In vitro and *in vivo* Evaluation of Two DNA Vaccine Candidates

Mihaela Zlei

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Division of Infection and Immunity,

Institute of Biomedical and Life Sciences.

University of Glasgow

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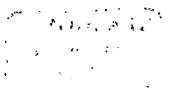

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Table of Contents

	Page
Table of contents	i-v
Abstract	vi
List of Abbreviation	vii-viii
List of Figures	ix-x
List of Tables	xi-xii
1. Introduction	1
1.1. General Introduction	2
1.2. Vaccination and Host Immunity	3
1.3. DNA Vaccination	6
1.3.1. Advantages and Disadvantages of DNA Immunisation	7
1.3.2. Activation of Host Immunity by DNA Vaccines	9
1.3.2.1. The Mechanism of Antigen Presentation Following DNA Immunisation	10
1.3.2.1.1. Direct Priming by Transfected Somatic Cells	10
1.3.2.1.2. The Role of APCs: Direct Transfection or Cross-Priming?	14
1.3.2.2. DNA Vaccines and Host Immunity	15
1.3.2.2.1. Cellular Immunity to DNA Vaccines	18
1.3.2.2.1.1. T Helper Cells Involved in Immune Response to DNA Vaccines	18
1.3.2.2.1.2. Cytotoxic T Lymphocyte Responses	18
1.3.2.2.2. Humoral Immunity to DNA Vaccines	20
1.3.3. Impact of Delivery Route on Immunological Properties of DNA Vaccines	21
1.3.4. Boosting Strategy and the Efficacy of DNA Vaccines	24
1.3.5. DNA Vaccines: Design and Optimisation	26
1.3.5.1. Immunostimulatory Elements	27
1.3.5.2. Factors Affecting Expression of Antigen in the Eukaryotic Host	29
1.3.6. Vaccine Candidates	29

1.3.6.1. Tetanus 30
1.3.6.2. Tuberculosis 33
1.3.6.2.1. Overview of the Immune Response to Mycobacteria 33
1.3.6.2.2. Bacillus Calmette-Guerin- the Current Available anti-Tuberculosis Vaccine 36
1.3.6.2.3. Importance of MPB-70 in Protective Immunity against Bovine Tuberculosis 37
1.4. Aims of the project 40
1.4.1. Objectives 40
2. Materials and Methods 41
2.1. Bacterial Strains 42
2.1.1. Growth Conditions 42
2.1.2. Master Seed Banking 42
2.1.3. Working Seed Banking 43
2.2. Plasmids 43
2.2.1. Plasmid Characterisation 43
2.2.2. Competent Cells and Transformation of *Escherichia coli* 48
2.2.3. Plasmid Purification 49
2.2.3.1. Preparation of Plasmids Using the Miniprep Kit 49
2.2.3.2. Preparation of Plasmids Using the Endotoxin-free Megaprep Kit 50
2.2.4. Qualitative and Quantitative Analysis of DNA Constructs 51
2.2.4.1. Restriction Enzymes Mediated Digestion of Plasmids 51
2.2.4.2. Agarose-Ethidium Bromide Gel Analysis of DNA 52
2.2.4.3. Spectrophotometric Analysis of Plasmid DNA 52
2.3. Expression of GFP, Fragment C and MPB-70 *in vitro* 54
2.3.1. COS-7 Cells - Growth Condition 54
2.3.2. Transfection of COS-7 Cells Using Lipofectamine 55
2.3.3. Harvest of Transfected Cells 55
2.3.4. Analysis of Protein Expression in COS-7 Cells 56
2.3.4.1. GFP Expression in Transfected COS-7 56
2.3.4.2. Fragment C and MPB-70 Expression in Transfected COS-7 Cells 56
2.3.4.2.1. SDS – Polyacrylamide Gel Electrophoresis 57
2.3.4.2.2. Staining Polyacrylamide Gels with Coomassie Blue 57

2.3.4.2.3. Western Blotting 58
2.3.4.2.4. Western Blot Detection Using Pico Chemiluminiscent Substrate 61
2.3.5. Glycosylation Inhibition 61
2.4. Intramuscular Immunisation of Mice 62
2.4.1. Mice 62
2.4.2. Vaccine Preparation 62
2.4.3. Evaluation of LPS Contamination in DNA Samples Used for Immunisation 63
2.4.4. Vaccination Regimen 63
2.4.5. Collection of Samples 65
2.5. Evaluation of the Immune Response Elicited by the Vaccine 65
2.5.1. Analysis of the Humoral Immune Response 65
2.5.1.1. Serum Preparation from Blood Samples 65
2.5.1.2. Measurement of Serum Specific Antibodies Using ELISA 65
2.5.1.3. Measurement of Antigen Specific Antibodies Using Western Blotting 66
2.5.1.4. Measurement of Specific Subtypes of Antibodies 67
2.5.2. Evaluation of Cellular Immune Response 67
2.5.2.1. T Cell Proliferation Assay 67
2.5.2.2. Cytokine Types in Culture Supernatant 68
2.6. Helios Gene-Gun Optimisation 69
2.6.1. Preparation of DNA/Gold Cartridges for Gene Gun Vaccination 69
2.6.2. Loading the DNA/Gold Suspension into Gold-Coat Tubing 71
2.6.3. Quantification of Plasmid DNA in Each Cartridge 73
2.6.4. Delivery of DNA by Gene-Gun 74
2.6.4.1. Transfection of Mammalian Target Cells *In Vitro* 74
2.6.4.2. Analysis of Protein Expression in COS-7 cells transfected by Gene-Gun 75
2.6.4.2.1. GFP Expression in COS-7 Cells transfected by Gene-Gun 75
2.6.4.2.2. TetC Expression in COS-7 Cells transfected by Gene-Gun 75
2.6.4.3. Transfection of Mammalian Cells *In Vivo* by Gene-Gun 75

2.6.4.4. Evaluation of the Immune Response Elicited by the Gene-Gun Method of Vaccination 76
3. Results - Section 1: Characterisation of Plasmid Constructs 78
3.1. Transformation of *E. coli* with DNA Vaccine Candidates 79
3.1.1. Initial Characterisation of pcDNA3/tetC and pcDNA3.1 79
3.1.2. Initial Characterisation of pCMV-70 and pCMV-link 82
3.2. Sequencing of the DNA Vaccine Candidates 82
3.3. Quantification of the DNA 82
3.4. Analysis of LPS Contamination 84
4. Results – Section 2: Transfection of COS-7 Cells Using Lipofectamine 86
4.1. Optimisation of *In Vitro* Transfection Experiments Using a Plasmid Encoding the Green Fluorescent Protein (GFP) 87
4.2. *In vitro* Expression of MPB-70 in COS-7 Cells 89
4.2.1. Optimising the Detection of MPB-70 Expressed *In Vitro* 90
4.2.2. MPB-70 Expression in COS-7 Lysates Following Transfection with pCMV-70 93
4.2.2.1. Reduction of Aggregate Forms of MBP-70 97
4.2.2.2. Role of N-Glycosylation in Post-Translation Modification of MBP-70 97
4.3. *In vitro* Expression of Fragment C 99
4.3.1. Detection of Fragment C Using a Chemiluminescent Substrate 99
4.3.2. Detection of *In Vitro* Expressed Fragment C 101
5. Results – Section 3: Gene-Gun Mediated Transfection of COS-7 Cells 103
5.1. Optimisation of Gene-Gun Cartridge Preparation and DNA Delivery 104
5.1.1. Impact of PVP Concentration and Ethanol Purity 104
5.1.2. Impact of Helium Pressure on Gene-Gun Driven Transfection 105
5.1.3. Inclusion of a Screen Diffuser on Gene-Gun Driven Transfection 107
5.1.4. Inter- and Intra-Variability of Transfection Using Different Batches of Coated Cartridges 107
5.1.5. Expression of GFP in COS-7 Cells Using Optimised Conditions of Delivery 109
5.1.6. Quantification of Amount of DNA Present in Each Cartridge 112
5.2. Expression of Fragment C of Tetanus Toxin in COS-7 Cells Transfected with pcDNA3/tetC Using the Gene-Gun 114

5.2.1. Optimisation of the Detection System 114
5.2.2. Transfection and Expression of Fragment C in COS-7 Cells 114
6. Results – Section 4: Vaccination with pcDNA3/tetC 117
6.1. Fragment C Specific Responses Following Immunisation with pcDNA3/tetC 118
6.1.1. Antibody Levels Following Intramuscular Immunisation with pcDNA3/tetC 118
6.1.2. Antibody Levels Following Gene-Gun Administration of pcDNA3/tetC 120
6.1.3. Impact of Route of Immunisation on IgG Isotype 120
6.1.4. Impact of Boosting Animals Previously Vaccinated Intramuscular with DNA 123
6.1.4.1. Impact of Prime/Boost Regimen on Specific Antibody Levels 123
6.1.4.2. Impact of Prime/Boost Regimen on IgG Isotype 125
6.1.5. Impact of Boosting Animals Previously Vaccinated with pcDNA3/tetC by Gene-Gun 128
6.1.5.1. Impact of Prime/Boost Regimen on Specific Antibody Levels 128
6.1.5.2. Impact of Prime/Boost Regimen on IgG Isotope 130
6.2. T Cell Proliferation Assay 133
6.2.1. T Cell Proliferation in Mice Immunised Intramuscularly with pcDNA3/tetC 133
6.2.2. T cell Proliferation in Mice Immunised by Gene-Gun with pcDNA3/tetC 136
6.3. Cytokine Secretion 136
6.3.1. Cytokine Secretion by Re-Stimulated Splenocytes and Regional Lymph Node Cells from Mice Immunised Intramuscularly With pcDNA3/tetC 136
6.3.2. Cytokine Secretion by Splenocytes and Lymph Node Cells from Mice Immunised with pcDNA3/tetC by Gene-Gun 137
7. Results – Section 5: Vaccination with pCMV-70 140
8. Discussion 142
8.1. pCMV-70 Plasmid, as an anti-Tuberculosis DNA Vaccine Candidate 144
8.2. pcDNA3/tetC Plasmid, as an anti-Tetanus DNA Vaccine Candidate 147
References I-XV

Abstract

DNA vaccinology, although still in its infancy, has raised a great wave of interest among researchers, due to its wide applicability and the potential advantages it appears to offer over traditional methods of vaccination. In this study two independent bacterially derived antigens were evaluated as DNA vaccine candidates. The main thrust being to determine the potential of these vaccines to express antigen *in vitro* and stimulate immune responses *in vivo*. In addition, the route of immunisation on the magnitude and orientation of the immune response to the encoded antigens was also considered, with mice immunized either intramuscularly or intradermally using a Gene Gun.

One of the candidates, a DNA vaccine encoding a *Mycobacterium bovis* antigen (MPB-70) expressed very effectively *in vitro* when transfected into COS-7 cells. However, it failed to stimulate an immune responses in mice, regardless the route of immunisation used. In contrast, a second bacterial antigen, Fragment C of tetanus toxin produced by *Clostridium tetani,* expressed poorly in eukaryotic cells but elicited a specific immune response in vaccinated mice. Although, the magnitude of these responses did not appear to differ when mice were immunized using intramuscular or intradermal immunisation using the Gene-Gun, the quality of the immune response generated varied considerably. These results demonstrated that vaccination of pcDNA3/tetC by intramuscular route was associated with a specific anti-Fragment C humoral and cellular response associated with Th1 CD4 cells, whilst immunisation using the Gene-Gun appeared to bias the response toward a CD4 associated Th2 profile. Moreover, both methods were able to prime a specific immune response with a magnitude that could be improved when the mice were boosted using the purified Fragment C antigen. Boosting these animals in such a manner resulted in an improvement in the magnitude of the immune response generated, however, the orientation of this response remained faithful to that originally initiated by the original route of DNA vaccination. Interestingly, the Gene-Gun mediated immunisation appeared to be far more efficient at stimulating immune responses as the magnitude of the immune response to the antigen was unaffected despite only a fiftieth of the amount of DNA used for each immunisation. This work highlights the potential impact of immunisation route on the quality and magnitude of an immune response to Fragment C when delivered as a DNA vaccine.

List of Abbreviations

Ab = antibody

APC = antigen presenting cells

APS = ammonium persulphate

BCG = bacillus Calmette-Guerin

BGH = bovine growth hormone

BSA = bovine serum albumin

CMI = cell-mediated immunity

CMV = cytomegalovirus

ctg. = cartridge

CTLs = cytotoxic T lymphocytes

CpG = cytidine-phosphate-guanosine motifs

DCs = dendritic cells

DLR = DNA loading ratio

DMEM = Dulbeco's modified Eagle medium

DMSO = dimethyl-sulph-oxyd

dsDNA = double stranded DNA

DTT = dithiothreitol

ELISA = enzyme linked immunosorbent assay

FBS = foetal bovine serum

GAD = glutamic acid decarboxylase

GFP = green fluorescent protein

HIV = human immunodeficiency virus

HBSS = Hanks' balanced salt solution

HRP = horse-radish peroxidase

IDDM = insulin-dependent diabetes miellitus

IFN = interferon

Ig = immunoglobulin

IL = interleukin

i. m. = intramuscular

LAL = Limulus Amebocysate lysate

L-broth = Luria Bertani broth

LNC = lymph node cells

LPM = litres per minute

LPS = lipopolysacharide

LT =heat labile toxin from *E. coli*

MES = 2-(N-morpholino) ethanesulphuric acid

MHC = the major histocompatibility complex

MLQ = microcarrier loading quantity

MOPS = 3-(N-morpholino) propanesulfuric acid

mRNAs = messenger RNAs

NK = natural killer

OD = optical density

PBMC = peripheral blood mononuclear cells

PBS = phosphate buffer saline solution

PMSF = phenylmethyl sulfonyl fluoride

pNA = p-nitroanile

PolyA = polyadenilation signal

PVP = polyvinyl pyrrolidone

ROS = reactive oxygen species

SDS-PAGE = sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SI = stimulation index

SOD = superoxide dismutase

SV40 = simian virus 40

TB = tuberculosis

TEMED = tetramethylendiamine

Th cells = T helper cells

WHO = World Health Organisation

List of Figures

	Page
Figure 1.1: Mechanism of immune response priming by direct transfection of somatic cells	11
Figure 1.2: Priming of immune response by direct transfection of antigen presenting cells	16
Figure 1.3: Cross-priming theory	17
Figure 1.4: A schematic representation of a plasmid-DNA vaccine	28
Figure 2.1: A schematic representation of pcDNA3.1 and pcDNA3/tetC plasmid maps	44
Figure 2.2: A schematic representation of pCMV-link and pCMV-70 plasmid maps	46
Figure 2.3: A schematic representation of pGFP plasmid map	47
Figure 2.4: A schematic representation of the main steps involved in cartridge preparation and Gene-Gun mediated delivery of DNA	72
Figure 3.1: Digestion of pcDNA3.1 and pcDNA3/tetC using *XbaI*	80
Figure 3.2: Restriction digest of pcDNA3.1 and pcDNA3/tetC with *HindIII* and *XbaI*	81
Figure 3.3: Digestion of pCMV-link and pCMV-70 with *Bgl-II*	83
Figure 4.1: *In vitro* expression of GFP by Lipofectamine	88
Figure 4.2: Detection limit of recombinant MPB-70	91
Figure 4.3: Analysis of recombinant MPB-70 using different reducing agents	92
Figure 4.4: Detection of MPB-70 in COS-7 cells transfected with pCMV-70 (polyclonal antibodies)	94
Figure 4.5: Detection of MPB-70 expression in COS-7 cells transfected with pCMV-70 (monoclonal antibodies)	96
Figure 4.6: Optimisation of antibody concentrations used in Western blot method using the SuperSignal substrate	100
Figure 4.7: Fragment C expression in COS-7 cells transfected	

with pcDNA3/tetC and Lipofectamine 102

Figure 5.1: The amount of unbound DNA present in solutions used to wash the gold particles during preparation of the firing cartridges 106

Figure 5.2: *In vitro* expression of GFP by Gene-Gun 111

Figure 5.3: Expression of Fragment C in COS-7 cells transfected using the Gene-Gun with pcDNA3/tetC 116

Figure 6.1: Titres of Fragment C specific IgG in the serum following two intramuscular doses of pcDNA3/tetC 119

Figure 6.2: IgG response against Fragment C following Gene-Gun immunisation with two doses of pcDNA3/tetC 121

Figure 6.3: Ratio of IgG1/IgG2a Fragment C specific antibody in the sera following intramuscular or Gene-Gun immunisation with pcDNA3/tetC 122

Figure 6.4: Titres of Fragment C specific IgG in the serum before and after boosting primed mice with purified Fragment C 124

Figure 6.5: Titres of Fragment C specific subclasses of IgG in mice primed with pcDNA3/tetC and boosted with purified Fragment C 126

Figure 6.6: Ratio of IgG1/IgG2a Fragment C specific antibody in the sera of mice primed i. m. with pcDNA3/tetC and boosted with Fragment C 127

Figure 6.7: Titres of Fragment C specific IgG in the serum before and after boosting Gene-Gun primed mice with purified Fragment C 129

Figure 6.8: Titres of Fragment C specific subclasses of IgG in mice primed with pcDNA3/tetC using the Gene-Gun and boosted with purified Fragment C 131

Figure 6.9: Ratio of IgG1/IgG2a Fragment C specific antibody in the sera of mice primed with pcDNA3/tetC and boosted with Fragment C 132

Figure 6.10: Stimulation of spleen cell and lymph node cells from vaccinated mice 135

List of Tables

	Page
Table 2.1. Primers used for pcDNA3/tetC and pCMV-70 sequencing	53
Table 2.2. Restriction enzymes used to linearise plasmid DNA Constructs	53
Table 2.3. Concentrations of primary and secondary antibody used for Western blot detection	59
Table 2.4. Concentrations of primary and secondary antibody used for Western blot detection using Pico Chemiluminiscent Substrate	60
Table 2.5. Vaccination regimen for mice immunised i. m. with pcDNA3/tetC or with the control plasmid, pcDNA3.1 and boosted with Fragment C	64
Table 2.6. Vaccination regimen for mice immunised i. m. with pCMV-70 or with the control plasmid, pCMV-link and boosted with MPB-70	64
Table 2.7. The main components needed for preparation of Gene-Gun cartridges	70
Table 2.8. Vaccination regimen for mice immunised by Gene-Gun with pcDNA 3/tetC or with the control plasmid, pcDNA3.1 and boosted with Fragment C	77
Table 3.1. Average amounts of plasmid DNA generated from miniprep and megaprep kits	85
Table 5.1. Number of fluorescent cells observed following transfection by Gene Gun using a helium pressure of 200 psi	108
Table 5.2. Number of fluorescent cells observed following transfection by Gene-Gun using a helium pressure of 100 psi	108
Table 5.3. Number of fluorescent cells observed following transfection by Gene-Gun using cartridges loaded with 0.05 mg/ml PVP, a helium pressure of 100 psi and a diffusion screen	110
Table 5.4. Analysis of the amount of DNA present in each cartridge	113
Table 6.1. Production of IFN-γ and IL-5 from spleen and lymph node	

cells from mice primed i. m. with DNA and boosted i. m. with purified Fragment C and LT 139

Table 6.2. Production of IFN-γ and IL-5 from spleen and lymph node cells from mice primed by Gene-Gun with DNA and boosted i. m. with purified Fragment C 139

Chapter 1

Introduction

1.1. General Introduction

From the late 18th century vaccines have had a significant impact on the prevention of a wide range of infectious diseases, including the world-wide eradication of smallpox. This success has led to the expansion of other global vaccination programmes, which, it is hoped, will lead to the eradication of other diseases such as poliomyelitis. The utility of other vaccines against serious diseases, such as tuberculosis, diphtheria, tetanus, pertussis and measles are now commonly accepted and adaptation of vaccination schedules to the epidemiological and social circumstances of each country has allowed widespread application of such vaccines. Given the remarkable success of this approach, vaccination has become one of the most cost-effective health interventions at our disposal.

The provenience of the word "vaccination" and the application of vaccination as a prophylactic method against infectious diseases are both attributed to the 18th century scientist Edward Jenner. In 1796 he took material from a pustular lesion on the hand of a milkmaid infected with cowpox and showed that by inoculation of this substance into the skin of a boy, the boy was protected from infection with smallpox. This result confirmed that immunisation of a related virus induced an immune response that could protect the host against subsequent infection. (The term vaccination has its roots in these experiments as the name "vacca" in Latin means "cow" and relates back to these early experiments using cowpox). The meaning of the word "vaccination" has by now extended to a larger context, implying the administration of substances of any origin capable of eliciting a protective response.

Today, our understanding of immunity and mechanisms of activation has extended to include the role of both humoral and/or cellular types of immunity in protection against disease. Most of the current vaccines available for clinical use today generate a strong

antibody or humoral immune responses that protect against acute phases of infection. Such vaccines are very effective against diseases in which the pathogenesis is dependent upon a single virulence factor such as tetanus toxin, where antibody is important for neutralisation of its effect. However, most existing vaccines do not provide protection against chronic infections or intracellular pathogens or protect the mucosal surfaces of the body, the site from which many pathogens gain access to the host.

Thus, despite remarkable successes of vaccination over several decades of history, new or improved vaccines are still urgently needed. These include ones against diseases for which no vaccines currently exist (for example, malaria and AIDS), or improved, more efficacious forms of currently available vaccines such as those against *Mycobacteria tuberculosis*.

1.2. Vaccination and Host Immunity

The efficacy of all vaccines is largely dependent on the type and magnitude of protective immune response generated in response to the vaccines. In vertebrates, the immune system is composed of non-specific (non-adaptive) and specific (adaptive) components. Non-adaptive components include a variety of cells, such as natural killer (NK) cells, eosinophils, and polymorphonuclear cells, and factors, such as the α-, and β-interferons (IFN) and complement. These cells and components are deployed rapidly and non-specifically, thus providing a first line of defence against the invading pathogen. In contrast, the specific, adaptive response is carried out by the lymphocytes, which upon activation show two critical characteristics: specificity and memory. These features are essential in vaccination, as prophylaxis is dependent on the rapid activation of a specific protective response to the organism, potentially years after the vaccine is given.

Lymphocytes are generally considered to fall into one of two classes, the first being the B lymphocyte, which following specific stimulation, replicate and differentiate into plasmocytes that either secrete specific antibodies or become memory cells, which can be efficiently evoked at a subsequent infection. The type of immunity governed by B cell activation, differentiation and antibody secretion was initially described as "humoral-mediated immunity".

The second class includes the T or "thymus derived" lymphocytes. They have been conventionally divided into two main groups, distinguished, in part by specific markers (CD4 and CD8) at the cell surface. These cells are responsible for the activation of the "cell-mediated" immune response (CMI), but they are also strongly involved in humoral immunity, by providing "help" or "assistance" to B cells.

Several functions have been attributed to $CD4^+$ T lymphocytes (or "helper" lymphocytes), including promotion of B-cell survival and antibody production through secretion of cytokines, such as interleukin (IL)-4, IL-5, IL-6, and IL-10 and through cell surface interactions (CD40L-CD40) (Banchereau *et al.*, 1994). They also provide "help" to $CD8^+$ T lymphocytes through production of IL-2 and/or through CD40L-CD40 interactions (Schoenberger *et al.*, 1998). Third, they secrete a myriad of cytokines, such as IL-2, IL-12, IFN-γ, which are important cytokines for the activation of cells such as macrophages, which are crucial for clearance of many bacterial infections.

Activated $CD4^+$T lymphocytes can subsequently be subdivided into two distinct subsets: T helper-1 (Th1) and T helper-2 (Th2) lymphocytes based on the cytokines they secrete. Th1 lymphocytes which preferentially secrete IL-2, IL-12, IFN-γ, support the raising of complement-binding subclasses of IgG (IgG2a), and activate phagocytic cells to recognise and kill opsonised-invading microbes. By contrast, Th2 cells that secrete IL-4, IL-5, IL-6, and IL-10, support the raising of IgE and non-complement binding subclasses of

IgG (IgG1 and IgG2b) and activate non-phagocytic defence mechanisms (such as mast cells). Many studies using infection of normal and transgenic animals, suggest that activation of Th1 cells are important in the control and clearance of mainly bacterial and chronic viral infections, whilst induction of Th2 cells appears important in the prevention of bacterial infections and in the control of parasitic infection.

Another group of T cells, distinguished from the first two groups by possession of receptor CD8 on the surface is represented by cytotoxic T cells, which have a broadly similar pattern of cytokine secretion to Th1 cells. These cells specifically mediate the direct killing of cells in particular the killing of virally infected cells.

Activation of both types of lymphocyte (helper and cytotoxic) is dependent upon their interaction with specialised cells, named antigen presenting cells (APC). These cells are able to internalise, process and present on their surface peptide fragments from antigens from infecting pathogen. These peptide fragments are presented on the cell surface of APCs in combination with molecules encoded by genes of the major histocompatibility complex (MHC). Class I MHC molecules are found on all nucleated cells in the body, whilst class II MHC molecules are only found on professional antigen presenting cells (macrophages, dendritic cells [DCs], B cells). The type of processing and presentation within APCs dictates the type of T-lymphocyte that interacts with the APC, affecting the subsequent downstream events and the type of immune response generated. Peptides bound to class I MHC molecules are typically derived from endogenously proteins, such as viral particles or proteins belonging to intracellular pathogens and are recognised by $CD8^+$ cytotoxic T lymphocytes. In contrast, peptides derived from proteins taken up by the APCs from the extracellular environment (exogenous antigens) are displayed in association with class II MHC molecules and are recognised by $CD4^+$ T cells.

The majority of vaccines in use today, induce high levels of long-lasting antibody responses that protect against disease by either prevention of infection or by control of pathogen replication. This type of response is dependent on B-cell activation and proliferation, which is dependent on help from $CD4^+$ Th2 cells. Antibody in this context is protective as it is able to neutralise the effects of toxins, or to kill bacteria by enhancement of the mechanisms of antibody dependent cellular cytotoxicity or complement dependent lysis. On the other hand, very few vaccines are available that activate the proliferation of $CD4^+$ Th1 or $CD8^+$ T cells that are effective in the control and clearance of intracellular infections. Therefore, new approaches are required to be able to specifically activate directly cell-mediated responses that can lead to the clearance of such pathogens.

1.3. DNA Vaccination

One approach that appears to preferentially enhance cell-mediated immunity is the use of 'naked' DNA directly to vaccinate individuals. This form of vaccination involves the direct transfer and expression of nucleic acid encoding a protective antigen from a pathogen, to an eukaryotic host, with the aim of activating a specific response to the encoded antigen.

The ability of DNA to activate such responses has only been recognised relatively recently and followed the observation that nucleic acid encoding the β-galactosidase enzyme could produce functional enzyme when injected into the muscle tissue of mice (Wolff *et al.*, 1990). Later, Tang and co-workers reported that plasmid DNA was able to mount a sustained immune response to such a plasmid-encoded antigen expressed in muscle cells (Tang *et al.*, 1992). This report led to an explosion of activity, which has resulted in the identification and generation of a number of new candidate vaccines. In addition, it has

led to a greater understanding of the mechanisms by which immune responses are activated and some of the factors that are likely to influence the type and magnitude of this response.

1.3.1. Advantages and Disadvantages of DNA Immunisation

Further extension of these studies has shown that DNA vaccination may have several advantages over current vaccines including the ability to raise protective and long-term cell mediated immune responses. Activation of these responses is particularly crucial for protection against intracellular pathogens such as HIV, malaria, leishmaniasis, listeriosis, and tuberculosis. DNA vaccines also have the potential to deliver multiple epitopes on one construct providing the potential to protect against several pathogens. Another advantage is that genes that confer pathogenicity or virulence or genes that lead to undesired inhibition of immunological responses or cross-reactivity can be excluded using this approach. In addition, manufacture of such vaccines can make use of a single production process, they can be stored by lyophilisation and transported without the need for cool storage, thus reducing cost considerably. Finally, this approach offers the opportunity to manipulate and explore the potential for the development of new immunotherapeutic vaccines and gene replacement therapy.

Nevertheless, a number of safety concerns remain, including the possible impact on the genetic mechanisms of carcinogenesis, which may result from the integration of bacterial DNA into the host genome. This event may increase the potential risk of malignancy, either by activating oncogenes, or by inactivating tumor suppressor genes. However, despite many attempts to measure such events experimentally, most date suggests that integration of injected DNA is unlikely, and, moreover, such events appear to occur less frequently than the rate of spontaneous mutation that occurs naturally in mammalian genomes (Martin *et al.*, 1999).

Another concern is the potential of DNA vaccination to induce autoimmunity by the generation of antibody to the host genomic DNA. However, despite the fact that bacterial DNA can induce the production of anti-double-stranded, dsDNA-autoantibodies, bacterial DNA has been shown to suppress autoimmune response in lupus-prone mice (Gilkeson *et al.*, 1995). In addition, hundreds of human volunteers have been exposed to plasmid DNA vaccines without serious adverse consequences (MacGregor *et al.*, 1998; Hanke *et al.*, 2000, Le *et al.*, 2000, Tacket *et al.*, 1999).

Finally, as the immune response to DNA vaccination appears to be relatively long-lived, persistence of this response may have consequences upon the host, such as induction of tolerance to the antigen or autoimmunity. Such consequences may be overcome by the use of an inducible promoter to control *in vivo* expression of the plasmid-coded (Dhawan *et al.*, 1995). For example, using a bacterial tetracycline (tet)-responsive system, Dhawan and co-workers were able to regulate the timing and expression level of an intramuscularly injected plasmid.

Most concerns regarding the potential induction of toleragenic responses has been associated with the potential use of these vaccines paediatrically, where factors such as the nature of the antigen, concentration of the plasmid as well as the age of the host appear important (Marodon *et al.*, 1994). As protein antigens encoded by a DNA vaccines are produced endogenously and expressed in the context of self-MHC, the potential exists for the neonatal immune system to recognise the antigen as "self", resulting in tolerance rather than immunity. For example, a DNA vaccine encoding the circumsporozoite protein of malaria was found to induce tolerance rather than immunity in new-born mice (Mor *et al.*, 1996). However, in this study, the induction of tolerance was observed only when vaccine was administered to mice less than 8 days of age. However, as most vaccines intended for

human use are administered to infants and children, such information may limit the application of such vaccines in a wider context.

Despite such queries regarding the safety of DNA vaccination, a number of volunteers have enrolled and taken part in a phase I and/or phase II clinical studies. These clinical trials are based on DNA vaccines encoding HIV antigenes, showing specific lymphocyte proliferation in patients, without eliciting local or systemic reactions, anti-DNA antibody or muscle enzyme elevations (MacGregor *et al.*, 1998; Hanke *et al.*, 2000), malaria antigens which triggered specific CTL responses without severe or serious adverse events or detectable anti-dsDNA antibodies (Le *et al.*, 2000), and hepatitis B surface antigen, suggesting that the vaccine may induce a booster response, but without eliciting primary immune responses at the lowest DNA dose used (Tacket *et al.*, 1999).

1.3.2. Activation of Host Immunity by DNA Vaccines

Induction of immune responses following DNA immunisation is a very complex process and begins when host cells are transfected with DNA. Following transfection DNA must travel to the cell nucleus, where is transcribed to an immature RNA transcript. The primary transcript undergoes the splicing process and is converted into mature messenger RNA (mRNA). The mRNA is subsequently transported to the cytosol where it is translated to the ribosomes into protein. The protein is then either associated with MHC class I molecules, which are transported to the cell surface for presentation to cytotoxic immune cells, or the protein is released (possibly from dying cells) allowing uptake by other professional APCs which present to T cells through MHC class II molecules.

1.3.2.1. The Mechanism of Antigen Presentation Following DNA Immunisation

The mechanism by which the antigen-encoded by plasmid DNA is processed and presented to the immune system is still under debate. However, it is possible that antigen encoded on DNA vaccines are processed and presented to the immune system using three different routes. These include:

1. Direct priming by somatic cells (myocytes in the case of intra-muscular administration and keratinocytes in the case of intra-dermal delivery of DNA).
2. Direct transfection of professional APCs (Langerhans cells found in the skin or other DCs found at the site of vaccine inoculation)
3. Cross-priming, in which plasmid DNA transfects a somatic cell and/or a professional APC and the released protein and/or apoptotic bodies containing the expressed protein are taken up by other professional APCs and presented to T cells.

1.3.2.1.1. Direct Priming by Transfected Somatic Cells

In the case of intra-muscular administration, myocytes have been shown to be able to take up the injected plasmid (Davis *et al.*, 1993[b]). However, the mechanism by which immune responses are generated in this context is unclear. Figure 1.1. illustrates the possible interaction, if any, of myocytes (as APCs) and $CD8^+$ T cells (as precursors of immune effector cells) in inducing immune response against an antigen encoded by a DNA vaccine.

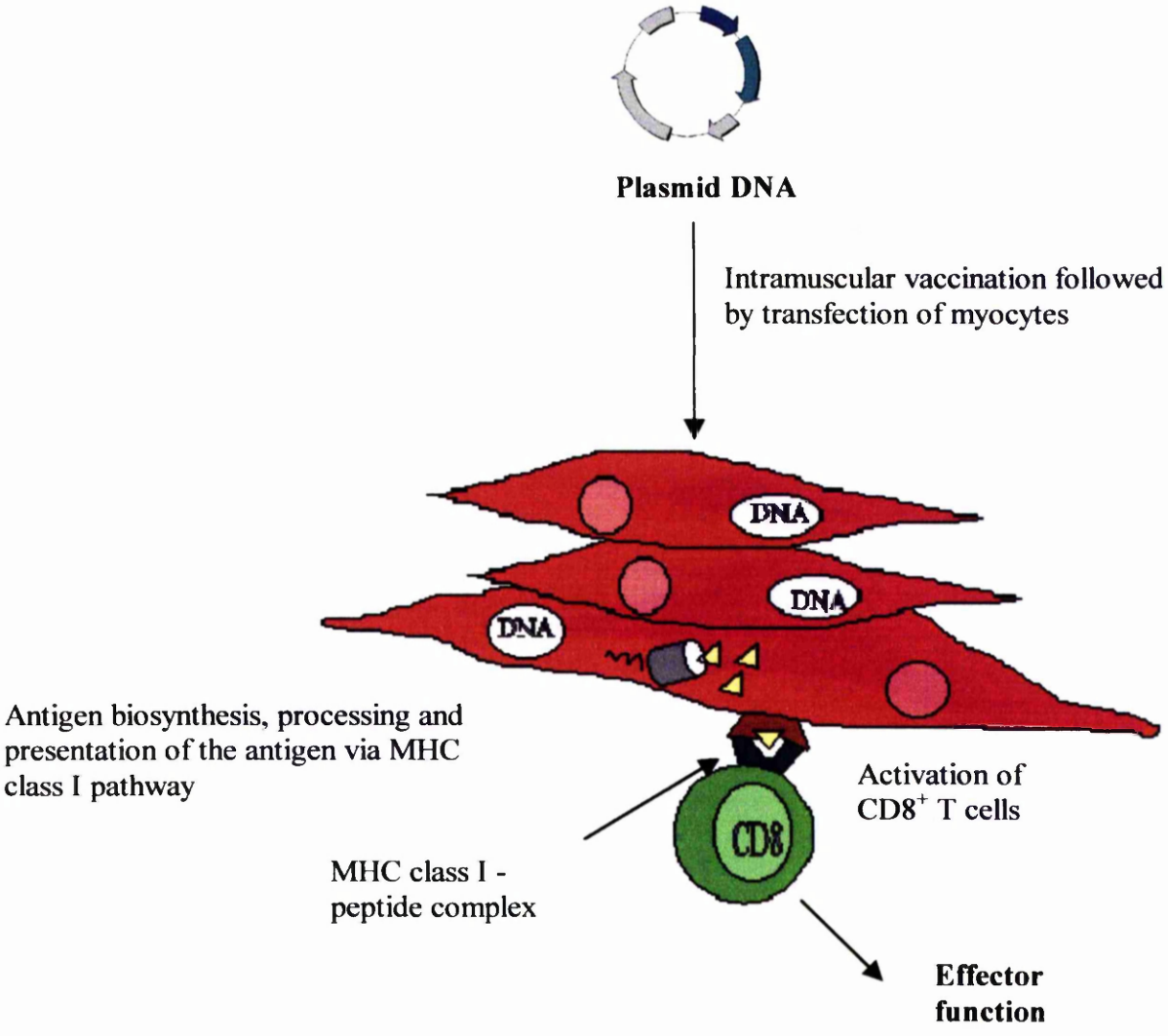

Figure 1.1: Mechanism of immune response priming by direct transfection of somatic cells

Directly transfected myocytes may induce activation of $CD8^+$ T cells by presentation of the endogenously synthesised antigen via MHC class I pathway. Though, muscle is not considered an immunologically active tissue, as they lack the characteristics of APCs (such as expression of MHC class II molecules, co-stimulatory molecules [B7.1/CD80 and/or B7.2/CD86] or cytokine secretion), they do express MHC class I molecules.

Muscle is not considered an immunologically active tissue, as although myocytes express MHC class I molecules, they constitutively lack the characteristics of APCs (such as expression of MHC class II molecules, co-stimulatory molecules [B7.1/CD80 and/or B7.2/CD86] or cytokine secretion). As co-stimulatory molecules, the role of CD80 and CD86 is to provide a second stimulatory signal for activation of antigen-specific $CD4^+$T cells. Their expression on the surface of APC is up-regulated after their activation. They interact with CD28 present on the cell surface of Th lymphocytes, concomitant with the primary activation signal provided by the interaction between antigen-specific T cell receptor and MHC-peptide complex on the surface of APC. Thus, myocytes alone are unlikely to be able to prime naive $CD4^+$ or $CD8^+$ T cells. Moreover, even the co-expression of co-stimulatory molecules as CD86 or of cytokines like GM-CSF or IL-12, has been shown to be insufficient to turn non-hematopoietic cells into efficient APCs for CTLs (Iwasaki *et al.*, 1997[a]).

By contrast, a separate study found that non-hematopoietic cells such as muscle cells could be engineered to function as APCs, by co-administration of CD86. This was demonstrated using bone-marrow chimeric animals, developed in β2 microglobulin (β2m) knockout mice. As β2m is essential for effective transport and expression of functional MHC class I molecules, normal mice irradiated and subsequently transplanted with bone-marrow from β2m knockout mice ($\beta2m^{-/-}$ to $\beta2m^{+/+}$) possess MHC class I positive muscle cells and MHC class I negative bone-marrow derived APCs. In contrast, $\beta2m^{+/+}$ to $\beta2m^{-/-}$ mice possess MHC class I positive bone-marrow derived APCs and MHC class I negative muscle cells. When these mice were co-immunised a plasmid encoding an HIV-1 antigen together with a second encoding CD86 or CD80, high levels of specific CTL responses were only observed in animals co-immunised with the plasmid encoding CD86. This

suggested that either the muscle cells or another non-hematopoietic cells became able to function as APCs when CD86 was co-administered (Agadjanyan *et al.*, 1999). In addition, Behrens and co-workers have described functionally active co-stimulatory molecules present on the surface of human muscle cells, which were distinct from B7.1/CD80 or from B7.2/CD86, but similarly involved in co-activation of antigen-specific $CD4^+$ T cells (Behrens *et al.*, 1998).

However, the fact that the injected muscle tissue can be removed from the mouse as little as 10 minutes after immunisation without affecting the subsequent immune response, suggests that the muscle is unlikely to be the major site of antigen presentation (Torres *et al.*, 1997).

The situation is much different in the case of intradermal injection of DNA vaccines where it has been shown that immediate removal of the skin after inoculation prevented immune response development (Torres *et al.*, 1997). In support of this, a separate study showed that transplantation of vaccinated skin 12 hours post vaccination could elicit an immune response in naive animals (Klinman *et al.*, 1998). This might suggest that transfected keratinocytes play a role in priming the immune response to DNA vaccines, but only for a short period of time, because when the period of transplantation exceeded 24 hours, little or no immune response could be initiated. However, it seems more likely that those intradermally located DCs, such as Langerhans cells, are being transfected and migrate to local lymph nodes within this 24h period. At such sites they can present antigen and activate naïve T-cells.

1.3.2.1.2. The Role of APCs: Direct Transfection or Cross-Priming?

The role of professional APC (such as macrophages and DCs) in the periphery includes capturing and processing antigens, which when expressed with suitable co-stimulatory molecules can activate naïve T-cells in appropriate draining lymphoid organs.

Several studies have conclusively demonstrated that bone-marrow derived APCs play a key role in initiating the immune response following DNA vaccination. Using bone-marrow chimeric mice, a number of workers (Corr *et al.*, 1996; Iwasaki *et al.*, 1997[b]; Doe *et al.*, 1996) have shown that priming of immune responses following DNA appears restricted to the haplotype of reconstituted bone-marrow. However, these studies failed to address the question of how these bone-marrow derived APCs obtained the antigen for presentation to T lymphocytes, i.e. by direct plasmid transfection of the APCs (as shown in Figure 1.2) or through uptake of the antigen released from transfected myocytes or keratinocytes (see Figure 1.3).

Evidence supporting the idea of direct transfection comes from several sources. First, plasmid DNA has been isolated from lymph node derived and skin-derived DCs after intra-muscular and intra-dermal DNA immunisation (Casares *et al.*, 1997). Second, only DCs (not B cells or keratinocytes) from vaccinated mice were capable of presenting antigenic epitopes to antigenic-specific T cells. However, it was estimated that only a small proportion of DCs (0.4%) were transfected with plasmid DNA (Porgador *et al.*, 1998; Akbari *et al.*, 1999). These data suggest that priming of an immune response by DNA vaccination predominantly involve the direct transfection of a small number of DCs.

Evidence supporting the cross-priming hypothesis in which antigen is transferred from transfected myocytes or keratinocytes to professional APCs also exists. In these experiments, myoblasts transfected with influenza nucleoprotein DNA were transplanted into F1 hybrid mice and induced CTL responses which were restricted by the MHC

haplotype of the recipient mice (Ulmer *et al.*, 1997; Ulmer *et al.*, 1996). These workers also showed that transfer of antigen from myocytes to professional APCs can occur *in vitro*. In addition, cross priming can occur when professional APCs phagocytose not only secreted protein (from somatic cells and/ or other APCs) but also apoptotic or necrotic bodies containing the antigen that are released following the death of a transfected cell (Albert *et al.*, 1998).

In conclusion, it would appear that APCs, but not somatic cells, are directly involved in priming immune responses following DNA vaccination, either by direct transfection, or by cross-priming.

1.3.2.2. DNA Vaccines and Host Immunity

DNA vaccination has been reported to induce long-term humoral and cellular immunity and protection against a variety of viral, bacterial, and eukaryotic (parasites and tumour) antigens. This includes the production of a strong antibody response in a number of animal species including mice, non-human primates and more recently, in man. In some cases, especially those involving antigens from viral pathogens, production of antigen specific antibody appeared sufficient to protect the animal against subsequent challenge (Fynan *et al.*, 1993). This probably reflects the production of high titres of neutralising antibody against the virus, which may reflect that mechanism of antigen processing following this form of vaccination more closely resembles that which occurs during the natural infection (such in the case of Herpes Simplex Virus, Ghiasi *et al.*, 1995). In contrast, recombinant proteins may produce antisera of relatively low neutralising activity as presentation of certain determinants or epitopes may be inhibited. In addition, antigens produced *in situ* inside cells are additionally subjected to appropriate post-translational modifications, including glycosylation. This means that for viral antigens, proteins produced

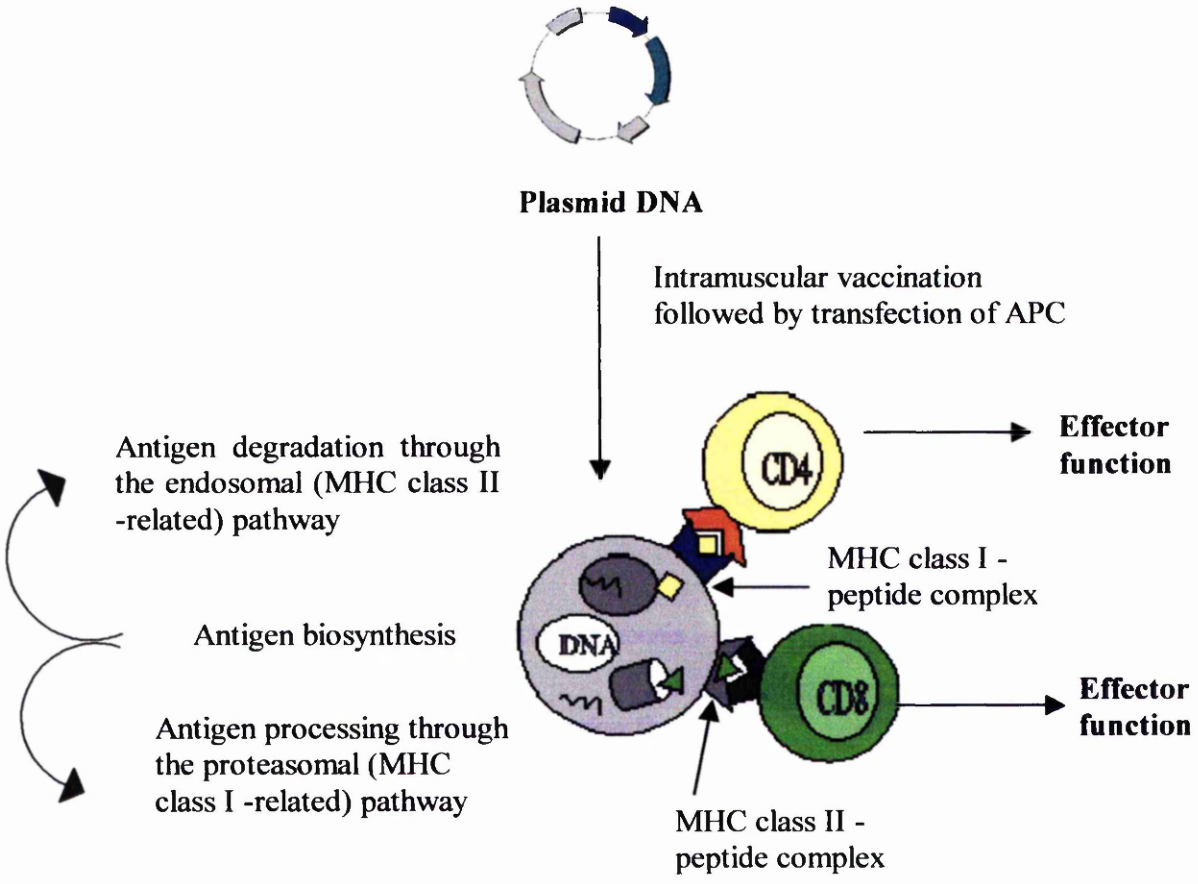

Figure 1.2: Priming of immune response by direct transfection of antigen presenting cells

Protein produced by transfected professional antigen presenting cells may be presented directly to $CD4^+$ T cells and $CD8^+$T cells via MHC class I and class II molecules respectively, leading to T cell activation.

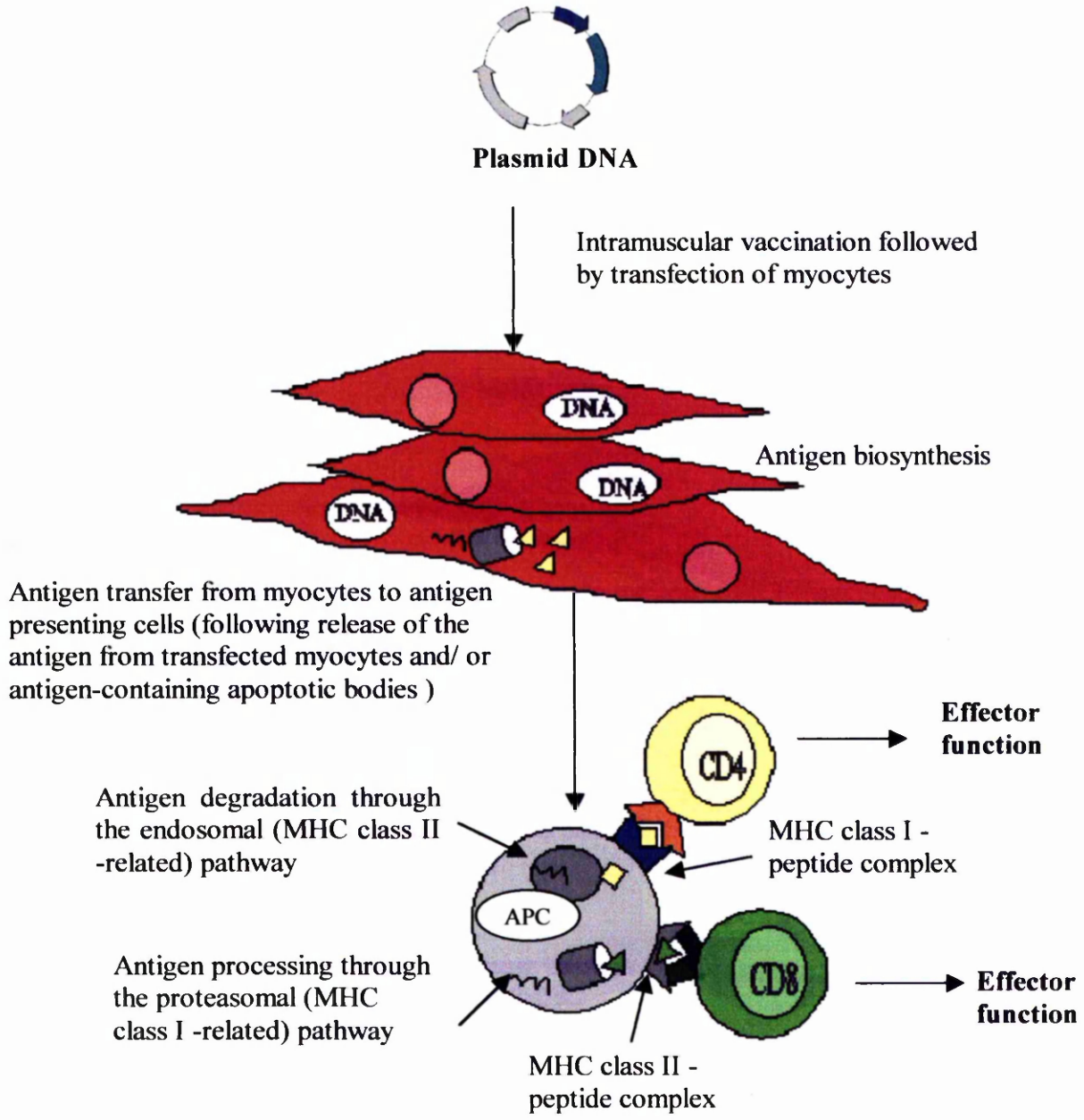

Figure 1.3: Cross-priming theory

Protein produced by transfected somatic cells may be taken up by professional antigen presenting cells (APCs) and then presented to $CD4^+$ T cells and $CD8^+$T cells via MHC class I and class II molecules respectively, leading to T cell activation.

following DNA vaccination more closely resemble those produced by the native virus. In contrast, bacterial glycoproteins expressed as DNA-vaccines may be subject to inappropriate glycosylation that may reduce the availability of immunodominant epitopes and affect immunogenicity.

1.3.2.2.1. Cellular Immunity to DNA Vaccines

1.3.2.2.1.1. T Helper Cells Involved in Immune Response to DNA Vaccines

Vaccination using DNA has the potential to preferentially stimulate a Th1 $CD4^+$T helper response to an antigen. Therefore, its application may be appropriate in the treatment of a number of conditions in which activation such a response would be beneficial, for example, in reducing intracellular infections, by inducing and optimising intracellular killing of infected cells. DNA vaccination may also be useful in preventing or limiting an ongoing Th2 response, associated with allergic or asthmatic diseases. Such an approach has been shown to be effective using a plasmid DNA encoding an antigen from the house dust mite allergen. Injection of this plasmid into rats intramuscularly resulted in long term expression of the allergen, which resulted in a reduction of histamine release in bronchoalveolar fluids and suppression IgE production. This effect appeared to be mediated by $CD8^+$ T cells (Hsu *et al.*, 1996).

1.3.2.2.1.2. Cytotoxic T Lymphocyte Responses

As already stated the main advantage of DNA vaccination appears to be the activation of specific $CD8^+$ cytotoxic T cells. Moreover, some studies introduced the concept of cross priming, in which triggering of $CD8^+$T cell responses can occur without *de novo* antigen synthesis within the APCs. Antigens released from the transfected cells either by secretion, or by transfection-mediated cell death, are taken up by professional APCs and somehow are

introduced into the MHC class I processing and presentation pathway, leading to the stimulation of $CD8^{+}$T lymphocytes (Harding *et al.*, 1995). In addition, it has been demonstrated that amongst the $CD4^{+}$ T-cell population, subsets of cells can exist that expresses cytolytic activity. These "killer" cells, which appear to produce Th1 cytokines, when activated, express Fas-ligand (FasL), which induces apoptosis in Fas-positive target cells (Hahn and Erb, 1999). It has also been proved that the magnitude of CTLs induced by DNA vaccination is sufficient for protection against disease. For example, a DNA vaccine encoding neuraminidase protected BALB/c mice from a lethal challenge with a heterologous influenza virus (Chen *et al.*, 1998).

Like antibody production, CTL responses appear to be long-lived. In addition, it was recently shown that the frequency of antigen-specific $CD4^{+}$ T lymphocytes (as measured by proliferation) remained elevated for up to 40 weeks post-vaccination (Akbari *et al.*, 1999). The authors proved that antigen-specific $CD4^{+}$ T lymphocytes are activated in the draining lymph nodes and migrate to the spleen where they can persist for up to 40 weeks in the absence of detectable antigen.

Persistence of this response probably reflects a low but continual production of antigen from the DNA construct. To support this idea some workers have demonstrated that intramuscular inoculation of a plasmid DNA encoding several different genes resulted in protein expression for about one year (Wolff *et al.*, 1992), whilst others have shown persistence of antigen-specific proliferative responses in the absence of detectable antigens (Akbari *et al.*, 1999). Therefore, it is possible that the antigen might be continuously present at low levels sufficient for antigen presentation but below possible levels of detection.

1.3.2.2.2. Humoral Immunity to DNA Vaccines

Immunisation with plasmid DNA is able to induce strong antibody response to a variety of proteins in animal species, including mice, non-human primates and more recently, human subjects. The first experiments describing the protective efficacy of intradermal, intramuscular, intravenous, intranasal, and Gene-Gun-delivered plasmid DNA in mice against a lethal challenge with influenza virus belong to Fynan and co-workers (Fynan *et al.*, 1993). DNA-vaccination also induced neutralising antibodies and provided protection against challenge with the relevant pathogen, such in the case of a herpes simplex protein (Ghiasi *et al.*, 1995), and influenza hemagglutinin (Larsen *et al.*, 2001).

The type of antigen, cellular location of antigen and method of administration of DNA vaccines can affect the magnitude and type of antibody produced (this is discussed in further detail elsewhere: Section 1.3.3). Such antibodies can be detected as early as one week after plasmid injection, with antibody titres reaching peak levels 8-12 weeks after immunisation. For example, life-long humoral immunity to influenza nucleoprotein (Yankauckas *et al.*, 1993), influenza hemaglutinin (Justewicz *et al.*, 1995), and hepatitis C core protein (Lagginng. *et al.*, 1995) has been demonstrated. These levels can then persist for several months (Davis *et al.*, 1993[a]; Davis *et al.*, 1996, Deck *et al.*, 1997), although in some studies, repeated administration of DNA were required to attain a long-lasting immune response (Xiang *et al.*, 1994).

Although antibody titres achieved by DNA vaccination, are generally long-lived, the magnitude of the response is often much lower than that elicited by a traditional subunit vaccine. To overcome this defect, several groups have shown that combining DNA with a more traditional vaccine may significantly increase antibody levels (Jones *et al.*, 2001; this is discussed in greater detail elsewhere: Section 1.3.4).

The ability of DNA vaccines to confer protection by inducing neutralising antibodies suggests that the antigen expressed *in vivo* following DNA vaccination assumes a native configuration. This is in contrast to some of recombinant proteins which may lack linear determinants or conformational epitopes required for activating of this type of response. In the case of viral antigens DNA vaccines provide further advantages over the conventional protein vaccines, because they use the mammalian-type pattern for post-translational modifications of the encoded antigen. In contrast, bacterial glycoproteins expressed as DNA-vaccines may be glycosylated differently from the proteins produced naturally by the bacteria. This may reduce the availability of immunodominant epitopes to which protective antibody may be generated.

1.3.3. Impact of Delivery Route on Immunological Properties of DNA Vaccines

Several studies have now indicated that the route of delivery of DNA vaccines can affect both quantitative and qualitative differences in the immune response generated. To date, DNA vaccines have been delivered using a number of routes including intra-muscular and intradermal injection, epidermal delivery by Gene-Gun, mucosal administration (oral, intranasal, vaginal) (McCluskie *et al.*, 1999, Fynan *et al.*, 1993), and more recently non-invasive vaccination to the skin (Shi *et al.*, 1999). However, the most widely used methods for gene-immunisation are intra-muscular and Gene-Gun delivery to the epidermis.

The main differences between these two methods include:

1. the way in which the plasmid DNA enter transfected cells,
2. the amount of plasmid DNA required for generating similar levels of specific immune responses,
3. the predominant type of immune response elicited in each case.

Direct injection of naked DNA into skeletal muscle is often carried out in saline solution and results in the delivery of DNA to the extracellular space from where it is taken up mainly by myocytes or blood cells found at the locality of the injection site. However immune responses can be enhanced if the tissue has previously been treated with a toxin or local anaesthetic (e. g. bupivacaine), which cause necrosis and regeneration of the injected muscle. Such treatments are believed to increase the expression of the encoded antigen and therefore to amplify the immune response. However, it is also possible that the observed enhancement of the immune response is attributed to the numerous professional APCs recruited to the site of tissue damage rather than elevated expression of the antigen in regenerated muscle.

On the other hand, delivery of DNA by Gene-Gun is dependent upon the direct immunisation of the skin with gold particles coated with plasmid DNA. These particles are driven into the skin under high pressure using helium as the gas. The main cell types transfected following this type of vaccination are keratinocytes and Langerhans cells, which are found in great numbers in the epidermal layer. Due to their small size, the gold particles can penetrate through the cell membrane, carrying the bound DNA directly into the cell, and thus circumventing its residence in the extracellular space.

Using this approach, several workers have reported similar levels of antibody and cellular responses generated using 100-5000- fold less DNA than traditional i. m. immunisations (Feltquate *et al.*, 1997). In one study, as little as 16 ng of plasmid DNA delivered epidermally via Gene-Gun could induce antibody and CTL responses in mice, whereas intradermal or intramuscular injection of the same plasmid required 10-100 μg of DNA to elicit comparable responses (Pertmer *et al.*, 1995).

Although, both systems are able to raise a strong humoral immune response, the type of T-cell help induced by each method appears to differ significantly. Using intra-muscular

injection of DNA, a predominantly Th1 type response can be generated (indicated by the increased production of Th1 cytokines such as IL-2, IFN-γ, IL-12 and elevated levels of antigen specific IgG isotype IgG2a. In contrast, immunisation using a Gene-Gun appears to induce $CD4^+$ T-cell, which secrete Th2 cytokines (generating less IFN-γ, more IgG1 antibodies and more IL-4). For example, intradermal immunisation of a plasmid vaccine expressing a *P. bergei* protein (CSP) raised a predominantly Th1 response with mostly IgG2a anti-CSP antibodies, while Gene-Gun DNA immunisation produced a predominantly Th2 response with mostly IgG1 specific antibodies (Weiss *et al.*, 2000).

One explanation for this difference may involve the quantity of plasmid DNA required to activate responses. Using the Gene-Gun, the amount of DNA is considerably lower than needed for intramuscular immunisation, which may affect the number of CpG motifs available for triggering the immune system. (Krieg *et al.*, 1998[b], O'Garra *et al.*, 1998). Therefore, the less DNA is used, the weaker the effect of the non-specific immunostimulatory sequences on the subsequent immune response.

In addition, it is assumed that immunisation using the Gene-Gun results in the direct transfection of cells, which may bypass the interactions that are now believed to take place between CpG motifs and specific cell membrane receptors such as the recently identified Toll-9 receptor (Budker *et al.*, 2000). Interaction with such receptors is believed to generate the transduction of an intracellular signal leading eventually to the observed adjuvant effects of bacterial CpG motifs. Thus, in the absence of such interactions, this CpG-mediated immunostimulatory "help" cannot take place. However it may be possible that other factors such as the host haplotype or the nature of processed antigen may also be involved in preferential induction of a certain type of immune response (Cohen *et al.*, 1998).

Similarly, by changing the form of the DNA expressed immunogen from cell-associated to secreted form, the same plasmid can be used to bias the type of CD4 helper response that is induced (Feltquate *et al.*, 1997).

1.3.4. Boosting Strategy and the Efficacy of DNA Vaccines

Although DNA vaccination has been shown to be effective at activation of immune responses to a wide range of pathogens, in many cases the level of immunity induced appears insufficient to afford protection. A novel strategy introduced to overcome this difficulty has involved the use of prime/boost strategies, in which an animal that has been primed with a DNA vaccine is subsequently boosted with a conventional vaccine such as an injected protein. Using this type of approach, improved protection was observed in mice that had been primed with a DNA plasmid encoding the protein E2 of hepatitis C virus and boosted with recombinant E2 protein (Song *et al.*, 2000). The level of protection observed was significantly higher than that observed when either vaccination strategies were used in isolation. The same strategy was also used to enhance antibody and Th cell proliferative responses for the antigen gD of herpes simplex virus-2 (Sin *et al.*, 1999). Similarly, three immunisations with a DNA vaccine encoding a *Plasmodium falciparum* erythrocyte binding protein, followed by a single immunisation with the recombinant protein induced high antibody levels that were equivalent to those induced by four recombinant protein immunisations. More significantly these animals showed improved protection against challenge with *P. falciparum* (Jones *et al.*, 2001).

Additionally, delivery of the same antigen multiple times using different carriers, with little or no immunogenic cross-reactivity (heterologous regimen) provides several advantages over the repeated delivery of an antigen with the same carrier (homologous boosting). For example, heterologous boosting yielded full protection in the *P. berghei*

malaria model when plasmid immunisation was followed by administration of recombinant poxvirus (Sedegah *et al.*, 2000) or recombinant vaccinia virus Ankara (Sedegah *et al.*, 1998). Homologous boosting was weak or ineffective in this model. Similarly, greatly improved immune responses and therapeutic effect were reported when a tumour model antigens were delivered in a heterologous prime-boost-regimen using recombinant cowpox or fowlpox expressing vectors (Irvine *et al.*, 1997). These differences may reflect the fact that the successful repeated use of any given vaccine may be dependent upon the generation of an immune response to the delivery vehicle itself.

In addition, the order in which the vaccines are delivered may affect the type and magnitude of immune response generated. This is particularly true when priming is performed using the antigenically more complex viral vector followed by boosting with a DNA vector. This difference might be due to the effect of immuno-suppressive viral products, which may interfere with the priming of T-cells against specific antigens (Levitskaya *et al.*, 1995). Such interference might have less impact on enhancement of a secondary response that has been primed against a specific antigen encoded by a DNA vaccine. In addition, the number of T-cells primed against an antigen may be increased by inclusion of several DNA priming steps. For example, McShane and co-workers compared the $CD4^+$ T cell immune response elicited after a combined regimen of immunisation using two types of vectors: naked plasmid DNA (D) and modified vaccinia virus Ankara (M) expressing a variety of antigens from *Mycobacterium tuberculosis*. Both D-M and M-D immunisation regimens produced significantly enhanced levels of $IFN\gamma^+$T cell responses, but the highest increase was noticed after a D-D-D-M immunisation regimen (McShane *et al.*, 2001).

Interestingly, when a prime/boost strategy was applied to enhance immune responses at mucosal sites, the maximal mucosal immunity was achieved when a recombinant vaccinia

virus encoding gB protein of Herpes Simplex Virus (HSV) was used for priming followed by boosting gB-encoding DNA vaccine. This suggests that activation of the responses at mucosal immune system maybe dependent upon a different set of conditions than those associated with systemic prime/boost strategies (Eo *et al.*, 2001).

1.3.5. DNA Vaccines: Design and Optimisation

DNA vaccination is essentially dependent on the cloning of the gene/genes of interest into a suitable plasmid vectors in which the expression of antigen is controlled by an eukaryotic promoter. Generally, such vectors have been engineered to ensure high productivity of plasmid without the expression of protein in the bacterial cell.

The elements of the vector that enable it to multiply in bacterial cells are:

1. an origin of replication allowing for growth in bacteria and
2. selectable markers, usually antibiotic-resistance markers (which maintain selection of plasmid-containing clones during bacterial culture)

However, to stimulate expression of the cloned antigen in eukaryotic cells, a different number of factors are required on the vector. These include:

1. a suitable cloning site, usually an endonuclease-restriction site, where the gene of interest can be inserted,
2. a strong promoter for optimal expression in mammalian cells, and
3. polyadenylation sequences (which provide for the stabilisation of messenger RNA transcripts).

In addition, DNA vaccines also may contain specific nucleotide sequences of bacterial origin consisting of unmethylated cytidine-phosphate guanosine (CpG) dinucleotides, which have a strong and unequivocal immunostimulatory role (Krieg *et al.*, 1998[b]).

Figure 1.4 illustrates a schematic representation of a plasmid DNA vector consisting of a transcription unit, and an adjuvant unit.

1.3.5.1. Immunostimulatory Elements

The efficacy of DNA-based immunisation has been partly attributed to the intrinsic adjuvant properties of bacterial DNA itself. This activity appears to be a general property of CpG motifs found in bacterial DNA, which are 20-fold more common in prokaryotic than eukaryotic DNA. In addition, eukaryotic DNA appears to be heavily methylated when compared to prokaryotic DNA, which in general is unmethylated (Sato *et al.*, 1996).

CpG motifs present on bacterial DNA appear to stimulate and activate several types of immune cells including monocytes, macrophages, DCs), and B cells. They can influence antibody production by B-cells and secretion of a large range of cytokines from T-cells including IL-6, IL-12, TNF-α, and IFN-γ production (Krieg, *et al.*, 1998[b]). This stimulatory effect is likely to be mediated by interactions which may take place between CpG motifs and membrane receptors such as Toll-like receptors {for example Toll 9 has been described to be specifically activated by prokaryotic DNA (Budker *et al.*, 2000)} or between CpG motifs and intracellular proteins (Krieg *et al.*, 1998[b]). Krieg and co-workers found that the earliest detectable effect of CpG on B cells and monocytes was the generation of reactive oxygen species (ROS). As production of ROS is one of the signals involved in the activation of nuclear factor κB (NFκB), a nuclear factor involved in the transcription of several immune factors, activation via these motifs is likely to affect the function of many genes in the immune cells.

In DNA based vaccination, these unspecific responses synergise with acquired immune responses against the DNA expressed protein and enhance the immune response generated. Thus, bacterial CpG sequences can be used as effective non-toxic adjuvants by

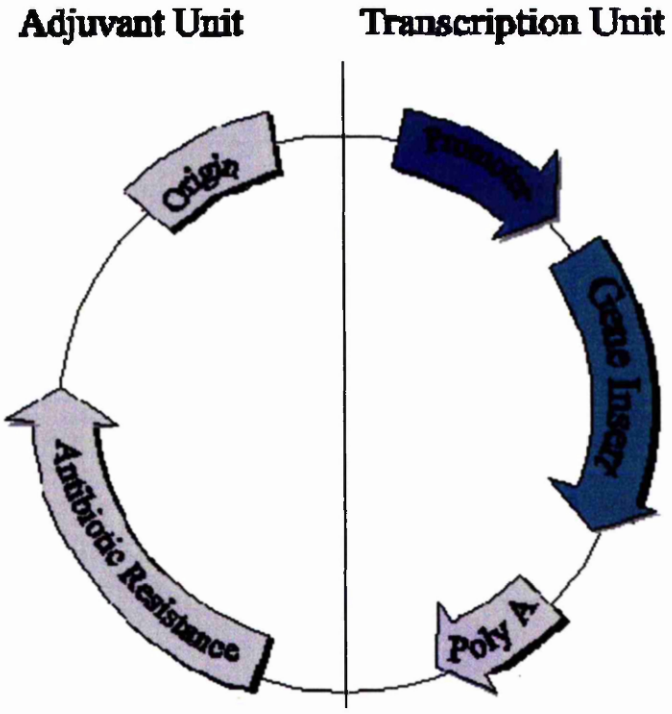

Figure 1.4: A schematic representation of the vector backbone of a DNA vaccine

The transcription unit includes a promoter that is activated in eukaryotic cells, a gene insert encoding the antigen of interest, and transcription/termination sequences (polyadenylation signals). The adjuvant unit includes an origin of replication that allows multiplication within a prokaryotic host and an antibiotic resistance marker. Both of these regions are generally rich in stimulatory CpG motifs.

either including a greater number of these sequences on the DNA vaccine vector itself or alternatively, by co-administration of these sequences in the form of synthetic oligonucleotides. In contrast, co-administration of mammalian DNA appears to be immuno-suppressive (Krieg, *et al.*, 1998[a]). Therefore, optimisation of immune activation in humans is likely to depend on the identification of specific motifs that can be used to regulate discrete elements of the human immune system. For example, Liang and co-workers found that a 6-base-pair motif that induces optimal stimulation in mice is less effective when tested on cells of primate origin (human, monkey, or chimpanzee) (Liang *et al.*, 1996).

1.3.5.2. Factors Affecting Expression of Antigen in the Eukaryotic Host

The main target of DNA-based vaccination is the activation of antigen specific immunity. Optimisation of this response is dependent upon a number of factors including level of antigen expression *in vivo*, which can be enhanced by the inclusion of a number of features on the vector backbone. These include introns to improve transcriptional efficiency, the design of the cloning site, codon optimisation for best usage in mammalian species, the nature of enhancer/ promoter region and the use of polyadenylation signals to enhance stability of the mRNA prior to translation.

1.3.6. Vaccine Candidates

Despite the fact that traditional vaccination strategies such as live, attenuated or whole inactivated agents have been very successful in the past, there are many microorganisms for which no effective vaccine currently exists. By using different strategies in the context of DNA vaccination it is possible to target the type(s) of the immune response required for protection against a particular disease. In the current study we intended to evaluate the impact of different immunisation strategies on the immunogenicity

of two independent DNA vaccine candidates, Fragment C of tetanus toxin and a *Mycobacterium bovis* antigen named MPB-70.

1.3.6.1. Tetanus

Tetanus, along with measles and pertussis, is one of the main vaccine-preventable infections that can kill within the first year of life. In many children infection with the organism *Clostridium tetani* can occur within the first month of life as a result of infection through the umbilical stump. Although not preventable by immunisation of the child, trans-placental transfer of circulating antibodies from a protected mother to the unborn child is sufficient to passively protect the child through the first few months of life. In contrast, adults are usually infected following inoculation of *C. tetani* spores through puncture wounds of the skin. This is especially true when tissue injury and necrosis accompany the generation of such wounds.

Clinical symptoms usually associated with tetanus are muscle spasms (the most dangerous being the ones involving muscles of the neck, thorax or glottis) and spasm related complications including asphyxia, fractures of the long bones and vertebrae, urinary retention, or pulmonary embolism. These symptoms are associated with the release of highly toxic protein of *C. tetani*, named tetanospasmin or tetanus toxin, which accumulates intracellularly during the growth of the organism and is released on lysis. Tetanospasmin binds to specific glycolipids in nerve cells in the peripheral nervous system, from where retrograde transport from the nerves in the periphery to the spinal cord occurs (Price *et al.*, 1975). The toxin blocks normal post-synaptic inhibition of spinal reflexes, leading to generalised muscular spasms.

Following toxin identification and purification in the 1890's, scientists were able to show that repeat inoculations of animals with minute quantities of toxin led to the

production of antibodies that neutralised the effect of the toxin. Preparation and passive immunisation of these antibodies particularly from horse sera, became the first means of reducing the impact of infection. In 1924, a chemically inactivated form of the toxin, named anatoxin or toxoid, was prepared, which was able to induce protective, neutralising antibodies to the toxin. This vaccine was so effective that it is still used today for the generation of active immunity, whilst Human Tetanus Immunoglobulin is available for passive protection.

Although, the current tetanus vaccine is efficient in the prevention of the disease, there are several reasons to suggest that this vaccine can be improved. These include the relatively limited duration of protection conferred by the vaccine, which has to be given every 10 years to maintain an appropriate level of neutralising antibodies. Second, immunisation is often accompanied by a local reaction following administration, which may be the result of the inclusion of the aluminium hydroxide or phosphate adjuvants (Stratton *et al.*, 1994). Thirdly, generation of the antigen is dependent upon the successful manufacture and inactivation of tetanus toxin from *C. tetani*, which, if performed incorrectly, may lead to the production of either an ineffective vaccine (Topsis *et al.*, 1996) or conversely immunisation of patients with active toxin (Gale *et al.,* 1994).

A suitable alternative to the vaccine is the production by recombinant technology of the non-toxic, 50 kDa carboxy-terminal fragment (Fragment C) of the tetanus toxin molecule. In 1990, immunisation of mice with recombinant Fragment C resulted in the production of antibodies that were able to protect the mice against a challenge with tetanus toxin (Halpern *et al.*, 1990, Figueiredo *et al.,* 1995). Fragment C of tetanus toxin was also proved to be strongly immunogenic and to elicit protective levels of serum anti-toxin in healthy human volunteers, when administered as a single-dose, orally, using as vector an attenuated strain of *Salmonella enterica* (Tacket *et al.*, 2000). Similarly, when mice were

immunised intragastrically or intranasally with live Fragment C-expressing lactobacilli, significant levels of circulating, specific IgG were induced, and moreover, specific IgA was detected in broncho-alveolar lavage fluids (Shaw *et al.*, 2000).

DNA vaccines encoding Fragment C under the control of the CMV promoter (Anderson *et al.*, 1996), or a RSV promoter (Saikh *et al.*, 1998) have also been generated and shown to induce protective responses when given intramuscularly to mice. The immunogenicity of the plasmid was found to be improved when the native gene for this protein was replaced with a synthetic, codon optimised version of the toxin (Stratford *et al.*, 2000). Although, substantial antibody titres were generated using these constructs, the level of antibody produced showed a reduced capacity to neutralise tetanus toxin when compared to polypeptide-based immunisation (Saikh *et al.*, 1998). In addition, the quality of the immune response varied considerably with the DNA vaccine apparently activating a CD4 Th1 response (indicated by high levels of IFN-γ and IgG2a production), whilst the Fragment C polypeptide appeared to stimulate CD4 Th2 type responses.

As protection to tetanus requires a strong humoral response rather than a cell-mediated immunity, immunisation strategies that improve the production of long-lasting neutralising antibodies should be considered. Within the context of this thesis, this includes combining the codon-optimised gene for Fragment C together with the use of Gene-Gun technology to:

1. reduce the amount of plasmid that can be used to generate antibody responses
2. to evaluate the potential of the Gene-Gun-administration to generate the production of neutralising antibodies (possibly of a Th2 subtype).

1.3.6.2. Tuberculosis

After a steady decline in the incidence of tuberculosis (TB) in industrialised countries, TB is re-emerging as an extremely serious worldwide public health problem. According to a global scale report of WHO, it is estimated that TB is responsible of about 3 million deaths per year, of which more than 95% occur in developing countries, associated with deteriorating social conditions. As the currently available vaccine against TB has its own limitations (see below, Section 1.3.5.2.2) and given the increasing incidence of multi-drug resistant strains of *M. tuberculosis* that hampers preventive programmes based on chemotherapy, a new, effective strategy is needed to address this re-emerging problem.

1.3.6.2.1. Overview of the Immune Response to *Mycobacteria*

Briefly, TB is caused by the pathogen *M. tuberculosis*, but another member of the family, *M. bovis*, which normally causes the disease in cattle, can be pathogenic for humans, when contracted through raw, unpasteurised milk. The initial infection is usually silent and may heal with no further symptoms, but some cases progress to pulmonary or disseminated TB, such as with meningeal or other extra-pulmonary involvement.

Tuberculosis is considered, in terms of its outcome and its clinical manifestations, a result of particular interactions between *Mycobacteria* and different components of the immune system. In most infected people, these interactions succeed to maintain a state of equilibrium between infection and immune response. On the other hand, disease pathology is thought to be a consequence of the disruption of this balance (Young and Thole, 1999).

Mycobacteria enter the body in the form of aerosolised droplets, initiating infection in the lung where alveolar macrophages represent their first serious encounter with the immune system. During a protective immune response, macrophages are probably the key

effector cells responsible for killing of *Mycobacteria*, although they also provide an environment for mycrobacterial replication.

Under normal circumstances, macrophages kill invading pathogens by either engulfing them in a phagosome, which is made toxic by acidification, generation of short-lived oxygen and nitrogen radicals. Finally the phagosome is fused to a lysosome and hydrolytic enzymes are released further, destroying the bacteria. Alternatively, *Mycobacteria* are killed by the triggering of programmed cell death of the macrophage (apoptosis) by a mechanism that results in killing of the intracellular organism (Molloy *et al.*, 1994). However, *Mycobacteria* have evolved two different strategies to circumvent the bactericidal activity of macrophages. The first strategy is based on the highly impermeable cell wall of *Mycobacteria*, which provides a defensive barrier against toxic solutes, hydrolytic enzymes, and free radicals encountered within host cells. In addition, *Mycobacteria* also express enzymes capable of detoxifying oxygen radicals, such as superoxide dismutase (SOD) and catalase. A second strategy, which prevents macrophage killing, involves occupation of a phagocytic compartment that is segregated from the conventional endosomal-lysosomal pathway. The factors that determine entry into this unusual phagosome are unknown. Live *Mycobacteria* might engage some specific receptors on the surface of the macrophage, activating a signal transduction mechanism that affects endosomal trafficking (Young and Thole, 1999). Alternatively, some functions expressed by the internalised mycobacterium might modify phagosome maturation and acidification (Gordon *et al.*, 1980).

The ability of macrophages to kill *Mycobacteria* or other intracellular organisms is dependent on the activation of these cells by specific T cells. T cell-mediated activation of macrophages is in turn a consequence of processing and presentation of mycobacterial antigens at the surface of the macrophage.

Mycobacterial antigens have been shown to be processed in a number of different ways, including presentation within the context of both MHC class I and II, and, of bacterial glycolipids in the context of CD1 molecules (Moody *et al.,* 2000). It is unclear how Mycobacterial proteins or peptides present in the segregated phagosome are able to enter these different antigen-processing pathways. One explanation is that *Mycobacteria* may escape into the cytoplasm, and provide a potential route for class I processing, while incorporation into the normal endosomal-lysosomal pathway would confer entry into the class II and CD1 pathway (Moody *et al.,* 2000).

The most important event of the immune response to *Mycobacteria* is antigen recognition by specific $CD4^+$ T cells in the context of MHC class II molecules and subsequent release of macrophage-activating cytokines (type 1 cytokines). In addition, specific $CD8^+$ T cells appear to kill *Mycobacteria*-infected cells, thus making the bacteria available to activated phagocytes.

Mycobacterial infection activates several other cellular subsets. These include natural-killer cells, which probably provide an important source of IFN-γ for the macrophage activation at the initial stage of infection. *Mycobacteria* also provide a potent stimulus for T cells with an antigen-specific γδT-cell receptor. The mechanism of antigen recognition by γδT cells and the molecular nature of their antigen repertoire remain poorly defined. The functional role of γδT cells is also unresolved, but it is possible that γδT cells are involved in protection by preferentially responding to non-proteineous ligands. Cytokines produced by γδT cell may also augment the role of NK cells in providing an early source of IFN-γ for macrophage activation (O'Brien *et al.*, 1989).

Although the antibody response against *Mycobacteria* is generally considered as a "side effect" of mycobacterial infection, antibodies directed to particular surface

components of mycobacterium may play a role in pathogenesis. This is because mycobacterial entry into host cells appears dependent on specific interactions with cell surface receptors. Therefore inhibition of such interactions could reduce the level of infection. In particular, antibodies generated and released in the alveolar space may at least eliminate the organism, before they invade macrophages (Kaufmann, 2000). However, a more likely prospect is vaccine- induced immunity that attacks the pathogen after it has entered macrophages, which is the exclusive function of in particular $CD8^+$ specific T cells.

1.3.6.2.2. Bacillus Calmette-Guerin- the Current Available anti-Tuberculosis Vaccine

The French scientists Calmette and Guerin developed the vaccine that is currently used in the fight against against TB. This vaccine known as Bacillus Calmette-Guerin (BCG), was developed by the in the first decade of the 19^{th} century and is based on an attenuated, avirulent strain of *M. bovis*. Although BCG has been delivered for over 70 years and is the most frequently administered vaccine (more than 3 billion adults and 100 million new-borns receive the vaccine annually), there still are some issues regarding its potential efficacy. These include the fact that, in large, controlled prospective studies, the response to the vaccine in different populations is inconsistent. Although its side effects are tolerable and it has been proved to be able to confer protection against miliary and meningeal TB to an appreciable degree, BCG fails to protect against the most prevalent disease form, pulmonary TB. The protective efficacy of BCG in adults ranges from 0% in South India to 80 % in the UK (Fine, 1995). In addition, the exact basis for the attenuation of the virulent *M. bovis* strain that led to BCG has not been yet established and also little is known about the duration of protection conferred by BCG. Protection after neo-natal BCG vaccination has been reported to be undetectable after about 15 years (Harboe *et al.,* 1996). Such information is essential mainly for rational decisions of the utility of repeat vaccination.

However, the only formal evaluation of the repeat BCG was carried out in Malawi, revealed that the second dose had no effect on incidence of pulmonary TB, but provided increased protection against leprosy (Karonga Prevention Trial Group, 1996). Finally, BCG given at birth was shown to interfere with prophylactic chemotherapy with isoniazid needed to confer protection in babies born to infected mothers (WHO vaccines and Biologicals- www.who.int/ vaccines/ intermediate/ tuberculosis.html).

Given all the above, further strategies for TB vaccine and alternative approaches for TB prophylaxis, such as subunit vaccines, DNA vaccination or combined strategies are currently being considered.

1.3.6.2.3. Importance of MPB-70 in Protective Immunity against Bovine Tuberculosis

For many years, considerable efforts have been made to identify appropriate targets of the *Mycobacteria* to which protective immune responses can be generated. One candidate antigen to be revealed by such studies is a mycobacterial protein known as MPB-70.

MPB-70 was initially isolated in 1981 by Nagai and colleagues (Nagai *et al.*, 1981). The term MPB was introduced for the designation of a protein purified from *M. bovis* BCG, and 70 denotes the relative mobility by electrophoresis on a 7.7% polyacrylamide gel run at pH 9.5 (Nagai *et al.*, 1981). MPT is used for similar designations of proteins purified from *M. tuberculosis* (Harboe *et al.,* 1998) but there are no differences between *M. bovis* and *M. tuberculosis* in their sequence of the expressed proteins encoded by the *mpb70* or *mpt70* genes (Wiker *et al.,* 1998).

MPB-70 is consistently present in virulent *M. bovis* strain, whereas it is secreted in markedly lower amounts by various substrains of avirulent *M. bovis* BCG (Harboe *et al.,* 1998). Interestingly, MPB-70 is highly expressed in *M. bovis* and minimally expressed in *M. tuberculosis*, probably reflecting differences in the regulation of these genes (Wiker *et al.,*

1998). However, there is evidence to suggesting that MPB-70 is upregulated during *M. tuberculosis* infection (Matsumoto *et al.*, 1995).

Cloning of *mpb70* gene revealed the presence of a typical signal peptide, which is commonly found upstream of the proteins naturally secreted from the bacteria (Harboe *et al.*, 1998; Wiker *et al.*, 1991). However, previously it had been described as an adhesion molecule, present on the outermost layer of the bacterial cell (Matsumoto *et al.*, 1995). The protein appears to be a completely soluble secreted antigen, which is secreted with a high efficiency by *M. bovis*. Differences in the localisation of individual proteins in relation to the mycobacterial cell are closely correlated to the mode of interaction between multiplying bacilli and the infected host. Secreted proteins are probably available for presentation to the immune system prior to cytoplasmic constituents, being therefore expected to be of grater interest in finding new vaccine candidates.

The rationale for testing of MBP-70 as a vaccine candidate comes from the following studies. First, bovine serum IgG antibodies from cattle experimentally infected with *M. bovis* appeared to strongly recognised six mycobacterial antigens, one of which was MPB-70 (Lyashchenko *et al.*, 1998). Second, antigen-specific T-cell proliferation, IFNγ and IL-2 responses to a panel of mycobacterial antigens, including MPB-70, in cattle experimentally infected with *M. bovis* have been reported (Rhodes *et al.*, 2000[a]). Third, MPB-70 induced a proliferative response in both PBMC (peripheral blood mononuclear cells), and LNC (lymph node cells of cattle experimentally infected with *M. bovis* (Rhodes *et al.*, 2000[b]).

This data suggests that MPB-70 appears to be an antigen to be considered as a vaccine candidate against tuberculosis.

In addition, candidate DNA vaccines using MPB-70 as the encoding antigen have also been tested. These were shown to be of greater benefit (considering the number of bacteria in lungs of vaccinated mice), than a plasmid expressing hsp65, when administered as

therapeutic vaccines in mice (Lowrie *et al.*, 1999). More recently, pCMV-70 (a plasmid encoding MPB-70) has been shown to induce significant proliferative responses, IFN-γ production and $CD4^+$ T cell response in peripheral blood in 50% cattle vaccinated with this construct. Moreover, DNA vaccination of cattle with pCMV-70 did not alter their sensitivity for tuberculin skin test (Vordermeier *et al.*, 2000).

As protection to tuberculosis requires mainly a cellular immune response rather than antibody-mediated immunity, immunisation strategies that improve the production of specific memory T cells should be considered. One of the aims of the current study was to evaluate the capacity of i. m. administration of pCMV-70 to trigger specific immunity to this antigen in BALB/c mice.

1.4. Aims of the Project

The aim of the project is to use two different bacterial antigens, Fragment C from tetanus toxin and MPB-70 from *Mycobacteria bovis* as model antigens in the context of DNA vaccination, to evaluate the potential for various immunisation strategies to alter the magnitude and orientation of the immune response generated. In particular, we wished to compare the type of immune response generated to these antigens when the DNA vaccine is delivered parenterally by injection o into the skin using the Gene-Gun. Previous data suggests that intramuscular immunisation induces a Th1 biased response, whilst immunisation using the Gene-Gun induces a Th2 response. Using these antigens, we hoped to establish whether this dogma was additionally true for these bacterially derived antigens.

1.4.1. Objectives

1. To establish whether these two DNA vaccine candidates expressed antigen in eukaryotic cells cultured *in vitro*.
2. To optimise parameters for transfection of cultured cells using the Gene-Gun
3. To determine the response to the two DNA vaccines following intramuscular injection of the plasmid into mice and compare these responses to those achieved using the same plasmids delivered using the Gene-Gun.

Chapter 2

Materials and Methods

2.1. Bacterial Strains

Escherichia coli XL-10 (kindly provided by Cathy Rush, Department of Infection and Immunity, Glasgow University) served as the bacterial host strain for all plasmids used in experiments described in this thesis.

2.1.1. Growth Conditions

Bacterial strains were cultured in Luria Bertani broth (L-broth: 10 g Bactotryptone, 5 g Bacto Yeast Extract, 5 g NaCl dissolved in 1 litre of distilled water and sterilised by autoclaving), or on solid media on L-agar plates (L-broth containing 15 g/L Bacto Agar). Culture medium was supplemented where appropriate with the following antibiotics: ampicillin (100 μg/ml), or kanamycin (30 μg/ml).

2.1.2. Master Seed Banking

Bacterial strains containing the plasmids were stored on arrival in a master seed bank from which working seed banks were generated. Master banks were produced by initially plating the culture on L-agar plates impregnated with the appropriate antibiotic. After overnight incubation, colony morphology was checked to ensure the purity of the culture (single colony shape and size). Once purity had been established, three individual colonies were inoculated into 5 ml of Luria broth, and incubated for 4-5 hours at 37°C with shaking, to the mid log phase (OD_{650} = 0.4-0.6). A single Microbank tube containing cryobeads (Pro-Lab) was then inoculated with 0.5 ml of bacterial growth. The contents were mixed thoroughly by multiple inversions, and then the liquid content of the tube was removed leaving only the beads coated with bacteria inside the tubes. The stocks were immediately stored at -70°C and only used once to generate material for the working seed bank.

2.1.3. Working Seed Banking

Working seed banks were prepared from each of the individual master seed banks by removing one bead, aseptically from the Microbank tube and placing it onto an L-agar plate impregnated with appropriate antibiotics. The bead coated with the bacteria was then spread from the bead over the surface of the plate using a sterile microbiological loop. After overnight incubation at 37°C, the colonies were checked for single morphology types, and then 2-3 identical colonies were inoculated into 5 ml of L-broth (containing appropriate antibiotics). The culture was grown with shaking at 37°C until it had reached mid log-phase (OD_{650}=0.4 - 0.6). At this time, 0.5 ml aliquots of culture were placed into 10 sterile 1.5 ml eppendorf tubes, 0.5 ml sterile glycerol added to each tube and the contents mixed. Stocks were immediately frozen and stored at -20°C. When required, an eppendorf was removed from –20°C, and the contents inoculated onto a L-agar plate using a sterile microbiological loop. Each eppendorf tube was used to recover bacteria on maximum three occasions before being discarded.

2.2. Plasmids

2.2.1. Plasmid Characterisation

Five previously constructed plasmids were used in the course of this study. These included pcDNA3/tetC (kindly provided by Gordon Dougan, Imperial College, London) and its corresponding vector control plasmid pcDNA3.1 (Invitrogen). The pcDNA3/tetC vector was initially–constructed by cloning the synthetic codon-optimised gene encoding Fragment C, the non-toxic binding domain of tetanus toxin into the eukaryotic expression vector pcDNA3.1 (Invitrogen). As can be seen in Figure 2.1 this vector contains the immediate early CMV promoter, the Bovine Growth Hormone polyadenylation signal (BGH polyA), a f1 origin for single stranded DNA replication, ColE1 origin of replication of the plasmid in *E. coli*, and a gene which encodes ampicillin-resistance in *E. coli*.

Figure 2.1 A

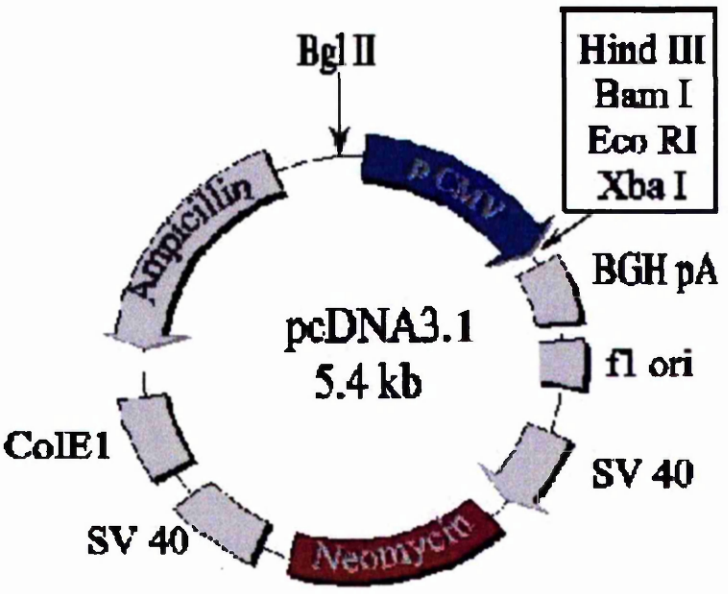

Figure 2.1B

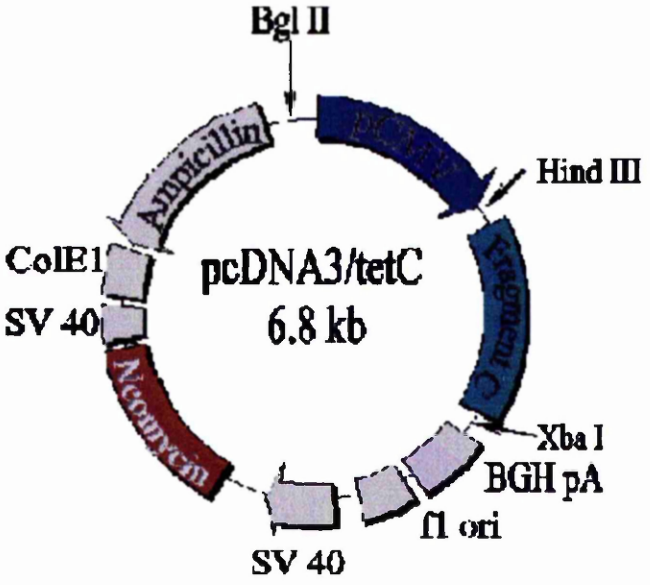

Figure 2.1: A schematic representation of pcDNA3.1 (A) and pcDNA3/tetC (B) plasmid maps

PCMV = CMV promoter; BGH pA = Bovine Growth Hormone polyadenylation signal; f1 ori = origin for single stranded DNA replication; SV40 = simian virus 40 origin of replication; ColE1 = origin of replication in *E. coli*; *XbaI, BamI, Bgl-II, EcoRI, HindIII* = restriction enzyme sites.

Between the CMV promoter and the polyA tail, a polylinker containing endonuclease restriction sites for *HindIII, BamHI, EcoRI,* and *XbaI.* Using the *HindIII* upstream of the start of the open reading frame and *XbaI* immediately downstream of the end of open reading frame, the Fragment C gene was inserted into the vector.

Two further plasmids used in the course of this work, named pCMV-70 and its vector control pCMV-link, were kindly supplied by Mark Chambers, Central Veterinary Laboratory, Weybridge. The vector pCMV-70 was initially constructed by inserting the *mpb70* gene from *Mycobacterium tuberculosis H37Rv* into the plasmid backbone pCMV-4. pCMV-4 is based on the plasmid pcDNA3.1 (Invitrogen) except that it has an intron A from the human CMV immediate-early gene inserted immediately downstream of the CMV promoter (Figure 2.2). Insertion of such introns has been shown to increase levels of antigen expression by the vector. The control plasmid CMV-link was obtained by removing *mpb70* from pCMV-70 using *XbaI* and *BamHI* restriction sites and replacing it with a polylinker containing *BamHI, EcoRI, PstI, HindIII,* and *XbaI* restriction sites.

The final vector used was plasmid pGFP-C3 (Clontech). This plasmid contains the immediate early promoter from CMV for the expression of the green fluorescent protein (GFP) from the jellyfish *Aequore victoria.* The backbone of this plasmid includes a pUC19 origin of replication for propagation in *E. coli*, a f1 origin for single stranded DNA production and neomycin/kanamycin-resistance cassette (neo^R) with a bacterial promoter upstream to this cassette (P_{amp}) that confers kanamycin resistance in *E. coli.* Restriction enzyme sites flanking the insert are *NheI, Eco47III,* and *AgeI,* upstream of the GFP gene insert and *Hind III* immediately downstream (Figure 2.3).

Figure 2.2 A

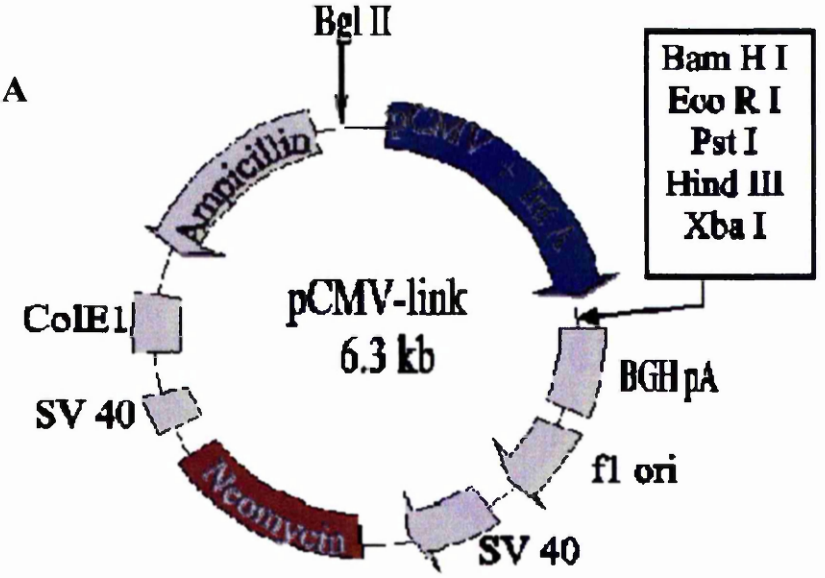

Figure 2.2B

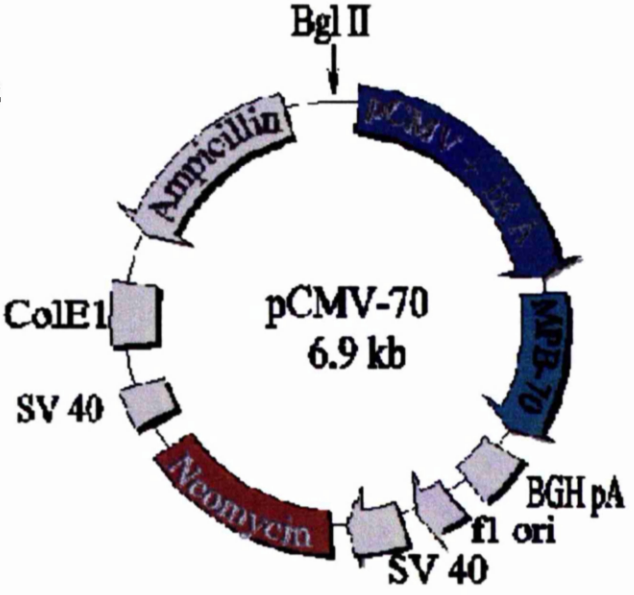

Figure 2.2: A schematic of pCMV-link (A) and pCMV-70 (B) plasmid maps

pCMV+Int A= CMV promoter and intron A; BGH pA = Bovine Growth Hormone polyadenylation signal; f1 ori = origin for single stranded DNA replication; SV40 = simian virus 40 origin of replication; ColE1 = origin of replication in *E. coli*; *XbaI, BamHI, Bgl-II, EcoRI, PstI, HindIII* = restriction enzyme sites.

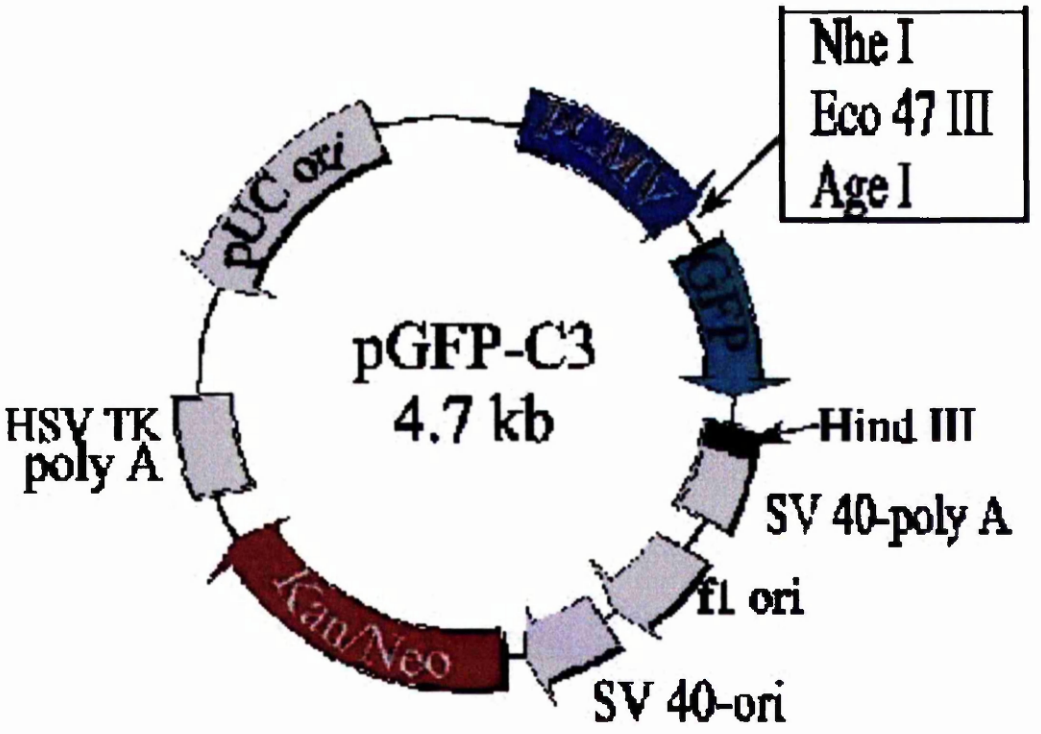

Figure 2.3: A schematic representation of pGFP plasmid map

pCMV= CMV promoter; polyA = polyadenylation signal; f1 ori = origin for single stranded DNA replication; SV40 = simian virus 40 origin of replication; pUC ori= origin of replication in *E. coli*; *Nhe I, AgeI, Eco47III, HindIII* = restriction enzyme sites.

2.2.2. Competent Cells and Transformation of *Escherichia coli*

To assure consistency in the potential contamination of purified plasmids, all constructs were first isolated and then used to transform the *E.coli* host strain XL-10. Competent bacterial cells were initially prepared using rubidium chloride method. In brief, a 25 ml culture of *E. coli* XL-10 was grown in L-Broth to early log phase (OD_{650}=0.45-0.55) and then chilled on ice for 15 minutes. The bacteria were then concentrated by centrifugation at 2000xg for 5 minutes, at 4°C and the bacterial pellet resuspended in 10 ml of ice-cold transforming buffer I (TFB I: 30mM potassium acetate, 100mM RbCl, 10mM CaCl2, 50mM MnCl2, 15% glycerol), and left to chill for 5 minutes on ice. The bacteria were then centrifuged again using the same conditions before being resuspended in 1 ml of transforming buffer II (TFB II; 10mM RbCl, 75mM CaCl2, 10mM MES - 2-(N-morpholino) ethanesulfuric acid, 15% glycerol). The bacterial suspension was aliquoted into ten aliquots of 0.1 ml bacterial culture. These were subsequently flash-frozen in dry ice/ ethanol bath. These aliquots were stored at -70°C until required.

The competent cells were transformed with plasmid DNA by mixing approximately 10-100 ng of plasmid DNA with an aliquot of competent cells, which had been thawed on ice. The mixture was stored on ice for 10 minutes and then heat shocked at 42°C for 2 minutes before being returned to the ice for a further 2 minutes. Transformed bacterial cells were allowed to recover in 0.9 ml of L-broth, which was added to the tube. The culture was then incubated at 37°C for 30-60 minutes before the bacteria were plated on L-agar plates containing the appropriate selection antibiotic.

2.2.3. Plasmid Purification

In order to characterise the transformed strains, small- or large-scale preparations of plasmid DNA were made from bacterial lysates. Plasmids were purified using the Qiagen Miniprep kit (Qiagen Ltd., UK), or, for immunisation experiments, the endotoxin – free Megaprep kit (Qiagen Ltd, UK).

2.2.3.1. Preparation of Plasmids Using the Miniprep Kit

Plasmid purification using these kits relies on the use of a modified alkaline lysis procedure followed by adsorption of DNA on silica in the presence of high salt. The protocol is designed to allow the preparation of up to 20 μg of plasmid DNA from a volume of 2.5 ml overnight bacterial culture. To purify plasmid, 2.5 ml of culture was initially centrifuged at 2000xg for 5 minutes, and the pellet resuspended in 250 μl provided buffer P1 in the presence of RNAse (50mM Tris-Cl, [pH 8.0]; 10 mM EDTA; 100 μg/ml RNAse A). The bacteria were then lysed with 250 μl buffer P2 (200 mM NaOH, 1% SDS {SDS solubilized the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents while the alkaline condition denatured the chromosomal and plasmid DNAs, as well as proteins}). The lysate was neutralised and adjusted to high-salt binding conditions by addition of 350 μl buffer N3 (3M potassium acetate, [pH 5.5]), which causes proteins to denature and chromosomal DNA, cellular debris and SDS to precipitate, whilst leaving the smaller plasmid DNA solution. The solution was then centrifuged for 10 minutes at 3000xg and the resultant supernatant placed in a spin column containing a silica gel membrane for selective adsorption of plasmid DNA in high-salt buffer and elution in low salt buffer (RNA, cellular proteins and metabolites are not retained on the membrane, and can be detected in the flow-through). After washing the column with 0.75 ml of provided buffer PE (1M NaCl, 50mM MOPS -

3-(N-morpholino) propanesulphuric acid , [pH 7.0], 15% isopropanol) to remove trace nuclease activity, the plasmid DNA bound to the column was eluted by pipetting 50 µl distilled H_2O in the centre of the column and centrifuging for 1 minute at 3000xg.

2.2.3.2. Preparation of Plasmids Using the Endotoxin-free Megaprep Kit

Qiagen Endotoxin-free Megaprep kit protocol for plasmid purification is also based on a modified alkaline lysis procedure, except that the protocol is designed for preparation of up to 2.5mg endotoxin-free plasmid from a volume of 2.5 litres of overnight bacterial culture. For preparation of plasmid DNA, endotoxin-free glass - and plastic-materials were used and, where appropriate, these were initially cleaned overnight in a 1% E-toxa clean solution (Sigma) prior to use.

The size of the culture used for preparation of the plasmid DNA varied between constructs, but, in general, between 1-2 L of cultures were used in each preparation. These bacteria were initially harvested by centrifugation at 6000xg for 15 minutes, at 4 °C, and the resultant pellet re-suspended in 50 ml of buffer P1, containing RNAse (50mM Tris-Cl, [pH 8.0]; 10 mM EDTA; 100 µg/ml RNAse A). After vortexing, the bacteria were lysed using 50 ml of buffer P2 (200 mM NaOH, 1% SDS) and then the lysate neutralised using 50 ml of the buffer P3 (3M potassium acetate, [pH 5.5]). The lysate was then poured into a Qiafilter cartridge and left at room temperature for 10 minutes to equilibrate. The cartridge was then connected to a vacuum source to allow the soluble fraction of the material to be separated from the precipitated genomic DNA and proteins. This solution was then mixed with 50 ml of buffer FWB (750mM NaCl, 50mM MOPS, [pH 7.0]; 15% isopropanol) followed by the addition of 12.5 ml of buffer ER. The mixture was left for 30 minutes at room temperature before being allowed to enter the resin of an equilibrated Qiagen column by gravity flow. The column was washed twice with 100 ml buffer QC (1M NaCl, 50mM MOPS, [pH 7.0], 15% isopropanol), and the plasmid DNA eluted using 35 ml of buffer

QN (1.25M NaCl, 50 mM Tris-Cl, [pH 8.5], 15% isopropanol) and precipitated with 24.5 ml of isopropanol. The precipitated DNA was then collected by centrifugation at 15,000 x g for 30 minutes, 4 °C. The pellet was then washed with 7 ml endotoxin-free 70% ethanol (15,000xg, 10 minutes), air-dried for 10-20 minutes and finally resuspended in 1 ml endotoxin-free distilled H_2O.

2.2.4. Qualitative and Quantitative Analysis of DNA Constructs

After purification, plasmids were checked in terms of their size, sequence, and concentration. Plasmids were sequenced at the Molecular Biology Sequencing Unit, Glasgow University, using primers from Table 2.1.

Two types of methods are widely used to quantify the amount of nucleic acid found in a sample. If the nucleic acid is pure (i. e. without significant amounts of contaminants such as proteins, phenol, agarose, or other nucleic acids), spectrophotometric measurement of the amount of ultraviolet radiation absorbed by the bases is simple and accurate. However, if the amount of DNA is very small or if the sample contains significant quantities of impurities, the amount of nucleic acid can be estimated by running serial dilutions of the sample on an agarose gel stained with ethidium bromide. Both methods were described and used in different sections of this study (Sections 2.2.4.2 and 2.2.4.3).

2.2.4.1. Restriction Enzymes Mediated Digestion of Plasmids

To confirm the size of the transformed plasmid, circular DNA was initially linearised by treatment with appropriate restriction enzymes (Table 2.2). This involved incubation of plasmid DNA with the appropriate restriction enzyme(s), a suitable buffer for the enzyme (1/10, v/v), and distilled water. Plasmid DNA was digested for 1 hour, at 37°C and then 20 µl of digested DNA admixed with 6x loading buffer (0.25% bromphenol blue, 0.25%

xylene cyanol, 40% w/v sucrose) was run on an agarose gel. Location of restriction enzyme sites and inserts in plasmids are also shown in Figure 2.1 (for pcDNA3.1 and pDNA3/tetC), Figure 2.2 (for pCMV-70 and pCMV-link) and Figure 2.3 (for pGFP).

2.2.4.2. Agarose-Ethidium Bromide Gel Analysis of DNA

The agarose gel was prepared by melting 0.8 g electrophoresis grade agarose (Biorad) in 100 ml TBE buffer [pH 8.0] (9mM Tris, 80mM boric acid, 0.5mM EDTA). After cooling the agarose-TBE solution to 60°C, 40 µl of a 1mg/ml stock solution of ethidium bromide was added to the mixture. Wells in which the samples were to be loaded were created using a suitable well comb. The gel was then cast into an appropriately sized carrier and allowed to set. Once set, the comb was removed and the gel placed in the tank together with an appropriate amount of TBE buffer. Samples were loaded into the wells and the gels run at 75 V, for 2 hours. A standard 1 kb DNA ladder (1 µg/µl solution) (Gibco - Life Technologies, UK) was included on each gel. This was suitable for sizing linear double stranded DNA fragments from 500 bp to 12 kb. In addition, as the 1636 bp band of this ladder was known to contain 10% of the mass applied to gel, the relative intensity of this band to the sample could be used for estimating the amount of DNA in each sample.

2.2.4.3. Spectrophotometric Analysis of Plasmid DNA

The amount of plasmid DNA in purified samples was also determined by reading the absorbency of 100 µl of plasmid DNA solution at 260 nm and 280 nm against a suitable blank control, using a Unicam UV/VIS-UV2 Spectrophotometer. The calculated amount of plasmid DNA in each preparation was estimated according to the fact that an OD_{260} of 1 corresponds to approximately 50 µg/ml of double stranded DNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provided an estimate of the purity of the nucleic acid with pure preparations having a value between 1.8 and 2.0.

Table 2.1: Primers used for pcDNA3/tetC and pCMV-70 sequencing

Location inside plasmids	**Sequence**
Forward primer pcDNA3 (homologous to T7 promoter)	**5' GAA ATT ACG ACT CAC TAT AGG G 3'**
Forward primer pCMV-70	**5' CGC AAA TGG GCG GTA GGC GTG 3'**
Reverse primer pcDNA3	**5' GTC GAG GCT GAT CAG CGA GC 3'**

Table 2.2: Restriction enzymes used to linearise plasmid DNA constructs

Plasmid	**Digestion**		**Digestion products expected**
	Enzyme	**Buffer**	
pcDNA3.1	*HindIII* (Promega)	Buffer E	5.4 kb
pcDNA3/tetC	*HindIII*	Buffer E	6.8 kb
	HindIII + *XbaI* (Promega)	Buffer B	5.4 kb + 1.4 kb
pCMV-link	*Bgl-II* (Promega)	Buffer M	6.3 kb
pCMV-70	*Bgl-II*	Buffer M	6.9 kb
pGFP-C_3	*HindIII*	Buffer E	7.4 kb

2.3. Expression of GFP, Fragment C and MPB-70 *in vitro*

2.3.1. COS-7 Cells - Growth Condition

The African green monkey kidney cell line known as COS-7 (kindly provided by Cathy Rush, Department of Infection and Immunity, Glasgow University) was used for *in vitro* expression of GFP, Fragment C and MPB-70.

COS-7 are adherent fibroblast-like cells and grow as monolayers in complete medium containing Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat inactivated Foetal Bovine Serum-FBS, 1% (w/v) L-glutamine and 1% (w/v) Penicillin/Streptomycin (all supplied by Gibco-Life Technologies). Prior to use, this media was filtered using a 0.20 μm Syringe Filter (Pall Corporation, UK) and the cells propagated at 37°C in 5% CO_2.

Cells were stored long-term as frozen stocks in liquid nitrogen. These were prepared by freezing $1x10^6$ cells in 1ml of storage medium: DMEM with 20% (v/v) FBS, 10% v/v Dimethyl-sulphoxide, DMSO (Sigma). When required, cells were quickly thawed at 37°C, and transferred into a 25 cm^2 culture flask containing 10 ml of complete medium. Five hours later, the medium was removed and replaced with fresh complete medium to ensure the removal of residual DMSO.

COS-7 cells were sub-cultured by splitting the confluent culture every 3-4 days. To split the cells, the monolayer was first washed with 3 ml Hanks' Balanced Salt Solution-HBSS (Sigma) and the cells detached from the flask by trypsination using 1.5ml Trypsin-EDTA. The detached cells were then washed in 5 ml of complete medium and pelleted by centrifugation (1000xg, for 3 minutes, at room temperature). The cells were then resuspended in 5 ml of HBSS, recentrifuged, and finally resuspended in 5 ml complete

medium. The number of viable cells was then determined by Trypan blue exclusion method using an Improved Neubauer Haemocytometer chamber.

2.3.2. Transfection of COS-7 Cells Using Lipofectamine

To induce protein expression, COS-7 cells were transfected with DNA plasmid using Lipofectamine as a carrier. Lipofectamine (Gibco-Life Technologies, UK) is a cationic lipid reagent, which is effective at inducing transfection of DNA into a variety of adherent eukaryotic cell lines.

Transfection using Lipofectamine was achieved as follows: the day before transfection, COS-7 cells were plated at $3x10^5$ cells/well, in a 6-well plate, a density that gives 70-90% confluence within 24 h. Plasmid DNA (1 μg) and the Lipofectamine reagent (10 μg) were diluted in 200 μl serum free DMEM and incubated at room temperature for 20 minutes to allow the formation of DNA/lipid complexes. The solution containing the complexes was then made up to 1 ml by the addition of 800 μl of serum free DMEM and the entire volume of solution added to one well on the 6 well plate. After 5 hours of incubation at 37°C/ 5%CO_2, the medium above the cells was replaced with fresh complete medium, and the cells were maintained in culture for about 72 hours.

2.3.3. Harvest of Transfected Cells

Following 72 hours of incubation, COS-7 cells were harvested by detaching the cells from the bottom of the flask using the trypsin/EDTA method described previously. The cells were collected and concentrated by centrifugation (1000xg, for 5 minutes at room temperature) and the resultant pellet re-suspended in 1 ml of complete medium. The number of viable cells in the suspension was then determined by Trypan blue exclusion. Once determined, approximately 1- $3x10^6$ cells were transferred to an eppendorf tube, and

the cells pelleted by centrifugation at 1000xg, for 5 minutes, room temperature. Finally, the pellet was lysed in 100 µl of 50mM Tris-Cl buffer, [pH 7.5], 150mM NaCl, 1mM phenylmethyl sulfonyl fluoride - PMSF (dissolved in 100% ethanol; Sigma). In order to establish the efficiency of transformation and antigen expression, 10-fold serial dilutions of these cells were prepared. These were admixed in an equal volume of 2xSDS protein buffer (12.5% v/v 1M Tris, [pH 6.8], 4% w/v SDS, 20% glycerol, 10% v/v beta-mercaptoethanol, 0.09% bromphenol blue) before being stored at - 20°C until required.

2.3.4. Analysis of Protein Expression in COS-7 Cells

2.3.4.1. GFP Expression in Transfected COS-7

Expression of GFP in COS-7 cells transfected with pGFP-C_3 was estimated by observing the cell 48–72 hours after transfection using an inverted microscope (Axiovert - Zeiss). The number of fluorescent cells per 10x microscopic field was determined and transfected cells were additionally visualised using an Imaging System equipped with a designated soft for picture processing (OpenLab-Improvision).

2.3.4.2. Fragment C and MPB-70 Expression in Transfected COS-7 Cells

Whole-cell lysates of transfected COS-7 cells were prepared as described above (Section 2.3.3), and separated by SDS-12.5% polyacrylamide gel electrophoresis (SDS-PAGE). The resultant protein bands were subsequently visualised by staining with Coomassie blue or by immunoblotting, using appropriate Fragment C or MPB-70 specific antibodies.

2.3.4.2.1. SDS – Polyacrylamide Gel Electrophoresis –(SDS-PAGE)

Separation of denatured proteins was performed using SDS-PAGElectrophoresis using a BioRad mini-gel system.

Prior to electrophoresis, a separating gel containing 12.5% polyacrylamide and a stacking gel containing 5% polyacrylamide were prepared as follows. 10 ml of separating gel were prepared by using 2.4 ml Lower Resolving Buffer (1.5mM Tris, 13,8mM Sodium Dodecyl Sulphate – SDS; pH adjusted to 8.8 with HCl), 3.35 ml distilled water, 4.15 ml 30% Acrylamide/Bis solution (Severn Biotech Ltd), 60 µl 10% Ammonium persulphate - APS, and 5 µl Tetramethylendiamine – TEMED (Sigma). To prepare 5 ml of stacking gel the following were used: 1.75 ml Upper Resolving buffer, [pH 6.8] (0.5M Tris, 13.8mM SDS, pH adjusted with HCl), 3 ml distilled water, 0.75 ml of 30% Acrylamide/Bis solution, 30 µl of 10% - APS, and 10 µl TEMED. Samples, mixed 1:1 with 2x sample buffer (12.5% v/v 1M Tris, [pH 6.8], 4% w/v SDS, 20% glycerol, 10% v/v beta-mercaptoethanol, 0.09% bromphenol blue) were boiled for 5 minutes and then loaded into the sample wells. See Blue Plus2 prestained markers (Invitrogen) were included on every gel and were used for sizing proteins or peptide fragments from 4 to 250 kDa. Loaded samples were run through the gel matrix at 200 V for 60 minutes using PAGE running buffer (27.25mM Tris, 0.2mM Glycine, 3.46mM SDS).

2.3.4.2.2. Staining Polyacrylamide Gels with Coomassie Blue

When the electrophoresis was complete, gels were transferred into a staining box containing 20 – 50 ml of Coomassie Blue Stain solution (1 $^{0}/_{oo}$ w/v Coomassie brilliant blue R-250, 45% v/v methanol, and 10% v/v glacial acetic acid). Gels were stained overnight at room temperature with gentle shaking. To remove the stain, gels were washed in destain solution (20% methanol, 7% acetic acid), until background of the gel was transparent.

In order to preserve the destained gels long term, gels were dehydrated using Gel Dry Solution (Novex). This required gels to be washed for 3x 15 minutes in deionised water to remove the methanol and then once in fresh Gel Dry Solution (Novex). After 15-20 minutes, the gels were placed between two sheets of Novex cellophane on a special drying frame, and left at room temperature for 24 - 48 hours to dry. After drying, gels were scanned or photographed.

2.3.4.2.3. Western Blotting

For immunoblotting, gels were removed from the electrophoretic apparatus and proteins transferred to wet nitrocellulose membranes (Amersham Hybond C) in 1x Transfer Buffer (25mM Tris, 192mM glycine, 20% methanol) using a mini-blot system (BioRad). Proteins were successfully transferred using 95 V for 75 minutes. After transfer, membranes were washed in PBST (0.05% Tween 20 (Sigma) in Phosphate Buffer Saline [pH 7.3] (PBS: 1.37M NaCl, 31mM KCl, 0.1M Na_2HPO4), for 5 minutes and then blocked in 3% skimmed milk in PBS, either at room temperature for 20-30 minutes, or, overnight at 4°C. Incubation with the primary and the secondary antibody used for detection lasted for at least one hour at room temperature. Antibody concentrations used for each of the various blots are given in Table 2.3. Between each incubation step, the blots were washed twice in PBST for 5 minutes, at room temperature, with shaking on a rotary shaker. In addition, prior to the addition of substrate, the membrane was washed additionally in PBS.

Blots were incubated with substrate (30 mg tablet of 4 chloro-1-naphtol (Sigma), 16.7% methanol, 0.015% v/v hydrogen peroxide in PBS), until the bands appeared. When appropriate, the reaction was stopped by soaking the membrane in distilled water.

Table 2.3. Concentrations of primary and secondary antibodies used in Western blot analysis of expression of MBP-70 and Fragment C in COS-7 cells

Antibody specificity		Concentration (in 3% skimmed milk in PBST)
Primary antibody	Rabbit anti-MPB-70 antibody	1:200
	Monoclonal anti- MPB-70 antibody	1:500
	Rabbit anti-Fragment C antibody (all of above kindly provided by Mark Chambers, Centre of Veterinary Laboratory, Weybridge)	1:500
Secondary antibody	Rabbit anti-mouse antibody Horse-Radish Peroxidase - HRP - conjugate (Dako)	1:1000
	Goat anti-rabbit IgG (whole molecule) HRP - conjugate (Sigma).	1:1000

Table 2.4: Concentrations of primary and secondary antibody used for Western blot detection of Fragment C expression of COS-7 cells using the Pico Chemiluminescent Substrate.

Blot	Fragment C concentration	Antibody concentration (in 3-5% Bovine Serum Albumine [BSA] in phosphate buffer saline[PBST])	
		Rabbit anti-Fragment C antibody	Goat anti-rabbit IgG -HRP
1	100ng, 1ng, 100pg, 1pg	1:500	1:50,000
2	100ng, 1ng, 100pg, 1pg	1:500	1:100,000
3	100ng, 1ng, 100pg, 1pg	1:1000	1:50,000
4	100ng, 1ng, 100pg, 1pg	1:1000	1:100,000
5	100ng, 1ng, 100pg, 1pg	1:5000	1:50,000
6	100ng, 1ng, 100pg, 1pg	1:5000	1:100,000

2.3.4.2.4. Western Blot Detection Using Pico Chemiluminescent Substrate

SuperSignal West Pico chemiluminescent substrate (Pierce), gives a highly sensitive signal enabling detection of picogram quantities of antigen, with relatively low levels of detecting antibody. To allow optimisation of antibody concentrations, six pieces of nitrocellulose membrane were used, on which four 2 µl spots containing 100 ng, 1 ng, 100 pg, 1 pg respectively of antigen were dotted. The antigen dilutions were allowed to dry on membranes for 10-30 minutes and then the remaining non-specific sites of the membrane were blocked with 3-5% Bovine Serum Albumin - BSA (Sigma) in PBST. The antibody dilutions (given in Table 2.4) were prepared in 3-5% BSA in PBST.

Between each step the membranes were washed at least 6 times for at least 5 minutes each time, in PBST. Following incubation with the secondary antibody and subsequent washes, blots were developed by addition of equal volumes of the Luminol/ Enhancer Solution and the stable peroxide solution (Pierce). The blots were incubated in the substrate for 5 minutes, washed again, placed in plastic membrane protectors, and exposed against a high performance chemiluminescence film (Pharmacia Biotech, UK) for 1 to 15 minutes. The films were developed using a X-Ray film processor (Compact X-4).

2.3.5. Glycosylation Inhibition

COS-7 cells were transfected on several occasions with pCMV-70 in the presence of the glycosylation inhibitor, Tunicamycin (Sigma). This experiment was designed to establish if glycosylation was responsible for the apparent difference in size between MPB-70 expressed in prokaryotic cells and the MPB-70 expressed in eukaryotic cells by the DNA construct. Tunicamycin, a nucleoside antibiotic produced by *Streptomyces sp.*, inhibits the biosynthesis of glycoproteins in eukaryotic cells. It is specific for a single step in the glycosylation pathway and easily penetrates eukaryotic cells. COS-7 cells were

transfected with 1 μg of pCMV-70 and 10 μg Lipofectamine, as described (Section 2.3.2). Glycosylation was inhibited by adding of 1, 5, 10, or 20 μg Tunicamycin/ml (stock prepared in DMSO and filtered using a 0.20 μm Syringe Filter) in the culture complete medium, immediately after the removal of the solution containing the Lipofectamine DNA complexes. Two different methods were then followed, in the first protocol the medium above the cells was changed every 24 hours (method I, see Mulero *et al.,* 2000), whilst in the second, the medium was only changed five hours before cell harvesting (method II, see Xu *et al.*, 1999).

2.4. Intramuscular Immunisation of Mice

2.4.1. Mice

Eight groups of five 6-8-week-old female BALB/c mice (Harlan/Olac, UK) were used in this study. To allow identification, mice were individually marked using an ear punch. The schedule of immunisation is outlined in Tables 2.5 and 2.6.

2.4.2. Vaccine Preparation

The four plasmids used for intramuscular immunisation of mice were purified using Endo-toxin free QIAgen Megaprep kit and characterised as described (Section 2.2.3.2.). For immunisations DNA was resuspended in endotoxin-free PBS [pH = 7.2] (Sigma) at a concentration of 1 mg/ml. For subsequent boosts with purified proteins, purified Fragment C (kindly provided by Neil Fairweather, Imperial College, London), and purified MPB-70 (kindly provided by Mark Chambers, Central Veterinary Laboratory, Weybridge) were resuspended in endotoxin-free PBS [pH = 7.2] (Sigma) at a concentration of 1 mg/ml.

2.4.3. Evaluation of LPS Contamination in DNA Samples Used for Immunisation

The level of contaminating lipopolysaccharide (LPS) in plasmid preparations was analysed using the Limulus Amebocysate Lysate (LAL) Assay (QCL, Biowhittaker), which makes use of the observation that Gram negative infection of *Limulus polyphemus*, the Horse shoe crab, results in fatal intravascular coagulation. This observation has now been developed into the generation of a quantitative, kinetic assay. This assay is based on the ability of Gram negative endotoxin to catalyse the activation of a proenzyme in the LAL that subsequently catalyses the splitting of p-nitroanile (pNA) from a colourless substrate. This change can be measured photometrically at 405 nm and the amount of endotoxin in the sample calculated by comparing the reaction time with those shown by solutions containing known amounts of endotoxin.

The assay was carried out as described in the manufacturer's instructions, with all dilutions of the standards and samples performed using endotoxin free plastic-ware and endotoxin-free water. Values in the samples were calculated using the LPS standard units, EU/ml, and concentrations in the samples calculated using a standard curve ranging from 50 EU/ml to 0.005 EU/ml.

2.4.4. Vaccination Regimen

All mice were immunised by inoculation in the quadriceps muscle of one hind leg, using a 27-gauge needle. Each dose was given in the same site regardless whether DNA or protein was being administered. Mice were immunised on days 0 and 8 with 100 μg of DNA in a volume of 100 μl. On day 37, mice were boosted with either a further dose of DNA (100 μg/100 μl) or a single dose of purified protein (10 μg/100 μl) as described in Tables 2.5 and 2.6.

Table 2.5: Vaccination regimen for mice immunised i. m. with pcDNA3/tetC or with the control plasmid, pcDNA3.1 and boosted with Fragment C

	Group 1	Group 2	Group 3	Group 4
Day 0	pcDNA3/tetC	pcDNA3/tetC	pcDNA3.1	pcDNA3.1
Day 8	pcDNA3/tetC	pcDNA3/tetC	pcDNA3.1	pcDNA3.1
Day 37	pcDNA3/tetC	Fragment C	pcDNA3.1	Fragment C

Table 2.6: Vaccination regimen for mice immunised i. m. with pCMV-70 or with the control plasmid, pCMV-link and boosted with MPB-70

	Group 5	Group 6	Group 7	Group 8
Day 0	pCMV-70	pCMV-70	pCMV-link	Naïve
Day 8	pCMV-70	pCMV-70	pCMV-link	Naïve
Day 37	pCMV-70	MBP-70	MBP-70	Naïve

2.4.5. Collection of Samples

Antibody responses in the sera were followed throughout the course of the experiment using sample bleeds taken from the tail veins. Samples were taken one day prior to the first immunisation (pre-immune sera), and subsequently on day 24 and 36 of the experiment (bleed 1 and bleed 2, respectively). Terminal bleeds taken by cardiac puncture were collected on day 50 (for the Fragment C groups) or on day 57 (for the MPB-70 groups). Following death by exsanguination, the spleens were removed and used in T-cell proliferative assays to assess the cellular immune response induced.

2.5. Evaluation of the Immune Response Elicited by the Vaccine

2.5.1. Analysis of the Humoral Immune Response

2.5.1.1. Serum Preparation from Blood Samples

To separate the serum from the blood, samples were first stored overnight at 4°C and then centrifuged at 3000xg, for 5 minutes, at room temperature. The serum was decanted from the pellet into a fresh tube and the serum re-centrifuged to pellet any remaining blood cells. The separated serum was then placed in a labelled tube, which was stored at -20°C.

2.5.1.2. Measurement of Serum Specific Antibodies Using ELISA

Individual sera were analysed in an enzyme linked immunosorbent assay (ELISA) against the purified protein (Fragment C or MPB-70). To detect antigen specific antibodies, 50 µl of either Fragment C (1 µg/ml) or MBP-70 2.5 µg/ml were used to coat the wells of a 96-well ELISA plate (Costar). Wells were coated overnight at 4°C with 50 µl antigen diluted in PBS or, for the negative control wells, with PBS alone. The plates were then incubated for one hour at 37°C with 100 µl blocking solution/well using 1% BSA in

PBST (0.05% Tween 20). After blocking, plates were washed three times in PBST, and then incubated for 2 hours with appropriate dilutions of sera, at 37°C. The starting sera dilution was 1:50 in PBS, followed by seven consecutive 5 fold dilutions of the same sample down the ELISA plate. As a positive control, the second column was incubated with the same dilutions from mouse-anti-Fragment C or anti-MPB-70 antisera. Inclusion of this control serum allows comparison of experiments carried out on different days to be possible. Following incubation, plates were again washed in PBST, and, then incubated for 90 minutes, at 37°C with rabbit anti-mouse - HRP conjugated antibody (Dako; 1:1000). This was then removed by PBST washes before addition of the substrate (50 µl/well; 48.48% v/v 0.1M citric acid, 51.52% v/v 0.25M Na2HPO4, 0.04% v/v hydrogen peroxide, and a 10 mg-tablet of O-phenylenediamine dihydrochloride for 25 ml final volume). The enzyme reaction was finally stopped with 50 µl 3 M HCl/well. The OD_{490} value was the determined using a EL x 808 ELISA plate reader (Bio-Tech Instruments, Inc). ELISA titres were determined arbitrarily as the dilution of serum that gave a value of 0.3 OD units above that level measured in equivalent negative control wells.

2.5.1.3. Measurement of Antigen Specific Antibodies Using Western Blotting

Serum from individual or pooled samples was also assessed for antigen specificity using Western blots. In these blots proteins from MPB-70 or Fragment C transfected COS-7 cells were transferred onto nitrocellulose and then probed with the relevant sera. As a negative control a similar concentration of COS-7 cells tranfected with control plasmids were used. The concentrations of sera used as primary antibodies in these experiments were between 1:150 and 1:20. The Western blot method is described in detail in Section 2.3.4.2.3.

2.5.1.4. Measurement of Specific Subtypes of Antibodies

Specific subtypes of antibodies were measured using antigen specific ELISA (described previously [Section 2.5.1.2]) except that Rat anti-mouse isotype specific biotinylated antibodies were used as secondary antibodies. The concentration of these antibodies had previously been standardised to detect equivalent amounts of monoclonal IgG1, IgG2a and IgG2b antibodies. In these assays they were used at concentrations of 1:5000, 1:1000 and 1:5000 for IgG1, IgG2a and IgG2b antibodies, respectively. The amount of bound biotinylated antibody was then detected using streptavidin- HRP conjugate (1:1000; Dako) diluted in 0.1%BSA/PBST. The conjugate was incubated on the plate for 1h at 37°C before the plates were washed and substrate added. When appropriate colour development was then stopped as described in Section 2.5.1.2 and absorbencies measured at OD_{490}nm. Titres of IgG1, IgG2a and IgG2b were then calculated as described previously.

2.5.2. Evaluation of Cellular Immune Response

2.5.2.1. T Cell Proliferation Assay

Using sterile techniques, spleens were removed from immunised mice and placed into 5 ml of fresh complete culture medium (RPMI-1640 containing 10% FBS, 1% Penicillin/ Streptomycin, 1% L-glutamine, 50 µl mercaptoethanol in 500 ml RPMI). The tissue was then crushed between sterile frosted microscope slides and released cells removed to clean 15 ml Falcon tubes, which were centrifuged at 1000xg for 7 minutes at room temperature. The cell pellets were washed in 3 ml RPMI (1000xg, 7 minutes, room temperature), and then lysed for 5 minutes in 2 ml of red blood cell lysis buffer (Gibco-Life Technologies). The activity of this buffer was then neutralised by adding 2x volumes of RPMI, and cells collected by centrifugation at 200xg for 10 minutes. The cells were washed for a final time before being re-suspended in 2 ml complete tissue culture medium.

The number of viable cells in each preparation was determined by Trypan blue exclusion using an Improved Neubauer Haemocytometer. The concentration of cells was adjusted at $2x10^6$ lymphocytes/ml, and 180 µl of suspension were added per well to a 96 well flat bottom tissue culture plate (Costar-Corning). Cells were then stimulated in triplicate using 20 µl of 3 x 10 fold dilutions of antigen (0.1, 1, and 10 µg/ml). Positive control wells in which samples were incubated with increasing concentrations of the mitogen Concanavalin A (Sigma) at concentrations of 0.5, 5, 50 µg/ml were also included. Negative controls of cells stimulated with 20 µl of media alone were also included in triplicate. After 72h of incubation at 37°C and 5% CO_2, 100 µl of the supernatant was removed from each well and stored for cytokine analysis. To the remaining 100 µl of supernatant, 20 µl of medium containing 1µCi of ^{3}H-thymidine was added to each well. The plates were then re-incubated for 6 hours. Levels of proliferation were then determined by harvesting the cells onto nitrocellulose mats using a cell harvester (Wallac) and then measuring incorporated Thymidine in a Beta plate counter (Wallac). Stimulation indexes (SI) were determined using formula:

SI = no of counts in stimulated wells ÷ no of counts in unstimulated wells

2.5.2.2. Cytokine Types in Culture Supernatant

The presence of IFN-γ and IL-5 in the culture supernatant taken 4 days after stimulation of spleen cells in vitro was determined using an ELISA kit (Pharmingen) according to the recommended protocol. 96 well flat bottomed Costar plates were incubated overnight at 4°C with 50 µl per well of Capture antibody (1:2000 anti-mouse IFN-γ and 1:250 anti-mouse IL-5 monoclonal antibody) diluted in freshly prepared Coating buffer (0.1M $NaHCO_3$, 33.5mM Na_2CO_3 [pH 9.5]). After washing, plates were blocked for

1 hour at 37°C with 100 µl/well of 1% BSA in PBST. A standard curve was created using doubling dilutions of recombinant IFN-γ and IL-5 cytokines (starting concentration: 2000 pg/ml for recombinant mouse IFN-γ and 1000 pg/ml for recombinant mouse IL-5), which were placed in duplicate in a final volume of 50 µl/well. Unknown samples were tested in triplicate and added undiluted to the plate (50 µl/well). Standards and samples were incubated at room temperature for 3 hours. After washing each plate 5 times, 50 µl of detection solution containing either 1:250 biotinylated anti-mouse IFN-γ or IL-5 antibody combined with 1:250 avidin HRP-conjugate in 1% BSA/PBST was added to each well. Plates were incubated for 1 hour at room temperature, washed and developed using 50 µl ELISA substrate in each well. The enzyme reaction was finally stopped with 50 µl 3M HCl/well. The OD_{450} values were determined using an ELx 808 ELISA plate reader (Bio-Teck Instruments, Inc) as described previously. Relative amounts of IFN-γ and IL-5 in test wells were then calculated using curves created from known standards diluted on the same plate.

2.6. Helios Gene-Gun Optimisation

2.6.1. Preparation of DNA/Gold Cartridges for Gene Gun Vaccination

All reagents used in the preparation of gold cartridges for Gene-Gun vaccination were purchased from BioRad, UK. The optimum quantities of polyvinyl pyrrolidone - PVP, microcarriers (1 µm diameter gold particles), spermidine (N-[3-aminopropyl]-1,4 butanediamine) (Sigma), 1 M CaCl2, and plasmid DNA are outlined in Table 2.7. The quantities described are appropriate for coating a single length of tubing placed in the tubing station, which is sufficient for the production of approximately 50 cartridges. To prepare the cartridges, gold microcarriers were weighed out in a 1.5 ml eppendorf tube.

Table 2.7: The main components needed for preparation of Gene-Gun cartridges

MLQ = Microcarrier Loading Quantity (the amount of microcarrier - gold - delivered per shot);

DLR = DNA Loading Ratio (the amount of DNA loaded per mg of microcarriers or per shot;

ctg. = cartridge.

MLQ		**DLR**			**0.05M Spermidine**	**1M $CaCl_2$**	**PVP sol. in ethanol**	
mg/ctg.	mg/ length	µg/ctg.	µg/mg gold	µg/ length			mg/ ml	ml sol./length
0.5	25	1-2	2-4	50-100	100 µl	100 µl	0 – 0.1	3.5

100 µl of 0.05M spermidine were added to the measured gold, and the mixture was vortexed for a few seconds. After the final ethanol wash, the pellet was re-suspended in ethanol/ PVP solution, which was then drawn into the tubing used for the production of cartridges. To the gold and spermidine solution, plasmid DNA was added, and the mixture was vortexed for a further 10 seconds. 100 µl of 1M $CaCl_2$ solution was then added dropwise to the solution, which was thoroughly vortexed between each addition. The mixture was then allowed to precipitate at room temperature for 10 minutes before centrifugation at 10,000g for 3 minutes to pellet the gold. The supernatant was removed and the pellet was resuspended in the remaining supernatant by vortexing briefly. The pellet was then washed three times with 1 ml of fresh endotoxin-free 100% ethanol, using 5-second bursts of centrifugation in a bench-top Microfuge. At each stage in the process, supernatants that were removed from the tube were stored at -20°C. These were checked at the end of the experiment to establish when and if DNA was being lost from the gold during the coating process.

2.6.2. Loading the DNA/Gold Suspension into Gold-Coat Tubing

A schematic representation of the main steps involved in cartridge preparation along with Gene-Gun mediated delivery of DNA is presented in Figure 2.4.

A piece of tubing of approximately 30 inches was inserted into the Tubing Prep Station. Prior to loading with gold, a flow of nitrogen (grade 4.8) from a compressed cylinder connected to the Prep Station was used to dry the interior of tube. The flow of nitrogen used was 0.3-0.4 LPM (litres per minute) for 15 minutes. The tubing was then removed from Prep Station and the flow of nitrogen was turned off. One end of the tubing was inserted into the end of a 16-18 inches of silicone adaptor tubing fitted to a 10 cc syringe, which was used to draw a homogenous DNA/gold suspension (vortexed immediately prior to aspiration) into the dried tubing.

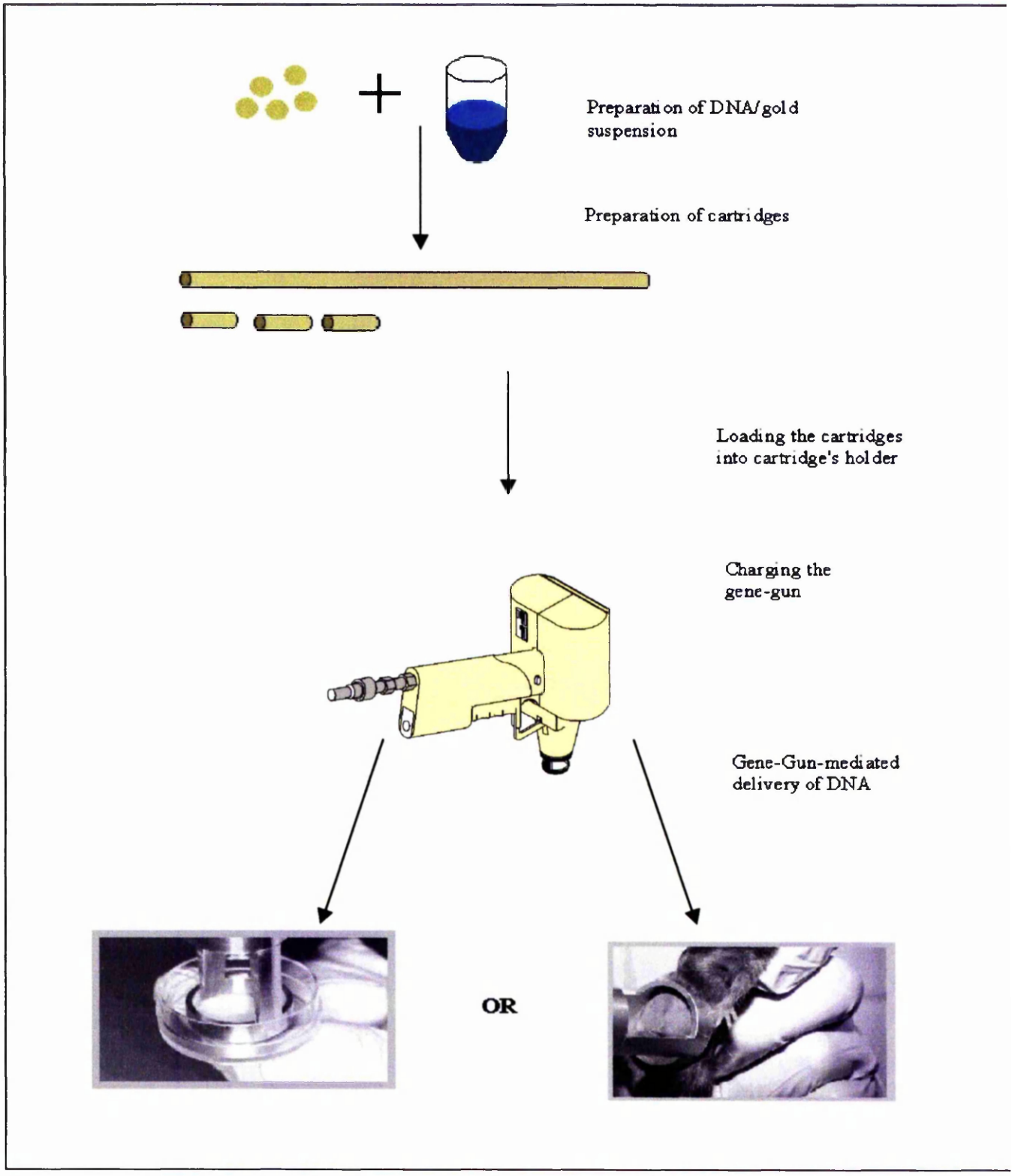

Figure 2.4: A schematic representation of the main steps involved in cartridge preparation and Gene-Gun mediated delivery of DNA: preparation of DNA/gold suspension; preparation of cartridges; charging the Gene-Gun; DNA delivery.

The tubing was then slid into the loading station and the gold was allowed to settle for 3-5 minutes. Ethanol was removed from the tubing using a 10 cc syringe fitted to a silicon adaptor tube at rate of 0.5-1 inches per second. (The removed ethanol was also stored and additionally checked for the presence of DNA at the end of the experiment).

After detaching the syringe, the tubing was immediately turned 180° and the gold was allowed to coat the inner surface of the tubing for 3 - 4 seconds. The bar of the Tube Preparation Station was then rotated and the gold was allowed to coat the tube for 20-30 seconds. Following coating and with the tube still rotating, the valve of the flowmeter was opened to allow nitrogen (0.3/0.4 LPM) to dry the gold onto the inner surface of the tubing. After approximately 30 minutes, the tubing was removed from the station and cut into 0.5 inch cartridges using a tubing cutter.

2.6.3. Quantification of Plasmid DNA in Each Cartridge

To quantify the average amount of DNA per cartridge, we used a method suggested by Craig Watkins (Department of Veterinary Pathology, Royal School of Veterinary Studies, Summerhall, University of Edinburgh). According to this method five ready for use cartridges were placed singly into five 0.5 ml eppendorf tubes, in the base of which a small hole had been created using a needle. These tubes were placed individually into 5x 1.5 ml lidless eppendorf tubes. 50 µl of 10mM Tris-Cl buffer (pH 8.5) was then added to the top of each cartridge, which was then left for 3 hours at 4°C. After incubation, the larger tubes containing the smaller stubs were vortexed, and the contents of the smaller tube collected into the larger tube by centrifugation at 3000xg for 3 minutes (RT). The recovered gold/DNA suspension was then decanted into a fresh 1.5 ml eppendorf tube (with a lid), and another 50 µl of 10mM Tris-HCl buffer added to the top of each cartridge. The tubes were then incubated for a second time overnight at 4°C, before the solution was collected using the vortexing and centrifugation step described above. Once the second collection was

complete, the suspensions from each extraction were pooled. The tubes were then centrifuged again to pellet the gold before the supernatant from each tube was removed carefully into a new 1.5 ml eppendorf tube. Recovery of DNA from the cartridges was determined by measurement of the light absorbency at 260 nm as described (Section 2.2.4.3). In these experiments 10mM Tris-Cl was used as the blank control sample. To estimate the mean amount of plasmid DNA per shot, the concentration of the 5 cartridges were averaged.

2.6.4. Delivery of DNA by Gene-Gun

2.6.4.1. Transfection of Mammalian Target Cells *In Vitro*

The day before Gene-Gun mediated transfection, COS-7 cells were seeded in small individual 35 mm dishes, at a density that ensured a cell monolayer with 70-90% confluence within 24 hours ($3x10^5$ cells/35 mm dish). 30 minutes prior to DNA delivery the complete medium, in which the cells were routinely grown, was replaced with DMEM without FBS, and immediately prior DNA delivery, the medium was aspirated from the dishes.

In order to activate the Gene-Gun for delivery, an empty cartridge holder was inserted into the Gene-Gun, and the Gun connected to a compressed helium cylinder (grade 4.5 [99.995%]). After the flow of helium was set up to the desired pressure the Gene-Gun trigger was depressed 2-3 times to discharge the device. This was done to establish the Gun at the appropriate discharge pressure.

For cell transfections, a loaded cartridge was inserted into the cartridge holder, and the holder introduced into the Gene-Gun. The Gene-Gun was then placed in proximity to the cells and the Gun discharged at helium pressures between 50- 200 psi (pounds per square inch), with an optimised pressure of 100 psi. After all cartridges were discharged,

the cartridge holder was removed, the cartridges were removed from the holder, and the gun was depressurised and shut down.

After transfection of the cells with the Gene-Gun, 2 ml of complete medium was added to each dish and the dishes were observed under the microscope to assess the extent of cell monolayer damage.

2.6.4.2. Analysis of Protein Expression in COS-7 cells transfected by Gene-Gun

2.6.4.2.1. GFP Expression in COS-7 Cells Transfected by Gene-Gun

GFP expression in COS-7 cells transfected by Gene-Gun was performed as described previously (Section 2.3.4.1) using an inverted microscope (Zeiss). Images of the cells were taken and stored on the Improvision Imaging System (OpenLab-Improvision).

2.6.4.2.2. TetC Expression in COS-7 Cells Transfected by Gene-Gun

TetC expression in COS-7 cells transfected by Gene-Gun was assessed by SDS PAGE and Western blotting of cell lysates, using the SuperSignal Chemiluminescent method as described (Section 2.3.4.2.4).

2.6.4.3. Transfection of Mammalian Cells *In Vivo* by Gene-Gun

The day before immunisation, all mice were sample bled from the tail vein. In addition, the fur from the abdomens of all mice was removed using appropriate clippers, to clean and expose the target site for the Gene-Gun-mediated immunisation.

For immunisations, the gun spacer was held against the skin and the DNA discharged from the cartridge at a pressure of 400 psi. The immunisation regimen used for this experiment is given in Table 2.8. For each immunisation, one cartridge containing

approximately 1-2 μg of DNA was used. For intramuscular immunisations 100 μg of plasmid DNA was delivered in a final volume of 100 μl.

2.6.4.4. Evaluation of the Immune Response Elicited by the Gene-Gun Method of Vaccination

Antibody responses in the sera were followed throughout the course of the experiment using sample bleeds taken from the tail veins. Blood samples were taken one day prior to the first immunisation (pre-immune sera), and subsequently on day 22 and 34 of the experiment (bleed 1 and bleed 2, respectively). Total anti-Fragment C IgG titres and specific antibody subtypes were tested using ELISA assay, as described in Section 2.5.1.2 Terminal bleeds taken by cardiac puncture were collected on day 46. Following death by exsanguination the spleens were removed and used in T-cell proliferative an cytokine secretion assays to assess the cellular immune response induced (see Sections 2.5.2.1. and 2.5.2.2, respectively).

Table 2.8: Vaccination regimen for mice immunised by Gene-Gun with pcDNA3/tetC or with the control plasmid, pcDNA3.1 and boosted with Fragment C

In addition, to groups primed by Gene-Gun, a group of mice were immunised with pcDNA3/tetC using the intramuscular route of immunisation.

	Group 1 (5 mice)	**Group 2 (5 mice)**	**Group 3 (3 mice)**	**Group 4 (2 mice)**
Day 1	pcDNA3/tetC - Gene-Gun	pcDNA3.1 - Gene-Gun	pcDNA3/tetC-i. m.	naive
Day 8	pcDNA3/tetC - Gene-Gun	pcDNA3.1 - Gene-Gun	pcDNA3/tetC-i. m.	naive
Day 35	Fragment C i. m.	Fragment C i. m.	Fragment C i. m.	Fragment C i. m.

Chapter 3

Results – Section 1

Characterisation of Plasmid Constructs

The aim of this section was the characterisation of all DNA vaccine constructs used in the course of this study. This included analysis of size, sequence, and sample concentration for each vaccine candidate.

3.1. Transformation of *E. coli* with DNA Vaccine Candidates

The work undertaken in the course of this thesis made use of a number of plasmids, all of which had been provided to us by collaborating scientists. To confirm that each plasmid was as described by those workers, each was initially subjected to a number of studies to confirm the size, sequence, and sample concentration.

All four DNA vaccine candidates used in this study (pcDNA3/tetC, pcDNA3.1, pCMV-70, pCMV-link) and the green fluorescent protein, GFP-encoding plasmid (pGFP-C3), used to optimise techniques, were initially transformed into competent *E. coli* XL-10 which had been prepared as described previously (Section 2.2.2). Using the appropriate antibiotic, successfully transformed bacteria where selected. Initially, several colonies from each transformation were chosen and screened for the presence of the appropriate plasmid initially by purification and restriction digest of the plasmid DNA.

3.1.1. Initial Characterisation of pcDNA3/tetC and pcDNA3.1

Using the restriction map of the plasmid, two restriction enzymes were identified for use. Both these enzymes (*XbaI* and *HindIII*) are able to cut the plasmid at a single site. However, when used together in a double digest, they are able to cut out the inserted Fragment C gene as *HindIII* and *XbaI* restriction sites are located immediately up and downstream of the end of open reading frame respectively. DNA purified from bacteria transformed with either pcDNA3.1 or pcDNA3/tetC were subjected to single (Figure 3.1) or double digest (Figure 3.2) using these enzymes and plasmid size was then determined following electrophoresis in a 0.8% agarose gel.

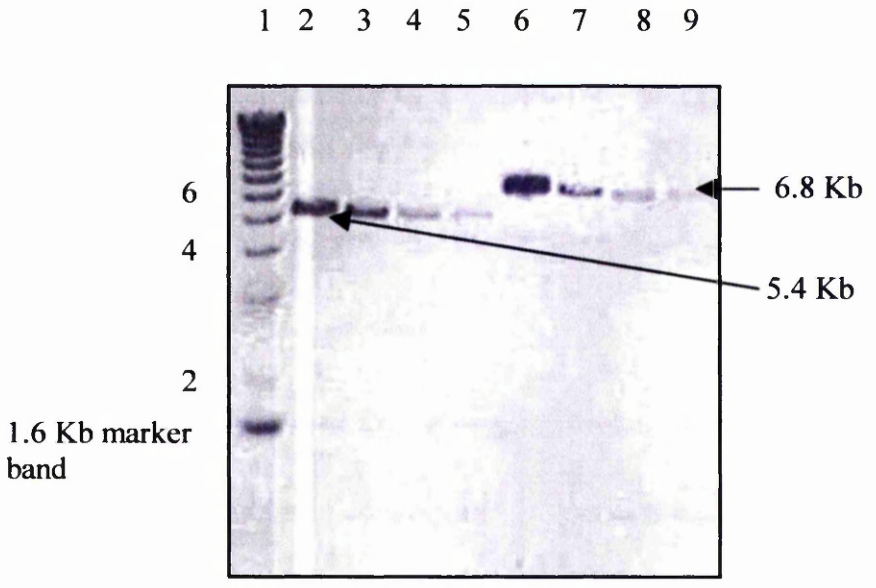

Figure 3.1: Digestion of pcDNA3.1 and pcDNA3/tetC using *XbaI*

Serial dilutions of digested pcDNA3.1 (1/20 - lane 2, 1/50 - lane 3, 1/100 - lane 4, 1/200 - lane 5) and pcDNA3/tetC (1/20 - lane 6, 1/50 - lane 7, 1/100 - lane 8, 1/200 - lane 9) were run on a 0.8% agarose gel containing 0.04% ethidium bromide. The difference in size between the 5.4Kb digestion fragment (pcDNA3.1) and the 6.8Kb fragment (pcDNA3/tetC) correlates with the size of the Fragment C gene insert (1.4Kb). An approximation of the correct concentration of the plasmids can also be determined by comparison with the 1.6Kb band of the DNA marker (lane 1), which contains 10% of the mass of the marker applied to the gel.

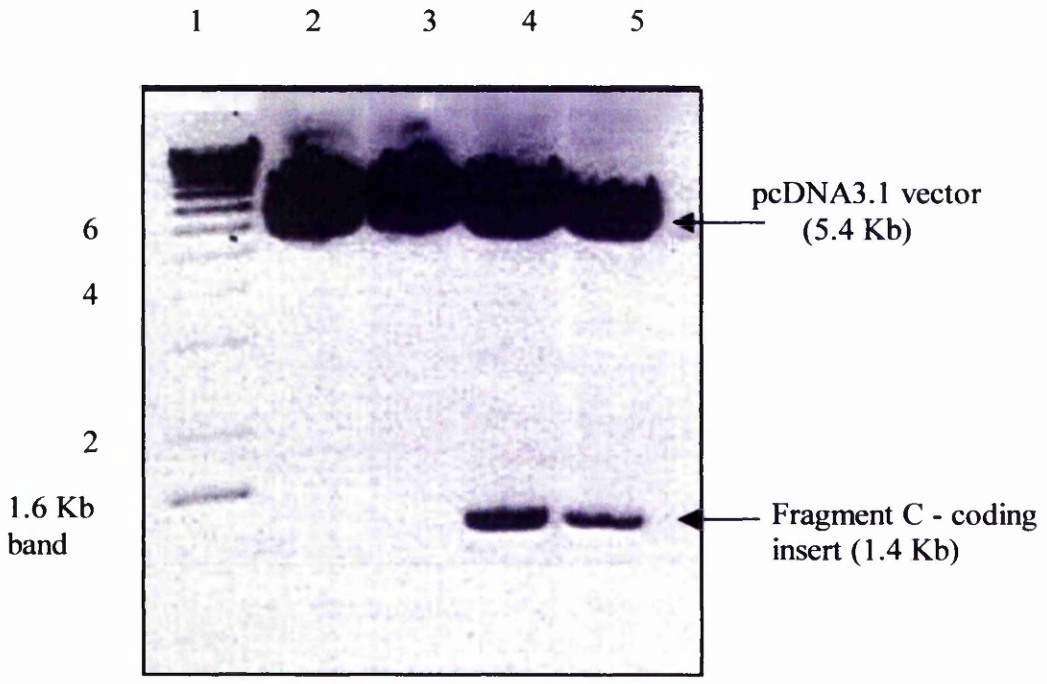

Figure 3.2: Restriction digest of pcDNA3.1 and pcDNA3/tetC with *HindIII* and *XbaI*

This figure shows plasmid pcDNA3.1 (lanes 2 and 3) and pcDNA3/tetC (lanes 4 and 5) on an agarose gel (0.8%) containing 0.04% ethidium bromide following digestion with *HindIII* and *XbaI* restriction enzymes. Control plasmid pcDNA3.1 is linearised by digestion of the two sites, which are close to each other inside the multiple cloning site. The digested plasmid is approximately 5.4 Kb in size and is identical with the larger fragment obtained by double digestion of pcDNA3/tetC. In pcDNA3/tetC *HindIII* and *XbaI* restriction sites flank the Fragment C gene insert, therefore double digestion leads to the production of a large fragment of 5.4 Kb (the vector) and a small one of 1.4 Kb (the insert).

3.1.2. Initial Characterisation of pCMV-70 and pCMV-link

Using restriction maps of these plasmids, the enzyme *Bgl-II* was identified as suitable for cutting the plasmid at a single site. The use of this enzyme resulted in the linearisation of the DNA of both plasmid and the production of a 6.9 kb fragment for pCMV-70, and a 6.3 kb fragment for pCMV-link respectively (Figure 3.3). However, due to the small size of the MPB-70 gene insert (~0.6 kb), identification of the inserted fragment by double digestion of the plasmid was found to be difficult. As the relative sizes of the singly digested plasmids appeared to suggest an insert, two clones were identified and the DNA subjected to sequencing, to confirm the presence of the MBP-70 coding region.

3.2. Sequencing of the DNA Vaccine Candidates

To confirm the sequence of the expressed protein in both pcDNA3/tetC and pCMV-70 respectively, appropriate primers were designed (see Table 2.1) and used to generate sequence data from both the 5' and 3' end of the expressed protein. Analysis of this data confirmed that both proteins were in frame and the sequence of the genes were as expected. The bacterial clone from which the plasmid had been purified for sequencing was then used to prepare a master seed bank for each individual plasmid. This seed bank was used for all of the experiments described in this thesis.

3.3. Quantification of the DNA

During the course of this study, DNA was prepared on a number of occasions for use both *in vitro* and *in vivo*. In order to determine the amount of DNA used in these experiments two different methods were adopted for quantification of the DNA (Section 2.2.4). In general, these methods were used in parallel, with data from one supporting analysis using the other.

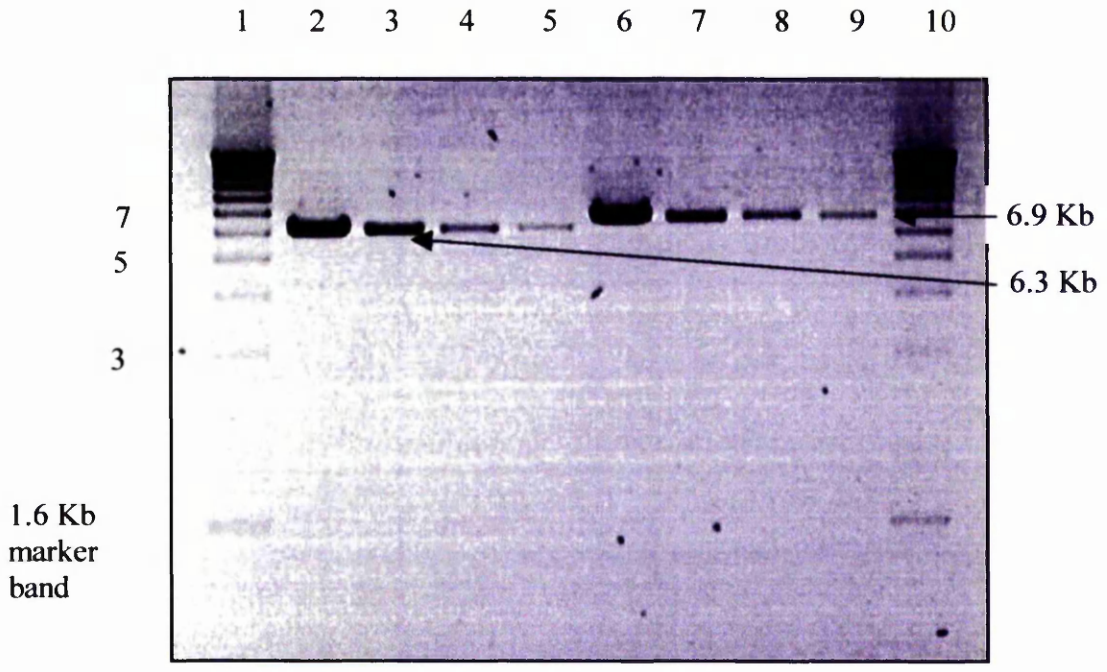

Figure 3.3: Digestion of pCMV-link and pCMV-70 with *Bgl-II*

Serial dilutions (1/20, 1/50, 1/100, 1/200) of plasmids pCMV-link (lanes 2, 3, 4, 5, respectively) and pCMV-70 (lane 6, 7, 8, 9, respectively) run on an agarose gel (0.8%) following digestion with *Bgl II*. The difference in size between the 6.9 Kb digestion fragment (pCMV-70) and the 6.3 Kb fragment (pCMV-link) correlates with the size of the MPB-70 gene insert of approximate 0.6 Kb.

The average amounts of plasmid DNA that were generated from miniprep and megaprep kits (see Section 2.2.3.1. and 2.2.3.2) are include in Table 3.1.

3.4. Analysis of LPS Contamination

Although all plasmid DNA for *in vitro* and *in vivo* experiments were prepared using Qiagen MegaPrep Endotoxin free kits, all batches of DNA were subject to LPS analysis using the LAL test prior to use (Section 2.4.3). In general, levels of LPS were less than 2 EU of LPS in 100 μg of DNA (levels of LPS acceptable in current paediatric vaccine <5 EU/dose). These levels of DNA were not considered to have any potential impact on any of the work subsequently carried out.

Table 3.1: Average amounts of plasmid DNA generated from miniprep and megaprep kits.

Preparation of plasmids using the Miniprep Kit	
pcDNA3.1	50-200 ng/μl
pcdna3/tetC	250 ng/μl
pCMV-link	160 ng/μl
pCMV-70	160 ng/μl
Preparation of plasmids using the Endotoxin-free Megaprep Kit	
pcDNA3.1	1.69 ng/μl
pcDNA3/tetC	1.76-2.35 ng/μl
pCMV-70	1.36-2.21 ng/μl

Chapter 4

Results – Section 2

Transfection of COS-7 Cells Using Lipofectamine

The aim of the work described in this chapter was to optimise transfection of COS-7 cells and measurement of antigen expression by DNA vaccine candidates using Lipofectamine as the transfection agent.

In this context, the plasmid expressing the green fluorescent protein was used to standardise methods, which were then used with the vaccine candidates, pcDNA3/tetC and pCMV-70.

4.1. Optimisation of *In Vitro* Transfection Experiments Using a Plasmid Encoding the Green Fluorescent Protein (GFP)

COS-7 cells used in transfection experiments adhere effectively to the surface of culture dishes, a property that makes them well suited for transfection, especially by Gene-Gun, when DNA delivery is carried out in complete absence of medium. In addition, the adherent nature of the cells, makes them suitable for the frequent replacement of medium that is required during transfection.

Initial experiments were carried out using a GFP-encoding plasmid (pGFP-C3 - Clontech). This plasmid was chosen as expression of the green fluoresence can be easily and precisely detected by fluorescence microspcopy. Once produced, it is stable in the cells (does not break down rapidly) and does not require additional proteins, substrates or co-factors to activate its natural fluoresence.

Initially, transfection of COS-7 cells was carried out using 7 or 10 μl Lipofectamine combined with 2μg DNA. This mixture was added to between 2 and $4x10^5$ cells and allowed to interact with the cells for various lengths of time, before the cells were washed and new media added. GFP expression was then determined by counting the number of fluorescent cells in an individual field of the microscope (as described in Section 2.3.4.1). These experiments revealed that the highest rate of transfection was observed when 2μg of DNA was incubated for 5 hours with 10μl of Lipofectamine/ $3x10^5$ cells.

The average number of fluorescent cells per 10x microscopic field (as can be seen in Figure 4.1), following 5 hours of transfection was about 115 with little variation between different culture dishes.

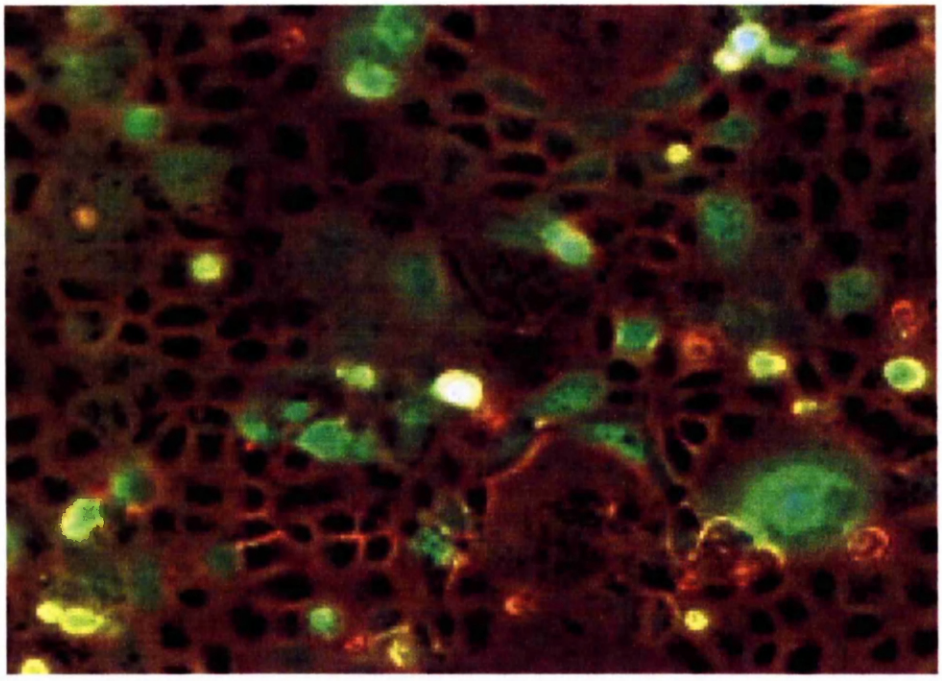

Figure 4.1: *In vitro* expression of GFP following transfection by Lipofectamine

Expression of GFP was quantified by counting the number of fluorescent cell per 10x microscopic field 72 hours after transfection of COS-7 cells with 2 μg pGFP-C3 (Clontech) and 10 μg Lipofectamine (Gibco-Life Technologies)/ 3x 10^5 cells. The number of fluorescent cells was established using an inverted microscope (Axiovert - Zeiss) connected to an Imaging System equipped with a designated software for picture processing (OpenLab-Improvision).

In comparison, following 8 hours of incubation, an average of only 72 fluorescent cells per field were observed. This was further reduced to 53 cells/field following overnight incubation of Lipofectamine and DNA. This reduction in numbers may reflect the toxic nature of Lipofectamine rather than transfection efficiency, with many potentially transfected cells unable to survive in this environment.

4.2. *In vitro* Expression of MPB-70 in COS-7 Cells

Following optimisation of expression of pGFP-C3 in COS-7 cells, work was extended to allow transfection of COS-7 cells with the MPB-70-encoding-plasmid pCMV-70.

MPB-70 is an immunogenic mycobacterial protein with very well documented structural and physiochemical properties, described in detail in (Section 1.3.5.2.3). Briefly, MPB-70 is a monomeric protein of a 163 amino acids (the mature form), which is preceded by a 30 amino acids peptide representing a secretory signal. However, several groups have reported that under different reducing conditions, the protein can display variations in its molecular mass. These variations have been attributed to the presence of two widely spaced cysteine residues in the MPB-70 polypeptide chain, which can alter the three dimensional structure of the protein under non-reducing conditions. Under reducing conditions (β-mercaptoethanol) the molecular weight of MPB-70 has been reported to be about 22 kDa (Wiker *et al.,* 1998). However, under non-reduced conditions the protein presents a different mobility on SDS-PAGE gels with a reported mobility of 15 kDa (Wiker *et al.,* 1998). This appears to additionally affect the availability of a number of conformational epitopes.

4.2.1. Optimising the Detection of MPB-70 Expressed *In Vitro*

To optimise the conditions for detection of the protein by immunoblots, various concentrations of purified recombinant MBP70 protein were loaded onto SDS PAGE gels. These gels were then either stained with Coomassie blue, or used in Western blot analysis to determine the optimum concentrations of the polyclonal rabbit anti MPB-70 antibody used as the primary antibody. Three gels were used to determined optimal primary antibody concentration (Concentrations tested included 1:200, 1:400 and 1:800). Figure 4.2. shows that between 100-50ng (per lane) of MBP-70 could be detected when the antibody was used at 1:200. In contrast, using Coomassie staining, the lowest concentration of MBP70 that can be detected is 1μg. Subsequent analysis of protein expression in transfected cells used the polyclonal antibody at this concentration. A similar approach was also taken to optimise the concentration of a monoclonal anti MPB-70 antibody (kindly provided by Mark Chambers, Centre of Veterinary Laboratory). A concentration of 1:500 of primary monoclonal anti-MPB-70 antibody gave the greatest level of sensitivity on a Western blot and was used at this concentration for subsequent analysis.

During the course of these experiments, the reported differences in the structure of this protein were observed. As can be seen in Figure 4.3, the molecular mass of fully reduced, denatured rMPB-70 was about 22 kDa (agreeing with the reported data (Wiker *et al.,* 1998)). This appeared to be the fully reduced form as when an alternative reducing agent, dithiothreitol (DTT), was added to the sample buffer in place of β-mercaptoethanol, only one band of 22 kDa was present. In the absence of a reducing agent, a 15k Da band corresponding to the described non-reduced form of the protein could be observed (Wiker *et al.,* 1998). Interestingly, in the absence of β-mercaptoethanol, both the 15k Da and the 22k Da protein could be seen suggesting that the protein exists in both forms in the boiled sample.

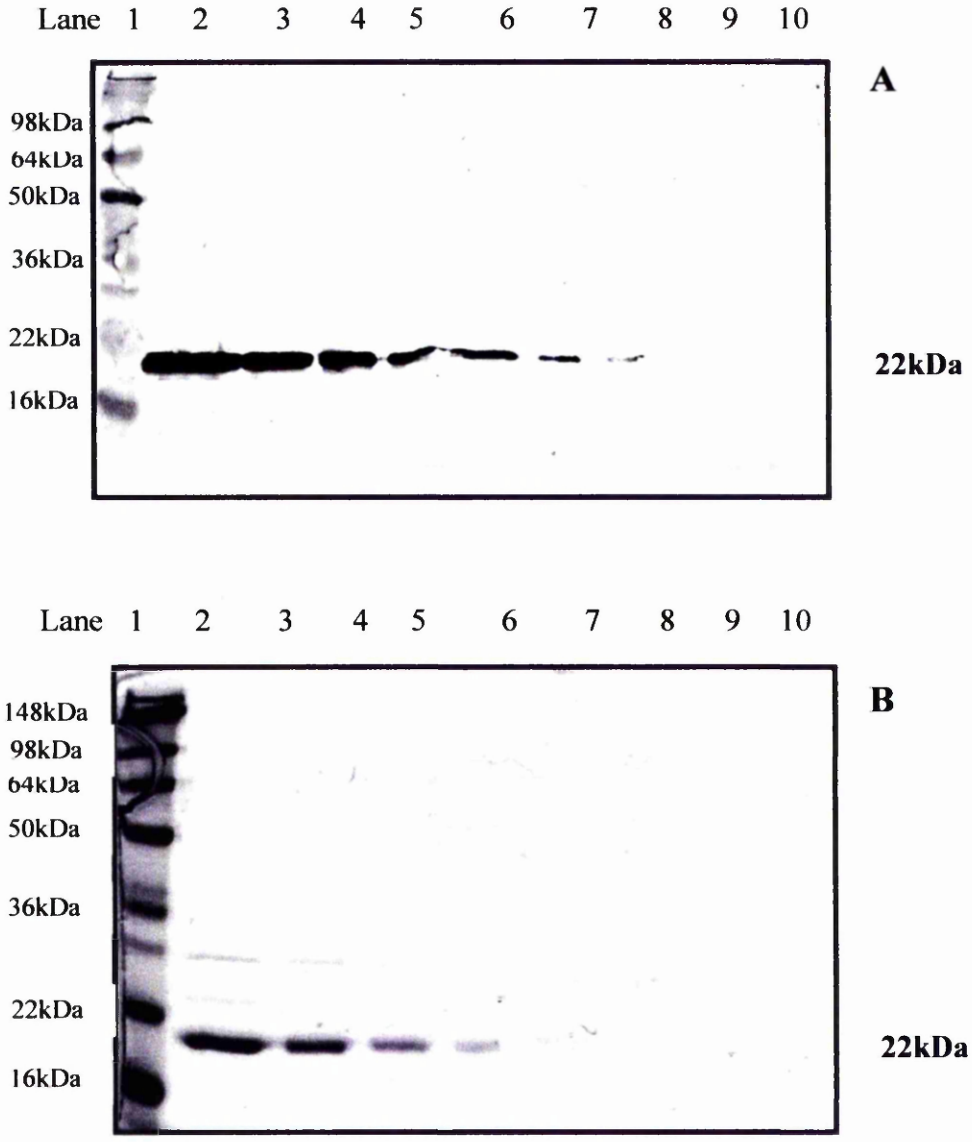

Figure 4.2: Detection limit of recombinant MPB-70

10, 5, 2.5, 1, 0.5, 0.1, and 0.05 µg of rMPB-70 were loaded into lanes 2, 3, 4, 5, 6, and 7, respectively on a 12.5% SDS PAGE gel.

A. This figure shows the detection of rMBP70 using Western blot, which was developed using concentrations of 1:200 for the primary antibody (rabbit anti-MPB-70) and 1:1000 for the secondary antibody (anti-rabbit - HRP conjugated).

B. This figure shows an identical gel stained with Coomassie Blue.

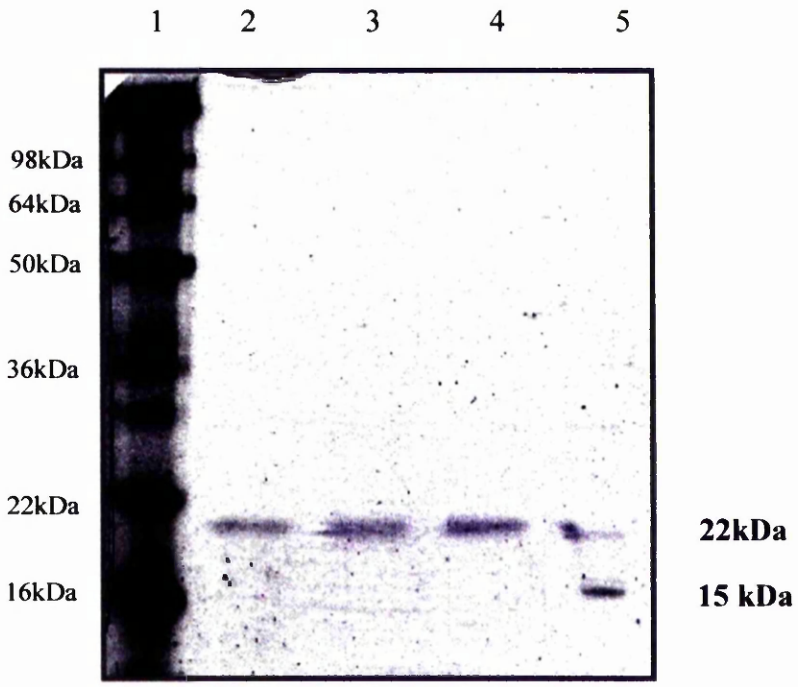

Figure 4.3: Analysis of recombinant MPB-70 using different reducing agents

1 μg of rMPB70 was admixed with sample buffers containing different reducing agents. The samples were then boiled for 10 mins, prior to analysis using a 12.5% SDS-PAGE gel. The samples loaded were treated as follows: lane 2: sample buffer with β-mercaptoethanol, lane 3: sample buffer with 2mM DTT, lane 4: sample buffer with 50mM DTT and lane 5: sample buffer without β-mercaptoethanol.

4.2.2. MPB-70 Expression in COS-7 Lysates Following Transfection with pCMV-70

Once the method for detection of MBP-70 had been established, COS-7 cells were transfected with the plasmids, pCMV-70 or pCMV-link. Following transfection using the optimised conditions, cells were harvested and a known number of lysed cells mixed with protein sample buffer and run on SDS page gels. Expression of the antigen was then determined by Western blot using the polyclonal and monoclonal antibodies described. As a positive controls, purified rMPB-70, *M. bovis* culture filtrate and sonicate were included on the gels. Cell lysates from COS-7 cells transfected with control plasmid pCMV-link were also included as a negative controls.

As observed previously, the polyclonal antibody detected the rMBP-70 as a band of about 22kDa in these gels (Figure 4.4). Similarly, a protein of an equivalent size was also observed in lanes loaded with material from either *M. bovis* filtered or sonicated material. In contrast, COS-7 lysates transfected with pcCMV-70, produced a number of proteins that were detected bands by the antisera, however, in this case the dominant protein appeared to have a lower mobility of about 28 kDa. In contrast, the apparent absence of any band in lanes loaded with an equivalent amount of COS-7 cells transfected with pCMV-link suggested that this protein was MBP-70 specific.

Equivalence of cell loading was demonstrated by Coomassie blue staining of a duplicated gel (Figure 4.4.B). One potential explanation for the difference in mobility observed is the potential for post-translation modification of protein in eukaryotic cells that do not occur naturally in prokaryotes.

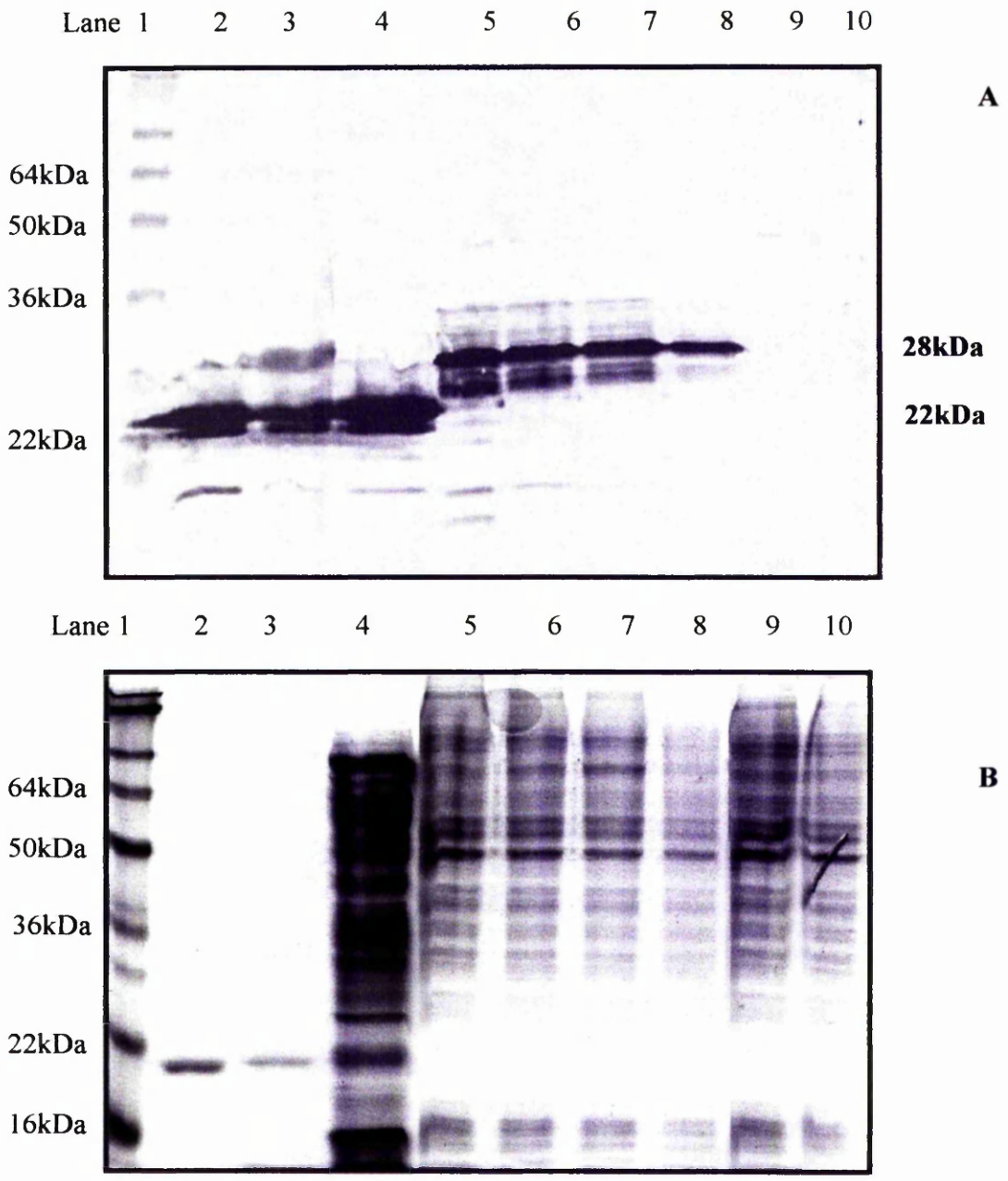

Figure 4.4: Detection of MPB-70 expression in COS-7 cells transfected with pCMV-70:

Figure A shows analysis of expression of MBP-70 by Western blot using MBP-70 specific polyclonal sera. The gel was loaded as follows: lane 1: molecular weight ladder; lane 2: 2.5 μg of recombinant MPB-70, lane 3: 10 μg of *M. bovis* filtrate; lane 4: 10 μg of *M. bovis* sonicate. Lanes 5, 6, 7, 8: $5x10^5$; $2.5x10^5$, $1.25x10^5$ $6.24x10^4$ COS-7 cells transfected with pCMV-70. Lanes 9, 10: $6x10^5$, $3x10^5$ COS-7 cells transfected with pCMV-link.

Figure B shows a duplicate gel loaded in parallel. Staining of the total protein by Coommassie blue shows that the number of COS-7 cells loaded per lane were approximately equivalent.

In contrast, experiments performed in parallel using the MBP-70 specific MoAb, failed to detect any MBP-70 specific band in lanes loaded with COS-7 cell lysates transfected with pCMV-70. In contrast, protein was readily detected in the lanes treated with either the recombinant or native version of the protein (Figure 4.5). This result suggests that the protein produced by COS-7 cells is either an unrelated protein that can react with the polyclonal antibody, or, the protein has been modified in such a manner as to mask important B-cell epitopes. The second explanation allows for the possibility that synthesis of MBP70 in the eukaryotic system results in posttranslational changes to the protein.

In an attempt to explain these apparent differences, two possible theories were considered, that:

1. the protein was failing to become fully reduced following treatment with a reducing agent due to the formation of additional disulphide bonds in the structure. However, as proteins with complex folded structures, would be expected to be more compact they would be also expected to have a higher rather than reduced mobility as in the case of the non-reduced form of rMBP-70 (Figure 4.3). However, if internal bonds mediate the formation of aggregates of the protein either in its complete or truncated form this could explain the larger molecular weight.
2. Alternatively, the difference in size between recombinant MPB-70 and *in vitro* - expressed protein after transfection with pCMV-70 may reflect post-translation modifications such as glycosylation and phosphorylation.

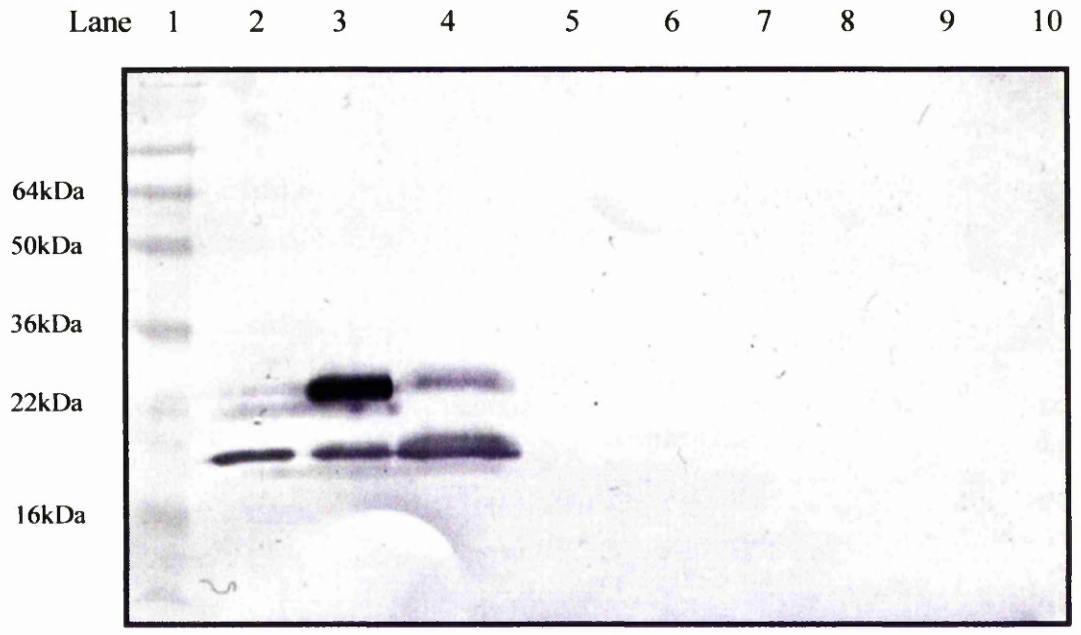

Figure 4.5: Detection of MPB-70 expression in COS-7 cells transfected with pCMV-70, using a monoclonal antibody

This Western blot shows expression of MPB-70 protein in COS-7 cells using antigen specific monoclonal antibodies. Lanes were loaded as follow: lane 1: 2.5 μg of recombinant MPB-70 (positive control), lane 2: 10 μg of *M. bovis* filtrate; lane 3: 10 μg of *M. bovis* sonicate. Lanes 5, 6, 7, 8: $5x10^5$; $2.5x10^5$, $1.25x10^5$ $6.24x10^4$ COS-7 cells transfected with pCMV-70. Lanes 9, 10: $6x10^5$, $3x10^5$ COS-7 cells transfected with the vector alone (pCMV-link).

4.2.2.1. Reduction of Aggregate Forms of MBP-70

To check the validity of the first hypothesis, cell lysates were loaded on SDS-PAGE gels in the presence of sample buffers containing different concentrations of DTT. As DTT is a very strong reducing agent, it was expected that any disulphide bonds present in this potentially aggregated form would eventually be reduced. This should be reflected by a reduction in the immunodominant band from 28Kda to approximately 22k Da. Although, three different concentrations of DTT (2, 20 and 50mM) were used in the sample buffer, none were able to modify the mobility of MPB-70. This suggests that either the formation of such bonds is not important in the formation of this structure or that DTT had no effect at the concentrations used.

4.2.2.2. Role of N-Glycosylation in Post-Translation Modification of MBP-70

In an attempt to determine whether N-glycosylation was responsible for the difference in size between purified MPB-70 and MPB-70 expressed *in vitro*, COS-7 cells were incubated during transfection with different concentrations of Tunicamycin, an inhibitor of the biosynthesis of N-linked glycoproteins. Tunicamycin is a nucleoside antibiotic produced by *Streptomyces sp.*, which is considered to be an ideal inhibitor of N-glycosylation, as it is specific for the first reaction in the glycosylation pathway and is able to easily penetrate cells *in vitro* and *in vivo*. It has been shown that cells incubated with Tunicamycin produce non-glycosylated forms of N-linked glycoproteins (Elbein, 1991).

In early experiments, Tunicamycin was included at a concentration of 10 μg per ml of culture medium (20 μg/ $3x10^5$ cells) during the whole period of transfection. Using these conditions MPB-70 expression could not be detected at all in the COS-7 cells.

However, under these conditions, a reduction in the number of harvested COS-7 cells was observed ($1.2x10^6$ harvested cells compared with $8.5x10^6$ cells in the absence of Tunicamycin). This suggested that the amount of 10 μg Tunicamycin might have had a toxic effect on the synthesis of all cellular proteins and, implicitly, on cell growth. This has been described previously in yeast (Elbein, 1991) and may be due to the following:

1. decreased stability and more rapid degradation of the protein in the absence of carbohydrate chains
2. decreased solubility and/ or aggregation of protein
3. increased susceptibility for proteolytic attack after cell lysis

To address the potential increased susceptibility of carbohydrate-depleted proteins to proteolytic attack, all subsequent experiments were performed using a protease inhibitor - 1mM phenylmethyl sulfonyl fluoride - PMSF (Sigma) added to the lysis medium used at the end of the experiment.

To address the problem of optimising the exposure of the cells to Tunicamycin, further experiments were attempted using lower concentrations of Tunicamycin (2.5 and 1μg/ml culture medium). These concentrations did not appear to affect the protein synthesis or the cell growth. Using these three concentrations of Tunicamycin (10, 2.5 and 1 μg) and two different methods (Tunicamycin present throughout the whole transfection, or added 5 hours before harvesting), the presence of the protease inhibitor in the lysis buffer did not appear to have any effect on the size of MPB-70 expressed by transfected COS-7 cells as detected on western blot. Together these data suggest that the difference in the size of MPB-70 when expressed in COS-7 cells could not be attributed to N-glycosylation.

4.3. *In vitro* Expression of Fragment C

In parallel but in isolation to the work on MBP-70, COS-7 cells were additionally transfected using Lipofectamine with pcDNA3/tetC. However, although the concentration of Fragment C specific antibody was initially optimised using purified Fragment C protein, initial experiments indicated that cells transfected with pcDNA3/tetC did not express Fragment C. Initially, it was unclear whether this indicated that

1. no trasfection had occurred
2. the vector was not capable of driving expression of the antigen in COS-7 cells, or,
3. the method used for detection of antigen expression lacked a suitable level of sensitivity.

As this vector had been used previously to successfully generate immune responses in animals we decided to investigate an alternative and potentially more sensitive method of antigen detection.

4.3.1. Detection of Fragment C Using a Chemiluminescent Substrate

SuperSignal West Pico (Pierce) is a chemiluminescent substrate that enables detection of picogram quantities of antigen. As this method is very sensitive, the concentration of primary and secondary antibodies must be carefully optimised to reduce the level of an unspecific background that may be observed. In an attempt to optimise conditions, decreasing concentrations of primary and secondary antibodies were tested to detect known concentrations of Fragment C, spotted on a nitrocellulose membrane. The range of concentrations varied from 100ng to 100pg. As it is shown in Figure 4.6, the combination of antibody concentrations of 1:5,000 for the primary antibody (polyclonal rabbit anti-Fragment C) and 1:50,000 for the secondary antibody (goat anti-rabbit HRP) correlated with the lowest background and the highest specific signal.

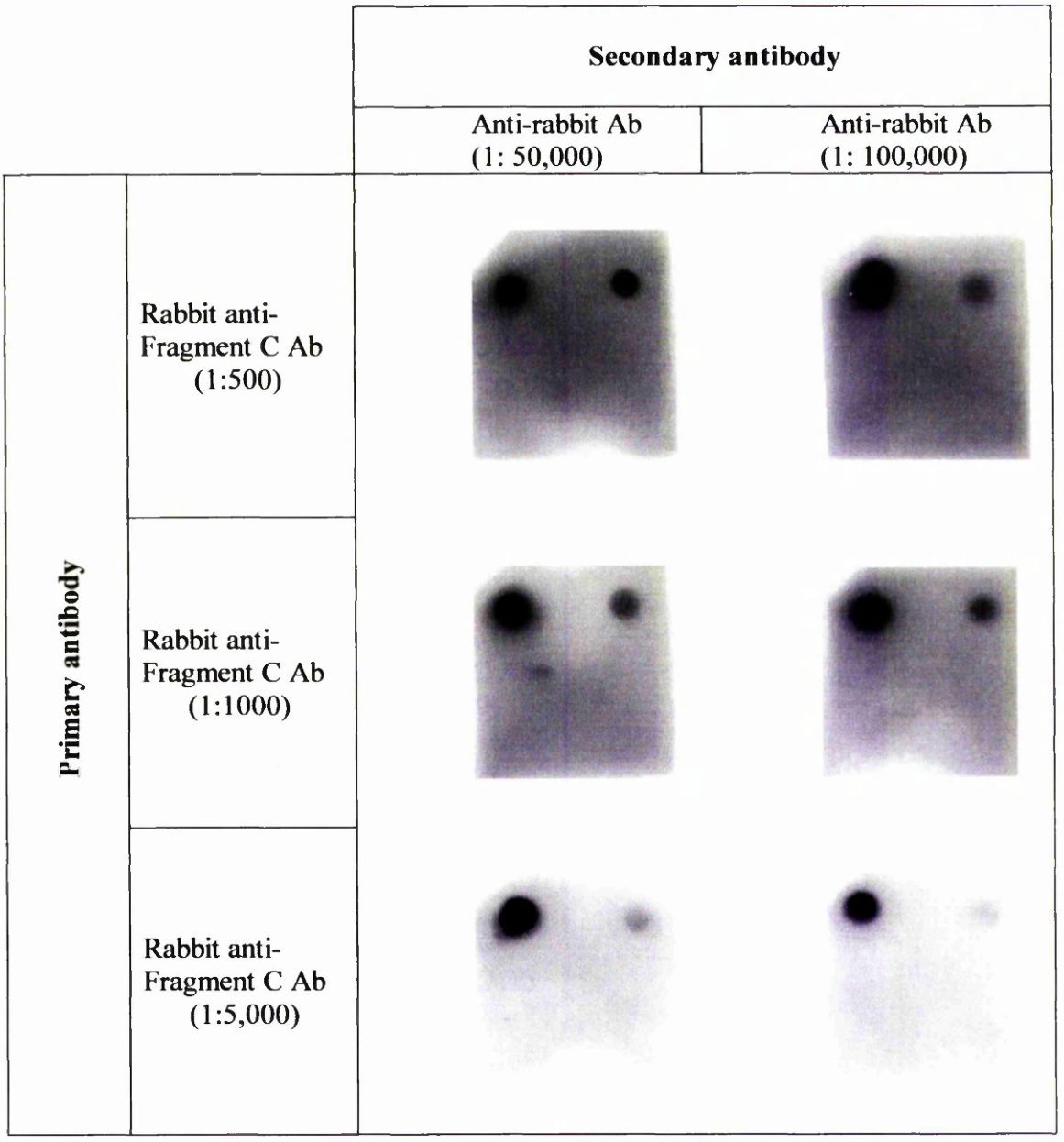

Figure 4.6: Optimisation of antibody concentrations used in Western blot method using the SuperSignal substrate

Each membrane contains four 2 µl spots of 100 ng (upper left), 1 ng (upper right), 100 pg (lower left), 1 pg (lower right) of antigen. The greatest sensitivity of the assay was shown to be at 100 ng of Fragment C, developed with concentrations of 1:5,000 of primary antibody and 1:50,000 of secondary antibody.

These conditions were therefore used to determine expression of Fragment C in transfected COS-7 cells. In addition, the exposure time of the radiographic film was also optimised, with the best results were obtained following the substrate incubation, the film was exposed to the filter for 15min.

4.3.2. Detection of *In Vitro* Expressed Fragment C

The level of detection of this method was initially demonstrated by running known concentrations of recombinant Fragment C (100 ng, 10 ng, 1 ng, 100 pg and 1 pg) on an SDS page that was used for immunoblot analysis with supersignal. Using this method only the 100ng band was detected.

Using the same method as outlined for MBP-70 (Section 2.3.2.), COS-7 cells were transfected with pcDNA3/tetC and following harvesting, different numbers of lysed cells (ranging from 3 x 10^5 to 3.8 x 10^4 transfected COS-7 cells) were loaded onto SDS-PAGE gels and level of Fragment Cexpression determined using the SuperSignal kit. As can be seen in Figure 4.7, an immunodominant band of approx 50kDa in size was expressed in the trasnfected cells. Using the positive control as a guide, in which 100ng of recombinant Fragment C was loaded, the level of expression was estimated to be up to 100 ng in 3.8 x 10^4 cells (about 70 ng/ 2.8 x 10^4 cells in comparison to about 10 ng / 2.8x104 cells transfected by Gene-Gun, see Section 5.2). In comparison, similar concentrations of COS-7 cells transfected with the control plasmid, pcDNA3.1, failed to produce any detectable band, suggesting that the band detected was dependent on expression of the Fragment C protein from the pcDNA3/tetC construct.

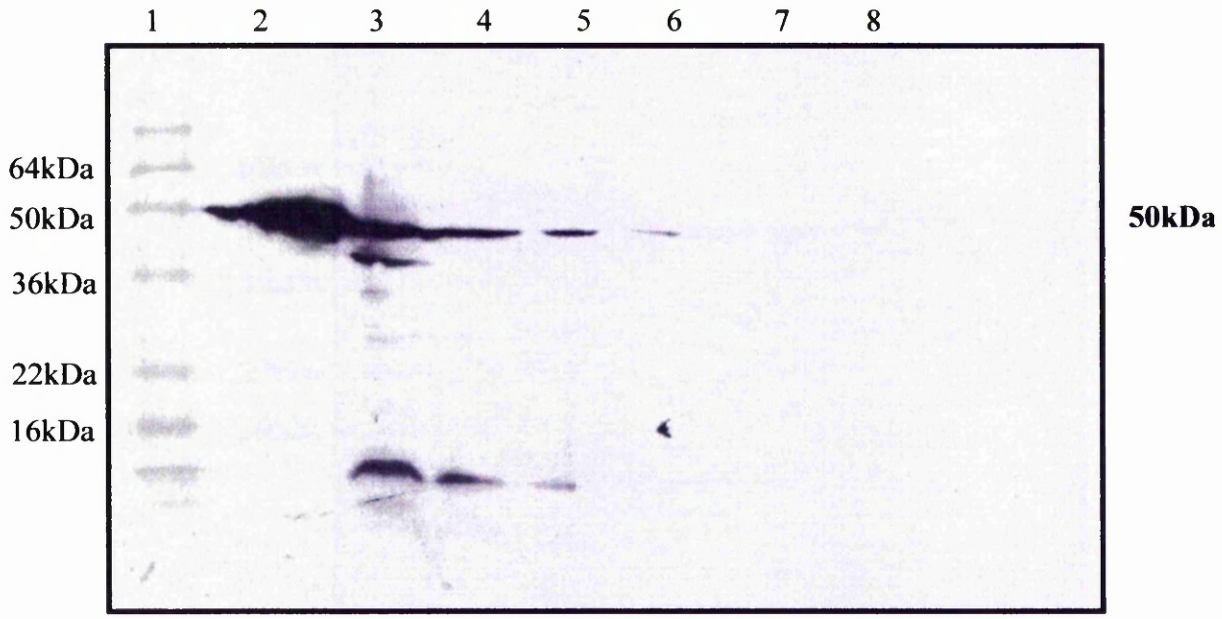

Figure 4.7: Fragment C expression in COS-7 cells transfected with pcDNA3/tetC.

Protein expression was detected by Western blot using Fragment C specific polyclonal antibody sera. The gel was loaded as follows: Lane 2, 100 ng Fragment C (positive control). Lanes 3, 4, 5, 6: 3 x 10^5, 1.5 x 10^5, 7.6 x 10^4, 3.8 x 10^4 COS-7 cells transfected with pcDNA3/tetC. Lanes 7 and 8 with 3.8 x 10^5 and 1.9 x 10^5 COS-7 cells transfected with pcDNA3.1 (negative control).

Chapter 5

Results – Section 3

Gene-Gun Mediated Transfection of COS-7 Cells

The aim of work outlined in this chapter was to optimise transfection of COS-7 cells and measurement of antigen expression by DNA vaccine candidates using the Gene-Gun as the agent of transfection.

Successful intradermal delivery of DNA vaccines has been shown to be possible using DNA coated gold particles fired through the skin using a Gene-Gun. As one of the aims of this project was to compare and contrast the type of immune response generated using a DNA vaccine delivered intramuscularly or via the Gene-Gun, it was first necessary to show that such gold particles could be used to deliver the plasmids successfully. In order to develop and optimise methods to be used in the successful production and firing of DNA coated gold particles, all work was initially carried out *in vitro*.

Most of the preliminary work was carried out using the GFP encoded plasmid to transfect COS-7 cells. This allowed development of protocols that produced cartridges containing a consistent amount of DNA (when cartridges were produced on either the same day or different days), which could additionally be quantified.

5.1. Optimisation of Gene-Gun Cartridge Preparation and DNA Delivery

5.1.1. Impact of PVP Concentration and Ethanol Purity

DNA coated gold particles are fired from the Gene-Gun using helium, which drives the vaccine from the pre-coated "delivery cartridges" that are loaded into the firing barrel of the Gun (see Figure 2.4). These cartridges are prepared by adsorbing known amounts of DNA coated gold particles onto the internal surface of the cartridge tubing, which can then be cut into single-use individual cartridges that are used for vaccination. During this adsorbtion process, PVP is added to the mixture to enhance the successful adherence of the suspension to the tubing. In fact the concentration of PVP added at this stage, appears to be crucial, as if the amount of PVP is too high, the DNA/gold will not detach from the tubing during firing of the Gene-Gun. However, when too low, some DNA will be lost during the coating process. To determine the impact of PVP concentration on the DNA vaccination, several concentrations of PVP (from 0.05 mg/ml to 0) were used to prepare cartridges

coated with pGFP-C3/gold suspension. These cartridges were then used to transfect COS-7 cells and the effect of PVP concentration assessed by measuring the amount of GFP expression. In addition, the amount of unbound DNA, present at each stage of the cartridge manufacture process, was measured.

Data from these experiments are shown in Figure 5.1 and revealed two important findings. First, that DNA could be lost during the ethanol washing stage prior to the addition of PVP (DNA can be detected in 2 of the 3 ethanol washes, used to wash the DNA coated gold particle prior to the addition of PVP) and second, that at the highest concentration of PVP, appeared to enhance binding of the DNA to the cartridge tubing. For example a large amount of DNA is detectable in the ethanol following adsorbtion of the DNA/gold to the cartridge tubing when either no PVP or less than 0.05 mg/ml was used. In contrast, cartridges prepared using 0.05 mg/ml showed no detectable loss of DNA.

These data also correlated with the number of fluorescent cells seen when these cartridges were used to transfect COS-7 cells, as cartridges prepared in the presence of 0.05 mg/ml PVP showed higher numbers of fluorescent cells than those prepared using a lower concentration of PVP (see Tables 5.1. to 5.3).

5.1.2. Impact of Helium Pressure on Gene-Gun Driven Transfection

A number of other parameters included the pressure of hellum used to drive the DNA/gold suspension from the cartridges into the cells also appeared to affetct the efficiency of transfection. To determine this effect, hellum pressures of 50, 100 and 200 psi were used when transfecting dishes of cells cultured in parallel.

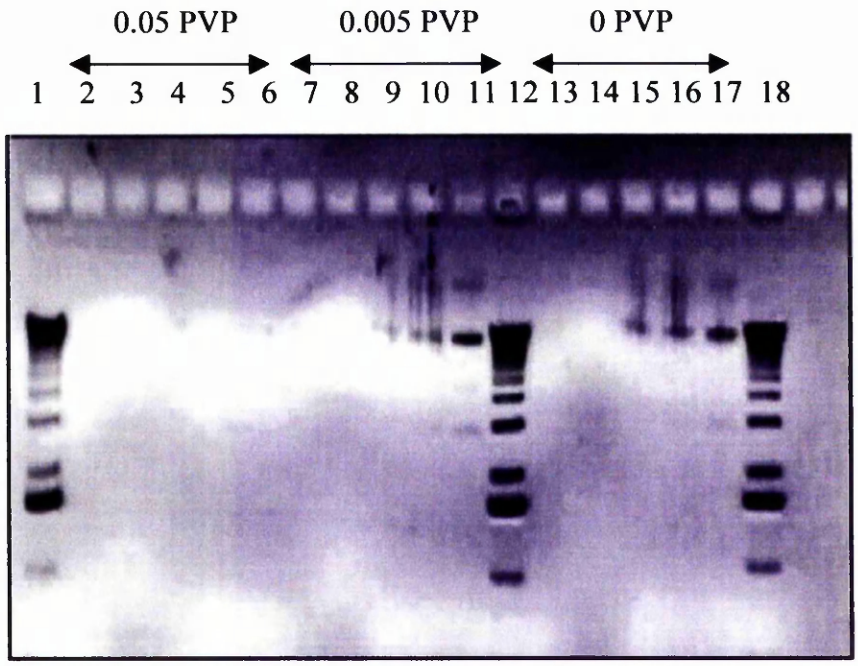

Figure 5.1: The amount of unbound DNA present in solutions used to wash the gold particles during preparation of the firing cartridges.

For each of the cartridge batches (prepared with no PVP, 0.005 or 0.05 mg/ml PVP) unbound DNA was measured at each of the 5 steps leading to the generation of the coated cartridge (Lanes 2-6 for 0.05 PVP; 7-12 for 0.005 PVP, 13-17 for no PVP). These include step 1, supernatant recovered from DNA/gold mixing step (lanes 2, 7, 13), step 2, supernatant recovered from first ethanol wash of gold particles (lanes 3, 8, 14), step 3, supernatant recovered from second ethanol wash of gold particles (lanes 4, 9, 15), step 4, supernatant recovered from third ethanol wash of gold particles of coated DNA (lanes 5, 10, 16) and step 5 ethanol removed from tubing station after coating of cartridge tubing (lanes 6, 11, 17).

This work showed that helium pressures higher than 100 psi produced large areas of disruption in the cell monolayer resulting in a lower number of fluorescent cells (Table 5.1 and 5.2). In contrast, at 50 psi most of the DNA/gold suspension remained on the inner surface of the cartridges and the number of cells fluorescing was low. It was therefore decided that for all subsequent *in vitro* experiments a pressure of 100 psi should be used.

5.1.3. Inclusion of a Screen Diffuser on Gene-Gun Driven Transfection

To try and reduce the level of damage to the cells, a screen diffuser was introduced to the front of the Gene-Gun. The purpose of this screen was to reduce any large aggregated particles that may cause cell damage. In addition, the Gene Gun was held at a distance of approximately 2 cm above the cell monolayer to try and enhance the diffusion of particles over the entire monolayer cell surface.

Using the diffusion screen, the area of fluorescent cells observed after transfection extended from a central concentrated spot to a larger zone, closer to the periphery of the culture dish. Finally, increasing the distance between the Gene-Gun and cells resulted in the highest number of fluorescent cells observed (Table 5.3).

5.1.4. Inter- and Intra-Variability of Transfection Using Different Batches of Coated Cartridges

Using these optimised conditions (0.05 mg/ml PVP, 100 psi, diffusion screen, and distance), three different batches of pGFP-C3 coated cartridges were prepared and used to transfect cells. The aim of this was to determine the level of variation observed within a batch of cartridges and between batches of cartridges.

Table 5.1: Number of fluorescent cells observed following transfection by Gene Gun using a helium pressure of 200 psi. This reflects the number of fluorescent cells in one microscopic field (x10 magnification). The impact of different concentrations of PVP are illustrated by the different number of fluorescent cells observed.

	Batch 1 (0 PVP)	Batch 2 (0.005 PVP)	Batch 3 (0.05 PVP)
Dish 1	3	7	5
Dish 2	2	3	7
Dish 3	5	2	2
Dish 4	3	6	4
Dish 5	3	4	8
Dish 6	4	2	2
Average	**3.33**	**4**	**4.66**
S-Dev	**1.03**	**2.09**	**2.50**

Table 5.2: Number of fluorescent cells observed following transfection by Gene-Gun using a helium pressure of 100 psi. This reflects the number of fluorescent cells in one microscopic field (x10 magnification) in the absence of a diffusion screen. Again, the impact of different concentrations of PVP are illustrated by the different number of fluorescent cells observed.

	0 PVP	0.005 PVP	0.05 PVP
Dish 1	0	8	19
Dish 2	6	5	7
Dish 3	5	6	9
Dish 4	2	11	21
Dish 5	3	6	8
Dish 6	6	2	9
Average	**3.66**	**6.33**	**12.16**
S-Dev	**2.42**	**3.01**	**6.14**

Table 5.3 shows the results of these transfections, which indicate that the variation in transfection efficiency using different cartridges of the same batch was less than that observed between different batches of cartridges.

5.1.5. Expression of GFP in COS-7 Cells Using Optimised Conditions of Delivery

Using conditions described above, the level of GFP expression was found to be about 3 times lower in cells transfected with the Gene-Gun compared to those in which Lipofectamine™ was used (with roughly the same amount of pGFP-C3), with the average of 37 fluorecent cells compared to 115 for the Lipofectamine experiment.

One explanation for the lower level of GFP expression *in vitro* following Gene-Gun transfection, is that the helium pressure may affect the viability of a number of cells. This explanation is supported by the differences in morphology of COS-7 observed following transfection by Gene-Gun (Figure 5.2) which was dramatically different in comparison to cells transfected by Lipofectamine (Figure 4.1). In dishes transfected with the Gene-Gun, most fluorescent cells were detached from the bottom of the dish, and were round and intensely green. In comparison, following Lipofectamine-mediated transfection the monolayer remained attached to the bottom wall of the dish, with adherent cells appearing pale-green.

An alternative suggestion for the lower level of GFP expression *in vitro* following Gene-Gun transfection, is that the helium pressure (100 psi) was not sufficient to efficiently drive the coated gold/DNA particles from the cartridges. However, increasing the pressure was not considered feasible as it produced, as mentioned, an even larger area of disruption to the monolayer even when a diffusion screen and distance from the monolayer was used.

Table 5.3: Number of fluorescent cells observed following transfection by Gene-Gun using cartridges loaded with 0.05 mg/ml PVP, a helium pressure of 100 psi and a diffusion screen. This table reflects the number of fluorescent cells observed in one microscopic field (x10 magnification). The difference between the efficiency of transfection (number of fluorescent cells) between cartridges prepared on the same day and those prepared as different batches on separate days does not appear to be significant.

	Batch 1	Batch 2	Batch 3
Dish 1	28	32	39
Dish 2	25	28	37
Dish 3	26	50	20
Dish 4	31	37	42
Dish 5	26	46	27
Dish 6	28	29	34
Average	**27.33**	**37**	**33.16**
S-Dev	**2.16**	**9.16**	**8.23**

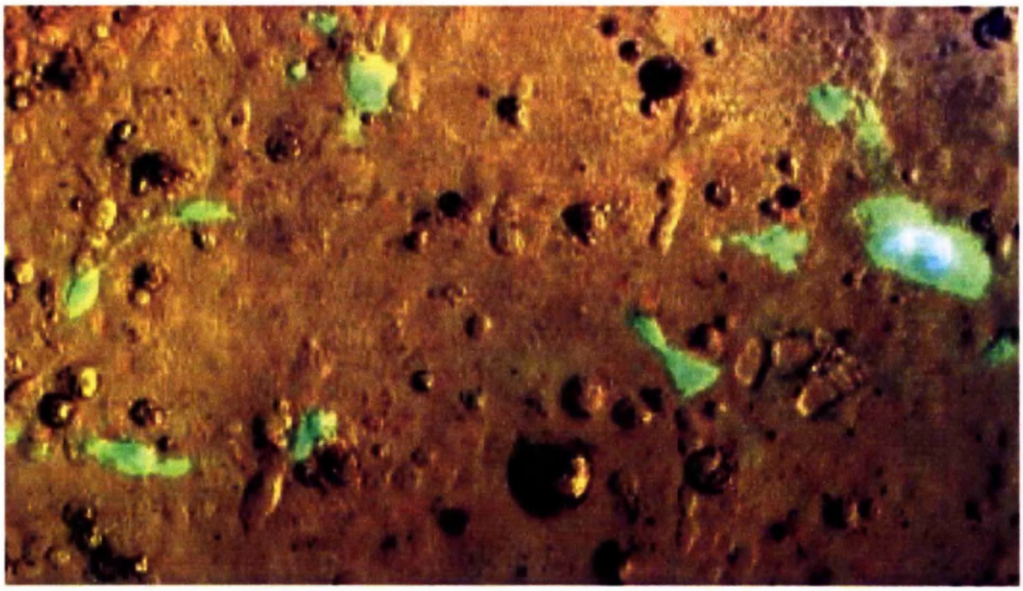

Figure 5.2: *In vitro* expression of GFP by Gene-Gun

Expression of GFP was visualised using an inverted microscope (Axiovert - Zeiss) connected to an Imaging System equipped with a designated software for picture processing (OpenLab-Improvision). The picture represents one microscopic field (x10 magnification).

5.1.6. Quantification of Amount of DNA Present in Each Cartridge

At the beginning of these experiments, one concern was the uneven coating of the gold/DNA particles in the cartridge tubing leading to differing amounts of DNA being delivered. In an attempt to overcome this problem, a number of methods of extraction and quantification were attempted, however, the most successful was that suggested by Watkins (personal communication, see Section 2.6.3). Using this method, the coated DNA was extracted from each cartridge and the DNA quantified by reading the absorbency of the resultant solution at 260 nm. Using this method, several cartridges taken at various intervals along the length of the tubing were tested. The results of this study showed that only very small variations in the amount of DNA were observed and gave confidence that all cartridges were capable of equal delivery of DNA.

Using the methods described above, gold coated cartridges were prepared for both pcDNA3/tetC (Table 5.4 A), or the control plasmid pcDNA3.1 (Table 5.4 B). These were used initially to transfect cells *in vitro* and as described later for immunising mice *in vivo*.

Due to time-limitations and the difficulty of interpretation of data generated using the pCMV-70 plasmid, this construct was not used in any experiments in which the Gene-Gun was used.

A

pcDNA3.1/tet C cartridges	OD 260	OD 280	DNA recovered / cartridge
1	0.213	0.147	1.065μg
2	0.247	0.186	1.230μg
3	0.225	0.123	1.120μg
4	0.215	0.105	1.068μg
5	0.244	0.114	1.220μg

B

pcDNA3.1 cartridges	OD 260	OD 280	DNA recovered/cartridge
1	0.237	0.200	1.185μg
2	0.244	0.189	1.22μg
3	0.215	0.143	1.075μg
4	0.206	0.209	1.03μg
5	0.214	0.206	1.07μg

Table 5.4: Analysis of the amount of DNA present in each cartridge

In table A cartidges were coated with approximate 2 μg of pcDNA3/tetC, whilst table B shows recovery from cartridges coated with an equal amount of pcDNA 3.1. About 1-1.22 μg of DNA was recovered from cartridges belonging to the same batch, suggesting that approximately half the DNA used had bound to the gold particles.

5.2. Expression of Fragment C of Tetanus Toxin in COS-7 Cells Transfected with pcDNA3/tetC Using the Gene-Gun

5.2.1. Optimisation of the Detection System

Using transfection conditions described above for the Gene-Gun, cells were transfected using pcDNA3/tetC and expression of the protein measured using the conditions previously described for the chemiluminiscent substrate SuperSignal West Pico (Section 2.3.4.2.4). However, during the course of these optimisation experiments, the goat anti-rabbit HRP conjugate antibody was finished and resulted in a new batch of this antibody being purchased. Unfortunately, if this antibody was used at the same concentration as those calculated previously, the blots produced were very dirty, with high background staining. Therefore the optimisation procedure was repeated and appropriate optimised dilutions of antibody calculated to produce very specific responses (1:10,000 concentration of the primary antibody, 1:250,000 concentration of the secondary antibody).

5.2.2. Transfection and Expression of Fragment C in COS-7 Cells

COS-7 cells were transfected using the Gene-Gun using the conditions as described above. Expression of antigen in these cells was then established by preparation of Western blots using whole COS-7 cell lysates. These results show that cells transfected with the Gene Gun produced a protein of approximate 50 kDa, which reacted specifically with the anti-Fragment C antibody (Figure 5.3). This protein was identified as Fragment C as it was exactly the same size as the recombinant Fragment C run on the same gel and a protein produced by COS-7 cells transfected with pcDNA3/tetC using the Lipofectamine method. In comparison, similar concentrations of COS-7 cells transfected with the control plasmid, pcDNA3.1, failed to produce any detectable band, suggesting that the presence of the

protein was dependent on expression from the pcDNA3/tetC construct. The level of expression of antigen by these cells was estimated to be around 10 ng in $2.8x10^4$ cells, based on the amount of recombinant protein loaded onto the gel, in comparison to about 70 ng/ $2.8x10^4$ cells transfected with Lipofectamine (Section 4.3.2.). Based on the success of these experiments we decided to test the immune response to Gene Gun vaccination of the pcDNA3/tetC construct in mice.

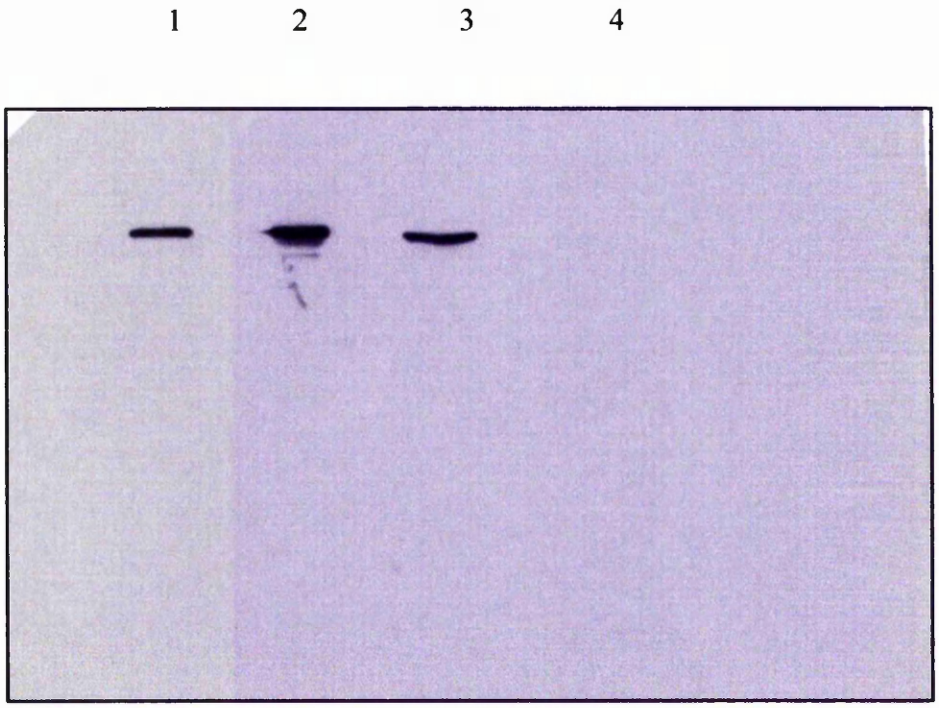

Figure 5.3: Expression of Fragment C in COS-7 cells transfected using the Gene-Gun with pcDNA3/tetC.

This figure shows the detection of Fragment C using a Fragment C specific polyclonal sera. The gel was loaded as follows: lane 1, 10 ng Fragment C (positive control); Lane 2, $4.6x10^4$ COS-7 cells transfected with pcDNA3/tetC with Lipofectamine; Lane 3, $2.8x10^4$ COS-7 cells transfected with pcDNA3/tetC by Gene-Gun; Lane 4, $2.8x10^4$ COS-7 cells transfected with pcDNA3.1.

Chapter 6

Results – Section 4

Vaccination with pcDNA3/tetC

The aim of the work outlined in this chapter was to determine whether the vaccine candidate pcDNA3/tetC could induce immune responses in BALB/c mice when delivered either parenterally or through the skin by Gene-Gun.

6.1. Fragment C Specific Responses Following Immunisation with pcDNA3/tetC

It has already been reported that mice vaccinated with pcDNA3/tetC activate strong humoral and cell mediated immune responses, which are protective against challenge with active tetanus toxin (Anderson *et al.*, 1996, Saikh *et al.*, 1998). However, these observations have only been made following parenteral immunisation of the muscle. To date, no data on the immunogenicity of Fragment C-encoding DNA vaccine administered by the Gene-Gun have been generated.

One of the aims of the study was to compare and contrast the immune responses generated when the two different routes of immunisation were used.

6.1.1. Antibody Levels Following Intramuscular Immunisation with pcDNA3/tetC

To determine the magnitude and specificity of the immune response generated by the DNA construct that expresses Fragment C, mice were initially immunised intramuscularly on day 1 and day 8, with either pc/DNA3/tetC, or the control plasmid (pcDNA3.1) (for immunisation schedule see Section 2.4.4, Table 2.5). Sixteen days after the second i. m. immunisation (day 24), sample bleeds were taken from all mice and tested by ELISA for the presence of Fragment C specific antibodies. As shown in Figure 6.1 and in agreement with previous experiments (Anderson *et al.*, 1996, Saikh *et al.*, 1998, Stratford *et al.*, 2000), all ten mice immunised with pcDNA3/tetC showed detectable levels of Fragment C specific antibodies (average titre of about $1x10^3$). In addition, this response was found to increase with time, as sample bleeds taken from the same animals twelve days later (day 36) showed an average 3 fold increase in titre compared to those measured on day 24 (Figure 6.1. B). In contrast, mice immunised with pcDNA3.1 only showed no detectable Fragment C specific antibody in the sera at either time point.

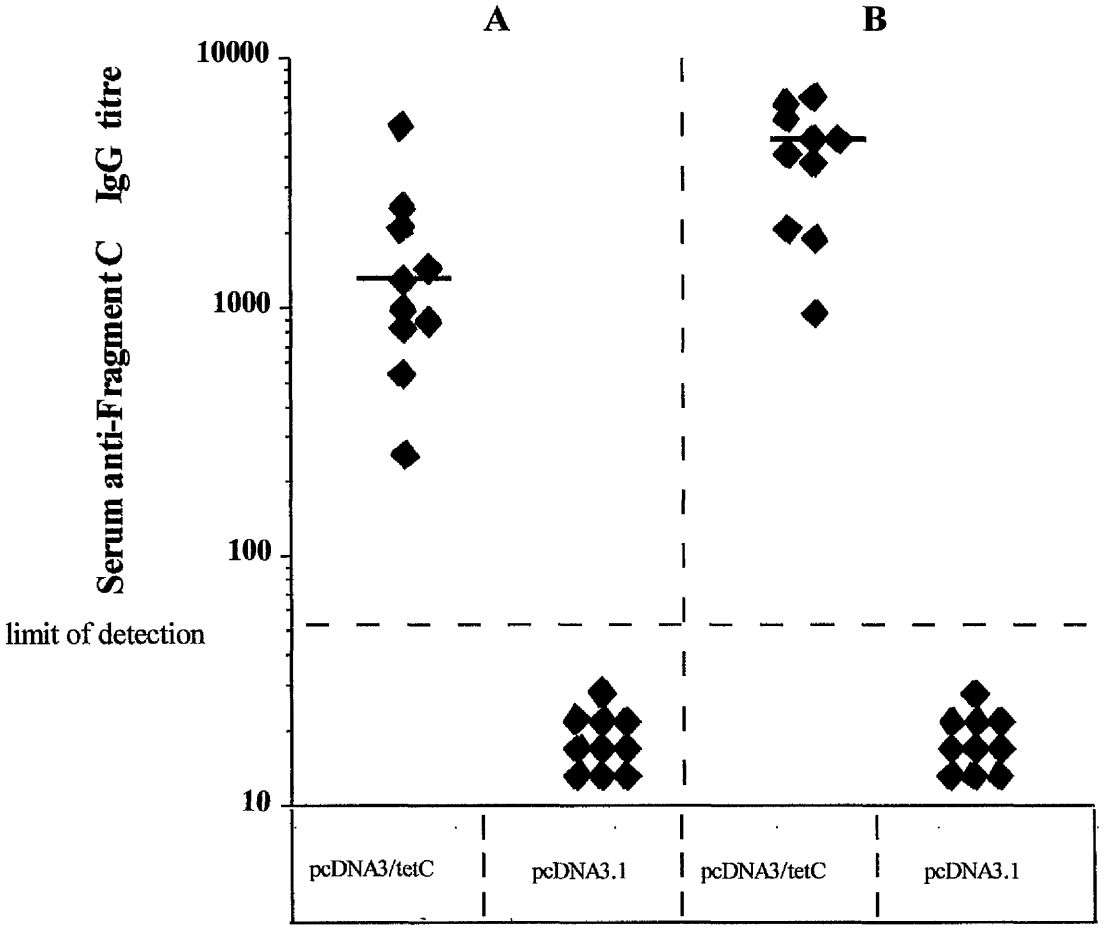

Figure 6.1: Titres of Fragment C specific IgG in the serum following two intramuscular doses of pcDNA3/tetC

Fragment C specific IgG titres were determined in sera of 10 individual mice on days 24 (A) and 36 (B) following intramuscular immunisation with two doses of 100 μg pcDNA3/tetC or pcDNA3.1. Mice were originally immunised on day 1 and 8 of the experiment, respectively. Each point (♦) represents the titres of Fragment C specific antibodies from individual sera with each of the horizontal lines representing the median values for each group.

6.1.2. Antibody Levels Following Gene-Gun Administration of pcDNA3/tetC

To determine the magnitude and specificity of the immune response, mice were immunised by the Gene-Gun as described in Section 2.6.4.3 (Table 2.8) on day 1 and day 8, with either pcDNA3/tetC, or the control plasmid (pcDNA3.1). Fourteen days following the second Gene-Gun immunisation (day 22), sample bleeds were taken from all mice and tested by ELISA for the presence of Fragment C specific antibodies. As shown in Figure 6.2 all five mice immunised with pcDNA3/tetC showed titres of Fragment C specific antibodies, which were comparable with those obtained following i. m. immunisation from the previous experiment. However, these titres were higher than those obtained in the three mice included as controls within this experiment.

In both experiments, mice immunised by either route showed an increase in the production of antibodies with time, as sample bleeds taken from the same animals twelve days later (day 34) had titres increased by about 2 fold for mice in the group immunised by the Gene-Gun, and about a 4 fold increase in the group immunised i. m.. In contrast, mice immunised with the empty vector pcDNA3.1 showed no detectable Fragment C specific antibody in the sera at any time point.

6.1.3. Impact of Route of Immunisation on IgG Isotype

Although the titre of response did not vary significantly between the two groups, the quality of the immune response was found to differ. This was highlighted by the differences in the antigen specific IgG isotypes measured. In agreement with most work found in the literature, mice immunised with pcDNA3/tetC intramuscularly produced a dominant IgG2a response in the sera, although antigen specific IgG1 was also detected. In contrast, mice immunised using the Gene-Gun produced a response that was completely dominated by antibody of the IgG1 subclass (Figure 6.3).

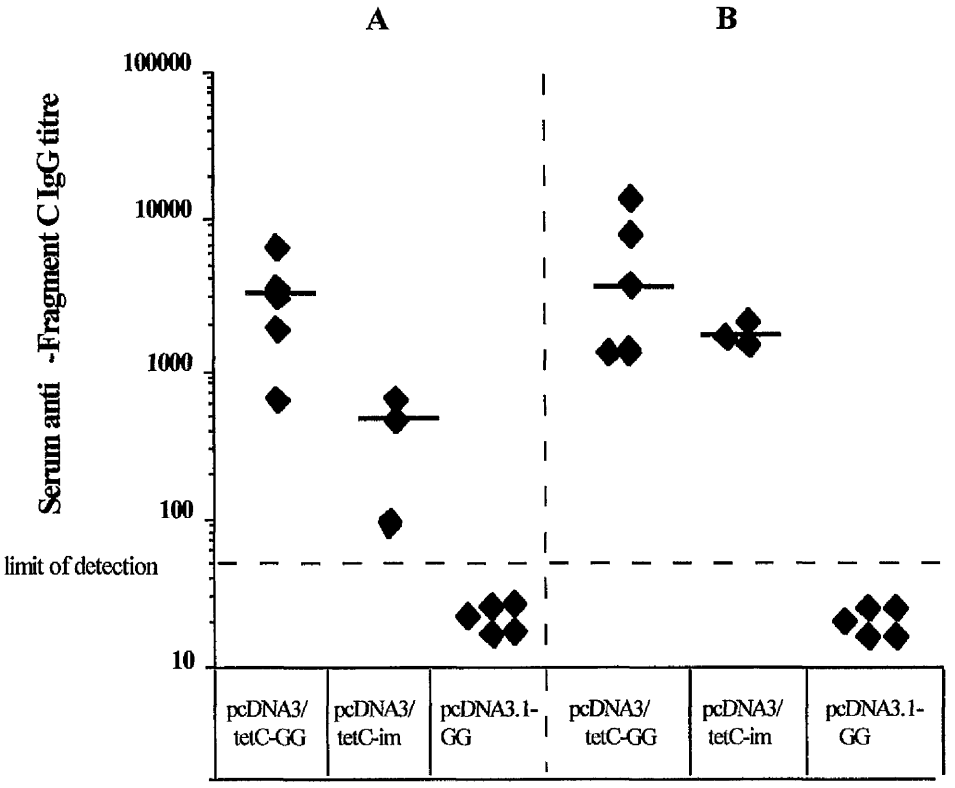

Figure 6.2: IgG response against Fragment C following Gene-Gun immunisation with two doses of pcDNA3/tetC

Titres of Fragment C specific IgG in sera of mice on days 22 (A) and 34 (B) following Gene-Gun (GG) immunisation with two doses of 2 μg pcDNA3/tetC or pcDNA3.1. In addition, as a control, three mice were immunised i. m. with two doses of 100 μg pcDNA3/tetC. Each rhombus represents individual sera and each horizontal line, median values for each group.

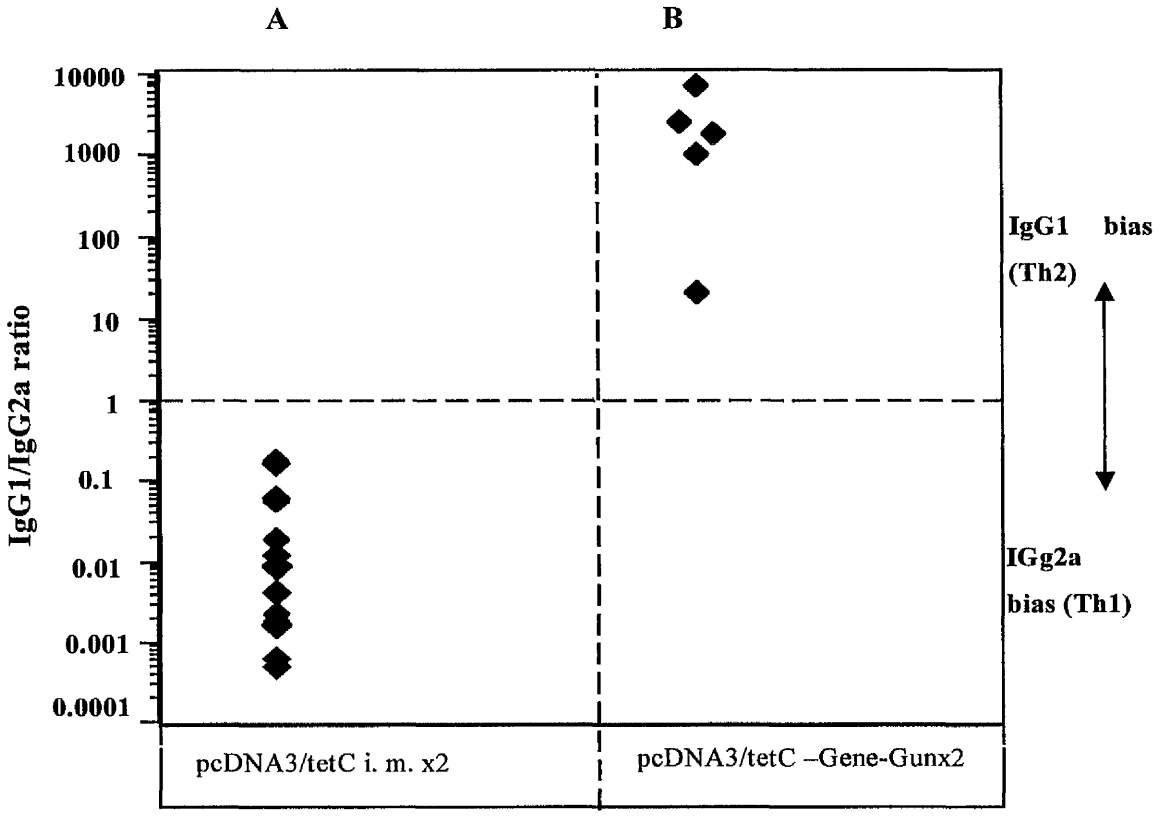

Figure 6.3: Ratio of IgG1/IgG2a Fragment C specific antibody in the sera following intramuscular or Gene-Gun immunisation with pcDNA3/tetC

Ratio of Fragment C specific IgG1/IgG2a in the sera of mice primed with two doses of pcDNA3/tetC delivered either i. m. (A) or by Gene-Gun (B). Figure shows the response measured 24 days after the second dose of DNA. Each point (♦) represents the titres of Fragment C specific antibodies from individual sera with each of the horizontal lines representing the median values for each group.

6.1.4. Impact of Boosting Animals Previously Vaccinated Intramuscularly with DNA

Although DNA vaccination generated measurable antibody in the blood using either route of immunisation, previous experience suggested that DNA vaccination with this construct resulted in the generation of a very low activation of antigen specific T-cells. However, amplification of DNA primed cells has been shown to be possible by boosting the animals with a low amount of the homologous purified antigen (Stratford *et al.,* 2001).

Using this approach, the 2 groups of 10 mice immunised intramuscularly with pcDNA3/tetC or pcDNA3.1 respectively, were split into 4 groups of 5. These mice were then subsequently immunised on day 37 with either a 3rd intramuscular immunisation with DNA or a 1st intramuscular immunisation with purified Fragment C. Table 2.5 in the Materials and Methods Chapter (Section 2.4.4) describes the specific prime/boost regimen. Fourteen days following this boost immunisation (day 50), mice were sacrificed, the blood taken for analysis of the Fragment C specific antibody and the T-cells analysed for antigen specific proliferative responses and cytokine release.

6.1.4.1. Impact of Prime/Boost Regimen on Specific Antibody Levels

Analysis of the sera established that mice given a third i. m. dose of pcDNA3/tetC had titres of Fragment C specific IgG which were about 7 times higher than those detected prior to the boost, as it can be seen in Figure 6.4. This increase maybe due to combination of factors including both the impact of a third dose of the pcDNA3/tetC construct and the fact that titres may have increased naturally with time following the 2nd immunisation.

In contrast, mice that had originally been primed with pcDNA3/tetC and then boosted with purified Fragment C showed a approximate 20 fold increase in Fragment C specific antibody in the sera, as illustrated in Figure 6.4. This increase was 3 times higher than that detected in similarly primed mice that had been boosted with pcDNA3/tetC.

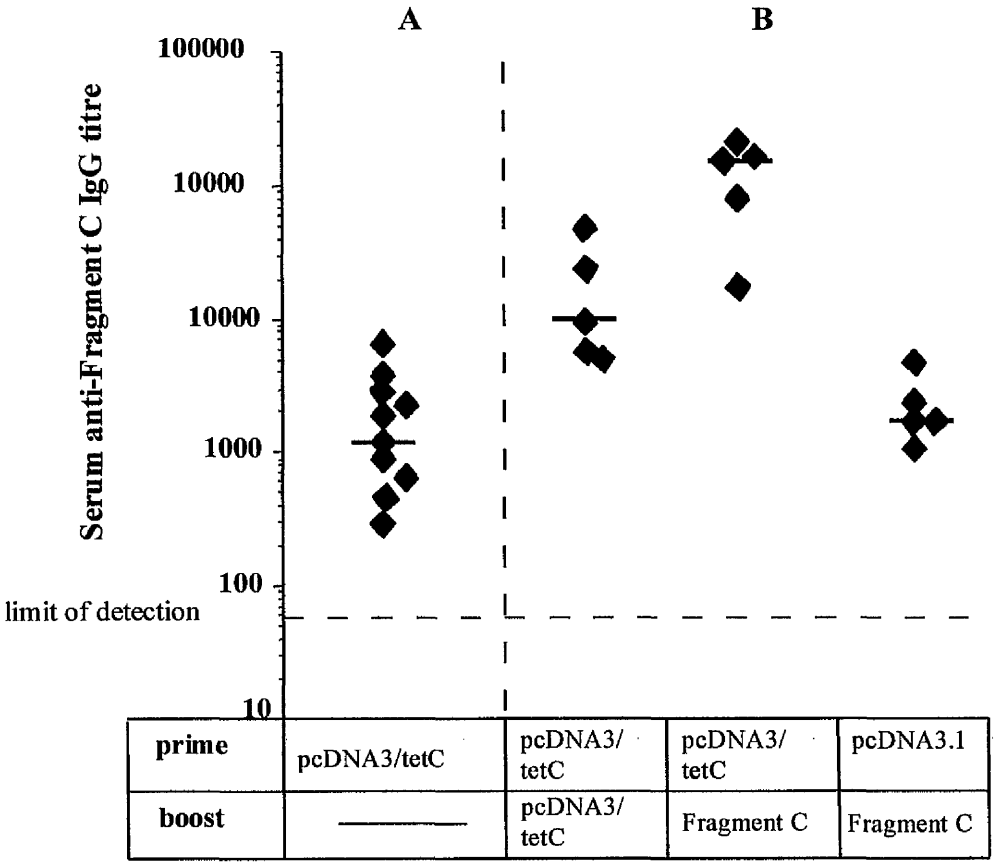

Figure 6.4: Titres of Fragment C specific IgG in the serum before and after boosting primed mice with purified Fragment C

Fragment C specific IgG titres were determined in the sera of individual mice primed intramuscularly with two doses of either pcDNA3.1 or pcDNA3/tetC and then boosted with either pcDNA3/tetC or with Fragment C. Figure A shows anti-Fragment C titres measured before boosting on day 36 and Figure B after boosting on day 50. Each point (♦) represents the titres of Fragment C specific antibodies from individual sera with each of the horizontal lines representing the median value for each group.

In contrast, mice which had been immunised with either 2 doses of pcDNA3.1 and 1 dose of purified Fragment C showed titres of specific antibody that were at least one and a half logs lower than those detected in mice primed with pcDNA3/tetC. This result confirms the impact of priming with pcDNA3/tetC on the magnitude of the antibody response generated.

6.1.4.2. Impact of Prime/Boost Regimen on IgG Isotype

The impact of priming animals with the DNA vector pcDNA3/tetC was most clearly shown in the antigen specific isotypes of IgG detected measured in the sera of vaccinated mice. These were studied both before (day 36) and after (day 50) the booster immunisations that were carried out on day 37. The isotypes of IgG present after vaccination with pcDNA3/tetC are shown in Figure 6.5. In these mice, although all isotypes were detected, the predominant antibody was IgG2a. This pattern of humoral immune response was not drastically altered by the third immunisation with another dose of DNA or a boosting protein dose. In fact, the antigen specific IgG2a remained dominant and titres were enhanced in mice boosted on day 37 with either pcDNA3/tetC or purified Fragment C. In contrast, mice primed with the pcDNA3.1 vector alone and boosted with purified Fragment C showed a predominant IgG1 subtype of specific antibody. These results suggest that in contrast to i. m. immunisation of purified protein, which induces a response that is strongly biased toward a Th2 type of response, pre-immunisation of the same antigen as a DNA construct result in the priming of Th1 specific CD4 helper cells, Figure 6.6 illustrating the IgG1/IgG2a ratio. In addition, boosting with purified protein appeared to enhance the size of the antibody response to the antigen.

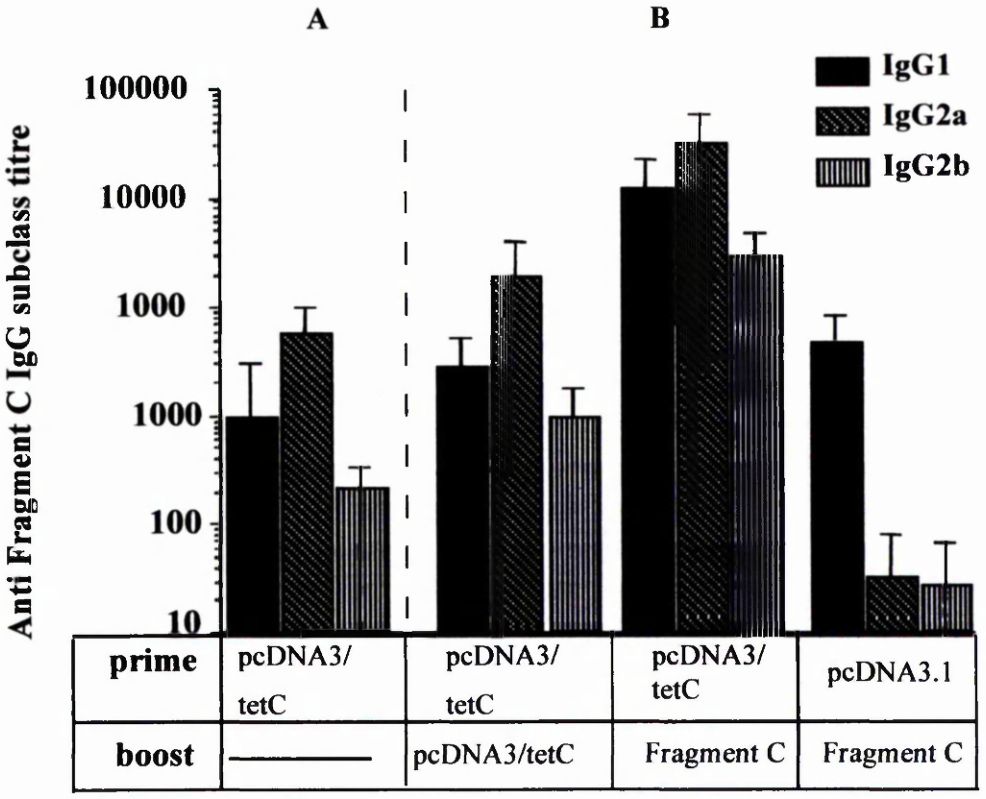

Figure 6.5: Titres of Fragment C specific subclasses of IgG in mice primed with pcDNA3/tetC and boosted with purified Fragment C

Fragment C specific IgG1, IgG2a, IgG2 b titres were determined in the sera of individual mice primed intramuscularly with two doses of either pcDNA3.1 or pcDNA3/tetC and then boosted with either pcDNA3/tetC or with Fragment C. Figure A shows the titres measured before boosting on day 36 and Figure B after boosting on day 50. The results shown represent the mean calculated titres for 5 mice per group and the error bars the standard deviation of the mean titre values.

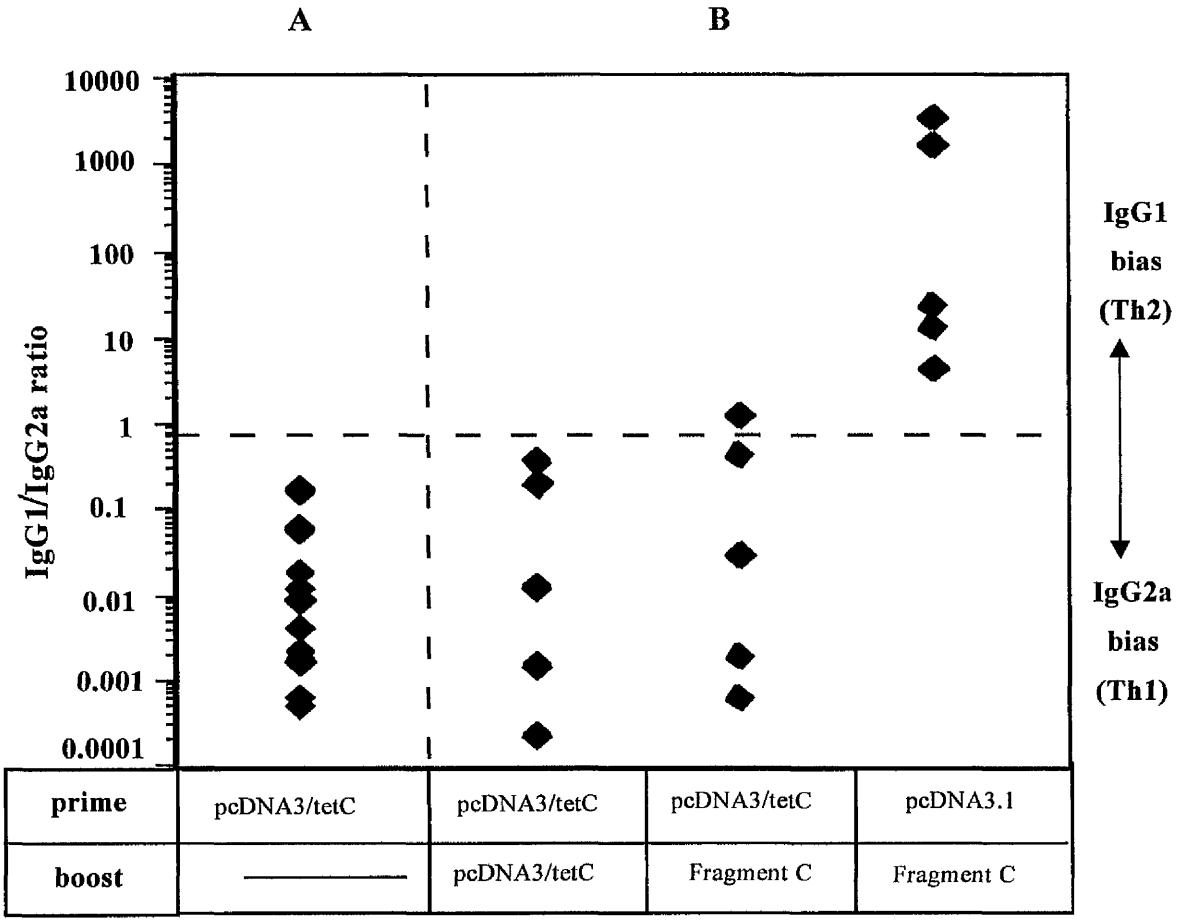

Figure 6.6: Ratio of IgG1/IgG2a Fragment C specific antibody in the sera of mice primed i. m. with pcDNA3/tetC and boosted with Fragment C

Ratio of Fragment C specific IgG1/IgG2a in the sera of mice primed with two doses of either pcDNA3.1 or pcDNA3/tetC and then boosted with Fragment C. Figure A shows the response before boosting (day 36) and Figure B the effect following boosting with Fragment C (day 50). Each point (♦) represents the ratio of IgG1/IgG2a Fragment C specific antibodies from individual sera.

6.1.5. Impact of Boosting Animals Previously Vaccinated with pcDNA3/tetC by Gene-Gun

Following the initial analysis of the antibody response to Gene-Gun administration of pcDNA3/tetC (Section 6.1.2), it was decided to boost these animals as previously with 10 μg i. m. of purified Fragment C. Table 2.8 in the Materials and Methods (Section 2.6.4.3) describes the specific prime/boost regimens employed. Twelve days following this boost immunisation (day 46), mice were sacrificed and the level of anti-Fragment C response measured by ELISA.

6.1.5.1. Impact of Prime/Boost Regimen on Specific Antibody Levels

The titre of specific antibody measured following the boost in mice initially primed with Gene-Gun delivered pcDNA3/tetC was about 6 times higher than those detected prior to the boost immunisation (Figure 6.7). Within the context of this experiment, an equivalent increase in the specific antibody response was also observed in mice boosted with protein that had initially been primed via the intramuscular route with pcDNA3/tetC. This result is in contrast to that observed in the initial experiment in which the increase of specific antibody was much higher (approximately 20 fold). These differences may reflect variation in the efficiency of immunisation between the two experiments and also the statistical effect of using a lower number of animals in the second experiment compared to the first. In particular, the result is skewed by the fact that one of the three mice used failed to respond to the initial immunisation, affecting the average titre within this group.

Immunisation by the Gene-Gun itself, did not appear to influence the level of antibody generated as mice immunised with the control plasmid (pcDNA3.1) and then boosted with Fragment C, had equivalent titres of Fragment C specific IgG as those obtained in naïve mice immunised with Fragment C alone.

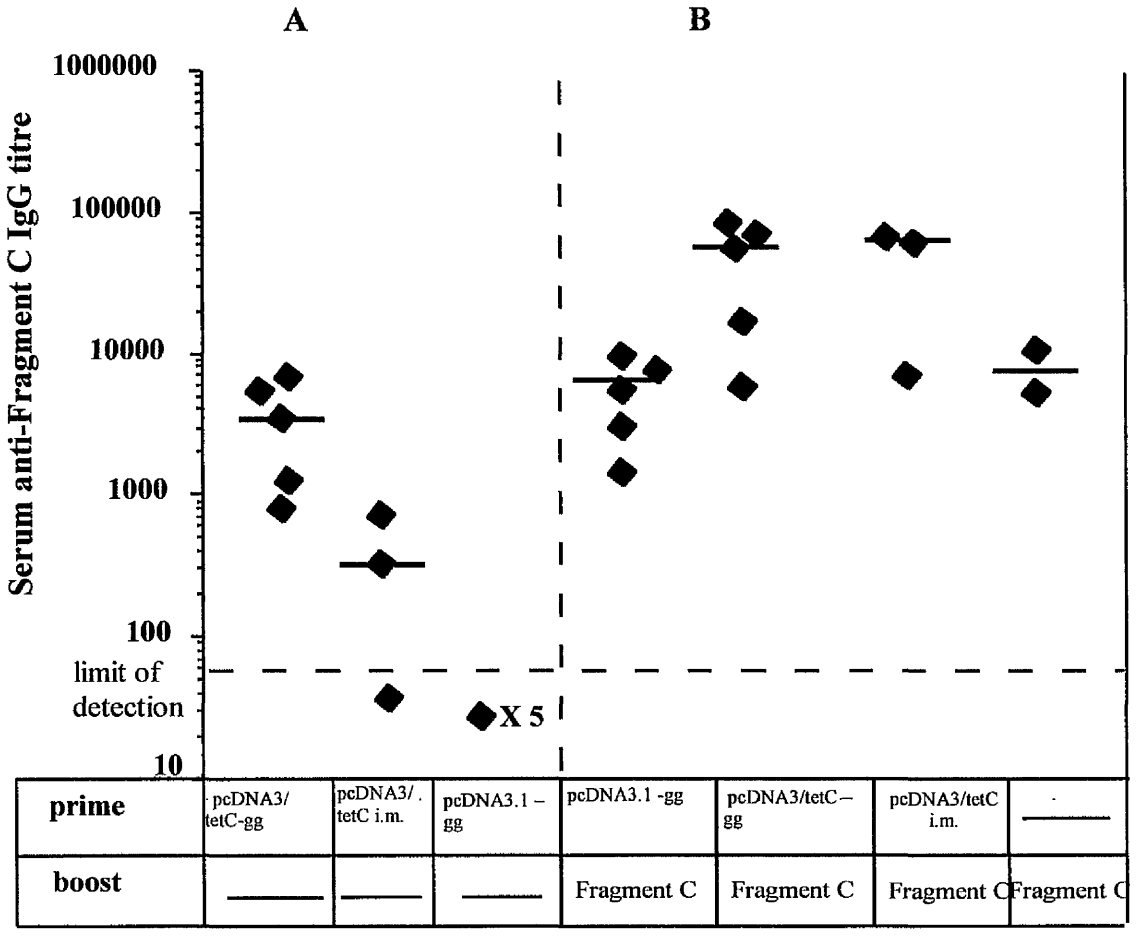

Figure 6.7: Titres of Fragment C specific IgG in the serum before and after boosting Gene-Gun primed mice with purified Fragment C.

Fragment C specific IgG titres were determined in the sera of individual mice primed with two doses of either pcDNA3.1 or pcDNA3/tetC delivered using the Gene-Gun and then boosted with Fragment C. Figure A shows anti-Fragment C titres measured before boosting on day 34 and Figure B after boosting on day 46. Each point (♦) represents the titres of Fragment C specific antibodies from individual sera with each of the horizontal lines representing the median values for each group.

6.1.5.2. Impact of Prime/Boost Regimen on IgG Isotype

One of the clearest differences between the two routes of immunisation was however clearly revealed by the antigen specific isotypes detected in the sera of vaccinated mice. These were studied both before (day 34) and after (day 46) the boost immunisations. The isotypes of specific IgG detected following Gene-Gun vaccination are shown in Figure 6.8.

In contrast with the pattern of IgG subtypes elicited in mice following i. m. immunisation, Gene-Gun mediated vaccination with pcDNA3/tetC was characterised by a predominant Th2 type of immune response, with a high titre of IgG1 and IgG2b both before and after boost with Fragment C. As in previous experiment, Fragment C boost did increase apparently significantly the level of specific antibody, but followed the same established Th2 orientation primed by the Gene-Gun immunisation, with the level of antigen specific IgG1 remaining dominant. Mice primed with the pcDNA3.1 vector alone and boosted with purified Fragment C and those immunised with Fragment C alone also showed a predominant IgG1 subtype of specific antibody. In contrast, the three mice immunised twice i. m. with pcDNA3/tetC and boosted with Fragment C had a mixed Th1/Th2 response, with predominant IgG2a titres, both before and after boost, which is consistent with the previous experiment.

Figure 6.9 shows the IgG1/IgG2a ratio for individuals within each group of mice, illustrating more clearly the orientation of the immune response (towards Th1 or Th2) activated by each immunisation protocol. These results suggest that, in contrast to i. m. immunisation with pcDNA3/tetC, the Gene-Gun induces a response that is strongly biased toward Th2. In agreement with the previous experiment, the immune response that is primed by i. m. or Gene-Gun is only enhanced following the boost with the purified protein.

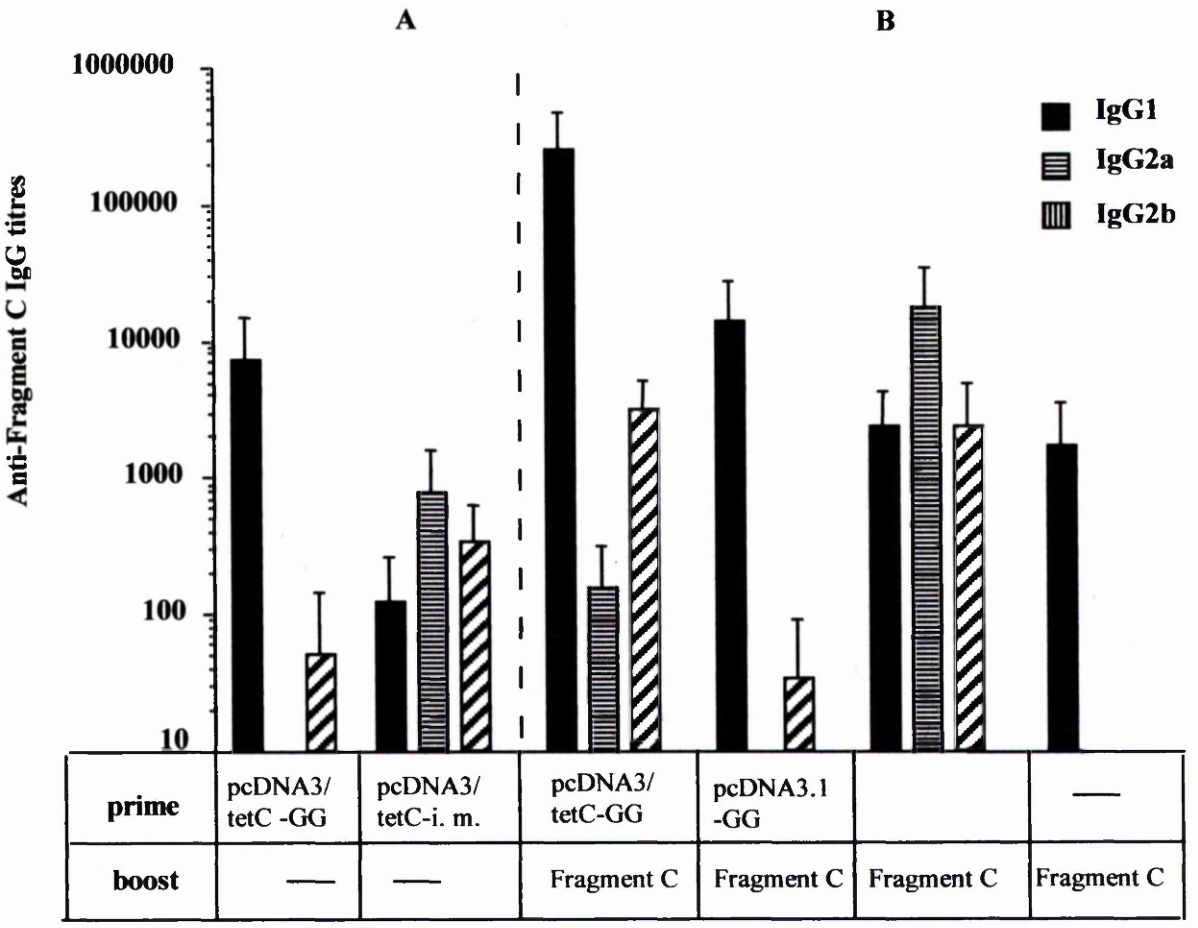

Figure 6.8: Titres of Fragment C specific subclasses of IgG in mice primed with pcDNA3/tetC using the Gene-Gun and boosted with purified Fragment C

Fragment C specific IgG1, IgG2a, IgG2b titres were determined in the sera of individual mice primed with two doses of either pcDNA3.1 or pcDNA3/tetC by Gene Gun and then boosted with either pcDNA3/tetC or with Fragment C. Figure A shows the titres measured before boosting on day 34 and Figure B, after boosting on day 46. The results shown represent the mean calculated titres for 5 mice per group and the error bars, the standard deviation of the mean titre values.

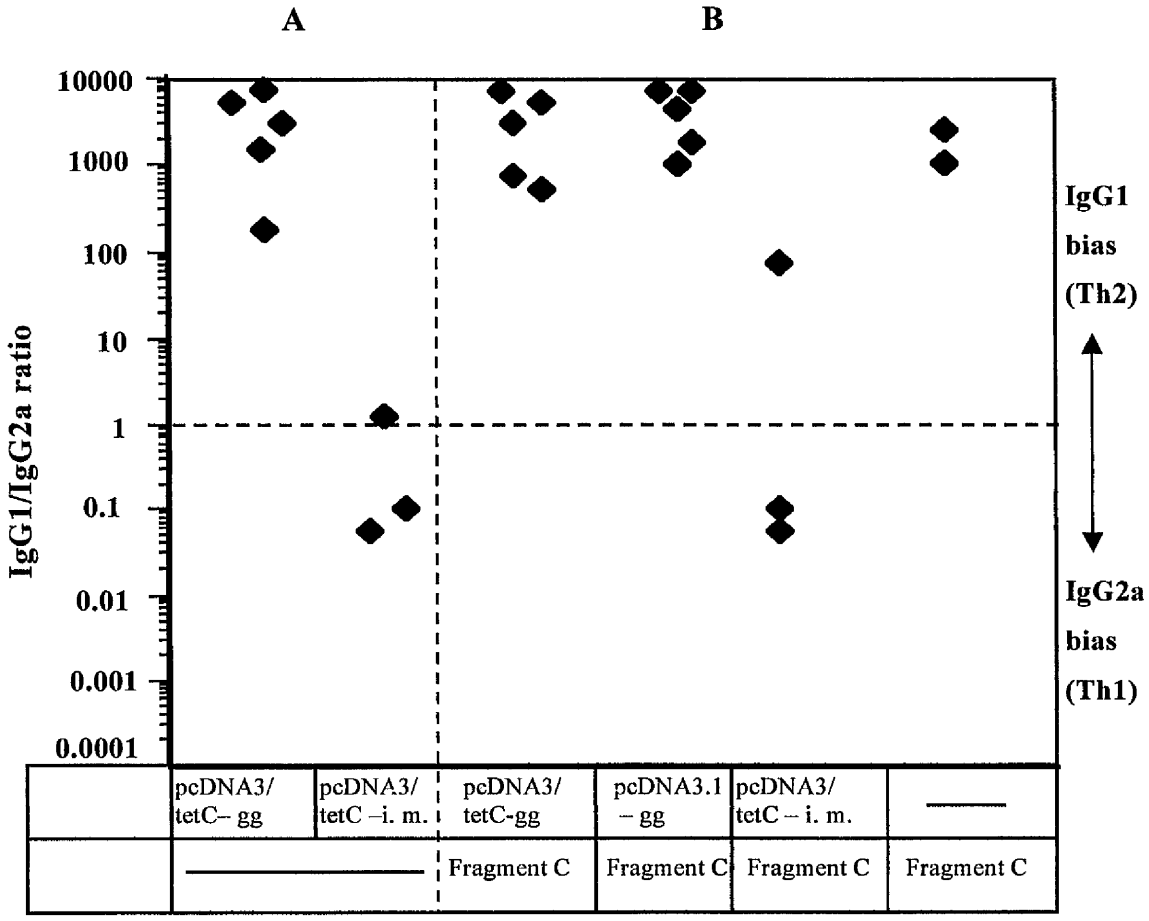

Figure 6.9: Ratio of IgG1/IgG2a Fragment C specific antibody in the sera of mice primed with pcDNA3/tetC and boosted with Fragment C

This figure shows ratio of Fragment C specific IgG1/IgG2a in the sera of mice primed with two doses of either pcDNA3.1 or pcDNA3/tetC and then boosted with Fragment C. Figure shows the response before (A, day 34) and after boosting with Fragment C (B, day 46) in mice primed by Gene-Gun or i. m. with pcDNA3/tetC. Each point (♦) represents the ratio of IgG1/IgG2a Fragment C specific antibodies from individual sera.

6.2. T Cell Proliferation Assay

6.2.1. T Cell Proliferation in Mice Immunised Intramuscularly with pcDNA3/tetC

Unfortunately, although T-cell assays were performed in an attempt to confirm the activation of specific $CD4^+$ T-cell populations for the first experiment, due to technical difficulties the data proved impossible to interpret. This is because the cells in all wells (stimulated and unstimulated) proliferated non-specifically. This was probably due to the presence of some mitogen in the media used during the course of the assay. It was therefore decided to repeat the experiment. However, as time was limited, it was decided that, to ensure strong proliferation of T-cells, an adjuvant should be included with the Fragment C boost phase of the experiment. The adjuvant chosen for these experiments was the heat labile toxin from *E. coli* (LT), which had been used previously in such prime/boost regimens. This toxin is able to stimulate the activation of both $CD4^+$ T Th1 and Th2 helper cells and it was felt would not skew the primed response toward the amplification of one particular cell population as is the case with many traditional adjuvants (example aluminium hydroxide activates specifically Th2 responses).

In the second experiment, four groups of mice were vaccinated in order to determine the proliferative response to Fragment C *in vitro*. Initially, all mice were primed twice with either pcDNA3/tetC or pcDNA3.1 and then one group from each boosted with Fragment C and LT. Ten days later the mice were sacrificed and the spleens and local lymph nodes around the site of intramuscular immunisation removed. Single cell preparations from both tissues were then stimulated *in vitro*. Seventy-two hours after stimulation, supernatants above the cells were removed for cytokine analysis and then the proliferative index determined by addition of tritiated thymidine, as described in Section 2.5.2.1.

As seen in Figure 6.10, cells from both the spleens and lymph nodes of mice primed with pcDNA3/tetC and boosted with Fragment C and LT proliferated strongly on restimulation. In contrast, mice primed with the control plasmid (pcDNA3.1) and boosted with Fragment C and LT showed no lymph node cell proliferation and only a very low level of thymidine incorporation in spleen cells. In agreement with previous observations, no proliferation was observed in cells taken from mice injected twice with pcDNA3/tetC (Stratford – personal communication). Similarly, cells from mice immunised with pcDNA3.1 failed to proliferate following restimulation with Fragment C. However, all cells from all groups of mice did respond to the mitogen, Concanavalin A (Con A), suggesting that this lack of response was not due to anergy within the cell population. (see Figure 6.10).

In conclusion, it would appear that boosting of animals with the Fragment C in the presence of the adjuvant LT, enhanced the proliferative response of T-cells apparently primed by the initial immunisation with pcDNA3/tetC. The boosting dose itself does not appear responsible for the large proliferative response observed in this group as mice immunised with pcDNA3.1 and boosted with Fragment C and LT failed to mount such a large response. However, vaccination with pcDNA3/tetC alone, did not appear sufficient to activate these responses. One explanation for this lack of response is time taken between DNA priming and completion of these experiments, which were chosen to maximise the effect of the boost immunisation rather than the DNA vaccination. With more careful study it may be possible to optimise the timing to maximise measurement of the response induced to DNA alone.

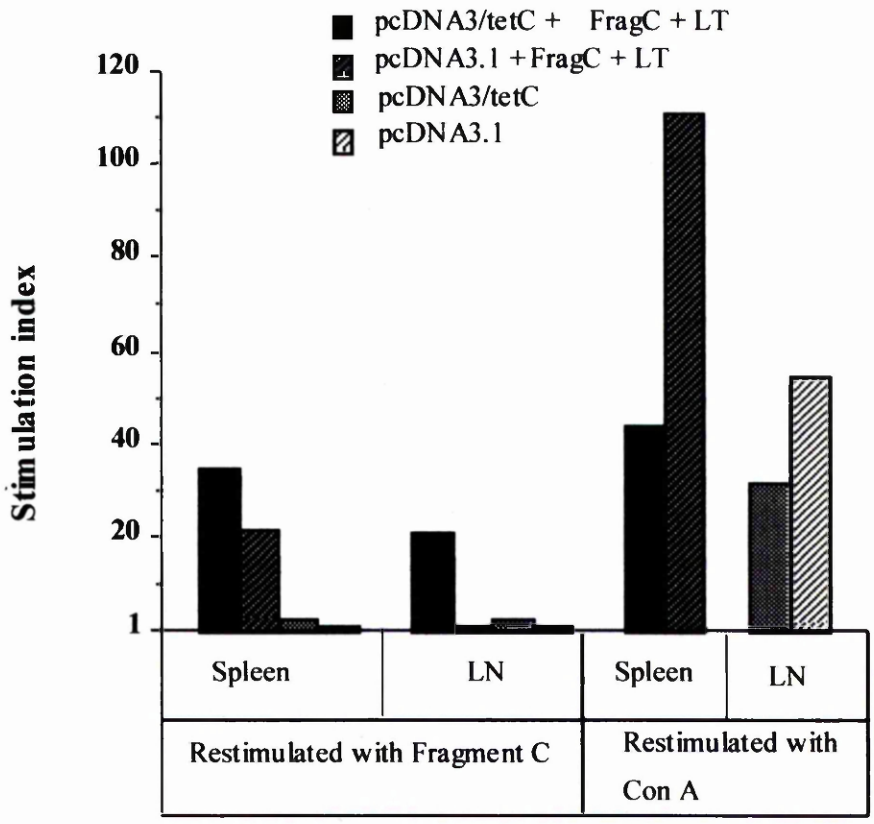

Figure 6.10: Stimulation of spleen cell and lymph node cells from vaccinated mice *in vitro*, cultured from mice primed intramuscularly with two doses of either pcDNA3.1 or pcDNA3/tetC and then boosted with Fragment C + LT

Proliferative responses were determined in spleen and lymph node cells from mice primed intramuscularly with two doses of either pcDNA3.1 or pcDNA3/tetC and then boosted with Fragment C + LT. Cells were restimulated with either Fragment C or Concanavalin A *in vitro* and stimulation indices were calculated as the ratio between the counts per minute of incorporated thymidine determined in stimulated wells divided by the counts per minute calculated from duplicate unstimulated cells. Assays were performed in triplicate using cell suspensions prepared from pooled tissues taken from 5 mice.

6.2.2. T cell Proliferation in Mice Immunised by Gene-Gun with pcDNA3/tetC

As for the previous experiments, spleens and lymph node tissues from mice in all four groups were removed and the proliferative response to stimulation *in vitro* with Fragment C investigated.

Unfortunately, the proliferation observed in these experiments was very low. Not even mice immunised i. m. with pcDNA3/tetC and boosted with Fragment C showed any significant level of response. In contrast, all cells responded strongly to the mitogen concanavalin A (Con A), suggesting that the cells in all cases were not anergic. The important difference between this experiment and that in which strong responses were observed is that no additional adjuvant was included in the booster regimen. This suggests that immunisation with the antigen alone is insufficient to initiate amplification of the T-cell population to a level that is detectable using this approach.

6.3. Cytokine Secretion

6.3.1. Cytokine Secretion by Re-Stimulated Splenocytes and Regional Lymph Node Cells from Mice Immunised Intramuscularly with pcDNA3/tetC

In order to confirm the type of $CD4^+$ T helper cells that were proliferating in response to *in vitro* stimulation, culture supernatant samples were tested for the presence of IL-5 and IFN-γ, which are cytokines associated with activation of Th2 or Th1 responses, respectively.

Supernatants from proliferating cells (splenocytes and regional lymph nodes) from each group were pooled and analysed in triplicate by ELISA assay in the presence of recombinant standard cytokines of known concentration (see Section 2.5.2.2).

The results indicated that a higher concentration of IFN-γ was released into the supernatants taken from stimulated splenic cells from mice primed with 2 doses of pcDNA3/tetC and boosted with Fragment C and LT than those primed with 2 doses of pcDNA3.1 and boosted with Fragment C and LT (Table 6.1). A similar pattern of IFN-γ secretion was also observed in culture supernatants taken from pooled regional lymph nodes from the same groups of mice. In contrast, levels of IL-5 were observed to be higher in the pooled supernatants stimulated lymph node cells from mice immunised with vector alone than those immunised with pcDNA3/tetC (Table 6.1). This could reflect a possible bias toward the secretion of Th1 type of cytokines in mice prime with DNA, which may inhibit the Th2 response usually elicited by the protein alone. However, the significance of this result is unclear, as the opposite pattern of IL-5 secretion was observed in the supernatants taken from spleen cells using the same groups of mice.

Although these findings appear largely to agree with the isotype of IgG observed (see Section 6.1.4.2), it is possible that the differences observed reflect either variability that is inherent in the assay itself, or the data is affected by the fact that pooled supernatants were used in the analysis. It is possible that high levels of cytokines produced by one animal may have skewed the pooled data in a particular direction. In addition, as the adjuvant LT has been shown to activate antigen specific Th1 and Th2 cells, its very use could have influenced specifically the production of IL-5.

6.3.2. Cytokine Secretion by Splenocytes and Lymph Node Cells from Mice Immunised with pcDNA3/tetC by Gene-Gun

Despite the poor T-cell responses generated in this experiment, the supernatants taken during the course of this experiment were additionally analysed for cytokine production.

As it can be seen in Table 6.2 the splenocyte culture from individual mice primed by Gene-Gun (with either pcDNA3/tetC or pcDNA3.1) and boosted i. m. with Fragment C showed similar levels of IFN-γ. As the mean values were almost equivalent between both groups, the impact of priming the animals with pcDNA3/tetC did not appear relevant.

However, this experiment lacked the control group of mice, which should have been immunised with pcDNA3/tetC using the Gene-Gun alone. Analysis of the responses from such a group compared to one in which a booster immunisation has been given may result in the production of a different pattern of cytokine production.

Mice immunised i. m. either with pcDNA3/tetC, and boosted with Fragment C, or with Fragment C alone had a slightly higher level of IFN-γ in both restimulated spleen cells and lymph node cells. These responses were higher than those observed in the Gene-Gun immunised groups. This difference might suggest the stronger induction of Th2 cytokines that are inhibiting production of IFN-γ in the Gene-Gun immunised mice, which would be consistent with serum IgG1/IgG2a values correlated with the same type of immune response in Gene-Gun- versus i. m - vaccinated mice (see Section 6.1.4.2).

The pattern of IL-5 secretion is also difficult to be interpreted, as, while in lymph node cells IL-5 secretion was difficult to detect in any of the groups tested, mice primed by Gene-Gun with pcDNA3/tetC and subsequently boosted had a slightly higher level of IL-5 production in splenocyte culture in comparison with all other groups of mice. Again, with the reservation of the lack of consistency between the situation in spleen and the one in lymph nodes, results might indicate a possible bias towards a Th2 type of cytokine secretion primed by the Gene-Gun in comparison to i. m. priming.

Due to the fact that the number of mice used in this experiment was small, the results cannot to be considered representative, but they suggest consistency with previous studies using different vaccine candidates and the same vaccination protocols.

Table 6.1: Production of IFN-γ and IL-5 from spleen and lymph node cells from mice primed i. m. with DNA and boosted i. m. with purified Fragment C and LT

	Group of mice	Primed i. m. pcDNA3/tetC x2 Boosted with Fragment C + LT x1	Primed i. m. pcDNA3.1 x2 Boosted with Fragment C + LT x1
IFN-γ (pooled supernatants)	Spleen cells	1195.6 pg/ ml	857.4 pg/ ml
	Lymph node cells	1541.8 pg/ ml	977.3 pg/ ml
IL-5 (pooled supernatants)	Spleen cells	215.6 pg/ ml	96.5 pg/ ml
	Lymph node cells	91.5 pg/ ml	173.3 pg/ ml

Table 6.2: Production of IFN-γ and IL-5 from spleen and lymph node cells from mice primed by Gene-Gun with DNA and boosted i. m. with purified Fragment C

		Group of mice			
		pcDNA3/tetC G.G. x 2 + Fragment C	**pcDNA3.1 G.G. x2 + Fragment C**	**pcDNA3/tetC i.m. x2 + Fragment C**	**Fragment C**
IFN-γ (averages)	Spleen cells	1246 pg/ ml	1326 pg/ ml	1900 pg/ ml	1750 pg/ ml
	Lymph node cells	348 pg/ ml	96 pg/ ml	766 pg/ ml	394 pg/ ml
IL-5 (averages)	Spleen cells	274 pg/ ml	84 pg/ ml	184 pg/ ml	55 pg/ ml
	Lymph node cells	51 pg/ ml	156 pg/ ml	186 pg/ ml	20 pg/ ml

Chapter 7

Results – Section 5

Vaccination with pCMV-70

The aim of this work was to establish whether the DNA vaccine construct pCMV-70 was able to induce immune responses when delivered to mice intramuscularly.

The immunogenicity of the second DNA vaccine candidate, pCMV-70, was also tested by intramuscular injection in BALB/c mice. This plasmid had previously been shown to be partially effective previously both in cattle and BALB/c mice. In cattle, i. m. administration of this plasmid had resulted in antibody seroconversion in 50% of the animals immunised (Vordermeier *et al.,* 2001), whilst in mice, four doses of pCMV-70 had resulted in a reduction in the number of live bacteria in spleen and lungs of mice challenged with the organism. (Lowrie *et al.,* 1999). Using a similar approach to that taken using Fragment C, we hoped to maximise the immune response generated.

In this experiment mice were immunised either three times i. m. with pCMV-70 or twice with the plasmid DNA and boosted once with the MPB-70 protein. Sera from the mice were subsequently analysed for the presence of MPB-70 specific antibodies. However, the results of these experiments proved to be very disappointing, as no or very low response was detectable either before or following boost either by ELISA using purified recombinant MPB-70 as the coating antigen, or by Western blot, using the purified antigen. However, as pCMV-70 produced a larger than expected product when expressed in COS-7 cells, it was unclear whether this lack of response indicated poor immunogenicity of the plasmid, or, whether the antibodies generated where able to recognise the aberrant form of the protein (28 kDa) and unable to recognise native MPB-70. In an attempt to resolve this problem the sera was tested for activity against both forms by Western blot using the recombinant material and lysates from transformed COS-7 cells. Unfortunately, even using this method of analysis, there did not appear to be any specific MPB-70 antibodies in the sera.

In conclusion, DNA vaccination using the MPB-70-encoding plasmid pCMV-70 did not result in detectable immune response in any of BALB/c groups, regardless the vaccination strategy used (3x pCMV-70, 2x pCMV-70+MPB-70, or 2x pCMV-link + MPB-70).

Chapter 8

Discussion

Although traditional vaccination strategies have been very successful at protecting man from a variety of infections, there still are many pathogens (such as malaria) for which effective vaccines have not yet been discovered. Failure to develop such vaccines can be attributed to a number of factors including the poor definition of protective antigens, low immunogenicity, availability of appropriate delivery vehicles to stimulate the immune system appropriately and issues relating to safety. For example, in the case of subunit vaccines weak immunogenicity frequently requires repeated boosts to induce long-term protective immunity whilst in the case of live attenuated vaccines, reversion to wild type phenotype maybe observed. In this regard, many challenges remain to be overcome and new, effective and potent vaccines to be developed.

Although vaccination using "naked" DNA is in its infancy, many features associated with this form of immunisation have been shown to induce protective immune responses in a number of animal models, in which difficulties had previously been encountered. In particular, as DNA vaccination appears able to induce both cellular and humoral immune responses, its use in the development of viral vaccines has been particularly significant. In addition, there is no danger of reversion to virulence, which has been the perceived problem associated with live attenuated organisms that stimulate similar cellular responses. The potential of this form of vaccination has resulted in rapid clinical testing with several vaccines currently in phase I/II trials for safety and efficacy. These include testing of DNA vaccines against hepatitis B (Tacket *et al.*, 1999), HIV infection (MacGregor *et al.*, 1998; Hanke *et al.*, 2000) and malaria (Le *et al.*, 2000). The therapeutic potential of DNA vaccines in cancer (Chen *et al.*, 1999) and autoimmune (Garren *et al.*, 2001) or allergic (Horner *et al.*, 2001) diseases is also being evaluated.

In the context of this study, the potential of two different DNA vaccine candidates to induce immune responses was tested in BALB/c mice. In particular, the aim was to

determine the type of immune response generated to these antigens when the naked DNA was introduced using two different routes of immunisation, namely by parenteral injection into the muscle or dermal inoculation into skin cells using a ballistic device. The downstream consequences of trying to alter the magnitude and type of response using alternative boosting strategies could then be considered. The two candidates used were the 50 kDa non-toxic binding domain from tetanus toxin from *Clostridium tetani,* known as Fragment C, and an outer membrane protein known as MPB-70 from *Mycobacterium bovis.*

8.1. PCMV-70 Plasmid, as an anti-Tuberculosis DNA Vaccine Candidate

In our study, plasmid pCMV-70 encoding the mycobacterial antigen MPB-70 was tested as a vaccine candidate against tuberculosis, first for its potential to induce protein expression in mammalian cells, *in vitro*, and then for its immunogenicity in BALB/c mice, when administered intramuscularly.

Although pCMV-70 successfully transfected COS-7 cells *in vitro*, and showed high levels of protein expression within these cells (see Section 4.2.2), the results were difficult to interpret. This is because the protein expressed in COS-7 cells was different in size to the recombinant or native form of the protein. In addition, its lack of reactivity with an appropriate monoclonal antibody suggested that expression of this antigen in the context of an eukaryotic cell resulted in a change to the protein that affected epitope availability. Though, the nature of this change could not be determined within the limits of this project, two potential hypotheses were tested and discounted. The first hypothesis, which considered N-glycosylation of the protein, appeared incorrect as treatment of the cells with Tunicamycin, and N-glycosylation inhibitor, did not affect the size of the protein produced. However, the second hypothesis, which considered the difference in size to reflect the

formation of additional disulphide bonds within the structure of the COS-7 expressed protein, was also irrelevant, as additional treatment of the protein with either DTT or fresh β-mercaptoethanol, failed to affect its size. It is possible that other post-translational modifications to the protein could have resulted in the observed changes in size. These include O-glycosilation, palmitoylation or phosphorylation. However within the context of this study no time was available for additional evaluation.

Although the cells transfected with pCMV-70 produced a protein that reacted with polyclonal sera against MPB-70, there is no direct evidence that this protein is MPB-70. It is possible that this maybe an artefact of the system, although cells transfected with the vector alone did not produce a similar sized protein. If further study were possible, the identity of this antigen could be confirmed by analysis of the protein separated by two-dimensional electrophoresis. Those protein spots that specifically react with the MPB-70 antisera could then be identified and isolated from such gels. It would then be possible to identify the composition of the protein using digested peptides analysed by matrix-assisted laser disruption/ ionisation mass spectrometry analysis (MALDI-TOF). If this methodology failed to identify the protein as MPB-70, its exact identity could then be determined by peptide sequencing using electrospray mass spectrometry (EMS).

In contrast to work described in the literature, in which i. m. administration of this plasmid to cattle had resulted in antibody seroconversion in 50% of the immunised cattle (Vordermeier *et al.,* 2001), the MPB-70-encoding plasmid pCMV-70, failed to induce any significant level of antibody responses in BALB/c mice. This also contrasts the work of Lowrie *et al.,* 1999 who showed that the number of live bacteria in spleen and lungs in BALB/c mice declined rapidly following four doses of i. m. pCMV-70. In our hands, the plasmid did not even appear to prime any responses, as when the protein was subsequently used to boost these animals, no detectable response was generated. However, this could

reflect the fact that the two proteins are not identical and may be being processed by the immune cell via different pathways. The fact that the monoclonal antibody failed to react with the protein suggested that the alternative post-translational folding of the protein maybe occurring, resulting in changes to the immunodominant epitopes. It is also possible that, by increasing the number of doses of DNA vaccine given, or by including an adjuvant with the protein boost, any immune responses that had been primed may have been enhanced (Lowrie *et al.,* 1999 used four i. m. injections). However, time limitations did not permit any further evaluation of this construct. It is also possible that the strain of mice chosen for this experiment are not the most appropriate for efficient processing and presentation of the H2 restricted epitopes of the MPB-70 protein. However, if the immunogenicity of this protein is highly dependent on the genetic background of the animal model chosen, it is unlikely that this protein will have application as a candidate for a human vaccine as the genetic background of this population is highly polymorphic. Thus, most likely, in the attempt to find a DNA vaccine against tuberculosis capable to cover all the limitations of currently available BCG vaccine, other structures, more immunogenic and less subjected to genetic variation within the host population have to be taken in consideration.

One point of interest that can be taken from this study regards the correlation between potential level of gene expression *in vitro* and immunogenicity *in vivo*. From the *in vitro* studies, the antigen appeared to be expressed at much higher levels than the Fragment C construct. However, the immunogenicity of the Fragment C construct appeared higher *in vivo*. It is therefore possible that this high level of expression is responsible for its low immunogenicity as over expression in the cell may lead to poor folding and rapid degradation. It is possible that using a different vector in which expression is not driven so strongly this protein would appear to be more immunogenic.

8.2. PcDNA3/tetC Plasmid, as an anti-Tetanus DNA Vaccine Candidate

Tetanus, along with measles and pertussis, is one of the main vaccine-preventable infections that can kill within the first year of life. Although, the current tetanus vaccine (based on a chemically inactivated form of the tetanus toxin, named anatoxin or toxoid) is efficient in the prevention of the disease, there are several reasons to suggest that this vaccine can be improved. These include the relatively limited duration of protection conferred by the vaccine, which has to be given every 10 years to maintain an appropriate level of neutralising antibodies. Second, immunisation is often accompanied by a local reaction following administration, which may be the result of the inclusion of the aluminium hydroxide or phosphate adjuvant included in the vaccine (Stratton *et al.*, 1994). Thirdly, as generation of the antigen is dependent upon the successful manufacture and inactivation of tetanus toxin from *Clostridium tetani*, failure in the manufacturing process can lead to the production of either an ineffective vaccine (Topsis *et al.*, 1996) or conversely immunisation of patients with active toxin (Gale *et al.,* 1994). A suitable alternative to the vaccine is the production by recombinant technology of the non-toxic, 50 kDa carboxy-terminal fragment (Fragment C) of the tetanus toxin molecule.

Although several plasmids encoding Fragment C under the control of different promoters (Anderson *et al.*, 1996; Saikh *et al.*, 1998; Stratford *et al.*, 2000) have already been used as DNA vaccines, the antibody titres generated in each case were not higher than those observed following immunisation with the purified protein. The main aim of the current study was to investigate how a prime-boost strategies may be used to optimise immune responses to antigen. This included consideration of the magnitude of this

response and the potential impact on the Th bias of this response. Such studies had previously been performed using animals primed i. m. with the plasmid construct boosted with the purified protein. However, no prime/boost evaluations have been performed using priming of animals using DNA delivered by the Gene-Gun. By carrying out i. m. and Gene-Gun experiments in parallel using the same plasmid, pcDNA3/tetC, allowed comparative analysis of the results generated.

Before *in vivo* experiments using the Gene-Gun could begin, it was essential to initially identify and standardise protocols that produced cartridges for the Gene-Gun that contained a consistent amount of DNA that could be accurately quantified. To eliminate ethical considerations and reduce cost, most of the preliminary work was performed *in vitro*, using the pGFP-C3 plasmid.

These optimisation experiments revealed several important findings. First, the concentration of PVP added to enhance the successful adherence of the DNA/gold suspension to the tubing, appeared crucial, as if the amount of PVP was too high, the DNA/gold failed to detach from the tubing during Gene-Gun firing. However, if it was too low, some of the DNA was lost during the coating process. Second, the efficiency of DNA appears dependent on the quality and purity of ethanol used for washings. Thirdly, pressure of helium used during transfection was crucial, as pressures higher than 100 psi produced large areas of disruption in the cell monolayer resulting in a lower number of fluorescent cells, whilst those lower than 50 psi failed to drive the DNA/gold from inner surface of the cartridges. Thus, in our *in vitro* experiments, a helium pressure of 100 psi appeared to be optimal. Finally, to reduce any further disruption to the cell monolayer, a protective diffusion screen was used and the Gene-Gun held at a distance of approximately 2 cm above the cell monolayer for firing.

Using these optimised conditions, the level of GFP expression in COS-7 transfected cells was found to be about 3 times lower than when a cationic lipid reagent, Lipofectamin, was used as a carrier with roughly the same amount of GFP-encoding DNA. The lower level of GFP expression *in vitro* following COS-7 transfection by Gene-Gun possibly reflects the greater damage to the cell membrane during entry by the gold particles. This can clearly be seen by the difference in morphology of the cells (Figures 4.1 and 5.2) transfected using the two different methods, with those in which Lipofectamine was used maintaining typical elongated morphology compared to the rounded cells observed in wells transfected using the Gene-Gun. However, using this method, we were able to show that pcDNA3/tetC could be delivered *in vitro* using the Gene-Gun, and resulted in the production of the Fragment C protein in these cells.

To investigate the magnitude of anti-Fragment C immune responses, in our initial studies, two i. m. or Gene-Gun doses of pcDNA3/tetC were given to BALB/c mice and the magnitude of the immune responses to Fragment C determined. These results showed that using either route of vaccination, a Fragment C specific immune response was detectable in the sera. These responses could be further enhanced when mice were given either an additional dose of pcDNA3/tetC (for the i. m. experiment), or Fragment C (for both experiments). Following three doses of i. m. DNA encoding Fragment C, a seven-fold increase in specific Ab titre was detected.

This study showed that the responses to pcDNA3/tetC could be enhanced when mice were subsequently boosted with purified protein, as following a boost with Fragment C, a 20 fold increase in Ab titre was observed in mice previously primed twice i. m.. In comparison, only a six- fold increase in Ab titres was observed in mice primed by Gene-Gun. This finding suggest that low Ab responses that might not afford protection in immunised subjects can be enhanced using appropriate prime-boost strategies, although

optimising the vaccination schedule (dose, interval between immunisations) may have impact on the magnitude and bias of the immune response and should be further considered.

Furthermore, studying the orientation of immune responses elicited by DNA vaccination, our results suggest that, in agreement with other studies (Anderson *et al.*, 1996, Saikh *et al.*, 1998), i. m. vaccination with pcDNA3/tetC plasmid encoding Fragment C of tetanus toxin stimulated a mixed Th1/Th2 immune response in BALB/c mice, with a predominance of the Th1 component. This was clearly demonstrated by a low IgG1/IgG2a ratio, in comparison with the group primed with the control plasmid and boosted with purified protein. Thus, DNA and protein based immunisations, clearly stimulated different types of immunity. Furthermore, the immune response established by i. m. immunisation with DNA, was not drastically altered when the purified protein was given as a booster immunisation. This was also consistent with studies describing the type of the immune response elicited by different DNA vaccines combined with protein boost (Levitskaya *et al.*, 1995, McShane *et al.*, 2001). Our results also showed that inclusion of the adjuvant LT with the purified Fragment C boost did not appear to alter the type of immune response generated, but only the magnitude of that response. All these results suggested that DNA administered i. m. may be successfully used to prime Th1 biased responses using vaccination strategies (as i. m. administration of protein) which, when given alone, would stimulate Th2 biased responses in naïve recipients.

As expected, clear differences in Th bias were observed when the type of response induced by the Gene-Gun delivered DNA was compared to that induced following i. m. injection. In contrast to the mixed Th1/Th2 response elicited following i. m. immunisation, Gene-Gun mediated vaccination with pcDNA3/tetC was characterised by a predominant Th2 type of immune response, with a high titre of IgG1 and IgG2b both before and after

the protein boost immunisation, and the highest production of IL-5 in supernatants from restimulated spleen cells.

These results suggest that in contrast to i. m. immunisation with pcDNA3/tetC, the Gene-Gun induces a response that is strongly biased toward a Th2, and, in agreement with the previous experiment, that Th bias was not drastically altered by the subsequent i. m. boost, although the magnitude of the antibody response to the antigen was enhanced.

One explanation for the difference in the orientation of Th response elicited by the two routes may involve the quantity of plasmid DNA required to activate responses. Using the Gene-Gun, the amount of DNA is considerably lower than needed for i. m. immunisation, which may affect the number of CpG motifs available for triggering the immune system (Krieg *et al.*, 1998[b], O'Garra *et al.*, 1998). In addition, it is assumed that immunisation using the Gene-Gun results in the direct transfection of DCs. This may bypass the interactions that are now believed to take place between CpG motifs and Toll-9 receptor found on the surface of antigen presenting cells (Budker *et al.*, 2000). It is this interaction that is believed to generate the transduction of an intracellular signal leading eventually to the observed adjuvant effects of bacterial CpG motifs.

Unfortunately, during the course of these experiments, we were unable to assess the relevance of prime-boost strategy on the production of tetanus toxin specific neutralising antibodies, which mediate protection. However, this would be very relevant for determining the protective capacity of the DNA vaccine administered using these two different routes of immunisation. In light of our data, the antibody response following Gene-Gun driven delivery of DNA, both in literature and in our study, suggests that this method of vaccination could be used as an alternative to traditional vaccines against tetanus. This is because using the Gene-Gun, very low amounts of DNA could be used to generate titres of antigen specific antibody. These titres were equivalent to those achieved

by i.m. immunisation in which much higher amounts of DNA were used. Despite this advance, the response to the Gene-Gun delivered DNA was still lower than that observed when the individual protein was given alone. However, it may be possible to further increase the magnitude of responses by optimising the immunisation schedule in terms of the number of immunisations given, the dose of DNA used and the time interval between prime and boost immunisations.

Our results and interpretation of Th modulation of the response were based on assessing specific IgG subclasses in serum and the type of cytokines present in culture supernatants from restimulated splenocytes and lymph node cells. Since measurement of IgG subclasses is not always considered the most appropriate measurement of Th bias due to the relative instability of antibody isotypes, alternative methods for following Th cell modulation should be considered in the future. These could include quantification of relative numbers of antigen specific Th1 versus Th2 cells using an ELIspot assay, intracellular staining of cytokines by FACs analysis or quantitative measurements of mRNA encoding Th1/Th2 related cytokines. Further studies using larger numbers of mice would also help to establish the statistical significance of the results achieved.

In our study Fragment C specific CTL responses were not measured as Fragment C specific T- cell epitopes have not yet been identified for BALB/c mice. Further studies may consider defining H-2K^{d} related-CTL epitopes of Fragment C and investigation of specific CTL responses by standard ^{51}Cr release assay from pulsed syngenic target cells.

Considering the great advantage of Gene-Gun which uses a small amount of DNA to induce similar responses to those obtained following i. m. route, DNA vaccination approaches against intracellular pathogens, requiring mainly Th1 mediated protection, should not neglect the Gene-Gun approach. Despite the fact that Gene-Gun delivery of DNA vaccines preferentially induces a Th2 type of immunity, a number of strategies can

be employed with the aim of improving the Gene-Gun effect on cellular immunity, these including:

- co-administration of GM-CFS, which is an important determinant of DC viability and activation, and was found to improve the immune response upon DNA vaccination (Fensterle *et al.*, 1999),

- co-immunisation with plasmid DNA encoding Th1 cytokines (Fensterle *et al.*, 1999),

- increasing the number of immunostimulatory sequences from the vector, which were identified as the main adjuvant component of bacterial DNA, shifting the immune response towards a type 1 response (Krieg *et al.*, 1998[b]),

- fusion of the antigen-coding insert with lysosome associated membrane protein, which has been shown to enhance the presentation of the antigen by MHC class II molecules (Ji *et al*, 1999),

- increasing the interval between the prime and boost, as apparently longer intervals tend toward type 1 polarisation. For example, a 45 day versus a 14 day schedule between the prime and the boost Gene-Gun immunisation was found to be protective against listeriosis (Fensterle *et al.*, 1999).

Alternatively, Gene-Gun-driven DNA inoculation may play an important role as a therapeutic tool in Th1-mediated autoimmune diseases, reverting the impact of immunopathology by down-regulating the pre-existing Th1 response toward a Th2 phenotype. However this has to be established by designing experimental studies in which factors controlling the ethiological Th1/Th2 balance of the autoimmune disorder have to be carefully defined.

In addition, there is evidence that, as Gene-Gun delivery of DNA vaccines in some models may be associated with both Th1 and Th2 responses, this route of immunisation

maybe appropriate in the treatment of malignant disease using naked DNA (Smahel *et al.*, 2001).

In summary, results presented in this thesis demonstrate that DNA vaccines delivered by either Gene-Gun or i. m. may provide an excellent tool for priming a particular type of immune response, whose magnitude and orientation may be further optimised using an appropriate boost immunisation. Moreover, as immunisation with the Gene-Gun is more likely to have application at the clinical interface, given the low amount of DNA that can be used, it is important to understand the type of the immune response elicited and the potential for modulating its orientation and magnitude. Such studies should provide information which will be essential if such DNA vaccines are to be used in the future as a preventive and therapeutic method against infections, immune disorders or even malignant diseases.

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