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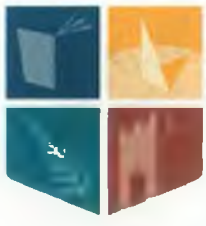
❖ REGULATION AND EFFECTOR FUNCTION OF ❖ TH1 AND TH2 CELLS IN IMMUNITY TO *BORDETELLA PERTUSSIS*

MIRIAM T. BRADY

A thesis submitted to the National University of Ireland
for the Degree of Doctor of Philosophy



NUI MAYNOOTH

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Supervisor: Prof. Kingston H. G. Mills
Infection and Immunity Group
Biology Department,
National University of Ireland,
Maynooth,
Co. Kildare.

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DECLARATION OF AUTHORSHIP

This thesis has not been previously submitted in whole or in part to this or any other university for any other degree. This thesis is the sole work of the author, with the exception of Figure 3.1 presented in chapter 3, which was reproduced from a study by Mills *et al.*, 1998.

Miriam Brady Date

DEDICATION

I dedicate this thesis to the people who have always been there for me;
who have given everything - without question, without exception, without a second thought, selflessly, unconditionally and constantly.

To the people…….

who have given me direction in life 

who have been with me through all my successes 

…….and all my failures!! 

who have always said 'yes'
here we are, no matter how,
no matter when,
no matter what! 

who laughed with me when I was happy 

…….and cried with me when sad 

who have always made me believe
I could make the best of every situation
be it bad or good…. 

who have always been there to see me through…...

Mam & Thomas, this one is for you…….

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ABSTRACT

The evaluation of vaccines for human use requires reliable models of infection, that are predictive of protective efficacy. Traditionally whole cell pertussis vaccines (Pw) have been controlled using the Kendrick test, which measures protection following intracerebral challenge with *Bordetella pertussis*. However, this test is unsuitable for assessing the potency of the new acellular pertussis vaccines (Pa). In this study, it was demonstrated that protection in a murine respiratory challenge model correlates with the protective efficacy of Pa and Pw in children, but vaccine potency could not be predicted on the basis of antibody responses against individual antigens. Furthermore, the murine model was shown to be a reliable method for the determination of consistency between different batches of Pa and Pw. This study highlights the possible applications of the murine model in the regulatory control and future development of pertussis vaccines.

The murine model of infection has been used in the elucidation of the protective mechanisms induced following immunization with Pw or Pa, revealing roles for both antibody and T-cells in protection against *B. pertussis*. It has been demonstrated that children still appear to be protected against infection, despite a rapid decline in antibody levels after vaccination. In the murine model, antibody levels quickly decline after immunization, but significant levels of protection were observed following aerosol challenge. Recall T-cell responses detectable for prolonged periods after immunization, together with an anamnestic antibody response post-challenge suggest that immunological memory is more significant in protection, than the induction of immediate antibody responses in vaccine-induced protection against *B. pertussis*.

It has been demonstrated that Th1 responses and particularly the cytokine IFN-γ, play a critical role in immunity to *B. pertussis*. In the present study, the role of IL-12 and IL-18 in the induction of a protective Th1 response to *B. pertussis* was examined. Lack of IL-12 during primary and secondary infection effected protection only at the early stages of infection, suggesting that IL-12 plays an important role at the induction stage of the immune response to *B. pertussis*, but also suggests that other cytokines may be involved. IL-18 is rapidly produced in the lungs of *B. pertussis* infected IL-$12^{-/-}$ and wild-type mice , which may compensate for the lack of IL-12 at the later stages of infection.

The importance of Th1 responses and IFN-γ were further highlighted in this study, through the investigation of the effect of helminth infection on the outcome of *B. pertussis* infection, or immunization with Pw. Infection with the parasite *Fasciola hepatica*, was shown to suppress *B. pertussis*-specific Th1 responses, and delay bacterial clearance from the lungs, and was also shown to inhibit this Th1 response after it had become established. Furthermore, it was demonstrated that infection with *F. hepatica* was capable of downregulating the Th1 response induced by systemic immunization with Pw, and reducing its protective efficacy. In addition, a role for IL-4 was demonstrated in the parasite-induced immunoregulation.

ABBREVIATIONS

ACT	Adenylate Cyclase Toxin
ADP	Adenosine Diphosphate
Aggs	Agglutinogens
APC	Antigen presenting Cell
ATP	Adenosine Triphosphate
cAMP	Cyclic Adenosine Monophosphate
CD	Cluster of Differentiation
CFU	Colony Forming Units
CNS	Central Nervous System
CR	Complement Receptor
CTL	Cytotoxic T-lymphocyte
CTLA	Cytotoxic T-lymphocyte Antigen
CTLL	Cytotoxic T-Lymphocyte cell Line
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DTH	Delayed Type hypersensitivity
EDTA	Ethylene-Diamine-Tetra-acetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked Immunospot
FCS	Foetal Calf Serum
FHA	Filamentous Hemagglutinin
FIM	Fimbriae
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HIV	Human Immunodeficiency Virus
HLT	Heat Labile Toxin
HRP	Horseradish Peroxidase
i.c	Intracerebral
i.n	Intranasal
i.p	Intraperitonal

IFN	Interferon
Ig	Immunoglobulin
IGIF	IFN Gamma Inducing Factor
IL-	Interleukin
IU	International Units
LFH	Liver Fluke Homogenate
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NEJ	Newly encysted Juvenile
NK	Natural Killer Cell
OD	Optical Density
Pa	Pertussis Acellular Vaccine
PBMC	Peripheral Blood Mononuclear Cells
PMA	Phorbal Myrisate Acetate
pNPP	p-Nitro Phenyl Phosphate
PRN	Pertactin
PT	Pertussis Toxin
Pw	Pertussis Whole Cell Vaccine
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
STAT	Signal Transducer and Activator of Transcription
TB	Tuberculosis
TCR	T-cell Receptor
TCT	Tracheal Cytotoxin
Th	T helper
UV	Ultra Violet
WHO	World Health Organization

❖ Chapter 1 ❖

General Introduction

INTRODUCTION

1.1 INNATE IMMUNITY

Protective immunity results from the interplay of two cardinal systems: non-specific innate immunity and antigen-specific adaptive immunity. Innate immunity involves a set of disease-resistance mechanisms that are not specific to a particular pathogen. Cells of the innate system recognize non-processed antigens, such as lipopolysaccharide (LPS), carbohydrates and nucleic acids expressed on pathogens, using a variety of receptors (Fearon & Locksley, 1996, Palucka & Banchereau, 1999) . This type of immunity provides the first line of defense after the host's exposure to the pathogen. Apart from the anatomical barriers such as the skin, and physiological barriers, such as temperature and pH which can restrict the growth of microorganisms, other components of innate immunity include complement, interferons α/β, NK cells and phagocytic cells.

Initiation of both the classical and alternative pathways of complement activation can contribute to innate immunity. A collection of germ-line encoded antigen recognition molecules, known as natural antibody, is important in the recognition of pathogens and activates the innate system via the classical pathway of complement activation (Carroll & Prodeus, 1998). Furthermore, cell wall constituents of both gram positive and gram negative bacteria, some viruses, fungal and yeast cell walls, and LPS are all initiators of the alternative pathway of complement activation, resulting in lysis of cells, bacteria and viruses and opsonization of antigen.

IFN-α and IFN-β play a particularly important role in the innate immune response to viral infection. Binding of IFN-α or IFN-β to the IFN-α/β receptor activates the synthesis of several factors responsible for the degradation of viral RNA and inhibition of viral replication. IFN-α enhances MHC class I expression, resulting in increased susceptibility to cytotoxic T-lymphocytes (CTL), and plays a role in the activation of macrophages (Palucka & Banchereau, 1999). The binding of IFN-α and IFN-β to NK cells induces lytic activity, making them very effective in killing virally infected cells. The activity of NK cells is also greatly enhanced by IL-12 and IL-18. Very early in infection IL-12 is produced and induces IFN-γ production from NK cells, which contributes to phagocytic cell activation and

inflammation. IL-12 has also been shown to act as a growth factor for NK cells and enhance their cytotoxicity (Trinchieri, 1995). IL-18 also induces IFN-γ production and enhances NK activity, activating the perforin/granzyme system (Okamura *et al.*, 1998), an action which does not require endogenous IL-12 (Akira, 2000). The IL-18 receptor is constitutively expressed on NK cells (Hyodo *et al.*, 1999), and functional NK cells can develop in the absence of IL-12. IL-12 and IL-18 also act in synergy in the development of Th1 cells, which are involved in cell-mediated responses necessary for clearance of intracellular microorganisms.

Of the many components of innate immunity perhaps the most important is the role played by phagocytic cells. Macrophages, neutrophils and other phagocytic cells are key components of the antimicrobial immune response. Organisms attach to long membrane evaginations on the phagocyte, called pseudopodia. Ingestion of the organism forms a phagosome which fuses with a lysosome. This fusion results in the release if lysosomal enzymes into the phagosome, destroying the ingested organism, by oxidative or non-oxidative mechanisms (Bogdan *et al.*, 2000).

1.2 Adaptive Immunity

In general, most of the microorganisms encountered by a healthy individual are cleared within a few days by non-specific defense mechanisms. However, if the pathogen eludes innate immunity, then the specific immune response of adaptive immunity is triggered to completely eradicate the pathogen. A defining feature of adaptive immunity is the development of immunological memory. This occurs once the immune system has recognized and responded to a specific antigen(s) on the infectious agent. Thus not only is acquired immunity responsible for the elimination of the invading pathogen, but it also ensures subsequent immunity to re-infection. Adaptive immunity is mediated by lymphocytes, that have the ability to recognize specific epitopes on a given pathogen. Lymphocytes are classified into two categories, B lymphocytes and T lymphocytes.

1.2.1 B-Lymphocytes

The B-lymphocyte derived it's letter designation from it's site of maturation, in the bursa of Fabricius in birds, a name which is also apt for bone marrow, it's major site

of maturation in many mammalian species. B-lymphocytes or B-cells are the primary cell type involved in antibody production, although they can also serve as potent antigen-presenting cells for $CD4^+$ T-cells and can produce a large number of cytokines (Pistoia, 1997). Mature B-cells are distinguished from other lymphocytes by their synthesis and display of membrane-bound immunoglobulin (antibody) molecules, which serve as antigen receptors. Antigen-specific proliferation of B-cell clones is elicited by the interaction of membrane antibody with antigen. Antibody molecules have a common structure of four peptide chains; two identical light (L) chains and two identical heavy (H) chains. Each chain contains constant (C) and variable (V) regions, the latter determining antibody specificity. Immunoglobulins (Ig) can be divided into five isotypes; IgG, IgM, IgA, IgE, and IgD, which participate in distinct effector functions.

1. IgG antibodies are the most abundant class in serum. There are four IgG subclasses in both humans and mice. This isotype has a wide variety of functions including activation of complement and opsonization of antigen. IgG readily crosses the placenta and plays an important role in protecting the developing foetus.
2. IgM is the first Ig class produced in primary response to antigen. Because of it's large size (pentamer), IgM does not diffuse well and is restricted almost entirely to the bloodstream. IgM is efficient in the agglutination of antigen and the activation of complement.
3. IgA is the predominant Ig class in mucosal secretions such as saliva, tears and mucus of the bronchial, genitourinary and digestive tracts. It plays an important role in prevention of bacterial/viral attachment at mucous membrane surfaces.
4. IgE antibodies mediate the immediate hypersensitivity reactions associated with allergic type responses. IgE binds to Fc receptors on basophils and mast cells. Cross-linkage of receptor-bound IgE with antigen causes the cells to release various mediators of allergic reactions, such as histamine.
5. No biological effector function has been identified for IgD, other than it's action as a membrane receptor for immunocompetent B-cells.

Mature B-cells can be activated by antigen that contains epitopes specifically recognized by their cell surface Ig. B-cell activation proceeds by two different

routes depending on the nature of the antigen. Thymus-independent antigens stimulate B-cells directly, and is dependent upon the cross-linking of membrane-bound Ig on B-cells. Most thymus-independent antigens are mitogens, and are able to activate B-cells regardless of their antigenic specificity. Thymus-dependent antigens require interaction with a T-helper cell to stimulate B-cells in the production of antibody. B-cells bind this type of antigen through their specific antigen receptors, and then process the antigen into peptides following endocytosis. The peptides are then exported to the cell surface where they are displayed in association with MHC class II molecules. $CD4^+$ T-cells with receptors specific for this class II-peptide complex interact with the B-cell. The T-cell in turn promotes B-cell activation, both by releasing cytokines and by direct intercellular contact. This contact between the T-cell and B-cell occurs through a number of molecules and plays an important role in B-cell activation. CD40 ligand (CD40L) and CD28 on T-cells bind to CD40 and B71/B72 respectively on B-cells. This crosslinking induces B-cell proliferation and antibody production (Clark & Ledbetter, 1994). Once activated, B-cells proliferate and differentiate into antibody-producing plasma cells. The cytokines produced by T-cells play a major role in the antibody isotype that is produced by the B-cell. In the mouse, IFN-γ promotes IgG2a production, but inhibits IgG1 and IgE production, the Ig classes associated with Th2 responses. On the other hand, the Th2-derived cytokine, IL-4, promotes production of IgG1 and IgE and suppresses production of IgM, IgG2a and IgG2b (Coffman, et al., 1993, Purkerson & Isakson, 1992).

1.2.2 T-Lymphocytes

T-lymphocytes, or T-cells, arise in the bone marrow and derive their name from their site of maturation in the thymus, where they undergo development and differentiation. Maturation in the thymus occurs along several developmental pathways, which generate functionally distinct subpopulations of mature T-cells, that migrate to peripheral lymphoid organs. During development in the thymus, T-cells come to express a unique antigen-binding molecule called the T-cells receptor (TCR) - a complex of polypeptides that includes CD3. T-cells can be divided into two distinct lineages based on the cell surface receptor expression. In humans and

most animals the vast majority of T-cell express a TCR composed of an α chain and a β chain, but there also exists a much smaller population of T-cells which possess a TCR comprising γ and δ chains. α/β T-cells can be further divided into subpopulations of cells, based on their expression of the co-receptors CD4 or CD8. These cells are fundamentally distinct from each other in their recognition of antigen and effector functions (Mills, 1989).

T-cell receptors do not recognize free antigen, but respond only to an antigenic peptide that is bound to cell membrane proteins called major histocompatibility complex (MHC) molecules. There are two types of MHC molecules: Class I MHC molecules are expressed on virtually all nucleated cells, whereas class II molecules are restricted to cells of the immune system. T-cells that express the membrane glycoprotein molecule CD8 are restricted to recognition of antigen bound to class I MHC molecules, whereas T-cells expressing CD4 are restricted to recognizing antigen bound to class II molecules. The class I antigen pathway typically begins with internally synthesized proteins, such as those from an invading virus, being reduced to peptides in proteasomes. These peptides are then carried to the endoplasmic reticulum where they are loaded onto MHC class I molecules to form a MHC-peptide complex. This complex is routed through the golgi apparatus and exported to the cell surface, where it can be recognized by $CD8^+$ T-cells with the appropriate TCR. In contrast, MHC class II molecules utilize an endocytic antigen pathway. Antigen-presenting cells (APC) ingest exogenous antigen, which is internalized by endocytosis. The antigen is degraded into peptides within endosomes. Peptides are then transported to the plasma membrane in association with MHC class II molecules, where they can be recognized by $CD4^+$ T-cells.

$CD8^+$ and $CD4^+$ T-cells have distinct immunological functions. $CD4^+$ T-cells or T-helper (Th) cells provide help for B-cells in antibody production. They also secrete a range of cytokines which activate macrophages and are involved in regulating immune responses. $CD8^+$ T-cells are cytotoxic to cells infected with bacteria or viruses and are referred to as cytotoxic T-lymphocytes (CTL). The CTL-mediated response involves a carefully orchestrated sequence of events culminating in the destruction of the target cells. There are two proposed

mechanisms of cell mediated cytotoxicity; one mechanism depends upon contact-mediated transmembrane signaling and the other on the exocytosis of toxic products by the CTL. The granule-exocytosis pathway is characterized by the secretion of the lytic protein perforin. This protein polymerizes to form a pore in the host cell, allowing the entry of the cosecreted granule contents, including granzymes, leading to apoptosis of the target cell. The second pathway is non secretory and instead involves the crosslinking of a surface-membrane ligand (Fas ligand) present on some antigen-activated CTL and a death receptor (Fas) on the target cell, which initiates a cascade of proteolytic enzymes, resulting in target cell apoptosis and lysis (Stenger & Modlin, 1998).

1.3 CD4+ T-cell Subsets

$CD4^+$ T-cells are considered to be the major regulators of the immune response during infection, and their developmental regulation is critically important to the form and effectiveness of acquired immunity. $CD4^+$ T-cells can be divided into two distinct subsets of effector cells based on their functional capabilities and the profile of cytokines they produce. These subsets, designated Th1 and Th2 cells, originally described by Mosmann and colleagues in 1986, have classically been considered to mediate cellular and humoral immunity respectively. Each subset produces cytokines that serve as its own autocrine growth factor, and promote differentiation of naïve T-cells to that subset. Mouse Th1 cells produce IL-2, IFN-γ, and TNF-β (lymphotoxin), whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann & Coffman, 1989). Human Th1 and Th2 cells produce similar patterns, although the synthesis of IL-2, IL-6, IL-10 and IL-13 is not as tightly restricted to a single subset as in mouse T cells. Several other cytokines are secreted by both subsets, including IL-3, TNF-α, and GM-CSF (Mosmann & Sad, 1996). Th1 cells are responsible for the induction of cell-mediated immunity, but are also associated with antibody production and Ig class switching. IFN-γ, a defining cytokine of the Th1 subset, activates macrophages; it stimulates these cells to increase microbicidal activity, to up-regulate the level of class II MHC, and to secrete cytokines such as IL-12, which induces Th cells to differentiate into the Th1 subset. IFN-γ secretion also induces antibody class-switching to IgG classes (such as IgG2a in the mouse),

that support phagocytosis and the fixation of complement. Th1 cells are associated with inflammatory phenomenon such as delayed type hypersensitivity, due to their secretion of cytokines that mediate inflammation such as IFN-γ and TNF-β. Th2 cytokines are associated with the induction of humoral immunity, promoting antibody production. The signature cytokines of Th2 cells are IL-4 and IL-5. IL-4 is the major inducer of B-cell switching to IgE production and is therefore a key initiator of IgE-dependent, mast-cell mediated reactions. IL-5 is the principal eosinophil-activating cytokine which play an important role in immunity to helminth parasites. The production of these two cytokines by the same Th subset accounts for the frequent presence of both IgE and activated eosinophils in Th2-dominated immune reactions, such as in allergies and helminthic infection. Th2 cells also stimulate antibody production very efficiently, such as IgG1 in mice, or it's homologue, IgG4, in humans.

A third subset of $CD4^+$ T-cells, called Th0 cells, have also been described, which produce a mixture of the two patterns of cytokine secretion exhibited by Th1 and Th2 cells (Firestein et al., 1989). The overlapping cytokine profiles associated with this subset, however, may be due to the presence of mixed populations of $CD4^+$ T-cell subsets rather than derived from individual Th cell clones. Analyses using in situ mRNA hybridization (Carding *et al.*, 1989) and intracellular protein staining (Openshaw *et al.*, 1995) in which cytokines can be detected in individual cells, favour the mixed population explanation.

1.4 Factors affecting the development of Th subsets

Studies of numerous animal and human models have revealed that the ability of a host to effectively eradicate an invading organism, depends on the class of effector-specific immune response that is mounted, and in particular the type of Th response elicited. Type-1 and type-2 immunity involve fundamentally different and opposing effector functions. T-cells, and the cytokines they produce, play a crucial role in determining the outcome of infections in terms of both protective immunity and immunopathology. The development of a particular Th subset has important implications for the outcome of infection, and has been demonstrated in disease conditions where the development of one Th subset is associated with susceptibility

to disease, whereas development of the corresponding Th subset is associated with disease resistance. The dual regulatory and effector roles of $CD4^+$ T-cells are best understood in murine *Leishmania major* infection. Cutaneous infection of most inbred strains with *L. major* results in a lesion that heals spontaneously and confers resistance to infection. However, in BALB/c mice, the infection progresses to a disseminated visceral disease that is usually fatal. Susceptible BALB/c mice develop a Th2 response and succumb to infection, whereas resistant strains such as C57BL/6 mice develop a Th1 response and survive (Scott *et al.*, 1989, Sher & Coffman, 1992). Therefore, the factors which are involved in the differentiation of naïve T-cells into either Th1 or Th2 subsets are critical. The selective differentiation of either subset is established during priming and the major variable in this differentiation is the cytokine environment (O'Garra & Murphy, 1994). However, many studies indicate that other factors including the APC type, co-stimulation and the nature and dose of antigen play a role in the selective development of a particular Th subset and are crucial to the progression towards either humoral or cell-mediated immunity (Constant & Bottomly, 1997).

1.4.1 Cytokines that selectively induce Th1 cells

A large body of evidence has accumulated over recent years to indicate that IL-12 is one of the most important cytokines in the differentiation of naïve $CD4^+$ T-cells to the Th1 subset (Germann *et al.*, 1993, Schmitt *et al.*, 1994, Manetti *et al.*, 1993, Abbas *et al.*, 1996, Trinchieri, 1994, Seder *et al.*, 1993). It's main function is to induce the canonical Th1 cytokine, IFN-γ. IL-12 is produced by macrophages and other APC (Hall, 1995), and serves to enhance proliferation, IFN-γ secretion and cytotoxic function by T-cells and NK cells (Gately *et al.*, 1998). IL-12 and IL-12-induced IFN-γ promotes the generation of Th1 cells by priming $CD4^+$ T-cells for high IFN-γ production and by favouring the expansion of Th1 cells (Wu et al., 1993, Seder et al., 1993). In doing so, IL-12 acts in antagonistic equilibrium with IL-4, which stimulates the development of Th2 cells. Both IL-12 and a functional IL-12 receptor are required for the development of fully competent Th1 cells, and IFN-γ mediates both IL-12Rβ2 expression (Szabo *et al.*, 1997), and actively

induces IL-12 production from macrophages (Ma *et al.*, 1996). The Th1 response modality is thus stabilized by a positive feedback loop.

Th1 responses are considered most relevant for the elimination of intracellular pathogens, and therefore it may also be assumed that IL-12, which favours the development of Th1 cells would play a role in the resolution of intracellular infections. Indeed, in experimental infections with intracellular pathogens such as *Leishmania*, *Toxoplasma* and *Listeria*, attenuation of the Th1 response through the elimination of IL-12 has revealed a compulsory role for this cytokine in the production of IFN-γ, and for the establishment of a Th1-type response (Mattner *et al.*, 1996, Gazzinelli *et al.*, 1993, Scharton-Kersten *et al.*, 1995). It has also been shown to be an important mediator in the generation of the type 1 response to *Mycobacterium leprae* in humans (Sieling *et al.*, 1994). In other systems, lack of IL-12 function resulted in a failure to produce a cytokine profile characteristic of a Th1 response, but rather directed the immune response towards Th2 responsiveness (Seder *et al.*, 1993). Thus, IL-12 seems to promote Th1 differentiation indirectly through the induction of IFN-γ. However, the status of IFN-γ as an inducer of the Th1 phenotype during primary Th stimulation remains controversial.

It is still unclear whether IL-12 can act directly on Th cells to cause them to differentiate and secrete IFN-γ, or whether the effect on Th cells is indirect, for example, NK derived. Several models have failed to provide a consensus view, with some indicating that the mechanism is IFN-γ independent, whilst others suggest a requirement for IFN-γ. A study by Scott (1991) revealed that treatment of mice with anti-IFN-γ antibody at the time of challenge with *Leishmania major*, resulted in reduced IFN-γ production from lymph node cells restimulated *in vitro*, suggesting a requirement for IFN-γ in the priming of Th1 cells. In contrast to this, Mountford and colleagues (1999) reported that Th1 differentiation can occur without the presence of NK-cell derived IFN-γ in an in vivo model of pulmonary granuloma formation, indicating that IL-12 can act independently of IFN-γ for the induction of Th1 cells. Similarly, in another study, it was shown that during infection with *Leishmania major*, Th1 cells, which are crucial in protection against

this parasite, can differentiate in vivo without exposure to IFN-γ from other cells, such as NK cells (Wakil *et al.*, 1998). Nevertheless, *in vitro* the presence of endogenously synthesized IFN-γ during the priming of naïve $CD4^+$ T-cells both accelerates and enhances the Th1-differentiating effects of IL-12 (Wenner *et al.*, 1996, Bradley *et al.*, 1996). In addition, IFN-γ and IL-12 can indirectly promote the induction of Th1 cells by inhibiting IL-4 production, thus generating optimal conditions for the priming of IFN-γ producing cells.

Not only does IL-12 synergize with IFN-γ in the development of Th1 cells, but it also acts in concert with another IFN-γ-inducing factor - IL-18. IL-18 is produced by a variety of different cells types including macrophages and T-cells and shares many biological properties with IL-12, such as IFN-γ induction, enhancement of cytotoxic function of NK cells and stimulation of Th1 differentiation. However, naïve T-cells only begin to express the IL-18 receptor (IL-18R) after IL-12 stimulation (Akira *et al.*, 2000). So, although IL-18 is a potent stimulus for the induction of IFN-γ from T-cells, it's function completely depends on the presence of IL-12, in the context of a second stimulus (Okamura *et al.*, 1995, Ushio *et al.*, 1996, Okamura *et al.*, 1998, Micallef *et al.*, 1996, Ahn *et al.*, 1997). In this way IL-18 potentiates IL-12-driven Th1 development. IL-18 has been shown to play a role in the development of a protective response to a variety of organisms including *Mycobacterium tuberculosis, Yersinia enterocolitica* (a gram negative bacterium which causes enteritis and enterocolitis), *Leishmania major* and *Salmonella typhimurium* (Sugawara *et al.*, 1999, Bohn *et al.*, 1998, Ohkusu *et al.*, 2000, Mastroeni *et al.*, 1999), all of which are associated with the generation of Th1 responses.

1.4.2 Cytokines that selectively induce Th2 cells

The development of Th2 cells from naïve precursor $CD4^+$ T-lymphocytes is induced by IL-4, through it's ability to activate signal transducer and activator of transcription (STAT) 6, a transcription factor which binds to the IL-4 gene (Murphy *et al.*, 2000). Although IL-4 induces Th2 cells and IL-12 induces Th1 cells, when both cytokines are present in the same culture, the effect of IL-4 is

dominant and Th2 cells develop in the presence of IL-12 (Seder *et al.*, 1993, Hsieh *et al.*, 1993). The critical role of signals from IL-4 in Th2 development is shown by the observation that knocking out the gene that encodes IL-4 prevents the development of this T-cell subset (Kuhn *et al.*, 1991, Kopf *et al.*, 1993). Similarly in STAT 6 knockout mice, IL-4 mediated processes are severely inhibited or absent (Kaplan *et al.*, 1996, Takeda *et al.*, 1996, Shimoda *et al.*, 1996).

Although Th2 cells are themselves a major source of IL-4, the source of early IL-4 production that mediates the development of Th2 cells remains to be clearly defined. It has been suggested that a number of different cell types including mast cells, basophils, eosinophils and $NK1.1^+$ T-cells (an atypical subset of T-cells not restricted by class I MHC), may serve as a source of early IL-4. However there is more convincing evidence to suggest that Th2 differentiation can be supported solely by IL-4 produced by antigen-primed (Schmitz *et al.*, 1994) or memory $CD4^+$ lymphocytes (Gollob & Coffman, 1994). Given the ability of threshold amounts of IL-4 to override signals for Th1 differentiation, even small amounts of IL-4 early in the immune response may be sufficient to direct the development of Th2 cells.

IL-13 has also been shown to play a role in the development of Th2 cells. Knocking out the gene that encodes for IL-13 has been shown to impair the development of Th2 responses (McKenzie *et al.*, 1998). In another study using a Th2-driven lung granuloma model induced with *Schistosoma mansoni* eggs, it was demonstrated that IL-4 and IL-13 act in concert with each other to initiate rapid Th2 responses, and that combined disruption of these cytokines can either abolish these responses or delay their onset, resulting in an inappropriate Th1 response (McKenzie *et al.*, 1999).

1.4.3 Th1/Th2 Crossregulation

The critical cytokines produced by Th1 and Th2 subsets have three characteristic effects on Th subset development. Firstly, they act as autocrine growth factors for the subset that produces them. IL-2 acts as a growth factor for Th1 cells, promoting their proliferation, and IL-4 promotes the expansion of Th2 cells. Secondly, they make members of the T-cell subset that releases them, less responsive to the cytokine that directs the differentiation of the other T-cell subset.

IL-4 enhances Th2 cell development by downregulating the β_2 subunit of the IL-12 receptor, thereby making T-cells unresponsive to IL-12, which would direct Th1 development. On the other hand, IFN-γ upregulates the expression of this β_2 subunit making T-cells more responsive to IL-12, and hence overriding the effects of IL-4 and favouring the differentiation and activity of Th1 cells (Gillespie *et al.*, 2000, Wu *et al.*, 2000). Finally, they inhibit the development and activity of the opposing subset, an effect known as cross-regulation. During many immune responses, antibody production and DTH responses are mutually exclusive. Since Th2 and Th1 cells appear to be at least partially responsible for antibody and DTH responses respectively, it is possible that the reciprocal relationship between these two responses is that Th1 and Th2 cells are mutually inhibitory and self-stimulatory. The signature Th1 cytokine IFN-γ, preferentially inhibits the proliferation of the Th2 subset (Manetti *et al.*, 1993), and IL-4 and IL-10 (secreted by Th2 cells) downregulate secretion of IL-12, the critical cytokine for Th1 differentiation, by both macrophages and dendritic cells (Sher *et al.*, 1992). Similarly, these cytokines have opposing effects on antibody-producing B-cells. IFN-γ promotes IgG2a production, but inhibits IgG1 and IgE production, the Ig classes associated with Th2 responses. On the other hand, IL-4 promotes production of IgG1 and IgE and suppresses production of IgG2a. Thus, the balance between IFN-γ, IL-12 and IL-4 plays a critical role in determining the outcome of the CD4 effector T-cell response (Nakamura *et al.*, 1997), and the net effect of this is that once a T-cell immune response begins to develop along either the Th1 or Th2 pathway, from a common precursor, it tends to become increasingly polarized in that direction

The crossregulation of Th1 cells by IL-10, secreted from Th2 cells and regulatory T-cells (Tr), appears to inhibit cytokine responses through an effect on macrophages and monocytes, rather than a direct interaction with T-lymphocytes. IL-10 interferes with the ability of APC to activate the Th1 subset to produce IFN-γ (Mosmann & Moore, 1991, Fiorentino *et al.*, 1991). This interference is thought to result from the ability of IL-10 to downregulate the expression of class II MHC molecules on APC, but has also been shown to inhibit costimulatory activity and B7 expression (Ding *et al.*, 1993). IL-10 can also downregulate IFN-γ dependent immunity by blocking this cytokine's ability to activate macrophages for the killing

of intra- and extra-cellular parasites (Gazzinelli *et al.*, 1992). This suppression is due to inhibition of IFN-γ-induced production of nitric oxide and other bactericidal metabolites known to be involved in parasite killing (Gazzinelli *et al.*, 1992). Since IFN-γ is an important mediator of the protective immune response against infection, downregulation of it's production by a Th2 cytokine maybe an important strategy promoting the survival of infectious agents. In this regard, IL-10 production has been shown to be associated with the decreased IFN-γ responsiveness accompanying helminth infection (Sher *et al*, 1991), and with the exacerbated disease occurring in BALB/c mice infected with *L. major* (Heinzel *et al.*, 1991).

1.4.4 DOSE OF ANTIGEN AND ROUTE OF ADMINISTRATION

Reports *in vivo* and *in vitro* have provided much evidence that the nature and availability of antigen, can participate in the commitment of $CD4^+$ T-cells to production of restricted or polarized patterns of cytokines. However, there is little consensus regarding a universal model of differentiation, with data suggesting that both low and high antigen dose favours Th2 responses. Some have suggested that this process not only involves the nature and dose of antigen, but also that the time period over which differentiation occurs can also be a major factor (Rogers & Croft, 1999). *In vitro* experiments have implicated antigen dose as an important factor in the Th1/Th2 decision. However, interpretation of these results is complex as increasing antigen dose may switch the immune response from Th1 to Th2 and visa versa, and when a wide range of doses is used a double switch can occur from Th2 to Th1 to Th2 (Mosmann & Sad, 1996).

Early *in vivo* studies showed that both high and low amounts of antigen primed for DTH, whereas moderate levels stimulated antibody production (Parish, 1972). Similar effects occur in infectious models and suggest that priming with high doses of an immunogen will induce humoral responses, whereas lower doses will lead to cell-mediated immunity. This particular dichotomy was demonstrated by Bretscher et al. (1992), by immunizing susceptible BALB/c mice with different numbers of *Leishmania major* parasites. They showed that immunization with low numbers of parasites induced DTH responses that protect mice against subsequent *L. major* infection. However, immunization with increasingly higher numbers of

parasites led to the progressive loss of DTH responses and protection. Similar to the *L. major* studies, low-level infections with the intestinal nematode *Trichuris muris*, polarize $CD4^+$ T-cell responses towards the Th1 phenotype (Bancroft *et al.*, 1994). In addition, low-dose infections with the parasitic helminth *F. hepatica*, gives rise to a more mixed Th1/Th2 cytokine profile compared with a high dose infection, which generates a highly polarized Th2 response (O'Neill *et al.*, 2000).

Other studies have demonstrated that priming to different doses of an immunogen can induce the opposite effect, with high doses of antigen inducing Th1 responses and low doses of antigen priming for the development of Th2 cells. For example Pfeiffer et al. (1995) immunized mice using a peptide antigen derived from the sequence of human collagen IV protein and demonstrated that immunization with 50μg of peptide led to the generation of Th1 cells, whereas 2μg induced the development of the reciprocal T-cell subtype.

These studies with seemingly contradictory conclusions, however, have one key difference, which is the type of antigen used. Most of the studies in which high doses of antigen induced Th2-like responses used parasites as immunogens (Bretscher *et al.*, 1992, Bancroft *et al.*, 1994, O'Neill *et al.*, 2000), whereas high doses of soluble peptide tended to skew toward the Th1 phenotype (Pfeiffer *et al.*, 1995). Therefore, it may be that the soluble or particulate nature of the antigen itself can influence the type of response initiated. At low concentrations of a soluble antigenic peptide, each APC should present a low-density of peptide-MHC complexes. In contrast, although relatively low numbers of APC will capture an infectious agent at a low dose, each successful APC will present a large amount of antigen because of the digestion of a whole particle.

The route of immunization may also be critical in influencing the type immune response that develops, which has important implications for disease outcome and protection against infection. It has been demonstrated that a pertussis vaccine administrated via the intraperitoneal route resulted in an enhanced Th1 response, whereas immunization by the subcutaneous route produced a predominantly Th2 response (Barnard *et al.*, 1996). Immunization by mucosal routes also appears to favour the induction of Th2 cells (Conway *et al.*, 2001).

1.4.5 Co-stimulatory Molecules

Naïve T-cells use the CD3-associated TCR α/β complex to recognize bound antigen to MHC molecules on the surface of the APC. The engagement of the TCR provides the first activation signal to the T-cell. However, for optimal activation to occur, a second activating signal, or co-stimulus is required. The development of both Th1 and Th2 cells is dependent on co-stimulation. The most studied co-stimulatory molecules in T-cell activation are the B7 family of molecules. B7-1 (CD80) and B7-2 (CD86) molecules can be expressed on activated B- and T-cells, dendritic cells (DC) and activated monocytes. It has been shown in some systems that B7-1 and B7-2 may differentially regulate Th cell differentiation (Kuchroo *et al.*, 1995, Lenschow *et al.*, 1995). It has been suggested that the dominant expression of B7-2 during priming may direct T-cell activation towards the Th2 phenotype (Bottomly *et al.*, 1997). However, other studies have reported that B7-1 and B7-2 can have the opposite or no effect on Th1/Th2 differentiation (Lanier *et al.*, 1995, Lenschow *et al.*, 1995).

Of the ligands for B7-1 and B7-2, CD28 is found on both resting and activated T-cells, whereas the other, cytotoxic T-lymphocyte antigen 4 (CTLA-4), is only found on activated T-cells (Clark & Ledbetter, 1994). The binding of B7 molecules to CD28 on T-cells previously stimulated through their antigen receptors stimulates IL-2 production and T-cell proliferation and the generation of cytotoxic activity (Zhang *et al.*, 1997). Analysis of CTLA-$4^{-/-}$ T-cells have revealed an important role for CTLA-4 in the regulation of Th cell differentiation. In the absence of CTLA-4, naïve $CD4^{+}$ T-cells differentiate into Th2 cells, producing large amounts of IL-4, IL-5 and IL-10 (Oosterwegel *et al.*, 1999). This skewing towards Th2 differentiation does not appear to be dependent on the strength of the TCR signal, as the effect can be observed over a broad range of antigen concentration (Oosterwegel *et al.*, 1999).

It is well known that CD40-CD40L interactions are required for B-cell activation (Clark & Ledbetter, 1994), but it is now accepted that they are also essential for the initiation of antigen-specific T-cell responses (Grewal & Flavell, 1996). CD40L is a type II membrane protein, and is preferentially expressed on activated T-cells and mast cells. CD40 is expressed on DC, activated macrophages,

follicular dendritic cells (FDC) and activated B-cells. CD40-CD40L interaction during T-cell activation by APC results in IL-12 production (Grewal & Flavell, 1996). It is widely accepted that IL-12 plays a crucial role in the development of Th1 cells, and thus it can be concluded that the CD40-CD40L interaction is important in this process.

The CD4 activation antigen, OX40 and its ligand (OX40L) are upregulated during $CD4^+$ T-cell priming and it has been shown that this molecule may also play a role in directing Th differentiation. One model suggests that the CD28 signal upregulates OX40 after antigen activation of $CD4^+$ T-cells, and that both molecules may act in synergy to induce the rapid expansion of $CD4^+$ T-cells and the preferential production of Th2 cytokines (Lane, 2000).

1.4.6 Antigen-Presenting Cells

In addition to the other contributory factors that determine Th1/Th2 commitment, the type of APC present at the site of T-cell activation plays a central, but not mutually exclusive role in Th cell differentiation. APC which express both TCR and co-stimulatory molecules, inform naïve Th cells about invading organisms, providing an antigen-specific 'signal 1' and a co-stimulatory 'signal 2'. Macrophages, DC and B-cells all have the ability to perform this important function.

The major variable in the differentiation of precursor T-cells towards either the Th1 or Th2 phenotype, as discussed earlier, is the cytokine environment. However, due to the relatively low frequency of specific T-cells secreting either Th1-inducing IFN-γ, or Th2-inducing IL-4, present in the local milieu during priming, the cytokines produced by APC are considered to play an important role in this process. Macrophages are potent producers of IL-12 and also produce IL-18, both of which favour the development of Th1 cells (Abbas *et al.*, 1996, Trinchieri, 1994, Micallef *et al.*, 1996). It has been suggested that some intracellular pathogens may inhibit the production of IL-12 by macrophages, and hence delay or prevent the development of cell-mediated immunity in order to potentiate their own survival (Mosser & Karp, 1999). They mechanism by which this occurs is thought to involve the crosslinking of surface receptors on the macrophages, such as CR3,

and confirms the importance of macrophage-secreted IL-12 in the development of Th1 responses. DC are also capable of producing IL-12, and stimulating Th1 responses (Macatonia *et al.*, 1995). Furthermore, DC can influence Th cell differentiation by preferentially inducing Th1 or Th2 type responses. This differential polarization of $CD4^+$ T-cells appears to be mediated by two discrete DC subsets; DC's of lymphoid origin induce Th1 differentiation and DC's of the myeloid lineage induce Th2 differentiation (Pulendran *et al.*, 1999, Rissoan *et al.*, 1999, Reid *et al.*, 2000).

1.5 *BORDETELLA PERTUSSIS*: HISTORICAL OVERVIEW

B. pertussis is a Gram negative bacterium and the causative agent of pertussis or 'whooping cough', a respiratory disease which remains a significant cause of morbidity and mortality in infants worldwide. Pertussis was not differentiated from other respiratory entities with any certainty until 1578 when Guillaume de Baillou described the first epidemic (Linneman, 1979), which primarily affected young children, and was characterized by paroxsyms of cough occurring about every five hours. His depiction is dramatic:

> *The lung is so irritated be every attempt to expel that which is causing the trouble it neither admits the air nor again easily expels it. The patient is seen to swell up and as if strangled holds his breath tightly in the middle of his throat....For they are without the troublesome coughing for the space of four or five hours at a time, then this paroxsym of coughing returns, now so severe that blood is expelled with force through the nose and through the mouth. Most frequently an upset stomach follows....For we have seen so many coughing in such a manner, in whom after a vain attempt semiputrid matter in an incredible quantity was ejected.*

In 1679 Sydenham (Lapin, 1943), writing about the disease gave it the name "pertussis", meaning "a violent cough". However it was not until 1906 when Bordet and Gengou first isolated the causative agent of pertussis, from the sputum of children suffering from the disease. They used a medium consisting of potato

starch infusion, glycerol, and defibrinated blood, which is still frequently used for the isolation of *B. pertussis* today. The bacterium was initially known as *Haemophilus pertussis*, but later became the type species of the genus *Bordetella* (Pittman, 1984). All members of the genus *Bordetella* have an absolute requirement for niacin or nicotinamide. Unsaturated fatty acids are toxic to the organism and, therefore media usually contain substances that absorb fatty acids such as starch, charcoal, ion exchange resins, or albumin. In Bordet-Gengou medium blood is used as a source of albumin.

1.6 *B. PERTUSSIS*: CLASSIFICATION AND MORPHOLOGY

B. pertussis belongs to the genus *Bordetella* in the family Alcaligenaceae, which contains several species of closely related bacteria with similar morphology. The genus *Bordetella* contains six species: *B. pertussis* which is the agent responsible for human pertussis; *B. parapertussis*, which causes a mild pertussis-like disease in humans; *B. bronchiseptica*, which is primarily a veterinary pathogen that causes atrophic rhinitis in swine and kennel cough in dogs, but may infect humans; *B. avium* and *B. hinzii* which both cause respiratory disease in poultry and are very rarely found in humans (Kersters *et al.*, 1984, Cookson *et al.*, 1994). A recent addition to the genus is *B. holmesii* which has been shown to cause bactereremia in an immunocompromised individual without respiratory infection (Weyant *et al.*, 1995). The four species are differentiated from each other by their phenotypic characteristics. DNA homology studies have shown that *B. pertussis, B. parapertussis* and *B. bronchiseptica* are genetically similar and therefore, might more appropriately be designated biotypes of the same species (Cherry *et al.*, 1988).

B. pertussis is a gram negative coccobacillus that measures 0.2 to 0.8μm in size. The bacillus has a slime sheath and filaments extruding from the cell wall. In 1931, Leslie and Gardner noted four phases (phases I, II, III, IV) when *B. pertussis* was cultured on artificial medium. The antigenically competent, smooth virulent form (phase I) which requires 3 to 4 days to form colonies, can mutate to the antigenically incomplete, nonvirulent, rough form (phase IV). This spontaneous phase variation is associated with a loss of capacity to synthesize pertussis toxin, filamentous haemagglutinin, heat-labile toxin, adenylate cyclase toxin, agglutinogens

and certain outer membrane proteins, and a concomitant loss of virulence in laboratory animals. There are also two intermediate forms, called phases II and III, but these are less well defined. The colonies when grown aerobically on Bordet-Gengou medium is small, punctiform, convex, glistening and translucent. A hazy zone of hemolysis surrounds the colony.

1.7 VIRULENCE FACTORS OF *B. PERTUSSIS*

In this section the properties of the bacterial components that contribute to the pathogenicity and immunogenicity of *B. pertussis* will be described. *Bordetella pertussis* is a complex organism with multiple virulence determinants. To establish efficient colonization of the respiratory tract, this bacterium produces a variety of virulence factors that contribute to it's adherence to the respiratory epithelium, and to the development of clinical pertussis. Recent studies have demonstrated that active or passive immunization of mice with various *B. pertussis* antigens confers a high level of protection against subsequent aerosol challenge (Mills *et al.*, 1998a). The contribution of each of these components to the disease process is presented below.

1.7.1 AGGLUTINOGENS

In the genus *Bordetella* the agglutinogens are highly immunogenic surface protein antigens, which induce the production of antibodies that can cause bacterial agglutination. A total of 14 agglutinogens are now recognized within the genus (Eldering *et al.*, 1957, Anderson, 1953). Agglutinogens (aggs) 1 and 7 are found in all strains of *B. pertussis* with agglutinogen 7 being common to all *Bordetella* species. It is now believed that in *B. pertussis* there are 3 major Aggs, 1, 2 and 3, and three minor Aggs, 4, 5, and 6 (Robinson *et al.*, 1985 (a)). Aggs 1 is specific for pertussis and is associated with lipooligosaccharide. Although it is common to all strains the nature of Aggs 1 is unclear, and it is not considered to be a significant mediator of protection. Most interest has centred on Aggs 2 and 3 which have been identified as immunogenically distinct fimbriae (Ashworth *et al.*, 1982, Irons *et al.*, 1985, Zhang *et al.*, 1985a). Due to their fimbrial nature and given the proven involvement of fimbriae in adherence of other bacteria to mammalian cells, it has

been suggested that agglutinogens 2 and 3 may act as adhesins. Antibodies to Aggs have been shown to block the binding of bacteria to Vero cells (Gorringe *et al.*, 1985) and HeLa cells (Redhead *et al.*, 1985). Alternatively, it has been found that non-fimbriated strains of *B. pertussis* are capable of binding to human ciliated cells (Tuomanen & Weiss, 1985).

Active immunization with purified Aggs 2 or mixtures of Aggs 2 and 3 have protected mice against respiratory infection with *B. pertussis* (Robinson *et al.*, 1985b, Zhang *et al.*, 1985b), and human antibodies to Aggs have been shown passively to protect mice from aerosol challenge (Oda *et al.*, 1985). Probably the strongest evidence for Aggs being protective antigens stems from the Medical Research Council vaccine trials in England in the 1950's. These trials revealed a good correlation between serum agglutinin titres in mice and vaccine efficacy in children (MRC, 1959). Taken together these findings suggest that agglutinogens may be important pertussis vaccine components as reflected by the WHO recommendation that vaccines should include the three major agglutinogens.

1.7.2 Filamentous Haemagglutinin (FHA)

FHA is a high molecular weight rod-shaped protein of approximately 220 kDa, named for it's ability to cause the agglutination of erythrocytes. It is a surface associated and secreted protein, easily purified in high yields from culture supernatants (Arai & Sato., 1976, Cowell *et al.*, 1981, Arai & Munoz., 1979), and is unusually susceptible to breakdown into a number of polypeptide species ranging from 95 kDa to 126 kDa (Arai *et al.*, 1976, Cowell *et al.*, 1981). When examined under an electron microscope, the protein appears as fine filaments measuring 2nm in diameter and 40-100nm in length (Arai *et al.*, 1976). FHA is now considered to be the major attachment factor of virulent *Bordetella* Spp. (Locht, 1993). Because FHA is not only a surface associated protein, but is also readily secreted from *B. pertussis* cells during growth (Tuomanen, 1988), raises an interesting possibility that FHA may play another role in the pathogenesis of whooping cough. This secreted FHA may be a component of an immunoevasive strategy of *B. pertussis*. FHA contains an arginine-glycine-aspartic acid (RGD) sequence which promotes binding to the integrin CR3 expressed on macrophages, PMN and other cell types

(Relman *et al.*, 1990). Ligation of CR3 has recently been demonstrated to suppress IL-12 production by macrophages (McGuirk & Mills, 2000). Pre-incubation of macrophages with FHA was shown to suppress IL-12 in response to LPS and low dose IFN-γ. Furthermore injection of mice with FHA suppressed IL-12 and IFN-γ levels in the serum, and that this suppression was mediated by an IL-10 dependent mechanism (McGuirk & Mills, 2000). It is possible that FHA may play a role in inhibiting the development of a cell-mediated response, which has been shown to be critical for the development of protection against *B. pertussis* (Mills *et al.*, 1993, Redhead *et al.*, 1993, Ryan *et al.*, 1997).

Active or passive immunization with FHA has been shown to protect mice against lethal infection with virulent *B. pertussis* (Oda *et al.*, 1984) and reduce the numbers of bacteria infecting the lungs of mice (Oda *et al.*, 1984). Serum antibodies to FHA have been detected in children following immunization with acellular pertussis vaccines (Pa), or whole cell pertussis vaccines (Pw), and in pertussis convalescent children (Sato & Sato, 1984). Furthermore, the inclusion of FHA in acellular vaccines has been correlated with lower rates of initial infection (Storsaeter *et al.*, 1990). Due to its non-toxicity, immunogenicity, and protective effects FHA is considered an important constituent of all current acellular pertussis vaccine preparations.

1.7.3 Pertactin (PRN)

Pertactin, originally known as "69 kDa protein", is a non-fimbrial protein closely associated with the outer membrane of *B. pertussis*. Although this protein can be detected and purified from the surface of *B. pertussis* as a single polypeptide of molecular mass close to 69 kDa, the pertactin gene was found to encode a 93 kDa protein (Charles *et al.*, 1989), which is cleaved to give a mature protein of 60.5 kDa.(Makoff *et al.*, 1990). The biological activities of this component remain to fully defined, but it has been demonstrated that PRN may play a role in adherence to monocytes (Hazenbos *et al.*, 1994). PRN, like FHA contains an RGD sequence which mediates binding to CR3, and it has been shown that PRN can augment the suppressive effect of FHA on LPS-induced IL-12 production *in vitro* (McGuirk & Mills, 2000). Active or passive immunization of mice with PRN has been shown to

protect mice against respiratory and lethal challenge with *B. pertussis* (Shahin *et al.*, 1990, Mills *et al.*, 1998a). PRN is a component of two Pa licensed for use in humans.

1.7.4 Pertussis Toxin (PT)

Pertussis toxin is a protein exotoxin, secreted during in vivo and in vitro growth. The toxin is a hexamer of five dissimilar subunits - S1, S2, S3, S4 and S5, present in the molar ratio of 1:1:1:2:1. Although PT is synthesized solely by *B. pertussis*, both *B. parapertussis* and *B. bronchiseptica* possess genes for PT without expressing them. Like other bacterial AB toxins, pertussis toxin consists of an A subunit that carries the biologic activity and a B subunit that binds the complex to the cell membrane. In pertussis toxin the S1 subunit constitutes the A protomer which is linked to the remaining subunits that make up the pentameric binding or B oligomer. PT binds to cell receptors by two dimers, one consisting of S2 and S4 and the other of S3 and S4 (Figure 1.1). Upon entry to the cell, S1 ADP-ribosylates a cysteine residue located near the carboxyl terminus of the α-subunit of several heterotrimeric G proteins including G_i which is involved in the inhibitory control of mammalian cellular adenylate cyclase. ADP-ribosylation uncouples signal transduction between G proteins and as a result the conversion of ATP to cAMP cannot be checked and intracellular levels of cAMP increase disrupting normal cellular function. This enzymatic activity also produces systemic effects, from which the toxin has acquired a number of synonyms: elicitation of a profound leucocytosis resulting from an accelerated maturation and release of thymocytes into the periphery and inhibition of migration of lymphocytes and neutrophils to the white pulp of the spleen (leucocytosis-promoting factor), stimulation of pancreatic islet cells to secrete insulin which induces hypoglycemia (islet-activating protein), and sensitization to histamine (histamine-sensitizing factor) (Burnette, 1992).

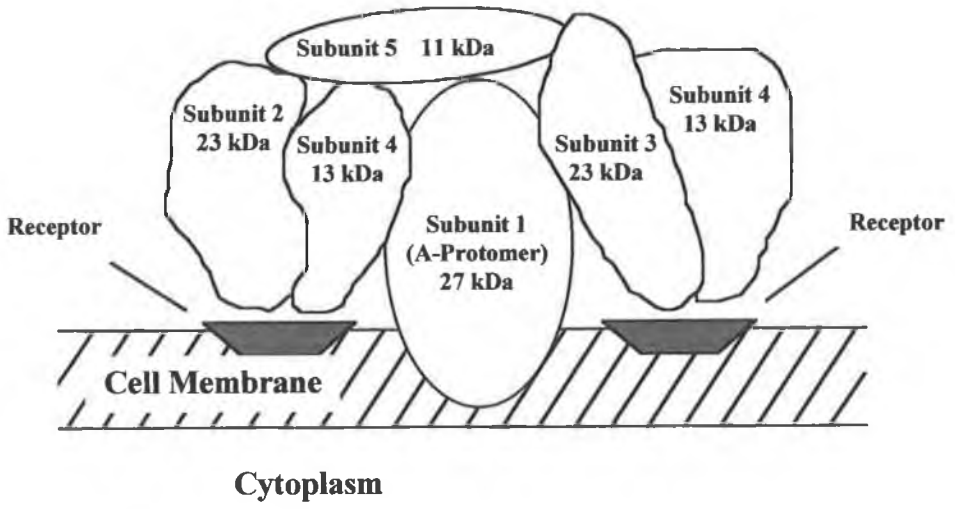


Figure 1.1 Binding of pertussis toxin to cell membrane

It is now widely accepted that PT is a crucial factor in the pathogenesis of pertussis and is the major cause of the harmful systemic affects (Pittman *et al.*, 1979, Pittman *et al.*, 1984). In a chemically or genetically detoxified form PT is considered an essential component of any effective vaccine. Active or passive immunization with PT is protective in animal models against respiratory infection with virulent *B. pertussis* (Oda *et al.*, 1984, Mills *et al.*, 1998a). Furthermore, serum antibodies to PT have been detected in children following immunization with Pa or Pw and in pertussis convalescent children (Sato & Sato, 1984).

1.7.5 Tracheal Cytotoxin (TCT)

TCT is a low molecular weight disaccharide-tetrapeptide containing diaminopimelic acid and muramic acid (Cookson *et al.*, 1989), and is secreted during the logarithmic growth of *B. pertussis*. This toxin is derived from the peptidoglycan of the bacterial cell envelope and induces ciliostasis and specific extrusion of ciliated cells from the respiratory epithelium (Goldman *et al.*, 1982). In the absence of ciliary activity, coughing becomes the only means in which to clear the airways of bacteria and inflammatory debris. Thus, TCT-mediated destruction of ciliated cells may trigger the violent coughing episodes symptomatic of pertussis. In addition, the destruction of cilia predisposes patients to secondary pulmonary infections

which are major causes of pertussis mortality. Furthermore, recent studies by Heiss et al. (1994) have shown that the deleterious effects of TCT are dramatically attenuated by nitric oxide synthase inhibitors, which suggests that TCT toxicity in the lung may be mediated through the induction of nitric oxide production. While TCT is almost certainly an important virulence factor, no immune response to it in animal or human infections has been reported and its possible value in a vaccine formulation is unknown.

1.7.6 Heat-Labile Toxin (HLT)

HLT, also known as dermonecrotising toxin, is a heat-sensitive (56°C for 10 min) toxin first described in 1909 by Bordet and Gengou and is now known to be produced by all species of *Bordetella*. This toxin is a simple protein with a total molecular weight of 102 kDa with sub-units of 20 and 30 kDa (Endoh et al., 1986). HLT may be responsible for some of the symptoms associated with pertussis but no defined role has been established for it in the pathogenesis of the disease and evidence of a protective role is lacking.

1.7.7 Adenylate Cyclase Toxin (ACT)

ACT is an extracytoplasmic enzyme that is released by *B. pertussis* organisms into the surrounding medium during exponential growth. The activity of the enzyme is markedly enhanced by calmodulin, a protein that is unique to eukaryotic cell systems (Wolff *et al.*, 1980). On entering cells, ACT catalyses the production of extremely high levels of cyclic adenosine monophosphate (cAMP), from endogenous cellular ATP, which then interferes with normal cellular metabolism and cell function (Confer & Eaton, 1982). ACT has been found to inhibit the phagocytic activity of macrophages and neutrophils (Confer & Eaton, 1982) in this way, and has also been linked to the induction of apoptosis in macrophages infected with *B. pertussis* (Guierard *et al.*, 1998). Molecular analysis has shown the gene for ACT to encode for a 200 kDa protein, with a calmodulin-sensitive adenylate cyclase domain at the N-terminus, and a hemolysin domain at the C-terminus (Glaser *et al.*, 1988a, Glaser *et al.*, 1988b). The hemolysin domain functions in the internalization of ACT into the cell. It has since been demonstrated that *B.*

pertussis mutants devoid of ACT, or those that lack either the adenylate cyclase activity or the hemolysin activity display a dramatic reduction in the pathogenicity of the bacterium (Weiss *et al.*, 1984, Gross *et al.*, 1992). Mutants deficient in hemolysin had a reduced virulence in the infant mouse respiratory infection model (Weiss & Goodwin, 1989). Active and passive immunization of mice with ACT has been shown to protect mice from lethal infection with *B. pertussis* (Brezin *et al.*, 1987). Furthermore, antibodies to ACT have been detected in the sera of children who had been vaccinated with a Pw, thus implicating a role for ACT in immunity to pertussis (Arciniega *et al.*, 1991). ACT is not present in any current Pa and further studies will be required before it is possible to determine whether it should be considered for inclusion.

1.7.9 Lipopolysaccharide (LPS)

The LPS of *B. pertussis* possesses many of the properties common to LPS of other Gram-negative bacteria in being heat stable, antigenic, pyrogenic and toxic (Ayme *et al.*, 1980). However, *B. pertussis* LPS is composed of two chemically and immunologically distinct lipids, lipid A and lipid X, and two different oligosaccharide chains (type I and type II) containing 12-16 monosaccharide units each (Ayme *et al.*, 1980). *B. pertussis* LPS is immunogenic and anti-LPS antibodies have been detected in animals and humans following vaccination with Pw or infection (Ashworth *et al.*, 1983, Li *et al.*, 1988). Nevertheless, LPS is not considered to be a protective antigen and has been shown to contribute directly to the reactogenicity of the whole cell vaccine in humans (Baraff *et al.*, 1989). Therefore, it is the current view that LPS be excluded from any Pa preparations.

1.8 Pathogenesis of *B. pertussis*

Pertussis has two phases, the first being colonization and the second, disease. It is a highly contagious disease, and can occur at any age, though severe illness is more common in young unimmunized children. The bacterium is spread by aerial transmission, and infection starts with it's arrival in the upper respiratory tract of a susceptible host. *In vivo B. pertussis* has a marked tropism for ciliated cells of the respiratory epithelium. Bacteria attach and multiply at the tips of, between, and at

the base of the cilia and this leads to ciliostasis, cell death and the shedding of the epithelial cells. Consequently, secondary infections are very common following *B. pertussis* infection, because the damaged respiratory epithelium may be more easily colonized by other respiratory pathogens. *Bordetella* organisms are thought to be non-invasive and remain on the respiratory surfaces where they multiply, but *B. pertussis* does have the ability to survive within alveolar macrophages and other mammalian cells types, which may contribute to the persistence of infection (Friedman *et al.*, 1992, Saukkonen *et al.*, 1991). *B. pertussis* adherence to cilia is mediated primarily by two non-fimbrial bacterial proteins, filamentous haemagglutinin (FHA) and pertussis toxin (PT) which are thought to act in synergy (Figure 1.2), each acting as a bivalent bridge between the bacterium and the ciliary receptor (Relman *et al.*, 1990, Locht *et al.*, 1993). Pertussis toxin mediates binding through the S2 and S3 subunits. The S2 subunit binds to lactosylceramide found primarily on ciliated cells, while S3 binds to a glycoprotein found mainly on phagocytic cells (Saukkonen *et al.*, 1992). FHA binds to galactose-containing glycoconjugates on both cilia and macrophages (Locht *et al.*, 1993, Relman *et al.*, 1990).

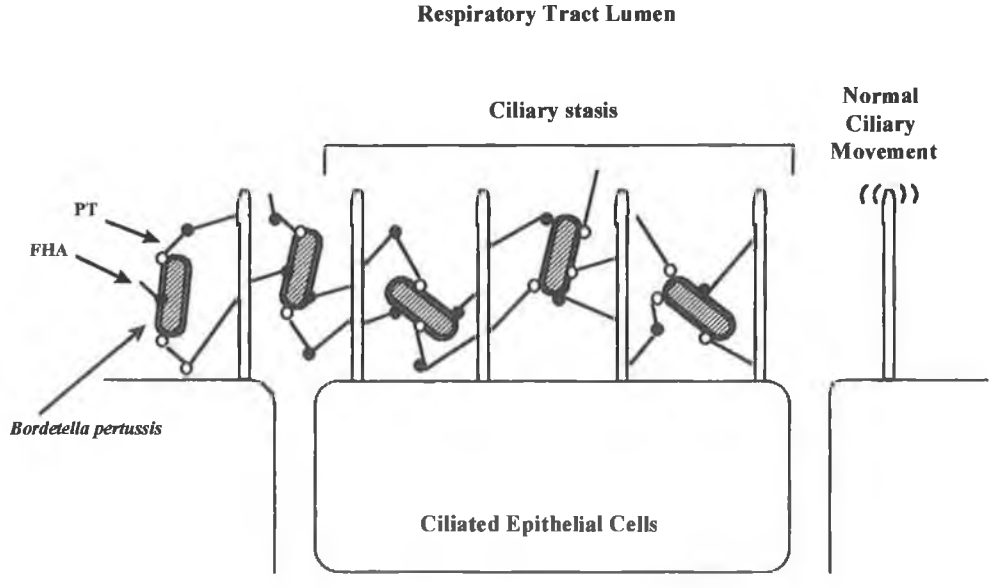


Figure 1.2

Synergy between PT and FHA in binding of *B. pertussis* to ciliated respiratory epithelial cells

In addition, the FHA gene contains an arginine-glycine-aspartate (RGD) sequence, which promotes adherence of *B. pertussis* to the macrophage integrin CR3 (CD11b/CD18) (Relman *et al.*, 1990). Although the non-fimbrial protein pertactin contains an RGD sequence like FHA, its exact role in adherence has yet to be clearly defined. One study has shown that PRN can mediate bacterial adhesion, even in the absence of a functional RGD motif, and that *Salmonella* strains expressing PRN were significantly more invasive than isogenic parental strains (Everest *et al.*, 1995). Agglutinogens may also be important in attachment, whereas HLT, in association with TCT and ACT may be important in the cause of local tissue damage in the respiratory tract during infection.

Generally, the typical clinical disease can be divided into four phases: incubation, catarrhal, paroxysmal and convalescent. The incubation period involves adherence of the bacterium to the cilia of respiratory tract epithelial cells where it multiplies, and then colonizes the ciliated mucosa of both the upper and lower respiratory tract. The length of this phase can vary from between 6 to 20 days (Friedman, 1988). The onset of disease is subtle, resembling a mild upper respiratory tract infection. This is known as the catarrhal phase, and lasts from seven to 14 days. Initially the infection induces cold-like symptoms including a mild cough, sneezing, rhinorrhea and a mild fever, and is the most infectious period, with a high risk of transmission. It is during this phase that organisms can frequently be cultured from nasopharyngeal swabs. The use of antibiotics, such as erythromycin, can reduce the duration and severity of disease if administered at this stage of infection. However, due the difficulty in identifying the disease prior to the development of the characteristic paroxysmal cough, pertussis is rarely diagnosed in the early stages of infection. The simple dry cough develops, gradually increases in frequency and intensity, leading to the paroxysmal phase. This phase can last up to six weeks and is typified by episodes of violent coughing, or paroxysms, which are more frequent at night. The characteristic "whoop", from which the disease gets it's name, immediately follow these paroxysms, and is due to rapid inspiration through a narrowed glottis. Antibiotic treatment is not effective at this stage (MRC, 1953). A wide range of complications are associated with pertussis during the paroxysmal phase. Violent coughing may result in umbilical or inguinal hernias,

and subconjuctival or cerebral hemorrhages. In addition, severe anoxia during paroxysms can lead to seizures, coma, encephalopathy, and in rare cases permanent brain damage (Cherry *et al.*, 1988). Paroxysms decline in frequency and severity, as the disease enters the convalescent stage, although a residual cough may persist even in the absence of *B. pertussis* organisms during this phase.

Therapeutic treatment of pertussis is only a viable option in the treatment of pertussis before the onset of the onset of the paroxysmal cough. Thus, because of the difficulty in identifying the disease before this stage, the most applicable control measure for the prevention of the disease is now considered to be through the development and widespread use of safe and effective vaccines.

1.9 Pertussis Whole-Cell Vaccines

Following the initial isolation of *B. pertussis* in 1906, attempts to produce a pertussis vaccine quickly followed. The majority of vaccines developed after this time contained intact bacteria, killed or inactivated by physical or chemical methods. This form became known as whole cell pertussis vaccine. It is generally accepted that the current Pw are effective at providing protection and are of substantial benefit to the recipient. The first studies to demonstrate significant protection against pertussis were carried out by Madsen (1933) in the Faroe islands. These studies clearly showed that a significant degree of protection was provided with Pw prepared from freshly isolated strains. This was an important observation in light of the discovery by Leslie and Gardner (1931) that phase I strains (which were used by Madsen) undergo phase variation and become avirulent in phase IV. Clinical trials conducted by the Medical Research Council in the 1950's showed that Pw was effective in children (MRC, 1959). A striking observation of pertussis efficacy resulted from reduced vaccine uptake in the UK, Japan and Sweden in the mid to late 1970's (Cherry *et al.*, 1988). Adverse publicity concerning pertussis vaccine reactions or efficacy caused a sudden fall in vaccine acceptance rates. The incidence of pertussis following this increased dramatically, resulting in two major epidemics in England and a huge increase in the incidence of disease and death in Japan and Sweden (Pollard, 1980). As acceptance rates rose again, case notifications of whooping cough fell to extremely low levels.

Although whole-cell vaccines are highly effective in controlling pertussis, they have been associated with a variety of adverse reactions. Local reactions such as pain, swelling and redness at the site of injection are usually mild and transitory. Mild to moderate systemic reactions include vomiting, fever, persistent crying and spasm, but a more serious cause of concern has been severe reactions such as shock, febrile and non-febrile convulsions, permanent brain damage and death. It has been suggested that residual active toxins, especially PT and LPS are responsible for vaccine reactogenicity (Cherry *et al.*, 1988, Sidley *et al.*, 1989). A recent study by Loscher et al. (1998) has suggested that these neurological reactions may be due to the presence of proinflammatory cytokines, such as IL-1β in the brain following parenteral immunization with Pw (Loscher *et al.*, 1998). Furthermore, immunization with Pw is associated with the induction of antigen-specific Th1 cells, which are critical in host resistance to infection (Redhead *et al.*, 1993, Ryan *et al*, 1997).

1.10 Pertussis Acellular Vaccines

The reactogenicity and toxicity of the whole-cell vaccine has prompted the development of non-toxic acellular pertussis vaccines, from purified components of the bacteria. The adhesion molecules FHA, PRN and FIM are suitable for inclusion in the vaccine because of their availability and immunogenicity. PT is also highly immunogenic and is the only toxin used in acellular vaccines.

The first Pa was developed by Yugi and Hirko Sato in the mid 1970's and since 1981 Japan has used this form of vaccine for routine primary immunization. Pa are produced by six manufactures in Japan, using slightly varying processes. Four manufactures produce the T-type (named after the manufacturer Takeda) vaccines which contain formalin treated preparations of FHA, PT, pertactin, and agglutinogen 2 in an approximately 90:10:10:1 ratio. One manufacture (Biken) produces a B-type vaccine, containing equal parts of formalin treated FHA and PT with the remaining manufacturer (Kitasato) producing a formalin-toxoided PT and FHA in a ratio of 1:4 (Aoyama, 1996).

Although the safety profile of Pa are considerably improved, when compared with Pw, in general the level of protection induced with most Pa does not

surpass that achieved with potent Pw. In fact, the residual toxins PT and LPS which are largely responsible for the reactogenicity of Pw, are also the same components that enhance the efficacy of the vaccine, but are absent from Pa. Immunization with Pa induces Th2 responses in mice (Redhead *et al.*, 1993, Mills *et al.*, 1998a, Barnard *et al.*, 1996) and a mixed Th1/Th2 response in children (Ryan *et al.*, 1997, Ausiello *et al.*, 1997). However, one study has shown that it is possible to mimic the effect of the toxins by adding exogenous IL-12 to Pa. This improves the protective efficacy of the vaccine by activating IFN-γ secreting cells (Mahon *et al.*, 1996). Recent field trials in Sweden, Italy, Senegal, Munich and Mainz of various Pa preparations have yielded estimated efficacies of 84%- 93%, (Gustafsson *et al.*, 1996, Greco *et al.*, 1996, Simondon *et al.*, 1997, Belohtadsky, 1997, Schmitt, 1997). The results of these and other trials suggest that routine immunization with Pa with the gradual elimination of whole cell vaccine, may be more beneficial to the recipient, with less adverse reactions and acceptable efficacy.

1.11 Immunity to *B. pertussis*

Acquired immunity to *B. pertussis* develops after natural infection and confers relatively long-lived protection against subsequent infection. However, it has not been possible to correlate protection against disease with a quantifiable immune response against a single protective antigen, or indeed with a single arm of the immune response. *B. pertussis* not only binds and multiplies extracellularly in the respiratory tract, but can occupy an intracellular niche within macrophages and other cells types. Therefore, it is not surprising that optimal immunity to *B. pertussis* is considered to be conferred by both cellular and humoral mechanisms.

1.11.1 Humoral Immunity

Antibody may function in immunity to *B. pertussis* by neutralizing toxins, inhibiting the binding of the bacteria to the ciliated epithelial cells of the respiratory tract, and by enabling bacterial uptake and destruction by macrophages and neutrophils. Levels of circulating antibodies that are critical for protection induced with certain vaccines such as *Haemophilus influenzae* type b (Hib), tetanus, diphtheria and polio (Robbins *et al.*, 1995, Kayhty *et al.*, 1998), have been identified. Consequently,

many studies have been carried out with the aim of also defining a level of protective antibody against *B. pertussis*. Although it has not been possible to correlate protection with a quantifiable immune response against a single protective antigen, recent studies have suggested that antibody to a number of antigens may confer protection (Storsaeter *et al.*, 1998, Cherry *et al.*, 1998).

In a murine model of respiratory infection with *B. pertussis*, where protection correlates with pertussis vaccine efficacy in children, it has been shown that pertussis vaccine efficacy could not be predicted on the basis of ELISA antibody responses against individual components of the vaccine (Mills *et al.*, 1998b). Immunization of mice with Pa induced high antibody titres against each component of the vaccine. The strongest response was detected against PT, even in the lower efficacy vaccine. On the other hand, antibodies to PT were low or undetectable in mice immunized with Pw. These data shows that there is no clear relationship between antibody responses and protection against infection. These results are consistent with the findings from human clinical trails which have also failed to demonstrate an immunological correlate of protection in children based on monotypic antibody responses against putative protective antigens (Greco *et al*,, 1996, Simondon *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997).

The results of passive immunization experiments have demonstrated that antibodies raised against PT and one or more additional antigens can confer a reasonable level of protection against respiratory challenge (Mills *et al.*, 1998a). However an active immunization schedule which induced the same levels of antibody in the circulation at the time of challenge conferred higher levels of protection, especially with the Pw vaccine (Mills *et al.*, 1998a). Furthermore, B cells have been shown to be required to confer immune mediated protection (Mahon *et al.*, 1997, Mills *et al.*, 1998a, Leef *et al.*, 2000). For example, *B. pertussis* challenge of naïve $Ig^{-/-}$ mice results in the development of a chronic infection; mice fail to clear the bacteria from their lungs for at least 6 months post-challenge, compared to wild-type mice which clear the infection after 8 -10 weeks (Mahon *et al.*, 1997). Moreover, immunization with Pa or Pw does not induce a significant protective effect in $Ig^{-/-}$ mice (Mills *et al.*, 1998a). However, $Ig^{-/-}$ mice do not make mature B-cells, and T-cell responses in these mice are defective, it cannot

be excluded that impairment of protection was due to a failure to produce antibodies. This is supported by a study by Leef and colleagues (2000), who demonstrated that intranasal immunization of $Ig^{-/-}$ mice with formal-fixed *B. pertussis* did not protect mice against aerosol challenge, which was restored by transfer of pertussis-immune B-cells. These results suggest an alternative role in for B-cells in protection against *B. pertussis* other than the production of antibody (Leef *et al.*, 2000).

1.11.2 Cellular Immunity

The first evidence of the importance of a direct role for T-cells in acquired resistance the *B. pertussis* was the demonstration that nude mice (which are deficient in T-cells), fail to clear an infection with *B. pertussis*, whereas normal BALB/c mice resolve the infection after 35 days (Mills *et al.*, 1993). Antibody responses in normal BALB/c mice did not develop until the infection was almost cleared, indicating that another mechanism of immunity was involved. It was subsequently shown that T-cell responses to PT, FHA PRN and *B. pertussis* sonic extract are induced earlier in infection. Furthermore, adoptive transfer of enriched T-cells or purified $CD4^+$ T-cells, but not $CD8^+$ T-cells from immune convalescent mice into nude or irradiated recipient mice conferred a high level of protection, in the absence of a detectable antibody response. The protective T-cells were shown to secrete IFN-γ and IL-2, but not IL-4, a cytokine profile characteristic of Th1 cells (Mills *et al.*, 1993). This characteristic response has been confirmed by the generation of *B. pertussis*-specific T-cell lines and clones (Barnard *et al.*, 1996). Since then it has also been demonstrated that PBMC from acutely infected or convalescent children secrete type 1 cytokines in response to *B. pertussis* antigens, indicating the preferential induction of $CD4^+$ Th1 cells by *B. pertussis* following infection in humans (Ryan *et al.*, 1997). In addition to this, other studies have indicated that the signature Th1 cytokine IFN-γ, can be detected in serum and bronchoalveolar lavage fluid of *B. pertussis*-infected mice (Torre *et al.*, 1993), and this cytokine has been shown to be critical in the appropriate control of bacterial dissemination, pathology and bacterial clearance after aerosol pertussis infection of adult mice (Mahon *et al.*, 1997, Barbic *et al.*, 1997). Furthermore exogenous

administration of recombinant IFN-γ and IL-12 has been shown to enhance resistance of mice to *B. pertussis* infection, either by the direct activation of alveolar macrophages by IFN-γ, or through IL-12-induced generation of Th1 cells (Torre *et al.*, 1995).

A role for cellular immunity in vaccine-induced immune responses has also been defined. Studies in mice have shown that spleen cells from mice immunized with Pw, or from convalescing mice secreted cytokines with the typical Th1 profile, with high levels of IFN-γ and IL-2, but not IL-4 or IL-5 (Redhead *et al.*, 1993). In contrast, immunization with Pa was shown to induce a Th2 response with the production of IL-4 and IL-5 from spleen cells in the absence of IFN-γ and IL-2. Following aerosol challenge with *B. pertussis*, Pw-immunized mice eliminated the bacteria from the lungs at a much faster rate than Pa-immunized mice. The authors concluded that the protracted clearance of bacteria reflected the failure of Pa to induce a potent Th1 response. It was suggested that high antibody levels induced by Pa resulted in an early decline in the numbers of viable bacteria in the lungs, but that complete and rapid clearance was dependent on cell-mediated immunity. A study by Mahon et al. (1996), is in agreement with this hypothesis, showing that addition of recombinant IL-12 to Pa significantly enhanced vaccine efficacy by promoting the induction of Th1 cells. In another study, Leef and co-workers (2000) defined specific roles for both B- and T-cells in protective immunity to *B. pertussis* following intranasal immunization with formalin-fixed *B. pertussis*. Following immunization, athymic *nu/nu* mice (lacking TCR-α/β^+ T-cells) and mice lacking all TCRs were aerosol challenged and bacterial recoveries from the lungs were assessed. Both athymic mice and mice lacking TCR were unable to clear the bacteria, indicating an important role for T-cells in the generation of protective immunity. Furthermore, CD4-depleted or CD4/CD8-depleted mice were shown to have significantly higher bacterial counts in the lungs and tracheae compared with controls, following aerosol challenge. B-cells were also shown to play an important role in the development of protective immunity as assessed by clearance of bacteria from the lungs and tracheas of B-cell knockout mice. Intranasal immunization of B-cell knockout mice with killed bacteria, or pertussis antigens in microspheres did not protect against aerosol challenge, with significantly higher numbers of bacteria recovered from the lungs and tracheae of knockout mice compared with controls.

B. pertussis-specific T-cell responses have also been defined in humans following immunization with Pw or Pa. One of the first studies to compare cytokine profiles after primary vaccination of children with Pw or Pa, suggested that pertussis vaccines induce a basic type 1 response in infants with constitutive expression of IFN-γ, which is accompanied by some production of type 2 cytokines (Ausiello *et al.*, 1997). They proposed that Th2 cytokines are expressed to a greater extent following immunization with Pa, and minimally expressed after immunization with Pw. A study by Ryan et al. (1998) further clarified the nature of *B. pertussis*-specific immune responses following immunization of children with Pw and Pa, and provided convincing evidence that these vaccines activate different populations of T-cells. Using peripheral blood lymphocytes isolated from children vaccinated with Pw or Pa, Ryan et al. (1998) examined the production of T-cell cytokines and proliferative responses specific for the *B. pertussis* antigens, PT, FHA, and PRN. They demonstrated that immunization with Pw, like natural infection, selectively induced the activation of pertussis-specific T cells that secreted high levels of IFN-γ but not IL-5, a cytokine profile indicative of Th1 cells. In contrast immunization with Pa was found to induce a more mixed Th1/Th2 or Th0 response.

1.12 Animal models for *B. pertussis* infection

Humans are the only natural host for *B. pertussis*, but a number of animal species including mice, rats, rabbits and monkeys can be infected with *B. pertussis*, and have been used for studies in immunity to this bacterium. However, these animal model systems are generally regarded as models of infection rather than disease, since *B. pertussis* is not a natural pathogen of animals.

A coughing rat model of *B. pertussis* infection, where the animals are challenged by intrabronchial challenge with *B. pertussis* embedded in fine agarose beads, resembles the human disease in the relationship between the time course of infection and cough production (Hall *et al.*, 1999). Pa and Pw have been found to be protective against paroxysmal coughing and leucocytosis (Hall *et al.*, 1998) indicating that this model may be useful for the comparative testing of different formulations of pertussis vaccines before they reach clinical trials.

The use of murine models of infection have played an important role in defining the mechanisms of protective immunity to *B. pertussis*, and have also been utilized to assess the efficacy of pertussis vaccines. Kendrick (1947) devised the original active mouse protection test for Pw, which measured survival following intracerebral (i.c.) challenge of immunized mice. Kendrick and colleagues demonstrated that protection in this model correlated with Pw efficacy in clinical trails (MRC, 1959). Since then the i.c. model or Kendrick test has been used to assess the potency of Pw. However, it has several disadvantages in that it does not predict the efficacy of the new generation Pa. Furthermore, the route of challenge and the lack of similarity to many aspects of the human model of disease is of little use to dissect the immune mechanisms to *B. pertussis*.

Respiratory challenge by aerosol or intranasal (i.n.) administration of the bacteria has been extensively used for studies of pathogenesis and immunity to *B. pertussis*. In the aerosol challenge model, animals are subjected to an aerosol containing concentrated live virulent *B. pertussis* bacteria, which has been reported to induce a more uniform and reproducible infection compared with the intranasal model (Peterson *et al.*, 1995). In the intranasal model of infection, live *B. pertussis* organisms are administered as droplets onto the nasal cavity. Although the characteristic cough or "whoop" is absent in these models of infection, the course of infection and many of the systemic effects, including leucocytosis and CNS effects, are similar to that observed in infants (Pittman *et al.*, 1980, Sato *et al.*, 1980, Loscher *et al.*, 2000). It has also been demonstrated that bacterial clearance from the lungs of mice immunized with Pa or Pw, following aerosol challenge, correlates with pertussis vaccine efficacy in children (Mills *et al.*, 1998a, Mills *et al.*, 1998b). Furthermore, measurement of antibody responses to pertussis vaccines in the murine respiratory challenge model, failed to show a correlation between antibody responses and vaccine efficacy (Mills *et al.*, 1998b). Similarly, results from human clinical trails have failed to demonstrate an immunological correlate of protection in children based on monotypic antibody responses against putative protective antigens (Greco *et al.*,, 1996, Simondon *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997). These observations further validate the respiratory challenge model for studies on the mechanisms of immunity to *B. pertussis*.

1.13 *FASCIOLA HEPATICA*: AN INTRODUCTION

Fasciola hepatica, and obligate, endoparasitic trematode is the causative agent of fasciolosis or liver fluke disease. The disease has worldwide distribution but is located primarily in the temperate zones (Europe, the Americas and Asia). The most common definitive hosts are sheep and cattle, but *F. hepatica* is also considered to be an important pathogen of humans, with approximately 2.5 million people infected worldwide (Spithill & Dalton, 1998). Fasciolosis can be a chronic disease with adult worms surviving for up to 12 years in the host, and is also associated with susceptibility to secondary infection. Infection in mice results in 100% mortality within approximately 2-6 weeks, depending on worm burden. The major pathology caused by *F. hepatica* includes fibrosis due to severe trauma induced by juvenile worms migrating through the liver, and inflammation, edema and fibrosis of the bile ducts due to the presence of the adult flukes. *F. hepatica* also secretes a number of excretory-secretory products like many other parasites.

1.14 THE LIFE CYCLE OF *FASCIOLA HEPATICA*

The life cycle of *Fasciola hepatica* is both cyclical and indirect, requiring an invertebrate intermediate host, the snail *Lymnaea truncatula*, and a definitive mammalian host (Fig. 1.3). The monoecious adult lives in the biliary tracts where it feeds on the lining of the biliary ducts. It is here that the sexually mature adult lays eggs which are carried by bile into the intestine. Although flukes are hermaphroditic and can self-fertilize, it's preferred means of reproduction is by cross fertilization. Eggs are void with the faeces and embryonation will only occur in the presence of suitable conditions. Moreover eggs must be liberated from faecal matter as it inhibits embryonation.

Free-swimming phototropic miracidia are liberated from the egg and must invade the secondary host within 24 hours (Hope-Cawdery *et al.*, 1978). It is believed that this adaptation has developed in order that the miracidia have the advantage of always continually migrating to the edge of the water source where the amphibious intermediate host resides. Once present in the host's habitat it is attracted to the snail by chemotaxis. Transmission into the intermediate host is active and may require proteolytic enzymes to penetrate the body wall.

On reaching the snail the miricidia attach to the intermediate host by it's apical gland. The final penetration is by a young sporocyst which migrates via the lymph of blood vessels to the digestive gland. In favourable conditions one sporocyst develops into 5-8 first generation rediae. First generation rediae in turn give rise to about 40 daughter rediae. Further development occurs and the rediae develop into approximately 600 cercariae which are shed via the birth pore (Andrews, 1998). The development of larval stages in the snail is temperature dependent.

After the release of cercariae some will encyst at the waters surface but most attach to aquatic plants below the water level. The tails from the cercariae are shed and the cyst wall is secreted within two minutes to two hours to form metacercariae, the infectious stage of the life cycle (Smyth and Halton, 1983). Transmission to the definitive host is passive, by the ingestion of aquatic plants or by drinking contaminated water.

Metacercariae excystment is initiated in the stomach of the mammalian host in the presence of high concentrations of carbon dioxide, reducing conditions and high temperatures (Fried, 1994). Liberation of the newly encysted juvenile (NEJ) fluke migrate through the intestinal wall into the peritoneal cavity, subsequently penetrating the gilsons capsule and migrating across the liver parenchyma to the biliary passages. It is during this phase that the parasite causes extensive damage. Once present in the bile duct the worms become sexually mature in four-five weeks and commence egg production.

1.15 Immunology of *F. hepatica* infection

The physiological components of the innate immune system, such as the skin, mucous layers and gastric acid may provide resistance to some infectious agents, but they are generally ineffective against helminth infections. The metacercariae of *F. hepatica* can not only have the ability to survive the acid environment of the stomach, but the gastric acid actually aids in the excystment process of the parasite (Mulachy *et al.*, 1999). With regard to acquired immunity, helminth infections are generally considered to induce a cytokines polarized to the Th2 subtype. It has been demonstrated that mice infected with *F. hepatica* display an early and

persistently polarized Th2 response (O'Neill *et al.*, 2000), with highly elevated levels of IL-4 and IL-5, and an absence of the type 1 cytokines IFN-γ and IL-2. In addition, it was found that mice produced antifluke IgG1 antibodies, without the production of the Th1-associated subtype, IgG2a, which is also indicative of a Th2 response (O'Neill *et al.*, 2000). The induction of type 2 responses in mice are in agreement with earlier studies which collectively suggest that type 2 responses predominate in *F. hepatica*-infected rats, sheep and cattle (Mulcahy *et al.*, 1999). Furthermore, *F. hepatica* infection has been shown to be accompanied by high levels of IgE and eosinophilia (Mulcahy *et al.*, 1999, Finkelman *et al.*, 1991). The induction of cytokines characteristic of the Th2 subtype involves the early production of IL-4. The cells that produce this early IL-4 remain to be elucidated, but it has been suggested that mast cells, basophils, eosinophils or $NK1.1^+$ T-cells, may be capable of fulfilling this role.

The induction of a Th2 response in many helminth infections is frequently accompanied by a downregulation in Th1 responses or cell-mediated immunity (Sher *et al.*, 1992). Inhibition of lymphocyte proliferative responses has been reported during nematode infections (Allen *et al.*, 1998). Similarly, infection with *F. hepatica* has also been associated with impaired proliferative responses (Cervi *et al.*, 1998), and the downregulation of Th1 cytokine responses in mice (Brady *et al.*, 1999, O'Neill *et al.*, 2000). The parasite-induced production of IL-4 is believed to play a role in this suppression, but may not be mediated solely by the production of inhibitory cytokines. Immunomodulatory molecules secreted by the liver fluke may also be critical in suppressing a potentially protective Th1 response and prolonging survival of the parasite. Cervi et al. (1996) showed that excretory-secretory antigens of *F. hepatica* suppressed the delayed hypersensitivity responses, which are regulated by Th1 cytokines, both specifically and non-specifically. Other candidate immunosuppressive molecules include glycoconjugates sloughed from the parasite surface glycocalyx (Mulcahy *et al.*, 1999), and/or cysteine proteinases (Smith *et al.*, 1993, Carmona *et al.*, 1993).

During the migratory phases, the flukes survive even though they are in contact with the host circulation. It was demonstrated that during these phases the flukes secrete a proteolytic enzyme capable of cleaving immunoglobulin (Chapman

& Mitchell, 1982). They proposed that *in vivo*, this enzyme may cleave anti-fluke antibody, and hence prevent the adherence of immune effector cells, such as macrophages and eosinophils, protecting the flukes from immune destruction. Since then it has been shown that *F. hepatica* secretes approximately 11 different proteinases (Dalton & Heffernan, 1989). One of these proteinases (cathepsin L1) was shown to cleave IgG at or near the hinge region, and to prevent the antibody-mediated attachment of immune effector cells to the parasite (Smith *et al.*, 1993, Carmona *et al.*, 1993). This effect can be blocked in vitro by the addition of anti-cathepsin L antibodies (Smith *et al.*, 1994). Another cathepsin L proteinase (cathepsin L2), with a different substrate specificity was also purified (Dowd et al., 1994). The authors suggested that the action of both of these enzymes may be the prime mechanism by which the parasites penetrate tissue, but that they may also play an immunoevasive role due to their ability to cleave host Ig (Smith *et al.*, 1993, Carmona *et al.*, 1993).

1.16 Parasite-induced immunosuppression

Possibly the most distinguishing feature if parasitic infection is its chronicity. In endemic areas, parasitic infection implies a lifelong intimate association, where individuals are infected from a very young age, and can expect to harbour worms for most of their lives, due to an inability to develop protective immunity. In both filarial and schistosome infections, adult worms are, in general, more resistant to immune effector mechanisms than the larval stages. This phenomenon is known as concomitant immunity and is considered a general feature of helminth infection (Maizels *et al.*, 1993). In concomitant immunity the host is protected against newly invading larvae while tolerating an established adult worm load. This suggests that adult worms have developed immune evasion strategies, such as stage-specific antigen expression, and/or an ability to counteract immune effector mechanisms that would kill immature forms.

Many parasitic infections are associated with the induction of Th2 type responses (Finkelman *et al.*, 1991, Grencis, 1993, O'Neill *et al.*, 2000). However, whether the production of these cytokines plays a role in protection against infection remains controversial. The cytokines produced in response to parasitic

protozoa and helminths can have opposing effects on the parasite, resulting in the resolution of infection or promotion of disease and immunopathology (Finkelman *et al.*, 1991, Grencis, 1993, Scott *et al.*, 1989, Sher & Coffman, 1992). Despite this apparent paradox regarding protection and immunopathology, type 2 cytokines induced by parasitic infection have been implicated in the process of immunosuppression, which is a frequently encountered feature of parasitic infection (Pearce *et al.*, 1991, Mosmann & Moore, 1991, Kullberg *et al.*, 1992, Maizels *et al.*, 1993, Actor *et al.*, 1993, Cervi *et al.*, 1997, Allen *et al.*, 1998, Cervi *et al.*, 1998, O'Neill *et al.*, 2000), and is almost certainly responsible for the long-term survival of parasites.

Parasite-induced cytokines produced by the Th2 subset, have the ability to suppress the production or activity of Th1 cytokines. The establishment of this state of cross-regulation has particular relevance where individuals may be exposed to multiple infections, or when vaccines are administered during chronic parasitic infection. Indeed, there have been several reports of a downregulation of Th1 cytokines to both parasite and non-parasite antigens during helminth infection with *S. mansoni* and *F. hepatica* (Kullberg *et al.*, 1992, Pearce *et al.*, 1991, O'Neill *et al.*, 2000), and an exacerbation of concurrent infections (Actor *et al.*, 1993, Njunda *et al.*, 1996, Brady *et al.*, 1999). IL-4, IL-10 and TGF-β, have all been implicated in the downregulation of cell-mediated immune responses (Sher *et al.*, 1992). These cytokines have well-defined effects on Th lymphocyte responses. IL-4 promotes the expansion of the Th2 subset, and acts as an antagonist to IL-12, therefore inhibiting Th1 development (Abbas *et al.*, 1996). IL-10 impairs ability of APC to activate the Th1 subset to produce IFN-γ (Mosmann & Moore, 1991, Fiorentino *et al.*, 1991), and can also inhibit macrophage activation and IFN-γ-induced nitric oxide production, which is involved in parasite killing (Gazzinelli *et al.*, 1992). Studies with the helminth *S. Mansoni* have implicate IL-10 in the suppression of Th1 responses (Sher *et al.*, 1991), and may also be responsible for immune suppression in *F. hepatica* infection (O'Neill *et al.*, 2000). IL-4 and IL-10 can act synergistically to inhibit the production of reactive nitrogen oxides, which are known to upregulate inflammatory responses (Liew, 1993). IL-4, IL-10 and TGF-β all downregulate macrophage functions involved in the effector phase of

cell-mediated immunity to *L. major*, *Trypanosoma cruzi* and *Toxoplasma gondii* (Sher *et al.*, 1992).

Immunosuppression by parasites may not be mediated solely by the production of inhibitory cytokines. Molecules contained in the excretory-secretory products of parasites may also play a critical role in immunoevasion and immunomodulation. These include phosphorlycholine (Harnett and Harnett, 1999), which has been shown to modulate lymphocyte responsiveness, immunosuppressive glycoconjugates (Mulcahy *et al.*, 1999), and cysteine proteinases, which have been implicated in tissue degradation, hemoglobin digestion (Dalton *et al.*, 1995), the induction of allergenic-like reactions (Dowd *et al.*, 1994), and antibody cleavage (Smith *et al.*, 1993, Chapman & Mitchell, 1982).

OBJECTIVES

The aims of this project were to demonstrate that the murine respiratory challenge model of infection with *Bordetella pertussis* is a reliable method for the assessment of pertussis vaccine potency, the determination of consistency between different clinical batches of Pa and Pw, and in the elucidation of the mechanisms mediating protection. This model was utilized to define the contributions of B- and T-cells in long term protection against *B. pertussis*, following immunization with Pa and Pw. In addition, because IFN-γ and Th1 cells have been shown to be critical in host protection to *B. pertussis*, the contribution of the IFN-γ-inducing cytokines IL-12 and IL-18 to protection was investigated. Finally, as Th1 and Th2 cells produce cytokines that are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype, it was examined whether the cross-regulation of Th1 and Th2 cells occurs *in vivo*, using a coinfection model with a Th2 inducing parasite *F. hepatica* and the Th1-inducing bacterium *B. pertussis*.

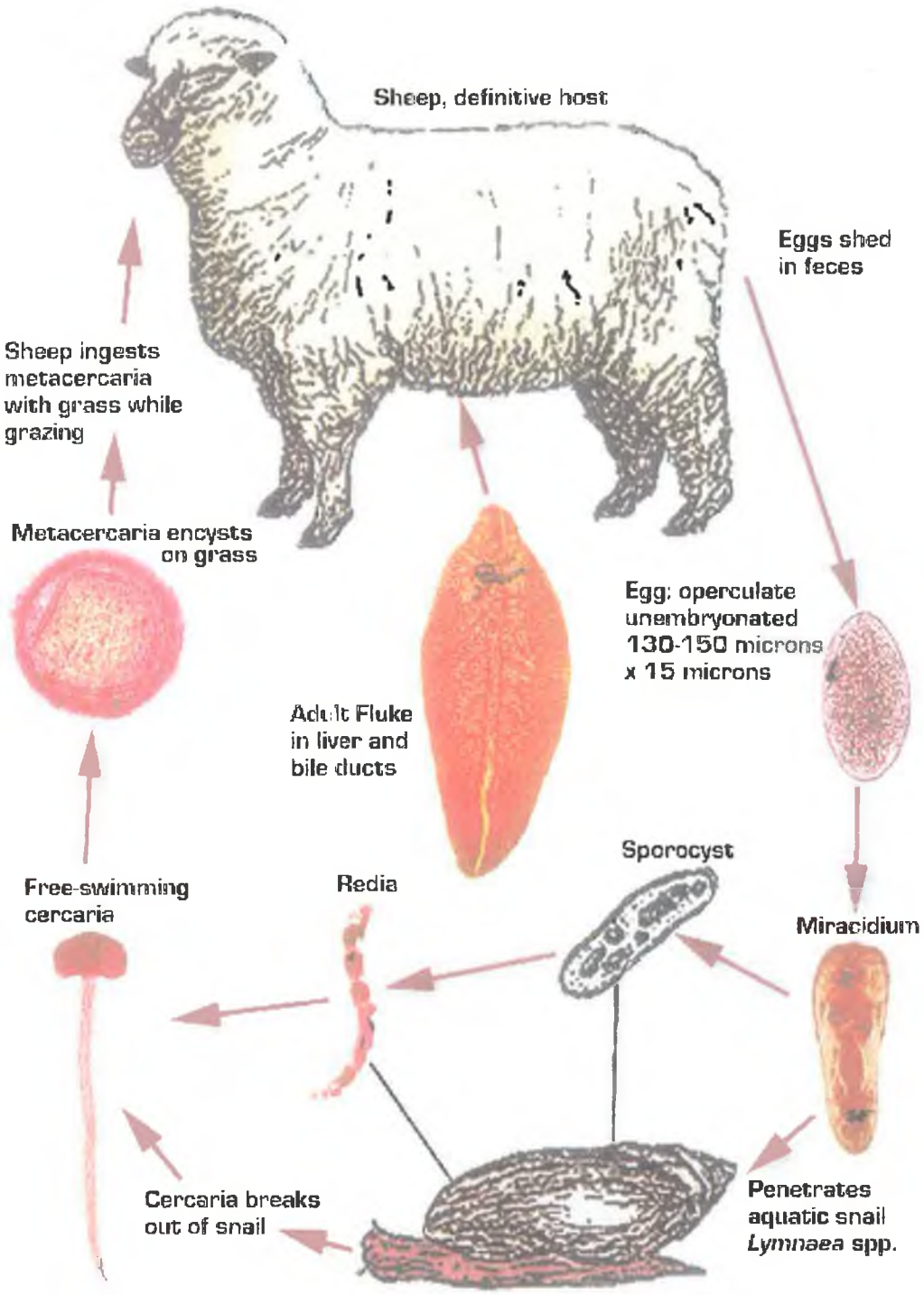


Figure 1.3 The Life Cycle of *Fasciola hepatica*

❖ CHAPTER 2 ❖

MATERIALS AND METHODS

2.1 Preparation Of Media And Buffers

2.1.1 Bordet-Gengou Medium

250 ml ddH_20 (distilled/deionised water)

2.5 ml Glycerol

7.5 g Bordet Gengou Agar (Difco)

The medium was then autoclaved (121°C for 20 mins.), allowed to cool and supplemented with 1 ml of cephalexin (Sigma, 10 mg/ml) and 50 mls of pre-warmed sterile defribrinated horse blood (E+O Laboratories, Bonnybridge, Scotland). The medium was then poured into 9 cm petri dishes (20 ml aliquots) and stored at 4°C for up to 5 weeks.

Cephalexin

0.1 g Cephalexin dissolved in ddH_20 and then filter sterilized.

1% Casein

6.0 g Sodium Chloride (NaCl)

10.0 g Casamino acids

Dissolved in 1 Litre ddH_20 pH 7.2 and autoclaved 115°C for 20 mins.

2.1.2 Phosphate-Buffered Saline (PBS)

80 g Sodium Chloride (NaCl)

11.6 g Sodium hydrogen phosphate (Na_2HPO_4)

2 g Potassium dihydrogen phosphate (KH_2PO_4)

2 g Potassium Chloride (KCl)

Dissolved in 1 L of ddH20 and pH adjusted to 7.0.

2.1.3 Tris-Buffered Saline (PBS)

2.42g Trizma base

8.76g Sodium Chloride (NaCl)

Dissolved in 1 L of ddH_2O and pH adjusted to 7.3

2.1.4 Stainer and Scholte Medium

Basal Medium

10.72 g L-Glutamic acid (monosodium salt)

0.24 g L-Proline

2.5 g NaCl

0.5 g KH_2PO_4

0.2 g KCl

0.3 g Magnesium Chloride ($MgCl_2.6H_20$)

0.4 g Calcium Chloride ($CaCl_2.2H_20$)

1.525 g Tris (hydroxymethyl) methylamine

Dissolved in 1 L ddH_20 and pH adjusted to 7.2. The medium was then autoclaved at 121°C for 20 minutes, allowed to cool and 1 ml of supplement added per 100 ml of basal medium before use.

Supplement

0.4 g L-Cysteine (dissolved first in 1 ml of concentrated HCl)

0.5 g Ferrous Sulphate ($FeSO_4.7H_20$)

0.6 g Ascorbic acid

0.7 g Nicotinic acid

1.0 g Glutathione

Dissolved in 100 ml of ddH_20 and filter sterilized and stored at 4°C for up to 5 weeks.

2.1.5 Cell Culture Medium

Complete RPMI medium: Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco BRC, Life Technologies, Paisley, Scotland), was used to culture all cell lines, and primary cells isolated from murine spleens and lymph nodes. This was supplemented with 8% heat inactivated (56°C for 30 mins) foetal calf serum (FCS), 100mM L-Glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-Mercaptoethanol.

2.1.6 Diethanolamine Buffer

97 ml Diethanolamine

0.1 g Magnesium Chloride (MgCl2.6H20)

0.2 ml 1M Sodium Azide (NaN3)

made up to 1 L with ddH20 and pH adjusted to 7.2

2.1.7 Sodium acetate buffer

Acetic acid (0.2M)

11.55ml of glacial acetic acid was added to 1 L of ddH_20

Sodium acetate (0.2M) (CH_3COONa)

16.4 g of sodium acetate was added to 1 L of ddH_20

Sodium acetate buffer

This was prepared by adding 148ml of 0.2M acetic acid to 352ml of 0.2M sodium acetate. The volume was brought up to 1 L with ddH_20 and the pH adjusted to 5.0

2.1.8 3-amino-9-ethylcarbazole (AEC) substrate buffer

10mg of AEC

1ml dimethylformamide (DMF)

30ml 0.1M sodium acetate buffer (pH 5.0)

15µl 30% Hydrogen peroxide (H_2O_2)

First, the AEC was dissolved well in 1ml of DMF in a glass tube. 30ml of sodium acetate buffer (pH 5.0) was then added, and the resulting solution was then passed through a 45µm filter. Finally 15µl of hydrogen peroxide was added. This substrate was freshly prepared prior to use.

2.2 Bacterial antigens

B. pertussis Wellcome 28 phase 1 stocks were provided by Keith Redhead at the National Institute for Biological Standards and Control (NIBSC), Herts, UK. Heat killed *B. pertussis* for use in T-cell assays were prepared by incubation of cells at

90°C for 20 mins. Purified native pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN) for use in T-cell assays were provided by Connaught Laboratories Ltd., Ontario, Canada. Purified filamentous haemagglutinin (FHA), pertactin (PRN) and non-toxic recombinant pertussis toxin (PT-9K/129G), were also provided by Chiron Corporation, Siena, Italy. PT was inactivated by heating at 80°C for 20 minutes. This abolished mitogenic activity prior to use in T-cell assays *in vitro*. All other antigens were used as native preparations without chemical or heat-inactivation.

2.3 Preparation of Liver Fluke Homogenate (LFH)

Adult liver flukes were obtained from infected livers of slaughtered cattle at a local abbatoir. In order to remove bile and debris the flukes were washed in sterile PBS. Mature liver flukes were homogenised in 10 ml of sterile PBS. The liver fluke homogenate was centrifuged at 13,000 g for 30 min. The supernatant containing soluble antigens was removed and aliquoted into 1 ml vials and stored at –20°C until use. Protein concentration was determined using bicinchoninic protein assay (BCA). This assay was used to quantitatively determine protein concentrations from 20µg/ml to 2000µg/ml. Bovine serum albumin, at a concentration range of 25µg/ml to 2000µg/ml were used to plot a standard curve.

2.4 Mouse Strains

Specific pathogen-free BALB/c (H-2^d), C57BL/6 (H-2^b), and mutant C57/BL6 mice deficient in the IL-4 gene (IL-$4^{-/-}$) were bred and maintained under the guidelines of the Irish Department of Health and were 8-12 weeks old at the initiation of experiments. IL-12 knockout (IL-12-/-) mice were purchased from Harlan Olac Ltd., Blackthorn, United Kingdom.

2.5 Antibodies

The specificities and source of monoclonal and polyclonal antibodies used are detailed in Table 2.1

2.6 Cytokine Standards

Murine cytokines were obtained from the Division of Immunobiology in NIBSC and from commercial sources as outlined in Table 2.2

2.7 Cell lines

The adherent murine macrophage cell line J774 and the IL-2 dependent T-cell line CTLL-2 were obtained from the American Type Culture Collection (ATCC) Rockville, USA.

2.8 CTLL Cell Culture

CTLL cells were cultured under sterile conditions in RPMI-1640 medium (as described in 2.2.5), supplemented with 5 U/ml of recombinant IL-2 at 37°C and 95% humidity in a CO_2 incubator. Cells were seeded at a density of 2 x 10^5/ml and passaged every two to three days of culture.

2.9 Cryopreservation of cells in liquid nitrogen

Cells were frozen in RPMI-1640 medium containing 10% (v/v) dimethysulphoxide (DMSO) and 20% FCS (v/v). The cells to be cryopreserved were first pelleted by centrifugation, resuspended with the freezing mix at 4°C and then transferred to cryo tubes in 1.2 ml volumes. Cells were frozen under conditions that permitted a gradual reduction of temperature (1°C/min) before transferral to liquid nitrogen. Recovery of viable cells from liquid nitrogen was performed by thawing the cells quickly in a 37°C water bath. The cells were then transferred to 10 ml centrifuge tubes and washed 3 times in RPMI-1640 to ensure that all traces of DMSO were removed from the cell suspension. A viability count was performed and cells resuspended in complete medium and cultured at 37°C in a CO_2 incubator as described above.

2.10 Assessment of cell viability

A staining solution was prepared by dissolving 0.1 mg of Acridine Orange and 0.1 mg of Ethidium Bromide (Sigma) in 100 ml of PBS and stored at 4°C. To assess cell viability 980 µl, 180 µl, or 20 µl (1/50, 1/20, ½ dilution, depending on cell

density) of this stain was mixed with 20µl of cell suspension and placed in a haemocytometer. The number of viable cells (green) and non-viable cells (orange) were counted using a fluorescent microscope.

2.11 Formaldehyde detoxification of bacterial antigens

The detoxification of bacterial antigen was achieved by addition of Gelatin (0.02%, v/v) and Tween 20 (0.05%, v/v) to the antigen preparation followed by addition of formaldehyde (0.2%, v/v) and incubated overnight at 37°C. Following this treatment the sample was exhaustively dialysed against 0.01% formaldehyde/PBS. The concentration of protein in the formaldehyde treated antigen sample was then assessed by the Bio-Rad protein assay.

2.12 Bio-Rad protein microassay

Protein concentrations were determined by the use of the Bio-Rad protein assay (Bio-Rad, Herts, UK). 800 µl of either the test sample, a serially diluted protein standard (Bovine serum albumin (BSA)), or PBS were mixed with 200 µl of Bio-Rad dye reagent. After 5 minutes, absorbance at 595nm was measured against the reagent blank and protein concentration of the test sample was determined after reference to a standard curve prepared using BSA in the range of 0 to 200 µg/ml.

2.13 UV-Sterilization

Protein antigens or other preparations were sterilized by exposure to UV-irradiation at 254nm for 10 minutes at a distance of 8 cm from the light source. Only preparations in medium free reagents were UV-irradiated as the phenol red dye present in RPMI-1640 medium generates toxic free radicals upon exposure to UV light.

2.14 Preparation of acellular vaccines

Alum adsorbed antigens were prepared by mixing 5µg each of FHA, pertactin and PTd to 150µg of alhydrogel and incubated overnight at 4°C with constant agitation. All other acellular vaccines used were provided as freeze dried preparations which were reconstituted in sterile PBS, or as full liquid formulations.

2.15 Preparation of whole cell vaccines

0.2 human dose of whole cell vaccines (88/522) or (94/532) reconstituted in sterile PBS were used for all immunizations, except where otherwise stated.

2.16 Immunizations

All mice were 8-12 weeks old at the initiation of experiments. Mice immunized by the intraperitoneal (i.p.) route received a maximum volume of 0.3 ml per mouse. In experiments to assess the protective efficacy of pertussis vaccines mice received two i.p. immunizations (weeks 0 and 4) and were aerosol challenged with *B. pertussis* (as described in section 2.14) 2 weeks after the second immunization.

2.17 Aerosol infection of mice

Respiratory challenge of mice was induced by aerosol challenge using a modified version of the method described by Sato et al., 1980. *B. pertussis* Wellcome 28 phase 1 was grown at 37°C under agitation conditions in Stainer-Scholte liquid medium (Stainer and Scholte, 1971). Bacteria from a 48hr. culture were concentrated by centrifugation to 2 x 10^{10} CFU/ml in physiological saline containing 1% casein. Mice were exposed for 15 minutes to the challenge inoculum using a nebulizer directed into an aerosol chamber containing groups of 20-30 mice. Four mice form each experimental group were sacrificed two hours after aerosol challenge and at various other times to assess the number of viable bacteria in the lungs.

2.18 Enumeration of viable bacteria in the lung

Lungs were removed aseptically and homogenized in 1 ml of sterile PBS with 1% casein on ice. One hundred microlitres of neat or serially diluted homogenate were plated out on Bordet-Gengou plates. The plates were incubated at 37°C for 4 days and the numbers of colony forming units (CFU) were estimated. Results are given as the mean number of *B. pertussis* CFU for individual lungs from four mice from each experimental group.

2.19 *FASCIOLA HEPATICA* INFECTION OF MICE

Metacercariae were obtained from Compton Paddock Laboratories, Berkshire, UK. Mice were infected by placing a very small amount of cotton wool which contained 10 metacercariae into the mouth, followed by a few drops of water to aid swallowing.

2.20 DETECTION OF T-CELL CYTOKINES

Spleen mononuclear cells (2 x 10^6/ml) were stimulated *in vitro* with *B. pertussis* antigens, mitogens (positive control) or medium alone (negative control). Alternatively, lymph node cells (1 x 10^6/ml) were cultured in vitro using the same stimuli. Supernatants were removed after 24h for analysis of IL-2 concentrations and after 72h for IL-4, IL-5 and IFN-γ. IL-2 was assayed by testing the ability of supernatants from antigen stimulated T-cells to support the growth of the IL-2 dependent CTLL-2 cell line. 50μl of test supernatants, removed after 24hrs of culture were added in triplicate to 96-well microtiter plates and incubated with 50μl of CTLL-2 cells (1 x 10^5/ml). Cells were cultured for 24hrs and pulsed with [^{3}H]-thymidine for the final 4hrs of culture. Following the 4h pulse period, the wells contents were harvested onto glass fiber paper with an automatic cell harvester. The filters were then dried using an IR lamp and placed in plastic bags with non-aqueous scintillation fluid and [^{3}H]-thymidine incorporation measured using a Beta-plate counter (Wallac). The levels of IL-2 were determined after reference to a standard curve prepared with recombinant murine IL-2 and results expressed as international units per milliliter (IU/ml).

The concentrations of IL-4, IL-5 and IFN-γ were measured by enzyme linked immunosorbent assay (ELISA) using the antibodies described in table 2.1 Cytokine specific capture antibodies (50μl/well at 1μg/ml in PBS) were added to 96-well microtitre plates (Nunc) and incubated overnight at 4°C. The plates were washed 4-5 times with wash solution (PBS/0.05% Tween 20) and then incubated with 200μl/well of blocking solution (PBS supplemented with 10% w/v of dried milk (marvel®)) at room temperature for 2h to block non-specific binding sites. Following washing, plates were incubated overnight at 4°C with 50μl/well of the

test supernatant or the corresponding cytokine standard. The plates were then washed and incubated with 50µl/well of a biotinylated anti-cytokine antibody and 1µg/ml in PBS at room temperature for 1h. After washing, the plates were incubated for 20 minutes at room temperature with 50µl/well of extravidin alkaline phosphatase (Sigma Chemical Co. Ltd, Poole, Dorset, UK) at 1/2500 dilution in PBS. Finally, after washing, the plates were incubated with 100µl/well of p-nitrophenyl phosphate (pNPP) substrate (Sigma) at 1mg/ml in 1M diethanolamine buffer pH 9.8. The O.D. value of test samples and cytokine standards were measured at 405nm using a microtiter plate reader (Titertex) and cytokine concentrations for test samples determined after reference to a standard curve prepared from recombinant cytokines of known concentration and potency. The limits of the assays were 0.5 ng/ml for IFN-γ and 20 pg/ml for IL-4 and IL-5.

2.21 Isolation of PBMC from murine blood

A stock solution of (35.3% w/v) of analytical grade metrizamide (Nyegaard) was prepared in distilled water. This was filter sterilized and kept in the dark at 4°C. Prior to use the stock metrizamide solution was diluted to 18% v/v in PBS. Fresh blood samples obtained by thoracic bleed were collected into tubes containing 1M EDTA (10µl/tube). This was to prevent the blood from clotting. Blood was then carefully layered onto the metrizamide gradient (2 ml) and centrifuged at 1500 rpm for 30 mins. PBMC were removed from the interface, washed twice and resuspended in RPMI-1640 (8% FCS), and a viable cell count was performed

2.22 Detection of cytokine-secreting cells by ELISPOT

Antibody-forming cells were quantified by an insoluble substrate *B. pertussis*-specific Enzyme-Linked Immunospot assay (ELISPOT). The wells of 96-well nitrocellulose plates (Multiscreen HA: Millipore, Bedford, MA) were coated overnight at 4°C with 100µl of IFN-γ or IL-5 capture antibody, at a concentration of 2µg/ml. The plate and the supporting manifold were washed 5 times aseptically with PBS and then were blocked for 2hr with RPMI, 8%FCS (vol/vol). Supernatants were removed from spleen cells ($1x10^5$ cells/ml), which had been

stimulated for 72 hours with *B. pertussis* antigens, and the cells were resuspended in fresh medium. Resuspended cells were added to the nitrocellulose plates and incubated overnight at 37°C with 5% CO_2. Plates were washed three times with PBS, followed by a further three washes with PBS/Tween. After washing, 100µl/well biotinylated detection antibody in PBS/Tween/ 1% BSA, was added at a concentration of 1µg/ml for IFN-γ and 5µg/ml for IL-5, and plates were incubated overnight at 4°C. Following incubation, plates were washed with PBS and peroxidase-conjugated polyclonal goat-anti-biotin (PAB) antibody was added at 100µl/well, and at a concentration of 1mg/ml in PBA/Tween/1% BSA for both cytokines, and plates were again incubated for 24 hours at 4°C. Plates were then washed 3 times more with PBS/Tween, before the addition of 200µl/well 3-amino-9-ethylcarbazole substrate buffer (AEC). After maximal spot development (5-30 minutes), this was removed, and the plates were washed once with distilled water and allowed to air-dry. Spots representing individual cytokine-secreting cells were counted using a zoom stereo microscope.

2.23 ELISPOT ASSAY FOR THE IDENTIFICATION OF ANTIBODY-SECRETING CELLS

Antibody-forming cells were quantified by an insoluble substrate *B. pertussis*-specific ELISPOT. The wells of 96-well nitrocellulose plates were coated overnight with 100µl of sonicated *B. pertussis* or inactivated pertussis toxin (5µg/ml). Both surfaces of the membrane were washed 5 times aseptically with PBS and then were blocked for 2hr with RPMI, 8%FCS (vol/vol). After blocking, spleen or peripheral blood mononuclear cell preparations were added to quadruplicate wells at concentrations from 1×10^4 to 1×10^7 cells/ml and were incubated at 37°C with 5% CO_2 for 2 days. After washing 6 times in sterile PBS, horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, Dorset, UK) was added for 2 hours. Plates were then washed 3 times more before the addition of 200µl/well 3-amino-9-ethylcarbazole substrate buffer (AEC). After 45 min, this was removed, and the plates were washed once with distilled water and allowed to

air-dry. Spots representing individual antibody-forming cells were counted by use of a zoom stereo microscope.

2.24 Determination of serum IgG antibody levels

Serum samples were prepared from peripheral blood of mice removed from the thoracic cavity after sacrifice. Blood was allowed to clot for 1 hour at room temperature and then placed at 4°C for 2-4 hours. Serum was then removed into fresh tubes and stored at -20°C. The levels of antigen-specific IgG antibodies in the sera of naïve, immunized or infected mice was determined by ELISA. PT, FHA and PRN (1μg/ml in PBS) or *B. pertussis* sonicate (5μg/ml in PBS), was added to 96-well microtiter plates and incubated overnight at 4°C. Excess antigen was washed with wash buffer (PBS/0.05% Tween 20) and non-specific binding sites were blocked by incubating the plates with PBS supplemented with 10% w/v of dried milk for two hours. Sera were serially diluted in PBS on the washed plates to determine the endpoint titres. The initial dilution used for analysis of total IgG titres depended on the number of immunizations and the time after immunization or infection. In general, an initial dilution of 1/75 or 1/100 was used for serum from mice which were infected or which had received two immunizations. The plates with the serum samples were incubated overnight at 4°C or for 2h at room temperature. After washing, plates were incubated for 1h at room temperature with 50μl/well of an alkaline phosphatase conjugated rat anti-mouse IgG monoclonal antibody (1/10,000 dilution in PBS/2%FCS), with a specificity for the whole IgG molecule. The plates were then washed and incubated with 100μl/well of p-nitrophenyl phosphate in diethanolamine buffer. The absorbance was measured at 405nm. Results are expressed as $\log_{10}$ endpoint antibody titres determined by extrapolation to the OD405nm value of the control, naïve serum. Control wells in which the antigen, serum sample or detection antibodies were omitted gave negligible absorbances with range 0.1-0.2.

2.25 The detection of IL-12 by immunoassay

IL-12 was detected by immunoassay using commercially available antibodies (Genzyme) specific for the IL-12 p40 subunit. These antibodies recognise IL-12

p40 either as a monomer, heterodimer, or as part of the p70 heterodimer. 96-well microtiter plates were coated with 50µl/well of a capture anti-IL-12 monoclonal antibody (as described in Table 2.1) at a concentration of 2µg/ml in PBS and incubated overnight at 4°C. Following washing non-specific binding sites were blocked at room temperature for 2hr using 200µl/well of PBS with 10% w/v dried milk. After washing 50µl of the test sample or diluted IL-12 standards in the range of 0-5000pg/ml were added and incubated overnight at 4°C. The plates were then washed and incubated with the detecting antibody (rat anti-mouse IL-12) at a concentration of 2µg/ml in PBS (50µl/well) for 1hr at room temperature. An alkaline phosphatase conjugated mouse anti-rat IgG2a (PharMingen) antibody was then used to detect the second anti-IL-12 antibody. Plates were incubated with 50µl/well of a 1/1000 dilution of the alkaline phosphatase antibody for 2hr at room temperature. Finally, plates were washed and incubated with 100µl/well of pNPP substrate at 1mg/ml prepared in diethanolamine buffer (pH 9.8). OD values were measured at 405nm and IL-12 concentrations were determined from a standard curve prepared using recombinant murine IL-12 of known concentration.

2.26 IL-18 ELISA

The concentrations of IL-18 were determined using an enzyme-linked immunosorbent assay (ELISA), using the antibodies described in Table 2.1. IL-18 specific capture antibody (50µl/well at 1µg/ml in PBS) was added to 96-well microtitre plates and incubated overnight at 4°C. The plates were washed 5 times with PBS/0.05% Tween 20 and then incubated with 200µl/well of blocking solution (1%BSA, 5% sucrose, and 0.05% NaN_3 in PBS) for 2 hours at room temperature, to block non-specific binding sites. Following washing, plates were incubated overnight with 50µl/well of the test supernatant, or lung homogenates, and the appropriate cytokine standard. The plates were then washed again and incubated with 50µl/well of a biotinylated detection antibody at 200ng/ml in Tris Buffered Saline (TBS) pH 7.3, at room temperature for 2 hours. After washing the plates were incubated with for 30mins at room temperature with 100µl/well streptavidin (horseradish peroxidase conjugated) (Sigma chemical Co. Ltd., Poole, Dorset, UK),

at a 1/1000 dilution in TBS as recommended by the manufacturer. Finally, after washing, the plates were incubated with 100µl/well of tetramethylbenzidine (TMB) substrate (Sigma). The plates were allowed to develop for approximately 20mins, and then the reaction was stopped with the addition of 1M H_2SO_4. The O. D. value of test samples and cytokine standards were measured at 450nm using a microtiter plate reader (SpectraMax, Gemini) and cytokine concentrations for test samples determined after reference to a standard curve prepared from recombinant cytokines of known concentration and potency. The limit of the assay was 31 pg/ml.

2.27 Statistics

Statistical analysis of data was performed using the computer based statistical package StatWorks. Statistical significance of difference was assessed using the Mann Whitney U test or Student's *t* test as indicated in the text and figure legends.

TABLE 2.1 THE ORIGIN AND SPECIFICITIES OF ANTIBODIES

Antibody clone	Specificity	Conjugate	Supplier
RA-6A2	mouse IFN-γ	-	PharMingen, San Diego, USA
XMG1.2	" IFN-γ	biotin	"
11-B-11	" IL-4	-	National Institute of Health, USA
BVD6-24G2	" IL-4	biotin	PharMingen, San Diego, USA
TRFK5	" IL-5	-	"
TRFK4	" IL-5	biotin	"
C17.8	" IL-12	-	Genzyme Diagnostics, MA, USA
C15.6	" IL-12	-	"
R19-15	" IgG2a	AKP	PharMingen, San Diego, USA
51817.111	" IL-18	-	R&D Systems, UK
-	" IL-18	biotin	"
-	goat Biotin	peroxidase	Sigma Chemical Co. Ltd, Poole, Dorset, UK

TABLE 2.2 RECOMBINANT CYTOKINES

Cytokine	Source
Mouse IFN-γ	NIBSC, Herts, UK
" IL-2	NIBSC, Herts, UK
" IL-4	NIBSC, Herts, UK
" IL-5	PharMingen, San Diego, USA
" IL-12	Stanley Wolf, Genetics Institute Inc., Mass., USA
" IL-18	R&D Systems, UK

❖ CHAPTER 3 ❖

A RESPIRATORY CHALLENGE MODEL FOR INFECTION WITH *BORDETELLA PERTUSSIS*: APPLICATION IN THE ASSESSMENT OF PERTUSSIS VACCINE POTENCY AND IN DEFINING THE MECHANISM OF PROTECTIVE IMMUNITY

3.1 Introduction

The last decade has seen the emergence of an abundance of information regarding pathogenesis and immunity to *B. pertussis*; data which has improved our current understanding of the disease, and contributed to the development of new less reactogenic vaccines. Although immune responses elicited following vaccination or infection in humans has provided important insights on immunological correlates of protection, a more precise examination of the mechanisms of protective immunity is very often dependent on the utilization of animal models. Furthermore, licensure of vaccines usually requires an evaluation of potency and safety in pre-clinical models of infection. Studies in a murine respiratory challenge model of infection with *B. pertussis* have made a significant contribution to the understanding of the immunological mechanisms that determine the resolution of infection or progression to disease.

Aerosol inoculation of mice with *B. pertussis* results in a uniform, self-limiting infection with many similarities to that observed in children. Although the characteristic cough is absent in this model, other disease manifestations such as local pathology, systemic reactions, including leucocytosis and CNS effects have been documented (Pittman *et al.*, 1980, Sato *et al.*, 1980, Loscher *et al.*, 2000). Because *B. pertussis* does not occupy a purely extracellular niche, but can invade and survive within several mammalian cell types, protection in both mice and children involves a complex dual T-cell and antibody mechanism. The murine model of *B. pertussis* infection has provided vital information on the roles of both humoral and cell-mediated immunity (Mills *et al.*, 1993, Mills *et al.*, 1998a, Redhead *et al.*, 1993, Barnard *et al.*, 1996, Mahon *et al.*, 1996, Mahon *et al.*, 1997, McGuirk *et al.*, 1998) to *B. pertussis* during infection and immunization. Antibody may be important for limiting infection and disease by preventing bacterial adherence to ciliated epithelial cells, may play a role in the neutralization of toxins, and may also be required for the elimination of bacteria through opsonization. Th1 cells and particularly the cytokine IFN-γ, appears to be required for the activation of macrophages to kill intracellular bacteria. However, despite the numerous reports on different aspects of immunity to *B. pertussis* after infection or immunization, in both mice and humans, there is still no clear consensus as to what constitutes a

protective immune response to *B. pertussis*. Not only does protection involve both humoral and cellular mechanisms, but optimum immunity to *B. pertussis* also appears to incorporate multiple protective antigens. However, a major goal of clinical studies on pertussis to correlate a critical level of antibody, or a quantifiable T-cell response against a single antigen or combination of antigens, with protection against *B. pertussis*, has to date remained unattainable.

The active mouse protection test originally devised by Kendrick (1947) has been used to assess the potency of Pw. This involves intracerebral (i.c) challenge of mice 2-3 weeks post immunization with decreasing doses of the vaccine. Although studies in the U.K. in the 1950's revealed that protection against i.c challenge in mice correlated with the estimated efficacy of Pw in clinical trials (MRC, 1959), the Kendrick test fails to predict the efficacy of Pa in humans, and is unsuitable for dissecting the mechanisms of immunity to *B. pertussis*, due to the route of challenge and the dissimilarity of the model to human disease (Corbel & Xing, 1997). Prediction of the potency of Pa based on immunogenicity, especially antibody responses to individual *B. pertussis* antigens is also questionable. The measurement of antibody to the putative protective antigens of *B. pertussis* by ELISA following immunization of children, have failed to correlate with the estimated vaccine efficacy in human clinical trials (Greco *et al*, 1996, Simondon *et al*., 1997, Gustafsson *et al*., 1996, Olin *et al*., 1997). Other drawbacks exist with regard to the evaluation of pertussis vaccine efficacy based on the measurement of antibody responses. Detection of *B. pertussis*-specific antibodies by ELISA does not consequently mean that these antibodies are functionally relevant, or play a role in protection. It may be argued that antibody levels detected by this method demonstrate that the antigenic proteins are immunogenic and are capable of inducing an acquired immune response, but does not automatically indicate that the antibody elicited functions in limiting infection *in vivo*. Furthermore, measurement of circulating IgG may not necessarily reflect what is happening at the local mucosal surface in the lung. It is reasonable to expect that antibodies to adhesins such as FHA will exert a stronger protective effect when present at the site of attachment than in the circulatory system. In addition, measurement of total IgG does not impart significant information on the induction of specific Th subtypes,

immunological memory, or their role in protective immunity. The inherent disadvantages in using antibody responses as a predictor of vaccine efficacy, together with the demonstration that cellular immunity has also plays a major role in the protective mechanisms against *B. pertussis*, suggests that murine respiratory challenge model, in which both T-cell and antibody responses to different components can be easily monitored, offers the most definitive method of evaluating pertussis vaccine potency. In this study, the murine respiratory challenge model was employed to evaluate the efficacy and immunogenicity of a number of acellular and whole cell pertussis vaccines.

3.2 Results

3.2.1 Serum antibody levels detected following immunization with Pa or Pw does not predict pertussis vaccine efficacy

It has previously been shown that there is a highly significant correlation between vaccine-induced protection against *B. pertussis* in children, and protection in the murine respiratory challenge model (Mills *et al.*, 1998a). Mills and colleagues evaluated clinical lots of different Pw and Pa using the murine respiratory challenge model, and demonstrated that the efficacy of these vaccines, which had previously been evaluated in human clinical trials, were markedly similar when assessed in the murine model. Vaccines of high efficacy in children were shown also to be highly protective in mice (Table 3.1). Immunization with PM Pw, which has an estimated efficacy of 95%, conferred the highest level of protection in mice. The lower efficacy CLI Pw, which protected 36 and 48% of children in the Italian and Swedish trials respectively (Greco *et al.*, 1996, Gustafsson *et al.*, 1996), also showed significantly poorer clearance in immunized mice. Mice immunized with the 3-component SmithKline Pa, had a higher rate of bacterial clearance than in those immunized with SB Pa2. This correlated well with the results from the Italian and Swedish trials (Greco *et al.*, 1996, Gustafsson *et al.*, 1996), which showed an estimated effiacacy of 84% for SB Pa3 and 59% for SB Pa2. The efficacy of both the 5-component Pa manufactured by Connaught laboratories (CLL Pa5) and the Pasteur Merieux 2-component Pa (PM Pa2), was estimated at 85%, in the Swedish (Gustafsson *et al.*, 1996) and Senegal (Simondon *et al.* 1997),and these vaccines generated an equivalent level on protection in immunized mice. The high level of protection (84%) conferred by the CB Pa3a in the Italian trial (Greco *et al.*, 1996), showed a similar level of protection in the mouse model, which was comparable to the level of protection afforded by a different clinical lot of the same vaccine (CB Pa3b). Analysis of T-cell responses following vaccination clearly showed that a higher level of protection was provided through the induction of a more polarized Th1 response, both in children and mice (Table 3.1). In contrast, Pa induce a mixed Th1/Th2 cytokine profile in children and a more polarized Th2 response in mice (Table 3.1).

Given that vaccine-induced protection in mice correlates with estimated pertussis vaccine efficacy in children, the serum antibody responses from immunized mice used in the study discussed above, were examined in an attempt to define the relative contribution of humoral immunity to vaccine-induced protection against *B. pertussis*, and to correlate levels of *B. pertussis*-specific antibody with protection. The antibody response of mice immunized with the Pasteur-Mérieux (PM) 2-component Pa combined with Hepatitis-B, Hib and IPV vaccines either as a full liquid formulation (PM 94), or reconstituted before injection (PM 181) were also evaluated, in order to determine if the addition of other vaccines would effect antibody responses to *B. pertussis*. Mice were immunized with 0.2 human dose of either PM Pw, CLI Pw, SB Pa2, SB Pa3, PM Pa2, CLL Pa5, CB Pa3(a)/CB Pa3(b), or Pasteur Merieux (PM) 2-component Pa combined with Hepatitis-B, Hib and IPV vaccines, either as a full liquid formulation (PM 94) or reconstituted before injection (PM 181), and boosted 4 weeks later. Two weeks after the second immunization, blood was recovered from individual mice and serum antibody responses to killed *B. pertussis*, PT, FHA, PRN and FIM were evaluated by ELISA.

Antibody responses to the antigens in different lots of the CB Pa3 vaccine were not significantly different (Fig. 3.2). Similarly different formulations of the PM Pa2 combined with Hepatitis-B, Hib and IPV vaccines (PM 94 and PM 181) did not have a significant effect of the induction of antibody responses to PT and FHA. Antibody levels to full liquid formulation (PM 94) is almost identical to that of reconstituted vaccine (PM 181) (Fig. 3.2). However, antibody levels to PT and FHA in these vaccines were slightly lower when compared with the PM Pa2 used in the Senegal trial, which was combined with diphtheria and tetanus vaccines. In general all the Pa induced potent antibody responses to each of the antigens in the vaccine (Fig. 3.2). The strongest antibody responses observed were those to PT, however antibody levels to FHA were also quite considerable. In contrast, the antibody response to PT was weak in mice immunized with the higher efficacy PM Pw. FHA-specific antibody detected in the serum of mice immunized with both high (PM Pw) and low (CLI Pw) efficacy Pw were comparable to those induced in mice immunized with Pa. Antibodies specific for FIM were detected at low levels in the high efficacy PM Pw. Similarly, immunization with the high efficacy 5-

component Connaught Pa induced weak FIM-specific antibody responses. In contrast, a strong antibody response to FIM was observed in mice immunized with the low efficacy CLI Pw. These results clearly demonstrate that antibody responses detected by ELISA do not correlate with estimated pertussis vaccine efficacy, and an observation of high levels of antibody to the putative protective antigens of *B. pertussis* following immunization does not necessarily indicate high efficacy.

3.2.2 Application of the murine respiratory challenge model in the evaluation of the protective efficacy of five different Pa

The benefits of animal models for vaccine evaluation and for dissecting the mechanism of immunity are well illustrated by several studies using a murine model of infection with *B. pertussis*. Experiments carried out using this model have shown that there is a significant correlation between pertussis vaccine efficacy in children and protection in mice (Mills *et al.*, 1998a, Mills *et al.*, 1998b), and identified that T-cells play a significant role in the protective mechanisms leading to the resolution of infection with *B. pertussis*. It has been demonstrated that immunization with Pw induces a polarized Th1 response (Redhead *et al.*, 1993, Barnard *et al.*, 1996, Mills *et al.*, 1998a), whereas immunization with Pa induces a polarized Th2 response in mice and a more mixed Th1/Th2 response in children (Ausiello *et al.*, 1997, Ryan *et al.*, 1997, Ryan *et al.*, 1998).

In this study, five different Pa, the efficacy of which were previously evaluated in clinical trials (Greco *et al.*, 1996, Gustaffson *et al.*, 1996, Simondon *et al.*, 1997), were received from the World Health Organization (WHO), to be evaluated in the murine respiratory challenge model as a blinded trial (it is important to note that the vaccines received were not the actual batches used in the clinical evaluations). This collaborative study was organized to examine whether the murine respiratory challenge model was suitable to discriminate between different Pa, based on their protective ability and induction of appropriate immune responses, and to assess the consistency of the model between different laboratories. Mice were immunized with SB Pa2, SB Pa3, CLL Pa5, JNIH-3, PM Pa2 (composition and efficacy outlined in Table 3.1), and boosted 4 weeks later. T-cell cytokine and antibody responses were evaluated 2 weeks after secondary immunization. Mice

were then infected with *B. pertussis* and CFU counts were performed on individual lungs at intervals after challenge to examine the protective ability of each vaccine.

The Pa in this study provided a significant level of protection against respiratory challenge (Fig. 3.3). Non-immunized control mice showed a progressive increase in the numbers of viable bacteria in the lungs, up to 10 days post-infection, after which time bacterial numbers begin to decline (Fig. 3.3). In contrast, all immunized groups showed a sharp reduction in bacterial load 3 days post challenge, and the lungs were almost completely free from bacteria at day 14. All immunized groups displayed very similar clearance, and there was no significant difference between the numbers of viable bacteria in the lungs of mice immunized with the different vaccines. All vaccines provided a significant degree of protection against *B. pertussis* challenge, despite differences in the their composition (Fig 3.3).

Analysis of spleen cell cytokine production showed that mice immunized with the different Pa displayed a polarized but not particularly potent Th2-type cytokine profile, with very little IFN-γ production, low levels of IL-4 and moderate levels of IL-5 in response to *B. pertussis* antigens (Fig. 3.4). Although there was small differences in the estimated efficacies of the vaccines, the polarized Th2 response observed was consistent, and cytokine levels did not differ significantly between each vaccine (Fig. 3.4).

Total IgG levels in the serum of mice immunized with the different Pa were also examined. There was no antibody detected in mice immunized with alum alone (Control) (Fig. 3.5). All Pa induced potent antibody responses to the antigens in the vaccine, but the levels of *B. pertussis*-specific antibody varied considerably between vaccines, even in those that had the same component antigens (Fig. 3.5). The 5-component CLL Pa induced lower levels of antigen-specific antibody in general, compared to other Pa, except in response to FHA. JNIH-3 and the 2-component PM Pa, induced the most potent antibody responses to all antigens tested. Overall, the strongest responses induced were those to PT and FHA, levels of which were high in all vaccines tested. As expected, PRN-specific antibodies were detected only in mice immunized with either the Connaught 5-component Pa or SB Pa3, with considerably higher levels of PRN-specific antibody induced with the SB Pa3. Antibodies to BP were detected at high levels in vaccines JNIH 3 and

PM Pa2, but were significantly lower in all other vaccines tested. (SB Pa2, CLL Pa5, SB Pa3).

3.2.3 Use of the murine model to determine consistency between different batches of Pw

The protective efficacy of different clinical lots of the Pw manufactured by Pasteur Mérieux was determined using the murine respiratory challenge model. Mice were immunized twice, 4 weeks apart and T-cell and antibody responses were assessed 2 weeks after secondary immunization. Mice were then challenged and lungs were taken at intervals after challenge to assess the numbers of viable bacteria in the lungs. The PM Pw has been previously shown to have an estimated efficacy of 95% in clinical trials. However, difficulties had recently been experienced with this vaccine in it's ability to pass the Kendrick test. Although the clinical lots of the vaccines used in this study were not the same lots used in clinical trials, this vaccine was found to provide highly effective protection against *B. pertussis* infection in the murine model (Fig. 3.6). Three clinical lots of this vaccine were tested and there was good reproducibility between batches.

Examination of the cytokine responses of Pw-immunized mice, revealed that each of the clinical lots of this vaccine induced a polarized Th1 response. Splenic T-cells secreted high levels of IFN-γ in particular to killed *B. pertussis*, and no IL-4 or IL-5 upon restimulation *in vitro* (Fig. 3.7). Surprisingly, cytokine responses to PT were absent; an unusual observation given that previous studies examining cytokine responses post-immunization with Pw have demonstrated that PT is capable of inducing appreciable amounts of IFN-γ (Mahon *et al.*, 1996, Barnard *et al.*, 1996, Ausiello *et al.*, 1997, Ausiello *et al.*, 1999).

Serum antibody levels induced following immunization with the Pasteur Merieux Pw were also examined. The antibody response to whole bacteria was the strongest response, but high levels of FHA- and PRN-specific antibodies were also detected (Fig. 3.8). Consistent with the data on T-cell responses, immunization with these vaccines did not induce antibodies specific for PT. The absence of an immune response to PT may suggest that the bacteria used to prepare these batches of Pw have lost this virulence factor. This may explain their failure to pass the

Kendrick test, where an immune response to PT is considered to play a major role. The antibody responses observed were consistent between different batches.

3.2.4 Use of the murine model to determine consistency between different batches of Pa

The 2-component Pa manufactured by Pasteur Merieux has been previously shown to have an estimated efficacy of 85% in the Senegal trial (Simondon *et al.*, 1997). In this study the protective efficacy of different clinical lots of the Pasteur Merieux 2-component vaccine, formulated with the *Haemophilus influenzae* type B (Hib) vaccine (also manufactured by Pasteur Mérieux), was evaluated in the mouse model. Mice were immunized at 0 and 4 weeks and then infected with *B. pertussis* 2 weeks post secondary immunization. The course of infection in immunized mice was examined through the analysis of the numbers of viable bacteria in the lungs at intervals post-challenge. This vaccine provided effective protection against respiratory challenge with *B. pertussis* (Fig. 3.9), although the level of protection was not as good as that observed with Pw (Fig. 3.6). The numbers of viable bacteria in the lungs of immunized mice decline rapidly within 3 days post-infection. Despite an occasional rebound in CFU numbers recovered from infected mice, the lungs are almost completely free from bacteria by day 14. Evaluation of the protective efficacy of this 2-component Pa without the addition of the Hib vaccine, was carried out in a previous study (Mills *et al.*, 1998a). Compared to this previous evaluation in the murine model, formulation with Hib does not appear to affect the protective efficacy of the vaccine.

Cytokine responses following immunization with different lots of PM Pa2, were measured 2 weeks after secondary immunization, on the day of challenge. These vaccines induced a polarized Th2 cytokine profile from spleen cells restimulated *in vitro* with the relevant *B. pertussis* antigens (Fig. 3.10). Splenic T-cells secreted high levels of IL-5 and moderate levels of IL-4. High levels of IFN-γ were produced to heat-killed *B. pertussis* and PT. However, the potent induction of IFN-γ by heat-killed *B. pertussis*, is most likely due to the presence of residual LPS in the bacterial preparation, which induces IL-12 production from innate cells. The high levels of IFN-γ produced in response to PT, are due to a mitogenic effect

of the toxin, as a result of insufficient inactivation of this batch of the toxin prior to spleen cell stimulation.

DISCUSSION

Effective vaccines against disease caused by *B. pertussis* infection have been available and used for over 50 years (Cherry, 1996). Although Pw have been shown to be very effective at inducing protective immunity against whooping cough, they have been associated with a variety of adverse reactions (Cherry *et al.*, 1998, Sidley *et al.*, 1989, Loscher *et al.*, 1998). Consequently, concern about safety, rather than efficacy has been the major impetus for the development of new less reactogenic Pa vaccines. A major obstacle in the development of new pertussis vaccines, is that there is no serologic marker that correlates with clinical efficacy, and it is unclear which *B. pertussis* antigen (or antigens) induces protective immunity. This means that any candidate pertussis vaccine must undergo expensive trials involving a large number of participants to show that the vaccine is efficacious. Ideally, laboratory tests should enable us to predict the protective efficacy of new or modified Pa vaccines without large and costly efficacy trials. Currently, there is an urgent need for such tests, because the traditional Kendrick mouse protection assay for potency is of little or no value for the new Pa (Corbel & Xing, 1997), although it has been used to assess the potency of Pw (Kendrick, 1947).

A candidate model of infection which may be able to overcome some of the limitations of current vaccine assessment protocols, is the murine respiratory challenge model. This model provides a means by which we can assess pertussis vaccine potency, and allows us to investigate the mechanisms of protective immunity to *B. pertussis*. Data obtained from studies in the murine model (Mills *et al.*, 1998a, Mills *et al.*, 1998b, Mahon *et al.*, 1996, Mahon *et al.*, 1997, Barbic *et al.*, 1997, Redhead *et al.*, 1993, Barnard *et al.*, 1996), is consistent with the failure to identify a single correlate of protection in clinical trials, and suggest that immunogenicity studies against individual *B. pertussis* antigens in mice are unlikely to predict vaccine efficacy in children. This can be explained in part by the fact that protection is multifactorial and is mediated by both humoral and cellular mechanisms (Mills *et al.*, 1998a). In addition, there has been a lack of standardized functional assays for the various putative protective vaccine-induced antibodies (Corbel & Xing, 1997, Siber, 1997). The highly significant correlation between

bacterial clearance in immunized mice, and vaccine efficacy in children (Mills *et al.*, 1998a, Mills *et al.*, 1998b), suggests that the murine respiratory challenge model may have valuable applications in the evaluation and development of future vaccines prior to licensure. Comparisons of the clearance of bacteria in mice immunized with an experimental vaccine, to a standard vaccine of known efficacy, may allow a reliable prediction of the efficacy of this vaccine in children.

The murine respiratory challenge model has been successfully used as a method for assessing the protective efficacy of both Pa and Pw (Mills *et al.*, 1998a, Mills *et al.*, 1998b). In this study, the murine model has been utilized to evaluate the protective efficacy of a range of Pa, and one Pw, and to investigate the relative contributions of humoral and cellular immunity to protection. The acellular vaccines tested in the WHO study were either two component (Pasteur Mérieux, SmithKline Beecham, JNIH-3 - PT, FHA), three component (SmithKline Beecham, - PT, FHA, PRN), or five component (Connaught - PT, FHA, PRN, FIM 2, 3). The contribution of different *B. pertussis* antigens to protection has been investigated both in the mouse model and in clinical trials. Results of active and passive immunization experiments with individual antigens in mice have demonstrated that either PT or PRN can confer relatively high levels of protection (Mills *et al.*, 1998a). FHA is less protective, but does augment the protective efficacy of PT. The findings from clinical trials indicate that a monocomponent pertussis toxoid vaccine, comprised of chemically detoxified PT, can provide 71% protection against WHO-defined pertussis (Taranger *et al.*, 1997, Trollfers *et al.*, 1995). Further studies from clinical efficacy trials have shown that 3-component, 5-component and most 2-component vaccines have higher estimated efficacies (84-85%) when compared to the protective efficacy of the monocomponent vaccine (Gustaffson *et al.*, 1996, Simondon *et al.*, 1997, Olin *et al.*, 1997). Although, a direct comparison of the efficacies of these vaccines is difficult, as they were tested in different clinical trials, contained different doses of antigen and had different inactivation procedures, it does appear that vaccine efficacy increases with the number of antigenic components included (CDC, 1997, Gustaffson *et al.*, 1996, Simondon *et al.*, 1997, Miller *et al.*, 1997, Olin *et al.*, 1997). However the benefit

of addition of more than 2 component antigens in the vaccine formulation is not so significant (Mills *et al.*, 1998a).

The findings from this study are consistent with previous observations. The five different vaccines from the WHO study showed that inclusion of five antigens to the vaccine formulation, did not increase the efficacy of the 2-component vaccines in the mouse model. The three 2-component vaccines (SB Pa2, JNIH-3, and PM Pa2) showed significant protection against aerosol challenge (Fig. 3.4). Surprisingly, immunization with the three-component SB Pa3 or the 5-component CLI Pa5, afforded very similar degrees of protection to the 2-component vaccines; the addition of PRN or FIM did not enhance their protective ability. However, estimations of the efficacy of these vaccines in clinical trials showed that they all provided a high degree of protection (Table 3.1), and without the inclusion of a low efficacy vaccine in the experimental design, discrimination between vaccines was difficult. The efficacies of the 2-component vaccines SB Pa2 and PM Pa2 were previously evaluated in clinical trials and shown to have efficacies of 59% and 85% respectively (Greco *et al.*, 1996, Simondon *et al.*, 1997). The differences in their efficacy was an unusual observation, given that both vaccines contained the same components. Because of this, it has been speculated that SB Pa2 may have been over-toxoided, which reduced it's immunogenicity and protective ability. Hence, the observation that both SB Pa2 and PM Pa2 showed similar protection in the mouse model, may be a reflection of the fact that the vaccines tested in the present study were not the same clinical lots used in the efficacy trials, and hence displayed comparable protective ability.

Because the numbers of vaccines included in childhood immunization schedules are increasing, it has become impractical to deliver these vaccines individually, and has lead to an increased interest in the development of combination vaccines. Combination vaccines have the advantage of reducing the need for multiple injections in children, but an important consideration is the effect that one vaccine may have on the responses to another vaccine when delivered simultaneously. Immunization with three clinical lots of the Pasteur Merieux 2-component Pa, formulated with the Hib vaccine was associated with a slight delay in bacterial clearance from the lungs, and an occasional rebound in bacterial load,

when compared to immunization with the Pasteur Merieux Pw. However, it is unlikely that this is due to formulation with Hib, as is has been previously demonstrated that immunization of mice with Pa is associated with protracted bacterial clearance from the lungs, when compared with Pw (Mills *et al.*, 1998a, Mahon *et al.*, 1996). The protective efficacy of the same Pasteur Merieux Pa without the inclusion of Hib has been previously evaluated in the mouse model (Mills *et al.*, 1998a). Compared to this evaluation, the efficacy of PM Pa2 does not appear to be affected when Hib is administered in the same injection. Furthermore, the demonstration that immunization with these Pa induce a polarized Th2 cytokine response is consistent with previous reports in mice (Mahon *et al.*, 1996, Mills *et al.*, 1998a, Mills *et al.*, 1998b). Therefore, the slight delay in bacterial clearance observed in Pa-immunized mice is more likely due to the lack of stimulation of Th1 cells, as has been suggested by others (Mahon *et al.*, 1996). The examination of antibody responses to different formulations of the Pasteur Merieux (PM) 2-component Pa, either combined with Hepatitis-B, Hib and IPV vaccines as a full liquid formulation (PM 94) or reconstituted before injection (PM 181), did not have any obvious effect on the induction of antigen-specific antibody. However, this is not surprising as both vaccines have the same antigenic composition and inactivation procedures. Furthermore, previous studies in mice have demonstrated that there is no significant difference in the level of T-cell activation post-challenge (Ryan, 2000), or in the level of protection conferred by immunization with either PM 94 or PM 181 (Mills *et al.*, 1998a).

In addition to their role in the evaluation of vaccine efficacy, animal models may also have important applications in the quality control of pertussis vaccines, to ensure there is minimum variation in protective efficacy between batches of the same vaccine. In this study, three clinical lots of the Pasteur Mérieux Pw (PM Pw) were evaluated for protective efficacy in the mouse model. These vaccines induced a significant level of protection against bacterial challenge, that correlated well with the estimated efficacy of 96% for the vaccine from clinical trials. There was also good reproducibility between batches. All clinical lots of the vaccine induced a polarized Th1 response, which is consistent with previous reports that immunization with Pw results in the generation of Th1 response both in mice and children (Mills

et al., 1993, Redhead *et al.*, 1993, Ryan *et al.*, 1997). Furthermore, immunization of mice with different clinical lots of the PM Pw vaccine induced very similar antibody levels to the constituent antigens. However, although these results would be expected, due to the fact that inactivation procedures, vaccine composition and antigen dose are all the same for the clinical lots tested, the findings from this study further validate the murine respiratory challenge model for the assessment of pertussis vaccine potency.

Despite the fact that no serologic marker in pertussis vaccination or disease correlates with immunity, one would expect that antibody production would be important for immunity to *B. pertussis*. Passive and active immunization experiments in mice have demonstrated that antibody can induce varying degrees of protection against either intracerebral or aerosol challenge (Oda *et al.*, 1984, Robinson *et al.*, 1985a, Kimura *et al.*, 1990, Sato *et al.*, 1981, Sato & Sato, 1984, Storsaeter *et al.*, 1998). However, despite this, the exact contribution of antibody to vaccine-induced protection remains to be elucidated. Furthermore, in clinical trials of Pa and Pw it has been difficult to define quantitative correlations between specific anti-pertussis antibody levels and protection. In general, Pa induce more potent antibody responses to *B. pertussis* antigens than Pw. The clinical importance of these antibody differences is unknown, but it has been shown that Pa-immunized mice develop a polarized Th2 response post-immunization (Mahon *et al.*, 1996), and a mixed Th1/Th2 response in children (Ryan *et al.*, 1997, Ryan *et al.*, 1998). Some have suggested that this may be due to formulation of component proteins with alum (Barnard *et al.*, 1996, Moore *et al.*, 1995). Th2 responses are classically associated with the induction of humoral immunity, and perhaps the induction of type 2 responses after immunization with Pa formulated with alum, is responsible for the induction of higher levels of antibody than the Th1-inducing Pw vaccines.

Analysis of serum antibody responses to several Pa and Pw, the efficacy of which were previously evaluated in clinical trials (Greco *et al.*, 1996, Gustafsson *et al.*, 1996, Simondon *et al.*, 1997), and in mice (Mills *et al.*, 1998a), are consistent with the findings from clinical trials, which have failed to demonstrate an immunological correlate of protection in children based on monotypic antibody responses against putative protective antigens (Ad Hoc group for the study of

pertussis vaccines, 1998, Greco *et al*,, 1996, Simondon *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997). Immunization of mice with Pa induced high antibody titres against each of the components in the vaccine, whereas Pw was not as efficient at inducing anti-PT and anti-FIM antibodies. All the Pa, including the lower efficacy SB Pa2 induced potent antibody responses against each of the antigens in the vaccine. In general the strongest response was detected against PT. In contrast, the antibody response was weak in mice immunized with PM Pw, indicating that there was no clear relationship between the antibody response to either PT, FHA, PRN or FIM and vaccine efficacy. Similarly, an examination of antibody responses in mice immunized with the Pa from the WHO study, revealed an inconsistency between the levels of anti-pertussis antibodies and vaccine efficacy. In general high levels of anti-PT and anti-FHA antibodies were induced after immunization with all Pa, even the low efficacy SB Pa2. In contrast, the high efficacy 5-component CLI Pa5 induced weaker antibody responses overall, when compared with the other Pa. However, these observations do not argue against a role for antibody in protection. The results of passive immunization experiments have demonstrated that antibodies raised against PT and one or more additional antigens can confer a good level of protection against respiratory challenge (Mills *et al.*, 1998a). However, an active immunization schedule which resulted in the same levels of antibody in the circulation at the time of challenge, conferred higher levels of protection, especially with the Pw vaccine. Furthermore, it has been shown that B-cells are required to confer immune mediated protection (Mahon *et al.*, 1997, Mills *et al.*, 1998a, Leef *et al.*, 2000). Nevertheless, the results of this study suggest that although each of the vaccines differs in its antigenic composition, antigen dose and method of inactivation and formulation, the monitoring of the potency of Pa based on ELISA-detected antibody levels, is insufficient and does not take into account the complex multi-antigen, dual T-cell and antibody mechanism of protection.

Results from this and other studies in the murine model have demonstrated a highly significant correlation between bacterial clearance in immunized mice, and vaccine efficacy in children (Mills *et al.*, 1998a, Mills *et al.*, 1998b). Furthermore, the lack of correlation between antibody responses and protection against infection

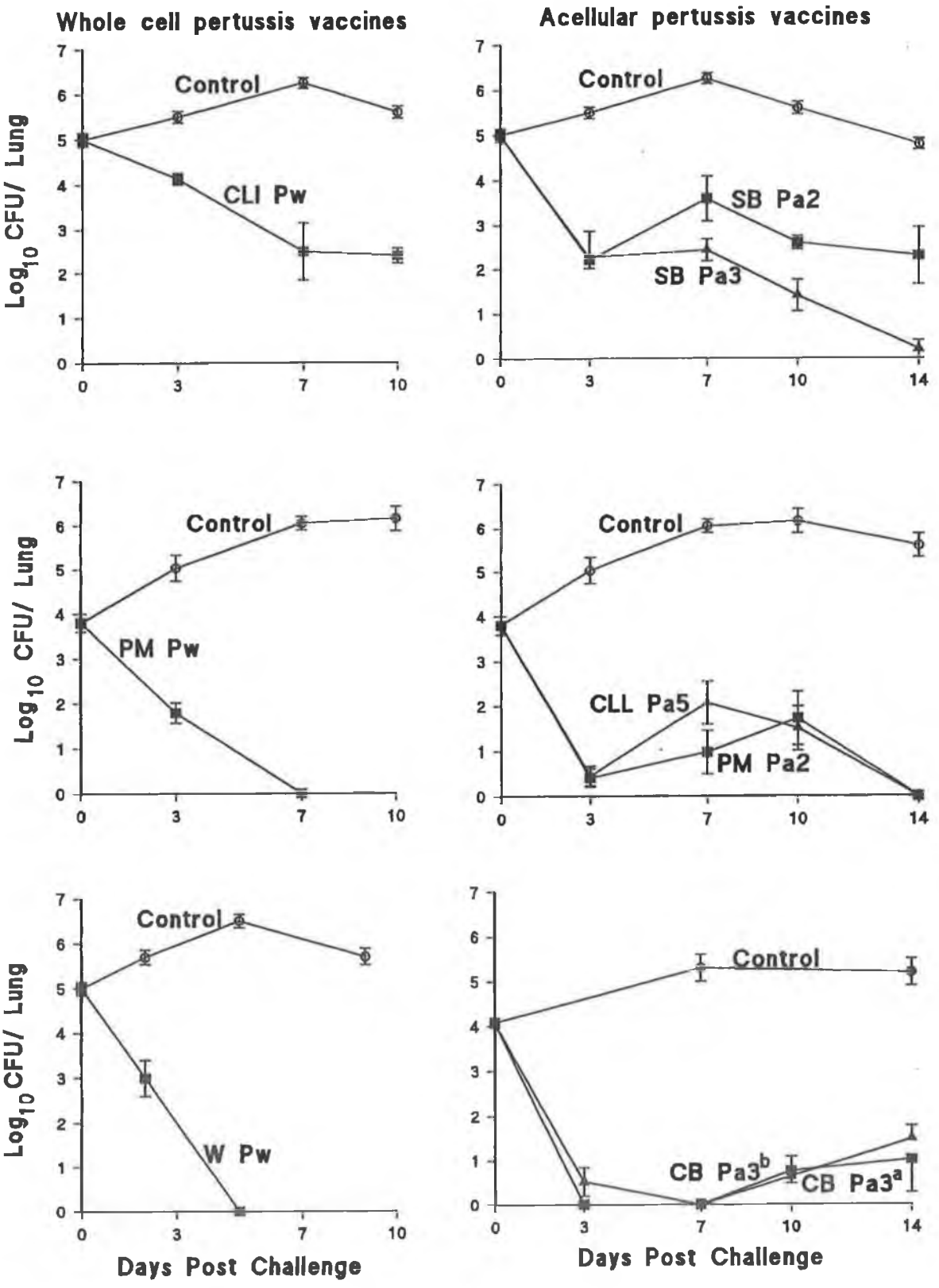
shown in this study, is consistent with the findings from clinical trials, which failed to demonstrate an immunological correlate of protection in children based on monotypic antibody responses against *B. pertussis* antigens (Trollfers *et al.*, 1995, Greco *et al.*, 1996, Gustafsson *et al.*, 1996). These results suggest that pertussis vaccine potency cannot be predicted solely on the basis of analysis of antibody responses to the putative protective antigens of *B. pertussis*, and suggest that evaluation of protection in the mouse model may offer a less complicated and more efficient method of evaluating pertussis vaccine potency, compared to large and costly clinical trials in humans. Furthermore, the murine respiratory challenge model is a valuable tool, not only in the prediction of pertussis vaccine efficacy, but may also be important in the regulatory control of pertussis vaccines, to ensure minimum variation in the protective efficacy between batches, and for the assessment of combined vaccine formulations.

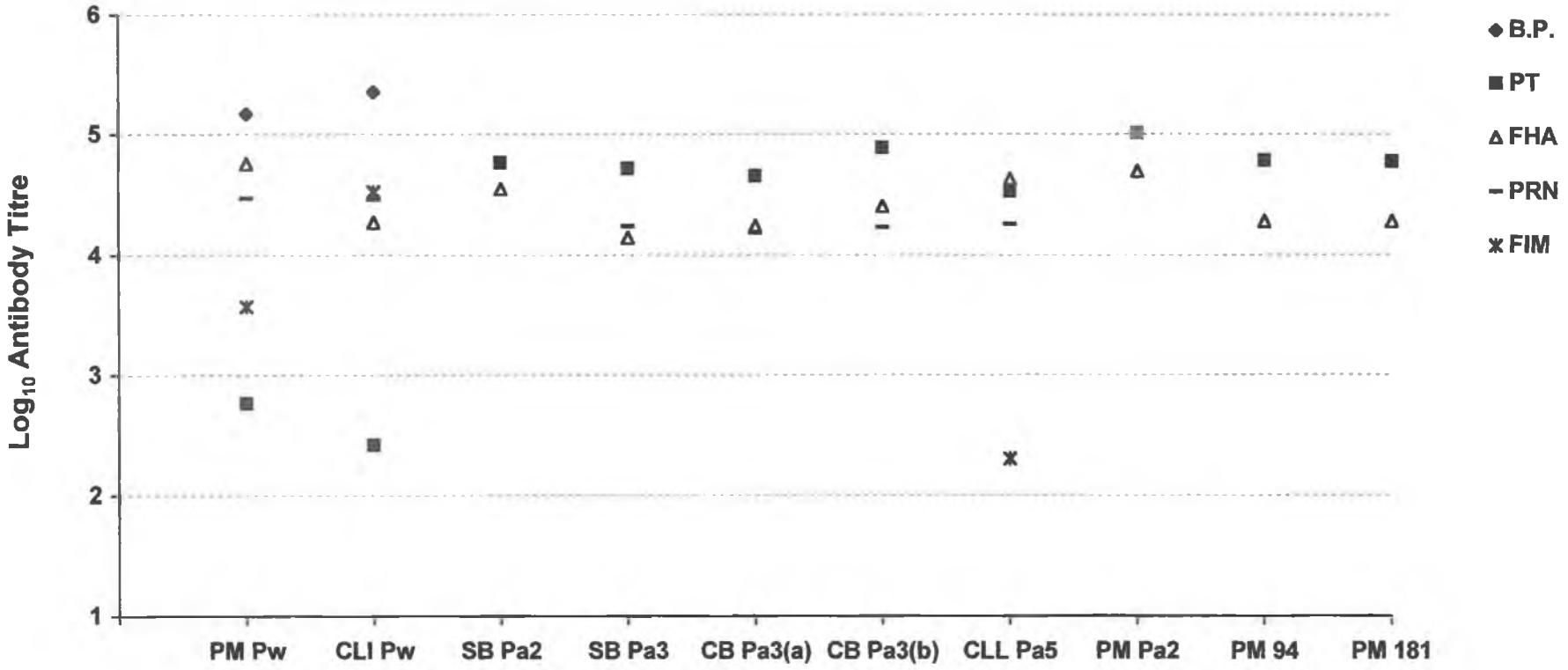
TABLE 3.1 VACCINE COMPOSITION, ESTIMATED EFFICACIES, AND T-CELL RESPONSES IN MICE AND CHILDREN.

Vaccine	*Composition*	*Trial/Study*	*Estimated Efficacy in Children (%)*	*Potency index in mice*	*T-cells in mice*		*T-cells in children*	
					Th1	Th2	Th1	Th2
PM Pw	Killed bacteria	Senegal	95	84	++++	-	nt	nt
CLI Pw	Killed bacteria	Italy/Sweden	36-48	44	nt	nt	+	-
SB Pa2	†PTd/FHA	Sweden	59	47	nt	nt	+	-
SB Pa3	PTd/FHA/PRN	Italy	84	75	+	+++	+++	++
CB Pa3	‡rPT/FHA/PRN	Italy	84	77	++	++	+++	++
JNIH-3	PTd, FHA	N/T	N/T	N/T	+	++	nt	nt
CLL Pa5	PTd/FHA/PRN/FIM	Sweden	85	75	+	+++	++	++
PM Pa2	PTd/FHA	Senegal	84	73	+	+++	++	++

Estimated efficacies for children are based on published data from clinical trials (Greco *et al.*, 1996, Gustaffson *et al*, 1996, Simondon *et al.*, 1997). T-cell responses in mice are reproduced from a study by Mills *et al*, 1998a. Th1/Th2 responses are arbitrarily designated - to ++++ on the basis of secretion of IFN-γ or IL-5 in response to antigen stimulation of spleen cells. T-cell responses were assessed in children as described (Ryan *et al.*, 1997, Ryan *et al.*, 1998).

†**PTd** Chemically detoxified pertussis toxin, ‡**rPT**, Genetically detoxified pertussis toxin.


Whole cell pertussis vaccines
Acellular pertussis vaccines
Log10 CFU/ Lung
Control
CLI Pw
SB Pa2
SB Pa3
PM Pw
CLL Pa5
PM Pa2
W Pw
CB Pa3b
CB Pa3a
Days Post Challenge


Log10 Antibody Titre
6
5
4
3
2
1
PM Pw
CLI Pw
SB Pa2
SB Pa3
CB Pa3(a)
CB Pa3(b)
CLL Pa5
PM Pa2
PM 94
PM 181
B.P.
PT
FHA
PRN
FIM

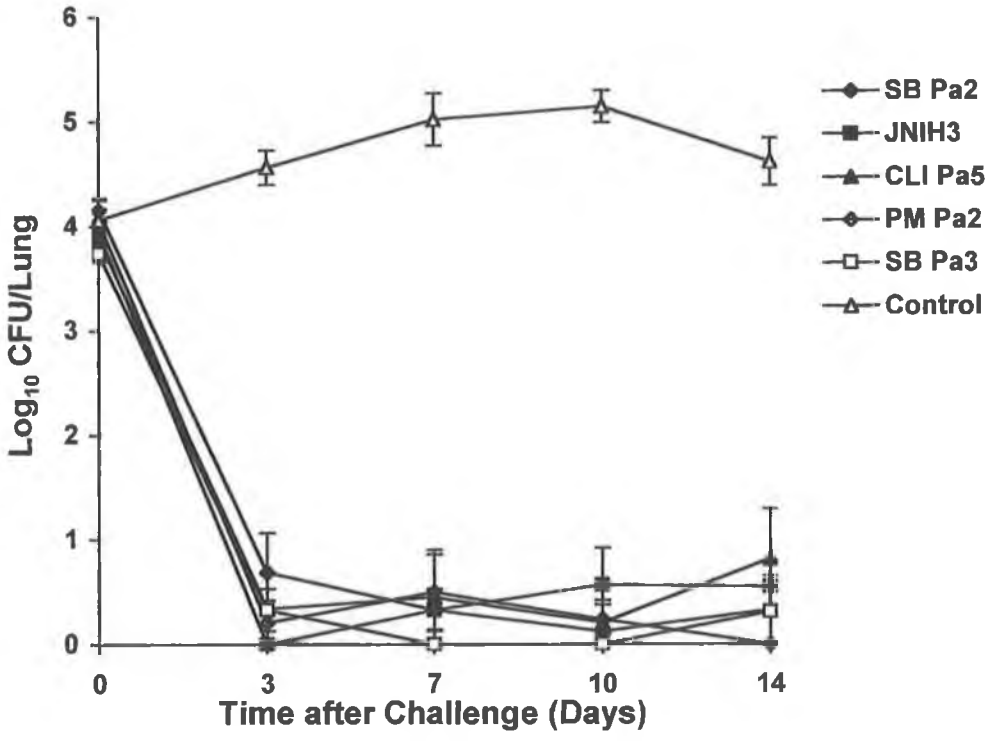


Figure 3.3

Kinetics of *B. pertussis* clearance from the lungs after respiratory challenge of mice immunized with different Pa. Groups BALB/c mice were immunized intraperitoneally with 0.2 human dose of Pa at 0 and 4 weeks. Control mice were immunized with alum only. Two weeks after secondary immunization, mice were challenged by aerosol inoculation with *B. pertussis*, and CFU counts were performed on individual lung homogenates at intervals after challenge. Results are mean (± SE) viable *B. pertussis* counts from four mice per group at each time point. Vaccine composition and estimated efficacies in children are outlined in Table 3.1.

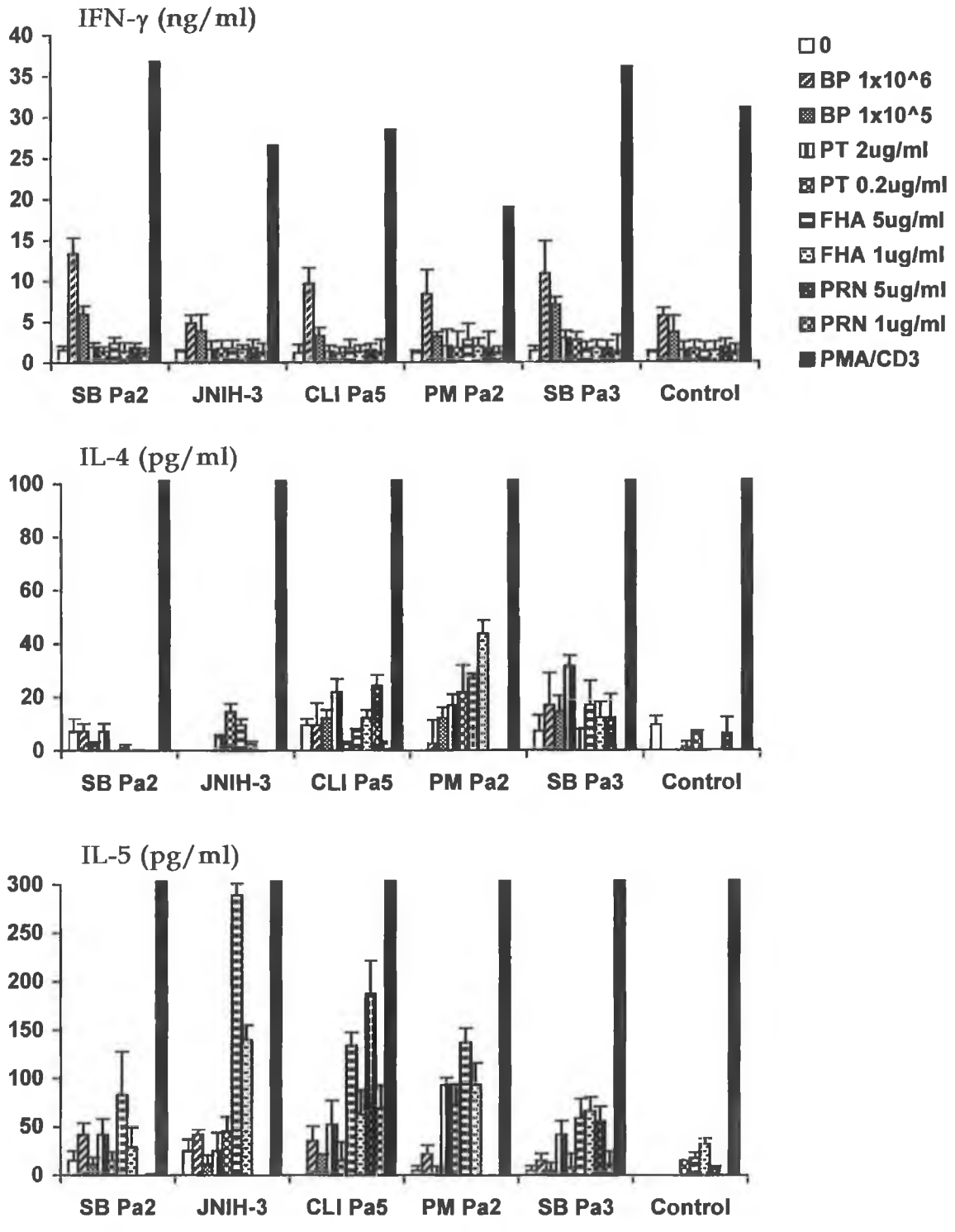


Figure 3.4

T-cell cytokine responses in mice immunized with Pa vaccines. Groups of BALB/c mice were immunized as described in the legend to Fig. 3.3. Two weeks after the second immunization spleen cells were isolated from immunized mice and restimulated *in vitro* with *B. pertussis* antigens. Supernatants were collected after 72 hours and levels of IFN-γ, IL-4 and IL-5 were determined by specific immunoassay. Results are representative of the mean responses (± SE) for four mice assessed individually in triplicate.

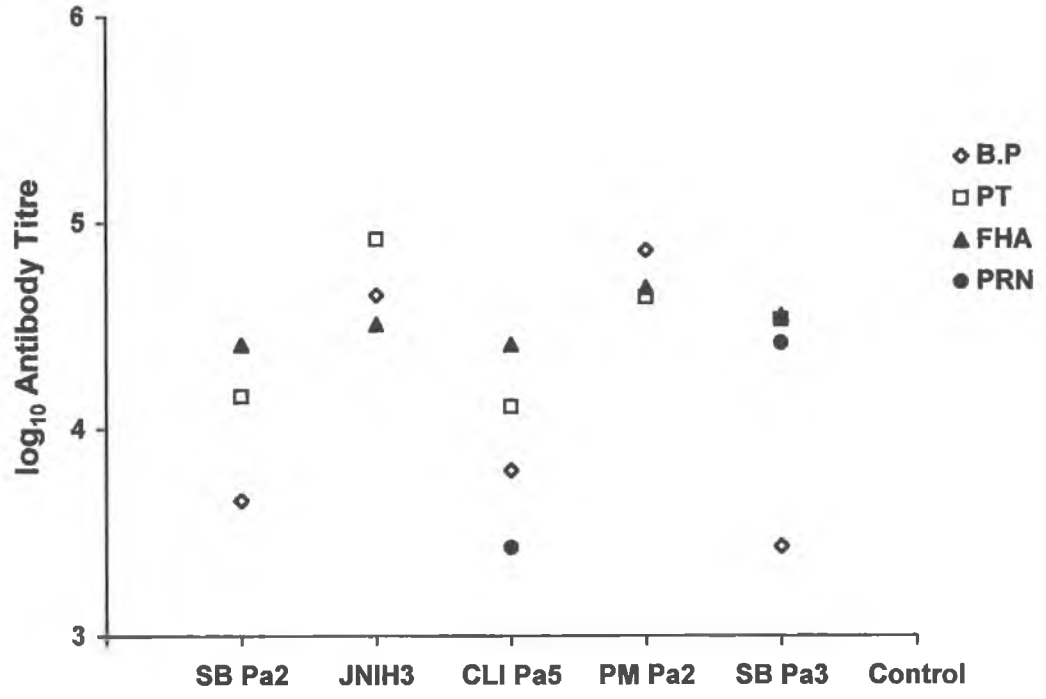


Figure 3.5

Serum antibody responses in mice immunized with Pa vaccines. BALB/c mice were immunized as described in the legend to Fig. 3.3. Two weeks after the second immunization antibody responses to killed *B. pertussis* (B.P), PT, FHA, and PRN were evaluated. Results are presented as the mean antibody titres from four mice per group. Vaccine composition and estimated efficacies in children are outlined in Table 3.1.

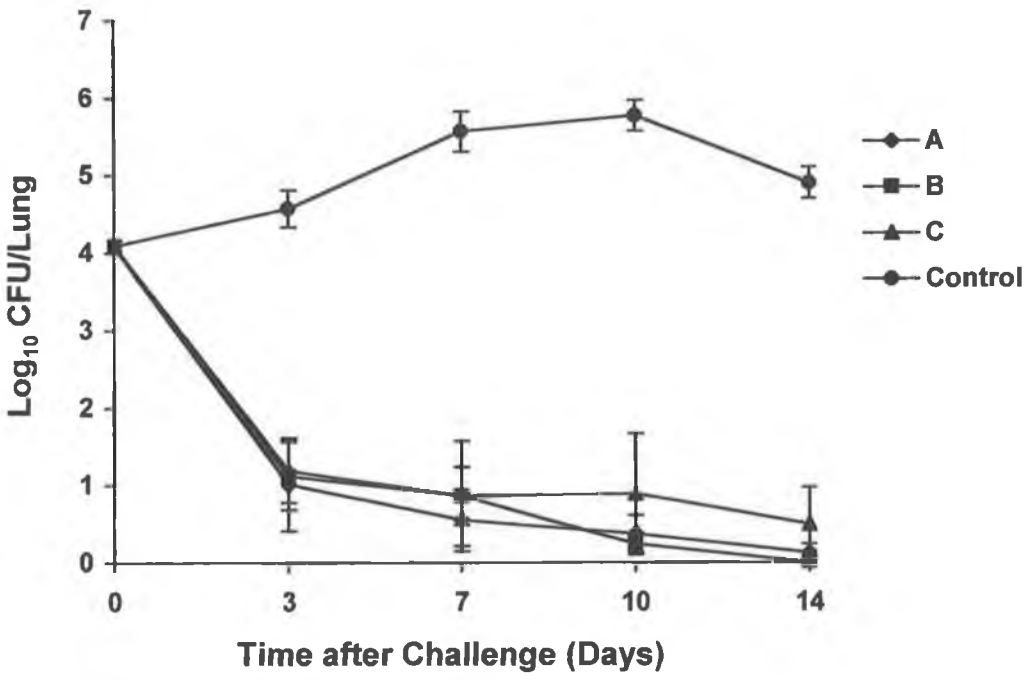


Figure 3.6

Kinetics of *B. pertussis* clearance from the lungs after respiratory challenge of mice immunized with different clinical lots (A, B and C) of the Pasteur Mérieux Pw, or alum only as control. Mice were challenged by aerosol exposure to *B. pertussis* two weeks after secondary immunization, and CFU counts were performed on individual lung homogenates at intervals after challenge. Results are mean (± SE) viable *B. pertussis* counts from four mice per group at each time point.

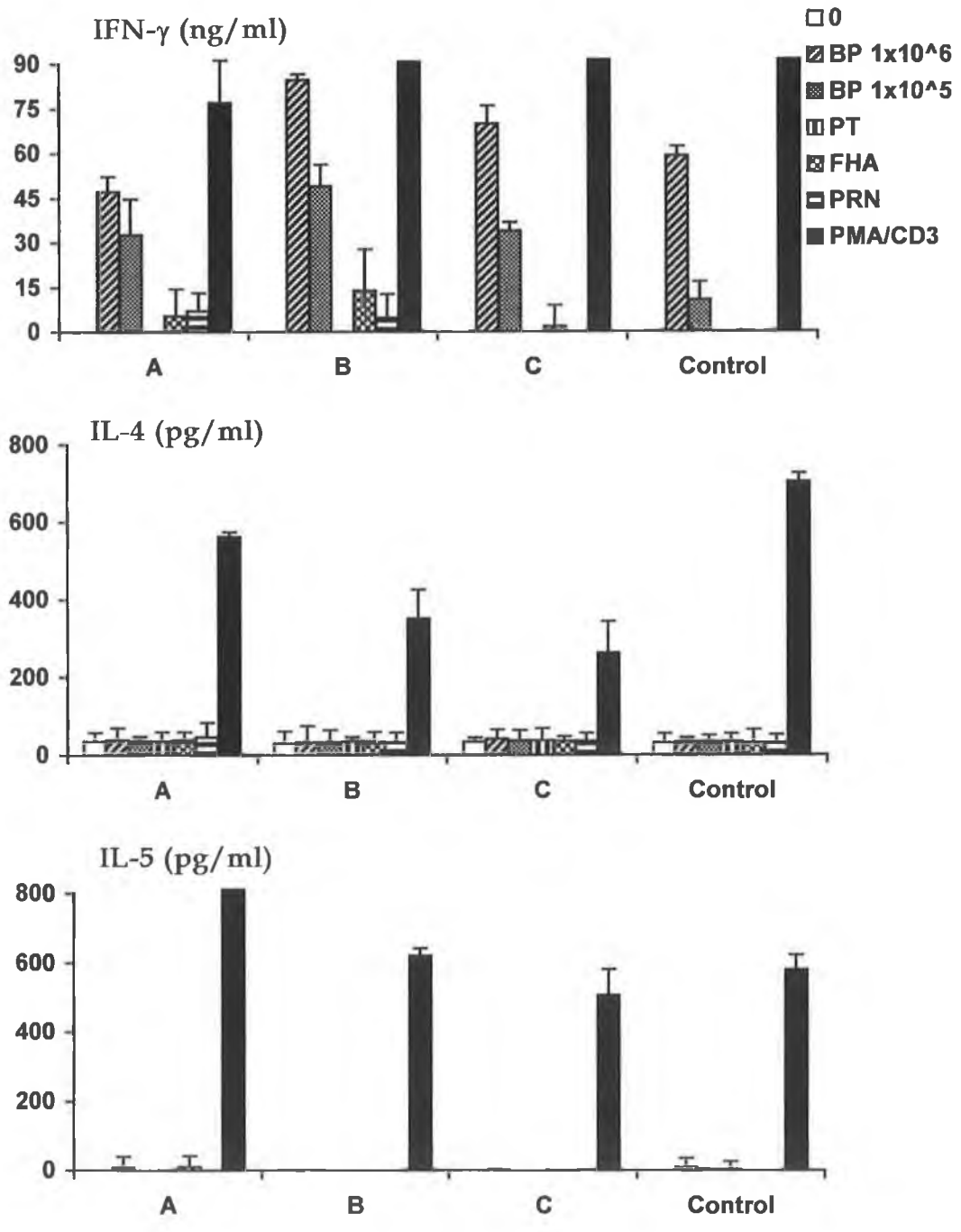


Figure 3.7

T-cell cytokine responses in mice immunized with different batches (A,B and C) of the same PM Pw. BALB/c mice were immunized intraperitoneally with 0.2 human dose at 0 and 4 weeks with Pw, or alum only as contol. Two weeks after the second immunization, spleen cells were isolated from immunized mice and were stimulated with *B. pertussis* antigens *in vitro* for 72 hours. Levels of IFN-γ, IL-4 and IL-5 in supernatants were determined by specific immunoassay. Results are representative of the mean responses (± SE) for four mice assessed individually in triplicate.

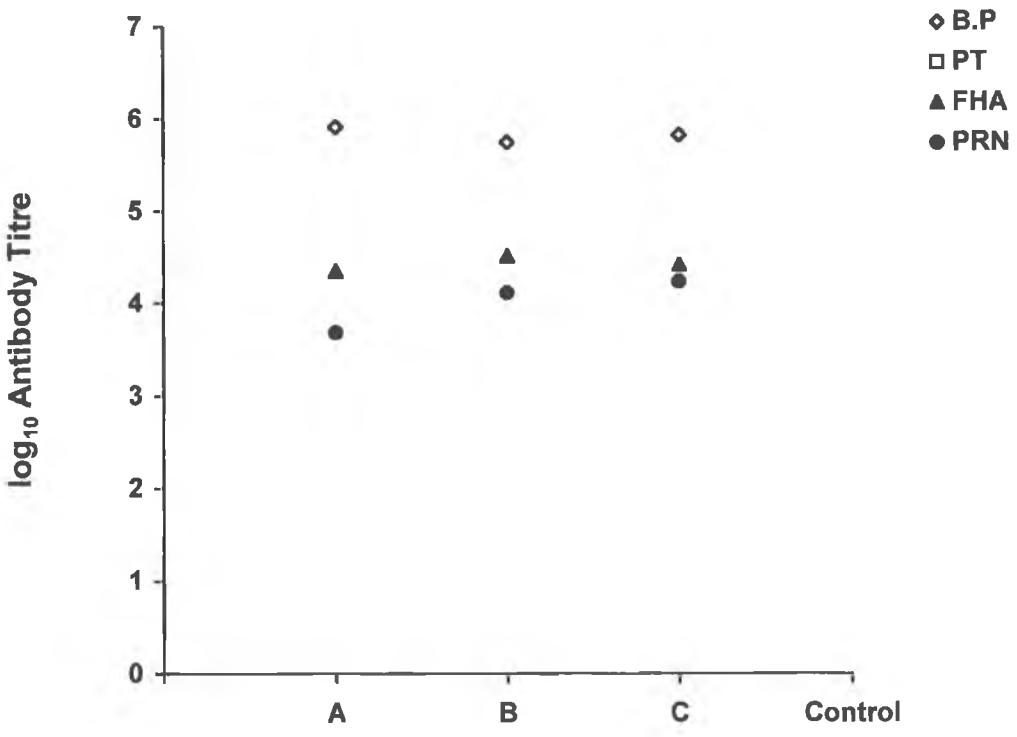


Figure 3.8

Serum antibody responses in mice immunized with different clinical lots (A, B and C) of PM Pw. BALB/c mice were immunized intraperitoneally at 0 and 4 weeks with 0.2 human dose of each vaccine. Control mice were immunized with alum only. Two weeks after the second immunization antibody responses to killed *B. pertussis* (B.P), PT, FHA, and PRN were evaluated. Results are presented as the mean antibody titres from four mice per group. Vaccine composition and estimated efficacy in children is outlined in Table 3.1.

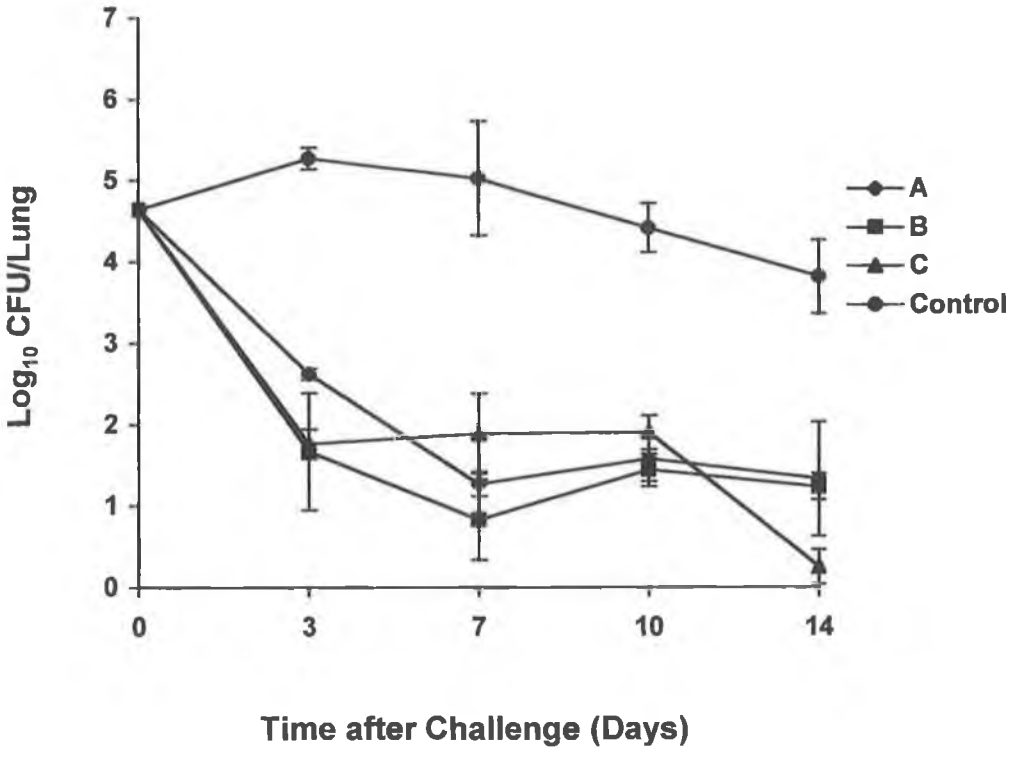


Figure 3.9

Kinetics of *B. pertussis* clearance from the lungs after respiratory challenge of mice immunized with different clinical lots (A, B and C) of PM Pa or alum as control. Two weeks after secondary immunization, mice were challenged by aerosol exposure to *B. pertussis*, and CFU counts were performed on individual lung homogenates at intervals after challenge. Results are mean (± SE) viable *B. pertussis* counts from four mice per group at each time point.

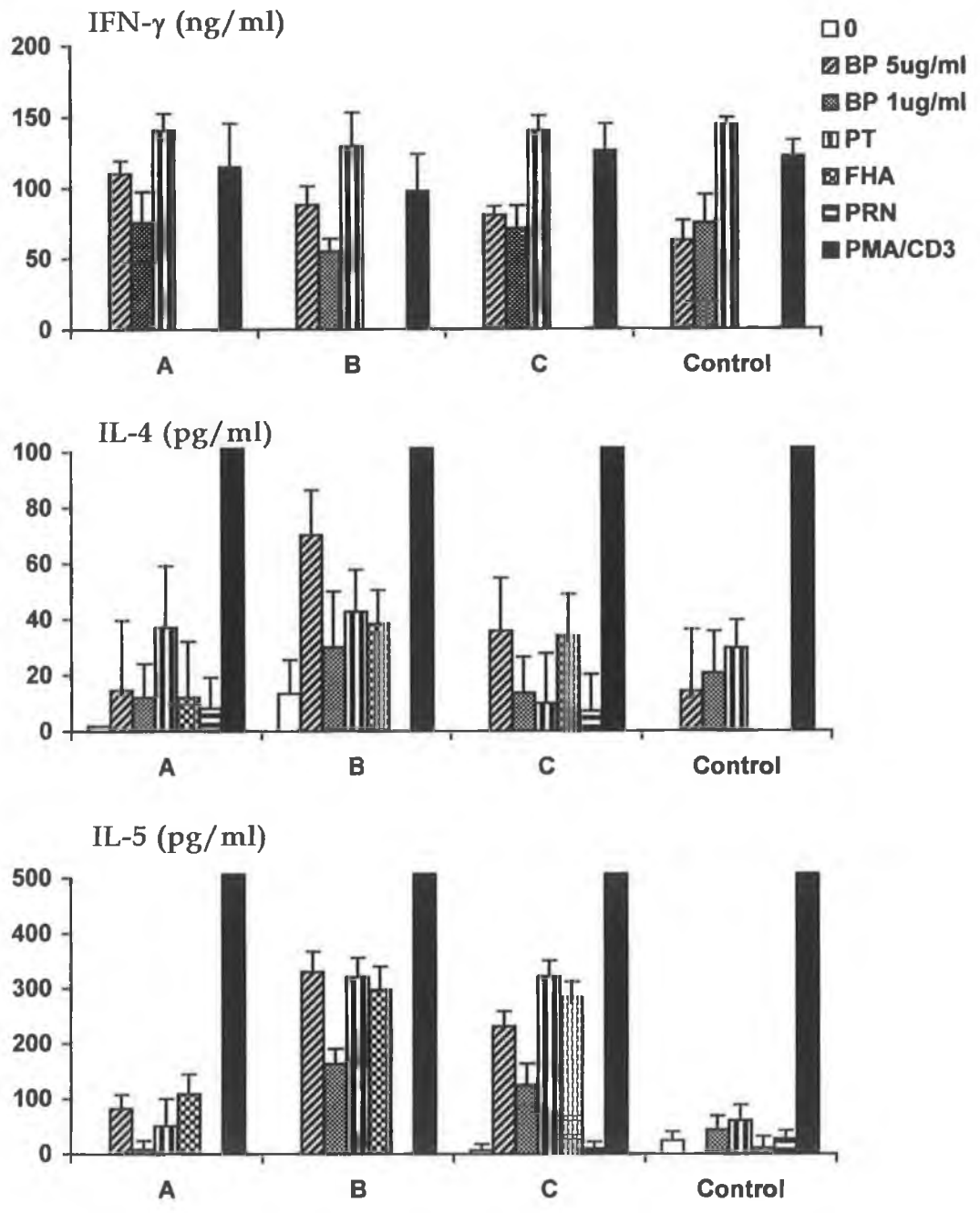


Figure 3.10

T-cell cytokine responses in mice immunized with different batches of the same PM Pa (A, B or C). BALB/c mice were immunized intraperitoneally with 0.2 human dose at 0 and 4 weeks with Pa, or alum only as a control. Two weeks after the second immunization, spleen cells were isolated from immunized mice and were restimulated with *B. pertussis* antigens *in vitro* for 72 hours. Levels of IFN-γ, IL-4 and IL-5 in supernatants were determined by specific immunoassay. Results are representative of the mean responses (± SE) for four mice assessed individually in triplicate.

❖ CHAPTER 4 ❖

PROTECTION AGAINST *BORDETELLA PERTUSSIS* IN MICE IN THE ABSENCE OF DETECTABLE CIRCULATING ANTIBODY : A ROLE FOR IMMUNOLOGICAL MEMORY

4.1 INTRODUCTION

Immunological memory develops in response to primary antigenic exposure, and is a defining feature of the adaptive immune response. It is widely accepted that once infected and therefore having mounted and immune response and survived the infection, the host is protected more efficiently against secondary infections through the induction of immunological memory. This memory response to previously encountered antigen surpasses that induced by an initial encounter both quantitatively and qualitatively, and is the rationale behind contemporary immunization strategies. The basic premise of vaccinology is that, by mimicking the effects of a primary exposure to a pathogen, immunization can induce protective immunity against a subsequent encounter with the same organism. Thus the generation of immunological memory and protective immunity are thought to be closely related (Ahmed *et al.*, 1996). Convincing evidence for the existence of identifiable T or B cells with specialized memory characteristics still remains ambiguous, and so, the phenotype of immunological memory correlates best with antigen-driven activation of low frequency effector T cells and plasma cells. Although the ability to maintain memory after an encounter with an antigen is one of the central features of the immune system, the mechanisms that generate and maintain a memory response, or the relative contributions of memory T- and B-cells to protection against certain infectious disease have still to be elucidated.

Long term antibody production is one of the hallmarks of effective vaccination and is an important characteristic of immunological memory. The identification of levels of circulating antibodies that are critical for protection induced with certain vaccines such as *Haemophilus influenzae* type b (Hib), tetanus, diphtheria and polio (Robbins *et al.*, 1995, Kayhty *et al.*, 1998), has led to the belief that it may be possible to define a level of protective antibody against *B. pertussis*. However, results from clinical trials have not provided definitive information on a relationship between antibody levels against a putative protective antigen and immune protection (Greco *et al.*, 1996, Simondon *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997).

A paradox exists with regard to the role of circulating antibody and immune protection. For most vaccines in human use, the induction of humoral immunity has

been considered to be the major effector mechanism mediating protection against viral and bacterial pathogens, and that this protection can be attributed to a critical level of specific serum IgG. However, certain vaccines appear to protect with low or undetectable pathogen-specific circulating antibody. It has been difficult to identify a serological correlate of protection induced with group B meningococcal vaccines (Goldschneider *et al.*, 1969, Kayhty *et al.*, 1998). Protection against poliovirus is primarily mediated via specific antibody supported by a $CD4^+$ T cell response (Mahon *et al.*, 1995), and yet protection can be conferred by very low antibody titres (McHammond *et al.*, 1953). There emerges a similar scenario with *Bordetella pertussis* vaccination. Recent reports from household contact studies have indicated that antibody responses to PT, FHA and fimbriae may correlate with absence of severe disease (Storsaeter *et al.*, 1998, Cherry *et al.*, 1998). However, results from phase 3 clinical trials have failed to demonstrate an immunological correlate of protection in children based on monotypic antibody responses against putative protective antigens (Ad hoc group for the study of pertussis vaccines, 1998, Greco *et al.*, 1996, Simondon *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997).

The clinical studies are supported by experiments in a murine model where pertussis vaccine efficacy could not be predicted on the basis of ELISA antibody responses against individual components of the vaccine (Mills *et al.*, 1998b). Furthermore bacterial clearance during primary infection of mice occurs prior to the development of IgG responses (Mills *et al.*, 1993), revealing a role for cell-mediated mechanisms in protection against *B. pertussis*. Paradoxically B cells are required to confer immune mediated protection (Mahon *et al.*, 1997, Mills *et al.*, 1998a, Leef *et al.*, 2000). For example, *B. pertussis* challenge of naïve $Ig^{-/-}$ mice (which do not make mature B-cells) results in the development of a chronic infection that does not clear for at least 6 months post-challenge, compared to wild-type mice which clear the infection after 8 -10 weeks (Mahon *et al.*, 1997). Nevertheless vaccination strategies have focused on the induction of short term responses following immunization and the generation of high titer antibody in serum. However, other factors are more relevant to our understanding of the mechanisms of protective immunity and the rational design of efficacious vaccines. These include the

definition of correlates of immunity, and the persistence or longevity of the protective immune response.

It has been assumed that natural infection with *B. pertussis* confers long-lived protection against subsequent infection. Furthermore, the traditional pertussis whole cell vaccines also appear to induce long-lived immunity against severe disease in children and young adults. However exposure to sub-clinical infection, especially in countries with a high incidence of disease, may contribute to persistence of protection by periodic boosting of immune responses (Isacson *et al.*, 1995, Rota *et al.*, 1998). Concern about the safety of these traditional vaccines has resulted in the development of acellular pertussis vaccines (Pa) prepared from highly purified components of *B. pertussis* (Rappuoli *et al.*, 1997) and these vaccines, which have a higher safety profile, have now replaced Pw in many developed countries. Results from clinical trials indicated that Pa conferred a level of protection against severe disease, which approached that of the European Pw (Greco *et al.*, 1996, Simondon, *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997). However recent follow up studies from the clinical trials have indicated that antibody responses in immunized children decline to almost undetectable levels 15-33 months after a complete course of vaccination (Giuliano *et al.*, 1998, Salmaso *et al.*, 1998). Although it appears that these children are not developing overt disease, without a history of exposure and sensitive indicators of infection, in large post-immunization surveillance programs, it is impossible to determine if they are immune to infection or to establish the mechanisms mediating protection.

With the utilization of a well-established murine respiratory challenge model of *B. pertussis* infection where protection correlates with vaccine efficacy in human clinical trials (Mills *et al.*, 1998, Mills *et al.*, 1993, Mahon *et al.*, 1997, Mills and Ryan *et al.*, 1998), together with gene knockout mice, active and passive immunization and adoptive transfer techniques, complementary roles for cellular and humoral immunity in protection against *B. pertussis* have been identified (Mills *et al.*, 1998, Mills *et al.*, 1993, Mahon *et al.*, 1997, Redhead *et al.*, 1993). In this chapter, the relationship between the persistence of the immune response and the development of immunological memory and protection against respiratory infection have been examined using this murine model of infection. The results demonstrate

that protective immunity persists in the absence of a detectable antibody response and imply that the induction of memory T and B cells may be more significant to vaccine efficacy than the initial serum IgG response.

4.2 Results

4.2.1 Analysis of serum antibody responses in mice immunized with acellular or whole cell pertussis vaccines

In pertussis vaccine clinical trials evaluation of antibody levels in the serum of immunized children, revealed high levels of antibody against the antigen components of the vaccine early after vaccination. This was true for both the acellular vaccines even those of low efficacy (Simondon *et al.*, 1997, Olin *et al.*, 1997, Greco *et al.*, 1996, Gustafsson *et al.*, 1996, Trollofers *et al.*, 1995) and for the whole cells vaccines (Simondon *et al.*, 1997, Olin *et al.*, 1997, Greco *et al.*, 1996, Gustafsson *et al.*, 1996, Miller *et al.*, 1997), although these were more variable, due to differences in concentrations of individual antigens in the preparations. However, antibody responses decline rapidly after a complete course of vaccination (Giuliano *et al.*, 1998, Salmaso *et al.*, 1998), yet immunized children appear to remain protected against severe disease. In order to quantify the contribution of circulating antibody to immune-mediated protection against *B. pertussis*, we exploited the well-characterized murine respiratory challenge model (Mills *et al.*, 1993, Mills *et al.*, 1998a). Mice received two immunizations with low doses of either a traditional Pw or a new generation Pa, equivalent to 0.1-0.2 μg recombinant pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN), and the persistence of the serum antibody response was measured by ELISA. Both Pw and Pa induce strong antibody responses against the *B. pertussis* antigens, PT, FHA and PRN (Fig. 4.1A, 4.1B). Anti-*B. pertussis* antibody responses were undetectable in control mice, immunized with adjuvant only (not shown). Serum antibody responses are particularly strong 6 weeks after immunization with Pa (Fig. 4.1B), but decline rapidly in both groups of mice after three months (Fig. 4.1A, 4.1B). Six to nine months after immunization, specific antibody can not be detected in the serum of immunized mice. So, in the murine model, which correlates with that in children, serum antibody responses do not persist following immunization with pertussis vaccines.

4.2.2 Pa and Pw protect against *B. pertussis* in the absence of circulating antibody

Mice immunized twice with 0.04 human doses of Pw or Pa were aerosol challenged with *B. pertussis* either at the peak of the antibody response 6 weeks after primary immunization or once circulating antibody titers had waned, 44 weeks after primary immunization. Although both vaccines conferred a high level of protection, the rate of bacterial clearance was marginally slower with this low dose of vaccine when compared with that induced with a higher dose (0.2 of human dose) of the same vaccines in earlier studies (Mills *et al.*, 1998). Following challenge of mice at week 6 the numbers of CFU recovered from the lungs declined to low levels 7-10 days later, and after a small rebound on day 14, were undetectable 21 days after challenge (Fig. 4.2A). In contrast, the numbers of viable bacteria in the lungs were 3-5 logs higher 7, 10 and 21 days after challenge in unimmunized control mice. Despite the decline in *B. pertussis*-specific circulating antibody to undetectable levels, both vaccines conferred protection against *B. pertussis* when challenged 44 weeks after immunization (Fig. 4.2B). In particular mice immunized with Pw displayed a high level of protection, which approached that observed at the peak of the antibody response 6 weeks after immunization. Thus while antibody declined, immunity persisted for at least 9 months after immunization.

4.2.3 Immunization induces long-lived T-cell recall responses

In contrast to the circulating antibody response, recall T-cell responses to *B. pertussis* antigens persisted in Pw and Pa immunized mice for long periods following immunization. Spleen cells from immunized or control mice were prepared at varying times post immunization or challenge and cytokine responses to *B. pertussis* antigens examined (Fig. 4.3, Fig. 4.4, Fig. 4.5, Fig. 4.6, Fig. 4.7, Fig. 4.8). The results demonstrate that recall T-cell responses persist for long periods following immunization. There is a clear dichotomy in the nature of the T-cell response induced either by Pw or Pa. Pw induces a Th1 dominated response, whereas Pa induces a Th2 dominated response and these patterns of cytokine secretion persist with time (Fig. 4.3-4.8). Forty six weeks after immunization with Pa or Pw, 3 weeks after challenge, *B. pertussis*-specific cytokine responses are

clearly detectable in immunized mice (Fig. 4.7). Furthermore, memory T-cells secreting IFN-γ and IL-5 were detected by ELISPOT in the spleen at 18 and 32 weeks post-immunization (Fig. 4.9, Fig. 4.10). 400-600 memory Th2 cells per 10^6 cells were detected in Pa immunized mice at 18 weeks (Fig. 4.9), and this had declined to 100-200 per 10^6 cells by week 32 (Fig. 4.10). The levels of memory Th1 cells induced by Pw appeared to increase from 100-150 per 10^6 cells (Fig. 4.9) to 350-400 per 10^6 at week 32 (Fig. 4.10). It has been previously reported that the Th1 population confers optimal protection against *B. pertussis* (Mills *et al.*, 1993, Brady *et al.*, 1998, Barnard *et al.*, 1996, Mahon *et al.*, 1996). The polarized Th2 response induced by Pa may result in impaired bacterial clearance particularly in the absence of a persisting antibody response early after challenge, as seen in Fig. 4.2. However, whilst these patterns of cytokine secretion are stable, they are not immutable or "locked in". Following bacterial challenge, there is a broadening of the pattern of cytokine responses observed in immunized mice, from a Th2 response at 7 days after challenge to a mixed Th1/Th2 or Th0 response thereafter (Fig. 4.7, Fig. 4.8). However, the initial immunization is not without influence, because challenge of Pa immunized animals does not result in the polarized Th1 responses normally observed after immunization with Pw or *B. pertussis* challenge of control animals.

4.2.4 Immunization induces persistent memory B-cells

Although *B. pertussis*-specific T cells persist after immunization and $CD4^+$ T cells are critical to vaccine-mediated immunity against *B. pertussis*, the long-term protection observed is not necessarily an exclusively cell-mediated phenomenon. Memory B cells specific for *B. pertussis* are also induced by immunization with Pw and Pa and persist for at least 44 weeks after vaccination. Using an ELISPOT technique (Czerkinsky *et al.*, 1983), specific antibody forming cells were detected in peripheral blood mononuclear cells and spleen cells following stimulation with PT or *B. pertussis* sonicate, and in the bone marrow following stimulation with BP, PT, FHA or PRN, (Fig. 4.11, Fig. 4.12). Significantly greater numbers of *B. pertussis*-specific B cells could be detected from the blood, spleens and bone marrow of immunized mice when compared to controls. The importance of memory induction is confirmed by the observation of an anamnestic antibody response to *B. pertussis*

antigens in immunized mice post challenge (Fig. 4.13). As previously reported (Mills *et al.*, 1993), IgG responses against *B. pertussis* could not be detected in unimmunized control mice for at least 21 days after challenge. In contrast, 7 days after challenge, circulating antibodies were detected against PRN and *B. pertussis* sonicate in Pw-immunized mice and against PRN and PT in Pa-immunized mice.

4.3 Discussion

The majority of the studies on the mechanisms of protective immunity against *B. pertussis* in both mice and humans have focused on the evaluation of immune responses early after immunization. However, the effector mechanisms controlling infection at the peak of the immune response are most likely to be different to those that maintain long term protection. The present study demonstrates that persistent circulating antibody need not be detectable at the time of exposure for the maintenance of vaccine-induced protective immunity against a bacterial pathogen of humans. The results from a murine model show that immunity against *B. pertussis* induced with Pw or Pa was maintained for a prolonged period after immunization, when serum antibody responses had declined to undetectable levels. The findings point to a role for immunological memory, at the T and B cell level, in the maintenance of long term protective immunity against *B. pertussis*.

Antibody has been considered to be the major effector mechanism mediating vaccine-induced immunity against viral and bacterial pathogens. Protective levels of circulating antibody have been identified for certain pathogens, such as poliovirus, diphtheria, tetanus and *Haemophilis influenzae* (Robbins *et al.*, 1995, Goldschneider *et al.*, 1969, Kayhty *et al.*, 1998, McHammond *et al.*, 1953). However there is some uncertainty about the accuracy or indeed relevance of these markers, and certain individuals with antibody levels below these threshold values appear to be protected. It has been more difficult to establish an immunological correlate of immunity against other pathogens, including *B. pertussis*. The clinical trials of pertussis vaccines could not establish a critical level of antibody against putative protective antigens that would confer protection (Ad hoc group for the study of pertussis vaccines, 1998, Greco *et al.*, 1996, Simondon *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997). However two recent reports from household contact studies indicated that antibody responses against PT, PRN and fimbriae may correlate with absence of severe disease (Storsaeter *et al.*, 1998, Cherry *et al.*, 1998). Although these studies suggested that antibodies against PRN and fimbriae are the most important, a monocomponent pertussis toxoid vaccine conferred 71% protection against severe disease in the Göteburg trial (Taranger *et al.*, 1997). Investigations in mice have demonstrated that passive transfer of a

combination of antibodies against two or more of the antigens can confer a high level of protection (Mills *et al.*, 1998a). However the mouse studies also demonstrated that T cell responses, in particular Th1 cells also play a critical role in protective immunity, especially that induced by natural infection or immunization with Pw (Mills *et al.*, 1993, Mahon *et al.*, 1997, Mills *et al.*, 1998a, Redhead *et al.*, 1993, Mahon *et al.*, 1996). Furthermore, follow up studies from the clinical trials have demonstrated that antibody levels decline quite rapidly after vaccination, yet these children still appear to be protected against whooping cough (Giuliano *et al.*, 1998, Salmaso *et al.*, 1998, Storaseter *et al.*, 1998). However in the clinical setting it is difficult to establish the level of exposure to infection and it is not clear whether these children would remain free from disease if exposed to *B. pertussis* in the future.

Animal models provide an alternative approach to dissecting mechanisms of immunity and allow us to address fundamental questions that cannot be answered from clinical studies. Protection against intracerebral (i.c.) challenge in immunized mice has been used as an indication of the potency of Pw (Kendrick *et al.*, 1947). However, most Pa do not protect mice against lethal i.c. challenge (Corbel & Xing, 1997). In contrast, it has been demonstrated that Pw and Pa can protect mice against respiratory challenge and that the rate of bacterial clearance from the lungs of immunized mice correlates with vaccine efficacy in clinical trials (Mills *et al.*, 1998a). In the present study we have demonstrated that protection against *B. pertussis* in mice is possible in the absence of circulating antibody. Following immunization with two low doses of either Pw or Pa, the serum IgG level specific for PT, FHA, PRN and *B. pertussis* lysate declined rapidly and had reached undetectable levels 6-9 months after immunization. However despite the waning antibody response, mice were still protected against respiratory infection when challenged 44 weeks after immunization. The course of infection in immunized compared with control naïve mice indicated persistence of vaccine-induced protection, especially with Pw.

There are a number of explanations for the maintenance of protection after the decline in circulating anti-*B. pertussis* antibodies. It is possible that antibody does not pay a role in protection and that immunity is conferred exclusively by

cellular mechanisms. Alternatively the vaccine may have induced immunological memory at the B and/or T cell level, which is recalled after *B. pertussis* challenge. Analysis of cell mediated immunity demonstrated that specific T cell responses were maintained up to 3 weeks post-challenge, 46 weeks after immunization. As previously reported for short-term immunization, Pw selectively induced *B. pertussis*-specific Th1 cells, whereas Pa induced T cells more polarized to the Th2 subtype (Barnard *et al.*, 1996, Mills *et al.*, 1993). Although this dichotomy in the cytokine profile was maintained up to 46 weeks after immunization, the response with the Pa broadened to a mixed Th1/Th2 after challenge and this is consistent with observations in immunized children (Ausiello *et al.*, 1997, Ryan *et al.*, 1998, Ryan *et al.*, 1997). The results suggest that the Th1/Th2 dichotomy does not impinge on memory induction. It has been previously reported that the Th1 sub-population confers optimal protection against *B. pertussis* (Mills *et al.*, 1998a, Redhead *et al.*, 1993, Mills *et al.*, 1993, Mahon *et al.*, 1996). The polarized Th2 response induced by Pa may result in impaired bacterial clearance particularly in the absence of a persisting antibody early after challenge. However, whilst these patterns of cytokine secretion are stable, they are not permanently fixed. Following bacterial challenge, there is a broadening of the pattern of cytokine responses observed in Pa immunized mice, from a Th2 response at 7 days after challenge to a mixed Th1/Th2 or Th0-type response thereafter.

The selective induction and maintenance of the Th1 response with Pw, together with their superior protection against *B. pertussis* infection, is consistent with previous reports that Th1 cells play an important role in protective immunity (Redhead *et al.*, 1993, Mills *et al.*, 1998a, Mills *et al.*, 1993, Mahon *et al.*, 1997). Although activation of cellular mechanisms against intracellular *B. pertussis* (1997, Mahon *et al.*, 1999, Friedman *et al.*, 1992), including the stimulation of bacterial uptake and killing by macrophages and PMN, may be one mechanism whereby Th1 cells mediate their protective function, we cannot rule out their role in stimulating protective humoral immunity. Th1 cells provide such a function in immunity to certain viral infections (Mahon *et al.*, 1995). Indeed studies with Ig defective mice and passive transfer of IgG have indicated that antibody can play a crucial role in protection against *B. pertussis* (Mahon *et al.*, 1997, Mills *et al.*, 1998a). In the

present study, an analysis of serological responses after challenge demonstrated anamnestic antibody responses against each of the *B. pertussis* antigens examined. Furthermore antibody forming cells specific for *B. pertussis* antigens were detected in the bone marrow, spleen and circulation at the time of challenge. These results demonstrate that immunological memory is maintained despite the decline in circulating antibodies and suggest that memory B and T cells are responsible for the maintenance of long term protection against *B. pertussis.*

Our findings are not incompatible with recent suggestions that antibodies against multiple antigens can mediate protection against *B. pertussis* (Olin et al., 1997, Mills *et al.*, 1998a, Storsaeter *et al.*, 1998, Taranger *et al.*, 1997). However it appears that current pertussis vaccines do not confer sterilizing immunity. Vaccine efficacy data from clinical trials was largely based on the WHO case definition of whooping cough based on 21 or more days of paroxysmal cough. However with a less stringent case definition of 7 or more days of non-paroxysmal cough, the pertussis vaccine efficacy was lower (Gustafsson *et al.*, 1996, Olin *et al.*, 1997). Thus pertussis vaccines are capable of preventing severe disease, but are less efficient when judged on the criteria of preventing infection or mild disease, even in the presence of high levels of circulating antibody at the time of infection.

The results of this present study have important implications for pertussis vaccination policy in relation to the level of protection in the face of waning antibody levels following vaccination and the need for booster immunization. The data from the mouse model suggest that persistence of circulating antibody is not a prerequisite for the maintenance of protective immunity. These findings have substantial ramifications for the evaluation and development of vaccines against a range of infectious diseases and demonstrate that a simple quantification of circulating antibodies may be a misleading correlate of immunity against *B. pertussis* and other pathogens. Although circulating neutralizing antibodies are clearly important in sterilizing immunity, especially against viral infections, it is also evident that certain vaccines can protect by non-humoral mechanisms or by the recall of immunological memory at the T and B cell level.

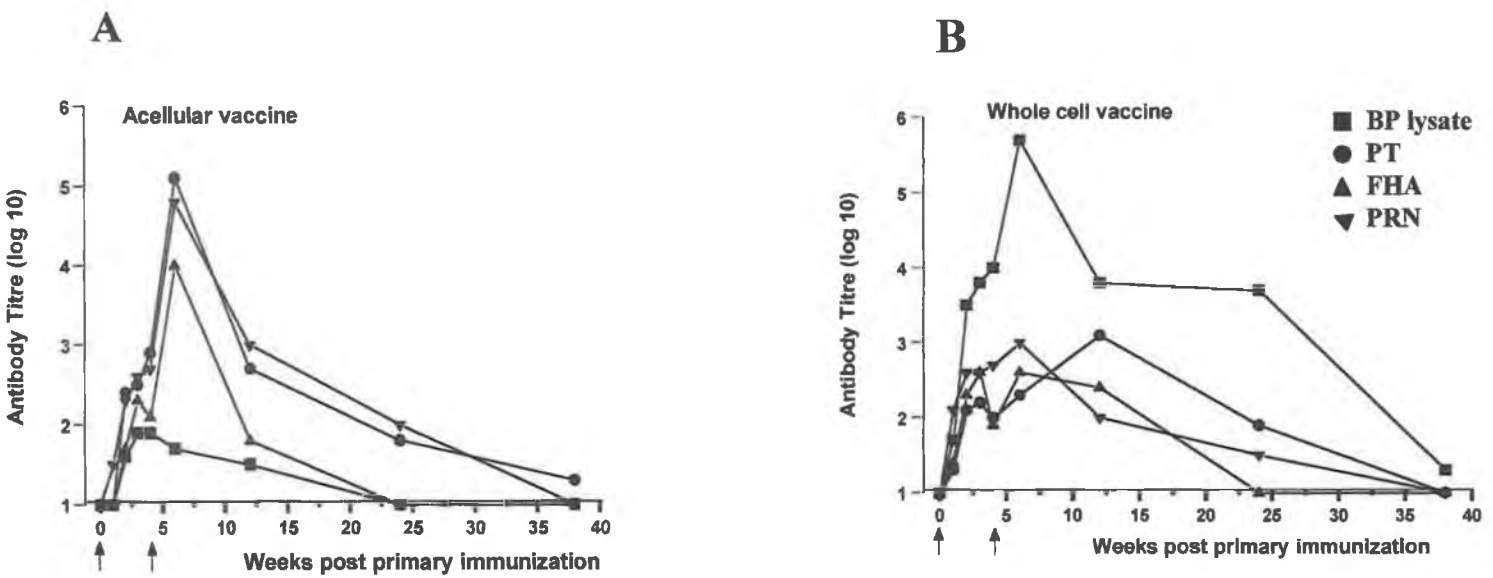


Figure 4.1

Decline in antibody responses to antigens of *B. pertussis* following immunization with whole cell (Pw) or acellular (Pa) pertussis vaccines. Mice were immunized at week 0 and 4 with 0.04 human dose of Pw or Pa (indicated by arrows), and the circulatory antibody responses to *B. pertussis* (BP) sonicate, PT, FHA and PRN were detected by ELISA at intervals after immunization.

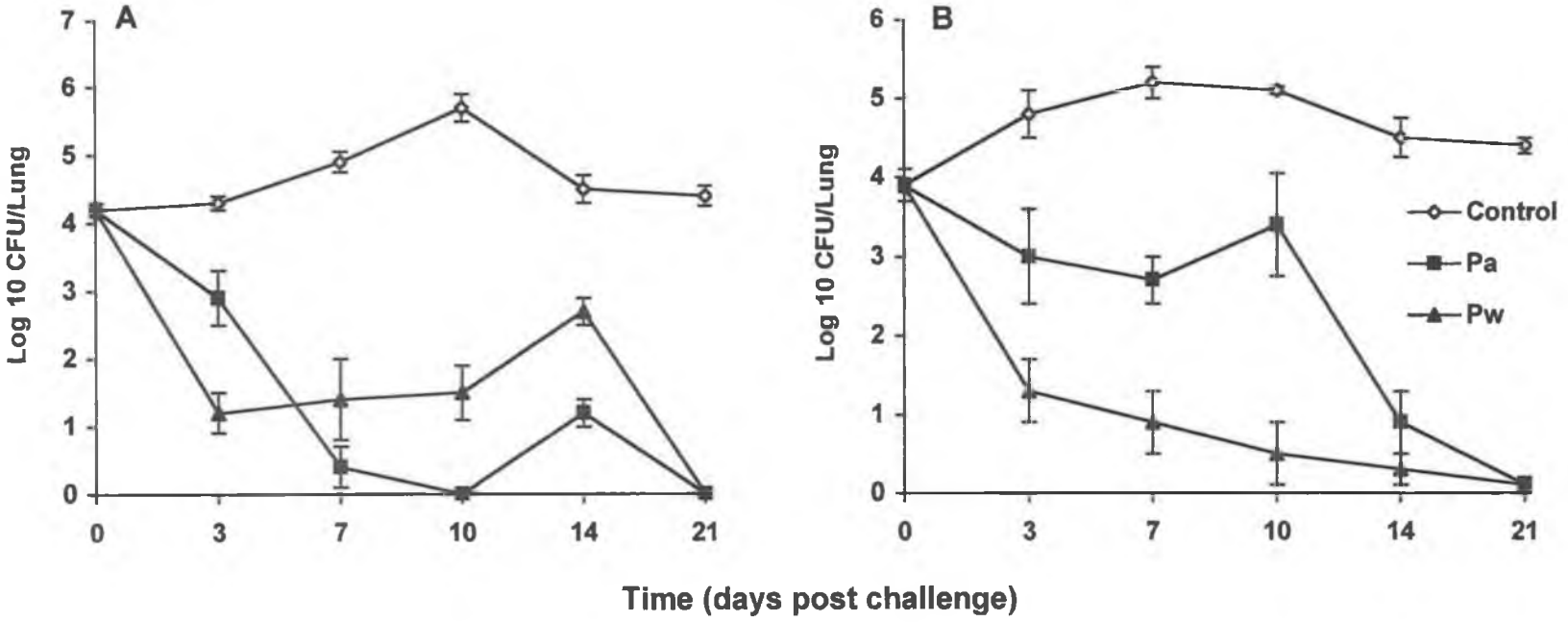
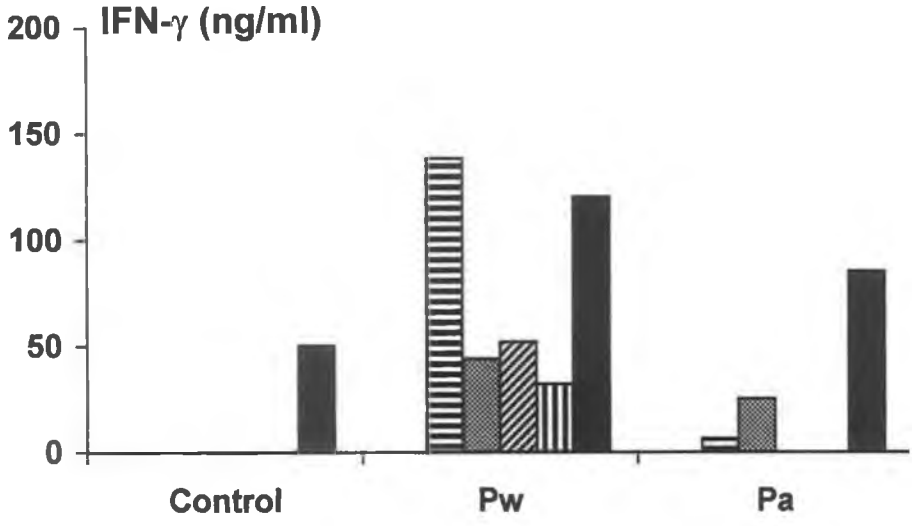
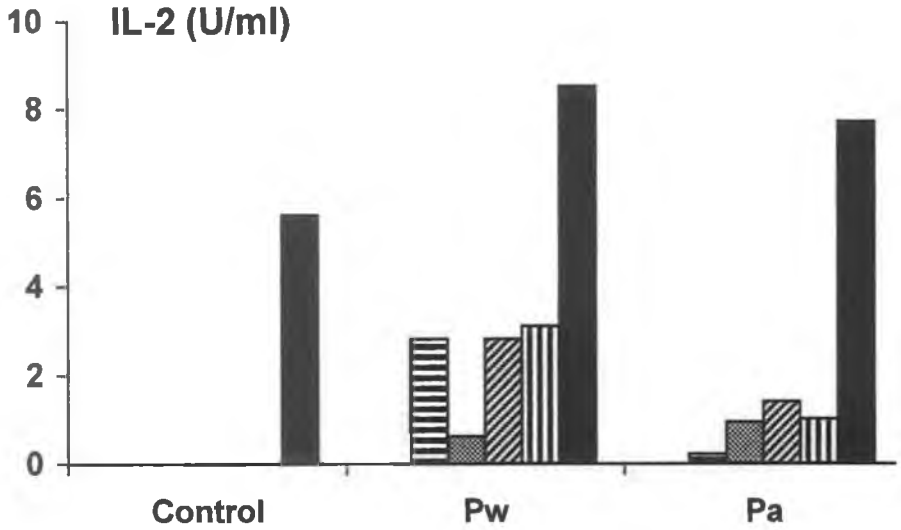
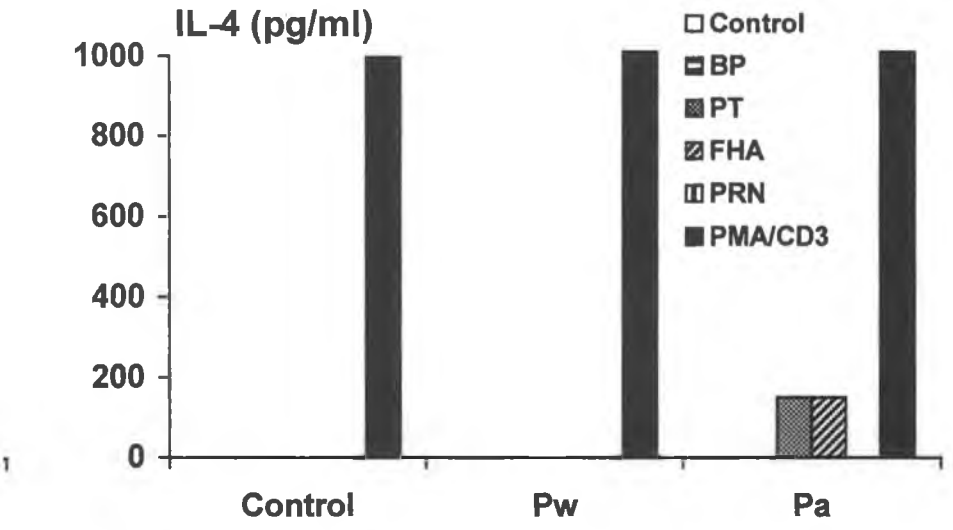


Figure 4.2

Persistence of protection against *B. pertussis* respiratory infection in long term immunized mice. Mice were immunized at week 0 and 4 with 0.04 human dose of Pw, Pa, or adjuvant only as control, and were exposed to an aerosol challenge of *B. pertussis* 6 weeks (A) or 44 weeks (B) after the primary immunization.


IFN-γ (ng/ml)
200
150
100
50
0
Control
Pw
Pa


IL-2 (U/ml)
10
8
6
4
2
0
Control
Pw
Pa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800
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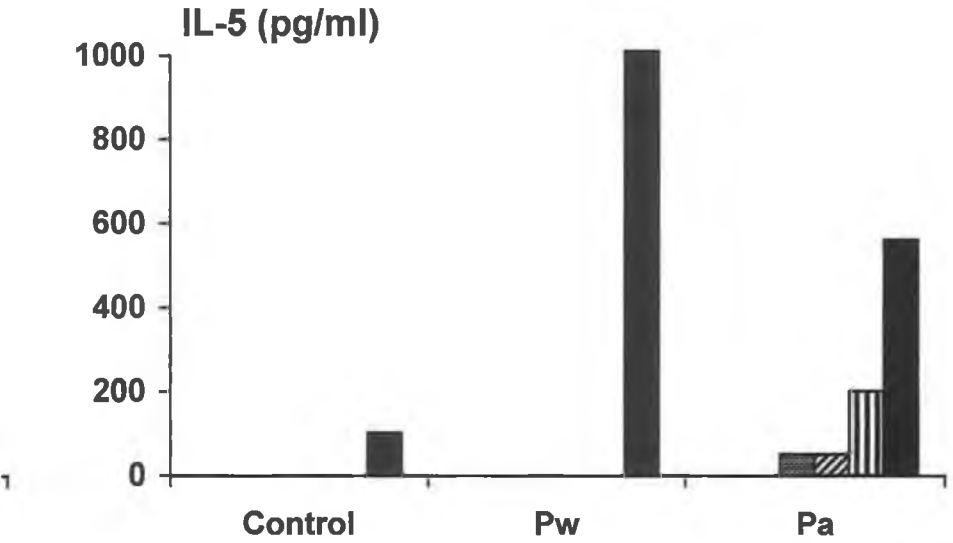
400
200
0
Control
Pw
Pa
Control
BP
PT
FHA
PRN
PMA/CD3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800
600
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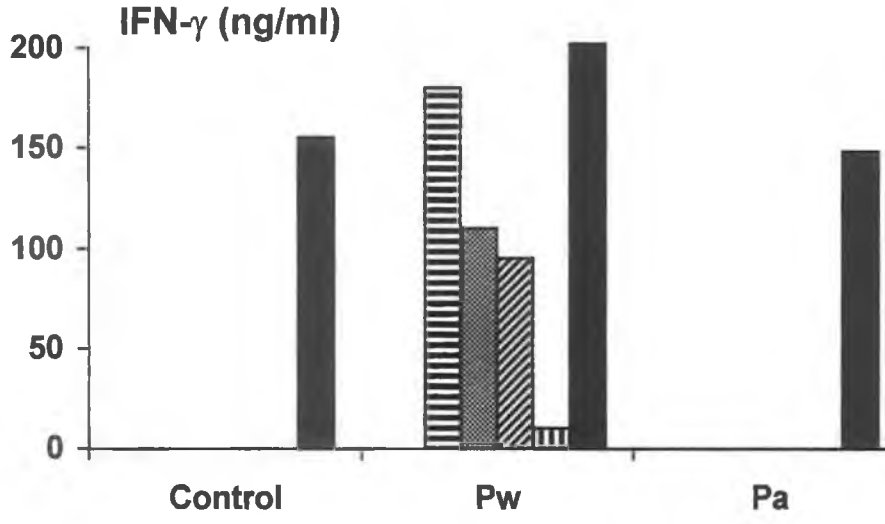
0
Control
Pw
Pa


IFN-γ (ng/ml)
200
150
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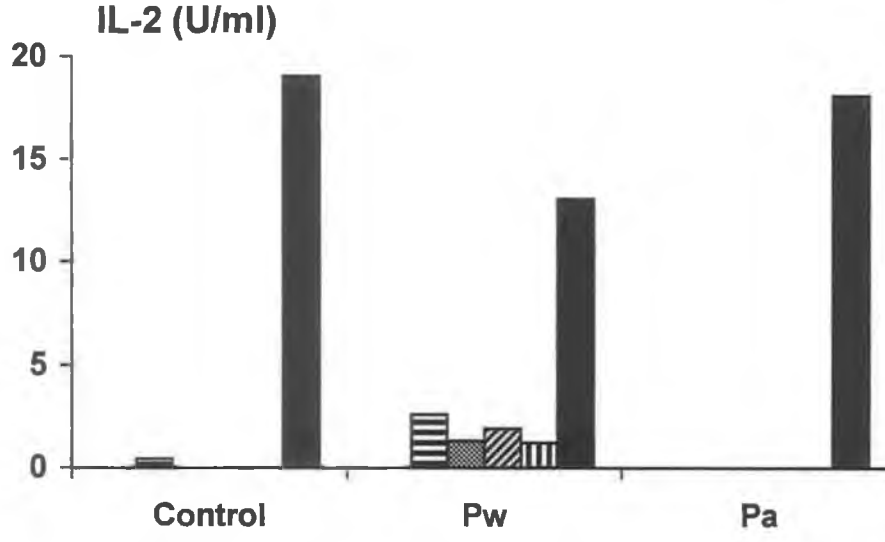
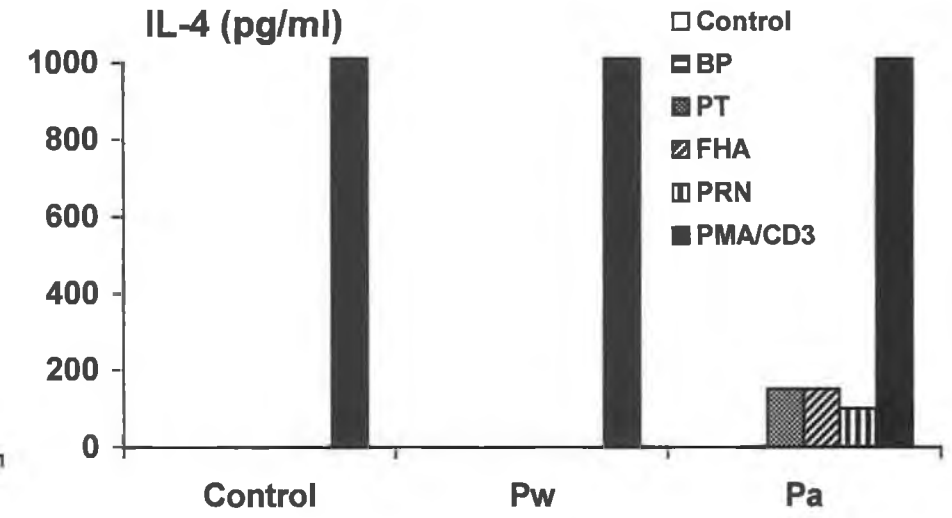
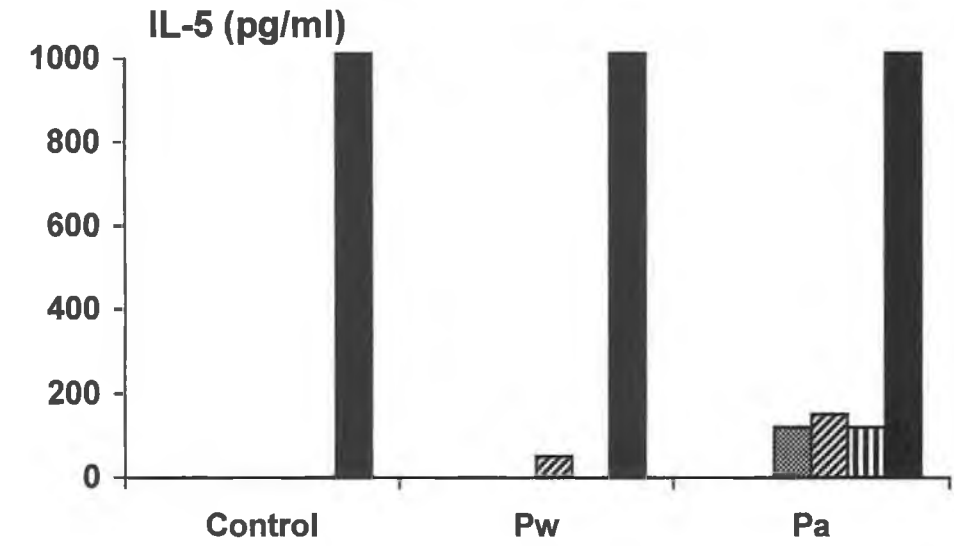
Control
Pw
Pa


IL-2 (U/ml)
20
15
10
5
0
Control
Pw
Pa


IL-4 (pg/ml)
Control
BP
PT
FHA
PRN
PMA/CD3
1000
800
600
400
200
0
Control
Pw
Pa


IL-5 (pg/ml)
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0
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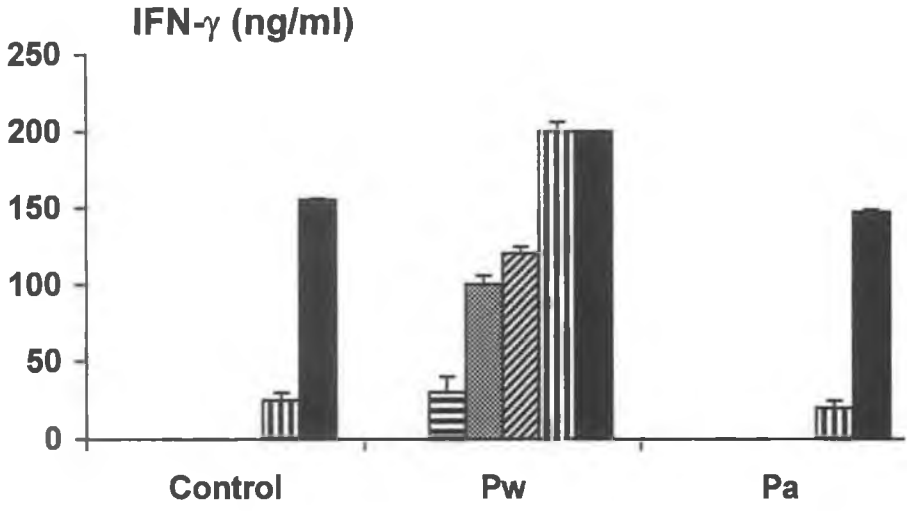
Pw
Pa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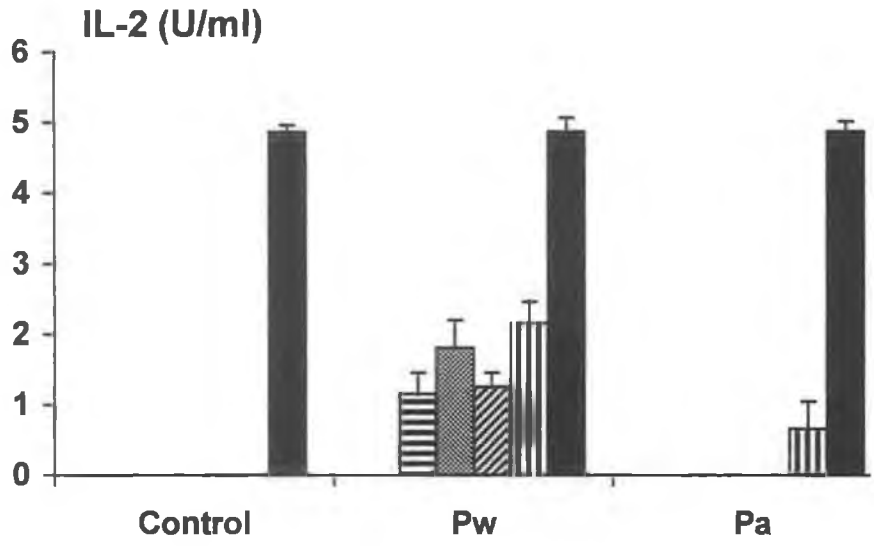
250
200
150
100
50
0
Control
Pw
Pa


IL-2 (U/ml)
6
5
4
3
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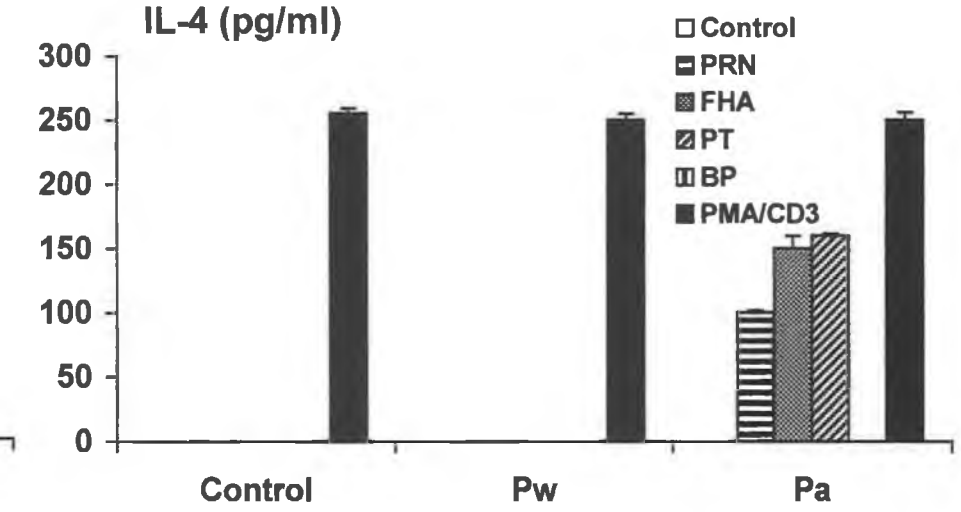
1
0
Control
Pw
Pa


IL-4 (pg/ml)
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200
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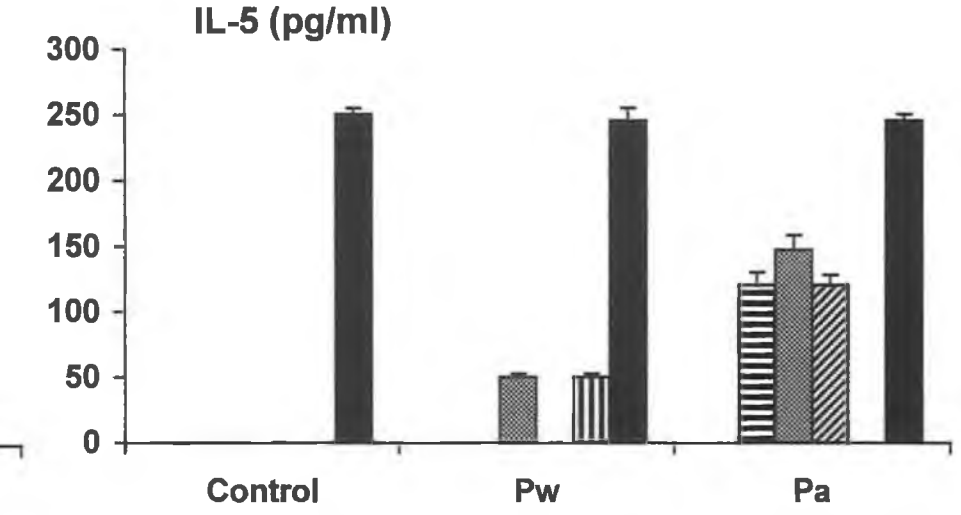
Control
PRN
FHA
PT
BP
PMA/CD3
Control
Pw
Pa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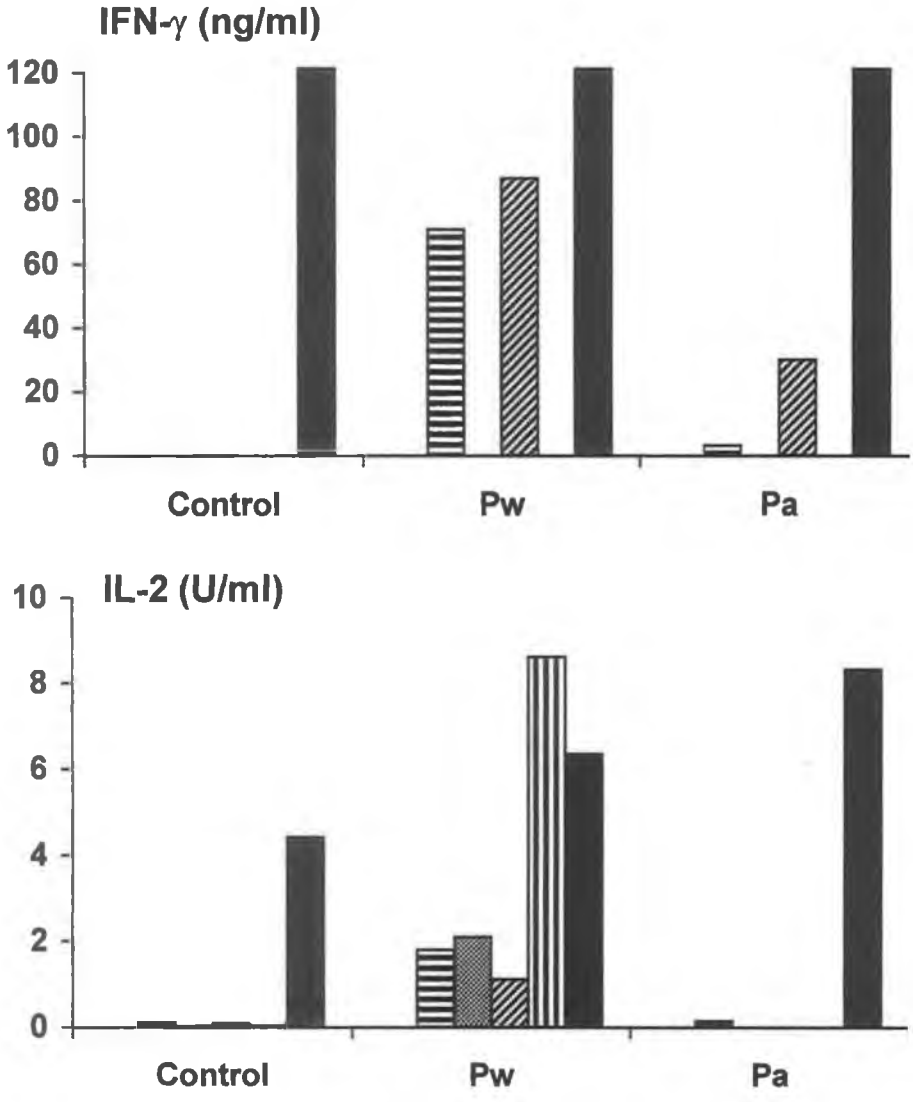
Pw
Pa


IFN-γ (ng/ml)
120
100
80
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Control
Pw
Pa
IL-2 (U/ml)
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8
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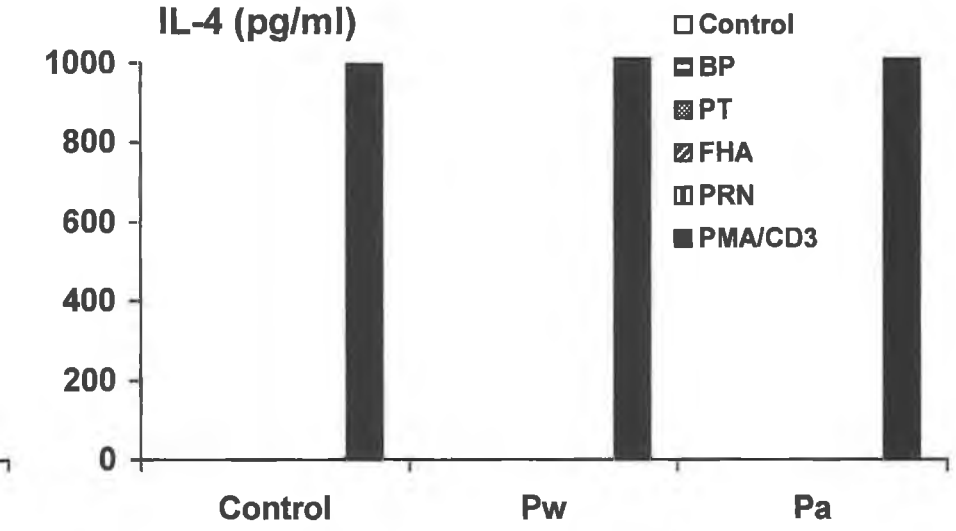
0
Control
Pw
Pa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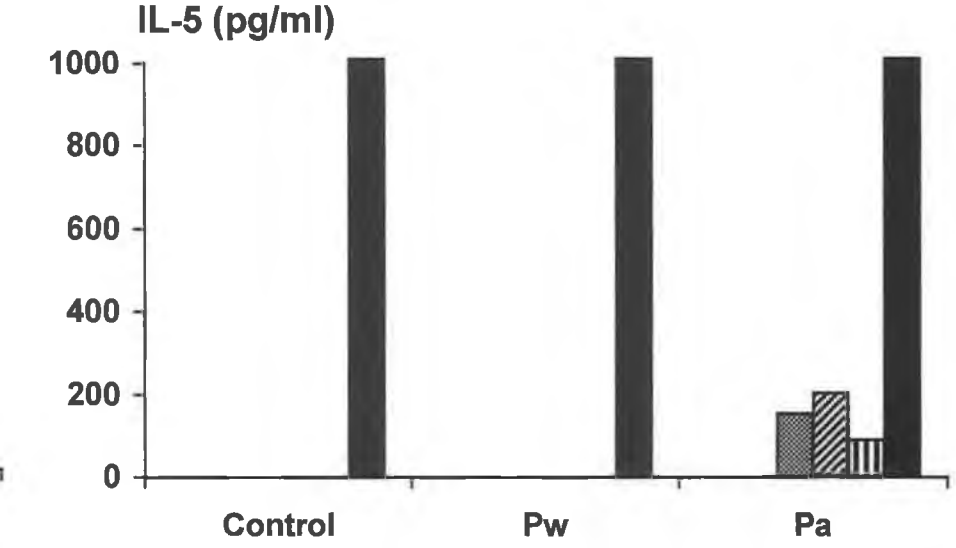
Control
Pw
Pa
Control
BP
PT
FHA
PRN
PMA/CD3


IL-5 (pg/ml)
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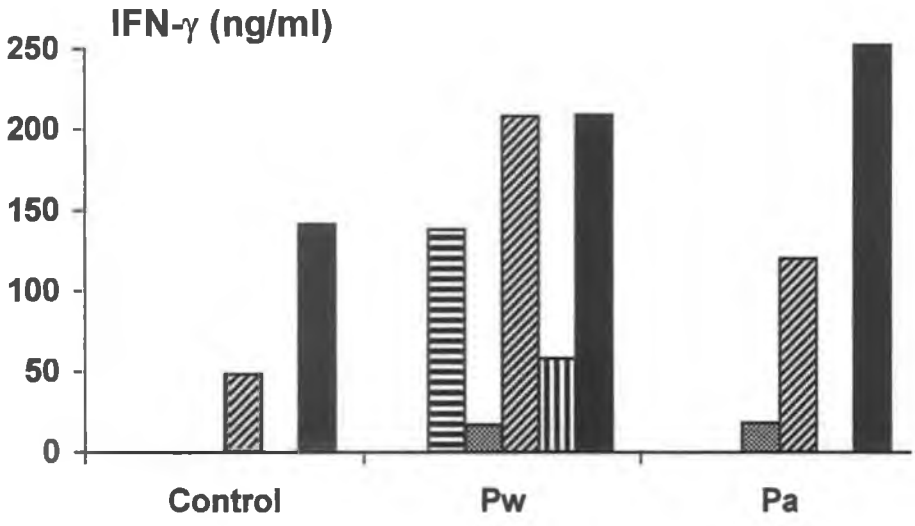
Pw
Pa


IFN-γ (ng/ml)
250
200
150
100
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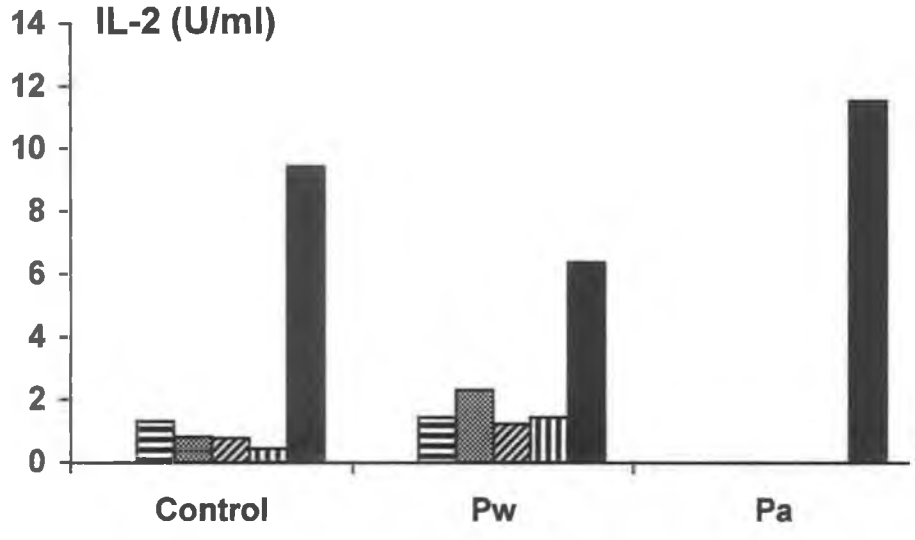
0
Control
Pw
Pa


IL-2 (U/ml)
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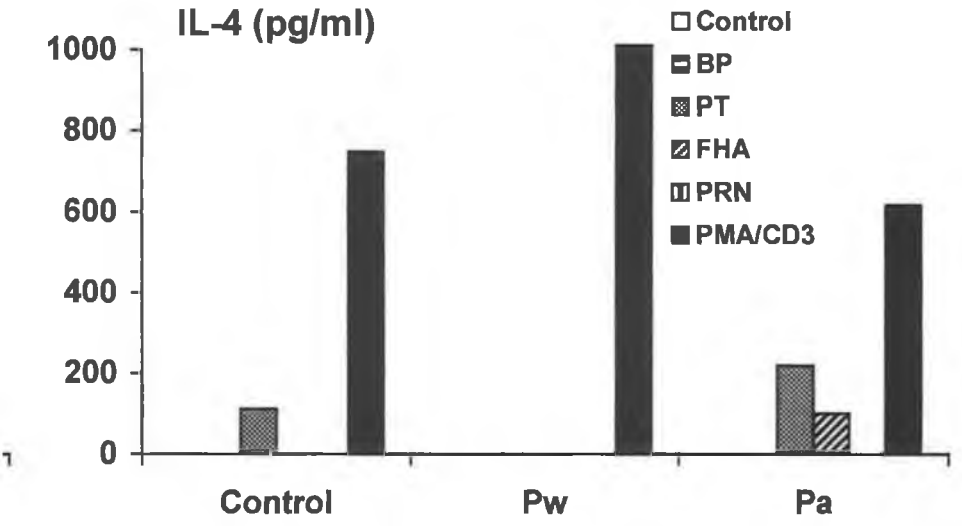
12
10
8
6
4
2
0
Control
Pw
Pa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800
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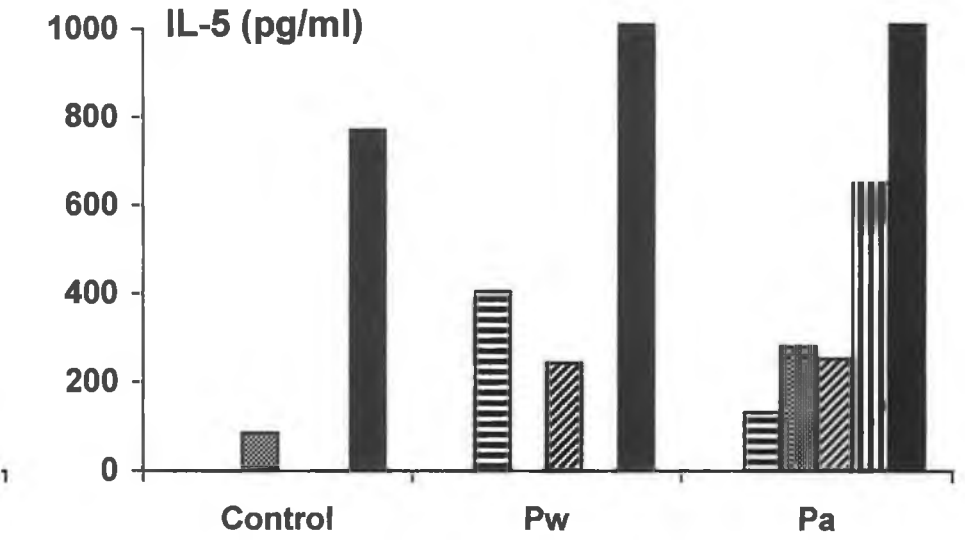
0
Control
BP
PT
FHA
PRN
PMA/CD3
Control
Pw
Pa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1000
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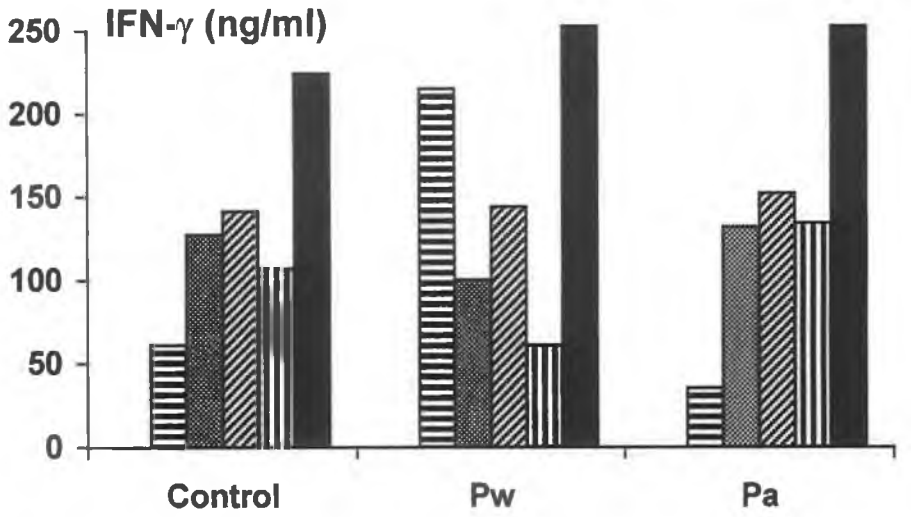
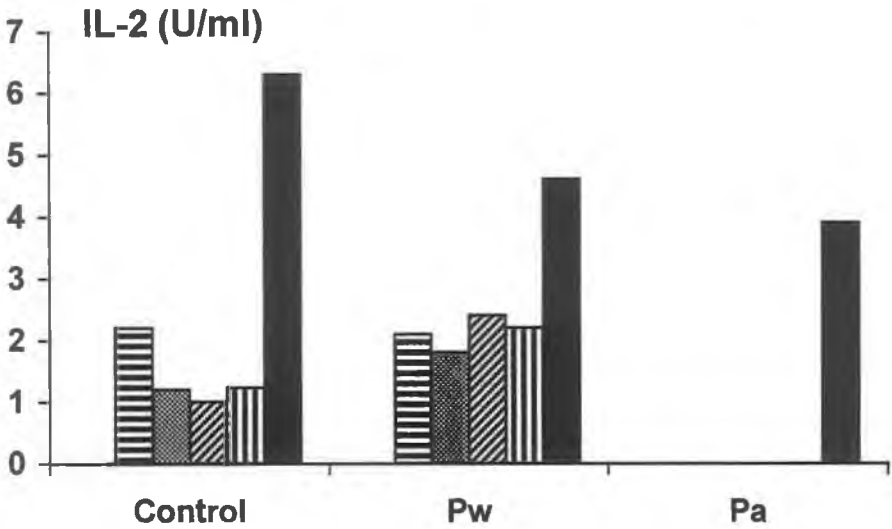
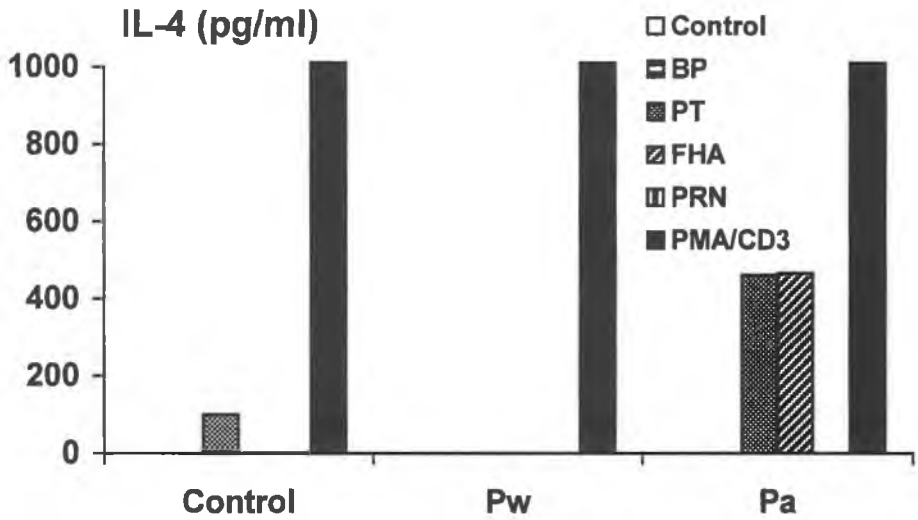
Pw
Pa


IFN-γ (ng/ml)
250
200
150
100
50
0
Control
Pw
Pa


IL-2 (U/ml)
7
6
5
4
3
2
1
0
Control
Pw
Pa


IL-4 (pg/ml)
1000
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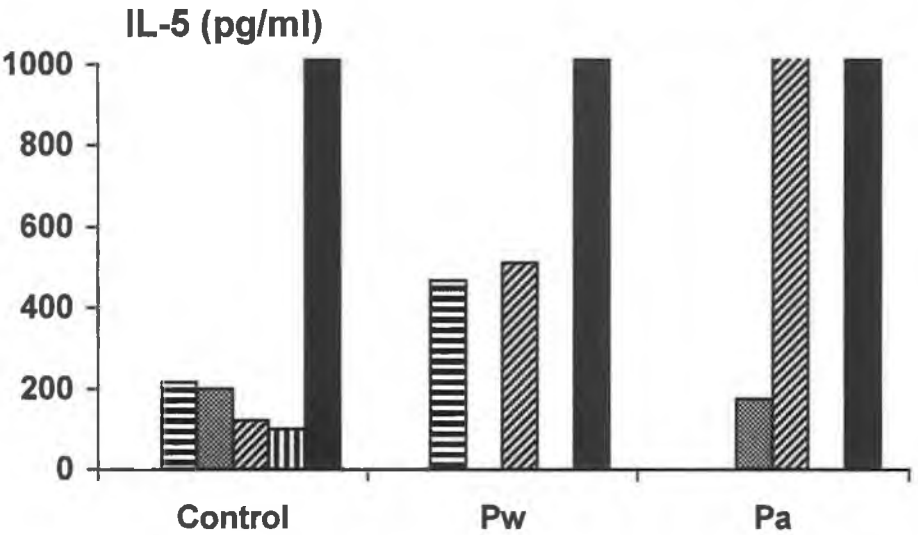
200
0
Control
BP
PT
FHA
PRN
PMA/CD3
Control
Pw
Pa


IL-5 (pg/ml)
1000
800
600
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0
Control
Pw
Pa

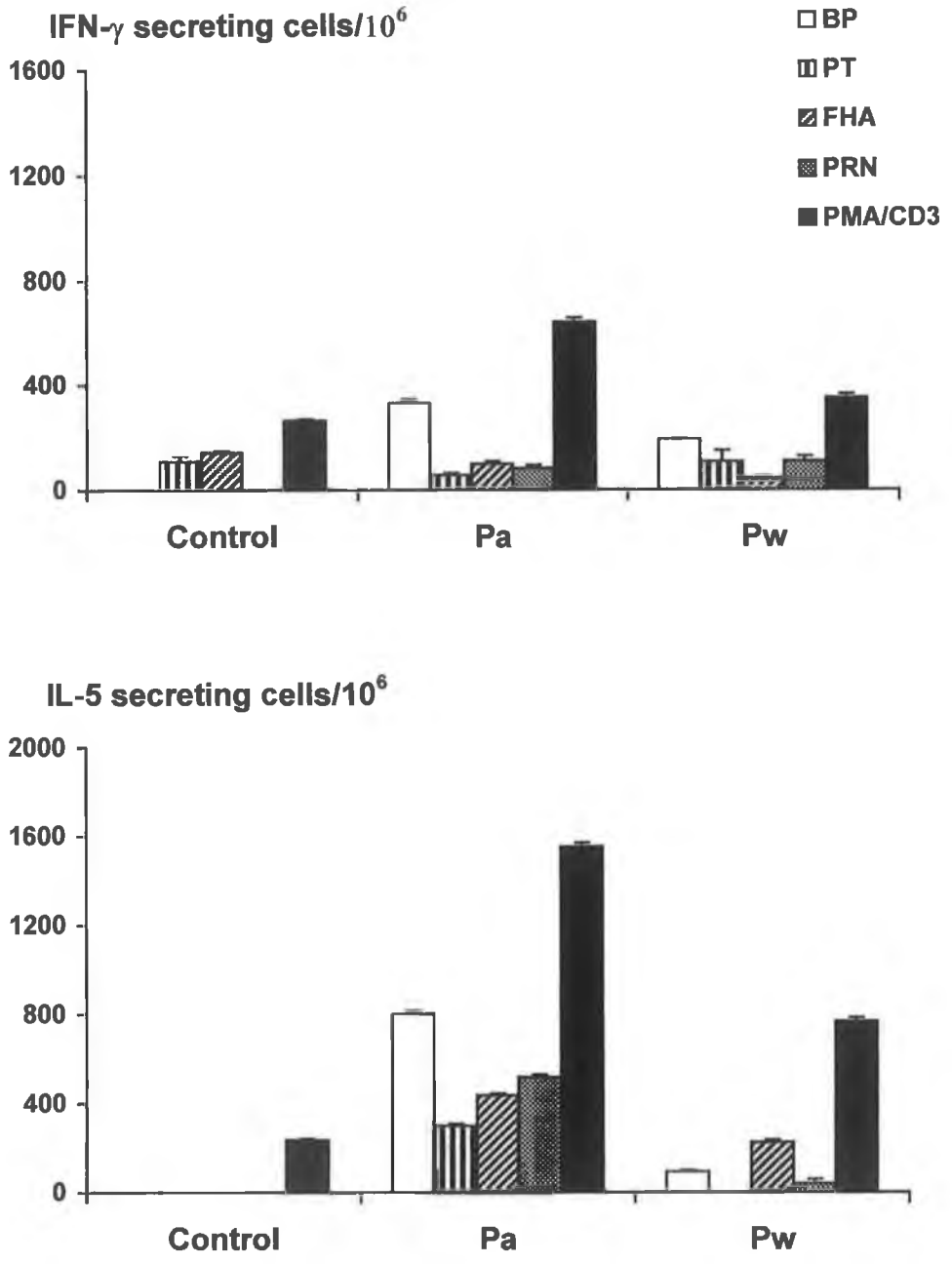


Figure 4.9

Detection of *B. pertussis*-specific memory T-cells 18 weeks after immunization with Pa or Pw. Spleen cells were stimulated with heat-inactivated PT, FHA or PRN (5.0μg/ml), heat killed *B. pertussis* (BP) (1x10^{6}cells/ml), and PMA/CD3 or medium as positive and negative controls respectively. The numbers of IFN-γ and IL-5 secreting cells were determined by ELISPOT. Results are the mean numbers of cytokine secreting cells (±SE), after subtraction of background control values with medium only (IFN-γ, 464-641 x 10^6 cells, IL-5, 359-424 x 10^6 cells) for 4 mice per group assayed in triplicate.

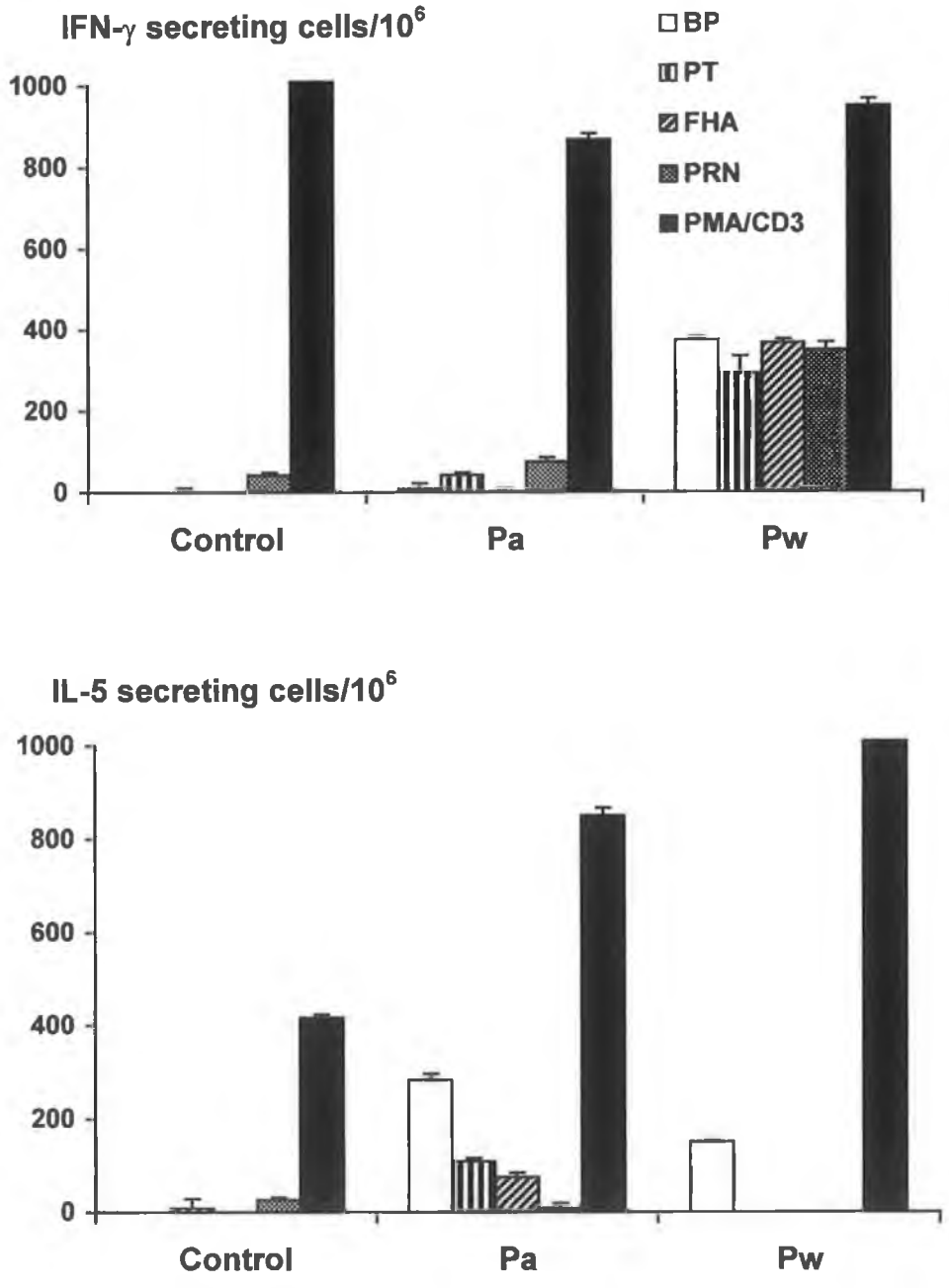


Figure 4.10

Detection of *B. pertussis*-specific memory T-cells 32 weeks after immunization with Pa or Pw. Spleen cells were stimulated with heat-inactivated PT, FHA or PRN (5.0μg/ml), heat killed *B. pertussis* (BP) (1x10^6cells/ml), and PMA/CD3 or medium as positive and negative controls respectively. The numbers of IFN-γ and IL-5 secreting cells were determined by ELISPOT. Results are the mean numbers of cytokine secreting cells (±SE), after subtraction of background control values with medium only (IFN-γ, 12-19 x 10^6 cells, IL-5, 3-6 x 10^6 cells) for 4 mice per group assayed in triplicate.

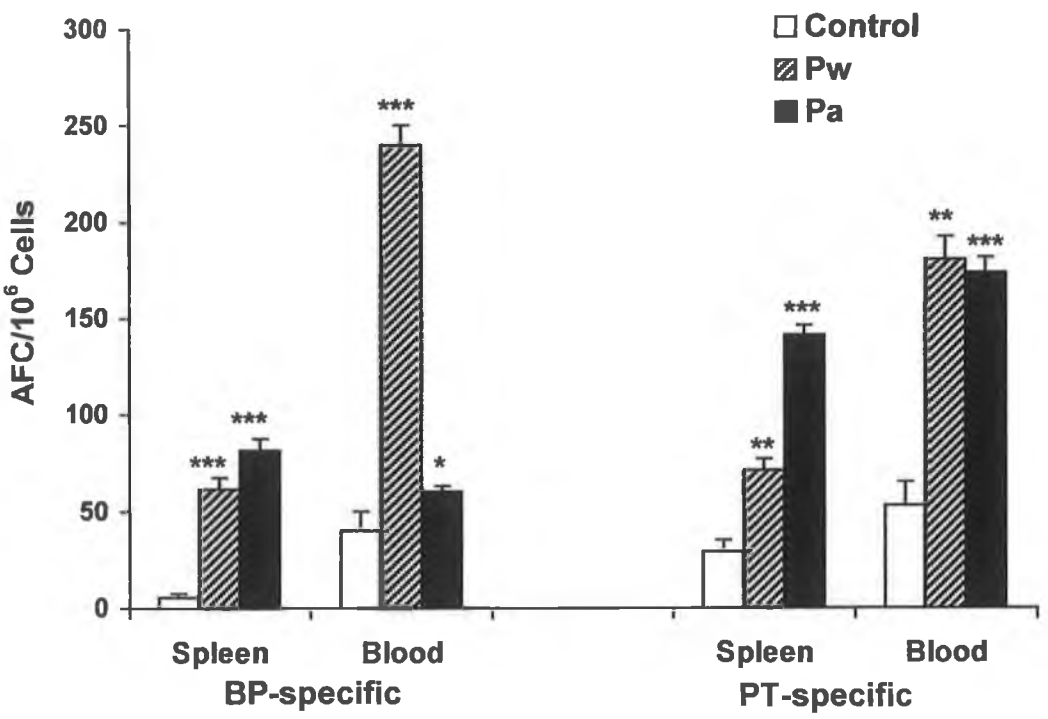


Figure 4.11

Detection of *B. Pertussis*-specific memory B-cells 44 weeks after immunization. Mice were immunized at week 0 and 4 with 0.04 human dose of Pw, Pa or adjuvant only (control). Peripheral blood mononuclear cells and spleen cells were incubated in plates coated with *B. pertussis* lysate (BP) or inactivated PT and the numbers of specific AFC determined by ELISPOT. Results are mean values for 4-6 mice per group. *, $P < 0.05$ versus mice immunized with alum only; **, $P < 0.01$ versus mice immunized with alum only; ***, $P < 0.001$ versus mice immunized with alum only.

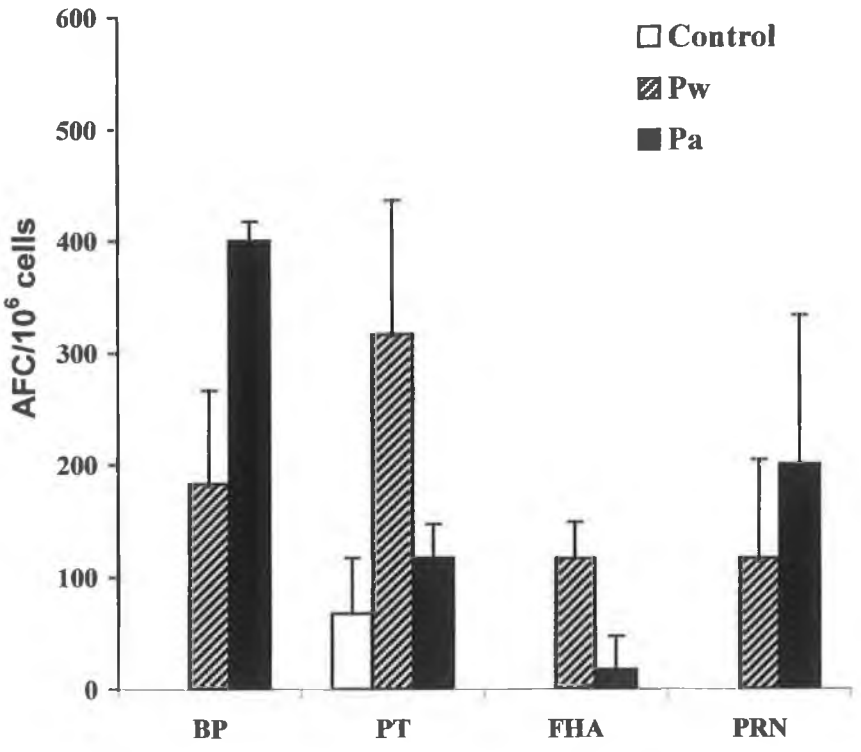


Figure 4.12

B. Pertussis-specific memory B-cells are detectable in bone marrow, up to 44 weeks after immunization with Pa or Pw. Mice were immunized at week 0 and 4 with 0.04 human dose of Pw, Pa or adjuvant only (control). Cells isolated from the bone marrow of immunized mice were incubated in plates coated with *B. pertussis* lysate (BP), inactivated PT, FHA or PRN, at a concentration of $1x10^6$/ml The numbers of specific AFC were determined by ELISPOT. Results are mean values for 4 mice per group, after subtraction of background values (72-90 AFC/10^6).

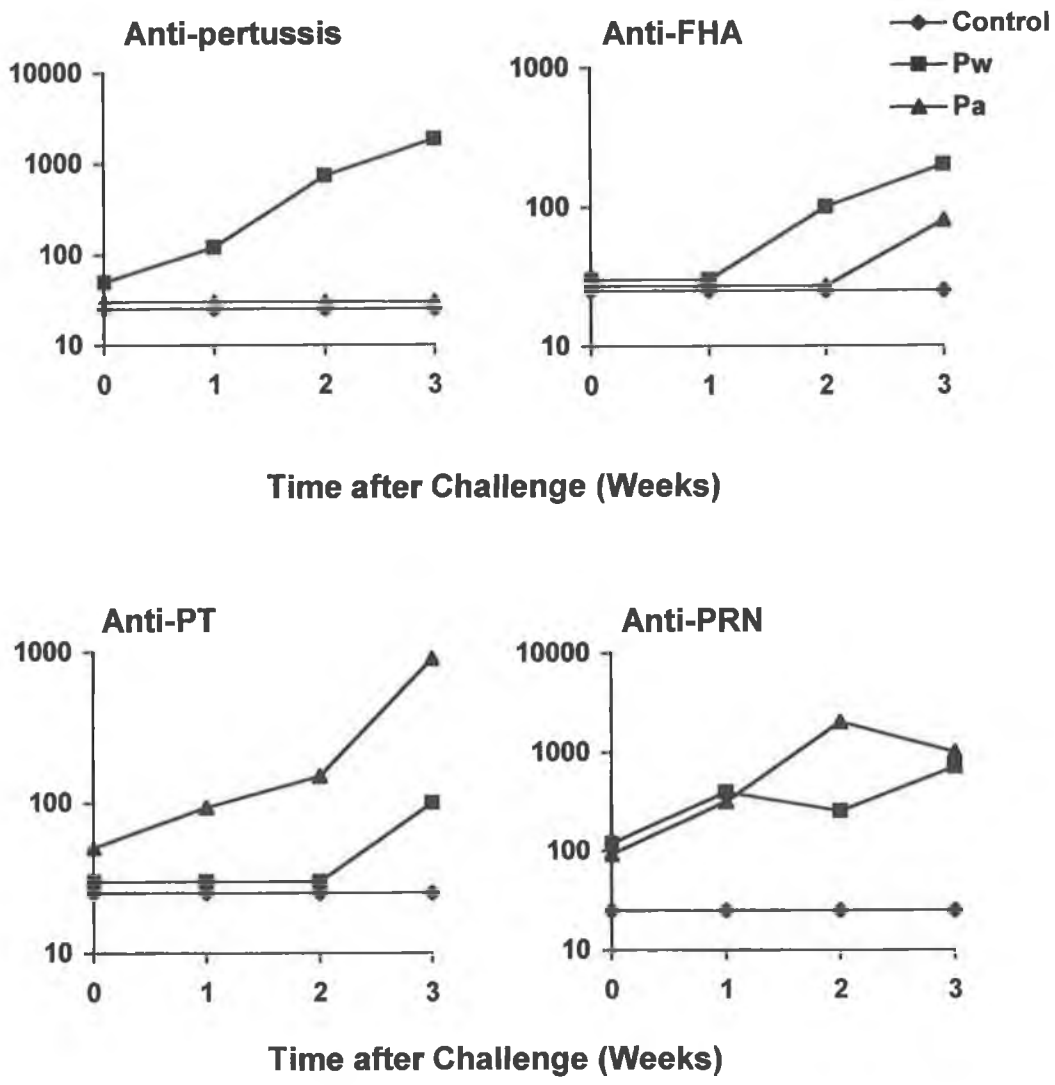


Figure 4.13

Long-term immunized mice develop an anamnestic antibody response on exposure to *B. pertussis*. Mice were immunized at week 0 and 4 with 0.04 human dose of Pw, Pa or adjuvant only as control, and were challenged with *B. pertussis* 44 weeks after the primary immunization. Antibody responses to *B. pertussis*, PT, FHA and PRN were determined by ELISA at days 7, 14 and 21 after challenge.

❖ Chapter 5 ❖

The role of IL-12 and IL-18 in the induction of a protective Th1 response to *Bordetella pertussis*

5.1 Introduction

The generation of Th1 responses and IFN-γ production are important in promoting immunity to a number of infectious pathogens, particularly those which occupy an intracellular niche. Optimal immunity to *Bordetella pertussis* is associated with the induction of cell-mediated immunity. Cheers and colleagues demonstrated more than 30 years ago that *B. pertussis* had an intracellular phase and suggested that cellular immunity may be required to eliminate intracellular bacteria (Cheers *et al.*, 1969, Gray *et al.*, 1969). These observations have been confirmed by the demonstration that *B. pertussis* has the ability to survive within macrophages, polymorphonuclear leukocytes and respiratory epithelial cells (Saukkonen *et al.*, 1991, Steed *et al.*, 1992, Friedman *et al.*, 1992, Bassinet *et al.*, 2000, Weingart *et al.*, 2000, Hellwig *et al.*, 1999). Although there is a large body of evidence that antibody plays a role in protection against *B. pertussis* (Mills *et al.*, 1998, Storsaeter *et al.*, 1998), it's dual extra- and intra-cellular location is congruous with a role for cellular as well as humoral mechanisms for the resolution of infection.

It has been shown that *B. pertussis*-specific T-cells induced by infection in mice are largely confined to IFN-γ and IL-2 secreting $CD4^+$ T-cells and that these cells play a critical role in the clearance of bacteria after a primary infection and in the development of protective immunity against subsequent challenge (Mills *et al.*, 1993). Similarly, infection in children or immunization with whole cell pertussis vaccine is associated with the induction of antigen-specific Th1 cells, which are critical in host resistance to infection (Redhead *et al.*, 1993, Ryan *et al.*, 1997). In particular, the Th1 cytokine IFN-γ plays a major role in controlling *B. pertussis* infection and in containing the bacteria to the mucosal site (Barbic *et al.*, 1997, Mahon *et al.*, 1997). Given the importance of IFN-γ and the induction of Th1 cells in immunity to *B. pertussis*, the factors which control the induction of IFN-γ and Th1 responses are worthy of investigation; namely IL-12 and IL-18. Indeed, it has already been shown that IL-12 is produced following exposure to live or killed *B. pertussis*, and that the addition of IL-12 to an acellular vaccine enhances it's protective efficacy by promoting type 1 cytokine production (Mahon *et al.*, 1996).

IL-12 was first described in 1989 (Kobayashi *et al.*, 1989) and was initially called natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte

maturation factor. Since then, IL-12 has been shown to be produced primarily by activated macrophages, but also by monocytes, neutrophils, dendritic cells and antibody-producing B-cells. (Hall, 1995). IFN-γ and IL-12 are thought to be major cytokines governing the promotion of Th1 differentiation. IL-12 is a 70- to 75-kDa heterodimeric protein consisting of disulphide-bonded 35-kDa (p35) and 40 kDa (p40) subunits. *In vivo*, IL-12 acts primarily at three stages during the innate resistance/adaptive immune response (Gately *et al.*, 1998). Firstly, IL-12 is produced early in infection, and activates NK and T-cells to produce IFN-γ, which contributes to phagocytic cell activation and inflammation. Secondly, IL-12 promotes the generation of Th1 cells by priming $CD4^+$ T-cells for high IFN-γ production and by favouring the expansion of Th1 cells. In doing so, IL-12 acts in antagonistic equilibrium with IL-4 which stimulates the development of Th2 cells. Thirdly, IL-12 determines optimal IFN-γ production and proliferation of Th1 cells, in association with other stimuli. Thus IL-12 represents a functional bridge between early non-specific innate immunity and the subsequent antigen specific adaptive immunity. In the case of IFN-γ, it's effect may be to prevent the outgrowth of Th2 cells rather than to promote directly the selective development of Th1 cells. In contrast, the presence of IL-12 during priming directly augments Th1 differentiation.

In addition to IL-12, IL-18 may have important relevance in the induction of Th1 cells, and in protection against intracellular pathogens. IL-18 was originally identified as IFN-γ inducing factor (IGIF) that was induced in the sera of mice treated with heat-killed *Propionibacterium acnes*, followed by lipopolysaccharide (LPS) challenge (Nakamura *et al.*, 1989, Okamura *et al.*, 1995a). IL-18 is a 24-kDa, non-glycosylated polypeptide and is structurally similar to IL-1β. Like IL-1β, IL-18 is first synthesised as a bioinactive propeptide, requiring the IL-1β converting enzyme (ICE) for cleavage into a bioactive 18-kDa molecule (Gu *et al.*, 1997). IL-18 shares many biological properties with IL-12 such as stimulation of IFN-γ production, enhancement of natural killer (NK) cell cytotoxicity and stimulation of Th1 cell differentiation, and is produced by a variety of different cell types including macrophages and T-cells. Although IL-18 is a stimulator of the induction of IFN-γ

production by T-cells, IL-12 is a prerequisite to exert the action of IL-18 on these cells (Okamura *et al.*, 1995b, Ushio *et al.*, 1996, Okamura *et al.*, 1998, Micallef *et al.*, 1996, Ahn *et al.*, 1997). This is because naïve T-cells do not express IL-18 receptor (IL-18R) but only begin to express IL-18R after IL-12 stimulation. (Akira *et al.*, 2000). Thus the function of IL-18 on T-cells completely depends on the presence of IL-12. IL-18 has been shown to play a role in the generation of protective immunity to a number of organisms including *Mycobacterium tuberculosis, Yersinia enterocolitica* (a gram negative bacterium which causes enteritis and enterocolitis), *Leishmania major* and *Salmonella typhimurium* (Sugawara *et al.*, 1999, Bohn *et al.*, 1998, Ohkusu *et al.*, 2000, Mastroeni *et al.*, 1999). Since Th1 cells play a pivotal role in host defence against infection with *B. pertussis*, we investigated whether these two major cytokines involved in Th1 induction, namely IL-12 and IL-18, play a role in the mechanism of protective immunity.

5.2 Results

5.2.1 *B. pertussis* induces IL-12 and IL-18 production from macrophages *in vitro*

It has previously been reported that murine macrophages produce IL-12 after stimulation with live or killed *B. pertussis* (Mahon *et al* ., 1996). The results of this study are in agreement with these findings. *B. pertussis* induced the production of IL-12 from J774 macrophages *in vitro*. J774 cells were incubated with killed *B. pertussis* in the range of $1x10^5$ CFU/ml to $1x10^8$ CFU/ml, and IL-12 levels measured after 24hrs. J774 macrophages produced high levels of IL-12 in response to stimulation with heat-killed *B. pertussis* in a dose dependent manner (Fig. 5.1).

To examine whether *B. pertussis* could induce production of IL-18 a modification of the technique employed by Mastroeni *et al.*, 1999 was utilized. A spleen cell suspension was prepared from naïve mice and incubated for 4hrs in petri dishes to allow macrophages to adhere. To assess whether IL-18 acts via IFN-γ induction, adherent cells were washed once and cultured overnight with medium alone or in the presence of IFN-γ. The cells were then infected with *B. pertussis* at a bacteria to cell ratio of 5:1 (0 hrs) and supernatants were collected for measurement of IL-18, for up to 4 hours post-infection. *B. pertussis* infection induced the release of high levels of IL-18 (Fig. 5.2) from both untreated (no IFN-γ) and treated splenic macrophages 1h after infection and IL-18 levels remained high throughout the course of the experiment. Treatment with IFN-γ significantly enhanced IL-18 production (Fig. 5.2).

5.2.2 Blocking IL-12 *in vitro* abrogates *B. pertussis*-induced IFN-γ production

It has previously been reported that murine macrophages produce IL-12 after stimulation with live or killed *B. pertussis* (Mahon *et al.*, 1996). Therefore, it was important to assertain whether the generation of IL-12 was responsible for stimulating the production of *B. pertussis*-induced IFN-γ. Spleen cells from naïve mice were prepared and stimulated with a range of concentrations of killed *B. pertussis* alone, or together with either a neutralizing anti-IL-12 or anti-IL-18

antibody. Following incubation for 72hrs, supernatants were removed and IFN-γ levels assessed. Spleen cells incubated with *B. pertussis* alone produce high levels of IFN-γ (Fig. 5.3A). However, following stimulation of cells with *B. pertussis* in the presence of a neutralizing anti-IL-12 antibody, IFN-γ production is completely abrogated (Fig. 5.3A). In contrast, IFN-γ production in response to PMA/CD3 was not affected by the presence of anti-IL-12 antibody. However, stimulation of spleen cells with *B. pertussis* in the presence of anti-IL-18 did not have downregulatory effect IFN-γ production induced by killed bacteria (Fig. 5.3B), but when added simultaneously with anti-IL-12, diminished IFN-γ production significantly (Fig. 5.3C). The results show that at least *in vitro* that *B. pertussis* induced IFN-γ production is dependent on IL-12.

5.2.3 Decreased Th1 and enhanced Th2 cytokine production in IL-12-deficient mice

Cytokine production by spleen cells from naïve mice was used to establish the Th1/Th2 profile of IL-12 deficient (IL-12$^{-/-}$) mice. Spleens from naïve IL-12$^{-/-}$ and wild-type BALB/c mice were prepared and stimulated with the polyclonal stimulator PMA and anti-CD3 or medium only. Following incubation for 72hrs supernatants were removed and assayed for the presence of IFN-γ and IL-4. IL-12$^{-/-}$ mice produced significantly less IFN-γ compared to wild-type mice, but they also produce significantly higher levels of IL-4 (Fig. 5.4).

Given that neutralizing IL-12 has the ability to block *B. pertussis* IFN-γ production *in vitro*, it was important to determine the *in vivo* function of IL-12 in resistance to *B. pertussis* infection. IL-12 defective mice were used to examine the role of IL-12 in shaping T-cell responses to *B. pertussis* and on the outcome of infection. IL-12$^{-/-}$ and wild-type BALB/c mice were infected with *B. pertussis* by aerosol inoculation. Spleen cells from IL-12$^{-/-}$ and wild-type BALB/c mice were restimulated in vitro with medium or PMA and anti-CD3 as negative and positive controls respectively, and killed *B. pertussis* in the range of $1x10^5$ to $1x10^7$ CFU/ml. Supernatants from 72hr. cultures were collected on days 14, 21, 63 and 70 post-challenge and analyzed for presence of IFN-γ and IL-5. An examination of T-cell

responses revealed that infection of BALB/c mice with *B. pertussis* induced a predominantly Th1 response with high levels of IFN-γ and little IL-5 (Fig. 5.5). IL-12$^{-/-}$ mice display a significant reduction in IFN-γ production from spleen cells at all timepoints tested. IL-5 levels in knockout mice are slightly higher compared to wild-type mice (Fig. 5.5). Although, there is no evidence of a complete switch from a Th1 response to a Th2 response in these mice, there was a consistent move towards a more mixed Th1/Th2 response. Groups of four mice were sacrificed at intervals post-challenge to estimate the numbers of viable bacteria in the lungs. Analysis of bacterial counts from the lungs of wild-type and knockout mice, during the course of primary infection with *B. pertussis*, show that there are significantly higher numbers of viable bacteria in the lungs of IL-12$^{-/-}$ mice 7 days post-challenge compared with wild-type mice (Fig. 5.6). However, later in infection there is no significant difference between the two groups, and IL-12$^{-/-}$ mice maintain the ability to clear the bacteria from the lungs as efficiently as wild-type mice (Fig. 5.6). Since it has previously been shown that IFN-γ receptor defective mice have reduced IFN-γ production and develop a disseminated infection (Mahon *et al.*, 1997), the blood and livers of wild-type and IL-12$^{-/-}$ mice were assessed for the presence of *viable B. pertussis* bacteria. However, *B. pertussis* was not detected in any other organ other than the lung.

5.2.4 The role of IL-12 in bacterial clearance and T-cell responses during secondary infection with *B. pertussis*

In a separate experiment, the role of IL-12 in secondary infection with *B. pertussis* was examined. IL-12$^{-/-}$ and wild-type BALB/c mice were infected with *B. pertussis* and allowed to recover from infection. Ten weeks after primary challenge, both groups of mice were rechallenged with *B. pertussis*. Groups of four mice were sacrificed at intervals post-challenge to estimate the numbers of viable bacteria in the lungs. Splenic T-cell responses were also assessed after restimulation *in vitro* with *B. pertussis* antigens. Spleen cells from IL-12$^{-/-}$ and wild-type BALB/c mice were restimulated *in vitro* with medium or PMA and anti-CD3 as negative and positive controls respectively, *B. pertussis* sonicate at 1 and 5μg/ml, PT (2μg/ml), FHA (5μg/ml) and PRN (5μg/ml). Spleen cell IFN-γ and IL-5 production was

assessed on the day of challenge (Day 0) and 2 weeks post-challenge. Examination of T-cell cytokine responses showed that rechallenge of IL-12$^{-/-}$ and wild-type BALB/c mice previously infected with *B. pertussis*, induced a mixed Th1/Th2 response with high levels of IFN-γ and IL-5 production in response to bacterial antigens (Fig. 5.7). Spleen cells from IL-12$^{-/-}$ mice secreted significantly lower amounts of IFN-γ compared to wild-type mice, which is similar to what is seen at day 70 after primary infection. However, knockout mice still displayed a mixed response, producing both Th1 and Th2 cytokines (Fig. 5.7). 14 days after secondary challenge, IFN-γ production from spleen cells of knockout mice is also significantly reduced. The complete clearance of bacteria after secondary infection was delayed in IL-12$^{-/-}$ mice (Fig. 5.8). However, the difference in CFU counts between knockout and wild-type mice was not statistically significant.

5.2.5 IL-18 IS RAPIDLY INDUCED IN THE LUNG FOLLOWING RESPIRATORY CHALLENGE WITH *B. PERTUSSIS*

In order to determine if IL-18 was induced at the site of infection, lungs from *B. pertussis* infected wild-type BALB/c and IL-12$^{-/-}$ mice were taken at 4 hours, and at intervals up to 70 days post-challenge, and IL-18 levels in lung homogenates were determined by ELISA. The results show that IL-18 is induced within 4 hours of infection. The most potent IL-18 induction is observed early in infection and significantly higher levels were detected in IL-12$^{-/-}$ mice at day 7 post-challenge. Similar levels of IL-18 in both wild-type and IL-12$^{-/-}$ mice were seen at all other timepoints (Fig. 5.9). IL-18 levels begin to decline after day 7 and reach levels similar to those observed in uninfected control mice by day 70 post-infection.

5.2.6 THE ROLE OF IL-12 IN BACTERIAL CLEARANCE AND T-CELL RESPONSES IN PA AND PW- IMMUNIZED MICE

It has already been demonstrated that immunization with Pw, like natural infection, induces Th1 cells, whereas Pa appear to protect by inducing antibody and Th2 cells (Mills *et al.*, 1993, Mills *et al.*, 1998a, Redhead *et al.*, 1993). Therefore, the role of IL-12 in the induction of vaccine-induced immunity was investigated. The development of systemic cell-mediated immune responses in *B. pertussis*-infected

animals that had been previously immunized with Pa or Pw were evaluated. Wild-type BALB/c or IL-12$^{-/-}$ mice were immunized with Pa or Pw and boosted 4 weeks later. Two weeks after the second immunization, mice were challenged with *B. pertussis*. T-cell responses in immune wild-type and gene-disrupted mice were assessed on the day of challenge, and on days 7 and 14 post-challenge. Pw-immunized IL-12$^{-/-}$ and wild-type mice exhibited a polarized Th1 response with high levels of IFN-γ and little or no IL-5 in response to killed *B. pertussis*, PT, FHA and PRN (Fig. 5.11). Compared with wild-type mice, there was a reduction in antigen-specific IFN-γ levels in Pw-immunized IL-12$^{-/-}$ mice at all timepoints tested, with a significant decrease at day 7 to killed bacteria and PT, and to FHA at day 14. A concomitant increase in IL-5 was not observed (Fig. 5.11). Immunization with Pa in knockout and wild type mice induced a T-cell response polarized to the Th2 subtype, with low levels of IFN-γ and high levels of IL-5 (Fig. 5.12).

An examination of bacterial clearance from the lungs of these mice revealed no significant difference between the numbers of bacteria recovered from the lungs of Pw-immunized IL-12$^{-/-}$ and wild-type mice (Fig. 5.10). This was particularly surprising for Pw-immunized mice, given that Pw selectively induces Th1 cells (Redhead *et al.*, 1993) and is associated with a cytokine profile of high levels of IFN-γ production and little IL-4 or IL-5. It might be predicted that Pw-immunized IL-12$^{-/-}$ mice would display impaired protection or somewhat protracted clearance of the bacteria from the lungs, especially when *B. pertussis*-specific IFN-γ production was reduced in these mice. Following immunization with Pa, bacterial clearance from the lungs remains unaffected in IL-12$^{-/-}$ mice (Fig. 5.10).

5.3 Discussion

Resistance or susceptibility to infection is strongly determined by the pattern of cytokines produced in response to the infection (Scott *et al.*, 1991). The proinflammatory cytokine IL-12 is mandatory for the induction of IFN-γ production, and is important in anti-microbial defense (Trinchieri, 1995, Trinchieri, 1994, Locksley, 1993, Jouanguy *et al.*, 1999). In a murine respiratory challenge model of infection with *B. pertussis*, it has been demonstrated that mice with a defective IFN-γ receptor (IFN-γR$^{-/-}$) gene, develop an atypical disseminated disease which is lethal in a proportion of animals (Mahon *et al.*, 1997). In comparison with wild-type 129 Sv/Ev mice that resolved the infection, gene-disrupted mice showed abnormal pathology characterized by pyogranulomatous inflammation and postnecrotic scarring in the livers, mesenteric lymph nodes and kidneys. Furthermore, viable virulent bacteria were detected in the blood and livers of diseased animals. In another study it was reported that murine macrophages produce IL-12 after stimulation with live or killed *B. pertussis* (Mahon *et al.*, 1996).

In this study, it was demonstrated that IL-12 and IL-18 production is induced *in vitro* following stimulation with *B. pertussis*. IL-12 was readily produced at high levels from J774 macrophage stimulated with killed *B. pertussis*. Furthermore, IL-18 production was precipitately induced from splenic macrophages isolated from naïve mice and subsequently infected with live *B. pertussis*. The addition of a neutralizing anti-IL-12 antibody (or a combination of anti-IL-12 and anti-IL-18) to a culture of spleen cells from naïve mice stimulated with *B. pertussis*, completely abrogated pertussis-induced IFN-γ production. However, addition of anti-IL-18 to a similar culture did not have any effect on *B. pertussis*-induced IFN-γ, indicating that at least *in vitro* IFN-γ is induced via IL-12.

Endogenous IL-12 has been shown to be necessary for IFN-γ production, and for the establishment of a Th1-type response after infection with *Listeria*, *Leishmania*, *Toxoplasma gondii* and *Candida albicans* (Scharton-Kersten *et al.*, 1995, Mattner *et al.*, 1996, Gazzinelli *et al.*, 1993, Mencacci *et al.*, 1998). IL-18 has also been shown to be important in the induction of Th1 responses, through its synergistic action with IL-12 (Ahn *et al.*, 1997). In other systems, lack of IL-12 function results in a failure to direct immune responses towards Th1 responsiveness,

and to produce a cytokine profile characteristic of a Th2 response (Seder *et al.*, 1993). Indeed, stimulation of naïve spleen cells from IL-12$^{-/-}$ mice with the mitogen PMA/CD3 yielded significantly reduced IFN-γ and increased levels of IL-4 in 72hr culture supernatants, when compared with responses from wild-type animals that readily produced high levels of IFN-γ and minimal IL-4.

In this study, the *in vivo* role of IL-12 in *B. pertussis* infection was examined, to determine whether IL-12 deficiency leads to a shift from the normally observed Th1 response to *B. pertussis*, to a more polarized Th2 response. It was important to determine the significance of IL-12 in the regulation of anti-bacterial cytokine expression, particularly when IFN-γ has such an important function in the induction of Th1 cells and in confining the bacteria to the respiratory tract (Barbic *et al.*, 1997, Mahon *et al.*, 1997). The responses of IL-12$^{-/-}$ mice infected with *B. pertussis* were examined, and compared with the responses of wild-type BALB/c mice. In primary infection of naïve mice with *B. pertussis*, a shift from a polarized Th1 response toward a mixed Th1/Th2 response was observed in IL-12$^{-/-}$ mice. IFN-γ production was significantly reduced and IL-5 levels elevated in response to killed *B. pertussis* in knockout mice. Bacterial titres in the lung were significantly elevated in IL-12$^{-/-}$ mice early in infection (day 7), compared to wild-type mice. However, these mice still maintained the ability to eventually clear the bacteria from the lungs with the same efficiency as wild-type mice. Furthermore, IL-12$^{-/-}$ mice did not exhibit an atypical disease pattern as was previously observed in IFN-γR$^{-/-}$ mice. Blood and livers from IL-12$^{-/-}$ infected mice were tested for the presence of viable virulent bacteria and revealed that the bacteria still remained confined to the respiratory tract and there was no dissemination of infection.

IL-18 was found to be rapidly induced in the lungs of both wild-type and IL-12$^{-/-}$ mice, just hours after respiratory challenge. The reduction in IFN-γ, together with the elevated numbers of bacteria in the lungs of IL-12$^{-/-}$ mice at day 7 post-challenge, coincides with an increase in IL-18. A possible explanation for this is that in the absence of IL-12 early in infection, an alternative pathway of IFN-γ induction comes into play, and this is mediated by IL-18. Lack of endogenous IL-12 during infection with *B. pertussis*, did not have a significant effect on complete clearance of bacteria from the lungs, as both wild-type and IL-12$^{-/-}$ mice cleared the

bacteria at the same rate. However, IL-12-deficiency results in a significant enhancement of the bacterial load in knockout mice at the early stages of primary infection. Studies in IFN-γ $R^{-/-}$ mice have shown that dissemination of infection is observed prior to the development of acquired immune responses (Mahon *et al.*, 1997), suggesting that innate immunity plays a role in limiting early infection in the respiratory tract. IL-12-induced IFN-γ, secreted by NK cells and γδ T-cells may help to contain the infection to the respiratory tract prior to the development of specific T-cell responses. Hence, the observation that bacterial load is elevated early in infection in knockout mice, may therefore reflect the influence of IL-12 on IFN-γ production by innate cells, rather than T-cells.

Similarly, IL-12 appears to play a role in the induction of a protective Th1 response during a secondary infection with *B. pertussis*, with a significant reduction in bacterial specific IFN-γ production observed in knockout mice on the day of challenge, and on day 14 post-challenge. This decrease in IFN-γ production may have affected complete clearance of the bacteria from the lungs during secondary infection with *B. pertussis*, which was slightly, but not significantly protracted in knockout mice. Spleen cells from wild-type and IL-$12^{-/-}$ mice consistently secreted both IFN-γ and IL-5, indicating a mixed Th1/Th2 response. Although, natural infection with *B. pertussis* normally induces a polarized Th1 response in mice and children (Mills *et al.*, 1993, Ryan *et al.*, 1997, Mahon *et al.*, 1997), it has been demonstrated that a Th2 response can also be protective, although this is mediated through a distinct mechanism (Mills *et al.*, 1998a, Mahon *et al.*, 1996). This may explain why the lack of IL-12-induced IFN-γ production did not impact the course of infection to a significant extent.

In Pa- and Pw-immunized IL-$12^{-/-}$ and wild-type mice, which were subsequently infected with *B. pertussis*, lack of functional IL-12 mice had no effect on protection against infection. Knockout mice immunized with Pa or Pw cleared the infection within the same time span as wild-type mice, and did not have significantly higher numbers of bacteria in the lungs. Pa-immunized wild-type mice exhibited a polarized Th2 response, and this response was unaffected in knockout mice. A decrease in IFN-γ levels was observed in Pw-immunized mice lacking IL-

12, without a significant concomitant increase in the levels of the Th2 cytokine IL-5. Previous studies have demonstrated that immunization with Pw induces a protective Th1 response in mice (Redhead *et al.*, 1993), and an exacerbated disseminated disease in IFN-γR-deficient mice (Mahon *et al.*, 1997). Therefore, the undiminished anti-bacterial resistance of Pw-immunized mice lacking IL-12, is rather surprising in view of the significant decrease in the Th1 response in these mice, and the critical role for IL-12 in the induction of IFN-γ and Th1 development. It has been demonstrated that immunization with Pa induces Th2 cytokine profile in mice (Mills *et al.*, 1998a), and addition of IL-12 to Pa, increases its protective efficacy through the induction of Th1 cells (Mahon *et al.*, 1996). Furthermore, stimulation of macrophages with FHA, detoxified PT and PRN (the components of Pa) does not induce IL-12 production (Mahon *et al.*, 1996). Therefore, the observation that Pa-immunized IL-12$^{-/-}$ mice do not display increased susceptibility to *B. pertussis* infection is not so surprising, as it appears that IL-12 does not play a major role in protection in this system.

Lack of IL-12 in this system appears only to effect protection against *B. pertussis* early in infection. This suggests that IL-12 may play a role in confining the bacteria to the respiratory tract through the induction of IFN-γ production by innate cells, during the early stages of infection. Furthermore, these data indicate that during *B. pertussis* infection, the ability of CD4^{+} T-cells to develop into Th1 type cells is diminished, in the absence of endogenous IL-12. The fact that the Th1 response is not completely abrogated in knockout mice, suggests that alternative cytokines or host factors may compensate for the lack of endogenous IL-12. The observation of an IL-12 redundance for IFN-γ production supports Schijns et al. 1998, who reported that IL-12 is not essential for the generation of a Th1 response for the control of Coronavirus-induced acute hepatitis. In another study, it was shown that *in vivo* alloreactive Th1 development may occur independent of IL-12 (Piccotti *et al.*, 1998). Piccotti and colleagues also suggested that in their system IL-18 may act as an alternative sensitizing pathway *in vivo*. So, in the absence of IL-12, we suggest from our observations that an alternate IFN-γ-inducing pathway may occur *in vivo* which is mediated by IL-18.

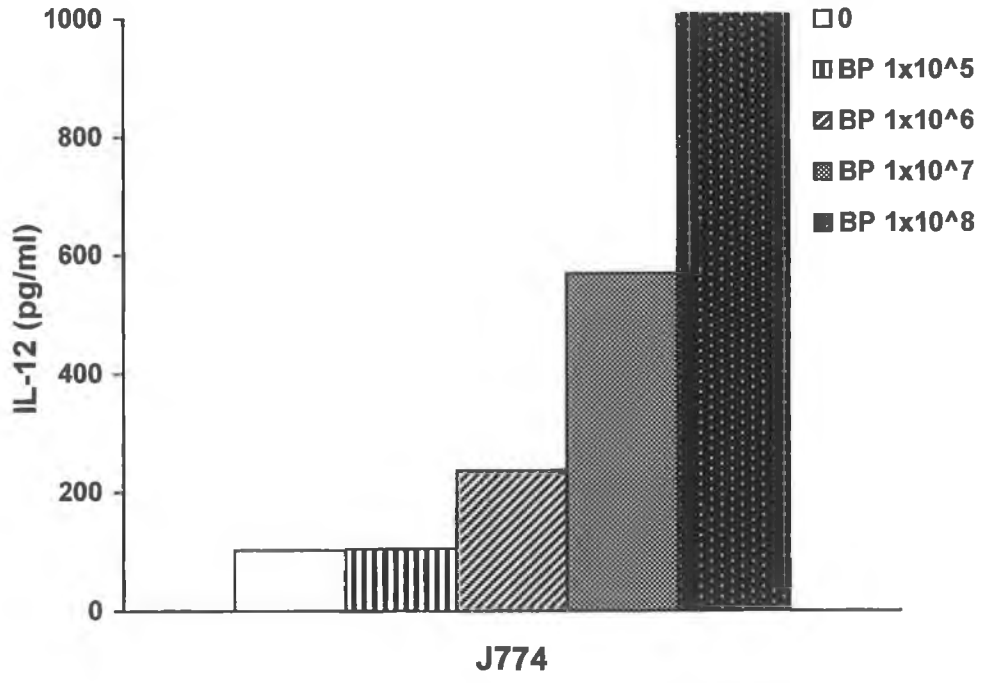


Figure 5.1

J774 Macrophages produce IL-12 in response to heat-killed *B. pertussis*. Macrophages were incubated with increasing doses of *B. pertussis* (10^5 to 10^8 CFU/ml) for 24 hours, after which supernatants were collected and assessed for IL-12 p40 by immunoassay. Results are expressed as the mean concentration of IL-12 from triplicate cultures.

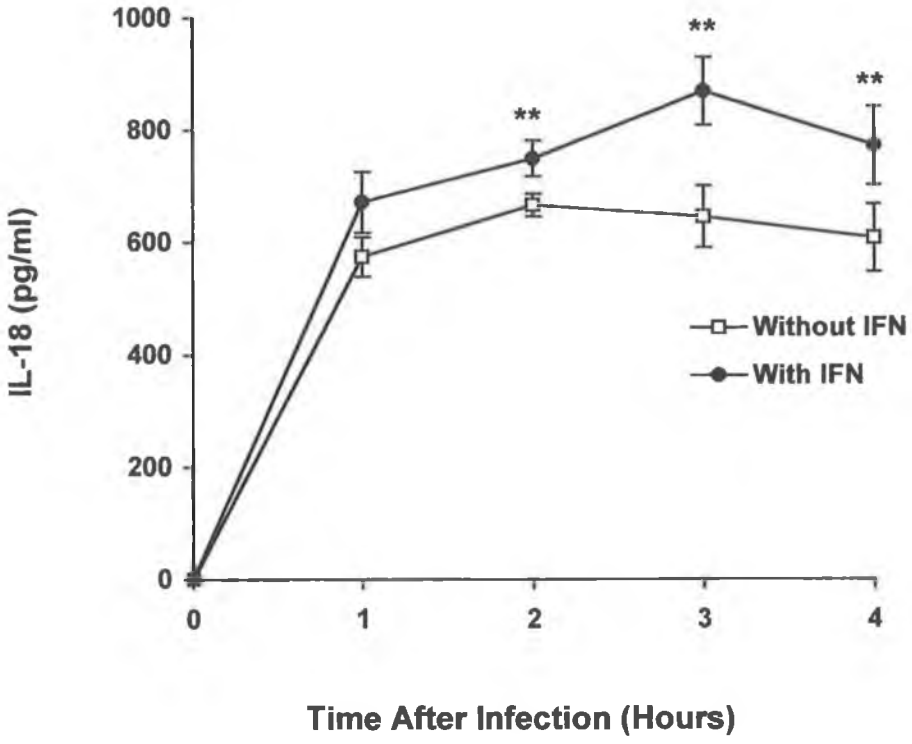


Figure 5.2

Splenic macrophages produce IL-18 in response to live *B. pertussis*. Macrophages were isolated from the spleens of naïve mice and incubated for 24 hours with (●) or without (□) rmIFN-γ. The cells were then infected with *B. pertussis* (0hrs) at a bacteria to cell ratio of 5:1. Supernatants were collected every hour for 4 hours post-infection, and IL-18 levels assessed by ELISA. Results are expressed as the mean (±SE) concentration of IL-18 from triplicate cultures. **, $P < 0.01$ versus cells stimulated with IFN-γ.

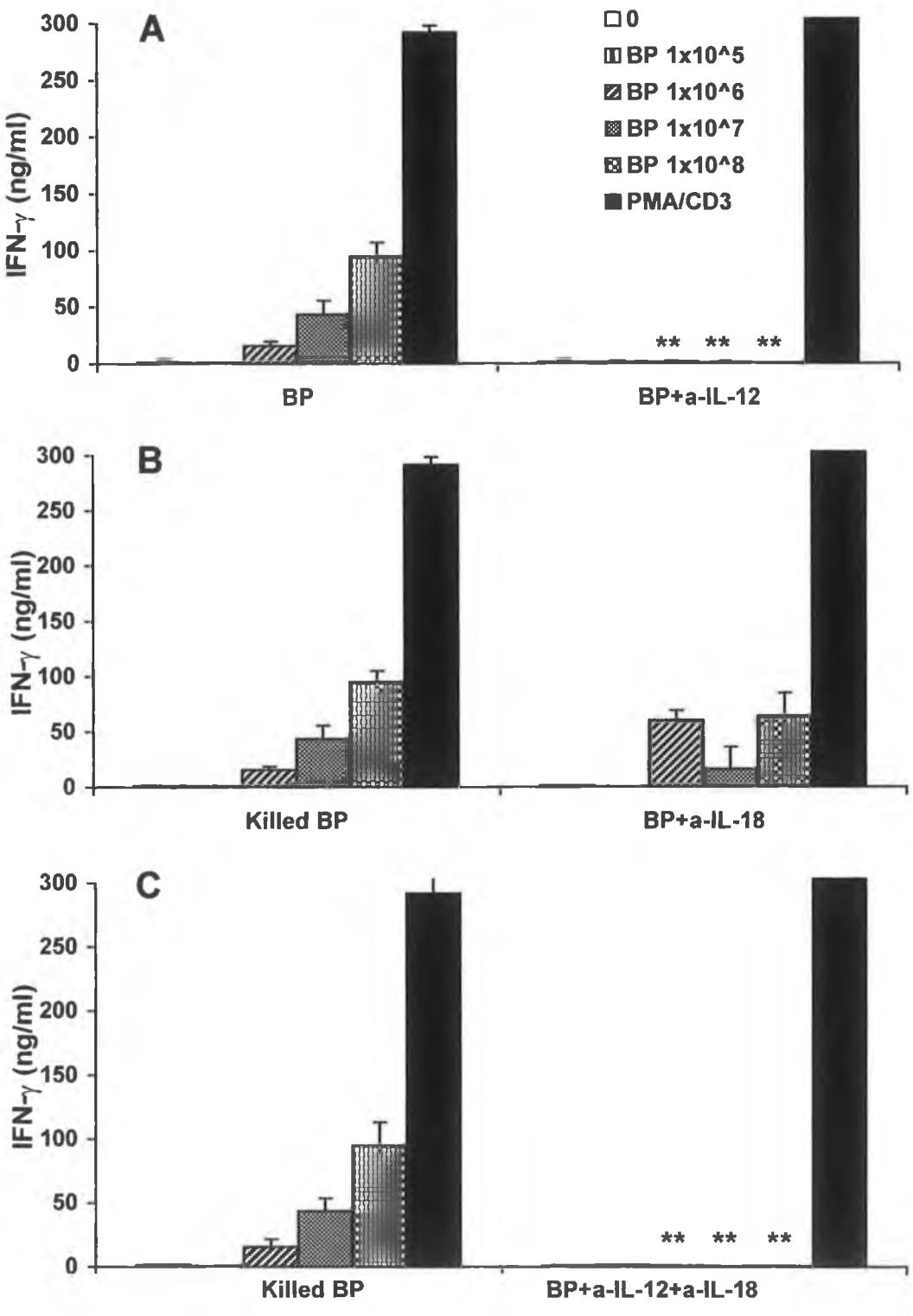


Figure 5.3

B. pertussis-induced IFN-γ is blocked by neutralizing anti-IL-12 *in vitro*. Spleen cells from naïve mice were stimulated *in vitro* with killed *B. pertussis* (BP) only, ($1x10^5$ to $1x10^8$ CFU/ml), or in the presence of a neutralizing anti-IL-12 antibody (A), neutralizing IL-18 (B), or a combination of both anti-IL-12 and anti-IL-18 (C). Supernatants were removed after 72hrs and IFN-γ levels measured by ELISA.

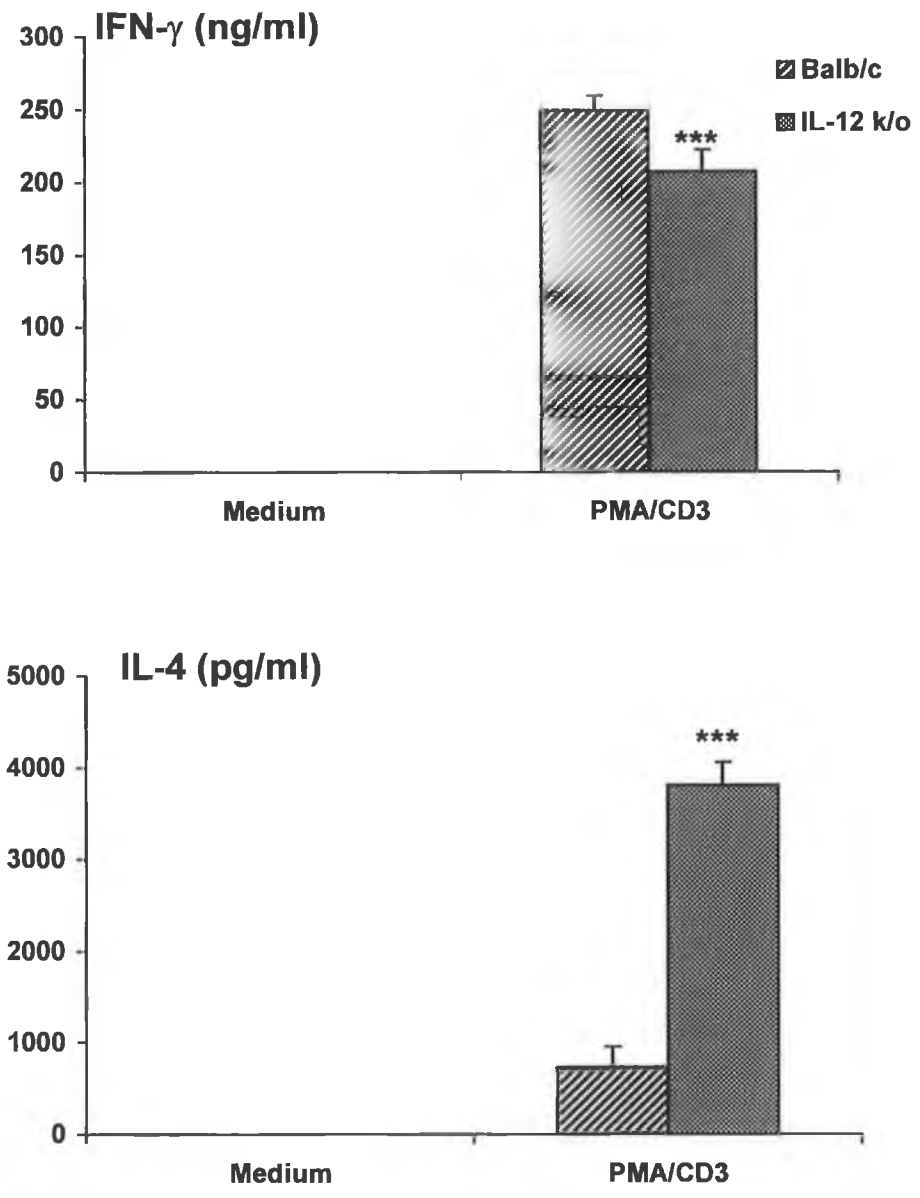
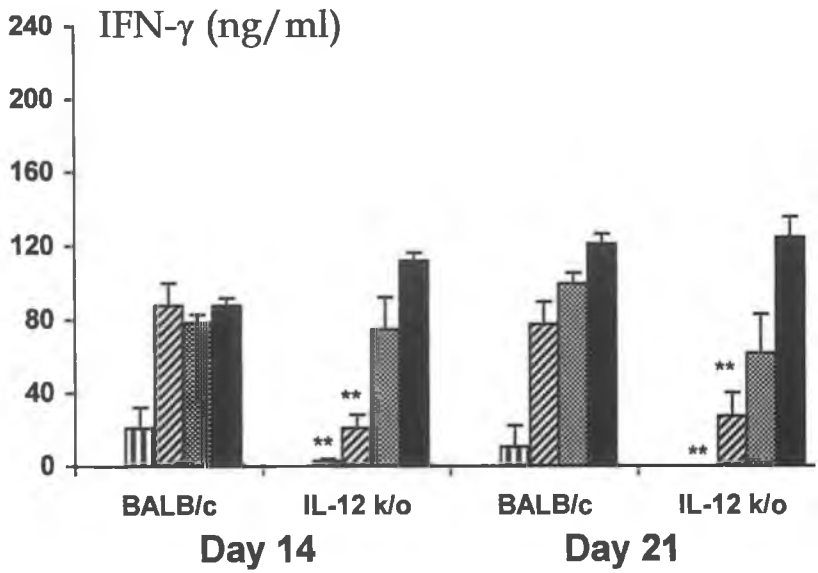
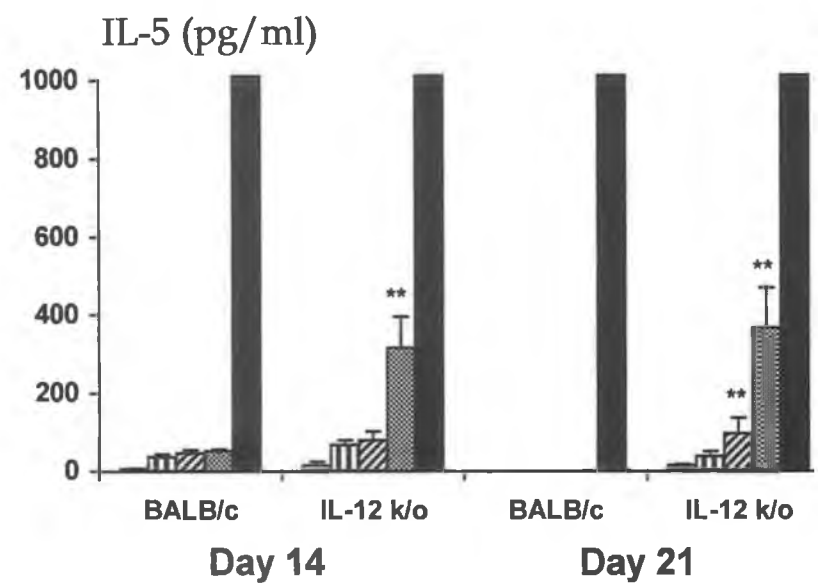
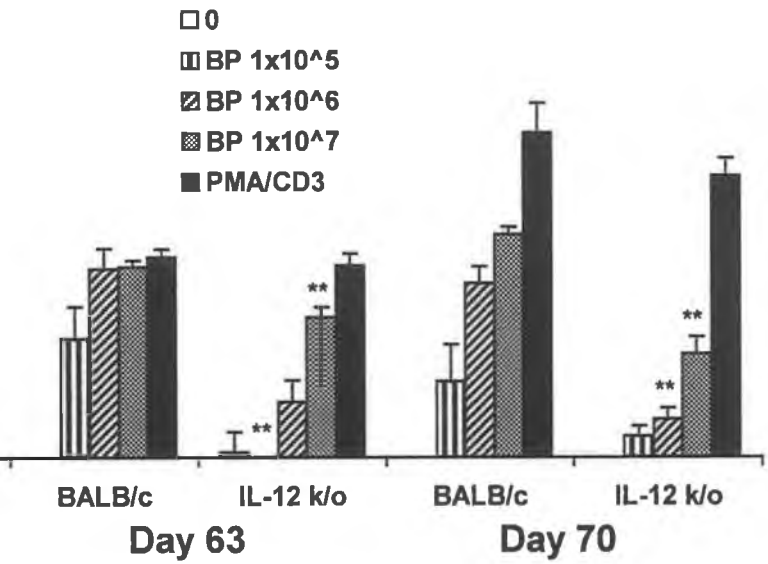


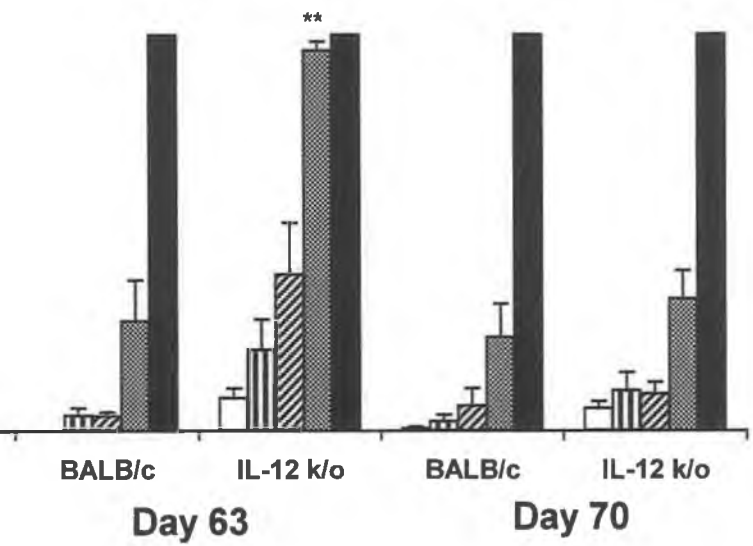
Figure 5.4

Decreased Th1 and enhanced Th2 cytokine production in IL-12-deficient mice. Spleen cells were isolated from naïve IL-12$^{-/-}$ and wild-type BALB/c mice, and stimulated with PMA and anti-CD3 or medium only. Supernatants were removed after 72 hours and assessed for IFN-γ and IL-4. Results are the mean (±SE) cytokine concentrations for four mice per group assayed in triplicate. ***, $P < 0.001$ versus wild-type mice.


IFN-γ (ng/ml)
240
200
160
120
80
40
0
**
**
**
**
BALB/c
IL-12 k/o
BALB/c
IL-12 k/o
Day 14
Day 21


IL-5 (pg/ml)
1000
800
600
400
200
0
**
**
**
BALB/c
IL-12 k/o
BALB/c
IL-12 k/o
Day 14
Day 21


0
BP 1x10^5
BP 1x10^6
BP 1x10^7
PMA/CD3
**
**
**
**
BALB/c
IL-12 k/o
BALB/c
IL-12 k/o
Day 63
Day 70


**
BALB/c
IL-12 k/o
BALB/c
IL-12 k/o
Day 63
Day 70

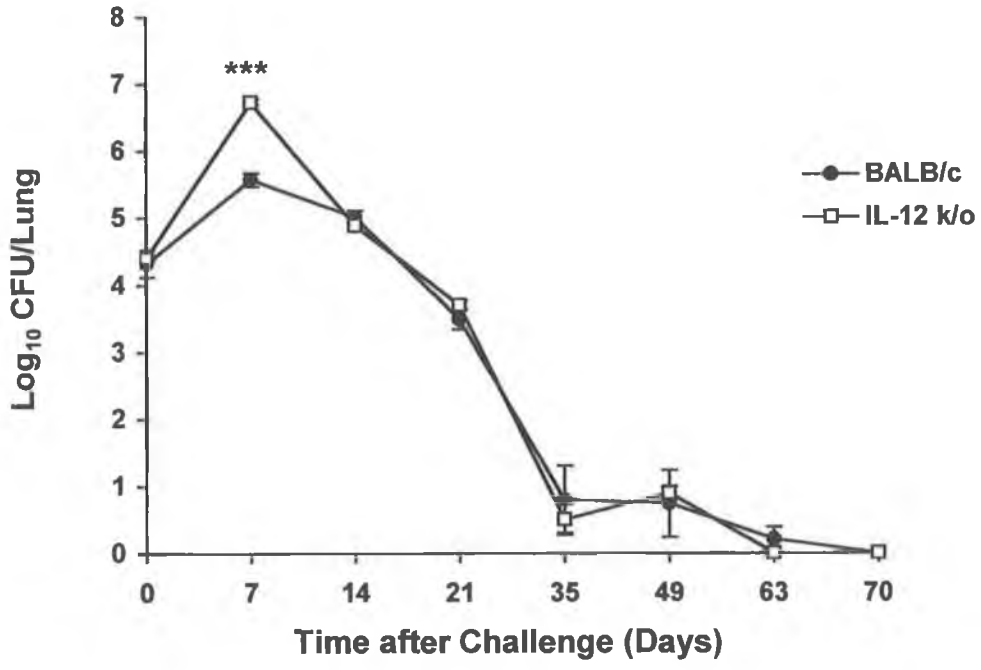
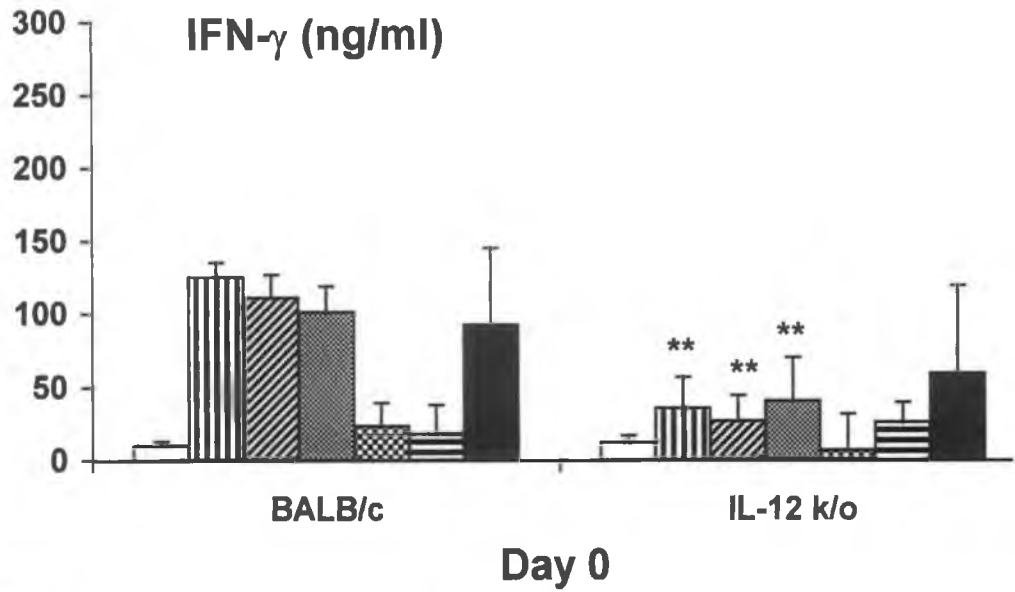
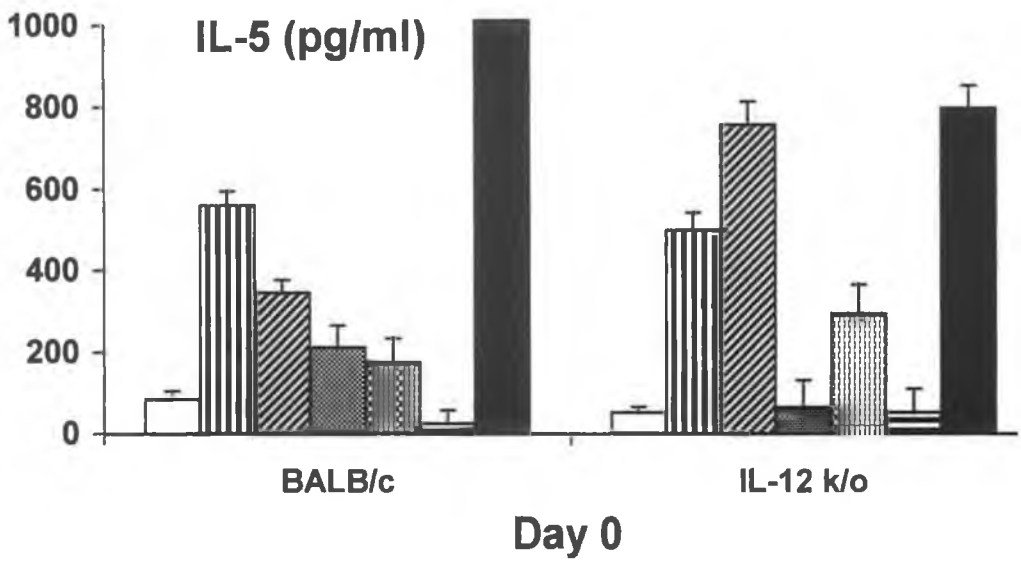
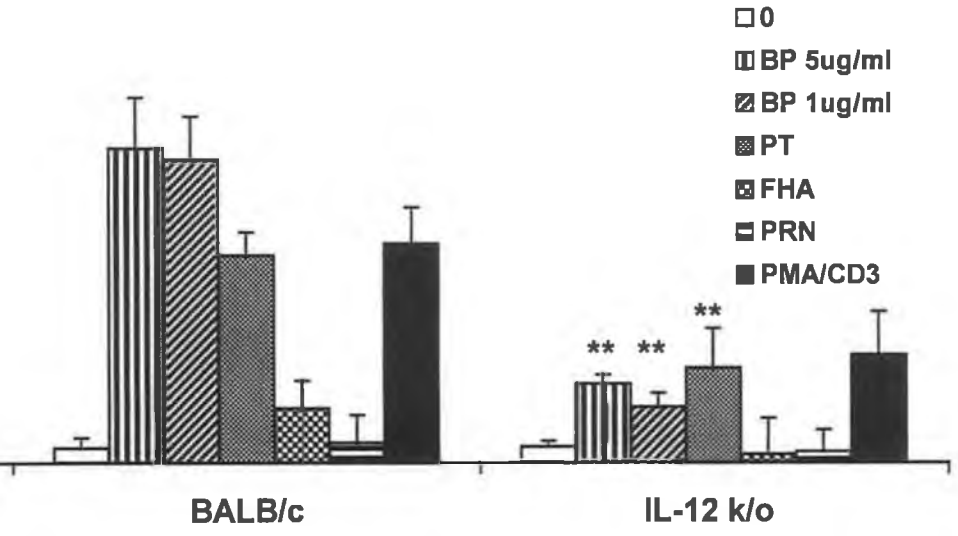


Figure 5.6

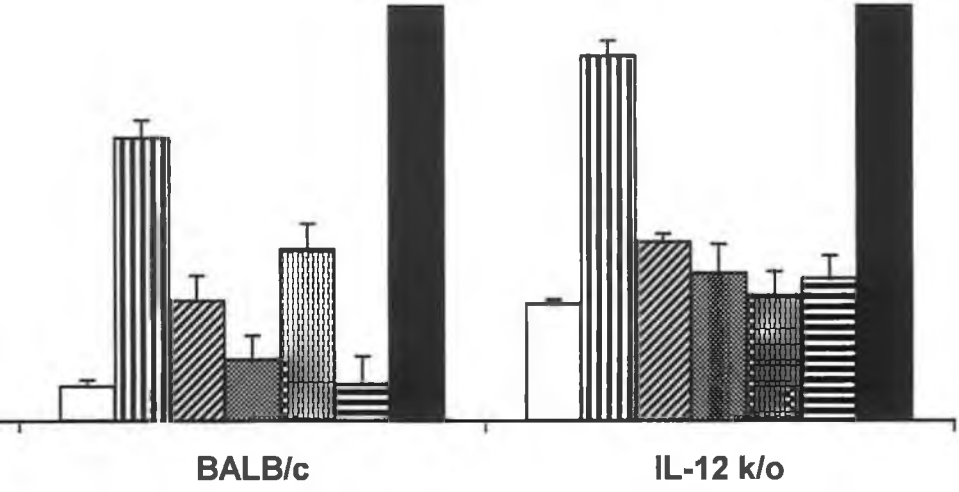
Clearance of *B. pertussis* from the lungs of knockout and wild-type mice during primary challenge. Both IL-12$^{-/-}$ (□) and W. T BALB/c mice (●) were infected with *B. pertussis* by aerosol challenge. Lungs were removed at intervals post-challenge and numbers of viable bacteria assessed. Results represent mean (± SE) CFU per lung for four mice per time point.


IFN-γ (ng/ml)
300
250
200
150
100
50
0
**
**
**
BALB/c
IL-12 k/o
Day 0


IL-5 (pg/ml)
1000
800
600
400
200
0
BALB/c
IL-12 k/o
Day 0


0
BP 5ug/ml
BP 1ug/ml
PT
FHA
PRN
PMA/CD3
**
**
**
BALB/c
IL-12 k/o

Day 14


BALB/c
IL-12 k/o

Day 14

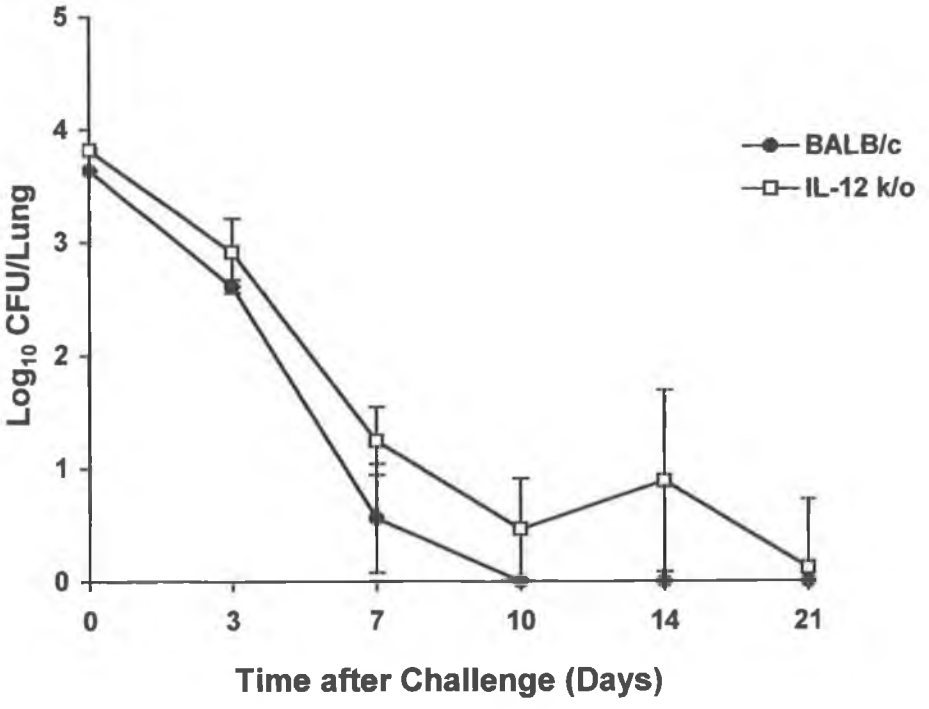


Figure 5.8

Clearance of *B. pertussis* from the lungs of BALB/c and IL-12$^{-/-}$ mice during secondary challenge. Both IL-12$^{-/-}$ (□) and W. T BALB/c mice (●) were infected with *B. pertussis* by aerosol inoculation, and allowed to clear the infection. 10 weeks post-primary challenge, mice were rechallenged with *B. pertussis*. Lungs were removed at intervals post-challenge and numbers of viable bacteria assessed. Results represent mean (± SE) CFU per lung for four mice per time point.

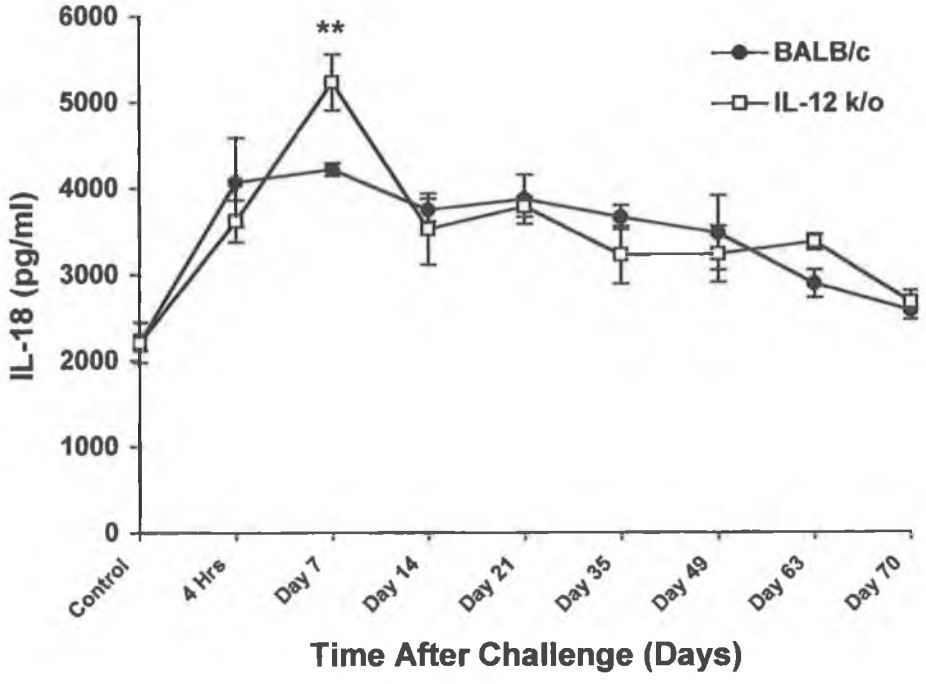


Figure 5.9

IL-18 is rapidly induced in the lung following respiratory challenge with *B. pertussis* and persists throughout the course of infection. Lungs from *B. pertussis* infected wild-type BALB/c (●) and IL-12$^{-/-}$ (□) mice, were taken at intervals post-primary challenge and homogenised. IL-18 levels in lung homogenates were then determined by ELISA. Results are expressed as the mean concentration (±SE) of IL-18 for four mice per experimental group.

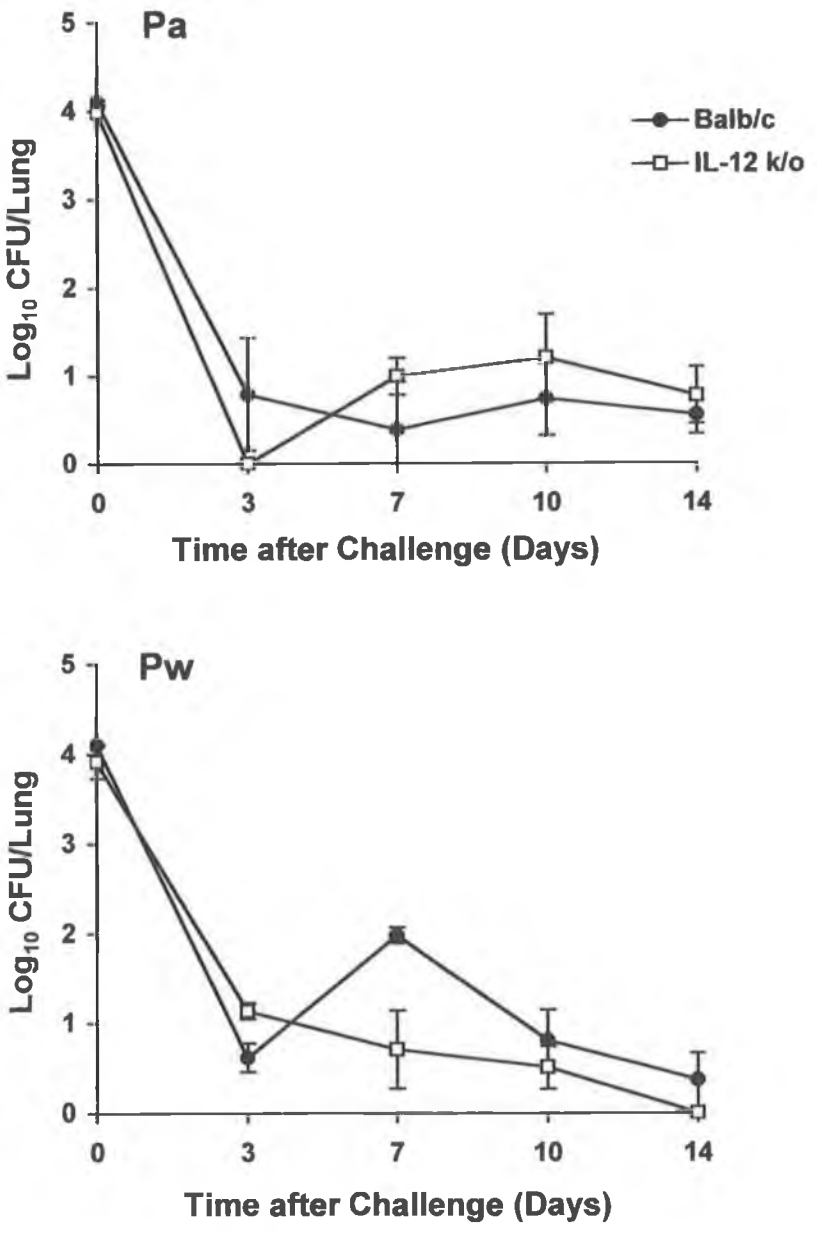
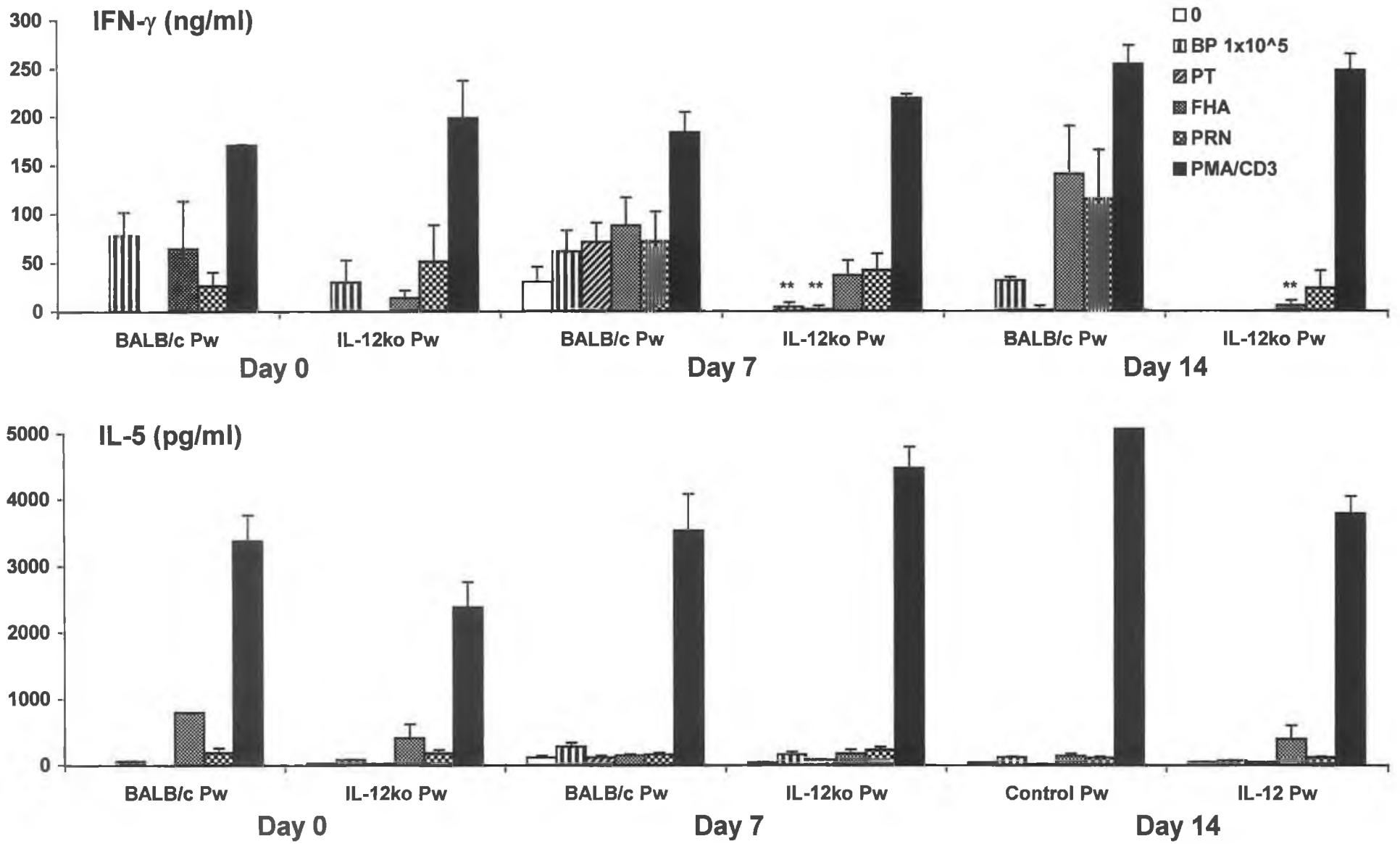


Figure 5.10

Absence of IL-12 does not affect the efficacy of Pa or Pw. Wild-type BALB/c (●) and IL-12$^{-/-}$ (□) mice were immunized with 0.2 Human dose of Pa or Pw and boosted 4 weeks later. Two weeks after secondary immunization, mice were infected with *B. pertussis*. Mice were sacrificed at various times after challenge to assess the numbers of viable bacteria in the lungs. Results are reported as the mean numbers of *B. pertussis* CFU (±SE) for individual lungs from 4 mice per group at each timepoint.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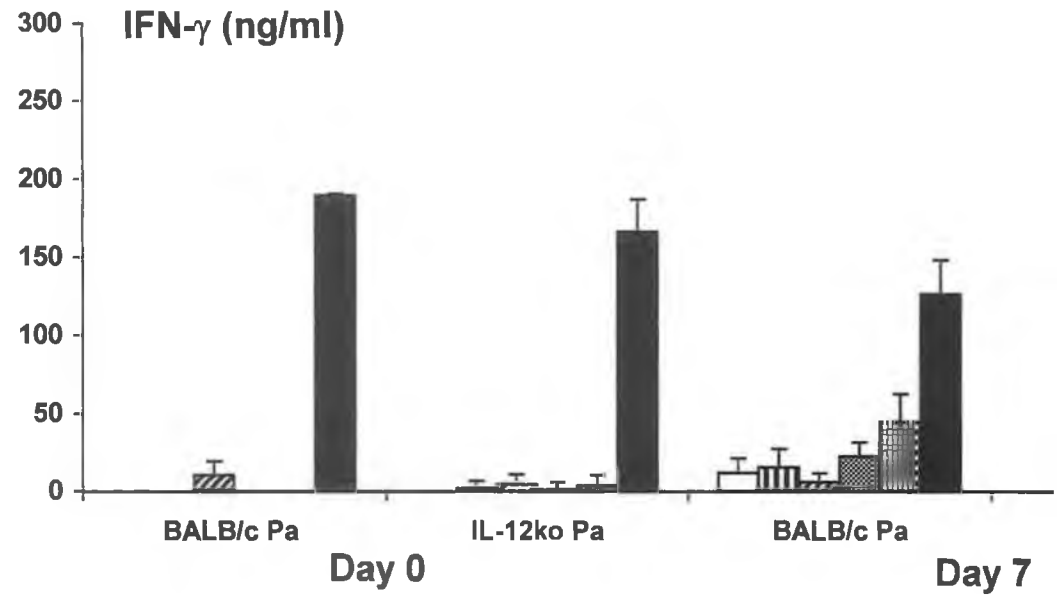
250
200
150
100
50
0
0
BP 1x10^5
PT
FHA
PRN
PMA/CD3
**
**
**
BALB/c Pw
IL-12ko Pw
BALB/c Pw
IL-12ko Pw
BALB/c Pw
IL-12ko Pw
Day 0
Day 7
Day 14
IL-5 (pg/ml)
5000
4000
3000
2000
1000
0
BALB/c Pw
IL-12ko Pw
BALB/c Pw
IL-12ko Pw
Control Pw
IL-12 Pw
Day 0
Day 7
Day 14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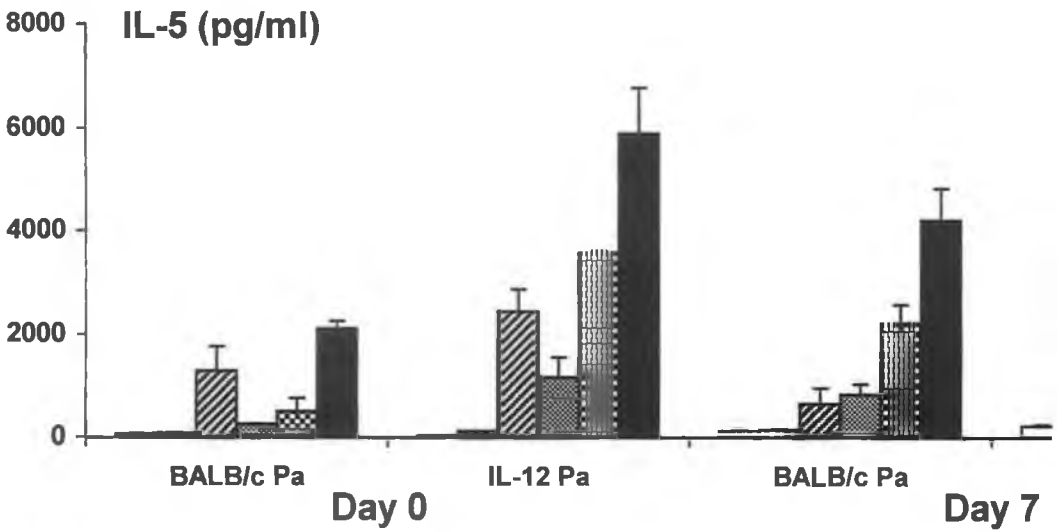
250
200
150
100
50
0
BALB/c Pa
IL-12ko Pa
BALB/c Pa
Day 0
Day 7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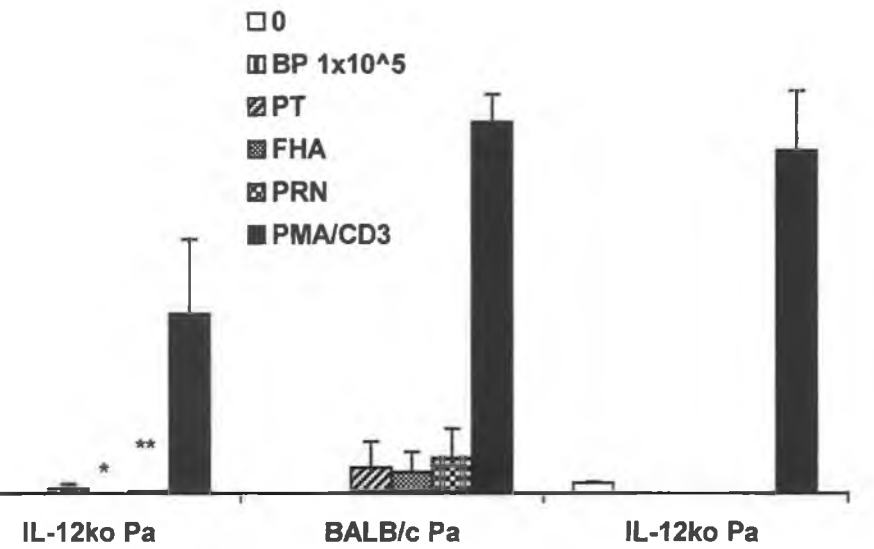
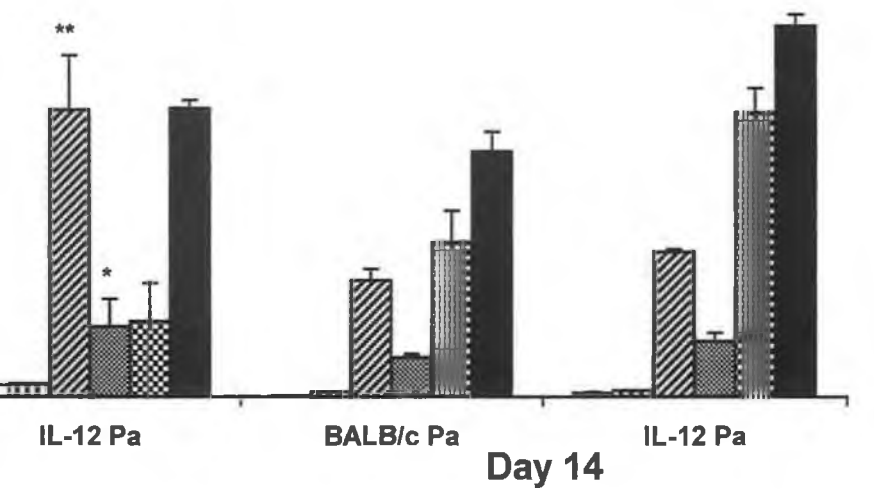
6000
4000
2000
0
BALB/c Pa
IL-12 Pa
BALB/c Pa
Day 0
Day 7


0
BP 1x10^5
PT
FHA
PRN
PMA/CD3
*
**
IL-12ko Pa
BALB/c Pa
IL-12ko Pa
Day 14


**
*
IL-12 Pa
BALB/c Pa
IL-12 Pa
Day 14

❖ CHAPTER 6 ❖

DOWNREGULATION OF A PROTECTIVE TH1 RESPONSE TO *BORDETELLA PERTUSSIS* BY A TH2-INDUCING PARASITE - *FASCIOLA HEPATICA*

6.1 Introduction

The identification of Th1 and Th2 cells has provided a useful model for our understanding the selective induction, polarization and reciprocal regulation of distinct arms of the immune response (Mossman *et al.*, 1986, Abbas *et al.*, 1996). Although immunity to infectious disease involves a complex interplay between both humoral and cellular immune responses against antigens on the infectious agent, $CD4^+$ T-cells appear to be the key regulators of the immune response. Th1 cells are normally induced following infection with intracellular bacteria and viruses, whereas Th2 responses are generated in response to allergens and helminth parasites (Abbas *et al.*, 1996, O'Garra *et al.*, 1994). The early decision to polarize the immune response towards type 1 or type 2 is controlled by a number of factors. Gram negative bacteria and viruses stimulate the production of IL-12 and IL-18 by dendritic cells and macrophages, which favours the induction and expansion of Th1 cells (Trincheiri *et al.*, 1994, Bohn *et al.*, 1998). Conversely, early IL-4 acts as a potent stimulus for Th2 differentiation (Abbas *et al.*, 1996, O'Garra *et al.*, 1994). Th1 and Th2 cells produce cytokines that act as their own autocrine growth factors and that are mutually inhibitory for the differentiation and effector functions of the reciprocal subtype. Thus, once a T-cell immune response begins to develop along either a Th1 or Th2 lineage, from a common precursor, it tends to become increasingly polarized in that direction.

The prevalent manifestation of multiple infections has led to an increased interest in the effects that one infection, immunization or antigenic stimulus may have on another. Circumstantial evidence exists to suggest that cross-inhibitory immunomodulation occurs between Th1 and Th2 cells *in vivo*. The incidence of IgE-mediated allergies, such as eczema, hay fever and asthma, has risen steadily in Westernized societies, doubling in the last twenty years (Seaton *et al*, 1994). The short time over which this change has occurred and continues to occur, indicates that the environment plays a central role in these disorders. Children are born with a tendency to show allergic-type Th2 cytokine responses, a pattern that disappears in the first few years of life. However, this maturation might be delayed or absent in individuals predisposed to allergy. The theory is that depriving the immune system of the early input historically provided by Th1-inducing childhood infections might

lead to inefficient maturation and a prevalence of the Th2 type responses normally associated with allergy. This proposal is referred to as the 'hygiene hypothesis' or 'Westernization' (Folkerts *et al.*, 2000, Cookson *et al.*, 1997). Further evidence for this Th1/Th2 cross-regulation *in vivo* has emerged through the study of the immunobiology of pregnancy. Normal pregnancy is characterized by a lack of strong maternal anti-fetal immunity and a bias toward Th2-type reactivity. A shift in this response to a Th1 bias, which may be caused by infection, or by the preponderance of certain cytokines in the local milieu during T-cell activation, has been shown to result in pregnancy loss (Raghupathy, 1997, Mellor *et al.*, 2000, Chaouat *et al.*,1997). Furthermore, a recent study suggests that helminth infection, which results in highly polarized Th2 responses, can impair the immune response of the host to HIV and TB, and may be a contributory factor in the spread of these diseases (Bentwich *et al.*, 1999).

This dichotomy into reciprocally regulated Th1 and Th2 cell type responses provides a simple framework in which we categorize immune responses and their role in dealing with distinct pathogens that require different effector mechanisms for their control. However, the real situation, especially in the developing world, is one where individuals may be exposed to multiple infections or where vaccines may be administered in the face of chronic parasitic infection. The aim of the present investigation was to examine the reciprocal, cross-inhibitory influences of a Th1-inducing bacterial pathogen and a Th2-inducing parasite *in vivo*.

Bordetella pertussis is a gram negative coccobacillus that primarily infects infants and young children via inhalation of aerosol droplets. It preferentially associates with ciliated respiratory epithelial cells, but can also invade and survive within alveolar macrophages and polymorphonuclear leucocytes (Friedman *et al.*, 1992). Colonization of the upper respiratory tract results in the disease whooping cough, a significant cause of morbidity and mortality worldwide. Respiratory infection or immunization with Pw is associated with the induction of antigen-specific Th1 cells, which are critical in host resistance to infection (Mills *et al.*, 1993, Redhead *et al.*, 1993, Ryan *et al.*, 1997). In particular, the type 1 cytokine IFN-γ plays a major role in controlling *B. pertussis* infection, and in containing the bacteria to the mucosal site (Barbic *et al.*, 1997, Mahon *et al.*, 1997).

The parasitic trematode *Fasciola hepatica* infects a wide variety of mammals, including cattle, sheep and humans, causing liver fluke disease or fasciolosis. Infection is usually acquired by the ingestion of encysted larvae that contaminate vegetation or water. The metacercariae excyst in the intestine, migrate through the intestinal wall into the peritoneal cavity an then migrate across the body cavity to the liver parenchyma, where they cause extensive tissue damage and haemorrhaging. After approximately eight weeks the parasites move into the biliary passages, become sexually mature and commence egg production. Infection with *F. hepatica*, like other helminths, is accompanied by elevated IgE levels, eosinophilia, immune responses associated with the Th2 subtype (Mulcahy *et al.*, 1999, Finkelman *et al.*, 1991), and it has been recently demonstrated that *F. hepatica* infection of mice results in an early and persistently polarized Th2 response (O'Neill *et al.*, 2000). This has provided an ideal model to examine the cross-regulatory effect of a Th2-inducing pathogen following prior or simultaneous exposure to Th1-inducing pathogen.

6.2 Results

6.2.1 *B. pertussis* - A Th1-inducing bacterium and *F. hepatica* - a Th2-inducing parasite

Previous studies have demonstrated that infection with *B. pertussis* induces a polarized Th1 response in mice and children (Mills *et al.*, 1993, Ryan *et al.*, 1997). The results of this study are in agreement with these findings. Naïve mice were infected with *B. pertussis* by aerosol inoculation of live virulent bacteria into the lungs. Three weeks after infection, spleen cells were prepared and incubated with heat-killed bacteria. *B. pertussis*-specific T-cells secreted high levels of IFN-γ and IL-2, with no IL-4 or IL-5 (Fig. 6.1), indicating that *B. pertussis* induces a Th1 response in mice.

Infection with the helminth parasite *F. hepatica* was shown to induce a Th2 response in mice. Mice were orally infected with 10 metacercariae. 3 weeks post-infection, spleen cells secreted IL-4 and IL-5, in the absence of IFN-γ and IL-2 following restimulation *in vitro* with liver fluke homogenate (LFH) (Fig. 6.2).

6.2.2 *F. hepatica* suppresses the type 1 response induced by respiratory infection with *B. pertussis*

To examine the effect of *F. hepatica* infection on the immune response induced by infection with *B. pertussis*, BALB/c mice were co-infected with both parasite and bacteria on the same day. Mice infected with either *F. hepatica* or *B. pertussis* only served as controls. Spleen cells prepared from mice 3 weeks after infection with *B. pertussis* alone secreted high IFN-γ levels, and undetectable IL-4, in response to *B. pertussis* sonicate and to the purified *B. pertussis* antigens filamentous haemagglutinin (FHA) and pertactin (PRN) (Fig. 6.3). This is consistent with previous reports (Mills *et al.*, 1993, Ryan *et al.*, 1997), that *B. pertussis* infection selectively induces Th1 cell responses. The production of *B. pertussis*-specific IFN-γ production is almost completely abrogated in mice co-infected with *F. hepatica*. In contrast, infection with *F. hepatica* results in a polarized Th2 response, with high levels of IL-4 and undetectable IFN-γ produced by spleen cells in response to liver fluke homogenate (LFH). However, the profile of *F. hepatica*-specific cytokine

production was not altered in mice co-infected with *B. pertussis* (Fig. 6.3) and there was no effect on the severity of fasciolosis, as determined by liver pathology.

Coincident with the suppression of the Th1 response, concurrent infection with *F. hepatica* also resulted in delayed *B. pertussis* clearance from the lungs. Mice infected with *B. pertussis* alone began to clear the bacteria at a steady rate after 7 days, whereas clearance was protracted in co-infected mice; the numbers of bacteria were significantly higher in the co-infected mice 14 and 21 days after challenge (Fig. 6.4).

6.2.3 *F. HEPATICA* SUPPRESSES AN ESTABLISHED *B. PERTUSSIS*-SPECIFIC TH1 RESPONSE

Having established that *F. hepatica* infection could suppress the *B. pertussis*-specific Th1 response during the induction phase, it was determined whether the same suppressive effect could be observed on an established Th1 response. BALB/c mice were infected with *B. pertussis* by aerosol challenge, and allowed to recover. After 6 weeks, by which time the *B. pertussis*-specific Th1 response was established and the mice had recovered from infection (the lungs were completely free from bacteria), the mice were infected with *F. hepatica*. Spleen cells from mice infected with *B. pertussis* alone secreted high levels of IFN-γ and low levels of IL-4, whereas mice infected with *F. hepatica* alone secreted IL-4 and low levels IFN-γ, typical Th1 and Th2 responses respectively (Fig. 6.5). However IFN-γ production in response to *B. pertussis* antigens was markedly diminished in the mice that cleared the *B. pertussis* infection and were subsequently infected with *F. hepatica* (Fig. 6.5), demonstrating suppression of the already established bacterial-specific Th1 response.

6.2.4 INFECTION WITH *F. HEPATICA* RESULTS IN SUPPRESSION OF THE *B. PERTUSSIS*-SPECIFIC TH1 RESPONSE IN MICE IMMUNIZED WITH PW

Since immunization with Pw also induces a potent Th1 response and confers a high level of protection against a *B. pertussis* respiratory challenge, we examined the effect of *F. hepatica* infection on this protective vaccination. Mice were immunized twice with Pw (0.8 iu, i.p. 0 and 4 weeks) and 4 weeks later were infected with 10

metacercariae of *F. hepatica.* As expected mice immunized with Pw alone or infected with *F. hepatica* only, developed Th1 and Th2 responses respectively. The production of IL-4 and IL-5 in response to *F. hepatica* was not affected by prior immunization with Pw. However, *B. pertussis*-specific IFN-γ and IL-2 production in Pw immunized mice is completely inhibited following *F. hepatica* infection, demonstrating that infection with *F. hepatica* severely decreases *B. pertussis*-specific Th1 cytokine production (Fig. 6.6). IFN-γ, but not IL-4 production from cells stimulated with PMA and anti-CD3 was also suppressed in mice infected with *F. hepatica*. Moreover infection with *F. hepatica* reduced the protective efficacy of the Pw in the respiratory challenge model. The numbers of viable bacteria in the lungs 7 days after *B. pertussis* challenge were 40 fold higher in immunized mice infected with *F. hepatica,* compared to those that received the vaccine only (Fig. 6.7).

6.2.5 *F. HEPATICA*-INDUCED SUPPRESSION OF TH1 RESPONSES INVOLVES IL-4

IL-4 plays a major role in directing the immune response to the Th2 subtype and has also been implicated in the reciprocal downregulation of Th1 responses. Therefore, we examined the role of IL-4 in the *F. hepatica*-induced suppression of *B. pertussis* specific Th1 responses by using IL-4 defective (IL-$4^{-/-}$) mice. IL-$4^{-/-}$ and wild-type C57BL/6 mice were immunized with Pw, and boosted 4 weeks later. Immunized and control naïve mice were then infected with 10 *F. hepatica* metacercariae and T-cell cytokine production was assessed two weeks later. Spleen cells of wild-type C57BL/6 mice immunized with Pw alone exhibited a strong Th1 response, characterized by high levels of IFN-γ production and low IL-4 to *B. pertussis* antigens. Interestingly the levels of *B. pertussis*-specific IFN-γ secreted by spleen cells were lower in IL-$4^{-/-}$ compared with the wild-type mice. However this is consistent with previous observations (Mahon *et al.*, 1997) and with a recent report which suggested that IL-4 is required in the priming phase of Th1-associated tumour immunity (Schuler *et al.*, 1999). Following infection with *F. hepatica*, a complete switch from type 1 to a type 2 response was observed. *B. pertussis*-specific IFN-γ production was markedly suppressed to undetectable levels, and significant levels of IL-4 were now detected in response to *B. pertussis* antigens

(Fig. 6.8). In contrast, *F. hepatica* infection did not suppress IFN-γ or elevate IL-4 production by *B. pertussis*-specific T cells from IL-4$^{-/-}$ mice immunized with Pw (Fig. 6.8).

In a separate experiment, IL-4$^{-/-}$ mice were infected with *B. pertussis* only, or coinfected with *B. pertussis* and *F. hepatica* and the course of infection was monitored through the enumeration of viable bacteria in the lungs at various times following challenge (Fig. 6.9). In contrast to the delay in bacterial clearance observed in wild-type mice coinfected with *B. pertussis* and *F. hepatica*, the numbers of viable bacteria recovered from the lungs of singly infected and coinfected IL-4$^{-/-}$ mice did not differ significantly (Fig. 6.9)

6.3 Discussion

The results of this study demonstrate that immune responses dominated by one T cell subtype, evoked at one mucosal surface in the body, can exert bystander modulation on the reciprocal T-cell subtype induced at another site in the body. Furthermore in an experimental exposure to simultaneous Th1 and Th2-inducing stimuli, suppression of Th1 responses was observed, without a reciprocal effect on Th2 responses, suggesting that at least in this model system that the Th2 cell may have a dominant effect in Th1-Th2 cross-regulation *in vivo*. In addition the results provide the first evidence that the immunosuppressive effect of helminth parasites can also operate on an established Th1 response and that the immunoregulatory mechanism involves IL-4.

In general, parasitic infections do not cause high mortality, but counteract the host's immune defenses by developing a variety of strategies to evade protective immune responses (Maizels *et al.*, 1993). It has been well documented that parasitic infection is frequently accompanied by a downregulation in cell-mediated immunity (Sher *et al.*, 1992). Inhibition of lymphocyte proliferative responses has been reported during nematode (Allen *et al.*, 1998, Lawrence *et al.*, 1995) and trematode infections (Cervi *et al.*, 1998). Parasitic infections also provide some of the clearest examples of how the nature and protective capacity of the host's immune system is dependent upon the polarized development of T-lymphocytes of either the Th1 or Th2 subsets. It is well established that the emergence of an immune response dominated by a Th2-type profile is characteristic of many helminth infections, and it has been reported that Th2 responses are essential for resistance to these parasites (Finkelman *et al.*,1991, Grencis, 1993). However, it remains unclear whether these responses are protective to the host. There is evidence from studies with other helminth parasites that Th1 stimulation may be associated with protection and that Th2 stimulation is associated with chronic disease (Scott *et al.*, 1989, Sher and Coffman, 1992). The adoptive transfer of a $CD4^+$ Th1 clone, obtained from mice protectively immunized against the blood fluke *Schistosoma mansoni*, has been shown to convey protection against this parasite (Jankovic *et al.*, 1996). In mice, resistance to *Trichinella spiralis* correlates with the early activation of IFN-γ secreting cells, and little activation of

Th2 cells (Pond *et al.*, 1992). Although Th1 or Th2 cells may play a role in protection against different parasites, it would be beneficial to the parasite to induce immune responses capable of suppressing the host's immune protective mechanisms.

In the present investigation two infection models have been exploited, which have been shown to be capable of generating highly polarized Th1 or Th2 responses in mice, in order to examine the influence the cross regulation of cell subtypes *in vivo*. Consistent with previous reports (Mills *et al.*, 1993, Redhead *et al.*, 1993, Ryan *et al.*, 1997), this study has demonstrated that respiratory infection with *B. pertussis* or immunization with Pw selectively stimulated Th1 responses. In contrast, infection with the parasitic helminth *F. hepatica* evoked a potent Th2 response and was capable of downregulating Th1 responses induced either by respiratory infection with *B. pertussis* or systemic immunization with Pw. Downregulation of Th1 cytokine responses to both parasite and non-parasite antigens has also been reported during infection with *S. mansoni* (Kullberg *et al.*, 1992, Pearce *et al.*, 1991). This Th2-inducing parasite has also been shown to exacerbate the outcome of *Salmonella typhi* infection, in concurrent infections (Njunda *et al.*, 1996). However, since the response has shifted from predominantly Th1 to Th2 at the egg stage of infection with *S. mansoni* (Finkelman *et al.*, 1991), this model is limited to an examination of the effects of an established parasite-specific Th2 response on the induction of a Th1 cells to other antigens or pathogens. In the *F. hepatica* model a highly polarized Th2 response is detected throughout the infection (O'Neill *et al.*, 2000), providing a model to examine the influence of the Th2-inducing pathogen at different stages of response to the Th1-inducing pathogen.

The data clearly indicates that the liver fluke has the ability, not only to alter the development of *B. pertussis* Th1 response during infection and vaccination, but also to modulate this response after it has become polarized. The modulatory effect of the parasitic infection could be observed when it was delivered either at the induction phase or during an established *B. pertussis*-specific Th1 response. Significantly the results also demonstrate that the modulation of the cytokine profile by *B. pertussis* specific T cells was accompanied by a reduction in host resistance to

the bacterial infection after challenge. The finding that protective immunity was not completely abrogated in the mice infected with *F. hepatica* can be explained by the fact that Th2 or a mixed Th1-Th2 response, such as that induced with an acellular pertussis vaccine, can also confer a level of protection against *B. pertussis* challenge by a distinct mechanism (Mills *et al.*, 1998a). The results suggest that the systemic response can influence protective effector mechanisms in the lung.

Our observations suggest that the suppression of the anti-bacterial immunity during the *F. hepatica* infection is a consequence of bystander down-regulation of the *B. pertussis*-specific Th1 cells by the parasite-specific Th2 cells. Nevertheless it is possible that the liver fluke infection may have exerted other effects on anti-bacterial immunity, independent of Th2 cells. It has been suggested that *S. mansoni* may induce apoptosis of IFN-γ-producing cells (Estaquier *et al.*, 1997). Excretory-secretory components of *F. hepatica* may also exert direct immune suppressive effects through the activity of proteinases on immunoglobulin molecules (Carmona *et al.*, 1993). However the abrogation of the modulatory effect of the *F. hepatica* infection in IL-4 defective mice argues against these possibilities and points to an important role for IL-4 in Th2-mediated immunoregulation. *F. hepatica* infection of C57BL/6 mice that had been immunized with Pw, resulted in a significant reduction in *B. pertussis*-specific IFN-γ production. In contrast, IFN-γ production was not significantly altered following *F. hepatica* infection of IL-4$^{-/-}$ mice immunized with Pw. Furthermore, a significant difference in the bacterial load in IL-4$^{-/-}$ mice coinfected with *F. hepatica* was not observed, suggesting that abrogation of the suppressive effect on IFN-γ production translates into restoration of full protection. However, interpretation of the effect of IL-4 on the outcome of infection in IL-4$^{-/-}$ mice is complicated by the fact that IFN-γ production in the absence of *F. hepatica* infection is also partially suppressed in these mice (Mahon *et al.*, 1997, Schuler *et al.*,1999, and this study).

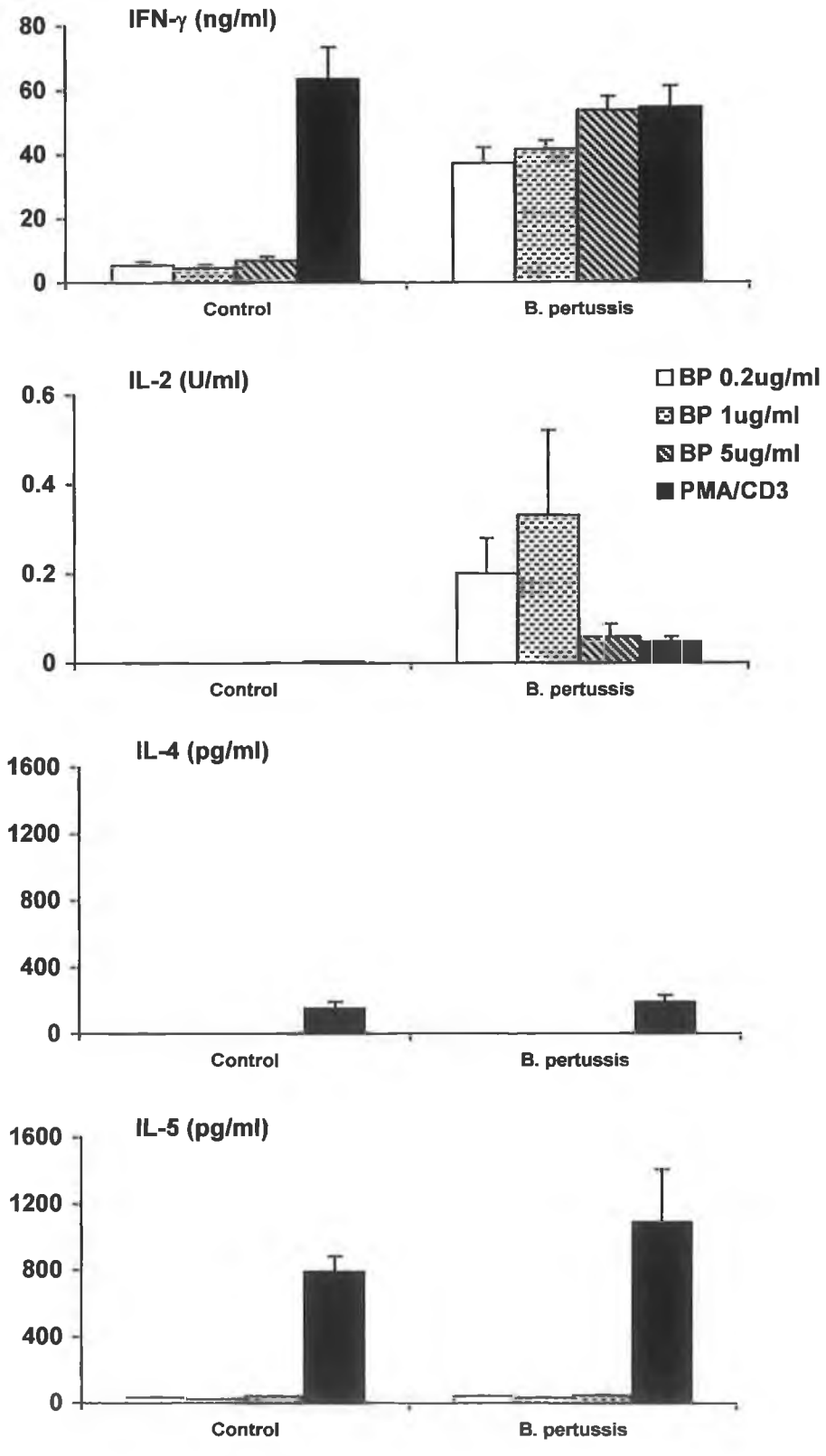
In addition to IL-4 other inhibitory cytokines may also be involved in the Th1 response inhibition by *F. hepatica.* Like IL-4, IL-10 can inhibit cytokine production by Th1 cells (Fiorentino *et al.*, 1991) and the ability of IFN-γ to activate macrophage killing of both intracellular and extracellular parasites (Gazzinelli *et al.*, 1992). It has been suggested that this inhibitory cytokine may be responsible for the

suppression of Th1 responses in *S. mansoni* infection (Sher *et al.*, 1991). IL-4 and IL-10 can act synergistically to inhibit the production of reactive nitrogen oxides, which are known to upregulate IL-12 production, and as a consequence, inflammatory responses (Liew, 1993). It has been shown that the excretory-secretory products produced during *F. hepatica* infection can decrease nitrite production by rat peritoneal cells (Cervi *et al.*, 1996). It has also been demonstrated that spleen cells from *F. hepatica* infection mice secrete high levels of IL-4 and IL-10 in response to liver fluke antigens *in vitro* (O'Neill *et al.*, 2000). Thus *F. hepatica* may, through the induction of IL-4 and perhaps IL-10, inhibit the activation of macrophages and suppress IFN-γ production by Th1 cells.

The present investigation demonstrated that *F. hepatica* infection could downregulate *B. pertussis*-specific IFN-γ production at both the induction and effector phases of the Th1 response. In C57BL/6 mice immunized with Pw and then infected with *F. hepatica*, the Th1 response completely switched to a Th2 response. The appearance of Th2 cytokines in C57BL/6 but not BALB/c mice was reproducable and is surprising in view of the observations that responses tend to be more polarized to Th1 in C57BL/6 mice. One possible explanation for this is that it may reflect complex differences in sensitivity to regulatory cytokines. As well as the recognized role for early IL-4 in directing the immune response to the Th2 pathway, there is some evidence to suggest that IL-4 is capable of converting committed Th1 cells to the Th2 subtype. In one study, a highly polarized *Leishmania*-specific Th1 cell population switched to a Th2 phenotype, following *in vitro* culture with IL-4, especially when added early in culture (Mocci *et al.*, 1995). In another study it has been demonstrated that ectopic expression of GATA-3 (a transcription factor differentially expressed on Th2 cells) converted committed Th1 cells and a Th1 clone to express Th2 cytokines (Lee *et al.*, 2000). Signaling through IL-4 induces high-level expression of GATA-3 which in turn blocks IL-12 signaling by inhibiting expression of IL-12Rβ2 (Ouyang *et al.*, 1998). Transcripts for IFN-γ have been shown to be dominant, in the skin-draining lymph nodes of mice vaccinated with irradiation-attenuated cercariae of *S. mansoni,* but following a challenge infection IL-4 becomes dominant and IFN-γ mRNA levels are barely detectable (Betts *et al.*, 1998). Thus while early IL-4 production probably plays a

major role in driving the immune response to a Th2 phenotype, and may be important in the maintaining the polarization of this response, it can also influence the profile of cytokines secreted in response to unrelated antigens.

In conclusion, the results show that infection the Th2-inducing parasite *F. hepatica*, can suppress a Th1 response induced by *B. pertussis*-infected or immunized mice. As well as suppressing IFN-γ and IL-2 production, *F. hepatica* infection also delayed clearance of the bacteria from the lungs following *B. pertussis* challenge. Suppression of Th1 responses was observed without a reciprocal effect on Th2 responses suggesting, that at least in the present model system, the Th2 cell may have a dominant effect in Th1/Th2 cross-regulation *in vivo*. Most importantly the demonstration of bystander immunomodulation of protective type 1 responses during infection with Th2-inducing organisms has profound implications for the outcome of concurrent bacterial infections and on protective efficacy of vaccines against intracellular pathogens.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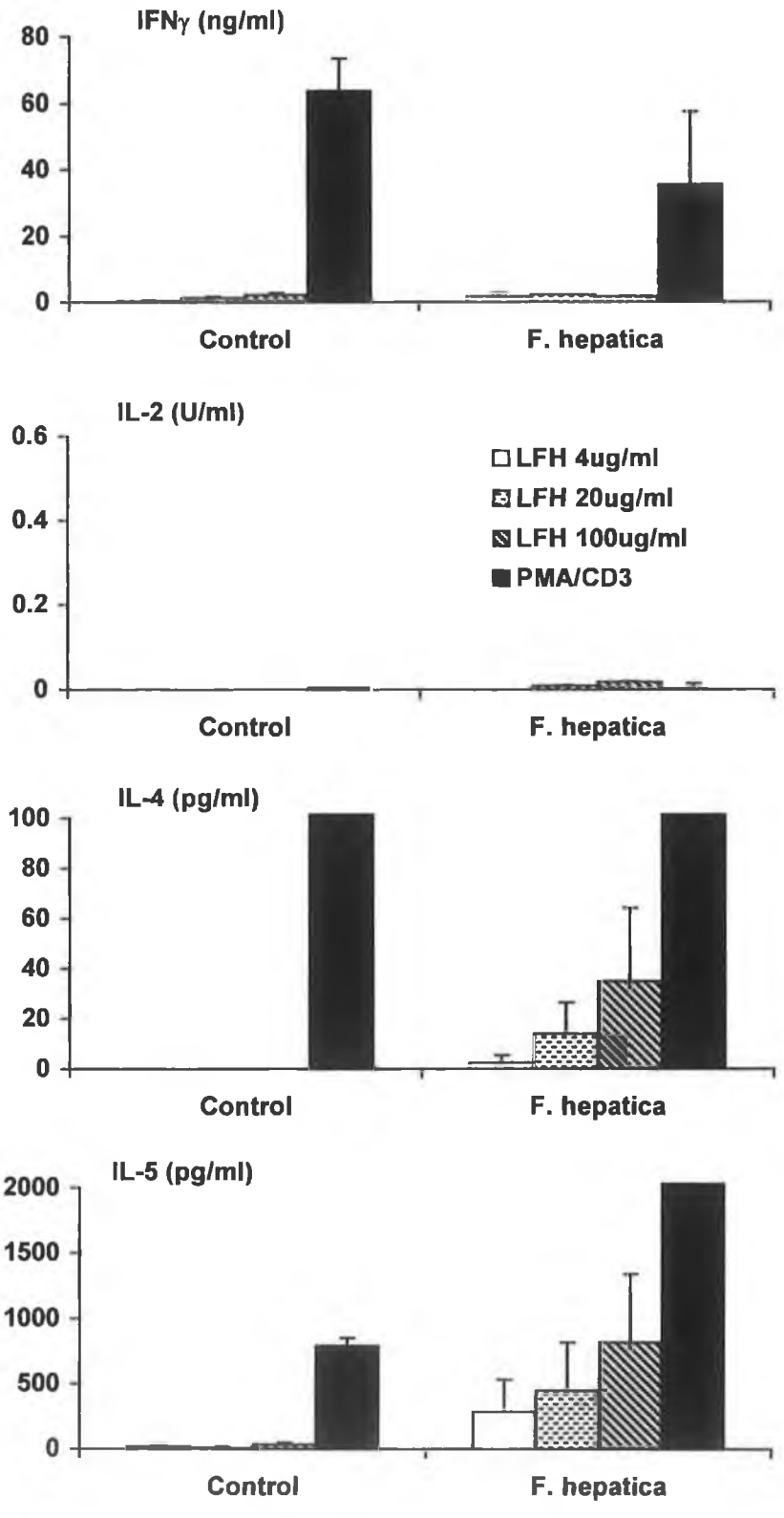
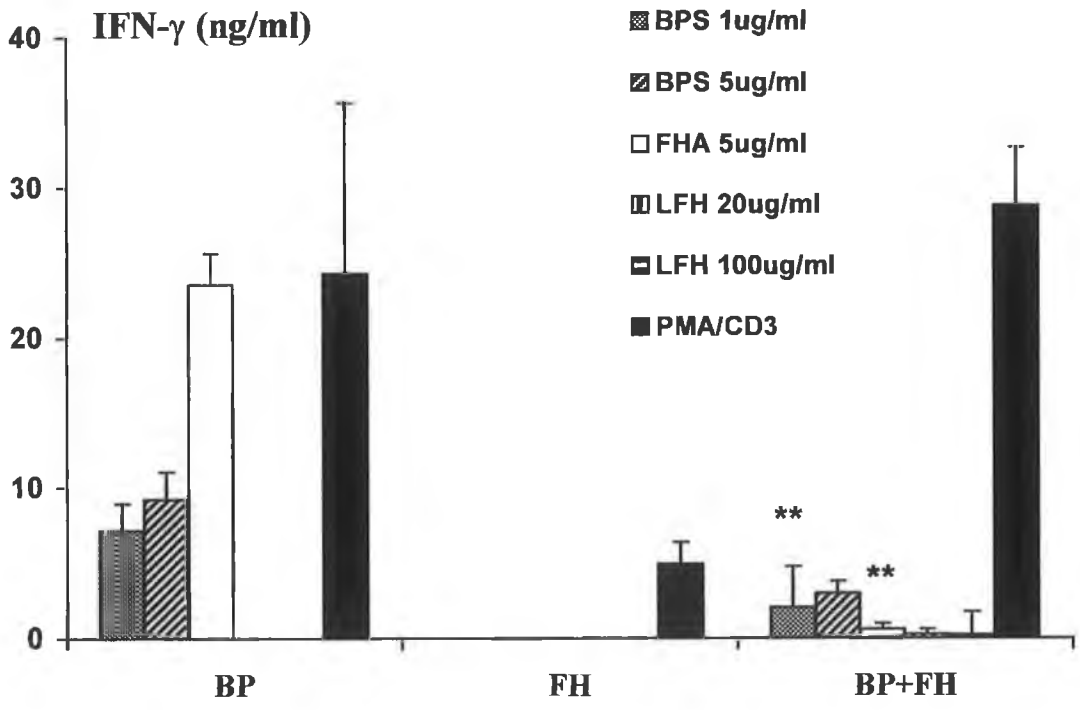
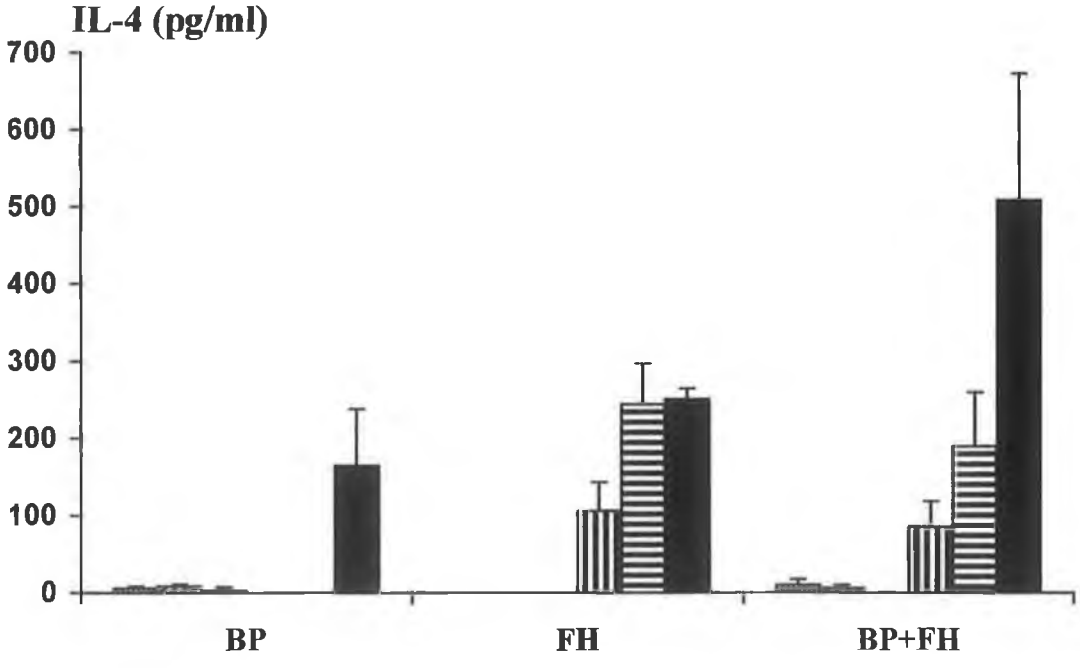
60
40
20
0
Control
B. pertussis
IL-2 (U/ml)
0.6
0.4
0.2
0
Control
B. pertussis
BP 0.2ug/ml
BP 1ug/ml
BP 5ug/ml
PMA/CD3
IL-4 (pg/ml)
1600
1200
800
400
0
Control
B. pertussis
IL-5 (pg/ml)
1600
1200
800
400
0
Control
B. pertussis


IFNγ (ng/ml)
80
60
40
20
0
Control
F. hepatica
IL-2 (U/ml)
0.6
0.4
0.2
0
LFH 4ug/ml
LFH 20ug/ml
LFH 100ug/ml
PMA/CD3
Control
F. hepatica
IL-4 (pg/ml)
100
80
60
40
20
0
Control
F. hepatica
IL-5 (pg/ml)
2000
1500
1000
500
0
Control
F. hepatica


IFN-γ (ng/ml)
40
30
20
10
0
BPS 1ug/ml
BPS 5ug/ml
FHA 5ug/ml
LFH 20ug/ml
LFH 100ug/ml
PMA/CD3
**
**
BP
FH
BP+FH


IL-4 (pg/ml)
700
600
500
400
300
200
100
0
BP
FH
BP+FH

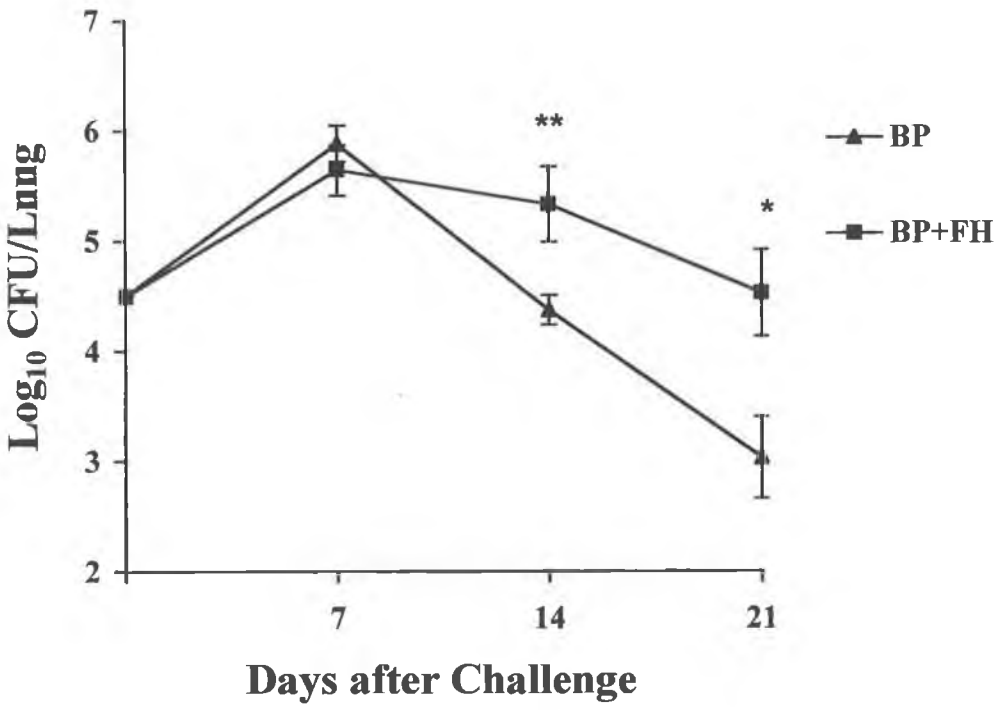
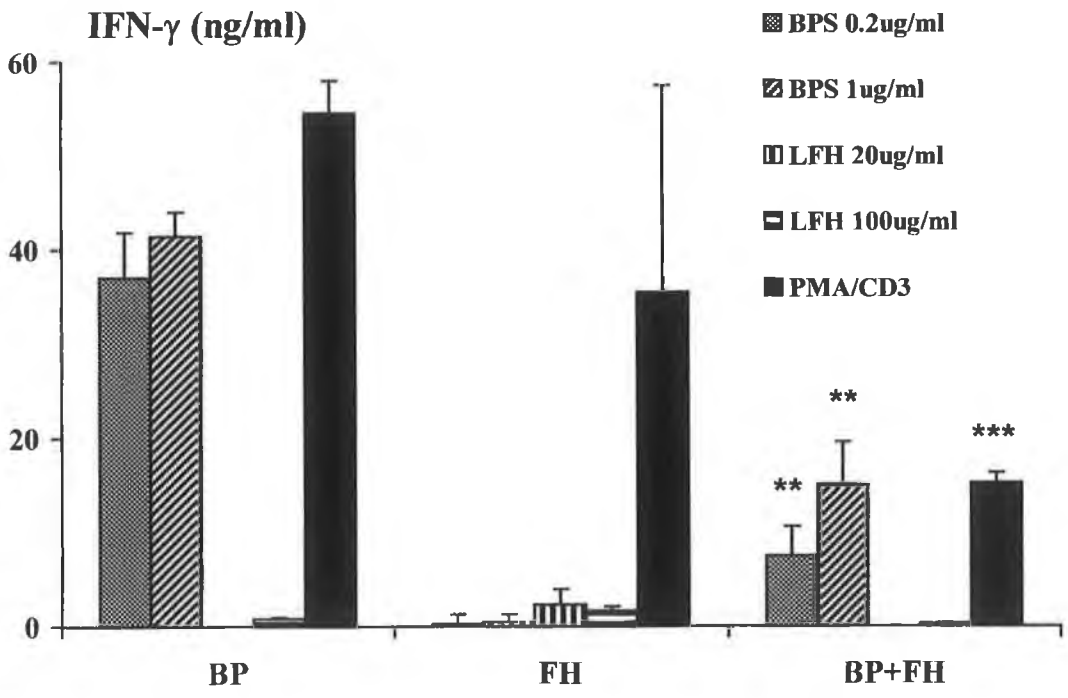
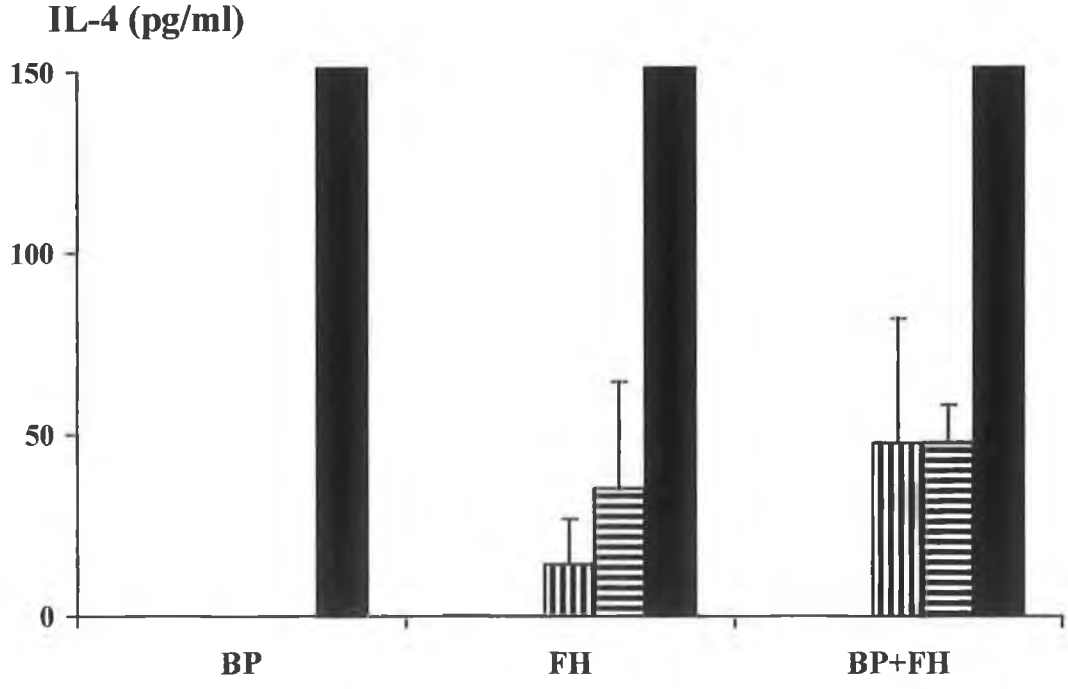
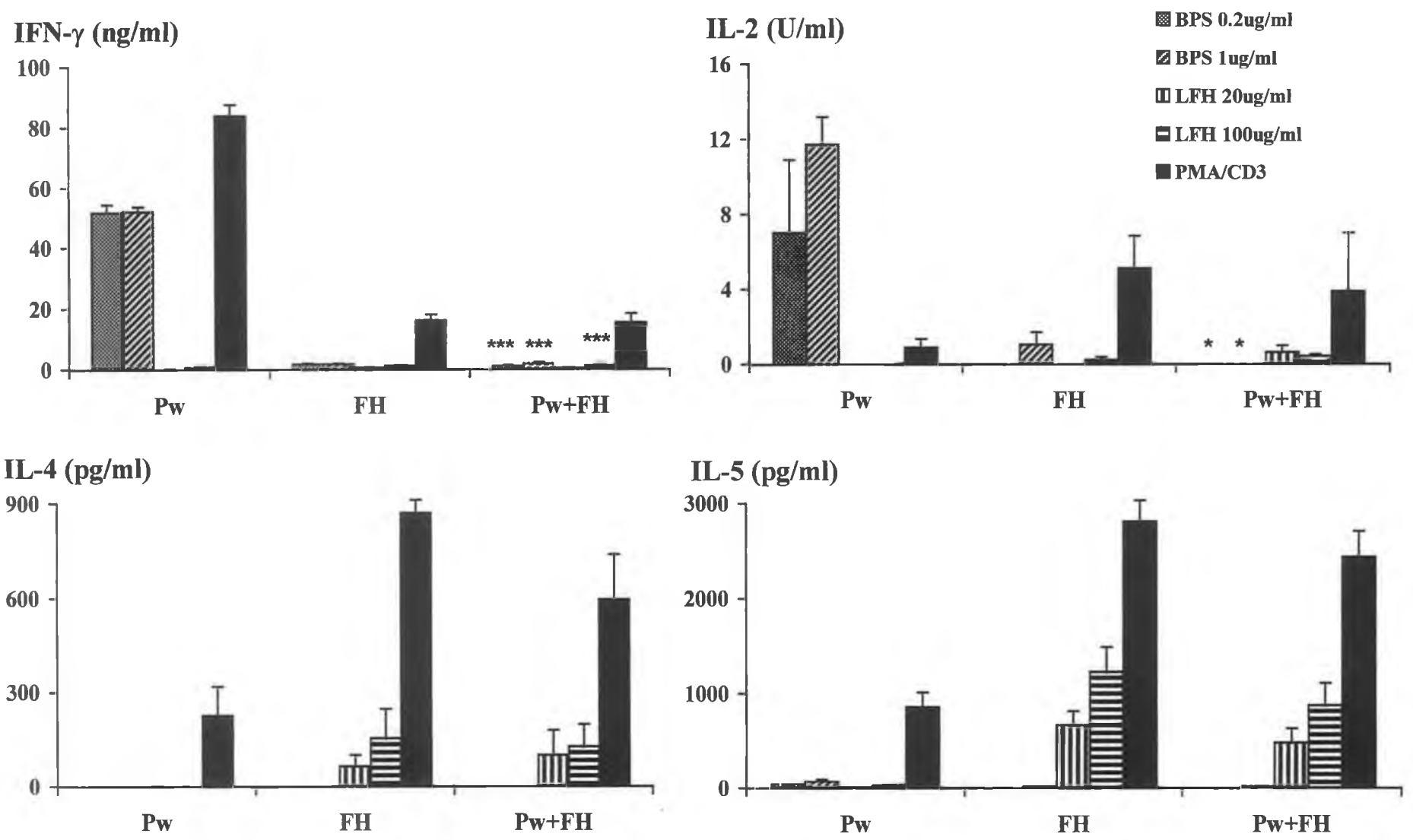


Figure 6.4

F. hepatica infection delays bacterial clearance in mice infected with *B. pertussis*. BALB/c mice were infected with either *B. pertussis* alone (▲) or concurrently with *F. hepatica* (■). Subsequently, mice were sacrificed at various times, to assess the numbers of viable bacteria in the lungs. Results are reported as the mean numbers of *B. pertussis* CFU for individual lungs from 4 mice from each group at each time point. *, $P < 0.05$ versus mice infected with *B. pertussis* alone; **, $P < 0.01$ versus mice infected with *B. pertussis* alone.


IFN-γ (ng/ml)
60
40
20
0
BPS 0.2ug/ml
BPS 1ug/ml
LFH 20ug/ml
LFH 100ug/ml
PMA/CD3
**
**
***
BP
FH
BP+FH


IL-4 (pg/ml)
150
100
50
0
BP
FH
BP+FH


IFN-γ (ng/ml)
100
80
60
40
20
0
***
***
***
Pw
FH
Pw+FH
IL-2 (U/ml)
16
12
8
4
0
*
*
Pw
FH
Pw+FH
BPS 0.2ug/ml
BPS 1ug/ml
LFH 20ug/ml
LFH 100ug/ml
PMA/CD3
IL-4 (pg/ml)
900
600
300
0
Pw
FH
Pw+FH
IL-5 (pg/ml)
3000
2000
1000
0
Pw
FH
Pw+FH

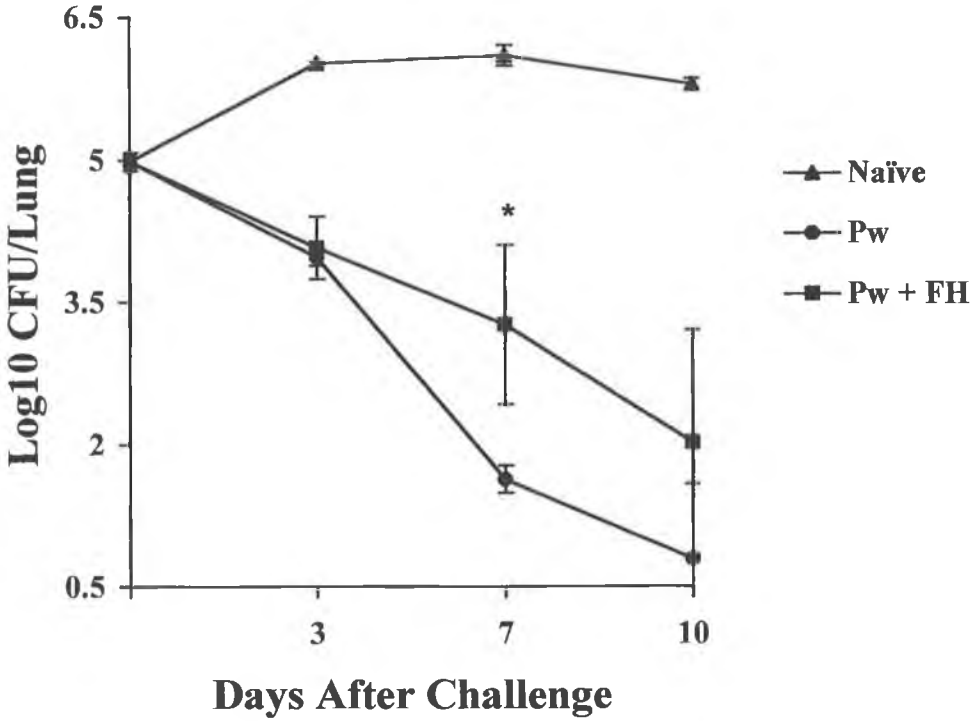
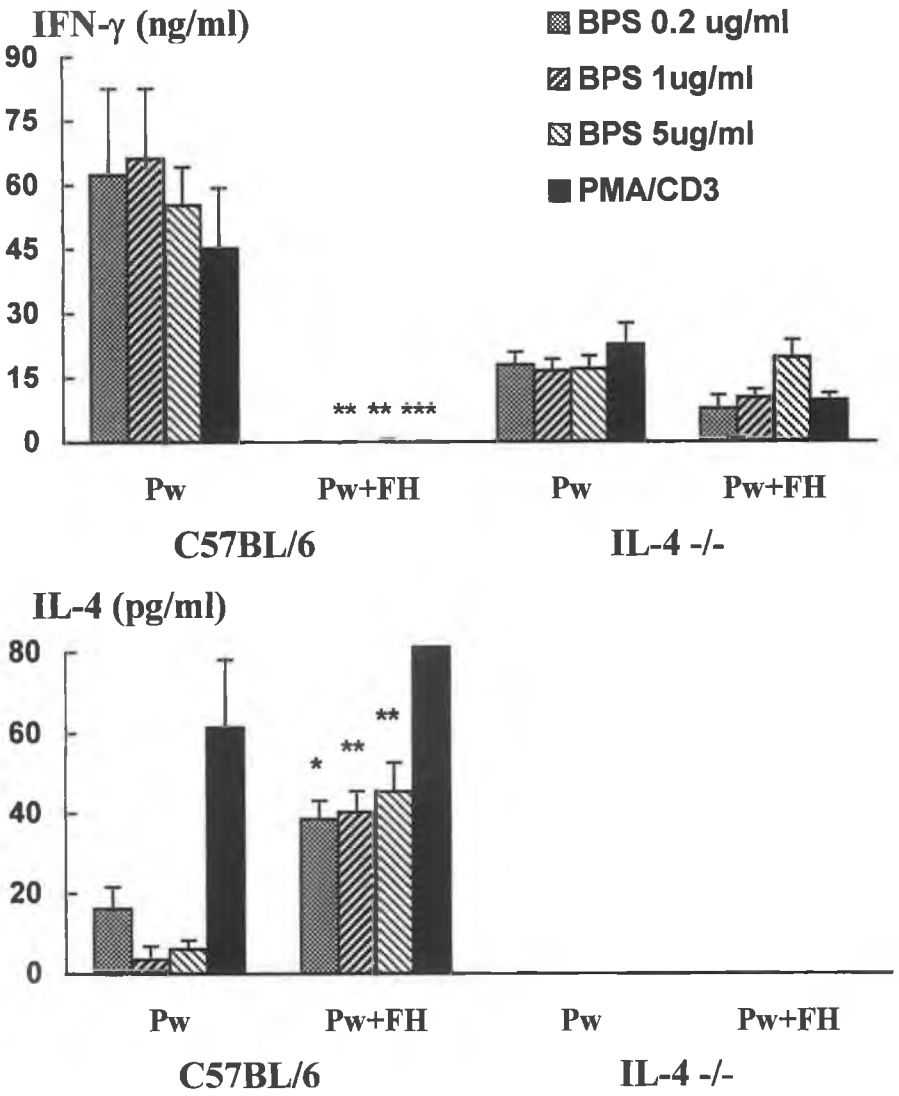
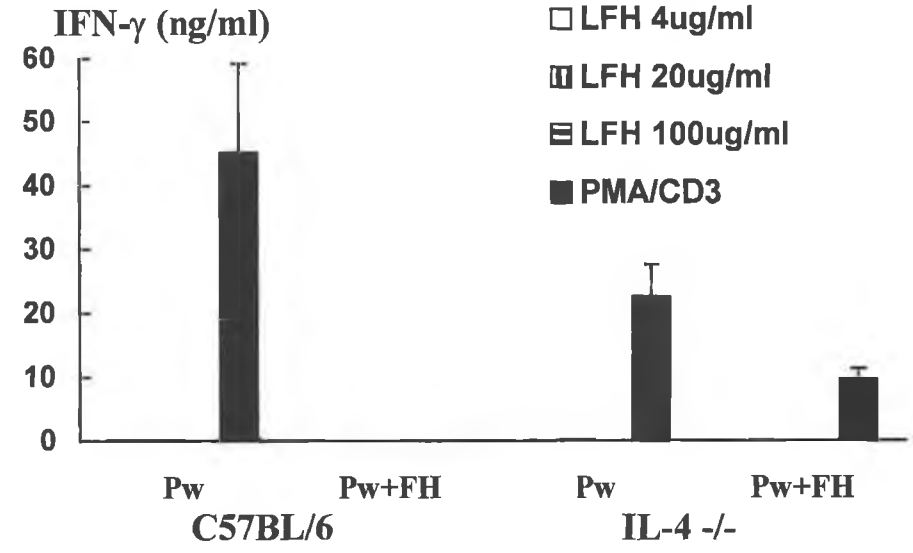
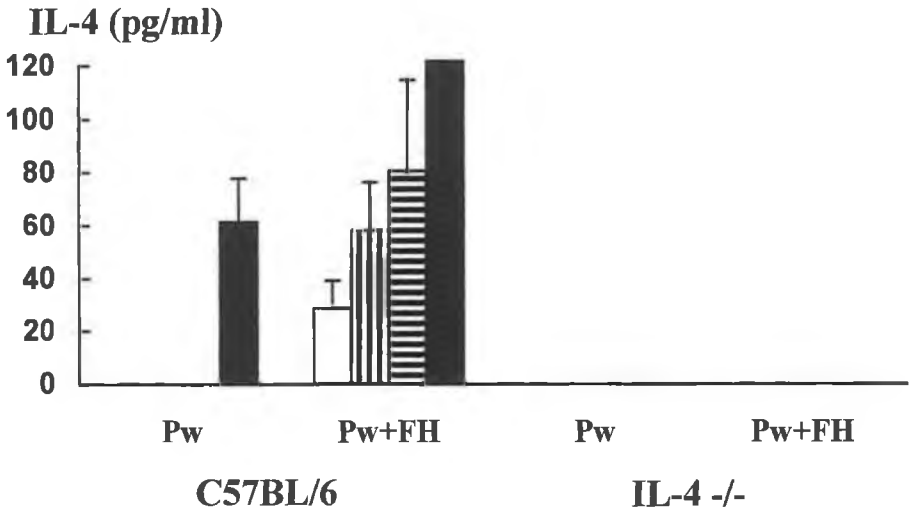


Figure 6.7

F. hepatica infection reduces the protective efficacy of a whole cell pertussis vaccine in mice. BALB/c mice were immunized with Pw (0 and 4 weeks), and one week later a proportion of these was infected with 10 metacercariae of *F. hepatica* (FH). Respiratory infection of mice with *B. pertussis* was performed by aerosol challenge 1 week after infection with *F. hepatica*. Naïve mice and mice that were immunized with Pw, and subsequently infected with *B. pertussis*, without a preceding *F. hepatica* infection served as controls. Mice were killed from all groups at various times after aerosol challenge to assess the numbers of viable bacteria in the lungs. Results are reported as the mean numbers of *B. pertussis* CFU for individual lungs from 4 mice at each time point. *, $P < 0.05$ versus mice immunized with Pw alone.


IFN-γ (ng/ml)
BPS 0.2 ug/ml
BPS 1ug/ml
BPS 5ug/ml
PMA/CD3
90
75
60
45
30
15
0
** ** ***
Pw
Pw+FH
Pw
Pw+FH
C57BL/6
IL-4 -/-
IL-4 (pg/ml)
80
60
40
20
0
*
**
**
Pw
Pw+FH
Pw
Pw+FH
C57BL/6
IL-4 -/-


IFN-γ (ng/ml)
LFH 4ug/ml
LFH 20ug/ml
LFH 100ug/ml
PMA/CD3
60
50
40
30
20
10
0
Pw
Pw+FH
Pw
Pw+FH
C57BL/6
IL-4 -/-


IL-4 (pg/ml)
120
100
80
60
40
20
0
Pw
Pw+FH
Pw
Pw+FH
C57BL/6
IL-4 -/-

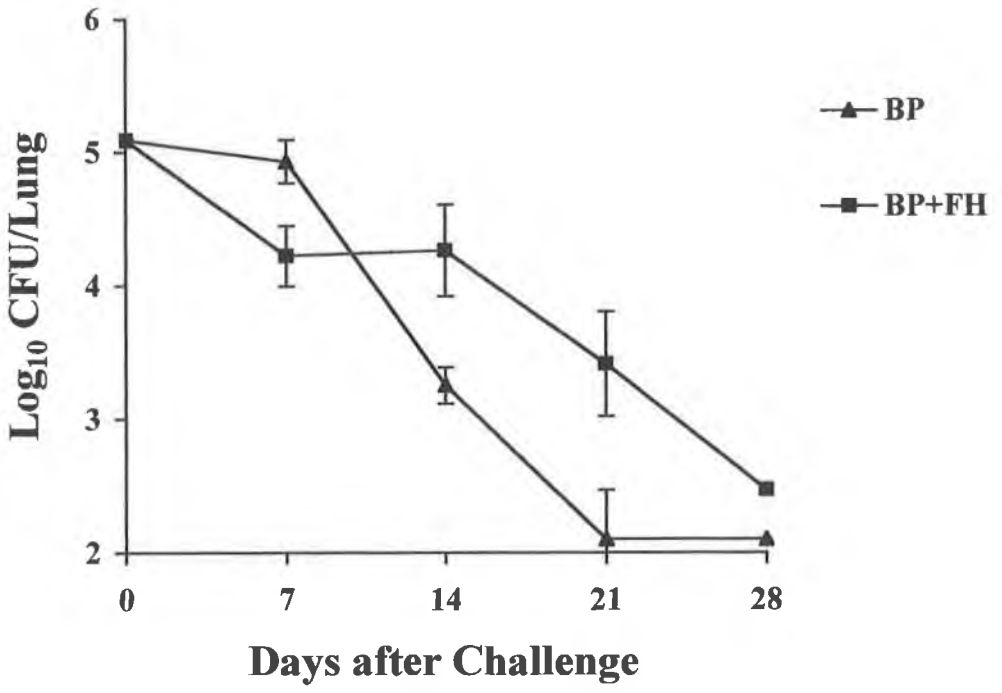


Figure 6.9

F. hepatica does not exacerbate infection with *B. pertussis* in IL-4$^{-/-}$ coinfected mice. IL-4$^{-/-}$ mice were infected with *B. pertussis* alone (▲) or concurrently with *F. hepatica* (■). Subsequently, mice were sacrificed at various times to assess the numbers of viable bacteria in the lungs. Results are reported as the mean numbers of *B. pertussis* CFU for individual lungs from four mice from each group at each time point.

❖ CHAPTER 7 ❖

GENERAL DISCUSSION

General Discussion

As we enter a new millennium, infectious diseases remain a major burden on both industrialized and developing countries. Although the advent of antibiotics has helped prevent death in individuals infected with bacteria, the greatest impact on the prevention of infection has come from new developments of vaccination (Bloom, 1999). Vaccines have led to the eradication of smallpox from the world and polio from the Northern Hemisphere. The World Health Organization has identified a number of infectious diseases for global eradication, although surprisingly pertussis is not one of them. Infection with *B. pertussis* results in whooping cough, a significant cause of morbidity and mortality in human infants. The shortcomings of the current preventative program resides in the nature and efficacy of current vaccine preparations. Immunization requires a 3-dose primary series and two additional boosters for continued protection. This immunization schedule is impractical in developing countries and difficult even where public health clinics are readily available. Furthermore, it has been recommended that pertussis immunization should be carried out every 10 years after the initial vaccination schedule, as pertussis does occur in older individuals, and this age group may be an important reservoir for the organism. Moreover, although Pa and Pw provide effective protection against pertussis (Lang *et al.*, 1995), the efficacy of pertussis vaccines is well below that achieved by with other vaccines, especially polio, tetanus, diphtheria and measles. With approximately 80% protection representing the maximum threshold for a fully immunized population, disease caused by *B. pertussis* is likely to continue.

Pw have been very effective in reducing the incidence of pertussis, but are associated with adverse events, including fever, persistent crying, seizures and hypotonic-hyporesponsive episodes post-immunization. Concerns regarding the adverse reactions associated with Pw, together with the recommendation that the risk/benefit ratio of Pw is thought to be too high for individuals over 7 years of age, led to the development of Pa (Lang *et al.*, 1995). The new generation Pa composed of purified bacterial components, have now replaced the traditional whole cell vaccine against whooping cough, in most developed countries (Rappuoli, 1996). Although Pa have a higher safety profile when compared with the Pw (Trollfers *et*

al., 1995, Greco *et al.*, 1996, Gustafsson *et al.*, 1996, Simondon *et al.*, 1997, Miller *et al.*, 1997), the estimated efficacy of Pa did not rival that of most European Pw (Gustaffson *et al.*, 1996). Although considerable advances have been made in understanding the pathogenesis and immunological mechanisms involved in the prevention and control of infection by *B. pertussis*, development of a more effective vaccine has been hampered by the inability to identify an immunological correlate of protection against *B. pertussis*. Further investigation of the relative contributions of T- and B-cells and their products in immunity to *B. pertussis*, and the mechanisms involved in bacterial elimination, may accelerate the development of more efficacious vaccines. In this study, the role of Th1 and Th2 cells in immunity to *B. pertussis* has been examined using a murine respiratory challenge model of infection.

Animal models, particularly the murine respiratory challenge model, have been pivotal in delineating the immunological mechanisms of protection against *B. pertussis*. It was initially presumed that protection against *B. pertussis* was mediated entirely by antibodies, due to the fact that the bacterium was considered to be non-invasive, and occupied a purely extracellular niche. Although antibody does play a role, both in toxin neutralization and in preventing bacterial attachment, indirect evidence of a role for T-cells in immunity to *B. pertussis* has been provided by the fact that specific serum antibodies do not rise to significant levels until about 5 weeks after challenge of mice (Mills *et al.*, 1993), when live bacteria are no longer detectable in the lungs. This suggests that prior to the appearance of serum antibodies, an alternative mechanism of immunity must mediate clearance of the bacteria from the lungs. This alternative protective mechanism has been shown to be mediated by *B. pertussis*-specific T-cells, and by cells of the innate immune system. Using a murine respiratory challenge model of infection Mills *et al.* (1993) demonstrated that normal naïve mice clear a respiratory infection after approximately 5 weeks, whereas nude mice which are deficient in T-cells, fail to eliminate the bacteria after aerosol inoculation, and develop a persistent infection. Furthermore, adoptive transfer of immune spleen cells, or purified $CD4^+$ T-cells into nude recipient mice, restored their ability to clear the bacteria. It was shown that these protective *B. pertussis*-specific T-cells induced by infection were $CD4^+$ T-

cells, which secreted IFN-γ and IL-2, but not IL-4 or IL-5; a characteristic Th1 response.

The use of animal models has also played an important role in the elucidation of the protective mechanisms induced following vaccination with Pa or Pw, and in the evaluation of pertussis vaccine efficacy prior to clinical trials. As well as being an important tool for vaccine efficacy evaluation, animal models can be effectively used in the quality control of pertussis vaccines, to ensure there is minimum variation in protective efficacy between different batches of vaccines. The traditional animal model used for testing the potency of Pw was the mouse protection assay devised by Kendrick (1947). This test, while sufficient for the evaluation of Pw, was not suitable for assessing the efficacy of Pa. New generation Pa, despite having relatively high efficacy in clinical trials, do not pass the original Kendrick test (Corbel & Xing, 1997). Furthermore, because the bacteria is delivered intracerebrally, the Kendrick test is of little use in the investigation of pathology, or protective mechanisms in acquired immunity to *B. pertussis*. The aerosol challenge model developed by Sato et al. (1980), has been important in dissecting the mechanisms of protection against *B. pertussis* (Mills *et al.*, 1998a, Mills *et al.*, 1998b, Mahon *et al.*, 1996, Mahon *et al.*, 1997, Barbic *et al.*, 1997, Redhead *et al.*, 1993, Barnard *et al.*, 1996, McGuirk *et al.*, 1998).

In this study the murine respiratory challenge model was employed in an attempt to validate the model for predicting vaccine efficacy and to examine the role of antibody and T-cell subtypes in protection. These studies were made possible by access to Pa and Pw vaccines that had been tested in the National Institute of Allergy and Infectious Disease-sponsored phase 3 clinical trials in Italy (Greco *et al.*, 1996), Sweden (Gustafsson *et al.*, 1996) and Senegal (Simondon *et al.*, 1997), and five vaccines tested in a WHO collaborative study on the mouse model, together with different clinical lots of Pa manufactured by Chiron Corporation (CB Pa3(a), CB Pa3 (b)), and different formulations of the Pasteur Merieux 2-component vaccine (PM 94, PM 181).

In previous studies comparing the protective efficacy of individual antigens of *B. pertussis* in mice, PT and PRN alone were found to be the most protective antigens, but also demonstrated increasing protection with two, three or five

component vaccines (Mills *et al.*, 1998a). Results from clinical trials have also demonstrated that increasing the number of antigens increased vaccine efficacy (Olin *et al.*, 1997, Gustafsson *et al.*, 1996). However, examination of the protective efficacy of the different Pa from the WHO study showed that increasing the number of antigens in the vaccines did not appear to have a significant effect on their protective ability. The 3-component SmithKline Beecham vaccine provided a very similar degree of protection to the 2-component vaccine in the mouse model. In contrast, when evaluated in clinical trials, the SmithKline vaccine that included PRN was more protective than a 2-component vaccine with PT and FHA alone (Greco *et al.*, 1996, Gustafsson *et al.*, 1996). Similarly, the 5-component Connaught Pa did not appear to be significantly more protective in the mouse model than 2- or 3-component vaccines, although studies in children have found 5-component Pa to be more efficacious than Pa with lesser numbers of antigenic components (Olin *et al.*, 1997, Gustafsson *et al.*, 1996). However, the vaccines evaluated in this study all provided a high degree of protection when estimated in clinical trials, and similarly, we have shown that these vaccines provided a significant degree of protection in the mouse model. Furthermore, the cytokine profile induced after immunization with the different Pa in this study is typical of a Th2 response, and this is in agreement with previous studies which showed that immunization with Pa results in the generation of T-cells that secrete Th2 cytokines (Barnard *et al.*, 1996, Mahon *et al.*, 1996). Similarly, Pw-immunized mice displayed a polarized Th1 response, in agreement with previous observations (Redhead *et al.*, 1993)

Evaluation of the antibody responses to the putative protective antigens of *B. pertussis* in immunized mice revealed that there was no correlation between the antibody titres generated to the component antigens in the vaccines, and estimated vaccine efficacy in children. This observation was consistent in all experiments. Highly efficacious Pw induced low levels of pertussis-specific antibody, and high antibody titres were induced by the Pa, even those of low efficacy. These results are congruous with the findings from clinical trials, which have failed to demonstrate an immunological correlate of protection in children based on antibody responses against the putative protective antigens of *B. pertussis* (Greco *et al.*,

1996, Simondon *et al.*, 1997, Gustafsson *et al.*, 1996, Olin *et al.*, 1997). These results, together with the demonstration of a highly significant correlation between bacterial clearance in immunized mice, and vaccine efficacy in children (Mills *et al.*, 1998a, Mills *et al.*, 1998b), suggests that the murine respiratory challenge model is a valuable animal model for the regulation and future development of pertussis vaccines, and also for the elucidation of protective mechanisms against *B. pertussis*.

The 3-component Pa manufactured by Chiron Corporation contains a genetically detoxified pertussis toxin (Rappuoli, 1996, Pizza *et al.*, 1989, Rappuoli, 1997), while the pertussis toxin present in all other vaccines is chemically inactivated. The genetically detoxified pertussis toxin retains conformational epitopes which are destroyed by chemical treatment, therefore the mutant toxin is more effective at generating anti-PT neutralizing antibodies than chemically treated toxins (Rappuoli, 1997). Furthermore, a lower dose of the mutant PT is present in the final vaccine formulation which has been shown to confer effective protection in clinical trials (Greco *et al.*, 1996). Analysis of antibody responses to different clinical lots of this vaccine in this study showed that immunization with CB Pa3 induced high antibody titres to the genetically detoxified PT, which were comparable to those induced with other Pa, even though the concentration of rPT was lower (5μg versus 25μg) in this vaccine. This is in agreement with previous findings suggesting that recombinant PT induces more potent antibody responses relative to antigen content (Rappuoli, 1997).

The numbers of vaccines included in childhood vaccination programs are increasing, and consequently there is much interest in increasing the number of vaccines administered in the same injection. However, an important consideration is the effect that one vaccine might have on the immune responses generated by other vaccines when delivered simultaneously. For example PT (Ryan *et al.*, 1998) and FHA (McGuirk and Mills, 2000) have been shown to possess immunomodulatory properties, and it is likely that these components of current Pa may effect the response to coinjected antigens in a combined vaccine formulation. Immunization with different clinical lots of Pasteur Merieux 2-component Pa combined with Hib, was associated with a slight delay in bacterial elimination and occasional rebound in bacterial load. This is consistent with previous studies which

demonstrated that immunization with Pa results in protracted clearance of the bacteria from the lungs when compared with Pw (Mills *et al.*, 1998a, Mahon *et al.*, 1996). The protective efficacy of this 2-component Pa without the addition of Hib in the formulation, was previously evaluated in mice (Mills *et al.*, 1998a). Compared to this evaluation in the mouse model, formulation with Hib does not appear to effect the efficacy of the Pa. Therefore, the slightly impaired bacterial clearance in these mice is unlikely to be due to the inclusion of Hib in the formulation, and is probably a result of the lack of induction a Th1 response, which has been shown to confer optimal immunity to *B. pertussis* (Mahon *et al.*, 1996, Mills *et al.*, 1993, Redhead *et al.*, 1993). Different formulations of the 2-component PM Pa (PM 94, PM 181) did not have any obvious effect on the induction of antigen-specific antibody. However, this is not surprising as both vaccines have the same antigenic composition and inactivation procedures. Furthermore, previous studies have shown that there was no difference in the level of T-cell activation post-challenge, in mice immunized with either PM 94 or PM 181 (Ryan, 2000).

The majority of the studies on the mechanisms of protective immunity against *B. pertussis* in both mice and humans have focused on the evaluation of immune responses early after immunization. However, the principle of effective vaccination in long term protection is dependent on the induction of immunological memory, maintained by antigen-specific B- and T-cells (Zinkernagal *et al.*, 1996). Protective levels of circulating antibody have been identified for certain pathogens, such as poliovirus, diphtheria, tetanus and *Haemophilis influenzae*. However, levels of circulating antibody that are critical for protection against *B. pertussis* have to date remained impossible to define both in children and in mice (Storsaeter *et al.*, 1998, Cherry *et al.*, 1998, Taranger *et al.*, 2000, Mills *et al.*, 1998a, Mills *et al.*, 1998b). Furthermore, it has been shown that *B. pertussis*-specific IgG levels in children wane to low or undetectable levels soon after immunization with Pa (Giuliano *et al.*, 1998, Storsaeter *et al.*, 1998). The effector mechanisms that maintain long term protection are most likely different from those controlling infection at the peak of the immune response. Anamnestic antibody production after exposure to the pathogen is considered to be an important characteristic of

immunological memory, which provides the host with the first line of defence against reinfection. It has been suggested that long-lived antibody-secreting plasma cells play a role in the maintenance of persistent antibody responses (McHeyzer-Williams & Ahmed, 1999, Slifka & Ahmed, 1998). Whether, the decline in serum antibody responses observed in children occurs as a result of premature apoptosis of plasma cells following immunization with Pa remains to be elucidated. Nevertheless, these children still appear to be protected against *B. pertussis* despite the decline of specific antibody (Storsaeter *et al.*, 1998). So, what are the mechanisms mediating protection? This study attempted to answer this question.

In this study, levels of anti-pertussis antibodies were measured following immunization of mice with low doses of Pw or a 3-component Pa. Serum antibodies to PT, FHA and PRN peaked 5-6 weeks after primary immunization, and in concordance with observations in immunized children, declined rapidly and reached undetectable levels after 6-9 months. Despite the decline in specific serum antibody responses, significant levels of protection were still observed following bacterial challenge. When Pw-immunized mice were challenged by aerosol inoculation at 38 weeks post-immunization, the bacterial clearance curves were not significantly different to that observed at the peak of the immune response, 6 weeks after immunization. Persistent protection was also observed in mice immunized with Pa, although the course of infection was slightly more protracted. However, immunization with Pa has previously been associated with a delay in bacterial clearance, when compared with Pw-immunized mice (Mills *et al.*, 1998a, Mahon *et al.*, 1996), and it is has been speculated that this effect is due to the lack of induction of Th1 cells, known to play a critical role in protection (Mills *et al.*, 1993, Redhead *et al.*, 1993).

In contrast to serum antibody levels, T-cell responses were detectable using *in vitro* stimulated spleen cells for prolonged periods after immunization. As had been previously reported there is a clear dichotomy in the nature of the T-cell responses induced by either Pa or Pw, which generate Th2 and Th1 cells respectively. Extensive analysis of splenic T-cell production of characteristic Th1 and Th2 type cytokines show that these patterns of cytokine secretion persist with time, and are detectable up to 46 weeks post-immunization, suggesting that

memory T-cells may maintain long term immunity. Following bacterial challenge, there was a broadening of the pattern of cytokine responses in Pa-immunized mice from a Th2 response 7 days after challenge to a mixed Th1/Th2 response thereafter. This indicates that although the Th phenotypes induced by pertussis immunization are stable, they are not immutable or "locked in", and is consistent with observations in immunized children (Ryan *et al.*, 1997, Ryan *et al.*, 1998).

Despite the inferior long-term protection in Pa-immunized mice, this vaccine still afforded a high level of protection, and this may reflect the switch from the Th2 response to a more mixed Th1/Th2 cytokine profile after challenge. In addition to the induction of persistent recall T-cell responses detected by cytokine secretion by antigen-stimulated spleen cells in vitro, *B. pertussis*-specific cytokine-secreting cells from the spleens of immunized mice were also enumerated by ELISPOT. At 3 months post-immunization, mice immunized with Pa and Pw had memory T-cells present in the spleen which secreted both the Th1 cytokine (IFN-γ) and the Th2 cytokine (IL-5). However, after 6 months, Pw-immunized mice had significantly greater numbers of IFN-γ secreting cells compared to IL-5 producing cells, and mice immunized with Pa had high numbers of IL-5 secreting cells relative to cells producing IFN-γ. These results correlate well with the T-cell responses detected by cytokine ELISA on supernatants of spleen cells stimulated with antigen *in vitro*.

Although *B. pertussis*-specific T-cells persist after immunization and $CD4^+$ T-cells are critical to vaccine-mediated immunity to *B. pertussis*, the long term protection observed is not necessarily an exclusively cell mediated phenomenon. In addition to the detection of memory cytokine-producing T-cells, memory B-cells specific for *B. pertussis* were also induced by immunization with Pa and Pw. These specific antibody-secreting cells were detected in the bone marrow at 9 months post-immunization, and persisted for at least 44 weeks after vaccination in peripheral blood and spleen. Taken together the data suggest that memory B- and T-cells can mediate persistent vaccine-induced protection against *B. pertussis* after the disappearance of specific IgG in the serum, and also suggest that cellular immunity may contribute to long term protection induced with Pa, as well as Pw.

The selective induction and maintenance of the Th1 response after immunization with Pw, together with their superior protection against *B. pertussis*

infection, is consistent with previous reports that Th1 cells play an important role in protective immunity (Redhead *et al.*, 1993). Activation of cellular mechanisms against intracellular *B. pertussis* (Mahon *et al.*, 1999, Friedman *et al.*, 1992), including stimulation of bacterial uptake and killing by macrophages and PMN, may be one mechanism whereby Th1 cells mediate their protective function. The Th1 cytokine IFN-γ has particular relevance for the immune protective mechanisms against *B. pertussis*. This cytokine plays a major role in controlling *B. pertussis* infection and in confining the bacteria to the mucosal site (Barbic *et al.*, 1997, Mahon *et al.*, 1997). Given the importance of IFN-γ in host resistance to *B. pertussis*, this study examined the contributions of two cytokines known to be involved in the induction of Th1 responses and IFN-γ production - IL-12 and IL-18. A large body of evidence has accumulated over recent years to indicate that IL-12 is one of the most important cytokines in the differentiation of naïve $CD4^+$ T-cells to the Th1 subset, and for stimulating the production of IFN-γ (Germann *et al.*, 1993, Schmitt *et al.*, 1994, Manetti *et al.*, 1993, Abbas *et al.*, 1996, Trinchieri, 1994, Seder *et al.*, 1993). Th1 responses are considered most relevant for the elimination of intracellular pathogens, and therefore it may also be assumed that IL-12, which favours the development of Th1 cells would play a role in the resolution of intracellular infections. Indeed, in experimental infections with intracellular pathogens such as *Leishmania*, *Toxoplasma* and *Listeria*, attenuation of the Th1 response through the elimination of IL-12 has revealed an obligatory role for this cytokine in the production of IFN-γ, and for the establishment of a Th1-type response (Mattner *et al.*, 1996, Gazzinelli *et al.*, 1993, Scharton-Kersten *et al.*, 1995). Similarly, IL-18, which is also an inducer of IFN-γ has been shown to play a role in the development of protective Th1 responses to a variety of pathogens (Sugawara *et al.*, 1999, Bohn *et al.*, 1998, Ohkusu *et al.*, 2000, Mastroeni *et al.*, 1999).

It has been demonstrated that IL-12 is induced following *in vitro* stimulation with live or heat-inactivated *B. pertussis*, and the addition of IL-12 to a Pa preparation enhances its protective efficacy by promoting the induction of Th1 cells and IFN-γ production (Mahon *et al.*, 1996). However, the exact role of IL-12 in the induction of immune responses responsible for the resolution of infection with

B. pertussis has not yet been clearly defined, and to date there is no evidence to suggest protection against *B. pertussis* involves the IFN-γ-inducing cytokine IL-18. In this study, it has been shown that addition of neutralizing IL-12 antibody to culture of spleen cells stimulated with *B. pertussis* completely inhibits *B. pertussis*-induced IFN-γ production. However, a similar finding was not observed with the addition of a neutralizing IL-18 antibody. This preliminary evidence demonstrated that *B. pertussis*-specific IFN-γ is induced via an IL-12 dependent mechanism, at least *in vitro*.

In certain systems, deficiency in IL-12 function leads to a shift from the normally observed Th1 response, to a more polarized Th2 response (Seder *et al.*, 1993). Given this and the fact that anti-IL-12 has the ability to block *B. pertussis*-induced IFN-γ production *in vitro*, we examined the role of IL-12 in *B. pertussis* infection *in vivo*, in an attempt to determine whether a lack of endogenous IL-12 would lead to impaired IFN-γ production and hence defective Th1 responses. During primary infection IL-12 deficient (IL-12$^{-/-}$) mice displayed a significant reduction in antigen-specific IFN-γ production compared to wild-type mice, with a concomitant increase in IL-5 levels. Although IL-12$^{-/-}$ mice displayed a shift towards a more mixed Th1/Th2 cytokine profile, compared to the characteristic Th1 response normally seen during infection of wild-type mice, a complete switch from a Th1 to a Th2 response was not observed. Lack of IL-12 in this system effected the clearance of the bacteria from the lungs, only very early in infection, indicating that IL-12 probably plays an important role during the induction phase of the immune response. Later in infection, IL-12$^{-/-}$ mice maintained the ability to clear the bacteria from the lungs with the same efficiency as the wild-type mice. Furthermore, in contrast to observations in IFN-γ R$^{-/-}$ mice, mice lacking IL-12 did not develop a disseminated infection, and blood and livers of these mice remained free of viable bacteria throughout the course of infection. Aerosol challenge of IL-12$^{-/-}$ and wild-type mice immunized with Pa or Pw demonstrated that lack of functional IL-12 did not effect protection against *B. pertussis* infection. Although IFN-γ production was significantly reduced in Pw-immunized IL-12$^{-/-}$ mice compared to wild-type mice, both knockout and control mice had antigen-specific

Th1 responses and displayed similar ability to clear the bacteria from the lungs. Pa-immunized knockout mice showed enhanced production of the Th2 cytokine IL-5, and these mice eliminated viable bacteria from the lungs, with the same efficiency as wild-type mice. Th2 responses have also been shown to be protective against respiratory challenge with *B. pertussis* (Mills *et al.*, 1998a). Furthermore, the results of this and other studies suggest that protection induced by Pa does not appear to be mediated by an IL-12-dependent mechanism (Mahon *et al.*, 1996). However, the observation that knockout mice immunized with the Th1-inducing Pw display undiminished anti-bacterial resistance is surprising, particularly in relation to the critical role attributed to IL-12 in the induction of IFN-γ and Th1 responses. The results suggest that in this system, $CD4^+$ T-cells have the ability, although impaired, to develop into Th1 cells and protect against challenge infection, despite the lack of IL-12.

In another series of experiments the role of IL-12 in clearance of a secondary infection was examined. IL-$12^{-/-}$ and wild-type BALB/c mice were infected with *B. pertussis* and were allowed to recover from infection. 10 weeks after the initial challenge, both groups of mice were rechallenged with *B. pertussis*, and then analyzed for cytokine production and their ability to clear the bacteria. Surprisingly, the IL-12 knockout group cleared the challenge infection at the same rate as wild-type mice. Although bacterial clearance was slightly delayed in the knockout mice, the numbers of viable bacteria in the lungs were not significantly different to those in wild-type mice. Cytokine responses were evaluated on the day of challenge and two weeks post-challenge. IL-$12^{-/-}$ mice and wild-type mice displayed a mixed Th1/Th2 cytokine profile on the day of challenge and 2 weeks post-challenge, with responding T-cells producing both IFN-γ and IL-5. However, knockout mice produced significantly lower levels of IFN-γ compared to BALB/c at these timepoints. This may account for the slight delay in bacterial clearance from the lungs of knockout mice.

The results of this study show that IL-12 does not have a significant effect on the protective mechanisms involved in the clearance of a primary infection with *B. pertussis*, or in the resolution of infection in mice primed by immunization or infection. This suggests that there is some degree of redundancy for the induction

of Th1 responsiveness, and alternative factors may function in the absence of IL-12. Therefore, the production of IL-18 by macrophages in response to *B. pertussis* was examined to assess if this cytokine played a role in IFN-γ induction in this system. The results showed that IL-18 is rapidly produced by splenic macrophages when infected with pertussis. Furthermore, this cytokine rapidly produced in the lungs of both BALB/c and IL-12$^{-/-}$ mice following respiratory challenge. High levels of IL-18 were detected in both groups of mice 4hrs post-challenge, and these levels remained elevated throughout the course of infection, up to 70 days after challenge. It is interesting to note that IL-18 production in the lungs of IL-12$^{-/-}$ mice is slightly elevated at day 7 post-challenge when compared with wild-type mice. At day 7, the numbers of viable bacteria in the lungs of these mice are significantly higher than those of wild-type mice, suggesting that in the absence of IL-12, IL-18 may be recruited for the induction of IFN-γ and the subsequent elimination of the bacteria from the lungs. Studies in other systems have also suggested a redundancy for IL-12 in IFN-γ production, and have speculated that IL-18 may act as an alternative sensitizing pathway in vivo (Schijns *et al.*, 1998, Piccotti *et al.*, 1998). Similarly, it appears that IL-12 is not a critical requirement for the differentiation of naïve T-cells towards a polarized Th1 phenotype during *B. pertussis* infection, and in this system, IL-12, although induced by the bacterium, is dispensable for the resolution of challenge infection. This suggests that an alternative IFN-γ inducing pathway occurs *in vivo* in the absence of IL-12, and may be mediated by IL-18.

Further evidence for the importance of IFN-γ and Th1 cells in immunity to *B. pertussis*, has been highlighted in this study through the investigation of the effect of helminth infection of the outcome of infection with *B. pertussis* or immunization with Pw. Access to mouse models of infection with *F. hepatica and B. pertussis*, which induce Th2 and Th1 responses respectively, provided an ideal system to examine the concept of Th1/Th2 cross-regulation *in vivo*. The helminth parasite *Fasciola hepatica* has been shown to induce a potent polarized Th2 response in this and other studies (O'Neill *et al.*, 2000). As previously discussed, recovery from natural infection with *B. pertussis* or immunization with Pw selectively induces Th1 cells (Mills *et al.*, 1993, Redhead *et al.*, 1993, Ryan *et al.*, 1997). Due to the prevalent occurrence of polyparasitosis or multiple infections,

particularly in developing countries, there has been an increased interest in the effect that one infection may have on another. It is well accepted that Th1 and Th2 cells have the ability to cross-regulate each others cytokine secretion and development *in vitro*. However, whether this crossregulation occurs between these two subsets of cells *in vivo* is less clearly defined. Although circumstantial evidence exists in favour of the latter, studies on the reciprocal regulation of Th subsets during infection *in vivo* have been rare both in humans and in animal models.

To examine the effect of *F. hepatica* infection on the immune response induced by infection with *B. pertussis*, mice were coinfected with both pathogens on the same day, and the course of infection was examined. Infection with *F. hepatica* resulted in delayed clearance of *B. pertussis* from the lungs of coinfected mice, and numbers of viable bacteria were significantly higher in mice infected with both parasite and bacteria. Examination of cytokine responses in these mice demonstrated that *B-pertussis*-specific IFN-γ production was almost completely abrogated in coinfected mice compared to mice infected with *B. pertussis* only. Given the importance of IFN-γ in the resolution of *B. pertussis* infection, the delayed bacterial clearance observed in these mice is almost certainly due to the downregulation of the protective Th1 response normally seen during infection. *F. hepatica* infection also has the ability to downregulate the protective Th1 response to *B. pertussis* after it has already become established. Mice infected with *B. pertussis* and allowed to recover form infection, and develop a Th1 response. They were then infected with *F. hepatica*. Infection with *F. hepatica* was also shown to significantly inhibit pertussis-specific IL-2 and IFN-γ production in these mice, indicating that the parasite has the ability not only to impair the bacterial-specific Th1 response at the induction phase, but can also debilitate this protective response after it has become established. Similarly, infection with *F. hepatica* was capable of downregulating the Th1 response induced by systemic immunization with Pw, and reducing its protective efficacy.

In IL-4 knockout mice the modulatory effect of *F. hepatica* infection *on B. pertussis*-specific IFN-γ production is abrogated, suggesting that IL-4 plays a major role in Th1 suppression and exacerbation of *B. pertussis* infection. These findings suggest that the suppression of anti-bacterial immunity during *F. hepatica* infection

is a result of bystander downregulation of *B. pertussis*-specific Th1 cells by the parasite-specific Th2 cells. *F. hepatica* has also been shown to induced the production of IL-10 *in vivo* (O'Neill *et al.*, 2000). This cytokine has been implicated in downregulation of cell-mediated immunity in response to *S. mansoni* (Sher *et al.*, 1991), and it is possible that IL-10 may act with IL-4 in this system in the suppression of *B. pertussis*-specific cytokine production, and Th2 immunomodulation. It is important to note that *B. pertussis* infection, or immunization with Pw had no effect on parasite-specific cytokine responses, or on the severity of fasciolosis determined by liver pathology. This indicates that at least in this model system Th2 responses have a dominant effect in Th1/Th2 crossregulation *in vivo*. However, protective immunity was not completely abrogated in mice infected with *F. hepatica*, and this can be explained that a mixed Th1/Th2 or a Th2 response may also confer protection against *B. pertussis* infection by a distinct but less effective mechanism, such as that shown by immunization with Pa (Mills *et al.*, 1998a).

Parasitic infections have frequently been associated with the preturbation of cell-mediated immunity and Th1 responses (Kullberg *et al.*, 1992, Pearce *et al.*, 1991, O'Neill *et al.*, 200 0). This immunosuppression may be mediated by cytokines, but immunoevasion strategies that downregulate host cell-mediated immunity and prolong parasite survival may also play a role. Molecules contained in the excretory-secretory products of *F. hepatica*, such as cysteine proteinases have been shown to cleave IgG at or near the hinge region, and to prevent the antibody-mediated attachment of immune effector cells to the parasite (Smith *et al.*, 1993, Carmona *et al.*, 1993). However, whether the excretory-secretory products of *F. hepatica* play an additional role to IL-4 in the suppression of Th1 responses to *B. pertussis* remains to be defined. Nevertheless, the observation of immunomodulation of protective type 1 responses during infection with Th2-inducing parasites has important implications for the outcome of concurrent infections and immunizations.

❖ Chapter 8 ❖

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A little learning is a dang'rous thing;
Drink deep, or taste not the Pierian spring:
There shallow draughts intoxicate the brain,
And drinking largely sobers us again

- ALEXANDER POPE