Effect of *Mycobacterium vaccae* NCTC 11659 (standard or recombinant) on cytokine production by human and murine cells

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Abstract

Killed *M. vaccae* is in clinical trials as an immunotherapeutic agent and adjuvant, but understanding of its mode of action is incomplete.

To test its potential as a recombinant carrier organism and the nature of the responses evoked, recombinant strains were generated that expressed p27 of SIV. In mice these primed for specific production of IFNγ in the presence of p27, and induced serum IgG2a and, at higher doses, IgG1 responses to *M. vaccae* sonicate.

Flow cytometry for intracellular cytokines after a single subcutaneous injection of 10^9 *M. vaccae* revealed accumulation of IFNγ-secreting CD8+ cells in lymph nodes. This finding, together with data generated simultaneously by another research group, implied an unusual adjuvant effect, not shared by other killed mycobacteria.

In order to investigate this adjuvanticity the effects of killed *M. vaccae* on cytokine release from the human THP-1 monocyte line were investigated. *M. vaccae*, BCG and soluble bacterial preparations were all able to induce IL-12, IL-10 and TNFα production *in vitro* to varying extents. Dose of mycobacterium and the addition of IFNγ influenced the balance of cytokines. Attempts to define active components in the IL-12 induction system were not successful, but it was noted that *M. vaccae* differed strikingly from the other killed mycobacteria in that its induction of IL-12 was greatly enhanced by exposure to lysozyme. The induction of IL-12 proved sufficiently reproducible to be used as a potency assay for material manufactured for clinical use.

In conclusion, *M. vaccae* has been shown to be a potent Th1 inducer at appropriate doses, with the added ability to induce expansion of the CD8+ IFNγ+ population, perhaps via IL-12 release following exposure to lysozyme *in vivo*. CD8+ cells are strongly implicated in the clinical situations where *M. vaccae* has shown benefit.

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Table of Contents

List of Tables

List of Illustrations

Abbreviations

ABTS	azino-ethylbenzthiazoline sulfonic acid
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cell(s)
BCG	Bacille Calmette Guerin
b.p.	base pairs
BSA	bovine serum albumin
c.p.m.	counts per minute
CD markers	clusters of differentiation
CD40L	CD40 ligand
CIAP	calf intestine alkaline phosphatase
CMI	cell mediated immunity
Con A	concanavalin A
CPB	citrate phosphate buffer
CTL	cytotoxic T lymphocyte
DC	dendritic cell(s)
DHEA	dehydroepiandrosterone
DMSO	diemethyl sulphoxide
DTH	delayed type hypersensitivity
ELISA	enzyme-linked immunoadsorbent assay)
FACS	fluorescent activated cell sorter
FCS	foetal calf serum
Fig.	figure
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
hsp	heat shock protein
i.p.	intraperitoneal
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-12R	IL-12 receptor
kDa	kiloDalton
kB	kilobase

LAM	lipoarabinomannan
LN	lymph node(s)
LPS	lipopolysaccharide
M. vaccae	*Mycobacterium vaccae*
mAb	monoclonal antibody
MHC	major histocompatibility complex
MVSA/MVTA	*M. vaccae* vaccine codes
NCU	*M. vaccae* vaccine code
NK	natural killer
NO	nitric oxide
NOS	nitric oxide synthase
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBL	peripheral blood lymphocyte
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbal 12-myristate 13-acetate
rBCG	recombinant Bacille Calmette Guerin
RE	restriction enzyme
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SOD	superoxide dismutase
3H-	tritiated
TFA	trifluoroacetic acid
TGF	transforming growth factor
Th	T helper
TMB	tetramethyl benzidine
TNF	tumour necrosis factor

Chapter 1 Introduction

1.1 Lymphoid system organisation

In simple terms, lymphoid organs can be differentiated into primary and secondary (peripheral) types. Generally, the primary tissues (thymus and bone marrow) are regions of lymphopoiesis, where T lymphocytes differentiate from stem cells, proliferate (under conditions that regulate self-recognition) and mature. In secondary tissues (spleen, lymph nodes and mucosa associated tissues) the lymphocytes interact with antigen presenting cells (APCs) and other groups of lymphocytes, within special T and B cell areas. Lymphocytes recirculate through these areas via blood and lymph.

The spleen is a highly vascularised organ which filters aged erythrocytes, platelets and particulate materials from the circulation by phagocytosis in the red pulp. The white pulp consists of lymphoid sheaths organised into T and B dependent zones around branches of the splenic artery. Recirculating T and B cells enter from the marginal zone where they are selectively released from the circulation. Blood-borne antigens are also taken up via macrophages and antigen presenting cells found in the marginal zone. The spleen provides the environment for specific humoral and cellular immune responses against circulating antigens, especially antibody production.

Lymph nodes (LN) contain numerous cells in specific regions capable of producing both humoral and CMI responses. B cells exist in the primary and secondary follicles and germinal centres of the cortex (with some CD4 cells) and antibody producing cells in the medulla. Resting and activated T cells are present in the paracortex, with APCs (including macrophages, dendritic cells interdigitating cells and veiled cells) and are continually renewing their cell populations through active lymphocyte trafficking through the lymphatics. Generally 2-3 days after the arrival of an immunogenic antigen, there is increased blood flow through the node and production of chemokines and upregulation of adhesion molecules enhances recruitment of sub-sets of lymphocytes. Antigen-specific naive lymphocytes react to antigen on APC and transform into immunoblasts, which proliferate but have limited effector functions. Some cells differentiate and mature to carry out functions within the node, but others are discharged in large numbers into efferent lymph after 4 days to colonise other lymphoid tissue and take up effector functions. Antigens from natural infections and intradermal or subcutaneous vaccination usually arrive at the LNs, while foreign soluble proteins

mostly pass into the blood unless they are localised by precipitation or mixing with adjuvants.

Cytokines are soluble polypeptides responsible for cell-to-cell communication and have a major role in maintaining normal immune homeostasis, by reciprocal regulatory processes. Many have multiple cellular sources, multiple targets and a broad overlapping range of activities.

Some aspects are discussed further below.

1.2 Innate and adaptive immunity

The activation of macrophages and NK cells represents one of the first events in the innate resistance to intracellular infection. They have germline encoded receptors capable of recognising molecular patterns (Medzhitov 1997) associated only with pathogens or their secreted products, e.g. formyl pepides, LPS, peptidoglycan from mycobacterial walls, unmethylated CpG, dsRNA; many are potent adjuvants. Upon receipt of these signals, inflammatory mediators i.e. cytokines and chemokines, are released which stimulate innate immunity and draw effector cells to the site of infection. These early responses of innate immunity are very effective at eliminating pathogens, or at least keeping their numbers limited (Dai 1997). However, often the complete elimination of pathogens is mediated by the antigen specific adaptive immunity, which becomes operative several days after infection when antigen-specific T cells have expanded.

Consequently cytokines controlling subsequent adaptive immune responses and MHC molecules and costimulators of T cell activation on APCs are also upregulated during the early innate inflammatory response; these influence the environment in the infected tissues or draining lymph nodes, between which the APCs and specific T cells migrate. The requirement for a concerted effort by innate and adaptive immunity helps ensure that immune responses are directed to pathogens rather than self-antigens. Furthermore, careful regulation of the response pattern is crucial to specific immunity: antibody mediated responses are most effective against soluble toxins and extracellular pathogens; CD8 CTLs are most effective against altered somatic cells, killing the infected/tumour cell by means of any of the pathways of apoptosis (TNF, FasL, perforin/granzyme); T cells/cytokines are vital for induction of macrophage activation.

Naive lymphocytes have antigen receptors of random specificity and are pluripotent; they can differentiate along different pathways to become distinct effector cells, depending on the additional signals they receive during activation (Kamogawa 1993). T

cells stimulated through T cell receptors, in conjunction with CD4 or CD8 molecules, respond to the specific peptide/MHC complex and costimulators: they express surface molecules including TNF-α and -β, Fas ligand, CD40L, CD30L and secrete cytokines, which in turn act on target cells.

CD4 T cell subsets were divided into two phenotypes in mice based on their synthesis of mutually exclusive sets of cytokines (reviewed in Mosmann 1989 and Heinzel 1995). Th1 cells produce IL-2, IFNγ and lymphotoxin, and support cellular responses, mediating delayed type hypersensitivity, CTL activity and providing some help for B cells for synthesis of opsonising and complement fixing IgG2a antibodies. Th2 cells produce IL-4, -5, -6, -10 and -13 and support phagocyte-independent humoral responses, primarily by inducing B cell differentiation and expansion and providing help for IgE and IgG1 isotype switching and synthesis. These subsets, along with the less polarised Th0, were then identified in humans (Romagnani 1991 and 1994a). The term was broadened to type 1 and type 2 cytokines (Clerici 1994) to include those made by CD8 T cells (Kemeny 1994, Sad 1995), and non-T cells including APCs which directed differentiation of T cell subsets (e.g. IL-12). It was noticed that there may be a heterogeneous effector response, with cells making varying absolute levels of each cytokine associated with their (or even the other) subset (Kelso 1995) and the levels of complexity in this system are still being increased e.g. some cells use non-classical MHC molecules, such as CD1 induced on APCs by pathogens, to recognise lipoid moieties (Kaufmann 1997) and show unusual patterns of response.

1.3 Polarised type 1 or type 2 responses and disease

Type 1 responses are in general more protective than type 2 responses against most infectious agents and particularly against intracellular parasites including viruses. When Th2 responses dominate, infections sustained by intracellular parasites usually progress, or show a spectrum of activity related to Th type. However, in the experimental absence of one protective mechanism, the existence of multiple overlapping alternative systems becomes apparent.

Only a small percent of people infected with *M. tuberculosis* show clinical symptoms, so the immune response is generally capable of controlling this infection. However, a competent T cell response is required for control of *M. tuberculosis* (Britton 1994, Rook 1996). This is apparent during reactivation of latent infection, which may occur due to protein-calorie malnutrition, viral infection (measles), immunosuppression and rapidly after co-infection with HIV.

In leprosy, the spectrum of disease is loosely associated with divergent expression of type 1 and type 2 cytokines, which is reflected by specific antibody levels in serum (Filley 1994, Sousa 1998). Cytokine profiles of T cells from leprosy lesions helped establish the presence of polarised type 1 and type 2 cells in humans. Tuberculoid leprosy lesions contained abundant macrophage and type 1 cytokines and were self-healing, while lepromatous leprosy patients had lesions containing type 2 cytokines and specific unresponsiveness causing permissive infection. The predominance of CD4 or CD8 cells also differed (Yamamura 1991, Salgame 1991). In unstable borderline leprosy lesions, Th0 and additional phenotypes exist (Verhagen 1998). The levels of specific cytotoxicity and the relative contribution to this of CD4 and CD8 cells also vary across the disease spectrum (de la Barrera 1997). IL-12 appears to be a pivotal regulatory factor in leprosy (Sieling 1994).

Leishmania, protozoan parasites that replicate exclusively in macrophages, also induce a wide spectrum of clinical infection. With mild cutaneous and visceral diseases, there are strong DTH and proliferative responses, while with the serious forms with widespread involvement, patients lack DTH and T cell proliferation and have significant specific antibody production. Destructive mucosal disease has exaggerated cellular responses and may be due to hypersensitivity reactions (Reed 1993). Similar patterns occur in primary experimental *Leishmania major* infection in inbred mice where ability to eradicate parasites is genetically controlled. Resistant mice develop strong Th1 responses, expanding IFNγ-producing T cells in draining lymph nodes and DTH responses, control parasite multiplication and heal. Susceptible mice have a dominant Th2 response with IL-4 and IL-10, hyperglobulinemia and IgE, uncontrolled lesion development, metastasis of the parasite and death of the animal.

A protective role for exclusive Th2 responses exists. Parasitic helminths e.g. the intestinal nematodes *Trichuris muris*, are large multicellular organisms which mostly exist extracellularly. The expulsion of intestinal helminths is the best documented example of protective immunity mediated by Th2 T cell responses (Else 1994). It is possible that IL-4 and mast cells influence the intestinal mucosa, while the role of IgE and eosinophilia is uncertain. However, for tissue dwelling helminths and schistosomes, the issue is not as clear, and the protective response may depend on the parasite developmental stage and even differs between mice and humans (Allen 1997).

However, regulation of the response is essential. Uncontrolled Th1 activation may result in autoimmunity (Romagnani 1994) and uncontrolled Th2 responses in allergy. Atopic allergy is characterised by an increased ability of B lymphocytes to form IgE

antibodies to certain allergens after ingestion, inhalation or skin sensitisation, leading to diseases such as allergic asthma and atopic dermatitis. IgE antibodies bind to high affinity Fcε receptors on mast cells/basophils, which triggers release of vasoactive mediators, chemotactic factors and cytokines. IL-3, -4 and -10 are growth factors for mast cells and IL-5 activates/differentiates eosinophils, which accumulate at sites of allergic inflammation. Activated Th2 cells accumulate in the target organs and Th2 cells induce IgE production via a combination of IL-4 (and IL-13) signals and CD40/CD40Ligand interactions.

Allergens may have a peculiar physiochemical structure (e.g. proteases) and preferentially expand T cells showing a Th2 profile, but possibly some genetic abnormality favours the preferential Th2 response in atopic individuals. One or more polymorphisms occur in a coding or regulatory region of the IL-4 gene, and overexpression of other cytokine genes, such as IL-13 and IL-5, may also occur. Alternatively, there may be a deficiency in the regulatory activity of cytokines such as IL-12 and IFN, since in recent decades the prevalence of atopic allergy has increased in all developed countries, which may be partly related to the strong reduction in Th1-inducing childhood infections.

1.3.1 The possibility of a *'switch'* in predominant immune responses during chronic disease

Polar responses are often regarded as stable, but in some chronic diseases there is a detrimental 'switch' from a type 1 pattern of response to type 2. For example, in syphilitic infection, most *Treponema pallidum* are killed by a vigorous DTH/macrophage response (with opsonising antibody) during the acute stage. However, this appears to be down regulated too soon by a suppressive activity involving PGE_2 whose over-synthesis by macrophages is stimulated by *T. pallidum*. This leads to an asymptomatic period during which antibodies (and predominant Th2 responses) develop to a variety of treponema epitopes but the organisms are able to multiply slowly and disseminate despite this, causing chronic syphilis with some autoimmunity lasting 5-30 years until it is fatal (Fitzgerald 1992).

In murine schistosomiasis, a Th2 response develops to egg antigens, which partially and transiently suppresses the Th1 response (including CTL generation) already evoked by the Schistosomula. However, after 6 weeks the Th2 component starts to wane and the Th1 response again becomes dominant. During the period of mixed responses, lesions become necrotic. In chronic tuberculosis, mixed Th1 and Th2 responses are also

seen, along with considerable TNFα production and necrosis. Thus benefit may be afforded by reduction of the Th2 component that develops (Hernandez-Pando 1994).

1.4 Factors polarising T cell responses

Many factors polarise naive lymphocytes, including:

The nature of the antigen-presenting cell

Activated mature DCs and not macrophages may be early APCs and the initial source of IL-12 *in vivo* to some infectious agents, especially before IFNγ is available (Sousa 1997 and comment) or if pathogens fail to trigger IL-12 production by macrophages (Gorak 1998). B cells also act as important APC, by uptake of specific antigen by their membrane immunoglobulin and subsequent processing and presentation on MHC class II. However, they have a limited capacity to secrete IL-12 and this may explain their propensity to selectively drive T helper 2 cell development (Guery 1997).

The type of antigen

Mycobacteria-specific human CD4 clones from diverse sources are generally Th1 (Haanen 1991)

Hormonal effects

Immunologically active hormones include DHEA and glucocorticoids (Rook 1994) and progesterone during pregnancy (Wegmann 1993).

The activity of cytokines in the microenvironment

Addition of IL-4 early during bulk T cell culture can switch the normal response seen to a polarising antigen such as tuberculin towards Th2 (Maggi 1992). This is a very important effect and will be discussed further.

1.5 Cytokine stimulation of type 1 responses:

1.5.1 Interleukin-12

1.5.1.1 IL-12 and IL-12 receptors

IL-12 was originally identified as a natural killer cell stimulatory factor, mediating effects on NK and T cells. IL-12 is a heterodimeric protein comprised of two disulphide linked subunits designated p35 and p40, encoded by unrelated genes (reviewed in Gately 1998). The 35kDa subunit shares homology with IL-6 and the 40kDa subunit somewhat resembles part of the extracellular domain of the IL-6 receptor. Production of

IL-12 results in secretion of a 5- to 500-fold excess of p40 relative to the active heterodimer. In mice, a small percent of p40 is present as homodimers, which may antagonise some IL-12 activities, while facilitating some responses that involve IFNγ production by murine CD8 cells (Piccotti 1997). There are less data on human p40 dimers, and they may show a lower tendency to dimerise.

IL-12R are primarily expressed on activated T and NK cells. IL-12R β1 and β2 subunits are transmembrane glycoproteins that form dimers or oligomers and although β2 is the signal transducing component, expression of both β1 and β2 leads to high affinity IL-12-binding and activation of kinases to phosphorylate STAT3 and STAT4. Expression of IL-12R β2 subunits may regulate responses (Rogge 1997 and Murphy 1998), as they are induced by IFNs during differentiation of Th1 cells and down regulated by IL-4, IL-10 and TGF-β and on Th2 cells. Down regulation of cytokine receptors on polarised cells may help maintain a stable phenotype after the initial differentiation to Th1 or Th2.

1.5.1.2 Production of IL-12 and its biologic activities

Most cell types constitutively express the p35 gene at low levels, but expression of the p40 gene is restricted. EBV-transformed B cells can produce variable amounts of IL-12 heterodimer. Adherent monocytes and HLA-DR+ cells from PBMC populations, neutrophils and several myeloid leukaemia-derived cell lines produce high levels of IL-12 after stimulation with bacteria, viruses or their products, in a T cell independent pathway. A T cell dependent pathway of IL-12 induction is mediated through CD40-CD40 ligand interaction (Trinchieri 1997). Under certain conditions, dendritic cells also produce IL-12.

IL-12 regulates NK cells and also the balance between Th1 and Th2 cells, acting in antagonism with IL-4 (reviewed by Trinchieri 1995). It promotes the development of NK and antigen-stimulated naive T cells into IFNγ-producing cells and then induces maximum IFNγ production from the differentiated Th1 cells. IL-12 also stimulates the development of IFNγ producing Th1 cells from antigen-stimulated resting memory cells. IFNγ in turn is a cofactor for phagocytic cell activation (important in early defence against infections) and optimal IL-12 secretion and IL-12Rβ2 expression.

IL-12 synergises with TNFα, IL-1 and IL-2 to elicit IFNγ production by T and NK cells (in a positive feedback loop), while IL-4, IL-10 and TGFβ may down-regulate IL-12 and IL-12-induced IFNγ production. IL-12 can be a costimulus for IL-10 production later by T cells and this may represent a negative feedback system.

Besides inducing IFNγ production, IL-12 can rapidly enhance the lytic activity of NK and LAK cells and promote specific CTL responses. Then IL-12 acts as a short-term growth factor for activated NK and T cells (additive with low levels of IL-2 and synergising with B7/CD28 during TCR stimulation) and may also inhibit activation-induced apoptosis in T cells. IL-12 also enhances Th1-associated IgG responses, possibly via IFNγ.

1.5.1.3 IL-12 in host defence

Leishmania major infection of different mouse strains has proved a useful model of cytokine involvement in disease. C57BL/6 and BALB/c mice both have *bcg*S alleles, but the BALB/c background is extremely susceptible to disease. It is interesting that IL-1α, IL-18 or TNFα may be required as co-factors to IL-12 for development of Th1 responses in BALB/c but not C57BL/6 mice (A. O'Garra, British Cytokine Group meeting, June 1998). Genetically resistant mice selectively initiate a Th1 response upon infection with *Leishmania major* and resolve the infection, while susceptible mice develop Th2 responses and disease, unless the infecting dose is extremely low (Bretscher 1992a). IL-12 is critical for early NK activation and IFNγ production (with phagocyte activation and NO production important in innate resistance) and generation of a subsequent protective Th1 response. However, the promastigote stage that initiates mammalian infection may not stimulate IL-12 synthesis, although the intracellular amastigotes do later (Reiner 1994). Instead, the T cell dependent route of IL-12 induction may be important as mice defective in CD40 or CD154 lack IFNγ and do not restrict parasite growth (Grewal 1997). Fas is also crucial to this response, possibly via the Th1 cells expressing FasL inducing apoptosis of infected macrophages expressing Fas, resulting in increased parasite destruction (Huang 1998).

Resistant mice treated with anti-IL-12 antibodies or IL-12 deficient mice become susceptible to even low inocula of *L. major* (Mattner 1997). Interestingly, highly resistant C3H mice treated with anti-IL-12 for 3 weeks develop a full Th2 response to *L. major,* but also continue to produce IL-12 (and possibly maintain a population of T cells able to respond to IL-12); when antibody treatment is withdrawn they revert to a Th1 healing phenotype (Hondowicz 1997). Thus not all Th2 responses are qualitatively similar and it may be possible to down-regulate certain Th2 responses by immunotherapy.

Conversely treatment of susceptible mice, by IL-12- or anti-IL-4-induced neutralisation of the IL-4 burst occurring between 16-48 hours after infection, does

result in a protective Th1 cell response (Launois 1997). If IL-12 treatment in BALB/c mice is delayed by 3 weeks, healing does not occur (unless *L. major* parasite load is reduced by drugs), suggesting the induction of a stable established Th2 response. The production of factors such as IL-1, IL-4, PGE2 and TGFβ favour Th2 development (Soares 1997) and may inhibit the ability of IL-12 to induce a Th1 response, possibly by rapid down regulation of T cell IL-12 responsiveness.

However IL-4 deficient BALB/c mice do not always spontaneously develop a healing response to *L. major* and still down regulate CD4 cells able to respond to IL-12. They may have increased alternate type 2-inducing mechanisms or endogenous IL-4 may sometimes potentiate Th1 responses (Mencacci 1998a). Thus the presence of an appropriate Th1 response besides the absence of a Th2 response may be necessary (Kropf 1997).

The models of mycobacterial disease in mice are also complex, depending on mouse strain, route and dose of infection and aggressiveness of the organism. Studies in IL-12 p40 deficient mice have demonstrated the essential role for endogenous IL-12 in promoting the generation of type 1 responses and IFNγ production leading to protection from *Mycobacterium tuberculosis* (Cooper 1997). In pulmonary BCG infections in resistant mice, if the IL-12 response is intact, both CD4 and to a lesser extent CD8 cells can separately contribute to the protection (Xing 1998). In CD8 deficient mice, CD4 cells can mount a full and appropriate protective response; CD4 deficient mice have high levels of IL-12 and mount a very rigorous CD8 response, but reduced levels of IFNγ result in a lower level of protection. However, to optimally overcome infections with *M. tuberculosis* requires the co-ordinated activity of macrophages, CD4 (Orme 1992) and CD8 (Flynn 1992) T cells, IFNγ, TNFα, IL-12 and NO (Kumararatne 1997).

IL-12 treatment of BALB/c for around 10 days at the beginning of infection with *M. tuberculosis* doubles their survival time, and this is dependent on IFNγ production (although not entirely, as IFNγ treatment itself does not enhance resistance, probably due to limited effects on Th1 induction). Some protection by IL-12 may be due to reduction of initial bacterial load. However, lung pathology and bacillary load still increase during infection and the mice die (Flynn 1995). Possibly an IL-4 producing population of CD4 T cells may emerge later in infection. This is unlike *L. major* infection, where IL-12 treatment induces a stable healing Th1 response and mice are resistant to rechallenge. Also unlike *L. major*, Fas may not be crucial in murine resistance to *M. tuberculosis* (Laochumroonvorapong 1997).

In humans, lack of IL-12Rβ1 leads to severe infections with intracellular bacteria (de Jong 1998). With mycobacterial infections, mature granulomas are seen surrounded by T cells and centred with epithelioid and multinucleate giant cells, but reduced IFNγ is secreted by the activated NK and T cells (Altare 1998). This allows disseminated infections with poorly pathogenic mycobacteria to be established (although the infections are not as severe as in IFNγ-R1 deficiency, due to IL-12-independent pathways of IFNγ production).

In some cases several pathways may be operational, depending on circumstances. Late, efficient IFNγ production by T cells during a secondary response is largely IL-12 independent. Memory T cell responses may not be dependent on early NK activation/IFNγ production (Kemp 1997); instead a weak cross-reactive memory response producing IFNγ may enhance macrophage and NK responses to a new organism. Furthermore in p35 deficient mice, p40 is still produced and may provide some protection from disease (e.g. *Listeria monocytogenes*) by an unknown mechanism (Gately 1998).

The role of IL-12 in some viral infections is less clear. Mouse hepatitis virus (coronavirus) infection in IL-12 deficient mice induces a polarised type 1 T cell response (including strong IFNγ production by CD4 cells) and normal survival rates. This is in contrast to increased susceptibility in IFNγR deficient mice. Possibly other cytokines make up the deficiency (Schijns 1998). However, patients with HIV infection may have a defect in IL-12 production from macrophages (possibly due to IL-10 production or prior chronic activation) that does play a role in the general immunodeficiency (review; Trinchieri 1997).

1.5.1.4 IL-12 in immunopathology

IL-12 excess may be harmful. In the generalised Shwartzman reaction, priming with endotoxin induces production of IL-12 and cofactors like TNFα, which then stimulate the secretion of IFNγ, resulting in priming of macrophages and other cell types for a lethal response to the following endotoxin challenge.

Similarly, IL-12 deficiency may reduce Th1 responses and cause pathology due to an inappropriate Th2 response (e.g. asthma). IL-12 deficient mice develop an eosinophilic airway inflammation, with enhanced IL-4, TNFα and IgE. An anti-inflammatory effect of exogenous IL-12 given to allergic mice may be due to reduction of IL-4 and IL-5 (Gavett 1995). Importantly, *M. vaccae* given to allergic mice has also

been shown to reduce IL-5 (Wang 1998), although the mechanism of action is not known.

1.5.2 Interferon γ

Cellular responses to IFNγ have been fully reviewed (Boehm 1997 and Murphy 1998). Bioactive IFNγ is a noncovalent 34kDa homodimer which plays a complex and central role in resistance to pathogens. The main cellular producers are CD4 and CD8 type 1 cells activated by IL-12/cross-linking of the T cell receptor, and NK cells, activated by macrophage derived cytokines, especially TNFα and IL-12, and also by IFNγ itself. IL-18, a relative of IL-1, released by APCs during interaction with T cells, does not activate Th1 cells alone, but increases IFNγ and IL-2 production from stimulated Th1 cells.

IFNγ interacts with a ubiquitous receptor, consisting of an α- and β-chain each associated with a Janus kinase. Interaction of IFNγ causes association of two receptor dimers resulting in phosphorylation steps and formation of STAT1α-homodimers. These translocate to the nucleus and initiate transcription of a number of genes, some of which are themselves transcription factors. Interaction of the latter and NF-κB (activated by TNFα) may cause the synergistic effect of these cytokines on inducible genes. IFNγ can down-regulate its receptor after signalling and in Th2 cells.

IFNγ is one of the type 1 cytokines, showing antagonism with IL-4 and synergy with TNFα. IFNγ is essential for control of immunoglobulin class switching, and is antagonistic to IL-4 in this (i.e. in mice IFNγ induces IgG2a and IgG3 and represses IgG1 and IgE).

IFNγ also stimulates innate immunity through NK cells, specific cytotoxic immunity by recognition of antigen with MHC molecules, leukocyte-endothelium interactions and activation of macrophages. Administration of IFNγ results in a widely disseminated increase in MHC class I expression (along with β_2-microglobulon) on many tissues. IFNγ is strongly synergistic with TNFα in class I induction. IFNγ also induces TAP and some proteasome proteins, to increase the yield of optimal peptides for loading to class I.

The MHC class II pathway is constitutively active only in professional antigen presenting cells, but all the key genes required for normal expression of class II (including the class II α and β transmembrane polypeptides, invariant chain and DM chains of the MIIC endosomal compartment) are regulated by a single IFNγ-inducible

transcription factor. Several lysosomal cathepsins implicated in the generation of peptides in the lysosomal/endosomal compartment are inducible by IFNγ in macrophages. Alongside, IFNγ also upregulates costimulators B7.1 and B7.2 (and IL-12); the activated APCs better stimulate T cell activation.

Activated macrophages also have greatly enhanced microbicidal activity for many intracellular and phagocytosed organisms. IFNγ increases levels of some components of the membrane-associated NADPH-dependent oxidase (that forms superoxide anions) and also iNOS in mice (so stimulation with TNFα results in NO production, which is toxic to pathogens and functions as intracellular messenger). IFNγ may even play a role in cancer surveillance, as a critical gene (interleukin-1β-converting enzyme) related to apoptosis after DNA damage is IFNγ inducible.

The expression of flexible patterns of chemokine receptors (Sallusto 1997) in a co-ordinated fashion ensures efficient functioning of immune responses e.g. CCR3 expression on Th2 cells, eosinophils and basophils, which all respond to eotaxin and home to allergic sites. Following activation, tissue APCs down regulate receptors for inflammatory chemokines and migrate to lymphoid tissues expressing constitutive chemokines, while activated T cells respond in the opposite pattern. IFNγ, with TNFα and IL-1, helps co-ordinate monocyte and lymphocyte migration through the endothelium to sites of inflammation. This is achieved by IFNγ-induced upregulation of chemokines such as RANTES and IP-10 (chemotactic for memory T cells and monocytes), MCP-1 (for monocytes) and MIP-1β (probably for CD8 T cells). Chemokines trigger high affinity integrin activation on recirculating leukocytes. These integrins then bind integrin ligands on endothelium, such as ICAM-1, which are upregulated by IFNγ and CD40:CD154 interactions, and the leukocytes transmigrate.

Convincing evidence for the important role of IFNγ in protective cell mediated responses exists. This can be illustrated for many diseases in mice and mycobacterial diseases in humans.

Mice that normally develop Th1 cells in response to *L. major* infection default to the Th2 pathway when the IFNγ gene is disrupted (Wang 1994). IFNγ deficient mice are also highly susceptible to mycobacterial infections, and although they produce normal levels of TNFα and granulomas after infection with *M. tuberculosis*, they cannot restrict the growth of the bacilli and develop widespread necrosis (Cooper 1993 and Flynn 1993). Interestingly, they can mount an apparent DTH response in footpads and produce little IL-4. Exogenous IFNγ can partially prolong survival, which may be of clinical

importance in HIV infected individuals with low CD4 counts who develop tuberculosis. Children with IFNγR deficiency are susceptible to infections with some mycobacteria including BCG and atypical mycobacteria (reviewed, Bellamy 1998). Interestingly, susceptibility to *M. tuberculosis* in human IFNγR1 deficiency has not been noted and several theories have been proposed to account for this difference between mice and humans (Rook 1997).

Murine systems may not translate to the human system (Paul 1996), as virulent and avirulent *M. tuberculosis* strains show widely discrepant growth rates in murine macrophages (53 versus 370 hours respectively) but nearly equal timings in human macrophages (80 hours).

Induction of anti-tuberculous activity in murine macrophages requires IFNγ and according to one view involves NO production (Yoshida 1995). Although human macrophages may express iNOS (Nicholson 1996), many combinations of stimuli failed to generate NO. However, macrophage treatment with IL-4 then stimulation of CD23 (low affinity IgE receptor) may lead to human NO production; this would suggest a role for the Th2 responses seen in human tuberculosis. Recently, in *M. tuberculosis*-infected human macrophages dual signalling, by high doses of IFNγ as well as autologous lymphocytes primed with IFNγ/*Mtb* lysate, achieved anti-tuberculous activity (possibly involving NO) accompanied by a high IL-12 p70: IL-10 ratio (Bonecini-Almeida 1998). In mice, contact of APC and activated CD4 cells via CD40:CD154 interactions (Grewal 1997) activates APCs, leading to induction of co-stimulatory molecules (CD80 and CD86), production of cytokines (TNFα, IL-12), chemokines (IL-8, MIP-1α) and NO. Triggering of apoptosis in infected macrophages sometimes leads indirectly to reduced viability of the organisms.

Activated cytotoxic lymphocytes may also directly contribute to anti-tuberculous activity, as granulysin, a protein found in granules of CTLs, can directly kill extracellular *M. tuberculosis* by altering its membrane integrity given time (Stenger 1998). The combination of granulysin and perforin was effective in CTL activity against intracellular bacilli.

1.5.3 Tumour necrosis factor α

TNFα is a 17kDa protein that normally forms noncovalently bound homotrimers. The major source of TNFα is activated macrophages, and it has wide ranging signalling and proinflammatory effector functions. In the immune system, TNF is involved in organisation of lymphoid structures and in Th1 development. Its absence renders mice

more susceptible to infection (Mencacci 1998). There are two membrane bound TNFα receptors, I (p55) and II (p75); soluble receptors can limit acute TNFα effects. TNF receptor I deficient mice are protected from the toxic effects of excess TNFα (endotoxic shock) but succumb to infection with intracellular pathogens. Similarly TNFα is therapeutic against malignancies but pathologic in inflammatory diseases such as rheumatoid arthritis (Eigler 1997). Even within one disease, such as murine tuberculosis, the regulation of TNFα and its balance with other cytokines can determine whether protective or pathological effects prevail (Hernandez-Pando 1997).

1.5.4 Regulators of Th1 responses: IL-4, IL-13 and IL-10

Important proteins in signalling are Jaks (Janus family tyrosine kinases) and STATs (signal transducers and activators of transcription). Cytokines induce STAT-mediated transcriptional regulation (Leonard 1998). Stat4 is essential for IL-12-mediated Th1 development and Stat6 for activation by IL-4 towards Th2. By 48 hours, the Th1 or Th2 cells are polarised and rapidly extinguish the expression of cytokines/receptors related to the opposite lineage. Thus the presence of IL-12 or IL-4 during priming becomes a critical point of control (Hu Li 1997) and mice deficient in IL-4 have impaired Th2 responses (Kopf 1993). The sources of IL-4 production promoting Th2 development (NK 1.1 T cells, basophils, mast cells (Romagnani 1992, Garside 1995) or pre-activated Th2 cells) are still debated (Murphy 1998).

IL-13 is an anti-inflammatory cytokine with homology to IL-4, produced by Th2 cells, which suppresses macrophage cytotoxicity and IL-12 production, but not antigen presentation, and hence favours Th2 responses (Zurawski 1994).

IL-10 is produced by many cells including stimulated macrophages (Malefyt 1991), NK cells stimulated with IL-2 and IL-12 for 3-6 days (Mehrotra 1998) and in PHA activated CD4 and CD8 cells treated with IL-6 and IL-12 (Daftarian 1996). IL-10 reduces B7 and MHC class II expression (Malefyt 1991a), TNFα and IL-12 production and/or activities and hence negatively regulates inflammatory and type 1 responses and in some circumstances its own production. Upsets in this regulatory balance may lead to some autoimmune diseases (Segal 1998, Bettelli 1998) or the toxic effects of inflammatory cytokine overproduction during infections (Gazzinelli 1996). However IL-10 may have a more complex regulatory role, as illustrated by responses in mice to *Schistosoma mansoni* egg antigens. Normal mice develop a dominant pathological Th2 response; IL-10 deficient mice develop a mixed response, so IL-10 is important for polarisation to type 2 responses; IL-4 deficient mice display diminished type 2

responses but do not default fully to type 1; mice doubly deficient in IL-4 and IL-10 develop polarised type 1 responses; mice doubly deficient in IL-12 and IL-10 generate exacerbated type 2 responses, while IL-12 depletion alone has little effect. Thus IL-10 can down-regulate type 2 as well as type 1 cytokine synthesis to limit pathology (Wynn 1997).

Other homeostasis mechanisms include macrophage production of TGFβ; also Fas/FasL or TNF/TNF-R interactions, which can lead to activation-induced T cell apoptosis (Wong 1997).

1.6 Multiple antigen processing pathways in immunity

Traditional dogma stated that there were two distinct types of antigen processing and presentation. Most CD4+ T cells recognise peptides derived from exogenous proteins endocytosed by professional APCs, which are degraded to peptide fragments in the acidic environment of late endosomes or prelysosomes and loaded onto MHC class II molecules. CD8+ CTLs generally recognise peptides derived from endogenous proteins presented in the context of MHC class I molecules. By this pathway, intracellular proteins are processed by the proteasome and the resulting peptide fragments transported into the lumen of the endoplasmic reticulum by membrane-associated transporters of antigenic peptides (TAP1 and TAP2). Once there the peptides, which are around 9 amino acids long and have appropriate termini, bind to nascent MHC class I complexes and are transported to the cell membrane (reviewed in Pamer 1998). The MHC class I pathway is operative in almost all cells and allows the immune system to monitor tissues for the presence of viral infections or tumours. If foreign rather than autologous peptides are bound to the MHC class I groove, then the cell is targeted for cytolysis by specific CD8 T cells. But MHC class I-restricted CD8 T cells also have a role in monitoring and protection against bacterial infections (Rock 1996).

Priming of an antigen-specific CTL requires two signals, one conferred by recognition of the appropriate peptide on MHC class I at the cell surface and the second by a non-specific costimulation signal. Provision of the second signal normally requires the presence of a professional APC, otherwise tolerance results. This scenario would be most easily achieved if the professional APC was directly infected by an organism residing in the cytosol, but this is rarely the case. Furthermore, to provide help CD4 cells must also recognise peptide/MHC class II complexes on the same APC. This may occur simultaneously so the CD4 cell produces IL-2 to stimulate the CD8 cell, or sequentially, with the CD4 cell first modifying the APC via CD40/CD40L interaction to

convert it from one liable to induce tolerance to one capable of supporting CTL priming (Ridge 1998). However some viral infections (e.g. lymphocytic choriomeningitis virus), certain antigens (e.g. Gram positive intracellular bacterium *Listeria monocytogenes* (Grewal 1997)) or DNA adjuvants (Sparwasser 1998) may bypass these requirements for CD8 CTL responses.

Recent information shows that in some circumstances exogenous proteins can prime MHC class I-restricted CTL responses, either after endocytosis of an antigen initially presented on somatic cells or after phagocytosis of an organism or particle-associated antigen. The terms cross-presentation and cross-priming have been applied to the acquisition of exogenous antigens and their presentation on MHC class I to naive CD8 T cells in LN (Carbone 1998). Cells capable of this include macrophages and DCs, although each may require special conditions to facilitate efficient presentation; also phagocytic mast cells and B cells may cross-present antigen in some cases.

Bacterial egression into the cytoplasm or pore formation may efficiently target exogenous antigens to class I rather than class II pathways (Darji 1995, Hess 1998), but this is not an essential event in all APCs. Leakage of digested proteins into the cytosol due to phagocytic overload (Reis e Sousa 1995) or through a phagosome to cytoplasm shuttle that feeds into the conventional TAP-dependent pathway (Kovacsovics-Bankowski 1995, Shen 1997) have been proposed. Stress proteins of the hsp70 and hsp90 family may facilitate class I loading by other pathways (Reimann 1997). Peptides may also be loaded externally onto the small number of 'empty' MHC class 1 molecules after regurgitation from phagosomes of the same or neighbouring cells (Harding 1996). Possibly endogenous and exogenous peptides may be exchanged during transit or recycling of surface-derived MHC class I molecules through the endolysosomal vesicles. A special case may exist for cross-presentation of antigens derived from engulfed apoptotic cells, although this may be limited to DC (Albert 1998).

MHC-Ib molecules present certain peptide motifs from endosomes and have a low degree of polymorphism but it is not certain whether they have a role in protective immunity. *M. tuberculosis*-reactive memory human CD8 T cell lines produced IFNγ when stimulated by infected DC, but this was not MHC I A,B,C or CD1 restricted and the processing route was unusual; consequently MHC-Ib may have been involved (Lewinsohn 1998).

Nonpolymorphic CD1 molecules are encoded outside the MHC region and are cell surface transmembrane glycoproteins that consist of a 43-49kDa heavy chain usually associated with β2-microglobulin. MHC class I and II molecules are not necessary for

CD1-restricted presentation. CD1 molecules are found on APCs and B cells. Generally the unusual binding groove may accommodate hydrophobic peptides or intact glycosylphosphatidylinositol (GPI) in mice, and in humans CD1 molecules are loaded endosomally with processed lipids and glycolipids containing mannosides and phosphatidylinositol (Moody 1997) like mycobacterial mycolic acid and LAM (Maher 1997). Several related CD1 molecules exist and they can elicit cytolytic/IFNγ responses or IL-4 production from responding T cells and NKT cells (Schofield 1999).

1.6.1 Dendritic cells and T cell stimulation

Macrophages can present exogenous antigen on MHC class I, especially when associated with pore forming proteins/organisms or live *Mycobacterium tuberculosis* (Mazzaccaro 1996), and much interest has recently centred on the ability of DC to perform this function. Uptake of bacteria but not latex beads can induce activation and maturation of murine DC, and epitopes from antigen expressed on the bacterial surface can be presented efficiently on MHC class I (Rescigno 1998).

The skin and mucous membranes are the anatomical sites where most exogenous antigens are normally encountered. Dendritic cells and Langerhans cells of the skin carry antigen to the draining lymph nodes, where they are potent activators of naive T cells. To mimic this, intradermal vaccination is most frequently used.

Dendritic cells in the periphery exist as immature cells. They capture antigen via macropinocytosis, receptor mediated endocytosis (e.g. mannose receptor) and phagocytosis (e.g. of apoptotic cells, using CD36 and an integrin and possibly other receptors), but have low motility and costimulatory functions. Processed antigens can be loaded onto MHC class I or II molecules (Shen 1997). DC can respond within a day to maturation signals (Winzler 1997) such as microbial components, cytokines and signalling molecules like CD40L; these trigger changes in chemokine receptors and migration via the afferent lymphatics to T cell areas of lymphoid organs, upregulation of molecules including B7, CD40 and ICAM-1, expression of cytokines such as IL-12 and TNFα and enhanced viability of the DC. However, these DC may induce cross-priming or cross-tolerance: this may depend on the method of antigen delivery to the DC, or possibly the nature of the DC involved e.g. myeloid DC may prime while lymphoid DC may tolerize the CD4 or CD8 cells (Albert 1998).

Efforts have been made to identify the source of tissue DC. Monocytes continuously emigrate from the blood into tissues and even in the absence of stimuli, DC traffic through lymph. In an *in vitro* model, monocytes could cross an endothelial monolayer,

but by 48 hours later half had reverse-transmigrated back across the layer (as if entering lymphatics). If phagocytic particles had been present, then many of these reverse-transmigrated cells had features of mature DCs, while the cells remaining sub-endothelial became macrophages (Randolph 1998). Thus *in vivo* migration and stimulation may rapidly direct differentiation to a DC phenotype.

In culture it has been demonstrated that monocytes when cultured with macrophage-CSF differentiate into macrophages while when cultured with granulocyte macrophage-CSF and IL-4 differentiate into immature DC. However, the phenotypes are somewhat reversible, although maturation leads to terminal differentiation in DC populations (Palucka 1998). If IL-10 is present during maturation of dendritic cells, the resultant cells are more macrophage like and secrete less IL-12 and have impaired capacity to induce a Th1 response (de Smedt 1997). DC may also secrete high levels of the chemokine IL-8 (Buelens 1997). The cytokine environment has been shown to be relevant *in vivo*: strong expression of either GM-CSF or IL-8 in LN granulomas is correlated with the presence of florid granulomatous lesions or the presence of neutrophil infiltration respectively (Bergeron 1997).

1.6.2 Possible roles for all presentation pathways in mycobacterial infections

Such flexibility in antigen presentation is essential in protection against infection with mycobacteria, which have many mechanisms to avoid killing by macrophages (Portillo 1995). Following phagocytosis, bacilli are directed to phagolysosomes, although some may prevent fusion or acidification (Sturgill-Koszycki 1994 and Xu 1994) and cause maturational arrest (Clemens 1996). This may occur if the macrophage was not rapidly activated and possibly allows mycobacteria to survive in the macrophage and contributes to failure of antigen presentation.

It is possible that mycobacteria evade recognition during chronic infection by sequestering their antigens away from sensitised CD4 T cells (Pancholi 1993), which would otherwise produce cytokines such as IFNγ and IL-2 and activate macrophages and non-MHC restricted NK and LAK cell cytotoxicity (Ravn 1994). *M. tuberculosis*-induced down-regulation of MHC class II antigens can be somewhat offset by simultaneous enhancement of presentation of antigens through MHC class I (Mazzaccaro 1996), along with the upregulation of accessory molecules such as B7.1 and 2 and CD40 upon stimulation with live mycobacteria (Henderson 1997), which would activate CD8 T cells. Specific memory CD8 cells can start to secrete IFNγ within

hours of antigen stimulation, and such cells have recently been identified in tuberculosis patients and contacts directed against mycobacterial products (Lalvani 1998), and cell lines later developed cytotoxicity. Susceptibility to *M. tuberculosis* in β2microglobulin deficient mice, which developed caseating necrosis and high bacillary load in the lungs even in the presence of granulomas with normal levels of TNFα and IFNγ (Flynn 1992), suggested the CD8 T cells' role in protective immunity was to lyse infected macrophages that were failing to eliminate the bacilli (Orme 1993). Thus, the function of CD8 cells as a source of IFNγ (especially if the CD4 T cell source of IFNγ is absent) may be as important as their cytolytic activities (Tascon 1998, Cooper 1997, Laochumroonvorapong 1997). It is unclear whether or not all modes of CTL cytotoxicity and macrophage death will reduce bacillary viability (Oddo 1998, Stenger 1998) and whether these apply *in vivo* as well as *in vitro*; some mechanisms may only become important after failure of others.

Additionally, CD1-restricted T cells may have a role, although some infected APCs have disrupted endosomal acidification and may down-regulate their CD1 (Stenger 1998a). Exogenous LAM may be taken-up into newly recruited macrophages and DCs via the mannose receptor, and transported to the endosomes to encounter CD1 molecules for presentation to broadly specific CD1-restricted T cells. These can express CD4 or CD8 molecules or be double negative; they produce IFNγ and show specific cytotoxicity, although the co-receptor expression may correlate with the use of degranulation/perforin or Fas-FasL mediated killing (Stenger 1997).

Consequently control of the infection by adequate macrophage activation (by IFNγ and TNFα) or cytotoxic lymphocyte activity may still be achieved, by utilising at least one mechanism of antigen presentation.

1.7 Vaccines

1.7.1 Attenuated vaccines

The historical success of attenuated organisms to induce an immune response resulting in protection from pathogenic infection has had great influence on modern vaccines. Attenuated organisms are avirulent yet still capable of infection and growth and can induce an immune response. The organisms themselves influence the polarisation of the specific immune response initiated and they are chosen to ensure this will be protective against subsequent challenge with the pathogenic form. Some 'attenuated' vaccines are simply close relatives of a pathogen which do not grow well in

human hosts or alternatively, a pathogenic organism may be weakened by serial passage through a non-optimal culture system. Because attenuated microbes grow in the host, they generate a long-lasting immune response to appropriate surface and secreted antigens. Attenuated vaccines are relatively easy to produce in bulk, unlike subunit vaccines. Furthermore many subunit vaccines require expensive multiple boosts.

However, the greatest disadvantage of attenuated vaccines lies in the potential for insufficient attenuation of the organism (e.g. some early Sabin poliovirus vaccines had insufficient genetic mutations), especially in the face of weakened immune responses. Inactivated virus (e.g. Salk poliovirus vaccine) is more expensive because of the greater antigenic mass required and may be less effective.

Most vaccines are to 'acute' infections; chronic infections are more of a challenge, partly due to a lack of understanding of the nature of infection and persistence.

1.7.2 Designer vaccines

When attenuated or killed forms of the pathogenic agent are not practical or fail to give protection, individual protein subunits can be selected for use and a vaccine designed.

Several aspects are considered in the following sections

1.7.2.1 The relevant protective antigen(s) or epitopes, which may be a complex mix

Peptides/subunits are safe and stable, but costly to produce and purify. Problems include glycosylation and discontinuous epitopes and the requirement for a carrier protein. Multiple epitopes may be required to accommodate the diverse MHC class I and II binding sites in the human population.

1.7.2.2 The immunological mechanisms required and the anatomical site where the response is needed

Vaccines aim to generate a large pool of memory T and/or B cells, although some (e.g. BCG) can boost innate, non-specific responses as well. Different routes of administration can be used to generate different responses, as intravenous or intraperitoneal vaccination may result in B cell tolerance and T cell suppression, while the subcutaneous or intradermal route can induce dose dependent DTH. The physical form and the dose of the antigen also influence polarisation.

The mucosal membrane surface is greater than the surface of the skin. It has a distinct immune system divided into inductive areas, such as gut Peyer's patches and nasal-associated lymphoreticular tissue, and effector tissues including lamina propria of respiratory and reproductive tracts and glandular tissues. Successful induction of the

mucosal immune response is effective both in preventing infection at mucosal surfaces (via secretory IgA) and in triggering a systemic immune response including CTL activity, although high doses are required to prevent tolerance e.g. oral delivery of live attenuated Sabin polio vaccine.

1.7.2.3 A safe adjuvant

An essential role of adjuvants is to direct T cell subset differentiation towards protective responses after immunisation (Linblad 1997), since vaccination with protein alone can lead to tolerance. Adjuvants function in several ways, including by acting as a depot to slow the release of antigen, targeting antigen to macrophages/DC and activating them, and inducing chemokines and T cell stimulatory cytokines. Many of the most effective adjuvants include bacteria or their products e.g. mycobacterial cell walls. However, it may be advantageous to separate adjuvant properties from some of the inhibitory molecules or those that induce unacceptable side effects. To this end, cytokines may augment or replace older adjuvants, e.g. inclusion of IFNγ in soluble protein vaccines against *L. major* in susceptible mice enhanced protective immunity but an adjuvant (*Corynebacterium parvum*) was still required for protection (Afonso 1994). However, 0.1-1μg of IL-12 could replace both *C. parvum* and IFNγ in the vaccine, influencing CD4 Th1 cell development and inducing IFNγ responses. Inhibition of IL-12Rβ2 transcription by soluble antigen is prevented by the presence of IL-12 (Galbiati 1998). Some caution is necessary with IL-12 administration however, as it can initially induce a period of immune suppression (Kurzawa 1998). It may become possible to express a cytokine from genes carried by a recombinant organism, either with an antigen as part of a vaccine, or alone to locally alter the cytokine balance (Rolph 1997).

1.7.2.4 A novel delivery system

Novel vaccine delivery systems can be categorised for simplification into particulate vaccine carriers, recombinant vaccine vehicles and gene vaccination.

Particulate vaccine carriers. These include virus-like particles, liposomes and immune stimulating complexes (ISCOMS). These are used to enhance the immunogenicity of sub-unit antigens. Linking an antigen to synthetic microspheres targets presentation by phagocytic cells but does not polarise the specific immune response to Th1 unless IL-12 is coadministered (Sedlik 1997). Interestingly, *M. tuberculosis* and *C. parvum* stimulate IL-12 production by macrophages and this may be a key component to their adjuvanticity for CMI.

Recombinant vaccine vehicles. Nucleic acid sequence for the antigen is inserted into the genome of a virus (e.g. vaccinia virus, adenovirus; reviewed in Rolph 1997) or bacterium (e.g. BCG, Salmonella; reviewed in Stover 1994, Staats 1994) from which protein is expressed. This may preclude the need to purify the antigen and association with the pathogen may improve immunity.

Most recombinant vaccines are live, as these are believed to be more immunogenic: replicating virus induces both humoral and CD8 cytotoxic T cell responses; T cells from viable BCG-immunised mice are stimulated to produce IFNγ (possibly correlating with protection, Kawamura 1992) from a wider range of mycobacterial products than T cells from killed BCG-immunised mice (Kawamura 1994) although other mycobacteria may be active when killed. Also, levels of expression of the foreign antigen may be more important than the persistence of the bacterial vehicle in tissues (Cardenas 1993).

However, a difficulty with live recombinant vaccines arises in maintaining the presence of the insert, both pre- and post-vaccination. This is often achieved by antibiotic selection, but the possibility of introducing antibiotic resistance to environmental organisms may favour development of gene complementation systems. Similarly, the safety of attenuated recombinant vaccines, especially in immunocompromised hosts, creates problems with licensing.

Gene vaccination. This is the induction of immune responses to antigens encoded by and delivered as plasmid DNA (Tighe 1998), usually given intradermally or intramuscularly. It is effective at inducing MHC I-restricted cytotoxic T cells (Lowrie 1997, Bonato 1998), as there is intracellular synthesis of antigen, in dendritic and other cells. Additionally it induces CD4 Th1 cells that secrete IFNγ. Small amounts of antigen are produced but the plasmid DNA backbone acts as a Th1 adjuvant, via the CpG motifs and their flanking sequences, which are frequently palindromic hexamers following the formula: 5'-purine-purine-CG-pyrimidine-pyrimidine-3'. These immunostimulatory sequences (ISS) induce type 1 cytokine production (IFNs, IL-12 and IL-18 from macrophages, IFNγ from NK cells and thence more IFNγ from the antigen-driven Th1 cells). Little antibody is induced unless protein is co-injected with DNA containing ISS. The type 1 inducing activity of DNA immunisation is dominant to a Th2/IgE response, so it may also have applications in therapy of allergic diseases.

1.7.3 Considerations for vaccine design

1.7.3.1 The requirement for a type 1/ type 2 balance?

In some circumstances, Th1 and Th2 play a complementary role (Taylor-Robinson 1993). Plasmodium in mice causes acute primary parasitaemia around day 10 and another (recrudescence) after 25 days. During the blood stage of malaria, both types of immune response are present and both confer protection. CD4 Th1 cells secrete IFNγ and give protection by a nitric-oxide dependent mechanism (especially during primary parasitaemia), whereas CD4 Th2 cells protect by the enhancement and accelerated production of specific IgG1, during secondary infection. This pattern of differential dependence on Th1 and Th2 responses during initial and subsequent infections may also be true in humans. While lymphocytes from human subjects with single or few past malaria infections produced high levels of IFNγ in response to malaria antigen, lymphocytes from adults exposed since birth to malaria displayed poor IFNγ production. This reduced IFNγ production could be beneficial in avoiding the immunopathological reactions associated with TNF overproduction, especially in cerebral malaria.

Viral infections often result in increased CTL activation, cytokine production by Th1 cells and complement fixing, cytotoxic IgG2a antibodies (Romagnani 1994). Responses to hepatitis viruses tend to be Th1, and the TNFα and IFNγ favour elimination of virus-infected hepatocytes, but also increase the sensitivity to destruction of normal hepatocytes and thus contribute to both viral clearance and liver cell injury in viral hepatitis. Similarly, in mice with respiratory syncytial virus, combinations of CD4 Th2 and Th1 cells and cytolytic CD8 T cells were required to reduce the viral load, while Th2 responses alone caused severe disease. The correct balance may be difficult to achieve in a vaccine.

1.7.3.2 Vaccines and immunotherapy against HIV: should they preferentially induce type 1 responses?

Prevention of the spread of HIV is difficult. Education has had limited usefulness. Consequently multidrug therapy of infected individuals remains vital (Aldhous 1995), as production of virus has been shown to be extremely high throughout infection (Wei 1995, Ho 1995). However rapid drug resistance and high expense restrict the use of drugs.

Some resistance to HIV is due to mutations in the HIV co-receptors (Abel 1997). Macrophage tropic strains use the chemokine receptor CCR5, while T cell tropic

viruses, frequently found in late stage HIV disease, also utilise CXCR-4. A change in coreceptor usage is associated with progression to AIDS (Connor 1997). Truncation of CCR5 is associated with reduced transmission risk and delayed progression (Michael 1997), but some subjects can be infected using alternative co-receptors.

CD4 responses (Rosenberg 1997) and CD8 CTL responses (Cao 1997) are stimulated during HIV infection and have a role in limiting viral replication: following early viraemia, some early down-regulation of viral load occurs although it is not sterilising (Pantaleo 1994). As the T cell response declines later in infection, viral load again rises and HIV mutants that escape CD8 T cell recognition are selected during infection. Some healthy exposed adults generate HIV-specific T cell responses (Shearer 1996).

However, it is unclear what causes the loss of T cell numbers during HIV infection. Although a large number of virions are produced each day, this may not involve cytolysis of a large number of infected CD4 T cells and their replacement may only require a few-fold increase in normal turnover. However, CTL activity and bystander mechanisms may add to the depletion, HIV may interfere at the level of T cell development or the limited renewal rate of the immune system may not keep pace with the chronic cell loss (Wolthers 1998). Clerici (1994) originally claimed a Th1 to Th2 switch triggered progression to AIDS, but chronic activation of many cell types (Graziosi 1994 and 1994a, Alonso 1997), apoptosis and disregulation of the immune response occurs, even in early in HIV infection (Fakoya 1997, Romagnani 1994a, Meyaard 1994), with increasing numbers of Th0 cells being generated (Maggi 1994) and macrophages specifically producing less IL-12 to some stimuli (Chehimi 1994). Furthermore, infected dendritic cells and follicular dendritic cells may infect migrating CD4 T cells and/or cause immune disregulation (Grouard 1997).

Interactions with other infections may also play a role (Bentwich 1995). Opportunistic infections occur in a specific order: immunity to tuberculosis is compromised early and TNFα production possibly contributes to HIV pathogenesis (via NF-κB) and raised susceptibility to malignancies occurs later, after depletion of the naive T cell precursor pool (Mosier 1994, Strobel 1995). If late in HIV infection effective antiretroviral therapy is started, some illnesses resolve and CD4 T cell numbers can increase but this may be due to an expansion of existing cell types, rather than a restoration of the T cell repertoire, as naive and memory populations may be regulated separately. Thus it may be important to start drug therapy early in HIV

infection and compliment it with immune stimulating therapies, such as IL-2, while the immune system still retains the potential to be fully reconstituted (Emery 1997).

Vaccines have been the major weapons against many diseases, but neutralising antibodies to HIV gp120 cannot reliably be induced (Fust 1995, Kohler 1995, Poignard 1996) and vaccination trials failed to show efficacy (Roberts 1994, Brown 1994). A newer 'prime-boost' strategy where the immune response is primed with a recombinant viral vector, followed by boosting with the corresponding recombinant protein, may improve vaccine efficacy (Rolph 1997). It may be necessary to use vaccines inducing mucosal immunity, but these still remain unreliable (Lu 1998). Various live attenuated HIV vaccines have been proposed (Bonhoeffer 1995) as they elicit a longer lasting, broader protection than inactivated or subunit vaccines; however, the risk in the normal population is too great.

If Th2 dominance may be associated with rapid progression (Del Prete 1995), vaccination to selectively induce type 1 immunity to HIV may be preferred. Addition of cytokines such as IL-12/GM-CSF or IL-12/TNFα to an HIV-1 peptide and adjuvant vaccine could promote CD8 CTL or Th1 responses respectively (Ahlers 1997) and induction of CTL or type 1 immunity may be achieved by recombinant viral or bacterial vaccines.

An alternative may be an immunotherapy that maintains general Th1 responses and corrects the aberrant immune activation. The aim would be to extend the symptomless phase indefinitely and help HIV infected individuals to remain clear of opportunistic infections for longer.

1.7.3.3 Vaccines that substitute for natural childhood infections in general education of the immune response

Allergy has been increasing in developed countries over recent years. It is possible that environmental factors are playing an additive role with genetic susceptibility. Increased pollution may bring forward the onset and exacerbate symptoms of asthma and allergy, but may not be responsible for the increased prevalence. The latter could instead be related to the strong reduction of childhood infections, including measles and tubercular infections (Erb 1998), which induce the production of cytokines antagonistic to Th2 development. Differences in intestinal microflora might affect the development and priming of the immune system in early childhood (Bjorksten 1999). Even the effect of common mild infections, such as those frequently picked up when a child attends a nursery or is part of a large family, may help reduce atopy if exposure is from a young age (6-11 months) (Kramer 1999).

Vaccines also may replace recovery from infections with a different, even opposite, type of stimulus e.g. influenza vaccines. Additionally, saprophytes and gut microflora may educate the immune system and the density and character of these organisms can be affected by life style (such as hygiene) and oral antibiotics. Besides influencing the cytokine balance, the nature of the T cell repertoire itself may be regulated by exposure to infections; bacterial homologues of autoantigens, such as heat shock proteins, may even be important in regulation of autoimmunity. New vaccines could be designed not only to prevent infections but replace them with a beneficial equivalent educational stimulus to the immune system (Romagnani 1997, Rook 1998).

1.8 History of vaccination with BCG and *M. vaccae*

One third of the world's population is latently infected with *M. tuberculosis* (Bloom 1992). However, generally the immune response to tubercle infection is very effective; only 5% of infected persons develop overt primary disease and only a further 5% develop post-primary disease later in life, due to the presence of 'persisters', which often heals spontaneously. Knowledge of the protective immune responses to tuberculosis is incomplete; protective mechanisms are complex, involving orchestration of many cells and cytokines (as discussed in previous sections) which may lead to specific recognition and lysis of ineffectual infected cells, resulting either directly in death of the mycobacteria or their efficient uptake by fresh activated macrophages.

Necrosis also occurs as a result of the action of T cell and macrophage cytokines on sensitised infected tissues, but may still lead to control of established infection by lowering the oxygen tension and preventing bacillary multiplication. If the lesions erode into bronchi, the bacilli multiply rapidly extracellularly, but are susceptible to chemotherapy. If they are walled off with fibrous tissue, this induces a state of minimal metabolism and drug-reistance in the bacilli, which requires a long period of chemotherapy to overcome, with abnormalities still present in the immune response.

A person dually infected with HIV and tubercle bacilli is at much greater risk of developing overt tuberculosis: 10% annually or 50% during their shortened life span and even then chemotherapy has reduced effectiveness. A *combination* of cell mediated immune responses (involving IFNγ) and chemotherapy is required for elimination of pathogenic mycobacteria (Jouanguy 1996). Therefore the aim of vaccination or immunotherapy is to boost the protective form of immune response (Stanford 1994b).

BCG (Bacillus Calmette Guerin) was attenuated between 1906-1919 from *M. bovis*, but was never cloned and hence modern 'strains' are not bacteriologically identical.

They were first used in the 1920s, amongst doubts as to their safety due to contamination with virulent tubercle bacilli. However, BCG gained widespread use in the 1950s and 1960s, except in Holland and USA. Schedules varied from one dose at birth or 13 years to multiple vaccinations in Eastern Europe. BCG vaccines are the most widely used and the most controversial, but the extensive use of BCG (more than 3 billion doses worldwide in four decades) means that they have had substantial impact on tuberculosis and leprosy (Fine 1988). Since BCG was derived from neither tubercle or leprosy bacilli, the protection is heterologous.

Vaccination with BCG has a highly variable record as a protective measure against both these mycobacterial diseases. Protection ranges from 80% (to tuberculosis in Britain and parts of America) to none or -50% (the latter reflecting increased susceptibility following vaccination) in India and other regions of USA. The last large trial in South India between 1968 and 1971 led to the conclusion that BCG had no overall protective effect, with protection of some volunteers and susceptibility of others.

Several different mechanisms may underly this variability: 1) interactions of BCG with the immune response to other naturally acquired mycobacterial infections (endemic and environmental). Different sorts of interactive effects may apply for different environmental mycobacteria, leading to the complex geographic trends. 2) differences between BCG preparations. However, the same vaccine gave different efficacies in leprosy trials in Uganda (80% protection) and Burma (none), whereas different vaccines both worked in Britain. 3) protection by BCG may depend on the strain of subsequently infecting TB and its incidence amongst the population (presence of boosting by natural infection). 4) BCG shows better protection against certain forms of disease (miliary/meningitic) and mortality (Colditz 1994). 5) There may also be genetic control of host responses, nutritional differences, or methodological differences in trials.

Bretscher suggested an improvement to current BCG protocols by analogy with *Leishmania major* (Bretscher 1992 and 1992a). The dose of antigen given in a vaccine is crucial to determine the type of immunity generated. Strains of mice (CBA/J) resistant to *L. major* mount a DTH response on challenge with both a large or small dose of parasites, but mice susceptible to *Leishmania major* (Balb/c) mount different kinds of immune response, depending on the number of parasites with which they are infected. Small numbers induce a sustained DTH response, whereas larger numbers induce antibody. Intermediate doses often caused induction of immunity sufficient to contain the spread of the parasite, but insufficient immunity to eliminate it. Several months after low dose exposure the susceptible mice are resistant to a stronger

challenge, producing IFNγ and an increased IgG2a/IgG1 ratio. Overt DTH was lost even while mice remained resistant and regained upon challenge. Importantly, the innoculation of a low dose of parasites into mice belonging to 'resistant' strains increases their long-term resistance. Thus a low dose vaccination procedure is effective independently of the genetic background of the host. In the case of a 'live' vaccine, if the low dose effective in susceptible mice is below the immunogenic threshold for DTH in resistant mice, the low dose will not be checked until the organisms reach a number immunogenic for a cell-mediated response in each individual. However a high dose will automatically result in some individuals producing antibody or a mixed response which may increase their susceptibility. This may explain some of the BCG failures, since the general policy was to use the largest acceptable dose. Possibly administration of lower doses would improve its efficacy.

The cross-reactive protection of BCG against leprosy demonstrated the potential for vaccines of more distantly related organisms e.g. most of the fifty or so species of mycobacteria exist as harmless environmental saprophytes, the balance of species present depending on the local conditions. Mycobacteria are not part of the normal human bacterial flora; thus the nature, route and extent of exposure to them depends on where a person lives, lifestyle features, the purity of the water and other local factors.

If administration of standard doses of BCG simply boosts the type of response present rather than changing it, the state of immunity existent before vaccination would determine the efficacy of BCG. The earliest responses may be to the common antigens (including heat shock proteins, LAM and some low molecular weight secreted antigens) shared by all mycobacterial species, Nocardiae and some Corynebacteria and it is suggested that these responses form the basis of protective immunity. Some group antigens are found either on slow or fast growing species, while all mycobacteria have specific antigens limited to their own species (Stanford 1983).

While post BCG tuberculin reactivity is not a direct correlate of immunity to tuberculosis, small non-necrotising responses to tuberculin in persons who have not received BCG do correlate with protection. It was observed that healthy persons in contact with either tuberculosis or leprosy react in skin testing to agents prepared from any species of mycobacterium, indicating recognition of shared epitopes, while patients themselves respond to a limited range of reagents, indicating recognition of species-specific epitopes but diminished responsiveness to the shared epitopes. Slow-growing mycobacterial species can initiate necrotic Koch responses, which are much less commonly developed to fast growers. Non-necrotising responses may form to of some

fast growing environmental mycobacteria, but if protective immunity is broken by infection with a pathogen like *M. tuberculosis*, responses to common antigens are lost or suppressed and a Koch response develops to the species specific antigens of *M. tuberculosis*.

From this model, frequent contact with potentially pathogenic environmental slow growing species such as *M. scrofulaceum* (Burma) and *M. intracellulare* (South India; BCG trials in both countries showed a lack of protective immunity), can induce Koch responses and once this has occurred further development of protective responses is blocked and BCG vaccination is ineffectual. However, where BCG vaccination works, children without a prior Koch response can be protected from developing one subsequently. Upon encountering tubercle bacilli, they develop protective, non-necrotic tuberculin tests. Thus contact with different species of mycobacteria in the environment may have two distinct effects, one promoting type 1 responses and protective immunity and the other the Koch phenomen, thus predetermining the effects of subsequent BCG vaccination.

1.8.1 The selection of *M. vaccae* for investigation as an immunotherapeutic agent and vaccine.

The selection of the fast growing environmental mycobacterium *M. vaccae* was based upon the following criteria:

- It was inducing skin-test positivity in an area where BCG vaccination was effective (Lake Kyoga in Uganda), and was therefore supposed to evoke the appropriate protective type of response (now known to be a 'pure' Th1 with cytotoxic T cells).
- It was relatively poor in the irrelevant species-specific antigens (identified in immunodiffusion) but rich in the common antigens that are the major targets of the protective response (such as hsp, and the 30kDa mycolyl transferases)
- Killed preparations were found to be more immunogenic in animals than live ones. A new live mycobacterial vaccine would be difficult to test in humans. This property is unique to *M. vaccae* and *M. leprae*. Other mycobacteria tend to evoke Th2-biased responses if injected as killed preparations.
- Soluble antigen from *M. vaccae* inhibited the Koch phenomenon when mixed with tuberculin used for Mantoux testing.
- The killed preparation was found to induce early recognition of *M. leprae* in the mouse foot-pad model and immunisation of people with *M. vaccae* (with and

without BCG) caused enhanced recognition of pathogens such as *M. leprae* upon later contact.

More recent work (apart from that which forms the results section of this thesis) has shown that killed *M. vaccae* has additional properties that are relevant to its exploitation as an adjuvant and immunotherapeutic agent:

- It can non-specifically down regulate pre-existing Th2 responses (i.e. to ovalbumin), by a mechanism that is apparently independent of its ability to induce Th1 (Wang 1998).
- It induces CD8 cytotoxic T cells that kill macrophages infected with *M. tuberculosis* (Skinner 1997)
- If injected intraperitoneally with a protein antigen (ovalbumin) it causes the development of cytotoxic CD8 T cells that will lyse recombinant cell lines expressing ovalbumin (Watson 1998; also announced at a conference, Skinner 1997a) A range of other mycobacteria fail to do this when used as killed preparations in the same system (*M. phlei, M. smegmatis, M tuberculosis*, BCG).
- It primes a large population of CD8 T cells that recognise hsp65, and home to the lung when hsp65 is injected into the trachea (Hernandez-Pando and Wang, unpublished data).
- Very low doses (10^7 killed organisms; 10μg wet weight) that stimulate a 'pure' Th1 response and downregulate the background Th2 activity, protect Balb/c mice against intratracheal infection with *M. tuberculosis* (Rook 1996).

The *M. vaccae* vaccine strain is a rough, mucoid scotochromogen R877R (NCTC 11659). It is grown on Sauton's medium solidified with 1.5% agar, at 32°C. At the end of logarithmic growth, it is scraped from the surface of the medium, weighed and suspended in M/15 borate-buffered saline at 10mg wet weight per ml and suitable volumes are autoclaved at 121°C for 15 minutes.

Use as a vaccine, begun in 1978, was replaced by use as an immunotherapy along with chemotherapy against leprosy in 1981 and later against pulmonary tuberculosis. It was hoped that *M. vaccae* could suppress tissue destruction, reintroduce protective immunity and lead to eradication of persisting bacilli, thus shortening drug treatment. A dose of 10^9 or $2x10^9$ autoclaved baccilli appeared to be effective in pilot studies in humans (not carried out to GCP standard) and induced little local side effects (reviewed by Grange 1994, Stanford 1991 and Stanford 1990). The lower dose, sometimes given

repeatedly, has since been adopted. There may also be scope for 'preventive immunotherapy': a vaccination given to contacts of tuberculosis patients to promote elimination of infecting bacilli and protect from reinfection (Stanford 1991a).

1.9 Recent uses of *M. vaccae* immunotherapy

About 30 million people in the world today are infected with *M. tuberculosis*. Of these, 10 million are significantly incapacitated and 3 million are estimated to die every year. Leprosy affects a further 15 million. The worldwide increase in tuberculosis demands novel therapies that can reduce the duration and cost of chemotherapy as the current 6 month schedule is not practical proposition in the many countries with wars or collapsing health care systems (Stanford 1991a). There is also an increasing need for therapies that can substitute for chemotherapy in patients with multi-drug resistant disease and that are safe for HIV infected recipients (Weltman 1993, Fine 1994).

1.9.1 Pilot studies in tuberculosis

M. vaccae has been used in trials as a potential immunotherapeutic agent in pulmonary tuberculosis where it is given as a single injection to patients within the first few weeks of starting chemotherapy. Pilot studies suggested that it can improve the cure rate and reduce the number of deaths (Stanford 1994). In one African trial, immunotherapy helped more patients survive chemotherapy and put on weight faster, and rapidly reduced their shedding of bacilli (73% receiving additional immunotherapy versus 19% with placebo, became sputum negative for acid-fast bacilli by 3 weeks after onset of available chemotherapy) even against a background of poor medication and coinfection of some patients with HIV (the latter underwent resolution of generalised HIV lymphadenopathy along with improvement of tuberculosis) (Onyebujoh 1995, Stanford 1993). Many trials were performed in centres where tuberculosis was severe, drug resistance was widespread, conventional therapy was not optimal and where there is a relatively high mortality rate. Therefore mortality rates and rate of sputum clearance were available as outcome measures.

In properly treated newly diagnosed pulmonary tuberculosis, chemotherapy can be very effective and there may be only small differences between patient groups receiving placebo and *M. vaccae* (Corlan 1997a). It is even more difficult to show efficacy in developed countries where mortality is very low and sputum often minimal, and proxy indicators of disease activity, such as weight, chest radiographs, erythrocyte sedimentation rates, C reactive protein, and soluble IL-2 receptor levels must be used.

These have a high natural variability and outcomes may lack statistical significance (Kon 1998).

However, pilot studies suggest that *M. vaccae* immunotherapy may be useful in drug-resistant tuberculosis (Etemadi 1992, Stanford 1994a, Corlan 1997). Improved outcomes included bacteriological success and resolution of chest lesions. Primary drug resistant disease may respond to a single injection of *M. vaccae* while chronic acquired resistance is harder to treat, requiring more injections and having a lower success.

1.9.2 GCP trials in tuberculosis and/or HIV

Some of the early trials suffered from methodologic problems including small sample sizes, non-random treatment allocation, high losses to follow-up and use of varying drug regimens. Further trials were carried out to GCP standard.

A phase III trial of *M. vaccae* immunotherapy as an adjunct to chemotherapy in tuberculosis in the Republic of South Africa, carried out under strict conditions of volunteer entry and monitoring of medication failed to show differences between placebo and active groups, although some analysis is still awaited.

A phase I/II randomised, placebo-controlled trial of *M. vaccae* as an adjunct to chemotherapy in drug-susceptible pulmonary tuberculosis (without HIV) was performed in Uganda, with 120 subjects. There was evidence of a possible early increase in sputum culture conversion and greater improvement in radiographic extent of disease at the end of treatment and at 1 year follow-up among *M. vaccae* treated patients (J.J. Ellner). X-rays at this late stage of follow-up were not available in the South African trial.

Multiple doses of *M. vaccae* were shown to be safe in HIV infected adults (Marsh 1997, von Reyn 1998) and children (Johnson 1999) and these patient groups may be important due to their increased susceptibility to mycobacterial diseases.

A phase III trial in Zambia/Malawi of immunotherapy in tuberculosis patients with and without HIV is ongoing and will recruit at least 1200 patients. There are no results available.

1.9.3 Use in other conditions

Patients with diverse diseases such as active tuberculosis, lepromatous leprosy, inflammatory bowel disease, rheumatoid arthritis, HIV and chagas' disease (due to *Trypanosoma cruzi)* lack responses to common antigens (Bottasso 1994) and may share similar underlying pathology. Possibly reconstituting these responses would improve their health by reducing immunopathology and inducing a 'switch' from type 2 or mixed responses towards protective type 1 T cell responsiveness (Grange 1994). By a

similar mechanism, suppression of IgE and IL-5 synthesis after *M. vaccae* treatment in a murine allergy model suggests it may have a clinical application in the immunotherapy of allergy (Wang 1998).

40 adults with grass pollen allergy and summer asthma were included in a placebo controlled trial of a 3 dose protocol of *M. vaccae* immunotherapy. Subjects receiving *M. vaccae* had fewer asthmatic symptoms and less need for a bronchodilator inhaler (Hopkin 1999). Also their production of IFNγ in response to tuberculin was increased.

BCG and mycobacterial DNA have been used as a preventative or therapy for cancer in animal models with variable success (Shimada 1985), depending on site of vaccination and tumour type. In humans, only in countries where neonatal BCG vaccination affords protection from tuberculosis is there also protection against leukaemia and other childhood cancers. In a manner similar to the Koch phenomenon in tuberculosis, some tumours would effectively and safely be killed by induction of necrosis of an entire region of tissue; but others may be more effectively removed by direct killing of tumour cells. Thus there may be a role for enhanced recognition of these tumour cells induced by *M. vaccae* immunotherapy; theoretically this may occur via improved type 1 and decreased type 2 responses in patients, along with increased recognition of heat-shock or cancer proteins leading to efficient CTL activity (Grange 1995). *M. vaccae* also lacks some risks associated with use of live BCG. Results using *M. vaccae* as an adjuvant in trials of prostate cancer in rats (Hrouda 1998) or using multiple doses of *M. vaccae* alone in phase I/II trials in human prostate cancer (Hrouda 1998a) were encouraging, as were those of a phase I/II trial of immunotherapy in malignant melanoma (Maraveyas, submitted). An improved prognosis after immunotherapy compared to historical controls was linked with sustained increase in the percent of blood lymphocytes producing IL-2 *in vitro*.

In NSC lung cancer or mesothelioma patients receiving chemotherapy, repeated injections of *M. vaccae* were well tolerated and there was a small but significant increase in mean survival of the patients (Webb 1998). A larger phase III GCP trial is beginning and the *M. vaccae* may also be tested as an adjuvant with allogenic cells lines.

1.10 Aims of the project

1. To establish the type 1 nature of the immune response to autoclaved *M. vaccae*.
2. To study the nature of the immune response to recombinant *M. vaccae*; specifically, its ability to evoke type 1 responses to an SIV protein.
3. To identify early events in lymphoid tissue draining sites of injection of autoclaved *M. vaccae*
4. To study the profile of cytokines released from macrophages in the presence of *M. vaccae*, with particular emphasis on IL-12, since it is a potent inducer of Th1 responses.
5. To seek potential potency assays needed for future commercialisation and standardisation.
6. To use the selected immunological parameters to compare manufacturing methods and study the sensitivity of the vaccines to denaturation.

Chapter 2 Materials and Methods

2.1 Materials and methods used in molecular biology

2.1.1 Cutting vectors and inserts with restriction enzymes

Type II restriction enzymes (RE) bind specifically to double stranded DNA sequences that are four, five or six nucleotides in length and display twofold symmetry. Some cleave at the axis of symmetry and create blunt ends, while others cleave each strand at similar locations on opposite sides of the axis, creating fragments of DNA that carry protruding single-stranded termini. *Pst1* cut as follows:

$^{5'}$NNCTGCA pGNN$^{3'}$

$^{3'}$NNGp ACGTCNN$^{5'}$

Isoschizomers *Pst1* and *Nsi1* cleaved within slightly different target sequences but produced identical protruding termini, i.e.

CTGCA G *Pst1* or ATGCA T *Nsi1*.

However, ligation of the different fragments created hybrids that could not be recognised by either enzyme.

RE (New England Biolabs) were stored at -20^{0}C at around 10units/µl in a buffer containing 50% glycerol. 1 unit is defined as cutting 1µg of DNA in 1 hour at 37^{0}C.

For vector or fragment preparation, a 50µl final volume contained 10µg of purified DNA, 30 units of stock RE and the appropriate 1x buffer (for ECoRV and Cla1: buffer B and Pst1: buffer H (Boehringer Manheim)), in a sterile eppendorf. For screening purposes, 20µl samples contained 1-2µg of DNA, 2-5 RE units and diluted buffer. Samples were incubated for 90 minutes (Grant heating block BT1) and then 500ng of the DNA was checked on an agarose gel.

2.1.2 Wizard DNA clean up system

Various enzymes required different buffering conditions or salts and when requirements were not compatible, the DNA was purified. Purification also removed small nucleotides from the system. Wizard kits relied on binding and elution of appropriately sized DNA from a resin column and rapidly purified up to 10µg of DNA in solutions of between 50 and 500µl.

The DNA sample was mixed with 1ml of Wizard (Promega) DNA-binding resin and pipetted into the Wizard minicolumn. This was washed with 2ml of 80% isopropanol

and the column centrifuged (MSE eppendorf centrifuge) for 20 seconds at 12,000g to dry the resin. 30-50µl of prewarmed (65-70^{0}C) water or TE was added, incubated for 1 minute and centrifuged over an eppendorf tube to elute the DNA.

Although DNA will undergo some degradation in the absence of a buffering agent, water was preferable to TE (10mMTris/HCl, 1mM EDTA pH8) when further manipulations were planned.

2.1.3 Calf intestine alkaline phosphatase treatment of cut vector DNA

This phosphomonoesterase hydrolysed 5'-phosphate groups from DNA to prevent circularisation of fragments cut with a single RE or with blunt ends. Then ligation would only occur between intact 5'P of the insert and the 3'OH of the vector, although the insert could still be incorporated in either direction.

Dephosphorylation used 0.01 units of CIAP (GibcoBRL, stored at 4^{0}C) per pmol of DNA 5'-protruding ends at 37^{0}C for 30 minutes or up to 1 unit per pmol of DNA 5'-recessed (e.g. Pst1) or blunt (e.g. ECoRV) ends at 50^{0}C for 1 hour. In 10µg of a cut 5kb plasmid there were 5pmol of 5'-ends so 5 units were required.

Hence:

40µl vector (10µg)

5µl of 10x CIP dephosphorylation buffer

µl CIAP diluted to 1U/µl in enzyme dilution buffer containing $MgCl_2$ and $ZnCl_2$.

The sample was incubated at 50^{0}C for 1 hour, then purified immediately on a Wizard mini-column.

2.1.4 Amplification by PCR of SIVp17/27

The linear SIVp17/27 sequence to be inserted (about 750bp) was amplified from the DNA plasmid pKA27, provided by MRC ADP at more than 500µg/ml. As target sequences are amplified slightly less efficiently when they are carried in closed circular DNAs rather than linear DNAs, 1µl of template was used per reaction. The necessary restriction enzyme sites were incorporated onto the ends of the p17/p27 sequence by using 30mer primers (1600µg/ml, Pharmacia Biotech) with 'floppy ends', structured as follows: protective bases, RE site, priming sequence

These were either identical to the coding strand of RE and p17-start (Forward primers, anneal to non-coding strands) or backwards and complementary to the coding strand of RE and p27-end (Reverse primers, anneal to coding strands)

Primer F2 (forward)

5'CCCATGCATGAAACTATGCCAAAAACAAGT

Nsi1

Primer R2 (reverse)

5'CCCATGCATTCATAATCTAGCCTTCTGTCC

Nsi1

Primer F3 (forward)

5'CCCGATATCGAAACTATGCCAAAAACAAGT

ECoRV

Primer R1 (reverse)

5'CCCATCGATTCATAATCTAGCCTTCTGTCC

ClaI

Reaction mix (total 100µl, in sterile PCR mini-eppendorfs, without oil):

10µl	10x reaction buffer
16µl	dNTP (stock 1.25mM each)
0.6µl	primer F (stock 1600µg/ml)
0.6µl	primer R (stock 1600µg/ml)
1µl	template pKA27
2-4µl	$MgSO_4$ (stock 100mM)
69.8-67.8µl	dH_2O
0.5µl	Vent polymerase (New England Biolabs; added after the initial 5 minute denaturation at 95^0C, which removes any proteases and decreases non-specific annealing before the first enzymatic extension)

For ligation into pSMT3 use primers F3 and R1 which would yield the product:

5'CCC - *ECoRV* - p27 - *ClaI* - CCC

For ligation into p16R1/SOD use primers F2 and R2 which would yield:

5'CCC - *NsiI* - p27 - *NsiI* - CCC

The PCR settings (Perking Elmer thermal cycler) were:

Preheat	5 minutes	95^0C	
25 cycles:	1 minute	94^0C	(denaturation)
	1 minute	55^0C	(annealing)
	1 minute	72^0C	(extension)
Final cycle	10 minutes	72^0C	(extension completion)

After the PCR was complete, 5µl of product was run on a 0.7% agarose gel with DNA markers. Conditions were not optimised and several minor contaminating bands

were produced. Consequently the entire PCR mix was run on a preparative gel and the appropriate band was excised and eluted from the agarose.

2.1.5 Separation of DNA on agarose gels

Large agarose gels which could accommodate 2 rows of 14 well were run in a horizontal configuration (Horizon 11.14, Gibco)

Briefly, open edges of the casting tray were sealed with autoclave tape and the comb(s) positioned. 0.7-1% agarose was dissolved by heating in 1x TBE (10.8g tris base, 5.5g boric acid, 0.74g EDTA in 1 litre dH_2O). Once the solution had cooled to 60^0C, ethidium bromide (a fluorescent dye that intercalates between the stacked bases of DNA) was added to 1µg/ml (10mg/ml stock) and the solution poured into the tray on a level surface until 3-5mm thick. Once set, the tape was removed, the gel was transferred to the tank which contained sufficient 1x TBE buffer (without EtBr) to just cover the gel and the comb was removed.

Samples containing 50-500ng of DNA were mixed with 5x loading buffer (0.25% bromophenol blue, 40%(w/v) sucrose in water; the tracking dye migrates at the same rate as DNA 300bp long) and 3-15µl pipetted into the wells. The 1kb DNA ladder (GibcoBRL) had clear bands at around 0.5, 1, 1.6, 2, 3, 4, 5 and 6kb; the 1.6kb band contained 10% of the mass applied to the gel (generally 0.3µg total mass) and hence was useful for quantification of linear DNAs.

100V was applied for around 1hour 20 minutes (Pharmacia EPS600 power pack). DNA is negatively charged at neutral pH and migrates to the anode. After electrophoresis bands in the gels were examined by EtBr fluorescence on a UV transilluminator (Eagle-eye, Stratagene) and photographic records taken.

2.1.6 QIAEX DNA gel extraction protocol

This was a reliable method to isolate the desired fragment from a heterogeneous mix of DNA (e.g. after PCR) separated by preparative agarose gel electrophoresis with ethidium bromide.

The band was excised under UV illumination, weighed and 300µl of QX1 (a complex buffer that disrupts the agarose polymer and releases DNA and then promotes DNA binding to silicagel particles) was added per 100mg of gel. This was warmed to 50^0C until the agarose melted.

Then 10µl of well vortexed QIAEX silicagel particles were added for every 5µg of DNA. This was placed at 50^0C and mixed every 2 minutes for 10 minutes for optimal DNA binding.

The sample was centrifuged in eppendorf tubes at 12,000g for 30 seconds and the supernatant discarded. The pellet was resuspended and washed twice in 500µl of QX2, (a high salt buffer to remove agarose contaminants), then twice with 500µl of QX3, (an ethanol containing buffer to remove salts and promote drying). The pellet was air-dried for 10-15 minutes.

To elute the bound DNA 20µl of water was added per 10µl of QIAEX, mixed and incubated for 10 minutes at room temperature. After centrifuging for 30 seconds the supernatant containing the DNA was transferred to a clean tube. Elution steps were repeated once and the eluates combined to improve the yield.

2.1.7 Ligation using T4 DNA ligase

This enzyme resealed the phosphodiester bonds between the juxtaposed 5'phosphate and 3'hydroxyl termini of the duplex DNA. The vector and insert DNA were comparatively quantified on an agarose gel and two to four times more insert than vector DNA was used for the ligation reaction with around 500ng of vector being desirable for subsequent transformations.

T4 DNA ligase (New England Biolabs, 400U/µl) and a 10x tris-based reaction buffer containing $MgCl_2$ and ATP were stored frozen. Supplier's information suggested 1 unit should yield 50% ligation of cohesive ends (using DNA at 330µg/ml in 20µl) in 30 minutes at 16^0C although 50 times more enzyme and a longer incubation was necessary for blunt ends. Consequently 0.01µl should have been adequate but this did not show complete ligation when checked by agarose gel (the two fragments should disappear and create a circular plasmid DNA) and the transformation rate was poor. Hence in a 10µl final volume, 0.5-1µl of the ligase and 1µl of 10x buffer was added to the DNA mix and incubated 16 hours at 16^0C. Ligation of cohesive ends is carried out at this temperature to maintain a good balance between annealing of the ends (cooler optimum) and activity of the enzyme (warmer optimum). The control for self-annealing of the vector was a ligation reaction with the insert DNA replaced by water. This was assayed by agarose gel for circularisation and in bacteria for transformation.

2.1.8 Culture of *E. coli* K12 DH5α, transformation and freezing

2.1.8.1 Culture

The *Esherichia coli* host strain used to propagate plasmids can affect the quality of the DNA. The host strain DH5α is a recombination deficient strain with low endonuclease activity, low carbohydrate levels and a good calcium chloride transformation efficiency (generally in about 0.1% of the cells the plasmid becomes stably established). The normal growth medium for *E. coli* was LB (Luria-Bertani) liquid medium (10g tryptone, 5g yeast extract, 5g NaCl per litre water, pH7 autoclaved) or LB agar (LB with 15g agar autoclaved, which was later melted at low power in a microwave, brought to 50^0C for addition of selective agents and poured into petri dishes).

Bacterial cultures for plasmid preparation were grown from a single colony picked from a selective plate (i.e. LB agar with 200μg/ml of hygromycin or 100μg/ml of sodium ampicillin), grown overnight, inverted at 37^0C. The single colony was inoculated into 2-5ml of liquid LB containing the appropriate selective agent (e.g. 200μg/ml of hygromycin or 200μg/ml of ampicillin) and grown for 5-16 hours at 37^0C, with shaking for aeration. This yielded up to 1-4x10^9cells/ml. The miniculture was diluted 1:100 into a larger volume of selective medium and regrown to saturation for greater plasmid requirements. Antibiotic selection was applied at all stages of growth to prevent overgrowth of any daughter cells which did not receive plasmids. On plates, antibiotics may be hydrolysed, resulting in small "satellite colonies" of non-resistant bacteria later on in the vicinity of an established colony.

2.1.8.2 $CaCl_2$ transformation

Making competent bacteria

An overnight culture was diluted 1:100 in 250ml of LB and incubated at 37^0C shaking vigorously until it was just turbid and in log phase.

The bacteria were cooled in ice, centrifuged at 0^0C for 10 minutes at 8,000g in sterile tubes and resuspended in 250ml 50mM $CaCl_2$ at 0^0C. The suspension was put on ice for 30 minutes and then centrifuged again.

The pellet was resuspended in 25ml of 50mM $CaCl_2$ containing 15% glycerol at 0^0C, frozen quickly in dry ice in 500μl aliquots and stored at -70^0C.

Transformation

An aliquot of cells was thawed slowly on ice. 100μl of cells were added to 10μl of plasmid DNA (10-100ng) in an eppendorf cooled on ice and incubated for 60 minutes

for the DNA to adhere. Besides the sample, a positive (original plasmid without an insert) and negative (water) control were included.

Subsequent incubation of the cells in a water bath at 42^0C for 60 seconds allowed DNA entry. Then 500µl of prewarmed LB without antibiotics was added and bacteria incubated for 60 minutes at 37^0C (orbital incubator, Jencons) to allow induction of the enzyme systems for antibiotic resistance encoded on the plasmid.

10µl and 100µl of cells were plated on pre-dried LB agar containing antibiotics and incubated overnight at 37^0C and surplus liquid culture stored at 4°C. One of the plates generally had single colonies visible which were picked and transferred to liquid LB containing antibiotics. After screening, a second round of cloning was sometimes performed.

Frozen stocks of transformed clones were made by mixing small aliquots (500µl) of an LB suspension 1:1 with sterile 30% glycerol, and snap freezing in dry ice prior to storage at -70^0C. To recover the bacteria, the frozen surface of the culture was scraped with a sterile loop and streaked onto a plate.

2.1.9 Plasmid isolation protocols

2.1.9.1 Small scale

Small scale plasmid isolation from 1-1.5ml of bacterial culture was used for screening. Single colonies of recombinant bacteria were grown overnight in bijoux in 3-4ml of appropriate media, then aliquotted for freezing, protein analysis by SDS-PAGE or plasmid isolation using the Qiagen buffers:

An aliquot of culture was transferred to an eppendorf and spun at 12,000g for 1 minute. The pellet was resuspended in 100µl of buffer P1 and incubated for 2 minutes at room temperature.

200µl of P2 (SDS/NaOH solution to lyse cells and denature DNA) was added, mixed gently and incubated for 5 minutes at room temperature. Then 150µl of cold P3 (potassium acetate buffer) was added to precipitate chromosomal DNA or cell debris and incubated 15 minutes on ice. After spinning for 5 minutes at 12,000g, 400µl of supernatant containing plasmid DNA was transferred into a new eppendorf.

400µl of phenol/chloroform/isoamylalcohol (Sigma, UK) were added, vortexed twice for 1minute and spun for 2 minutes. The upper DNA phase (400µl) was transferred to a new eppendorf and 1ml of pure ethanol (2-3 volumes) added and incubated for 30 minutes at -20^0C for precipitation.

The plasmid DNA was centrifuged for 15 minutes and the supernatant discarded. The pellet was washed in 1ml of 70% ethanol and spun 5 minutes 12,000g. The pellet was dried for around 10 minutes at room temperature, before being resuspended in 20µl of water. 3µl was enough for RE analysis and the remainder was frozen.

A more rapid, expensive small scale isolation used the QIAprep-spin plasmid miniprep protocol (QIAGEN) to produce higher quality DNA for transformations:

The pellet was resuspended in 250µl P1 in an eppendorf and 250µl of P2 added and incubated for 5 minutes at room temperature. 350µl of chilled N3 was added, mixed by inversion and incubated on ice for 5 minutes to induce precipitation of debris and spun at 12,000g for 10 minutes.

The supernatant was applied to a QIAprep-spin column in a 2ml tube, then spun for 30-60 seconds to draw the cleared lysate over a silica membrane to which the DNA adsorbs in high salt conditions. The column was washed with 0.75ml buffer PE, and transferred to a clean eppendorf tube.

80-100µl of water was added and the column centrifuged 30 seconds to elute the pure DNA.

2.1.9.2 Large scale

This method was for 10ml (or 100ml) of pelleted bacterial culture, for sequencing.

The pellet was resuspended in 300µl (5ml) of P1, incubated for 2 minutes at room temperature. 600µl (5ml) of P2 was added and incubate 5 minutes at room temperature. 450µl (5ml) of cooled P3 was added, mixed, and incubated 15 minutes on ice then centrifuged at 15,000g for 15 (30) minutes.

The supernatant was added to a Qiagen tip20 (tip100) column containing an anion-exchange resin, pre-equilibrated with 1ml (4ml) of buffer QBT. The column was washed with 2x1ml (2x10ml) of buffer QC. The DNA was eluted with 0.8ml (5ml) of buffer QF and precipitated with 0.7 volumes of isopropanol at room temperature. After centrifuging at 15,000g for 15 (30) minutes the supernatant was discarded. The pellet was washed with 1ml (5ml) of cold 70% ethanol, and the pellet dried, then redissolved in 50µl (250µl) water. This yielded high purity supercoiled plasmids.

2.1.10 Culture and electroporation of *M. vaccae*

Fast growing mycobacteria can be grown on plates with 7H11 agar containing glycerol and glucose at 30^{0}C for at least 5 days. Selection for hygromycin resistance plasmids uses 50µg/ml of hygromycin.

Mycobacteria have impossibly low calcium chloride transformation efficiencies, so high voltage electroporation must be used.

M. vaccae taken from vaccine stock (R877R, NCTC11659) grown on a simple salts agar (sauton) was prepared for electroporation as follows:

Approximately 1g of *M. vaccae* from a 6 day old sauton slope was suspended in 10ml of 10% cold glycerol and converted to a single bug suspension by shearing the culture using a syringe pressed against the base of a universal. This was made to 50ml with cold 10% glycerol and incubated for 10-15 minutes on ice. After gentle centrifugation for 20 minutes at 4^0C the supernatant was removed and another 50ml cold glycerol added and the incubation and centrifugation repeated. These washes reduced the salt content of the culture so the bacteria were not "burnt" by the current during electroporation. The competent cells were resuspended in 5ml of 10% glycerol and frozen to -70^0C in 400µl aliquots.

For electroporation, 200µl of competent cells were mixed with 10µl (containing 0.1-1µg) of pure plasmid DNA on ice and transferred to a cooled electroporation cuvette (low temperatures improve efficiency). Electroporation of mycobacteria required a voltage of 2.5kV, capacitance 25 µF and resistance at 1000ohm. Then 0.4ml of cold 7H9 was added and the culture transferred to a bijou for incubation at 37^0C with moderate shaking for two hours for induction of resistance.

50 and 300µl of culture were spread on two 7H11 plates containing hygromycin and incubated for 5 to 8 days at 30^0C. Individual colonies were assayed for expression and positive clones were spread onto further plates for vaccine production or storage at 4^0C. Frozen glycerol stocks were also prepared.

2.1.11 DNA sequencing using T7 polymerase.

Sequencing was necessary to check that PCR had not introduced mutations into the SIV p17/p27 gene. Reagents and methods were provided in the T7 sequencing kit (Pharmacia Biotech).

2.1.11.1 Sequencing reactions

Preparation of ssDNA and binding of primers:

32µl of template containing 2-3µg of supercoiled plasmid DNA was put in an eppendorf and 8µl of 2M NaOH was added and incubated at room temperature for 10 minutes for alkali denaturation. 7µl of 3M sodium acetate (pH 4.8) and 4µl of distilled water were added for neutralisation. Then 120µl of 100% ethanol was added and the

sample placed on dry ice for 15 minutes before centrifugation to collect the precipitated DNA. The pellet was washed with cold 70% ethanol, recentrifuged, dried and redissolved in 10µl of distilled water.

2µl of primer (adjusted to 2.5-5µM; equivalent to 15-30µg/ml of an 18mer) and 2µl of annealing buffer were added and incubated at 65^0C for 5 minutes, then transferred to a 37^0C water bath for 10 minutes and then at room temperature for 5 minutes.

Meanwhile the T7 DNA polymerase was diluted 1:4 in cold dilution buffer.

Sequencing-labelling reactions:

To 14µl of freshly annealed template/primer were added 3µl of labelling mix-dATP (this contained limiting amounts of dCTP, dGTP and dTTP for use with labelled dATP), 1µl of ^{35}S-labelled dATP (at 10mCi/ml, Redivue Amersham life Science, handled appropriately) and 2µl of diluted T7 polymerase. Incubation was at room temperature for 5 minutes for extension and labelling.

Termination reactions:

Four rows of a microsample plate were labelled A,C,G and T. 2.5µl respectively of the "A mix-short", "C mix-short", "G mix-short" and "T mix-short" were pipetted into the corresponding wells. (Each "mix-short" is the termination mix that contains all the dNTPs and the specific chain-terminating dideoxynucleotide (ddNTP) and is for reading up to 500bp on a 40cm gel).

The 4 sequencing termination mixes were warmed by floating the microsample plate in a 37^0C water bath for a minute. 4.5µl of the labelling reaction was transferred to each of the four pre-warmed termination mixes, and incubated at 37^0C for 5 minutes for further elongation and base-specific termination reactions. Then 5µl of "Stop solution" (containing formamide and dyes) was added to each well, mixed and an aliquot (3-5µl) transferred to a new sample plate. The stock plate was frozen for up to a week. The aliquot was heated at 75-80^0C for 2 minutes (by floating the plate in a water bath) and 1.5-2µl of each reaction was loaded immediately onto the appropriate well of a sequencing gel, under conditions that can discriminate between individual DNAs that differ in length by a nucleotide.

2.1.11.2 Sequencing gel preparation

A pair of clean glass sequencing plates, separated by wedge shaped spacers were taped together, and propped at 45° angle for pouring. Wedge shaped gels are thicker at the bottom than at the top and this reduces the otherwise large spacing between bands at the bottom of the gel, so more sequence can be read.

Pre-mixed sequencing solutions were used to prepare the 6% acrylamide and 48% urea gels. (The urea is a denaturing agent and suppresses base pairing so the DNA migrates independently of its base composition). 20ml of Sequagel complete buffer reagent, 80ml of Sequagel 6 (sequencing gel solution) and 800ul of fresh 10% ammonium persulphate were mixed thoroughly and poured carefully between the plates, avoiding air bubbles. The flat edge of a shark's toothcomb was inserted into the top of the gel. Complete polymerisation required 1 hour after which the tape was removed and the plates put onto the vertical stand. TBE buffer was poured into the upper and lower chambers, the comb was removed and the well was rinsed. The shark's tooth comb was replaced inverted so the teeth just dented the top of the gel to create closely adjacent sample loading slots. The gel was pre-run for 45 minutes to warm and equilibrate. Then sample wells were flushed with buffer to remove diffused urea just before loading the reaction mixes with each A, C, G and T set of mixes in adjacent wells. Outer lanes were not used.

The electrophoresis voltage and current were adjusted to about 80W so the 50^0C optimum was maintained. This kept the DNA fragments denatured without cracking the gel plates. Electrophoresis continued for about 2 hours until the bromophenol blue front line reached the bottom of the gel. The apparatus was dismantled and the gel was stuck to a sheet of filter paper and transferred to the gel dryer. The gel was dried to the filter paper under vacuum at 80^0C for 2 hours, then transferred to a film cassette in the darkroom and a piece of scientific imaging film (Kodak BioMax MR 35x43cm) placed next to the gel. The b-particles interact with the silver halide crystals in the emulsion. The film was exposed for 24-72 hours and then developed in an automatic processor (Agfa, Curix 60) to produce a permanent image of the distribution of radioactive atoms.

The products of the extension and termination reactions are a series of oligonucleotide chains whose lengths are determined by the distance between the terminus of the primer used to initiate DNA synthesis and the sites of premature termination. Because every base in the DNA has an equal chance of being the variable terminus, and by using the four different ddNTPs in four separate reactions, populations of oligonucleotides are generated that terminate at positions occupied by every A, C, G or T in the template strand.

The sequence of the target DNA was read from the autoradiograph, with lanes labelled A, C, G, and T accordingly. The order of nucleotides was deduced from the order in which successively larger fragments occurred in the four lanes. For forward primers on the sense strand, the start of the sequence was about 50 bases from the

primer, read upwards (i.e. starting at the smallest fragments). For reverse primers, the sequence reflected the bases in the non-coding strand in a backward direction.

2.1.12 SDS-PAGE and immunoblotting

Sample preparation: an aliquot of an *E. coli* suspension was spun down in an eppendorf and 50µl of 1x sample buffer (SB) added to the small pellet. This was boiled in a water bath for 5 minutes and centrifuged to pellet the DNA if necessary. For *M. vaccae*, part of a colony was resuspended in 50µl of water and sonicated for 20-30 seconds with high power before 12.5µl of 5x SB is added and the sample boiled. (5xSB: 250mM Tris. HCl pH8.3, 10% SDS, 25% 2-mercaptoethanol, 50% glycerol and 0.05% bromophenol blue. SB denatures the protein and gives it a net negative charge).

For polyacrylamide gel electrophoresis, the mighty-small minigel system (Hoefer Scientific instruments) was used, with chemicals from BDH "electran" or Sigma. A two layer system of discontinuous stacking and running gels was followed (Laemmli 1970).

The 10x8cm glass plate and alumina plate were clamped around two 0.75mm spacers in a casting chamber. The gel solutions were prepared as follows:

	Running (ml)		Stacking (ml)
	15%	12%	
Acrylamide 30% (+ bis 0.8%)	5.0	4.0	0.66
RGB 1.5M Tris. HCl, pH 8.8	2.5	2.5	
SGB 0.5M Tris. HCl, pH6.8	1.0		
Distilled water	2.5	3.5	2.3
SDS 10%	0.1	0.1	0.04
Ammonium persulphate 10%	0.1	0.1	0.04
TEMED	10 µl	10 µl	4 µl

A 12% gel has optimal separation for proteins in the range of 15-60kD and 15% for 12-45kD. The resolving gel was poured between the plates and allowed to polymerise and then the stacking gel solution was poured above and the comb inserted. After this has polymerised the plates were transferred to the electrophoresis apparatus. The chambers were filled with electrophoresis buffer (50mM Tris, 380mM glycine, 0.1% SDS) and once the comb was removed 10-15µl of sample (up to 4µg) or prestained molecular weight markers (Biorad prestained SDS-PAGE standards "broad" or "low" range or kaleidoscope) was applied to the wells. The electrophoresis was run at 20mA per gel until the bromophenol blue front line reached the bottom of the resolving gel (about 1 hour). When finished the gel was removed from the plates.

Proteins were stained using 0.1% Coomassie blue (BDH) in 40% methanol and 10% acetic acid for 30 minutes, then destained with 10% methanol and 10% acetic acid for several hours and dried.

Alternatively proteins were transferred to nitrocellulose (Schleicher and Schuell) using Fastblot (Biometra) apparatus. 5 sheets of filter paper were soaked in blotting buffer (25mM tris.HCl, 192mM glycine, 20% ethanol) and placed on the carbon base-plate (anode). Wet nitrocellulose was placed on top and the gel lowered onto this. Five more soaked filter papers were put above the assembly and the lid (with cathode electrode) attached. Electroblotting was carried out at 5mA per cm^2 of gel (200mA for one 8x5cm gel, limited to10W) for about 30 minutes. Transfer of prestained markers to the nitrocellulose indicated that electroblotting was complete, and lanes were identified by reversible staining with Ponceau S (0.2% Ponceau S in 3% TCA). The nitrocellulose blots were washed 3 times for 10 minutes in washing buffer (10mM Tris.HCl pH7.4, 150mM NaCl, 0.5% tween-20) and then ink stained (Pelican Indian ink at 1/1000 in washing buffer, rocked for 1-16 hours can detect 50ng of protein per band) or prepared for immunostaining.

Immunstaining: Unused binding sites on the nitrocellulose blots were blocked using 1% BSA in washing buffer for 1 hour. Then 7ml of antibody solution (as described below) was added per blot and incubated for 90 minutes at room temperature, shaking in a covered container. A mix of monoclonal antibodies (MAb) avoided the possibility of one epitope being hidden. Two murine IgG2a monoclonal antibody supernatants were available that bound to SIV gag p27 after Western blotting (ADP392 and 393). A 1:300 dilution of each antibody in washing buffer could detect 100-200ng of a glutathione transferase/p27 fusion protein. A prediluted solution of mouse MAb to *M. tuberculosis* superoxide dismutase (SOD), which was also cross-reactive with *M. vaccae* SOD, was donated by Sarah Ely. Prepared solutions were reusable six times with freezing in between.

After 3 washes, blots were incubated for 60-90 minutes with HRP labelled anti-mouse Ig (Dako) at 1/1000 in washing buffer. Following 3 final washes and a rinse in water blots were incubated for a few minutes with a sensitive colour substrate, prepared fresh as follows for each blot:

10 ml	Citrate phosphate buffer (5mM citric acid, 10mM Na_2HPO_4, 25% ethanol)
100 µl	Stock TMB solution (60mg/ml tetramethylbenzidine and 200mg/mldioctylnatriumsulphosuccinate (DONS, Merck) dissolved together in DMSO)
6 µl	30% H_2O_2

Colour developed as a result of localised enzyme activity and deposition of a dye precipitate. Blots were placed on filter paper in the dark to dry and photographed for a permanent record (Stratagene, Eagle Eye II).

2.1.13 Production of autoclaved recombinant *M. vaccae* vaccine

Recombinant *M. vaccae* was scraped from 7H11 plates containing hygromycin after 5 days growth at 30^0C. It was transferred into preweighed universals and sheared to a nearly single bug suspension in about 10ml of sterile borate buffered saline pH8, (borax 3.63g, boric acid 5.25g, sodium chloride 6.19g, tween 80 0.0005%, water to 1000ml; autoclaved) before being diluted to 20mg/ml (1mg wet weight of *M. vaccae* represents approximately 10^9 bacteria). 5ml aliquots in vaccine vials were then autoclaved for 20 minutes at 120^0C. The vaccine was stored at 4°C until injection of 10^7-10^9 killed recombinant *M. vaccae* per mouse.

2.2 Materials and methods used in analysis of murine responses to recombinant *M. vaccae*

2.2.1 Antigen preparation for spleen cell stimulation and antibody detection.

2.2.1.1 Sonicate of autoclaved *M. vaccae*

M. vaccae strain R877R NCTC11659 was grown on sautons medium solidified with agar. (This was prepared using 6g l-asparagine, 30ml glycerol, 2g citric acid, 1.5g K_2HPO_4, 0.25g magnesium sulphate, 0.05g ferric ammonium citrate, adjusted to pH6.2 with ammonia; then 1% agarose and distilled water to 1 litre. The solution was autoclaved 15lb/15minutes, cooled to 50°C and 25ml sterile 40% glucose added). This took 8-14 days at 32°C until strong growth was visible. The organisms were collected into a glass container and phosphate buffered saline added (PBS: 8g NaCl, 0.2g KH_2PO_4, 1.135g Na_2HPO_4, 0.2g KCl, distilled water to 1 litre) using a ratio of about 3g wet weight of *M. vaccae* for 10ml PBS. This preparation was autoclaved at 15lb/sq.in pressure for 25 minutes and left to cool.

The autoclaved *M. vaccae* was transferred to a cooled sonicating chamber, and sonicated with good efficiency (in a 100W ultrasonicator, with wave peak distance at 8μm) for around 30 minutes until the sonicate appeared 'milky', to ensure most organisms were disrupted. The sonicate was divided into 1.5ml screw cap eppendorf

tubes and spun at high speed (11000g) in an eppendorf centrifuge for 10 minutes to pellet the insoluble material. The supernatant was collected using a syringe and fine needle and passed through a 0.2μm filter (Millex-GV, Millipore) to sterilise the solution and further remove particulate matter. The sample protein content was estimated by the Biorad protein estimation system and diluted to 10mg/ml. Aliquots were stored frozen until use.

2.2.1.2 Additional antigens

The p27GST fusion protein was from recombinant *E. coli* (MRC ADP 643) and the *M. tuberculosis* superoxide dismutase (SOD) protein was produced in recombinant *M. vaccae* and purified (kindly provided at 2mg/ml by Dr. Y. Zhang, St.Mary's Medical School, London).

2.2.2 Culture of spleen cells and collection of supernatants for cytokine measurement.

Mice were killed by cervical dislocation and the fur was cleaned by short immersion in 70% alcohol prior to surgical removal of the spleen using sterile dissecting instruments. Procedure/culture medium throughout was: bicarbonate buffered RPMI 1640 (Imperial Laboratories) containing penicillin and streptomycin, L-glutamine (2mM Gibco), 2-mercaptoethanol (50mM Gibco) and nutridoma SR (Boehringer). This is a buffered, serum-free supplemented medium which helps cell survival and can protect the cells from proteases released by damaged cells which otherwise may attack the cell surfaces and lead to non-specific responses.

The spleen was placed in a sterile petri dish and the cells collected by injection of 5ml of culture medium under the capsule, which led to bursting of the capsule and gentle expulsion of the spleen cells (generally 6 x 10^7 cells/spleen). The burst capsule was then gently squeezed with forceps a few times to release the remaining cells. Spleen cells were immediately transferred to a universal and centrifuged (Heraeus Christ centrifuge, 200g, 5 minutes) and the supernatant removed.

The pellet was resuspended in 5ml of haemolysis solution (0.83g ammonium chloride, 0.1g potassium hydrogen carbonate, distilled water to 100ml, filtered) for 7 minutes to lyse erythrocytes. The cell suspension was made up to 25ml with medium and spun. The pelleted cells were resuspended in 10ml medium, and the number of viable cells in an aliquot counted using trypan blue exclusion in a haemocytometer, whilst the rest was spun. The pellet was resuspended at 2 x 10^7 cells/ml in medium and

the cell suspension added in 250µl volumes to the wells of a 24 well costar plate for cytokine production assays.

Medium or soluble antigens were then prepared at double-strength in medium and 250µl added to the cells. This yields a final cell concentration of $5x10^6$/0.5ml total volume with antigens at the appropriate concentration. Concanavalin A at 1ug/ml in medium stimulated production of IFNγ and IL-2 from vaccinated and unvaccinated spleen cells alike and acted as a positive control to demonstrate good survival. The autoclaved-*M. vaccae* sonicate was used at a final concentration of 10µg/ml to demonstrate specific responsiveness in vaccinated mice to the mycobacterial carrier. Earlier experiments (not-shown) showed greater concentrations resulted in increasing non-specific stimulation of IFNγ production from spleen lymphocytes. Specific responses to SIV p27 (as a GST fusion protein) and *M. tuberculosis* superoxide dismutase (SOD) were tested using the antigens at a range of dilutions (from 2 to 0.2µg/ml, diluted in medium and filter sterilised).

The cells were incubated humidified at 37°C with 5% CO_2 for 24 hours (IL-2 and 4) and 48 hours (IFNγ) for cytokine yield. The suspensions were collected into eppendorf tubes and spun at high speed for 5 minutes to pellet the cells. The supernatants were removed and immediately frozen, prior to assay for cytokines.

2.2.3 Capture ELISA for the detection of murine IFNγ in supernatants

The murine IFNγ capture ELISA uses the Pharmingen kit of paired purified monoclonal rat anti-mouse IFN antibodies (protein G purified primary mAb, clone R4-6A2, cat. no 18181D and biotinylated secondary mAb, clone XMG1.2, cat. no 18112D; both supplied at between 0.5-1mg/ml) following the suggested general protocol with small adjustments to timings and reagent concentrations for optimisation of IFNγ detection. Briefly, the primary capture antibody was diluted to 3µg/ml in coating buffer (0.1M sodium bicarbonate, pH 8.2) and coated at 50µl/well in Easywash plates (Corning) overnight at 4°C in a moist chamber.

Uncoated binding sites on the plates were blocked with 200µl of 3% bovine serum albumin in phosphate buffered saline (PBS/BSA) for 1 hour at 37°C to reduce non-specific responses, before the plates were washed 3 times in PBS containing 0.05% tween 20 (PBS/t) over a period of 30 minutes at room temperature. Then the standards or supernatant samples were added in duplicate in 100µl, and incubated for 2 hours at 37°C. The purified recombinant murine IFN standard (Pharmingen 19301U) was used

from 64ng/ml to 0.5 ng/ml in doubling dilutions in complete medium to establish a dose response curve for the assay. Supernatants were used neat and at 1 in 4 dilutions.

The plates were washed 4 times in PBS/t before addition of 100µl/well of the biotinylated secondary antibody, diluted to 1µg/ml in PBS/BSA. Incubation was for 90 minutes at 37°C. Following 4 washes, HRP conjugated streptavidin (Dako) diluted 1/1000 in PBS/BSA was added at 100µl/well and incubated for 1 hour at 37°C, before the final 4 washes in PBS/t. Detection of the bound HRP was by the peroxidase sensitive chromogen ABTS (2,2'-azino-bis(3'ethylbenzthiazoline sulfonic acid) Sigma), freshly diluted to 1mg/ml in citrate phosphate buffer pH 4 containing 0.05% hydrogen peroxide (20vol) and added at 100µl/well. After a 20-30 minute incubation in the dark at 37°C, the colour was read at 405nm in a Dynatech MR5000 ELISA plate reader, linked directly to a Macintosh computer running the 'assay-zap 2' program (Biosoft). Results of the paired samples were related to the standard curve and expressed as amount of IFNγ in ng/ml. The sigmoid curve was satisfactory between 2 and 40ng/ml of IFNγ; concentrations above this required dilutions of the samples and multiplication of the results by the factor.

2.2.4 Capture ELISA for the detection of murine interleukin 2 and interleukin 4 in supernatants

The basic assay procedure was similar to that described for IFNγ. The paired IgG monoclonal antibodies for IL-2 detection from Pharmingen were: purified rat anti-mouse IL-2 (18161D, from clone JES6-1A12) used as a capture antibody at 4µg/ml and biotin rat anti-mouse IL-2 (18172D, from clone JES6-5H4) used for detection at 2µg/ml. The timings were as described above and the supernatants were applied neat. An appropriate range of concentrations of the mouse IL-2 standard (Pharmingen 19211U) for obtaining a linear standard curve was 32ng/ml to 250pg/ml mouse IL-2 set out in doubling dilutions.

Similarly the IL-4 ELISA used the Pharmingen pairs 18031D (coating, 4µg/ml) and 18042D (detecting, 2µg/ml) and the standard curve from 4000pg/ml to 31pg/ml recombinant mIL-4 (Pharmingen 19004T). These values were low but agreed with other claims for the sensitivity of ELISAs for murine IL-4 (between 260-67pg/ml; Rao 1995).

2.2.5 ELISA to detect specific immunoglobulins in the mouse sera.

ELISA plates (Nunc maxisorp) are coated at 4°C overnight with 50µl of antigens at 3µg/ml in 0.05M carbonate/ bicarbonate coating buffer (1.59g Na_2CO_3, 2.93g $NaHCO_3$,

distilled water to 1 litre, pH9.4). The antigens are those also used in CMI studies: autoclaved *M. vaccae* sonicate, the p27GST fusion protein isolated from recombinant *E. coli* and *M. tuberculosis* superoxide dismutase (SOD). Additionally, dose response curves using doubling dilutions of standard IgG1 and IgG2a (a kind gift from C. Hetzel, Imperial College) are prepared between 2 and 0.16μg/ml.

The plates are blocked using 200μl of 1%BSA in PBS/t for 1 hour at 37°C and then washed twice with PBS/t before banging dry. Sera are diluted in BSA/PBS/t and 50μl of each added in duplicate to the plates which are incubated at 37°C for 2 hours. After washing, bound immunoglobulins are detected using antisera diluted in BSA/PBS/t and 50μl/well incubated at 37°C for 60-90 minutes. Following three washes, the 50μl/well of chromogen (ABTS in CPB) is applied and incubated in the dark for 15 minutes for colour development prior to reading the absorbance at 405nm (Dynatech MR). Duplicates are averaged and background binding levels of antibodies to coating buffer only are subtracted. When appropriate, the optical densities are converted to μg/ml by comparison with the standard curves.

Slightly different concentrations are used for detection of different antibody classes or antigens as follows:

Antibody class	Serum concentration	Conjugate concentration
Total immunoglobulin	1/50-1/500 (Ag dependent)	1/1000 HRP-rabbit-anti-mouse Ig (Dako)
IgG1	1/500	1/1000 HRP-goat anti-mouse IgG1 (seralab SBA 1070-05)
IgG2a	1/500	1/500 HRP-goat anti-mouse IgG2a (seralab SBA1080-05)

Table 2-1 : Detection of antibody classes in murine sera

2.3 Materials and methods used in flow cytometric analysis of murine responses to standard *M. vaccae.*

2.3.1 Vaccination of mice

Mice were anaesthetised (Fluothane) and injected at the base of the tail with 100μl of standard or 1/100 diluted autoclaved *M. vaccae* vaccine or placebo. After the appropriate time, mice were sacrificed and the spleen and bilateral inguinal lymph nodes were removed. Animal work was performed during the morning, with the help of Dr C C Wang. Only one mouse was used for each dose at each time point in each strain:

this was to minimise expense and simplify the FACS protocol, which required many tubes of cells being permeabilised and double stained per organ.

2.3.2 Cell preparation

2.3.2.1 Spleen cells

This has been previously described. The procedure was followed to the point where counted spleen cells were pelleted.

2.3.2.2 Lymph node cells

The pair of inguinal lymph nodes was collected into 0.5ml of RPMI/nutridoma. The nodes were transferred to a sterile 100μm nylon cell strainer (Falcon 2360) held over a 50ml sterile plastic centrifuge tube (Greiner). The top of the plunger of a 1ml syringe was used to grind the nodes and release the cells. 5ml of RPMI/nutridoma was poured over the cells to wash them through the strainer (lymph nodes cells are approximately 30μm large). Cells were counted and pelleted prior to stimulation.

2.3.2.3 Whole blood

0.7ml of blood was diluted 1/8 into RPMI/nutridoma containing heparin and stimulated (as below). Then 0.7ml aliquots were prepared for staining (as below).

2.3.3 Cell stimulation and staining

Pelleted cells were resuspended at 1 x 10^6/ml in RPMI/nutridoma containing PMA (Sigma P-8139) at 50ng/ml and ionomycin (calcium salt, Calbiochem 407952) at 500ng/ml in lidded FACS tubes (Falcon 2054). These were incubated for 1 hour at 37°C. Then brefeldin A (Sigma B-7651) was added at 10μg/ml for a further 3 hours. Unstimulated cells were incubated in brefeldin A for 3 hours.

0.5ml aliquots of cell suspension were added to 2ml volumes of staining buffer (1% BSA plus 0.1% sodium azide in PBS) in FACS tubes and centrifuged (390g, 5 minutes). All but 50μl of buffer was poured off the pellet and the viable cells were surface stained for 30 minutes, in the dark at 4°C as follows:

Monoclonal anti-mouse CD4 Quantum Red (Sigma R-3637), 10μl of 1/40 dilution

Monoclonal anti-mouse CD8a Quantum Red (Sigma R-3762), 10μl of 1/40 dilution.

Then 2ml of FACS lysing solution (BD 92-0002) was added and the tube vortexed briefly and incubated dark for 10 minutes at room temperature. This lysed RBC and

prepared cells for permeabilisation. Tubes were centrifuged, supernatant poured off and pellets flicked.

Then 0.5ml of BD permeabilising solution (BD 340457) was added, vortexed and incubated in the dark for 10 minutes at room temperature. This induced permanent pores. 2ml of staining buffer was added, lidded tubes were mixed and centrifuged. Supernatants were poured off to leave 50µl. Anti-cytokine antibodies were added to surface stained cells as follows, mixed and incubated at room temperature, in the dark for 30 minutes:

FITC rat IgG1 isotype (Pharmingen 0.5mg/ml, 11014C) 6µl of 1/40 dilution (0.075µg)

FITC rat IgG2b isotype (Pharmingen 0.5mg/ml, 20634A) 10µl of 1/40 dilution (0.125µg)

FITC rat anti-mouse IFNγ (Pharmingen IgG1 0.5mg/ml, 18114A) 6µl of 1/40 dilution

FITC rat anti-mouse IL-2 (Pharmingen IgG2b 0.5mg/ml, 18174A) 10µl of 1/40 dilution.

Then 2ml of staining buffer was added, mixed, centrifuged and supernatants poured off. Pellets were resuspended and 0.25ml of 1% paraformaldehyde in PBS added. Fixed suspensions could be stored for 24 hours at 4°C in the dark prior to analysis.

2.3.4 Cell analysis

Cells were analysed on the Becton Dickinson FACSCalibur. Fluorocromes were excited with a 488nm laser; FITC emission was measured as a green signal (530nm peak fluorescence) by the FL1 detector; Quantum Red, a tandem fluorescent dye created by coupling R-phycoerythrin with Cy5 reactive dye, was detected as a violet signal at 670nm (FL3). Acquisition was performed using CELLQuest software with the following settings:

Parameter	Detector	Voltage	AmpGain	Mode
P1	FSC	E00	2.91	Lin
P2	SSC	420	1.0	Lin
P3	FL1(FITC)	530		Log
P5	FL3(QR)	615		Log

Threshold FSC: 75
No compensation FL1/FL3 was available.

10000 events were stored. (As the samples were from spleen and LN, no granulocyte population as seen in whole blood staining was present.) A lymphocyte gate and appropriate quadrants were fitted during analysis.

2.4 Materials and methods used in macrophage cell culture and cytokine analysis

2.4.1 Culture and stimulation of THP-1 cells

The THP-1 cell line (donated by J. Friedland) consists of non-adherent macrophage-like cells, which take the form of small clumps and irregularly sized cells when dividing vigorously. They were cultured in flasks (Nunc, 250ml) in 20ml of RPMI containing glutamine, antibiotics and 10% foetal calf serum (FCS; Imperial Labs) (culture medium) at 37°C, 5%CO_2. Cell suspensions were divided into three and fresh medium added, twice a week. THP-1 can be further differentiated by culture for 24 hours in medium containing 1.2% dimethyl sulphoxide (DMSO, Sigma D-5879) with 10% additional FCS: this results in a higher cytokine yield for a few days, but is accompanied by reduced proliferation.

For assay, serial dilutions of samples were prepared (these will be the concentrations quoted in results). These were added to 24 well plates (Nunc) at 5% (32.5µl) of the final total assay volume of 650µl per well (so for LPS quoted at 20µg/ml, its final dilution will be 1µg/ml; 'undiluted' vaccine actually has a final concentration of 5%). THP-1 cells were resuspended at 2.1 x 10^6/ml in culture medium containing 20% FCS and 617.5µl volumes added per well.

Plates were incubated generally for 18 hours, prior to harvesting into eppendorf tubes then centrifugation (11000g, 5 minutes; MSE Micro Centaur) of the suspensions and collection of the supernatants, which were stored at -20°C till cytokine assay.

Assays were usually performed several times. Although absolute values varied between assays, probably due to the culture conditions of the THP-1 shortly prior to assay, sample trends were always reproducible and the best example of each series is shown in the results.

2.4.2 Assessment of THP-1 viability after assay

This was based on a 20 hour proliferation assay, using uptake of tritiated thymidine as the read-out. Fresh THP-1 (with DMSO) were resuspended at 1.1 x 10^6/ml in

medium with 20% FCS and plated in triplicate in a 96 well plate using 140µl per well. 7.5µl volumes of samples were added (5%). Methyl ^{3}H-thymidine (Amersham TRA 120, 37MBq/ml or 1mCi) was added after 1-3 hours to a final dilution of 1/300 (0.0185MBq/well or 0.5µCi/well). After 18 hour incubation, the cells were harvested (SkatronAS) onto glass microfibre filters (Whatman 1822 849 GF/C) and read by β-scintillation counter (Ecoscint, LKB).

It was assumed that cells with medium and no sample would give the normal level of proliferation, and reduction in levels from this would indicate either direct toxicity of the sample (usually visible by microscopy e.g. where pH or salt conditions were not favourable) or an anti-proliferative effect/ secondary cell death (e.g. due to the cellular response to the sample such as high TNF production).

Viable cell counts after THP-1 culture with samples were performed by trypan blue exclusion. THP-1 were pre-treated with 1.2% DMSO. A cell suspension at 1.1 x 10^{6}/ml was prepared in medium with 20% FCS and 140µl/well plated in 96 well plates. 0µl, 2.5µl or 6µl of MVSA was added per well, with or without IFNγ at 1000U/ml final concentration. After 22 hour incubation, the cells were mixed and a 20µl aliquot was transferred to 20µl of 0.4% trypan blue solution (Sigma T-8154) and loaded onto a haemocytometer. Live (transparent) and dead (blue) cells in the central field were counted and recorded; e.g. 55 total cells. This could be calculated as cells per ml as follows: 55 x 2 x 10^{4}/ml = 1.1 x 10^{6}/ml.

2.4.3 Culture and stimulation of human monocytes

50ml of venous blood was collected into heparinised tubes (250µl monoparin). Tubes were centrifuged at 1600g for 8 minutes, plasma was collected and the cells were resuspended in RPMI to double the original volume. Cell suspensions were layered over Ficoll (LSM, Flowgen) and centrifuged at 500g for 28 minutes. Meanwhile, a 250ml flask was coated with 4ml plasma or FCS. Mononuclear cells were collected from the Ficoll interface and spun at 200g for 10 minutes, then washed once, resuspended in 16ml RPMI and poured into the prepared flask. This was incubated for 18 hours, 37°C, 5%CO_2.

Non-adherent cells were removed by rinsing the flask several times in RPMI. The monocytes were removed from the plastic by 30 minute incubation in a solution of 2ml 3.3%EDTA/ 2ml FCS or plasma/ 6ml RPMI. Cells were counted, centrifuged and plated in 96 well flat bottom tissue culture plates (Nunc) at 1 x 10^{5}cells/well in 200µl of complete RPMI containing 20% FCS or plasma. Spare cells were analysed by flow

cytometry after staining with anti-CD14 PE (Dako R0864). Human IFNγ was added to some wells at 1000U/ml final concentration. Samples were added in 10µl volumes, from stock preparations at 20x strength (similarly to THP-1 methodology). Incubation was 24 hour, before supernatants were collected, centrifuged and frozen, prior to cytokine measurement. Undiluted supernatants were collected after each cytokine ELISA and reused for the next one.

The confluency of monocytes still stuck to the wells was assayed by the crystal violet cell staining method. Briefly, cells were fixed to the plates by gentle treatment with 70% ethanol for 1 minute. Then 100µl of 1% crystal violet in water was added to each well for 5 minutes and the excess washed of under running water. Crystal violet trapped in stained cells was solublised in 50µl of 33% acetic acid and read on the Dynatech plate reader at 570nm. Any reduction in optical density compared to controls indicated a reduction in adherence (often indicating reduced viability) of the monocytes.

2.4.4 Cytokine production in a simple murine model

Female 5 week old C57bl/6 mice were purchased and settled into the animal facilities. Mice were anaesthetised and injected via the intraperitoneal route with 100µl or 300µl of vaccine preparation or placebo, in duplicate groups. Mice were bled by cardiac puncture at 3 hours or 6 hours post injection and sacrificed. In some cases, 2ml of RPMI was injected into the peritoneal cavity and then 1ml recovered. All the animal work was kindly performed by Professor G Rook. Peritoneal washes were centrifuged (11000g, 5 minutes) and supernatants frozen for assay. Blood samples were allowed to clot for 1 hour at 37°C and centrifuged to collect serum, which was frozen until assay. (This protocol was based on work from Heinzel 1994).

2.4.5 Cytokine detection by ELISA

2.4.5.1 Human IL-12

Bioactive IL-12 is a 75kDa glycoprotein heterodimer composed of two genetically unrelated subunits (p35 and p40) linked by a disulphide bond. Upon stimulation of macrophages and B cells an excess of the p40 subunit is produced in addition to the heterodimer and detection of either molecule can be achieved by ELISA.

Human IL-12 p40

Paired antibodies were obtained from R&D, with minor variations on their suggested method. Capture antibody MAB609 (anti-human IL-12) was used at 2.5µg/ml in 0.1M sodium bicarbonate coating buffer to coat ELISA plates (Nunc Maxisorb)

overnight at 4°C in a damp box. The assay volume was 100µl, in duplicate. Plates were washed three times in PBS/tween 20 0.05% prior to blocking with 2% BSA in PBS at 37°C for 60 minutes. Samples were added undiluted and a dose response curve between 8000pg/ml and 62pg/ml was constructed using recombinant IL-12 p40 (R&D 309-IL) in RPMI/20%FCS. These were incubated 120 minutes at 37°C, before washing. Biotinylated anti-human IL-12 (R&D BAF219) was used at 0.33µg/ml in PBS/2%BSA for 90 minutes at 37°C, before washing. Dako streptavidin HRP was used at 1/2000 dilution in PBS/2%BSA for 60 minutes at 37°C, before washing. The substrate was TMB (Sigma T-8665) for 30 minutes. The plate was read at 630nm, with background subtracted, then stopped with 0.5M sulphuric acid and re-read with test filter 450nm with ref filter 570nm (Dynatech MR5000 ELISA plate reader). This expanded the dose response curve. Interpolation was performed using AssayZap.

Human IL-12 p70

A high sensitivity kit was purchased from R&D systems (Quantikine HS120) and the instructions were followed exactly, using reagents from the kit. The dose response curve ranged from 40 to 0.625pg/ml. (R&D literature quotes concentrations in normal serum from undetectable to 3.58pg/ml, while in mitogen stimulated 3 day PBMC culture supernatants this reached 30.4pg/ml)

2.4.5.2 Human IL-10

Antibody pairs to human IL-10 were purchased from Pharmingen. The ELISA followed the method described for IL-12 p40 with the following exceptions: capture antibody, purified rat anti-human IL-10 (Pharmingen 18551D) used at 2µg/ml; detection antibody, biotinylated rat anti-human IL-10 (Pharmingen 18562D) used at 1µg/ml; Strepavidin HRP (Dako) used at 1/1000; recombinant IL-10 (Pharmingen 19701V) used to construct the standard curve from 20000pg/ml to 310pg/ml.

2.4.5.3 Human TNF

Paired antibodies to human TNFα were purchased from Genzyme (Duoset 80-3933-00). The basic ELISA was as described for IL-12 p40 with the following exceptions: assay volume was 50µl; mouse anti-human TNFα coating antibody used at 2µg/ml; rabbit anti-human TNFα biotinylated antibody at 0.4µg/ml, 60 minute incubation; strepavidin HRP 1/2000, 30 minute incubation; standard provided used to construct curve from 6000pg/ml to 2.7pg/ml using tripling dilutions, 90 minute incubation.

2.4.5.4 Murine IL-12 p40

A Quantikine M kit for detection of murine IL-12 p40 sub-unit (R&D, M1240) was used to measure the cytokine in murine serum. All reagents were provided and full instructions provided in the kit were followed. Sample volume was 50µl, which was added to 50µl diluent in wells for assay. The standard curve was linear from 750 to 11.7pg/ml. The control sample fell within acceptable limits.

2.4.5.5 Murine TNFα

Antibody pairs were purchased from Genzyme (Mouse TNFα Duoset 80-3807-00). Hamster capture antibody was coated at 6µg/ml overnight in 0.1M carbonate buffer. 80µl of TNFα standards (provided) or samples diluted 1:1 in buffer were incubated for 90 minutes at 37°C. Detection antibody was HRP linked goat antibody used at 4µg/ml, 60 minutes, 37°C. TMB 30 minutes. The standard curve was 2000 - 7pg/ml

Chapter 3 Generation of recombinant *M. vaccae* vaccines

3.1 Introduction

3.1.1 Recombinant mycobacteria as potential vaccines

Effective vaccination of a population prevents contraction and spread of disease, and hence vaccination programs reduce the requirement for extensive drug treatment of patients and contact tracing. Although vaccines are among the most cost-effective medical interventions known to prevent disease, with immunisation saving millions of children's lives each year, there are significant problems with the use of vaccines in the third world. Among these are the need for repeated immunisations often by injection, and the heat instability and cost of the vaccines. Hence the need for development of a recombinant vaccine vehicle that would allow simultaneous expression of multiple protective antigens from different pathogens. However, running parallel with this is the need for effective treatment when the vaccine cover breaks down, and for this immunotherapy may supplement drug treatment thus reducing the cost.

Many authors already advocate the use of BCG (bacille Calmette-Guerin) as a recombinant vehicle. BCG is a live attenuated bovine tubercle bacillus used extensively worldwide to immunise over 2.5 billion people against tuberculosis and leprosy (Stover 1991). Throughout its use to date, BCG has only a low incidence of complications and a single injection given at or any time after birth can sensitise an individual for up to 50 years. It has a large DNA capacity. BCG is also very stable and cheap and the possibility exists to administer it as an oral vaccine that is important for avoiding the spread of blood-borne viruses via contaminated needles. Moreover, like many mycobacteria BCG is a potent adjuvant for induction of cell-mediated and cytotoxic immune responses.

The adjuvant properties of BCG and its cell wall components (e.g. muramyl dipeptide) have been extensively exploited (e.g. Freund's complete adjuvant and Ribi adjuvant). The ability to engineer BCG to produce one or more foreign pathogen antigens has several advantages over mixes: the antigen continues to be produced in replicating BCG and hence may give a more long lived immune response; it may be more cost effective to engineer BCG recombinants than produce the mixture; genetic manipulation may tailor the vaccine to maximise the desired immune responses.

Alterations of the rBCG involving alternative forms of antigen presentation and vaccine preparation might augment the immune response it elicits. For instance signal peptides derived from mycobacterial lipoproteins may be used to direct export of the antigen through the mycobacterial cell wall and subsequent surface expression may enter the endogenous processing pathway and improve immunogenicity. Lipoproteins of many pathogens are amongst the most immunogenic antigens expressed *in vivo* and some studies show that when some antigens are expressed on the surface of BCG as lipoproteins, rather than as cytoplasmic proteins a 2- to 3-log increase in antibody titre can be achieved (Stover 1993). This may be relevant when humoral immunity is the protective response, such as in Lyme disease (*Borrelia burgdorferi*).

However, because of the risk of live vaccines in immunocompromised people, there is also interest in the use of killed vaccines and immunotherapies, such as *M. vaccae*, to deliver heterologous antigens to the immune system, either by mixing purified antigen with existing vaccines or by recombinant DNA techniques to achieve expression of the antigen. It is possible that many of the arguments provided for rBCG are also relevant for *M. vaccae* e.g. good storage, cheap, strong Th1 adjuvant, slow breakdown of the mycobacteria may give a long lasting response even though the bacteria are not actively multiplying, surface expression versus cytoplasmic may modulate the type of response.

Both in peripheral blood lymphocytes of HIV-1 infected humans and SIV_{mac} infected rhesus monkeys, AIDS virus replication was inhibited by a population of CD8+ cytotoxic T cells (Tsubota 1989). Hence CMI and CTL responses may be important in immunity to HIV and SIV, but these responses are not well stimulated by immunisation with viral subunits or killed virus. Thus there is considerable interest in non-pathogenic organisms as vaccine vectors in HIV.

The potential use of BCG as a live recombinant antigen delivery system for HIV and SIV has been illustrated by studies in the murine system. Aldovini (1991) expressed a variety of HIV-1 polypeptides in BCG recombinants under the control of the mycobacterial hsp70 promoter and they induced antibody and T cell responses (including IFNγ and IL-2 and CD8 cytotoxicity) in mice. Yasutomi (1993) expressed SIV_{mac} gag extrachromosomally in BCG under the control of hsp60 and 70 regulatory sequences. Gag was expressed relatively well in the cytoplasm and immunisation with the hsp70 construct elicited MHC 1-restricted CD8+ *gag*-specific CTL in monkeys. The CTL recognised gag protein endogenously processed by target cells and the epitope recognised was the same as that seen in infected monkeys. Interestingly even following boosting the immunised monkeys did not generate an anti-SIV gag antibody response.

3.1.2 History of development of plasmid vectors for expression of foreign proteins in *M. vaccae* and other mycobacteria.

The potent adjuvant properties of mycobacteria make them attractive candidate vaccines for delivery of recombinant antigens from pathogenic mycobacteria or other infectious agents. The development of gene transfer systems for mycobacteria provided an important tool for the generation of novel recombinant vaccines (Stover 1991).

Generation of *E. coli* containing new recombinant plasmids and subsequent screening is extremely rapid, due to the ease of transformation, fast growth of the transformants and the simple extraction of the DNA or protein for testing. However, none of these benefits exist in mycobacteria and to attempt to incorporate new recombinant plasmids directly into mycobacteria is difficult. However, a 'shuttle plasmid' can be screened and grown to high density in *E. coli*, purified and subsequently incorporated into mycobacteria.

The most commonly used gene transfer systems employ plasmid vectors carrying the origin of replication from a naturally occurring mycobacterial plasmid from *M. fortuitum*; pAL5000. Many modern plasmids have features in common with the original shuttle vectors; a 1.8kb segment (*oriM*) from the plasmid pAL5000 supported plasmid replication in mycobacteria and modification by PCR mutagenisis eliminated undesirable restriction sites so it could be combined with the *E. coli* plasmid replicon derived from pUC19 (*oriE*) to create a shuttle vector, which also carried a kanamycin antibiotic resistance marker. An expression cassette was added, containing 404bp of the 5' regulatory region (promoter) of the BCG hsp60 gene including codons for the first six amino acids, a multiple cloning site with 10 unique RE sequences and finally the rrnABt1 transcriptional terminator aligned in the same transcriptional orientation as the other elements, resulting in the *E. coli*-mycobacteria shuttle vector pMV261. This could transform BCG with high efficiency and maintain about five plasmid copies per genome equivalent. (Stover 1991). Although it is difficult to maintain recombinant plasmids *in vivo* without selection, *M. vaccae* is a killed vaccine so does not present this problem.

Transformation efficiency can be varied e.g. wild-type *M. smegmatis* can only be transformed with low efficiency, whereas transformants cured of their plasmid are subsequently able to act as recipients with a relatively high efficiency of transformation and have become laboratory strains. Transfection and transformation efficiencies observed with *M. avium* complex and *M. paratuberculosis* hosts were between 10- and 1000-fold lower than with *M. smegmatis* mc^2155, for kanamycin resistance plasmids

(Foley-Thomas 1995). Using the shuttle plasmid pYUB180 there was no rearrangement after one passage in *M. paratuberculosis*. Thus these mycobacterial species also have the machinery necessary for replication of pAL5000 type shuttle plasmids.

However, Garbe (1994) was unable to demonstrate transformation of *M. w* and *M. vaccae* with vectors employing kanamycin resistance as a selectable marker, and they recently introduced hygromycin as a selectable marker. They developed a novel shuttle vector by inserting a fragment from pYUB12 containing the origin of replication of pAL5000 into the hygromycin resistance expressing plasmid pIJ963. The latter is a pUC (*E. coli*) based plasmid. One colony (p16R1) was capable of replication in *E. coli* and *M. smegmatis* in the presence of hygromycin but the ampicillin resistance associated with the pUC component was deleted during the transformation. This vector gave greater rates of transformation than previously available kanamycin based vectors in many mycobacterial systems and transformants appeared earlier. In *M. vaccae* the p16R1 yielded 10^3-10^5 transformants per μg DNA, with no spontaneous drug resistance in controls. The hygromycin resistance gene (hyg^R) from *Streptomyces hygroscopicus* may be more efficiently expressed in mycobacteria than the *E. coli*-derived aminoglycoside phosphotransferase gene involved in kanamycin resistance. Furthermore hygromycin is useful in providing a resistance marker which avoids the use of a clinically applicable drug. This p16R1 *E. coli*-mycobacterium shuttle vector formed the basis for many available hygromycin resistance containing expression vectors.

Mycobacteria have advantages over other bacteria for the expression of cloned mycobacterial antigens and enzymes in active form. Superoxide dismutase from *M. tuberculosis* was used to investigate the use of hyg vectors for the expression of a heterologous protein in *M. vaccae*. The recombinant *sod*A gene was already expressed in a fully active form in *M. smegmatis* (but not in *E. coli*) and this gene was inserted into the unique Kpn1 site of p16R1 (to form p16R1SOD) and used to transform *M. vaccae*. The 23 kDa protein sub-units (visualised by SDS-PAGE) accounted for more than 10% of the total protein in extracts from transformed cells and the foreign SOD (a tetrameric enzyme) did not form hybrids with the endogenous *M. vaccae* SOD present at low levels.

Little is known about mycobacterial gene expression, particularly in the intracellular environment of the host. However the abundant and highly conserved hsp60 stress protein is a major antigen, essential under all growth conditions but upregulated in response to stress. The *E. coli lacZ* gene when placed under the control of hsp60 in

pMV261 (extrachromosomal) showed striking constitutive expression in excess of 10% of the total BCG protein, but was not upregulated in response to any stress.

Antigens of the human immunodeficiency virus type-1 (HIV-1) are relatively difficult to express at high amounts in many bacterial systems, but could be expressed in rBCG, although expression quantities for most of the hsp60-driven HIV-1 antigen genes (except the HIV-1 *pol* coding region minus its protease which had good expression) did not rival those of hsp60-β-gal (all fused to the sixth codon of the hsp60 gene). Furthermore extrachromosomal expression sometimes appeared to be lethal and it was only possible to express HIV-1 gp120 in the single copy integrative vectors while for HIV-1 gag extrachromosomal and integrative expression were comparable (Stover 1991).

M. smegmatis is a useful model system for mycobacterial genetic experiments because of its ease of laboratory manipulation (non-pathogenic and rapid growing). However, other relatively rapid growing species such as *M. w* and *M. vaccae* (R877R) are currently being administered as immunotherapeutic agents to patients with leprosy and tuberculosis. These could be used as non-living carriers to deliver heterologous antigens to the immune system as an alternative to the *M. bovis* BCG systems already widely proposed.

3.1.3 The aim and practical considerations of cloning SIVp27 into *M. vaccae*

SIVmac is a lentivirus related to HIV-1 and 2 and part of a large group of RNA retroviruses. The group antigen of lentiviruses (*gag*) codes for structural nucleocapsid matrix protein (p17, which lies under the lipid bilayer), inner core protein (p27) and another protein (p15). The gag polyproteins are cleaved by the virion protease into mature proteins.

The aim was to produce a recombinant *M. vaccae* that expressed sufficient SIV *gag* core protein p27 plus some of p17 to induce an immune response to this protein when injected into mice or primates. The nature of this response and how it related to the mode of delivery could then be studied in detail. If the results were encouraging, it would then be possible to test the recombinant *M. vaccae* in the SIV monkey model.

Several vectors were considered, either to increase the level of expression or to alter the compartmentalisation of the SIVp27 within the *M. vaccae*. Finally, with the help of J. Thole, three plasmid vectors were chosen from which to express inserted SIVp27 in bacteria. These plasmids are described next. The results will be summarised later and then presented as three annotated flow diagrams of the sequence in which various

methods were used to generate the final recombinant mycobacteria, followed by annotated photographic scans.

3.1.3.1 Description of expression vectors based on p16R1

Expression vectors based on p16R1 (Garbe 1994) were already available in the laboratory and fully described and hence only a summary is given. Each has an Mrep, Erep and hygR region, plus the appropriate expression module some with a polycloning site (pc). A cartoon of the three plasmids is shown in Figure 3-1.

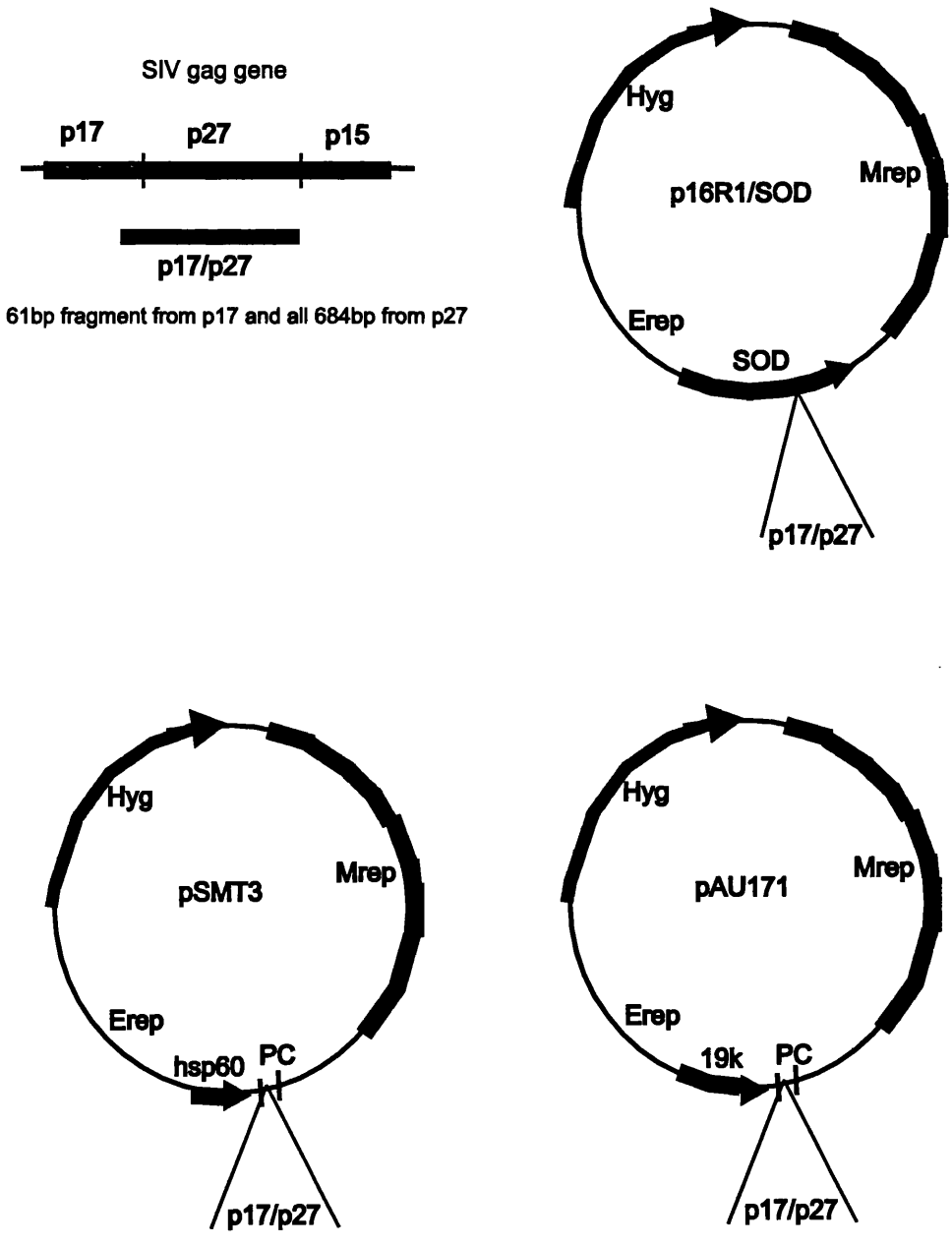


Figure 3-1: Insertion site of SIV p17/p27 in three plasmids

Appropriate regions of sequence for each plasmid are shown in subsequent figures.

p16R1/SOD is the SOD expression vector. It contains a 790bp region (Zhang 1991) including *M. tuberculosis* SOD (bases 112-723, shown as a schematic in Figure 3-2, with important RE sites and the positions of primers for sequencing indicated; each bar represents 10 bases) and a potential ribosome-binding site directly preceding the start codon, cloned as a 1.1kb fragment into the Kpn1 site of p16R1. The SOD gene is strongly expressed and has a number of unique RE sites documented throughout it and hence it is possible to generate a fusion protein of the required length by cloning at an appropriate "in frame" RE site. The high expression of SOD protein within *M. vaccae* cytoplasm made a fusion of SIV p27 to a long region of SOD desirable, since the latter might also confer stability at the mRNA or the protein level and hence allow large overexpression of the fusion protein. The insert occurs after 624 base pairs at Pst1 site. Thus SOD contributes 21kDa to the SOD/p27 fusion protein. Also shown are primer positions for the sequencing primers SOD F4 and SOD R3 used.

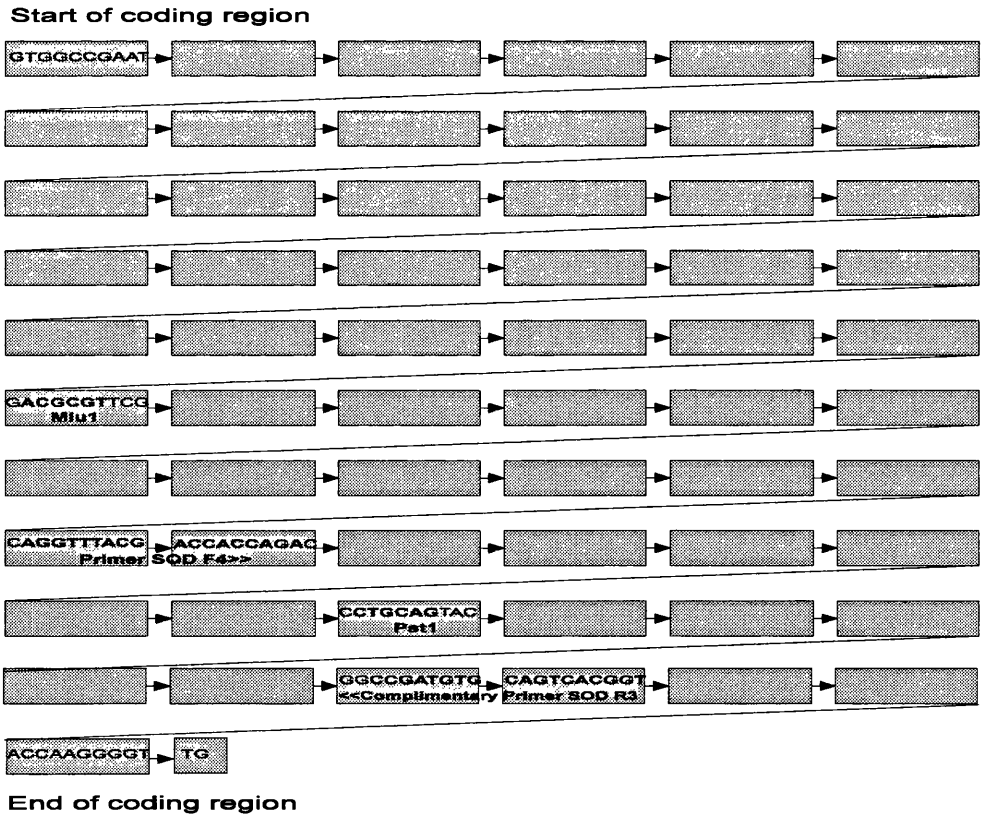


Figure 3-2: Cloning site in plasmid p16R1/SOD

pSMT3. The polycloning site of pBluescript was cloned into p16R1 to generate a flexible vector pOLYG. Then the mycobacterial hsp60 promoter region (350bp) from pMV261 was cloned near the beginning of the polylinker region of pOLYG between Xba1 and BamH1. This is pSMT3 which generates expression of seven amino acids of hsp60 before the first RE site (BamH1) of the remaining polylinker region (shown in Figure 3-3). Proteins expressed from hsp promoters are generally well expressed although not inducible.

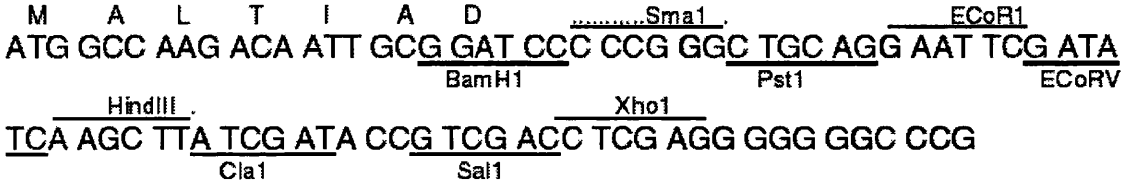


Figure 3-3 : Cloning site in plasmid pSMT3

The RE sites in the polylinker sequence are shown. The first amino acids of the 65k protein are shown. The ECoRV and Cla1 sites were used for cloning (they are sufficiently far apart for efficient RE cutting).

pAU171. High antibody responses can be elicited to an antigen when it is expressed as a membrane associated lipoprotein from the *M. tuberculosis* 19kDa promoter (Stover 1993). It is of interest to see whether altered compartmentalisation of SIVp27 expression in *M. vaccae* would change the immune response evoked in mice.

Part of the gene for the 19kDa lipoprotein of *M. tuberculosis* (representing approximately 30 amino acids) has been cloned into pOLYG using the same sites as those employed for pSMT3 (i.e. Xba1 followed by BamH1). This is pAU171 and the polycloning sites are in the same frame as in pSMT3, which allows straightforward cloning between the two vectors (shown in Figure 3-4). The sequence includes the codons for the acylation signal "LSGCSS" and should generate a secretory and/or membrane bound form of the insert as a fusion protein. The 19k gene segment contributes 4kDa to the 19k/SIVp27 fusion protein.

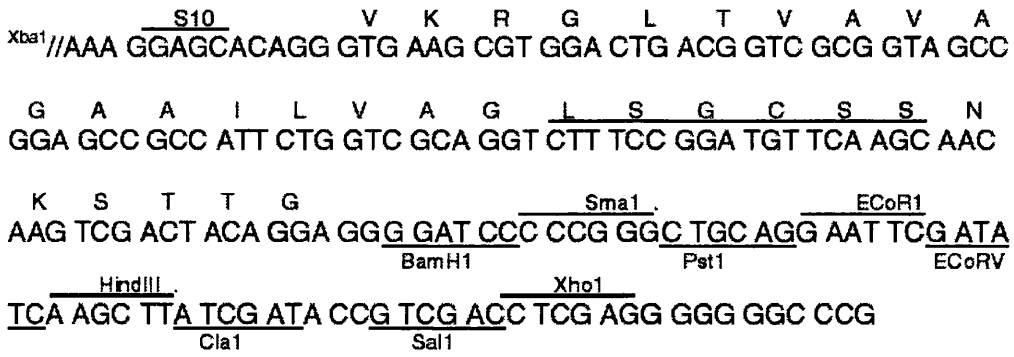


Figure 3-4: Cloning site in plasmid pAU171

3.1.3.2 Source of SIV p27 DNA

Immune responses are already seen to core protein in both animal models and humans infected with HIV. Core protein antigenemia and anti-core protein antibody levels provide an important prognostic indicator of disease progression. SIV is similar to HIV in its nucleotide sequence and its tropism for CD4 lymphocytes and macrophages and after infection with a number of SIV isolates, macaque monkeys develop an immune deficiency syndrome similar to that seen in HIV-infected humans (Letvin 1990).

The source of SIVp17/27 was the recombinant plasmid pKA27 generated by Almond 1990 (obtained from MRC, ADP221). The complete SIV gag gene encompasses three regions arranged consecutively coding for the core proteins p17, p27 and p15. DNA for part of p17 and the whole of the p27 were amplified by PCR from the genomic DNA of cells infected with the SIVmac251 isolate. PCR circumvents the initial need for a clone, and the 767 bp product was fully sequenced (there were 9 silent mutations) and cloned into the expression vector pUC19 before subsequently being expressed from pKA27 as a lacZ-p27 fusion protein in *E. coli* cytoplasm.

Purification by affinity chromatography yielded 500µg of recombinant lacZ-p27 from 1.2g of *E. coli* wet paste (Almond 1990). Consequently it appeared the fusion protein was not toxic for *E. coli* and gave a good yield and hence we would clone the same fragment i.e. p17/27 into our vectors, as the portion of p17 may stabilise the core protein in some way.

3.2 Results

3.2.1 Summary of cloning procedures

Some RE sites adjacent to SIVp17/27 within pUC19 (pKA27) were already available and were checked for suitability. A restriction site map for the published sequence of p17/27 and the silent mutations listed for pKA27 was prepared by Jelle Thole. All RE that cut within the sequence could therefore be excluded from further consideration. Then compatible RE sites were searched for in the chosen shuttle vectors. Ligation of the insert at the RE site must keep the start of the foreign gene in frame with the end of the truncated gene from which the fusion protein will arise. However, no such sites were available so simple sub-cloning was not possible. Consequently desired RE sites had to be added onto either end of the p17/27 region by use of PCR amplification using "floppy extensions".

In frame directional cloning (which is very efficient) into the polycloning (PC) region of pSMT3 was achieved, cutting the PCR product and plasmid with *ECoRV* which generated blunt ends, and *ClaI* which generated 5' four base-pair overhangs. The latter improved circularisation and ligation, reducing some of the problems associated with normal blunt end ligations. After introduction of the recombinant plasmid to *E. coli*, any colonies growing on selective plates would contain a plasmid and re-excision of the insert with ECoRV/ClaI or antibody staining of the *E. coli* lysate would check for the presence of the insert. pSMT3/p27 plasmids were given the codes **'H..'** and expressed the SIVp27 from the hsp60 promoter.

A limitation on the use of PCR for molecular cloning is the risk of misincorporation of nucleotides. Vent polymerase was chosen as it has a low error rate, but the inserts were sequenced after incorporation of correct recombinant plasmids into *E. coli.*

Since pSMT3 and pAU171 had the same polycloning region, simple subcloning of a sequenced SIV insert from the former to the latter was also possible. The plasmids were grown in *E. coli* and expression from the 19k promoter checked. pAU171/p27 plasmids were given codes **'L..'** and expressed SIVp27 attached to several amino acids of the *M. tuberculosis* 19kDa protein, which included the acylation signal and may allow insertion into the membrane.

On this basis several plasmids were transferred to *M. vaccae*. Expression of SIVp17/p27 in mycobacteria was not always equivalent to that in *E. coli.* However, the colonies with best expression were grown and converted to vaccine.

Using p16R1/SOD, the desire to insert p27 towards the end of the coding region of the SOD-A gene (to take maximum advantage of the possible stabilising effect of this protein on inserts within it) limited the choice of RE available that did not cut within the p27 insert. Finally, Pst1 could be used to cut the vector somewhat over 4/5 of the way along the SOD gene, but Pst1 would generate a cut in the p17/27 insert at 324 bp. However, an isoschizomer Nsi1 which did not cut the insert was available and was used to cut the RE sites introduced by floppy extensions to the SIVp17/p27 PCR fragment. This generated cohesive ends but after ligation the fragment could no longer be re-isolated using either enzyme for screening. Furthermore the singly cut vector could self-ligate (which was prevented somewhat by CIAP treatment) and the p17/27 fragment could insert in either orientation only one of which would generate correct expression of the SOD/27kDa fusion protein.

A method existed for ascertaining the orientation of the insert in p16R1/SOD after plasmid purification from the newly transformed *E. coli* clones on selective medium. A fragment was cut out with two RE, one of which cut once within the vector close to the insert, and the other cut asymmetrically within the insert. In this way the cut fragment appeared as one of two different length bands on agarose gels of the RE cut plasmids, thus indicating the orientation. In p16R1/SOD/p27 Mlu1 cut once within SOD, after 414 bp. This left 204bp attached to the following p27 gene. Hind111 cut at 566 bp along from the 5' start in the p27 which is 179 bp from the 3' end. Consequently in the correct orientation the fragment was 770bp long, but in the wrong orientation it was only 383bp long, these differences being clearly visible on a 1% agarose gel. When no insert was present Mlu1 cut once within the SOD gene and the plasmid was simply linearized (i.e. no small fragment will be present). In all cases a larger band was present at approximately 6.4kb representing the rest of the vector DNA.

This method showed there were a large number of incorrect plasmids so only selected *E. coli* lysates were screened for SIVp27 and SOD expression. Then the chosen plasmids were sequenced and electroporated into *M. vaccae*, where expression of SIVp27 and SOD were checked before single colonies were cultured and converted to vaccine. p16R1SOD/p27 plasmids were given codes '**S1..** and **S2..**' and expressed SIVp27 as a long fusion protein with *M. tuberculosis* SOD.

3.2.2 Full details of the cloning procedures

The complete series of steps involved in generating each recombinant *M. vaccae* are shown in 3 flow diagrams (Figure 3-5, Figure 3-6, Figure 3-7). Important steps have

figure numbers attached and these relate to scanned images of the gels or blots which are shown subsequently.

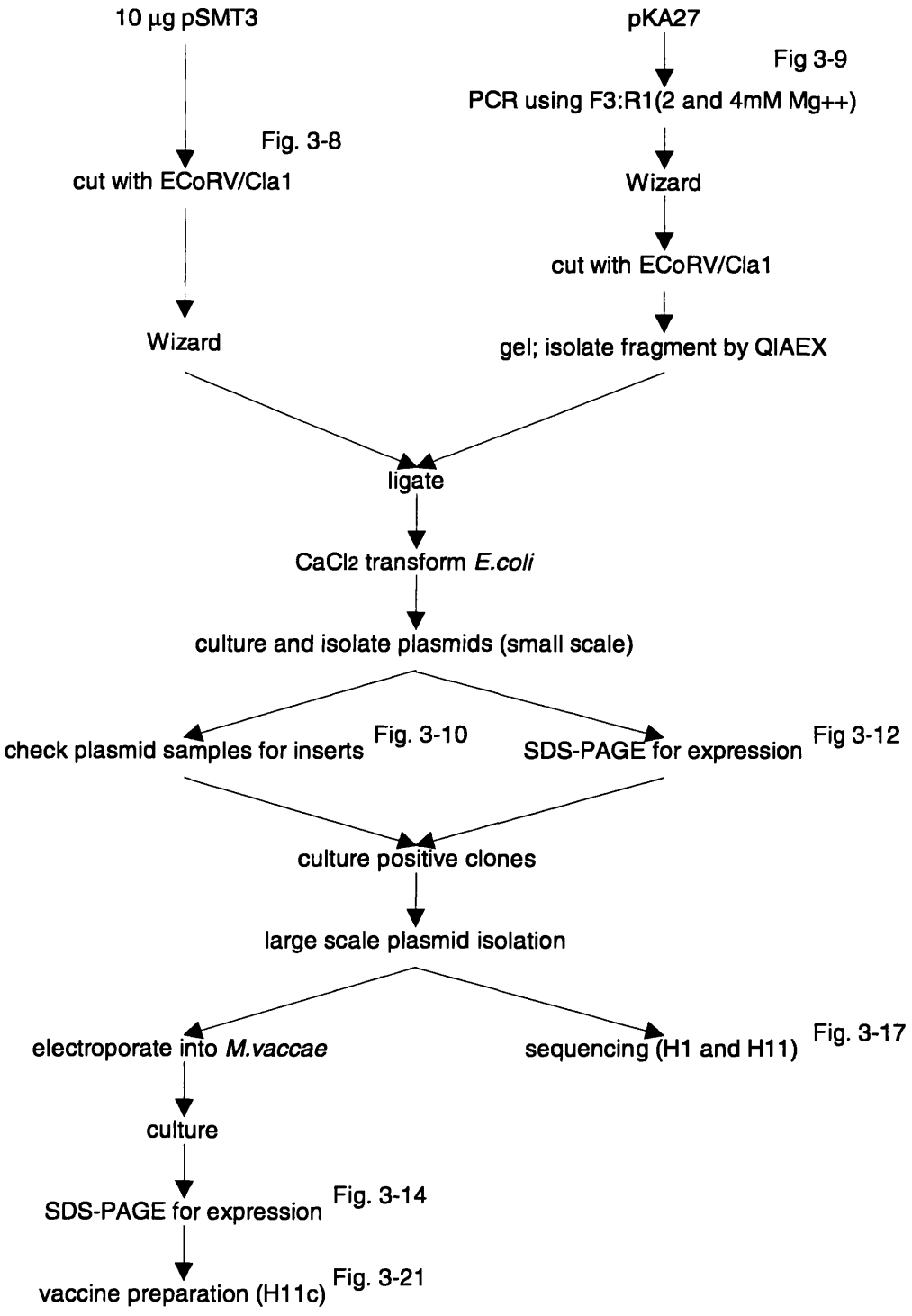


Figure 3-5: Flow chart of introduction of SIVp27 into pSMT3 and subsequent culture in various bacteria

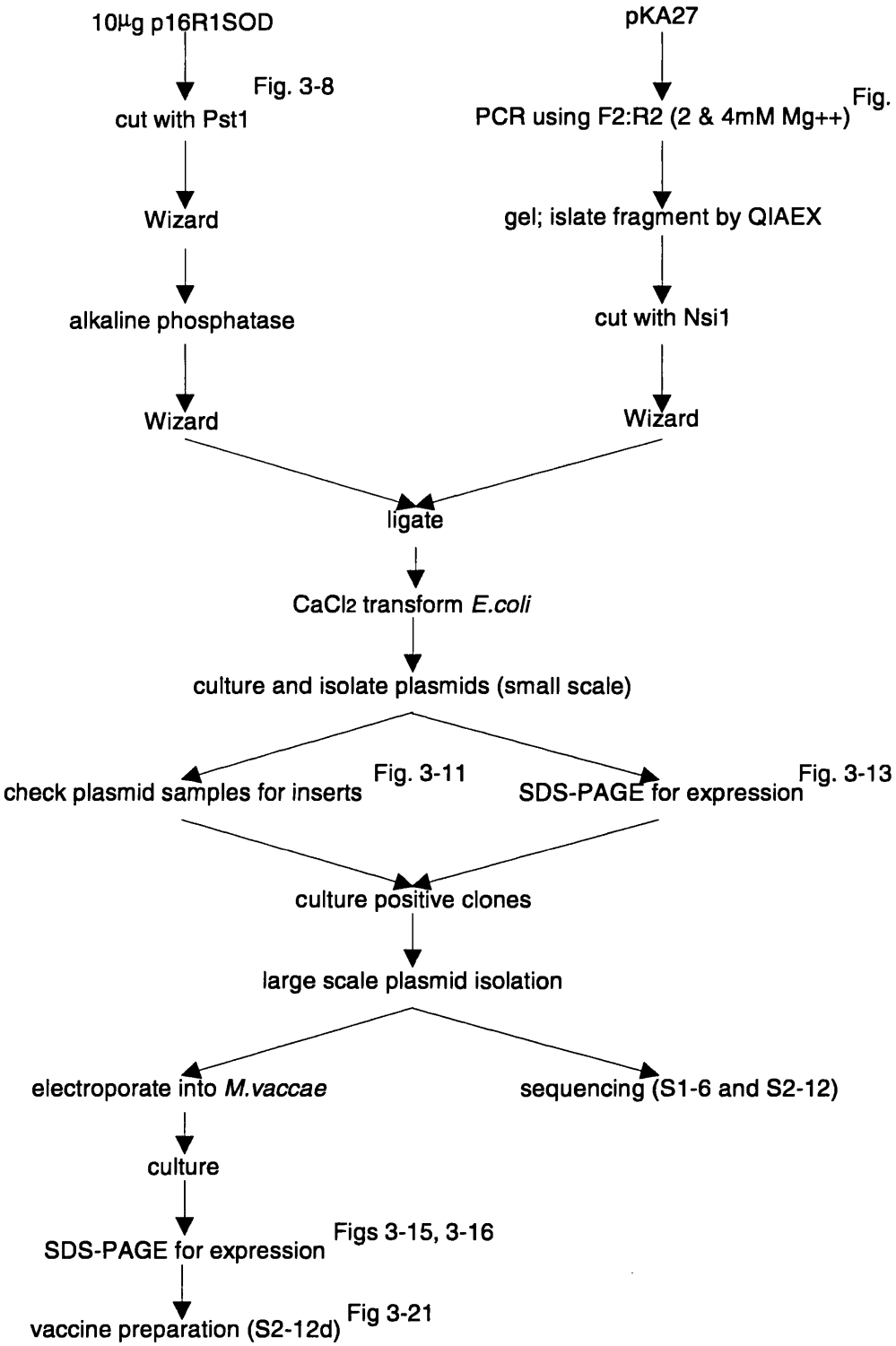


Figure 3-6: Flow chart of introduction of SIVp27 into p16R1SOD and subsequent culture in various bacteria

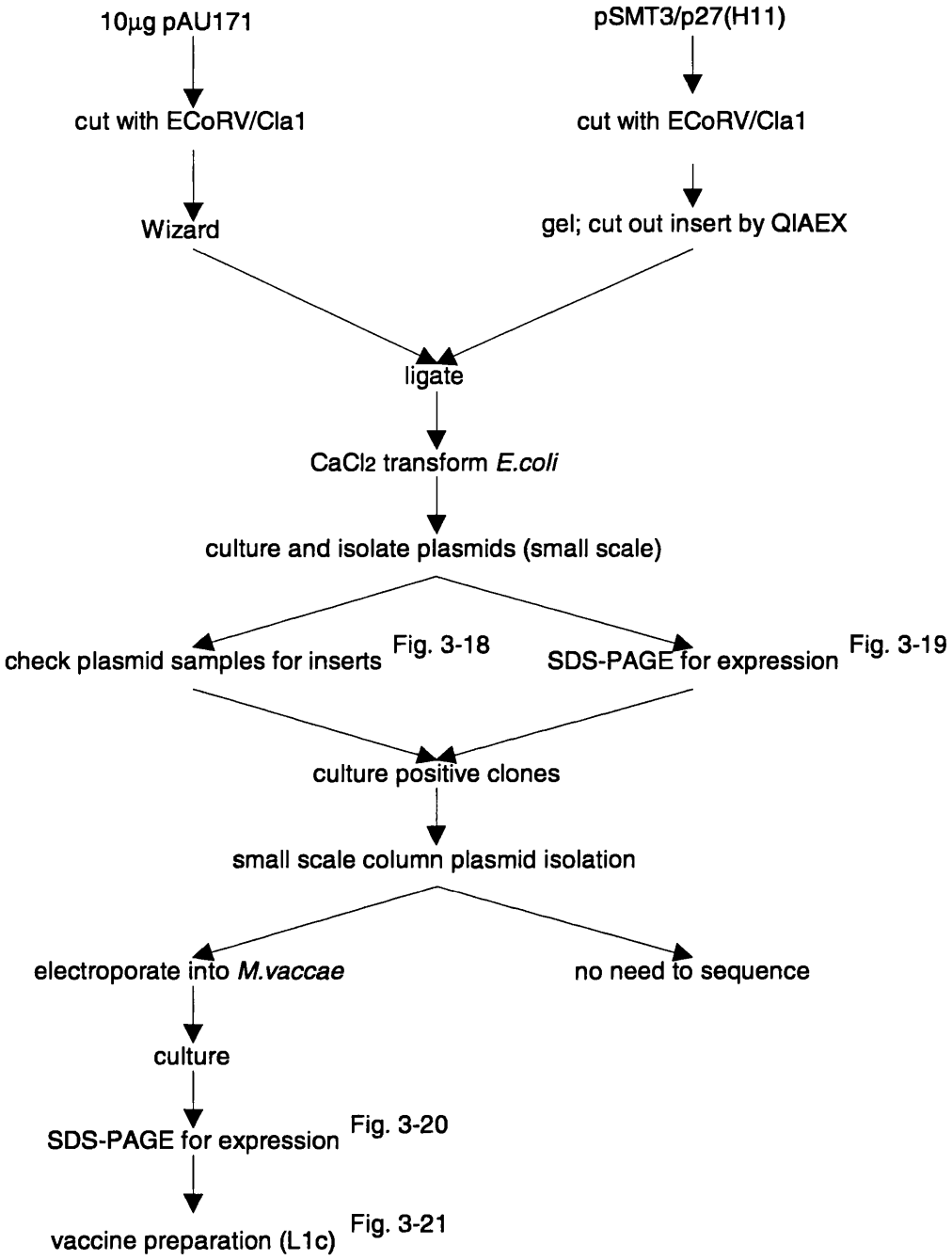
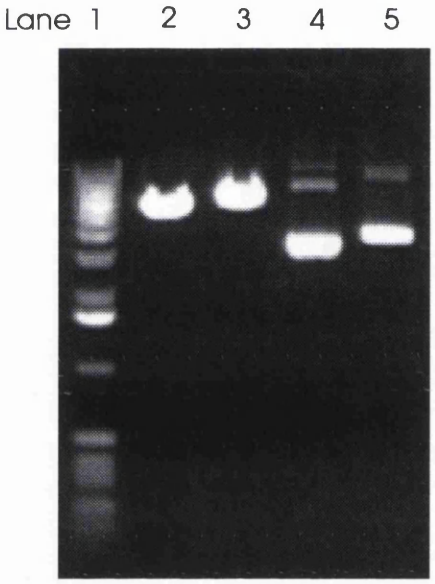


Figure 3-7: Flow chart of introduction of SIVp27 into pAU171 and subsequent culture in various bacteria

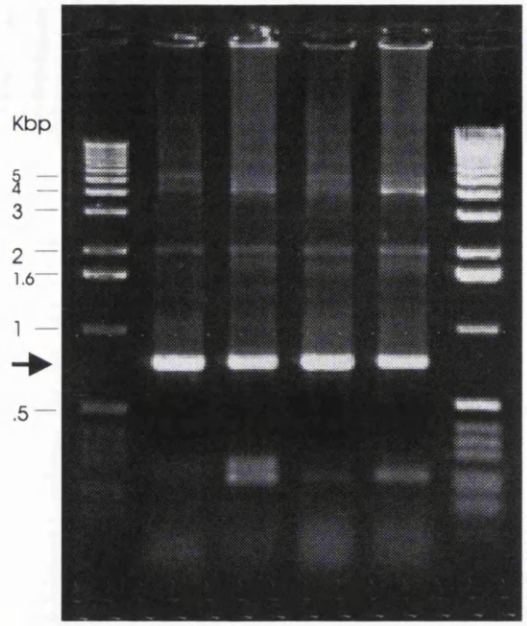
Enzyme reactions, primer sequences, PCR conditions, plasmid purification, agarose gels, sequencing, Western blotting and bacterial culture protocols are all fully described in the Materials and Methods chapter.



Lane 1: DNA Markers (5µl of 0.1µg/µl)
Lane 2: pSMT3 (5.7kb) successfully cut with ECoRV /Cla1
Lane 3: p16R1SOD (6.4kb) successfully cut with Pst1
Lane 4: Uncut pSMT3 (showing the fast running supercoiled DNA and the mutimeric plasmids above it)
Lane 5: Uncut supercoiled p16R1SOD, which is a somewhat larger plasmid that, even supercoiled, runs less far in the gel.

Figure 3-8: 0.7% agarose gel showing vectors before and after cutting by RE.

The vectors are opened at specific RE sites, an aliquot checked by electrophoresis and the remainder treated and cleaned as indicated on the flow charts ready to receive the insert DNA.

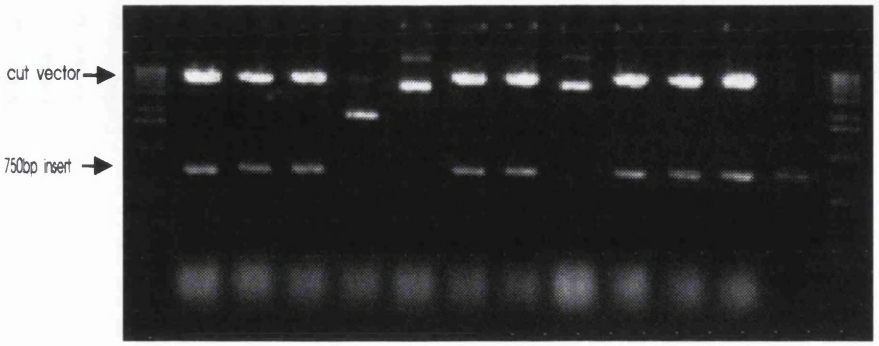


Lane 1: DNA Markers (3µl).
Lane 2: Primers R1 and F3 (ECoRV/ClaI termini) with 2mM Mg^{++} for ligation to pSMT3
Lane 3: Primers R1 and F3 (ECoRV/ClaI termini) with 4mM Mg^{++} for ligation to pSMT3
Lane 4: Primers R2 and F2 (NsiI termini) with 2mM Mg^{++} for ligation to p16R1SOD
Lane 5: Primers R2 and F2 (NsiI termini) with 4mM Mg^{++} for ligation to p16R1SOD
Lane 6: Markers (5µl)

Figure 3-9: 0.7% agarose gel showing the results of several PCR reactions.

3µl of 0.1µg/µl DNA ladder markers produced clearer results and were used for all future experiments. The DNA sizes (in kilo-base pairs) are indicated next to the bands.

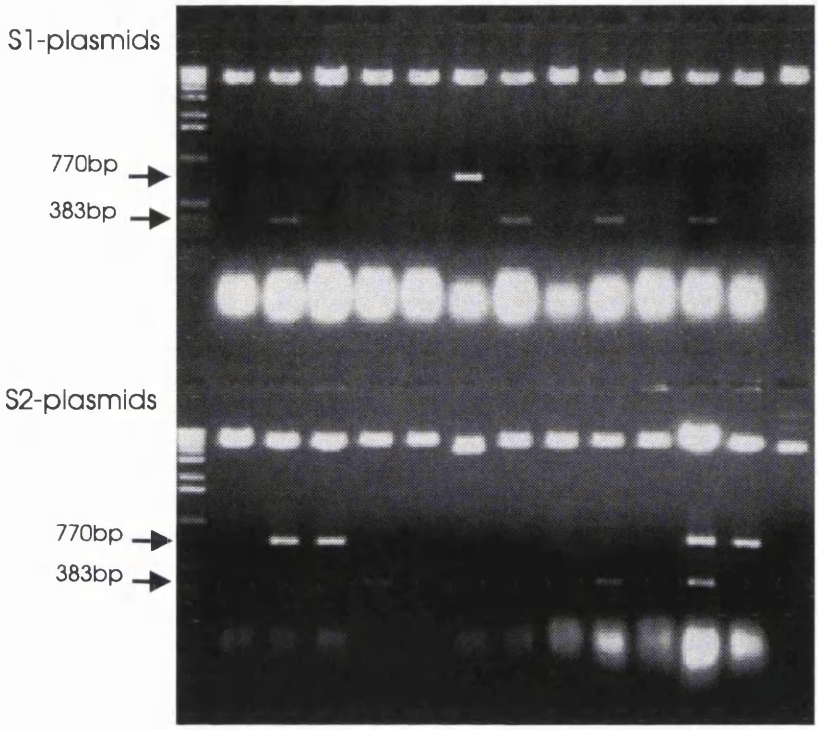
The p17/27 insert was amplified by PCR from the pKA27 plasmid using the primers with 'floppy extensions' R1 and F3 or R2 and F2. 5µl of product was loaded. From the gel it was estimated that 100µl contained 20µg most of which was the specific 750bp product (indicated by an arrow), so half was used for further reactions and ligations. The concentration of magnesium sulphate did not affect the PCR greatly, although 4mM did appear to generate slightly higher concentrations of non-specific bands. Hence the 2mM products were used for the RE cutting (although problems with p16R1SOD meant finally both 2 and 4mM products were cloned). The presence of mis-primed bands indicates that prior to ligation, the entire cut PCR sample must be loaded onto a gel and the 750bp fragment cut out and recovered by QIAEX to purify the DNA and prevent incorrect fragments being ligated to vector and transforming the *E. coli* (not shown).



Lanes 1 and 14	Markers
Lanes 2-12	Recombinant plasmid DNA cut with ECoRV/ClaI for analysis (called H1 to 8; H9 not tested; then H10 to 12 respectively)
Lane 13	3µl of PCR R1 F3 (from prep used for ligation) to confirm size of insert

Figure 3-10: 1% agarose gel of cut plasmids from *E. coli* transformed with pSMT3/p27.

H 1, 2, 3, 6, 7, 10, 11 and 12 were clones of *E. coli* that contained the correct recombinant pSMT3/p27 plasmids since all had correct size inserts recut from purified plasmid by the REs. H4 was an uncut supercoil with no insert while H5 and H8 were uncut supercoils with inserts. These had been mutated and lost their RE sites during plasmid preparation or transformation. H9 was not gelled. The transformation rates of these and other plasmids in *E. coli* are shown in Table 3-1.



Upper half (S1- plasmids)

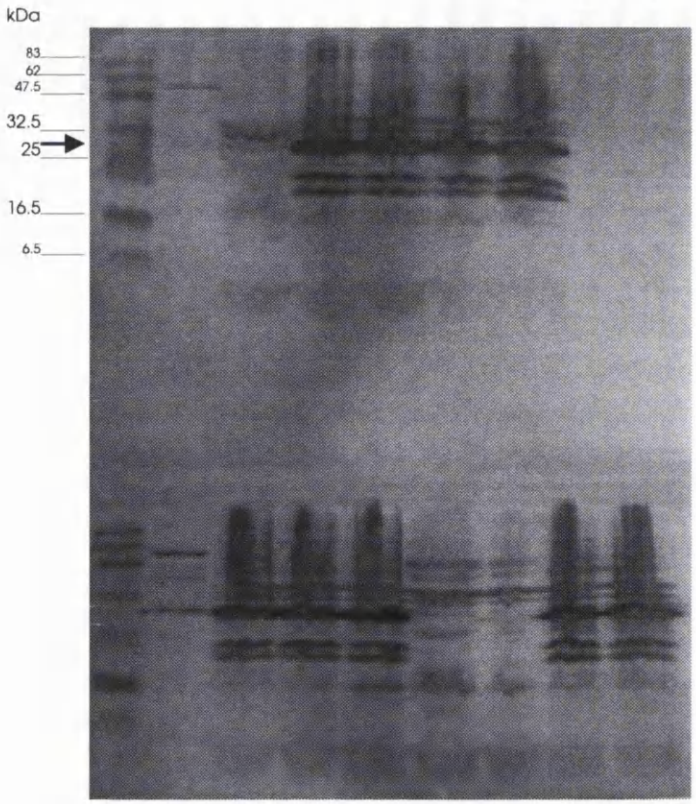
Lane 1:	Markers
Lanes 2-13:	Plasmid DNA cut with Mlu1/HindIII (called S1 1-12, PCR 2mM Mg)
Lane 14:	p16R1SOD vector cut with Mlu1/HindIII (control)

Lower half (S2- plasmids)

Lane 1:	Markers
Lanes 2-13:	Plasmid DNA cut with Mlu1/HindIII (called S2 1-12, PCR 4mM Mg)
Lane 14:	uncut p16R1SOD vector (control)

Figure 3-11: A 1% agarose gel showing plasmids from *E. coli* transformed with p16R1SOD/p27, and cut to determine orientation of inserts

All the plasmids were successfully cut. Those without inserts were simply linearized. From upper half, clones S1-2,7,9,11 have inverted p27 inserts (383bp fragment). S1-6 has the p27 in the correct orientation (770bp fragment). From lower half, S2-4,5,9 have inverted inserts and S2-2, S2-3 and S2-12 have p27 in the correct orientation. S2-6 has mutated somehow. S2-11 has both plasmids at this stage.



Proteins from all twelve H type recombinants were run on two 15% SDS-PAGE mini-gels. They were fast blotted onto a single sheet of nitrocellulose and the blot was stained with anti-p27, to check for expression from the recombinant plasmids.

Top

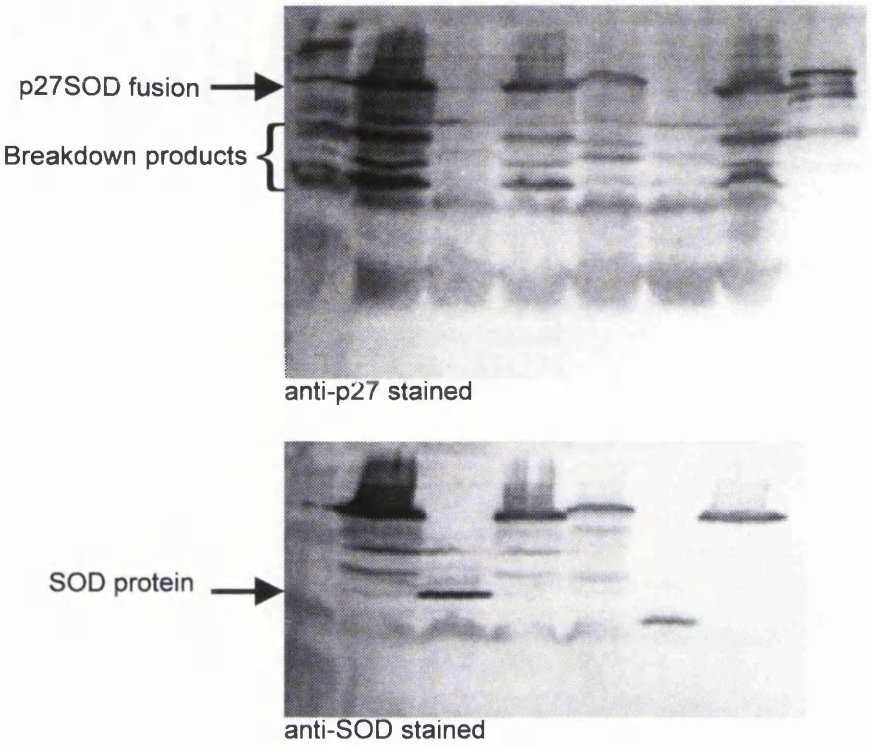
Lane 1	Prestained broad range protein markers.
Lane 2	1μg of SIV p27 expressed as a glutathione -s-transferase fusion protein (SIVp27GST) of 54kD (ADP643/628). Some breakdown products are also present.
Lane 3-7	H8 to H12. 1ml of culture, pelletted and boiled in 75μl of SB. 10μl loaded.

Bottom

Lane 1	Prestained broad range protein markers.
Lane 2	1μg of SIV p27. As above
Lane 3-9	H1 to H7 respectively. As above.

Figure 3-12: Immunoblot of H plasmid (pSMT3/p27) expression in *E. coli*

Expression of the SIVp27 peptide is present in H1, H2, H3, H6, H7, H9, H10, H11 and H12 and appears as a band between 25 and 32.5kD (indicated with an arrow in the top gel), with a breakdown product at about 20kD. This correlates with the presence of inserts seen in the plasmids by agarose gel. The SIVp27GST control was also positive (limit of detection between 0.23 and 0.07μg; not shown). The immunoblot showed some background staining, but the negative samples were clearly distinguishable.



Proteins from selected S1 and S2 type recombinants (based on the RE pattern) were run on two 15% SDS-PAGE mini-gels, blotted onto nitrocellulose and checked by Ponceau S. One blot was stained with anti-p27 (upper part) and the other (lower) with anti-SOD (D2D). For both gels the samples were:

Lane 1:	Prestained broad range protein markers.
Lane 2:	S1-6
Lane 3:	S2-1
Lane 4:	S2-2
Lane 5:	S2-3
Lane 6:	S2-4
Lane 7	S2-12
Lane 8 (upper only):	1μg of SIV p27 as positive control

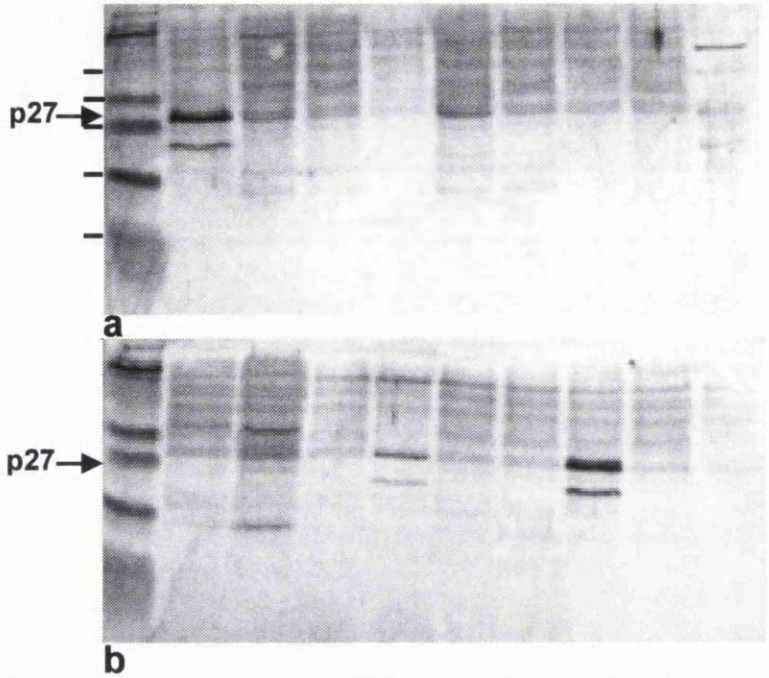
Figure 3-13: Immunoblots of p27 and SOD expression from S1- and S2- plasmids (p16R1SOD/p27) in *E. coli*

Previously it was uncertain whether *E. coli* could express proteins from the mycobacterial SOD promoter, as it had been shown in *functional* assays (using non-denaturing gels) not to yield an active SOD molecule. (Ying Zhang, personal communication). However, in agreement with the agarose gel results of the plasmid inserts, S1-6, S2-2 and S2-12 expressed a SODp27 fusion protein at about 50kD which was recognised by anti-p27 and anti-SOD mAbs.

S2-1 had been previously shown to have no insert (the original plasmid either self-annealed due to incomplete alkaline phosphatase treatment, or was never cut) and did not express a SODp27 fusion protein. However, it did express an intact SOD at around 23kD, at a similar concentration to the fusion protein.

S2-3 has a mutated SODp27 fusion protein of a different size and with altered breakdown products. This was not recognised at the DNA level, and the recombinant was discarded.

S2-4 had been shown to have an inverted insert and hence no band was present after anti-p27 staining. There was a truncated band in the anti-SOD stained blot; this was due to the part of SOD up to the Pst1 site being expressed, but where the inverted insert was present it possibly introduced a stop codon or other termination signal and hence the shorter SOD was expressed.



The protein markers at 8.3, 18.8, 25.5, 31.4 and 47.1kD are indicated along with the position of the SIVp27 protein expressed from the 65kD hsp promoter

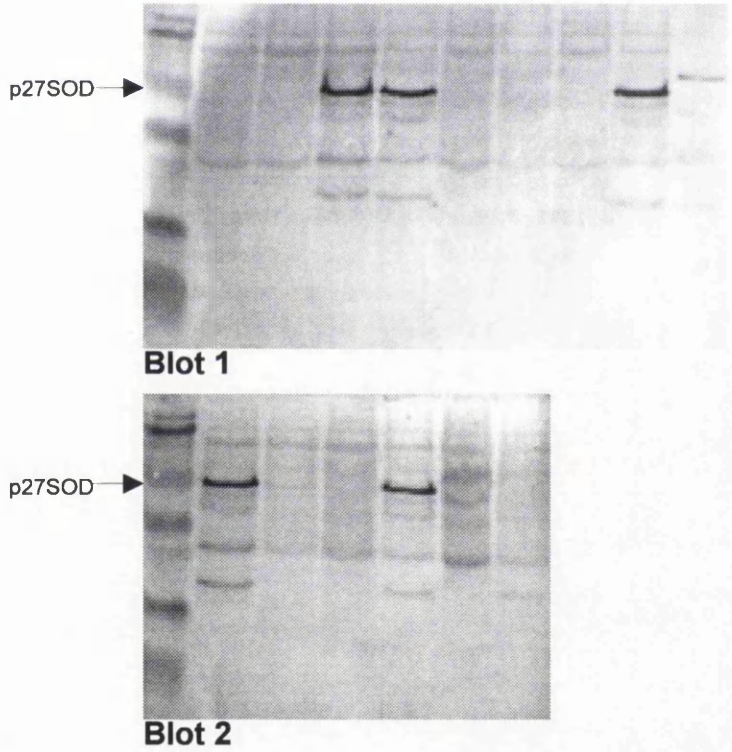
	Blot a	**Blot b**
Lane 1:	protein markers	protein markers (broad range)
Lane 2:	H1a	H3a
Lane 3:	H1b	H3b
Lane 4:	H1c	H3c
Lane 5:	H1d	H3d
Lane 6:	H2a	H11a
Lane 7:	H2b	H11b
Lane 8	H2c	H11c
Lane 9:	H2d	H11d
Lane 10:	purified GSTp27	pSMT3

Figure 3-14: Expression of SIVp27 from pSMT3/p27 (H-type plasmids) in *M. vaccae*

Plasmids purified from *E. coli* clones H1, H2, H3 and H11 were electroporated into *M. vaccae* and cultured on separate plates for 7 days (this is two days longer than necessary for the positive control pSMT3 plasmids) with hygromycin selection until discrete colonies were visible on each. 4 colonies were tested from each plasmid type. The electroporation efficiencies for each plasmid in *M. vaccae* are shown in Table 3-2.

Part of each small colony was sonicated and boiled in sample buffer, before being loaded on a 15% SDS-PAGE and immunoblotted with anti-p27. Although in theory all plasmids were identical and hence any colony that grows should contain the correct plasmid, it was shown that different clones had different levels of expression in *M. vaccae*.

Only clones H1a, H11c and possibly H3d showed adequate expression. The other colonies either had expression below the limit of resolution by immunoblotting, or somehow failed to express the p27 protein even though they were resistant to hygromycin. (The negative control plates however showed no evidence of spontaneous mutation). The overall level of expression in *M. vaccae* did not appear as great as in *E. coli*, since the band staining with anti-p27 was less intense. A number of technical points, such as the need for sonication to release the cytoplasmic proteins from the stronger *M. vaccae* cell wall and total number of bacteria lysed may explain this. Alternatively, differences in plasmid copy number and expression or protein stability between *E. coli* (selected for its suitability for cloning) and *M. vaccae* (registered for use in humans as a vaccine) may also contribute to levels of expression of p27.



Blot 1:
Lane 1: protein markers (broad range)
Lanes 2-5: S1-6 a-d
Lanes 6-9: S2-2 a-d

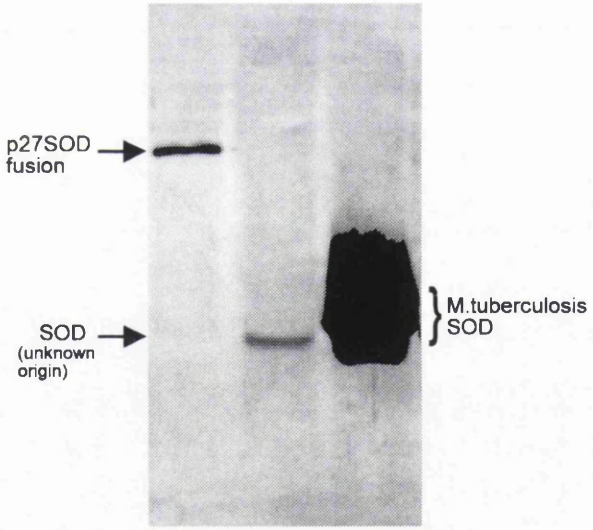
Blot 2:
Lane 1: protein markers
Lanes 2-5: S2-12 a-d
lane 6: S2-3
lane 7:p16R1SOD

Figure 3-15: Expression of SIVp27 from p16R1SOD/p27 (S-type plasmids) in *M. vaccae*

4 colonies of *M. vaccae* transformed with each purified S-type plasmid were prepared for p27 immunoblotting as just described. Two out of four colonies from the electroporation with S1-6 (namely c and d) expressed SIVp27 and one from S2-2

(namely d; as shown on blot 1). Two of four expressed p27 from the S2-12 set (a and d, blot 2). The controls containing irrelevant plasmids S2-3 (which had shown an altered p27 pattern in *E. coli*) and p16R1SOD in *M. vaccae* showed no non-specific binding of anti-p27 with normal bacterial proteins.

When a suspension from the original S2-12d colony was made and plated at a concentration to yield further single colonies by eight days, all six tested were as strongly positive as the original. However, with more than two successive sub-cultures (by streaking) and growth to confluency (more than 13 days) on 7H11 with hygromycin expression gradually declined until there was no detectable SIVp27/SOD fusion protein band. (A similar trend was seen in the other constructs). No clear reason for this was established due to lack of time (data not shown).



Lane 1: *M. vaccae* expressing S2-12d (original colony)
Lane 2: *M. vaccae* expressing S2-12c (original colony)
Lane 3: *M. vaccae* expressing p16R1SOD

Figure 3-16: Immunoblot stained with D2D (anti-SOD) to show relative abundance of the *M. tuberculosis* SOD produced from various plasmids in *M. vaccae*

The samples were run on a 12% gel and immunoblotted. The mAb D2D showed good specificity and identified the large SOD/p27 fusion protein in lane 1 (that was also detected by anti-p27 run on the previous gel). Lane 3 shows the far greater expression of the unaltered *M. tuberculosis* SOD from the original vector p16R1SOD, which was claimed to yield up to 10% of the bacterial proteins as foreign peptide. The expression in my hands is sufficiently large to be visible on Coomassie blue staining of the gel

itself whereas the fusion proteins could not be distinguished from other proteins of *M. vaccae* (not shown). S2-12c (lane 2) did not express p27 and the band identified by anti-SOD ran between the 18 and 25kDa markers as expected for SOD alone. Old cultures of *M. vaccae* did express endogenous SOD detected weakly by D2D, (Garbe 1994) and if this is *M. vaccae*'s own SOD the recombinant SOD from the plasmid is not expressed, which may mean loss of the plasmid despite growth as a single colony on selective medium. However it is possible that the SOD band is that of *M. tuberculosis* SOD from the plasmid, since no such *M. vaccae* band is apparent in lane 1. If this is the case then the expression has been severely reduced presumably in the recombination process that also prevented the expression of p27 (either loss of part of the DNA or some form of protein termination). Interestingly, when reduction of p27 expression is seen, simultaneous immunoblotting with anti-SOD shows a similar reduction of the high molecular weight fusion protein band and an increase in this lower molecular weight band, possibly indicating that some conversion has taken place during culture.

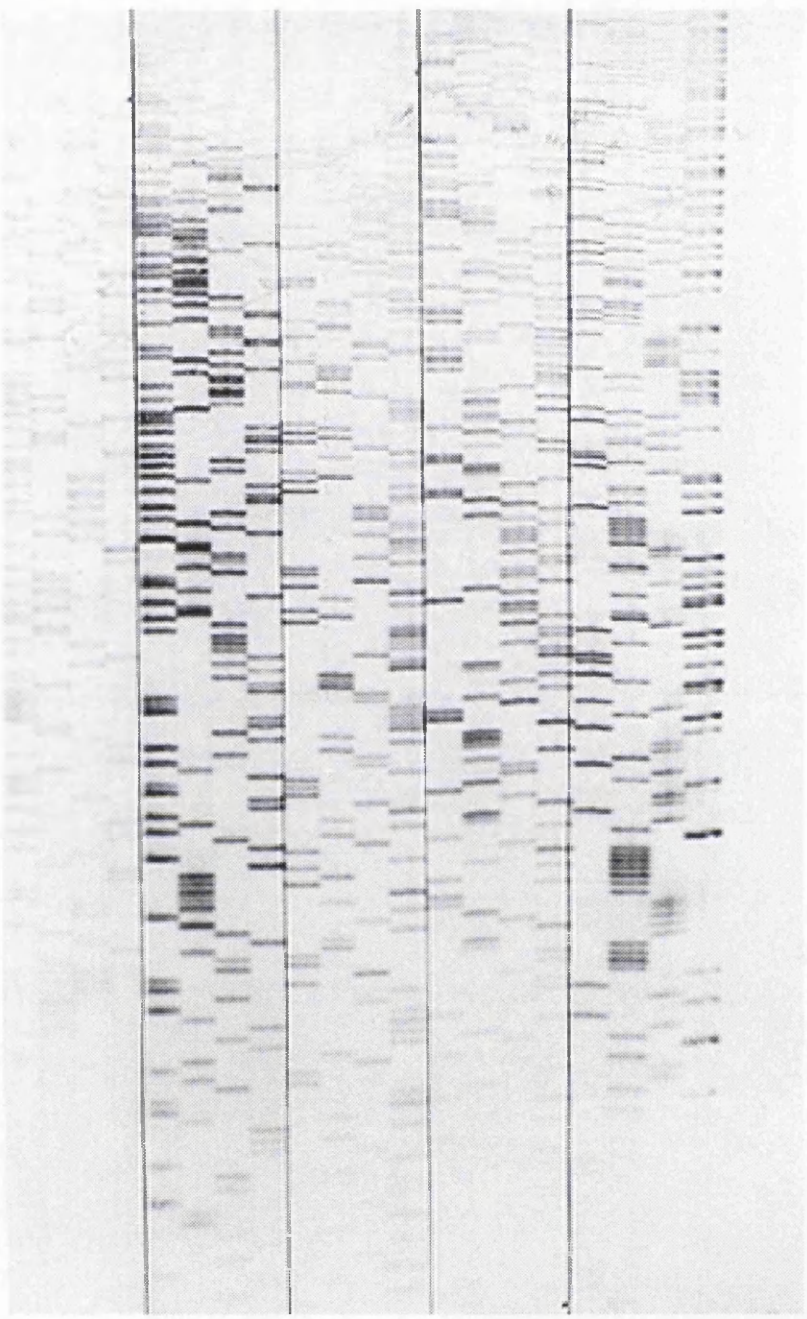


Figure 3-17: Results of sequencing the H11 plasmid

The photograph shows the autoradiograph of the sequence of the insert in the H11 plasmid purified from *E. coli*. The other sequences are not shown. They were prepared using the following primers, and corresponding lanes are labelled.

18mer sequencing primers reading inside p17/27

P27 F4: 5'TAAATGCCTGGGTAAAAT

p27 R3: 5'CATTTTGAATCAGCAGTG

p27 R4: 5'CTGTAAATGTTGCCTACT

18mer sequencing primers reading in SOD flanking the Pst1 site

SOD F4: 5'AGGTTTACGACCACCAGA

SOD R3: 5'ACCGTGACTGCACATCGG

18mer sequencing primers reading in pSMT3 flanking the insert

The codes were 93-668 (forward) and 9002 (reverse).

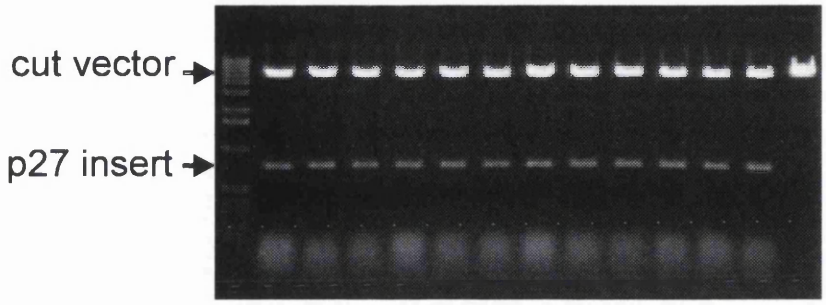
These were provided by Sarah Ely (sequences not available).

Sequencing autoradiographs are read by tracking up from the bottom of the gel checking each of the four lanes (labelled a, c, g, t) for the next occurring band. The sequence reads forwards on the coding strand from forward primers (called F..) and backwards on the non-coding strand from reverse primers (labelled R..). This is compared base by base to the known sequence of the p17/27 (pNIBSC1). To initially identify your position in the sequence it is possible to look for obvious groupings of bases. For example reading from 9002 backwards in a pSMT3/p27 plasmid the region containing multiple Gs and Cs at the end of the plasmid polylinker sequence (after the end of p27 at Cla1) was very clear.

Plasmids H1 and H11 (pSMT3/p27) and plasmids S1-6 and S2-12 (p16R1SOD/p27 constructed from different PCR samples) were sequenced, using 5 primers each. The complete gene was readable with some overlap occurring between primers.

Since the plasmids all expressed peptides of the correct length and immunogenicity (by immunoblotting of SDS-PAGE), small areas of compression where the exact number of a single base type present was not clear (e.g. AAAA versus AAA) could be disregarded, since it was unlikely that Vent DNA polymerase would cause a deletion or insertion. These problems were not common, usually occurring towards the top or bottom of the autoradiograph and could usually be resolved by checking an overlapping region from another primer. It appeared that no incorrect base pairs had been incorporated and my p17/27 sequences were identical to that of pKA27. Hence all clones tested were suitable for further investigation as potential vaccines.

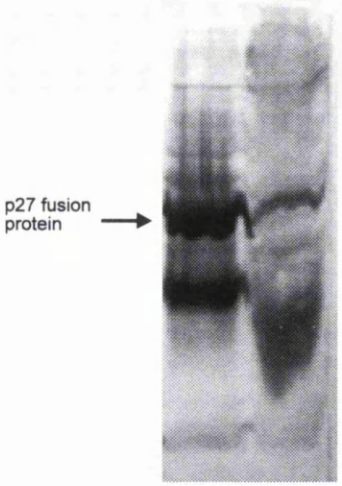
Furthermore this region from plasmid H11 was suitable for sub-cloning directly into the pAU171 vector.



Lane 1: Markers
Lanes 2-13: recombinant plasmids L1-12 cut with ECoRV/Cla1
Lane 14: vector alone cut with ECoRV/Cla1

Figure 3-18: 0.7% agarose gel showing cut plasmids (called L-) from *E. coli* transformed with pAU171/p27.

Purified DNA from the H11 plasmid in *E. coli* (pSMT3/p27) was cut with ECoRV /Cla1 and the insert extracted from a 0.7% gel prior to insertion in cut pAU171 (the 19k expression vector) and transformation of *E. coli*. This eliminated the need for sequencing these new plasmids (called **L-**) since they were generated by simple cloning. All the recombinants on the selective medium contained p27 inserts in the 19k vector. This shows good efficiency and may reflect the quality of the DNA or the fact that the insert had already been successfully propagated in *E. coli* prior to simple cloning into a new vector.

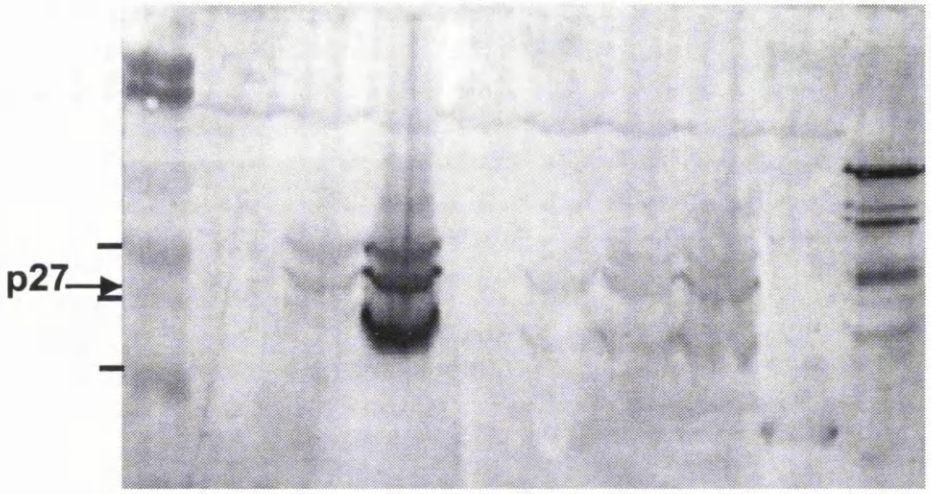


First lane: L1 expressed in *E. coli* (as indicated) and a breakdown product
Second lane: pAU171 control in *E. coli*

Figure 3-19: Immunoblot of plasmid L1 (pAU171/p27) expression in *E. coli*.

Proteins were run on a 12% SDS-PAGE and immunoblotted with anti-p27. The SIVp27 is expressed to a very high level attached to the signal region of the 19kD protein. Non-specific bands in the control lysate do not correspond to the heavily stained p27 band and its breakdown product in lane 1.

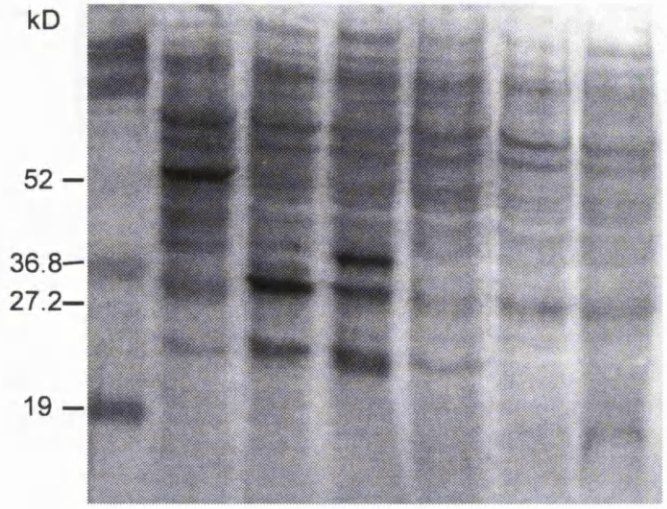
All twelve *E. coli* recombinants with plasmids L1-12 showed similar expression of the partial 19k/p27 fusion protein (whole gel not shown) which agreed with the DNA gel.



Lane 1: Protein markers (low range; 19, 27.2 and 36.8 kD markers indicated)
Lanes 2-9: L1a-d and L2a-d respectively; position of expressed SIV p27 shown
Lane 10: purified p27 1µg

Figure 3-20: Immunoblot of expression of SIVp27 in pAU171/p27 plasmids L1 and L2 in *M. vaccae* following sub-culture

Individual colonies of *M. vaccae* growing soon after electroporation with plasmids L1 and L2 showed no p27 expression (gel not shown), but technical difficulties may have been responsible since the colonies were small, or perhaps the expression required longer to become apparent from this plasmid (e.g.. if it was acylated and secreted). Hence eight colonies were sub-cultured onto 7H11-hygromycin plates and grown for a further 5 days at 30^0C. These results are shown in Figure 3-20, after immunoblotting of a 12% SDS-PAGE with anti-p27. Only one colony (L1c) had strong expression of SIVp27 from the 19kD promoter (seen as several bands, the higher one possibly being acylated/glycosylated/secreted), the others having levels at least below the limit of detection, even though the gel was overloaded. This is at odds with the ease of expression in *E. coli*, and may again indicate a toxicity of highly expressed proteins for *M. vaccae*.



Lane 1: Protein markers (low range)
Lane 2: *M. vaccae* with p16R1SOD/p27 (S2-12d)
Lane 3: *M. vaccae* with pSMT3/p27 (H11c)
Lane 4: *M. vaccae* with pAU171/p27 (L1c)
Lane 5: *M. vaccae* with p16R1SOD
Lane 6: *M. vaccae* with pSMT3
Lane 7: *M. vaccae* with pAU171

Figure 3-21: Immunoblot of all recombinant *M. vaccae* (expressing SIVp27 and controls) just prior to conversion into vaccine.

Each original recombinant *M. vaccae* colony expressing SIVp27 (S2-12d and H11c or the sub-culture for L1c) was sub-cultured onto 10 7H11 plates containing hygromycin and grown for 5-6 days at 30^0C. Samples were sonicated, boiled in sample buffer, run on a 12% SDS-PAGE, blotted and stained lightly with ink before staining with anti-p27. The blue TMB colour (seen here as dark bands) clearly identified the SIVp27 (and breakdown products) as being quite uniformly expressed, against a background black staining used to pin-point the p27 band against the other proteins and to show an even level of protein loading. The relative positions of the protein markers are shown. This showed that all recombinants were expressing p27 protein to a reasonable level, which was probably similar to the best that had been seen to date, and hence the mycobacteria were ready for immediate conversion to vaccine. They were not cultured until late logarithmic phase (as for standard vaccine) as this resulted in decreased SIVp27 expression.

3.2.3 Tables showing relative abundance of recombinant plasmids in various bacteria

These indicate the transformation efficiencies of various plasmids into *E. coli* and mycobacterial hosts.

E. coli DH5α				
ligation mix	µl plated	n° colonies	plasmid name	remarks
pSMT3/PCRlane2	10	15	H (1-12)	Ligation mixes transformed directly into *E. coli* and 12 colonies tested for insertion and expression
	100	crowded		too many to count, with some colonies being very small due to inhibition and some merged
pSMT3/H_2O	10	23		Cut vector should have unmatched ends and not transform, but possibly both REs did not cut 100% efficiently
	100	crowded		
pSMT3 (original)	100	crowded		positive control confirms competence of *E. coli*
H_20	100	none		negative control confirms antibiotic sensitivity of *E. coli*
p16R1SOD/PCR lane4	10	4	S1 (1-12)	these recombinant plasmids transformed less well than pSMT3/PCRlane2; influence of alkaline phosphatase (plasmid circular not superhelical) and size?
	100	47	ditto	12 colonies taken randomly from both plates
p16R1SOD/PCR lane5	10	7	S2 (1-12)	(A large number of colonies were tested as the p27 gene could insert in either orientation)
	100	41	ditto	12 colonies taken randomly from both plates
p16R1SOD/H_2O	10	1		Alkaline phosphatase treatment greatly reduced the self-ligation of vector cut with one RE, to decrease non-recombinant clones
	100	19		
p16R1SOD (original)	100	covered		p16R1SOD had similar efficiency of transformation as pSMT3
H_2O	100	none		
pAU171/H11 insert	10	43	L (1-12)	All colonies expressed p27
	100	500		
pAU171/H_2O	10	15		
	100	240		
pAU171 (original)	10	850		Vector treatments and gene insertion reduce the transformant number by 20 fold
	100	covered		pAU171 had similar efficiency to other vectors
H_2O	100	none		

Table 3-1:Transformation efficiencies in *E. coli*

M. vaccae				
plasmid name	µl plated	n° colonies	colony code	remarks
H1 (pSMT3/p27 from *E. coli*)	50	2	H1 (a-d)	Different volumes were plated after electroporation (for *M. vaccae*) compared to transformation. Recombinant colonies took 3 days longer to appear than controls
	300	17	ditto	4 colonies from each electroporation (either plate) were tested for p27 expression and sub-cultured
H2	50	4	H2 (a-d)	
	300	21	ditto	
H3	50	1	H3 (a-d)	
	300	5	ditto	
H11	50	2	H11 (a-d)	A selection of positive plasmids (H1,2,3&11) were transferred to *M. vaccae*, in case each had different levels of expression in mycobacteria
	300	10	ditto	
pSMT3 (original)	50	>1500	control	Using this batch of competent *M. vaccae*, 1ng of pSMT3 yields 20 colonies in 5 days (electroporate 1µl of 720µg/ml vector; use 50 out of 600µl)
H_2O	300	none	control	no spontaneous mutants to antibiotic resistance
S1-6 (p16R1SOD/p27)	50	3	S1-6 (a-d)	Individual colonies tested for p27 and SOD expression (since a large part of the SOD peptide was present in the fusion protein)
	300	10	ditto	
S2-2	50	7	S2-2 (a-d)	
	300	13	ditto	
S2-12	50	0		
	300	12	S2-12 (a-d)	
p16R1SOD	50	covered	control	
H2O	300	none	control	
L1 (pAU171/p27)	50	4	L1 (a-d)	Only L1 and L2 of the 12 positive plasmids were chosen for electroporation. 4 colonies from each were tested for p27 expression. After sub-culture only L1c was positive
	300	10	ditto	
L2	50	3	L2 (a-d)	
	300	6	ditto	
pAU171	50	covered	control	There are far fewer colonies with all my recombinant plasmids than original vector, even though all were purified from *E. coli* clones and had similar concentrations. This may indicate a toxicity of the insert for *M. vaccae*
H_2O	300	none	control	

Table 3-2: Electroporation efficiencies in *M. vaccae*

M. smegmatis plasmid name	µl plated	n° colonies	colony code	remarks
H1	50	115	Ms H1 (a-d)	The recombinant plasmids yielded similar numbers of colonies to the control, so the insert did not seem toxic in the *M. smegmatis* laboratory strain
	300	covered		
H2	50	190	Ms H2 (a-d)	All 8 recombinant *M. smegmatis* showed p27 expression to varying degrees (not shown), some equal to that seen with *M. vaccae*
	300	covered		
pSMT3	50	140	control	Recombinant and original plasmids grew at the same rate. The *M. smegmatis* suspension (from S. Ely) contained less competent cells than my *M. vaccae* one
H_2O	300	none	control	
L1	50	58	?	Not tested for expression
	300	covered		
pAU171	50	21	control	Again the insert had little effect on the number of colonies
H_2O	300	none	control	

Table 3-3: Electroporation efficiencies in *M. smegmatis* mc^{2}6

3.3 Conclusions

Several recombinant shuttle plasmids were prepared containing SIVp27, and used initially to transform *E. coli*. Good transformation efficiencies were seen with pSMT3 vectors in *E. coli*. Although many colonies were present in the control where RE cut pSMT3 was ligated with 'H_2O', 9 out of 12 *E. coli* clones sampled after transformation with pSMT3/p27 (from PCR lane 2) were positive indicating that the presence of the insert reduced self-ligation of incorrectly cut vectors. These recombinant plasmids (called H1, H2, H3 etc.) supported p27 expression, when tested on immunoblots with monoclonal antibodies.

p16R1SOD based vectors containing inserts showed poor transformation compared to the uncut original in *E. coli*. This may be due to the effect of alkaline phosphatase treatment, necessary as the plasmid was only cut with one RE. However, it had the desired effect since there were less colonies of cut p16R1SOD ligated to water than those with PCR inserts and thus a sufficient percentage of colonies contained an insert when tested. Since immunoblotting was time consuming, only those plasmids shown to probably contain an insert in the correct orientation (and some controls) were tested with anti-p27 and anti-SOD. Of 24 *E. coli* colonies chosen, only 3 correctly expressed SIVp27.

Sequencing was performed on plasmids containing SIVp27 from different PCR reactions, in case an error had occurred at an early stage. All DNA sequences were

shown to be identical to that of the original plasmid pKA27 and the hence the published amino acid sequence for SIVp27.

Selected recombinant plasmids expressing correct SIVp27 were then purified from bulk *E. coli* cultures and electroporated into *M. vaccae*. As can be seen from Table 3-2, relatively low numbers of slowly growing recombinant colonies were obtained containing pSMT3/p27 and p16R1SOD/p27 plasmids. Furthermore only some of these expressed SIVp27 and then to a low level, even though hygromycin selection pressure was maintained.

These experiments were amongst the first to express non-mycobacterial genes in *M. vaccae* and this poor outcome was disappointing.

SIVp27 was also sub-cloned into pAU171 and expressed from the 19kD control elements. Very good transformation and expression was seen in *E. coli*, but again very poor results were achieved with all except one clone of *M. vaccae*. Triton-X-114 partitioning, (in which sonicated mycobacteria were mixed with 2% triton in tris buffer, then warmed to 37°C to yield separate aqueous and detergent phases from which proteins were precipitated with acetone and immunoblotted) was used to determine whether the protein in *M. vaccae* was membrane bound (lipid phase) or cytoplasmic. Partitioning revealed the SIVp27 in H11c (pSMT3/p27) to be absent from the detergent phase and strongly present in the aqueous phase, while p27 from L1c (pAU171/p27) only gave very faint staining in both phases (my data, not shown). It is possible that the acetone precipitation from the detergent phase was not efficient and the p27 was lost. Although the results are not conclusive, they may indeed indicate a difference in the compartmentalisation of the SIVp27 expressed from these vectors.

Generally, the SIVp27 expression in *E. coli* was good even though the mycobacterial promoter used may not be adapted to *E. coli*, whereas expression in *M. vaccae* from the mycobacterial promoters was poor. Differences in the level of expression between different *M. vaccae* colonies also occurred, even though the positive plasmids purified from *E. coli* and used for electroporation into *M. vaccae* were clonal. It may be due to some minor recombination events that occur during transformation that can reduce either plasmid copy number or protein expression. Alternatively it may be explained by increased degradation of the foreign RNA or protein compared to the native mycobacterial products, although it had initially been hoped to capitalise on the stability that SOD may impart on the SIVp27 fused to it. However, this was unsuccessful, as is especially clear when the strong native SOD expression is compared to the relatively poor recombinant fusion protein expression. Furthermore, expression of

SIVp27 in *M. vaccae* on a 7H11-hygromycin plate remained stable for only 13 days before gradually declining in level and finally decreasing beyond the limit of detection. When a positive colony was grown for more than 2 sub-cultures expression also began to wane.

Although this problem was overcome by sub-culture of each original recombinant *M. vaccae* colony on several plates for only 6 days prior to conversion into an autoclaved vaccine where continued expression was not a factor, this would not be compatible with the long term culture of organisms for commercial vaccine production if the HIV p27 homologue were to be introduced for human use. Good expression of the foreign protein may be necessary for generation of an immune response. Hence low expression of SIVp27 was of great concern but efforts failed to correct this problem.

The anti-p27 monoclonals used throughout to identify bacterial extracts expressing correct recombinant plasmids, failed to recognise the epitopes of SIVp27 in immunoblots of autoclaved recombinant *M. vaccae* vaccines, while a reduced level of recognition by D2D antibodies to the *M. tuberculosis* SOD expressed from p16R1SOD was still seen after autoclaving. This may purely relate to the sensitivity of the technique, since SOD was always present in greater quantities, but it does cast doubt on the suitability of autoclaved recombinants, including pAU171/p27, to induce antibody responses. However, polyclonal responses may be better since Abou-Zeid (1997) did observe an anti-19kDa IgG2a response after immunisation of mice with autoclaved *M. vaccae* expressing high levels of the whole 19kDa protein.

3.4 Discussion

All recombinant plasmids prepared showed only an unstable, low level expression of SIVp27 in *M. vaccae*. For a positive clone to gradually lose expression is unusual, but not unheard of in other systems. For example, DNA cloned into the intergenic region of filamentous bacteriophages tends to be unstable, the longer the cloned segment the greater the rate at which deletions occur. Hence the bacteriophage cannot be grown by serial growth of infected cells, instead small-scale cultures being frequently set up from single plaques taken from frozen stocks. Recombinant bacteriophages carrying larger segments of foreign DNA almost always grow more slowly than those carrying smaller inserts and deletion of part of the foreign DNA therefore confers a selective advantage that can result in elimination of bacteria synthesising the original recombinant within a few serial passages (from 'Molecular cloning, a laboratory manual'. 2nd ed. Sambrook, Fritsch and Maniatis. Book 1, 4.16 'Single stranded filamentous bacteriophage

vectors'). With my plasmids, loss of expression may indicate a particular toxicity or growth retardation of SIVp27 expressed in *M. vaccae*, more than *M. smegmatis* or *E. coli*. The relative toxicity of the expressed protein determines how great is the selective pressure to down regulate the plasmid. The viral SIVp27 protein may indeed be slightly toxic since:

- when the control vectors were cloned into *M. vaccae* over 1500 colonies developed from 50µl of electroporated culture, whereas recombinant plasmids only transformed a few (less than 10). Since the plasmids had all been grown up in *E. coli* previously and purified extensively, there should not have been technical difficulties with the plasmid quality
- the recombinant colonies took 3 days longer to appear than the control colonies, indicating a growth retardation by the foreign DNA in the plasmid at or before the expression level.

Interestingly, when the plasmids were electroporated into the laboratory strain of *M. smegmatis* (mc^26), the colonies took three days to appear and the control and recombinant plasmids yielded colonies with very similar growth characteristics (Table 3-3). All 12 recombinant colonies from *M. smegmatis* expressed p27 from pSMT3/p97 to varying degrees upon immunoblotting, with the strongest being equivalent to *M. vaccae* expression (blots not shown). However, it has already been demonstrated that it is easier to transform *M. smegmatis* than *M. vaccae*, since only the former can be transformed using kanamycin based vectors (Garbe 1994).

Further it was not a problem with the *M. vaccae*, since the control plasmid p16R1SOD was successfully electroporated and maintained very strong expression of the *M. tuberculosis* SOD during all subcultures (possibly because mycobacterial proteins even expressed to high levels lack inherent toxicity). Also another gene cloned into pAU171 (the DERP2 house dust mite antigen) and electroporated into competent *M. vaccae* prepared by me showed good expression in all *M. vaccae* colonies tested, whereas DERP1 seemed to be toxic to both *E. coli* and *M. vaccae* since no colonies could be obtained on either (Sarah ELY, personal communication).

M. vaccae grows in clumps and was hard to disrupt into single cells and it was suggested (Barry Bloom, personal communication) that possibly non-transformed bacteria remained attached to recombinant ones and grew as the antibiotic hygromycin in the medium was degraded. This would result in overgrowth by the *M. vaccae* not expressing the p27 protein. However, when these low expression sub-cultures were plated onto new 7H11-hygromycin and only grown to low density, the expression was

not increased, as would be expected if numbers of the non-resistant bacteria decreased as the fresh antibiotic killed them. This also ruled out any possibility of differences in stationary and log phase growth altering the expression by merely generating technical anomalies e.g. the strength of the cell wall affecting the efficiency of sonication, or the build up of secreted proteins preventing the p27 binding the nitrocellulose after SDS-PAGE or masking the p27 from its antibodies. When the original S2-12d colony was diluted in 7H9, gently agitated to dissociate the clumps into single bacteria and plated to obtain single colonies, all showed similar SIVp27 expression for the first week of growth. Thus it did not immediately appear to be a problem of contamination of the positive colony with p27-negative ones that contained only original vector. It is possible that the temporary presence of antibiotic resistance plasmids enabled natural mutations within *M. vaccae* to become expressed and induce chromosomal resistance prior to plasmid loss, since there was no spontaneous drug resistance in controls without DNA. Alternatively, the antibiotic resistance and p27 expression may be regulated independently in the plasmid.

As an alternative method, freshly ligated plasmids (PCR product into cut vector) were electroporated directly into *M. vaccae* without prior selection in *E. coli.* Clearly selection in *E. coli* may not be ideal for further growth in mycobacteria and plasmid selection or recombination events during the transformation or electroporation may influence the ability of the plasmid to grow and express its foreign protein. This procedure has many difficulties since it is harder to obtain plasmids from mycobacteria and thus screening would have to be by expression only, followed by sequencing of the plasmid to check for mutations introduced by PCR (the latter may involve small scale isolation of *M. vaccae* plasmids and back-transformation of *E. coli* to amplify enough plasmid for this sequencing). However, very few antibiotic resistant *M. vaccae* colonies were obtained by this method for any recombinant vector, and although all the colonies were tested for expression of p27, none were positive and this approach was abandoned.

To clone very toxic products it is sometimes necessary to cut the gene into sections and clone each piece into the bacteria, since in this way the protein may have decreased toxicity. Then the different clones can be mixed at the vaccine stage to cover all regions of the gene, apart from those epitopes flanking the cuts which may be altered by being incomplete. This is most effective for CMI (rather than humoral) responses where the conformation of the protein is not as important. Thus although it would have been possible to clone the first part of the p27 only into p16R1SOD by cutting with Pst1 (which cuts after around 430 bp of the p27) this was not undertaken, due to a shortage

of time. If epitopes shown to active in either T or B cell assays are known a structured approach can be taken e.g. each small epitope can be inserted at an exposed position in a loop of the SOD protein (Hetzel 1998). However, this was not the done for SIVp27 in the mouse as no epitopes had been published (J.Thole, A Vyakarnam; personal communication).

There was some debate about the level of expression of a foreign antigen within a recombinant bacterium that is necessary to induce an immune response. Stover (1991) immunised mice intradermally with BCG (10^{4-6}) expressing hsp60-β-gal protein (as 10% of the total protein from BCG), hsp60-tet toxC protein (only 0.1% of total protein from BCG), and HIV pg120 from an integrating vector (very low levels of expression as this protein was toxic). High levels of antibodies were produced to β-gal and antibody responses were also induced to tetanus toxoid C but not to gp120 and hence level of expression may be important. These antibody responses were assumed to indicate T cell activation for 'help' and this was confirmed by IFNγ production to β-gal in appropriate mice. The group also showed that heat killed BCG (10^6 id.) were not effective at immunisation. The ability of rBCG expressing foreign protein (HIV-gag) to only ~0.1% of total BCG protein to induce T cell responses was also demonstrated by Aldovini (1991), using HIV-gag to demonstrate cell mediated immunity including cytotoxic and Th1 responses. This was encouraging since this may be around the level of my SIVp27 expression. However, *M. vaccae* is used as an autoclaved vaccine and this results in denaturation of the incorporated protein. Although *M. vaccae* is used in greater quantities (10^7-10^9 instead of 10^4-10^6 per dose) and will take a considerable time to be cleared by the immune system, BCG provides a long term depot of multiplying cells expressing the non-denatured foreign protein.

Whether even low level expression of a denatured foreign protein in dead *M. vaccae* induces a specific immune response in vaccinated mice is approached in the next chapter.

Chapter 4 Immunisation of mice with recombinant *M. vaccae*

4.1 Introduction

There is some evidence that immunity to HIV/SIV requires type 1 cell mediated immunity. Macaques rendered resistant to SIV show strong specific cell mediated but not humoral immunity (Clerici 1994). Also Th1 activity protects against apoptosis and HIV+ individuals with a Th2 biased immune response have a rapid rate of CD4 loss and progress faster to AIDS (Heinzel 1995). However, many common anti-viral vaccines induce antibody based immunity.

Mycobacteria have long been known to be capable of priming type 1 T helper responses, by virtue of provoking delayed type hypersensitivity responses and CMI to mycobacterial derivatives (e.g. PPD) and heat shock proteins. This property is extensively exploited by use of complete Freund's adjuvant to direct responses in mice.

Immune responses to heterologous antigens have been successfully induced by recombinant BCG (rBCG) in mice. Recombinant vaccines are very cost effective as they obviate the need to purify the antigen but to be generally useful they must satisfy several criteria:

Live vaccines must be stable *in vivo*. Although some rBCG have been shown to be able to grow *in vivo* and the extrachromosomal plasmid was stable for several weeks without selection (Stover 1991), other constructs (e.g. rBCG expressing OspA as a membrane associated lipoprotein; Stover 1993) are attenuated in their ability to replicate and persist compared to normal BCG and hence the viability and stability of antigen expression for each construct would have to be evaluated during prolonged storage and *in vivo*.

The level of expression of foreign proteins in recombinant vaccines must be sufficient to induce good immunity.

The recombinant vaccine must have good efficacy. In humans BCG itself has variable efficacy, probably because of variable prior exposure to mycobacteria and in a human population this is likely to affect both the size and nature of the response to recombinant BCG. A large percentage of the human population has been immunised with BCG, approximately 2.5 billion people, and although the very low rate of serious side effects argues for the development of recombinant vaccines in BCG, this priming could modify the immune response to future recombinant BCG vaccines.

For example, the growth of recombinant BCG in spleen or lymph nodes of BCG primed mice was strongly limited compared to naive mice, when the subcutaneous vaccinations were within 6 months of each other (Gheorghiu 1994). This reduction in recombinant BCG growth was associated with decreased proliferative responses (although suppression never exceeded 50%) but in contrast to these reduced T-cell responses, BCG-primed mice developed higher levels of anti b-galactosidase antibodies than naive mice after immunisation with recombinant BCG expressing this antigen. This was not due to a non-specific adjuvant effect of BCG. The increased antibody responses observed in BCG-primed animals may be related to the activation of BCG-specific T cells, which could lead to macrophage activation and enhanced antigen presentation of both the mycobacterial and recombinant proteins. It may also be explained by increased killing of the recombinant organisms and release of the foreign antigen under a soluble form. Thus priming with BCG would not limit the use of recombinant BCG vaccines when a strong antibody response was desirable for immunity, but in diseases when an antibody response was not advantageous a tendency towards reduced CMI could be detrimental. The authors proposed a sequence of successive vaccinations with rBCG vaccines where the first injection would be performed with rBCG expressing foreign antigens for which cellular immunity was required and subsequent injections would be performed with rBCG expressing antibody-dependent protective antigens. However this is some way in the future!

Additional problems exist. BCG can sometimes be poorly tolerated (e.g. it gives an unpleasant reaction in people with necrotic skin-test reactivity or after a previous BCG vaccination and cannot be given to patients with tuberculosis as it can make the disease worse). Also because recombinant BCG is a live vaccine, there may be problems licensing it with regulatory authorities for use world wide as the safety of live recombinant vaccines is uncertain in regions with high levels of endemic HIV.

Some of these problems associated with live rBCG may be avoided by use of dead recombinant *M. vaccae*, which remains immunogenic when killed by autoclaving. Multiple dosing with dead *M. vaccae* in humans at 1-2 month intervals can induce measurable immunologic responses to mycobacterial antigens in some healthy adults and appears not to cause unacceptable increments in local reaction (von Reyn 1997) and the vaccine is safe for use in individuals infected with *M. tuberculosis* (Kon 1998) or HIV (Marsh 1997). Thus recombinant *M. vaccae* may be of most relevance where use of live vaccines is not advisable.

Importantly, the dose of recombinant protein can also be properly controlled since there is no replication or change in expression *in vivo* from a killed vaccine. Autoclaved *M. vaccae* has been used with carefully controlled doses to induce type 1 responses to mycobacterial antigens in mice (Hernandez-Pando 1994 and 1997). A low dose (10^7) is protective against *M. tuberculosis* in mice and primes a slowly developing Th1 response without a Th2 component, that after 14 days results in DTH that does not undergo tissue damage in response to TNFα. The TNFα may play a supplementary macrophage activating role (in granuloma formation and synergising with IFNγ in nitric oxide release). However, in this model, a high dose (10^9) primes a mixed response which may result in IL-4 mediated down regulation of activated macrophage function and cause TNFα to mediate local necrosis. In a murine allergy model, *M. vaccae* has also been shown to reduce some Th2 responses, such as IgE and IL-5 production (Wang 1998). Thus *M. vaccae* is a well characterised CMI-inducing vaccine and although the pattern of response is dose related it probably always has a Th1 component.

4.1.1 Aims and considerations

Previously we expressed simian immunodeficiency virus p27 protein in *M. vaccae* using a number of plasmid systems similar to those already tested in rBCG. The systems are summarised here:

pSMT3 is the vector only control

pSMT3/p27 expresses p27 from the mycobacterial hsp65 promoter

pAU171/p27 expresses p27 from the 19kDa lipoprotein promoter, which may direct its acylation and secretion

p16R1SOD (SOD) expresses *M. tuberculosis* SOD at high levels

p16R1SOD/p27 (SOD/p27) has p27 as a large fusion protein with much of *M. tuberculosis* SOD

SIV p27 is an analogue of HIV p24 and part of the gag core protein complex, which has low mutation rates but is immunogenic in mice. Use of the SIV protein makes it possible to move to the monkey protection model later. Now we attempt to evaluate the cell mediated and humoral immune responses to SIVp27 and *M. vaccae* antigens in immunised mice.

The dose of *M. vaccae* used in immunisation is known to influence the generation of type 1 or type 2 responses and it is of interest to compare the outcomes in relation to the foreign protein. A single vaccination with 10^7 organisms should be optimum for Th1 induction (as assessed by high levels of IFNγ and IL-2) while 10^9 produces a mixed

Th1/Th2 response (IFNγ and IL-2, with additional IL-4 and TNF-induced tissue damage) although neither induce strong antibody responses to *M. vaccae* (Hernandez-Pando 1994 and 1997). However, for these experiments with recombinant vaccines a boost at 30 days was included to increase exposure to the SIVp27, although this protocol had not been previously evaluated (Abou-Zeid (1997) has subsequently used a boost at 21 days and sacrifice at 31 days).

Since it was known that the expression of the SIVp27 from any construct was not strong, additional protocols d and e were devised to attempt to increase T and/or B cell responses. Initial vaccination was with autoclaved *M. vaccae* expressing the *M. tuberculosis* SOD in large amounts, which should induce a strong T cell response to SOD as well as *M. vaccae* antigens. This was followed by two boosts with autoclaved recombinant *M. vaccae* expressing SODp27 (or SOD as non-specific control), with the aim that the SOD would then act as a carrier molecule in immune mice and strong responses to the SIVp27 associated with it would be elicited. For T cell responses, degradation of antigen during autoclaving should not be a problem.

Different routes of delivery may produce different results and although testing SIVp27 as a membrane lipoprotein (from the 19k promoter) may not be relevant to protective immunity in SIV, it may help assess the role of *M. vaccae* as a therapy or vaccine for other diseases where antibodies have been shown to play a role. It could also act as a control to test whether specific antibody production is possible from an autoclaved recombinant vaccine.

Although *M. vaccae* vaccines containing the 'vector only' controls were available for all recombinant plasmids used, the complexity of the mouse vaccination protocols made it possible only to include the pSMT3 vector control. It was planned to repeat the experiments with matched control and recombinant *M. vaccae* vaccines when optimised vaccination and boost protocols had been devised.

4.2 Results

4.2.1 Mouse vaccination protocol.

Four week old Balb/c mice were vaccinated with 10^7 or 10^9 organisms of the recombinant *M. vaccae* vaccine under test and then boosted as indicated in the table below.

Vaccination-day0	Boost 1-day31	Boost 2-day60	Short code
10^7	**10^7**		**a**
pSMT3	pSMT3		pSMT3 a
p16R1SOD/p27	p16R1SOD/p27		SOD/p27 a
10^7	**10^9**		**b**
pSMT3	pSMT3		pSMT3 b
pSMT3/p27	pSMT3/p27		pSMT3/p27 b
pAU171/p27	pAU171/p27		pAU171/p27 b
p16R1SOD/p27	p16R1SOD/p27		SOD/p27 b
10^9	**10^9**		**c**
pSMT3	pSMT3		pSMT3 c
pSMT3/p27	pSMT3/p27		pSMT3/p27 c
pAU171/p27	pAU171/p27		pAU171/p27 c
p16R1SOD/p27	p16R1SOD/p27		SOD/p27 c
10^7	**10^9**	**10^9**	**d**
p16R1SOD	p16R1SOD	p16R1SOD	SOD d
p16R1SOD	p16R1SOD/p27	p16R1SOD/p27	SOD/p27 d
10^9	**10^9**	**10^9**	**e**
p16R1SOD	p16R1SOD	p16R1SOD	SOD e
p16R1SOD	p16R1SOD/p27	p16R1SOD/p27	SOD/p27 e

Table 4-1: Vaccination protocol for mice

Each group contained 3 mice. 10^9 represents a subcutaneous injection at the base of the tail of 50µl of appropriate stock vaccine. 10^7 represents an injection of 50µl of a 1/100 dilution of vaccine in sterile saline. All mice were bled by cardiac puncture with recovery 30 days after vaccination, just before the first boost. Mice boosted only once were bled again 10 days later, prior to sacrifice and collection of the spleens. Mice boosted twice (at 30 days then 60 days after vaccination) were bled and the spleens removed at sacrifice, 10 days after the second boost. This is summarised in Figure 4-1:

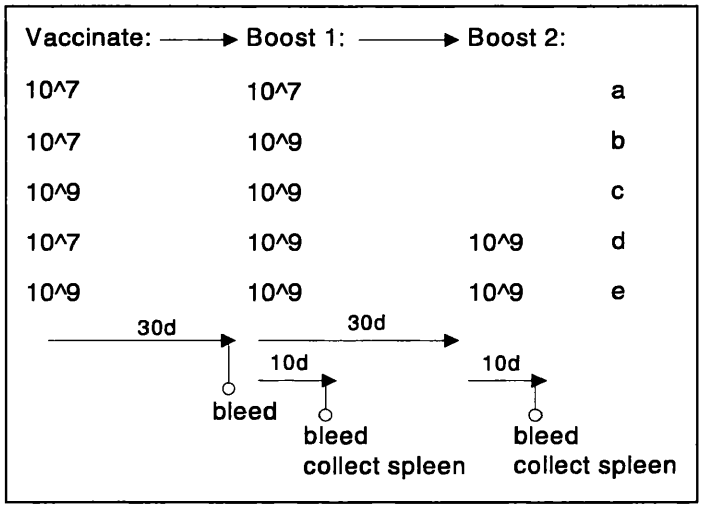


Figure 4-1: Schematic of vaccination protocol

4.2.2 IFNγ production in response to Concanavalin A or autoclaved *M. vaccae* sonicate

Sonicated non-autoclaved *M. vaccae* stimulated cytokine production *in vitro* from spleen cells of mice singly immunised with standard vaccine (10^7 autoclaved *M. vaccae*), but an autoclaved sonicate induced stronger responses that consistently gave greater differentiation between vaccinated and naive mice (not shown). The presence of 'common' antigens in the environment and water of the mice may lead to sensitisation of some normal mice to the preparations. However vaccination with the autoclaved *M. vaccae* vaccine possibly favours responses to the autoclaved sonicate preparation.

To assess the health and reproducibility of the splenocytes in each group and check the effectiveness of the five vaccination protocols (a to e) at inducing a Th1 component, the production of IFNγ to medium alone, Concanavalin A and autoclaved *M. vaccae* sonicate was examined. Results from the different recombinant vaccines could be pooled for this purpose since the basic *M. vaccae* components should be alike. There were additionally 9 unimmunised control mice included for the various experiments.

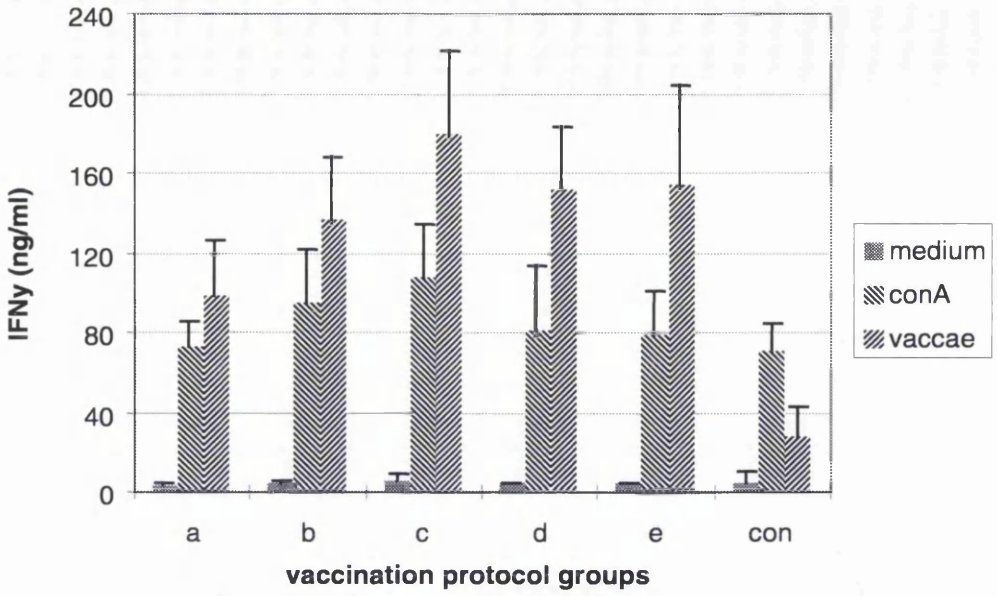


Figure 4-2: IFNγ production from spleens of mice, 10 days after the final boost of each vaccination protocol

The mean concentrations with standard deviations of IFNγ (ng/ml) in the 48 hour supernatants from spleen cells cultured with various antigens are shown for each group in Figure 4-2. All mice gave satisfactory levels of IFNγ when induced by the mitogen Concanavalin A, and negligible amounts when unstimulated (medium), indicating the cells were all healthy. (Differences between Concanavalin A and medium were highly significant $p<0.001$ using 2 tailed T-Tests). Additionally, protocol c ($10^9/10^9$) appeared to have non-specifically primed the splenocytes to secrete higher levels of IFNγ in response to Concanavalin A than controls ($p=0.0013$). The cell-type involved was not investigated.

Unexpectedly, the unimmunised mice showed a small response to autoclaved *M. vaccae* sonicate when compared to medium only stimulation ($p=0.002$), indicating either a non-specific stimulation of cells by some component of the sonicate, or environmental immunisation of the mice prior to sacrifice, possibly with cross-reactive mycobacteria in the drinking water. However, vaccination with any protocol (a to e) induced significantly more IFNγ secretion from splenocytes stimulated with autoclaved *M. vaccae* sonicate ($p<0.001$) compared to unimmunised control mice, indicating induction of a strong Th1 component after immunisation.

Generally mice given the single boost protocols with higher doses of *M. vaccae* produced more IFNγ in response to sonicate (b more than a, $p=0.009$; c more than b, $p=0.011$). Thus using large quantities of recombinant *M. vaccae* to increase the relative

amount of foreign protein introduced to the mice does not inhibit, and even enhances, the Th1 response to *M. vaccae* sonicate as demonstrated by IFNγ production. However, multiple boosts did not further increase this (protocols b or c are not different to d or e).

4.2.3 Specific IFNγ production to SIVp27GST

Groups of 3-5 mice were given one of the 3 recombinant *M. vaccae* preparations containing the SIVp27 insert (pSMT3/p27, p27 expressed from 65k promoter; SOD/p27, p27 fusion with SOD; pAU171/p27, p27 expressed from 19k promoter) or *M. vaccae* with the pSMT3 vector only (as a representative control). 5 mice remained unvaccinated. The various immunisation protocols were followed as described. The IFNγ production from spleen cells of each vaccination group was analysed after stimulation with medium or several concentrations of purified p27GST (the glutathione-s transferase-linked SIVp27 protein generated from recombinant *E. coli*).

4.2.3.1 Protocols a, b and c

Protocol **a** ($10^7/10^7$) did not induce any difference in specific IFNγ to p27GST at 1.4μg/ml, between mice given recombinant *M. vaccae* containing pSMT3 vector control (mean 23.72ng/ml) or SOD/p27 (mean 25.16ng/ml) or the appropriate unvaccinated group (mean 12.48ng/ml). These data are not shown.

Results for protocols **b** ($10^7/10^9$) and **c** ($10^9/10^9$) are shown in Figure 4-3

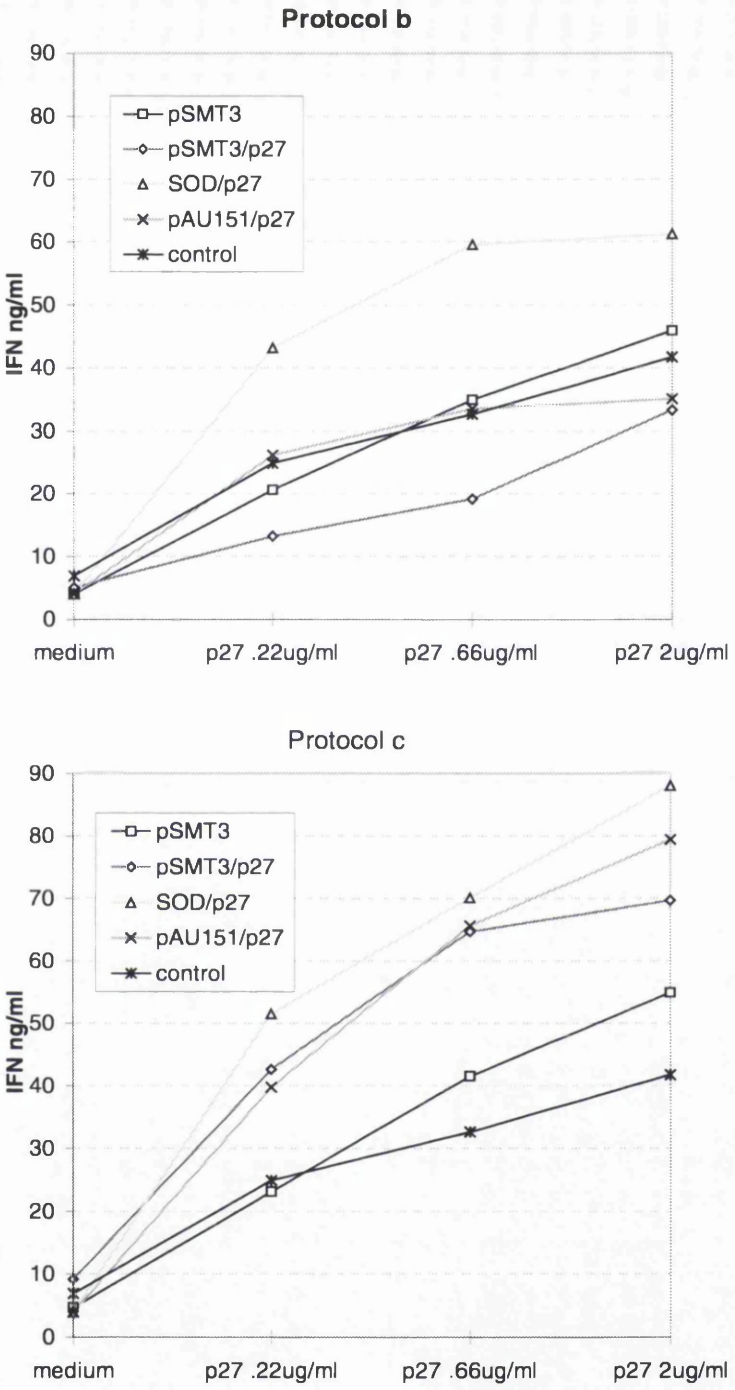


Figure 4-3: IFNγ production in response to p27GST

At higher antigen concentrations non-specific stimulation of IFNγ production was seen in unvaccinated control mice and mice vaccinated with *M. vaccae* containing pSMT3 vector only. This may be due to some reactivity with the glutathione-s-transferase part of the fusion protein or the recombinant p27GST being contaminated by *E. coli* products e.g. highly immunogenic lipopolysaccharides, which stimulate macrophages and may promote IFNγ from T cells and NK cells in naive mice.

Using immunisation protocol **b** ($10^7/10^9$), only SOD/p27 recombinants appeared to produce more IFNγ than controls, but this was not significant by T test (1 tail). Other groups gave very similar results to unvaccinated mice. Large standard deviations (not shown for clarity) may be partly responsible for lack of significance. For example, two of three mice given $10^7/10^9$ SOD/p27 gave the strongest specific responses at all dilutions of p27GST antigen, while one mouse gave suppressed responses (thus giving a standard deviation of around 80% of the mean). The latter may be due to inaccurate placing of the vaccine dose, since administration to the muscle near the spine at the tail (rather than subcutaneous) causes stress to the animal and the vaccine could engage the immune system from a different (detrimental) route of presentation. Other groups of mice were more consistent, generally giving standard deviations of 13 to 60% of their mean.

Using protocol **c** ($10^9/10^9$), all recombinants expressing SIVp27 appeared to induce higher mean specific IFNγ values to p27GST than unimmunised or pSMT3 vector controls. By 1 tail T test, SOD/p27 vaccinated mice produced significantly more IFNγ than unvaccinated control mice for all p27GST concentrations, with the highest concentration (2μg/ml) being most significant (p=0.017, 1 tail). pAU171/p27 narrowly missed significance (p=0.06, 1 tail) and all other groups were not significant compared to controls. Unfortunately, no group gave significantly more IFNγ than mice given pSMT3 vector without SIVp27; this may be due to the one mouse from the pSMT3 group which had unusually elevated levels of IFNγ to p27GST, or alternatively due to non-specific boosting of the immune response by *M. vaccae* as shown later.

4.2.3.2 Protocols d and e

Additional mice were given *M. vaccae* expressing high levels of TB SOD (as a carrier molecule) followed by two boosts (protocols **d** ($10^7/10^9/10^9$) and **e** ($10^9/10^9/10^9$)) with either SOD (as control) or SOD/p27. All groups showed strong IFNγ responses to p27GST, even when immunised with recombinant *M. vaccae* expressing SOD without the p27 insert. Mean IFNγ production ng/ml (standard deviations) is shown in response to SIV p27 in (Table 4-2). This is seen as significant responses (1 tail T test, $p<0.05$) when any vaccinated group is compared to the 2 unvaccinated control mice used in this experiment. Hence these responses may be due to strong non-specific stimulation of the whole immune response; this is a recognised feature of some mycobacteria, and can sometimes be used to advantage to induce enhanced recognition of cancer cells (Grange

1995). This triple immunisation protocol cannot therefore be used further for specific immune stimulation.

Double boost	Medium	SIVp27 2μg/ml	SIVp27 0.66μg/ml
SOD d	5.00	85.43 (39)	89.37 (29)
SOD e	5.00	64.57 (11)	85.00 (15)
SODp27 d	5.00	81.00 (53)	75.50 (36)
SODp27 e	5.00	67.97 (23)	67.13 (25)
control	5.00	17.18 (2)	5.98 (3)

Table 4-2: Interferon γ production to SIVp27 in mice from groups d and e

4.2.3.3 Use of SIVp27 overlapping peptides

In an attempt to improve the sensitivity of detection of specific IFNγ production to SIVp27, 22 overlapping peptides (20mers with 10 amino acid overlap) which covered the entire SIVp27 region of SIV_{mac251} were obtained (MRC ADP 714). These were dissolved in dimethyl sulphoxide, pooled in groups (1-6, 7-12, 13-17, 18-22) and diluted in RPMI to 100μg/ml each, before being sterile filtered, aliquotted and frozen. However, used at 5μg/ml per peptide in spare frozen cells from mice immunised by protocol **a** there was no IFNγ produced in response to any peptides (data not shown).

4.2.4 Specific IFNγ production to *M. tuberculosis* SOD

As a comparison with p27GST, responses to *M. tuberculosis* SOD purified from recombinant *M. vaccae* were tested and are shown in Table 4-3.

Immunisation protocol	IFNγ ng/ml (st.dev) to SOD 0.66μg/ml	Compare groups to controls (1 tail T test)
unvaccinated controls (for a,b,c)	17.10 (18.6)	
unvaccinated controls (for d,e)	12.36 (7.1)	
pSMT3 protocol a ($10^7/10^7$)	27.85 (10.3)	p=0.200
SOD/p27 protocol a ($10^7/10^7$)	33.00 (6.4)	p=0.085
SOD/p27 protocol b ($10^7/10^9$)	79.27 (37.2)	p=0.048
SOD/p27 protocol c ($10^9/10^9$)	95.63 (16.0)	p=0.001
SOD/p27 protocol d ($10^7/10^9/10^9$)	67.97 (37.5)	p=0.059
SOD/p27 protocol e ($10^9/10^9/10^9$)	75.17 (31.6)	p=0.033
SOD protocol d ($10^7/10^9/10^9$)	75.17 (30.9)	p=0.032
SOD protocol e ($10^9/10^9/10^9$)	99.20 (26.6)	p=0.011

Table 4-3: IFNγ production to SOD

There was relatively little IFNγ response to the SOD when mice were unvaccinated and this only increased insignificantly after vaccination with the lowest dose (protocol a) of *M. vaccae* with an irrelevant vector (pSMT3) or SOD/p27. As the immunising dose of SOD/p27 increased (protocols b and c), the IFNγ response to SOD also increased (and became significant), but this was not improved by additional boosts with SOD/p27 (protocols d and e) and use of recombinants with greater expression of SOD (i.e. without p27 fusion).

However, since results using a high dose of irrelevant (pSMT3) recombinant *M. vaccae* were not available, it is possible that *M. tuberculosis* SOD purified from recombinant *M. vaccae* would suffer the problem of non-specific stimulation of cells immune to autoclaved *M. vaccae*, due to mycobacterial contamination or maybe a lack of differentiation between the native *M. vaccae* and cloned *M. tuberculosis* SODs at the T cell level.

4.2.5 IL-2 and IL-4 production in response to Concanavalin A, autoclaved *M. vaccae* sonicate or p27GST

4.2.5.1 Interleukin-2

IL-2 was measured after 48 hours stimulation for groups b and c and at 24 hours for d and e. The values in Table 4-4 are in ng/ml with standard deviations in brackets. No IL-2 was produced to stimulation with medium only. There was no IL-2 detected after stimulation with the p27GST protein under any conditions.

Protocol Group	Concanavalin A	*M. vaccae* sonicate
b ($10^7/10^9$)	9.9 (4.0)	0
c ($10^9/10^9$)	12.5 (4.5)	0.51 (0.54)
d ($10^7/10^9/10^9$)	24.5 (4.5)	0.84 (0.55)
e ($10^9/10^9/10^9$)	23.6 (3.5)	1.10 (1.4)
control 48hr / 24 hr	9.1 / 26; mean 12.3 (8.6)	0

Table 4-4: Mean IL-2 production to Concanavalin A and *M. vaccae* antigens

It is unclear whether slight differences in IL-2 production to Concanavalin A were due to timings or immunisation protocols but there is clearly over 20 fold less IL-2 made in response to stimulation with *M. vaccae* sonicate antigen.

However, the presence of some IL-2 from spleens of mice receiving greater vaccine doses compared to its complete absence in unimmunised animals may mean that the sensitivity of the assay was simply insufficient for accurate quantitation.

4.2.5.2 Interleukin-4

Similarly to IL-2, samples were only available for IL-4 detection from groups b and c at 48 hours and d and e at 24 hours. The 48 hour time point was unsuitable for measurement of IL-4 (as IL-4 was being released spontaneously without stimulation) and only groups d and e are shown as pg/ml in Table 4-5 below (with standard deviations in brackets).

Protocol Group	Medium	Concanavalin A	*M. vaccae* sonicate
d ($10^7/10^9/10^9$)	0	460 (102)	92 (81)
e ($10^9/10^9/10^9$)	0	474 (134)	127 (134)
control (24 h)	0	589 (372)	0

Table 4-5: Mean IL-4 production to Concanavalin A and *M. vaccae* antigens

The groups boosted twice with 10^9 recombinant *M. vaccae* (d and e) and assayed at 24 hours had no IL-4 in the medium only samples but clear IL-4 in Concanavalin A stimulated samples. They also had IL-4 in samples from immunised but not control mice stimulated with sonicate, although it was 4 fold less than to mitogen. This indicates that these long protocols induced a Th2 component along with the Th1 response. This agrees with previous work showing that a dose of 10^9 induces a mixed response.

4.2.6 Results of antibody studies

4.2.6.1 Antibody responses to *M. vaccae* sonicate.

The autoclaved *M. vaccae* preparation was used for ELISA since this was being used simultaneously for splenic T cell studies, and gave a clear indication of responses to the immunising antigen. Althought harmless mycobacteria are ubiquitous in the environment, being in mouse food, water and bedding, they do not form part of the normal commensal flora. Only very low antibody levels were present to denatured mycobacterial components in unvaccinated mice. Initial studies using sera at 1/500 followed by an HRP-conjugated rabbit-anti-mouse-immunoglobulin, demonstrated detectable levels of total immunoglobulin to autoclaved *M. vaccae* sonicate compared to the background, in immunised mice. In singly vaccinated mice (i.e. blood taken just before the first boost) very little anti-*M. vaccae* antibody was generated, but in these experiments with boosts there was clearly a link between the immunisation protocol and the level of total antibody reached at death (not shown). Hence to further characterise the nature of the response to the autoclaved *M. vaccae* (and possibly its associated

inserts) IgG isotypes were tested. Murine IgG1 is produced during Th2 dominated responses (stimulated partly by IL-4), while IgG2a is the predominant antibody in Th1 responses and may be bacteriostatic (Rao 1995). Levels of IgG1 in murine sera are generally higher than IgG2a under normal circumstances, e.g. IgG1=4.6-6.5mg/ml, IgG2a =1.0-4.2mg/ml (Roitt Advanced Immunology).

Antibody responses to autoclaved *M. vaccae* sonicate in sera taken 30 days after the initial vaccination and at sacrifice 10 days after the final boost (i.e. day 40 or 70) were tested simultaneously and optical densities converted to µg/ml by comparison with the standard dose response curves for each isotype. Mean immunoglobulin levels (with standard deviations) are shown for each of the pooled protocol groups or unvaccinated controls in Figure 4-4. Note that the scales vary tenfold.

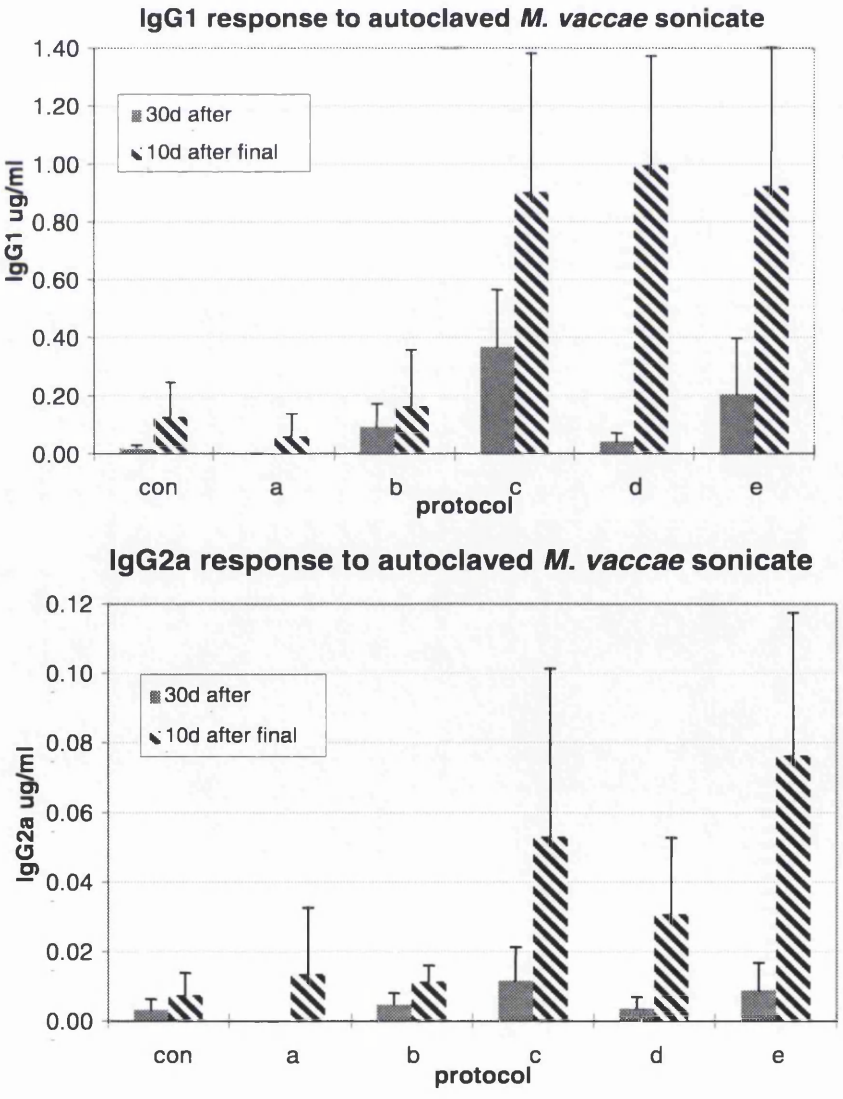


Figure 4-4: IgG isotype responses to autoclaved *M. vaccae* sonicate

The most interesting results were analysed using 2-tail T tests. Protocol a ($10^7/10^7$) did not induce increased antibody production compared to unvaccinated control mice. Protocol b ($10^7/10^9$) showed IgG1 levels were slightly raised after the first 10^7 vaccine ($p=0.01$) but this became non-significant after the 10^9 boost, suggesting significant antibody levels had not been induced; IgG2a had no significant differences. 2-tail T tests comparing protocols b ($10^7/10^9$) and c ($10^9/10^9$) for IgG1, show at both time points they were highly significantly different ($p<0.0005$). For IgG2a, protocols b and c again induced different amounts of antibody both after vaccination ($p=0.036$) and at death ($p=0.017$).

Protocols a and b induced little antibody of either isotype compared to unimmunised controls. This indicated that a vaccination with 10^7 organisms induced a sustained stable cell mediated response (Th1) without antibody production, even when boosted once with 10^9 organisms. However, initial vaccination with 10^9 organisms (protocols c ($10^9/10^9$) and e ($10^9/10^9/10^9$)) did induce antibody of both isotypes, and this increased up to a plateau after one (c) or two (e) boosts, indicating that specific antibody production with strong Th1 and Th2 components had been stimulated. Interestingly, in protocol d ($10^7/10^9/10^9$), vaccination with 10^7 organisms was unable to prevent generation of antibody responses following two boosts with 10^9 organisms, suggesting a limit to the 'stability' of the pure cell mediated (Th1) response.

4.2.6.2 Specific antibody responses to p27GST.

Specific antibody to p27GST could not be detected in sera (diluted to 1/50) from mice given any recombinant *M. vaccae* (expressing SIVp27 either cytoplasmically or possibly membrane associated) using any protocol. Similarly, no response was seen to purified *M. tuberculosis* SOD. (Data not shown). It may be relevant that the p27GST antigen in ELISAs is a native SIV p27 protein fused with an irrelevant molecule, both presumably displaying conformational epitopes, whereas in the vaccine it is SIVp27 (including a part of p17) or p27SOD fusion after expression in various recombinant *M. vaccae* systems and denaturation by autoclaving. B cell responses are very sensitive to antigenic denaturation and thus it is not surprising that specific antibody responses to native p27GST were not induced. (The murine anti-p27 mAbs (ADP 392/393) could not detect this antigen after *autoclaved* sonicates were run on SDS-PAGE and immunoblotted (data not shown). Different results may have been obtained if the p27GST was denatured prior to testing the sera by ELISA, but since it is uncertain whether denaturation of the antigen would expose relevant epitopes and the protein was

in limited supply, and as any response to its denatured form would be irrelevant in B cell immunity to a natural infection, this was not done.)

4.3 Conclusions

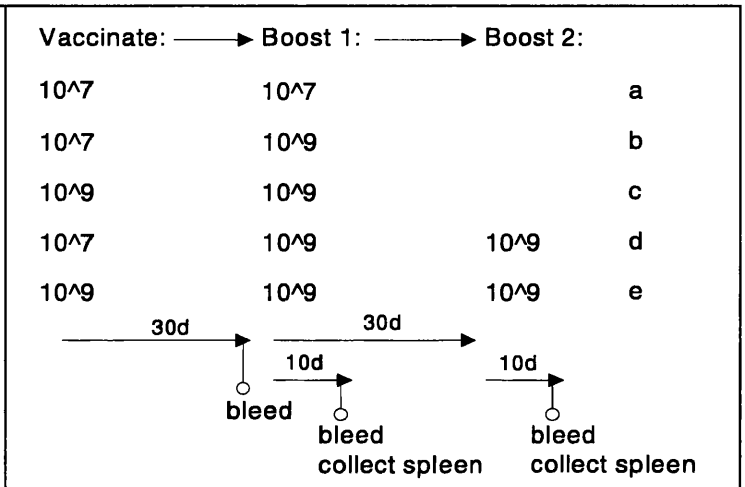
Nomenclature of *M. vaccae* plasmids	
pSMT3 is the vector only control	
pSMT3/p27 expresses p27 from the hsp65 promoter	
pAU171/p27 expresses p27 from the 19k lipoprotein promoter	
SOD/p27 has p27 as a fusion protein with much of *M. tb* SOD	

Figure 4-5: Vaccination protocols

The type of immune response after the various immunisation protocols (Figure 4-5) was initially examined by studying responses to mitogen and *M. vaccae* sonicate. In previous experiments using similar lymphocyte culture conditions, IFNγ production from spleens of mice which received a single dose of *M. vaccae* generally increased until 3 weeks after vaccination (Hernandez-Pando 1994) and a short-lived burst of IFNγ was also seen at around 6 days after the single vaccination with 10^9 organisms.

From my experiments, vaccination followed by a boost after 30 days, induced strong IFNγ production to sonicate in spleens 10 days later, indicating a strong Th1 response to *M. vaccae* had become established in the mice. Hence boosting did not appear to be detrimental to the aim of inducing a Th1 response. Furthermore, higher doses of *M. vaccae* in vaccination and/or single boosts produced stronger responses, which may reflect the increase in antigen load in protocol c and b over a, or possibly the presence of an IFNγ burst remaining at 10 days after the 10^9 boost. However, a second boost did not further increase the IFNγ production in response to mitogen or sonicate.

With the high dose ($10^9/10^9$ protocol c) of all recombinant *M. vaccae* preparations expressing SIVp27, IFNγ responses were induced to the p27GST protein, although only recombinant *M. vaccae* expressing SOD/p27 was effective after lower dose

immunisation (protocol b). Strong IFNγ responses to SOD were also induced under these conditions.

SOD is relatively heat stable and may protect the p27 during autoclaving and also from cellular proteases during culture and stages of stress leading to vaccine production. There may also be better responses to the fusion protein since SOD is antigenic. However p27 expressed from pSMT3 has no such protection as few amino acids of the heat shock protein are present. The p27/pAU171 which appears as two bands may be partially secreted and may separate away from the mycobacteria during vaccine production, thus reducing the effectiveness of the vaccine. Thus with foreign proteins in the SOD fusion form recombinant *M. vaccae* can induce immunity to heterologous antigens.

Antibody responses to the foreign proteins were probably not involved in immunity under these conditions. No antibodies recognising native p27(GST) were detected even when the antigen was expressed from the 19k lipoprotein promoter (pAU171/p27). The fusion protein may have been refractory to export or acylation, although it is more likely that autoclaving does prevent generation of a humoral response to native protein. Lack of antibody should not have been due to murine genetic factors as the balb/c strain of mice can develop strong antibody responses to β-galactosidase expressed in rBCG at different levels from several promoters (Lagranderie 1997). This was in contrast to IFNγ production in balb/c mice which remained strongly dependent on β-galactosidase expression levels.

Relatively high levels of IL-4 were produced after multiple boosting with 10^9 *M. vaccae* following a low or high dose vaccination (protocols d and e) and this means that the 'purity' of the Th1 response has been lost. The induction of IgG1 antibody responses to sonicate was useful in confirming the expected mixed type 1: type 2 status of the immune response after high dose vaccination and single boosting (protocol c, $10^9/10^9$), even though the IgG2a concentrations achieved were small. (Wang working on IFNγ knockout mice (Wang 1994) showed that a Th2 response induced IgG1 optical densities 8 fold higher than low IgG2a ones, but a predominantly Th1 response had intermediate, nearly equivalent, levels of both IgG1 and IgG2a.) Interestingly, vaccination with 10^7 *M. vaccae* could prevent induction of antibody responses (and by inference a type 2 response) by a single boost of 10^9 *M. vaccae*.

This can be summarised: immunisation of mice with low dose *M. vaccae* followed by a boost induces a type 1 response to soluble *M. vaccae* antigens (IFNγ, no antibodies) but poor responses to SIVp27. With high doses, a stronger type 1 response

is induced to *M. vaccae* antigens and also p27GST, but a type 2 response (IgG1 and IL-4) is also induced. Additional boosts are not beneficial for induction of specific immunity although they may generally stimulate the immune response.

This suggests a specific response with a strong type 1 component can be induced to the foreign protein in mice when sufficient recombinant material is used. However, to avoid induction of a type 2 component it would be preferable to use lower doses of the vaccine (protocol b), but for this it may be necessary to increase the levels of expression of the foreign protein.

There are some parallels with a schistosomiasis model (Caulada-Benedetti 1991). A single immunisation with irradiated cercarie led to a predominant protective cell mediated response, but continued immunisation additionally led to the emergence of Th2 cells in draining lymph nodes and antibody responses. In this model (like *M. vaccae*/SIV) the presence of a Th2 response did not suppress the Th1 reactivity. Boosting very slightly supplemented the resistance to challenge.

However, there was no 'protection' model available for SIV in mice and more conclusive results would be necessary to indicate tests in monkey models. Further experiments were therefore not performed on these recombinant *M. vaccae* constructs.

4.4 Discussion

4.4.1 Increasing the exposure to foreign antigens

The level of expression of foreign proteins in recombinant vaccines is important: several rapid-growing non-pathogenic recombinant mycobacteria that secrete large quantities of *M. tuberculosis* extracellular proteins have been developed, which may allow mass-production of these proteins (Harth 1997). In BCG, some non-mycobacterial proteins have also been expressed to a high level. Good expression may be essential to induce immunity to the foreign antigen (Stover 1991): mice immunised intradermally with 10^4 BCG expressing the hsp60-β-gal protein (as about 10% of the total protein) produced high levels of antibodies to β-gal that persisted longer than those to BCG. Relatively weakly expressed antigens (e.g. tetanus toxoid fragment C from hsp60 represented only about 0.1% of the BCG protein) could also induce some antibody responses but the HIV gp120 expressed on an integrating vector at very low levels could not. Actively growing rBCG provides continuous boosting to the foreign antigen from a single immunisation and this persistence may be important in BCG based recombinant vaccines, since heat-killed BCG-toxC was not effective at immunisation.

To increase the exposure to foreign antigen by conjugation or mixing of purified SIVp27 (GST fusion) protein with *M. vaccae* would require large quantities of SIVp27 (around 10μg/injection) and would be complicated by the presence of any minor contaminants present in the preparation. It is also unclear what type of immune response would be generated to a simple mixture since the soluble part may diffuse away from the *M. vaccae* and the Th1 adjuvant effect may be lost (Gheorghiu 1994). Most adjuvants are *emulsions* which prolong the half-life of the antigen by protecting it from degradation, minimise any direct toxic effects of the antigen and by allowing a slow sustained release from the site of immunisation, increase the efficiency of uptake of the antigen by macrophages.

Booster injections are essential for good CMI and humoral responses. When normal people were vaccinated with a live recombinant vaccinia virus expressing HIV envelope gp160, they expressed transient T cell responses and little antibody to envelope protein and consequently were boosted twice one year later with soluble purified gp160 in alum (Cooney 1993). They then developed neutralising antibodies, but also stronger T cell proliferative responses and both CD4 and CD8 cytolytic T cells. Thus to increase exposure to the antigen, immunisation of mice with recombinant *M. vaccae* expressing SIVp27 followed by boosting with p27GST (rather than SOD followed by SODp27 in *M. vaccae*) may induce a broader immunity than that described already. Again this required large quantities of purified SIVp27, which were not available.

An elegant method to improve the recognition of foreign antigens from *M. vaccae* involves incorporating known dominant epitopes in an exposed loop of SOD, which would remain well expressed in autoclaved *M. vaccae*. This has been achieved for Der p1 (Hetzel 1998), where IFNγ and IgG2a responses were generated to the peptide. However, this requires that the T cell epitopes are already well defined, which was not the case for SIVp27 in mice.

4.4.2 Humoral responses

The aims of this project included testing the ability of recombinant *M. vaccae* to induce humoral responses to the foreign protein. While cytoplasmic expression of proteins in rBCG generally induced CMI they only sometimes induced a humoral response; possibly use of membrane associated lipoproteins located at the surface of the bacterium would improve the potential for recombinant *M. vaccae* to elicit protective humoral responses.

In *M. tuberculosis* the 19kD lipoprotein is one of the most immunogenic antigens. It has a hydrophobic NH_2-terminal region which is a lipoprotein signal peptide, and the protein is lipid acylated during the export process and is abundant in the outer capsule and culture filtrate. The capsule determines what components come in contact with the host cells and immune system in early stages of infection. (Ortalo-Magne 1995). In Lyme disease humoral immunity is protective but a predominantly Th1 response is always elicited to infection (Rao 1995). Stover 1993 tested whether BCG expression of the outer surface (lipo)protein A (OspA) antigen of *Borrelia burgdorferi* as a membrane-associated lipoprotein could elicit high titre protective antibody responses. They used signal peptides (promoter, ribosomal binding site and secretion signal) and the first six processed codons from the *M. tuberculosis* 19kD antigen to direct the export and membrane associated surface expression of the target antigen. While expression from the 19kD signal was about 5 fold lower than pMV261, immunisation of mice with membrane lipoprotein versions resulted in consistent protective antibody responses that were elicited earlier and were higher (100-1000-fold in some mouse strains) than responses elicited by immunisation with recombinant BCG expressing OspA cytoplasmically (in excess of 10% of total rBCG protein) or as a secreted fusion protein. Also, only mice immunised with the membrane bound form showed good protection from challenge.

However, with pAU171/p27 in dead *M. vaccae*, no specific antibodies to p27GST were detected. Immunisation with a denatured antigen can generate a T cell response to both denatured and native antigen (T cells respond to linear fragments of peptide presented associated with MHC molecules), whilst only irrelevant antibodies may be produced. Hence by immunisation with autoclaved recombinant *M. vaccae* it may not be possible to generate antibody responses to entire native proteins.

It is also possible that a less severe form of killing the vaccine, for example by heating to 80°C for 30 minutes rather than autoclaving, may leave the foreign peptides in better condition for inducing both T cell and humoral immunity. However, while autoclaved *M. vaccae* vaccine has a license for use in humans and has been widely tested, a heat-killed vaccine has not and this would be another obstacle to eventually testing it as a potential recombinant vaccine in humans.

However many diseases exist where a clear beneficial role for antibodies has not been shown and specific antibodies may even be detrimental (e.g. enhancing antibodies or when the presence of antibodies forms part of a screening system e.g. anti-p24

antibodies as evidence of HIV infection in human blood samples), for which vaccine-induced antibody positivity would be a disadvantage.

4.4.3 Induction of different branches of immunity by mycobacteria

Vaccination with recombinant dead or non-pathogenic mycobacteria does not have a good history. *M. smegmatis* expressing a BCG genomic library has been generated (Falcone 1995). *M. smegmatis* only survives about 24 hours alive in macrophages (this is independent of an acquired T cell response) and thus it does not have the advantage of establishing a persistent, self-limiting infection like BCG. Even when various BCG antigens were well expressed and several injections of 10^8 live *M. smegmatis* were introduced into tissues, the recombinant was inactivated before it could induce a detectable cell mediated immune response to the foreign proteins in mice (assessed by resistance to a challenge with live *M. tuberculosis*). The only way to achieve protection with the recombinant was to suspend heat-killed organisms in incomplete Freund's adjuvant and administer two doses over a three week interval, three weeks prior to challenge. However, the need for this type of adjuvant rendered this vaccine unsuitable for humans.

It was hoped that *M. vaccae* may have some inherent adjuvant effect for type I cellular immunity which other non-pathogenic mycobacteria do not (J. Watson, patent document) and vaccination with dead *M. vaccae* did induce IFNγ production. rBCG has the ability to induce all branches of the immune response, including MHC Class I-restricted cytotoxic T lymphocytes, which are critical for resistance to many viral and bacterial infections (Stenger 1998). As mycobacteria are rapidly phagocytosed and most are contained within the endosomal compartment of phagocytic cells, rBCG is effective at inducing both helper T cells that induce IgG production and T cells that are capable of secreting lymphokines such as IFNγ via the generation of MHC Class II responses. To also induce potent MHC Class I responses, persistent infection of the macrophage may result in the release or leakage of antigen from the phagolysosome or a specific route for 'cross presentation' may be active (Carbone 1998 and Albert 1998). Similarly, a recombinant protein subject to proteolysis within the lysosomal compartment can not effectively elicit antibody responses, which generally recognise epitopes of intact antigens. However, rBCG vaccines must contain nonviable organisms capable of releasing relatively intact recombinant antigens recognised by B cells. Since killed rBCG failed to immunise, these dead mycobacteria are not effective immunogens and it

may be that the effectiveness of the live vaccine to produce long-lived responses is due to some antigen molecules being released from the live rBCG over long periods *in vivo*.

So far, *in vitro* IFNγ responses after immunisation of mice with foreign epitopes expressed in *M. vaccae* have been identified only in the CD4 cell subset (Hetzel 1998), although CD8 T cells from *M. vaccae* vaccinated mice can produce IFNγ when stimulated with live *M. tuberculosis, in vitro* (Skinner 1997). The cellular source of specific IFNγ in response to SIVp27 was not defined.

Interestingly, when Abou-Zeid (1997) tested recombinant *M. vaccae* expressing the *M. tuberculosis* 19kDa lipoprotein in mice, type 1 immunity was generated but in a tuberculosis challenge assay, the presence of the 19kDa reduced the limited protection shown by the *M. vaccae* alone. In contrast, the 19kDa antigen when delivered by a recombinant vaccinia virus provided some protection against tuberculosis infection (Zhu 1997). Thus choice of antigen and the natural adjuvanticity of the delivery system together are important criteria, as both may influence the pattern of cellular activation.

In conclusion, there was insufficient expression of the foreign protein to induce a strong 'pure' type 1 response in immunised mice. However, some IFNγ was produced *in vitro* in response to the foreign protein and *M. vaccae* components, but it is uncertain which cells were responsible for this production.

Poor responses to the foreign antigens in recombinant *M. vaccae* and the ability of standard *M. vaccae* vaccine to favourably influence the outcome of varied diseases in humans and animals (e.g. tuberculosis: Corlan 1997 and Hernandez-Pando 1997 and Abou-Zeid 1997; allergy: Wang 1998; psoriasis: Lehrer 1998) indicated that future work should attempt to define the factors involved in enhancement of non-specific type 1 responses after stimulation with standard dead *M. vaccae*.

Chapter 5 Flow cytometric analysis of murine type 1 T cell responses after vaccination with standard *M. vaccae.*

5.1 Introduction

It was of interest to look at the types of cells producing cytokines in response to *M. vaccae* immunotherapy or vaccination. The previous chapter showed IFNγ production after multiple *M. vaccae*/SIV vaccinations, but did not show the dynamics or the cell types involved. Enumeration of intracytoplasmic IFNγ positive T cells has been shown to correlate with quantitative measurement of IFNγ in culture supernatants. Additionally flow cytometry has the advantage of simultaneous detection of surface markers. Analysis of *ex vivo* IFNγ production at the single cell level may reflect more accurately T cell IFNγ production, by avoiding the polyclonal stimulation of IFNγ production observed after short term *in vitro* stimulation (Vikingsson 1994).

In human melanoma cancer trials of *M. vaccae* immunotherapy, a generalised stimulation of IL-2 production by circulating CD4 T cells correlated with improved prognosis (B. Baban, personal communication and in press) and it was of interest to examine whether a similar non-specific stimulation of type 1 responses occurred during vaccination of normal mice with autoclaved *M. vaccae.*

It was also possible that differences in the early cytokine response of T cells following vaccination of mice with low or high dose autoclaved *M. vaccae* could lead to the subsequent divergence towards type 1 or type 2 responses noted by Hernandez-Pando (1997).

Using flow cytometry, intracellular production of cytokines such as IL-2 and IFNγ by murine T lymphocyte subsets was evaluated, following a single injection with high or low dose autoclaved *M. vaccae.* This was a simple model designed to determine whether *M. vaccae* vaccination caused any gross changes in type 1 immune responses.

5.2 Results

5.2.1 Optimisation of cytokine detection

There was rapid down regulation of CD4 after PMA/ionomycin treatment, but positive and negative populations were still separate after a 4 hour stimulation.

Fluorescent antibody concentrations were optimised against their isotype controls and corresponded closely to manufacturer's recommendations (not shown). In each experiment, tubes containing surface stained cells were permeabilised and stained with isotype control or anti-cytokine antibody. The position of quadrants was based on the isotype control and fixed for each series of experiments. Any non-specifically stained cells were subtracted. PE labelled rat anti-mouse IL-4 (Pharmingen 18195A) had been purchased, but a clear population of positive cells was not identified in early experiments. In addition there was limited availability of cells from LN and IL-4 detection was abandoned.

Spleens from 6 month old C57Bl/GrFA mice were compared after stimulation with different transport inhibitors and permeabilisation with buffers from different manufacturers (BD or Ortho). Ortho Permeafix involved a single incubation of surface stained cells with 200µl of reagent, for 40 minutes, dark at room temperature, and had been shown to kill HIV (D. Wallace, personal communication). Results are shown in Table 5-1.

Stimulation	Permeabilising	% CD4	% CD8	% CD4 /IL-2	% CD4 /IFN	% CD8 /IFN
PMA/Ionomycin +Brefeldin A	BD lyse/perm	14.6	12.3	14	22	57
PMA/Ionomycin +Brefeldin A	Permeafix	15.5	10.8	9	13	44
PMA/Ionomycin +Monensin	BD lyse/perm	23	20.3	14	15	40

Table 5-1: Optimisation of cell stimulation and permeabilisation conditions

Ortho permeafix was discounted as some cytokine producing cells possibly were not identified by this method.

Use of monensin led to identification of a larger number of T cells, although not the same percent were secreting cytokines; but monensin was unacceptable because periodically in stimulated spleen cells, two separate cell populations became apparent in the lymphocyte region. The smaller lymphocytes produced less cytokines and this could lead to misinterpretation of results.

The combination of PMA/ionomycin stimulation with brefeldin A as transport inhibitor was considered to give the most representative and reproducible results.

In conclusion, variations occurred when different conditions were employed, so rigorous adherence to the final protocol (as listed in methods) was necessary.

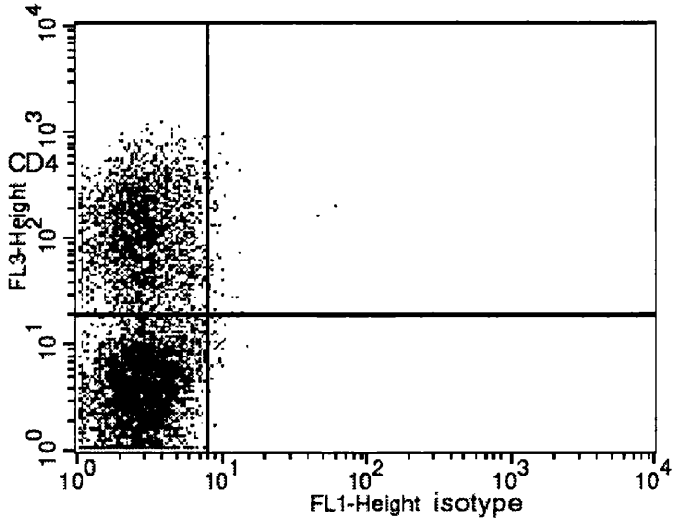
5.2.2 Preliminary time course in C57Bl/GrFA mice

The optimum time after vaccination with autoclaved *M. vaccae* to look for cytokine producing cells was not known, so a preliminary time course experiment was undertaken. Male 12 month old black and tan C57Bl/GrFA mice were vaccinated with 100µl of autoclaved *M. vaccae* batch NCU 003, undiluted (high dose, $1x10^9$ *M. vaccae*) or diluted 1/100 (low dose, approximately $1x\ 10^7$ mycobacteria) with borate buffer. There was one mouse per dose per time point, and two normal mice.

There was some inter-mouse variability, but mouse numbers were limited in this experiment due to restrictions on the numbers of FACS tubes that could be handled (because spleen and LN were tested for several cytokine combinations, sometimes with and without mitogen stimulation). It was planned that after an optimum time point was established, further experiments would include larger numbers of mice per group.

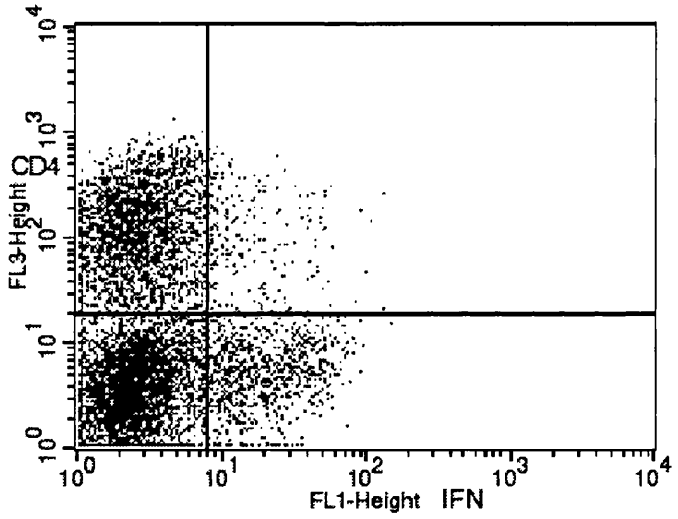
5.2.2.1 Cytokine production by cells from vaccinated C57Bl/GrFA mice

Representative FACS plots are presented (Figure 5-1), showing the comparison between isotype control and IFNγ staining in CD4 or CD8 cells from LN. Cells positive for surface marker and cytokine appear in the UR quadrant. The % gated for each quadrant are shown adjacent to the plots.



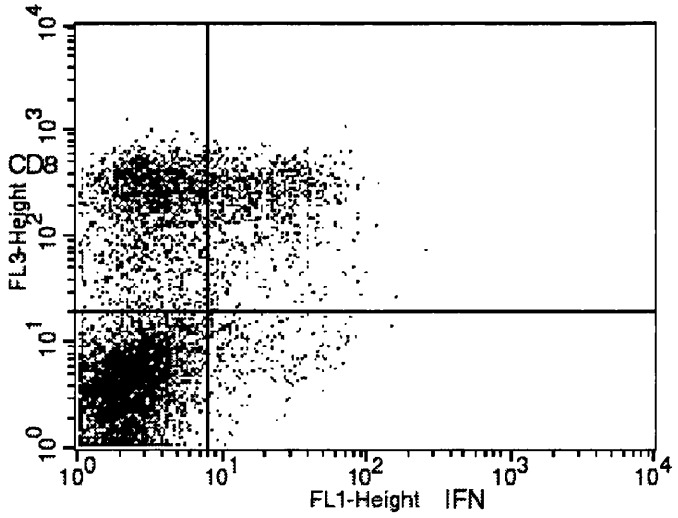
LN stained for CD4 and isotype

Quad	Events	% Gated	X Mean
UL	3068	36.06	3.06
UR	53	0.62	11.23
LL	5359	62.99	3.07
LR	28	0.33	9.49



LN stained for CD4 and IFN

Quad	Events	% Gated	X Mean
UL	2869	34.31	3.00
UR	221	2.64	21.17
LL	4412	52.77	2.81
LR	859	10.27	23.68



LN stained for CD8 and IFN

Quad	Events	% Gated	X Mean
UL	1940	23.48	3.66
UR	957	11.58	23.19
LL	5135	62.15	2.55
LR	230	2.78	21.90

Figure 5-1: Dot plots of IFN-gamma production by CD4+ or CD8+ LN cells from C57Bl/GrFA mice vaccinated with high dose *M. vaccae*

The percent of CD4 or 8 cells that express cytokine can be calculated by:

$$\frac{UR}{UR+UL}\times 100$$

An alternative way of presenting the data is to multiply the value of the UR quadrant by the number of cells in that organ to give a total number of cells expressing CD4 or 8 molecules with a particular cytokine.

The former calculation is influenced by changes in %CD4 or %CD8, while the latter is influenced by cell accumulation.

5.2.2.2 Time course of cytokine production by mitogen stimulated LN cells after vaccination

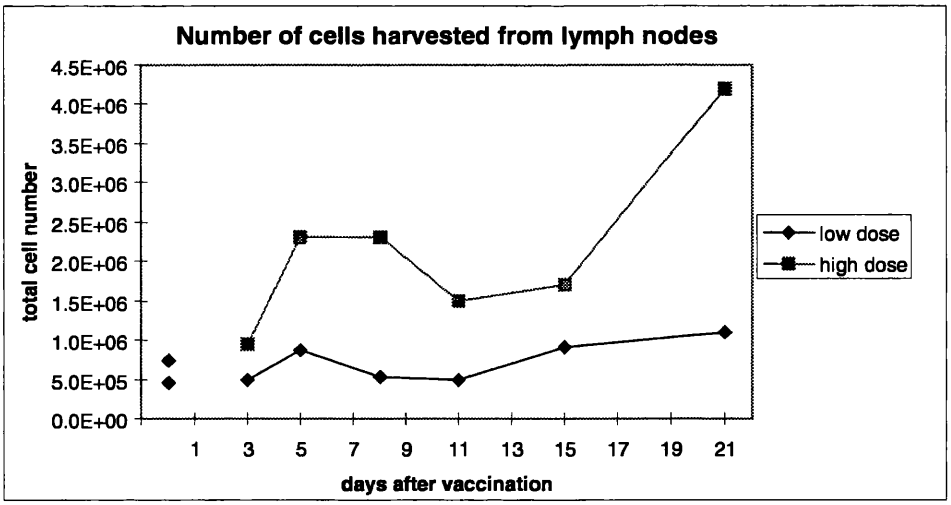
Figure 5-2 shows the results for LN cells from normal mice (on the left at day 0) and at various time points after vaccination with low or high dose autoclaved *M. vaccae*. High dose vaccination increased the total cell yield from lymph nodes (LN) at all time points and also increased the number of mitogen stimulated CD8+ LN cells producing IFNγ at all time points, compared to normal mice. Low dose vaccination did not show these effects. Furthermore, IL-2 and IFNγ production by CD4+ LN cells after high or low dose vaccination showed little change from normal levels (not shown) and no consistent effect was observed with spleen cells under any conditions (not shown).

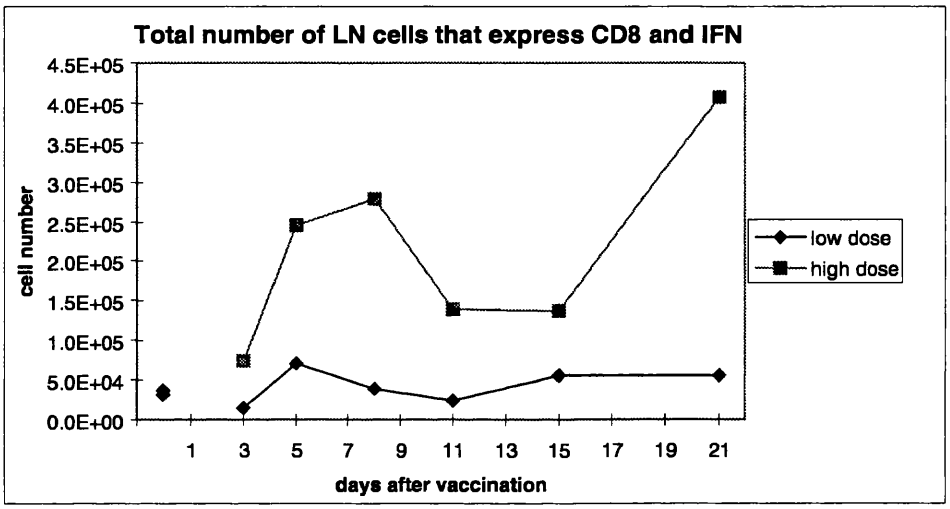
5.2.2.3 Unstimulated cells

Unstimulated CD4 or CD8 spleen cells produced very low levels of cytokines which were difficult to quantify accurately (not shown). There were insufficient LN cells to test unstimulated responses.

5.2.2.4 Conclusions from preliminary time-course of changes induced in T cell populations by *M. vaccae* in C57Bl/GrFA mice

IFNγ production by CD8+ cells appeared to be generally raised in LN at all points after vaccination. Additionally, there was some indication that peak production may be bi-phasic, possibly as a result of early CD8 cell stimulation, then migration of activated cells from the LN followed by a later re-entry phase. However a satisfactory time course was not achieved for IL-2 or IFNγ production by CD4+ cells (not shown). It was decided to repeat the work using young balb/c mice, as these may be more biased to type 2 responses, as they are susceptible to intracellular diseases and induction of Th2 responses to allergens and *M. vaccae* may have a greater effect on this background.





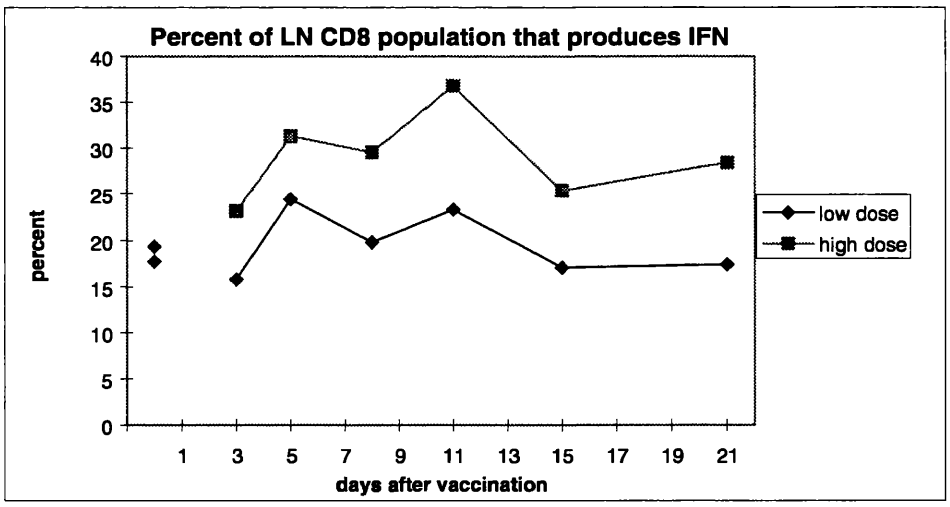


Figure 5-2: Production of IFN-gamma by CD8+ LN cells from C57B1/GrFA mice

5.2.3 Comparison of lymphocyte populations between mouse strains

It was observed that 8 week old female C57bl/6 mice had similar CD4 and CD8 populations to the older C57Bl/GrFA mice used previously, but these were markedly different to populations in 8 week female balb/c mice (Figure 5-3).

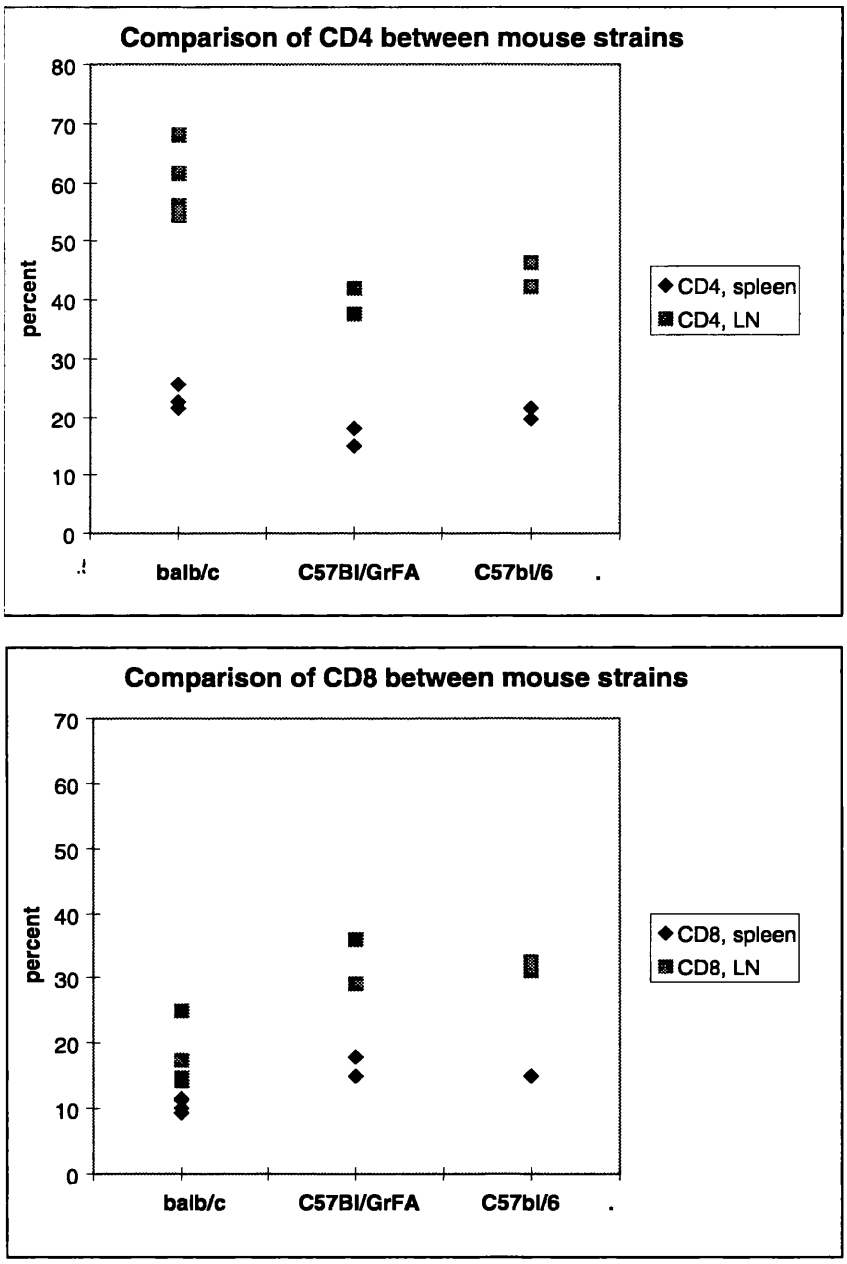


Figure 5-3: Comparison of the percentage CD4 and CD8 cells between normal mouse strains

All mice had a higher percent CD4 and CD8 cells in their LN than spleens. However, balb/c mice had about 3 fold more CD4 cells than CD8 cells in LN, while C57 mice had nearly equal percents of CD4 and CD8.

5.2.4 Expanded time course in young balb/c mice

The time course was repeated with young balb/c mice, and more early time points were included. Balb/c mice were vaccinated with 1 x 10^7 *M. vaccae* (low dose) or 1 x 10^9 *M. vaccae* (high dose) of batch NCU 003 and for each time point one of each group was used. Also three to seven normal mice were tested (on separate occasions).

5.2.4.1 Accumulation of T cells in LN and spleen and change in CD4:CD8 ratio

After vaccination with high dose *M. vaccae* a trend towards increased cell number was seen in the spleen and LN preparations (Figure 5-4). In LN, increased cellularity was often accompanied by decreased percent of CD4+ cells (Figure 5-5). This pattern was seen previously in C57bl/GrFA mice. The peaks and troughs may represent changes in cell trafficking, but as individual mice are represented some may be normal variation.

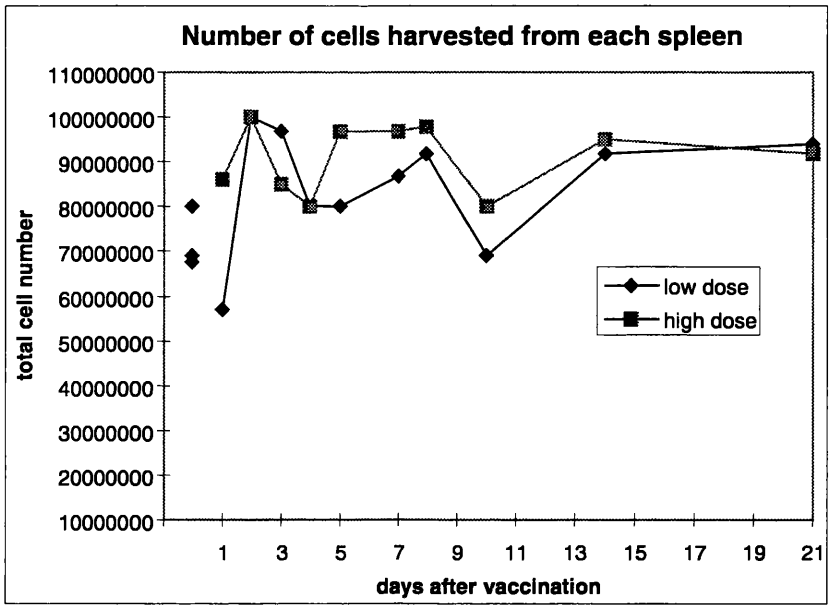
5.2.4.2 Time course of mitogen-stimulated cytokine production in balb/c mice

Representative dot plots of cytokine production from mitogen stimulated LN cells are shown (Figure 5-6 and Figure 5-7).

Time course results are shown as percent of CD4 or CD8 cells making cytokines and also as the total number of cytokine producing cells (Figure 5-8 and Figure 5-9). Results from normal mice are indicated to the left of the time courses.

Examining Figure 5-8 and Figure 5-9 together, it can be seen that vaccination had little effect on CD4 production of IFNγ in balb/c spleen or LN at any point. However, high dose *M. vaccae* appeared to increase the number of CD4+ cells producing IL-2 at 2 days and 5-7 days in LN and at 7 days in spleen. (These changes were not previously seen in C57Bl/GrFA mice, but balb/c mice tend to have a higher percent of CD4+ cells in their LN, which may have made this observation easier.) This may represent an initial priming event in the LN followed by a secondary proliferation in LN and spleen later. Similar peaks were also seen with CD8+ cells producing IFNγ after high dose vaccination. Importantly the percentage and number of CD8 cells making IFNγ remained greater in the high dose group than the low dose or control groups from 2 days to at least 2 weeks after vaccination. Such $CD8^+$ $IFN\gamma^+$ cells had been seen in C57Bl/GrFA mice. Peaks seen after low dose vaccinations were smaller and may have been natural variation.

Generally less than 2% of either unstimulated CD4 or unstimulated CD8 cells showed cytokine production, and this did not seem to be influenced by vaccination (data not shown).



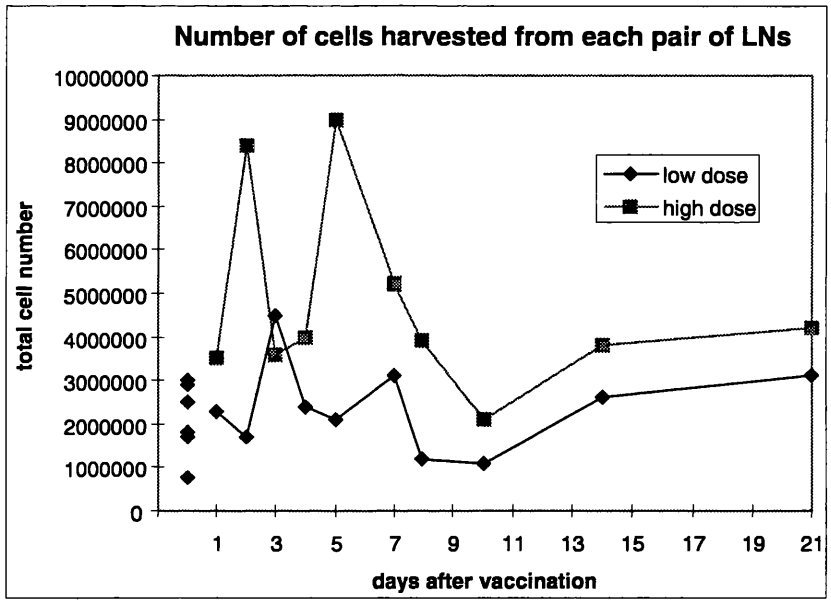
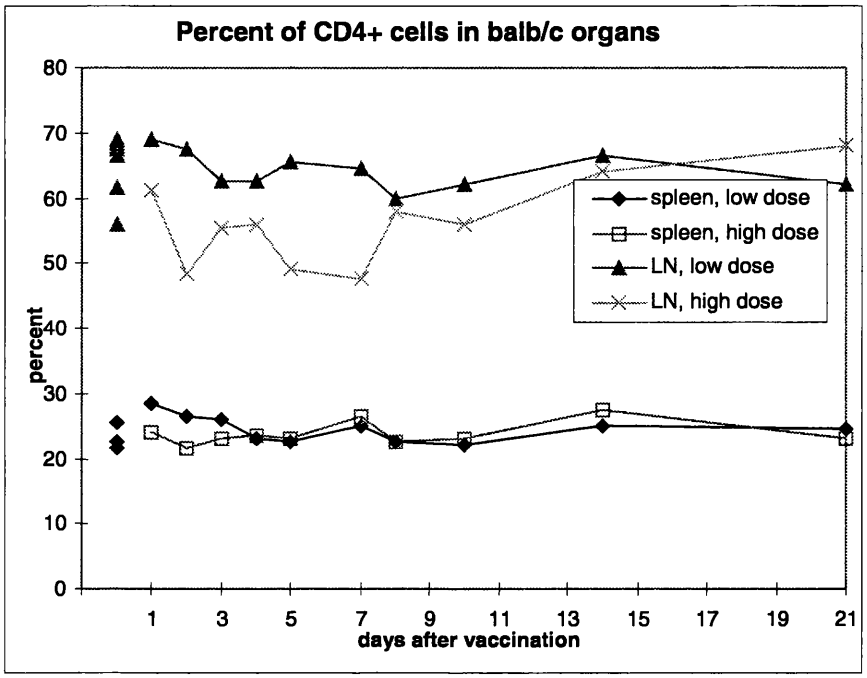


Figure 5-4: Number of cells harvested from spleen or LN of balb/c mice after vaccination



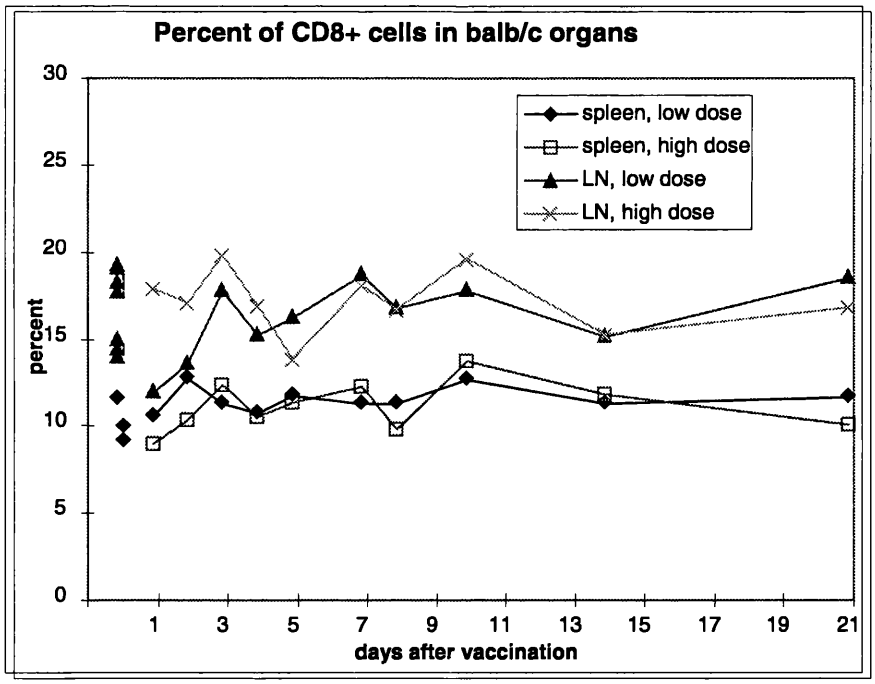
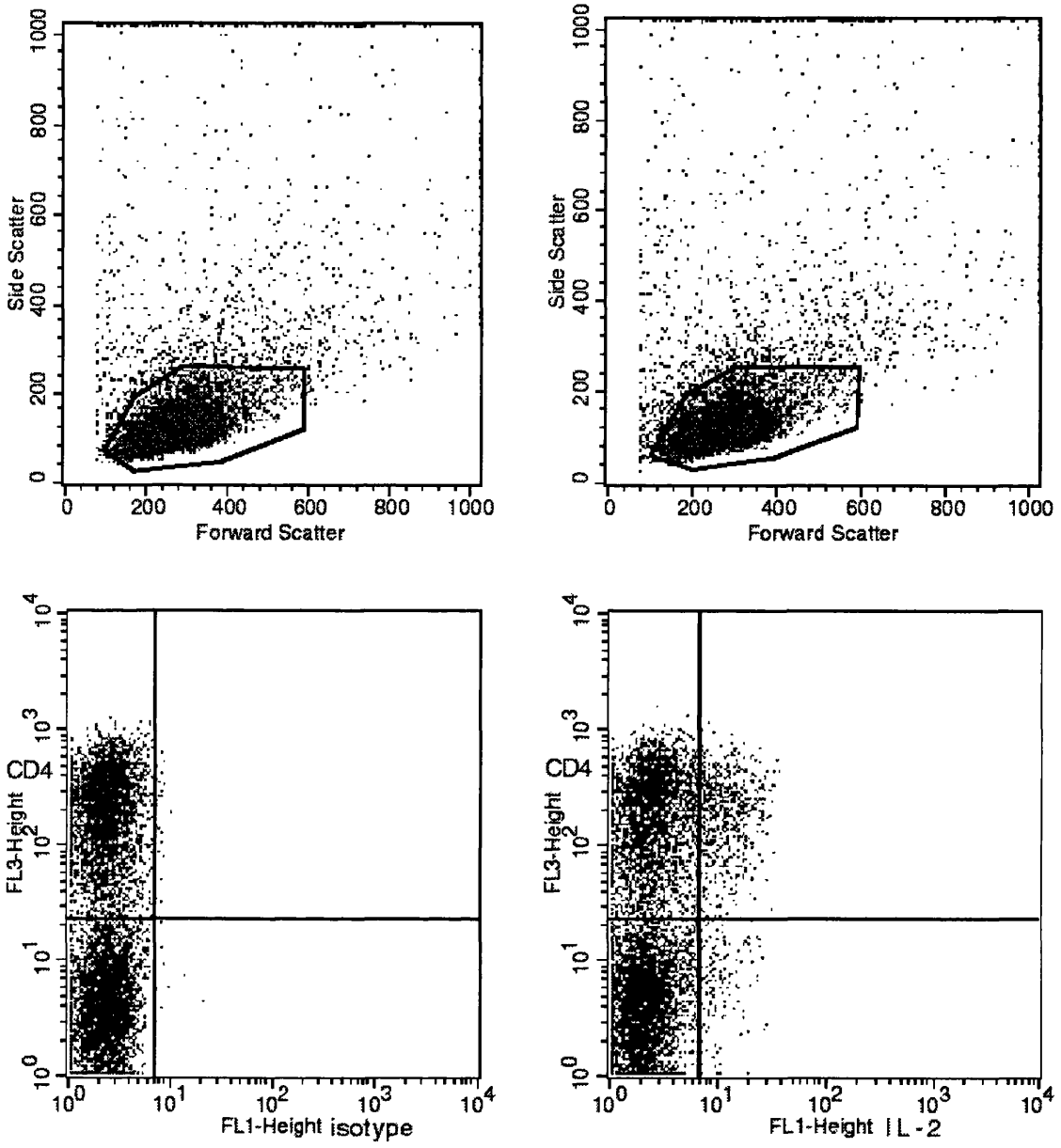


Figure 5-5: Percentage of CD4 or CD8 cells in vaccinated balb/c spleen or LN



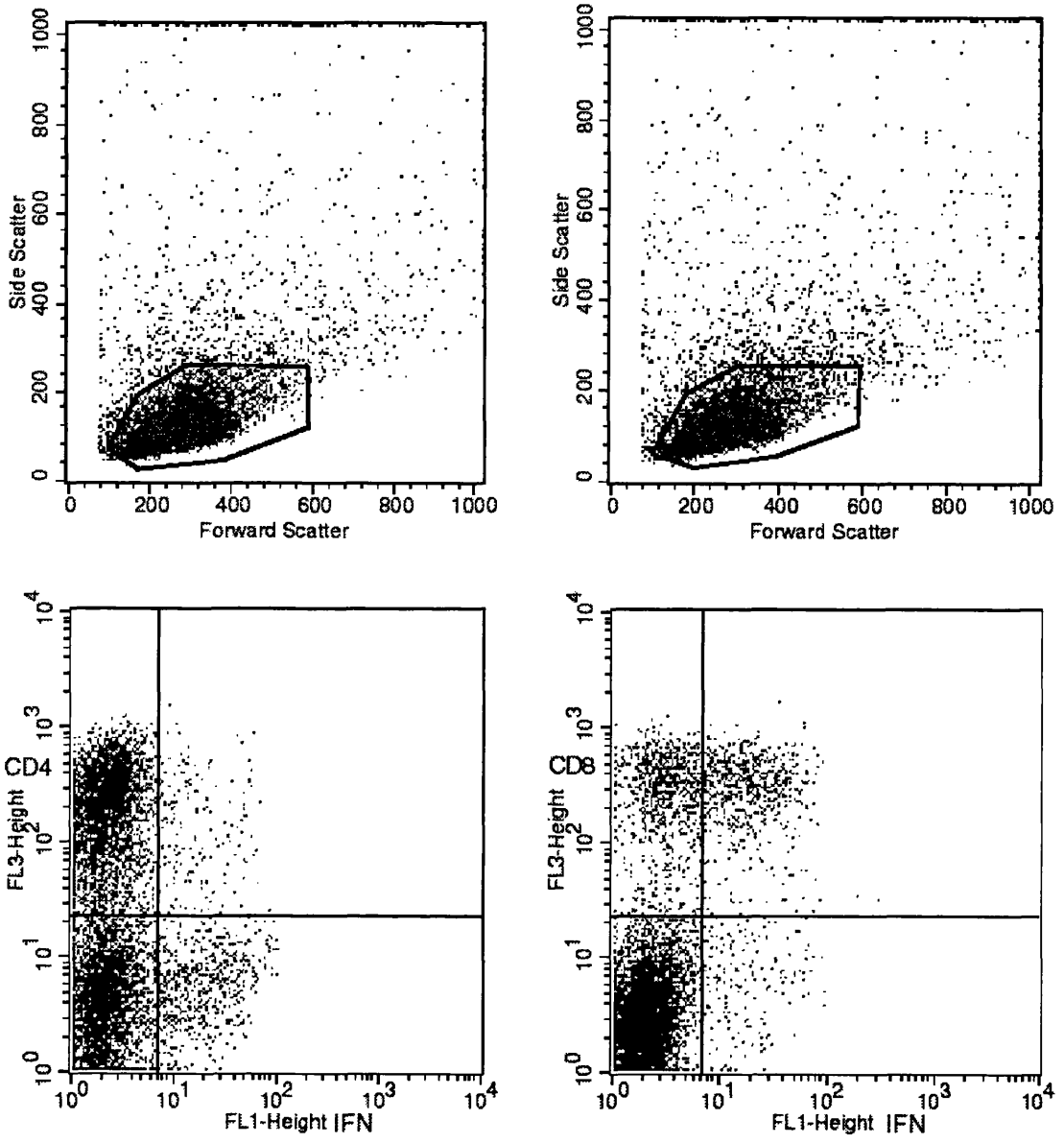
Stained for CD4 and isotype

Quad	Events	% Gated
UL	4212	47.30
UR	31	0.35
LL	4651	52.23
LR	11	0.12

Stained for CD4 and IL-2

Quad	Events	% Gated
UL	3438	39.34
UR	628	7.19
LL	4531	51.84
LR	143	1.64

Figure 5-6: Dot plots of IL-2 production by CD4+ cells from LN of a balb/c mouse vaccinated with high dose *M. vaccae* 7 days previously



Stained for CD4 and IFN

Quad	Events	% Gated
UL	4041	45.49
UR	181	2.04
LL	4065	45.76
LR	597	6.72

Stained for CD8 and IFN

Quad	Events	% Gated
UL	927	10.61
UR	657	7.52
LL	6978	79.89
LR	173	1.98

Figure 5-7: Dot plots of IFN-gamma production by CD4+ or CD8+ cells from LN of a balb/c mouse vaccinated with high dose *M. vaccae* 7 days previously

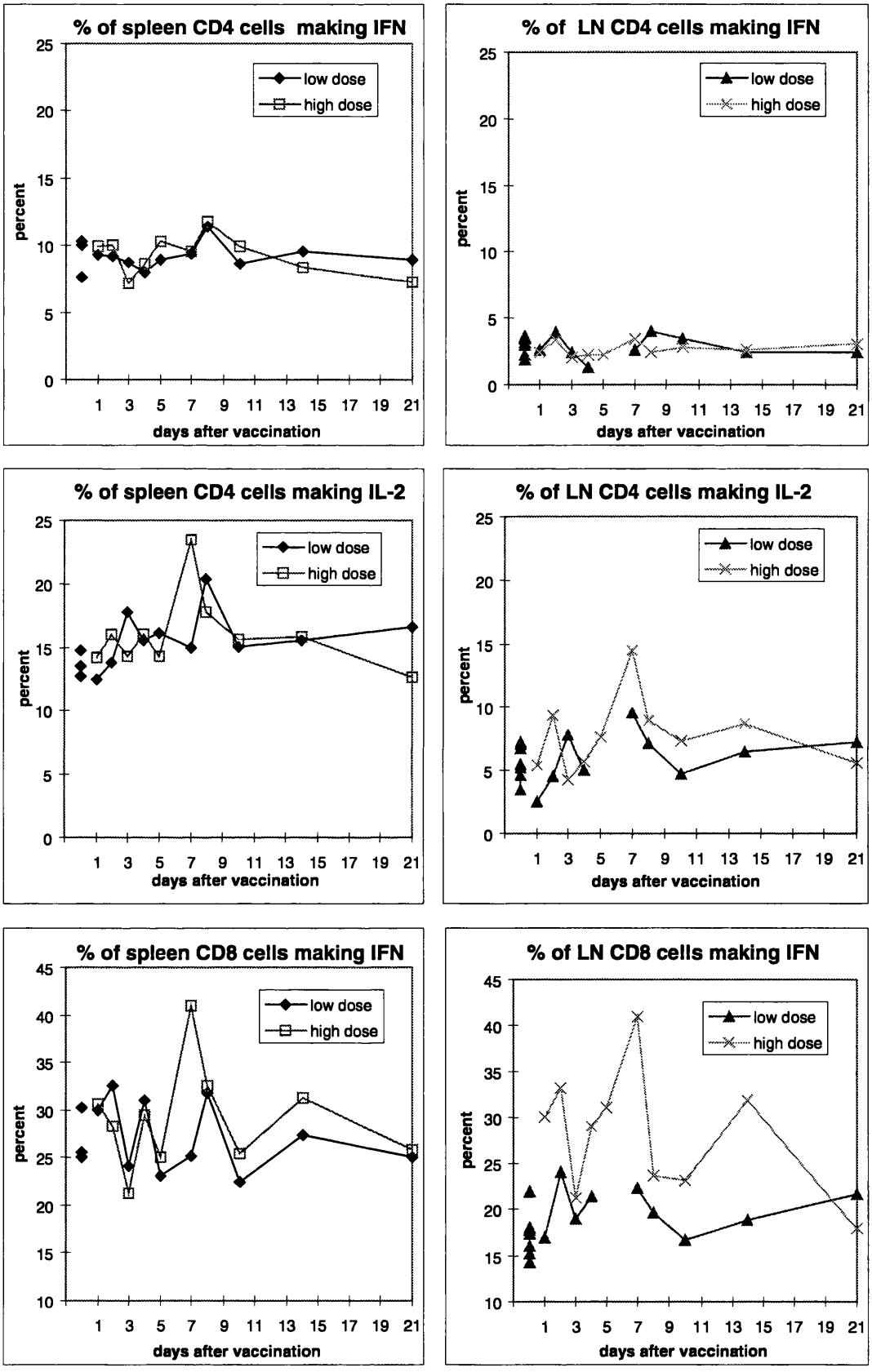


Figure 5-8: Percent cytokine producing cells in balb/c spleen or LN at intervals after vaccination

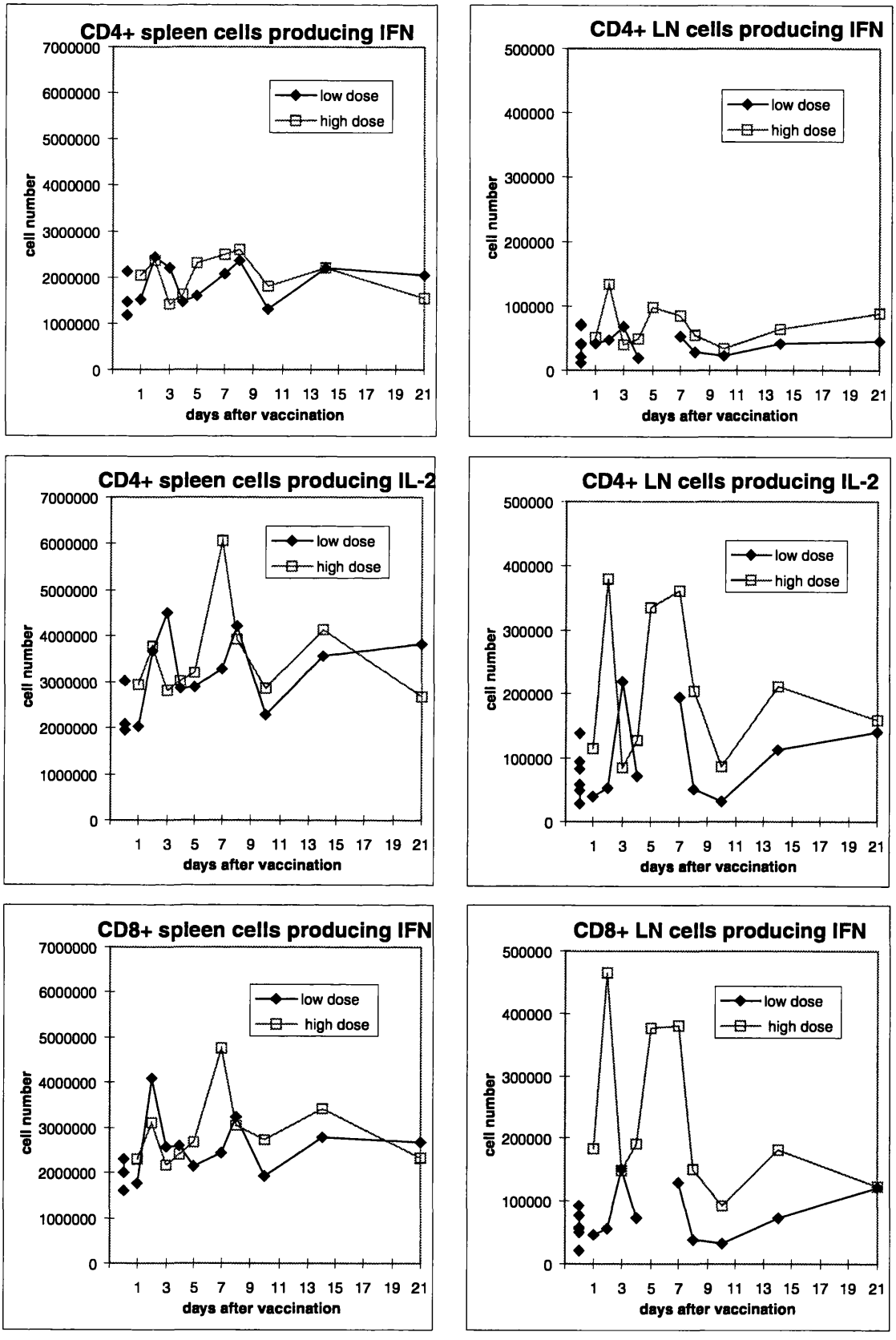


Figure 5-9: Total number of cells producing cytokines in balb/c spleen and LN at intervals following vaccination

5.2.4.3 Assay 12 mice for 7 day peak of LN cytokines

The apparent peak of cytokine production at 7 days was investigated further. 12 young female balb/c mice were vaccinated with placebo or high and low doses of *M. vaccae* batch NCU 001 (as NCU 003 was limited). NCU 001 had previously been used in humans, but in other assays it was shown to have the least activity of any batch of *M. vaccae*. Cytokines were tested in stimulated lymphocytes from paired LN, using 4 mice per group. Table 5-2 shows the average cytokine production (standard deviation) for each group.

	% of CD4+ with IL-2	% of CD8+ with IFNγ	Total cells	no. of cells with CD4/IL-2	no. of cells with CD8/IFNγ
Placebo	6.4 (1.2)	16.2 (1.7)	3075000 (1230000)	135423 (65200)	85753 (37200)
Low dose vaccine	5.6 (0.8)	14.8 (4.0)	3275000 (1340000)	131020 (80200)	81488 (61200)
High dose vaccine	6.9 (2.0)	16.8 (1.5)	4950000 (1440000)	200130 (53900)	**153415** (51100)

Table 5-2: Average cytokine production in LN, 7 days after vaccination.

Placebo mice showed similar results to normal mice tested previously but there was no increase in the percent of CD4+ cells making IL-2 nor in the percent of CD8+ cells making IFNγ after *M. vaccae* vaccination.

However, there was an increase in cell number after high dose vaccination and an increase in the total number of CD8 cells expressing IFNγ after high dose vaccination, as this takes into account the increased size of LN. (For the high dose vaccine group compared to placebo group, the number of CD8 cells producing IFNγ was significantly different (p= 0.04) using a 1 tail T test, but not significant by Mann-Whitney).

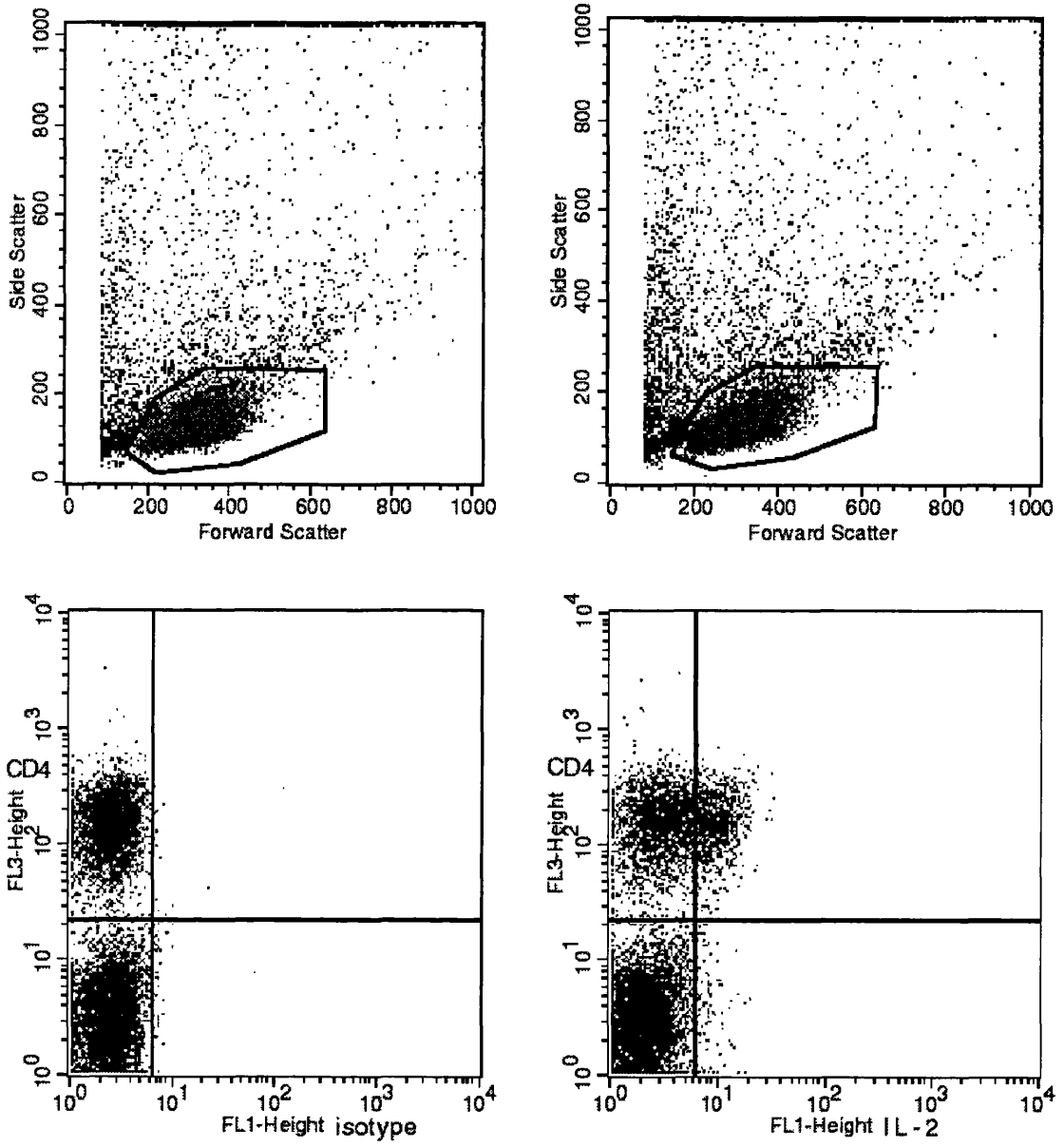
5.2.5 Compare blood and lymphoid organs in balb/c mice 8 days after vaccination

This was tested because IL-2 production had been observed in stimulated and unstimulated CD4 cells from the blood of cancer patients receiving *M. vaccae* immunotherapy, and blood and lymphoid organs represent different compartments.

Blood was taken from one balb/c mouse per group 8 days after vaccination and cells were mitogen stimulated and stained (spleen and LN results were also included in previous time courses).

In Figure 5-10 and Figure 5-11 dot plots show 38.5% of blood lymphocytes were CD4+ and 11% were CD8+ and that surprisingly large numbers of CD4 cells producing IL-2 could be identified. Smaller numbers of CD8 cells made IFNγ. There were also about 5% of blood lymphocytes that produced IFNγ but were not CD4 or CD8 positive (in R3). A similar population had been seen in spleens of both mouse strains, but never in LN. However, these cells were present in normal mice, with or without mitogen stimulation and were not investigated further.

The graphs (Figure 5-12) show the percent of CD8 cells making IFNγ rises in LN and spleen following vaccination, while this percent falls in blood of the same mice. The percent of CD4 cells making IL-2 in blood was higher than in spleen or LN, but *M. vaccae* vaccination still only marginally affected IL-2 production in these samples.



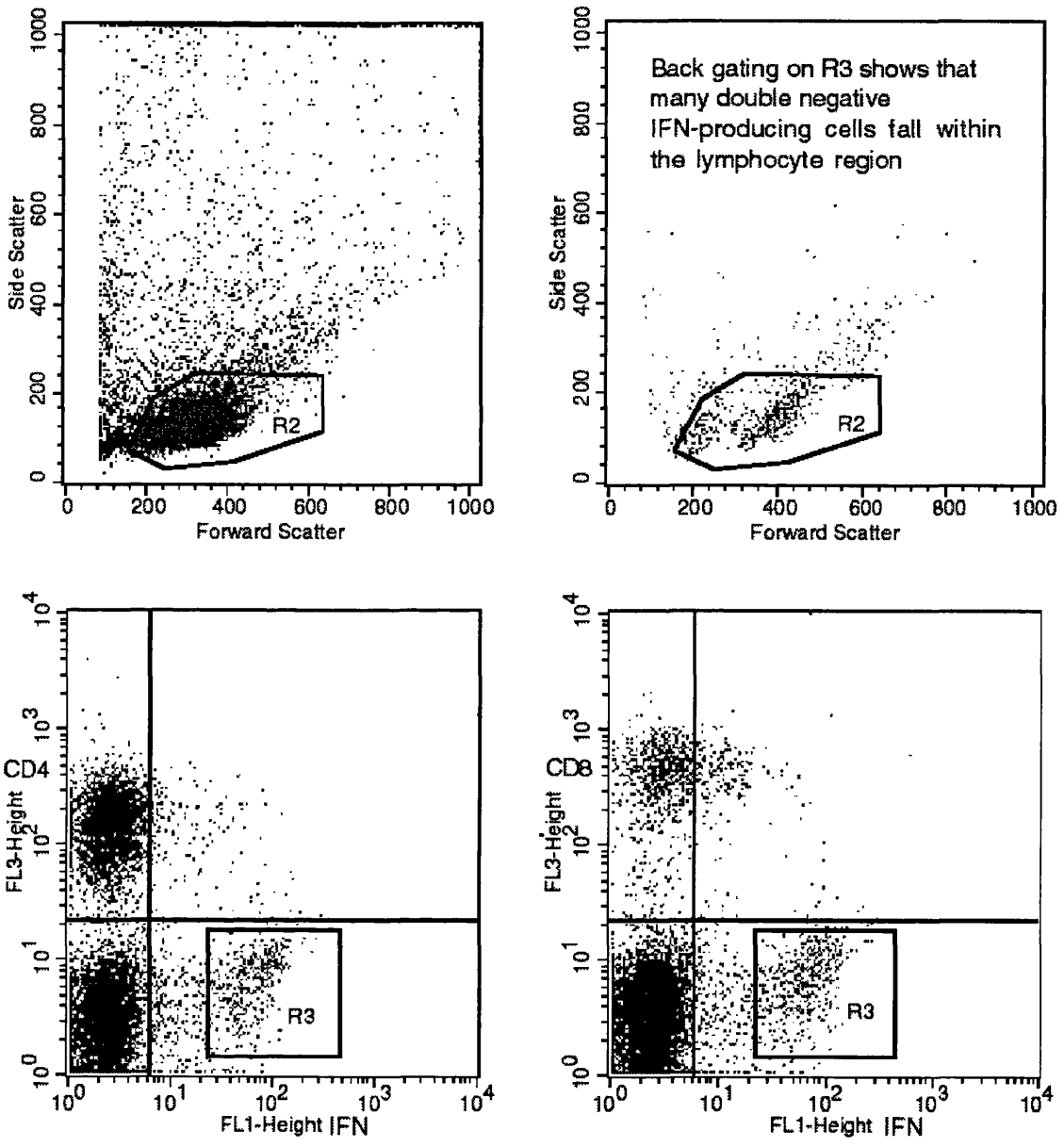
Stained for CD4 and isotype control

Quad	Events	% Gated
UL	3830	38.40
UR	19	0.19
LL	6075	60.91
LR	50	0.50

Stained for CD4 and IL-2

Quad	Events	% Gated
UL	2501	25.10
UR	1180	11.84
LL	6164	61.86
LR	120	1.20

Figure 5-10: Dot plots of IL-2 production by CD4+ cells from the blood of balb/c mice vaccinated with high dose *M. vaccae*



Quad	Events	% Gated
UL	3651	36.99
UR	177	1.79
LL	5313	53.84
LR	728	7.38

Quad	Events	% Gated
UL	843	8.53
UR	220	2.23
LL	8054	81.49
LR	767	7.76

Figure 5-11: Dot plots of IFN-gamma production by cells from the blood of balb/c mice vaccinated with high dose *M. vaccae*

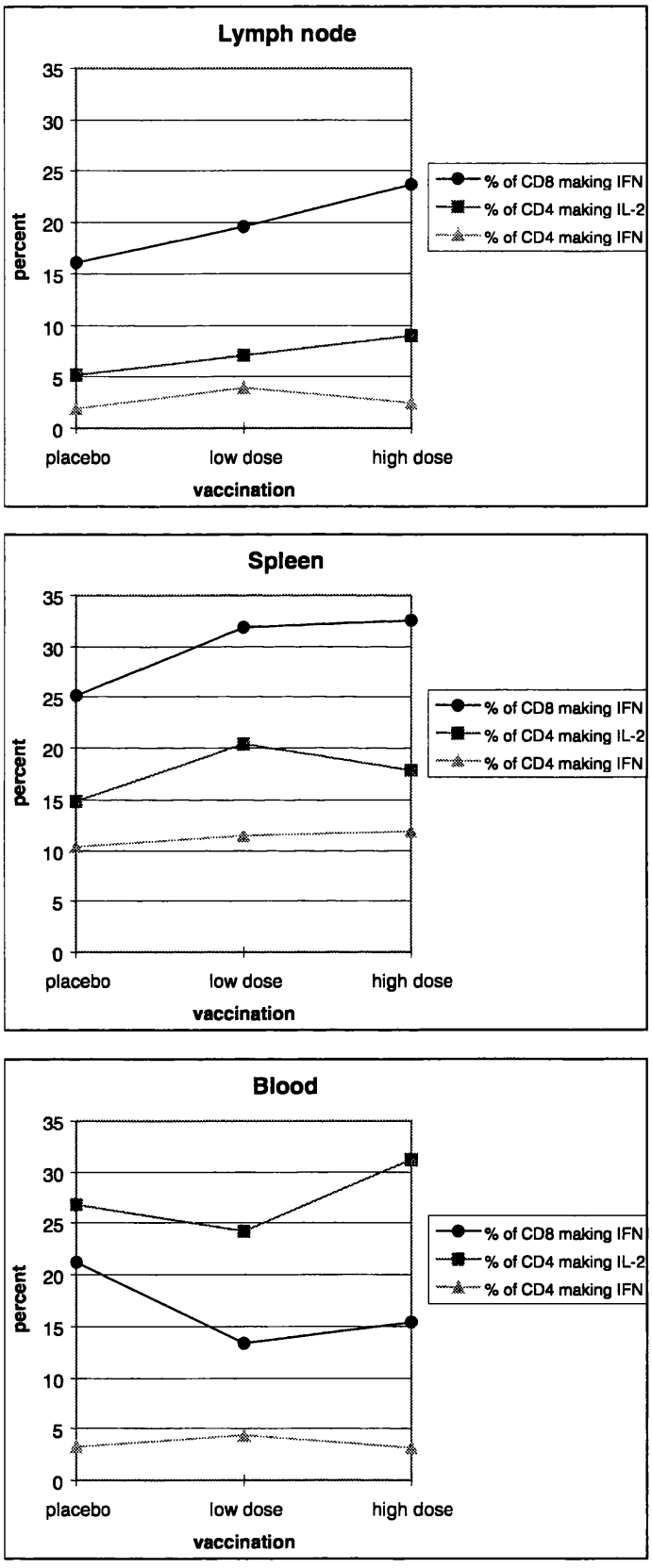


Figure 5-12: Cytokine-producing cells in LN, spleen and blood 8 days after vaccination

5.3 Conclusions

A general observation after vaccination with autoclaved *M. vaccae*, was the increased cell number. This was most apparent in lymph nodes and was dose dependent, being most obvious after injection with around 10^9 autoclaved organisms. Large fluctuations occurred in the initial stages, which may have been as a result of cell trafficking. The increased cellularity was selective, because as cell numbers in LN increased, the percent of CD4 positive cells decreased.

To enable detection of intracellular cytokines in this system, cells required brief stimulation with PMA/Ionomycin; this probably induces cytokine synthesis in cells that were activated *in vivo*. Periodic increases in the absolute numbers of CD4+ cells producing IL-2 were observed in balb/c mice shortly after a high dose vaccination with *M. vaccae*. However, this was not observed in every experiment nor in both mouse strains, which may indicate that IL-2 induction by CD4+ cells was at the limit of sensitivity in this type of assay. Furthermore, only a low percent of CD4 cells produced IFNγ and this changed little after vaccination, so it is unlikely that the same cells were producing both cytokines. Possibly the CD4 cells were not polarised to Th1 or they may have been naive, making mainly IL-2 prior to division and differentiation.

Both the percent and the absolute count of CD8 cells producing IFNγ were increased in LN after high dose vaccination and this was apparent from very early in the time course until around 2 weeks. Peak production appeared to be bi-phasic. Although variations would have been emphasised by using only one mouse per condition, raised levels of CD8 cells with IFNγ were not consistently seen when a dose of 10^7 *M. vaccae* was used. Nor were the trends as clear using spleen cells. In fact, when replicate mice were tested at 7 days after vaccination with an old batch of *M. vaccae*, a significant increase in the total number of LN CD8+ cells making IFNγ was demonstrated for 10^9 *M. vaccae* compared with placebo.

From a small experiment where percents of cells making cytokines in the blood, spleen and LN were compared, CD8+IFNγ+ levels in blood may fall after high dose vaccination, while those in LN rise. Thus some such fluctuations may have been as a result of vaccine-induced cell trafficking.

In spite of differences in broad cell balance between C57Bl/GrFA and balb/c mice, both strains demonstrated this general increase in CD8+IFNγ+ producing cells in LN following 10^9 *M. vaccae* vaccination.

5.4 Discussion

5.4.1 T cell traffic between blood and lymphoid compartments

Naive T cells circulate between secondary lymphoid organs, such as spleen and LN, via the blood and efferent lymphatics. To activate them, foreign antigens must traffic from the site of infection to the draining LN, where they can be presented by professional APCs. Once activated, T cells can enter peripheral tissues and perform effector functions.

Lymphocyte recirculation facilitates the detection and elimination of pathogens and the dissemination of immunologic memory. L-selectin, the lymph node homing receptor, is central to the control of lymphocyte recirculation. In the absence of L-selectin, B and T cells predominantly migrate to the spleen. T cells express 50-100% more L-selectin than B cells (Tang 1998) and this explains the more efficient migration of T cells to LNs (and hence their higher percent by flow cytometry). Furthermore the cytokine production patterns in blood and lymphoid tissues after *M. vaccae* vaccination varied; this may be explained by somewhat selective recirculation patterns of small lymphocytes between lymph and blood (Andrade 1998). However, even more complex recirculation patterns exist in activated T cells. Between 1-4 hours after T cell stimulation, T cells down regulate L-selectin and then over the course of the following 48 hours, surface expression increases markedly, before it is lost again by 5-7 days after stimulation (Chao 1997).

Thus chemokines produced in stimulated tissues induce up-regulation of integrins on various subsets of leukocytes, and thus migration partly depends on flexible programs of chemokine receptor expression (Sallusto 1997). For example, CXCR4 on naive T cells recognise stromal cell-derived factor 1 expressed in lymphoid organs; Th1 cells express CXCR3, respond to IFNγ-induced IP-10 and Mig and home to sites of DTH; Th2, eosinophils and basophils express CCR3 and respond to eotaxin and and thus home to allergic sites.

Immature DC express one set of chemokine receptors to enter inflammed tissue, then down regulate these as they mature and express new receptors such as CCR7, which binds ELC constitutively expressed in lymphoid organs (A. Mantovani, British Cytokine Group meeting, June 1998). Thus DC can migrate very rapidly to draining LN, where they present particulate antigens on MHC class I or class II, before the DC are cleared from the LN by 48 hours (Ingulli 1997, Rescigno 1998). The presence of

inflammatory cytokines in the lymphoid tissue also enhances T cell accumulation, proliferation and cytokine production (Pape 1997).

The relevance of rapid cytokine responses in LN was shown by others for IL-4; mRNA was produced within 16-48 hours of infection of susceptible BALB/c mice with *L. major* and this strongly influenced the course of infection (Launois 1997). A second wave of IL-4 mRNA was seen from day 5 onwards, reflecting the differentiation of cells towards Th2. This pattern is not dissimilar to the peaks of cytokine production seen in my model.

The waves of adhesion molecule, integrin and MHC expression on APCs and T cells, along with T cell proliferation and differentiation, may explain the waves of cytokine activity in LN and spleen which follow relatively rapidly after *M. vaccae* stimulation. However, receptor expression in response to *M. vaccae* was not examined.

5.4.2 Activation of CD8 T cells

5.4.2.1 Specific activation by mycobacterial antigens

Production of IFNγ by murine CD8 T cells after vaccination with autoclaved *M. vaccae* suggests an alternative form of antigen presentation of exogenous antigens may be occurring. One possibility is presentation on MHC class I molecules. Infection or immunisation of humans with live mycobacteria can elicit MHC class I restricted cytotoxic T lymphocytes that produce IFNγ and are directed against mycobacterial products (Lalvani 1998), and in murine macrophages, viable *M. tuberculosis* allows soluble antigens ordinarily unable to enter the cytoplasm, such as ovalbumin, to be presented through the MHC I pathway to T cells. At high multiplicities of infection, *M. tuberculosis* even inhibits MHC II presentation of ovalbumin (Mazzaccaro 1996).

However, it was held that immunisation with proteins or killed pathogens primed DTH responses but did not prime protective T cell responses *in vivo* (Orme 1988). However, in immunisation and immunotherapy regimens, dead *M. vaccae* was shown to influence the outcome of *M. tuberculosis* infection in mice (Hernandez-Pando 1997 and Rook 1996). Using several killed fast and slow growing mycobacteria as adjuvants, only *M. vaccae* was able to enhance presentation of soluble ovalbumin on MHC class I (Watson 1997, patent document). Thus killed *M. vaccae* may have special properties. Skinner (1997) vaccinated mice with a high dose of a similar preparation of heat-killed *M. vaccae*. By two to four weeks following immunisation, splenic CD8 T cells secreted IFNγ when cocultured for 72 hours with macrophages infected with live *M. tuberculosis*. The CD8 cells also showed strong specific cytotoxicity for infected

syngeneic macrophages, although by 3 weeks after immunisation this response was only observed after restimulation of the spleen cells, suggesting CD8 IFNγ and CTL activity were not linked. CD4 cells from both normal and immunised mice made IFNγ at high levels but showed inefficient cytotoxicity. Dead *M. vaccae* thus retains antigens that are cross-reactive with those of live *M. tuberculosis* and stimulates CD8 cells capable of producting IFNγ. The early IFNγ production by CD8 cells in my model may represent the initial stimulation stages of the memory CD8 cells seen at later time points in spleens by Skinner et al. The CD8 T cells producing IFNγ in my model were not assayed for cytotoxicity.

Additional studies by Skinner (1997) examined prior exposure to mycobacteria on the immune response in a subsequent infection with *M. tuberculosis*. If dead *M. tuberculosis* was used as immunisation 3 weeks prior to infection, spleen cells from mice infected with *M. tuberculosis* for 1 week could proliferate in response to *M. tuberculosis in vitro* but made little IFNγ, while when dead *M. vaccae* had been used, spleen cells from infected mice made large amounts of IFNγ in response to live *M. tuberculosis in vitro*. However, this beneficial effect was short lived, and when infection was allowed to continue for 6 weeks, preimmunisation no longer affected the amount of IFNγ that was produced by fresh or restimulated CD8 cells in response to infected macrophages, although only *M. vaccae* vaccinated mice showed any specific CTL activity (Skinner 1997).

5.4.2.2 Bystander stimulation

When complete Freund's adjuvant or CpG DNAs are used as adjuvants with protein antigens, they induce a strong response skewed towards type 1, which can include induction of CD8 accumulation and proliferation in LN and spleen (Sun 1998). Production of IL-12 and IL-18 along with T cell receptor stimulation, inducing IFNγ production and activation of many cell types, may be credited. However, during viral infections for example, large numbers of CD8 T cells selectively become activated, some by specific T cell receptor interactions, but many others by bystander stimulation of memory CD8 T cells by cytokines, such as type 1 IFNs (IFN-1). Non-specific stimulation of memory CD8 cells (but not CD4 cells) in mice also occurs in response to bacterial products such as LPS and CpG DNA. One model involves production of IFN-1 (by APCs or possibly fibroblasts and endothelial cells), which then induces IL-15 production by APCs, which binds IL-2Rβ and stimulates memory CD8 and NK cells to

proliferate to a limited extent. This response occurs within a few days of stimulation *in vivo* (Tough 1996, Zhang 1998).

IFN-1 may also act directly via IFN-1R on naive or memory CD8 T cells, without IL-15 induction (Sun 1998a). This causes rapid partial activation of the T cells (seen as upregulation of activation markers but not proliferation). However, if APCs are prevalent, the antiproliferative function of IFN-1 onT cells may be countered by an adjuvant effect on APCs (possibly via upregulation of costimulatory molecules) and hence IFN-1 inducing agents like CpG DNA can act as powerful adjuvants for antigen specific responses *in vivo*. IFN-1 can also affect lymphocyte trafficking.

The rapid kinetics of CD8 activation and proliferation to CpG/IFN-1/IL-15 somewhat resemble the rapid appearance of CD8 cells making IFNγ in draining LN after *M. vaccae* vaccination.

Interestingly, unpublished data quoted in two papers (Tough 1997 and Zhang 1998) suggest rIL-12 can cause strong and selective stimulation of memory ($CD44^{hi}$) CD8 T cells *in vivo* but not *in vitro*. IL-12 may not act directly on T cells and is independent of IFN-1, but the mechanism is not known (although it is possible it also acts via IL-15). Some infectious agents or their products may selectively stimulate the memory CD8+ populations (e.g. viruses, LPS and killed *B. abortus*) while certain bacteria induce strong proliferation of memory CD4+ cells *in vivo* (unpublished data quoted in Tough 1997). Also the injection of viable syngeneic tumour cells producing IFN-1 or IL-12 can cause proliferation of memory CD8 T cells in spleen and LN of mice (Belardelli 1998).

Thus sub-cutaneous vaccination with *M. vaccae* may induce production of a range of cytokines possibly including IFN-1, IL-15 and IL-12 and trigger these complex responses, thereby acting as a rapid non-specific adjuvant for CD8+ cells. *M. vaccae* may also provide cross-reactive antigenic stimulation for longer lasting T cell responses.

5.4.3 Effect of high and low dose vaccination

Low dose *M. vaccae* failed to induce cell accumulation in LN or cytokine production. The high dose of dead *M. vaccae* may provide special conditions that enhance cytokine production or access of exogenous antigens to the MHC class I pathway in APCs and therefore induce CD8 responses in draining LN. Other research indicated conditions including a sufficiently high antigen dose or antigen associated with inflammatory signals (e.g. following tissue destruction) to be important for naive CD8 activation in LN (Kurts 1998). DC may also take up apoptotic cells containing products of the vaccine, and present these antigens in LN on MHC class I. However

macrophages taking up apoptotic cells will instead produce IL-10 upon subsequent stimulation (Albert 1998). Thus induction of CD8 responses by *M. vaccae* may be influenced by the dose and route of vaccination. The transient production of IL-2 by CD4 T cells may also indicate that these cells were available to provide 'help' for the CD8 response.

Hernandez-Pando (1997) showed immunisation of mice with low and high doses of autoclaved *M. vaccae* had divergent effects on a subsequent challenge with *M. tuberculosis*: high dose lead to death within 28 days while low dose enhanced survival to over 120 days. Low dose immunisation had primed for Th1 responses and maintenance of high IL-2 to IL-4 ratio in infected lungs while high dose had primed for Th1 and Th2 responses accompanied by increased sensitivity of DTH sites to TNFα toxicity and an IL-2 to IL-4 ratio approaching 1 in the infected lungs. However, CD8 cells were not shown to be involved in this protection. In my experiments, only high dose vaccination produced a CD8/IFNγ response so there may be different dose requirements for CD4 and CD8 responses. Another explanation is that in my experiments, responses were rapidly induced after vaccination and visualised after a very short non-specific stimulation, rather than memory responses identified by *in vitro* restimulation. As IL-4 was not investigated, no differentiation into type 1 or type 2 bias after vaccination was identified by this method.

5.4.4 Importance of IFNγ production by CD8 T cells in diseases

Tascon demonstrated that unprimed purified CD4+ or CD8+ cells from donor spleens could partially protect athymic mice against *M. tuberculosis* infection. However if the donor cells came from IFNγ knockout mice, no protection was observed with total spleen cells or purified CD8+ cells (Tascon 1998). Thus IFNγ was essential to protection, and some CD8+ cells may exert their protective effects by classical cytokine-mediated macrophage activation, especially in the absence of a CD4 response.

As vaccination can alter a subsequent immune response to challenge, it is not surprising that vaccination often does not induce the same response pattern as infection. Immunisation of mice with plasmid DNA expressing mycobacterial hsp65 rather than infection with virulent *M. tuberculosis* generated more activated memory CD8 cells producing IFNγ and fewer weakly activated CD4+ cells producing IL-4 in response to hsp65. Activated IFNγ-producing CD8+ cells provided protection in adoptive transfer experiments; the most protective cells also displayed antigen-specific cytotoxicity

(Bonato 1998). Thus, if IFNγ production is already adequate, cytotoxicity may boost protection.

Thus in tuberculosis patients where protective immunity has failed and there is often evidence of a Th2 response, if immunotherapy with dead *M. vaccae* could induce IFNγ production by stimulated CD8 T cells (amongst other things), this may improve the outcome of treatment. This may have a direct effect, or cause a general shift to a more 'pure' type 1 response, thus reducing necrosis and increasing protection.

IFNγ plays an important role in diseases other than tuberculosis. In some mouse tumour models, tumours were naturally rejected in 1/3 cases. While all mice recruited lymphocytes to LN and made IL-2 and IL-3 responses, a high, IL-12-dependent IFNγ response was necessary for tumour rejection (Fallarino 1996). Treatment with IL-12 can decrease tumour growth and metastasis formation, and in some cases the effect requires CD8 cells (Brunda 1993). In another example, administration of recombinant IL-12 could expand CD8 T cells in LN, enhance IFNγ secretion and cause a T cell infiltration into a tumour. However, an earlier response was a profound increase in tumour-associated iNOS and tumour regression and this early direct killing may have served as a way of generating tumour antigen which enabled the host to mount a subsequent T cell response against the tumour (Hunter 1997). The observed IFNγ LN responses in *M. vaccae* vaccinated mice therefore support a role for *M. vaccae* immunotherapy in cancer, especially if *M. vaccae* were also shown to be a potent cytokine inducer. Immunotherapy with *M. vaccae* alone may improve upon the natural regression seen in some tumour models, but the antigen(s) to which an immune response is directed is not yet defined: shared antigens such as stress proteins, or tumour specific antigens. Autoclaved *M. vaccae* may be injected directly into the tumour or mixed with killed cancer cells or homogenates as an adjuvant, to improve specific responses to tumour antigens.

Further work

Experiments with stronger batches of vaccine or boosting to look at memory responses may help confirm these results. Mice could be vaccinated with live or autoclaved BCG or other mycobacteria to compare the production of IFNγ from CD8 T cells with that after vaccination with *M. vaccae*, but the system would require many mice to achieve reliable results.

A disease model may have provided useful data about the maximum levels of intracellular cytokine production that could be detected in a disease state compared to

normal or vaccinated mice, and the effect of *M. vaccae* vaccination upon these levels could be studied. In humans, *M. vaccae* is most fully documented as an immunotherapy, and using an indirect system in mice may allow changes after low dose vaccination or in cytokine production by CD4 T cells to be detected.

It would be interesting to document the CD8 population that responds to *M. vaccae* further, e.g. looking at surface receptor molecules for lymphocyte activation and homing and testing whether the cells showed cytotoxicity after *in vitro* restimulation.

The surprising production of IFNγ by CD8 T cells also suggests a study of APCs, to discover whether *M. vaccae* stimulates production of IL-12 and IFN-1 etc., up-regulation of accessory molecules or antigen presentation on classical MHC class I molecules.

Chapter 6 Early cytokine production by macrophages stimulated *in vitro* and *in vivo* by standard *M. vaccae* vaccine

6.1 Introduction

IL-12 is a heterodimeric cytokine that is produced mainly by antigen presenting cells and regulates T cells and NK cells. It controls the balance between Th1 and Th2 cells, and is essential for generating optimal Th1 responses in many systems, especially those involving intracellular parasites, where cell mediated immunity is required. IL-12 has therapeutic effects on tumours and airway inflammation and may have utility as a vaccine adjuvant (Gately 1998).

The efficacy of IL-12 adjuvanticity is not guaranteed, as IL-12 given at the same time as BCG does not improve its somewhat limited efficacy against subsequent tuberculosis infection in BALB/c mice, so BCG must induce IL-12 but the mice still respond inappropriately. In contrast, IL-12 given at the initiation of *M. tuberculosis* infection in BALB/c mice does reduce bacterial burden, delay lung pathology and increase survival time; this is dependent upon IFNγ (Flynn 1995).

Furthermore, a synergistic effect of IL-12 and antibiotics on reduction of bacterial numbers is seen in immunocompetent or SCID mice infected with *M. avium*. IL-12 activity is dependent on IFNγ production, but when IFNγ itself is used, it fails to enhance antibiotic clearance of bacteria. IL-12 may be a particularly potent adjunct for chemotherapy of *M. avium* infection in immunocompromised individuals (Doherty 1998).

Similarly, autoclaved *M. vaccae* is used as an adjunct to chemotherapy in mycobacterial diseases, and although the mechanism of action is not known, it may involve a 'switch' towards type 1 responses, reducing pathology and improving protective immunity.

Uptake of mycobacteria and their products (e.g. LAM) into macrophages has been variously shown to induce cytokine production by the macrophages (Barnes 1992) and to inhibit macrophage activation and antigen presentation and depress DTH responses (Chan 1991, Britton 1994). Murine bone marrow macrophages can produce IL-12 after infection with BCG, but require priming with IFNγ and TNFα first, possibly to alter the activation state of the macrophages and/or the IL-12 gene promoters (Flesch 1995). Kubin (1994) showed the human macrophage cell line THP-1 could produce IL-12 in

response to LPS or *S. aureus,* and this did not require IFNγ or TNFα, although maturation of the cells or addition of certain cytokines could influence the levels of IL-12 produced.

Consequently the cytokine inducing ability of standard *M. vaccae* vaccines on macrophages, and factors that influenced this, was investigated.

6.2 Results

6.2.1 Establishment of general principles for production of IL-12 from THP-1 cells

Initial experiments were designed to duplicate the work of Kubin (1994) with LPS (from *E. coli* 0127:B8, Sigma L-4516) on THP-1 cells and determine whether samples of standard *M. vaccae* vaccine (e.g. NCU 002) could also stimulate IL-12 production. Dimethyl sulphoxide (DMSO 1.2%) pre-treatment of THP-1 cells was shown by Kubin to enhance IL-12 production in the short term by inducing increased differentiation in the cells and induction of the p40 subunit was taken as representative of the presence of active p70 heterodimer.

Preliminary experiments showed *M. vaccae* vaccine could induce IL-12 from THP-1 cell lines (not shown). Optimal dilutions of LPS (starting at 20μg/ml with subsequent tripling dilutions, used 5% in the wells) and *M. vaccae* vaccine NCU 002 (starting undiluted with subsequent tripling dilutions, used 5% in the wells) were then established, to induce similar levels of IL-12 production from THP-1 cells within 18 hours of culture, and used in subsequent assays.

6.2.1.1 Enhancement of IL-12 production by DMSO and IFNγ.

Several assay modifications were tested for both LPS and for standard vaccine at several doses (Figure 6-1).

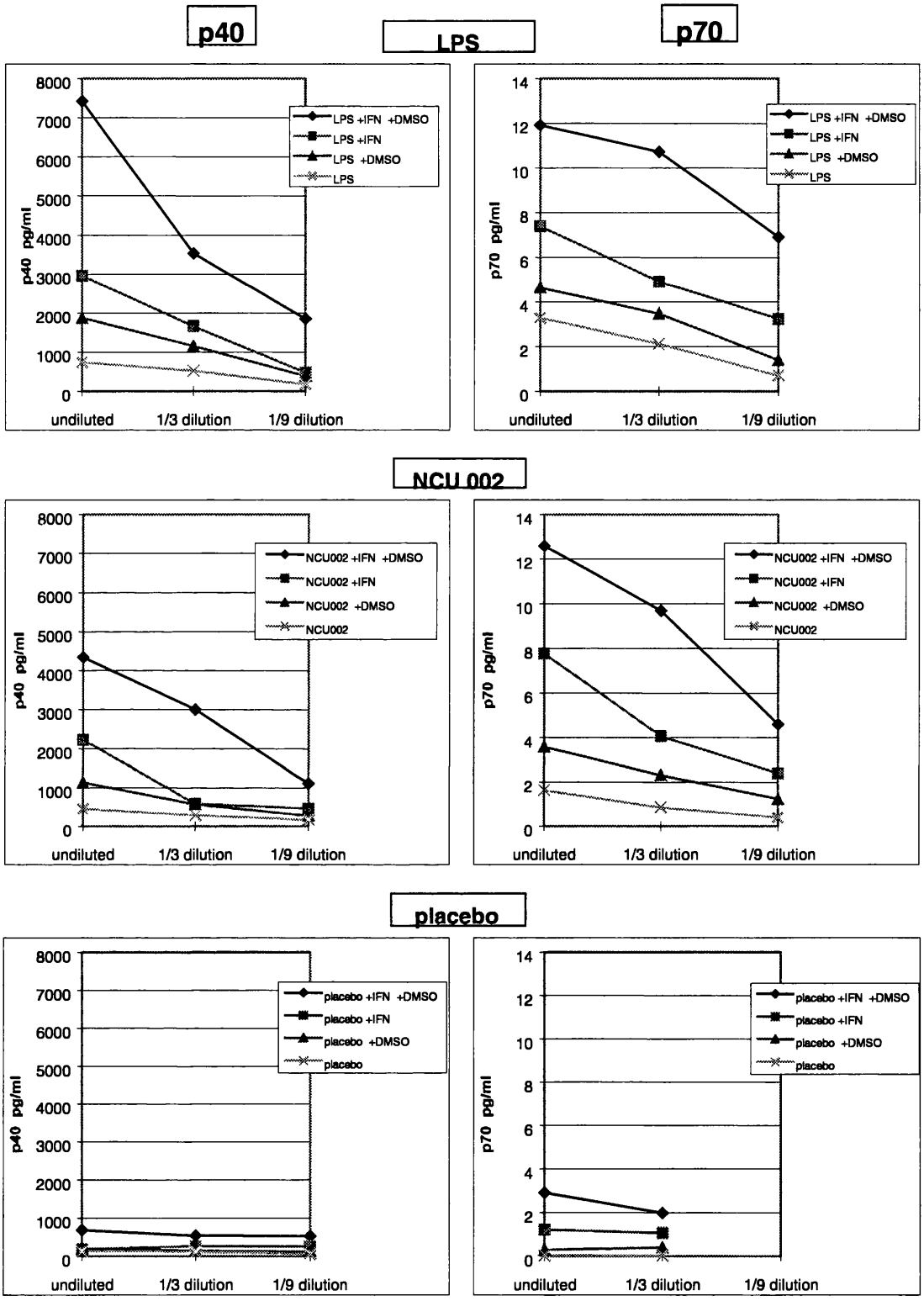


Figure 6-1: Production of IL-12 p40 and p70 under various conditions

IL-12 p70 heterodimer was produced at a low levels whenever the p40 subunit was produced. ELISA assay of p40 was chosen for subsequent work as the p40 antibody pairs were much less expensive than the p70 kit. The enhanced IL-12 levels in supernatants from 24 hour DMSO pre-treated cells could be more reliably assayed in the p40 ELISA, so THP-1 maturation by DMSO pre-treatment was routinely incorporated into the assay.

The production of IL-12 was greatly increased by the presence of excess recombinant human IFNγ (Pharmingen 19751N, 1000U/ml or 32ng/ml) throughout the 18 hour THP-1 incubation with samples.

IL-12 production in response to *M. vaccae* vaccine or sonicate was not due to contamination with endotoxin. Polymyxin B sulphate (Sigma P-1004) used at 5μg/ml reduced LPS-induced IL-12 levels to almost background, while *M. vaccae* preparations were not inhibited (data not shown).

6.2.1.2 Role of phagocytosis in IL-12 triggering

i) Polystyrene beads

Fulton (1996) demonstrated in peripheral blood monocytes that mycobacteria but not PPD could induce IL-12 production. However plastic adherence in the short term or ingestion of polystyrene beads later could also lead to IL-12 production. The role of phagocytosis in IL-12 induction had not previously been tested in THP-1 cells, but its non-specific contribution to cytokine production was not inevitable, as THP-1 are non-adherent and are routinely grown in plastic ware. The THP-1 may also exist in a different state of activation to these monocytes.

Suspensions (10% solid) of polystyrene (latex) beads ranging in diameter from 3μm (Sigma LB-30; approximately 4.5 x 10^9/ml) to 1.1μm (Sigma LB-11) and 0.5μm (Sigma LB-5) were diluted over a wide series from 1/30 to 1/1000 and included in a standard THP-1 assay. After 18 hours, supernatants were tested for cytokine production and cells were stained by Ziehl-Neelsen for the presence of beads or mycobacteria.

M. vaccae preparations after autoclaving retain their acid-fast properties upon Ziehl-Neelsen staining and 80% of cells incubated with undiluted NCU 002 had 3-20 mycobacteria associated with them, while in 1/3 diluted NCU 002, 60% of cells had 1-10 bacteria associated with them (undiluted vaccine is assumed to contain 1 x 10^{10} bacteria/ml, but many are in clumps).

With beads, the following was observed:

0.5μm, 1/300 optimum; very small beads adhering to cells and probably phagocytosed

1.1μm, 1/100 optimum; 5-20 small translucent beads polarised inside most cells

3μm, 1/30 optimum; large adherent beads, occasionally phagocytosed.

This is illustrated in Figure 6-2.

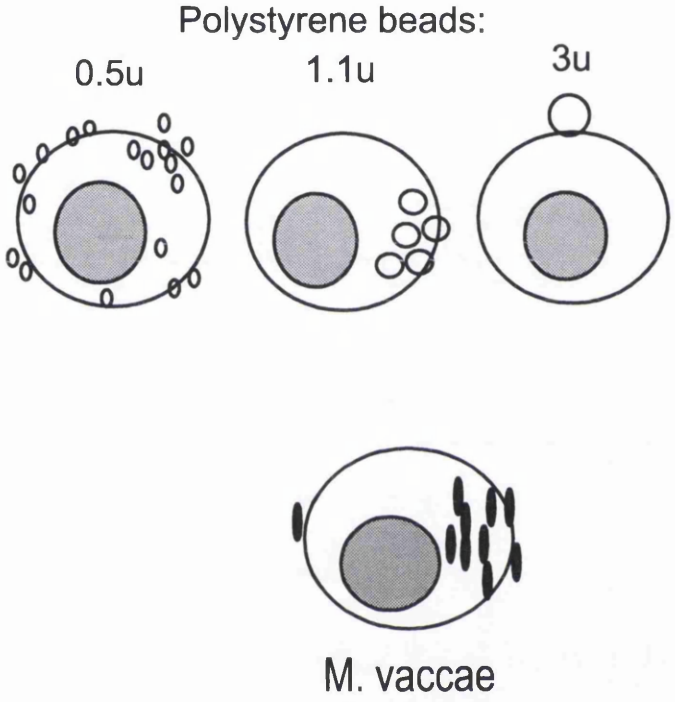


Figure 6-2: Microscopic appearance of THP-1 with associated particles

No IL-12 was produced in response to phagocytosis of inert beads (Figure 6-3) at any dilution, although the NCU 002 vaccine response was adequate, so it is probable that one more components of the *M. vaccae* stimulate cytokine release from THP-1.

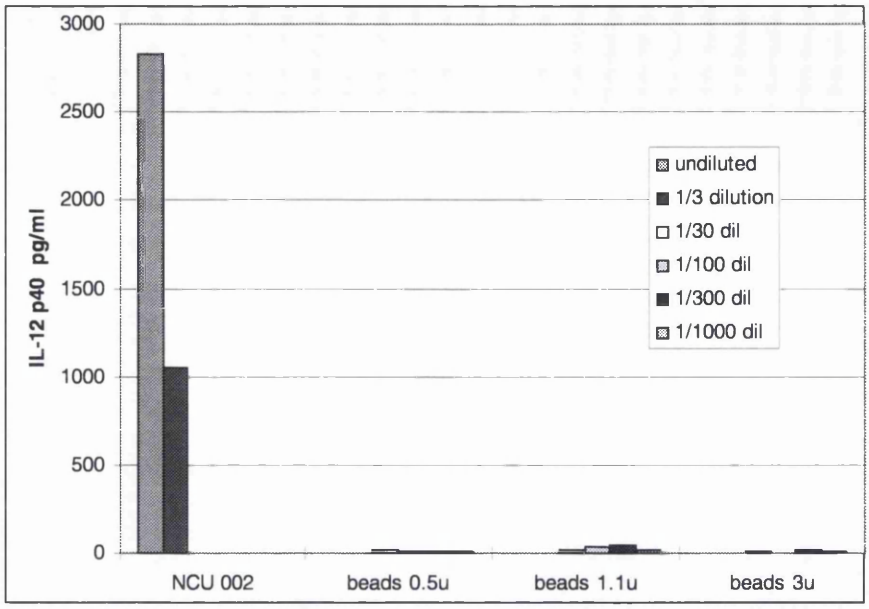


Figure 6-3: No IL-12 was produced in response to polystyrene beads

ii) Cytochalasin D

Since phagocytosis alone of inert beads was not sufficient for IL-12 production from THP-1, it was of interest to see whether phagocytosis of vaccine played a role. Cytochalasin D (Sigma C-8273) used at 5μg/ml final concentration can inhibit reorganisation of microfilaments, necessary for phagocytosis. Cytochalasin D had some inhibitory effects on IL-12 production when THP-1 were stimulated by vaccine (undiluted stock), especially in the presence of IFNγ (Figure 6-4), although the reduction was not complete (background was <50pg/ml). However, there was no effect on LPS-stimulated IL-12 production (LPS 20μg/ml stock). The experiment was repeated with several dilutions of NCU 002 with cytochalasin D at 10μg/ml (Figure 6-5). Again, vaccine activity at all dilutions was reduced, but not to background (<40pg/ml).

These findings may be explained. Adherence alone of gram-negative bacteria to macrophage surfaces can still induce some proinflammatory cytokines, such as TNF, IL-6 and IL-1 (Yamamoto 1994), thus adherence of vaccine particles or activity of soluble components in the supernatant may account for IL-12 induction by NCU 002. There is likely to be more than one pathway to IL-12 induction by the vaccine. The greater effect in the presence of IFNγ may be as a result of altered macrophage activation/phagocytosis states. Above 100ng/ml LPS activity becomes partially independent of CD14 and LPS-binding-protein (Troelstra 1997) and the reduced requirement for endocytosis may bypass cytochalasin D effects.

Thus vaccine activity is not simply due to particle phagocytosis; however, phagocytosis plays some part, possibly increasing the exposure of the THP-1 cells to antigens. Although IFNγ can up regulate IL-12 expression directly, it may also have indirect actions, such as increasing phagocytosis or the breakdown of phagocytosed bacilli.

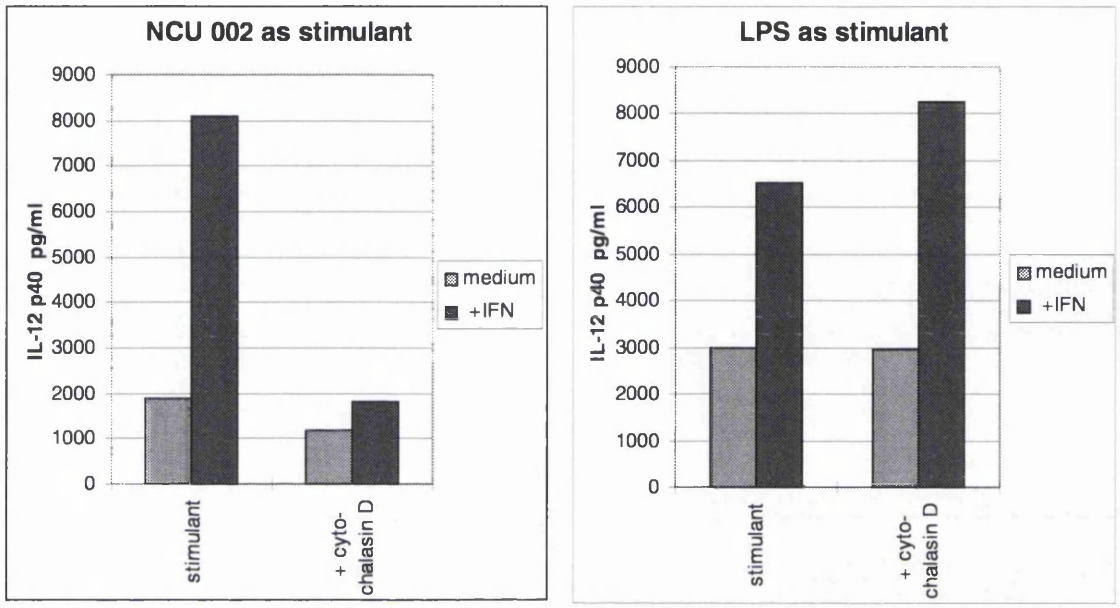


Figure 6-4: Effect of cytochalasin D on IL-12 induction by NCU002 and LPS

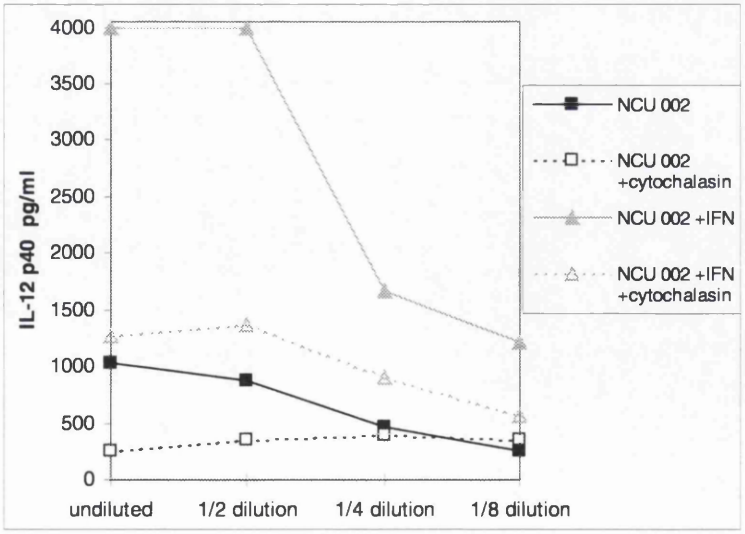


Figure 6-5: Effect of cytochalasin D and IFNγ on IL-12 induction by various doses of NCU002

6.2.2 Production by THP-1 of multiple cytokines and their interactions.

Use of *M. vaccae* vaccine *in vivo* shows induction of a Th2 response if the vaccine dose is too high. Similarly, an excessive dose in monkeys (> 5 x 10^8 killed mycobacteria) caused a Koch type reaction and no protection from a challenge with viable *M. tuberculosis* (J. Watson, discussion meeting). Thus the starting concentrations of soluble samples were increased (LPS 200μg/ml; sonicates 1mg/ml) and newer, more thoroughly disaggregated preparations of autoclaved mycobacteria were used. (These were called MVSA and MVTA and still had starting concentrations of 10mg wet weight per ml but were shown in an ELISA system (Prova) that detects antigen availability to be several times more potent than NCU 002).

6.2.2.1 Differential cytokine expression from THP-1 depends on the nature and quantity of the stimulus

DMSO-pretreated THP-1 were stimulated with various samples serially diluted across a very wide concentration range and used at 5% as already described. 18 hour supernatants were analysed for IL-12 p40, IL-10 and TNFα and results are shown (Figure 6-6). There was little background cytokine production.

Each preparation induced its own pattern of differential cytokine production. LPS and the sonicates induced dose-dependent production of IL-12, but little IL-10 or TNFα. Undiluted autoclaved BCG induced a high level of IL-12 (>10000pg/ml), moderate TNFα and little IL-10; responses showed a simple dose dependency. In contrast, the dose response curves of MVSA were complex. Undiluted MVSA had sharply reduced IL-12 production and elevated IL-10 and TNFα production, compared to the remaining dilutions. This was observed in several experiments and with other batches of 'highly-disrupted' autoclaved *M. vaccae* (more results are shown in a later chapter). This may be a direct effect of cytokine down-regulation by IL-10. Hence both the nature and the dose of the preparation can have profound consequences on final cytokine balance.

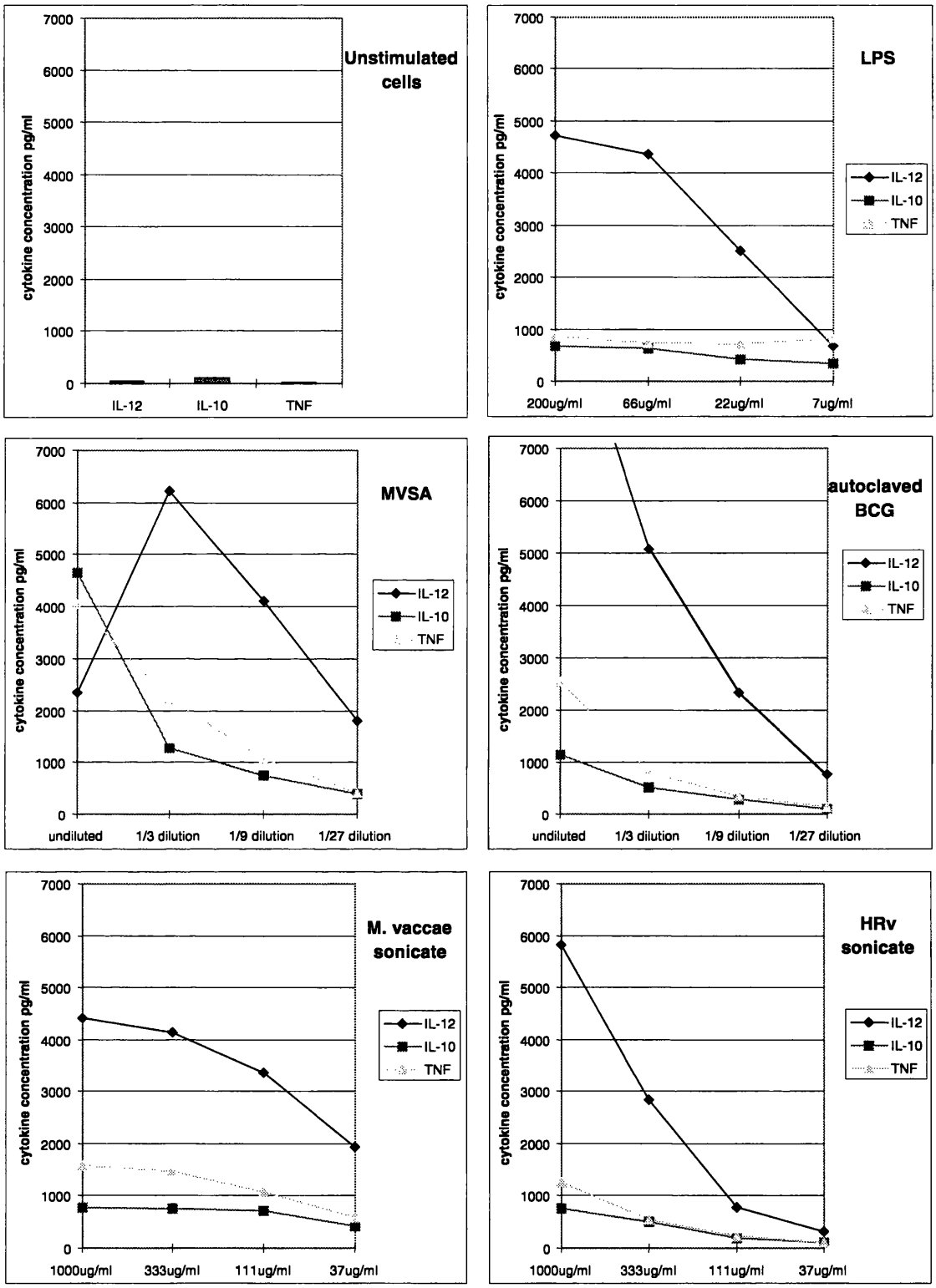


Figure 6-6: Differential Production of IL-12, IL-10 and TNF in response to various stimuli

6.2.2.2 Time course of cytokine production

A time course of cytokine production from stimulated THP-1 could clarify some cytokine relationships (Figure 6-7). Supernatants were collected after 7 or 24 hours incubation with 5% of serially diluted MVSA or LPS (starting 200μg/ml).

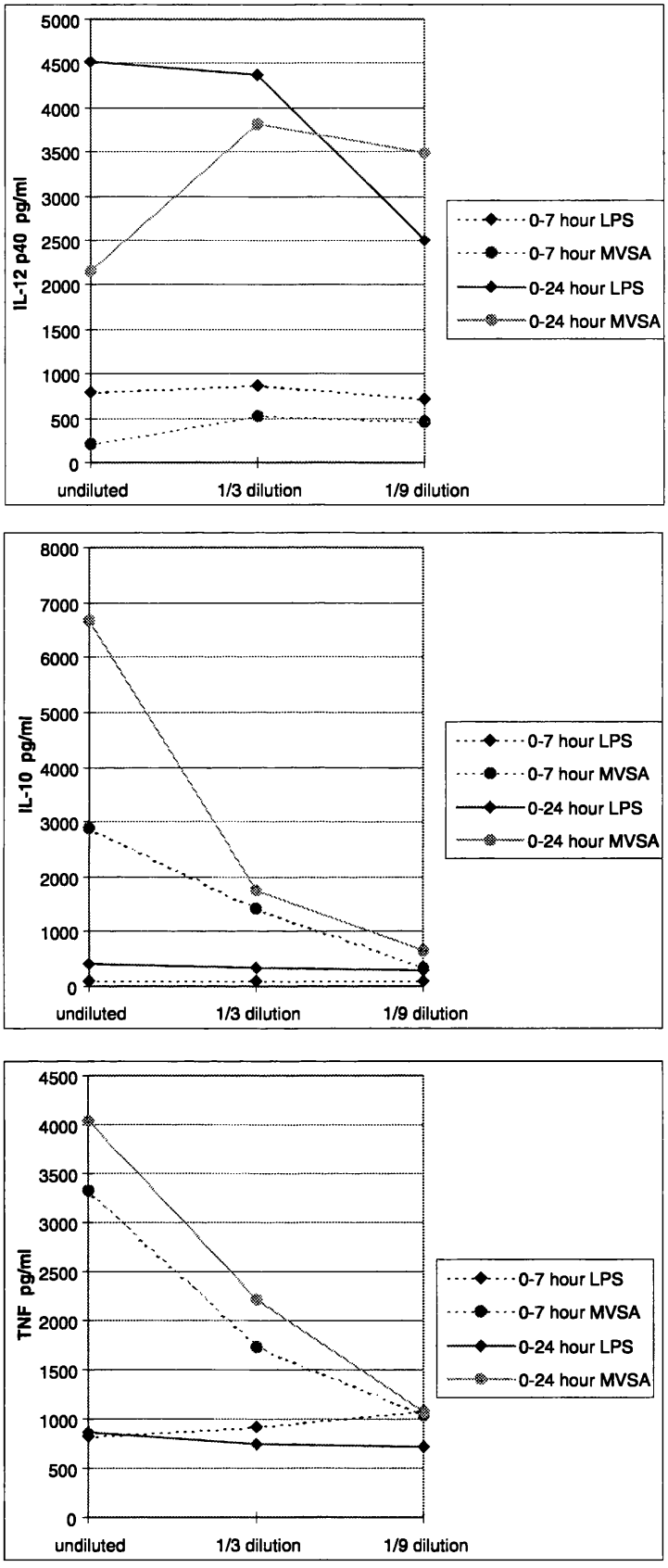


Figure 6-7: Time course of cytokine production after LPS or MVSA stimulation

By 7 hours, almost no IL-12 p40 is present in the supernatants, while production of IL-10 from MVSA stimulated THP-1 has begun. Levels of TNFα are almost maximal

by 7 hours which fits the pattern of release of preformed TNFα. TNFα may also induce IL-10 production in resting and stimulated macrophages (Daftarian 1996). Thus early IL-10 production may inhibit subsequent IL-12 production, although this was not proved using anti IL-10.

6.2.3 Effect of IFNγ on dose response relationships

Earlier data showed IFNγ at 1000U/ml enhanced levels of IL-12 to all stimuli. Therefore IFNγ was included in a duplicate set of wells as part of a previous experiment. Data are shown for LPS, MVSA and *M. vaccae* sonicate (Figure 6-8). IFNγ increased IL-12 p40 production and TNF production, and decreased IL-10 production, often to background. The IFNγ could overcome a very strong IL-10-inducing stimulus (undiluted MVSA) to switch the dominant cytokine to IL-12. This may be relevant to the presence *in vivo* of cells that rapidly produce IFNγ (e.g. memory CD8 T cells).

To demonstrate this was not an artefact, similar observations were made in THP-1 cells without DMSO maturation, using undiluted standard vaccine (NCU 002). This vaccine formulation induces less IL-10 than MVSA, but IL-10 levels were still reduced further by IFNγ (Table 6-1).

IL-10 induced by:	without DMSO	with DMSO
NCU 002	426pg/ml	566pg/ml
NCU 002 + IFNγ	93pg/ml	136pg/ml

Table 6-1 : Down regulation of IL-10 production by IFNγ

Supernatants from MVSA stimulated THP-1 were tested for the presence of both IL-12 p40 and p70, in case the dose related switching off of IL-12 p40 was not carried through to the active heterodimer. Although there was about 300 times more p40 produced than p70, both p40 and p70 were reduced in response to undiluted MVSA (which induces IL-10) compared to the 1/3 dilution of MVSA (Table 6-2).

MVSA:	undiluted	1/3 dilution
IL-12 p40	2163pg/ml	3810pg/ml
IL-12 p70	7.9pg/ml	11.5pg/ml

Table 6-2 : IL-12p70 is down regulated coordinately with p40

Thus the presence of IFNγ may act as a "switch" and looking at cell lines without inclusion of relevant factors from other cells may produce a biased result.

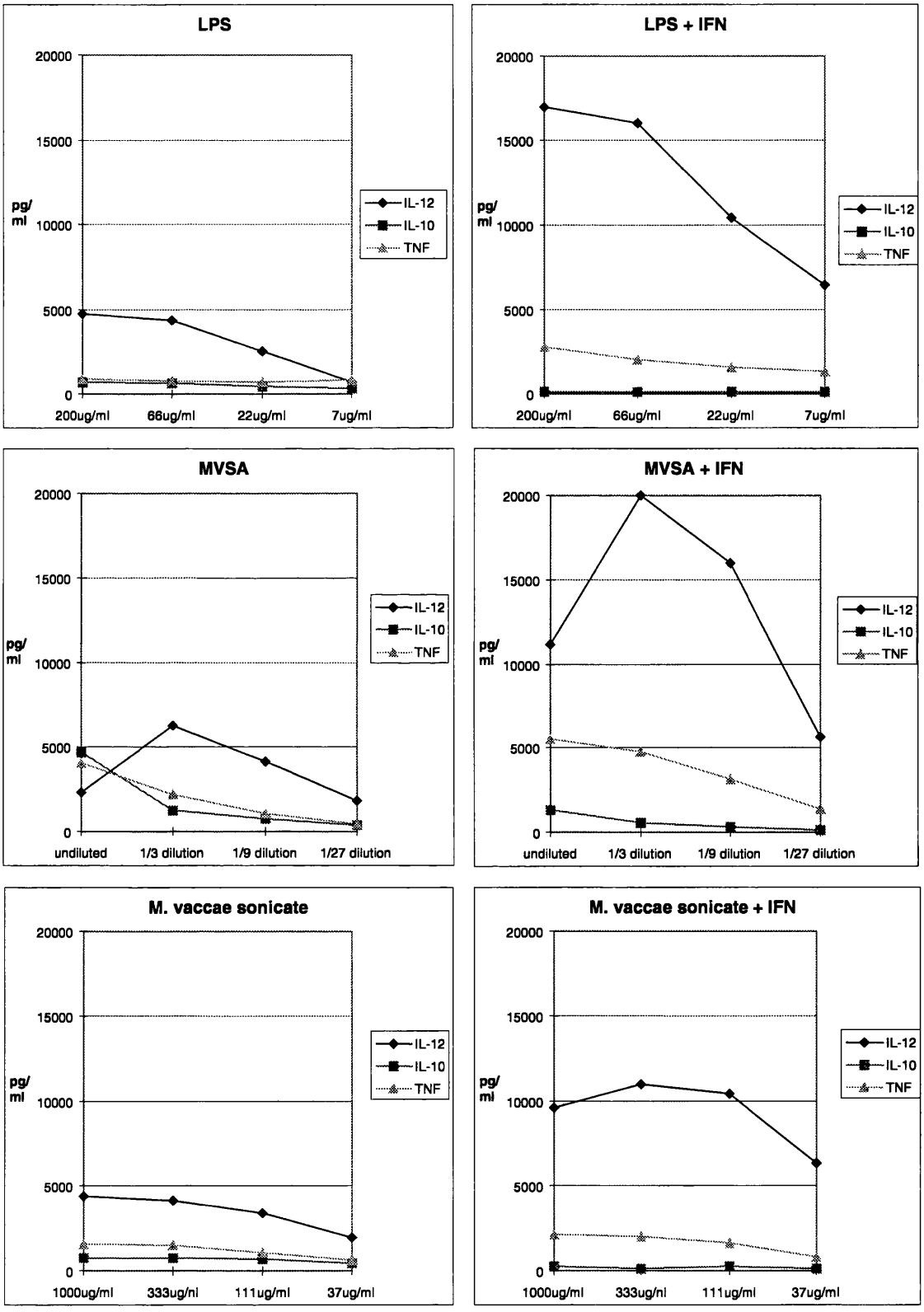


Figure 6-8 : Effect of IFNgamma on IL-12, IL-10 and TNF production in response to various stimuli

6.2.4 Effects of dose on THP-1 viability

The probability of a relationship between cytokine balance and THP-1 viability and proliferation could be tested in a tritiated thymidine incorporation assay. DMSO pretreated THP-1 were incubated for 24 hours with various samples and proliferating cells allowed to incorporate the radiolabel. Samples were 5% of the following stocks: LPS 20μg/ml, MVSA vaccine undiluted, *M. vaccae* sonicate 400μg/ml and 1.1μm beads 1/30 (Figure 6-9). There was no loss of viability in cells incubated with beads or LPS, but both high dose MVSA and sonicate had significantly reduced counts. This pattern suggested that production of high levels of TNF (produced in response to MVSA or sonicate) resulted at least in part in reduced counts in cells stimulated with mycobacterial preparations.

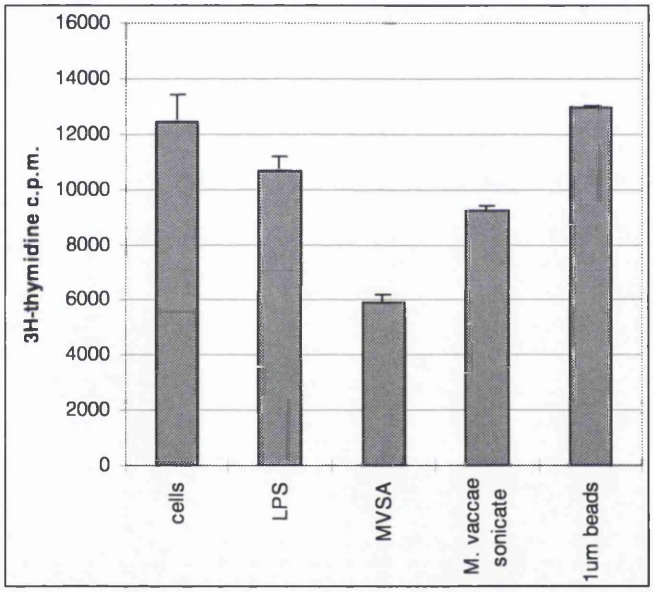


Figure 6-9: Thymidine incorporation by THP-1 during 24 hour incubation with samples

However, IL-10 has also been implicated in apoptosis. Kremer (Kremer 1997) showed that infection of monocytes with live or killed BCG prevented the cells from undergoing apoptosis. BCG infection impaired the ability of these monocytes to produce IL-10 and rendered them resistant to IL-10-induced apoptosis. When tritiated thymidine uptake by THP-1 was compared between autoclaved BCG or *M. vaccae*, BCG was always less toxic (not shown); similarly, autoclaved BCG had previously induced high levels of IL-12 and some TNFα but little IL-10, which is in agreement with Kremer's findings.

Thus an experiment was performed to determine which cytokine played the key role when *M. vaccae* was used. At high doses, MVSA has been shown to induce high IL-10

production, but if IFNγ is included the IL-10 is reduced and TNFα is dominant. A dilution series of MVSA with and without IFNγ on THP-1 was tested for thymidine incorporation during a 24 hour period and compared to a similar experiment measuring TNFα production in 18 hour supernatants (Figure 6-10). A clear inverse relationship is achieved between counts and TNF levels, which depends on MVSA concentration and the presence of IFNγ, indicating that TNF production probably contributes to reduced thymidine uptake. This could have been tested with a neutralising anti-TNF antibody.

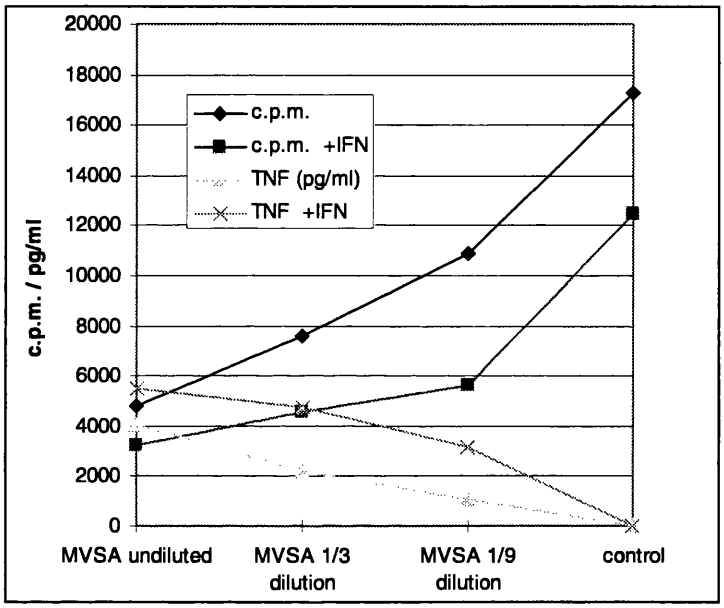


Figure 6-10: Inverse relationship between endogenous TNF concentration and thymidine incorporation after MVSA stimulation

To attempt to define whether reduced thymidine uptake was due to cell death or simply stasis, viable cell counts were performed using trypan blue exclusion after a 22 hour THP-1 culture with MVSA and/or IFNγ. The graph (Figure 6-11) shows total number of cells in the central squares of the haemocytometer (starting counts would have been 55) and the percent of those cells that were dead. Proliferation had occurred maximally in THP-1 without MVSA or IFNγ, but was slightly reduced upon addition of IFNγ or low dose MVSA; the former may have been due to THP-1 becoming more differentiated and the latter due to some cell death. Low dose MVSA with IFNγ or high dose MVSA more noticeably limited proliferation. Cell death increased as soon as MVSA was included, becoming much more pronounced with the addition of IFNγ or higher dose MVSA. Therefore it appears that both cytostasis and cytotoxicity play a role in reducing the viable cell counts.

Thus a complex balance of cytokine production by THP-1 may be reached, depending on the quality and quantity of *M. vaccae* stimulation. Addition of IFNγ enhances IL-12 production by THP-1, possibly by reducing the inhibitory effect of IL-10, but subsequently the IFNγ-stimulated IL-12 production is curtailed by reduction in cell number/viability probably correlating with enhanced TNF release.

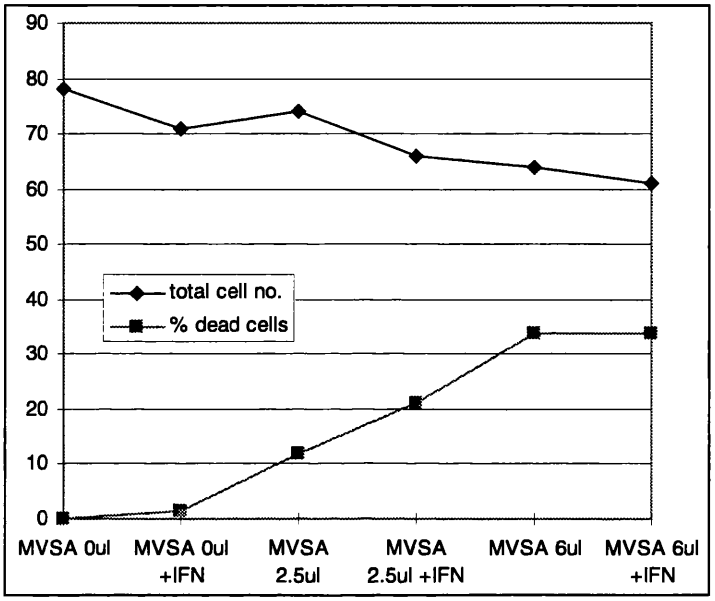


Figure 6-11: Trypan blue estimation of THP-1 cell number and viability after incubation with MVSA

6.2.5 Effect of human plasma on IL-12 production

The THP-1 work was carried out in FCS, but recent papers (Sutterwala 1997) indicate that ligation of macrophage Fcγ, complement or scavenger receptors inhibits induction of IL-12 (but not IL-10 or TNF) by LPS. Consequently there could be added complexity *in vivo*, where antibody responses to pathogens may play a role. Could specific antibodies in human plasma alter the activity of the vaccine? When 5% plasma from a BCG immunised donor was included in a single experiment, some reduction was seen in IL-12 response to *M. vaccae* sonicate (starting at 400μg/ml) but not to whole vaccine (Figure 6-12). There is no proof that the effect was due to antibody. This was not pursued further because there was no effect on NCU 002, which is the material used *in vivo*. However this may need consideration in work with peripheral blood monocytes.

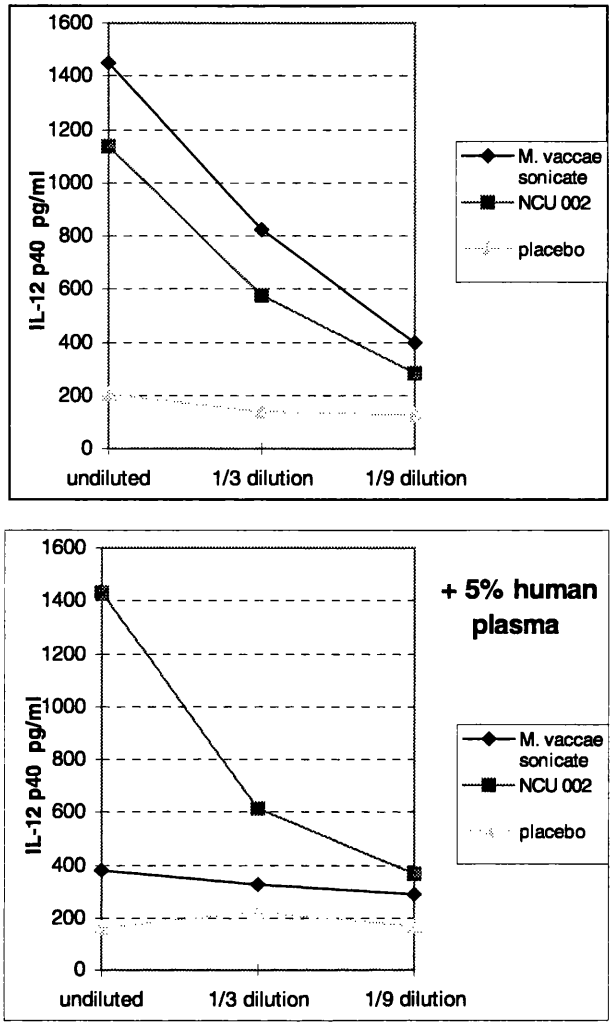


Figure 6-12 : Human plasma may alter IL-12 production

6.2.6 Verification of the THP-1 observations: Cytokine production in stimulated normal monocytes

6.2.6.1 Preliminary experiment

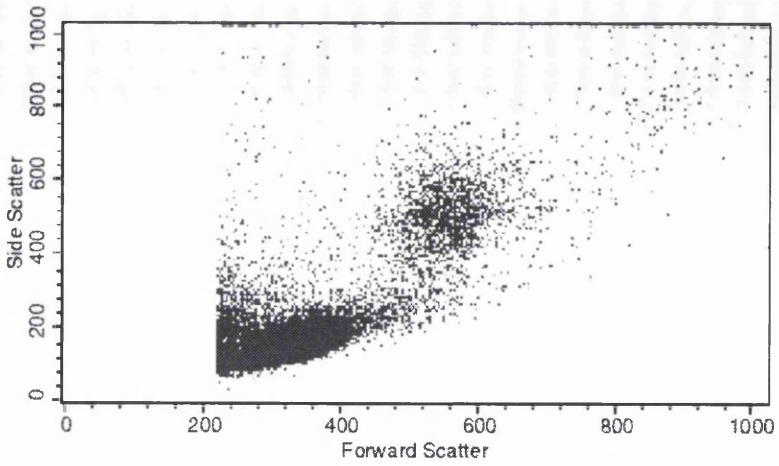
An initial experiment using 2 hour adherent macrophages was performed in 20% FCS to exclude any activity of serum cytokines or antibodies from the donor. This showed peripheral blood monocytes were extremely susceptible to death when exposed to higher doses (undiluted to 1/10 dilution) of whole mycobacteria, although they showed good survival when activated by soluble sonicated mycobacteria. This led to low and unreliable results for cytokine production by whole mycobacteria (not shown) as death may have occurred very rapidly before much cytokine accumulation had occurred. Control monocytes had an unstimulated production of IL-10 at 10000pg/ml, which was increased further by mycobacterial sonicates. Conversely, IL-12 production reached barely two fold over the high unstimulated background of 400pg/ml, although IFNγ increased IL-12 and TNFα as expected.

A subsequent experiment was performed using less MVSA and autologous plasma to remove non-specific stimuli. Additionally the macrophages were adhered overnight, which may allow them to mature slightly.

6.2.6.2 The cell population after overnight adherence

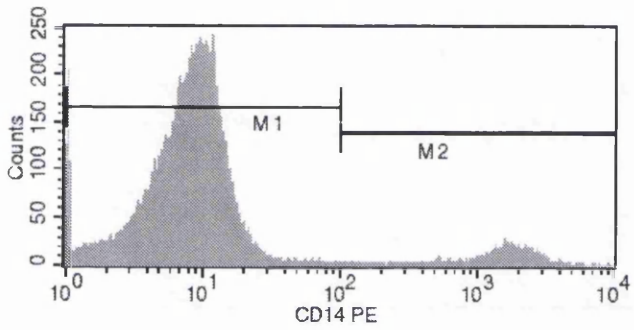
Analysis by flow cytometry showed 68% of the adherent cell population to be CD14 positive (Figure 6-13). These were about half of the total monocyte population present (around 10%) in the blood sample, so some CD14 cells were not collected. Thus not all peripheral blood monocytes were at identical stages of differentiation; variations could include phagocytosis levels or readiness to secrete cytokines. Also, 10^5cells/200µl was 4 fold less cells per ml than THP-1, so less particulate antigens should be added per ml. Monocyte cultures started as single, adherent non-confluent monolayer. After 20 hour stimulation, many had formed clumps, indicating that activation had occurred.

Non-adherent cells from PBMC population

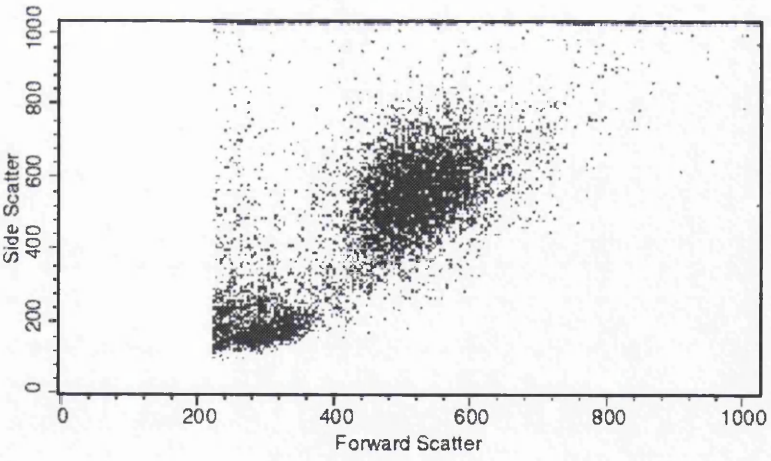


Histogram Statistics

Marker	Events	% Gated
All	31687	100.00
M1	29766	93.94
M2	1923	6.07



Plastic adherent cells from PBMC population



Histogram Statistics

Marker	Events	% Gated
All	8646	100.00
M1	2754	31.85
M2	5896	68.19

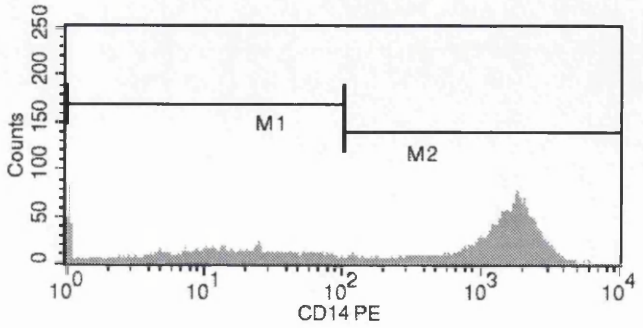


Figure 6-13 : The PBMC population after overnight adhernce

6.2.6.3 Stimulation of cytokine production by monocytes in autologous plasma

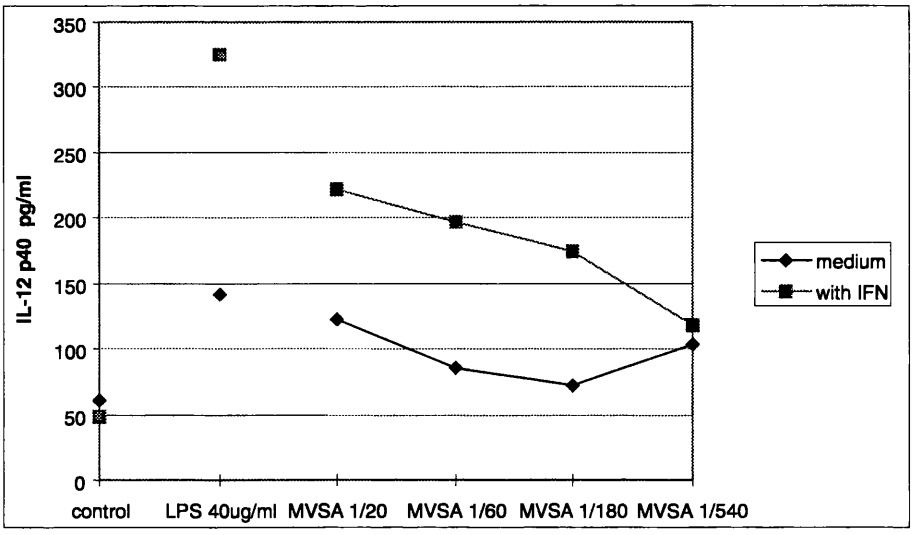
The monocyte stimulation was performed using 20% autologous plasma. Cell survival (estimated by adherent cell staining by crystal violet) in all cases was good (not shown). Cytokine levels in the plasma itself were undetectable. The results are shown in Figure 6-14.

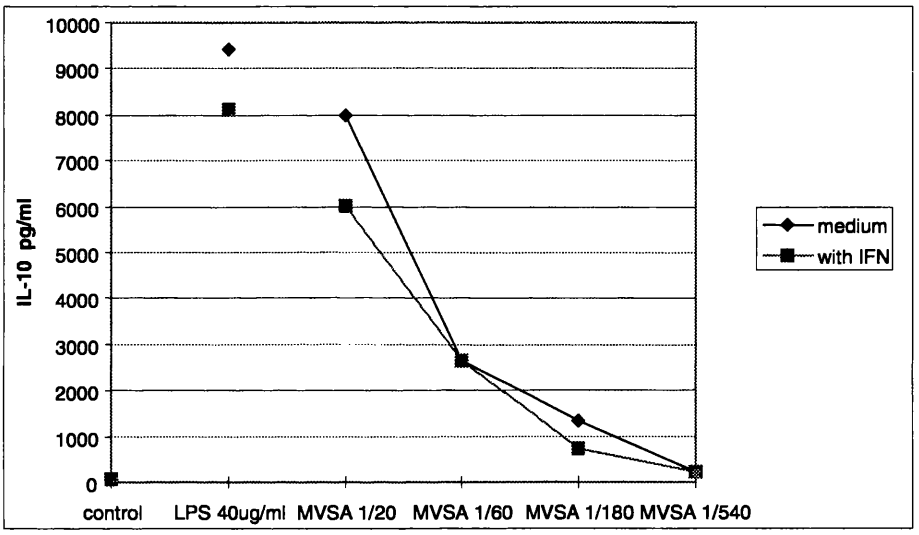
Unstimulated cells produced low levels of cytokines. LPS stimulation was included for comparison and generally MVSA induced cytokine production to a similar order of magnitude to LPS. IL-12 production by MVSA was dose related, but relatively low without the enhancement of IFNγ. High levels of IL-10 were produced, but were only reduced marginally by IFNγ. In this system, IL-10 may not have such a strong influence over IL-12 induction as in THP-1 cells. It was not demonstrated that the IL-10 was produced solely by monocytes, a possible contribution coming from contaminating cells.

Very high levels of TNFα were induced by treatment with MVSA and IFNγ although cell survival was good. It is possible that cell death in response to endogenously produced TNFα occurred in the previous experiment where higher MVSA doses were used.

With and without IFNγ, a 1/10 dilution of autoclaved BCG induced similar levels of IL-12 and TNFα to the 1/20 diluted MVSA, but about 3 fold less IL-10. Also mycobacterial sonicates (200μg/ml stock) induced similar levels of IL-12 and at least 3 fold lower IL-10 and TNFα than MVSA (not shown). In this experiment, IL-12 responses to whole mycobacteria were probably not due to phagocytosis alone (unlike Fulton 1996); also human serum did not block responses to sonicate.

In summary autoclaved *M. vaccae* could induce a dose-dependent production of IL-12 and other cytokines in this experiment using normal human monocytes. Other mycobacterial stimuli could induce different patterns of monocyte responsiveness. This work was not repeated using other donors, as its main point had been to establish IL-12 induction by *M. vaccae* products in normal cells, not just THP-1. Although results (such as IL-12 to IL-10 balance) were not identical to THP-1, variations may be due to the differentiation state of the monocytes; *in vivo* at an inflammatory site, monocytes leave the blood and mature into macrophages or dendritic cells, so peripheral blood monocytes may not be an appropriate model.





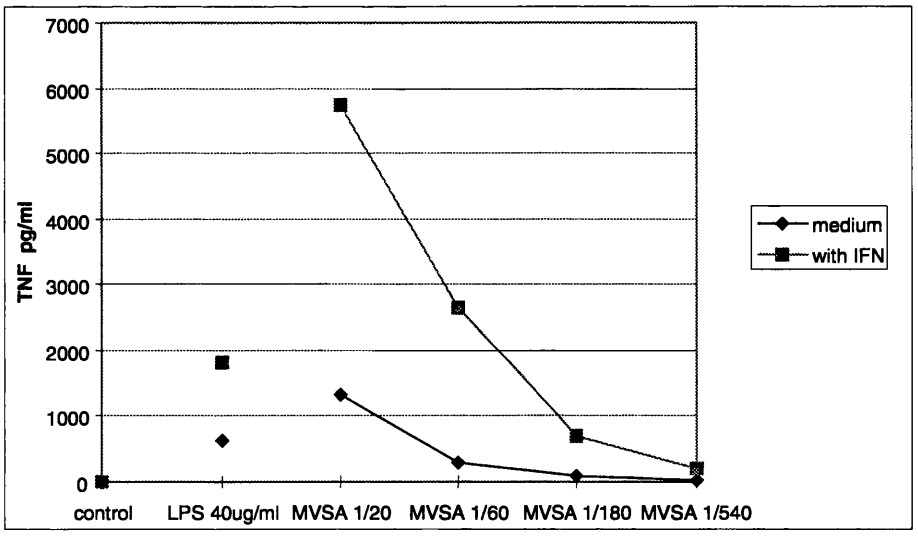


Figure 6-14: Cytokine production in 24 hour stimulated human monocytes

6.2.7 Verification of the THP-1 observations: Cytokine production in a simple mouse model

Heinzel (1994), using 100-300µg of *S. enteriditis* LPS intraperitoneally in C57bl/6 mice achieved peak IL-12 p70 in sera of around 0.38ng/ml after 2-4 hours, while 15ng/ml of p40 was achieved at 4-6 hours post injection.

The first experiment tested 3 and 6 hour time points post injection, using two 6 week old mice per group. (Figure 6-15)

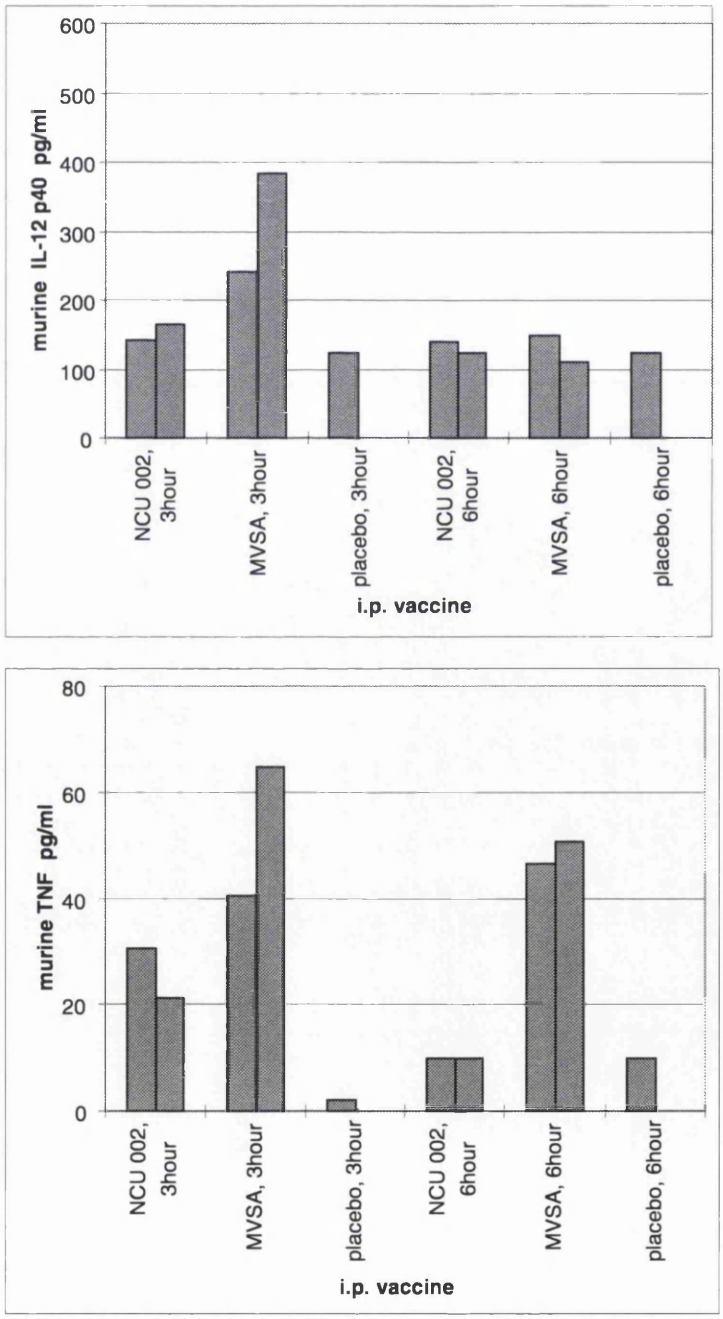


Figure 6-15: Time course of cytokine appearance in murine sera

Each bar represents serum from a single mouse, first for IL-12 and then in the same order for TNFα to allow comparison. Only the more disrupted preparation of autoclaved *M. vaccae* vaccine induced production of IL-12 p40 above normal levels in sera at 3 hours, and this declined by 6 hours. There was some inter-mouse variability. Both types of vaccine induced low levels of serum TNFα, which had peaked by 3 hours. Actually the peak serum TNFα may be as early as 1 hour post injection (for LPS), when pre-synthesised surface TNF is cleaved from macrophages. This peak may have been missed, and the TNFα may have been adsorbed out of the serum e.g. by responding cells or by soluble TNF-receptor.

A second experiment was designed using higher doses of vaccines. Sera and corresponding peritoneal washes were tested at 3 hours post injection (Figure 6-16).

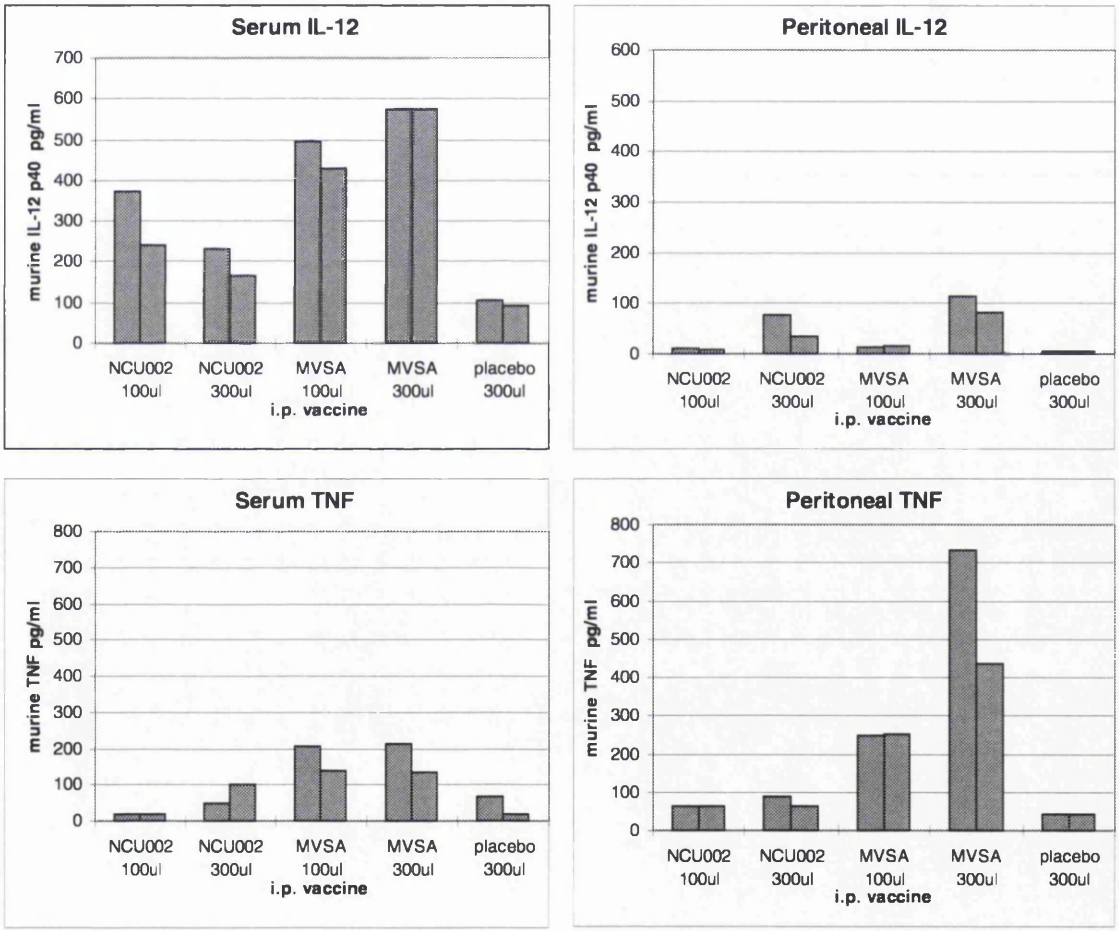


Figure 6-16: Local variations in murine cytokine production with dose and vaccine type

Each group contained two mice, which were 15 weeks old. Both vaccines induced serum IL-12 p40. The MVSA induced higher levels than the NCU 002, at whichever doses were compared. Hence in these mice, a saturating dose has been achieved for each vaccine. This may relate to the presentation; MVSA mostly presents as small clumps

and single or broken bacteria, with activity in the supernatant, while NCU 002 contains many larger clumps. This may limit uptake of NCU 002 in the very short term and form instead a depot of vaccine, or perhaps the mode of phagocytosis and treatment within the phagolysosome is different. Comparatively little IL-12 was detected in the peritoneal washes, even at the highest doses. Perhaps some of the activated macrophages had migrated away or the IL-12 may have been used by other cells in the peritoneum.

Background TNFα levels were higher in this assay, possibly because the mice were older or had lived for many more weeks in the animal house (note the scale is 10 fold more than the first experiment). Only mice given MVSA still had significant amounts of serum TNFα at 3 hours and in the case of 300μl of MVSA more was present in peritoneal washes than in serum. The peritoneal TNFα may have been released before macrophages migrated to the blood or be due to a new wave of synthesis and release of TNF, possibly even from a different cell source to the IL-12 (e.g. neutrophils are attracted to the peritoneal cavity by TNFα from macrophages and can themselves secrete TNFα upon stimulation); the extended presence of TNFα may cause harmful toxicity in the longer term. It should be noted that this highly disrupted form of vaccine is not used in humans, and a 300μl dose is 'enormous', but was used simply to elicit a clearly visible, quick read-out. Excess IL-12 or TNF in a response, without down-modulation by IL-10, can be dangerous, although this was not obvious during this short time course experiment.

In vivo in a mouse model, vaccines based on autoclaved *M. vaccae* do induce serum IL-12, although only in limited amounts when compared to LPS (Heinzel 1994). Further experiments using a greater range of vaccine dilutions, alternative sites of injection and measurements of additional cytokine were considered of low priority.

6.3 Conclusions

The cytokine production from THP-1 stimulated with LPS under various conditions correlated well with results from Kubin (1994), including the effects of DMSO maturation, and addition of exogenous cytokines such as IFNγ. Autoclaved *M. vaccae* was able to induce significant production of IL-12 (p40 and p70) and TNFα from the THP-1 human macrophage cell line. Addition of cytochalasin D partially inhibited this induction. However, phagocytosis of inert beads did not stimulate cytokine production

while soluble sonicates of mycobacteria did, so phagocytosis probably served to increase the exposure of the macrophages to *M. vaccae* products.

Importantly, high doses of some dead *M. vaccae* preparations (e.g. MVSA which does not meet standards for use in humans) were also capable of inducing early production of endogenous IL-10. This may have had regulatory effects upon the IL-12 production. Reduction in THP-1 viability by some *M. vaccae* preparations within 24 hours, possibly related to TNFα concentration, may also be expected to influence cytokine production.

Thus the cytokine balance was extremely delicate, even in this simple monoculture system, and the dose and formulation of the mycobacterial products influenced this balance. Addition of IFNγ to the system enhanced IL-12 and TNFα production, whilst inhibiting IL-10 production and this effect may be particularly important *in vivo*.

Limited results from normal peripheral blood monocytes generally supported the THP-1 findings.

Autoclaved *M. vaccae* given into the peritoneal cavity of mice was shown to induce rapid IL-12 and TNFα production *in vivo*. The IL-12 levels were not as high as may have been predicted from the THP-1 results, and the highest IL-12 p40 production was achieved with large doses of *M. vaccae* preparations which in THP-1 cells had instead induced IL-10. This demonstrates the difficulty in relating the doses used per macrophage *in vitro* to those that might be achieved *in vivo* or to predict the *in vivo* outcome of exposure to these preparations solely from THP-1 results. Outcome may depend on the differentiation state of the cells, the diffusion/uptake rate of the cytokines in blood or the peritoneal cavity and the interplay of other cytokines and chemokines.

When given to humans intradermally, local production of IL-12 may help *M. vaccae* vaccines to act as Th1 adjuvants for a variety of antigens, both specific and shared with other organisms. However, from THP-1 data, IL-12 induction alone probably does not represent the unique adjuvanticity of dead *M. vaccae* as dead BCG and mycobacterial sonicates were active in this respect. Interestingly, each stimulatory preparation induced a different pattern of cytokine induction from THP-1; these preparations may also stimulate different responses *in vivo*. Repeated treatment with LPS can cause endotoxic shock, soluble antigens of tuberculosis are not effective without adjuvants, dead BCG vaccine is inefficient compared to live BCG at induction of protective immunity against mycobacterial diseases and yet autoclaved *M. vaccae* may modulate an ineffective immune response against mycobacterial diseases, allergies and cancers towards a

protective one. Furthermore *M. vaccae* can be given to humans repeatedly without serious local side-effects.

Possibly the regulatory interplay of cytokines seen during stimulation of THP-1 with *M. vaccae* is also important *in vivo*, although it is not proven that the dose of bacilli used per cell is applicable in both cases. IL-10 has an important role in preventing pathology from IL-12-induced excess IFNγ, although conversely IL-10 may down regulate type 1 responses and possibly exacerbate diseases; however, if some IFNγ is present (e.g. from rapid responses of lymphocytes and NK cells responding to a challenge), subsequent IL-12 production from macrophages and consequently type 1 immunity could be boosted.

6.4 Discussion

Results of *M. vaccae* stimulation of cytokine production from THP-1 and human monocytes are in broad agreement with some other systems studied, which are illustrated here.

Human herpervirus-6 infection of THP-1 induced the expression and induction of IL-12 and IL-10 and kinetic study showed expression of IL-12 mRNA decreased with accumulation of IL-10. Addition of IFNγ enhanced expression of the IL-12 gene and down-regulated IL-10 mRNA accumulation, which suggests the IFNγ regulation was at the transcriptional level. Also IL-12 was enhanced by addition of anti-IL-10, so it was concluded that endogenous IL-10 production inhibited IL-12 production (Li 1997).

Using mycobacteria, IFNγ differentially up-regulated IL-12 and down-regulated IL-10 production in response to *M. leprae* stimulation of macrophages (Libraty 1997). *M. tuberculosis* infected human monocyte-derived macrophages produced high levels of IL-10, some IL-12 p40 and minimal IL-12 p70, unless IFNγ was added, in which case both forms of IL-12 were produced in greater quantitiy than the IL-10 (Bonecini-Almeida 1998). This agrees with my cytokine production from monocytes stimulated with *M. vaccae*.

Whereas LPS induced little IL-10 from my THP-1 even at high concentrations, it did induce IL-10 from the normal monocytes, which is in agreement with others (Malefyt 1991). The reason for this is unknown.

An IL-10-induced reduction in IL-12 production by macrophages may play a role in disease. For example, *M. tuberculosis*-induced IFNγ was reduced in PBMCs from tuberculosis patients and anti-IL-10 could enhance the IFNγ production (Gong 1996).

HIV-1 infection of macrophages induces IL-10 with loss of IL-12, and a failure to support T cell proliferation within a week (Yoo 1996). This may be due to gp120 activity (Taoufik 1997). Altered macrophage function may contribute to global suppresion in HIV infection e.g. HIV PBMC produce higher levels of IL-10 to *M. avium* complex preparations than normal PBMC and HIV/AIDS patients have higher circulating IL-10, so elevated monocyte-derived IL-10 production may contribute to the high susceptibility to MAC infection seen in patients with advanced HIV disease (Muller 1998).

Death of macrophages is also an important consideration as some forms of macrophage death may be beneficial e.g. in control of intracellular mycobacteria (Lammas 1997) and in cross-presentation of extracellular antigens after apoptosis. The mode of *M. vaccae* induced THP-1 death (as seen by reduced cell number, reduced thymidine uptake and altered morphology) was not determined. IL-10, high doses of IFNγ or abnormal activation can cause macrophage death by apoptosis within 48 hours (Carracedo 1995 and Estaquier 1997). This situation may be complicated by the differentiation state of the macrophages and the influence of mycobacteria e.g. BCG infection can prevent apoptosis in resting human monocytes (Kremer 1997), while *M. tuberculosis* increases apoptosis of alveolar macrophages, via a TNFα dependent mechanism (Keane 1997). Live *M. tuberculosis* promotes apoptosis in murine macrophages while dead *M. tuberculosis* and some structural components inhibit it (Rojas 1997). Some mycobacteria induce sensitivity to the toxic effects of TNFα in other cell types (Filley 1991 and 1992).

In my studies, it would be useful to examine the influence of each cytokine over the other or on cell viability by inhibition with specific neutralising monoclonal antibodies.

As the formulation and dose of *M. vaccae* appear so crucial in influencing the macrophage responses (and T cell responses in other systems), this topic will be addressed next.

Chapter 7 Susceptibility of *M. vaccae* vaccines to physical or chemical treatments

7.1 Introduction

Mycobacteria belong to a group of Gram-positive bacteria containing GC-rich DNA, and they have a complex, multilayered cell wall and capsule structure (Brennan 1995). Outside the plasma membrane the basic mycobacterial wall skeleton consists of chains of repeating dimers of N-acetylglucosamine (NAG) and N-glycolylmuramic acid (NGM). These polysaccharide chains are cross-linked through a proportion of the tetrapeptide side-chains which are attached to the muramic acid residues.

10-12% of the muramic acids residues bear branched arabinogalactan side chains, which are esterified at their termini by mycolic acids. These key molecules are tightly packed side by side perpendicular to the cell surface and determine much of the physiology of the inner cell wall, including its impermeability and the lipophilic behaviour of the whole bacteria (Draper 1982). Total lipids represent about 10% of the cell dry weight in each mycobacterium.

Also associated with the wall are immunologically important molecules such as lipoarabinomannan; this consists of a phosphatidylinositol anchor, a long branched mannan core, and arabinan side chains, sometimes capped with mannoses (Brennan 1997), which affect its biological properties. LAM can have a role in phagocytosis and evoke certain macrophage cytokines such as TNFα (Zhang 1993) and IL-6 and -10, and can inhibit activation of T cells. Extractable outer cell wall lipids include lipooligosaccharides, phenolic glycolipids (e.g. mycosides) and glycopeptidolipids (e.g. surface antigens which define separate serovars of the MAIS group).

The cell wall also contains porin proteins to allow transport of hydrophilic solutes.

Interwoven but not covalently linked is the capsule. This variable outer layer contains constituents that have been 'exported' from the cell, mostly polysaccharides and lipoproteins which may be species specific. In old cultures, some degradation products of the cell wall may be present. Capsular material is easily shed into the culture filtrate e.g. by gentle mixing with glass beads, which disrupts clumped bacteria without affecting their integrity (Ortalo-Magne 1995).

Adjuvant properties exist in cell wall extracts e.g. peptidoglycans, whose minimal active structure is a muramyl dipeptide (Stewart-Tull 1983). Disruption of the cell skeleton with lysozyme which cleaves the glycosidic bond between C-1 of NGM and C-

4 of NAG releases breakdown products of peptidoglycan which have water-soluble adjuvant activity. Although lysozyme has limited direct effects upon whole *M. tuberculosis*, tubercle bacilli ingested by monocytes in the presence of large amounts of extracellular lysozyme could be converted intracellularly into spheroplast-like forms, possibly by co-operation with other enzymes (Lowrie 1983).

Extractable glycolipids such as trehalose dimycolate or 'cord factor' have also been incorporated into adjuvants as they can increase macrophage functions such as phagocytosis and TNF production. Human CD1 proteins mediate specific T cell recognition of bacterial lipid and glycolipid antigens, such as mycobacterial mycolic acids, mycolyl glycolipids, PIMs and LAM (Moody 1997).

Within the cytoplasm exist many immunomodulatory proteins, including mycobacterial 'stress proteins' that stimulate macrophages (Zhang 1993) and may play a role in immunotherapies (Srivastava 1998). Single stranded bacterial DNA is also now recognised as having adjuvant effects for Th1 (Chu 1997) and cytotoxic T cell responses (Lipford 1997). Unmethylated CpG dinucleotides, often in palindromic sequence contexts, are common in bacterial DNA but are under-represented and methylated in vertebrate DNA. Bacterial DNA stimulates macrophages to produce IL-12, IL-18 and IFNα (Roman 1997) and dendritic cells undergo maturation and activation to become APC and produce large amounts of type-1 cytokines (Sparwasser 1998). NK cells then produce large amounts of IFNγ. Mice respond to injected CpGs with rapid production of IL-12 and IFNγ and for a time become resistant to listeria challenge (Krieg 1998).

Thus mycobacteria contain many immunologically active entities and it was of interest to attempt to 'dissect' the *M. vaccae* vaccines using simple physical and chemical means, to identify components active in the induction of IL-12 from THP-1 cells.

7.2 Results

7.2.1 Enhancement of cytokine-inducing activity by physical disruption

7.2.1.1 Different methods of clump disruption before autoclaving *M. vaccae* suspensions

All vaccines were prepared by collecting *M. vaccae* from Sauton's agar medium into borate buffer, disruption of bacterial clumps in the suspension and dilution to a final concentration of 10mg starting weight of bacteria per ml of buffer, followed by autoclaving at 120°C for 30 minutes. However, the disruption may be gentle, such as

passing the suspension through a syringe or pipette several times (coded NCU), or more vigorous, such as passing it through a small hole at high pressure (coded MVSA and MVTA).

Microscopically, *M. vaccae* are rods (mycobacteria measure about 1-4μm by 0.3-0.6μm), which largely retain their acid-fast properties after autoclaving. If a sample of NCU 002 is Ziehl-Neelsen stained and 100 'colony forming units' counted, the following groupings are seen: 5 large clumps (containing possibly 50 mycobacteria), 30 small clumps (3-5 bacteria), 30 individual/paired bacteria and 35 bacteria which are no longer acid-fast (this may occur with age in culture, or they may have been damaged during processing). Thus about half the mycobacteria are available to macrophages, the remainder being buried inside the clumps.

More precise differences between the two disruption methods can be detected by flow cytometry. After a standard 1/10 dilution of vaccine, the number of particles collected in a fixed time (10 seconds) can be estimated. If size (FSC, Voltage E02, AmpGain 4.0) and granularity (SSC, Voltage 375, AmpGain 2.0) are plotted (e.g. for NCU 002 (Figure 7-1) and MVTA (Figure 7-2), the particles fall into two regions: R2, small with low granularity (smallest may pass through a 0.45μm filter) or R1, larger and more granular (probably single and clumps). The logarithmic scale allows a considerable range of size, and mycobacteria or clumps can pass through the laser beam in any orientation. A 1/100 dilution of standard 1.1μm latex beads was run for comparison (Figure 7-3).

The particle profiles of several *M. vaccae* preparations are summarised in Table 7-1.

Vaccine	Total events	% R1	% R2
NCU 002	30945	48	52
NCU 003	45525	55	45
MVSA	64230	39	61
MVTA	58230	30	70

Table 7-1: Summary of vaccine particle profiles

The result is that after vigorous disruption (MVSA and MVTA), there are more events in total, and the ratio of small to large clumps has increased. As the weight of bacilli per ml at the outset was identical in all preparations, this suggests that single large clumps have been broken down to smaller clumps, individual bacteria and debris.

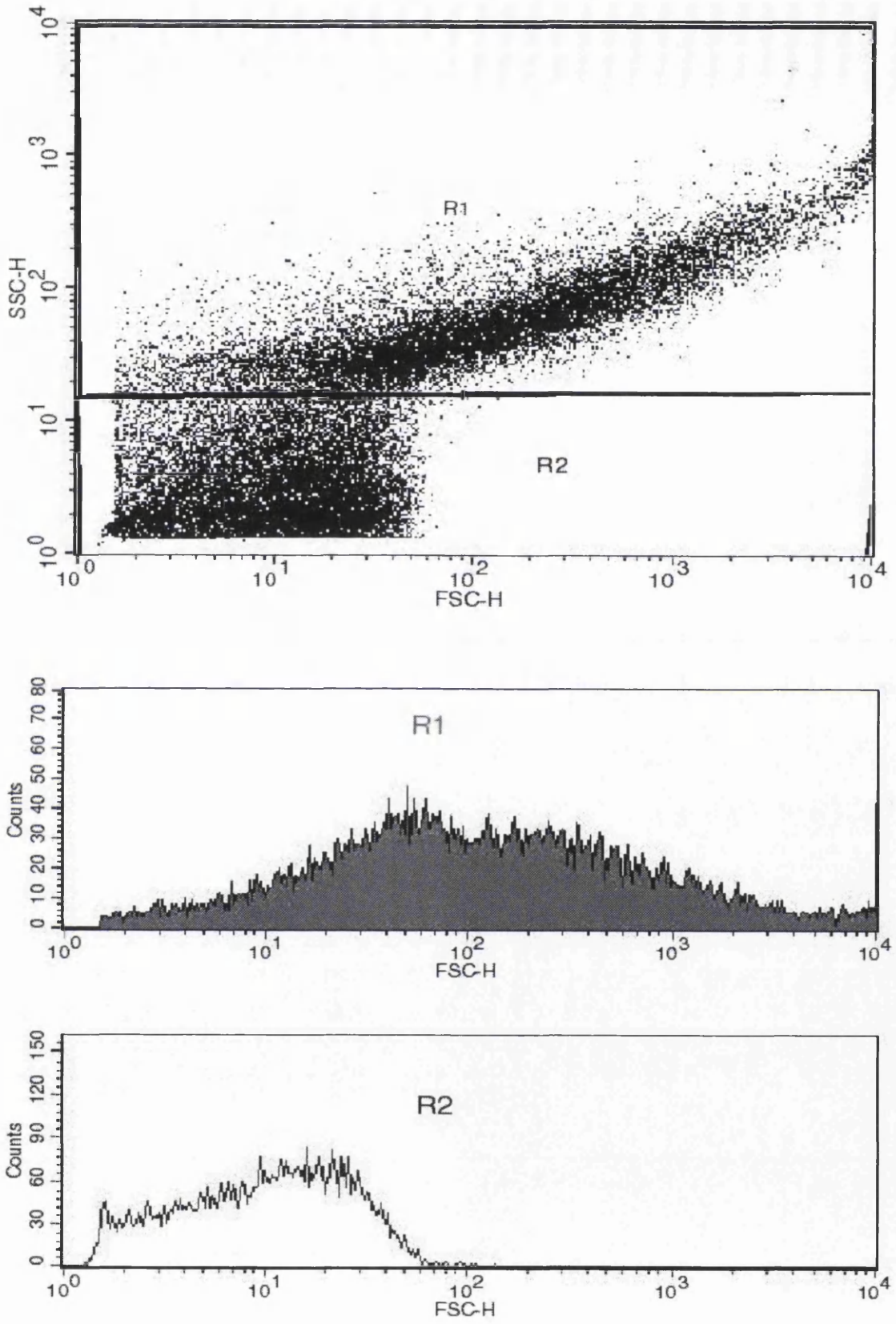


Figure 7-1: Particle profile of NCU 002 vaccine in regions R1 and R2

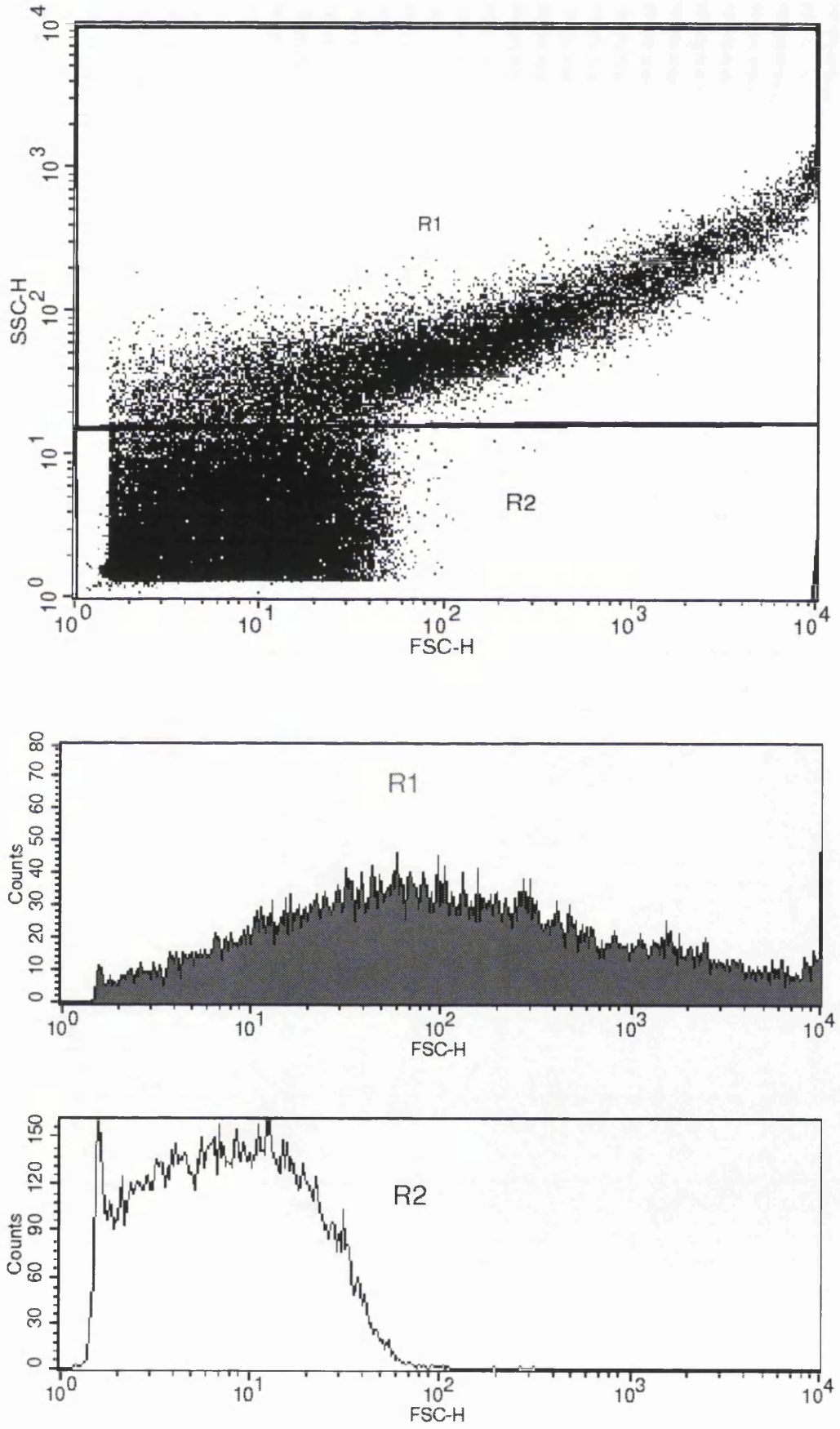


Figure 7-2: Particle profile of MVTA vaccine in regions R1 and R2

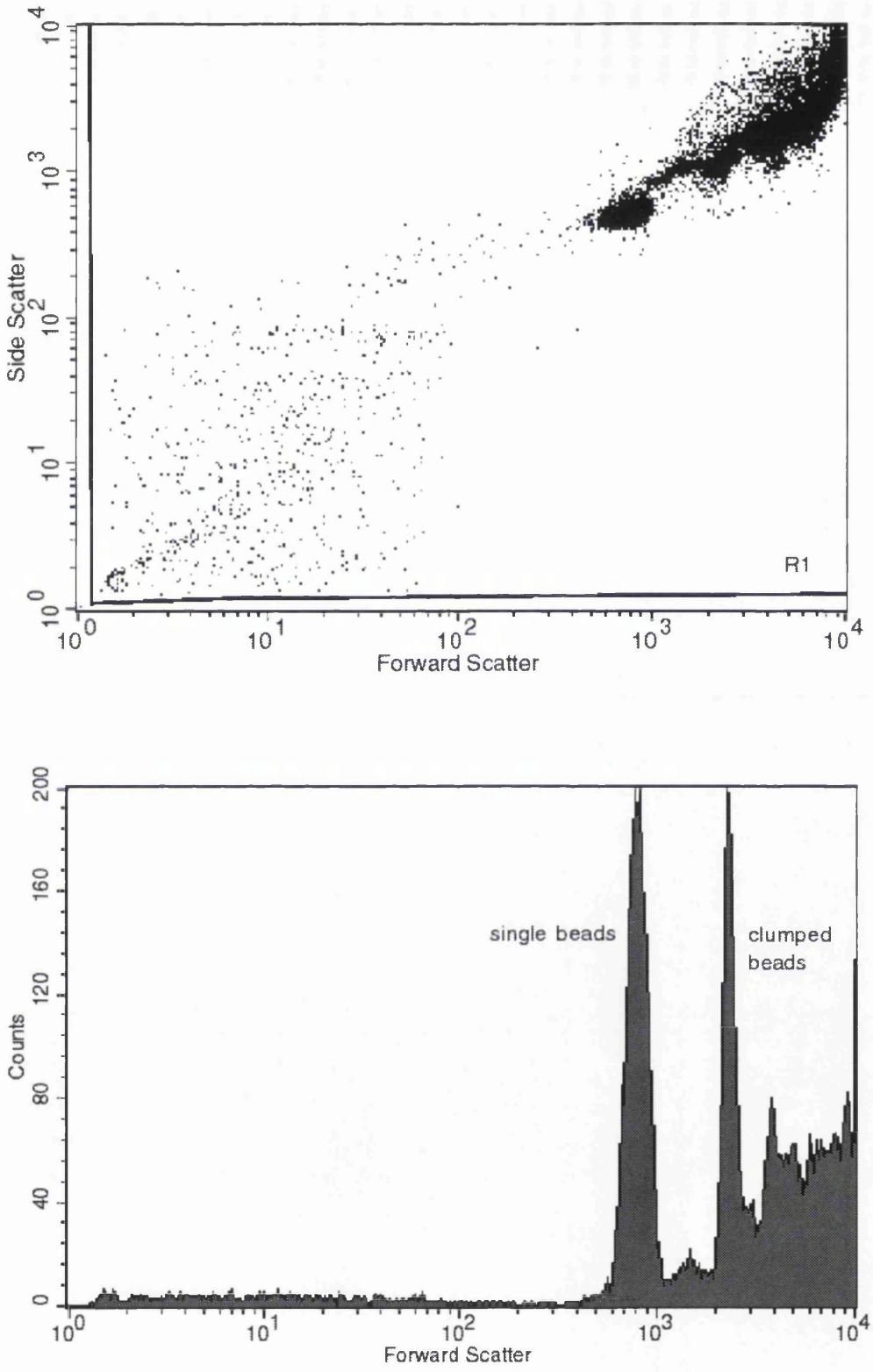


Figure 7-3: Particle profile of standard 1.1 μm latex beads

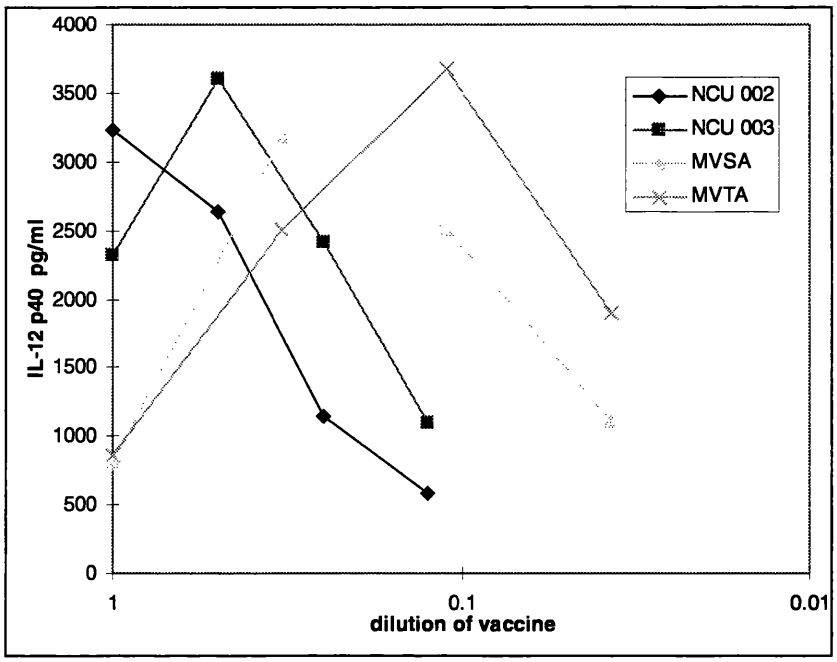


Figure 7-5: Physical characteristics of each vaccine determine the IL-12 induction

The overall cytokine-inducing potential increased with the increase in total events; this is seen as a continuation of IL-12 production further along the dilution series (Figure 7-5). However, the bell shape of some IL-12 curves suggests IL-10 production was also enhanced.

These changes may be due to increased accessibility of specific compounds associated with each bacterium (possibly different compounds for IL-12 and IL-10), or due to the availability of a larger number of phagocytosable particles per macrophage.

7.2.1.2 Sonication of prepared vaccine

Sonication of ready-made vaccine for 10 or 30 minutes progressively increased the cytokine induction from THP-1, probably by a similar mechanism to that described above (Figure 7-6). A small amount of activity was released into the supernatant.

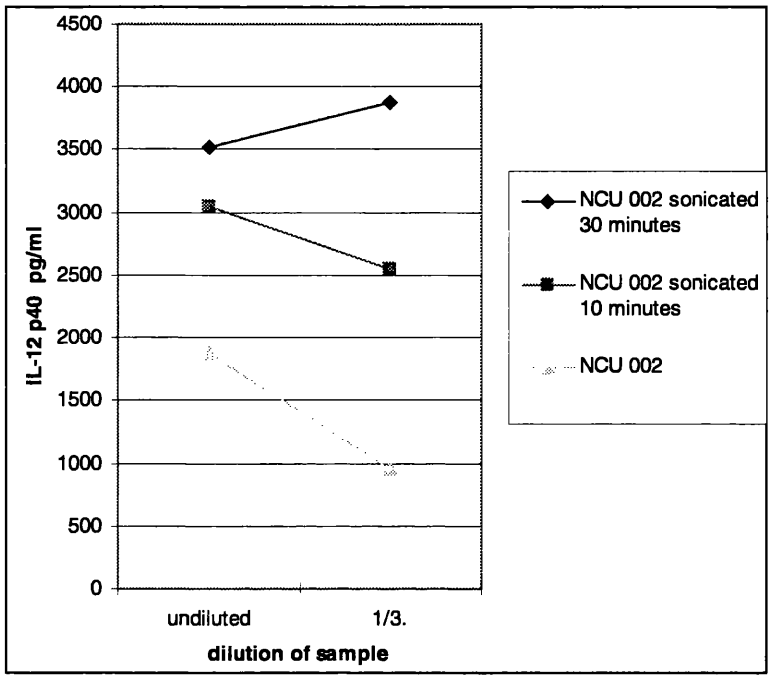


Figure 7-6: Sonication increases vaccine activity

Thus physical properties of the vaccine are extremely important. However, it was possible that by treatment with chemicals or specific enzymes, the biological activity may be assigned to a particular class of molecules. This may lead to changes in the vaccine formulation e.g. to isolate the Th1 and Th2 promoting effects.

7.2.2 Reduction of IL-12 inducing activity by hydrogen peroxide

An aliquot of NCU 003 was shaken for 20 minutes at room temperature in the dark with various concentrations of hydrogen peroxide. Then FCS was added (20%) and the aliquots spun at 11000g for 20 minutes. The pellet was washed once and resuspended in medium with FCS, ready for assay. By Ziehl-Neelsen, peroxide treatment caused the formation of large acid-fast clumps, while washed control samples showed reduced numbers of single bacteria but not increased clumpiness.

Brief exposure to 15% hydrogen peroxide can reduce the ability of NCU 003 to induce any cytokine from THP-1, although the effect is most obvious on IL-12 (Figure 7-7). This is not due to toxicity. As the concentration of peroxide is reduced, the effects decline. This appears to be a general non-specific denaturation of the vaccine. Reduction in activity may have been due to oxidation of active components or simple physical alteration.

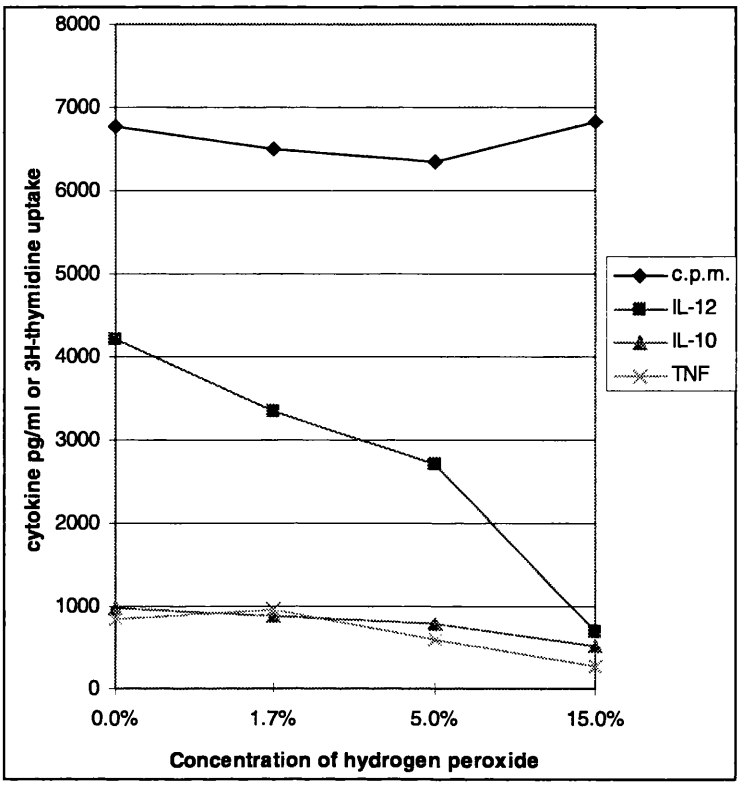


Figure 7-7: Dose dependent effect of hydrogen peroxide on NCU 003

7.2.3 Time dependence of deactivation by treatment with trifluoroacetic acid

This unusual acid was used due to its volatile nature, as treatment with hydrochloric acid and neutralisation with hydroxide left salt levels which were cytotoxic, but could not be washed away without loss of some of the hydrolysed vaccine pellet.

Briefly, NCU 002 was centrifuged (11000g, 10 minutes) and the pellet resuspended in an equivalent volume of distilled water. 3µl TFA (approximately 13M, Sigma T-1647) was added to all except one 400µl aliquot (to make 0.1M), then the glass vials were sealed and put into a heating block at 100°C for the following times: 30, 105, 300 and 1000 minutes. After heating, each vial was removed and frozen until all samples could be freeze-dried to remove the volatile acid. Dry samples were resuspended in 400µl of sterile PBS. A similar approach was used for LPS, prepared at 20µg/ml in distilled water.

Heating with TFA had a dramatic effect on mycobacterial morphology as seen by Ziehl-Neelsen staining: initially the bacilli exist as single or clumped acid fast organisms. After an hour small flocules were present with acid-fast flecks within them, and less free organisms were acid-fast. By 6 hours, the flocules were large and white,

with diffuse areas that stain in an acid-fast fashion. At 18 hours, only loose floccular debris remained. In theory (Prof. T Rademaker) TFA exposure for over 4 hours removes hexose sugars by 'nibbling' from the ends and with long treatment the effect would be complete hydrolysis.

NCU002 activity mainly resides in the pellet, and little is lost when the supernatant is removed. The effect of TFA treatment on the ability of NCU 002 and LPS to induce IL-12 production by THP-1 was dependent on the length of incubation of the sample with TFA at 100°C (see Figure 7-8). For NCU 002 between 30 and 105 minutes, there may be activation of a compound, an 'unmasking' of an active ingredient, or perhaps the TFA simply loosens inaccessible clumps of bacteria. By 6 hours, the activity is reduced, possibly by an effect on sugar-containing molecules, or more general hydrolysis. Overnight heating with TFA destroys the IL-12 inducing activity of NCU 002.

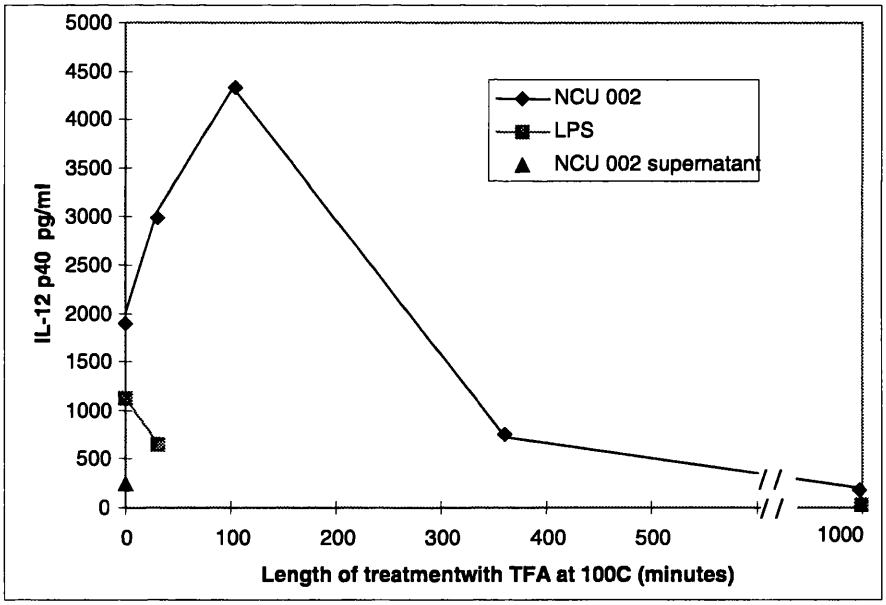


Figure 7-8: Effect of TFA on NCU 002 is time dependent

By contrast, TFA rapidly destroys the activity of LPS; this may be due to the presence of sugars in LPS (which consists of three regions: lipid A, a core oligosaccharide and an O side chain).

Loss of IL-12 induction was not due to toxic effects of samples (Figure 7-9). After 18 hours TFA reduced the levels of cell death and IL-12 induction.

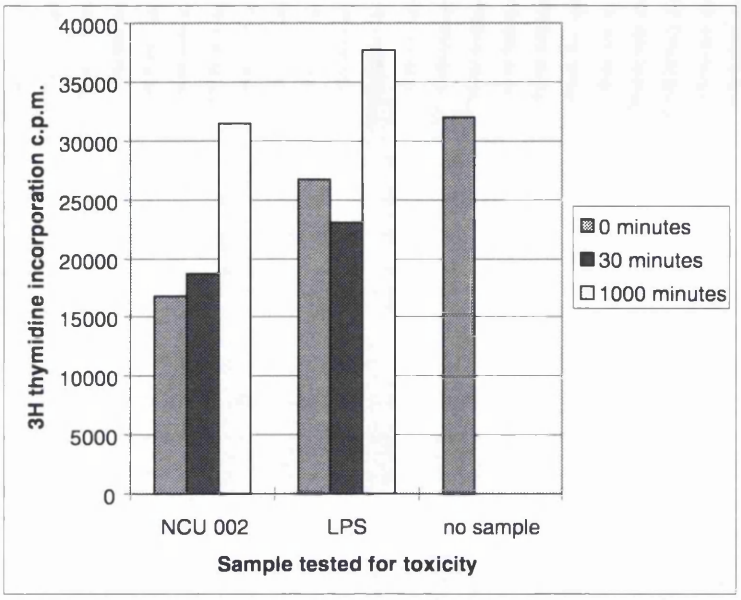


Figure 7-9: Loss of IL-12 inducing activity after TFA treatment is not due to toxicity

The enhancing effect on the vaccine is clearly interesting but complex, and such a biphasic activity would always be too difficult to incorporate into routine vaccine production.

7.2.4 Chloroform phase partitioning

Briefly, chloroform was added to whole vaccine suspension, shaken for 1-2 hours and centrifuged to separate the layers, which were dried and resuspended. An alternative method was to add chloroform to a vaccine pellet, mix for 2 hours then add water and mix a further hour, prior to separating the layers. Whether vaccine, pellet or supernatant were used, a precipitate formed at the phase interface, and this contained most of the cytokine-inducing activity. This was not pursued further.

7.2.5 No effect of DNase 1 on *M. vaccae* preparations

Bacterial DNA may be responsible for adjuvant effects, so various attempts to remove DNA from the *M. vaccae* vaccines were made. The most rigorous treatment is described briefly. 2 aliquots of 300µl of MVSA were acidified to less than pH6 with 0.5µl acetic acid and magnesium sulphate was added to make 0.006M. 150Kunitz Units (60µl of 1mg/ml stock) of DNase 1 (deoxyribonuclease 1, D-4527, a 5'hydrolase from bovine pancreas) or saline was added and the aliquots incubated for 3 hours at 37°C, then overnight at room temperature. (N.B. This represented a dilution to 80% of normal). The enzyme was denatured by autoclaving at 15lb for 15 minutes, with a 1

hour cooling time. Supernatants taken from enzyme treated samples no longer contained sizeable DNA fragments (not shown).

The aliquotting/incubation/autoclaving alone of the vaccine somewhat reduced all cytokine-inducing activity, but the DNase1 had very little additional effect on the cytokine induction measured (Figure 7-10).

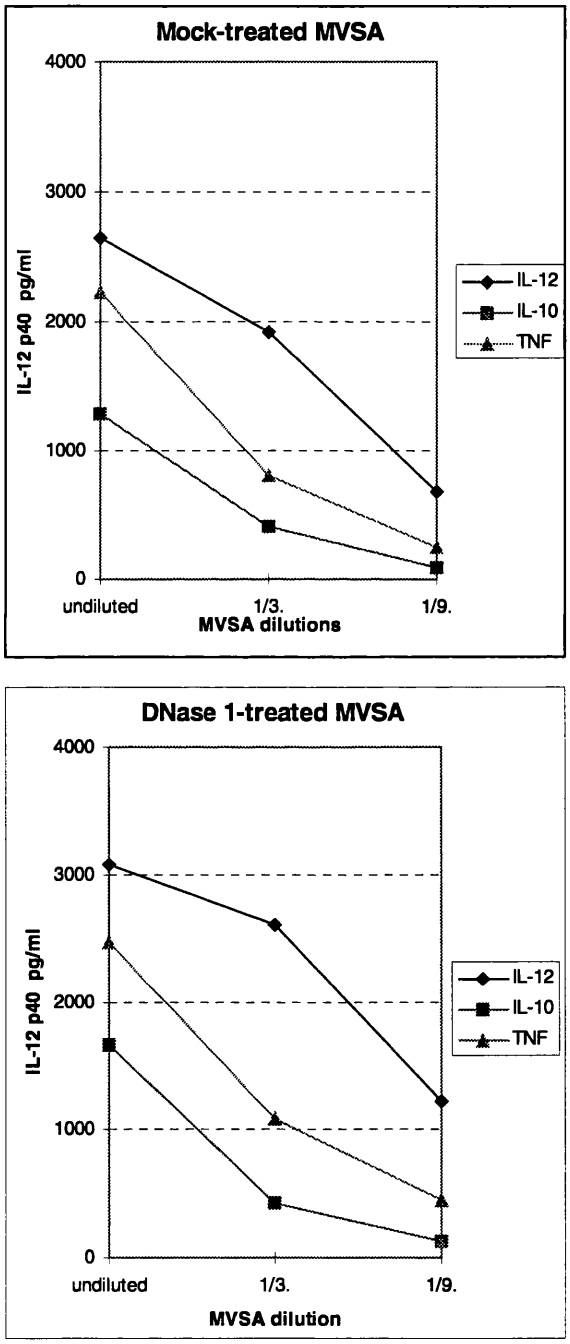


Figure 7-10: DNase 1 treatment of MVSA does not alter IL-12 induction

However, this may have been due to poor penetration of the cell wall by the enzyme and hence the chromosomal DNA may have remained uncut prior to uptake by THP-1.

To address this, *M. vaccae* sonicate was treated with DNase1. Sonicate has enormous quantities of DNA; when a volume containing 20μg of protein is loaded onto a 1% agarose gel containing ethidium bromide, some DNA is too large to enter the gel while some is present as a bright smear of fragments from 2000 to 50 base pairs long (not shown). This is due to disruption of bacteria and release of bacterial cell contents into the solution. (A similar smear is seen in the supernatant of MVSA but not NCU 002, while in unautoclaved *M. vaccae* only much larger fragments are seen).

To each aliquot of 1.5mg (protein estimate) of acidified *M. vaccae* sonicate containing magnesium sulphate was added either 75 Kunitz Units of DNase 1 or saline (total reaction volumes 150μl). These were incubated for 6 hours at 37°C, when 2μl was taken for analysis and 1350μl of saline added to the remainder to make 1mg/ml *M. vaccae* sonicate which was frozen till use. By agarose gel, the bright smear of DNA had been broken into small fragments which appeared as a smudge just under the 50b.p. marker (not shown).

When assayed for induction of IL-12 by THP-1, there was no effect of the DNase1 on *M. vaccae* sonicate. This is shown in Table 7-2.

IL-12 p40 induced by:	**5% of 1000μg/ml sample**	**5% of 330μg/ml sample**
sonicate	3482pg/ml	3329pg/ml
sonicate + saline	3525pg/ml	3639pg/ml
sonicate + DNase 1	3494pg/ml	3361pg/ml

Table 7-2: No effect of DNase 1 on IL-12 induction by *M. vaccae* sonicate

However, *M. vaccae* sonicate is a complex mixture of many compounds, and the contribution of DNA to cytokine induction may be masked by other components.

7.2.6 Varied effects of enzymatic treatments

A long and complex method had been established to fully disintegrate *M. vaccae* embedded in agar (G. McIntyre, personal communication). This was abbreviated to achieve partial disruption of various bacterial components as follows:

lysozyme (final concentration)	5 mg/ml, 3.5 hours, 37°C	and/or
lipase, typeX1 (L-4384)	18 μg/ml, 3.5 hours, 37°C	followed by
proteinase K (P-2308)	2 mg/ml, 1.5 hours, 55°C	followed by
enzyme denaturation	18 hours, 100°C	

In the graph (Figure 7-11) lysozyme alone had a strong IL-12 enhancing effect, while lipase alone reduced the activity of whole vaccine by half. The enzyme treated samples were not toxic for THP-1. This is shown in Figure 7-12; standard deviations are all less than 5% of the mean. Although this suggests cell wall lipids may have a role in cytokine induction, this function is complex because lipase did not affect the lysozyme-enhanced IL-12 induction. An additional lipase-insensitive (or non-lipid) compound may have been revealed or disruption of the bacteria may release many buried lipids and 'swamp' the enzyme. Proteinase K had no activity; however, alone it may not have gained access to intact autoclaved mycobacteria, and when added after lysozyme and lipase, its activity may have been neutralised by the presence of large amounts of irrelevant protein. Although this complex experiment was only performed once, the most dominant effect was that of enhancement of IL-12 induction by lysozyme-treated vaccine.

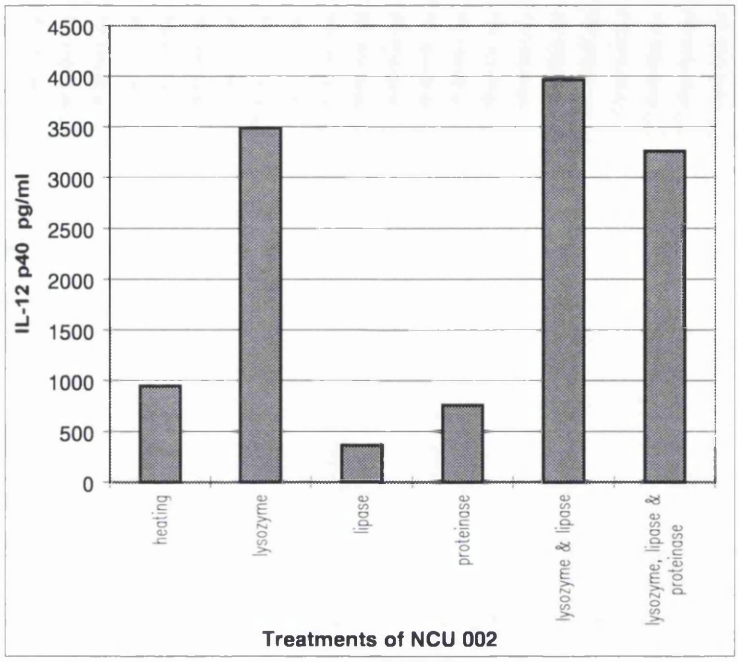


Figure 7-11: Effect of serial enzymatic treatments of the vaccine on IL-12 induction

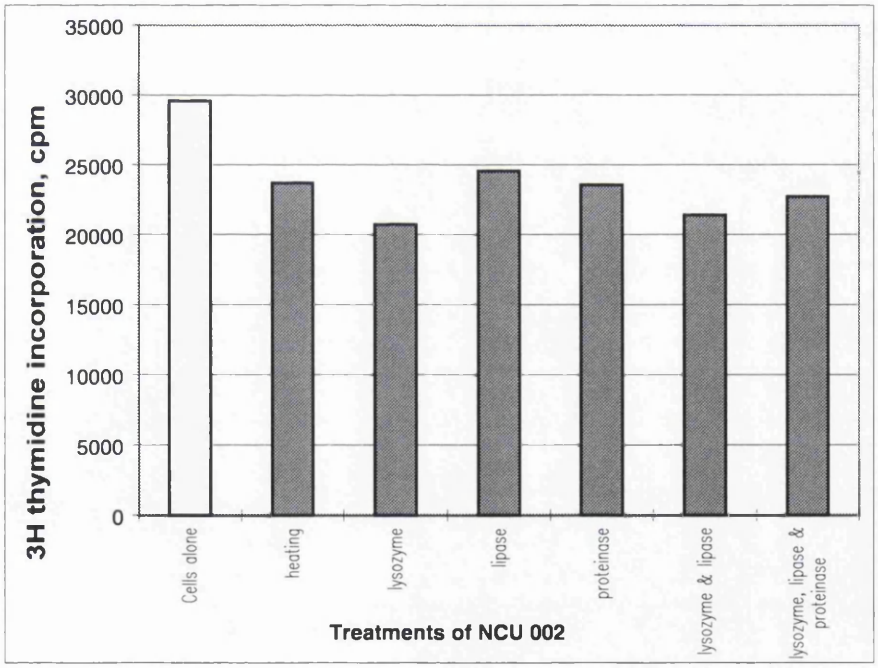


Figure 7-12: Enzyme treated vaccines are not toxic for THP-1

7.2.7 Enhancement of IL-12 inducing activity of *M. vaccae* by lysozyme

Vaccine was treated with 5mg/ml of lysozyme (from chicken egg white, Sigma L-6876, stock 50mg/ml in water) for 1 hour at 37°C, without subsequent denaturation; this may have altered the surface properties of the *M. vaccae* as it tended to coalesce. When

this treated vaccine was used in a THP-1 assay, there was enhanced production of IL-12, but no change to IL-10 production or cell survival. Lysozyme alone had no effect on THP-1 survival or cytokine output. At lower doses of lysozyme, the effect was somewhat reduced, possibly due to sub-optimal enzymatic activity (Figure 7-13).

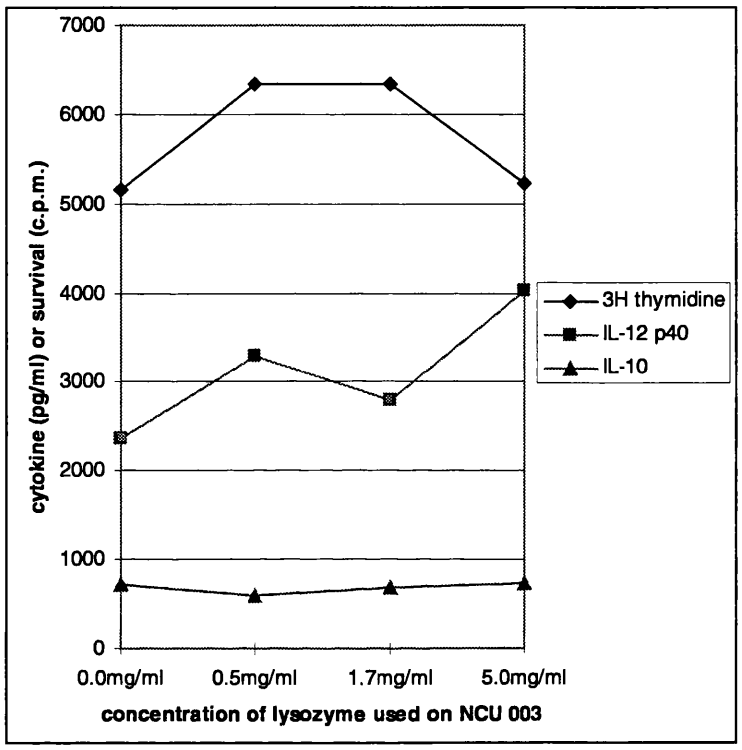


Figure 7-13: Lysozyme treatment of vaccine specifically increases IL-12 induction in THP-1

Lysozyme may be partially disrupting the cell wall and making some components more accessible to the THP-1, either before or after phagocytosis. If this is the case, lysozyme should have less effect on highly disrupted forms of vaccine. Thus NCU 002, MVSA and live *M. vaccae* (an *M. vaccae* suspension at 10mg/ml, reasonably disrupted in borate buffer but unautoclaved) were compared and cytokine results are shown for the 1/3 dilution of treated or untreated vaccines in Figure 7-14. Induction of IL-12 was enhanced after lysozyme treatment of NCU 002 by 3.4 fold, of MVSA by 1.5 fold and of live *M. vaccae* by 1.6 fold. However, induction of IL-10 and TNF were only mildly affected by lysozyme treatment of the sample.

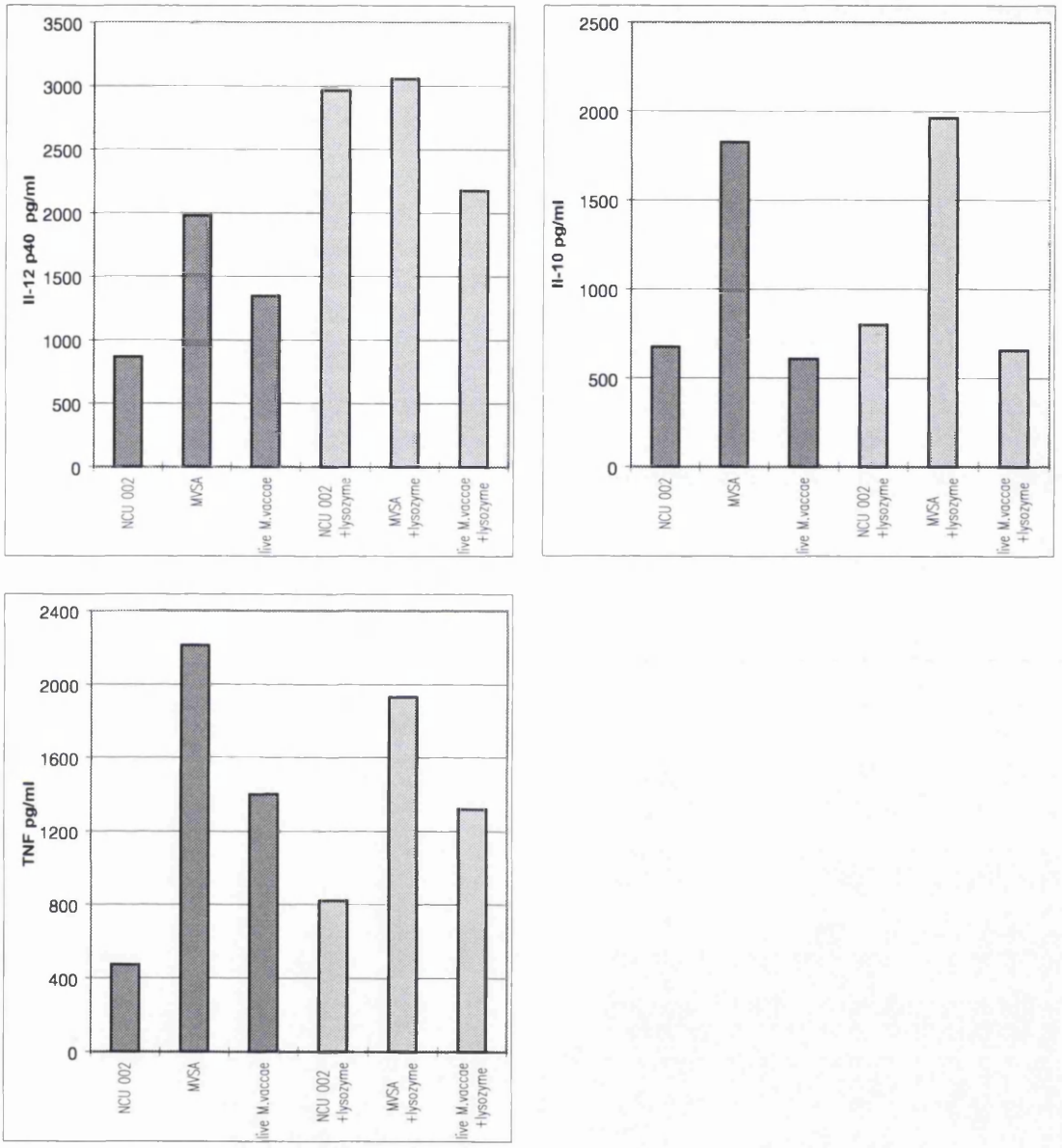


Figure 7-14: Manner of *M. vaccae* vaccine preparation effects the action of lysozyme on the vaccine

When live *M. vaccae* was autoclaved for the first time cytokine induction changed minimally (not shown), but autoclaving may increase the accessibility of peptidoglycan to lysozyme. Conversely, MVSA is highly disrupted and consequently IL-12 and IL-10 inducing factors may already be exposed and lysozyme has limited activity. It may be that in NCU 002 the balance of several independent cytokine-inducing factors has been altered by lysozyme, boosting IL-12 selectively. This is unlike the action of IFNγ, where the IL-12 production is boosted due to cellular mechanisms that increase IL-12 but the stimulus itself (vaccine) has not changed. Thus a combination of lysozyme treatment of *M. vaccae* and addition of IFNγ to the THP-1 culture greatly increased the level of IL-12 induction.(Table 7-3)

	No Treatment	**Lysozyme**	**IFNγ**	**IFNγ + lysozyme**
Medium	<30 pg/ml	<30 pg/ml	< 30 pg/ml	80 pg/ml
1/3 dilution of NCU002	748 pg/ml	3340 pg/ml	4088 pg/ml	15025 pg/ml

Table 7-3: Lysozyme treatment of *M. vaccae* and addition of IFNγ greatly enhances IL-12 production

7.2.7.1 Lysozyme does not alter IL-12 induction by BCG

The previous findings were extended in Figure 7-15 where several doses of NCU 002 and MVSA were compared to live BCG (a BCG Glaxo suspension at 10mg/ml, reasonably disrupted in borate buffer but unautoclaved) and autoclaved BCG (part of this suspension autoclaved for 30 minutes at 120°C) with and without lysozyme treatment in wells for 1.5 hours at 37°C.

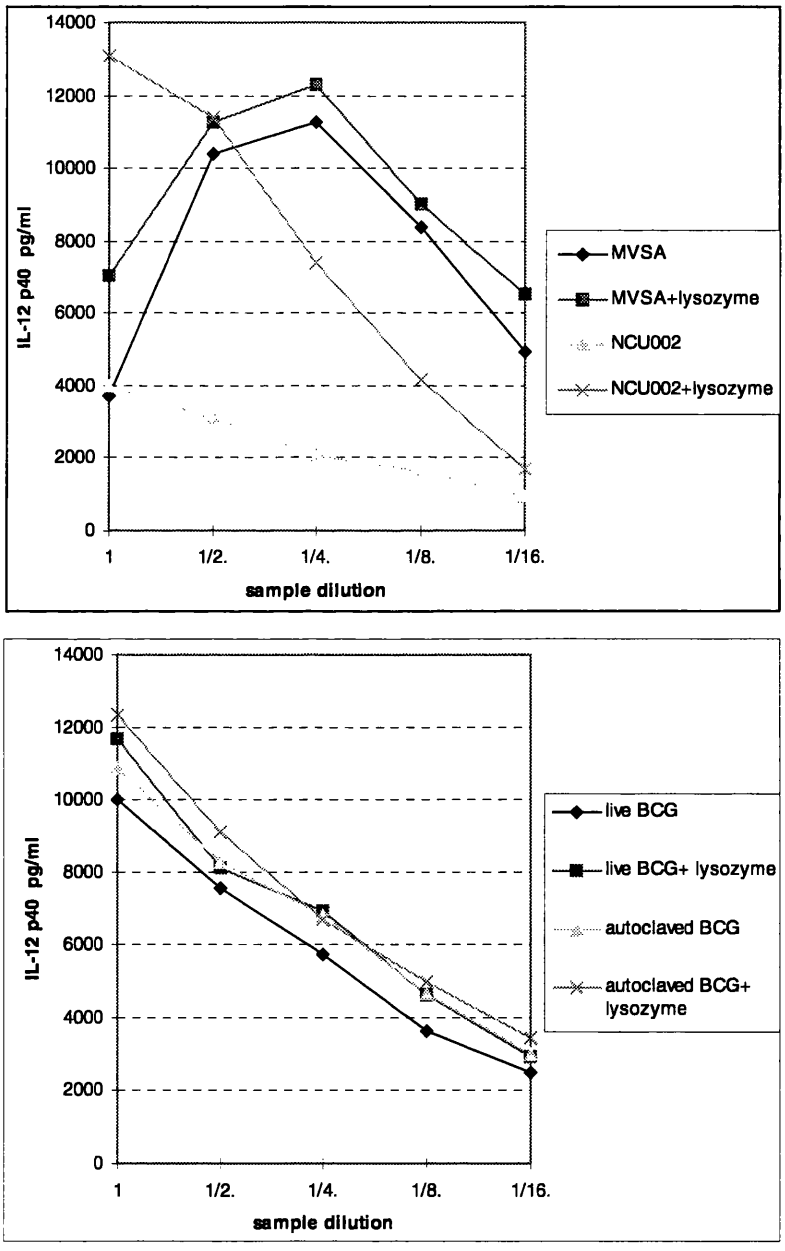


Figure 7-15: Effect of lysozyme on different preparations of BCG and *M. vaccae* vaccines

Lysozyme very strongly increased IL-12 induction by NCU 002 but had less effect on MVSA which was already highly disrupted. Lysozyme failed to prevent 'switching off' of IL-12 induction at high doses of MVSA. Live and autoclaved BCG were similar and strongly induced IL-12 production, but this was not enhanced by treatment with lysozyme. As the BCG preparations were not highly disrupted, the cell walls of BCG may have differences that either prevent lysozyme activity or cause the IL-12-inducing factors to already be accessible.

In a similar experiment, autoclaved *M. smegmatis* in borate buffer induced IL-12 production by THP-1 cells and lysozyme again failed to have an enhancing effect (not shown).

7.3 Conclusions

Attempts were made to partially disrupt or denature different components of whole *M. vaccae* vaccines. This involved physical and chemical treatments of the vaccine; IL-12 production by THP-1 cells provided a simple system for assessment of the treatments. It was possible to up or down regulate several cytokine inducing activities of the *M. vaccae* vaccine.

The simplest system, physical disruption, had considerable effect on *M. vaccae* vaccine preparations. Low doses (e.g. 1/9 – 1/27 dilutions) that had little activity using standard vaccine (possibly because there were too few available phagocytosable particles to stimulate enough cells or to pass a threshold required for activation of each cell) were sufficient to greatly stimulate production of IL-12 using more disrupted vaccines. This also had the result that the highest doses of the most disrupted vaccines down-regulated IL-12 production dramatically.

BCG and *M. smegmatis* preparations (made according to protocols for standard *M. vaccae* vaccine) were able to induce extremely high levels of IL-12 without causing this down-regulation. However, experiments to determine whether greater quantities of bacilli (e.g. from preparations of more than 10mg/ml wet weight of organism in borate buffer), or highly disrupted versions of BCG preparations, would eventually lead to down-regulation of IL-12 were not performed. It already appeared that the total weight of organism per ml of buffer was not a very important factor; instead accessibility of the active components to the macrophages was more critical, and this could be influenced by physical disruption or the species of mycobacterium.

Peroxidation, hydrolysis with TFA or some enzymatic treatments could also partially influence the activity of *M. vaccae*. However, the outcomes were rarely straight-forward. A single active ingredient was not identified, probably because there were several active compounds, and they may not have been in a single 'compartment' (soluble, exposed or internal) in the vaccine. Treatments may have increased or decreased the activity or accessibility of one compared to the other, but any redundancy in the system would mask the contribution of a single cytokine-inducing species.

Treatment of standard *M. vaccae* vaccines with lysozyme rendered them 3-4 fold more potent in induction of IL-12 (but not other cytokines) from THP-1 cells. However, this effect was not seen in highly disrupted *M. vaccae* vaccines or normal preparations from other mycobacteria. The restricted nature of this activity makes it of interest in future investigations into the actions of *M vaccae* vaccines.

7.4 Discussion

A better approach may be to partially fractionate the *M. vaccae* into cell wall fractions, DNA, proteins etc. and analyse each separately or in combinations; in this way, the full extent of their activity may be revealed. This would benefit from collaboration with biochemists. Such partial purifications have been employed by others using mycobacteria.

Cell wall components.

Mycobacteria extracted with chloroform/methanol (2:1) for 24 hours have been used to prepare lipids from mycobacterial walls (Ratledge 1982, pg 56). Filtrates contained the soluble phospholipids and neutral lipids, while the residue when acid hydrolysed with chloroform/methanol could be separated into 'bound lipid' and defatted cell material. The separation achieved is crude and furthermore in old cultures, autolysis can disrupt the cell envelope and prematurely release bound lipids into the soluble phase. The simple chloroform partitioning attempted by me was inadequate to draw conclusions, while lipids purified by more complex extractions and suitable for testing in tissue culture would require more time and more suitable materials than were available.

Surface exposed lipids vary widely between different mycobacteria, both in nature and distribution: for example trehalose dimycolate (cord factor) can be identified as a surface component of *M. aurum* (a relative of *M. vaccae*), but is only revealed after treatment with tween 80 and glass beads in *M. tuberculosis* (Ortalo Magne 1996). Thus

as disruption becomes more aggressive, lipids that were buried more deeply in the envelope become accessible. However, beyond a certain point, the activity may be destroyed. This may be relevant to physical disruption and also the bi-phasic action of TFA.

DNA.

CFA is prepared by mixing IFA (mineral oil) and nonviable *M. tuberculosis*. CFA and IFA create a local antigen depot, but CFA contains immunomodulatory substances that promote Th1 rather than Th2 differentiation. The ability of CpG-containing sequences added to IFA to induce Th1 dominated responses suggests a possible role for mycobacterial DNA in the adjuvanticity of CFA (Chu 1997). If this was the main mechanism of *M. vaccae* action, and as CpG motifs activate macrophages, denaturation of the *M. vaccae* DNA may be expected to severely reduce the IL-12 production from THP-1. The ineffectiveness of severe fragmentation of the DNA of *M. vaccae* or its sonicate by DNase 1 to reduce IL-12 induction indicates that intact bacterial DNA does not play the major role in this system. However, CpG motifs are short and some may have been present in the 50 base pair fragments, although the disruption of the DNA might still have been expected to reduce the quantity of suitable motifs and thus the activity (CpG dinucleotides are predicted in bacterial DNA at 1/16 bases, although the preference for two 5'purines and two 3' pyrimidines make efficient motifs less common). It can not be ruled out that other IL-12 inducers substituted for DNA after its disruption and masked the effect. This would require semi-purification of intact mycobacterial DNA to demonstrate its activity.

In some cases a combination of several such compounds is necessary. Regressin, a potential bladder cancer therapeutic agent (Bioniche Inc.), is an emulsion containing a combination of cell wall complex and DNA fragments from *M. phlei.* This preparation can induce IL-12 production from macrophages including THP-1 and inhibit proliferation of bladder tumour cells (communication from M.C. Filion).

Action of lysozyme.

Autoclaving *M. vaccae* may destroy cellular enzymes such as lipases and help maintain the activity of the vaccine over long periods. It may also alter the nature of the bacterial compounds including wall structures. Standard *M. vaccae* vaccines retain most activity in the bacteria not the supernatant, but vigorous physical disruption exposes and releases into the supernatant many active components, causing strong induction of IL-12, IL-10 and TNFα. Lysozyme may also disrupt *M. vaccae* cell walls but the situation

is more complex as lysozyme increases IL-12 induction but not that of other cytokines. In fact, if supernatant is taken from lysozyme treated vaccine, no additional activity is found compared to control supernatant, so lysozyme is not releasing active factors, they are remaining associated with the bacilli. Interestingly, the inability of lysozyme to affect BCG or *M. smegmatis* preparations may suggest that the ease with which the capsule and outer wall can be breached differs in these species of mycobacteria or the active component is already exposed, or these bacilli may be using different balances of compounds to induce macrophage cytokines.

Lysozyme may also affect the macrophages directly, although within the sensitivity of the ELISA used this does not include induction of IL-12. Many therapeutic benefits of hen egg lysozyme administration have been documented, some based on its ability to control the growth of susceptible bacteria but many due to the gentle interaction of lysozyme with the immune system (Sava 1996). One use is for immune restoration after treatment for neoplasia. It may enhance macrophage and lymphocyte responses and alter the tumour cell surface. However, the effects are only achieved by oral administration at present.

Human lysozyme constitutes up to 2.5% of the macrophage protein and can act both within the phagolysosome and as a secreted protein. Activity of an enhancer region correlates with lysozyme expression in mature macrophages (Schmitz 1997). Activated neutrophils can also release lysozyme from their granules, especially if pre-treated with TNFα (Burt 1997) so high concentrations of endogenous lysozyme may be relevant *in vivo* at *M. vaccae* vaccination sites, possibly boosting type 1 responses without type 2 or TNF toxicity.

M. vaccae immunotherapy is used in varied diseases and it may work in conjunction with unidentified antigens in the patient e.g. stress proteins, environmental bacteria, allergens to produce its effect. Thus efforts to identify a single active component, with the aim of purifying it to produce an 'improved vaccine', may not ever prove informative. Multiple inter-related responses to the whole bacilli may jointly 'reset the balance' of a failing immune response and promote recovery.

Chapter 8 Final discussion in relation to manufacture and commercial exploitation

These experiments were undertaken with two endpoints in mind:

1) To extend basic research on *M. vaccae*, possibly to broaden its applications, and to determine whether differences could be detected between experimental outcomes with *M. vaccae* and other mycobacteria, such as BCG. This has formed the main focus of the previous discussions of individual chapters.

2) To develop a standard assay to compare different manufacturing processes and batch to batch variations in potency of the vaccine, for regulatory authority purposes. For this, the assay would need to be relevant to the proposed protective mechanism of the vaccine, rapid and straight forward to perform, reproducible and sensitive to small changes in quantity and quality of the active component in different batches. These aspects are considered here.

Recombinant *M. vaccae* has already proved valuable in producing large quantities of mycobacterial products relatively easily. However it appears to be of less use for non-mycobacterial proteins e.g. SIVp27, which are difficult to express at high levels. This restricts its ability to elicit specific immune responses to these foreign proteins, and it is unlikely to become a serious candidate as a recombinant vaccine. Moreover, recombinant forms would all need to undergo independent safety evaluation and toxicity studies, greatly adding to development costs.

Responses to the *M. vaccae* itself were analysed simultaneously with SIVp27. The production of IFNγ by stimulated spleen cells of vaccinated mice demonstrated the ability of *M. vaccae* as a vaccine to induce a type 1 response. However, IFNγ was produced under a wide range of dose and immunisation conditions and consequently alone would not provide enough quantitative data for a potency assay. Induction of different IgG subclassses as the vaccination protocols varied would somewhat improve the distinction.

Production of intracellular IFNγ or IL-2 by lymphocytes after a single vaccination of mice with low or high doses of *M. vaccae* was studied. A cellular response and cytokine induction became apparent after the high dose of vaccine, especially in the CD8 T cell subset producing IFNγ, demonstrating the ability of *M. vaccae* to stimulate type 1 immunity, which may be relevant to immunotherapy of tuberculosis and cancer. However, even though broad trends were consistently observed the inter-assay variation

was unacceptible for a potency assay. Comparisons with other mycobacteria would be interesting, but have not yet been performed.

Interestingly, the findings have lead to a study by others in this laboratory of the possible role of *M. vaccae* in cross-presentation of antigens to human CD8+ cells cultured from PBLs.

Cytokine production, especially IL-12, in the THP-1 macrophage assay provided valuable basic information about *M. vaccae* and was useful in the attempts at its chemical 'dissection'. An *M. vaccae* dose dependent balance was observed between IL-12 and IL-10 induction (inverse), and also between TNFα concentration and cell death (proportional). The presence of IFNγ influenced these balances.

The assay defined certain differences between *M. vaccae* and BCG prepared in an identical fashion, such as the susceptibility to lysozyme pre-treatment, which only in standard *M. vaccae* caused a strong selective increase in IL-12 induction. This suggests that lysozyme revealed a substance that was masked in standard *M. vaccae*. In BCG the substance may already be accessible or it may not be present (the BCG induction of IL-12 possibly being due to other compounds), or lysozyme penetration may be inefficient. However, vigorous disruption of *M. vaccae* clumps and probably also bacterial integrity could itself increase the levels of IL-12 (and IL-10) induction and render lysozyme ineffective. The mechanism of lysozyme selective activity is to be investigated further.

Within macophages and under conditions of inflammation, human lysozyme may influence mycobacterial activity *in vivo*. This may have some bearing on the differences observed between injection of *M. vaccae*, BCG and soluble antigen *in vivo*.

Importantly, IL-12 production from THP-1 stimulated with *M. vaccae* fitted the criteria for a potency assay. As IL-12 is important in induction of type 1 immune responses, this may be one of the protective mechanisms of the immunotherapy. The assay was simple, gave incredibly detailed qualitative and quantitative information and by using a standard batch to provide a baseline in each assay was highly reproducible. This is likely to form the basis of a potency assay submitted for regulatory approval.

Using the THP-1 assay, it has been possible to show that all seven batches of commercially produced *M. vaccae* vaccine used in previous and ongoing trials are very similar in their ability to induce IL-12.

However, as attempts are made to scale up the production process, the THP-1 assay has demonstrated the importance of the method of bacterial clump dispersal to the activity of the final product. In an attempt to standardise the dispersal process (previously, simple mixing up and down with a pipette) *M. vaccae* was disrupted using

high pressure to force the suspension through a small orifice. The product was MVSA and this varied substantially from NCU 002 as described; it has subsequently been possible using the THP-1 assay to define lower conditions of pressure and a larger orifice diameter to achieve disruption similar to that of previous commercial batches.

In future, it may be desirable to produce the *M. vaccae* by fermentation rather than on solid medium; alongside simple assays checking the general chemical properties of the vaccine produced under different fermentation conditions for different lengths of time, IL-12 production by stimulated THP-1 cells will be used to provide vital information on the potency of the new-style vaccines.

Ongoing investigations

The THP-1 assay is being exploited further with ongoing investigations into the expression of accessory and adhesion molecules CD40, CD80 (B7.1), CD86 (B7.2) and ICAM-1 following *M. vaccae* stimulation. Upregulation appears to be dose dependent and increased by lysozyme treatment of the vaccine.

Adequate B7 expression on APCs stimulates CD28 costimulation and T cell activation; subsequent CTLA-4 upregulation after T cell stimulation down regulates the response and is vital for maintaining peripheral T cell homeostasis (Chambers 1997). CTLA-4 binds B7 molecules more strongly than does CD28 and mediates an inhibitory costimulation in the absence of sufficient B7. The combination of IL-12 and inhibition of CTLA-4 engagement converts a normally tolerogenic stimulus in mice to an immunogenic one (Parijs 1997). This may also have a role in tumour immunotherapy; progressing melanoma metastases produce IL-10 and melanoma-derived dendritic cells have low B7.2 (CD86) expression and induce T cell tolerance to tumour tissue. The anergy could be reversed by IL-12 (Enk 1997).

Thus establishment of conditions for *M. vaccae* induction of both IL-12 and costimulatory molecules in macrophages is important for the study of its stimulation of type 1 responses (CD4 Th1 and CD8 CTL).

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Appendix 1 Publications by the author during the period of study

Elaine Anne Bayley is known as Elaine Anne Filley
in these publications

RESPIRATORY MEDICINE (1998) 92, 597–598

Short Reports

Mycobacterium vaccae: a study of safety and outcome measures

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Background

Mycobacterium vaccae has been assessed as a potential immunotherapeutic treatment in tuberculosis (TB) (1–6) with apparent success. However, these trials were performed in centres where TB is severe, drug resistance is widespread, conventional therapy is not optimal and there is a relatively high mortality rate (7). As a prelude to further studies in a country where there is good response to standard chemotherapy and low mortality, a small double-blind, placebo-controlled study was undertaken to identify optimum indices of disease activity for use as outcome measures of efficacy and to assess the safety of *M. vaccae*.

Methods

Eleven patients (ten male, one female) with fully sensitive and bacteriologically proven active pulmonary TB participated (mean age, 40 years; range, 21–58 years). Six patients were randomized into the placebo group and five into the active group. All gave informed consent and the study was approved by the St. Mary's Local Research Ethics Committee.

This was a randomized double-blind, placebo-controlled study. Following diagnosis all subjects were started on conventional anti-tuberculous chemotherapy (rifampicin, isoniazid and pyrazinamide for 2 months followed by rifampicin and isoniazid for a further 4 months). From 1 to 2 weeks later, a single intradermal injection of 0·1 ml of *M. vaccae* or saline was given. Subjects were followed up by a 'blinded' physician and progress monitored by weight, chest radiographs, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) for a total of 4 months. In addition soluble interleukin-2 receptor (sIL-2R). the percentage of IgG that is of the agalactosyl glycoform [Gal(0) I(%)] and concentrations of antibody to lipoarabinomannan (anti-LAM) were analysed.

Chest radiographs were reported on and graded according to a modification of the WHO guidelines (8) by a radiologist blinded to the treatment code. This score ranged from trivial changes (score 1) to extensive disease (score 8). sIL-2R was measured with a kit in accordance with the manufacturer's instructions (Medgenix diagnostics SA; code 40 027 00). Anti-LAM and Gal(0) (%) measurements were performed as described previously with minor modifications (9).

Results

All patients who received active treatment tolerated the injection of *M. vaccae* well. Two subjects had a small, localized pustular reaction at the site of injection which resolved spontaneously over 72 h. There were no systemic reactions and no long-term scarring. One subject in the active group suffered from a myocardial infarction after 12 weeks but this was not felt to be related to the immunotherapy.

With the exception of two subjects from the placebo group, all patients had an increase in their weight. The median gain in the active group was 4·2 kg (range 1·8–10 kg) and placebo 6·15 kg (range −2·5 kg to+8·2 kg). All subjects demonstrated a decrease in ESR with a median fall in the active group of 24 mm h^{-1} (range 14–64 mm h^{-1}) and in the placebo group 19 mm h^{-1} (range 1–57 mm h^{-1}). There was an improvement in CRP concentrations in all subjects (Fig. 1) with a median reduction in the active group of 30 mg l^{-1} (range 0–99 mg) l^{-1} and in the placebo group of 31·5 mg l^{-1} (range 0–106 mg l^{-1}). Individuals with the highest initial CRP concentrations tended to have abnormal concentrations at 16 weeks but improvements were more clearly seen in these patients. The results for CRP exhibited the least fluctuation when results

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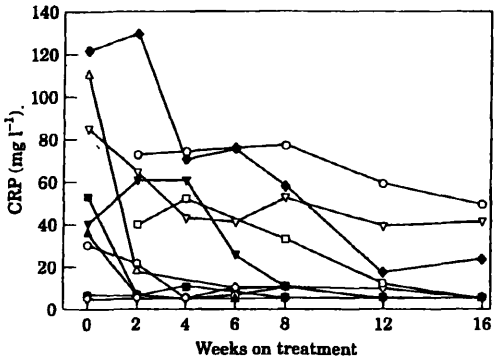

FIG. 1. Changes in CRP (mg l^{-1}) over 16 weeks in individual patients: open symbols, placebo (n=6); full symbols, *M. vaccae* (n=5).

from all parameters were compared. With the exception of one subject, there was an improvement in sIL-2R concentrations in all patients with a median reduction in the active group of 410 units ml^{-1} (range 72–1179 units ml^{-1}) and the placebo group 906 units ml^{-1} (range+299 to −1327 units ml^{-1}). There was a median improvement in chest radiograph scores in the active group of 1·0 (range 0–4) and in the placebo group of 1·5 (range 0–6). There was no consistent change in the level of Gal(0) (%) and anti-LAM.

As the majority of patients were unable to produce sputum after treatment we were unable to assess the rate of change in sputum positivity. Furthermore, two subjects had only been diagnosed after either a bronchoalveolar lavage or after sputum culture at 5 weeks.

There were no significant differences in the changes of the parameters measured, between active and placebo groups. Significant correlations were noted between the chest radiograph score and ESR (r=0·46, P=0·03), CRP (r=0·57, P=0·005) and sIL-2R (r=0·61), P=0·003) but not between the change in weight and these parameters.

Discussion

In this small study, *M. vaccae* appeared to be well tolerated and did not produce any serious adverse effects.

We also observed that surrogate markers of disease activity are unsatisfactory. Although there was a trend to improvement in the parameters measured there was a high noise-to-signal ratio and we were unable to demonstrate any statistically significant difference between treatment groups. There appeared to be a good correlation between the inflammatory markers and chest radiograph scores although this could not be demonstrated for the changes in weight.

This study illustrates the problems of studies in the developed world in establishing outcome measures to assess the efficacy of novel treatments of TB in the context of effective chemotherapy.

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HIV infection alters the production of both type 1 and 2 cytokines but does not induce a polarized type 1 or 2 state

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Objective: To test the T-helper (TH)1/TH2 cytokine paradigm in HIV infection.

Design and methods: Cytokine profiles in two separate studies of HIV patients and controls are presented: (i) a longitudinal study of HIV patients with CD4 counts $> 500 \times 10^6$/l tested at three timepoints compared with controls; (ii) a blinded cross-sectional study of controls and patients with high ($> 500 \times 10^6$/l) and low ($< 500 \times 10^6$/l) CD4 counts. Peripheral blood mononuclear cells (PBMC) from patients and controls were tested for the production of two type 1 [interleukin (IL)-2, interferon (IFN)-γ] and two type 2 (IL-4, IL-10) cytokines by enzyme-linked immunosorbent assay. Both spontaneous and mitogen-induced cytokine production was measured.

Results: HIV infection was noted to have the following effects on cytokine production: (i) it led to the *in vivo* activation of type 2 cytokines in a small group of individuals with high CD4 numbers characterized by the spontaneous release of IL-4 and IL-10. Longitudinal data showed high spontaneous IL-4 and IL-10 to be a consistent feature of the patient group (at each timepoint some patients were high producers) but to be variable in a given individual; (ii) HIV infection impaired the ability of PBMC to respond to stimuli (selected for their ability to optimally induce each cytokine) in terms of IL-2, IL-4 and IL-10 production in patients with both high and low CD4 cell counts; and (iii) conversely, HIV infection led to an overproduction of IFN-γ in patients with high CD4 counts; patients with low CD4 produced normal levels of IFN-γ.

Conclusions: Our observations did not suggest polarization of the type 1/type 2 cytokine profile in HIV patients. Instead, the data suggested more complex changes to type 1/type 2 cytokine patterns in HIV infection than originally proposed by the TH1/TH2 dichtomy.

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Introduction

Studies of type 1/type 2 cytokine production have been useful in establishing the nature of protective and pathogenic immune responses to pathogens [1,2]. In humans, type 1 cytokines [interferon (IFN)-γ, interleukin (IL)-2, and IL-12] induce cellular immunity [cytotoxic T lymphocytes delayed type hypersensitivity and immunoglobulin (Ig) G_1 antibody], whereas type 2 cytokines (IL-4, IL-6, IL-5 and IL-10) induce antibodies of the IgA, IgE and IgG_4 subclasses. Type 1/type 2 cytokines are reciprocally regulated, and immunity to

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many foreign antigens includes the preferential activation of one group or the other [3]. However, some pathogens have evolved mechanisms to induce a highly polarized type 1 or 2 state as a means of subverting the defence mechanisms of the host [1,2,4].

The earliest observations in HIV infection suggested that disease was associated with overproduction of type 2 cytokines and decreased production of type 1 cytokines [5,6]. However, these observations have been challenged. There is general agreement that one type 1 cytokine, IL-2, is decreased in HIV-infected individuals [7–10] and that the overall production of another type 1 cytokine, IFN-γ, is enhanced in peripheral blood mononuclear cells (PBMC), although IFN-γ production may be impaired at the CD4+ T-cell level [8,9,11]. There is also some agreement that the type 2 cytokine IL-10 is overproduced in some HIV-infected individuals [9,10]. However, there is controversy regarding the production of the key type 2 cytokine IL-4. Some studies have shown IL-4 to be enhanced [7,12,13], whereas others have shown this cytokine to be decreased [9,10,14]. Such diverse observations have resulted in two questions on type 1/type 2 cytokine production in HIV infection remaining unresolved. It is not clear whether (i) HIV infection results in a polarized type 1 or 2 state, and (ii) the production of type 1 or 2 cytokines governs the disease state of the individual.

In this study, we have suggested some answers to these questions. A pilot study to determine the optimal induction of key type 1/type 2 cytokines (IL-2, IFN-γ, IL-4 and IL-10) in seronegative controls showed that PBMC of some individuals spontaneously produced IL-4 and IL-10; the optimal mitogenic stimulus (in terms of inducing the highest level of cytokine and the number of responders) for these cytokine was as follows: IL-4 and IL-10, concanavalin A (Con A); IFN-γ, Con A or phytohaemagglutinin (PHA) plus phorbol myristate acetate (PMA); IL-2, anti-CD3 plus PMA or PHA plus PMA but not Con A. The optimal timepoint for measuring all four cytokines was 2 days (unpublished data).

On the basis of this study, we compared the levels of IL-2, IL-4, IL-10 and IFN-γ in PBMC cultures of HIV-infected patients and seronegative controls. Two groups of patients were studied: those with high ($> 500 \times 10^6$/l) and those with low ($< 500 \times 10^6$/l) CD4 counts. Cytokine levels in some of the patients with high CD4 were monitored over three timepoints. We asked the following questions. (i) Do patients and controls differ in cytokine production, and is the difference linked to CD4 number? (ii) Is there a difference in spontaneous and stimulated cytokine production between patients and controls? (iii) What is the variation over time in cytokine production in a given patient? Our data suggested that unlike other infectious diseases, HIV infection, despite causing the altered production of both type 1 and type 2 cytokines, did not lead to a polarized type 1 or 2 state that could be correlated with disease.

Materials and methods

Patients and controls

Two groups of HIV-seropositive patients were studied. Twenty health patients [Centers for Disease Control and Prevention (CDC) 1987 criteria stage II/III] with CD4 counts greater than 500×10^6/l. These patients were HIV p24 antigen-negative and had received no prior antiviral chemotherapy. Longitudinal studies were performed on PBMC from patients in this group because 16 patients were recruited as part of a trial of *Mycobacterium vaccae* immunotherapy. Heparinized blood samples were taken at three timepoints (0, 14 and 28 days) prior to immunotherapy. Seven patients with CD4 counts below 500×10^6/l were also studied. The control group consisted of 33 HIV-seronegative individuals. Eleven healthy HIV-seronegative individuals were recruited from an outpatient clinic, and 22 were individual blood donors. Blood from these individuals was collected in citrate phosphate dextrose buffer (blood packs from St George's Medical School, Tooting, London, UK) or collected in heparinized tubes.

Cell cultures

Heparinized blood (50 ml) diluted 1 : 1 with Hank's balanced salt solution (HBSS; Gibco, Paisley, Scotland, UK) was layered on 50 ml Ficoll-Paque (Pharmacia Biotech, St Albans, Hertfordshire, UK) and centrifuged at 500 g, for 30 min at room temperature. The interface containing mononuclear cells was harvested and washed three times in RPMI-1640 medium (Gibco), plus 2% human serum (First Link UK, Brierly Hill, West Midlands, UK). PBMC (1×10^6) were cultured at 37°C in a final volume of 500 µl in RPMI plus 10% human serum medium in 24-well tissue culture plates in the presence and absence of 20 µg/ml concanavalin A (Con A; Sigma, Poole, Dorset, UK) or anti-CD3 antibody (final concentration 1 : 100 tissue culture supernatant; kind gift from Prof. P.C.L. Beverley, UCL Medical School, London, UK) plus 1 ng/ml PMA (Sigma) or 1 µg/ml PHA (Sigma) plus 1 ng/ml PMA. Cell-free supernatants were harvested at 2 days post-stimulation and stored at −80°C until analysed.

Cytokine assays

Four cytokines were measured using commercially available paired antibodies: IL-4 and IFN-γ (AMS Biotechnology, Whitney, Oxfordshire, UK), IL-10 (Pharmingen, Cambridge Bioscience, Cambridge, UK),

and IL-2 (Medginex, Lifescreen Ltd, Watford, Hertfordshire, UK). A detailed description of the enzyme-linked immunoabsorbent assay (ELISA) conditions is described. Maxisorp plates (96-well; Nunc, Gibco, Paisley, Scotland) were coated at 4°C overnight with the following antibody concentrations: IL-2, 2.5 μg/ml in phosphate-buffered saline (PBS); IL-10, 2 μg/ml in 0.1 M $NaHCO_3$ (pH 8.2); IL-4, 1 mg/ml PBS; IFN-γ 0.5 mg/ml PBS. The plates were washed twice in PBS plus 0.05% Tween-20 (Sigma) and blocked for 2 h at room temperature with PBS plus 2% bovine serum albumin (BSA) with the exception of IL-10, which was blocked with PBS plus 10% fetal calf serum (FCS). Standards consisted of twofold serial dilutions (in PBS plus 2% BSA) of recombinant cytokines from the following sources: IL-4 (R&D Systems, Abingdon, Oxfordshire, UK), IL-10 (AMS Biotechnology), IFN-γ and IL-2 (UK AIDS Repository, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, UK; reference number ADP902 and ADP901, respectively). Plates were incubated with 100 μl test sample and standards at 4°C overnight, with the exception of IL-2, which was incubated for 2 h at room temperature on a plate shaker at 700 rpm. For the IL-2 assay, the detection antibody was added at the same time as the standard and test samples at a final concentration of 1 μg/ml in 2% PBS–BSA. For all other assays, plates were washed four times after the overnight incubation with samples/standards and 100 μl of detection antibody added as follows: IL-10, 1 μg/ml in PBS plus 10% FCS, IL-4, 1 μg/ml in 2% BSA–PBS, IFN-γ 0.5 μg/ml in 2% BSA–PBS. Plates were incubated for 1 h at room temperature, washed four times, and 100 μl of either streptavidin-alkaline phosphatase (AMS Biotechnology; used at 1 : 1000 in PBS plus 2% BSA) for IL-4 and IFN-γ or avidin-peroxidase (1 : 400, 1 μg/ml; Sigma) for IL-2 and IL-10. Plates were incubated at room temperature for between 30 min and 1 h followed by a further four washes and 100 μl substrate solution (Sigma substrate tablet sets) was added. The substrates used and the timings at which the plates were read on an UV_{max} ELISA reader (Molecular Devices, Coulter Electronics, Luton, Bedfordshire, UK) are as follows: IL-2, tetramethylbenzidine dihydrocholride stopped at 30 min and read at 450 nm (reference wavelength, 650 nm); IL-10, o-phenylenediamine dihydrocholride stopped at 30 min and read at 490 nm; IL-4 and IFN-γ, p-nitrophenyl phosphate read at 405 nm after 1 h for IL-4 and 30 min for IFN-γ. The IL-2 and IL-10 assays were stopped by the addition of 50 μl 2M H_2SO_4.

Statistical analysis

A non-parametric (Mann-Whitney U test) was used to analyse differences between cytokine levels in the HIV-infected groups and seronegative controls. Fisher's exact test was used to analyse differences in the number of high cytokine producers (calculated as those who produced greater than the mean + 2 SD of the control) in the patient versus control groups as well as determining the number of responders and non-responders to each mitogen. Responders were defined as those individuals whose PBMC produced more than twice the (same individuals) spontaneous cytokine level.

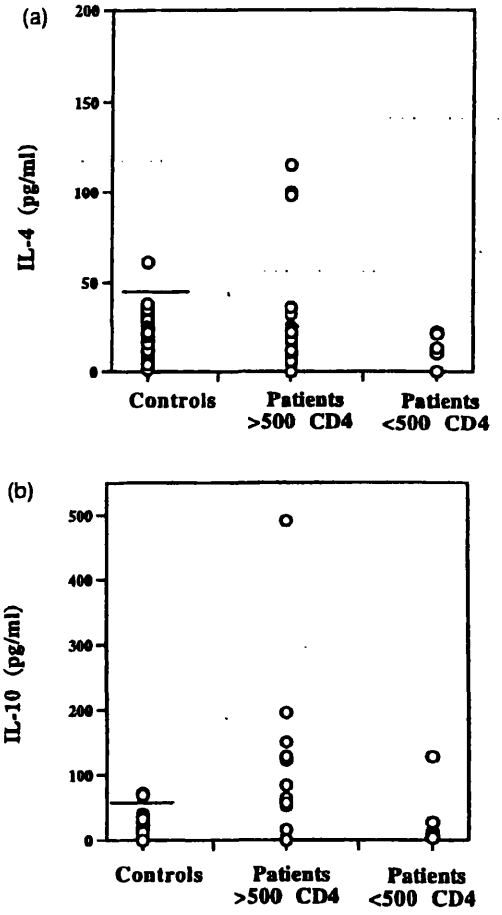

Fig. 1. Spontaneous interleukin (IL)-4 and IL-10 production by patients and controls. (a) IL-4 levels in culture supernatants harvested at 2 days from peripheral blood mononuclear cells (PBMC) of controls (n = 33) and patients with CD4 counts greater (n = 20) or less than (n = 7) 500×10^6/l. (b) IL-10 levels in culture supernatants harvested at 2 days from PBMC of controls (n = 32) and patients with CD4 greater (n = 16) or less than (n = 6) 500×10^6/l. Cells were cultured without stimulation.

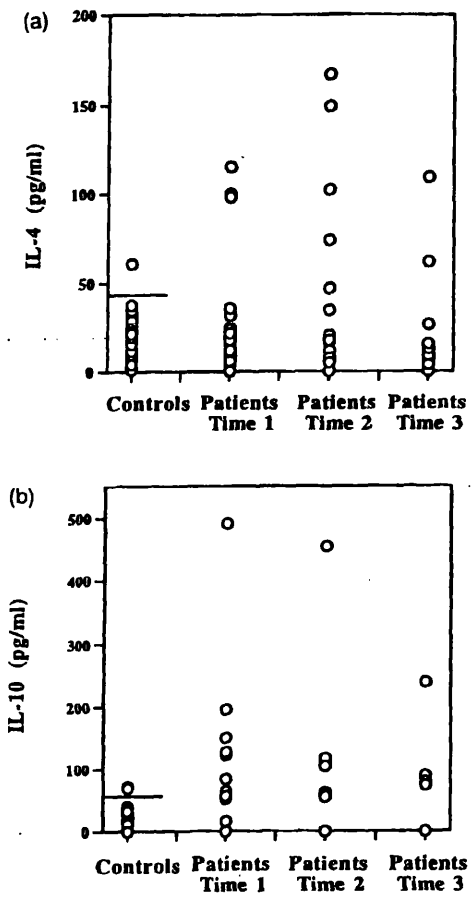

Fig. 2. Spontaneous interleukin (IL)-4 and IL-10 production over time by patients with high CD4 count. (a) Spontaneous production of IL-4 by patients with CD4 >500 × 10^6/l tested on three separate occasions (time 1, day 0; time 2, day 14; time 3, day 28) compared with controls. Spontaneous production of IL-10 by patients with CD4 >500 × 10^6/l tested on three separate occasions (time 1, day 0; time 2, day 14; time 3, day 28) compared with controls. The line across the control group in each case represents the mean + 2 SD of IL-4 or IL-10 produced by the control group. Cytokine production over this level indicates high producers.

Results

Type 2 cytokines: spontaneous IL-4 and IL-10 is enhanced in a subgroup of HIV patients

One indication of HIV infection directly inducing cytokine synthesis is the spontaneous secretion of cytokines (in the absence of any *in vitro* activation signals) from patients copared with controls. Figure 1 indicates the spontaneous levels of IL-4 and IL-10 in cultures of controls and patients with high (> 500 × 10^6/l) and low (< 500 × 10^6/l) CD4 counts. High producers were identified as those who produced more than the mean + 2 SD of the control group as a whole. For IL-4, the number of high producers (> 44 pg/ml) were one out of 33 controls, three out of 20 patients with CD4 counts > 500 × 10^6/l, and none out of seven patients with CD4 <500 × 10^6/l. For IL-10, the number of high producers (> 61 pg/ml) were three out of 32 controls, seven out of 16 patients with CD4 count > 500 × 10^6/l, and one out of six patients with CD4 counts <500 × 10^6/l.

To establish whether high constitutive IL-4 and IL-10 synthesis was a feature of patients as opposed to controls, a group of patients with high CD4 counts were followed for IL-4 and IL-10 production on a further two occasions (Fig. 2). Although the same control individual was not studied on three separate occasions, the levels represent data from cultures set up at four separate timepoints consisting of cells from a group of six, eight, nine and 11 healthy individuals. Analysis of the control data showed no significant difference in any of the cytokines between the timepoints (data not shown), and therefore the data from the four timepoints was combined. Fig. 2 and Table 1 show high spontaneous IL-4 and IL-10 production to be a consistent feature of the patient group; at each timepoint some HIV-infected patients were identified as high producers. Statistical analysis based on the number of high producers confirmed differences between patients and controls to be significant ($P < 0.05$) on one of the three timepoints for IL-4, whereas differences in IL-10 were significant at all three timepoints (Table 1). It was noted, however, that cytokine production in a given individual varied over time. Thus, of the six high IL-4 producers, four produced high levels on two of three timepoints, while two individuals produced high IL-4 only on one of the three timepoints. None of the six patients produced high IL-4 on all three timepoints. Of the 10 high IL-10 producers, seven did so on two of the three timepoints, whereas the rest produced high levels of IL-10 on only one of the three timepoints.

Having identified a small number of individuals who overproduced IL-4 and IL-10 spontaneously, it was important to establish whether these individuals were predisposed to a faster rate of CD4 decline. Serial CD4 counts performed over 18 months showed no apparent differences in rates of decline in patients with high compared with those with low spontaneous IL-4 production. The mean slopes in the two groups were −0.12 and 0.03, respectively (data not shown).

Type 2 cytokines: mitogen-induced IL-4 and IL-10 production is impaired in HIV patients

Cytokine levels in Con A-stimulated cultures were measured to establish whether the enhanced sponta-

Table 1. Cytokine overproduction by HIV-infected patients.

Group	Cytokine levels [median (range)]	*P* (Mann–Whitney)	High producers (n/total)	*P* (Fisher's exact test)
Spontaneous IL-4 (pg/ml)				
Control	18.00 (1–61.00)		1/33	
Patients > 500 × 10^6/l CD4				
Time 1	19.5 (0–115.0)	0.6011	3/20	0.1
Time 2	17.5 (0–167.0)	0.8902	5/16	0.01
Time 3	8.5 (0–109.0)	0.0297	2/16	0.2
Patients < 500 × 10^6/l CD4	12.0 (0–22.0)	0.2127	0/7	1.0
Spontaneous IL-10 (pg/ml)				
Control	16.0 (0–72.0)		3/32	
Patients > 500 × 10^6/l CD4				
Time 1	57.0 (0–491.0)	0.0223	7/16	0.01
Time 2	57.0 (0–455.0)	0.2969	5/12	0.02
Time 3	36.5 (0–239.0)	0.5636	5/11	0.02
Patients < 500 × 10^6/l CD4	12.0 (3–127.0)	0.8072	1/6	0.5
Con A-induced IFN-γ (IU/ml)				
Control	98.0 (0–646.0)		1/34	
Patients > 500 × 10^6/l CD4				
Time 1	644.0 (2–2926.0)	0.0019	10/19	0.0004
Time 2	656.5 (1–2894.0)	0.0210	10/16	0.00007
Time 3	95.0 (4–2558.0)	0.4860	7/16	0.0007
Patients < 500 × 10^6/l CD4	161.0 (0–267.0)	0.1363	0/7	1.0

Data expressed as the median (range) levels of spontaneous interleukin (IL)-4, spontaneous IL-10 and concanavalin A (Con A)-induced interferon (IFN)-γ in the control and patient groups. Patients with high CD4 count were studied at three timepoints. In addition, the number of individuals in the control and patient groups who produced high levels of IL-4 (> 44 pg/ml), IL-10 (> 61 pg/ml) and IFN-γ (>367 IU/ml) is shown. High producers were identified as those who produced greater than the mean + 2 SD of the control level of a given cytokine. Tests for significance between the patient and control groups were calculated using Mann–Whitney U test or by Fisher's exact test.

neous production of IL-4 and IL-10 by HIV-infected patients was associated with cytokine overproduction in response to stimuli. In contrast to spontaneous cytokine production, Fig. 3 and Table 2 show that stimulation of patient cells (irrespective of CD4 number) led to the reduced production of IL-4 (Fig. 3a) and IL-10 (Fig. 3b). Not only was the overall level of both cytokines lower in cultures of patient compared with control cells, but fewer patients responded to Con A (response was measured as twofold increase over the same individuals spontaneous production). In addition, the reduced IL-4 and IL-10 response was consistent over time (Table 2).

Type 1 cytokines: IL-2 production is impaired but IFN-γ is over-produced in patients with normal CD4 counts

The production of IL-2 and IFN-γ (type 1 cytokines), was measured in parallel to the two type 2 cytokines. Unlike type 2 cytokines, HIV-infected patients did not spontaneously produce IL-2 or IFN-γ (data not shown). The ability to produce these cytokines upon activation was studied by measuring IFN-γ production in Con A-stimulated cultures and IL-2 in anti-CD3 plus PMA-stimulated cultures.

IFN-γ production was associated with patients' CD4 cell counts. Patients with high CD4 counts produced six- to eightfold higher levels of IFN-γ than controls. By comparison, patients with low levels of CD4 cells produced similar levels to controls (Table 1). Thus, patients with high CD4 cell counts showed signs of immune activation by spontaneously secreting high levels of IL-4 and IL-10 and overproducing IFN-γ in response to stimulation (Table 1). The increased production of IFN-γ by patients was consistent over time. A group of patients with high CD4 counts were followed for IFN-γ over three timepoints. Differences between the control and patient groups calculated on the basis of the frequency of high producers (identified as those who produced greater than the mean + 2 SD of the control, >347 IU/ml) was significant ($P < 0.05$) at all three timepoints (Table 1) despite differences in the level of overall IFN-γ being significant on only two of the three timepoints (Table 1).

Several studies [7–10] have recorded reduced IL-2 production in patients with low CD4 counts, so we therefore focused on IL-2 production by patients with high CD4 counts. IL-2 production was significantly lower in patients compared to controls (Table 2). The mean overall level secreted by patients' cells was 2.5- to fourfold lower than control cells. Longitudinal data of IL-2 production over three timepoints showed these observations to be consistent over time. Not only was the overall level of IL-2 lower in patients than controls, but fewer patients responded to anti-CD3 plus PMA stimulation compared with the control group (Table 2).

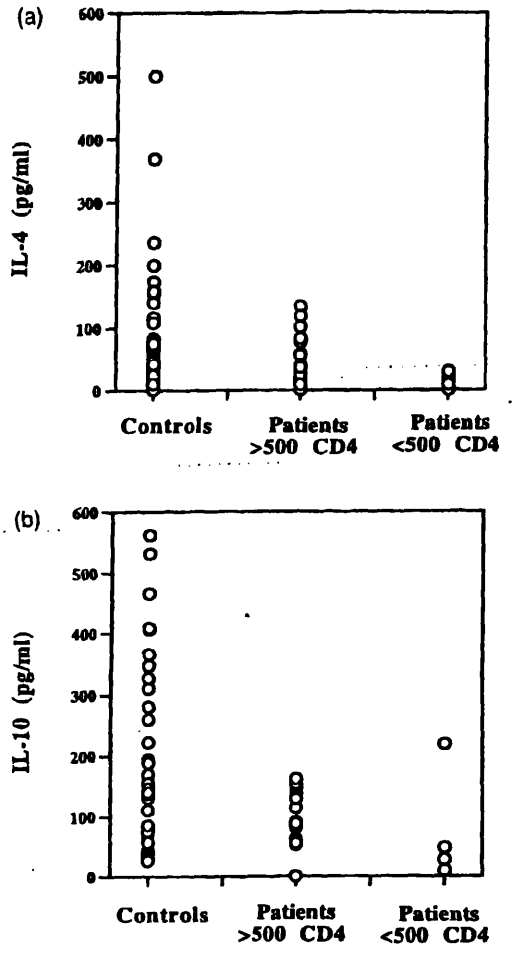

Fig. 3. Concanavalin A (Con A)-induced interleukin (IL)-4 and IL-10 in patients and controls. (a) IL-4 levels in culture supernatants harvested at 2 days from peripheral blood mononuclear cells (PBMC) of controls (n = 33) and patients with CD4 greater (n = 20) or less than (n = 7) 500 × 10^6/l. (b) IL-10 levels in culture supernatants harvested at 2 days from PBMC of controls (n = 32) and patients with CD4 greater (n = 16) or less than (n = 6) 500 × 10^6/l. Cells were cultured with 20 μg/ml Con A.

Discussion

A polarized type 1/type 2 cytokine state is characterized by two features: First, by the overproduction of type 1 or 2 cytokines (usually more than one of each category), which can be measured by an increase in the expression of cytokine-specific mRNA or protein, or both, before and after *in vitro* stimulation of cell mixtures (e.g., PBMC). Second, as the classification of the type 1/type 2 cytokine system was originally based on CD4+ T cells, polarization would lead to a shift from a predominantly large number of type 0 CD4+ T-cell clones to an increase in the number of either type 1 or 2 CD4+ T cells in the infected individual, and to an associated increase in the production of those cytokines by freshly isolated CD4+ T cells. Three studies have shown that HIV infection induces a shift to a type 2 or an activated type 0 state at the CD4+ T-cell level and to the overproduction of the type 2 cytokine IL-4 [7,12,13], whereas four studies have suggested reduced production of IL-4 [9,10,14,15]. Only one study has found no change in IL-4 production in HIV [16]. Our data paradoxically supported both opposing views. On the one hand, the spontaneous increase in IL-4 and IL-10 production supported increased type 2 cytokine production in HIV, and on the other, the decreased production of both these cytokines in response to stimulation supported the opposite view. Part of the answer to this paradox lies in how cells are cultured. We provide two novel observations regarding the production of type 2 cytokines. First, these cytokines can be spontaneously secreted (in controls and patients), with the frequency of high producers being higher in the patient group. Second, we have noted that both these cytokines are optimally induced by Con A as opposed to anti-CD3 plus PMA or PHA plus PMA (unpublished observation). Optimal stimulation of cells would be critical in comparing cytokine production between patients and controls. It is therefore possible that the failure to do so might have contributed to some of the contradictory observations in the literature.

The paradox of why cytokines activated *in vivo* (e.g., IL-4 and IL-10) are not overproduced when the cells are stimulated might be explained by the high levels of activation-induced cell death (AICD) observed in HIV-infected cultures [17]. HIV proteins such as Tat and gp120 enhance AICD of CD4+ T cells through the CD95 pathway [18]; this would explain why stimulation of cells from HIV-infected patients leads to the reduced production of type 1 (IL-2) and type 2 cytokines (IL-4, IL-10). The cytokines that are not dramatically lost in HIV infection, such as IFN-γ, are most likely to be produced by cells other than CD4 [e.g., CD8+ T cells or natural killer (NK) cells]; indeed, CD8+ T cells expressing high levels of IFN-γ mRNA have been found in the lymph nodes of HIV-infected individuals [19].

On the basis of the arguments presented above, we concluded that the cytokine profile of HIV-infected individuals does not fit a hypothesis based on a straight T-helper (TH)1 1/TH2 dichotomy, and that HIV infection is unlikely to lead to a clear polarization of the type 1/type 2 cytokine response. There are several

Table 2. Reduced cytokine production by HIV-infected patients.

Group	Cytokine levels [median (range)]	*P* (Mann–Whitney)	Responders (n/total)	*P* (Fisher's exact test)
Con A-induced IL-4 (pg/ml)				
Control	70.0 (1–500.0)		29/33	
Patients > 500 × 10^6/l CD4				
Time 1	30.25 (0–134.0)	0.03	5/20	0.00001
Time 2	34.5 (0–223.0)	0.046	5/16	0.0001
Time 3	13.5 (2–73.0)	0.0001	6/16	0.005
Patients < 500 × 10^6/l CD4	19.0 (0–30.0)	0.0017	2/7	0.003
Con A-induced IL-10 (pg/ml)				
Control	155.0 (25–562.0)		29/32	
Patients > 500 × 10^6/l CD4				
Time 1	89.5 (0–162.0)	0.0258	4/16	0.00001
Time 2	63.5 (0–210.0)	0.0018	3/12	0.00005
Time 3	66.5 (0–239.0)	0.0114	2/11	0.0001
Patients < 500 × 10^6/l CD4	36.5 (9–218.0)	0.0113	3/6	0.04
Anti-CD3+PMA-induced IL-2 (IU/ml)				
Control	50.05 (20.2–86.6)		10/10	
Patients > 500 × 10^6/l CD4				
Time 1	1.05 (0–99.9)	0.002	8/16	0.01
Time 2	3.65 (0.3–102.8)	0.0038	10/16	0.05
Time 3	0.85 (0–40.1)	0.0002	5/13	0.02

Data expressed as the median (range) levels of concanavalin A (Con A)-induced interleukin (IL)-4, Con A-induced IL-10 and anti-CD3 plus phorbolmyristate acetate IL-2 in the control and patient groups. Some patients with high CD4 counts were studied at three timepoints. In addition, the number of individuals in the control and patient groups who responded to stimulation by producing a minimum of twofold increase over spontaneous cytokine release is shown. Tests for significance between the patient and control groups were calculated using Mann–Whitney U test or by Fisher's exact test.

reasons for this. In addition to increased AICD (see above), recent data enumerating cytokine production at the single cell level by flow cytometry suggests that unlike the mouse, human memory cells can have complex cytokine patterns [20] and include cells that present a broad spectrum of cytokines as opposed to cells that fall into two clear subsets [20,21]. In particular, human TH1/TH2 cells cannot be classified on the basis of IL-10 production because they both can produce this cytokine [22]. Finally, cytokine profiles are likely to be governed by the differential replication of HIV in CD4+ T-cell subsets [13,23]. We have shown that CD4+ T-cell clones that fall in the type 1 category, unlike TH2 clones, fail to support HIV replication efficiently [23], are intrinsically more prone to AICD [24] (consistent with murine observations) [25], and are more prone to apoptosis when exposed to HIV [24]. Type 1 CD4+ T cells would therefore be most susceptible to apoptosis in HIV-infected individuals and a loss of this population would be consistent with a fall in IL-2 production. A fall in type 1 CD4+ T cells would also be consistent with an increase in type 2 cytokines [7,13]. However, we suggest that a loss of type 1 CD4+ T cells in HIV infection is unlikely to lead to the overproduction of type 2 cytokines. This could be due partly to the increased production of IFN-γ, which can downregulate IL-4 and IL-10, and partly to the reduced production of type 2 cytokines in response to stimulation.

A recent study by Meyaard *et al.* [26] lends support to the above discussion. Consistent with our findings, Meyaard *et al.* [26] noted that IL-2, IL-4 and IL-10 production (in response to stimulation) was impaired by PBMC of HIV-infected patients. By single cell analysis after intracytoplasmic cytokine staining. They found no differences between patients and controls in the number of T cells that produced IL-4. However, unlike our data, they observed reduced IFN-γ in patient cultures and fewer CD4 cells that produced this cytokine. Two possibilities might account for the disparity between our IFN-γ data and those of Meyaard *et al.* [26]. First, the stimulus used in the two studies was different. Secondly, they did not measure the total number of IFN-γ-producing cells. It is possible that although the frequently of IFN-γ producing CD4 cells was decreased, the same might not have occurred in other IFN-γ producing cell types (e.g., CD8+ T cells and NK cells). Taken together, their data indicated that reduced cytokine production in HIV is not necessarily due to a reduced frequently of CD4+ T cells that produce those cytokines (e.g., IL-4 production). In turn, this supports our suggestion that a polarization of the type 1/type 2 cytokine response does not occur in HIV infection.

Finally, in view of the accepted importance of type 1/type 2 cytokines in inducing different arms of the immune response, it is worth considering whether this factor should be taken into account in developing

strategies for prophylactic immunization against HIV [27]. Our data and those reviwed above [28,29] indicate that further studies are needed to answer this question. In particular, further data on the frequency and phenotype of cells that produce key type 1 and 2 cytokines in HIV infection is needed. Correlating, at the single cell level, cytokine production [21,26] with virus production (p24 staining) and apoptosis is likely to resolve some of the paradoxes in cytokine production in HIV infection. Such a study might also provide important information on those cytokines that are associated with disease and those associated with a disease-free state.

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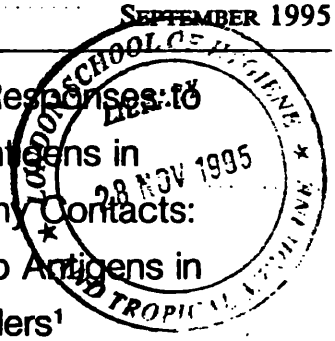

Analysis of T-Cell and B-Cell Responses to Recombinant *M. leprae* Antigens in Leprosy Patients and in Healthy Contacts: Significant T-Cell Responses to Antigens in *M. leprae* Nonresponders[1]

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It is believed that upon infection with *Mycobacterium leprae* the nature of the T-cell immune response in the exposed individual determines whether leprosy will develop or whether protection will be induced. Healthy exposed individuals and tuberculoid leprosy patients are both characterized by a strong T-cell response to *M. leprae* which limits infection in healthy individuals but which may be pathogenic to the host in tuberculoid patients. Lepromatous leprosy is typically characterized by a specific T-cell nonresponsiveness to *M. leprae* and is associated with dissemination of the leprosy bacillus throughout the host.

To identify the molecules of *M. leprae* involved in these immune responses, biochemical purification procedures and

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TABLE 1. M. leprae *antigens used in this study.*

Name	Description	Reference
M. leprae	Sonicate of *M. leprae* (batch CD179) derived from armadillo tissue	
M. tuberculosis	Sonicate of *M. tuberculosis*	
hsp10	Recombinant *M. leprae* 10-kDa heat-shock antigen	15
hsp18	Recombinant *M. leprae* 18-kDa heat-shock antigen	5
hsp65	Recombinant *M. leprae* 65-kDa heat-shock antigen	14
L1	Recombinant fusion protein containing amino acids 295–408 of a 45-kDa *M. leprae*-specific antigen	24, 25
L2	Recombinant fusion protein of 154-kDa	25
LL2	Recombinant fusion protein containing C-terminal 188 amino acids of the secreted *M. leprae* 30–31-kDa antigen 85 complex component A	22
L7	Recombinant fusion protein containing amino acids 55–266 of the secreted *M. leprae* 30–31-kDa antigen 85 complex component B	25, 28
L8	Recombinant fusion protein containing amino acids 23–112 of a *M. leprae* 15-kDa antigen	25, 26
L14	Recombinant fusion protein containing amino acids 58–287 of the secreted 25.5-kDa (43L) antigen of *M. leprae*	25, 31
L43	Recombinant fusion protein of 140-kDa	25
L44	Recombinant fusion protein containing amino acids 265–327 of the secreted *M. leprae* 30–31-kDa antigen 85 complex component B	25, 28

screening of recombinant DNA expression libraries with monoclonal antibodies and patient sera have led to the identification of a large number of protein antigens (reviewed in [33]). Analyses of T-cell responses of subjects exposed to *M. leprae* thus far have indicated that heat-shock proteins (hsp) of 10 kDa, 18 kDa, 65 kDa and 70 kDa, as well as a 36-kDa protein and a family of 30/31-kDa secreted fibronectin-binding proteins are important T-cell antigens ([2, 6, 8, 10, 11, 15–18]). However, the contributions of the various antigen-specific T-cell responses to protection or pathogenesis of leprosy remain unclear.

The present study was initiated to examine the ability of T cells as well as B cells to respond to a panel of *M. leprae* protein antigens in patients and healthy controls in leprosy-endemic regions of Ethiopia. The results demonstrate that distinct differences do exist between the abilities of individual antigens to stimulate proliferation (and antibody production) in the subjects studied. However, these antigen-specific responses did not show a clear association with leprosy status. Thus, no evidence was found for a potential role of particular antigens in the induction of protection or in the pathogenesis of leprosy. Secondly, it was established that the specific nonresponsiveness to *M. leprae* sonicates in lepromatous (LL) leprosy patients is not reflected by specific nonresponsiveness to any of the *M. leprae* antigens tested.

MATERIALS AND METHODS

Patients and contacts. Forty-seven leprosy patients, classified according to Ridley-Jopling criteria, were included in this study. Thirty-five patients were recruited from Addis Ababa and the surrounding Ethiopian highlands by the ALERT Leprosy Control Unit. Twelve patients were recruited from the Rift Valley near Sashamene by the National Leprosy Control Programme which is funded by the German Leprosy Relief Association (GLRA). Patients were grouped as paucibacillary [borderline tuberculoid leprosy (BT) = 26; polar tuberculoid leprosy (TT) = 3] and as multibacillary [lepromatous leprosy (LL) = 18]. Forty-two of the patients studied were receiving multiple drug treatment (MDT); five BT patients (BT20, 38, 63, 66, 70) and one TT patient (TT53) were studied before treatment. Twenty-one healthy household contacts (HC) of leprosy patients living in a rehabilitation village near the ALERT hospital in Addis Ababa were studied: 18 contacts were Mitsuda positive, 1 was negative and 2 were not tested. The household contacts were not related to any of the patients involved in this study.

Antigens. The antigens used in this study are listed in Table 1. Purified recombinant proteins *M. leprae* hsp10 and hsp65 were

obtained from J. van Embden through the WHO/TDR/IMMLEP Special Programme. *M. leprae* sonicates were obtained from R. Rees; *M. tuberculosis* sonicates from A. Kolk; purified *M. leprae* hsp18 from J. Watson. *Escherichia coli* strains carrying pEX2 containing *M. leprae* DNA inserts derived from λgt11 recombinants L1, L2, LL2, L7, L8, L14, L43, and L44 ([21, 24]) were established as described ([28]). Semipurified crobeta-galactosidase fusion proteins were prepared from induced lysates as described ([27]). Fusion proteins were approximately 50% pure, as estimated by the protein profiles from SDS/PAGE gels stained with Coomassie brilliant blue.

Peripheral blood mononuclear cells (PBMC) and sera. PBMC were collected by venipuncture using heparinized vacutainer tubes (Becton, Dickinson, Mechelen, Belgium). PBMC were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) centrifugation and resuspended in culture medium containing RPMI-1640 medium (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) supplemented with penicillin (100 IU/ml)BCO, Gaithersburg, Maryland, U.S.A.) and glutamine (2 mM; ICN Flow, High Wycombe, U.K.). Serum was separated from whole blood and stored, without preservative, at −70°C until use.

Proliferation assay. Isolated PBMC (10^5 cells/well) were cultured in 96-well, round-bottom, microtiter plates (Costar Corporation, Cambridge, Massachusetts, U.S.A.) in culture medium containing 10% pooled human serum and antigen. Sonicates of *M. leprae* and *M. tuberculosis* were used at two different concentrations of 1 μg/ml and 10 μg/ml; purified recombinant *M. leprae* antigens at concentrations of 2 μg/ml and 20 μg/ml. Semipurified fusion proteins were used at dilutions of 1/100 and 1/5000. Control wells contained either phytohemagglutinin (PHA, 2 μg/ml; Wellcome Diagnostics, Dartford, U.K.), semipurified crobeta-galactosidase (nonfused) proteins, or culture medium alone. Cultures were set up in triplicate and incubated for 6 days at 37°C in a fully humidified atmosphere containing 5% CO_2. For the last 18 hr, the cultures were pulsed with 1.0 μCi of ^{3}H-thymidine (specific activity, 5.0 μCi/mmol; Amersham International, Amersham, U.K.) per well. The cells were then harvested onto glass-fiber filters with a semi-automatic sample harvester. ^{3}H-thymidine incorporation was assessed by liquid scintillation spectroscopy. The results of the responses to sonicates of *M. leprae, M. tuberculosis* and to the purified proteins hsp10, hsp18 and hsp65 are expressed as a stimulation index (SI) which is the ratio of ^{3}H-thymidine incorporation of antigen-stimulated cultures to that of the control cultures, containing neither antigen nor PHA. The results of the responses to the (semi)purified recombinant fusion proteins L1, L2, LL2, L7, L8, L14, L43 and L44 are expressed as SI, which is the ratio of ^{3}H-thymidine incorporation of antigen-stimulated cultures to that of cultures containing semipurified crobeta-galactosidase proteins as expressed by pEX2 vector. A SI of ≥3 was taken as a positive response. The average PHA responses were 26, 805 counts per minute (cpm) for the 21 HC, 25,031 cpm for the 29 BT/TT patients, and 19, 453 cpm for the 18 LL patients.

Immunoblotting and ELISA. Eight percent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli ([9]). Either 200 ng purified hsp10 or 50 ng purified hsp65 were loaded per lane. Gels were blotted onto nitrocellulose membranes (BA-83; Schleicher & Schuell, Dassel, Germany) using a 0.2 amp current for 2 hr in blotting buffer according to Maniatis ([13]). The nitrocellulose membranes were blocked overnight with 50 mM Tris-HCl pH 8.0; 150 mM NaCl; 5% ovalbumin; 0.01% NaN3 at room temperature prior to incubation with antisera. Sera were tested at 1/100 (HC and BT/TT) or 1/500 (LL) dilutions.

ELISA was performed essentially as described previously ([12]). Sonicates of *M. leprae* and *M. tuberculosis* and hsp10 and hsp65 antigens were tested at 200 ng/well; and phenolic glycolipid-I (PGL-I) coupled to human serum albumin was tested at 50 ng/well and compared to uncoupled human serum albumin (50 ng/well). Sera were tested at 1/100 dilutions for antibodies reactive with hsp10 and hsp65, and at dilutions of 1/150, 1/400 and 1/600 for sonicates of *M. leprae* and *M. tuberculosis*. The serum samples were added to quadruplicate wells of the microtiter plate (Dynatech Immulon): two with antigen and two without antigen. The plates were incubated at room temper-

ature for 2 hr. Alkaline phosphatase conjugated F(ab')2 fragments of affinity isolated anti-human gamma or anti-human mu chain or anti-human gamma, alpha and mu chain (Sigma) were used as secondary antibodies. P-nitrophenylphosphate disodium (Sigma 104 phosphatase substrate tablets) was used as a substrate and the reaction was stopped by the addition of 4 N NaOH after 20 min. Absorbances were read at 405 nm using a Titertek Multiscan spectrophotometer (Flow Labs, Richmond, Virginia, U.S.A.), and mean absorbance values were calculated. The antibody reactivity to the coated antigen for each serum sample was calculated by subtracting the mean absorbance of duplicate samples in buffer-coated wells from the mean absorbance of duplicate antigen-coated wells. Delta absorbances of ≥ 0.20 were considered positive.

RESULTS

Proliferative T-cell responses to *M. leprae* antigens. Pilot experiments demonstrated that freshly isolated human peripheral mononuclear cells (PBMC) gave significantly higher T-cell proliferative responses than did frozen PBMC upon stimulation with *M. leprae* and control antigens (data not shown). Therefore, we performed our studies on fresh PBMC within 48 hr after collection of blood samples. Tables 2A-2C summarize the T-cell proliferative responses of the subjects studied, grouped according to their disease status (HC, BT/TT and LL). Three purified *M. leprae* antigens (hsp10, hsp18 and hsp65) and eight semipurified *M. leprae* proteins fused to *E. coli* crobeta-galactosidase were tested.

High frequencies of responses to *M. leprae* sonicates were found in HC (95%) and in BT/TT subjects (72%). Only 11% of the Ll patients studied responded to *M. leprae* sonicate, a finding in agreement with the previously reported *M. leprae*-specific nonresponsiveness at the lepromatous pole of the leprosy spectrum. In all three subject groups the frequency of responses to *M. tuberculosis* sonicates was high (90%).

A large variability in responses to the three purified and eight semipurified recombinant *M. leprae* proteins tested was found in the three subject groups. For example, BT patient 49 recognized 6 of 11 T-cell antigens versus BT patient 45 who recognized 0 of 11 T-cell antigens.

Among the purified *M. leprae* antigens, hsp65 was most frequently recognized in HC (33%), followed by hsp10 (19%) and hsp18 (10%). In patients the frequencies of proliferative T-cell responses to these three antigens were more similar (7%-10% in BT/TT patients; 11%-22% in LL patients). Among the semipurified *M. leprae* antigens the frequencies of T-cell responses were highest to antigens L14 (6%-19%), LL2 (14%-17%) and L7 (6%-14%), all of which are secreted. Frequencies of recognition to the other, nonsecreted antigens ranged from 0% to 11%. None of the antigens was shown to be exclusively recognized by any of the three subject groups, which would suggest that none of these antigens played a particular role in protection or in disease.

With respect to MDT treatment, no correlation could be demonstrated between duration of treatment and recognition profiles of any tested recombinant *M. leprae* antigen. No differences in T-cell responses to any *M. leprae* antigens could be detected between MDT untreated and treated individuals.

B-cell recognition of *M. leprae* antigens. To evaluate the antibody responses to purified hsp10 and hsp65 antigens, we employed ELISA and Western blotting techniques (Tables 3A–3C). All LL patients (100%) and the majority of BT/TT patients (86%) and HC (90%) reacted to *M. leprae* sonicates in ELISA. With respect to *M. tuberculosis*, serum antibodies were detected in 78% of LL patients, 41% of BT/TT patients and 60% of HC.

Combining the ELISA and Western blotting results, antibodies to *M. leprae* hsp10 and hsp65 appeared more frequently in the LL group than in the BT/TT and the HC groups. The fact that in the LL group antibodies to hsp65 were only detected by Western blotting and not by ELISA suggests that these antibodies are mainly directed to linear determinants. The existence of mainly linear determinants on hsp65 has been reported previously, and is thought to reflect its predominant existence as an unfolded molecule which is more amenable to proteolytic degradation ([34]). Comparisons of T-cell versus B-cell responses in the three groups of subjects studied did not reveal any

TABLES 2A–2C. *T-cell proliferative responses of Ethiopian HC (Table 2A), BT/TT (Table 2B) and LL (Table 2C) to purified recombinant* M. leprae *heat-shock proteins (hsp18, hsp19, and hsp65), semipurified recombinant* M. leprae *antigens (L1, L2, LL2, L7, L8, L14, L43 and L44) and (as controls) PHA, sonicates of* M. leprae *(M. lep) and* M. tuberculosis *(M. tub). Patient information is shown in terms of Mitsuda tests (Mit), years of multidrug treatment (MDT), the areas where the subjects live (H = highlands, L = lowlands) and IgM antibodies against PGL-I as tested by ELISA. T-cell responses are given as SI values, calculated as described in Materials and Methods. Of the various antigen concentrations tested, the highest SI values are shown. SI values of ≥3.0 are considered as positive responses; SI values of <3.0 are represented by −. % = Percent positivity in each subject group.*

TABLE 2A. *Healthy contacts.*

subject	Mit	area	PGL1	PHA	M.lep	M.tub	hsp10	hsp18	hsp65	L1	L2	LL2	L7	L8	L14	L43	L44
HC02	+	H	-	15.5	-	-	-	-	-	-	-	-	-	-	3.2	-	-
HC03	+	H	-	65.6	11.1	36.8	-	-	-	-	-	-	4.3	-	-	-	-
HC04	ND	H	-	123.6	6.5	23.8	-	-	-	-	-	-	-	-	-	-	-
HC05	+	H	-	18.4	11.2	12.1	-	-	-	-	-	-	-	-	-	-	-
HC10	-	H	-	40.9	20.4	37.2	6.1	5.2	9.4	-	-	-	-	-	-	-	-
HC11	+	H	-	14.7	10.9	24.2	-	-	3.6	-	-	-	-	-	-	-	-
HC12	+	H	-	46.6	4.3	15.3	-	-	-	-	-	-	-	-	-	-	-
HC13	-	H	-	37.2	8.2	37.4	-	-	3.2	-	-	3.8	-	-	-	-	-
HC14	+	H	-	29.0	3.1	19.4	-	-	-	-	-	-	-	-	-	-	-
HC15	+	H	-	10.9	3.8	25.2	-	-	-	-	-	-	-	-	-	-	-
HC16	+	H	-	16.0	10.7	27.7	-	3.3	-	-	-	-	-	-	-	-	-
HC17	+	H	-	46.6	25.0	28.4	-	-	-	-	-	-	-	-	-	-	-
HC21	+	H	-	1.7	21.2	26.2	-	-	5.8	3.7	5.9	7.2	-	-	7.8	-	-
HC23	+	H	-	13.1	4.4	7.5	-	-	-	-	-	-	-	-	-	-	-
HC24	+	H	-	2.3	27.2	22.2	10.1	-	-	-	-	-	4.3	-	-	-	-
HC25	+	H	-	5.6	9.0	34.4	-	-	-	5.1	-	-	-	-	3.7	3.4	-
HC40	+	H	-	16.7	17.6	51.1	3.9	-	7.2	-	-	3.2	4.0	-	-	-	-
HC41	ND	H	-	4.1	18.9	30.6	-	-	5.0	-	-	-	-	-	3.4	-	-
HC42	ND	H	-	9.6	51.9	72.2	5.2	-	12.8	-	-	-	-	-	-	-	-
HC43	+	H	-	3.0	20.4	50.9	-	-	-	-	-	-	-	-	-	-	-
HC44	+	H	-	6.8	13.1	18.7	-	-	-	-	-	-	-	-	-	-	-
%					95	95	19	10	33	10	5	14	14	0	19	5	0

TABLE 2B. *Tuberculoid leprosy patients.*

subject	MDT	area	PGL1	PNA	M.lep	M.tub	hsp10	hsp18	hsp65	L1	L2	LL2	L7	L8	L14	L43	L44
BT01	1	H	-	60.1	13.8	16.7	-	-	-	-	-	-	-	-	-	-	-
BT18	1.5	H	-	23.6	7.1	6.6	-	-	-	-	-	-	-	-	4.5	-	-
TT19	1	H	-	56.3	22.6	30.1	-	-	-	-	-	4.5	3.0	-	-	3.1	-
BT20	-	H	-	3.8	31.6	43.0	-	-	3.5	4.4	-	3.3	-	-	-	-	-
BT22	?	H	-	ND	18.3	24.8	-	3.0	-	-	-	-	-	-	3.0	-	-
BT38	-	L	-	14.1	3.5	7.7	-	-	-	-	-	-	-	-	-	-	-
BT39	0.5	L	-	15.7	4.9	37.2	-	-	-	-	-	3.6	-	-	-	-	-
BT45	0.4	L	-	66.3	8.7	9.2	-	-	-	-	-	-	-	-	-	-	-
BT46	1.5	L	-	210.4	-	-	-	-	-	-	-	-	-	-	-	-	-
BT47	0.2	L	+	120.7	-	18.9	-	-	-	-	-	-	-	-	-	-	-
BT48	0.2	L	-	132.5	7.7	11.8	-	-	-	-	-	4.1	-	-	-	-	-
BT49	0.2	L	-	29.8	10.0	8.7	-	-	4.0	3.1	3.8	-	-	3.6	3.9	-	4.6
BT50	2	L	-	63.0	13.3	44.2	-	5.7	5.7	-	-	-	-	-	-	-	-
BT51	1	L	-	23.6	-	7.7	-	-	-	-	-	-	-	-	-	-	-
BT52	1.5	L	-	62.3	-	3.1	-	-	-	-	-	-	-	-	3.2	-	3.8
TT53	-	L	-	30.9	3.9	28.3	-	-	-	-	-	-	-	-	-	-	-
BT54	0.2	L	-	114.1	9.5	3.7	-	-	-	-	-	-	-	-	-	-	-
BT55	0.2	L	-	27.3	-	5.9	-	-	-	-	-	-	-	-	-	-	-
BT56	1.5	L	-	70.3	-	71.4	-	-	-	-	3.1	-	-	-	-	-	-
BT62	0.2	H	-	36.4	8.4	5.3	-	-	-	-	-	-	-	-	-	-	-
BT63	-	H	-	44.1	6.6	9.8	3.4	-	-	-	-	-	-	-	-	-	-
TT65	0.1	H	-	10.1	3.4	6.0	-	-	-	-	-	-	-	-	-	-	-
BT66	-	L	-	31.6	3.2	41.8	-	-	-	-	-	-	-	-	-	-	-
BT67	0.5	L	-	109.6	31.3	18.1	-	-	-	-	-	-	-	-	-	-	-
BT68	0.5	L	-	361.7	4.2	44.7	3.3	4.2	-	-	-	-	-	-	-	-	-
BT69	0.5	L	-	145.0	9.4	13.8	-	-	-	-	-	-	-	-	-	-	3.5
BT70	-	L	-	103.1	-	-	-	-	-	-	-	-	5.6	-	-	-	-
BT71	0.5	L	-	60.2	-	42.6	-	-	-	3.3	-	-	-	-	-	-	-
BT72	0.5	L	-	29.8	23.5	46.3	-	-	-	-	-	-	9.6	-	-	-	-
\					72	93	7	10	10	10	7	14	10	3	14	3	10

TABLE 2C. *Lepromatous leprosy patients.*

subject	MOT	area	PGL1	PHA	M.lep	M.tub	hsp10	hsp18	hsp65	L1	L2	LL2	L7	L8	L14	L43	L44
LL06	8	H	+	76.9	-	22.3	-	-	-	-	-	-	-	-	-	-	-
LL07	3.2	H	-	60.9	-	34.3	-	-	-	-	-	-	-	-	-	-	-
LL08	6	H	-	72.4	-	5.3	-	-	-	-	-	-	-	-	-	-	-
LL09	4.5	H	-	74.5	-	38.0	-	-	-	-	-	3.4	-	3.2	-	-	-
LL26	0.8	H	+	14.9	-	27.7	-	-	-	-	-	-	3.4	-	-	-	3.2
LL27	1.5	H	+	20.9	-	37.6	6.0	3.8	3.3	-	-	-	-	-	-	-	-
LL28	5	H	+	11.5	-	-	-	-	-	-	-	-	-	-	-	-	-
LL29	7.5	H	-	15.6	-	18.8	-	-	-	-	-	-	-	-	-	-	-
LL30	3.5	H	-	26.9	-	10.1	3.0	-	-	-	-	-	-	-	-	-	-
LL31	0.3	H	+	5.1	-	29.1	-	-	-	-	-	-	-	-	-	-	-
LL32	6	H	+	6.6	-	7.0	-	-	-	-	-	-	-	-	-	-	-
LL33	8	H	-	8.3	7.0	56.6	6.5	4.7	7.2	-	-	-	-	-	-	-	-
LL34	4	H	+	8.2	3.8	5.8	3.3	-	3.6	-	-	3.0	-	-	3.3	-	-
LL35	1.5	H	+	14.5	-	11.2	-	-	-	-	-	-	-	-	-	-	-
LL37	2	L	-	50.5	-	43.3	-	-	-	-	-	-	-	-	-	-	-
LL57	1.8	H	+	97.3	-	-	-	-	-	-	-	-	-	-	-	-	-
LL58	4.5	H	+	29.2	-	3.7	-	-	-	-	-	3.2	-	6.6	-	-	-
LL60	21	H	-	62.5	-	39.8	-	-	-	-	-	-	-	-	-	-	-
%					11	89	22	11	17	0	0	17	6	11	6	0	6

TABLE 3A. *Healthy contacts.*

	ELISA				W.blot	
subject	M.lep	M.tub	hsp10	hsp65	hsp10	hsp65
HC02	0.49	0.32	-	-	-	-
HC03	0.39	0.28	-	-	-	-
HC04	0.29	0.26	-	-	-	-
HC05	0.22	-	-	-	-	-
HC10	0.27	-	-	-	-	++
HC11	0.25	-	-	-	-	-
HC12	0.68	0.50	-	-	-	-
HC13	0.45	0.37	-	-	-	+
HC14	-	-	-	-	-	-
HC15	0.54	0.21	-	-	-	-
HC16	0.40	0.28	-	-	-	-
HC17	0.24	-	-	-	-	-
HC21	0.33	0.28	-	0.28	-	-
HC23	ND	ND	-	-	ND	-
HC24	0.51	0.45	-	-	-	-
HC25	0.30	0.23	-	-	-	-
HC40	0.24	-	-	-	ND	-
HC41	0.32	0.23	-	-	-	-
HC42	-	-	-	-	-	-
HC43	0.30	0.27	-	-	-	-
HC44	0.23	-	-	-	-	+
%	90	60	0	5	0	14

TABLE 3B. *Tuberculoid leprosy patients.*

	ELISA				W.blot	
subject	M.lep	M.tub	hsp10	hsp65	hsp10	hsp65
BT01	0.21	0.25	-	-	+	-
BT18	0.41	-	-	-	-	-
TT19	0.38	0.32	-	-	-	-
BT20	0.32	0.24	-	-	-	+
BT22	0.28	0.21	-	-	-	-
BT38	0.33	-	-	-	-	-
BT39	0.23	-	-	-	-	-
BT45	-	-	-	-	-	-
BT46	0.51	0.29	-	-	-	-
BT47	0.62	0.37	-	-	-	-
BT48	0.25	-	-	-	-	-
BT49	-	-	-	-	-	-
BT50	0.38	0.21	-	-	-	-
BT51	0.30	-	-	-	-	-
BT52	0.23	-	-	-	-	-
TT53	0.39	0.22	-	-	-	-
BT54	0.25	-	-	-	-	-
BT55	0.24	0.21	-	-	-	-
BT56	0.25	-	-	-	-	-
BT62	0.31	0.21	-	-	-	-
BT63	-	-	-	-	-	-
TT65	-	-	-	-	-	-
BT66	0.41	0.33	-	-	-	-
BT67	0.21	-	-	-	-	-
BT68	0.32	-	-	-	-	-
BT69	0.32	0.28	-	-	-	-
BT70	0.25	-	-	0.21	-	+
BT71	0.24	-	-	-	-	-
BT72	0.20	-	-	-	-	-
%	86	41	0	3	3	7

TABLE 3C. *Lepromatous leprosy patients.*

	ELISA				W.blot	
subject	M.lep	M.tub	hsp10	hsp65	hsp10	hsp65
LL06	0.61	0.35	-	-	-	-
LL07	0.55	0.35	-	-	-	-
LL08	0.41	0.31	-	-	-	-
LL09	0.72	0.40	-	-	-	-
LL26	0.69	0.43	-	-	-	-
LL27	0.54	0.27	-	-	-	-
LL28	0.59	0.30	0.28	-	+	-
LL29	0.63	0.29	-	-	-	++
LL30	0.62	0.34	-	-	-	-
LL31	0.74	0.43	0.33	-	+	++
LL32	0.50	0.23	-	-	+	+
LL33	0.48	0.22	-	-	-	-
LL34	0.62	0.38	-	-	-	+
LL35	0.59	0.29	-	-	-	+
LL37	0.57	0.33	0.35	-	+	+
LL57	0.51	0.24	-	-	-	++
LL58	0.48	-	0.42	-	+	++
LL60	0.28	-	-	-	-	+
%	100	78	22	0	28	50

TABLES 3A–3C. *Antibody responses as measured by ELISA and Western blots of HC (Table 3A), BT/TT (Table 3B), and LL (Table 3C) to sonicates of* M. leprae *(M. lep), M. tuberculosis (M. tub) and purified* M. leprae *hsp10 and hsp65 proteins. Western blot information was interpreted by visual inspection; ELISA values are given in Δ absorbance values. Δ absorbances of <0.20, which are considered negative responses, are represented by −. ND = Not done.*

striking correlations (Tables 2A-2C versus 3A-3C).

DISCUSSION

In this study we have compared immune responses to 11 *M. leprae* antigens in 21 healthy household contacts and 47 leprosy patients from Ethiopia, a country where leprosy is endemic.

A major question we wanted to address by this study was whether there is a role for any particular *M. leprae* antigen in either immunopathology or protection as judged by T-cell proliferation. A role for secreted antigens in protective immunity has been proposed since these antigens are actively secreted by live bacteria and, thus, are available for immune recognition early in infection.

A limited amount of protection in an animal model for tuberculosis after immuni-

zation with secreted proteins was recently reported [20]. In the present study, we found that the response to secreted antigens, i.e., 30/31-kDa and 25.5-kDa antigens, is not restricted to any particular patient group and, thus, is not predictive for protection or pathogenesis. Our study confirms earlier data [3, 4, 11] that secreted antigens frequently induce proliferative responses [23, 31]. However, the responses toward heat-shock proteins (hsp) were found to be equally high, and this finding indicates that antigens from different compartments serve as frequent targets for proliferative T cells. Mycobacterial hsp have been implicated in immunopathological phenomena [21]. Autologous hsp may form a target for T cells that were originally raised to structurally similar *M. leprae* hsp molecules. Proliferative T cells specific for mycobacterial hsp65 have been shown to induce autoimmune phenomena in a rat model [30], and it has been hypothesized that a similar reactivity may be involved in some of the immunopathological phenomena associated with leprosy [11]. Although a considerable proportion of the subjects in our study recognized hsp65 and other hsp, no support for a particular role for hsp in the induction of immunopathology was found, and hsp were also frequently recognized by healthy individuals.

Thus, with regard to hsp and secreted antigens and the other antigens studied, we found an overall similarity in the antigenic repertoire recognized in healthy individuals and patients. Although a different picture may emerge when using different read-out systems, like cytotoxicity or cytokine production, this similarity in antigen recognition may indicate that the key to distinguishing protective from disease-associated immunity may lie in the exact nature of the T-cell response. It may be that the magnitude, quality and, perhaps, timing of the T-cell response rather than the recognized antigenic repertoire ultimately determines the outcome of an infection with *M. leprae*.

A potentially important finding in this study was that T-cell nonresponsiveness of 5 LL patients (LL09, 26, 27, 30, 58), 4 BT patients (BT52, 56, 70, 71) and 1 HC (HC02) to *M. leprae* sonicates is not reflected by nonresponsiveness to individual antigens of *M. leprae* (Tables 2B-2C). For example, the *M. leprae* nonresponder LL27 showed significant T-cell proliferation to all three purified *M. leprae* hsp and the nonresponders LL09, LL26, LL58 and BT52 responded to two semipurified crobeta-galactosidase *M. leprae* fusion proteins. The above finding confirms previous work for recombinant *M. leprae* antigens [16, 18, 26], and opens up possibilities to study the mechanisms underlying specific nonresponsiveness to whole *M. leprae*. The outcome of such studies may, ultimately, be helpful in the design of immunotherapy aimed at triggering antigen-specific immunity in nonresponsive leprosy patients.

SUMMARY

The recognition of a panel of recombinant *Mycobacterium leprae* antigens by T cells and B cells from 29 borderline tuberculoid/tuberculoid (BT/TT) and 18 lepromatous leprosy (LL) patients and from 21 healthy controls (HC) in leprosy-endemic regions of Ethiopia was examined. All 11 antigenic molecules tested (including *M. leprae* hsp10, hsp18, hsp65 and several novel *M. leprae* antigens) were shown to be recognized by T cells, but clear quantitative differences existed between reactivities induced by individual antigens. Similar quantitative differences were observed when antibody responses to hsp10 and hsp65 antigens were determined. No associations were found between the antigen-specific responses and the subject status of either BT/TT and LL patients or HC. Fifteen percent of the patients who were nonresponsive to sonicates of *M. leprae* showed significant T-cell responses to one or more individual *M. leprae* antigens. This indicates that *M. leprae* constituents other than the proteins tested are responsible for the *M. leprae*-specific nonresponsiveness in these patients, which may be exploited for the design of vaccines or immunotherapeutic modalities aimed at inducing *M. leprae*-specific immunity in nonresponders.

RESUMEN

Se examinó el reconocimiento de un panel de antígenos recombinantes de *Mycobacterium leprae* por las células T y B de 29 pacientes con lepra tuberculoide/tuberculoide subpolar (BT/TT), de 18 pacientes con lepra lepromatosa (LL), y de 21 controles sanos (HC)

de regiones con lepra endémica en Etiopia. Las 11 moléculas antigénicas probadas (incluyendo hsp10, hsp18, hsp65 y varios antígenos nuevos de *M. leprae*) fueron reconocidos por las células T, aunque existieron claras diferencias cuantitativas entre las reactividades inducidas por los antígenos individuales. También se observaron diferencias cuantitativas similares en el caso de las respuestas en anticuerpo con los antígenos hsp10 y hsp65. No se encontraron asociaciones entre las respuestas específicas para un antígeno y el estado BT/TT, LL, o HC de los individuos. Quince porciento de los pacientes que no respondieron a sonicados totales de *M. leprae* mostraron significantes respuestas celulares (Lc T) hacia uno o más antígenos individuales de *M. leprae*. Se concluye que no son las proteínas probadas en este estudio, sino otros constituyents de *M. leprae*, los responsables de la anergia específica que muestran los pacientes hacia el bacilo de la lepra. Esto podría ser explotado en el diseño de vacunas o de modalidades inmunoterapéuticas tendientes a inducir inmunidad específica hacia *M. leprae* en los individuos no respondedores.

RÉSUMÉ

La reconnaissance d'un panel d'antigènes recombinants de *Mycobacterium leprae* par des cellules T et B provenant de 29 patients lépreux borderline tuberculoides et tuberculoides (BT/TT), de 18 patients lépromateux (LL) et de 21 témoins en bonne santé de régions d'Ethiopie endémiques pour la lèpre a été examinée. On a montré que toutes les 11 molécules antigéniques testées (y compris hsp10, hsp18, hsp65 et plusieurs antigènes "nouveaux" de *M. leprae*) étaient reconnues par les cellules T, mais que des différences quantitatives évidentes existaient dans la réactivité induite par les antigènes individuels. De semblables différences quantitatives ont été observées quand on a déterminé les réponses en anticorps aux antigènes hsp10 et hsp65. Aucune association n'a été trouvée entre les réponses spécifiques pour l'antigène et le statut individuel des personnes, que ce soient des patients BT/TT ou LL ou des individuels en bonne santé. Quinze pourcents des personnes qui ne répondaient pas aux sonicats de *M. leprae* montraient des réponses significatives de leurs cellules T à un ou plusieurs antigènes individuels de *M. leprae*. Ceci indique que des constituants de *M. leprae* autres que les protéines testées sont responsables de la non-réponse spécifique pour *M. leprae* chez ces patients, ce qui pourrait être exploité pour la conception de vaccins ou de moyens immunothérapeutiques ayant pour but d'induire une immunité spécifique pour *M. leprae* chez les non-répondants.

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Identification of an Antigenic Domain on *Mycobacterium leprae* Protein Antigen 85B, Which Is Specifically Recognized by Antibodies from Patients with Leprosy

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Sixty-three overlapping 15-oligomer peptides covering the 30-kDa protein antigen 85B of *Mycobacterium leprae* were tested by ELISA to identify epitopes recognized by human antibodies. Serum samples from patients with lepromatous leprosy (LL) reacted mainly with peptides comprising amino acid regions (AA) 206–230, 251–280, and 291–325. Sera of patients with active tuberculosis who responded to the native 30-kDa antigen did not recognize these peptides. The antibody-binding specificity to the defined B cell regions was evaluated in a blind study with 71 serum samples from patients and household contacts living in Ethiopia where leprosy is endemic. The peptide of AA 256–280 was recognized by 88% of LL patients, 15% of patients with tuberculoid leprosy, and none of the contacts. These findings suggest that there are major linear B cell epitopes on the *M. leprae* 30-kDa protein that are recognized by lepromin-negative LL patients, whereas lepromin-positive patients respond preferentially to conformational epitopes.

Through the application of molecular biologic tools and recombinant DNA expression technology, a number of protein antigens of *Mycobacterium leprae* have been characterized [1]. Identification of antigenic determinants on immunodominant proteins will help in understanding the molecular mechanisms involved in the immune response to mycobacteria and may ultimately lead to the development of novel vaccines and immunodiagnostic reagents.

The 30- to 31-kDa proteins are major secreted antigens of *M. leprae*, as well as of *Mycobacterium tuberculosis* and *Mycobacterium bovis* bacille Calmette-Guérin, and correspond to the antigen 85 complex defined by crossed immunoelectrophoresis (reviewed in [2]). This group of proteins is secreted by mycobacteria during growth and constitute 20%–30% of all proteins in culture filtrates [3, 4]. The 85 complex consists of three antigenically and structurally related components, 85A, 85B, and 85C [5, 6]; the genes coding for the A–C components have been cloned from several mycobacterial species [7–14]. We have shown that members of the 30-kDa protein family have the ability to bind to fibronectin and, in a cell-associated form, can mediate attachment of whole bacteria to fibronectin-coated surfaces [12, 15, 16]. It has been suggested that binding to fibronectin may play a role in attachment and entry of mycobacteria into host cells.

The antigen 85 complex is a prominent target of the humoral and cellular immune responses against mycobacteria [17–22]. Recently, we reported the molecular analysis of *M. leprae* protein antigen 85B and the serologic activity of the recombinant fusion protein (amino acid [AA] 55–266) expressed in *Escherichia coli* with sera from patients with leprosy [12]. To define the epitopes of *M. leprae* antigen 85B recognized by human antibodies and to determine whether peptide sequences would be useful for serodiagnosis and monitoring of leprosy patients, we prepared overlapping synthetic peptides spanning the entire protein and tested them for antibody reactivity by ELISA. We used the *M. tuberculosis* homologous protein to analyze serum samples from patients with leprosy and from control subjects for antibody to the whole protein and to compare the reactivity of sera to native protein with that to peptides derived from the *M. leprae* 30-kDa protein.

Materials and Methods

Patients and controls. The preliminary study was done with serum samples obtained from a mixed population of 41 patients with lepromatous leprosy (LL) attending the Hospital for Tropical Diseases (London). Sera had been previously tested, and the

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Informed consent was obtained from the patients or their parents or guardians.

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13 sera selected were found to give a strong response to antigen 85B of *M. tuberculosis*. Sera were collected from newly diagnosed patients either before (patient LL3) or after a few days of treatment (LL1 and LL2). Patient LL4 suffered from a severe bacterial relapse, and blood was taken 1 year after the start of multidrug therapy and in subsequent years. The remaining sera were collected from patients receiving treatment for 2 years (LL7) or for several years (LL5, LL6, LL10, and LL11), 17 years (LL8), 21 years (LL9), and 28 years (LL12), and from patient LL13 who had already completed drug therapy. Control sera were donated by 5 healthy laboratory employees at University College London Medical School.

In the large study, serum samples were collected from Ethiopian patients at ALERT Leprosy Control (ALC) and the Armauer Hansen Research Institute in Addis Ababa. There were 17 LL patients who had had leprosy for an average of 8.7 years and 33 patients with borderline (BT) or tuberculoid leprosy (TT) for ~2 years. Also, 21 healthy household contacts who were not related to the patients studied were included. All patients with leprosy in both studies were classified clinically and histologically according to the Ridley-Jopling scale [23].

Serum samples from patients with smear-positive pulmonary or extrapulmonary tuberculosis were provided by J. Ivanyi (Medical Research Council Tuberculosis and Related Infections Unit, Hammersmith Hospital, London).

Peptide synthesis. A complete set of 63 overlapping biotinylated peptides covering the entire sequence of *M. leprae* 30-kDa protein antigen 85B [12] were synthesized by using solid-phase methodology [24] on an Abimed AMS 422 automated multiple peptide synthesizer (Abimed Analysen-Technik, Langenfeld, Germany). Repetitive coupling was done according to standard 9-fluorenylmethoxycarbonyl (FMOC) chemistry using BOP/NMM activation and 20% piperidine FMOC removal. Peptides were cleaved from the resin with aqueous trifluoroacetic acid, then isolated and purified by ether precipitation. Peptide purity was assessed by reverse-phase high-performance liquid chromatography and mass spectrometry. The localization of these peptides within the sequence of the 30-kDa protein, consisting of an N-terminal signal peptide of 38 AA and a mature protein of 289 AA, is shown in figure 1. Peptides were 15 residues long and overlapped by 5 AA. Three nonbiotinylated peptides, 25 residues long and corresponding to AA 206–230, 256–280, and 291–315, were synthesized as described above.

Protein antigens. Antigen 85B (MPT59) was purified from the culture filtrate of *M. tuberculosis* H37Rv as previously described in detail [6] by combining ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography on DEAE-Sepharose columns. The *M. leprae* 18-kDa antigen was a recombinant preparation (provided by J. D. Watson, University of Auckland, New Zealand). The recombinant 65-kDa heat-shock protein of *M. leprae* was a gift of M. J. Colston (National Institute for Medical Research [NIMR], London). The sonicate of armadillo-grown *M. leprae* (batch CD114) was provided by R. J. W. Rees (NIMR).

ELISA. In preparation, 15- and 25-oligomer (mer) peptides were coated overnight at 37°C onto wells of Nunc-Immunoplates MaxiSorp (Nunc, Roskilde, Denmark) in 0.05 *M* carbonate buffer (pH 9.6). Final concentrations of peptides ranged from 1 to 10 μg/mL. MPT59 was coated at 37°C in 0.01 *M* PBS (pH 7.4), while *M. leprae* antigens were coated at 4°C in carbonate buffer, at an optimal concentration of 5 μg/mL. Microtiter plates were blocked for 60 min with 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20 (PBST), washed with PBST, and incubated with sera at a 1:200 dilution in BSA-PBST in duplicate for 90 min at 37°C. After further washes with PBST, peroxidase-conjugated rabbit anti-human IgG (Dako, Copenhagen) was added at a 1:1000 dilution in BSA-PBST for 60 min. The washing process was repeated, and the substrate solution was 0.5 mg/mL 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, St. Louis) in 0.1 *M* citrate-phosphate buffer (pH 4.1) containing 0.35 μL/mL 6% hydrogen peroxide. After 30 min, absorbance was read at 630 nm with a Dynatech MR5000 microplate reader (Dynatech, Guernsey, UK). The results are expressed as mean absorbance of duplicates, or as change in absorbance (ΔA), which was calculated by subtracting the mean in control wells coated with buffer alone from the mean in test wells.

Statistical analysis. Antibody levels between different groups were compared using the Mann-Whitney *U* test.

Results

Identification of antibody-binding regions on M. leprae antigen 85B using synthetic peptides. We have standardized an ELISA using synthetic peptides to identify antigenic domains within antigen 85B recognized by antibodies from patients with leprosy. In the preliminary study, we screened the complete set of 63 15-mer peptides representing overlapping sequences of protein antigen 85B of *M. leprae* with 13 LL serum samples previously found to give a strong response to MPT59. Representative antibody response patterns obtained with sera from patients LL1, LL3, and LL5 are shown in figure 2. Sera from LL patients reacted mainly with peptides covering AA 206–230, 251–280, and 291–325, indicating that these regions represent major human linear B cell epitopes on antigen 85B. Occasionally, individual sera recognized additional peptide sequences, as shown for patients LL1 and LL3 in figure 2.

Control sera from healthy donors that were negative to MPT59 remained without reactivity to synthetic peptides; a representative antibody-binding profile is shown in figure 2 (N1). Furthermore, 3 patients with BT or TT who reacted to MPT59 did not respond to any of the peptides (data not shown). Antibody responses to peptides were reproducible using three independent batches of synthetic peptides. Moreover, the peptides were effectively coated onto microtiter wells, as all were found to react with streptavidin (data not shown).

The individual antibody response values of 13 LL patients and 5 healthy donors to the 15-mer peptides comprising the 3 main antigenic domains of *M. leprae* antigen 85B are presented in figure 3. Those data were obtained from a study we later undertook to compare the seroreactivity of patients and

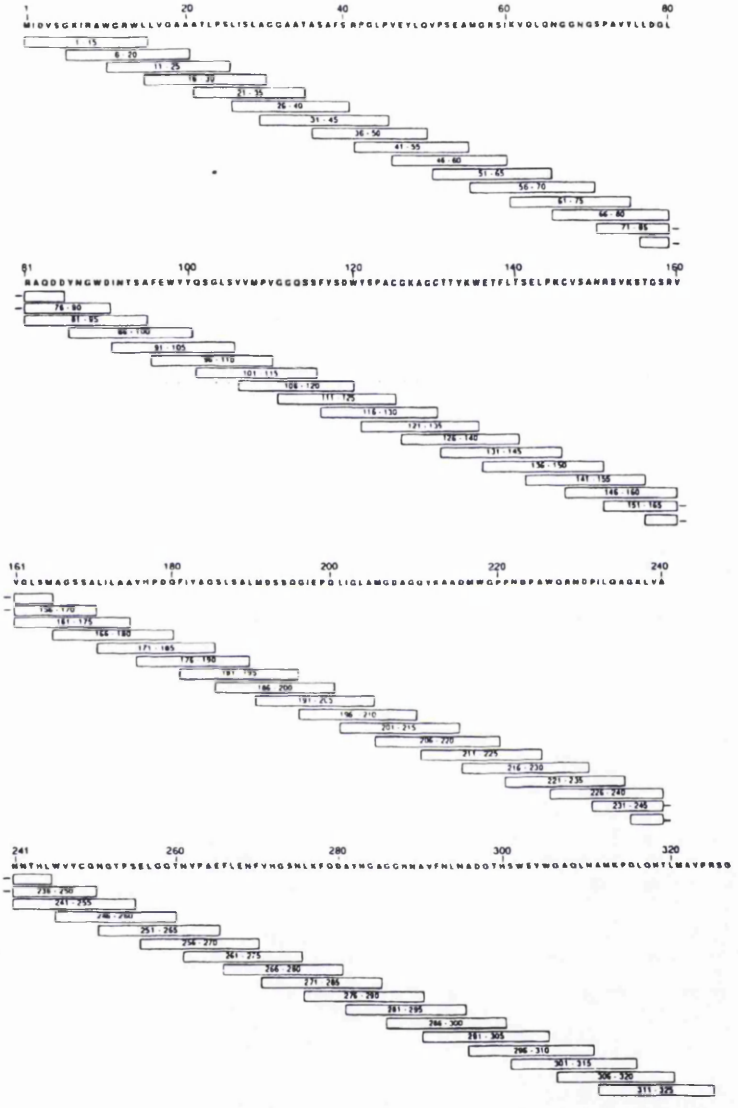

Figure 1. Amino acid (AA) sequences of overlapping peptides of *M. leprae* 30-kDa protein antigen 85B. Positions of synthetic peptides are indicated by boxes underneath sequence of 30-kDa protein, and nos. within boxes are AA residues.

control subjects to 15-mer peptides with their responsiveness to larger peptides, 25 residues long (see below).

Longitudinal studies of leprosy patients undergoing treatment. In a retrospective assay, the pattern of response to the entire set of 63 15-mer peptides was followed for patient LL4 1 year after the start of therapy (figure 4A) and at 1-year intervals during treatment (figure 4B, C). Although the antibody-binding profile remained constant during treatment, the antibody titer decreased substantially after 2 years. These data are in agreement with the decrease in antibody response to MPT59 (figure 4). In striking contrast, antibody levels to the sonicate, the 65- and 18-kDa proteins of *M.*

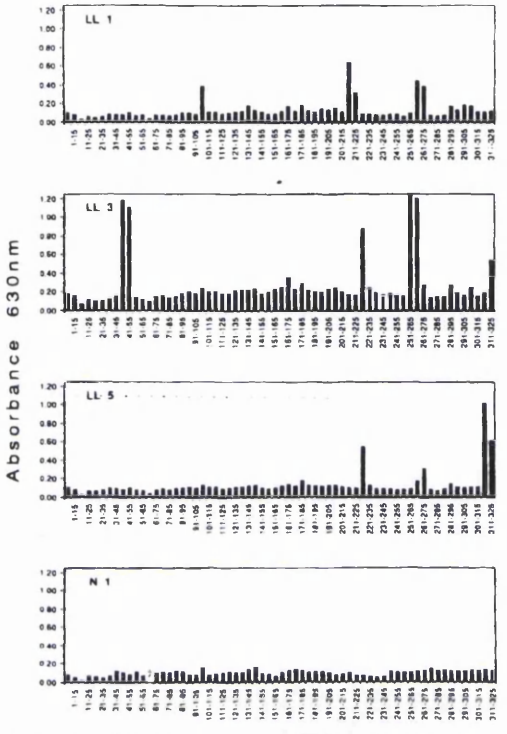

Figure 2. Antibody response patterns of 3 patients with lepromatous leprosy (LL1, LL3, and LL5) and normal healthy individual (N1) to 63 overlapping 15-mer peptides comprising entire sequence of the *M. leprae* 30-kDa protein antigen 85B. Peptide sequences are shown on horizontal scale; first bar represents mean absorbance of control wells coated with buffer alone. ND, not done.

leprae, decreased slightly after 2 years. Three other LL patients were studied. Again, we observed a decline in the reactivity of sera with peptides following treatment but no change in patterns of reactivity (data not shown).

Comparison of antibody responses to the 15- and 25-mer peptides comprising the immunodominant B cell regions of M. leprae antigen 85B. Having identified the main antigenic determinants on antigen 85B, it was of interest to test peptides >15 residues, as it has been suggested that native linear epitopes are better mimicked by long than by short peptides. Three peptides, 25 residues long and corresponding to AA 206–230, 256–280, and 291–315, were synthesized and probed with the same panel of serum samples from LL patients, together with the 15-mer overlapping peptides. Individual antibody responses are shown in figure 3.

Sera from patients with leprosy gave comparable patterns of reactivity with the 15- and 25-mer peptides that span AA 256–280; moreover, antibodies from those patients showed strong reactivity with that domain. Serum antibody responses to the 25-mer peptide of AA 291–315 were raised and were somewhat higher than antibody responses to the corresponding 15-mer peptides in 50% of patients tested. In addition, some sera that did not recognize the 15-mer peptides recognized the larger peptide much better, while a few weak responders to the former peptides lost their reactivity to the latter. Antibody responses to the 25-mer peptide, covering AA 206–230, were not demonstrable in serum samples that did react with the 15-mer peptide of AA 216–230. An explanation for this could be a difference in folding between the 15- and 25-mer peptides, making the epitope in the 25-mer peptide less accessible for antibody binding. No response was observed to the 25-mer peptides tested with control sera.

It is worthwhile to point out a few facts about the clinical

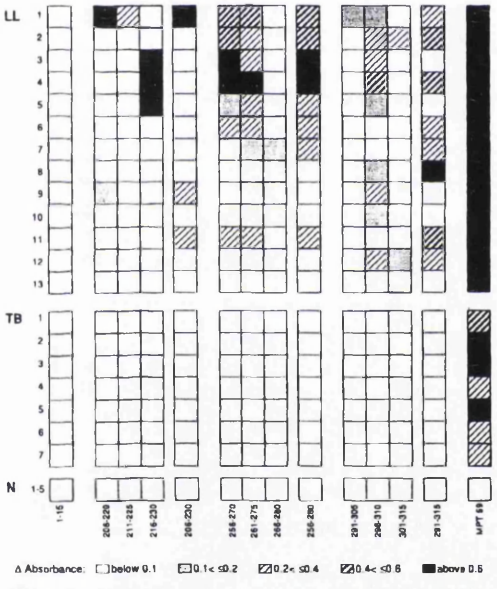

Figure 3. Comparison of reactivity of sera from patients with lepromatous leprosy (LL1–LL13) or tuberculosis (TB1–TB7) and 5 normal healthy donors (N1–N5) to 15- and 25-mer peptides comprising main antigenic domains of *M. leprae* antigen 85B and to MPT59. Sera were also assayed with 15-mer peptide (1–15) included as control. Peptide sequences are shown on horizontal scale. Individual antibody response values are represented by boxes, and degrees of reactivity are shown at bottom. Serologic responses of normal subjects are grouped in single box, although sera were always tested individually.

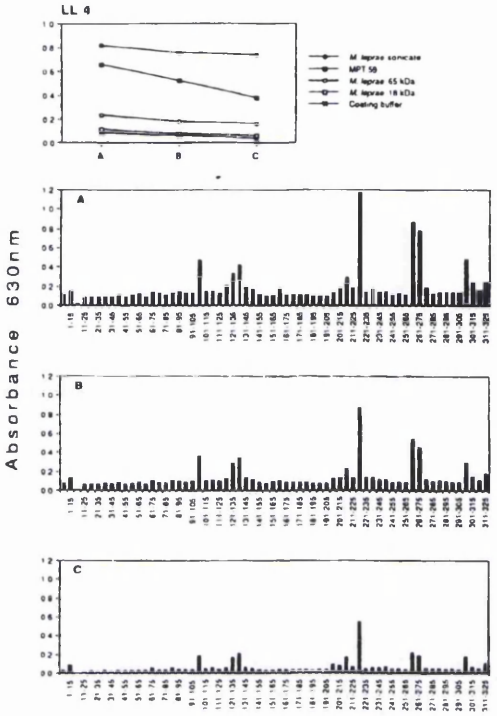

Figure 4. Serum antibody-binding profiles of patient LL4 1 year after beginning therapy (**A**) and at 1-year intervals during treatment (**B** and **C**). Top, binding curves to MPT59 and to *M. leprae* sonicate and purified recombinant proteins. A–C, Binding to 63 overlapping 15-mer peptides of sequence shown on horizontal scale; first bar, mean absorbance of control wells coated with buffer alone. Serum samples collected over time were tested on same day.

state, bacterial index (BI), and lepromin reaction of patients in this study. Patients LL1–LL4, who reacted strongly with peptides, were newly diagnosed or had undergone a recent drug resistance relapse; their skin biopsies yielded a high BI of 5+ on the Ridley-Jopling scale. Patients LL5–LL8, who responded to some of the peptides, remained lepromin-negative despite various lengths of treatment; their smear BI varied between 0.7 and 4.0. The other 5 patients (LL9–LL13) reacted poorly or not at all with synthetic peptides, though they responded strongly to the native protein MPT59. Those patients were smear-negative and lepromin-positive or have become lepromin-positive within 1 year of serum collection [25]. Our data suggested that LL patients with smear-positive lesions loaded with large numbers of bacilli responded not only to conformational epitopes on MPT59 but also to linear epitopes.

To further characterize the human B cell epitopes on *M. leprae* antigen 85B, we checked whether sera from patients with active tuberculosis would bind to the 15- or 25-mer peptides comprising AA 206–230, 256–280, and 291–315. None of the peptides were recognized by sera from 7 patients (TB1–TB7) with smear-positive tuberculosis (figure 3). Of interest, those sera were selected for their strong reactivity to MPT59 and many responded as well to the whole protein as did sera from patients with leprosy.

Recognition of the 25-mer peptides by antibodies in sera from Ethiopian patients with leprosy and their healthy contacts. To evaluate the antibody binding specificity to the B cell regions of *M. leprae* antigen 85B, it was of interest to screen large numbers of persons living in leprosy-endemic areas. Seventy-one sera collected from Ethiopian patients with LL, BT, or TT and from household contacts were tested in a blind study for their reactivity to MPT59 and to the 25-mer peptides comprising AA 206–230, 256–280, and 291–315. The 15-mer peptide, covering AA 216–230, was also included because we found that patients who reacted with that peptide did not respond to the larger peptide (see above).

The individual antibody-binding responses in the examined groups of patients and contacts are shown as ΔA in figure 5. Sera from all LL patients reacted with MPT59, and a strong antibody response was a dominant feature of this group. Also, MPT59 was recognized by less than half of the BT and TT patients and by few healthy contacts. Antibody responses to the 25-mer peptides showed a selective recognition of the peptide comprising AA 256–280, suggesting that this region contains the most dominant linear B cell epitope on *M. leprae* antigen 85B. Only a small number of patients responded to AA 206–230 and 291–315, and testing on the 15-mer peptide (AA 216–230) did not improve responsiveness to the former region. Of interest, patients' sera that reacted with peptides also responded to the entire protein MPT59. No reactivity was found to an irrelevant peptide included as a negative control in this study.

Antibody response values to AA 256–280 were considered positive when the ΔA was >0.171 (mean ΔA + 2 SD of 21 healthy contacts). Thus, 88% of the LL patients, 15% of the BT and TT patients, and none of the healthy contacts responded to the 25-mer peptide of AA 256–280. Antibody responses to MPT59 and to the *M. leprae* 25-mer peptide of AA 256–280 of LL patients, compared with those of BT and TT patients and contacts, were highly significant ($P < .001$), whereas the antibody responses of BT and TT patients were not significantly different from those of the contacts.

Discussion

We have sought to define the antigenic domains of *M. leprae* 30-kDa protein antigen 85B and synthesized peptides, which were then tested for antibody reactivity by ELISA.

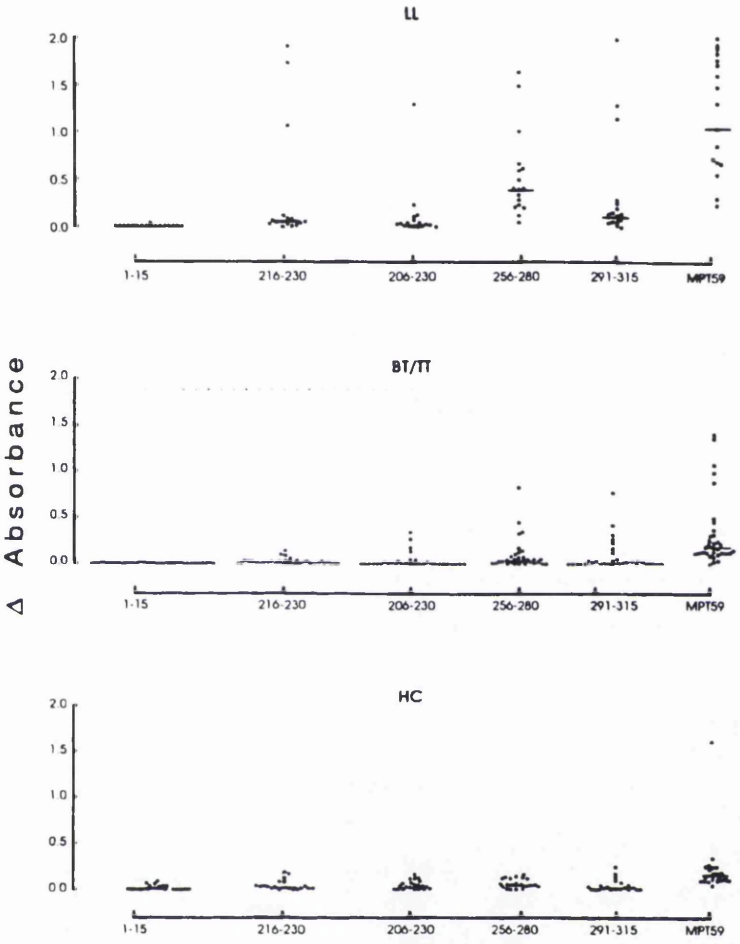

Figure 5. Serum antibody responses of Ethiopian patients with leprosy and healthy contacts to 25-mer peptides comprising immunodominant B cell regions of *M. leprae* 30-kDa protein and to MPT59; 15-mer peptides corresponding to amino acid regions 1–15 and 216–230 were also tested. Individual response values are shown as dots. Horizontal bars indicate geometric means in groups of patients and contacts.

These mapping studies have shown that there are linear antibody epitopes on *M. leprae* antigen 85B that are recognized by human antibodies.

Serum samples from patients with leprosy reacted mainly with peptides comprising AA 206–230, 251–280, and 291–325 of the 30-kDa protein. Binding of patients' sera to peptides was specific, as confirmed by the absence of any positive responses in healthy controls. Antibody response patterns remained constant for LL patients at the start of therapy and in subsequent years of treatment; only the antibody titers decreased over time, and this was reflected in the background as well (figure 4). Furthermore, we did not observe any novel immunoreactive peptide sequences emerging with time after treatment or with improved health and clinical upgrade of patients.

Our findings indicated a correlation of antibody levels to the linear B cell epitopes with the extent of disease or bacillary load in patients with leprosy and their lepromin reaction. Antibodies to peptides corresponding to the immunodominant B cell sites were demonstrable in patients at the lepromatous end of the spectrum, in whom the BI was the greatest (5+). In contrast, LL patients who became smear-negative after many years of treatment and converted to lepromin-positive before or shortly after time of serum collection reacted poorly or not at all with peptides. Similarly, peptides were not recognized by serum samples from pa-

tients with BT or TT, in which the bacterial content is minimal. It is suggested that antibodies in LL patients' sera react to linear and to conformational epitopes on the 30-kDa protein. Then, if the immune state of the patient improves from polar lepromatous and lepromin positivity is restored, antibody responses to the 30-kDa protein become mainly directed against conformational epitopes. This may represent an interesting change in the role of antigen-presenting cells and T cells in epitope selection.

These results were confirmed in a subsequent screening of a large panel of sera from Ethiopian patients and healthy household contacts. This blind study was undertaken to evaluate the utility of synthetic peptides for monitoring leprosy in patients from leprosy-endemic areas. Since the recombinant *M. leprae* protein is not yet available, antibody responses to conformational epitopes were assessed by testing sera to *M. tuberculosis* homologous protein MPT59. Binding to peptides was detected in serum samples also responding to native protein MPT59, and nonresponders to MPT59 did not react with peptides. The 25-mer peptide, corresponding to AA 256–280, contained the most dominant epitope, recognized by 15 (88%) of 17 LL patients, 5 (15%) of 33 BT or TT patients, and none of the 21 household contacts. A ΔA cutoff of >0.171 was applied to increase the specificity of our assay while maintaining sufficient sensitivity. Again, there was a direct relationship between antibody activity to the defined antigenic determinants on the *M. leprae* 30-kDa protein and the spectrum of disease.

The 30- to 31-kDa proteins are secreted antigens and are likely to become available for immune recognition at an early stage of infection. On the other hand, this antigen complex contains epitopes that are widely shared among mycobacteria [2], which raises the possibility of positive responses in persons with tuberculosis and of nonspecific reactions in healthy subjects exposed to environmental mycobacteria. However, patients with active pulmonary tuberculosis did not react to the epitopes on the *M. leprae* 30-kDa protein recognized by patients with leprosy, even though high levels of antibodies to *M. tuberculosis* MPT59 were present in sera from these patients. Also, we were unable to detect any response to the *M. leprae* 30-kDa protein-derived peptides in healthy household contacts from leprosy-endemic areas. A subsequent study suggests that conserved regions of the 30-kDa protein are recognized differentially by persons infected with *M. leprae* or *M. tuberculosis* (unpublished data).

This is the first report to our knowledge on the successful mapping of human B cell epitopes on an *M. leprae* protein antigen using synthetic peptides. Indeed, recent studies on the 36-, 65-, and 70-kDa proteins failed to detect linear epitopes with sera from infected patients [26–28]. In contrast, analysis of B cell epitopes on *M. tuberculosis* proteins showed an immunodominant B cell site within MTP40, and the epitope represented by a synthetic peptide was recognized by 55% of smear-positive tuberculosis patients and 5.5% of normal donors [29]. However, that was not the case with the *M. tuberculosis* 10-kDa protein; Verbon et al. [30] found that there was no relationship between the ability of patients' sera to inhibit binding of monoclonal antibody SA-12 to the 10-kDa antigen and the binding of these sera to peptides comprising the epitope recognized by SA-12.

Peptides would be very attractive to use in serodiagnostic tests because they can be easily prepared in sufficient purity and large quantities to allow their widespread use. In our studies, peptides in phosphate-buffered solutions were stable for at least 6 months in storage at 4°C and remained active after a few days at ambient temperature. Moreover, our results with three independent batches of peptides showed consistent recognition of the same domains by patients' sera with reproducible absorbance values. We attempted to simplify the procedure while improving the efficacy of our assay by coating microtiter wells with a pool of the three 25-mer peptides representing the immunodominant B cell sites within the 30-kDa protein. Patients who showed strong reactivity to one or more of the 25-mer peptides still responded to the pooled peptides, giving absorbance values similar to those seen with the dominant individual peptide(s), while the reactivity of weak responders remained poor. In conclusion, we did not see any difference between the antibody responses to the 25-mer peptides coated individually or as a mixture. The mixture of three 25-mer peptides described here are possible candidates for the development of a serologic test for follow-up not only of patients with leprosy who develop relapse due to drug resistance but also of LL patients who improve to convert from lepromin-negative to -positive.

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