ competent *E. coli* DH10B and *E. coli* BL21 were transformed with plasmids according to standard methods (Sambrook *et al.* 1989). Aliquots of ligation reactions or purified plasmids (5 or 10 µl) were mixed with $CaCl_2$ competent *E. coli* (50 µl), incubated on ice for 30 minutes, heat shocked at 42°C for 90 seconds, and incubated on ice for a further 2 minutes before LB broth (1ml) was added. After at least one hour of incubation at 37°C, aliquots of the cell suspensions (100 to 200 µl) were spread on LB agar containing the appropriate antibiotic (ampicillin 100 µg / ml or kanamycin 20 µg / ml). The plates were incubated overnight at 37°C, and eight to ten individual transformant colonies were restreaked onto a fresh plate of LB agar containing the appropriate antibiotic.

7.2.5 Verification of cloning.

E. coli transformants containing insert DNA were identified by PCR. The reaction mixtures (20 µl) consisted of 1 x PCR Buffer (Tris-HCl 20 mM, KCl 50 mM; Life Technologies), $MgCl_2$ (2.0 mM), dNTPs (0.2 mM each), primers (0.4 mM each), and 1 unit of *Taq* DNA polymerase (Promega Corporation, Madison, USA). Reactions with the primer pairs SM5f/SM12 and SM16/SM17 also contained DMSO (10%). Template DNA was added as whole *E. coli* transferred directly from colonies with a pipette tip. The reaction conditions were; 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 65°C for 45 seconds, 72°C for 1 minute; followed by 72°C for 5 minutes. For PCR with the primer pair SM13F/SM13R the annealing and extension temperatures were 60°C and 70°C respectively. Aliquots (10 µl) of the PCRs were separated by agarose gel electrophoresis

(1% TAE), and the PCR products were stained with ethidium bromide and visualized by UV transillumination. The nucleotide sequences of expression constructs were determined by automated DNA sequencing as described in Chapter 2.

7.2.6 Expression of recombinant fusion proteins in *E. coli.*

Primary cultures of *E. coli* BL21 clones (see Table 7.2) that had been freshly transformed with the plasmids pGEX-6P-3, pGEX-6P-3:8.4, pGEX-6P-3:8.4-DS(B), pGEX-6P-3:8.4-DS(T), pPROEX HT-CAT, pPROEX HTc:8.4 or pPROEX HTc:8.4-DS(B) (see Table 7.1) were grown overnight in LB broth (5 ml) containing ampicillin (100 μg / ml) at 37°C with shaking at 150 r.p.m. Expression cultures were grown in LB broth (20 ml) containing ampicillin (100 μg / ml), inoculated with primary culture (0.2 ml), and incubated at 37°C with shaking at 150 r.p.m. After 2 hours an aliquot (1 ml) was removed and isopropylthio-β-galactoside (IPTG, Life Technologies) was added (0.2, 0.5, or 1.0 mM final concentration). Further sample aliquots (1 ml) were removed from expression cultures approximately 4 and 18 hours after induction with IPTG and centrifuged at 12,600 x g for 1 minute. The cell pellets were resuspended in 100 μl of pPRO Lysis Buffer (Tris-HCl 50 mM, 2-ME 5 mM) and aliquots (10 μl) were mixed with reducing 2 x sample loading buffer (2xSLB+2-ME). The samples were heated at 100°C for 10 minutes, centrifuged at 12,600 x g for 10 seconds, loaded onto 0.75 mm thick SDS-Tris-glycine polyacrylamide (12% or 15%) gels (Laemmli 1970) and separated by electrophoresis (SDS-PAGE) at 150 volts in a Mini-PROTEAN II Cell (Bio-Rad Laboratories Pty Ltd, Auckland, NZ). The separated proteins were visualized by staining with Coomassie brilliant blue R-250 (Sigma-Aldrich).

7.2.7 Determination of the solubility of the GST-8.4 kDa antigen fusion protein.

Expression cultures of *E. coli* BL21 clones transformed with the plasmids pGEX-6P-3 or pGEX-6P-3:8.4-DS(B) (see Table 7.1) were grown in LB broth (100 ml) and induced with IPTG (1.0 mM) as described. Sample aliquots (1 ml) were removed before induction and 5 hours later. The cultures were transferred to 2 x 50 ml conical tubes and centrifuged at 750 x g for 15 minutes. The supernatants were discarded and the pellets were frozen at

-20°C. Thawed pellets were resuspended in PBS (5 ml total volume) and the cells were sonicated on ice (6 x 30 seconds) at power level 1.8 using the fine tipped probe of a Sonicator XL (Misonex, USA). Aliquots of sonicate (1.5 ml) were centrifuged at 15,800 x g for 30 minutes at 4°C. The supernatants were removed and the pellets were resuspended in PBS (150 µl). Samples (15 µl) of suspended whole cells, resuspended sonicate pellet and sonicate supernatant were separated by Tris-glycine SDS-PAGE (12% polyacrylamide) in reducing conditions (2xSLB+2-ME) and stained with Coomassie brilliant blue R-250.

7.2.8 Transformation of *M. smegmatis.*

Aliquots of *M. smegmatis* mc^2 155 (100 µl) were mixed with aliquots (15 µl) of the plasmid pSUPSSHTb:8.4-DS(B) (see Tables 7.1 and 7.2) and electroporated as described in Chapter 2. After LB broth (500 µl) was added, the transformed cells were incubated at 37°C for at least 3 hours. Aliquots of cells (100 to 200 µl) were spread on LB agar containing kanamycin (20 µg / ml) and incubated at 37°C in aerobic conditions for 72 hours until colonies appeared.

7.2.9 Overexpression of the 8.4 kDa antigen by *M. smegmatis.*

Primary cultures of *M. smegmatis* clones containing the plasmid pSU4511 or pSUPSSHTb:8.4-DS(B) (see Table 7.1) were grown in 7H9 minimal medium (15 ml) supplemented with D (+) glucose (0.03 g) and kanamycin (20 µg / ml) at 37°C for 48 hours with shaking at 150 r.p.m. Expression cultures were grown in supplemented 7H9 minimal medium (900 ml) inoculated with primary culture (900 µl), and incubated at 37°C for 72 hours with shaking at 150 r.p.m. The cultures were centrifuged at 5000 x g for 15 minutes and the supernatants were filtered through 0.22 µm GP Express membranes and pre-filters using the Steritop vacuum filtration system (Millipore, Bedford, USA). Sodium azide (0.5% final concentraion) (NaN_3, BDH) and phenylmethylsulfonyl fluoride (100 µg / ml final concentration) (PMSF; Sigma-Aldrich) were added. The CFs were concentrated to approximately 10 ml in an Amicon ultrafiltration stir-cell apparatus fitted with a YM3 membrane (MWCO ≥ 3 kDa) (Amicon Inc., Beverly, USA).

7.2.10 Purification of the 6 x Histidine tagged 8.4 kDa antigen by metal chelate affinity chromatography.

The concentrated CFs (10 ml) derived from *M. smegmatis* pSUPSSHTb:8.4-DS(B) were exchanged 1/250 into Phosphate Start Buffer (PO_4SB; Na_2HPO_4 20 mM, $NaCl_2$ 500 mM, PMSF 100 µg / ml, pH 8.4) by three dilution/concentration cycles in an Amicon ultrafiltration apparatus fitted with a YM3 membrane (MWCO ≥ 3 kDa). Metal chelate affinity columns (Hi-trap Chelating 1 ml; Amersham Pharmacia Biotech) were washed with dH_2O (5 ml), charged with 2 ml of $NiCl_2 \bullet 6H_2O$ 200 mM (Sigma-Aldrich), washed with dH_2O (5 ml) and equilibrated with PO_4SB (5 ml). Concentrated CF (10 ml) was centrifuged at 5000 x g for 2 minutes and an aliquot (5 ml) was loaded onto the charged metal chelate affinity columns at a flow rate of 1 ml / minute. The unbound proteins were eluted in 2.5 ml of Phosphate Wash Buffer (PO_4WB; Na_2HPO_4 20 mM, $NaCl_2$ 500 mM, pH 7.0), and the bound proteins were eluted in successive 2.5 ml fractions of PO_4WB containing 0.1, 0.2, 0.3, 0.4, 0.5 or 1.0 M imidazole (Sigma-Aldrich). Samples (10 µl) of concentrated CF, the loading flow through and the eluate fractions were separated by SDS-PAGE through Tris-tricine gels (0.75 mm thick) in reducing conditions (2xSLB+DTT). Gels were stained with Coomassie brilliant blue R-250. The separated proteins were transferred onto BioTrace™ PVDF membranes (Pall Corporation, Ann Arbor, USA) by the semi-dry method. The blots were probed with rabbit anti-8.4 kDa Ag polyclonal serum (1/1000), goat anti-rabbit IgG biotin conjugate (1/5000), streptavidin-POD (1/5000), and developed with DAB as described in Chapter 3.

7.3 RESULTS.

7.3.1 Construction of plasmids for overexpression of recombinant 8.4 kDa antigen in *E. coli.*

A DNA insert coding for the mature 8.4 kDa Ag of *M. bovis* was amplified by PCR from the 4.3 kb insert of *M. bovis* DNA in plasmid pUC18/4.3#2 with the primer pair SM5f/SM5r (see Table 7.3). DNA inserts that consisted of the coding sequence of the 8.4 kDa Ag plus the downstream intergenic regions that contained the 62 bp ETR loci of *M. bovis* (1.7 copies of 62 bp ETR) and *M. tuberculosis* (2.7 copies of 62 bp ETR) were amplified from plasmid pUC18/4.3#2 and *M. tuberculosis* H37Ra genomic DNA respectively using the primer pair SM5f/SM12 (see Tables 7.1 and 7.3).

The DNA inserts were ligated into the *Eco* RI and *Sal* I sites of the plasmids pGEX-6P-3 and pPROEX HTc to produce the plasmids pGEX-6P-3:8.4, pGEX-6P-3:8.4-DS(B) pGEX-6P-3:8.4-DS(T), pPROEX HTc:8.4 and pPROEX HTc:8.4-DS(B) (see Table 7.1). The nucleotide sequence of the expression construct in the plasmid pGEX-6P-3:8.4-DS(B) was determined by automated dideoxy chain-termination DNA sequencing initiated with the pGEX 3' sequencing primer (see Table 7.3). The coding sequence of the 8.4 kDa Ag was found to be in frame with the sequence coding for glutathione-S-transferase (GST) (see Appendix VIa).

7.3.2 Overexpression of recombinant 8.4 kDa antigen in *E. coli.*

Parallel expression cultures (20 ml) of *E. coli* BL21 transformed with either the plasmid pGEX-6P-3:8.4 that contained the coding sequence of the 8.4 kDa Ag or the plasmid pGEX-6P-3:8.4-DS(B) that contained the coding sequence of the 8.4 kDa Ag plus 243 bp of downstream sequence from *M. bovis* were induced by IPTG (1.0 mM) to overexpress a protein (see Figure 7.1). The apparent molecular weight of the overexpressed protein was consistent with it being the 8.4 kDa Ag fused to GST (26 kDa). The *E. coli* BL21 clones transformed with the plasmids pPROEX HT:8.4 and pPROEX HTc:8.4-DS(B) were not induced to overexpress any proteins (not shown).

7.3.3 Comparison of GST-8.4 kDa protein expression from *M. bovis* and *M. tuberculosis* sequences.

Parallel expression cultures (100 ml) of *E. coli* BL21 freshly transformed with either the plasmid pGEX-6P-3:8.4-DS(B) that contained the 8.4 kDa Ag coding sequence plus 243 bp of downstream intergenic sequence from *M. bovis*, or plasmid pGEX-6P-3:8.4-DS(T) that contained the 8.4 kDa Ag coding sequence plus 305 bp of downstream intergenic sequence from *M. tuberculosis* were induced with IPTG (1.0 mM) to overexpress a protein with an apparent molecular weight of 34 kDa (see Figure 7.2). There was no apparent difference between the *M. bovis* or *M. tuberculosis* sequences with respect to the amount of the GST-8.4 kDa fusion protein expressed in *E. coli* BL21.

7.3.4 Solubility of GST-8.4 kDa protein expressed in *E. coli* BL21.

To determine the solubility of the recombinant GST-8.4 kDa Ag fusion protein expression cultures (100 ml) of *E. coli* BL21 transformed with the plasmid pGEX-6P-

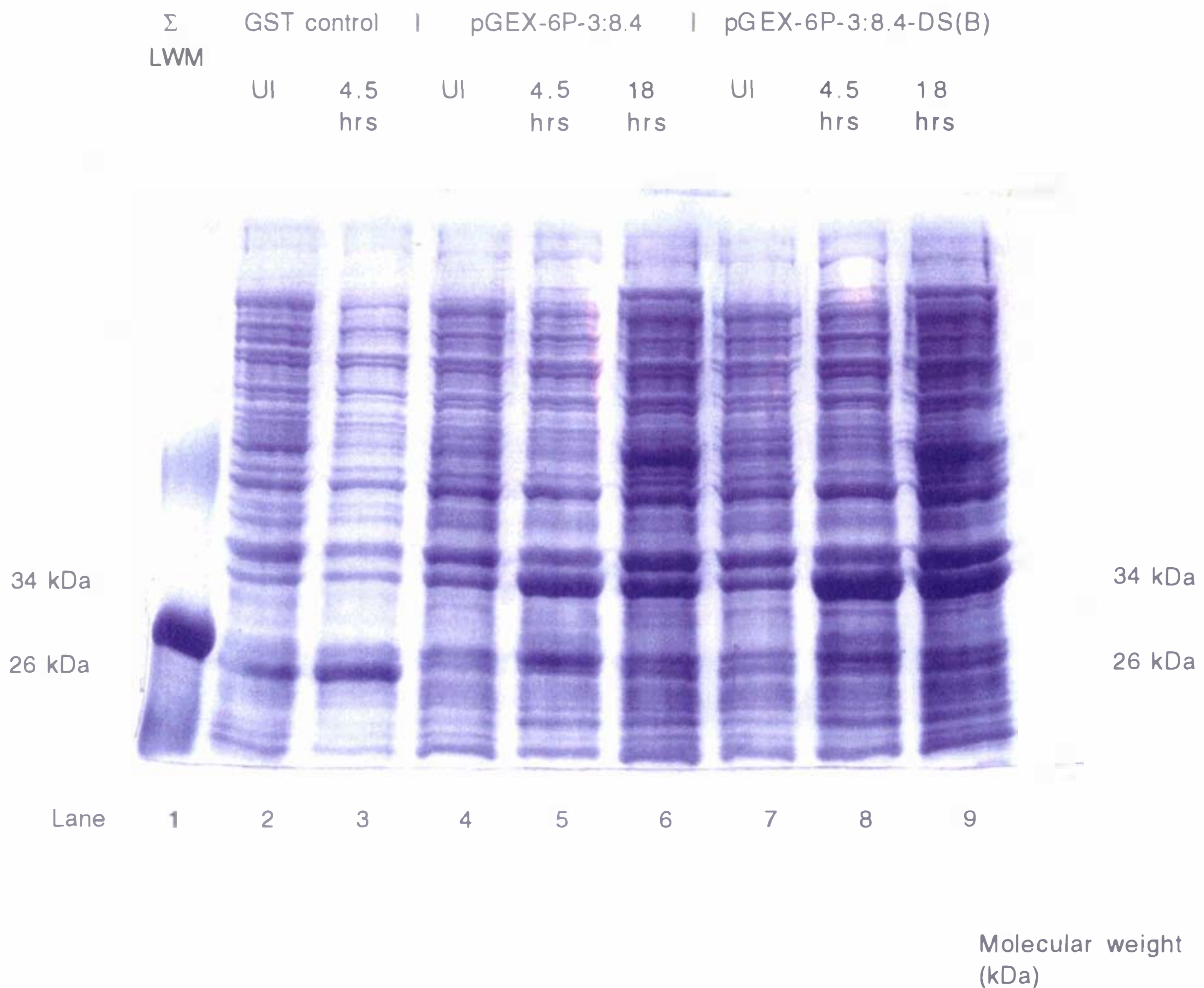

Figure 7.1 SDS-PAGE gel showing overexpression of a GST-8.4 kDa Ag fusion protein. *E. coli* BL21 were transformed with the control plasmid pGEX-6P-3, plasmid pGEX-6P-3:8.4 with insert coding for the mature 8.4 kDa Ag, and plasmid pGEX-6P-3:8.4-DS(B) with insert coding for the mature 8.4 kDa Ag and including the downstream intergenic region containing the 62 bp ETR locus of *M. bovis*. Expression was induced with IPTG (1.0 mM). Aliquots (10 μl) of whole cells removed prior to induction, 4.5 and 18 hours post-induction were separated in reducing conditions through a 12% polyacrylamide 0.75 mm thick Tris-glycine gel and stained with Coomassie brilliant blue R-250. The GST expressed in the *E. coli* clone containing the control plasmid pGEX-6P-3 is shown as a 26 kDa band in samples taken from cultures that were uninduced (lane 2) and 4.5 hours post-induction (lane 3). The GST-8.4 kDa Ag fusion protein expressed from pGEX-6P-3:8.4 is shown as a 34 kDa band in samples taken from cultures that were uninduced (lane 4), 4.5 hours post induction (lane 5) and 18 hours post-induction (lane 6). The 34 kDa GST-8.4 kDa Ag fusion protein expressed from pGEX-6P-3:8.4-DS(B) is shown in samples taken from cultures that were uninduced (lane 7), 4.5 hours post-induction (lane 8) and 18 hours post-induction (lane 9). Lane 1 contains a low molecular weight marker (Σ LWM) (Sigma-Aldrich).

Σ LWM | pGEX-6P-3:8.4-DS(B) | pGEX-6P-3:8.4-DS(T) | pGEX-6P-3:8.4-DS(B) | pGEX-6P-3:8.4-DS(T) | Σ LWM

uninduced

5 hours post-induction

34 kDa

Lane 1 2 3 4 5 6 7

Molecular weight (kDa)

Figure 7.2 SDS-PAGE gel showing expression of a GST-8.4 kDa Ag fusion protein from *M. bovis* and *M. tuberculosis* sequences. *E. coli* BL21 was transformed with the plasmid pGEX-6P-3:8.4-DS(B) containing an insert coding for the 8.4 kDa Ag and including the 62 bp ETR locus from *M. bovis* (1.7 copies of the 62 bp repeat) or plasmid pGEX-6P-3:8.4-DS(T) containing an insert coding for the 8.4 kDa Ag and including the 62 bp ETR locus from *M. tuberculosis* (2.7 copies of 62 bp repeat). Expression was induced with IPTG (1.0 mM). Aliquots (10 μl) of whole cells removed prior to and five hours post-induction were separated in reducing conditions through a 15% polyacrylamide Tris-glycine gel and stained with Coomassie brilliant blue R-250. Samples taken from uninduced cultures are shown in lanes 2 and 3. Samples taken five hours post-induction are shown in lanes 5 and 6. A band of overexpressed GST-8.4 kDa fusion protein with an apparent molecular weight of 34 kDa is shown in samples taken from cultures of pGEX-6P-3:8.4-DS(B) (lane 5) and pGEX-6P-3:8.4-DS(T) (lane 6). A low molecular weight marker (Sigma-Aldrich) is shown in lanes 1 and 7 (Σ LWM).

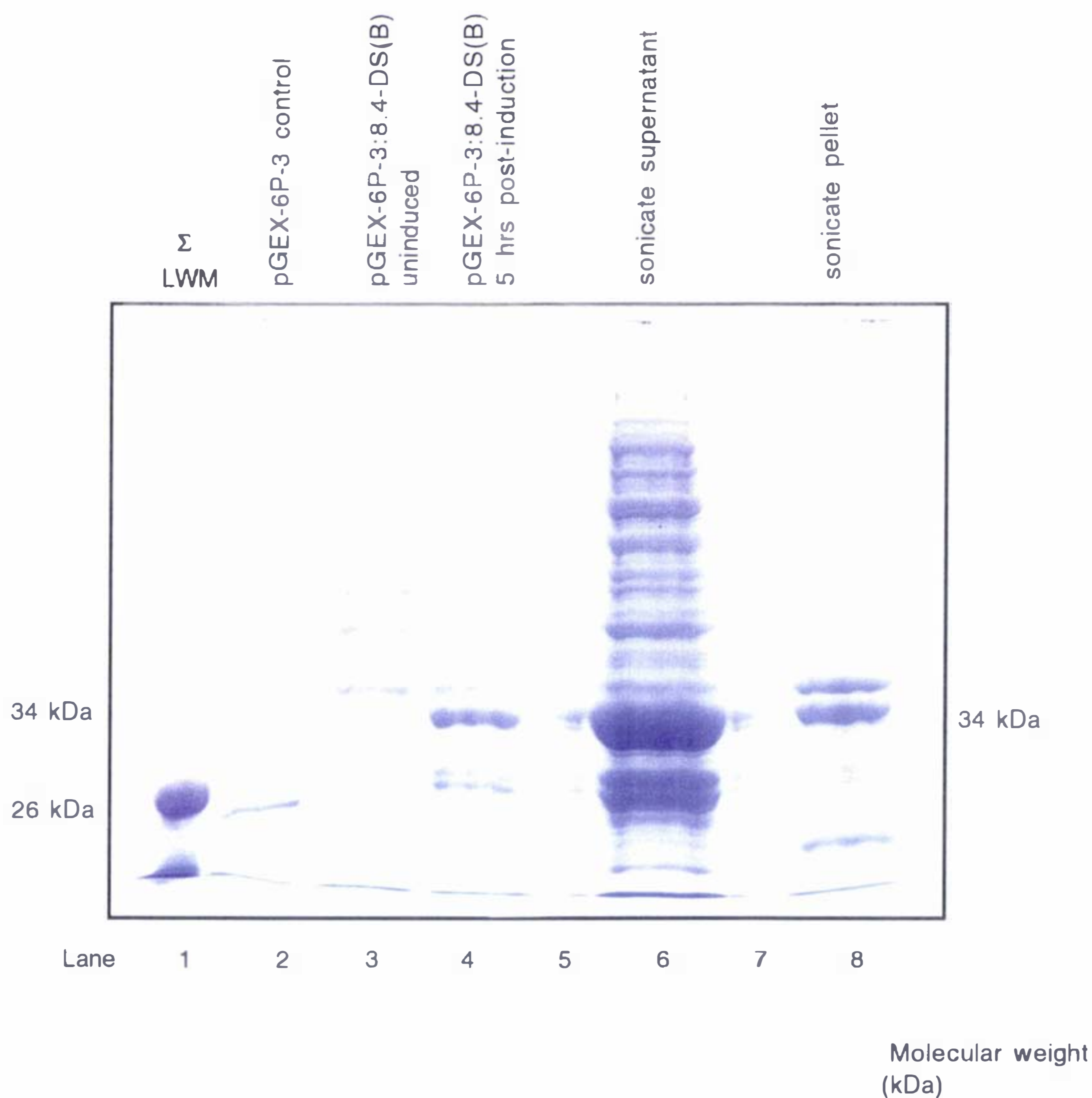

Figure 7.3 SDS-PAGE gel showing the soluble GST-8.4 kDa Ag fusion protein expressed in *E. coli* BL21 transformed with plasmid pGEX-6P-3;8.4-DS(B). Samples were removed from cultures of freshly transformed *E. coli* BL21 prior to and 5 hours after induction of expression with IPTG (1mM). The cells were pelleted, sonicated and centrifuged. Aliquots of the samples of culture, sonicate supernatant, and sonicate pellet were separated by 12% SDS-PAGE and stained with Coomassie. The 26 kDa GST expressed from the control plasmid pGEX-6P-3 is shown in the sample of culture taken 5 hours post-induction (lane 2). The GST-8.4 kDa fusion protein expressed from pGEX-6P-3:8.4-DS(B) is shown as a 34 kDa band in samples taken from cultures that were uninduced (lane 3) and 5 hours post-induction (lane 4). The soluble GST-8.4 kDa Ag fusion protein is shown in samples of sonicate supernatant (lane 6) and unwashed sonicate pellet (lane 8). Lane 1 contains a low molecular weight marker (Σ LWM) (Sigma-Aldrich)

3:8.4-DS(B) were grown, pelleted, sonicated and the cellular fractions were separated by SDS-PAGE as described (see Figure 7.3). The majority of the overexpressed GST-8.4 kDa Ag fusion protein was observed to be in the sonicate supernatant, which indicates that the overexpressed GST-8.4 kDa fusion protein was soluble. The recombinant 8.4 kDa Ag could not be enzymatically cleaved from the GST. Therefore, purification of recombinant 8.4 kDa Ag from *E. coli* was was not pursued.

7.3.5 Construction of the plasmid pSUPSSHTb:8.4-DS(B) for over-expression and secretion of the 8.4 kDa Ag by *M. smegmatis.*

The plasmid pSUPSSHTb:8.4-DS(B) was constructed in the mycobacteria/*E. coli* shuttle vector pSU4511. The expression construct consisted of the mutant strong promoter *blaF** from *M. fortuitum* (Timm *et al.* 1994), a sequence coding for a modified 8.4 kDa antigen signal peptide, the 6 x Histidine tag coding sequence and the multiple cloning site of plasmid pPROEX HTb, and the coding sequence of the mature 8.4 kDa Ag plus the downstream intergenic region of *M. bovis* (see Appendix VIb).

The nucleotide sequence of the expression construct in the plasmid pSUPSSHTb:8.4-DS(B) was determined by automated DNA sequencing initiated using the primer SM16 (see Figure 7.4). The expression construct inserted into the mycobacteria/*E. coli* shuttle vector pSU4511 to create the plasmid pSUPSSHTb:8.4-DS(B) contains the strong mycobacterial promoter *blaF** in frame with sequence coding for a protein consisting of an export signal sequence, a 6 x Histidine tag and the mature 8.4 kDa Ag of *M. bovis* (see Figure 7.4).

7.3.6 Overexpression and secretion of recombinant 8.4 kDa antigen with a 6 x Histidine tag by *M. smegmatis.*

The plasmid pSUPSSHTb:8.4-DS(B) was extracted from *E. coli* DH10B, and *M. smegmatis* mc^2 155 was transformed with pSUPSSHTb:8.4-DS(B) by electroporation as described. Expression cultures (900 ml) of the *M. smegmatis* clone containing the plasmid pSUPSSHTb:8.4-DS(B) were grown for 72 hours and the CFs were harvested, concentrated and exchanged into PO_4SB. Half the volume of the concentrated CFs were separated by metal chelate affinity chromatography. Samples of the CFs, and affinity column eluates were separated by Tris-tricine SDS-PAGE, transferred onto PVDF membranes, and the recombinant 8.4 kDa Ag was detected on Western blots with rabbit

```
                                                  Modified 8.4 kDa Ag signal peptide.
                                                   M   R   L   S   M   R   A
    C GGC TGA CCC GCT ACA CTG GGT CCA ATG AGG CTG AGC ATG | CGC GCA
(segment of blaF* promoter from M. fortuitum)                    Sph I

 L   S   A   G   V   G   A   V   A   M   S   L   T   V   G   A   G   V
TTG AGC GCC GGT GTA GGC GCC GTG GCA ATG TCG TTG ACC GTC GGG GCC GGG GTC
          (sequence coding for a modified 8.4 kDa antigen signal peptide)

                                   pPROEX HTb 6 x Histidine Tag, and spacer.
 A   S   A ## D   P   I   D   H   H   H   H   H   H   D   Y   D   I
GCC TCC GCA  GAT CCC AT|C GAT CAT CAC CAT CAC CAT CAC GAT TAC GAT ATC
                     Cla I

 P   T   T   E   N   L   Y   F   Q  **  G   A   M   G   S   G   I   E
CCA ACG ACC GAA AAC CTG TAT TTT CAG ** GGC GCC ATG GGA TCC GGA ATT CAA

 R   P   T   S   T   S   S   L   V   A   A   A   F   E   S   R   D
AGG CCT ACG TCG ACG AGC TCA CTA GTC GCG GCC GCT TTC GAA T | CT AGA GAT
                 (pPROEX HTb multiple cloning site)          Xba I

                 Mature 8.4 kDa Ag protein.
 P   V   D   A   V   I   N   T   T   C   N   Y   G   Q   V   V   A   A
CCC GTG GAC GCG GTC ATT AAC ACC ACC TGC AAT TAC GGG CAG GTA GTA GCT GCG

 L   N   A   T   D   P   G   A   A   A   Q   F   N   A   S   P   V   A
CTC AAC GCG ACG GAT CCG GGG GCT GCC GCA CAG TTC AAC GCC TCA CCG GTG GCG

 Q   S   Y   L   R   N   F   L   A   A   P   P   P   Q   R   A   A   M
CAG TCC TAT TTG CGC AAT TTC CTC GCC GCA CCG CCA CCT CAG CGC GCT GCC ATG

 A   A   Q   L   Q   A   V   P   G   A   A   Q   Y   I   G   L   V   E
GCC GCG CAA TTG CAA GCT GTG CCG GGG GCG GCA CAG TAC ATC GGC CTT GTC GAG

 S   V   A   G   S   C   N   N   Y  OCHRE
TCG GTT GCC GGC TCC TGC AAC AAC TAT TAA GCCATGCGGGCCCCATCCCGCGACCCG
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Figure 7.4 Nucleotide sequence of the expression construct in plasmid pSUPSSHTb:8.4-DS(B), and the translated amino acid sequence of the overexpressed nascent protein. The nucleotide sequence of the expression construct was determined by automated dideoxy chain-termination DNA sequencing, initiated with the primer SM16. The DNA sequence (IUPAC code) is shown by triplet codon below the deduced amino acid sequence. The ATG start codon and TAA stop codon are shown in boldface italics. The origins of the segments of DNA sequence are annotated below the nucleotide sequence (brackets), and the component regions of the nascent protein are labelled above the amino acid sequence (underlined). The restriction endonucleases used are shown (italics) below the corresponding endonuclease cleavage sites (vertical lines between nucleotides). The signal peptidase cleavage site (##) and the Tobacco Etch Virus enzyme cleavage site (**) are indicated between the appropriate amino acids.

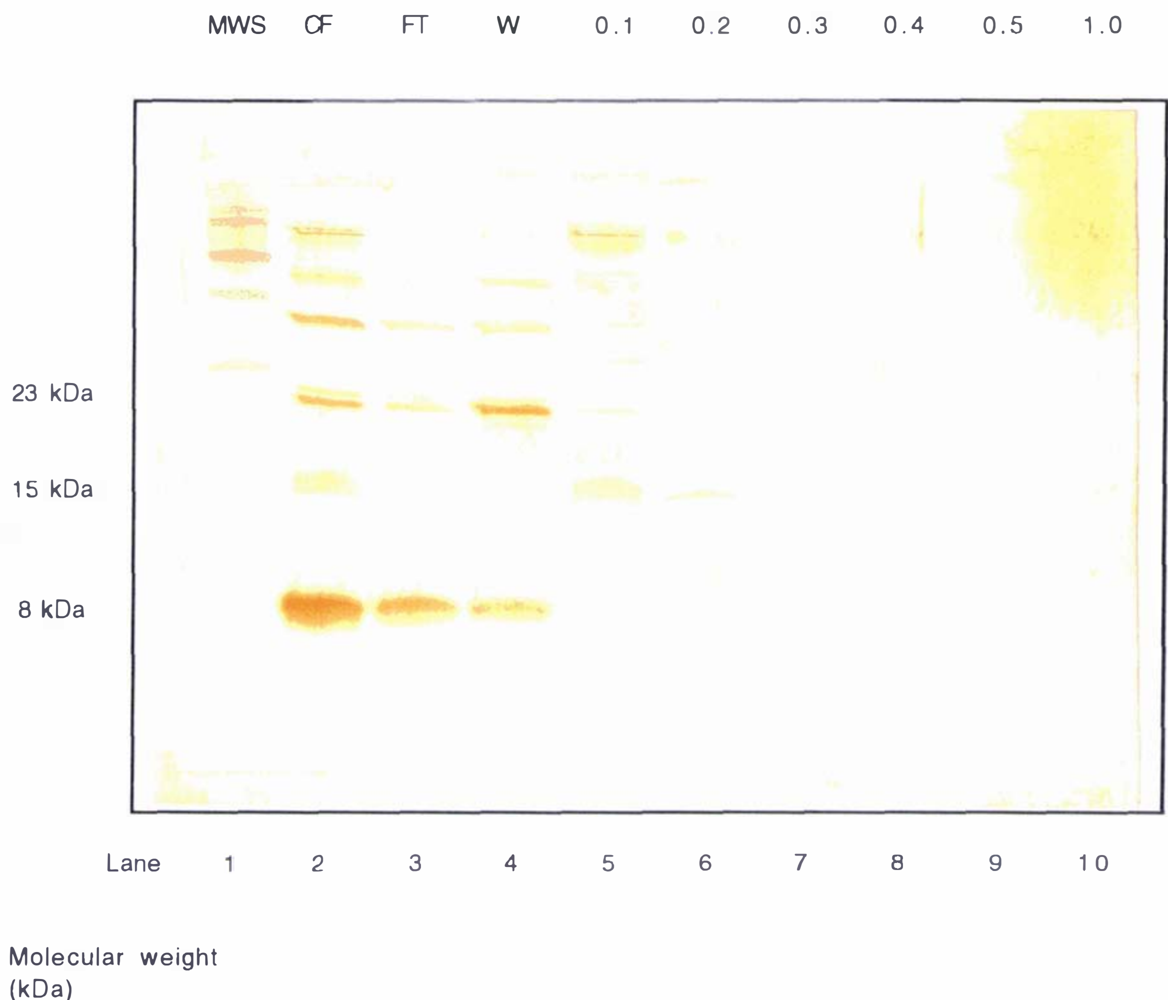

Figure 7.5 Western blot of recombinant 8.4 kDa Ag overexpressed and secreted by *M. smegmatis*. A culture of *M. smegmatis* containing the plasmid pSUPSSHTb:8.4-DS(B) was grown for 72 hours. The CF was harvested and separated by metal chelate affinity chromatography. Aliquots of CF (lane 2) and column eluate fractions (lanes 3 to 10) were separated by Tris-tricine SDS-PAGE and transferred onto PVDF membrane. Recombinant 8.4 kDa Ag was detected by rabbit anti-8.4 kDa polyclonal sera (1/1000), goat anti-rabbit biotin conjugate (1/5000), Streptavidin-POD (1/5000) and DAB. The apparent molecular weight was estimated by reference to the electrophoretic mobility curve. Recombinant 8.4 kDa Ag with an N-terminal 6 x His tag is shown as a 15 kDa doublet in fractions corresponding to the CF (lane 2), and 0.1 M imidazole (lane 5). The 8.4 kDa Ag without a 6 x His tag is shown as an 8 kDa band in the CF (lane 2), column flow through (lane 3) and column wash (lane 4). Purified 6 x His tagged 8.4 kDa Ag is shown as a 15 kDa band in the 0.2 M imidazole eluate fraction (lane 6). Precision prestained molecular weight standard (Bio-Rad) is shown in lane 1 (MWS). The bands at 23 kDa and greater were also detected by sera from unvaccinated rabbits (not shown).

anti-8.4 kDa Ag polyclonal serum, goat anti-rabbit IgG biotin conjugate, streptavidin-POD and DAB as described (see Figure 7.5).

The recombinant 8.4 kDa Ag present in the CF was detected as a doublet with an apparent molecular weight of 15 kDa and as another band at 8 kDa. The bands correspond to 6 x Histidine tagged 8.4 kDa Ag and 8.4 kDa Ag without a 6 x Histidine tag respectively. The column flow through and wash fractions contained only the 8 kDa Ag which is consistent with elution of untagged protein that was not bound by the column. The fraction that eluted in 0.1 M imidazole contained the 15 kDa doublet, consistent with elution of bound 6 x Histidine tagged 8.4 kDa protein. The eluate fractions described also contained other high molecular weight proteins, that were also observed on Western blots of CF probed with sera from non-vaccinated rabbits (not shown). A single 15 kDa protein was detected in the fraction eluted in 0.2 M imidazole. The N-terminal amino acid sequence of the protein was determined to be DPQDHHH. Six out of seven residues were consistent with the predicted sequence of N-terminally 6 x Histidine tagged 8.4 kDa Ag. The protein concentration of the 0.2 M imidazole eluate (2.5 ml) was estimated to be approximately 0.1 mg / ml, and equates to purification of approximately 0.5 mg 6 x Histidine tagged 8.4 kDa antigen / litre of expression culture.

7.3.7 Stimulatory activity of the recombinant 8.4 kDa antigen in IFN-γ assays.

Whole blood IFN-γ assays were performed as described (Chapter 3). Aliquots of whole blood from a BCG vaccinated steer (No. 5) and two control animals (Nos. 53 and 55) were incubated with PPD-B (20 μg / ml blood), PPD-A (20 μg / ml blood), concentrated CF from a culture of the *M. smegmatis* clone containing the plasmid pSUPSSHTb:8.4-DS(B) (20 μg protein / ml blood), and 6 x Histidine tagged 8.4 kDa Ag that had been purified by metal chelate affinity chromatography and exchanged into PBS (5 μg protein / ml blood).

The vaccinated animal No. 5 had positive responses to PPD-B (Δ OD_{450} 2052), PPD-A (Δ OD_{450} 1017), unfractionated CF (Δ OD_{450} 1112), 6 x Histidine tagged 8.4 kDa Ag (Δ OD_{450} 433) and ConA (Δ OD_{450} 2483). The responses of control animal No. 53 were

negative to all the mycobacterial antigen preparations, but positive to ConA (Δ OD_{450} 1931). Control animal No. 55 which was known to be sensitized to PPD-A had positive IFN-γ responses to PPD-B (Δ OD_{450} 340), PPD-A (Δ OD_{450} 151) and ConA (Δ OD_{450} 2439), but negative responses to unfractionated CF (Δ OD_{450} 77) and 6 x Histidine tagged 8.4 kDa Ag (Δ OD_{450} 21) (see Table 7.4).

Table 7.4 IFN-γ assay responses.

Anitgen	BCG vaccinated animal No. 5	Control animal No. 53	Control animal No. 55
PPD-B	+	—	+
PPD-A	+	—	+
CF	+	—	—
6 x His tagged 8.4 kDa Ag	+	—	—
ConA	+	+	+

Whole blood IFN-γ assay reponses positive (+) and negative (—) of a BCG vaccinated steer (No. 5) and two control cows (Nos. 53 and 55) to stimulation with PPD-B (20 μg / ml), PPD-A (20 μg / ml), CF derived from *M. smegmatis* containing the plasmid pSUPSSHTb:8.4-DS(B) (20 μg / ml), recombinant 6 x Histidine tagged 8.4 kDa Ag of *M. bovis* expressed and secreted by *M. smegmatis* (5 μg / ml), or ConA (20 μg / ml) are shown. Positive responses were defined as Δ OD_{450} x 1000 ≥ 100.

7.4 DISCUSSION.

Recently, it has been suggested that diagnostic tests for tuberculosis in cattle would have greater specificity if PPD tuberculin were replaced by preparations of *M. bovis* specific antigens (Lyashchenko *et al.* 1998a, Buddle *et al.* 1999, Vordermeier *et al.* 1999, Pollock *et al.* 2000, van Pinxteren *et al.* 2000). However, it is difficult to produce sufficient

quantities of antigens in a form suitable for use in immunological assays (Hewinson and Russell 1993, Colangeli *et al.* 1998).

This study demonstrated expression of the 8.4 kDa antigen of *M. bovis* as a GST fusion protein in *E. coli* from expression constructs with and without the downstream intergenic region that contains the 62 bp ETR locus. The downstream sequence of *M. bovis* contains 1.7 copies of the 62 bp exact tandem repeat unit and *M. tuberculosis* contains 2.7 copies. There was no apparent difference in the amounts GST-8.4 kDa fusion protein expressed from the *M. bovis* and *M. tuberculosis* sequences by *E. coli.* Attempts to purify recombinant 8.4 kDa Ag expressed in *E. coli* were unsuccessful, consequently overexpression of the 8.4 kDa Ag by *M. smegmatis* was investigated.

A unique expression system was constructed in the mycobacteria/*E. coli* shuttle vector pSU4511. The strong mutated promoter of the β-lactamase gene from *M. fortuitum* (*blaF**) was cloned in frame with a sequence coding for a modified 8.4 kDa antigen signal sequence. The modifications to the signal sequence were necessary to accomodate restriction endonuclease recognition sites used to construct the expression plasmid. Immediately downstream was sequence coding for a 6 x Histidine tag and spacer arm, followed by the multiple cloning site of plasmid pPROEX HTb. The plasmid into which the coding sequence of the mature 8.4 kDa antigen plus the downstream intergenic region of *M. bovis* was inserted contained five unique restriction sites in the multiple cloning site (*Bam* HI, *Spe* I, *Not* I, *Xba* I, *Kpn* I). The intention was that the signal peptide of the nascent protein would direct export of overexpressed N-terminally 6 x Histidine tagged 8.4 kDa antigen into the CF. Unpurified concentrated CF could then be used directly in cellular immunoassays, or if desired the 6 x Histidine tagged antigen could be purified in one step by metal chelate affinity chromatography.

Cultures of *M. smegmatis* transformed with the plasmid pSUPSSHTb:8.4-DS(B) constitutively expressed and secreted approximately 2 mg of recombinant 8.4 kDa antigen / litre of CF. Although, the signal sequence appeared to direct export of the overexpressed N-terminally 6 x Histidine tagged 8.4 kDa antigen, the 6 x Histidine tag was missing from approximately 80% of the antigen detected in the CF by Western blotting. The apparent molecular weight of the untagged protein (8 kDa) was not consistent with cleavage at the Tobacco Etch Virus cleavage site present within the spacer arm between the 6 x Histidine tag and the 8.4 kDa Ag. Thus, it appears that the 6 x Histidine tag detached from the

protein near the beginning of the mature 8.4 kDa antigen. Nevertheless, approximately 500 μg of 6 x Histidine tagged 8.4 kDa antigen was purified / litre of culture in a single step by metal chelate affinity chromatography. This level of recovery compares favourably with the 25 μg of 8.4 kDa antigen purified / litre of CF from cultures of the *M. smegmatis* subclone pSU151.43 after two steps of chromatography.

This study is the first report of overexpression and secretion of a recombinant *M. bovis* antigen with an amino terminal 6 x Histidine tag by *M. smegmatis.* Furthermore, both the recombinant 8.4 kDa antigen in unpurified *M. smegmatis* CF and purified 8.4 kDa antigen with an N-terminal 6 x Histidine tag were detected by specific anti-8.4 kDa Ag polyclonal sera and specifically elicited *in vitro* IFN-γ production in whole blood assays.

Conclusions and future directions.

In New Zealand cattle continue to become infected with *M. bovis* transmitted from feral reservoir hosts, primarily possums and ferrets (Cooke 2000). The national prevalence of tuberculosis in cattle is currently estimated to be in the order of 2%, but approximately one third of the total land area of New Zealand is as designated vector risk areas (VRA) (AHB 2000).

Consequent to annual intradermal testing of breeding herds and slaughter of reactors clinical tuberculosis is rare, and since the early 1990s cattle have not been considered to be an epidemiologically significant source of human *M. bovis* infection (Brett and Humble 1991, Crews 1991). Nevertheless, bovine tuberculosis remains of concern to the agricultural industries. The perceived threat is that New Zealand's beef and dairy products could be excluded from major export markets on phytosanitary grounds, or that market share could be lost due to negative consumer perceptions (AHB 2000).

In the early 1990s, as progressively more of the country became designated VRAs, the task of controlling bovine tuberculosis with the techniques available appeared to be insurmoutable. A 1992 report to the Minister of Research Science and Technology recommended coodinated research into alternative control measures, including vaccination of cattle (Allison 1992).

At Massey University a novel approach was employed to identify protein antigens of *M. bovis* that could be potential components of a sub-unit vaccine to protect cattle from tuberculosis. A cosmid library of the *M. bovis* genome had been constructed (Wilson *et al.* 1995), and was transformed into *M. smegmatis* (Carpenter *et al.* 1995). The culture filtrates (CF) from 356 cosmid clones were screened for stimulatory activity in lymphocyte proliferation and IFN-γ assays using PBMC from cattle that had been vaccinated with a low dose of *M. bovis* BCG. The CFs derived from 24 of the cosmid clones that were found to be stimulatory did not contain the genes for known antigens. The CF from one (Cos151) stimulated PBMC from seven out of eight BCG vaccinated animals to proliferate and produce IFN-γ (Gormley *et al.* 1999).

Subsequently, the *M. bovis* DNA insert from Cos151 was subcloned and the CF from a subclone containing a 4.3 kb insert of *M. bovis* DNA was also found to stimulate PBMC from BCG vaccinated cattle to proliferate and produce IFN-γ. This study investigated the nature of the immunoreactive component of CF derived from the *M. smegmatis* subclone containing the plasmid pSU151.43.

The nucleotide sequence of the 4.3 kb insert of *M. bovis* DNA was determined and potential open reading frames were identified. An 8.4 kDa protein that stimulated PBMC from a steer vaccinated with *M. bovis* BCG to proliferate and produce IFN-γ was purified from CF derived from the *M. smegmatis* clone pSU151.43. The N-terminal amino acid sequence and molecular mass of the purified 8.4 kDa protein corresponded with the deduced amino acid sequence of a predicted gene present within the 4.3 kb fragment of *M. bovis* DNA.

Polyclonal anti-8.4 kDa antisera were raised in rabbits and used to detect the recombinant 8.4 kDa antigen in the Western blot format. The anti-8.4 kDa antibodies could also be used for immunohistological studies to investigate expression of the 8.4 kDa Ag by intracellular *M. bovis* in both *in vitro* macrophage cultures and tissue sections from tuberculous lesions. However, the antisera were also found to contain antibodies that bound to a 23 kDa protein present in CFs derived from *M. smegmatis* clones containing plasmid without an insert. Therefore, the anti-8.4 kDa antibodies would need to be purified from the polyclonal antisera, but purification by affinity chromatography would require sufficient quantities of pure antigen.

The immune responses to the 8.4 kDa Ag of BCG vaccinated and unvaccinated cattle experimentally infected with virulent *M. bovis* were investigated. There was evidence that BCG vaccination had elicited an immune response to the 8.4 kDa antigen, but that *M. bovis* infection did not. The animals that had been vaccinated with BCG had significantly less severe tuberculous lesions at 17 weeks post-challenge. It is tempting to speculate that one mechanism by which BCG vaccination may exert its protective effect is by eliciting immune responses against some *M. bovis* antigens that animals fail to responded to following respiratory infection with *M. bovis.*

Whilst the final drafts of this thesis were in preparation, an article entitled 'Vaccination with the T Cell Antigen Mtb 8.4 Protects Against Challenge with *Mycobacterium*

tuberculosis.' was published by Coler *et al.* in May 2001. The authors wrote that their previous studies had identified antigens present in *M. tuberculosis* CF that elicited proliferation and IFN-γ production from the PBMC of healthy PPD positive human donors (Coler *et al.* 1998, Skeiky *et al.* 1999). Subsequently, an unspecified number of antigens were tested in animal models, for their ability to stimulate protective immune responses to *M. tuberculosis* infection. The article reports that immunization with either the 8.4 kDa Ag of *M. tuberculosis* (Mtb 8.4) formulated in Incomplete Freund's Adjuvant (IFA), or immunization with plasmid DNA encoding Mtb 8.4 induced mice to mount strong Mtb 8.4 specific Th 1 and CTL responses. In addition, C57BL/6 mice vaccinated with Mtb 8.4 or with *M. bovis* BCG had similarly reduced bacterial counts following subsequent intravenous challenge with *M. tuberculosis.*

It was also reported that Mtb 8.4 specific CTL from vaccinated or *M. tuberculosis* infected mice produced IFN-γ when stimulated *in vitro* with macrophages infected with *M. tuberculosis*, indicating that Mtb 8.4 is processed by infected APC and presented by MHC class I to CTL. The authors suggested that IFN-γ production by Mtb 8.4 specific CTL is a component of the Type 1 immune responses that are required to activate the bacteriostatic mechanisms of macrophages.

Interestingly, it was found that Mtb 8.4 specific CTL isolated from *M. tuberculosis* infected mice produced less IFN-γ when stimulated with *M. bovis* BCG infected macrophages than when stimulated with *M. tuberculosis* infected macrophages. Mice infected with *M. bovis* BCG also had lower Mtb 8.4 specific CTL activity than *M. tuberculosis* infected mice. The study did not report the relative levels of IFN-γ produced by Mtb 8.4 specific $CD4^+$ T-cells from *M. tuberculosis* and *M. bovis* BCG infected mice, nor were the IFN-γ responses of $CD4^+$ T-cells compared with CTL.

The study of Coler *et al.* (2001) has apparently demonstrated the importance of immune responses against the 8.4 kDa for protection against *M. tuberculosis* infection in mice. However, considerable further work needs to be done to determine the relevance of the 8.4 kDa antigen to the anti-tubercular immune response of natural hosts of TB complex bacilli.

To date, only a few of the 52 identified extracellular protein antigens of TB complex species have been investigated with respect to their ability to elicit a protective immune response against challenge infection (Andersen 2001). A practical method of screening such a large number of potential immunogens would be immunization of laboratory animals with DNA vaccines carrying the genes for the 52 protein antigens, followed by challenge with virulent *M. bovis* or *M. tuberculosis*. Subsequently, antigens that appeared to induce protective immune responses could be trialed in natural hosts of *M. bovis* such as cattle.

In recent years, some workers have expressed the opinion that widely occurring non-species specific antigens such as heat-shock proteins, could be at least as effective as secreted mycobacterial proteins at inducing protective immunity, due to their immunomodulatory effects (Silva 1999). Vaccination and challenge trials could be used to dissect the nature of the responses to various immunogens. Furthermore, the effects of combinations of antigens and vaccination shedules such as an initial vaccination with proteins or plasmid DNA encoding proteins that prime a Type I response, followed by specific immunization with secreted antigens could be explored. However, the definition of protection needs to be clarified. Currently vaccine efficacy is variously reported in terms of log counts of bacterial numbers, severity of tuberculous lesions, or survival time (Elhay and Andersen 1997, Orme *et al.* 2001).

Whilst an improved vaccine against tuberculosis would probably find application in humans, it is unlikely that vaccination of cattle would be considered as an adjunct to bovine tuberculosis control in New Zealand. The primary objection to vaccinating cattle against tuberculosis is that whereas humans with strong immune responses develop "closed" lesions containing a few so called dormant bacilli and do not transmit disease, in cattle tuberculosis is invariably progressive and even animals with small lesions confined to lymph nodes are potentially infectious (Francis 1958, Cassidy *et al.* 1998). Therefore in order to be considered, vaccination of cattle would have to not only prevent disease progression, but also confer sterilizing immunity.

The distinction between prevention of disease and elimination of infection is fundamental. The situation with respect to tuberculosis is quite different to Johne's disease caused by *M. avium* subsp. *paratuberculosis*, where the aim of vaccination is to prevent production losses from disease in the face of infection. New Zealand's export markets exclude beef originating from animals infected with *M. bovis* regardless of whether they

were clinically diseased or not. The current definition of *M. bovis* infection is the discovery of tuberculous lesions at slaughter or a positive intradermal tuberculin test. Consequently, even if vaccination could prime an immune response sufficient to eliminate *M. bovis* infection, following exposure to *M. bovis* the animal would be sensitized to tuberculin. Therefore, the infection status of the animal would be indeterminable *ante-mortem.*

The second main objection to vaccination of cattle is that it is considered unlikely that the regulatory authorities of importing countries would accept meat from animals vaccinated with a live vaccine such as *M. bovis* BCG (Livingstone and Davidson 1993, de Lisle *et al.* 1998). Although laboratory animal studies indicate that DNA vaccination shows great promise with regard to efficacy (Doherty and Andersen 2000) it can be anticipated that there would be considerable consumer resistance to food products derived from animals vaccinated with foreign DNA. The alternative is sub-unit vaccines using protein antigens, but so far the results of vaccination and challenge experiments in cattle have not been encouraging (Wedlock *et al.* 2000). However, cattle are a natural host of *M. bovis* and the bovine vaccination and challenge model could be useful for testing vaccination regimes; differences in the predominance of antigen recognition between humans and cattle notwithstanding (Lyashchenko *et al.* 1998b).

Despite the limitations imposed by current technology, during the 1990s the number of herds on movement control restrictions in New Zealand was reduced from over 1400 in 1994 to 358 at 30 June 2000, by application of the test and slaughter technique supplemented with vector control (AHB 2000). As the bovine tuberculosis control programme enters the eradication phase it can be predicted that the proportion of reactor animals that have no visible lesions (NVL) at slaughter will increase. Therefore, producer resistance and possibly disenchantment with the test and slaughter programme could become a factor that limits progress towards eradication. Furthermore, when the incidence of disease is low it will become increasingly difficult to detect newly infected herds. Thus, there is a need on one hand for tests of greater specificity to prevent wastage, and on the other for greater sensitivity to facilitate elimination of all infected animals.

Recently, a number of authors have suggested that if PPD tuberculins were replaced by *M. bovis* specific antigens the diagnostic tests would have greater specificity. The cellular immune responsiveness of infected cattle to the antigens ESAT-6, MPB70, MPB64, MPB83 and CFP10 have been investigated. It has been reported that the specificity of

diagnosis was greater than with PPD tuberculin, but the sensitivity was lower. Consequently, it has been suggested that tests of adequate sensitivity and specificity could be developed using 'cocktails' of TB complex specific antigens (Vordermeier *et al.* 1999, van Pinxteren *et al.* 2000, Andersen *et al.* 2000, Buddle *et al.* 2001).

To date nine secreted and three somatic protein antigens have been identified that are either exclusive to TB complex, or which are limited to slow growing pathogenic mycobacterial species (MTB32A, 28 kDa, MPB64, 27 kDa, MPB70, MPT63, MPT40, CFP10, ESAT-6, MTB39A, VirS and MTB9.9A), and others have TB complex specific epitopes (MPB83, MTB12, 38 kDa) (see Appendix I). Immunodiagnostic preparations composed of 11 of those antigens (MPT40 does not occur in *M. bovis*) could potentially be highly specific and probably have adequate sensitivity if a high dose were used. However, it is desirable to include as many targets of the immune response to *M. bovis* as possible (Andersen *et al.* 2000, Buddle *et al.* 2001).

In this study it was found that PBMC from cattle that produced IFN-γ when stimulated with PPD-A also produced small amounts of IFN-γ when stimulated by the 8.4 kDa antigen. Thus, although the test format differed from the whole blood IFN-γ assay and the number of animals studied was small, there is indirect evidence that *M. avium* expresses a protein that can sensitize cattle to the 8.4 kDa antigen of *M. bovis*. Furthermore, a nucleotide sequence was identified in the genome of *M. avium* that could potentially code for a protein with homology to the 8.4 kDa antigen of *M. bovis*. Thus, although further investigation is needed before any conclusions can be drawn regarding sensitization of animals to the 8.4 kDa antigen of *M. bovis* consequent to environmental exposure to *M. avium*, it appears that the 8.4 kDa Ag is not a candidate for specific diagnosis of *M. bovis* infection.

This study has demonstrated that screening CFs from a cosmid library of the *M. bovis* genome for antigens expressed and secreted into the culture media by *M. smegmatis* is a practical method for identifying genes coding for *M. bovis* protein antigens. In the future it is likely that bioinformatic techniques will supplement cosmid library screening. Alignment of the genomes of *M. avium* and *M. bovis* against the *M. tuberculosis* H37Rv genome will enable genes unique to each species to be identified. However, to determine the antigens that are candidates for specific diagnosis of tuberculosis, it will be necessary

to screen expressed gene products for recognition by T-lymphocytes from *M. bovis* infected and *M. avium* sensitized animals.

Currently, one of the main sources of producer disillusionment with the bovine tuberculosis control programme is the delay between the discovery of lesions at routine meat inspection and definitive bacteriological diagnosis. Australia, introduced a multiplex PCR based typing method which differentiated between *M. bovis*, *M. avium* or *M. intracellulare* to make rapid presumptive diagnoses of the cause of lesions found at slaughter (Wilton and Cousins 1992).

In the course of this study, an exact tandem repeat locus that is specific to TB complex species and polymorphic between *M. bovis* and *M. tuberculosis* was discovered. It was shown that PCR directed at the 62 bp ETR locus could be used to rapidly and simply differentiate between a large percentage of *M. bovis* and *M. tuberculosis* isolates with a high degree of accuracy. When the complete sequence of the *M. avium* genome becomes available, the ability to align it against the *M. bovis* genome will provide the opportunity to identify loci that are polymorphic between those species. It could then be possible to develop more refined techniques for rapid post-mortem bacteriological diagnosis based on variable number of tandem repeats (VNTR) typing.

A major practical impediment to developing diagnostic preparations to replace PPD tuberculins remains the inability to produce sufficient quantities of antigen without the need for extensive purification. This study has shown that *M. smegmatis* can overexpress and secrete a *M. bovis* antigen, and that concentrated CF was suitable for use in *in vitro* cellular assays without further purification. However, *M. smegmatis* CF appears to contain proteins recognized by antibodies present in the serum of normal animals. Therefore, for use in assays of humoral responses using an ELISA format the antigens would need to be purified.

This study demonstrated that *M. smegmatis* transformed with a novel expression construct overexpressed and secreted the 8.4 kDa antigen with a 6 x Histidine tag. The 6 x Histidine tagged protein was able to be purified from CF in one step by metal chelate affinity chromatography. If a number of antigens could be similarly expressed and purified, a high throughput ELISA assay for serum antibodies could be developed. Such an assay could find application as a parallel ancillary test, to identify the animals with advanced disease that are anergic in cellular assays.

The intradermal test using PPD-B tuberculin is well characterized and large numbers of animals can be tested quickly, easily and cheaply. Therefore, for routine surveillance the intradermal test is likely to remain the primary screening test. Nevertheless, in New Zealand the application of *in vitro* cellular assays for diagnosis of *M. bovis* infection is currently an area of active investigation. Although throughput is limited and the costs are prohibitive for routine surveillance, the BOVIGAM™ whole blood IFN-γ assay has proved useful for ancillary testing (AHB 2000). However, because the BOVIGAM™ test compares the responses to PPD-B and PPD-A, interpretation is not straightfoward (Ryan *et al.* 2000, Buddle *et al.* 2001).

The *in vitro* assays of a cellular response require considerably less antigen than the intradermal test, therefore more expensive preparations of species specific antigens could be used instead of PPD-B and PPD-A without markedly increasing the costs of testing. Thus, if biotechnological methods enabled preparations of species specific antigens to be produced commercially, it can be envisaged that highly sensitive and specific *in vitro* assays could be developed.

APPENDIX I. Compilation of TB complex species' secreted protein antigens.

Table I.A. Protein antigens identified in the CF of TB complex species.

Antigens with identifiable Type II signal sequences. [TB complex specific (+), common to many mycobacterial species (—), insufficient information (?)]

MW	Names	TB	Gene	Access No.	Comments	References.
40479 Da$_a$	CtaC, Mtb92	?	*ctaC* Rv2200c	SwP: Q10375	probable cytochrome C oxidase subunit II (PhoA)	Moran *et al.* 1999
38243 Da$_a$	Protein antigen B, PAB, antigen 5, antigen 78, 38kDa antigen	±	*phos1* Rv0934	SwP: P15712	TB complex specific epitopes phosphate binding	Andersen & Hansen 1989 Harboe & Wiker 1992
37953 Da$_a$	Phosphate binding protein 3, PBP-3, PSTS3, Ag88, Phos2	?	*psts-3* *phos2* Rv0928	TrE: O86343	phosphate transport lipoprotein anchor	Braibant *et al.* 1996b Lefèvre *et al.* 1997
37864 Da$_a$	Phosphate binding protein 2, PBP-2, PSTS-2	?	*psts-2* Rv0932c	TrE: O05870	phosphate transport	Braibant *et al.* 1996a Lefèvre *et al.* 1997
36771 Da$_a$	Antigen 85-C, MPT45	—	*fbpc* Rv0129c	SwP: P31953	fibronectin binding mycolyltransferase	Content *et al.* 1991
35686 Da$_a$	Antigen 85-A, MPT/MPB44, 32 kDa protein	—	*fbpa* Rv3804c	SwP: P17944	fibronectin binding mycolyltransferase	Borremans *et al.* 1989 de Wit *et al.* 1990
34926 Da$_a$	MTB32A	+	*pepA* Rv0125	TrE: O07175	serine protease	Skeiky *et al.* 1999
34403 Da$_a$	Antigen 85-B, MPT/MPB59, Extracellular alpha antigen, 30 kDa extracellular protein	—	*fbpb* Rv1886c	SwP: P31952 SwP: P12942	fibronectin binding mycolyltransferase	Matsuo *et al.* 1988
32720 Da$_a$	Ala-Pro rich 45/47kDa, MPT32, FAP-B, 45kDa glycoprotein	—	*modd* *apa* Rv1860	SwP: Q50906	belongs to MODD family. molybdenum transport	Romain *et al.* 1993 Laqueyrerie *et al.* 1995

APPENDIX I. Compilation of TB complex species' secreted protein antigens.

Antigens with identifiable Type II signal sequences continued.

MW	Names	TB	Gene	Access No.	Comments	References.
32244 Da[a]	Proline rich 28kDa antigen, MTC28	+	*mtc28* Rv0040c	SwP: P71697	similar to *M. tuberculosis* LPQT	Manca *et al.* 1997b
31119 Da[a]	MPT51	—	*mpt51*	TrE: O33176	cross-reactive with Ag85s	Oettinger & Andersen 1997*d*
31089 Da[a]	MPB51	—	Rv3803c	TrE: Q48923	mycolyltransferase	Ohara *et al.* 1995
27700 Da[a]	Exported repetitive protein, Cell surface protein PIRG, P36/P34, Exp53	?	*erp* *pirg* Rv3810	SwP: Q50793	required for multiplication and intracellular growth contains PGLTS repeats (PhoA)	Berthet *et al.* 1995 Bigi *et al.* 1995 Berthet *et al.* 1998
24855 Da[a]	MPT64 / MPB64	+	*mpt64* *mpb64* Rv1980c	SwP: P19996	RD2 deletion of BCG	Yamaguchi *et al.* 1989 Oettinger & Andersen 1994 Harth *et al.* 1997
24548 Da[a]	27 kDa lipoprotein, Antigen P27, Lipoprotein LPRG	+	*lprg* *lpp-27* Rv1411c	SwP: P71679	belongs to LPPX/LPRAFG family of lipoproteins	Bigi *et al.* 1997
24140 Da[a]	24.1 kDa secreted lipoprotein	—	*lppx* Rv2945c	SwP: P96286	homologue in *M. leprae* homology with 19 kDa lipoprotein	Oftung *et al.* 1997
24029 Da[a]	Mtb200	?	Rv0200	TrE: O53651	(PhoA)	Moran *et al.* 1999
22598 Da[a]	CFP25	?	Rv2301	SwP: Q50664	belongs to cutinase family	Weldingh *et al.* 1998
22070 Da[a]	MPT83, Lipoprotein P23	?	*mpt83* *mpb83* Rv2873	SwP: Q10790	61% homology with MPB70 highly immunogenic	Hewinson *et al.* 1996 Matsuo *et al.* 1996 Vosloo *et al.* 1997
21782 Da[a]	CFP21	?	Rv1984c	SwP: Q10837	RD2 deletion of BCG	Weldingh *et al.* 1998 Weldingh & Andersen 1999

APPENDIX I. Compilation of TB complex species' secreted protein antigens.

Antigens with identifiable Type II signal sequences continued.

MW	Names	TB	Gene	Access No.	Comments	References.
19072 Da[a]	MPT70 / MPB70	+	*mpt70* *mpb70* Rv2875	SwP:Q50769	high / low producing BCG strains major constituent of CF	Radford *et al.* 1988 Terasaka *et al.* 1989 Matsumoto *et al.* 1995
18383 Da[a]	soluble antigen MPT53	—	*mpt53* Rv2878c	SwP: Q10804	thioredoxin family	Wiker *et al.* 1999
16635 Da[a]	Low MW antigen MTB12, Low MW protein antigen 2, CFP-2	?	*mtb12* Rv2376c	SwP: O05822	TB complex and *M. leprae*, not non-TB complex by Southern	Bhaskar & Mukheriee 1998*d* Webb *et al.* 1998
16524 Da[a]	MPT63, 16 kDa antigen	+	*mpt63* Rv1926c	SwP: P97175	antigen	Manca *et al.* 1997a Harth *et al.* 1997
15147 Da[a]	19 kDa lipoprotein antigen	—	*lpqh* Rv3763	SwP: P11572	antigen	Ashbridge 1989 Collins *et al.* 1990
10881 Da[a]	8.4 kDa antigen SA-5K	—	Rv1174c	TrE: O50430	antigen	Coler *et al.* 1998 Freer *et al.* 1998b

Antigens without identifiable signal sequences.

MW	Names	TB	Gene	Access No.	Comments	References.
80604 Da	KatG, MPT35	?	*katG* Rv1908c	SwP: Q08129	catalase-peroxidase confers isoniazid susceptibility	Zhang *et al.* 1992 Heym *et al.* 1993
66699 Da	DnaK, hsp70	—	*dnaK* Rv0350	SwP: P32723	heat shock protein, chaperonin belongs to hsp70 family	Lathigra *et al.* 1991 Wiker *et al.* 1999
66129 Da	Mtb898	?	Rv1166	TrE: O50422	probable lipoprotein (PhoA)	Moran *et al.* 1999

APPENDIX I. Compilation of TB complex species' secreted protein antigens.

Antigens without identifiable signal sequences continued.

MW	Names	TB	Gene	Access No.	Comments	References.
56595 Da	60 kDa chaperonin 2, GroEL2, 65 kDa antigen, hsp65, antigen A, cell wall protein A	—	*GroEL2* *hsp65* Rv0440	SwP: P06806	heat shock protein, belongs to chaperonin (hsp60) family	Shinnick 1987 Thole *et al.* 1987
53826 Da	AppC, Mtb827	?	*appC* Rv1623c	TrE: O06140	(PhoA)	Moran *et al.* 1999
53570 Da	glutamine synthetase 1, GlnA	—	*glnA* Rv2220	SwP: Q10377	glutamine synthetase	Harth & Horwitz 1997
41439 Da	MTB41	?	Rv0915c	TrE: O05907	PPE family	Skeiky *et al.* 2000
38770 Da	Des	—	*des* Rv0824c	TrE: Q50824	homologies to stearoyl-acyl carrier protein desaturases (PhoA)	Jackson *et al.* 1997
38713 Da	L-alanine dehydrogenase, 40 kDa antigen, TB43	—	*ald* Rv2780	SwP: P30234	L-alanine dehydrogenase	Andersen *et al.* 1992
28860 Da	CFP29	—	*cfp29* Rv0798c	TrE: O07812 TrE: O07181	possible bacteriocin	Rosenkrands *et al.* 1997*d* Rosenkrands 1998
27212 Da	27 kDa antigen, CFP30B	?	*cfp30b* Rv0577	SwP: O53774	homologues in *Streptomyces.*	Oettinger 1998*d*
23034 Da	Superoxide dismutase(Fe), SodA, MPT58	—	*sodA* Rv3846	SwP: P17670	destroys peroxide radicals	Zhang *et al.* 1991 Harth & Horwitz 1999
19239 Da	CFP22, PPIase, rotamase	?	*ppia* Rv0009	SwP: P71578	belongs to cyclophillin-type PPIase family, isomerase, rotamase	Weldingh *et al.* 1998
18630 Da	TB 18.6	?	Rv2140c	SwP: Z95436		Weldingh *et al.* 2000
16896 Da	CFP20	?	*tpx* Rv1932	SwP: P95282	probable thiol peroxidase	Weldingh *et al.* 1998

APPENDIX I. Compilation of TB complex species' secreted protein antigens.

Antigens without identifiable signal sequences continued.

MW	Names	TB	Gene	Access No.	Comments	References.
16096 Da	14 kDa antigen, hsp16.3, 16kDa antigen	—	*hspx* Rv 2031c	SwP: P30223	belongs to small heat shock protein (hsp20) family	Verbon *et al.* 1992 Lee *et al.* 1992
14972 Da	MTP40	+	*mtp40* *plcC* Rv2349c	SwP: 04001	unique to *M. tuberculosis* phospholipase	Parra *et al.* 1991 Del Portillo *et al.* 1991
12223 Da	CFP-6, Low molecular weight protein antigen 6	?	*cfp6* Rv3004	SwP: 053251		Bhaskar & Mukherjee 1998*d*
12413 Da	Thioredoxin, MPT46, Trx	?	*trx* Rv3914	SwP: P52229	thioredoxin	Wieles *et al.* 1995
10794 Da	CFP10, MTSA-10	(+)	*lhp* Rv3874	TrE: O69739	member of ESAT-6 family RD1 deletion of BCG	Berthet *et al.* 1998 Colangeli *et al.* 2000
10673 Da	10 kDa antigen, GroES, MPB57	—	*groES* Rv3418c	SwP:P15020 SwP: P09621	chaperonin	Baird *et al.* 1989 Barnes *et al.* 1992
10259 Da	10 kDa antigen, CFP7, TB10.4	—	*cfp7* Rv0288	SwP: O53693	member of ESAT-6 family	Nielsen 1997*d* Skjøt *et al.* 2000
9773 Da	6 kDa early secretory antigenic target, ESAT-6	(+)	*esat-6* Rv3875	SwP: Q57165	RD1 deletion of BCG	Klausen *et al.* 1994 Sørensen *et al.* 1995
9425 Da	9.5 kDa antigen, CFP10A	?	*cfp10a* Rv1335	SwP: Q10646	belongs to UPF0084 family	Oettinger 1998*d*
7306 Da	TB7.3	—	Rv3221c	TrE: O05845		Skjøt *et al.* 2000
7240 Da	CspA	?	Rv3648c	SwP: Z95388	small cold shock protein	Weldingh *et al.* 2000

APPENDIX I. Compilation of TB complex species' secreted protein antigens.

Table I.B. Extracellular proteins of *M. tuberculosis* not identified as antigens.

MW	Names	TB	Gene	Access No.	Comments	References.
46452 Da[a]	MTB32B	?	Rv0983*	TrE: O53896*	probable serine protease	Skeiky *et al.* 1999
44417 Da	Enhanced intracellular survival protein, Eis	+	*eis* Rv2416*	TrE: P71727*	enhances intracellular survival surface location	Wei *et al.* 2000
23844 Da	SodC	—	*sodC* Rv0432	TrE: P96278	Cu, Zn superoxide dismutase	Wu *et al.* 1998
22819 Da[a]	25 kDa protein	?	Rv1815	SwP: Q50618	unknown	Sonnenberg & Belisle 1997
9917 Da	MTB10	?	Rv0916c	TrE: O05908	PE family	Skeiky *et al.* 2000

* The reported translated amino acid sequences are not identical.

Table I.C. Somatic protein antigens unique to TB complex species.

MW	Names	TB	Gene	Access No.	Comments	References.
40015 Da	Mtb39A	+	*mtb39a* Rv1361c	SwP: Q11031	member of the PPE family	Dillon *et al.* 1999
37789 Da	VirS	+	*virS* Rv3082c	SwP: Q06861	virulence factor, possible transcriptional regulator	Gupta *et al.* 1999
9942 Da	Mtb9.9A	+	*mtb9.9a* Rv1793	TrE: O53942		Alderson *et al.* 2000

APPENDIX I. Compilation of TB complex species' secreted protein antigens.

Legend for Tables I.A, I.B and I.C. MW; molecular weight in Daltons predicted from the translated data base amino acid sequence. Da_a; predicted molecular weight of the uncleaved precursor protein. In general, the export signal sequences have predicted molecular weights of 1.5 to 3 kDa. TB; + antigen unique to TB complex species; — antigen common to many mycobacterial species; ? insufficient information; (+) ESAT-6 & CFP10 do not occur in *M. bovis* BCG, *M. avium*, *M. intracellularae* , *M. fortuitum* or *M. scrofulaceum*, but do occur in *M. kansasii* and *M. marinum* . Gene; alternative names in italics, Rv numbers refer to the predicted *M. tuberculosis* H37Rv genes (Cole *et al.* 1998). Access No.; Swiss-Prot (SwP) or TrEMBL (TrE) data base accession numbers. (PhoA); proteins identified by *phoA* fusion techniques (Lim *et al.* 1995). Reference; year followed by *d* indicates a direct submission to the database and no further published information.

Table I.D. Extracellular enzyme activities of *M. tuberculosis* CF (Raynaud *et al.* 1998).

Group 1. extracellular in *M. tuberculosis* only:
Alanine aminopeptidase, Cystine aminopeptidase, Alanine dehydrogenase, Glutamine synthetase, Nicotinamidase, Isonicotinamidase, Superoxide dismutase, Alcohol dehydrogenase, Catalase, Peroxidase.

Group 2. extracellular in *M. tuberculosis* and fast growing mycobacterial species:
Arginine aminopeptidase, Leucine aminopeptidase, Proline aminopeptidase, Amylase, β-esterase, Esterase (C_4), Lipase esterase (C_8), Lipase (C_{14}), Acid phosphatase, Alkaline phosphatase, Phosphoamidase, β-lactamase.

APPENDIX II.A Nucleotide sequence of the 4.3 kb *Sph* I fragment of *M. bovis.*

CAGGGCGGTATCGATCCCGAGCTACCGGTCACCGGCTACGCCGATCTGGTTCGTGCCGTCAAGGCGCGGGT
GCCCTCCATGCATGTGCACGCGTTTTCCCCGATGGAGATCGCCAACGGCGTCACCAAGAGCGGGCTGAGCA
TTCGCGAGTGGCTGATCGGCCTGCGCGAGGCCGGGCTGGATACCATCCCGGGTACCGCCGCGGAAATCCTG
GACGACGAGGTTCGCTGGGTGCTGACCAAGGGCAAGCTGCCGACGTCATTGTGGATCGAAATCGTGACGAC
CGCCCACGAGGTGGGTCTGCGGTCATCATCGACGATGATGTACGGGCATGTGGACAGTCCACGGCACTGGG
TCGCCCATCTTAACGTGCTGCGCGATATTCAGGACCGTACCGGCGGCTTCACCGAGTTCGTCCCGTTGCCG
TTCGTGCACCAGAATTCACCGTTGTACCTGGCCGGTGCGGCGCGCCCCGGGCCCAGCCATCGCGACAACCG
CGCGGTACATGCTTTGGCGCGGATCATGTTGCACGGCCGCATCTCGCACATTCAGACCAGCTGGGTGAAAC
TTGGAGTGCGGCGCACCCAGGTGATGCTCGAAGGTGGCGCCAACGACCTGGGCGGCACGCTGATGGAGGAG
ACCATCTCGCGGATGGCCGGTTCCGAACACGGATCGGCCAAGACCGTCGCTGAGCTGGTCGCGATCGCCGA
AGGCATCGGCCGCCCGGCGCGCCAGCGCACTACCACATACGCCCTGCTTGCGGCCTAGCCCCGGCGACGAT
GCCGGGTCGCGGATGCGGCCCGTTGAGGAGCGGGGCAATCTGGCCTAGCCCCGGCGACGATGCCGGGTCG
CGGGATGGGGCCCGCATGGGCTTAATAGTTGTTGCAGGAGCCGGCAACCGACTCGACAAGGCCGATGTACT
GTGCCGCCCCGGCACAGCTTGCAATTGCGCGGCCATGGCAGCGCGCTGAGGTGGCGGTGCGGCGAGGAAAT
TGCGCAAATAGGACTGCGCCACCGGTGAGGCGTTGAACTGTGCGGCAGCCCCCGGATCCGTCGCGTTGAGC
GCAGCTACTACCTGCCCGTAATTGCAGGTGGTGTTAATGACCGCGTCCACGGGATCTGCGGAGGCGACCCC
GGCCCCGACGGTCAACGACATTGCCACGGCGCCTACACCGGCGCTCAATGCGGTCAACGACAGCCTCATTT
ATGGACACCTTCCCCAAACTATTGCACCGTCGTTAAGACGGCGACGACATCTGCCCAGCGGTTGCCGTCTG
CGGTCGAGGGTACCAGGCGCCGTGGGCTTGCTTCTCTCAAACTGGTTATCGGGCGACACTGCGCGGCCATA
CCAATCTGCAGGTCAGCAGCGATGAAACAACGTTGTTTACAGCCCGAGAAATGAGTTTATAGCCTGGCCGC
AAGTTCGGTGCCTTGCTTGATGGCGCGCTTGGCGTCCAACTCGGCGGCAACCGCCGCGCCACCGCTGATGT
GCGGGTTAATGCCGTGCCGGCGCAGTTCACTCTCCAGATCTCGCACCGGTTCCTGGCCGGCGCAGACCACT
ACGTTGTCCACCGCCAGCAGCTGGGGCCGCCTGCGCTTCGGGCCGAAGCTGATGTGTAGGCCGTCGTCGTT
GATCTGTTCGTAGTTCACCCCAGACAGCTGATGAACGCCCTTGGCCTTCAACGACGCCCGGTGGACCCATC
CGGTGGTCTTGCCGAGCCGCTTGCCCTGCGGGCCTTTGGTGCGCTGCAGTAGGTACACCTCACGGGCGGGC
GGCGCCGGCAGTGGAGTCGTCAACGCTCCGCGGGCTTCTCGCGGATCAGCGACCCCCCATTCGGCCTTCCA
CTCTTTGAGGTTGAGGGTGGGTGAGGAGTCGGTGACCAGCAGTTCGGTGACGTCGAAGCCAATGCCGCCGG
CGCCGACGACAGCCACGGTTCGCCCGACCGGTCTGACACCGGTGATGGCTTCGGCGTAGGTTAACACCATG
GGGTGGTCGATGCCGGGGATGGCCGGAATGCGCGGTGCCACGCCGGTGGCCAAGACGACCTCGTCGTAGCC
GGTCAACTCCTGGGCGGCCACCCGAGTGCCCAGTCGCACCTCGACACCGTGTTTGGCCAGAATCGTCGAGA
AATACCGGATGGTTTCGCTGAATTCCTCTTTGCCGGGAATGCGGCGGGCCATGTCAAACTGTCCACCGATA
AAGTCGTTGGCCTCGAACAGCGTGACCCGGTGACCCCGTTGCGCGGCGTTGGCCGCCGTGGCCAGCCCGGC
TGGTCCAGCCCCGACGACGGCCACCGAGCGGGCGCGCCGGGTCGGGGACAGCACCAACTGCGTCTCGCGCC
CGGCGCGTGGATTGAGCAGACACGACACCGTTTTCCTGGCAAATGCGTGGTCCAGGCAGGCTTGATTGCAG
GAGATGCAGGTGTTGATTTCGTCGACCCGATTGGACTGCGCCTTGAGCACCCAGTCCGGGTCGCTCAGCAT
CGGCCGGGCCATTGATATCAGCCGCACCTGGGTTTCGGCCAGAATCCGTTCCGCGGCCTGCGGCATGTTGA
TCCGGTTGGACGCCACCACCGGGATAGTGACGTGTTCGGCGACGGCGCTGCTGATGTCGACAAACGCGCCG
CCCGGCACTGAGGTGACGATAGTGGGCACCCGGGCCTCGTGCCAGCCGAAGCCGGAGTTGATGATGGTTGC
GCCTGCCCCTTCCACTTCGGTTGCCAGCGCGACGATTTCATCCCAACTCTGGCCTTCTGCAACGTAGTCGG
CCATTGACAGCCGGTAACAGATGATGAAGTCGGATCCGACGGCGGCGCGGCTGCGTCGGATGATCTCGACC
GGGAACCGGCGACGGTTGGCCGGTGTGCCGCCCCACGAGTCGGTGCGCTTGTTGGTGCGCGGCGCCAGGAA
CTGATTGAGCAGATACCCTTCGCTGCCCATGATTTCGACGCCGTCGTAGCCGGCATCGCGGCCAACTGCG
CGCAGCGGGCGAAATCCGCGATGGTCGCTTCGACCCCGCGAGCCGATAGTGCTCGCGGACGAAACGGGGTG
ATCGGCGCCTTGATCGGCGAGGCGCTGACCGCAAGTGGGTGGTAGGCGTAGCGTCCGGCGTGCAGGATTTG
CAGCAGGATCTTTGCACCCGAATCGTGGACCGCCCTGTTGATTCGGCGGTGCCGTCGGGCTTGCGCCGAAG
TGACGAGTTCGGAGGCGAACGGCAGCAGCCATCCGGTGCGGTTGGGCGCGTAGCCACCGGTGATGATCAGC
CCGACGCCGCCGCGTGCACGTTCGGCGAAGTAGTCGGCGAGCCGATCGATATGGCGGGCCCGGTCTTCCAG
TCCGGTGTGCATCGAACCCATAACCACCCGGTTGCGCAGCGTGGTAAACCCAAGGTCCAACGGGGACAGCA
GATTTGGGTATGGATTTGTCATCGCTTCTCCTGGAGCGCTTCAGCTACTTCGTCGAGCCAATCGATGGCAC
TTTCTTCGGCTCGGATTCCGCCGCGCAGCACGAGGTATTGATGCAGTGCGGCGCCATCGAGCGCCGACGGA
TCTGCGAAGGTGCGCTTCTCGATACCGCGATAGGTGTCCAGTGACTTGACACGCTCGGCGCGCAGCGCGGT
GACTTGGGTATACAGCGCGGCAACGTCTCCGTAGCCGGCGCCACGCAGCTTGACGGCGATATCGCGCGTGC

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TGCTGTCGGTCAGCGCACTGCCGCGGCCGGGCCTGGTCGGGCTGAGCGGCTCGGCGATCCAGCGAGCCAGC
TCGGCCCGGCCGCTGTCGGAGATCGCGTATACCTTCTTGTCGGGCCGGCCATGCTGGAGCACGGTCGTCGC
GCGCACCCAGTTGTTGTTCTCCATCACCCGTAACGTCCGATAGATCTGCTGATGGGTTGCGGTCCAGAAAT
AGCCGATGGAGCGATCGAATCGGCGGGCCAACTCGTAGCCCGAGCTGGCCTGTTCACACAGCGACACCAAG
ATCGCGTGGGGTAGCGCCATCCGGGCAGCATAGACGGCAAGCCGGATTGCTATGCAACTAGGTGCATATTG
ACCGTGTACGCCGACGCATGTGCCAAGTGGTCGACGTGTATGTGCAACGTCTAGTATCAGTAACCGAACGC
ATTGCCTCAGCAGGGCCCGGAGGAAGCCTTGGCGAGGTGGACAGCAGCCCACACATAGCGGTATCTGGAAG
ACATGTTGAGGAGACGTCCGTGACGTACACGATCGCCGAACCCTGTGTCGACATCAAGGACAAGGCATGCC
TGCAGG
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APPENDIX II.B Primers used to sequence the 4.3 kb fragment of *M. bovis* DNA.

M13/pUC foward	5'	CCC AGT CAC GAC GTT GTA AAA CG	3'
M13/pUC reverse	5'	AGC GGA TAA CAA TTT CAC ACA GG	3'
43 for A	5'	TTC ACC GAG TTC GTC CCG TTG C	3'
43 rev A	5'	GGT GAT GGA GAA CAA CAA CTG G	3'
43 for B	5'	CTC GAC AAG GCC GAT GTA CTG TGC	3'
43 rev B	5'	TTT ACC ACG CTG CGC AAC C	3'
43 rev del KS	5'	GCA CAG TAC ATC GGC CTT GTG GAG	3'
43 rev del LB	5'	CGA TGC CGG GTC GCG GGA TG	3'
43 for C	5'	CGC AAG TTC GGT GCC TTG CTT G	3'
43 rev C	5'	GCG CAC CAA CAA GCG CAC CG	3'
43 for D	5'	TGG CTT CGG CGT AGG TTA AC	3'
43 rev D	5'	GTC GTG TCT GCT CAA TCC AC	3'
43 for E	5'	ATT GAT ATC AGC CGC ACC TG	3'
43 rev E	5'	TCG CTG ATC CGC GAG AAG CC	3'
43 for F	5'	CGC GAT GGT CGC TTC GAC	3'
43 rev F	5'	TGA GAG AAG CAA GCC CAC	3'
43 for G	5'	GCG CAG CAC GAG GTA TTG	3'
43 for H	5'	GGC AGC ATA GAC GGC AAG	3'
43 rev H	5'	CCG CGC CAA AGC ATG TAC	3'

APPENDIX III. Published sequence details of the 8.4 kDa antigen of *M. tuberculosis* and *M. bovis* BCG.

CGGCAACCGCTGGGCAGATG**TCGTCG**CCGTCTTAACGACGGTGCAA**TAGTT**
-35 -10

TGG**GGAAGG**TGTCCA TAA ATG AGG CTG TCG TTG ACC GCA TTG AGC
SD *M R L S L T A L S*

GCC GGT GTA GGC GCC GTG GCA ATG TCG TTG ACC GTC GGG GCC GGG
A G V G A V A M S L T V G A G

GTC GCC TCC GCA GAT CCC GTG GAC GCG GTC ATT AAC ACC ACC TGC
V A S A **D P V D A V I N T T** C

AAT TAC GGG CAG GTA GTA GCT GCG CTC AAC GCG ACG GAT CCG GGG
N Y G Q V V A A L N A T D P G

GCT GCC GCA CAG TTC AAC GCC TCA CCG GTG GCG CAG TCC TAT TTG
A A A Q F N A S P V A Q S Y L

CGC AAT TTC CTC GCC GCA CCG CCA CCT CAG CGC GCT GCC ATG GCC
R N F L A A P P P Q R A A M A

GCG CAA TTG CAA GCT GTG CCG GGG GCG GCA CAG TAC ATC GGC CTT
A Q L Q A V P G A A Q Y I G L

GTC GAG TCG GTT GCC GGC TCC TGC AAC AAC TAT TAA GCCCATGCG
V E S V A G S C N N Y ochre

GGCCCCATCCCGCG

Nucleotide sequence and deduced amino acid sequence of the translated product of the *M. bovis* homologue of the *M. tuberculosis* gene Rv1174c. The 110 residue amino acid sequence is shown below the corresponding open reading frame. It is predicted that the 28 amino acid signal sequence is cleaved releasing the 82 amino acid mature protein to the exterior. The experimentally determined N-terminal sequence is in boldface. The signal sequence is in italics and the consensus cleavage peptidase recognition site is underlined. The potential ribosome binding site shown in boldface is denoted by SD for Shine-Dalgarno. The putative Pribnow boxes (-35 and -10 consensus sequences) are also shown in boldface (adapted from Cole *et al.* 1998, Coler *et al.* 1998, Freer *et al.* 1998).

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 4. Pre-challenge, seven weeks post-BCG booster vaccination										
Lymphocyte proliferation data (mean c.p.m of quadruplicate wells)										
Unvaccinated animals (Tag No.)										
	31	87	90	116	122	194	196	197	208	215
Unstimulated wells	475	53.1	199.4	171.5	2445.3	4470.5	571.5	363.1	7707.9	627.3
c.p.m 1st quadruplicate	746.2	40.4	269.3	202.1	2035.8	12282.4	646.2	489.5	6962.1	431.8
	1528.8	65.0	553.2	201.7	1951.2	4319.3	1081.9	2336.2	1763.3	619.0
	628.4	70.1	364.4	320.4	3339.9	13847.4	542.4	389.5	3716.5	829.6
Unstimulated wells	419.2	48.8	415.8	80.2	6501.2	15810.1	569.4	197.9	3756.8	489.2
c.p.m 2nd quadruplicate	380.7	34.2	173.2	123.5	2382.3	2943	515.5	914.6	2629	402.2
	558.4	48.0	168.5	111.5	2244.5	3207.0	369.5	238.8	1628.2	602.0
	409.6	56.1	275.6	174.2	1068.4	4540.4	396.1	822.0	1542.5	584.9
Mean unstimulated	643.3	52.0	302.4	173.1	2746.1	7677.5	586.6	717.7	3738.3	573.3
SD unstimulated (c.p.m)	378.8	11.9	134.5	73.9	1642.2	5334.7	220.3	703.5	2395.0	135.2
SE unstimulated (c.p.m)	133.9	4.2	47.6	26.1	580.6	1886.1	77.9	248.7	846.8	47.8
ConA 0.25µg/ml	14350.2	10731.3	28107.8	18279.9	25994.8	45080.5	45130.3	37750.4	24193.5	30263.3
SD (%)	12.4	9.76	9.35	24.69	5.33	11.16	13.53	9.19	7.2	10.03
SD (c.p.m)	1779.4	1047.4	2628.1	4513.3	1385.5	5031.0	6106.1	3469.3	1741.9	3035.4
SE (c.p.m)	889.7	523.7	1314.0	2256.6	692.8	2515.5	3053.1	1734.6	871.0	1517.7
Stimulation Index	22.3	206.5	92.9	105.6	9.5	5.9	76.9	52.6	6.5	52.8
Δ c.p.m	13707.0	10679.4	27805.4	18106.7	23248.8	37403.0	44543.7	37032.7	20455.2	29690.1
log 10 Δ c.p.m	4.1	4.0	4.4	4.3	4.4	4.6	4.6	4.6	4.3	4.5
SE Δ c.p.m (c.p.m)	1023.6	527.9	1361.6	2282.8	1273.4	4401.6	3131.0	1983.4	1717.7	1565.5
SE Δ c.p.m (%)	7.5	4.9	4.9	12.6	5.5	11.8	7.0	5.4	8.4	5.3
Geomean Δ c.p.m	24030.1									
PPD-B 6.25µg/ml	577.0	351.5	845.2	454.2	90882.7	4579.8	703.5	476.6	1581.4	777.8
SD (%)	46.2	32.1	45.7	24.9	7.9	33.1	24.5	35.8	27.5	14.6
SD (c.p.m)	266.4	112.8	386.3	113.2	7197.9	1516.4	172.5	170.5	434.9	113.7
SE (c.p.m)	133.2	56.4	193.2	56.6	3599.0	758.2	86.3	85.2	217.4	56.9
Stimulation Index	0.9	6.8	2.8	2.6	33.1	0.6	1.2	0.7	0.4	1.4
Δ c.p.m	-66.3	299.6	542.8	281.1	88136.6	-3097.8	117.0	-241.1	-2156.9	204.5
log 10 Δ c.p.m	1.0	2.5	2.7	2.4	4.9	1.0	2.1	1.0	1.0	2.3
SE Δ c.p.m (c.p.m)	267.1	60.6	240.7	82.7	4179.6	2644.3	164.2	334.0	1064.2	104.7
SE Δ c.p.m (%)	402.8	20.2	44.4	29.4	4.7	85.4	140.3	138.5	49.3	51.2
Geomean Δ c.p.m	125.4									
PPD-B 1.25µg/ml	485.6	50.6	470.3	217.1	78756.8	3189.4	634.5	572.9	3661.3	748.0
SD (%)	19.1	43.5	65.0	47.5	20.9	34.1	28.2	53.3	47.4	13.3
SD (c.p.m)	92.9	22.0	305.4	103.2	16468.0	1088.2	178.9	305.1	1735.1	99.3
SE (c.p.m)	46.4	11.0	152.7	51.6	8234.0	544.1	89.4	152.6	867.5	49.6
Stimulation Index	0.8	1.0	1.6	1.3	28.7	0.4	1.1	0.8	1.0	1.3
Δ c.p.m	-157.7	-1.4	167.8	43.9	76010.7	-4488.1	47.9	-144.8	-77.0	174.8
log 10 Δ c.p.m	1.0	1.0	2.2	1.6	4.9	1.0	1.7	1.0	1.0	2.2
SE Δ c.p.m (c.p.m)	180.3	15.2	200.3	77.7	8814.6	2430.2	167.3	401.3	1714.3	97.4
SE Δ c.p.m (%)	114.4	1100.8	119.3	177.0	11.6	54.1	349.3	277.1	2225.6	55.8
Geomean Δ c.p.m	58.5									
PPD-A 6.25µg/ml	6793.7	9659.8	4222.2	17890.6	79861.3	29729.0	1761.2	769.4	34449.0	1788.8
SD (%)	147.1	97.9	14.8	64.9	10.9	39.5	72.7	80.2	17.8	34.1
SD (c.p.m)	9992.1	9452.1	626.6	11618.1	8672.9	11734.0	1280.4	616.6	6131.9	610.2
SE (c.p.m)	4996.1	4726.0	313.3	5809.1	4336.5	5867.0	640.2	308.3	3066.0	305.1
Stimulation Index	10.6	185.9	14.0	103.3	29.1	3.9	3.0	1.1	9.2	3.1
Δ c.p.m	6150.4	9607.8	3919.7	17717.5	77115.2	22051.5	1174.7	51.7	30710.7	1215.6
log 10 Δ c.p.m	3.8	4.0	3.6	4.2	4.9	4.3	3.1	1.7	4.5	3.1
SE Δ c.p.m (c.p.m)	5130.0	4730.2	360.8	5835.2	4917.1	7753.1	718.1	557.1	3912.7	352.9
SE Δ c.p.m (%)	83.4	49.2	9.2	32.9	6.4	35.2	61.1	1078.3	12.7	29.0
Geomean Δ c.p.m	5246.7									
PPD-A 1.25µg/ml	3571.9	1239.1	449.1	2936.3	75535.2	14989.0	1227.0	1069.4	17680.0	906.0
SD (%)	12.0	95.4	45.8	24.3	11.9	38.6	24.9	49.2	53.5	90.4
SD (c.p.m)	429.7	1182.6	205.7	713.2	8950.9	5791.7	305.8	525.6	9457.0	818.7
SE (c.p.m)	214.8	591.3	102.8	356.6	4475.5	2895.9	152.9	262.8	4728.5	409.4
Stimulation Index	5.6	23.8	1.5	17.0	27.5	2.0	2.1	1.5	4.7	1.6
Δ c.p.m	2928.6	1187.1	146.7	2763.1	72789.1	7311.5	640.5	351.7	13941.7	332.7
log 10 Δ c.p.m	3.5	3.1	2.2	3.4	4.9	3.9	2.8	2.5	4.1	2.5
SE Δ c.p.m (c.p.m)	348.8	595.5	150.4	382.7	5056.1	4782.0	230.8	511.5	5575.3	457.2
SE Δ c.p.m (%)	11.9	50.2	102.5	13.9	6.9	65.4	36.0	145.5	40.0	137.4
Geomean Δ c.p.m	1947.2									
8.4 kDa 6.25µg/ml	732.3	48.5	640.9	322.1	25664.6	4270.5	1444.0	477.0	2733.2	1647.4
SD (%)	29.6	19.1	51.1	93.4	33.5	50.2	75.4	55.4	126.0	60.5
SD (c.p.m)	216.4	9.3	327.2	300.8	8595.1	2142.1	1089.3	264.3	3444.0	997.0
SE (c.p.m)	108.2	4.6	163.6	150.4	4297.5	1071.0	544.7	132.1	1722.0	498.5
Stimulation Index	1.1	0.9	2.1	1.9	9.3	0.6	2.5	0.7	0.7	2.9
Δ c.p.m	89.1	-3.4	338.4	148.9	22918.5	-3407.1	857.4	-240.7	-1005.1	1074.2
log 10 Δ c.p.m	1.9	1.0	2.5	2.2	4.4	1.0	2.9	1.0	1.0	3.0
SE Δ c.p.m (c.p.m)	242.1	8.8	211.1	176.5	4878.2	2957.1	622.6	380.9	2568.8	546.3
SE Δ c.p.m (%)	271.9	257.4	62.4	118.5	21.3	86.8	72.6	158.2	255.6	50.9
Geomean Δ c.p.m	125.2									
8.4 kDa 1.25µg/ml	915.7	46.7	470.3	164.0	11717.7	9029.9	635.4	763.7	1894.0	510.6
SD (%)	46.6	41.3	12.8	24.3	111.7	73.3	28.9	55.6	28.3	27.3
SD (c.p.m)	426.9	19.3	60.0	39.8	13085.1	6618.0	183.6	424.6	535.6	139.5
SE (c.p.m)	213.4	9.6	30.0	19.9	6542.6	3309.0	91.8	212.3	267.8	69.8
Stimulation Index	1.4	0.9	1.6	0.9	4.3	1.2	1.1	1.1	0.5	0.9
Δ c.p.m	272.4	-5.3	167.9	-9.2	8971.6	1352.4	48.8	46.0	-1844.3	-62.7
log 10 Δ c.p.m	2.4	1.0	2.2	1.0	4.0	3.1	1.7	1.7	1.0	1.0
SE Δ c.p.m (c.p.m)	347.4	13.9	77.5	46.0	7123.2	5195.1	169.7	461.0	1114.6	117.6
SE Δ c.p.m (%)	127.5	263.7	46.2	500.8	79.4	384.1	347.6	1003.1	60.4	187.6
Geomean Δ c.p.m	81.2									

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 4. Pre-challenge, seven weeks post-*BCG* booster vaccination

Lymphocyte proliferation data. (mean c.p.m of quadruplicate wells)

Vaccinated animals (Tag No.)	44^	61^	118^	138^	145^	158^	167^	176^	195^	203^
Unstimulated wells	244.1	1064.8	45.6	590.6	180.7	379	1218.6	207.1	417.2	378.5
c.p.m 1st quadruplicate	235.3	670.6	79.8	3478.1	205.2	457.3	1473	192.9	467.7	530.6
	135.0	537.2	80.0	937.9	236.7	388.1	1899.3	153.9	449.9	498.4
	172.5	719.7	67.1	386.9	168.0	404.5	1184.2	246.8	550.7	322.6
Unstimulated wells	180.2	438.5	59.5	412.6	128.9	349.3	807.1	148.2	267.5	269.7
c.p.m 2nd quadruplicate	175.2	347.1	70.5	361.4	152.3	541.3	1816.4	136.8	436.6	239.3
	150.2	336.5	36.2	386.7	75.4	266.5	835.4	121.0	327.3	315.7
	176.6	265.5	133.2	435.9	133.8	611.6	1680.3	113.0	306.4	270.5
Mean unstimulated	183.6	547.5	71.5	873.8	161.4	412.2	1364.3	165.0	402.9	353.2
SD unstimulated (c.p.m)	37.9	264.2	29.3	1069.6	49.6	89.0	421.9	46.4	94.8	108.4
SE unstimulated (c.p.m)	13.4	93.4	10.4	378.2	17.5	31.5	149.2	16.4	33.5	38.3
ConA 0.25µg/ml	51504.8	13344.3	31776.4	11837.3	25826.3	42430.4	36053.5	63840.0	36826.4	22732.1
SD (%)	8.5	18.6	17.5	12.2	5.4	26.2	13.0	24.8	28.7	29.0
SD (c.p.m)	4352.2	2480.7	5554.5	1443.0	1389.5	11108.3	4697.8	15813.2	10580.2	6587.8
SE (c.p.m)	2176.1	1240.4	2777.3	721.5	694.7	5554.1	2348.9	7906.6	5290.1	3293.9
Stimulation Index	280.5	24.4	444.5	13.5	160.0	102.9	26.4	387.0	91.4	64.4
Δ c.p.m	51321.1	12796.8	31704.9	10963.5	25665.0	42018.2	34689.2	63675.0	36423.5	22379.0
log 10 Δ c.p.m	4.7	4.1	4.5	4.0	4.4	4.6	4.5	4.8	4.6	4.3
SE Δ c.p.m (c.p.m)	2189.5	1333.8	2787.6	1099.6	712.3	5585.6	2498.1	7923.0	5323.6	3332.2
SE Δ c.p.m (%)	4.3	10.4	8.8	10.0	2.8	13.3	7.2	12.4	14.6	14.9
Geomean Δ c.p.m	29151.7									
PPD-B 6.25µg/ml	5256.6	82049.8	2801.5	64870.8	274.8	6034.8	48359.9	1873.7	3643.2	9231.7
SD (%)	85.2	14.0	69.1	9.0	3.3	84.0	15.8	86.4	62.8	70.9
SD (c.p.m)	4476.5	11519.8	1935.6	5851.3	9.1	5069.2	7621.5	1618.8	2287.9	6547.1
SE (c.p.m)	2238.3	5759.9	967.8	2925.7	4.6	2534.6	3810.8	809.4	1144.0	3273.5
Stimulation Index	28.6	149.9	39.2	74.2	1.7	14.6	35.4	11.4	9.0	26.1
Δ c.p.m	5073.0	81502.3	2730.1	63997.0	113.4	5622.6	46995.6	1708.7	3240.3	8878.5
log 10 Δ c.p.m	3.7	4.9	3.4	4.8	2.1	3.7	4.7	3.2	3.5	3.9
SE Δ c.p.m (c.p.m)	2251.7	5853.3	978.2	3303.8	22.1	2566.1	3959.9	825.8	1177.5	3311.9
SE Δ c.p.m (%)	44.4	7.2	35.8	5.2	19.5	45.6	8.4	48.3	36.3	37.3
Geomean Δ c.p.m	6349.1									
PPD-B 1.25µg/ml	2995.3	69179.3	1132.5	15807.7	193.0	1949.6	28668.3	3194.4	2652.4	842.4
SD (%)	130.7	21.8	150.8	94.1	36.4	79.5	14.1	164.2	96.3	26.9
SD (c.p.m)	3915.5	15101.8	1707.9	14876.7	70.3	1550.7	4067.5	5244.2	2553.0	226.3
SE (c.p.m)	1957.8	7550.9	854.0	7438.3	35.2	775.4	2033.8	2622.1	1276.5	113.1
Stimulation Index	16.3	126.4	15.8	18.1	1.2	4.7	21.2	19.4	6.6	2.4
Δ c.p.m	2811.7	68631.8	1061.0	14934.0	31.6	1537.4	27504.0	3029.4	2249.5	489.3
log 10 Δ c.p.m	3.4	4.8	3.0	4.2	1.5	3.2	4.4	3.5	3.4	2.7
SE Δ c.p.m (c.p.m)	1971.2	7644.3	864.3	7816.5	52.7	806.8	2182.9	2638.5	1310.0	151.5
SE Δ c.p.m (%)	70.1	11.1	81.5	52.3	166.8	52.5	7.9	87.1	58.2	31.0
Geomean Δ c.p.m	2590.6									
PPD-A 6.25µg/ml	13149.1	106176.9	3771.4	78729.8	595.6	19968.0	28591.1	12672.3	9453.3	28324.2
SD (%)	72.5	9.6	80.5	2.1	85.0	32.3	22.3	79.8	71.6	32.5
SD (c.p.m)	9534.4	10235.5	3035.6	1684.8	506.5	6445.7	6361.5	10113.8	6769.5	9191.2
SE (c.p.m)	4767.2	5117.7	1517.8	842.4	253.2	3222.8	3180.8	5056.9	3384.7	4595.6
Stimulation Index	71.6	193.9	52.8	90.1	3.7	48.4	21.0	76.8	23.5	80.2
Δ c.p.m	12965.5	105629.4	3699.9	77856.1	434.2	19555.8	27226.8	12507.3	9050.4	27971.1
log 10 Δ c.p.m	4.1	5.0	3.6	4.9	2.6	4.3	4.4	4.1	4.0	4.4
SE Δ c.p.m (c.p.m)	4780.6	5211.1	1528.2	1220.6	270.8	3254.3	3329.9	5073.3	3418.3	4633.9
SE Δ c.p.m (%)	36.9	4.9	41.3	1.6	62.4	16.6	12.2	40.6	37.8	16.6
Geomean Δ c.p.m	13997.8									
PPD-A 1.25µg/ml	6101.3	102454.4	1568.0	81838.0	222.9	14635.6	11992.5	2529.2	3762.4	8275.2
SD (%)	77.9	12.9	55.1	8.4	11.2	60.8	58.3	86.9	86.2	76.9
SD (c.p.m)	4751.7	13185.9	864.4	6907.1	24.9	8895.5	6991.6	2198.1	3241.7	6359.5
SE (c.p.m)	2375.8	6592.9	432.2	3453.6	12.4	4447.8	3495.8	1099.1	1620.8	3179.8
Stimulation Index	33.2	187.1	21.9	93.7	1.4	35.5	8.8	15.3	9.3	23.4
Δ c.p.m	5917.6	101907.0	1496.5	80964.2	61.5	14223.4	10628.2	2364.2	3359.5	7922.1
log 10 Δ c.p.m	3.8	5.0	3.2	4.9	1.8	4.2	4.0	3.4	3.5	3.9
SE Δ c.p.m (c.p.m)	2389.2	6686.4	442.6	3831.7	30.0	4479.2	3645.0	1115.5	1654.4	3218.1
SE Δ c.p.m (%)	40.4	6.6	29.6	4.7	48.7	31.5	34.3	47.2	49.2	40.6
Geomean Δ c.p.m	5795.6									
8.4 kDa 6.25µg/ml	1153.6	5096.6	162.7	36797.3	277.3	821.4	21053.1	584.9	1803.4	3146.7
SD (%)	55.8	65.7	22.3	72.9	11.4	86.3	34.9	84.8	21.9	51.3
SD (c.p.m)	643.3	3345.9	36.3	26810.5	31.7	708.6	7351.8	495.8	394.9	1613.6
SE (c.p.m)	321.7	1673.0	18.1	13405.3	15.8	354.3	3675.9	247.9	197.5	806.8
Stimulation Index	6.3	9.3	2.3	42.1	1.7	2.0	15.4	3.5	4.5	8.9
Δ c.p.m	969.9	4549.2	91.2	35923.6	116.0	409.2	19688.9	419.9	1400.5	2793.5
log 10 Δ c.p.m	3.0	3.7	2.0	4.6	2.1	2.6	4.3	2.6	3.1	3.4
SE Δ c.p.m (c.p.m)	335.1	1766.4	28.5	13783.4	33.4	385.8	3825.1	264.3	231.0	845.2
SE Δ c.p.m (%)	34.5	38.8	31.2	38.4	28.8	94.3	19.4	62.9	16.5	30.3
Geomean Δ c.p.m	1363.4									
8.4 kDa 1.25µg/ml	639.0	2309.4	147.8	29961.3	293.8	242.5	18996.5	287.8	2600.6	742.8
SD (%)	67.5	90.9	32.1	70.2	31.8	37.2	35.5	49.1	69.1	53.5
SD (c.p.m)	431.4	2098.8	47.5	21044.8	93.3	90.2	6736.2	141.3	1796.2	397.2
SE (c.p.m)	215.7	1049.4	23.7	10522.4	46.6	45.1	3368.1	70.6	898.1	198.6
Stimulation Index	3.5	4.2	2.1	34.3	1.8	0.6	13.9	1.7	6.5	2.1
Δ c.p.m	455.3	1761.9	76.3	29087.5	132.4	-169.7	17632.2	122.9	2197.7	389.6
log 10 Δ c.p.m	2.7	3.2	1.9	4.5	2.1	1.0	4.2	2.1	3.3	2.6
SE Δ c.p.m (c.p.m)	229.1	1142.8	34.1	10900.5	64.2	76.6	3517.2	87.1	931.6	237.0
SE Δ c.p.m (%)	50.3	64.9	44.7	37.5	48.5	45.1	19.9	70.9	42.4	60.8
Geomean Δ c.p.m	580.8									

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 4. Pre-challenge seven weeks post-BCG booster vaccination

IFN-γ data. (OD450 x 1000)

Unvaccinated animals (Tag No.)

	31	87	90	116	122	194	196	197	208	215
unstimulated 1	275	120	149	113	542	487	151	260	402	117
unstimulated 2	140	84	105	93	434	320	125	177	257	105
Mean unstimulated	207.5	102	127	103	488	403.5	138	218.5	329.5	111
ConA 0.25µg/ml	1524	416	1474	1425	3000	2236	1524	686	1664	1519
Δ OD450	1316.5	314	1347	1322	2512	1832.5	1386	467.5	1334.5	1408
SI	7.34	4.08	11.61	13.83	6.15	5.54	11.04	3.14	5.05	13.68
mean Δ OD450	1324.00									
Geomean Δ OD450	1152.25									
PPD-B 6.25µg/ml	674	118	210	116	2842	372	169	273	367	541
Δ OD450	466.5	16	83	13	2354	-31.5	31	54.5	37.5	430
SI	3.25	1.16	1.65	1.13	5.82	0.92	1.22	1.25	1.11	4.87
mean Δ OD450	345.40									
Geomean Δ OD450	61.51									
PPD-B 1.25µg/ml	222	91	137	93	2421	298	128	214	294	160
Δ OD450	14.5	-11	10	-10	1933	-105.5	-10	-4.5	-35.5	49
SI	1.07	0.89	1.08	0.90	4.96	0.74	0.93	0.98	0.89	1.44
mean Δ OD450	183.00									
Geomean Δ OD450	6.60									
PPD-A 6.25µg/ml	919	247	380	430	3000	702	176	347	823	493
Δ OD450	711.5	145	253	327	2512	298.5	38	128.5	493.5	382
SI	4.43	2.42	2.99	4.17	6.15	1.74	1.28	1.59	2.50	4.44
mean Δ OD450	528.90									
Geomean Δ OD450	299.93									
PPD-A 1.25µg/ml	389	131	158	210	2013	512	187	348	682	118
Δ OD450	181.5	29	31	107	1525	108.5	49	129.5	352.5	7
SI	1.87	1.28	1.24	2.04	4.13	1.27	1.36	1.59	2.07	1.06
mean Δ OD450	252.00									
Geomean Δ OD450	92.37									
6.4 kDa 6.25µg/ml	485	92	179	110	1356	251	176	254	285	378
Δ OD450	277.5	-10	52	7	868	-152.5	38	35.5	-44.5	267
SI	2.34	0.90	1.41	1.07	2.78	0.62	1.28	1.16	0.86	3.41
mean Δ OD450	133.80									
Geomean Δ OD450	25.28									
6.4 kDa 1.25µg/ml	229	93	132	99	625	256	132	206	244	154
Δ OD450	21.5	-9	5	-4	137	-147.5	-6	-12.5	-85.5	43
SI	1.10	0.91	1.04	0.96	1.28	0.63	0.96	0.94	0.74	1.39
mean Δ OD450	-5.80									
Geomean Δ OD450	4.85									

Vaccinated animals (Tag No.)

	44^	61^	118^	138^	145^	158^	167^	176^	195^	203^
unstimulated 1	127	350	97	292	103	119	267	87	112	92
unstimulated 2	94	132	93	179	114	116	187	90	109	100
Mean unstimulated	110.5	241	95	235.5	108.5	117.5	227	88.5	110.5	96
ConA 0.25µg/ml	1580	1466	583	2515	1612	1077	3000	1135	536	663
Δ OD450	1469.5	1225	488	2279.5	1503.5	959.5	2773	1046.5	425.5	567
SI	14.30	6.08	6.14	10.68	14.86	9.17	13.22	12.82	4.85	6.91
mean Δ OD450	1273.7									
Geomean Δ OD450	1073.0									
PPD-B 6.25µg/ml	385	2179	213	1973	119	193	1045	141	383	170
Δ OD450	274.5	1938	118	1737.5	10.5	75.5	818	52.5	272.5	74
SI	3.48	9.04	2.24	8.38	1.10	1.64	4.60	1.59	3.47	1.77
mean Δ OD450	537.1									
Geomean Δ OD450	193.8									
PPD-B 1.25µg/ml	242	1236	140	712	98	141	620	136	157	99
Δ OD450	131.5	995	45	476.5	-10.5	23.5	393	47.5	46.5	3
SI	2.19	5.13	1.47	3.02	0.90	1.20	2.73	1.54	1.42	1.03
mean Δ OD450	215.1									
Geomean Δ OD450	55.1									
PPD-A 6.25µg/ml	387	2253	247	3000	144	330	852	239	325	356
Δ OD450	276.5	2012	152	2764.5	35.5	212.5	625	150.5	214.5	260
SI	3.50	9.35	2.60	12.74	1.33	2.81	3.75	2.70	2.94	3.71
mean Δ OD450	670.3									
Geomean Δ OD450	313.8									
PPD-A 1.25µg/ml	236	1733	193	2404	93	253	491	120	175	141
Δ OD450	125.5	1492	98	2168.5	-15.5	135.5	264	31.5	64.5	45
SI	2.14	7.19	2.03	10.21	0.86	2.15	2.16	1.36	1.58	1.47
mean Δ OD450	440.9									
Geomean Δ OD450	106.9									
6.4 kDa 6.25µg/ml	254	509	131	1455	117	262	607	116	263	167
Δ OD450	143.5	268	36	1219.5	8.5	144.5	380	27.5	152.5	71
SI	2.30	2.11	1.38	6.18	1.08	2.23	2.67	1.31	2.38	1.74
mean Δ OD450	245.1									
Geomean Δ OD450	108.9									
6.4 kDa 1.25µg/ml	139	473	110	878	127	110	449	110	192	133
Δ OD450	28.5	232	15	642.5	18.5	-7.5	222	21.5	81.5	37
SI	1.26	1.96	1.16	3.73	1.17	0.94	1.98	1.24	1.74	1.39
mean Δ OD450	129.1									
Geomean Δ OD450	43.7									

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 5. Five weeks post-challenge

Lymphocyte proliferation data. (mean c.p.m of quadruplicate wells)

Unvaccinated animals (Tag No.)	31	87	90	116	122	194	196	197	208	215
Unstimulated wells	2874.3	94.5	294.2	1718.7	1624.5	413.7	668.9	344	156.7	370.5
c.p.m	510.4	130.7	205.3	2785.2	1503.8	483.6	379.7	318.4	173.2	860.6
	1011.8	108.7	270.0	2542.1	1500.2	336.0	609.4	218.6	167.5	975.7
	4357.0	102.1	213.3	2799.4	197.2	2860.8	315.3	601.8	122.5	3050.5
Mean unstimulated	2188.4	109.0	245.7	2461.4	1206.4	1023.5	493.3	370.7	155.0	1314.3
SD unstimulated (c.p.m)	1767.6	15.6	43.3	509.0	675.3	1226.3	172.1	163.3	22.7	1186.8
SE unstimulated (c.p.m)	624.9	5.5	15.3	180.0	238.8	433.6	60.9	57.7	8.0	419.6
ConA 0.25µg/ml	34229.8	21089.4	18667.6	37836.4	7460.5	58441.6	34750.1	14376.4	33041.4	36573.8
SD (%)	14.4	23.0	10.1	7.7	30.6	16.2	17.4	21.6	16.1	16.3
SD (c.p.m)	4918.8	4850.6	1892.9	2913.4	2283.7	9473.4	6046.5	3103.9	5332.9	5961.5
SE (c.p.m)	2459.4	2425.3	946.4	1456.7	1141.8	4736.7	3023.3	1551.9	2666.4	2980.8
Stimulation Index	15.6	193.5	76.0	15.4	6.2	57.1	70.4	38.8	213.2	27.8
Δ c.p.m	32041.4	20980.4	18421.9	35375.1	6254.1	57418.1	34256.8	14005.7	32886.4	35259.5
log 10 Δ c.p.m	4.5	4.3	4.3	4.5	3.8	4.8	4.5	4.1	4.5	4.5
SE Δ c.p.m (c.p.m)	3084.4	2430.8	961.8	1636.7	1380.6	5170.3	3084.1	1609.7	2674.5	3400.4
SE Δ c.p.m (%)	9.6	11.6	5.2	4.6	22.1	9.0	9.0	11.5	8.1	9.6
Geomean Δ c.p.m	24786.3									
PPD-B 6.25µg/ml	104530.9	78721.8	4132.5	101770.5	1042.3	52805.3	1255.0	3764.0	2721.5	43921.8
SD (%)	2.3	16.2	31.4	5.8	60.3	11.4	51.6	46.7	122.0	16.4
SD (c.p.m)	2362.4	12745.1	1296.0	5862.0	628.2	6019.8	647.2	1759.3	3318.9	7220.7
SE (c.p.m)	1181.2	6372.5	648.0	2931.0	314.1	3009.9	323.6	879.7	1659.4	3610.4
Stimulation Index	47.8	722.2	16.8	41.3	0.9	51.6	2.5	10.2	17.6	33.4
Δ c.p.m	102342.5	78612.8	3886.8	99309.2	-164.1	51781.8	761.7	3393.3	2566.5	42607.4
log 10 Δ c.p.m	5.0	4.9	3.6	5.0	1.0	4.7	2.9	3.5	3.4	4.6
SE Δ c.p.m (c.p.m)	1806.1	6378.0	663.3	3110.9	552.9	3443.5	384.5	937.4	1667.5	4030.0
SE Δ c.p.m (%)	1.8	8.1	17.1	3.1	336.9	6.6	50.5	27.6	65.0	9.5
Geomean Δ c.p.m	7341.0									
PPD-B 1.25µg/ml	102122.0	55031.5	2088.4	86331.2	632.0	37544.6	565.5	3415.4	858.2	37632.3
SD (%)	2.9	42.8	64.5	9.2	56.9	34.2	82.0	67.9	66.6	28.7
SD (c.p.m)	2941.1	23548.0	1346.8	7968.4	359.7	12840.3	463.8	2318.0	571.2	10789.2
SE (c.p.m)	1470.6	11774.0	673.4	3984.2	179.9	6420.1	231.9	1159.0	285.6	5394.6
Stimulation Index	46.7	504.9	8.5	35.1	0.5	36.7	1.1	9.2	5.5	28.6
Δ c.p.m	99933.7	54922.5	1842.7	83869.9	-574.5	36521.1	72.2	3044.7	703.3	36318.0
log 10 Δ c.p.m	5.0	4.7	3.3	4.9	1.0	4.6	1.9	3.5	2.8	4.6
SE Δ c.p.m (c.p.m)	2095.5	11779.5	688.7	4164.1	418.6	6853.7	292.7	1216.7	293.6	5814.2
SE Δ c.p.m (%)	2.1	21.4	37.4	5.0	72.9	18.8	405.6	40.0	41.8	16.0
Geomean Δ c.p.m	4207.5									
PPD-A 6.25µg/ml	69189.0	68011.5	4940.1	98258.5	1482.1	31615.5	627.7	3109.1	10398.9	12421.4
SD (%)	4.1	28.3	45.8	10.4	93.0	13.7	27.9	34.6	84.5	98.2
SD (c.p.m)	2850.6	19260.8	2263.6	10258.2	1378.9	4318.7	175.1	1074.2	8788.1	12202.7
SE (c.p.m)	1425.3	9630.4	1131.8	5129.1	689.5	2159.3	87.6	537.1	4394.0	6101.4
Stimulation Index	31.6	624.0	20.1	39.9	1.2	30.9	1.3	8.4	67.1	9.5
Δ c.p.m	67000.6	67902.5	4694.4	95797.2	275.6	30592.0	134.4	2738.4	10243.9	11107.0
log 10 Δ c.p.m	4.8	4.8	3.7	5.0	2.4	4.5	2.1	3.4	4.0	4.0
SE Δ c.p.m (c.p.m)	2050.2	9635.9	1147.1	5309.0	928.2	2592.9	148.4	594.8	4402.1	6521.0
SE Δ c.p.m (%)	3.1	14.2	24.4	5.5	336.8	8.5	110.5	21.7	43.0	58.7
Geomean Δ c.p.m	7689.1									
PPD-A 1.25µg/ml	68290.0	50589.7	1217.9	51365.2	606.1	3410.8	645.8	2389.4	1762.6	3657.7
SD (%)	7.6	28.7	39.6	36.8	37.7	88.0	55.4	37.4	62.9	88.7
SD (c.p.m)	5176.4	14499.0	482.0	18912.7	228.7	3001.8	357.9	892.9	1108.8	3243.6
SE (c.p.m)	2588.2	7249.5	241.0	9456.3	114.3	1500.9	178.9	446.5	554.4	1621.8
Stimulation Index	31.2	464.1	5.0	20.9	0.5	3.3	1.3	6.4	11.4	2.8
Δ c.p.m	66101.6	50480.7	972.2	48903.9	-600.3	2387.2	152.5	2018.7	1607.6	2343.3
log 10 Δ c.p.m	4.8	4.7	3.0	4.7	1.0	3.4	2.2	3.3	3.2	3.4
SE Δ c.p.m (c.p.m)	3213.1	7255.0	256.3	9636.3	353.1	1934.5	239.8	504.2	562.4	2041.4
SE Δ c.p.m (%)	4.9	14.4	26.4	19.7	58.8	81.0	157.2	25.0	35.0	87.1
Geomean Δ c.p.m	2313.5									
6.4 kDa 6.25µg/ml	27170.8	560.7	487.0	1522.2	173.1	6199.8	360.2	538.6	306.4	2060.1
SD (%)	72.5	65.7	24.7	82.6	57.7	92.5	57.7	34.5	40.9	29.6
SD (c.p.m)	19701.6	368.2	120.2	1257.7	99.9	5737.2	207.9	185.6	125.4	609.8
SE (c.p.m)	9850.8	184.1	60.1	628.8	50.0	2868.6	103.9	92.8	62.7	304.9
Stimulation Index	12.4	5.1	2.0	0.6	0.1	6.1	0.7	1.5	2.0	1.6
Δ c.p.m	24982.5	451.7	241.3	-939.1	-1033.3	5176.2	-133.1	167.9	151.4	745.8
log 10 Δ c.p.m	4.4	2.7	2.4	1.0	1.0	3.7	1.0	2.2	2.2	2.9
SE Δ c.p.m (c.p.m)	10475.7	189.6	75.4	803.8	288.7	3302.2	164.8	150.5	70.7	724.5
SE Δ c.p.m (%)	41.9	42.0	31.2	86.1	27.9	63.8	123.8	89.7	46.7	97.1
Geomean Δ c.p.m	220.1									
6.4 kDa 1.25µg/ml	26522.8	253.7	272.9	698.3	80.8	4351.8	176.9	1545.2	140.1	2251.5
SD (%)	74.1	44.4	63.7	48.2	27.9	46.0	8.6	95.4	35.2	82.6
SD (c.p.m)	19640.1	112.7	173.9	336.6	22.5	2002.7	15.2	1474.5	49.2	1858.6
SE (c.p.m)	9820.1	56.4	87.0	168.3	11.3	1001.4	7.6	737.2	24.6	929.3
Stimulation Index	12.1	2.3	1.1	0.3	0.1	4.3	0.4	4.2	0.9	1.7
Δ c.p.m	24334.4	144.7	27.2	-1763.1	-1125.6	3328.3	-316.5	1174.5	-14.9	937.1
log 10 Δ c.p.m	4.4	2.2	1.4	1.0	1.0	3.5	1.0	3.1	1.0	3.0
SE Δ c.p.m (c.p.m)	10445.0	61.9	102.3	348.2	250.0	1434.9	68.5	795.0	32.6	1348.9
SE Δ c.p.m (%)	42.9	42.7	376.0	19.8	22.2	43.1	21.6	67.7	218.7	143.9
Geomean Δ c.p.m	142.7									

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 5. Five weeks post-challenge

Lymphocyte proliferation data. (mean c.p.m of quadruplicate wells)

Vaccinated animals (Tag No.)

	44^	61^	118^	138^	145^	158^	167^	176^	195^	203^
Blank 1	74.6	428.3	56.3	497.9	3227.6	636	412.6	107.6	403.4	136.5
	75.1	723.7	109.9	536.7	2001	227.1	337.4	159.2	116.1	113.1
	159.4	1502.4	206.8	392.8	1784.9	118.3	252.7	76.4	415.8	153.3
	181.7	500.9	138.2	345.0	2197.8	163.2	514.5	196.0	210.3	91.9
Mean Blanks	122.7	788.8	127.8	443.1	2302.8	286.2	379.3	134.8	286.4	123.7
SD Blanks	56.0	492.0	62.7	89.3	639.2	237.5	111.3	53.2	147.5	26.9
SE Blanks	19.8	174.0	22.2	31.6	226.0	84.0	39.4	18.8	52.1	9.5
ConA 0.25µg/ml	41315.1	20876.4	18577.3	30710.2	47094.4	40697.2	42417.4	46150.9	36096.2	19101.2
SD (%)	5.3	18.9	23.9	18.7	5.6	19.2	17.7	21.0	21.7	6.7
SD (c.p.m)	2193.8	3939.4	4447.4	5739.7	2613.7	7813.9	7490.9	9710.1	7822.0	1276.0
SE (c.p.m)	1096.9	1969.7	2223.7	2869.9	1306.9	3906.9	3745.5	4855.1	3911.0	638.0
Stimulation Index	336.7	26.5	145.4	69.3	20.5	142.2	111.8	342.4	126.0	154.4
Δ c.p.m	41192.4	20087.6	18449.5	30267.1	44791.5	40411.0	42038.1	46016.1	35809.8	18977.5
log 10 Δ c.p.m	4.6	4.3	4.3	4.5	4.7	4.6	4.6	4.7	4.6	4.3
SE Δ c.p.m (c.p.m)	1116.7	2143.7	2245.9	2901.4	1532.9	3990.9	3784.8	4873.9	3963.2	647.5
SE Δ c.p.m (%)	2.7	10.7	12.2	9.6	3.4	9.9	9.0	10.6	11.1	3.4
Geomean Δ c.p.m	31924.1									
PPD-B 6.25µg/ml	16733.1	68170.1	22095.1	7729.9	108496.1	65139.5	40552.0	2570.1	6534.1	355.4
SD (%)	120.7	6068.0	33.8	69.7	5.0	20.9	14.9	58.9	50.8	38.1
SD (c.p.m)	20201.9	4136561.1	7463.7	5383.9	5435.7	13627.2	6030.1	1512.8	3320.6	135.6
SE (c.p.m)	10100.9	2068280.5	3731.9	2691.9	2717.8	6813.6	3015.0	756.4	1660.3	67.8
Stimulation Index	136.4	86.4	172.9	17.4	47.1	227.6	106.9	19.1	22.8	2.9
Δ c.p.m	16610.4	67381.3	21967.3	7286.8	106193.3	64853.3	40172.7	2435.3	6247.7	231.7
log 10 Δ c.p.m	4.2	4.8	4.3	3.9	5.0	4.8	4.6	3.4	3.8	2.4
SE Δ c.p.m (c.p.m)	10120.7	2068454.5	3754.0	2723.5	2943.8	6897.5	3054.4	775.2	1712.4	77.3
SE Δ c.p.m (%)	60.9	3069.8	17.1	37.4	2.8	10.6	7.6	31.8	27.4	33.3
Geomean Δ c.p.m	13311.9									
PPD-B 1.25µg/ml	4479.0	62348.6	16924.0	3895.6	107687.0	25938.7	15953.2	669.3	1839.4	103.7
SD (%)	97.8	16.2	55.6	149.4	6.1	66.2	49.7	73.1	35.3	39.4
SD (c.p.m)	4382.3	10088.0	9416.5	5819.7	6547.4	17161.0	7922.3	489.1	649.7	40.8
SE (c.p.m)	2191.1	5044.0	4708.2	2909.8	3273.7	8580.5	3961.2	244.5	324.8	20.4
Stimulation Index	36.5	79.0	132.4	8.8	46.8	90.6	42.1	5.0	6.4	0.8
Δ c.p.m	4356.3	61559.8	16796.2	3452.5	105384.2	25652.6	15573.9	534.5	1553.0	-20.0
log 10 Δ c.p.m	3.6	4.8	4.2	3.5	5.0	4.4	4.2	2.7	3.2	1.0
SE Δ c.p.m (c.p.m)	2210.9	5218.0	4730.4	2941.4	3499.7	8664.5	4000.5	263.3	377.0	29.9
SE Δ c.p.m (%)	50.8	8.5	28.2	85.2	3.3	33.8	25.7	49.3	24.3	149.3
Geomean Δ c.p.m	4715.4									
PPD-A 6.25µg/ml	19714.8	85729.1	35621.1	34426.4	98962.2	53389.1	16136.0	6115.5	2278.9	1080.3
SD (%)	103.2	11.2	70.6	32.8	7.2	37.2	20.9	66.4	14.7	119.2
SD (c.p.m)	20343.7	9593.1	25159.2	11298.8	7135.2	19839.4	3375.6	4058.3	334.3	1288.0
SE (c.p.m)	10171.8	4796.5	12579.6	5649.4	3567.6	9919.7	1687.8	2029.1	167.2	644.0
Stimulation Index	160.7	108.7	278.7	77.7	43.0	186.6	42.5	45.4	8.0	8.7
Δ c.p.m	19592.1	84940.3	35493.3	33983.3	96659.4	53102.9	15756.7	5980.7	1992.5	956.6
log 10 Δ c.p.m	4.3	4.9	4.6	4.5	5.0	4.7	4.2	3.8	3.3	3.0
SE Δ c.p.m (c.p.m)	10191.6	4970.5	12601.8	5680.9	3793.6	10003.6	1727.2	2047.9	219.3	653.5
SE Δ c.p.m (%)	52.0	5.9	35.5	16.7	3.9	18.8	11.0	34.2	11.0	68.3
Geomean Δ c.p.m	16855.0									
PPD-A 1.25µg/ml	5658.0	89207.0	13867.8	19589.5	95376.2	42041.5	3002.9	2058.1	1385.2	240.5
SD (%)	81.2	3.3	49.5	119.3	11.4	22.9	36.9	127.8	62.4	87.4
SD (c.p.m)	4592.6	2952.8	6857.6	23368.3	10872.9	9627.5	1107.8	2630.5	864.7	210.2
SE (c.p.m)	2296.3	1476.4	3428.8	11684.2	5436.4	4813.8	553.9	1315.2	432.3	105.1
Stimulation Index	46.1	113.1	108.5	44.2	41.4	146.9	7.9	15.3	4.8	1.9
Δ c.p.m	5535.3	88418.2	13740.0	19146.4	93073.4	41755.4	2623.6	1923.3	1098.8	116.8
log 10 Δ c.p.m	3.7	4.9	4.1	4.3	5.0	4.6	3.4	3.3	3.0	2.1
SE Δ c.p.m (c.p.m)	2316.1	1650.3	3451.0	11715.7	5662.4	4897.7	593.2	1334.0	484.5	114.6
SE Δ c.p.m (%)	41.8	1.9	25.1	61.2	6.1	11.7	22.6	69.4	44.1	98.1
Geomean Δ c.p.m	7096.8									
8.4 kDa 6.25µg/ml	7533.0	3439.4	122.6	6211.1	18197.1	1369.2	11080.8	368.4	3394.8	194.3
SD (%)	97.3	55.7	50.7	121.0	58.7	17.5	29.5	32.7	76.2	63.9
SD (c.p.m)	7332.7	1916.8	62.1	7516.7	10679.9	238.9	3267.7	120.6	2586.2	124.1
SE (c.p.m)	3666.3	958.4	31.1	3758.4	5339.9	119.5	1633.9	60.3	1293.1	62.0
Stimulation Index	61.4	4.4	1.0	14.0	7.9	4.8	29.2	2.7	11.9	1.6
Δ c.p.m	7410.3	2650.6	-5.2	5768.0	15894.3	1083.1	10701.5	233.6	3108.4	70.6
log 10 Δ c.p.m	3.9	3.4	1.0	3.8	4.2	3.0	4.0	2.4	3.5	1.8
SE Δ c.p.m (c.p.m)	3686.1	1132.4	53.2	3789.9	5565.9	203.4	1673.2	79.1	1345.2	71.5
SE Δ c.p.m (%)	49.7	42.7	1017.6	65.7	35.0	18.8	15.6	33.9	43.3	101.4
Geomean Δ c.p.m	1267.4									
8.4 kDa 1.25µg/ml	3927.3	1354.4	84.1	2222.6	8739.2	209.9	7926.1	423.9	3343.9	91.0
SD (%)	78.1	81.2	64.1	42.9	74.2	52.4	69.6	79.9	74.4	15.5
SD (c.p.m)	3068.0	1099.5	53.9	953.5	6485.3	109.9	5518.9	338.5	2486.8	14.1
SE (c.p.m)	1534.0	549.7	27.0	476.8	3242.7	55.0	2759.5	169.3	1243.4	7.1
Stimulation Index	32.0	1.7	0.7	5.0	3.8	0.7	20.9	3.1	11.7	0.7
Δ c.p.m	3804.6	565.5	-43.7	1779.5	6436.3	-76.3	7546.8	289.1	3057.5	-32.7
log 10 Δ c.p.m	3.6	2.8	1.0	3.3	3.8	1.0	3.9	2.5	3.5	1.0
SE Δ c.p.m (c.p.m)	1553.8	723.7	49.1	508.3	3468.6	138.9	2798.8	188.1	1295.5	16.5
SE Δ c.p.m (%)	40.8	128.0	112.4	28.6	53.9	182.1	37.1	65.1	42.4	50.6
Geomean Δ c.p.m	418.4									

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 5. Five weeks post-challenge.										
IFN-γ data. (OD450 x 1000)										
Unvaccinated animals (Tag No.)	31	87	90	116	122	194	196	197	208	215
Unstimulated	450	158	111	84	87	125	82	117	85	146
ConA 0.25µg/ml	820	391	669	1236	360	711	231	245	228	626
Δ OD450	370	233	558	1152	273	586	149	128	143	480
SI	1.8	2.5	6.0	14.7	4.1	5.7	2.8	2.1	2.7	4.3
mean Δ OD450	407.2									
Geomean Δ OD450	321									
PPD-B 6.25µg/ml	1962	716	259	1289	145	404	101	174	132	582
Δ OD450	1512	558	148	1205	58	279	19	57	47	436
SI	4.36	4.53	2.33	15.35	1.67	3.23	1.23	1.49	1.55	3.99
mean Δ OD450	431.9									
Geomean Δ OD450	188									
PPD-B 1.25µg/ml	1967	549	164	978	147	266	94	158	104	484
Δ OD450	1517	391	53	894	60	141	12	41	19	338
SI	4.37	3.47	1.48	11.64	1.69	2.13	1.15	1.35	1.22	3.32
mean Δ OD450	346.6									
Geomean Δ OD450	122									
PPD-A 6.25µg/ml	1656	833	239	884	134	360	86	189	224	350
Δ OD450	1206	675	128	800	47	235	4	72	139	204
SI	3.68	5.27	2.15	10.52	1.54	2.88	1.05	1.62	2.64	2.40
mean Δ OD450	351.0									
Geomean Δ OD450	154									
PPD-A 1.25µg/ml	1214	444	167	428	115	158	92	218	141	231
Δ OD450	764	286	56	344	28	33	10	101	5	85
SI	2.70	2.81	1.50	5.10	1.32	1.26	1.12	1.86	1.6	1.58
mean Δ OD450	176.3									
Geomean Δ OD450	85									
6.4 kDa 6.25µg/ml	871	405	133	124	80	173	108	183	132	228
Δ OD450	421	247	22	40	-7	48	26	66	47	82
SI	1.94	2.56	1.20	1.48	0.92	1.38	1.32	1.56	1.55	1.56
mean Δ OD450	99.2									
Geomean Δ OD450	46									
6.4 kDa 1.25µg/ml	908	106	89	89	133	250	111	164	99	164
Δ OD450	458	-52	-22	5	46	125	29	47	14	18
SI	2.02	0.67	0.80	1.06	1.53	2.00	1.35	1.40	1.16	1.12
mean Δ OD450	66.8									
Geomean Δ OD450	20									
Vaccinated animals (Tag No.)	44^	61^	118^	138^	145^	158^	167^	176^	195^	203^
Unstimulated	111	249	94	219	711	110	93	81	100	80
ConA 0.25µg/ml	584	489	320	666	1422	719	504	260	316	186
Δ OD450	473	240	226	447	711	609	411	179	216	106
SI	5.26	1.96	3.40	3.04	2.00	6.54	5.42	3.21	3.16	2.33
mean Δ OD450	361.8									
Geomean Δ OD450	311									
PPD-B 6.25µg/ml	316	1031	224	234	1716	412	312	93	219	101
Δ OD450	205	782	130	15	1005	302	219	12	119	21
SI	2.85	4.14	2.38	1.07	2.41	3.75	3.35	1.15	2.19	1.26
mean Δ OD450	281.0									
Geomean Δ OD450	120									
PPD-B 1.25µg/ml	163	726	199	179	1787	244	183	91	144	80
Δ OD450	52	477	105	-40	1076	134	90	10	44	0
SI	1.47	2.92	2.12	0.82	2.51	2.22	1.97	1.12	1.44	1.00
mean Δ OD450	194.8									
Geomean Δ OD450	45									
PPD-A 6.25µg/ml	464	874	284	664	1951	532	245	123	204	104
Δ OD450	353	625	190	445	1240	422	152	42	104	24
SI	4.18	3.51	3.02	3.03	2.74	4.84	2.63	1.52	2.04	1.30
mean Δ OD450	359.7									
Geomean Δ OD450	209									
PPD-A 1.25µg/ml	242	779	196	413	1732	489	172	98	163	105
Δ OD450	131	530	102	194	1021	379	79	17	63	25
SI	2.18	3.13	2.09	1.89	2.44	4.45	1.85	1.21	1.63	1.31
mean Δ OD450	254.1									
Geomean Δ OD450	127									
6.4 kDa 6.25µg/ml	427	282	108	281	960	136	219	103	241	137
Δ OD450	316	33	14	62	249	26	126	22	141	57
SI	3.85	1.13	1.15	1.28	1.35	1.24	2.35	1.27	2.41	1.71
mean Δ OD450	104.6									
Geomean Δ OD450	65									
6.4 kDa 1.25µg/ml	284	168	142	173	600	250	172	108	198	109
Δ OD450	173	-81	48	-46	-111	140	79	27	98	29
SI	2.56	0.67	1.51	0.79	0.84	2.27	1.85	1.33	1.98	1.36
mean Δ OD450	35.6									
Geomean Δ OD450	22									

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 6. Ten weeks post-challenge

Lymphocyte proliferation data (mean c.p.m of quadruplicate wells)

Unvaccinated animals (Tag No.)	31	87	90	116	122	194	196	197	208	215
Unstimulated wells	627.3	101.9	342.2	419.2	843	3405.9	397.9	199.6	326.2	2121.7
c.p.m 1st quadruplicate	6568.6	96.4	1435.8	459.3	303.8	1213.6	256.2	256.1	300.4	765.6
	4722.40	390.20	712.60	468.20	645.00	769.40	303.60	248.40	210.10	770.80
	1371.00	83.10	680.80	436.60	345.40	1280.10	318.40	167.20	282.80	2559.80
Unstimulated wells	1340.9	54.1	2457.7	293.3	472.4	3883.8	101.5	599.9	154.6	590.8
c.p.m 2nd quadruplicate	2077.4	69.5	524.6	256.2	353.6	1595.1	144.9	629.5	302.4	1146.4
	1609.50	108.70	434.40	394.90	770.80	2046.60	160.30	164.20	146.20	1496.00
	704.10	76.10	412.40	439.70	373.40	2197.00	108.90	89.10	365.40	1653.10
Mean unstimulated	2377.65	122.50	875.06	395.93	513.43	2048.94	223.96	294.25	261.01	1388.03
SD unstimulated (c.p.m)	2127.87	109.64	726.86	78.75	210.84	1092.97	110.26	204.75	81.05	702.61
SE unstimulated (c.p.m)	752.32	38.76	256.98	27.84	74.54	386.42	38.98	72.39	28.66	248.41
ConA 0.25µg/ml	24428.59	40097.48	32772.54	45516.55	33383.64	60545.51	49063.21	28285.11	38180.99	31026.19
SD (%)	20.82	17.62	13.36	8.93	10.8	14.64	11.2	10.05	9.21	13.8
SD (c.p.m)	5086.03	7065.18	4378.41	4064.63	3605.43	8863.86	5495.08	2842.65	3516.47	4281.61
SE (c.p.m)	2543.02	3532.59	2189.21	2032.31	1802.72	4431.93	2747.54	1421.33	1758.23	2140.81
Stimulation Index	10.27	327.33	37.45	114.96	65.02	29.55	219.07	96.13	146.28	22.35
Δ c.p.m	22050.94	39974.98	31897.48	45120.63	32870.22	58496.57	48839.25	27990.86	37919.98	29638.17
log 10 Δ c.p.m	4.34	4.60	4.50	4.65	4.52	4.77	4.69	4.45	4.58	4.47
SE Δ c.p.m (c.p.m)	3295.33	3571.35	2446.19	2060.16	1877.26	4818.35	2786.52	1493.72	1786.89	2389.22
SE Δ c.p.m (%)	14.94	8.93	7.67	4.57	5.71	8.24	5.71	5.34	4.71	8.06
Geo mean Δ c.p.m	36089.30									
PPD-B 6.25µg/ml	132075	61364.39	46995.99	133791.64	3340.26	43105.72	292.65	314.46	1500.04	4529.99
SD (%)	8.9	17.57	50.23	6.35	60.6	34.93	18.96	51.95	55.57	40.15
SD (c.p.m)	11754.68	10781.72	23606.09	8495.77	2024.20	15056.83	55.49	163.36	833.57	1818.79
SE (c.p.m)	5877.34	5390.86	11803.04	4247.88	1012.10	7528.41	27.74	81.68	416.79	909.40
Stimulation Index	55.55	500.93	53.71	337.92	6.51	21.04	1.31	1.07	5.75	3.26
Δ c.p.m	129697.35	61241.89	46120.93	133395.72	2826.84	41056.78	68.69	20.21	1239.03	3141.97
log 10 Δ c.p.m	5.11	4.79	4.66	5.13	3.45	4.61	1.84	1.31	3.09	3.50
SE Δ c.p.m (c.p.m)	6629.65	5429.63	12060.03	4275.73	1086.64	7914.84	66.72	154.07	445.44	1157.80
SE Δ c.p.m (%)	5.11	8.87	26.15	3.21	38.44	19.28	97.14	762.36	35.95	36.85
Geo mean Δ c.p.m	5605.87									
PPD-B 1.25µg/ml	123199.25	43712.61	38585.18	125733.37	1743.99	46077.68	163.63	243.88	593.22	3819.35
SD (%)	4.88	35.99	30.56	10.22	48.41	23.53	30.53	42.94	72.41	29.59
SD (c.p.m)	6012.12	15732.17	11791.63	12849.95	844.27	10842.08	49.96	104.72	429.55	1130.15
SE (c.p.m)	3006.06	7866.08	5895.82	6424.98	422.13	5421.04	24.98	52.36	214.78	565.07
Stimulation Index	51.82	356.84	44.09	317.57	3.40	22.49	0.73	0.83	2.27	2.75
Δ c.p.m	120821.60	43590.11	37710.12	125337.45	1230.57	44028.74	-60.33	-50.37	332.21	2431.33
log 10 Δ c.p.m	5.08	4.64	4.58	5.10	3.09	4.64	1.00	1.00	2.52	3.39
SE Δ c.p.m (c.p.m)	3758.38	7904.85	6152.80	6452.82	496.68	5807.46	63.96	124.75	243.43	813.48
SE Δ c.p.m (%)	3.11	18.13	16.32	5.15	40.36	13.19	106.01	247.67	73.28	33.46
Geo mean Δ c.p.m	3189.45									
PPD-A 6.25µg/ml	83462.45	39810.58	28478.48	60068.63	15357.03	20339.21	344.34	360.11	3796.83	3059.29
SD (%)	10.73	37.76	51.36	29.25	57.15	65.35	64.87	58.36	82.8	29.46
SD (c.p.m)	8955.52	15032.48	14626.55	17570.07	8776.54	13291.67	223.37	210.16	3143.78	901.27
SE (c.p.m)	4477.76	7516.24	7313.27	8785.04	4388.27	6645.84	111.69	105.08	1571.89	450.63
Stimulation Index	35.10	324.98	32.54	151.72	29.91	9.93	1.54	1.22	14.55	2.20
Δ c.p.m	81084.80	39688.08	27603.42	59672.71	14843.61	18290.27	120.38	65.86	3535.82	1671.27
log 10 Δ c.p.m	4.91	4.60	4.44	4.78	4.17	4.26	2.08	1.82	3.55	3.22
SE Δ c.p.m (c.p.m)	5230.08	7555.00	7570.26	8812.88	4462.82	7032.26	150.67	177.47	1600.54	699.04
SE Δ c.p.m (%)	6.45	19.04	27.43	14.77	30.07	38.45	125.16	269.47	45.27	41.83
Geo mean Δ c.p.m	6065.69									
PPD-A 1.25µg/ml	80342.26	21021.14	20942.51	22460.08	3936.59	13135.13	153.83	479.27	2377.82	1735.08
SD (%)	8.2	81.53	63.65	57.35	95.26	35.96	17.42	37.41	83.51	65
SD (c.p.m)	6588.07	17138.54	13329.91	12880.86	3750.00	4723.39	26.80	179.29	1985.72	1127.80
SE (c.p.m)	3294.03	8569.27	6664.95	6440.43	1875.00	2361.70	13.40	89.65	992.86	563.90
Stimulation Index	33.79	171.60	23.93	56.73	7.67	6.41	0.69	1.63	9.11	1.25
Δ c.p.m	77964.61	20898.64	20067.45	22064.16	3423.17	11086.19	-70.13	185.02	2116.81	347.06
log 10 Δ c.p.m	4.89	4.32	4.30	4.34	3.53	4.04	1.00	2.27	3.33	2.54
SE Δ c.p.m (c.p.m)	4046.35	8608.03	6921.94	6468.27	1949.54	2748.12	52.38	162.04	1021.51	812.31
SE Δ c.p.m (%)	5.19	41.19	34.49	29.32	56.95	24.79	74.69	87.58	48.26	234.06
Geo mean Δ c.p.m	2864.64									
8.4 kDa 6.25µg/ml	54608.13	158.69	616.42	1129.04	392.69	7597.4	253.28	363.56	181.83	1821.21
SD (%)	34.03	26.42	37.2	77.81	51.74	36.75	29.86	52.91	5.83	49.23
SD (c.p.m)	18583.15	41.93	229.31	878.51	203.18	2792.04	75.63	192.36	10.60	896.58
SE (c.p.m)	9291.57	20.96	114.65	439.25	101.59	1396.02	37.81	96.18	5.30	448.29
Stimulation Index	22.97	1.30	0.70	2.85	0.76	3.71	1.13	1.24	0.70	1.31
Δ c.p.m	52230.48	36.19	-258.64	733.12	-120.74	5548.46	29.32	69.31	-79.18	433.19
log 10 Δ c.p.m	4.72	1.56	1.00	2.87	1.00	3.74	1.47	1.84	1.00	2.64
SE Δ c.p.m (c.p.m)	10043.89	59.73	371.64	467.09	176.13	1782.45	76.80	168.57	33.96	696.70
SE Δ c.p.m (%)	19.23	165.04	143.69	63.71	145.68	32.13	261.95	243.21	42.88	160.83
Geo mean Δ c.p.m	152.42									
8.4 kDa 1.25µg/ml	47712.06	86.11	1392.73	1713.11	647.8	7103.08	211.52	553.35	229.63	1224.12
SD (%)	10.29	50.78	49.87	123.86	83.32	61.74	36.81	62.11	29.96	65.86
SD (c.p.m)	4909.57	43.73	694.55	2121.86	539.75	4385.44	77.86	343.69	68.80	806.21
SE (c.p.m)	2454.79	21.86	347.28	1060.93	269.87	2192.72	38.93	171.84	34.40	403.10
Stimulation Index	20.07	0.70	1.59	4.33	1.26	3.47	0.94	1.88	0.88	0.88
Δ c.p.m	45334.41	-36.39	517.67	1317.19	134.38	5054.14	-12.44	259.10	-31.38	-163.91
log 10 Δ c.p.m	4.66	1.00	2.71	3.12	2.13	3.70	1.00	2.41	1.00	1.00
SE Δ c.p.m (c.p.m)	3207.10	60.63	604.26	1088.77	344.42	2579.14	77.91	244.23	63.05	651.51
SE Δ c.p.m (%)	7.07	166.61	116.73	82.66	256.31	51.03	626.17	94.26	200.92	397.49
Geo mean Δ c.p.m	187.74									

Wallaceville Trial Sampling time 6. Ten weeks post-challenge

Lymphocyte proliferation data. (mean c.p.m of quadruplicate wells)

Vaccinated animals (Tag No.)	44^	61^	118^	138^	145^	158^	167^	176^	195^	203^
Unstimulated wells	152.4	321.8	314.2	348.6	1487.1	363.1	516	126.9	137	114.1
c.p.m 1st quadruplicate	174.2	407.3	355.7	409.5	1706.9	398.2	691.9	120.4	146.2	174.2
	245.60	492.60	366.70	276.10	2197.90	1351.90	330.90	176.20	132.20	94.40
	169.40	694.80	518.60	500.20	1962.20	539.70	374.60	235.40	167.20	91.90
Unstimulated wells	109.8	287.2	231.4	249.5	785.7	674.2	193	107.6	366.5	85.3
c.p.m 2nd quadruplicate	133.2	368.5	244.7	284.3	2011.2	703.1	443.6	112.3	85	58.1
	218.20	932.10	461.60	468.90	1516.20	221.70	261.90	126.30	129.00	62.90
	110.20	579.70	350.40	423.60	2670.70	323.40	328.70	165.90	185.20	106.20
Mean unstimulated	164.13	510.50	355.41	370.09	1792.24	571.91	392.58	146.38	171.04	98.39
SD unstimulated (c.p.m)	48.76	218.14	96.12	94.34	560.97	357.63	157.00	43.62	91.88	36.19
SE unstimulated (c.p.m)	17.24	77.13	34.69	33.36	198.33	126.44	55.51	15.42	32.48	12.79
ConA 0.25µg/ml	48315.3	38653.1	47374.62	30587.22	47533.12	41049.72	36432.31	57428.46	38028.75	27494.21
SD (%)	9.66	35.99	7.96	23.89	8.23	12.25	19.31	15.81	16.29	10.51
SD (c.p.m)	4667.26	13911.25	3771.02	7307.29	3911.98	5028.59	7035.08	9079.44	6194.88	2889.64
SE (c.p.m)	2333.63	6955.63	1885.51	3653.64	1955.99	2514.30	3517.54	4539.72	3097.44	1444.82
Stimulation Index	294.38	75.72	133.29	82.65	26.52	71.78	92.80	392.34	222.34	279.45
Δ c.p.m	48151.18	38142.60	47019.21	30217.13	45740.88	40477.81	36039.74	57282.09	37857.71	27395.82
log 10 Δ c.p.m	4.68	4.58	4.67	4.48	4.66	4.61	4.56	4.76	4.58	4.44
SE Δ c.p.m (c.p.m)	2350.97	7032.75	1920.20	3687.00	2154.32	2640.74	3573.05	4555.14	3129.93	1457.62
SE Δ c.p.m (%)	4.88	18.44	4.08	12.20	4.71	6.52	9.91	7.95	8.27	5.32
Geomean Δ c.p.m	39945.76									
PPD-B 6.25µg/ml	30409.5	114821.23	51193.96	65201.82	121899.42	5731.51	2802.93	187.97	177.86	166.75
SD (%)	22.06	7.83	20.13	31.47	9.77	57.18	99.07	26.1	24.62	33.7
SD (c.p.m)	6708.34	8990.50	10305.34	20519.01	11909.57	3277.28	2776.86	49.06	43.79	56.19
SE (c.p.m)	3354.17	4495.25	5152.67	10259.51	5954.79	1638.64	1388.43	24.53	21.89	28.10
Stimulation Index	185.28	224.92	144.04	176.18	68.02	10.02	7.14	1.28	1.04	1.69
Δ c.p.m	30245.38	114310.73	50838.55	64831.73	120107.18	5159.60	2410.36	41.60	6.82	68.36
log 10 Δ c.p.m	4.48	5.06	4.71	4.81	5.08	3.71	3.38	1.62	0.83	1.83
SE Δ c.p.m (c.p.m)	3371.41	4572.38	5187.36	10292.86	6153.12	1765.08	1443.94	39.95	54.38	40.89
SE Δ c.p.m (%)	11.15	4.00	10.20	15.88	5.12	34.21	59.91	96.05	797.05	59.82
Geomean Δ c.p.m	3563.52									
PPD-B 1.25µg/ml	14450.81	95809.9	45543.16	27422.32	128306.09	3196.33	1202.71	155.27	140.64	103.83
SD (%)	35.89	9.97	45.75	67.79	9.53	85.86	107.99	16.96	42.27	32.64
SD (c.p.m)	5186.40	9552.25	20836.00	18589.59	12227.57	2744.37	1298.81	26.33	59.45	33.89
SE (c.p.m)	2593.20	4776.12	10418.00	9294.80	6113.79	1372.18	649.40	13.17	29.72	16.95
Stimulation Index	88.05	187.68	128.14	74.10	71.59	5.59	3.06	1.06	0.82	1.06
Δ c.p.m	14286.69	95299.40	45187.75	27052.23	126513.85	2624.42	810.14	8.90	-30.40	5.44
log 10 Δ c.p.m	4.15	4.98	4.66	4.43	5.10	3.42	2.91	0.95	1.00	0.74
SE Δ c.p.m (c.p.m)	2610.44	4853.25	10452.69	9328.15	6312.12	1498.62	704.91	28.59	62.21	29.74
SE Δ c.p.m (%)	18.27	5.09	23.13	34.48	4.99	57.10	87.01	321.40	204.65	546.43
Geomean Δ c.p.m	1712.35									
PPD-A 6.25µg/ml	23017.1	114103.11	58939.07	99034.05	104445.48	15716.81	1729.9	225.12	165.91	140.3
SD (%)	60.08	10.05	40.28	13.37	9.53	58.48	87.04	71.02	35.6	30.27
SD (c.p.m)	13828.67	11467.36	23740.66	13240.85	9953.65	9191.19	1505.70	159.88	59.06	42.47
SE (c.p.m)	6914.34	5733.68	11870.33	6620.43	4976.83	4595.60	752.85	79.94	29.53	21.23
Stimulation Index	140.24	223.51	165.83	267.60	58.28	27.48	4.41	1.54	0.97	1.43
Δ c.p.m	22852.98	113592.61	58583.66	98663.96	102653.24	15144.90	1337.33	78.75	-5.13	41.91
log 10 Δ c.p.m	4.36	5.06	4.77	4.99	5.01	4.18	3.13	1.90	1.00	1.62
SE Δ c.p.m (c.p.m)	6931.58	5810.81	11905.02	6653.78	5175.16	4722.04	808.36	95.36	62.02	34.03
SE Δ c.p.m (%)	30.33	5.12	20.32	6.74	5.04	31.18	60.45	121.10	1209.48	81.19
Geomean Δ c.p.m	3992.70									
PPD-A 1.25µg/ml	8980.27	108388.49	42611.22	75851.78	98504	4472.93	1046.98	200.18	166.65	88.27
SD (%)	123.02	8.29	19.66	44.3	12.21	74.81	93.25	10.34	33.84	30.37
SD (c.p.m)	11047.53	8985.41	8377.37	33602.34	12027.34	3346.20	976.31	20.70	56.39	26.81
SE (c.p.m)	5523.76	4492.70	4188.68	16801.17	6013.67	1673.10	488.15	10.35	28.20	13.40
Stimulation Index	54.72	212.32	119.89	204.96	54.96	7.82	2.67	1.37	0.97	0.90
Δ c.p.m	8816.15	107877.99	42255.81	75481.69	96711.76	3901.02	654.41	53.81	-4.39	-10.12
log 10 Δ c.p.m	3.95	5.03	4.63	4.88	4.99	3.59	2.82	1.73	1.00	1.00
SE Δ c.p.m (c.p.m)	5541.00	4569.83	4223.37	16834.53	6212.00	1799.54	543.66	25.77	60.68	26.20
SE Δ c.p.m (%)	62.85	4.24	9.99	22.30	6.42	46.13	83.08	47.90	1383.05	258.94
Geomean Δ c.p.m	2293.65									
8.4 kDa 6.25µg/ml	1751.84	2931.25	548.18	5196.28	12618.03	964.93	1444.95	143.48	179.41	112.19
SD (%)	37.45	133.93	31.88	76.15	83.23	46.59	30.38	48.91	56.49	77.45
SD (c.p.m)	656.06	3925.82	174.76	3956.97	10501.99	449.56	438.98	70.18	101.35	86.89
SE (c.p.m)	328.03	1962.91	87.38	1978.48	5250.99	224.78	219.49	35.09	50.67	43.45
Stimulation Index	10.67	5.74	1.54	14.04	7.04	1.69	3.68	0.98	1.05	1.14
Δ c.p.m	1587.72	2420.75	192.77	4826.19	10825.79	393.02	1052.38	-2.90	8.37	13.80
log 10 Δ c.p.m	3.20	3.38	2.29	3.68	4.03	2.59	3.02	1.00	0.92	1.14
SE Δ c.p.m (c.p.m)	345.27	2040.04	122.07	2011.84	5449.33	351.22	275.00	50.51	83.16	56.24
SE Δ c.p.m (%)	21.75	84.27	63.33	41.69	50.34	89.37	26.13	1744.71	993.24	407.46
Geomean Δ c.p.m	336.30									
8.4 kDa 1.25µg/ml	1679.85	803.99	578.09	14021.28	4652.93	285.28	501.19	155.87	170.55	105.29
SD (%)	58.81	11.49	34.78	43.76	43.81	90.82	55.11	39.95	13.7	18.06
SD (c.p.m)	987.92	92.38	201.06	6135.71	2038.45	259.09	276.21	62.27	23.37	19.02
SE (c.p.m)	493.96	46.19	100.53	3067.86	1019.22	129.55	138.10	31.14	11.68	9.51
Stimulation Index	10.24	1.57	1.63	37.89	2.60	0.50	1.28	1.06	1.00	1.07
Δ c.p.m	1515.73	293.49	222.68	13651.19	2860.69	-286.63	108.62	9.50	-0.49	6.90
log 10 Δ c.p.m	3.18	2.47	2.35	4.14	3.46	1.00	2.04	0.98	1.00	0.84
SE Δ c.p.m (c.p.m)	511.20	123.31	135.22	3101.21	1217.56	255.99	193.61	46.56	44.17	22.30
SE Δ c.p.m (%)	33.73	42.02	60.72	22.72	42.56	89.31	178.25	490.33	9059.89	323.10
Geomean Δ c.p.m	139.31									

APPENDIX IV. Chapter 6. Raw Data.

Wallaceville Trial Sampling time 6. Ten weeks post-challenge

IFN-γ data. (OD450 x 1000)

Unvaccinated animals (Tag No.)

	31	87	90	116	122	194	196	197	208	215
Unstimulated	886	115	361	183	370	270	172	323	136	450
ConA 0.25µg/ml	3000	1443	2905	3000	3000	1615	1077	1055	1013	1361
Δ OD450	2114	1328	2544	2817	2630	1345	905	732	877	911
SI	3.39	12.55	8.05	16.39	8.11	5.98	6.26	3.27	7.45	3.02
mean Δ OD 450	1620.3									
Geomean Δ OD 450	1437.5									
PPD-B 6.25µg/ml	3000	1345	2205	3000	972	964	245	463	253	784
Δ OD450	2114	1230	1844	2817	602	694	73	140	117	334
SI	3.39	11.70	6.11	16.39	2.63	3.57	1.42	1.43	1.86	1.74
mean Δ OD 450	996.5									
Geomean Δ OD450	543.6									
PPD-B 1.25µg/ml	3000	1044	1698	3000	705	865	201	280	173	842
Δ OD450	2114	929	1337	2817	335	595	29	-43	37	192
SI	3.39	9.08	4.70	16.39	1.91	3.20	1.17	0.87	1.27	1.43
mean Δ OD 450	834.2									
Geomean Δ OD450	232.2									
PPD-A 6.25µg/ml	3000	1282	1892	1628	1816	968	340	622	449	1081
Δ OD450	2114	1167	1531	1445	1446	698	168	299	313	631
SI	3.39	11.15	5.24	8.90	4.91	3.59	1.98	1.93	3.30	2.40
mean Δ OD 450	981.2									
Geomean Δ OD450	747.8									
PPD-A 1.25µg/ml	3000	696	1124	727	693	596	199	431	315	549
Δ OD450	2114	581	763	544	323	326	27	108	179	99
SI	3.39	6.05	3.11	3.97	1.87	2.21	1.16	1.33	2.32	1.22
mean Δ OD450	506.4									
Geomean Δ OD450	278.2									
8.4 kDa 6.25µg/ml	3000	139	373	355	532	367	344	524	157	760
Δ OD450	2114	24	12	172	162	97	172	201	21	310
SI	3.39	1.21	1.03	1.94	1.44	1.36	2.00	1.62	1.15	1.69
mean Δ OD 450	328.5									
Geomean Δ OD450	114.0									
8.4 kDa 1.25µg/ml	2417	97	249	254	402	312	164	377	114	346
Δ OD450	1531	-18	-112	71	32	42	-8	54	-22	-104
SI	2.73	0.84	0.69	1.39	1.09	1.16	0.95	1.17	0.84	0.77
mean Δ OD 450	146.6									
Geomean Δ OD450	12.0									

Vaccinated animals (Tag No.)

	44^	61^	118^	138^	145^	158^	167^	176^	195^	203^
Unstimulated	191	308	433	513	1056	183	213	139	234	204
ConA 0.25µg/ml	2016	1548	2206	2808	3000	1450	1435	982	939	527
Δ OD450	1825	1240	1773	2295	1944	1267	1222	843	705	323
SI	10.55	5.03	5.09	5.47	2.84	7.92	6.74	7.06	4.01	2.58
mean Δ OD450	1343.7									
Geomean Δ OD 450	1181.9									
PPD-B 6.25µg/ml	824	2603	2302	1962	3000	433	331	194	380	178
Δ OD450	633	2295	1869	1449	1944	250	118	55	146	-26
SI	4.31	8.45	5.32	3.82	2.84	2.37	1.55	1.40	1.62	0.87
mean Δ OD 450	873.3									
Geomean Δ OD450	277.6									
PPD-B 1.25µg/ml	553	1917	1652	1021	3000	274	230	137	294	150
Δ OD450	362	1609	1219	508	1944	91	17	-2	60	-54
SI	2.90	6.22	3.82	1.99	2.84	1.50	1.08	0.99	1.26	0.74
mean Δ OD 450	575.4									
Geomean Δ OD450	103.9									
PPD-A 6.25µg/ml	932	2737	2515	3000	3000	656	372	233	618	221
Δ OD450	741	2429	2082	2487	1944	473	159	94	384	17
SI	4.88	8.89	5.81	5.85	2.84	3.58	1.75	1.68	2.64	1.08
mean Δ OD 450	1081.0									
Geomean Δ OD 450	492.3									
PPD-A 1.25µg/ml	435	1906	1451	2229	3000	415	290	246	307	182
Δ OD450	244	1598	1018	1716	1944	232	77	107	73	-22
SI	2.28	6.19	3.35	4.35	2.84	2.27	1.36	1.77	1.31	0.89
mean Δ OD 450	698.7									
Geomean Δ OD 450	220.9									
8.4 kDa 6.25µg/ml	438	550	898	638	1542	358	381	190	360	162
Δ OD450	247	242	465	125	486	175	168	51	126	-42
SI	2.29	1.79	2.07	1.24	1.46	1.96	1.79	1.37	1.54	0.79
mean Δ OD 450	204.3									
Geomean Δ OD 450	116.9									
8.4 kDa 1.25µg/ml	274	271	496	659	1083	204	168	133	237	99
Δ OD450	83	-37	63	146	27	21	-45	-6	3	-105
SI	1.43	0.88	1.15	1.28	1.03	1.11	0.79	0.96	1.01	0.49
mean Δ OD 450	15.0									
Geomean Δ OD 450	9.6									

Appendix Va. Chapter 6. Comparison of Lymphocyte proliferation and IFN-γ assays. Positive / negative responses; Fisher's exact test.

Difference between proportion of positive / negative responses in LoP and IFN-γ assays. Fisher's exact test.

Sampling 4 Unvacc	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
LoP	8	2	10	LoP	1	9	10	LoP	9	1	10
IFN-γ	7	3		IFN-γ	1	9		IFN-γ	7	3	
	15	5	20		2	18	20		16	4	20
		P value	0.3483			P value	0.52632			P value	0.24768

Sampling 4 BCG vacc	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
LoP	1	9	10	LoP	0	10	10	LoP	3	7	10
IFN-γ	4	6		IFN-γ	1	9		IFN-γ	4	6	
	5	15	20		1	19	20		7	13	20
		P value	0.13545			P value	0.5			P value	0.32508

Sampling 5 Unvacc	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
LoP	2	8	10	LoP	2	8	10	LoP	7	3	10
IFN-γ	4	6		IFN-γ	3	7		IFN-γ	8	2	
	6	14	20		5	15	20		15	5	20
		P value	0.24381			P value	0.3483			P value	0.3483

Sampling 5 BCG vacc	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
LoP	1	9	10	LoP	0	10	10	LoP	3	7	10
IFN-γ	3	7		IFN-γ	2	8		IFN-γ	6	4	
	4	16	20		2	18	20		9	11	20
		P value	0.24768			P value	0.23684			P value	0.15004

Sampling 6 Unvacc	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
LoP	2	8	10	LoP	3	7	10	LoP	8	2	10
IFN-γ	1	9		IFN-γ	0	10		IFN-γ	4	6	
	3	17	20		3	17	20		12	8	20
		P value	0.39474			P value	0.10526			P value	0.07502

Sampling 6 BCGvacc	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
LoP	3	7	10	LoP	3	7	10	LoP	5	5	10
IFN-γ	2	8		IFN-γ	2	8		IFN-γ	2	8	
	5	15	20		5	15	20		7	13	20
		P value	0.3483			P value	0.3483			P value	0.14628

$\chi 2$ test of independence; number of positive LoP and IFN-γ responses

	-	+						
LoP	241	119	241	119	0.00104	0.0021	Chi square	0.00628
IFN-γ	241	119	241	119	0.00104	0.0021	P value	0.93685

Appendix Vbi. Chapter 6. Lymphocyte proliferation assay, comparison of responses to each antigen. Positive/negative responses; Fisher's exact test.

Difference between proportion of positive / negative *responses to antigen pairs in LoP assays. Fisher's exact test.*

Sampling 4
Unvacc

	-	+	
PPD-B	8	2	10
PPD-A	1	9	
	9	11	20
	P value		0.002679

	-	+	
8.4 kDa	9	1	10
PPD-B	8	2	
	17	3	20
	P value		0.394737

	-	+	
8.4 kDa	9	1	10
PPD-A	1	9	
	10	10	20
	P value		0.000541

Sampling 4
BCG vacc

	-	+	
PPD-B	1	9	10
PPD-A	0	10	
	1	19	20
	P value		0.5

	-	+	
8.4 kDa	3	7	10
PPD-B	1	9	
	4	16	20
	P value		0.247678

	-	+	
8.4 kDa	3	7	10
PPD-A	0	10	
	3	17	20
	P value		0.105263

Sampling 5
Unvacc

	-	+	
PPD-B	2	8	10
PPD-A	2	8	
	4	16	20
	P value		0.417957

	-	+	
8.4 kDa	7	3	10
PPD-B	2	8	
	9	11	20
	P value		0.032151

	-	+	
8.4 kDa	7	3	10
PPD-A	2	8	
	9	11	20
	P value		0.032151

Sampling 5
BCGvacc

	-	+	
PPD-B	1	9	10
PPD-A	0	10	
	1	19	20
	P value		0.5

	-	+	
8.4 kDa	3	7	10
PPD-B	1	9	
	4	16	20
	P value		0.247678

	-	+	
8.4 kDa	3	7	10
PPD-A	0	10	
	3	17	20
	P value		0.105263

Sampling 6
Unvacc

	-	+	
PPD-B	2	8	10
PPD-A	3	7	
	5	15	20
	P value		0.348297

	-	+	
8.4 kDa	8	2	10
PPD-B	2	8	
	10	10	20
	P value		0.01096

	-	+	
8.4 kDa	8	2	10
PPD-A	3	7	
	11	9	20
	P value		0.032151

Sampling 6
BCG vacc

	-	+	
PPD-B	3	7	10
PPD-A	3	7	
	6	14	20
	P value		0.371517

	-	+	
8.4 kDa	5	5	10
PPD-B	3	7	
	8	12	20
	P value		0.240057

	-	+	
8.4 kDa	5	5	10
PPD-A	3	7	
	8	12	20
	P value		0.240057

Appendix Vbii. Chapter 6. IFN-γ assay, comparison of responses to each antigen. Positive/negative responses; Fisher's exact test.

Difference between proportion of positive / negative responses to antigen pairs in IFN-γ assays. Fisher's exact test.

Sampling 4
Unvacc

	-	+			-	+			-	+	
PPD-B	7	3	10	8.4 kDa	7	3	10	8.4 kDa	7	3	10
PPD-A	1	9		PPD-B	7	3		PPD-A	1	9	
	8	12	20		14	6	20		8	12	20
		P value	0.009526			P value	0.371517			P value	0.009526

Sampling 4
BCGvacc

	-	+			-	+			-	+	
PPD-B	4	6	10	8.4 kDa	4	6	10	8.4 kDa	4	6	10
PPD-A	1	9		PPD-B	4	6		PPD-A	4	6	
	5	15	20		8	12	20		8	12	20
		P value	0.135449			P value	0.350083			P value	0.350083

Sampling 5
Unvacc

	-	+			-	+			-	+	
PPD-B	4	6	10	8.4 kDa	8	2	10	8.4 kDa	8	2	10
PPD-A	3	7		PPD-B	4	6		PPD-A	3	7	
	7	13	20		12	8	20		11	9	20
		P value	0.325077			P value	0.075018			P value	0.032151

Sampling 5
BCGvacc

	-	+			-	+			-	+	
PPD-B	3	7	10	8.4 kDa	6	4	10	8.4 kDa	6	4	10
PPD-A	2	8		PPD-B	3	7		PPD-A	2	8	
	5	15	20		9	11	20		8	12	20
		P value	0.348297			P value	0.150036			P value	0.075018

Sampling 6
Unvacc

	-	+			-	+			-	+	
PPD-B	1	9	10	8.4 kDa	4	6	10	8.4 kDa	4	6	10
PPD-A	0	10		PPD-B	1	9		PPD-A	0	10	
	1	19	20		5	15	20		4	16	20
		P value	0.5			P value	0.135449			P value	0.043344

Sampling 6
BCGvacc

	-	+			-	+			-	+	
PPD-B	2	8	10	8.4 kDa	2	8	10	8.4 kDa	2	8	10
PPD-A	2	8		PPD-B	2	8		PPD-A	2	8	
	4	16	20		4	16	20		4	16	20
		P value	0.417957			P value	0.417957			P value	0.417957

Appendix Vc. Chapter 6. Post-challenge responses (Sampling times 5 & 6) compared with pre-challenge (Sampling 4) responses. Positive / negative; Fisher's exact test.

Lymphocyte proliferation (SI) difference between proportions of positive / negative responses following challenge. Fisher's exact test.

Unvaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 5	2	8	10	Sample 5	2	8	10	Sample 5	7	3	10
Sample 4	8	2		Sample 4	1	9		Sample 4	9	1	
	10	10	20		3	17	20		16	4	20
	P value		0.01096		P value		0.39474		P value		0.24768

Unvaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 6	2	8	10	Sample 6	3	7	10	Sample 6	8	2	10
Sample 4	8	2		Sample 4	1	9		Sample 4	9	1	
	10	10	20		4	16	20		17	3	20
	P value		0.01096		P value		0.24768		P value		0.39474

BCG vaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 5	1	9	10	Sample 5	0	10	10	Sample 5	3	7	10
Sample 4	1	9		Sample 4	0	10		Sample 4	3	7	
	2	18	20		0	20	20		6	14	20
	P value		0.52632		P value		1		P value		0.37152

BCG vaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 6	3	7	10	Sample 6	3	7	10	Sample 6	5	5	10
Sample 4	1	9		Sample 4	0	10		Sample 4	3	7	
	4	16	20		3	17	20		8	12	20
	P value		0.24768		P value		0.10526		P value		0.24006

IFN-γ (Δ OD450) difference between proportions of positive / negative responses following challenge. Fisher's exact test.

Unvaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 5	4	6	10	Sample 5	3	7	10	Sample 5	8	2	10
Sample 4	7	3		Sample 4	1	9		Sample 4	7	3	
	11	9	20		4	16	20		15	5	20
	P value		0.15004		P value		0.24768		P value		0.3483

Unvaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 6	1	9	10	Sample 6	0	10	10	Sample 6	4	6	10
Sample 4	7	3		Sample 4	1	9		Sample 4	7	3	
	8	12	20		1	19	20		11	9	20
	P value		0.00953		P value		0.5		P value		0.15004

BCG vaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 5	3	7	10	Sample 5	2	8	10	Sample 5	6	4	10
Sample 4	4	6		Sample 4	1	9		Sample 4	4	6	
	7	13	20		3	17	20		10	10	20
	P value		0.32508		P value		0.39474		P value		0.23869

BCG vaccinated

	PPD-B				PPD-A				8.4 kDa		
	-	+			-	+			-	+	
Sample 6	2	8	10	Sample 6	2	8	10	Sample 6	6	8	14
Sample 4	4	6		Sample 4	1	9		Sample 4	4	6	
	6	14	20		3	17	20		10	14	24
	P value		0.24381		P value		0.39474		P value		0.32154

Appendix Vd. Chapter 6. Comparison of the unvaccinated group and the BCG vaccinated group. Positive / negative responses; Fisher's exact test.

Lymphocyte proliferation (SI) difference between proportion of unvaccinated & BCG vaccinated animals with positive / negative responses. Fisher's exact test.

Sampling 4

PPD-B	-	+	
unvacc	8	2	10
BCGvacc	1	9	
	9	11	20
		P value	0.00268

PPD-A	-	+	
unvacc	1	9	10
BCG vacc	0	10	
	1	19	20
		P value	0.5

8.4 kDa	-	+	
unvacc	9	1	10
BCGvacc	3	7	
	12	8	20
		P value	0.00953

Sampling 5

PPD-B	-	+	
unvacc	2	8	10
BCGvacc	1	9	
	3	17	20
		P value	0.39474

PPD-A	-	+	
unvacc	2	8	10
BCG vacc	0	10	
	2	18	20
		P value	0.23684

8.4 kDa	-	+	
unvacc	7	3	10
BCGvacc	3	7	
	10	10	20
		P value	0.07794

Sampling 6

PPD-B	-	+	
unvacc	2	8	10
BCGvacc	3	7	
	5	15	20
		P value	0.3483

PPD-A	-	+	
unvacc	3	7	10
BCG vacc	3	7	
	6	14	20
		P value	0.37152

8.4 kDa	-	+	
unvacc	8	2	10
BCGvacc	5	5	
	13	7	20
		P value	0.14628

IFN-γ (Δ OD450) difference between proportion of unvaccinated & BCG vaccinated animals with positive / negative responses. Fisher's exact test.

Sampling 4

PPD-B	-	+	
unvacc	7	3	10
BCGvacc	4	6	
	11	9	20
		P value	0.15004

PPD-A	-	+	
unvacc	1	9	10
BCGvacc	1	9	
	2	18	20
		P value	0.52632

8.4 kDa	-	+	
unvacc	7	3	10
BCG vacc	4	6	
	11	9	20
		P value	0.15004

Sampling 5

PPD-B	-	+	
unvacc	4	6	10
BCG vacc	3	7	
	7	13	20
		P value	0.32508

PPD-A	-	+	
unvacc	3	7	10
BCGvacc	2	8	
	5	15	20
		P value	0.3483

8.4 kDa	-	+	
unvacc	8	2	10
BCGvacc	6	4	
	14	6	20
		P value	0.24381

Sampling 6

PPD-B	-	+	
unvacc	1	9	10
BCG vacc	2	8	
	3	17	20
		P value	0.39474

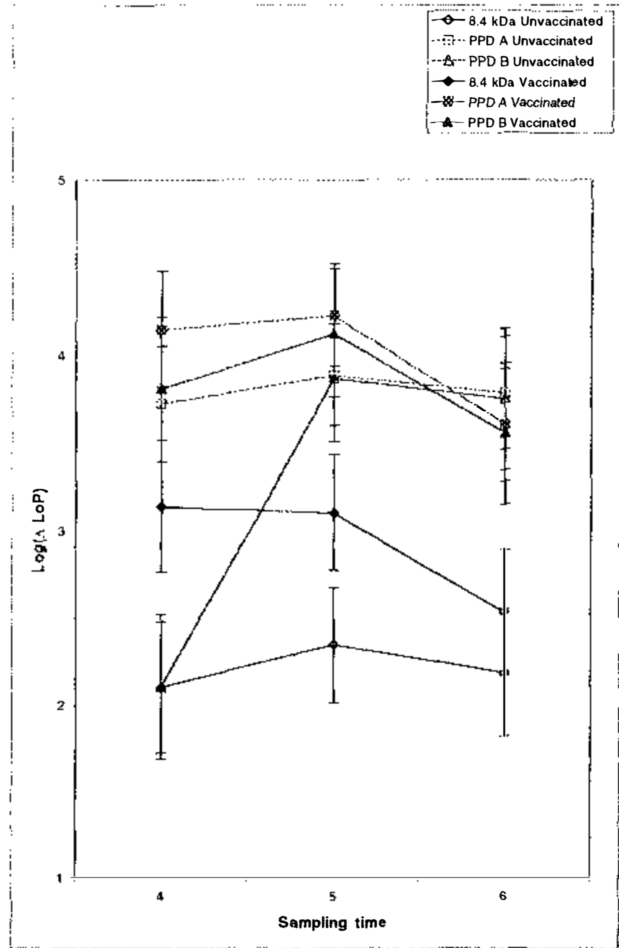
PPD-A	-	+	
unvacc	0	10	10
BCG vacc	2	8	
	2	18	20
		P value	0.23684

8.4 kDa	-	+	
unvacc	4	6	10
BCGvacc	2	8	
	6	14	20
		P value	0.24381

Appendix Ve. Chapter 6. ANOVA - Lymphocyte proliferation (Δ c.p.m)

Least Squares Means

TREAT	ANTIGEN	SAMPLING	Estimate	Standard Error
Unvaccinated	8.4 k	4	2.0977	0.3742
Unvaccinated	8.4 k	5	2.3427	0.3301
Unvaccinated	8.4 k	6	2.183	0.3639
Unvaccinated	ppd a	4	3.7199	0.3299
Unvaccinated	ppd a	5	3.8859	0.2923
Unvaccinated	ppd a	6	3.7829	0.321
Unvaccinated	ppd b	4	2.0984	0.4138
Unvaccinated	ppd b	5	3.8658	0.364
Unvaccinated	ppd b	6	3.7486	0.4022
Vaccinated	8.4 k	4	3.1346	0.3742
Vaccinated	8.4 k	5	3.1029	0.3301
Vaccinated	8.4 k	6	2.5267	0.3639
Vaccinated	ppd a	4	4.1461	0.3299
Vaccinated	ppd a	5	4.2267	0.2923
Vaccinated	ppd a	6	3.6013	0.321
Vaccinated	ppd b	4	3.8027	0.4138
Vaccinated	ppd b	5	4.1242	0.364
Vaccinated	ppd b	6	3.5519	0.4022



Significance of Differences Between LS Means

TREATMENT	ANTIGEN	TREATMENT	ANTIGEN	Pr > |t|
SAMPLING 4				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	8.4 k	Unvaccinated	ppd b	0.9982
Unvaccinated	**8.4 k**	**Vaccinated**	**8.4 k**	**0.0520**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0027**
Unvaccinated	**ppd a**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	ppd a	Vaccinated	8.4 k	0.2427
Unvaccinated	ppd a	Vaccinated	ppd a	0.3625
Unvaccinated	ppd a	Vaccinated	ppd b	0.8759
Unvaccinated	**ppd b**	**Vaccinated**	**8.4 k**	**0.0653**
Unvaccinated	**ppd b**	**Vaccinated**	**ppd a**	**0.0002**
Unvaccinated	**ppd b**	**Vaccinated**	**ppd b**	**0.0042**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0036**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0448**
Vaccinated	ppd a	Vaccinated	ppd b	0.1028
SAMPLING 5				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	8.4 k	Vaccinated	8.4 k	0.1056
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0004**
Unvaccinated	ppd a	Unvaccinated	ppd b	0.9119
Unvaccinated	**ppd a**	**Vaccinated**	**8.4 k**	**0.0779**
Unvaccinated	ppd a	Vaccinated	ppd a	0.4110
Unvaccinated	ppd a	Vaccinated	ppd b	0.6104
Unvaccinated	ppd b	Vaccinated	8.4 k	0.1228
Unvaccinated	ppd b	Vaccinated	ppd a	0.4407
Unvaccinated	ppd b	Vaccinated	ppd b	0.6163
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0002**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0005**
Vaccinated	ppd a	Vaccinated	ppd b	0.5732
SAMPLING 6				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	8.4 k	Vaccinated	8.4 k	0.5053
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0040**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0127**
Unvaccinated	ppd a	Unvaccinated	ppd b	0.8661
Unvaccinated	**ppd a**	**Vaccinated**	**8.4 k**	**0.0106**
Unvaccinated	ppd a	Vaccinated	ppd a	0.6897
Unvaccinated	ppd a	Vaccinated	ppd b	0.6542
Unvaccinated	**ppd b**	**Vaccinated**	**8.4 k**	**0.0258**
Unvaccinated	ppd b	Vaccinated	ppd a	0.7750
Unvaccinated	ppd b	Vaccinated	ppd b	0.7299
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0015**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0017**
Vaccinated	ppd a	Vaccinated	ppd b	0.8078

SAMPLING	SAMPLING	P value
UNVACCINATED 8.4 kDa		
4	5	0.5854
4	6	0.8566
5	6	0.6262
UNVACCINATED PPD A		
4	5	0.6699
4	6	0.8778
5	6	0.7170
UNVACCINATED PPDB		
4	**5**	**0.0006**
4	**6**	**0.0021**
5	6	0.7490
VACCINATED 8.4 kDa		
4	5	0.9437
4	6	0.1996
5	**6**	**0.0802**
VACCINATED PPD A		
4	5	0.8359
4	6	0.1851
5	**6**	**0.0290**
VACCINATED PPD B		
4	5	0.5217
4	6	0.6348
5	6	0.1194

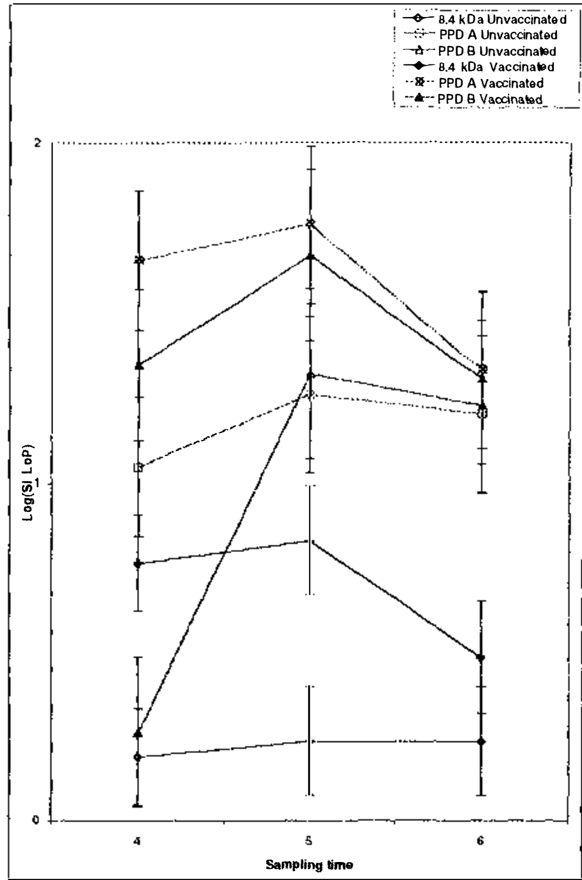
Appendix Vf. Chapter 6. ANOVA - Lymphocyte proliferation (SI).

Least Squares Means

TREATMENT	ANTIGEN	SAMPLING	Estimate	Standard Error
Unvaccinated	8 4 k	4	0 1874	0 1444
Unvaccinated	8 4 k	5	0 235	0 1629
Unvaccinated	8 4 k	6	0 2335	0 1642
Unvaccinated	ppd a	4	1 047	0 2042
Unvaccinated	ppd a	5	1 2609	0 2303
Unvaccinated	ppd a	6	1 2004	0 2322
Unvaccinated	ppd b	4	0 2604	0 2224
Unvaccinated	ppd b	5	1 321	0 2508
Unvaccinated	ppd b	6	1 2258	0 2529
Vaccinated	8 4 k	4	0 7622	0 1444
Vaccinated	8 4 k	5	0 8306	0 1629
Vaccinated	8 4 k	6	0 4833	0 1642
Vaccinated	ppd a	4	1 6553	0 2042
Vaccinated	ppd a	5	1 7609	0 2303
Vaccinated	ppd a	6	1 332	0 2322
Vaccinated	ppd b	4	1 3471	0 2224
Vaccinated	ppd b	5	1 6707	0 2508
Vaccinated	ppd b	6	1 3066	0 2529

Significance of Differences Between LS Means

TREATMENT	ANTIGEN	TREATMENT	ANTIGEN	Pr > |t|
SAMPLING 4				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	8.4 k	Unvaccinated	ppd b	0.6650
Unvaccinated	**8.4 k**	**Vaccinated**	**8.4 k**	**0.0056**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**<.0001**
Unvaccinated	**ppd a**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	ppd a	Vaccinated	8 4 k	0 2567
Unvaccinated	**ppd a**	**Vaccinated**	**ppd a**	**0.0369**
Unvaccinated	ppd a	Vaccinated	ppd b	0 3218
Unvaccinated	**ppd b**	**Vaccinated**	**8.4 k**	**0.0604**
Unvaccinated	**ppd b**	**Vaccinated**	**ppd a**	**<.0001**
Unvaccinated	**ppd b**	**Vaccinated**	**ppd b**	**0.0007**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**<.0001**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0007**
Vaccinated	**ppd a**	**Vaccinated**	**ppd b**	**0.0071**
SAMPLING 5				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	**8.4 k**	**Vaccinated**	**8.4 k**	**0.0107**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**<.0001**
Unvaccinated	ppd a	Unvaccinated	ppd b	0 6378
Unvaccinated	ppd a	Vaccinated	8 4 k	0 1293
Unvaccinated	ppd a	Vaccinated	ppd a	0 1269
Unvaccinated	ppd a	Vaccinated	ppd b	0 2308
Unvaccinated	ppd b	Vaccinated	8.4 k	0 1032
Unvaccinated	ppd b	Vaccinated	ppd a	0.1984
Unvaccinated	ppd b	Vaccinated	ppd b	0.3258
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**<.0001**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**<.0001**
Vaccinated	ppd a	Vaccinated	ppd b	0 4797
SAMPLING 6				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	8.4 k	Vaccinated	8 4 k	0 2839
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0002**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0005**
Unvaccinated	ppd a	Unvaccinated	ppd b	0 8435
Unvaccinated	**ppd a**	**Vaccinated**	**8.4 k**	**0.0128**
Unvaccinated	ppd a	Vaccinated	ppd a	0 6892
Unvaccinated	ppd a	Vaccinated	ppd b	0 7574
Unvaccinated	**ppd b**	**Vaccinated**	**8.4 k**	**0.0150**
Unvaccinated	ppd b	Vaccinated	ppd a	0.7575
Unvaccinated	ppd b	Vaccinated	ppd b	0 8214
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**<.0001**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**<.0001**
Vaccinated	ppd a	Vaccinated	ppd b	0 8437

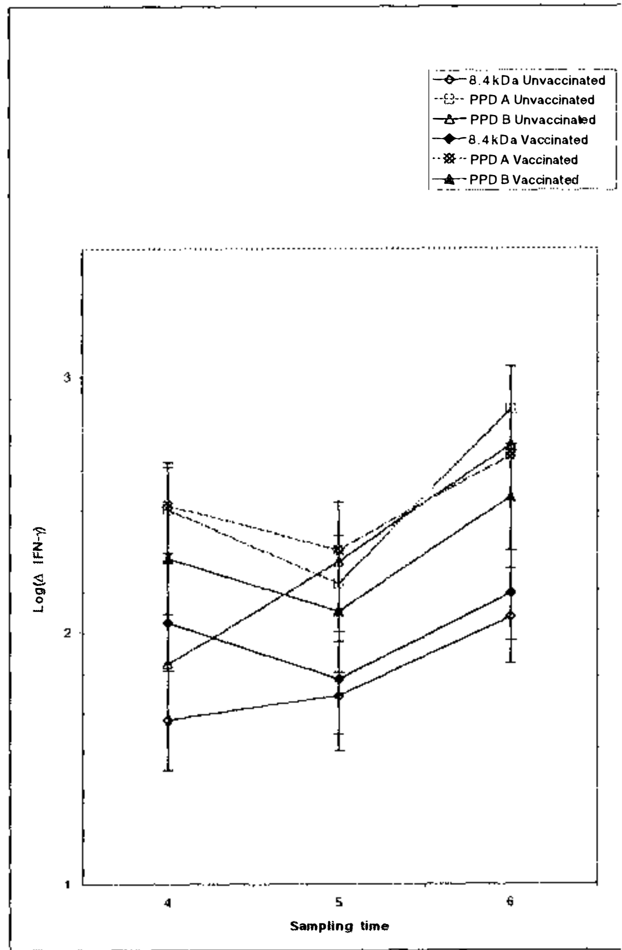


SAMPLING	SAMPLING	P value
UNVACCINATED 8.4 kDa		
4	5	0 7910
4	6	0.8100
5	6	0 9824
UNVACCINATED PPDA		
4	5	0 3995
4	6	0.5718
5	6	0.7739
UNVACCINATED PPDB		
4	**5**	**0.0002**
4	**6**	**0.0013**
5	6	0 6782
VACCINATED 8.4 kDa		
4	5	0 7030
4	6	0 1472
5	**6**	**0.0209**
VACCINATED PPDA		
4	5	0 6772
4	6	0.2342
5	**6**	**0.0432**
VACCINATED PPDB		
4	5	0 2424
4	6	0 8908
5	6	0 1140

Appendix Vg. Chapter 6. ANOVA - IFN-γ (Δ OD_{450}).

Least Squares Means

TREAT	ANTIGEN	SAMPLING	Estimate	Standard Error
Unvaccinated	8.4 k	4	1.6499	0.1947
Unvaccinated	8.4 k	5	1.7463	0.2158
Unvaccinated	8.4 k	6	2.0569	0.1867
Unvaccinated	ppd a	4	2.477	0.1696
Unvaccinated	ppd a	5	2.1876	0.1876
Unvaccinated	ppd a	6	2.8738	0.1628
Unvaccinated	ppd b	4	1.8713	0.2173
Unvaccinated	ppd b	5	2.2733	0.2411
Unvaccinated	ppd b	6	2.7353	0.2081
Vaccinated	8.4 k	4	2.037	0.1947
Vaccinated	8.4 k	5	1.8116	0.2158
Vaccinated	8.4 k	6	2.1504	0.1867
Vaccinated	ppd a	4	2.4966	0.1696
Vaccinated	ppd a	5	2.3192	0.1876
Vaccinated	ppd a	6	2.6922	0.1628
Vaccinated	ppd b	4	2.2874	0.2173
Vaccinated	ppd b	5	2.0795	0.2411
Vaccinated	ppd b	6	2.5258	0.2081



Significance of Differences Between LS Means

TREATMENT	ANTIGEN	TREATMENT	ANTIGEN	Pr > |t|
SAMPLING 4				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	8.4 k	Unvaccinated	ppd b	0.1944
Unvaccinated	8.4 k	Vaccinated	8.4 k	0.1620
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0013**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0305**
Unvaccinated	**ppd a**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	**ppd a**	**Vaccinated**	**8.4 k**	**0.0906**
Unvaccinated	ppd a	Vaccinated	ppd a	0.9350
Unvaccinated	ppd a	Vaccinated	ppd b	0.4927
Unvaccinated	ppd b	Vaccinated	8.4 k	0.5709
Unvaccinated	**ppd b**	**Vaccinated**	**ppd a**	**0.0248**
Unvaccinated	ppd b	Vaccinated	ppd b	0.1778
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0052**
Vaccinated	8.4 k	Vaccinated	ppd b	0.1424
Vaccinated	**ppd a**	**Vaccinated**	**ppd b**	**0.0694**
SAMPLING 5				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**0.0158**
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd b**	**0.0061**
Unvaccinated	8.4 k	Vaccinated	8.4 k	0.8310
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0470**
Unvaccinated	8.4 k	Vaccinated	ppd b	0.3049
Unvaccinated	ppd a	Unvaccinated	ppd b	0.5031
Unvaccinated	ppd a	Vaccinated	8.4 k	0.1905
Unvaccinated	ppd a	Vaccinated	ppd a	0.6206
Unvaccinated	ppd a	Vaccinated	ppd b	0.7238
Unvaccinated	ppd b	Vaccinated	8.4 k	0.1558
Unvaccinated	ppd b	Vaccinated	ppd a	0.8807
Unvaccinated	ppd b	Vaccinated	ppd b	0.5706
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0056**
Vaccinated	8.4 k	Vaccinated	ppd b	0.1595
Vaccinated	ppd a	**Vaccinated**	**ppd b**	**0.0623**
SAMPLING 6				
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd a**	**<.0001**
Unvaccinated	**8.4 k**	**Unvaccinated**	**ppd b**	**<.0001**
Unvaccinated	8.4 k	Vaccinated	8.4 k	0.7237
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0113**
Unvaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0957**
Unvaccinated	ppd a	Unvaccinated	ppd b	0.2070
Unvaccinated	**ppda**	**Vaccinated**	**8.4 k**	**0.0041**
Unvaccinated	ppd a	Vaccinated	ppd a	0.4316
Unvaccinated	ppd a	Vaccinated	ppd b	0.1900
Unvaccinated	**ppd b**	**Vaccinated**	**8.4 k**	**0.0382**
Unvaccinated	ppd b	Vaccinated	ppd a	0.8708
Unvaccinated	ppd b	Vaccinated	ppd b	0.4778
Vaccinated	**8.4 k**	**Vaccinated**	**ppd a**	**0.0006**
Vaccinated	**8.4 k**	**Vaccinated**	**ppd b**	**0.0221**
Vaccinated	ppd a	Vaccinated	ppd b	0.1299

SAMPLING	SAMPLING	P value
UNVACCINATED 8.4 kDa		
4	5	0.6907
4	6	0.1029
5	6	0.1595
UNVACCINATED PPD A		
4	5	0.1663
4	6	**0.0651**
5	6	**0.0004**
UNVACCINATED PPDB		
4	5	0.1411
4	6	**0.0023**
5	6	**0.0632**
VACCINATED 8.4 kDa		
4	5	0.3524
4	6	0.6483
5	6	0.1251
VACCINATED PPD A		
4	5	0.3951
4	6	0.3609
5	6	**0.0504**
VACCINATED PPD B		
4	5	0.4451
4	6	0.3937
5	6	**0.0726**

APPENDIX VIa. Sequence of expression construct pGEX-6P-3:8.4-DS(B)

```
                    Vector sequence                 Eco RI           Insert sequence
 E   V   L   F   Q   G   P   L   G   S   P   N   S   D   P   V   D   A
GAA GTT CTG TTC CAG GGG CCC CTG GGA TCC CCG^AAT TCA GAT CCC GTG GAC GCG

 V   I   N   T   T   C   N   Y   G   Q   V   V   A   A   L   N   A   T
GTC ATT AAC ACC ACC TGC AAT TAC GGG CAG GTA GTA GCT GCG CTC AAC GCG ACG

 D   P   G   A   A   A   Q   F   N   A   S   P   V   A   Q   S   Y   L
GAT CCG GGG GCT GCC GCA CAG TTC AAC GCC TCA CCG GTG GCG CAG TCC TAT TTG

 R   N   F   L   A   A   P   P   P   Q   R   A   A   M   A   A   Q   L
CGC AAT TTC CTC GCC GCA CCG CCA CCT CAG CGC GCT GCC ATG GCC GCG CAA TTG

 Q   A   V   P   G   A   A   Q   Y   I   G   L   V   E   S   V   A   G
CAA GCT GTG CCG GGG GCG GCA CAG TAC ATC GGC CTT GTC GAG TCG GTT GCC GGC

 S   C   N   N   Y  ochre
                                      62 bp direct repeat partial copy (43/62).
TCC TGC AAC AAC TAT TAA GCCCATG * CGGGCCCCATCCCGCGACCCGGCATCGTCGCCGGGGC

                          62 bp direct repeat copy.
TAGGCC * AGATTGCCCCGCTCCTCAACGGGCCGCATCCCGCGACCCGGCATCGTCGCCGGGGCTAGGCC

                    downstream intergenic region of M. bovis.
* GCAAGCAGGGCGTATGTGGTAGTGCGCTGGCGCGCCGGGCGGCCGATGCCTTCGGCGATCGCGACCAGC

                                                                 Sal I
TCAGCGACGGTCTTGGCCGATCCGTGTTCGGAACCGGCCATCCGCGAGATGGTCTCCTCCAT|CG ^ TCG

  vector sequence →
ACTCGAGCGGCCGCATCGTGACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGACGGNGAAAACCTCT

GACACATGCAGCTTCCGGAGACGGTCACAAGCTTGTCTGTAAGCGGATGCCCGGGAGCAAACAAACCCGTC

ANGGGCGCGTCAACNGGTGTTGGGCNGGGTGTCGGGGCNCAACCATGACCCAATNCACNTTANCGATAGGG

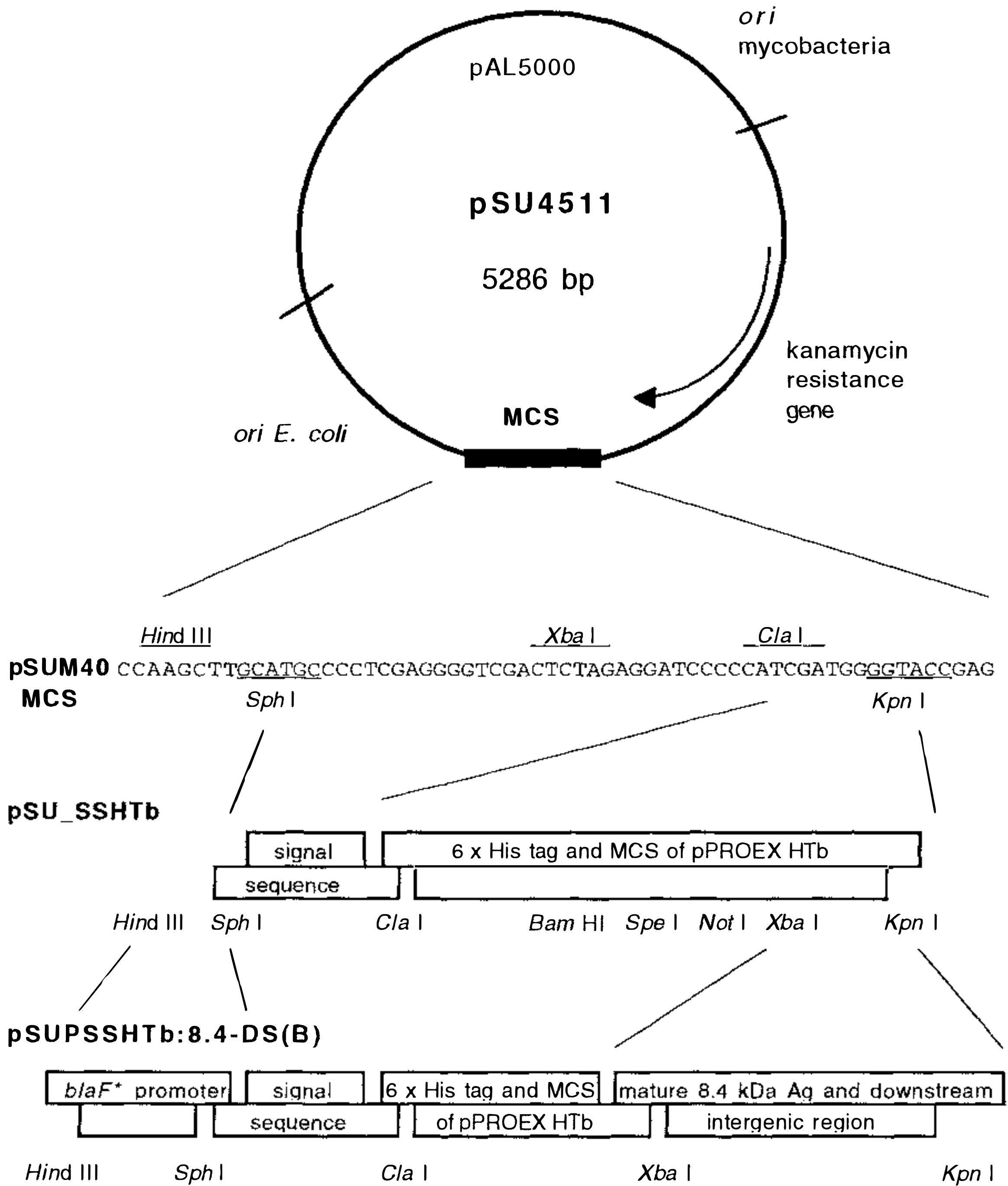
GGAGTGTNTAATTNTTGAANAANAAANGGCCTTCGNGATACCCCTATTTTTATTAGGNTAANGGCATGAAA

AANAATGGGTTNTTAAAACNNNAGGNGGGNCCTTTTTNGGGGAAAANGTGCCCCGGAANCCCTNTTTGTTT

NTTTTTNTNAAAAACATNNAAAANTNTNTTCCNNTNATTAAAAAANTAANCC
```

The nucleotide sequence was determined by the automated dideoxy chain termination method and initiated with the primer pGEX 3'. The nucleotide sequence of the vector with insert between the *Eco* RI and *Sal* I sites (^) and the corresponding translated amino acid sequence of the C terminus of the GST protein with the attached 8.4 kDa antigen are shown. The 62 bp tandem repeat copies are marked at each end by asterisks (*).

APPENDIX VIb. Plasmid map showing construction of pSUPSSHTb:8.4-DS(B).



The plasmid pSU_SSHTb was constructed by inserting a signal sequence modified from the *M. bovis* 8.4 kDa antigen gene, and the sequence coding for a 6 x histidine tag and multiple cloning site (MCS) from plasmid pPROEX HTb (Life Technologies), into the mycobacteria/*E.coli* shuttle vector pSU4511 (Ainsa *et al.* 1996). The plasmid pSUPSSHTbB:8.4-DS(B) was constructed by inserting the sequence coding for the mature 8.4 kDa antigen of *M. bovis* plus the downstream intergenic region, and the strong mutant promoter *blaF** from *M. fortuitum*, into pSU_SSHTb. Unique restriction sites are shown.

APPENDIX VII. Media, buffers and commonly used reagents.

Lauria-Bertani (LB) broth (1 litre).

Tryptone	10.0 g
NaCl	10.0 g
Yeast Extract	5.0 g
dH_2O	1 litre
(autoclave)	

LB agar (1 litre).

as for LB broth	
Bacto-Agar (1.5%)	15 g
(autoclave)	

7H9 minimal medium (900 ml).

Middlebrook 7H9 broth	4.7 g
dH_2O	900 ml
(autoclave)	
D (+) -Glucose	0.03g
kanamycin (20 µg / ml)	900 µl of kanamycin 20 mg/ml

25 x TAE gel running buffer (1 litre).

Tris-HCl	121 g
EDTA	18.5 g
Glacial acetic acid	28.5 ml
dH_2O	to 1 litre
(pH 8.5)	

6 x Agarose gel loading dye (1 ml).

Glycerol (30%)	300 µl
Bromophenol blue	700 µl 0.05% bromophenol blue

Ethidium bromide stain.

Ethidium bromide 0.5 mg / ml dH_20.

TE Buffer (100 ml).

Tris-HCl (10 mM)	0.12 g
EDTA (Na^+ salt) (1mM)	0.037 g
dH_2O	100 ml
(pH 8.0 with NaOH pellets ~ 2 g, autoclave)	

20 x SSC (1 litre).

NaCl (3 M)	175.2 g
Sodium citrate (0.3 M)	88.2 g
dH_2O	1 litre

Denaturing solution (1 litre).

NaOH (1.0 M)	40 g
NaCl (0.5 M)	29.2 g
dH_2O	1 litre

Neutralizing solution (1 litre).

Tris-HCl (1 M)	121.1 g
NaCl (3 M)	175.2 g
dH_2O	1 litre
(pH 5.5 with conc HCl)	

Phosphate buffered saline (1 litre).

NaCl	8.0 g
KCl	2.0 g
$Na_2HPO_4.7H_2O$	1.44 g
KH_2PO_4	0.24 g
dH_2O	1 litre
(autoclave)	
($MgCl_2$ to prevent PBMC clumping	0.05 g)

RPMI-1640.

RPMI medium 1640 powder with L-glutamine, without $NaHCO_3$	1 sachet (10.4 g)
Hepes	6.5 g
$NaHCO_3$	2.0 g
2-ME	1 ml 2-ME 0.39 g/ml
Glutamax I	10 ml
dH_2O	to 1 litre

(pH 7.2 to 7.4 with conc HCl. Filter sterilize through 0.2 μm syringe filter. Incubate at 37°C one week to check sterility, store at 4°C. Prior to use add 5% FCS and gentamicin 50 μg / ml).

Trypan blue stain (10 ml).

Trypan blue (0.2%)	0.02 g
PBS	10 ml

Tris-glycine SDS-PAGE gels (2 x minigels •.75 mm thick).

Separating gel (10 ml).	(12%)	(15%)
dH_2O	3.3 ml	2.3 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml	2.5 ml
30% Acrylamide/Bis 29:1	4 ml	5 ml
10% SDS	0.1 ml	0.1 ml
10% APS	0.1 ml	0.1 ml
TEMED	4.0 μl	4.0 μl

Stacking gel (4 ml).

dH_2O	2.8 ml
1.0 M Tris-HCl (pH 6.8)	0.5 ml
30% Acrylamide/Bis 29:1	0.67 ml
10% SDS	40 μl
10% APS	40 μl
TEMED	4.0 μl

Overlay solution.

Butanol saturated in dH_2O

5 x Electrode running buffer (1 litre).

Tris-HCl	15 g
Glycine	72 g
SDS	5.0 g
dH_2O	to 1 litre

Tris-tricine SDS-PAGE gels (2 x minigels 0.75 mm thick).

Separating gel (10 ml)	(16.5%)
30% Acrylamide/Bis 29:1	5.5 ml
Gel buffer	3.8 ml
Glycerol	1.3 ml
10% APS	50 μl
TEMED	5.0 μl

Sample gel (4 ml).	
30% Acrylamide/Bis 29:1	0.53 ml
Gel buffer	1.0 ml
dH_2O	2.47 ml
10% APS	33 μl
TEMED	3.3 μl

Gel buffer (500 ml).

Tris-HCl (3 M)	181.7 g
conc HCl (1 M)	50 ml
SDS (0.3%)	1.5 g
dH_2O	to 500 ml

Gel overlay solution (250 ml).

Tris-HCl (1 M)	30.3 g
conc HCl (0.33 M)	8.25 ml
SDS (0.1%)	0.25 g
dH_2O	242 ml

Anode buffer (1 litre).

Tris-HCl (0.2 M)	24.22 g

Cathode buffer (1 litre).

Tris-HCl (0.1 M)	12.11 g
Tricine (0.1 M)	17.92 g
SDS (0.1%)	1.0 g

2 x Sample loading buffer (1 ml).

SDS (5%)	500 μl 10% SDS
Glycerol (15%)	150 μl
Tris-HCl (50 mM pH 6.8)	50 μl 1M Tris-HCl pH 6.8
Bromophenol blue (0.003%)	60 μl 0.05% bromophenol blue
dH_2O	220 μl
(2-ME (2%)	20 μl)
(DTT (0.2 M)	0.03 g)

Coomassie stain (400 ml).

Methanol (40%)	160 ml
Acetic acid (10%)	40 ml
Coomassie brilliant blue R-250 (0.25%)	1.0 g

Western blotting transfer buffer (1 litre).

Tris-HCl (48 mM)	5.82 g
Glycine (39 mM)	2.93 g
SDS (0.015%)	1.5 ml 10% SDS
Methanol	200 ml
dH_2O	to 1 litre

(Dissolve solids in some dH_2O, add methanol, make up to 1 litre with dH_2O. Check pH 9.0 to 9.3, if not discard. Store at 4°C).

pPROEX lysis buffer (40 ml).

Tris-HCl (50 mM)	0.24 g
2-ME (5 mM)	14.1 μl

Mycobacterial lysis buffer (40 ml).

NaCl (100 mM)	0.234 g
EDTA (25 mM)	0.372 g
Tris-HCl (10 mM)	0.048 g
SDS (0.5%)	2 ml of 10% SDS
dH_2O	30 ml

(pH 8.0 with NaOH, adjust to 40 ml with dH_2O).

TTSB for Western blots (1 litre).

Tris-HCl (25 mM)	3.0 g
NaCl (0.9%)	9.0 g
Tween 20 (0.1%)	1.0 ml
milliQ H_2O	1 litre

(pH to ~7.4 with conc HCl)

For blocking add 2.5 g non fat skim-milk / 100 ml TTSB.

Phosphate buffers PO_4SB and PO_4WB (250 ml).

Na_2HPO_4 (20 mM)	0.71 g
NaCl (500 mM)	7.25 g

(For PO_4WB pH to 7.0 with HCl)

PMSF 10 mg / ml stock solution.

PMSF	0.1 g
methanol	10 ml

Maleic acid buffer (500 ml).

Maleic acid (0.1 M)	5.8 g
NaCl (0.15 M)	4.3 g

(pH 7.5 with ~ 35 NaOH pellets)

Maleic acid wash buffer (500 ml).

Maleic acid buffer	500 ml
Tween 20 (0.3%)	1.5 ml

DIG-Detection buffer (500 ml).

Tris-HCl (0.1 M)	6.05 g
NaCl (0.1 M)	2.9 g
(pH 9.5 with HCl)	

Anion exchange buffer A (1 litre).

Tris-HCl (20 mM)	2.42 g
NaN_3 (0.5%)	2 ml 10% NaN_3
dH_2O	1 litre
(pH 8.2)	

Anion exchange buffer B (1 litre).

Anion exchange buffer A	1 litre
NaCl (1 M)	58.4 g

PreScission Cleavage Buffer (100 ml).

Tris-HCl (50 mM)	0.61 g
NaCl (150 mM)	0.88 g
EDTA (1 mM)	0.037 g
DTT (1 mM)	0.015 g
dH_2O	100 ml
(pH 7.0 with HCl)	

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