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MODULATION OF INNATE AND ADAPTIVE IMMUNE RESPONSES BY INTRANASALLY DELIVERED ADJUVANTS

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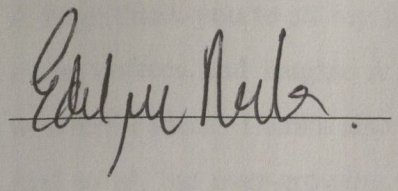

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Summary

Vaccines have had a significant impact on human health for over a hundred years, resulting in the prevention of many millions of deaths each year from infectious diseases. However in recent decades, the evolution of antibiotic resistant strains of bacteria, the emergence of new pathogens and widespread concerns over the safety of traditional vaccines have highlighted the requirement for the continued development and improvement of vaccination strategies. Alternatives to parenteral routes of vaccine delivery, such as intranasal immunization, may increase patient compliance and may assist in the elimination of pathogens that replicate on or enter via mucosal surfaces due to the induction of local immunity. New generation vaccines, based on recombinant proteins or DNA, are likely to be less reactogenic than traditional vaccines composed of live attenuated or killed pathogens or inactivated toxins. However, these vaccines are often less immunogenic, especially following mucosal delivery. There is therefore an urgent requirement for the development of potent and safe adjuvants that are effective for mucosal immunization.

This study examined the influence of chitosan and mutants of *E. coli* heat labile toxin (LT) as adjuvants for the induction of immune responses to intranasally delivered foreign antigens. It was found that chitosan augmented immune responses to diphtheria when delivered nasally to mice with a highly purified mutant of diphtheria toxin, CRM_{197}. Furthermore, formulation of CRM_{197} with chitosan induced potent anti-diphtheria toxin neutralizing antibody responses following intranasal delivery to adults previously immunized with conventional diphtheria toxoid. A non-toxic mutant, LTK63, and a partially toxic mutant, LTR72, of LT also significantly enhanced local and systemic immunity to CRM_{197} and to two novel meningococcal type B antigens following intranasal delivery in mice. Chitosan and lower doses of LTR72 were found to selectively enhance Th2 type responses to intranasally delivered CRM_{197}, whereas higher doses of LTR72 and LTK63 augmented both Th1 and Th2 type responses. However, the type of immune response induced using chitosan or mutants of LT as adjuvant was also influenced by additional factors, including the route of immunization, the nature and dose of the antigen and the dose of the adjuvant.

The adjuvanticity of molecules, such as chitosan and mutants of LT, may be related to their ability to enhance antigen uptake at mucosal surfaces. However, chitosan, LTR72 and LTK63 also augmented immune responses to parenterally delivered CRM_{197}. Furthermore, LTR72 and LTK63 induced the maturation of murine DC *in vitro*, indicating that the potent adjuvanticity of these molecules may also be related to their ability to non-specifically activate cells of the innate immune system. LTR72 and LTK63 differentially regulated LPS-induced pro-inflammatory cytokine and chemokine production by murine DC *in vitro*, which may partially explain the different T cell subtypes induced by these toxins *in vivo*.

The development of new mucosally delivered vaccines relies primarily on the identification of adjuvants that can enhance the immunogenicity of antigens without the induction of adverse effects. New generation vaccines may be possible using highly purified toxins or recombinant proteins delivered intranasally with adjuvants such as chitosan or mutants of LT. Furthermore, advances in our understanding of the immunological mechanisms of action of adjuvants such as these, may make it possible to design effective adjuvant-antigen combinations that are capable of inducing protective immunity against a variety of infectious diseases.

Abbreviations

ADP	Adenosine Diphosphate
AKP	Alkaline Phosphatase
APC	Antigen Presenting Cell
ARF	ADP-Ribosylation Factor
BMDC	Bone Marrow-derived Dendritic Cell
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CpG	cysteine-phosphate-guanosine motif
cpm	counts per minute
CREB	cAMP Response Element Binding Protein
CRM	Cross Reacting Material
CT	Cholera Toxin
CTL	Cytotoxic T Lymphocyte
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DT	Diphtheria Toxoid
ELISA	Enzyme Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Scanning
FCS	Foetal Calf Serum
FHA	Filamentous haemagglutinin
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HRP	Horse Radish Peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.m.	intramuscular
i.n.	intranasal
i.p.	intraperitoneal
ISCOM	Immune Stimulating Complexes
KLH	Keyhole Limpet Haemocyanin
LPS	Lipopolysaccharide

LT	Heat labile toxin of *Escherichia coli*
LTK63	Site-directed mutant of *E. coli* heat labile toxin, substitution of alanine for serine at position 63 of the catalytic site
LTR72	Site-directed mutant of *E. coli* heat labile toxin, substitution of arginine for alanine at position 72
MALT	Mucosal Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
MIP	Macrophage Inhibitory Protein
MPL	Monophosphoryl Lipid A
NALT	Nasal Associated Lymphoid Tissue
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor-κB
ng	nanogram
NK	Natural Killer Cell
OD	Optical Density
PBA	PBS/BSA/Azide
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
pg	picogram
PKA/C	Protein Kinase A/C
PLG	Poly(lactide-co-glycolide)
PMA	Phorbal Myristic Acid
PRN	Pertactin
PT	Pertussis Toxin
RANTES	Regulated on Activation Normal T-cell Expressed
RNA	Ribonucleic Acid
RPMI	Rosewell Park Memorial Institute
SE	Standard Error
STAT	Signal Transducer and Activation of Transcription
TCR	T Cell Receptor
TGF	Transforming Growth Factor
Th	T helper cell

TLR	Toll Like Receptor
TNF	Tumour Necrosis Factor
UV	Ultra-Violet

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CHAPTER 1

INTRODUCTION

1.1 General introduction

Vaccines have been one of the major success stories of the twentieth century. Since the discovery by Edward Jenner in 1796 that cowpox, or vaccinia, could induce protection against human smallpox, vaccines have been developed against a broad range of diseases, including diphtheria, whooping cough, polio, measles, mumps and rubella and have prevented millions of deaths each year from these infectious diseases. Due to the immunization programmes of the World Health Organization, smallpox disease was eradicated in 1979 and it is hoped poliomyelitis will be eliminated in the next few years. A new and very effective conjugated meningococcal serogroup C vaccine has been developed and introduced into Ireland and the UK in the last four years and used to vaccinate adolescents against the highly infective meningococcal C disease. However, there are still a number of diseases, including HIV, hepatitis C, meningococcus B and malaria, for which there are no effective vaccines available.

Traditional vaccines, generally based on live attenuated pathogens, killed whole cell organisms or inactivated toxins, were designed using an empirical approach, with little knowledge of the protective antigens or correlates of immunity. Nevertheless, this approach was highly successful and these vaccines were responsible for eliminating or significantly reducing the incidence of a number of infectious diseases. However, increases in antibiotic-resistant bacteria, the emergence of new pathogens and concerns over the safety of killed and attenuated vaccines have highlighted the requirement for new vaccines and for the refinement of existing vaccines to render then safer and more effective. In particular, the major obstacle to the continued use of live viral, whole-cell bacterial or inactivated toxin vaccines relates to safety concerns. Adverse reactions associated with traditional vaccines may result in a decrease in immunization rates and a related rise in infectious diseases. This was the case in Russia where concerns over adverse reactions, associated with diphtheria vaccines, resulted in a switch to a lower dose vaccine for primary vaccination of children and a concomitant resurgence in the disease (Hardy *et al.*, 1996, Galazka, 2000). More recently, outbreaks of measles in the U.K. have occurred due to a decrease in the uptake of the measles, mumps and rubella vaccine in infants, related to safety concerns of the vaccine.

Recent advances in our understanding of immunity to infectious diseases and in the identification of protective antigens on a range on pathogens, together with gene cloning and expression, have enabled a more rational approach to the development of

safer subunit vaccines consisting of specific antigenic components of the pathogen. Recently, an acellular pertussis vaccine has been developed that is composed of specific components of *Bordetella pertussis*, such as filamentous haemagglutinin and pertussis toxin, and is now widely used in a number of countries instead of the highly reactogenic whole-cell pertussis vaccine (Mills, 2001). However, for subunit vaccines to reach their full potential they require alternative methods of vaccine delivery, such as mucosal administration.

1.2 Mucosal immunization

Since the majority of human pathogens initially infect through the mucosal tissues, nasal or oral delivery of vaccines may facilitate the prevention of certain infectious diseases through the induction of local immune responses. In addition, mucosal immunization abrogates the need for injections and may reduce certain of the adverse reactions seen when the vaccines are administered by parenteral routes. However, to date only a few mucosal vaccines, mostly oral, have been approved for human use. These include poliovirus, *Salmonella typhi*, tetravalent rotavirus vaccine and intranasally delivered influenza vaccine. The nasal route has certain advantages over other mucosal routes of vaccine administration in that it allows for the rapid transport of proteins across the epithelium and it has an extensive lymphatic system and local blood supply. In addition, nasal immunization has often been shown to require lower doses of antigen compared to oral delivery and potent immune responses may be elicited in both local and distal mucosal sites, such as the genital tract (Nugent *et al.*, 1998, Partidos, 2000).

1.3 Immunity to infection

The immune system may be classified into both the innate and adaptive arms of immunity. Innate responses provide the first line of defence against pathogens, while the acquired or adaptive immune system, involving both humoral and cell-mediated immunity, usually develops after infection is established and may help in preventing infiltration of pathogens and assist in their elimination. In particular, acquired immunity is associated with the activation of antigen-specific T and B lymphocytes. Some of these lymphocytes may develop into memory cells that have the capacity to mount a rapid and effective immune response upon subsequent exposure to antigen.

The induction of a potent memory response is considered the basis of successful vaccination.

1.4 Innate immunity

The initiation of an immune response relies on innate immunity not only to provide the first line of defence against infection but also to direct the adaptive immune response. Innate defence against pathogens consists of both innate barriers, such as epithelium and mucous, and innate cells including macrophages and dendritic cells. Mucosal epithelial cells are joined by tight junctions, which allow the passage of ions and water but exclude larger peptides and macromolecules including those derived from bacteria, viruses and parasites (Madara *et al.*, 1990). In addition, the microvilli on mucosal surfaces are covered by mucin-like glcoproteins (Maury *et al.*, 1995), resulting in a thick layer that acts as a diffusion barrier and prevents direct contact of most bacterial and viral particles with the microvillus membrane (Frey *et al.*, 1996). Mucus secreted by glands and goblet cells of the airways assists in the elimination of foreign material and pathogens (Wanner, 1986).

If pathogens breach the epithelial defences, innate cells and soluble protein effectors may assist in their elimination. Innate cellular responses are mediated to a large extent by phagocytic cells such as polymorphonuclear neutrophils (PMN), macrophages and dendritic cells, which rely primarily on cytokines to regulate their effector functions. These cells may phagocytose and destroy pathogens, but additionally may secrete a variety of inflammatory mediators and cytokines that are important in the regulation of adaptive immune responses. Human PMNs have been shown to release a range of cytokines and chemokines including TNF-α, IL-1β, TGF-β1, IL-8, MIP-1α and MIP-1β (Cassatella, 1995). Recognition of pathogens by innate cells is achieved by means of cell-surface receptors expressed on the immune cells, including CD14, macrophage mannose receptor, scavenger receptors, integrins, C-reactive protein and Toll-like receptors (Medzhitov and Janeway, 1997, Aderem and Underhill, 1999). The most important killing mechanisms within phagocytes are the activation of the respiratory burst and the production of reactive oxygen intermediates, formed from the superoxide radical ($O2^-$) by decomposition, or reactive nitrogen intermediates, produced through the activity of inducible nitric oxide synthase (iNOS) (MacMicking *et al.*, 1997, Bogdan *et al.*, 2000).

Natural killer cells (NK), activated in response to type I interferons (IFN-α/β) or macrophage-derived cytokines, also play an important role in innate host defence responses to microbial infections (Biron and Brossay, 2001). These cells express a functional IL-2 receptor and once activated by IL-2 acquire enhanced cytotoxic activity (Frederick *et al.*, 1997, Yu *et al.*, 2000). In addition to IL-2, cytokines such as IL-12, IL-15 and IL-18 have been shown to be involved in the regulation of NK cell activity (Okamura *et al.*, 1998, Trinchieri, 1998, Liu *et al.*, 2000). NK cells may themselves provide an early source of immunoregulatory cytokines, including IFN-γ that may be induced by IL-2 (Trinchieri, 1995a). It has been shown that NK cells destroy target cells by at least three different mechanisms: secretion of cytotoxic cytokines and calcium-dependent or calcium-independent cytotoxicity. In the calcium-dependent pathway, cytotoxic granules, containing mainly perforin and granzymes, are secreted on to the surface of the target cell resulting in membrane and DNA damage. In contrast, calcium-independent cytotoxicity has been shown to cause apoptosis of target cells via receptor-ligand interactions between the target cell and NK cell (Moretta, 1997, Shresta *et al.*, 1998). The γδ T cell population, which is present in the gut, lungs and skin, is also involved in regulation of innate and acquired immune responses (Mak and Ferrick, 1998). Mice deficient in γδ T cells have been shown to develop fatal responses to the bacterium *Nocardia*, which may be due to their inability to mobilize neutrophils following aerosol delivery of bacteria to the lungs (King *et al.*, 1999).

The soluble effector components of innate immunity include type I interferons (IFNα/β), which are involved in innate protection against viruses (Durbin *et al.*, 2000), collectins such as mannose binding lectin (MBL) and mucosal-associated surfactant proteins A and D that promote phagocytosis (Lawson and Reid, 2000, Gadjeva *et al.*, 2001), natural antibodies and complement. The sera of non-immunized human and mice contain natural or spontaneous antibodies to toxins, bacteria and erythrocytes (Avrameas, 1991, Herzenberg and Kantor, 1993). These natural antibodies, so called because they are present even in the absence of apparent antigenic stimulation, have been shown to mediate direct neutralization of bacteria or viruses present in the circulation (Gobet *et al.*, 1988, Ochsenbein *et al.*, 1999) and may activate the complement cascade (Carroll, 1998). Most natural antibodies belong to the IgM isotype, although some natural antibodies of IgG and IgA isotype have also been identified (Avrameas, 1991). Natural antibodies have been shown to be important in

protection from mucosal influenza virus infection (Baumgarth *et al.*, 2000). The major soluble protein effector of innate immunity, complement, comprises an array of plasma proteins that are involved in opsonization of pathogens. Complement may be activated by three alternative pathways: the binding of collectin to certain antibody-antigen complexes or carbohydrates initiates the classical pathway (Holmskov *et al.*, 1994); the alternative pathway is triggered by interaction with microbial carbohydrate-rich particles lacking sialic acid (Fearon and Austen, 1980) and the mannose binding lectin (MBL) pathway is mediated by binding of MBL to carbohydrates on the pathogen surface (Gadjeva *et al.*, 2001). One of the cleavage products of the complement cascade is C5a, which acts as a potent mediator of inflammation. This peptide has been shown to act as a powerful chemoattractant for neutrophil and monocytes and to activate local mast cells and phagocytic cells (Harvath, 1991, Erdei *et al.*, 1997). In addition, C5a has been shown to be essential for mucosal host defence to *Pseudomonas aeruginosa* (Hopken *et al.*, 1996).

1.4.1 Pattern recognition receptors

Recognition of pathogens is mediated by a set of germline-encoded receptors that are referred to as pattern-recognition receptors (PRRs). These receptors recognize conserved molecular patterns, or pathogen-associated molecular patterns, which are shared by large groups of microorganisms (Medzhitov and Janeway, 1997). Toll-like receptors (TLRs) have been identified to function as PRRs and are essential components of the innate immune system (Akira *et al.*, 2001). The first Toll receptor was identified during the study of embryonic development in the fruit-fly *Drosophila melanogaster*. Subsequently, the role of Toll in innate immunity of adult flies was demonstrated, as Toll mutants were unable to mount effective anti-microbial responses and succumbed to overwhelming fungal infection (Lemaitre *et al.*, 1996). To date, at least 10 mammalian TLRs have been identified and shown to be required for responses to components of gram-positive and gram-negative bacteria, mycobacteria and fungi.

The first mammalian TLR identified, TLR4, was shown to be responsible for inducing inflammatory responses to LPS using strains of mice such as C3H/HeJ which are resistant to LPS (Poltorak *et al.*, 1998a, Hoshino *et al.*, 1999). C3H/HeJ mice and the C3H/HeN substrain were both derived from the progenitor strain, C3H/He. In contrast to both C3H/He and C3H/HeN mice, the C3H/HeJ strain exhibits natural tolerance to otherwise lethal doses of LPS, as well as an altered inflammatory

response. Macrophages from C3H/HeJ mice fail to produce inflammatory cytokines, including TNF-α, IL-1 and IL-6, in response to LPS (Morrison and Ryan, 1979). In addition, their splenic B cells do not proliferate after exposure to LPS (Hoshino *et al.*, 1999). Positional cloning of the *lps* gene in C3H/HeJ mice revealed a proline to histidine mutation within the cytoplasmic signalling domain of TLR4 (Poltorak *et al.*, 1998b, Qureshi *et al.*, 1999), and subsequent specific deletion of the gene confirmed the role of TLR4 in mediating inflammatory signals from LPS (Hoshino *et al.*, 1999). In addition, corroborating evidence of a genomic deletion and absent TLR4 transcription was observed in C57BL/10ScCr mice, another endotoxin-tolerant strain known to have a mutation at the *lps* locus (Poltorak *et al.*, 1998a). There have been conflicting reports on the ability of TLR2 to also function as a receptor for mediating responses to LPS (Kirschning *et al.*, 1998, Yang *et al.*, 1998, Heine *et al.*, 1999, Takeuchi *et al.*, 1999). Highly purified LPS was shown to be unable to stimulate cells through TLR2, and therefore it was suggested that some commercially available LPS contained small quantities of contaminants that are recognized by TLR2, and may explain any inflammatory responses induced by activating TLR2 with LPS (Hirschfeld *et al.*, 2000). However, another study has shown that murine TLR2 was activated by synthetic lipid A treatment, indicating that LPS could activate TLR2 with its lipid A portion (Musikacharoen *et al.*, 2001). The most convincing evidence that LPS signals through TLR4, and not TLR2, has come from studies using knockout mice. A knockout mutation of TLR2 had no effect on LPS signal transduction (Takeuchi *et al.*, 1999). In contrast, a knockout mutation of TLR4 inhibited LPS signalling (Hoshino *et al.*, 1999), indicating that TLR2 is not required for LPS signal induction. In addition to LPS, TLR4 is involved in the recognition of other bacterial pathogen-associated molecular patterns and a viral fusion protein (Underhill and Ozinsky, 2002). Since the discovery that TLR4 could mediate immune responses to LPS, several other TLRs have been discovered and their activators identified. These include TLR2, which mediates responses to peptidoglycan, zymosan and microbial lipoproteins (Schwandner *et al.*, 1999, Underhill *et al.*, 1999, Yoshimura *et al.*, 1999), TLR5, involved in the recognition of flagellin (Hayashi *et al.*, 2001), and TLR9, which recognizes CpG (Hemmi *et al.*, 2000). More recently, TLR7 has been shown to respond to small anti-viral imidazoquinoline compounds (Hemmi *et al.*, 2002) and it has been reported that TLR3 is required for mediating responses to double-stranded RNA (Alexopoulou *et al.*, 2001). Co-operation between different TLRs, such as

dimerization of TLR2 and TLR6, allows this restricted family of receptors to recognize an even broader spectrum of microbial molecules (Ozinsky *et al.*, 2000). Mucosal epithelial cells have been shown to express several TLRs, although they respond poorly to LPS stimulation (Frendéus *et al.*, 2001).

The intracytoplasmic domains of Toll and the mammalian IL-1R (Toll/IL-1R homology domain or TIR domain) are homologous, and both activate signalling pathways resulting in the recruitment of the cytoplasmic adaptor protein MyD88, activation of serine/threonine kinases of the IRAK family and ultimately the induction of proinflammatory cytokines and co-stimulatory molecules under the regulation of NF-κB (Rock *et al.*, 1998, Means *et al.*, 2000, Medzhitov and Janeway, 2000, Akira *et al.*, 2001). More recently, an additional molecule called Tollip has been proposed to interact with TIR domains and participate in the signalling events (Burns *et al.*, 2000). In addition to the MyD88-dependent pathway of NF-κB activation, there is evidence that other signalling pathways may be activated by TLRs. NF-κB activation is delayed, but not absent, in MyD88 deficient cells activated through TLR4, indicating that a MyD88-independent pathway may exist (Kawai *et al.*, 1999). A molecule called TIRAP (TIR adaptor protein) or MAL (MyD88-adapter-like) has been identified that interacts specifically with TLR4 and is thought may be responsible for this alternative pathway of NF-κB activation (Fitzgerald *et al.*, 2001, Horng *et al.*, 2001). The importance of TLRs in both innate and acquired immunity has been demonstrated in many studies. Signalling through TLRs results in the upregulation of inflammatory mediators such as TNF-α and IL-12, which may have important instructive roles in the regulation of adaptive immunity (Medzhitov and Janeway, 2000, Kadowaki *et al.*, 2001). MyD88-deficient mice injected with OVA in complete Freund's adjuvant failed to produce T cells that proliferated in response to antigen, indicating the importance of TLR signalling in the development of adaptive immune responses (Schnare *et al.*, 2001). TLR signalling may also result in the upregulation of microbial killing mechanisms of the innate immune system, through the induction of iNOS mRNA and the production of reactive nitrogen intermediates (Brightbill *et al.*, 1999, Byrd-Leifer *et al.*, 2001).

It was generally thought that the immune system functions in discriminating between self and non-self antigens and mounts a response against foreign or exogenous antigens (Janeway, 1992). However, it has also been suggested that endogenous danger signals, such as products released from damaged, stressed and

dying cells, may be capable of triggering the innate immune response (Gallucci and Matzinger, 2001). TLR4 may be activated by fibronectin, a molecule released by damaged tissue (Okamura *et al.*, 2001). In addition, heat shock protein 60, which is released from stressed and damaged cells, is recognized by the same TLR (Ohashi *et al.*, 2000, Vabulas *et al.*, 2001). It would therefore now appear that both exogenous and endogenous signals mediate the activation of the innate immune system.

The TLRs are likely not the only PRRs involved in the innate immune system. TREM-1 receptor is activated by exposure to bacteria such as *Pseudomonas aeruginosa* or *Staphylococcus aureus*, triggering a signalling cascade that results in inflammation through a pathway that does not involve NF-κB (Bouchon *et al.*, 2001).

1.4.2 Dendritic cells

Macrophages, in addition to their role as phagocytic effector cells, play a critical role in regulating innate and acquired immunity by acting as antigen-presenting cells (APC). Targeting of antigen to scavenger receptors on macrophages has been shown to increase the antigen's immunogenicity (Abraham *et al.*, 1995). In addition, B cells may capture antigen via surface immunoglobulin and process it for presentation with MHC class II to $CD4^+$ T cells (Watts *et al.*, 1991). Dendritic cells (DC), however, are the most potent types of APC specialized in antigen capture and T cell priming. The induction of DC maturation is critical for the generation of antigen-specific primary T cell responses (Hart, 1997, Banchereau and Steinman, 1998). DCs have been shown to express TLR-2, -3, -4, -5, -6 and -9, and respond to various pathogen-associated molecular patterns such as LPS, bacterial lipoproteins, peptidoglycan and CpG dinucleotides, which reflects their role in detecting microorganisms and activating the acquired immune system (Muzio *et al.*, 2000, Hertz *et al.*, 2001, Krug *et al.*, 2001, Visintin *et al.*, 2001). Indeed, TLR3, which has been implicated in the recognition of double-stranded RNA, is uniquely expressed on DCs (Muzio *et al.*, 2000, Alexopoulou *et al.*, 2001).

Immature DC reside in unperturbed tissues where they capture and process antigen and are found to preferentially express inflammatory chemokine receptors, such as CCR1, CCR2, CCR5, CCR6 and CXCR1. Upon exposure to inflammatory mediators, such as TNF-α, IL-1 or LPS, to danger signals delivered by the innate immune system or to T cell-derived stimuli, like CD40L, DC transform into cells that are capable of delivering co-stimulatory signals to naïve T cells. This transformation

correlates with a downregulation of inflammatory chemokine receptors, such as CCR5 and CCR6, and an upregulation of the chemokine receptors CCR7 and CXCR4, which enables homeostatic chemokines such as CCL19 and CCL21 to recruit mature DC to draining afferent lymphatics and subsequently to T cell areas of the lymph nodes (Dieu *et al.*, 1998, Sallusto *et al.*, 1998a, Sozzani *et al.*, 1998, Sozzani *et al.*, 2000). Mature DC lose their ability to acquire and process antigen but acquire potent antigen-presenting capacity as a consequence of enhanced surface expression of MHC class II and co-stimulatory molecules (Steinman, 1991, Banchereau and Steinman, 1998). Maturation of DCs is strengthened during interactions with T cells via CD40-CD40L signalling (Caux *et al.*, 1994, Kitajima *et al.*, 1996).

Although all DCs function in antigen processing and T cell activation, there is evidence that they differ in lineage origin, phenotype and in aspects of their function. To date, at least two distinct murine DC subsets have been identified: the myeloid and lymphoid subsets. These subsets differ in phenotype, localization and function. Splenic myeloid DC are preferentially located in the marginal zone bridging channels of the spleen, whereas lymphoid DC are found localized in T cell rich areas of the periarteriolar lymphatic sheaths (PALs) (Steinman *et al.*, 1997, Leenen *et al.*, 1998). Both myeloid and lymphoid murine DC express high levels of CD11c, MHC class II and the co-stimulatory molecules CD80 and CD86, but may be separated on the basis of their expression of CD11b and CD8α respectively. CD8α is expressed as a homodimer on the lymphoid, but not the myeloid, subset. The splenic CD11b+ myeloid DC subset has been subdivided further into $CD4^+$ and $CD4^-$ subsets (Wu *et al.*, 1996, Vremec *et al.*, 2000). Flt3ligand (Flt3L) has been shown to induce the expansion of both lymphoid and myeloid DC from bone marrow progenitors (Maraskovsky *et al.*, 1996, Pulendran *et al.*, 1997). In contrast, GM-CSF preferentially expands the myeloid DC subset *in vivo* (Pulendran *et al.*, 1999). Murine lymphoid DC, in contrast to myeloid DC, have been shown to secrete significant levels of IL-12 and to promote predominant Th1 type responses (Maldonado-Lopez *et al.*, 1999, Pulendran *et al.*, 1999, Hochrein *et al.*, 2001). Paradoxically in humans, myeloid DC (DC1) induce Th1 type responses whereas plasmacytoid-cell-derived DC (DC2) of lymphoid origin stimulate polarization of naïve T cells towards a Th2 phenotype (Rissoan *et al.*, 1999). However, viruses and CD40 ligation may activate plasmacytoid DCs to induce a Th1 response (Cella *et al.*, 2000), indicating that the capacity of DCs to polarize naïve T cell responses may be regulated primarily by the nature of maturation signals

and not by the lineage or subset of DC (Fig. 1.1). Whelan *et al.* (2000) showed that LPS was capable of inducing DC to acquire a phenotype (DC1) that promoted the differentiation of naïve T cells towards a Th1 phenotype, whereas a filarial nematode-secreted product induced the maturation of dendritic cells capable of driving Th2 responses (DC2) (Whelan *et al.*, 2000). Another study has shown that the kinetics of DC maturation may also influence their ability to polarize T cell responses. Activated DCs only secreted IL-12 up to 16 hours after stimulation and at later time-points these DCs may therefore be able to prime Th2 and non-polarized T cell responses (Langenkamp *et al.*, 2000). It has also been shown that DCs may be activated to polarize naïve T cells towards a regulatory phenotype that characteristically produces high levels of IL-10. Filamentous haemagglutinin (FHA) from *Bordetella pertussis* stimulated murine DCs to direct naïve T cells into a regulatory (Tr1) phenotype (McGuirk *et al.*, 2002).

Monocytes have the capacity to differentiate into either macrophages or DCs *in vitro*. It has been shown that exogenously added IFN-γ and endogenously produced IL-10 may function as mutually opposed regulators that govern whether $CD14^+$ monocytes differentiate into DCs or macrophages respectively (Koski *et al.*, 2001). In particular, IL-10 may inhibit the upregulation of co-stimulatory molecules and TNF-α and IL-12 production by DCs, and thereby impair their ability to generate Th1 responses (Cavani *et al.*, 2000, Corinti *et al.*, 2001). IL-10 treated DCs may also induce a state of antigen-specific tolerance (Steinbrink *et al.*, 1997), and it has been suggested that autocrine IL-10 may prevent spontaneous DC maturation *in vivo* (Corinti *et al.*, 2001).

1.4.3 Signal transduction in innate cells

The transcription factor NF-κB is one of the key mediators of innate inflammatory responses. The members of the NF-κB family include p50, p52, p65 (RelA), RelB and c-Rel. NF-κB is maintained in the cytoplasm of cells as inactive homo- or hetero-dimers, via interactions with a class of inhibitory proteins called IκBs. In response to a variety of stimuli, IκBs are phosphorylated and degraded, allowing the translocation of NF-κB dimers to the nucleus where they are involved in inducing the transcription of target genes including those encoding inflammatory cytokines and chemokines and adhesion molecules (Baldwin, 1996, Ghosh *et al.*, 1998). NF-κB is

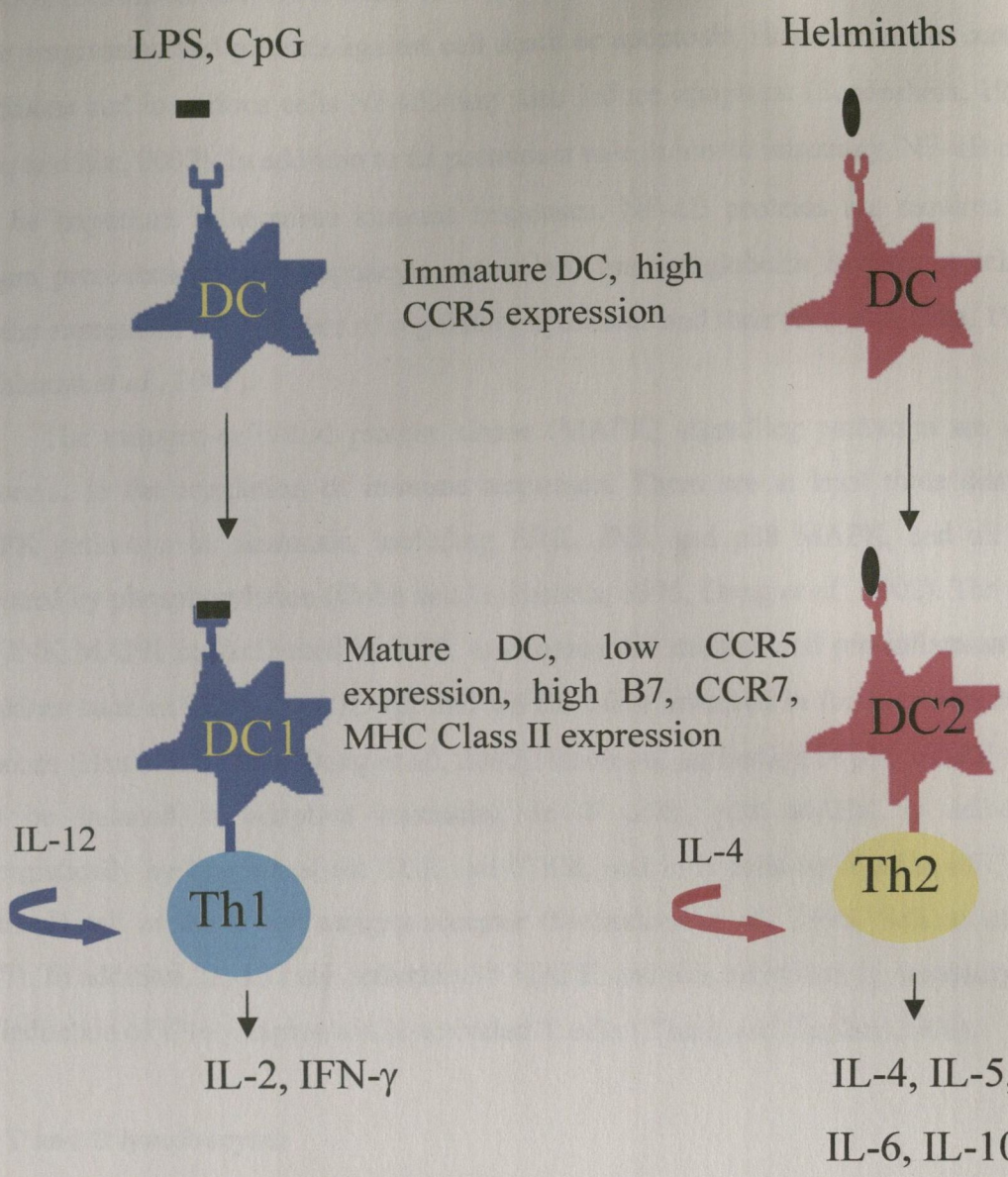


Figure 1.1. Pathogen-derived molecules stimulate dendritic cells to direct Th1 or Th2 polarization. Immature DC express high levels of the chemokine receptors, CCR5 and CCR6, and readily acquire antigen. Pathogen-derived molecules such as LPS may stimulate DC maturation, which is accompanied by a loss in CCR5 and CCR6 expression, enhanced expression of CCR7, B7 molecules and MHC Class II and an increased ability to process and present antigen. The ability of DC to direct distinct Th1 or Th2 type responses appears to depend on distinct activation signals provided by different pathogens, for example, LPS and glycoproteins from helminths may seletively drive Th1 and Th2 responses respectively.

also involved in antiviral immune responses through the regulation of interferon gene expression (Pitha *et al.*, 1998, Stark *et al.*, 1998). In most cells, NF-κB supports cell cycle progression and protects against cell death or apoptosis. However, under certain conditions and in various cells NF-κB may also induce apoptosis (Sonenshein, 1997, Karin and Lin, 2002). In addition to its prominent role in innate immunity, NF-κB may also be important in acquired immune responses. NF-κB proteins are required for antigen presentation and lymphocyte activation, immunoglobulin isotype switching and the expression of a number of regulatory cytokines and their receptors (Sha, 1998, Yoshimura *et al.*, 2001).

The mitogen-activated protein kinase (MAPK) signalling pathways are also important in the regulation of immune responses. There are at least three distinct MAPK pathways in mammals, including ERK, JNK and p38 MAPK, and all are activated by phosphorylation (Cobb and Goldsmith, 1995, Dong *et al.*, 2002). The p38 and JNK MAPK are activated by LPS, environmental stresses and pro-inflammatory cytokines such as TNF-α and IL-1β, and are therefore involved in the innate immune response (Han *et al.*, 1998, Dong *et al.*, 2002). However, activation of p38 MAPK may also be induced in adaptive immunity. In T cells, p38 MAPK is activated synergistically by ligation of the TCR and CD28, and in B cells by ligation of CD40 on the B cell or the B cell antigen receptor (Sutherland *et al.*, 1996, Salmon *et al.*, 1997). In addition, IL-12 may activate p38 MAPK and this activation is necessary for the induction of IFN-γ expression in activated T cells (Zhang and Kaplan, 2000).

1.5 T and B lymphocytes

B and T lymphocytes use somatic gene arrangement to generate diverse surface antigen receptors, enabling them to target specific pathogenic antigens with high affinity. The receptors on B lymphocytes recognize conformations of native antigen and respond by secreting immunoglobulin or antibody of a certain specificity (Rajewsky, 1996). Interaction of a heterodimeric T cell receptor (TCR αβ) with an antigenic ligand or peptide bound to a self MHC molecule, in the presence of co-stimulation, is necessary for T lymphocyte activation, clonal expansion and a variety of effector functions (Davis and Bjorkman, 1988, Crabtree, 1989).

1.5.1 Local antibody responses

Immunoglobulins (Igs) are produced by B lymphocytes in a vast range of antigen specificities, each B cell secreting Ig of a single specificity. Most pathogens elicit a humoral immune response characterised by an early rise of IgM, followed by affinity maturation and isotype switching to IgG, IgA, IgD and IgE. CD40-CD40L interactions have been shown to be crucial for Ig switching (Kawabe *et al.*, 1994, Castigli *et al.*, 1995). In addition, a number of cytokines are involved in isotype switching. IL-4 induces class switching to IgE and IgG4 in humans (Lundgren *et al.*, 1989) and IgE and IgG1 in mice (Stavnezer, 1996), whereas IFN-γ has been shown to preferentially induce isotype switching to IgG1 in humans and IgG2a and IgG3 in mice (Stavnezer, 1996). TGF-β is also involved in murine IgG2b induction (McIntyre *et al.*, 1993).

IgA is the most abundantly produced immunoglobulin in mammals. It constitutes a small component of serum Ig, predominately monomeric IgA, but most IgA is secreted across mucosal membranes into external fluids (Kerr, 1990). IgA secreting plasma cells are found predominately in the lamina propria tissue beneath the epithelium and from here IgA antibodies can be transported across the epithelium to its external surface. The polymeric Ig receptor (pIgR), also called the secretory component, mediates the generation of secretory antibodies. The polymeric form of IgA binds specifically to pIgR, resulting in internalisation of the complex and transport through the cytoplasm of the epithelial cell via a transport vesical to the apical surface in a process known as transcytosis. The pIgR is then cleaved at the surface releasing secretory IgA (sIgA), composed of dimeric IgA covalently associated with the extracellular portion, or secretory component, of pIgR (Mostov, 1994, Brandtzaeg, 1995). The Fc region of sIgA is wrapped within the secretory component and as a result this secretory immunoglobulin is highly resistant to proteases (Mestecky and Russell, 1986). Secretory IgA may protect against pathogens that replicate on or enter via mucosal surfaces by blocking their attachment or colonization and by neutralizing surface-acting toxins (Vaerman *et al.*, 1985, Brandtzaeg, 1995, Lamm *et al.*, 1995). In addition, intraepithelial IgA may inhibit intracellular virus replication (Mazanec *et al.*, 1992). Monomeric serum IgA, on the other hand, has been shown to activate complement and respiratory activity in phagocytic cells (Kerr, 1990, van Egmond *et al.*, 2000). A number of studies have shown that IgA antibodies, as the sole effectors of immunity, can mediate protection against a broad range of viral and bacterial

infections (Apter *et al.*, 1993, Czinn *et al.*, 1993, Blanchard *et al.*, 1995, Cotter *et al.*, 1995). However, other reports using IgA knockout mice have demonstrated that IgA is not essential for protective mucosal immunity to several infections including influenza (Parr *et al.*, 1998, Mbawuike *et al.*, 1999).

The anti-microbial action of antibodies may involve interactions with cellular receptors that bind to the Fc portion of Igs. Interaction of IgA with Fc_α receptors is considered important for effective protection against pathogens. In particular, IgA may interact with $Fc_\alpha R1$ (CD89) on human monocytes, macrophages, granulocytes and eosinophils (Maliszewski *et al.*, 1990, Monteiro *et al.*, 1990, Morton *et al.*, 1996). Immature DCs have also been shown to take up sIgA, independently of CD89, via a C-type lectin pathway (Heystek *et al.*, 2002).

Either Th1 or Th2 cells or a combination of both may be important in supporting antigen-specific IgA responses. Both IL-4 and TGF-β promote isotype switching to IgA *in vitro* (Kunimoto *et al.*, 1988, Coffman *et al.*, 1989). IL-10 and IL-2 may synergise with TGF-β to increase the efficiency of Ig switching (Coffman *et al.*, 1989, Defrance *et al.*, 1992). However, TGF-β would appear to be the crucial factor in inducing a switch to IgA. CD40-CD40L interactions, in the absence of other stimuli, are sufficient to efficiently induce switching to IgA due to the action of endogenous TGF–β, secreted by CD40L-induced B cells, on DNA recombination events (Zan *et al.*, 1998). More recent *in vivo* studies have shown that TGF-β or TGF-β receptor knockout mice exhibit low levels of IgA antibody forming cells (van Ginkel *et al.*, 1999, Cazac and Roes, 2000). In addition, the Th2 type cytokines, IL-4 and IL-5, have been shown to increase the numbers of IgA^+ B cells post isotype switching and to enhance secretion of IgA (Beagley *et al.*, 1988, Kunimoto *et al.*, 1988, Harriman *et al.*, 1988). The Th1 cytokine, IFN-γ, has been implicated in the induction of pIgR, required for transport of sIgA (Sollid *et al.*, 1987, Piskurich *et al.*, 1997).

Although IgA is the most important immunoglobulin in resolving mucosal infections, other classes of antibodies have been shown to interact with antigens and generate mucosal immune responses. IgM, like IgA, is polymeric and can bind to the pIgR, resulting in the production of secretory IgM. Secretory IgM antibodies may contribute to mucosal protection, especially in neonates and adults with IgA deficiency (Mellander *et al.*, 1984, Brandtzaeg *et al.*, 1987, Brandtzaeg, 1995). IgG has also been shown to play a role in mucosal immunity. A higher specific activity of IgG has been

observed during intestinal inflammation, suggesting that IgG may be synthesised locally (Macpherson *et al.*, 1996). Despite its low concentrations in extracellular fluid, IgE is a significant mucosal immunoglobulin (Negrao-Correa *et al.*, 1996) and is specificially involved in responses to parasites and in allergic conditions (Bell, 1996, Oettgen and Geha, 2001).

1.5.2 T cell mediated immunity

Cellular immunity, mediated by $CD4^+$ and $CD8^+$ T cells, plays a major role in protection against infectious pathogens. $CD4^+$ and $CD8^+$ T cells respond to antigens processed and presented by APCs by distinct mechanisms. $CD4^+$ T cells recognize exogenous antigen in association with major histocompatability complex (MHC) class II molecules whereas $CD8^+$ cytotoxic T lymphocytes (CTL) recognize endogenous antigen in association with MHC class I molecules. CTL represent an important immune effector mechanism for clearance of many intracellular viruses, bacteria and parasites. Following recognition of processed viral and bacterial fragments, presented on the infected cell surface, CTL destroy the infected cells by the release of granule contents such as perforin and granzyme or by a process of Fas-mediated lysis (Kuwano and Arai, 1996). The induction of local mucosal CTL responses may be critical for the elimination of some pathogens that are transmitted through mucosal surfaces. Belyakov *et al.* (1998) showed that gp160-specific CTL in the mucosa was essential for resistance against mucosal viral challenge with a recombinant virus strain expressing gp160 from HIV. In contrast, parenterally elicited gp160-specific CTL was ineffective at preventing viral transmission from the mucosa into the deeper tissues (Belyakov *et al.*, 1998). $CD4^+$ T helper cells, on the other hand, provide help for B cells in antibody production, but also function in cell mediated immunity as producers of cytokines that are involved in a variety of immunoregulatory functions or have a direct effect on invading pathogens.

Naïve $CD4^+$ or $CD8^+$ peripheral T cells circulate through the blood and peripheral lymphoid organs until they encounter an APC bearing an antigenic ligand bound to a self-MHC molecule. These naïve T cells require two signals for their activation. The first is provided by MHC-bound antigen and TCR/CD3 interactions (Davis and Bjorkman, 1988). The second co-stimulatory signal involves interactions between CD28 receptor, expressed on naïve T cells, and its ligands B7.1 (CD80) and B7.2 (CD86) on APCs (Linsley and Ledbetter, 1993, June *et al.*, 1994). The co-

stimulatory signal provided by CD28, in conjunction with TCR stimulus, induces IL-2 production by precursor T helper cells, resulting in subsequent cell division and a variety of effector functions and thereby preventing apoptosis (Crabtree, 1989, Boise *et al.*, 1995). More recently, a third member of the B7 family has been identified called B7-H1 that does not bind to CD28. The B7-H1 co-stimulation pathway mainly induces the production of IL-10, with lesser amounts of IL-2 induced (Dong *et al.*, 1999). Interruption of the CD28-B7 interaction, both *in vivo* and *in vitro*, has been shown to inhibit immune responses and in some instances to result in T cell tolerance or anergy (Sperling and Bluestone, 1996). CTLA4, a second ligand for CD80 and CD86, provides a negative signal to the T cell, thus limiting immune responses (Chambers and Allison, 1997). Another novel gene has been identified in mice belonging to the B7 family called B7 homolog (B7h) or B7-related protein 1 (B7RP-1), that can also function as a co-stimulatory ligand for T cells (Swallow *et al.*, 1999, Yoshinaga *et al.*, 1999). In contrast to CD80, CD86 and B7-H1, B7h does not bind to CD28 or CTLA4, but binds to a CD28 homolog called inducible T cell costimulator (ICOS). ICOS is not expressed on naïve T cells but is induced after activation (Hutloff *et al.*, 1999), suggesting that B7h may be involved in sustaining local inflammatory responses in mice, by providing co-stimulatory signals to T cells previously activated through the CD28 pathway. Additional interactions, such as CD40-CD40L signalling, are also important in T cell activation. In particular, CD40-CD40L interactions are thought to play a critical role in CD4-dependent generation of $CD8^+$ T cell responses (Bennett *et al.*, 1998, Schoenberger *et al.*, 1998).

1.5.3 T cell differentiation

The $CD4^+$ T cell population may be divided into type 1 and type 2 T helper (Th1/Th2) subpopulations, first described by Mosmann and colleagues, on the basis of their function and the cytokines they secrete (Mosmann *et al.*, 1986). $CD8^+$ T cells may also differentiate into Tc1 or Tc2 cell subsets, which produce corresponding cytokines to their $CD4^+$ counterparts but in addition possess cytotoxic activity (Sad *et al.*, 1995). Th1 cells secrete IFN-γ and TNF-β, and mediate cell-mediated immunity such as delayed-type hypersensitivity, macrophage activation and inflammatory responses (Mosmann and Coffman, 1989). However, Th1 cells can also provide helper function for specific IgG subclasses, particularly those involved in opsonization and virus neutralization (Mahon *et al.*, 1995). Th2 induction is considered to be the default

pathway and in the absence of microbial products that stimulate IL-12 production, antigens that lack immunomodulatory activity, such as many soluble recombinant proteins, will induce Th2 type cells. Type 2 Th cells secrete IL-4, IL-5, IL-10 and IL-13 and support IgG1 subclass and IgE secretion (Mosmann *et al.*, 1986, Romagnani, 1994, Stavnezer, 1996). The Th1 and Th2 subpopulations of $CD4^+$ T cells are reciprocally regulated by a range of cytokines produced by the T cells themselves or by cells of the innate immune system. Up-regulation of one type of response has also been shown to often result in down-regulation of the other (O'Garra and Murphy, 1994, Seder and Paul, 1994, Abbas *et al.*, 1996).

In addition to Th1 and Th2 cell types, Th0 populations have also been described that secrete combinations of Th1 and Th2 cytokines (Firestein *et al.*, 1989, Elson *et al.*, 1995). However, it has also been suggested that the Th0 population may represent a transitory precursor subset, which will eventually undergo terminal differentiation into Th1 or Th2 cells (Nakamura *et al.*, 1997). Exogenously added IL-4 and endogenously produced IFN-γ have been shown to promote the development of these cells *in vitro* (Miner and Croft, 1998). More recently, a number of regulatory T cell subsets have been identified. Activation of human or mouse CD^+ T cells, *in vivo* or *in vitro*, in the presence of IL-10 results in the generation of T cells that secrete significant amounts of IL-10, moderate amounts of IFN-γ, TGF-β and IL-5, but low amounts of IL-2 and no IL-4. This novel $CD4^+$ T cell subset, called Tr1, has been shown to inhibit antigen-specific activation of naïve autologous T cells (Groux *et al.*, 1997). IL-10 blocks the nuclear translocation of the p65, but not p50, subunit of NF-κB, leading to an accumulation of inhibitory p50 homodimers in the nucleus and an associated inhibition of the expression of pro-inflammatory genes (Volk *et al.*, 2001). This inhibition of NF-κB activity may lead to an increased generation of IL-10 producing regulatory T cells. In addition, another regulatory T cell type that secretes high levels of TGF-β, termed Th3, has been described (Roncarolo and Levings, 2000). These cells have been shown to provide protection in a murine model of experimental autoimmune encephalomyelitis (EAE) (Chen *et al.*, 1994).

Protective immunity against certain diseases is dependent on the differential induction of Th1 or Th2 responses. It has been shown that for several intracellular pathogens, protective immunity requires the development of Th1 responses and pathology may develop if Th2 type cytokines are induced. While the induction of Th1 responses is desirable for immunization against many intracellular pathogens, Th2

responses may be more important in protection against pathogens such as *Helicobacter pylori* and helminth parasites and in the control of immune-mediated diseases such as organ-specific autoimmune disorders and Crohn's disease. Th2 type responses have also been associated with allergic and atopic manifestations (Romagnani, 1994, Spellberg and Edwards, 2001).

A variety of factors appear to be important in the regulation of T cell differentiation *in vivo*. Several studies have shown that the antigen dose (Mosmann and Sad, 1996, Sakai *et al.*, 1999, Ruedl *et al.*, 2000) and route of administration (Barnard *et al.*, 1996, Conway *et al.*, 2001) may differentially regulate the induction of $CD4^+$ Th subtypes. In addition, co-stimulatory molecules may provide signals for the selective differentiation of Th1 or Th2 cells. Antibodies against CD80 and CD86 have been shown to selectively inhibit the development of Th1 and Th2 responses *in vivo* and *in vitro*, suggesting that CD80 and CD86 differentially enhance Th1 and Th2 type responses (Freeman *et al.*, 1995, Kuchroo *et al.*, 1995). However, B7 molecules have also been reported to have the opposite, or no, effect on T cell differentiation (Lanier *et al.*, 1995, Lenschow *et al.*, 1995). OX40, a member of the TNF receptor family, co-stimulates T cell proliferation and cytokine production *in vitro* (Godfrey *et al.*, 1994, Gramaglia *et al.*, 1998). It has been proposed that OX40 co-stimulation favours the development of Th2 responses, through enhancement of IL-4 production (Flynn *et al.*, 1998, Ohshima *et al.*, 1998). Indeed, OX40-deficient mice are severely impaired in their ability to induce a Th2 type response (Jember *et al.*, 2001). However, OX40- and OX40 ligand (L)-deficient mice also exhibit impaired IFN-γ production, indicating that OX40/OX40L interactions participate in enhancing both Th1 and Th2 responses (Gramaglia *et al.*, 2000, Murata *et al.*, 2000). Cytokines, which act through various transcription factors, are considered the key mediators in the selective differentiation of Th1 and Th2 cells. In addition, chemokines have been shown to play an important role in the induction of $CD4^+$ Th subtypes.

1.5.4 Cytokine milieu in T cell differentiation

The cytokines in the environment in which T cell differentiation take place have a significant influence on the initial response (O'Garra, 2000). IL-12 is required for the development of IFN-γ secreting CTL *in vitro* and for driving the differentiation of $CD4^+$ T cells into Th1 type cells (Hsieh *et al.*, 1993, Trinchieri, 1994, Sad *et al.*, 1995). In addition, IL-12 may be important in the maintenance of Th1 responses. In

several studies, Th1 responses have been shown to decrease rapidly in the absence of IL-12, resulting in a loss of protective immunity to intracellular pathogens such as *Leishmania* and *Toxoplasma* (Park *et al.*, 2000, Yap *et al.*, 2000). Microbial products such as LPS stimulate IL-12 production from APCs through the activation of PRRs. This IL-12 then induces the production of IFN-γ from NK cells and antigen-activated T cells, resulting in the differentiation of Th precursors towards a Th1 phenotype (Trinchieri, 1995b). IL-12 may also induce expression of CD40 on DCs. Further IL-12 production may be sustained during communication of DCs with activated T cells via CD40-CD40L interactions, resulting in the augmentation of the developing Th1 response (Cella *et al.*, 1996, van Kooten and Banchereau, 2000). Indeed, CD40 and CD40L knockout mice are unable to induce protective Th1 responses following infection with *Leishmania major*, which is related to their inability to induce the production of IL-12 from macrophages (Campbell *et al.*, 1996). Recently it has been shown that IL-1β may act conjointly with CD40L to induce high levels of IL-12 mRNA from human monocyte-derived DC *in vitro* (Wesa and Galy, 2001). IL-12 is a heterodimer of p35 and p40 subunits and therefore to achieve the production of bioactive IL-12 diverse stimuli must coordinate the expression of both the IL-12 subunits. IL-12 p40 is usually made in excess of p35 and consequently the amount of IL-12 heterodimer assembled is restricted by the extent to which APCs make p35 (Schoenhaut *et al.*, 1992, Snijders *et al.*, 1996).

IL-4, on the other hand, is generally thought to be responsible for the differentiation of naïve precursor T cells into Th2 type cells. IL-4 may also inhibit the development of Th1 type responses, even in the presence of IL-12 (Hsieh *et al.*, 1993) or IL-12 signalling (Nishikomori *et al.*, 2000). The cellular source of early IL-4 production remains to be defined. Cells of the innate immune system, including NK cells, basophils and mast cells, may secrete IL-4 after stimulation (Seder and Paul, 1994, Chen and Paul, 1997). Naïve T cells have also been shown to secrete significant quantities of IL-4, prior to receiving an IL-4-inducing signal, and thereby driving their own differentiation towards a Th2 phenotype (Noben-Trauth *et al.*, 2000, Ouyang *et al.*, 2000). However, it has been shown that *Leishmania* infected IL-$4^{-/-}$ mice are still capable of generating Th2 type responses (Noben-Trauth *et al.*, 1996). Furthermore, type II DC can induce Th2 cell development *in vitro* in the absence of detectable IL-4 (Rissoan *et al.*, 1999).

IL-18, in synergy with IL-12, has been shown to be important in the development of Th1 responses *in vivo* (Kohno *et al.*, 1997, Robinson *et al.*, 1997). However, it has also been reported that IL-18 either alone or with IL-4 may induce $CD4^+$ Th2 differentiation and exacerbate Th-2 mediated disease in *Leishmania* infected BALB/c mice (Xu *et al.*, 2000). IL-13 has also been shown to promote Th2 responses during *Leishmania major* infection (Matthews *et al.*, 2000). Analogous to the role of IL-12 in driving the polarization of Th1 type responses, IL-10 appears to be a crucial cytokine for the generation of Tr1 cells *in vivo* and *in vitro*. It has been shown that a virulence factor of *Bordetella pertussis*, filamentous haemagluttinin, inhibited IL-12 production and augmented IL-10 secretion by immature DC, enabling the DC to direct naïve T cells into a regulatory Tr1 type phenotype (McGuirk *et al.*, 2002).

1.5.5 Signalling in T cell differentiation

The differentiation and function of the T lymphocyte lineage are regulated by signalling pathways, and involve lineage-restricted cell-surface receptor, intracellular signalling molecules and transcription factors. TCR ligation induces three major signalling pathways in T cells: protein kinase C (PKC) activation, calcium/calcineurin signalling and mitogen activated protein kinase (MAPK) signalling (Cantrell, 1996). The patterns of TCR signalling induced during the activation of naïve T cells may subsequently influence their differentiation into Th1 or Th2 effector cells. Stimulation of PKC activity or partial blockade of calcineurin has been shown to favour type 2 differentiation, while stimulation of calcium signalling, partial inhibition of PKC, or high levels of MAPK activity have been shown to favour the type 1 pathway in both $CD4^+$ and $CD8^+$ T cells (Noble *et al.*, 2000, Noble *et al.*, 2001). It would now appear that the TCR complex is associated with cytoplasmic tyrosine kinases, which initiate signals that determine the specific outcome of the immune response. In the presence of CD28 co-stimulation, the generation of plasma membrane microdomains called rafts at the site of TCR triggering may increase the stability of TCR-induced tyrosine phosphorylation of signal-transduction molecules. This in effect causes amplification of TCR-induced signals and may allow naïve T cells to respond to lower levels of triggered TCRs (Simons and Toomre, 2000, Viola, 2001).

Some of the STAT family of proteins and other transcription factors are important signalling effectors for the development of Th1 and Th2 responses and may represent in part the transcriptional basis for differential cytokine expression by Th

subsets (Murphy *et al.*, 2000). Induction of STAT4 by IL-12 is required for Th1 differentiation (Jacobson *et al.*, 1995, Kaplan *et al.*, 1996a, Thierfelder *et al.*, 1996), whereas STAT6 induced by IL-4 (Kaplan *et al.*, 1996b, Takeda *et al.*, 1996) and, as more recently shown, STAT5a (Kagami *et al.*, 2001) are important for the development of Th2 cells. $CD8^+$ T cells have also been shown to require STAT4 activation for IL-12-dependent IFN-γ production (Carter and Murphy, 1999). Other transcription factors, such as T-box expressed in T cells (T-bet) (Szabo *et al.*, 2000) and GATA-3 (Zheng and Flavell, 1997), have been reported to be required for Th1 and Th2 cell differentiation respectively. GATA-3 has been shown to induce a wide range of Th2 type cytokines in developing (Zheng and Flavell, 1997, Ferber *et al.*, 1999) and committed (Lee *et al.*, 2000) Th1 cells, and may inhibit the production of IFN-γ (Lee *et al.*, 2000, Ouyang *et al.*, 2000). Similarly, T-bet has been shown to inhibit Th2 differentiation (Szabo *et al.*, 2000). Another transcription factor, C-Maf, augments the induction of IL-4 via transactivation of the IL-4 promoter (Ho *et al.*, 1996). It would appear that cytokines and cytokine-induced STAT factors, such as STAT-4 and STAT-6, are important in the initiation of antigen-dependent Th cell differentiation (Wurster *et al.*, 2000), while transcription factors, such as GATA-3, C-Maf and T-bet, are required for the maintenance of the differentiated phenotype in the absence of further antigen stimulation (Glimcher and Murphy, 2000, Murphy *et al.*, 2000). Gene transcription by differentiated Th1 and Th2 cells is elicited by secondary contact of differentiated T cells with antigen and is mediated by inducible transcription factors such as NFAT (Rao *et al.*, 1997, Agarwal *et al.*, 2000).

T1/ST2, an orphan receptor with sequence homology to IL-1R, is found preferentially expressed on murine Th2 type cells, and is important for the regulation of Th2 effector functions *in vivo* (Lohning *et al.*, 1998, Xu *et al.*, 1998a, Coyle *et al.*, 1999). The expression of T1/ST2 on Th2 cells may be enhanced by the addition of exogenous IL-5, IL-6, IL-1 and TNF-α *in vitro* (Meisel *et al.*, 2001). Inhibition of T1/ST2 signalling has been shown to abrogate Th2, but not Th1, cytokine production *in vivo* and to reduce the severity of Th-2 mediated eosinophilic immunopathgology in a murine model of respiratory syncytial virus infection (Coyle *et al.*, 1999, Walzl *et al.*, 2001). In contrast, the IL-18 receptor (IL-18R) is expressed on activated Th1 cells and is involved in the regulation of IFN-γ production, IL-12R expression and Th1 type responses *in vivo* (Xu *et al.*, 1998b).

1.6 Chemokines

Chemokines are 8- to 10-kDa inducible and secreted proteins that comprise the largest mammalian cytokine superfamily, and are required to attract monocytes and immature DCs to sites of inflammation, direct maturing APC to lymphatic vessels, bring T cells and APCs together within lymphoid organs and recruit effector T cells to sites of inflammation (Cyster, 1999, Campbell and Butcher, 2000). Recent studies have also supported the role of chemokines in B cell development and maturation (Ngo *et al.*, 1998, Ghia *et al.*, 1999). Chemokines have been classified into two major (CC and CXC) and two minor (C and CX_3C) families, based on the configuration of cysteine residues near the NH_2-terminus (Zlotnik and Yoshie, 2000). Alternatively, they may be classified as homeostatic (constitutive) or inflammatory (inducible) chemokines in accordance with their cell-surface expression and function. Homeostatic chemokines are expressed constitutively on cells and are thought to be responsible for the trafficking of lymphocytes under conditions of homeostasis. In contrast, inflammatory chemokines are specifically induced at sites of inflammation and appear to play a role in the recruitment of lymphocytes to peripheral tissues in response to immunological challenge (Baggiolini, 1998, Sallusto *et al.*, 1998b).

Naïve T cells express the chemokine receptors CXCR4 and CCR7, whereas subsets of effector or memory T cells express CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CXCR5 and CCR9 (Bleul *et al.*, 1997, Sallusto *et al.*, 1997, Campbell *et al.*, 1999, Sallusto *et al.*, 1999, Zabel *et al.*, 1999, Sallusto *et al.*, 2000, Ebert and McColl, 2002). The prototypic chemokine associated with Th2 type responses is CCL11 (eotaxin-1), although IL-4 has been shown to stimulate the production of other chemokines such as CCL24 (eotaxin-2), CCL26 (eotaxin-3) and CCL13 (MCP-4) (Li *et al.*, 1999, Shinkai *et al.*, 1999). In addition, there is evidence that the chemokine CCL2 (MCP-1) induces IL-4 expression and is critical for the development of Th2 responses (Lukacs *et al.*, 1997, Gu *et al.*, 2000). In a similar manner to IL-4, CCL2 may also negatively regulate Th1 type responses. The CCL11 receptor, CCR3, is expressed on some Th2 cells, but not Th1 cells, and is regulated by IL-4 (Sallusto *et al.*, 1997). CCR4 and CCR8 chemokine receptors have also shown to be preferentially expressed on Th2 type cells (O'Garra *et al.*, 1998), although CCR4 may also be expressed on skin-homing Th1 and Th2 cells (Campbell *et al.*, 1999). In contrast, some receptors such as CCR1, CCR5 and CXCR3 are mainly expressed on Th1 type cells

(Bonecchi *et al.*, 1998, Sallusto *et al.*, 1998b, Sallusto *et al.*, 2000). More recently it has been shown that the chemokine receptor CXCR6 is a differential marker for Th1 and Tc1 cell subsets (Kim *et al.*, 2001).

Chemokines may also be important in mediating mucosal immune responses. Infection with respiratory syncytial virus has been shown to induce the secretion of chemokines such as IL-8, MCP-1 and CCL5 (RANTES) from human nasal- and adenoid-derived epithelial cells (Saito *et al.*, 1997, Olszewska-Pazdrak *et al.*, 1998). In addition, CCL5 has been shown to induce lymphocyte migration into the nasal mucosa of allergic subjects (Kuna *et al.*, 1998). Lymphotactin and CCL5 are also capable of enhancing mucosal and systemic immunity to co-delivered antigens in mice, suggesting they may play important roles in the induction and development of mucosal immunity (Lillard *et al.*, 1999, Lillard *et al.*, 2001).

1.7 Mucosal immunity

Mucosal immune responses are elicited mainly in organised mucosal lymphoid follicles, called mucosal-associated lymphoid tissue (MALT), which lack a lymphatic supply of antigen but can sample antigen directly from epithelial surfaces. Foreign material is preferentially taken up by MALT through specialised areas of the follicle-associated epithelium, the membrane (M) cells (Neutra, 1998). These specialised epithelium cells sample antigen unspecifically and by receptor-mediated uptake and are thought to be capable of processing and presenting antigen. However, MHC class II expression is very weak on human intestinal M cells (Bjerke and Brandtzaeg, 1988) and there is little evidence for APC function of these cells. The best-studied mucosal inductive sites are the gut-associated lymphoid tissues (GALT) but additional mucosal responses may be elicited in other tissues including bronchus-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT). Small soluble antigens may penetrate the nasal epithelium and interact with immune cells, including dendritic cells and macrophages. Drainage is then to the superficial cervical lymph node and subsequently to the posterior lymph nodes. Mucosal epithelial cells have been shown to secrete a range of cytokines, including IL-1, IL-6, IL-18 and IL-8, which may be involved in the induction and regulation of mucosal immunity (Schuerer-Maly *et al.*, 1994, McGee *et al.*, 1995, Panja *et al.*, 1995).

1.8 Mucosal adjuvants and delivery systems

Mucosally delivered subunit vaccines are often poorly immunogenic because they are highly purified and therefore do not contain the immunomodulatory bacterial or viral components, which are often present in traditional live or killed vaccines. These components provide the danger signals for activation of innate cells, such as DC, the maturation of which is required for the induction of specific subtypes of antigen-specific T cells. The development of effective mucosal vaccines is dependent on the use of antigen delivery systems that protect the antigen from degradation and enzymatic digestion and enhance uptake by targeting to epithelial and M cells at mucosal surfaces and on the inclusion of adjuvants that will stimulate innate immune responses and direct the appropriate acquired immune responses.

Immunological adjuvants were originally described by Ramon as 'substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone' (Ramon, 1924). It is now more than 70 years since it was shown that substances such as oil and lecithin could increase the activity of anti-toxin to diphtheria and tetanus. Since then many compounds have been evaluated as adjuvants for commonly used vaccines. However, toxicity and adverse reactions have precluded the use of many of these candidates and aluminium-based mineral salts, or alum, remain the only adjuvants approved by the FDA and used routinely in human vaccines (Gupta and Siber, 1995). Although alum has gained universal approval for use in human vaccines, it is often ineffective for purified or recombinant antigens, it is not desirable for mucosal delivery and it is generally poor at inducing cell-mediated immunity (Gupta, 1998a). Alum has been shown to preferentially enhance Th2 type responses to co-administered antigen (Brewer *et al.*, 1996), and may therefore not be suitable for the generation of protective immunity to many intracellular pathogens and viruses. Furthermore, alum has been reported to elicit toxic effects such as stimulation of granulomas and enhanced IgE antibody responses and has been associated with allergic reactions in some subjects (Gupta, 1998a, Relyveld *et al.*, 1998, Malakoff, 2000). Therefore, mucosal administration of antigens requires the development of adjuvants and delivery systems to augment the strength of the immune response and influence the type of cellular and antibody responses induced, so as to direct the immune response to the desired route as part of vaccine design strategy, while at the same time minimizing reactogenicity and adverse effects.

1.8.1 Microparticles

Microparticles, prepared from the biodegradable polymer poly (lactide-co-glycolide) (PLG), may act as efficient delivery systems for entrapped protein or polysaccharide antigens, viruses, plasmid DNA or peptides. The potential of PLG microparticles as a delivery system relies on their capacity to release entrapped antigen over extended periods (Gupta *et al.*, 1998b) and on their ability to deliver antigen directly to phagocytic APC. Following mucosal administration, microparticles are taken up by M cells and allow exogenous antigen to gain access to the endogenous route of processing for presentation to Class I restricted T cells (Moore *et al.*, 1995, Thomas *et al.*, 1996). In mice, oral immunization with PLG microparticles has been shown to induce potent mucosal and systemic immunity to a range of entrapped antigens (Brayden and Baird, 2001). Furthermore, in systemically primed animals, oral immunization with microparticles containing entrapped simian immunodeficiency virus (SIV) induced protective immunity against intravaginal challenge with SIV (Marx *et al.*, 1993). Although to date microparticles have generally been used for the oral delivery of vaccines, they also show potential for nasal delivery of antigens, through activation of APC and T cells in nasal associated lymphoid tissue and draining lymph nodes (Heritage *et al.*, 1998, Vajdy and O'Hagan, 2001). Intranasal immunization with microparticles has been shown to induce protection against challenge with *Bordetella pertussis* (Cahill *et al.*, 1995), ricin toxin (Yan *et al.*, 1996), *Streptococcus pneumoniae* (Trolle *et al.*, 2000) and pneumonic plague (Eyles *et al.*, 2000).

Entrapment of antigen in PLG microparticles has been shown to switch the CD4+ T cell response induced with soluble protein antigens from a predominant Th2 to a Th1 subtype (Moore *et al.*, 1995, Conway *et al.*, 2001). It has also been shown that intranasal or intragastric immunization with HIV-1 gp120 (Moore *et al.*, 1995) or peptides representing measles virus CTL epitopes (Partidos *et al.*, 1999), entrapped in microparticles, may effectively prime CTL responses *in vivo*. Microparticles may therefore be important mucosal adjuvants for the induction of cellular immune responses to intracellular pathogens. However, the size of the particles may also affect the outcome of the immune response; larger particles were found to be more effective for the induction of cellular immunity while smaller particles favoured the induction of antibody responses (Conway *et al.*, 2001).

Microparticles have also shown potential as adjuvants for DNA vaccine delivery. Cationic PLG microparticles with adsorbed plasmids have previously been shown to strongly enhance the potency of DNA vaccines following intramuscular immunization (Singh *et al.*, 2000). More recently, cationic microparticles have been shown to enhance local and systemic cell-mediated and humoral immunity against HIV-1 gag following intranasal immunization (Vajdy and O'Hagan, 2001). *In vitro* studies demonstrated that bone marrow-derived DC may take up microparticles encoding HIV-1 gag DNA and present the antigen to a gag-specific T cell hybridoma (Denis-Mize *et al.*, 2000), indicating that the effectiveness of microparticles *in vivo* may be as a consequence of efficient delivery of the adsorbed plasmid into DC or other APC.

1.8.2 Monophosphoryl lipid A (MPL)

MPL, a derivative of LPS, retains much of the immunostimulatory properties of LPS without the inherent toxicity. It is primarily derived from *Salmonella minnesota* and has been widely used as an adjuvant for the parenteral administration of antigens. MPL has also been extensively evaluated in the clinic and Phase III trials are now underway for several infectious diseases. The adjuvant activity of MPL is primarily attributed to its ability to activate APC and elicit cytokine production, in particular IL-12, by DC and macrophages, resulting in the induction of antigen-specific cellular immunity and enhancement of complement fixing antibodies (Moore *et al.*, 1999, Ismaili *et al.*, 2002). Calcium mobilization, mitogen-activated protein kinase activation and NFκB transcription factor were induced after stimulation of human monocyte-derived DC with high doses of MPL. In addition, MPL induced up-regulation of surface molecules, such as CD80 and CD86, on these DC (Ismaili *et al.*, 2002). MPL is also capable of acting directly on T cells, likely through interaction with TLRs, by increasing their intracellular calcium and upregulating CD40L expression (Ismaili *et al.*, 2002).

Due to its ability to stimulate the release of cytokines such as IL-12 by APC, MPL may be particularly beneficial in immunizations against many viruses and intracellular bacteria, where Th1 type responses are required for pathogen elimination. Intranasal immunization with influenza virus antigens formulated with MPL induced antigen-specific IgA, CTL responses and antibody responses suggestive of Th1-type immunity (Baldridge *et al.*, 2000). However, both Th1 and Th2 type cytokines have

been induced *in vitro* using MPL-treated human (Ismaili *et al.*, 2002) and murine (De Becker *et al.*, 2000) DC. While MPL alone can augment humoral and cellular immune responses to foreign antigens, it has been found to be more effective when combined with other adjuvants or delivery systems. For example, MPL with QS21 was found to significantly enhance Th1 responses to HIV gp120 recombinant glycoprotein (Moore *et al.*, 1999) and to activate CTL to tumour-specific peptides (Silla *et al.*, 1999) or herpes simplex virus protein (Mikloska *et al.*, 2000) *in vitro*. MPL has also been shown to be effective as an adjuvant for the augmentation of mucosal and systemic immune responses to intranasally delivered liposome-formulated *Streptococcus mutans* crude glucosyltransferase (Childers *et al.*, 2000).

1.8.3 Saponins

Triterpenoid glycosides or saponins, derived from the bark of a Chilean tree *Quillaja saponaria*, have been shown to have immunostimulatory and adjuvant properties. Quil A, a purified mixture of Quillaja saponins, is known to have potent adjuvant activity and has been widely used as an adjuvant for veterinary vaccines. However, Quil A is a surface-active agent and has been shown to display potent haemolytic activity *in vitro* (Kensil, 1996). Saponins have been shown to intercalate into cell membranes forming pores (Glaueri *et al.*, 1962), and thereby possibly allowing antigens to gain access to the endogenous route of antigen presentation for CTL induction. QS21, a purified saponin with low toxicity, has been reported to be a potent adjuvant for CTL induction and for the enhancement of Th1 type cytokines and antibodies of the IgG2a subclass (Kensil, 1996, Moore *et al.*, 1999). A number of clinical trials have been performed using QS21 as an adjuvant for cancer vaccines and for infectious diseases, including HIV-1, influenza, herpes, malaria and hepatitis B (Kensil, 1996). However, higher doses (200 μg or higher) have been associated with significant adverse reactions in humans. Saponins have also been purified from root extracts of *Polygala tenuifolia*, termed onjisaponins A, E, F and G. These saponins have been shown to display potent adjuvant activity when administered intranasally in combination with influenza vaccines or diphtheria, tetanus and pertussis vaccines. In addition, haemolytic activity could only be detected at higher doses compared to QS21, suggesting that onjisaponins may be safer than both Quil A and QS21 as vaccine adjuvants (Nagai *et al.*, 2001).

Quil A saponins have been incorporated into lipid vesicles to generate another class of lipid-based adjuvant termed immune stimulatory complexes (ISCOMs). Within the ISCOM structure, the Quil A is bound to cholesterol and is not free to interact with cell membranes, resulting in significantly reduced haemolytic activity of the saponins (Soltysik *et al.*, 1995). To date, ISCOMs have also been shown to induce protective immunity to more than 20 pathogens encompassing viruses, bacteria and parasites (Morein *et al.*, 1999). ISCOMs are capable of promoting the induction of CD8+ CTL and CD4+ Th1 cells. This appears to reflect the ability of these complexes to facilitate transport of antigen into the cytoplasm for access to the endogenous route of processing and to stimulate IL-12 production from cells of the innate immune system (Smith *et al.*, 1999). Th1 type or mixed Th1/Th2 type responses have been observed following immunization with ISCOMs and influenza virus antigens (Villacres-Eriksson *et al.*, 1992, Sjolander *et al.*, 1997a), ovalbumin (Maloy *et al.*, 1995, Sjolander *et al.*, 1997b), herpes simplex virus type 1 glycoproteins (Hassan *et al.*, 1996) and Epstein-Barr virus (Dotsika *et al.*, 1997). In most studies the levels of antigen-specific Th2 type cytokines were low. However, ISCOMs may also induce high levels of Th2 type cytokines; OVA-ISCOMs activated T cells have been shown to produce levels of IL-4 comparable to those obtained from cells primed with OVA in alum (Sjolander *et al.*, 1997b).

1.8.4 Live vectors

Live vector vaccines, consisting of attenuated strains of bacteria or viruses that express foreign antigens of unrelated pathogens, have been shown to be effective mucosal delivery systems (Morrow *et al.*, 1999, Mielcarek *et al.*, 2001). Due to their inherent adjuvant activity, live attenuated microbial vectors are highly immmunogenic and have potential for the expression of many unrelated foreign antigens. The use of live attenuated micro-organisms as vectors has not always been very successful at generating antibody responses to the foreign insert (Killeen *et al.*, 1999), but, because they undergo limited replication at target sites of mucosal immunization, live attenuated vectors provide an effective means of inducing CD8+ T cell responses. Attenuated *Salmonella* spp. are particularly attractive as potential live vectors because of their potent immunogenicity and their ability to deliver antigens via mucosal surfaces. Intranasal immunization with attenuated *Salmonella* spp. vectors, carrying a plasmid encoding fragment C of tetanus toxin, has been shown to generate high

proliferative T cell responses against the foreign antigen (Pasetti *et al.*, 2000). Two *Salmonella* serovars have been most widely used as vectors, *Salmonella typhimurium* in mice and *Salmonella typhi* in humans. Recombinant *Salmonella typhimurium* strains producing human papillomavirus type 16 virus-like particles (Benyacoub *et al.*, 1999) or hepatitis B virus core antigen (Hopkins *et al.*, 1995) have been shown to induce antibodies specific to the heterologous antigen following intranasal immunization in mice. Protective immunity has been generated against *Helicobacter pylori* following intranasal immunization with attenuated *Salmonella typhimurium* producing urease A and B subunits (Corthesy-Theulaz *et al.*, 1998).

However, safety concerns and environmental considerations still negates the use of many mucosally delivered vector candidates such as *Salmonella* spp., therefore commensal bacteria have begun to receive attention as safe non-invasive vectors. Nasal delivery of fragment C of tetanus toxin, expressed as a surface-exposed protein on *Lactobacillus plantarum*, was shown to induce secretory IgA antibody responses and antigen-specific T cell activation in draining lymph nodes (Shaw *et al.*, 2000). Powerful expression systems for the surface display of recombinant proteins have been developed in *Staphylococcus carnosus* and *Staphylococcus xylosus* and used for the display of various epitopes, including *Plasmodium falciporum* peptides (Hansson *et al.*, 1992, Samuelson *et al.*, 1995), a fragment of diphtheria toxin (Fromen-Romano *et al.*, 1999), epitopes from human respiratory syncytial virus (Cano *et al.*, 2000) and the cholera toxin B subunit (Liljeqvist *et al.*, 1997). Antibody responses to the surface-displayed immunogens have been reported following intranasal administration of the recombinant bacteria. A new approach to live vector vaccines has been the insertion of antigens from human viruses or bacteria into plant viruses. Nasal or oral administration of chimeric plant virus particles induced potent mucosal and systemic immune responses to a fibronectin-binding protein of *Staphylococcus aureus* (Brennan *et al.*, 1999).

1.8.5 Lipid vesicles

Liposomes, or bilayered phospholipid membrane vesicles have the capacity to adjuvant entrapped, surface-linked or admixed antigens. Antigen-specific IgG and local IgA responses have been shown to be enhanced after intranasal administration of inactivated measles virus with liposomes (de Haan *et al.*, 1995) and following nasal delivery of BSA-associated liposomes (Aramaki *et al.*, 1994) in mice. Formulation of

Streptococcus mutans antigen into liposomes has also been shown to enhance local secretory IgA responses following intranasal delivery in humans (Childers *et al.*, 1999). Virosomal influenza vaccine, comprising antigens from influenza virus incorporated in the membranes of phosphatidylcholine-containing liposomes, administered intranasally with *Escherichia coli* heat labile toxin (LT), enhanced virus specific IgA responses in human volunteers (Glück *et al.*, 1999). Formulation of antigen into liposomes may also induce antigen-specific cellular immune responses, including CD4+ Th1 cells and CD8+ CTL. Liposome-formulated *Yersinia pestis* vaccine induced strong IFN-γ responses, with no IL-4 detected, after intranasal administration in mice (Baca-Estrada *et al.*, 2000). Liposomes encapsulating OVA were shown to elicit IFN-γ secretion that was considerably stronger using negatively charged liposomes compared to neutral liposomes (Aramaki *et al.*, 1995).

1.8.6 CpG motifs of bacterial DNA

Bacterial plasmid DNA may constitute a very powerful adjuvant for systemic and mucosal immunity due to the presence of unmethylated CpG motifs, which are generally absent from mammalian DNA. These adjuvants are most often given in the form of synthetic oligodeoxynucleotides (CpG-ODN) that provide a broad adjuvant effect, including stimulation of B cells to proliferate and secrete immunoglobulin and direct activation of monocytes and macrophages to upregulate the expression of surface molecules such as MHC II, CD80 and CD86 and to secrete various cytokines and chemokines (Davis *et al.*, 1998, McCluskie and Davis, 2000). CpG-ODN have been shown to induce the maturation of DC and to trigger the secretion of cytokines, such as TNF-α, IL-6 and IL-12, from these cells *in vitro* (Jakob *et al.*, 1998, Sparwasser *et al.*, 1998, Hartmann *et al.*, 1999). It has recently been shown that cellular responses to CpG DNA are mediated by binding to TLR9 (Hemmi *et al.*, 2000). CpG-ODN have been shown to induce cytokines such as IL-12, TNF-α and IFN-γ, with little secretion of Th2 type cytokines, and therefore may be considered as immunoadjuvants where a Th1 type response is desired. In contrast, non-CpG–ODN, which also has adjuvant activity at mucosal sites, would appear to selectively induce Th2 type responses (McCluskie *et al.*, 2001a). It has been shown that CpG DNA may augment humoral and cell-mediated systemic and mucosal responses to hepatitis B surface antigen following intranasal administration to mice. It was also found that CpG

and cholera toxin could act synergistically to provide potent antibody responses, which were stronger than when either adjuvant was used alone (McCluskie and Davis, 1998).

1.8.7 Cytokines

Soluble cytokines regulate many of the molecular and cellular events during an immune response, including the selective induction of Th1 and Th2 cells, and therefore recombinant cytokines have been assessed as vaccine adjuvants. Indeed, cytokines themselves are likely to play an important role in the enhancement and modulation of immune responses by adjuvants (Brewer and Alexander, 1997). In theory, certain cytokines should be capable of selectively enhancing the induction of IgG, IgA, CTL, Th1 or Th2 responses at mucosal surfaces. Boyaka *et al.* (1999) showed that mucosally delivered IL-6 and IL-12 enhanced antigen-specific serum antibody responses to co-administered protein antigen, but only IL-12 was able to trigger antigen-specific secretory IgA responses. Furthermore, when tetanus toxoid was used as antigen, both IL-6 and IL-12 promoted protective immunity against lethal challenge with the toxin (Boyaka *et al.*, 1999). The mucosal adjuvant activity of IL-12 was also demonstrated using an influenza vaccine consisting of haemagglutinin and neuraminidase, where IL-12 was shown to enhance IgG and secretory IgA antibody responses and to offer protection against challenge with influenza virus (Arulanandam *et al.*, 1999). Intranasal administration of a DNA vaccine, encoding HIV antigens, together with an IL-2 expression plasmid has been shown to induce a strong antigen specific Th1 response (Xin *et al.*, 1998). IL-12 has also been shown to augment the Th1 type response when administered nasally with a tetanus toxoid and cholera toxin vaccine (Marinaro *et al.*, 1999). The potent pro-inflammatory cytokines, IL-1α and IL-1β, were found to enhance antigen-specific serum IgG, vaginal IgG and IgA, systemic delayed-type hypersensitivity and T cell responses when administered intranasally with OVA or tetanus toxoid antigens (Staats and Ennis, 1999). The use of IL-12, IL-1α and GM-CSF as nasal adjuvants for an HIV immunogen has been shown to be associated with the increased expression of MHC II and CD80 on nonlymphocytes within the NALT (Staats *et al.*, 2001).

Mucosal delivery of certain cytokines is also being assessed for the treatment of Th1 mediated inflammatory responses. Intranasal delivery of DNA encoding IL-10 resulted in diminished delayed type hypersensitivity reactions in mice primed with herpes simplex virus (Chun *et al.*, 1999). In addition, combined nasal administration of

autoantigen and IL-10 suppressed experimental allergic encephalomyelitis in rats (Xu *et al.*, 2000).

Lymphotactin and RANTES chemokines have also been evaluated as adjuvants for mucosal vaccines. Nasal administration of lymphotactin and OVA has been shown to enhance OVA-specific systemic IgM, IgG, IgA and IgE and secretory IgA antibody responses and to induce mixed Th1/Th2 type responses (Lillard *et al.*, 1999). Similarly, RANTES enhanced OVA-specific serum IgM, IgG and IgA, but not IgE, antibody responses and secretory IgA responses following intranasal administration. Analysis of antibody subclasses demonstrated that IgG2a was the main IgG subclass induced, indicating a predominant Th1 type response (Lillard *et al.*, 2001).

1.8.8 Chitosan

Chitosan is a polysaccharide comprising copolymers of glucosamine and N-acetylglucosamine that can be derived by the partial deacetylation of chitin, a natural material found in the exoskeletons of various crustaceans and insects. Chitosan itself is also found in some yeast and fungi (Illum, 1998). Chitosan is insoluble at neutral and alkaline pH values but forms salts with organic and inorganic acids. In solution, the amino functions of chitosan are protonated and as a result the soluble polysaccharide carries a positive charge. Chitosan salts may therefore bind strongly to negatively charged materials such as cell surfaces and mucus. These properties of chitosan have been exploited for the delivery of a variety of pharmacological drugs (Illum *et al.*, 1994, Illum, 1998, Thanou *et al.*, 2001). Chitosan-based formulations have been reported to greatly improve the absorption of drugs from the nasal cavity, thought to be due to the ability of chitosan to bind to negatively charged sites on cell surfaces and tight junctions and allow the paracellular transport of large hydrophilic compounds by opening the tight junctions of mucosal membrane barriers (Borchard *et al.*, 1996, Schipper *et al.*, 1996, Dodane *et al.*, 1999). In addition to enhancing drug delivery, chitosan has been shown to have many beneficial effects, including wound-healing properties (Biagini *et al.*, 1991, Ueno *et al.*, 2001) and candidacidal (Suzuki *et al.*, 1987) and anti-viral activities (Iida *et al.*, 1987). It has been shown that 70% deacetylated chitin could non-specifically confer protection against Sendai virus and *E. coli* infections when administered intranasally or intravenously to mice prior to infection (Iida *et al.*, 1987).

Chitosan is non-toxic in humans and is widely used in the food industry as a food additive and as a weight-loss product (Hirano, 1996, Riccardo and Muzzarelli, 1996, Ylitalo *et al.*, 2002). Studies have shown that when chitosan is administered nasally only transient decreases in mucociliary transport rates are detected *in vitro*, leaving no permanent damage to this defence system (Aspden *et al.*, 1997). Its safety, proven ability to deliver drugs across the nasal tract and recent FDA approval have meant that chitosan has begun to receive more attention in recent years for its use as a mucosal adjuvant (van der Lubben *et al.*, 2001). Roy *et al.* (1999) demonstrated the potency of chitosan in immunological protection against peanut allergy using DNA nanoparticles containing plasmid DNA coding for a dominant peanut allergy gene complexed with chitosan. Following oral administration of these nanoparticles, enhanced secretory IgA and serum IgG2a antibodies were observed and mice showed a substantial reduction in allergen-induced anaphylaxis compared to animals immunized with plasmid DNA alone (Roy *et al.*, 1999). Enhanced antibody responses have been observed following systemic immunization of mice with recombinant beta-human chorionic gonadotropin and a zinc-chitosan particle formulation or an emulsion formulation containing chitosan. Immunization with chitosan particles was shown to induce IgG antibodies of an IgG1 subtype while mixed IgG1, IgG2a and IgG2b antibody responses were observed following immunization with the emulsion (Seferian and Martinez, 2000). Le Buanec *et al.* (2001) demonstrated enhanced mucosal immunity towards Tat protein using nanoparticles of chitosan with Tat toxoid following oral or intranasal administration in mice (Le Buanec *et al.*, 2001). Chitosan solutions have also been shown to augment mucosal and systemic immune responses to mucosally delivered influenza virus vaccines (Bacon *et al.*, 2000) and *Bordetella pertussis* haemagluttinin (Jabbal-Gill *et al.*, 1998). Enhanced immune responses have also been observed following intranasal delivery of tetanus toxoid with a non-ionic block copolymer and chitosan as adjuvant (Westerink *et al.*, 2002).

By opening the tight junctions between epithelial cells, chitosan and chitosan derivatives allow for paracellular transport of drugs following mucosal administration (Borchard *et al.*, 1996). In a similar manner chitosan may also increase the absorption of antigens following mucosal immunization, thereby allowing the antigen to gain rapid access to the nasal associated lymphoid tissue. In support of this, Seferian and Martinez (2001) reported that when chitosan and the antigen were administered separately in two different stages, no enhancement of the immune responses was

observed (Seferian and Martinez, 2000). However, chitosan and related molecules have previously been described as potent adjuvants or immunomodulatory compounds following parenteral administration (Azuma, 1992). Therefore, the adjuvant effects of chitosan may extend beyond antigen uptake at mucosal surfaces. Chitosan has been reported to bind to CD14, an LPS glycoprotein receptor (Otterlei *et al.*, 1994), and may therefore stimulate innate immunity through a similar pathway to LPS. Chitosan may also stimulate innate immunity though interaction of its N-acetylglucosamine subunits with mannose-binding protein, an acute phase protein that can activate complement, act as opsonin and link the innate and adaptive immune systems (Stahl and Ezekowitz, 1998). Nishimura *et al.* (1986) reported that chitosan stimulated the production of IL-1 by macrophages (Nishimura *et al.*, 1986). In addition, expression of activation markers, such as MHC classes I and II, Fc receptors and mannose receptor were induced following treatment of murine peritoneal macrophages with chitosan (Ueno *et al.*, 2001). Chitosan has also been shown to promote the production of transforming growth factor (TGF-β1) and platelet derived growth factor from human monocyte-derived macrophages and IL-8 from primary cultured dermal fibroblasts (Ueno *et al.*, 2001).

1.8.9 Cholera toxin and *Escherichia coli* heat labile toxin

The most powerful mucosal adjuvants described to date are the enterotoxin from *Vibrio cholerae* (CT) and heat labile toxin (LT) produced by some enterotoxigenic strains of *E. coli*. The mucosal immune system is able to recognize these molecules as a threat and a short time after they make contact with a mucosal surface a powerful immune response is generated against them. These anti-toxin responses are so potent that strong responses may also be stimulated against foreign bystander molecules that are present simultaneously at mucosal surfaces (Rappuoli *et al.*, 1999). As a consequence of these properties, CT and LT have been extensively used as mucosal adjuvants with a wide variety of antigens in animal models and in a number of human clinical trials. CT and LT delivered by the nasal, oral or other mucosal routes have been shown to significantly enhance IgG and IgA antibody responses to a broad range of antigens (Holmgren *et al.*, 1993, Marinaro *et al.*, 1995, Takahashi *et al.*, 1996, Williams *et al.*, 1999, Tsuji *et al.*, 2000). The parenteral adjuvant effects of CT and LT have also been demonstrated in studies involving immunization via the subcutaneous, intraperitoneal, intravenous, intradermal and

transcutaneous routes (Sverremark and Fernandez, 1997, Glenn *et al.*, 1999, Scharton-Kersten *et al.*, 2000, Weltzin *et al.*, 2000).

1.8.10 Structure of CT and LT

CT and LT are composed of two subunits: an enzymatically active A subunit with adenosine-diphosphate (ADP)-ribosyltransferase activity, that is responsible for the toxicity, and a B subunit that mediates binding of the molecules to their receptors on eukaryotic cell surfaces (Fig. 1.2), (Spangler, 1992, Zhang *et al.*, 1995). The B subunits of LT (LTB) and CT (CTB) are formed by five monomers that are arranged in a cylinder-like structure with a central cavity (Sixma *et al.*, 1993a, Sixma *et al.*, 1993b). Interaction of LTB and CTB with their cell surface receptors is necessary for internalisation of the globular A subunit (Holmgren *et al.*, 1973). In the case of intranasal delivery, the specific interaction of the B subunit with its receptor on epithelial cells is necessary for uptake from the respiratory tract. The receptor binding site is specific for a variety of galactose-containing molecules, CTB and LTB bind with high affinity to the glycosphingolipid, GM1-ganglioside [Gal(β1-3)GalNAc(β1-4)(NeuAc(α2-3))Gal(β1-4)Glc(β1-1)ceramide], present on the surfaces of mammalian cells (Holmgren *et al.*, 1973). CTB and LTB can also bind to GD1b-ganglioside, but with a lower affinity than to GM1. In addition, LTB may bind to other glycosphingolipids (asialo-GM1), to glycoprotein receptors present in the intestine of rabbits and humans, to polyglycosilceramides and to paragloboside (Pizza *et al.*, 2001). The different receptor binding specificities of LT and CT indicates that the two toxins should not be considered identical as mucosal adjuvants.

The A subunit of LT and CT is composed of a globular enzymatically active A_1 domain and an A_2 domain formed by a long α helix whose carboxy-terminus enters into the central cavity of the B oligomer. The 2 domains are attached via a trypsin-sensitive loop and a disulphide bridge between A_1 –cys187 and A_2 –cys199. To become activated, the loop must be proteolytically cleaved and the disulphide bond reduced (Gill and Rappaport, 1979). The A1 fragment enters the cell cytosol and ADP-ribosylates the stimulatory α subunit of a GTP-binding protein (Gsα) that causes permanent activation of adenylate cyclase resulting in an elevation in cyclic AMP (cAMP) levels (Holmgren, 1981, Field *et al.*, 1989, Gill and Woolkalis, 1991, Spangler, 1992). As a result, protein kinase A (PKA) phosphorylates and opens the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel, resulting in the osmotic movement of water into the gut lumen and diarrhoea. The major function of the A_2-fragment is in

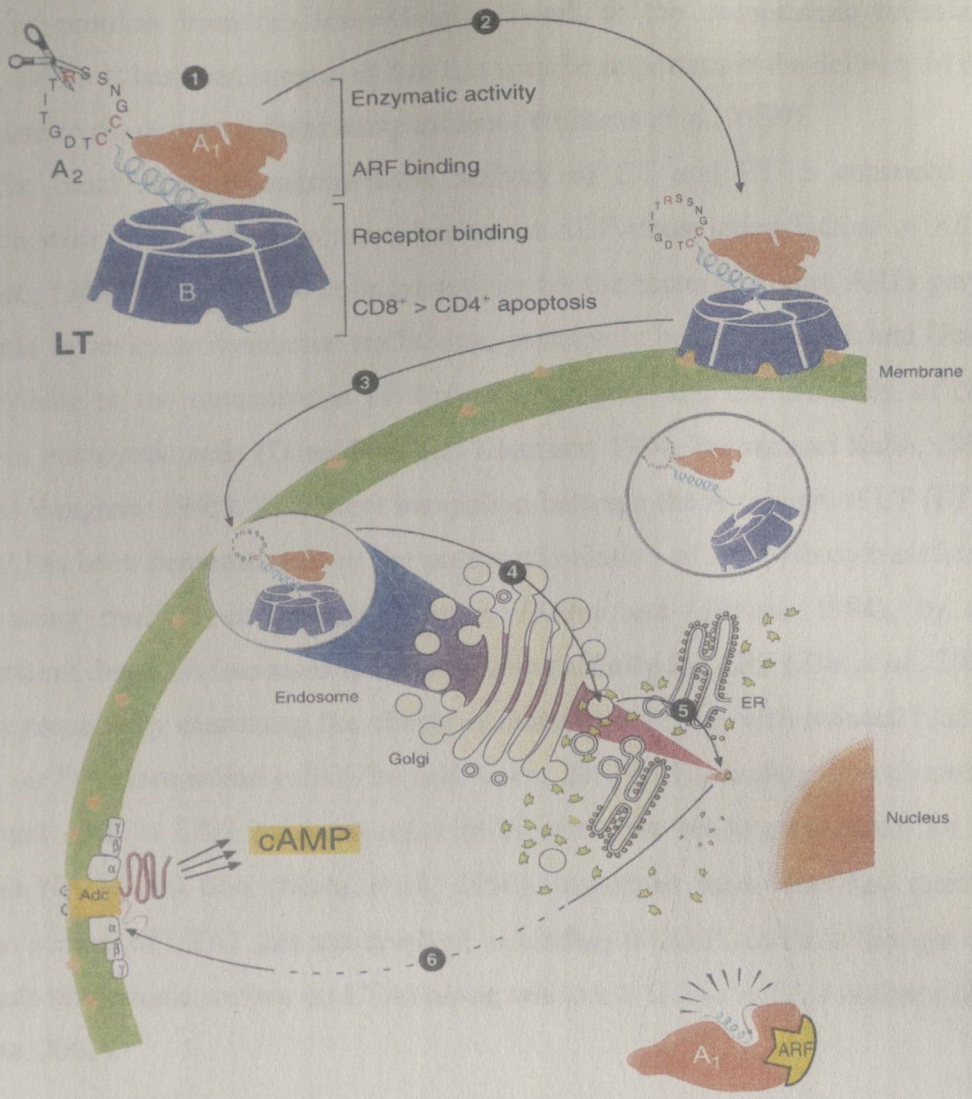


Figure 1.2. Sequence of events during intoxication of eukaryotic cells by CT or LT. (1) Schematic representation of CT or LT showing the A and B subunits and the site of proteolytic cleavage of the A1 fragment. (2) The toxin binds to its receptor on the surface of mammalian cells. (3) The toxin is then internalised into vesicles and (4) transported to the Golgi apparatus. (5) The A subunit is then transported from the Golgi to the endoplasmic reticulum (ER) where it interacts with soluble ADP-ribosylating factors (ARF). (6) Finally the A1 subunit migrates to the plasma membrane and ADP-ribosylates the α subunit of a GTP binding protein, Gs, resulting in the permanent activation of adenylate cyclase (Adc) and the accumulation of intracellular cAMP. Reproduced from Immunology Today. 1999. Vol. 20 p494.

interaction with the B subunits. However, the C-terminus of the A_2-fragment also contains a sequence motif (RDEL for CT, KDEL in the case of LT) associated with retrieval of proteins from the *trans*-Golgi network to the endoplasmic reticulum (Pelham, 1992). It has been suggested that this may be important in the delivery of the A_1-fragment to the correct cellular compartment (Williams *et al.*, 1999).

The basal ADP-ribosyltransferase activity of CT and LT is enhanced by interaction with GTP-binding proteins, known as ADP-ribosylation factors or ARFs (Tsai *et al.*, 1988). In addition to cofactor activity for the bacterial toxins, ARFs play a crucial role in vesicular membrane trafficking, principally between the ER and Golgi, and contribute to the maintenance of organelle integrity and the assembly of coat proteins in eukaryotic cells (Donaldson and Klausner, 1994, Boman and Kahn, 1995, Moss and Vaughan, 1995). The direct interaction between the A subunit of LT (LTA) and ARF has been demonstrated by the potent stimulation of ADP-ribosyltransferase activity using purified compounds *in vitro* (Kahn and Gilman, 1984), by the demonstration that LTA increases the GTP binding affinity for ARF (Zhu *et al.*, 2000) and more recently by examining the effects of mutants of LTA1, with reduced binding to ARF, on live mammalian cells (Zhu and Kahn, 2001). The binding of activated or GTP-bound ARF to LTA has been reported to lower the binding constants for the substrates NAD+ and Gsα (Noda *et al.*, 1990). Mutations have identified residues along the surface of LTA1 that are involved in binding to ARF, and it is thought that ARF binds to the same surface on LTA1 along which LTA2 lies in the holotoxin (Zhu and Kahn, 2001).

1.8.11 Adjuvant action of CT and LT

CT and LT have been shown to enhance local and systemic humoral and cellular immune responses to numerous co-administered antigens following mucosal delivery. Most studies indicate that CT induces a mixed Th1/Th2 type response with a biased Th2 type response to itself and to bystander antigens. This is based on the findings that IL-4, IL-5 and IL-10 are predominately produced with little IFN-γ following mucosal administration of CT (Xu-Amano *et al.*, 1993, Marinaro *et al.*, 1995, Yamamoto *et al.*, 1997a). In addition, CT has been shown to induce antigen-specific IgE responses (Marinaro *et al.*, 1995, Yamamoto *et al.*, 1997b, Simecka *et al.*, 2000) and IgG antibodies predominately of the IgG1 subclass (Pierre *et al.*, 1992, Marinaro *et al.*, 1995, Ruedl *et al.*, 1996, Yamamoto *et al.*, 1997b) following mucosal

immunization. In contrast, LT has been shown to induce a more mixed Th1/Th2 type response, with the production of antigen-specific IL-4, IL-5 and IFN-γ, IgG antibodies of the IgG1, IgG2a and IgG2b subclasses and significantly less antigen-specific IgE than CT, following mucosal delivery (Ruedl *et al.*, 1996, Takahashi *et al.*, 1996, Cheng *et al.*, 1999, Douce *et al.*, 1999).

LT or CT have also been used to adjuvant antigen-specific Class I restricted CTL responses, usually by intranasal immunization (Bowen *et al.*, 1994, Simmons *et al.*, 1999, Simmons *et al.*, 2001a). OVA-specific CTL has been detected in the spleen and local draining lymph nodes following intranasal or oral immunization of mice with OVA or OVA peptide together with CT or LT. LT has also been shown to elicit CTL responses to OVA independently of CD4+ T cell help, suggesting that it may activate host antigen presenting cells directly or indirectly such that they are able to prime an OVA-specific CD8+ T cell response (Simmons *et al.*, 1999).

In addition to the studies carried out in experimental animals, CT and LT have also proven to be strong mucosal immunogens and adjuvants in humans. Two nasal vaccinations with LT-adjuvanted virosomal influenza vaccine were shown to induce antigen-specific immune responses comparable to those induced after a single parenteral vaccination with influenza virus haemagluttinin in adult volunteers (Glück *et al.*, 1999). Although LT has been shown to be tolerated by adults following mucosal delivery (Michetti *et al.*, 1999), humans are exquisitively sensitive to LT and CT; diarrhoeagenic responses may be triggered at low doses that are non-toxic in mice (Levine *et al.*, 1983, Spangler, 1992). Therefore, although LT and CT have proven to be effective mucosal adjuvants their clinical use may be restricted due to their toxicity, and this has led to attempts to separate the toxic effects of these molecules from their adjuvanticity.

1.8.12 Mutations in the A subunit of CT and LT

A number of attempts have been made to alter the toxicity of LT and CT, without affecting their adjuvanticity, most of which have focused on eliminating or attenuating the enzymatic activity of the A subunit. The majority of efforts have involved the use of site-directed mutagenesis to change the amino acids associated with the active site of the toxins that are critical for enzymatic activity. Some of the mutant derivatives obtained are efficiently assembled into the AB structure, are stable on storage and have either greatly reduced or undetectable toxicity. Among the several

LT and CT mutants generated by site-directed mutagenesis of the A subunit, those referred to as LTK7 (Arg7 → Lys), LTK63 (Ser63 → Lys), LTR72 (Ala72 → Arg), CTK63 (Ser63 → Lys), CTE112K (Glu112 → Lys) and CTS61F (Ser61 → Phe) have been characterised in detail for the induction of systemic and local immune responses to co-administered antigens (Douce *et al.*, 1995, Yamamoto *et al.*, 1997b, Giuliani *et al.*, 1998, Marchetti *et al.*, 1998, Douce *et al.*, 1999, Ryan *et al.*, 1999, Olszewska *et al.*, 2000, Barackman *et al.*, 2001). It was initially thought that ADP-ribosylation and accumulation of cAMP were necessary for the adjuvanticity of LT and CT (Lycke *et al.*, 1992). More recently mutants lacking ADP-ribosyltransferase activity have been shown to retain the ability to act as mucosal adjuvants (Douce *et al.*, 1995, Yamamoto *et al.*, 1997b, Douce *et al.*, 1999, Ryan *et al.*, 1999, Barackman *et al.*, 2001). However, in several studies it has been shown that LT and CT or derivatives with higher enzymatic activity exert stronger adjuvant effects compared to those with little or no enzymatic activity (Giuliani *et al.*, 1998, Douce *et al.*, 1999, McCluskie *et al.*, 2001b).

LTK63, a non-toxic mutant completely devoid of enzymatic activity, and LTR72, a partially toxic mutant that retains approximately 1% of the wild-type enzyme activity, have been extensively evaluated as mucosal adjuvants using a wide range of antigens. Mucosal delivery of LTK63 or LTR72 together with pertussis (Ryan *et al.*, 1999), pneumococci (Jakobsen *et al.*, 1999), *Helicobacter pylori* (Marchetti *et al.*, 1998), *Shigella flexneri* (Koprowski *et al.*, 2000), *Toxoplasma gondii* (Bonenfant *et al.*, 2001) and a peptide from respiratory syncytial virus (Simmons *et al.*, 2001a) has been shown to confer protection in the appropriate animal models. LTK63 and LTR72 have also been shown to induce CTL responses to a number of antigens, including synthetic peptides (Partidos *et al.*, 1999), OVA (Simmons *et al.*, 1999), HIV gag p55 protein (Neidleman *et al.*, 2000) and peptides from respiratory syncytial virus (Simmons *et al.*, 2001a). LTR72 would appear to exert the strongest mucosal adjuvanticity of these two mutant toxins, with properties similar to those of wild type LT (Giuliani *et al.*, 1998, Greer *et al.*, 2000, Olszewska *et al.*, 2000, Barackman *et al.*, 2001). However, the deficiency in the response generated by LTK63 may be overcome by altering the dose given, as has been demonstrated in studies in which the dose of the mutant toxin was increased (Giuliani *et al.*, 1998), or by administering the toxin via an alternative route (Neidleman *et al.*, 2000, McCluskie *et al.*, 2001b). Hartman *et al.* (1999) found that LTK63 was as effective as native LT for the mucosal delivery of *E. coli-Shigella flexneri* hybrid vaccine strains to guinea pigs (Hartman *et al.*, 1999). In

addition, higher systemic antibody responses to intranasally delivered pneumococcal conjugate vaccines have been achieved using LTK63, rather than LTR72, as mucosal adjuvant (Jakobsen *et al.*, 1999).

Mutations have also been constructed in the protease sensitive loop of the toxins, rendering the loop insensitive to proteases and hence eliminating the susceptibility of the toxins to the cleavage required for activation of the enzymatic activity and toxicity. Mucosal immunization with one such mutant, LTR192G that is devoid of ADP-ribosyltransferase activity, has been shown to enhance humoral immunity to a range of antigens (Dickinson and Clements, 1995, Douce *et al.*, 1999, Guillobel *et al.*, 2000, Morris *et al.*, 2000). In addition, LTR192G has been shown to induce protective immunity against several viral and bacterial infections in murine models (Chong *et al.*, 1998, O'Neal *et al.*, 1998, Cárdenas-Freytag *et al.*, 1999). LTR192G also enhanced IgA responses and Th1 type cytokines (in *H. pylori* negative subjects) following mucosal delivery with formalin-inactivated and *Helicobacter pylori* whole-cell vaccine in human volunteers (Kotloff *et al.*, 2001).

Recently novel mutants of LT displaying low levels of toxicity have been identified. These mutants retain basal ADP-ribosylation activity but are defective in ARF binding. However, it remains to be seen if these mutants retain the adjuvant properties of wild-type LT (Zhu and Kahn, 2001).

The mutant CT molecules CTS61F, CTE112K and CTE29H have been shown to retain the adjuvant property of CT by inducing CD4+ Th2 type responses (Yamamoto *et al.*, 1997a, Yamamoto *et al.*, 1997b, Tebbey *et al.*, 2000). However, the subtype of T cell responses induced with certain LT mutants has been shown in some studies to differ from that induced by native LT, and would appear to be affected by numerous factors including the nature and dose of toxin used, the route of administration, the strain of mice and the nature and dose of the co-administered antigen (Cárdenas-Freytag *et al.*, 1999, Cheng *et al.*, 1999, Ryan *et al.*, 1999, Olszewska *et al.*, 2000, Neidleman *et al.*, 2000, Bonenfant *et al.*, 2001). It was shown that IgG1 was the predominant antibody in mice immunized with LT, LTR192G, LTK63 or LTB and KLH. However, as the toxicity of the mutant decreased there was an associated loss of detectable IgG2a in the sera and IFN-γ in the culture supernatants (Douce *et al.*, 1999). Nasal delivery of the non-toxic mutant LTK63 has been shown to induce mixed Th1/Th2 type responses to co-administered pertussis antigens. In contrast, the partially toxic mutant preferentially enhanced Th2 type responses at low

doses of the toxin, although at higher doses the T cell subtype responses were more mixed (Ryan *et al.*, 1999). However, IgG2a was the dominant subclass of antibody induced following intranasal immunization with influenza virus haemagluttinin and LTR72, indicating a predominant Th1 type response (Barackman *et al.*, 1999).

1.8.13 Use of A or B subunits alone of LT or CT as mucosal adjuvants

Initial commercial preparations of CTB in which the B subunit was purified from the active toxin had strong adjuvant activity. However, it was later found that much of this activity was derived from contamination with active CT (Tamura *et al.*, 1994). Recombinant LTB and CTB have now become available and studies using these molecules have shown that rLTB and rCTB are weaker mucosal adjuvants than the native toxins (Clements *et al.*, 1988, Douce *et al.*, 1995, Yamamoto *et al.*, 1997a, Cheng *et al.*, 1999), although supplementation of rLTB or rCTB with a trace amount of LT or CT has been shown to result in a synergistic enhancement of antibody responses, up to levels observed following immunization with the holotoxins alone (de Haan *et al.*, 1996). Nonetheless, rLTB and rCTB have been used as mucosal adjuvants to a range of antigens following mucosal administration. Enhanced antigen-specific immunity has been demonstrated using LTB and a number of antigens including herpes simplex virus glycoproteins (Richards *et al.*, 2001) and glycoprotein D (Hazama *et al.*, 1993), influenza antigens (de Haan *et al.*, 1998a, Verweij *et al.*, 1998), KLH (de Haan *et al.*, 1998b) and DT (Kozuka *et al.*, 2000).

LTB has also been evaluated as an adjuvant for antigens expressed in transgenic plants. LTB and the spike protein of swine transmissible gastroenteritis virus have been expressed at high levels in corn, and have been shown to elicit protective immune responses when delivered in the seed (Streatfield *et al.*, 2001). In addition, subcutaneous immunization with an extract of potato tubers expressing LTB elicited high anti-LT antibody titres. Oral immunization was also seen to significantly augment IgA responses in subcutaneously primed animals (Lauterslager *et al.*, 2001).

CTB has also been shown to enhance immune responses to co-administered antigens. Intranasal immunization of tetanus toxoid (TT) with rCTB was shown to induce high levels of TT-specific serum IgG antibodies and provide protection in mice challenged with lethal doses of tetanus toxin (Isaka *et al.*, 1998). An oral vaccine containing cholera and CTB has been thoroughly evaluated in animal models and humans (Holmgren *et al.*, 1987, Holmgren *et al.*, 1992). A CTB-mediated GM1 vesicle

delivery system has been designed to enhance localization of protein antigens at mucosal cells, due to the ability of the pentameric CTB to bind to GM1 on both epithelial cells and GM1 vesicles. Intranasal administration of a recombinant HIV envelope protein formulated in CTB-associated GM1 lipid vesicles was shown to enhance IgA antibody responses in comparison to control animals immunized with the antigen formulated in GM1-free vesicles with CTB. In addition, CTB in GM1 vesicles enhanced both IgG1 and IgG2a antibodies while CTB in an alum formulation only enhanced IgG1 antibodies (Lian and Ho, 1997, Lian *et al.*, 1999). The importance of receptor binding by the B subunit for immunogenic responses has been demonstrated using a non-receptor binding mutant of LTB, LTBG33D, which is poorly immunogenic at mucosal surfaces (Nashar *et al.*, 1996a). However, another study has shown that LTB monomer expressed in *Streptococcus gordonii*, despite being unable to bind GM1, was highly immunogenic in mice following parenteral and mucosal delivery (Ricci *et al.*, 2000).

A completely non-toxic novel fusion protein has been constructed that combines the enzymatic activity of CTA1 with a dimer of an Ig-binding fragment of *Staphylococcus aureus* protein A, DD. The fusion protein, CTA1-DD, retains the enzymatically active CTA1 moiety, while the CTB subunit and the ability to bind to the GM1-ganglioside receptor were removed (Ågren *et al.*, 1997, Ågren *et al.*, 1998). CTA1-DD has been shown to be an effective mucosal and parenteral adjuvant, capable of enhancing IgG1 and IgG2a antibodies to co-administered antigens (Ågren *et al.*, 1997, Mowat *et al.*, 2001). Enzymatically inactive CTA1-DD derivatives fail to induce an adjuvant response following systemic immunization, showing that the adjuvant effect of this molecule depends on the enzymatic activity (Ågren *et al.*, 1999).

1.8.14 CT- and LT-mediated apoptosis

In vitro, the B subunits of CT and LT cause the selective depletion of CD8+ T cells from cell cultures by a process of apoptosis (Nashar *et al.*, 1996a, Nashar *et al.*, 1996b, Pitman *et al.*, 1998). It has now been established that recombinant CTB and LTB are capable of exerting immunomodulatory effects *in vivo*. CTB conjugated to myelin basic protein was shown to prevent the establishment of the multiple sclerosis like disease, experimental allergic encephalomyelitis (EAE), in rats (Sun *et al.*, 1996). It was initially thought that the antigen had to be coupled directly to the B subunit of the toxin to exert this effect (Simmons *et al.*, 2001b). However, more recent studies

have revealed that both LTB and CTB can modulate autoimmune diseases when they are administered alone in the absence of added antigen. Parenteral delivery of CTB has been shown to delay the onset of insulin dependent diabetes mellitus (Sobel *et al.*, 1998) and subcutaneous administration of LTB completely prevented the induction of collagen- induced arthritis (CIA) in animal models (Williams *et al.*, 1997). These studies demonstrated that the B subunits of LT and CT have broad immunomodulatory properties *in vivo* that are not limited to mucosal surfaces.

The prevention or treatment of autoimmune diseases is thought to be associated with immune deviation from a pathological Th1 dominated response to a Th2 associated response. Alternatively, the generation of a CD4+ regulatory cell population may be capable of suppressing the disease-associated Th1 response, in the absence of increased Th2 type responses (Luross and Williams, 2001, Simmons *et al.*, 2001b). The importance of receptor binding by the B subunit in mediating disease protection has been demonstrated in the case of LTB by the failure of a non-receptor binding mutant of LTB, LTBG33D, to prevent or treat CIA (Williams *et al.*, 1997).

1.8.15 Mechanism of adjuvant action of CT and LT

The mechanism of adjuvant action of CT and LT has long been controversial, although a number of hypotheses have been put forward to explain the potent immunogenicity and adjuvanticity of these toxins. Studies using non-toxic or partially toxic mutants or the use of the A or B subunits alone have suggested that the adjuvant effect of the toxins is derived from independent contributions of the enzymatic activity, the non-toxic AB complex, the binding activity of the B subunit and the A and B subunits themselves (Nashar *et al.*, 1996a, Ågren *et al.*, 1997, Giuliani *et al.*, 1998, Isaka *et al.*, 1998, Ågren *et al.*, 1999, Douce *et al.*, 1999, Ryan *et al.*, 1999, Guillobel *et al.*, 2000, Ricci *et al.*, 2000, Richards *et al.*, 2001). The A and B subunits provide a large number of B and T cell epitopes and in addition the A subunit may influence events such as intracellular antigen processing and presentation, interact with the vesicular transport system and be translocated to the cytosol where it can interact with ADP-ribosylation factors (ARF). One of these activities or a combination of them may be responsible for the adjuvanticity of the non-toxic AB complex.

Enhanced uptake at mucosal surfaces is another mechanism by which the toxins may enhance the immunogenicity of co-administered antigens. Following oral delivery of KLH, dextran and CT it has been shown that CT, but not CTB, strongly

increased the gut permeability for dextran concomitantly with a strong enhancing effect on the KLH-specific immune response in the lamina propria (Lycke *et al.*, 1991). The enzymatic activity may therefore play a role in gut permeability, facilitating access of luminal antigens to the gut mucosal immune system, and thereby enhancing mucosal immune responses. It has been demonstrated that membrane lipid rafts selectively enriched in GM1, which are believed to play an important role in signal transduction and membrane trafficking, were selectively targeted to the MHC II peptide-loading compartment after B cell receptor cross-linking (Cheng *et al.*, 1999). Therefore the ability to preferentially target GM1 may also increase antigen delivery and subsequent MHC II presentation by B cells.

Potentiation of APC function may also explain some of the adjuvant and immunogenic properties of CT and LT. It has been reported that both native CT and a fusion protein with an intact A1 subunit enhance CD80 and CD86 expression on B cells (Ågren *et al.*, 1997). CT has also been shown to enhance expression of CD80 and CD86 on murine Flt3L-expanded DC (Williamson *et al.*, 1999) and on human DC (Gagliardi *et al.*, 2000). In contrast, other studies have shown that CT enhances CD86, but not CD80, expression by bone marrow-derived macrophages (Cong *et al.*, 1997) and by macrophages and B cells obtained from Peyer's patches (Yamamoto *et al.*, 1999, Yamamoto *et al.*, 2000, Yamamoto *et al.*, 2001). LT on the other hand has been reported to selectively up-regulate the expression of CD80, but not CD86, on bone marrow-derived DC (Simmons *et al.*, 2001b). In another study, it was shown that LT, LTR72 and LTK63 enhanced CD80 expression, whereas only LT enhanced CD86 expression on murine macrophages *in vitro* (Ryan *et al.*, 2000). CT- and LT-treated APC enhance proliferative responses in T cells stimulated with anti-CD3 monoclonal antibody. These T cell proliferative responses may be blocked by anti-CD86 monoclonal antibody (Cong *et al.*, 1997, Yamamoto *et al.*, 1999, Yamamoto *et al.*, 2000). Furthermore, administration of anti-CD86 monoclonal antibodies has been shown to inhibit KLH-specific antibody responses in mice given KLH and CT orally (Cong *et al.*, 1997). Thus, an enhancement of the co-stimulatory activity of mucosal APC, by up-regulation of B7 expression, would appear to be important for the mucosal adjuvanticity and immunogenicity of these toxins. *In vitro* studies have shown that rCTB fails to enhance CD86 expression on macrophages (Cong *et al.*, 1997, Yamamoto *et al.*, 1999, Yamamoto *et al.*, 2001). In addition, treatment of macrophages with dibutyryl cAMP has been shown to upregulate CD86 expression

(Cong *et al.*, 1997), indicating that enhancement of CD86 expression may be linked to the enzymatic activity of the A subunit. Pre-treatment of native or mutant CT with GM1 has been shown to block CD86 expression (Yamamoto *et al.*, 1999), indicating that binding of the B subunit to APC via a specific receptor is also necessary for enhancement of CD86 expression. The stimulatory effect of CT and LT on B7 expression has been shown to be independent of CD40-CD40L co-signalling. However, oral immunization of $CD40^{-/-}$ mice with CT or LT as adjuvant failed to generate mucosal or systemic antibody responses to co-administered antigen (Yamamoto *et al.*, 2000). These studies suggest that although CD40-CD40L co-signalling is not directly involved in the effect of LT and CT on upregulatuion of B7 expression, it is an essential signal for the initiation of antigen-specific immune responses. CT, either alone or with TNF-α and IL-1β, has been shown to induce phenotypic and functional maturation of DC, by upregulating the expression of HLA-DR and co-stimulatory molecules on their surface and enabling the DC to induce Th2 polarization of naïve T cells *in vitro* (Gagliardi *et al.*, 2000, de Jong *et al.*, 2002). However, the B subunit of CT was unable to induce DC maturation (Gagliardi *et al.*, 2000). CT-induced maturation of DC was associated with a rapid and sustained upregulation of the constitutive chemokine receptors, CXCR4 and CCR7 (Gagliardi *et al.*, 2000).

CT and LT have also been shown to modulate cytokine production and antigen-presentation by APC. CT enhances alloantigen presentation by cultured intestinal epithelial cells (Bromander *et al.*, 1993). Paradoxically however, CT and LT inhibit antigen processing of soluble protein antigen by APC. LT toxin-treated macrophages exhibited an impaired ability to present protein antigen via MHC class II to cognate T cells, but had only limited effect on the presentation of peptide. Inhibition of antigen processing would appear to be mediated via the ADP-ribosyltransferase activity, as mutants lacking enzymatic activity were unable to induce the same effect (Matousek *et al.*, 1998). Pre-treatment of APCs with LT or LTR72, but not LTK63, has been shown to suppress IFN-γ production by a Th1 clone, but enhance IL-5 production by a Th2 clone (Ryan *et al.*, 2000). CT and LT toxins have also been shown to modulate the production of regulatory cytokines and chemokines by cells of the innate immune system. CT has been shown to suppress IL-12 p70 production induced by human monocytes and DC in response to a variety of stimuli (Braun *et al.*, 1999, Gagliardi *et al.*, 2000). Similarly, LT suppressed IL-12 production by stimulated human monocytes

(Braun *et al.*, 1999) or murine macrophages (Ryan *et al.*, 2000). In addition, CT has been shown to inhibit the expression of the β1 and β2 chains of the IL-12 receptor on activated T cells, leading to a suppression of Th1 cell differentiation and driving Th2 responses (Braun *et al.*, 1999). In contrast, CTB was shown to have minimal effects on IL-12 p70 production by stimulated human DC (Braun *et al.*, 1999, Gagliardi *et al.*, 2000) and LTK63, which is completely devoid of enzymatic activity, has been shown to actually enhance IL-12 levels *in vivo* and *in vitro* (Ryan *et al.*, 2000). Furthermore, LTB has been shown to enhance IL-12 p40 mRNA following oral administration (Bost and Clements, 1995). In addition to suppressing IL-12 p70 production, CT and LT have also been found to inhibit TNF-α production from stimulated monocytes and DC (Braun *et al.*, 1999, Gagliardi *et al.*, 2000) or murine macrophages (Ryan *et al.*, 2000), respectively, but have minimal effects on IL-6 and IL-10 production. Forskolin and other direct activators of adenylate cyclase have been shown to mediate similar effects to CT (Kalinski *et al.*, 1997, Panina-Bordignon *et al.*, 1997, Gagliardi *et al.*, 2000), suggesting that cAMP is responsible for the effects. Furthermore, it has been demonstrated that the enzyme activity of the A subunit of LT leads to the accumulation of intracellular cAMP (Cheng *et al.*, 1999, Ryan *et al.*, 2000), which is a potent inhibitor of pro-inflammatory cytokines (Zidek, 1999) and may therefore have enhancing effects on Th2 type responses. In contrast, LTK63 enhanced the activation of NFκB (Ryan *et al.*, 2000), the activation of which is required for the transcription of several immune response genes, including IL-12 and B7, in innate cells (Baldwin, 1996, Ghosh *et al.*, 1998).

It has also been established that CT and LT may directly affect T cells. Studies using Th1 and Th2 clones demonstrated that CT inhibited proliferative responses and cytokine production of Th1 clones, but had no effect on Th2 clones activated by TCR-mediated signalling (Munoz *et al.*, 1990). It has also been shown that CT directly affects Peter's patches CD4+ T cells activated via the TCR-CD3 complex, with selective inhibition of Th1 responses (Yamamoto *et al.*, 1999, Yamamoto *et al.*, 2000, Yamamoto *et al.*, 2001). In contrast, LT maintained Th1 type responses by CD4+ TCR-activated T cells, but significantly inhibited IL-4 production (Yamamoto *et al.*, 2000, Yamamoto *et al.*, 2001). These studies may explain in part the different T cell subtype responses that have been reportedly induced with the two toxins. In addition, oral immunization of IL-$4^{-/-}$ mice with protein antigen and LT, but not CT, has been shown to induce antigen-specific Th2 type responses including IL-5, IL-6 and IL-10,

suggesting a role for IL-4 in the induction of Th2 type responses by CT (Okahashi *et al.*, 1996, Yamamoto *et al.*, 2000, Yamamoto *et al.*, 2001).

1.8.16 Implications for the safety of CT and LT holotoxins and mutants

There is limited data available on the effects of LT, CT and mutant toxins in humans. It has been shown that oral immunization of *Helicobacter pylori* infected human volunteers with *H. pylori* urease and LT enhanced antigen-specific IgA antibody responses and reduced gastric *H. pylori* density. However, immunization also induced diarrhoea in 66% of the subjects (Michetti *et al.*, 1999). Phase I clinical trials have been performed with an LT-adjuvanted influenza vaccine. It was reported that LT enhanced antigen-specific local and systemic immunity and the vaccine was initially well tolerated (Glück *et al.*, 1999, Glück *et al.*, 2000). However, more recently this vaccine has been withdrawn from clinical trials as a limited number of patients temporarily developed facial paralysis following vaccination. It is hoped that the side effects associated with the administration of holotoxins to humans may be abolished or reduced using B subunits of the toxins or mutant molecules. A number of clinical trials have been performed with CTB and killed enterotoxigenic *E. coli* or inactivated *Vibrio cholerae* as oral vaccines against diarrheal diseases (Holmgren *et al.*, 1992, Cohen *et al.*, 2000, Taylor *et al.*, 2000). CTB has been shown to be safe at 1 mg/dose after nasal administration (Holmgren *et al.*, 1992). Kotloff *et al.* (2001) reported enhanced mucosal and systemic immune responses in humans to *H. pylori* antigens using an oral *H. pylori* whole-cell vaccine administered with LTR192G. However, diarrhoea, low-grade fever and vomiting were observed in some of the volunteers following immunization (Kotloff *et al.*, 2001). In another study the LTR192G mutant was found to be safe at doses of 5, 25 and 50 μg, but induced diarrhoea in some volunteers receiving 100 μg/dose (Conference Coverage (ICAAC), 1997). The non-toxic mutant of LT, LTK63, is currently being tested in a clinical trial for nasally delivered influenza vaccine.

Nasal vaccine formulations with CT, LT or mutant toxins as antigens or adjuvants may pose a risk of neurological side effects, associated with direct transport of the toxins to the brain via the olfactory bulb. Van Ginkel *et al.* (2000) showed that both CT and CTB accumulated in the olfactory nerves and bulbs when given intranasally, however, neither molecule was present in nasal-associated lymphoreticular tissue beyond 24 hours. This uptake into olfactory regions was shown

to be GM1-dependent since accumulation could be inhibited by pre-incubation of the molecules with GM1 prior to intranasal administration (van Ginkel *et al.*, 2000). Intranasal vaccination with tetanus toxoid together with CT as adjuvant resulted in antigen uptake into the olfactory nerve/epithelium, whereas tetanus toxoid alone did not enter the central nervous system (CNS) (van Ginkel *et al.*, 2000). In contrast, a recent report has demonstrated that intranasal administration of CTB or LTB, supplemented with small amounts of holotoxin, did not localize to the brain or cause any histological changes to brain tissue in mice (Hagiwara *et al.*, 2001). Further studies are required to clarify the possible neurological effects of these toxins following nasal administration.

1.9 Aims of this study

The major obstacle to the development of mucosally delivered vaccines is the identification of safe and effective adjuvants that can enhance the immunogenicity of co-administered antigens. The aims of this study were firstly to evaluate the effectiveness of chitosan and two mutants of *E. coli* heat labile toxin, LTK63 and LTR72, as adjuvants for the nasal delivery of diphtheria and meningococcal type B antigens in mice. Furthermore, the immunogenicity of an intranasally delivered diphtheria vaccine, consisting of a highly purified mutant of diphtheria toxin, CRM_{197}, formulated with chitosan, was examined in human volunteers during a Phase I clinical trial. Understanding the mechanism of action of adjuvants, such as LTK63 or LTR72, may make it possible to design protective vaccines based on prior knowledge of the correlates of protective immunity. Therefore, this study also investigated the influence of LTK63 and LTR72 on DC maturation and cytokine or chemokine production *in vitro*, which may provide some insight into the ability of these molecules to induce specific types of immune responses *in vivo*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Media and Buffers

2.1.1 Diethanolamine buffer

97 ml Diethalonamine (0.9M)

0.1 g Magnesium chloride ($MgCl_2.6H_2O$, 0.0005M)

2 mls of 2% Sodium azide (NaN_3, 0.0006M)

Made up to 1L with ddH_2O and adjusted to pH 9.8

2.1.2 Phosphate-Buffered Saline (PBS)

80 g Sodium chloride (NaCl, 1.4M)

11.6 g Sodium hydrogen phosphate (Na_2HPO_4, 0.08M)

2 g Potassium dihydrogen phosphate (KH_2PO_4, 0.01M)

2 g Potassium chloride (KCl, 0.03M)

Dissolved in 1L of ddH_2O and adjusted to pH 7.0

2.1.3 PBS/BSA/Azide (PBA)

500 ml PBS pH 7.0

2.5 g Bovine serum albumin (BSA)

0.5 ml 1M Sodium azide (NaN_3)

2.1.4 Sodium hydrogen carbonate buffer

4.2 g Sodium hydrogen carbonate ($NaHCO_3$, 0.05M)

Dissolved in 1L of ddH_2O and adjusted to pH 8.2

2.1.5 Sodium carbonate/sodium hydrogen carbonate buffer

5.3 g Sodium carbonate (Na_2CO_3, 0.05M)

4.3 g Sodium hydrogen carbonate ($NaHCO_3$, 0.05M)

Dissolved in 1L of ddH_2O and adjusted to pH 9.4

2.1.6 Tris-Buffered Saline (TBS)

2.42 g Trizma base (0.02M)

8.76 g Sodium chloride (NaCl, 0.15M)

Dissolved in 1L of ddH_2O and adjusted to pH 7.3

2.1.7 Cell culture media

The J774 cell line, human peripheral blood mononuclear cells (PBMC) and all primary cells isolated from murine spleen, lymph nodes or bone marrow were maintained in RPMI-1640 medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 8% (v/v) heat inactivated (56^{0}C for 30 mins) foetal calf serum (FCS), 100 mM L-Glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-Mercaptoethanol, unless otherwise stated. The Vero cell line was cultured in M199 medium (Gibco BRL) containing 2.2 g/L $NaHCO_3$ and 25 mM HEPES. The medium was supplemented with 8% (v/v) heat inactivated FCS, 2 mM L-Glutamine and 50 µg/ml gentamycin.

2.2 CULTURING OF CELL LINES

The adherent murine macrophage cell line, J774, and the monkey kidney epithelial cell line, Vero cell line, were obtained from the American Type Culture Collection (ATCC), Rockville MD, USA. J774 and Vero cell lines were cultured under sterile conditions in complete RPMI-1640 or M199 medium, respectively, at 37°C and 95% humidity in a CO_2 incubator. The cells were seeded at a variety of densities in flasks and passaged every 2-3 days depending on cell type, viability and rate of growth.

2.2.1 Trypsinization of cells

Strongly adherent Vero cells, to be sub-cultured or used experimentally, were removed from the base of the flask by trypsinization. M199 medium in the flask was removed and cells were washed with sterile PBS. The cells were then incubated at 37°C for approximately 5 mins with 0.25% trypsin-EDTA solution. The trypsinization reaction was stopped by the addition of ice-cold M199 medium containing 8% (v/v) FCS. Any cells that still adhered to the flask were detached by banging the flask gently. The cell suspension was then pelleted by centrifugation (1200 rpm for 5 mins) and resuspended in fresh complete M199 medium.

2.2.2 Cryopreservation of cells in liquid nitrogen

Cells were harvested in the same manner as for subculturing and their cell viability assessed. Cells were then centrifuged (1200 rpm for 5 mins) and the pellet resuspended in ice-cold medium containing 10% (v/v) dimethylsulphoxide (DMSO) as

cryoprotectant and 20% (v/v) FCS to give a final cell concentration of 2-4 x 10^6 cells/ml. The cells were then transferred in 1-1.5 ml volumes to cryotubes and frozen in a freezing plug over a liquid nitrogen (N_2) tank, conditions that permitted a gradual decrease in temperature (1°C/min), before being transferred to liquid N_2. To maximise the recovery of cells from liquid N_2, the cells were thawed quickly in a 37°C water-bath and immediately transferred to ice-cold medium. Cells were washed twice in medium to ensure all traces of DMSO were removed from the cell suspension. The final pellet was resuspended in complete medium containing 8% (v/v) FCS and cell viability assessed.

2.3 Assessment of cell viability

An Ethidium bromide/Acridine orange (EtBr/AO) staining solution was prepared by dissolving 0.1 mg Acridine orange (Sigma, Poole, UK) and 0.1 mg Ethidium bromide (Sigma) in 100 ml of PBS and stored in the dark at 4°C. To assess cell viability 20 µl of cell suspension was diluted in an appropriate volume of EtBr/AO and one drop was placed on a haemocytometer. The number of viable cells (green) and non-viable cells (orange) were counted using a fluorescent microscope with a combination of UV and visible light.

2.4 Bio-Rad protein assay

Protein concentrations were determined by the Bio-Rad protein assay (BioRad, Herts, UK). 150 µl of diluted test sample or a serially diluted protein standard (BSA, Sigma) were mixed with 40 µl of Bio-Rad dye reagent in a 96-well microtitre plate. After 5 mins incubation absorbance at 595 nm was measured against the reagent blank. Protein concentration of the test sample was determined with reference to a standard curve prepared using BSA (0-200 µg/ml).

2.5 LPS analysis

The levels of LPS present in samples were assessed using the *Limulus amebocyte* lysate (LAL) test, obtained commercially as E-Toxate™ kit from Sigma-Aldrich. Endotoxin levels were measured according to manufacturer's instructions and semi-quantified by a simple equation to give endotoxin amounts in EU/ml.

2.6 UV STERILIZATION

Protein antigens or other preparations were sterilized by exposure to UV-irradiation at 254 nm for 15 mins at a distance of 8 cm from the light source. Only medium-free preparations were UV-irradiated as the phenol red dye present in RPMI-1640 medium generates toxic free radicals upon exposure to UV light.

2.7 MICE

Specific pathogen-free BALB/c (H-2^d), C3H/HeN and C3H/HeJ (H-2^k) mice were obtained from Harlan UK Ltd., Bicester, Olac, UK and were maintained according to the regulations and guidelines of the Irish Department of Health. Experiments were performed under license from the Department of Health and with the approval of the university ethics committee. All mice used were female 6-12 weeks old at the initiation of each of the experiments.

2.8 BACTERIAL ANTIGENS

Preparations of CRM_{197}, that were untreated (native) or had been pre-treated with 0.06%, 0.18% or 0.6% concentrations of formaldehyde, diphtheria and tetanus adsorbed to alum and purified filamentous haemagglutinin (FHA), pertactin (PRN), recombinant pertussis toxin (PT-9K/129G) and tetanus toxoid (TT) were supplied by Chiron Corporation, Siena, Italy. Two meningococcal B antigens, ORF1 and ORF40, were also supplied by Chiron Corporation, Siena as protein solutions in 10% glycerol, 0.5 M arginine, 5 mM reduced glutathione, 0.5 mM oxidate glutathione, 50 mM phosphate buffer, pH 9.8. Keyhole Limpet Hemocyanin (KLH) was obtained from CalBiochem®, SanDiego, USA. PT was heat inactivated (20 mins at 80°C) prior to use in T cell assays *in vitro* in order to abolish mitogenic activity.

2.9 ADJUVANTS

Chitosan glutamate (UPG210) was obtained from Pronova Biomedical and supplied by West Pharmaceutical Services, Nottingham, UK as a 5 mg/ml solution in 10% sucrose, 10 mM phosphate buffer, pH 5.6. *E. coli* heat labile toxin (LT) and mutants of LT, LTK63 and LTR72 created by site-directed mutaganesis as previously described (Magagnoli *et al.*, 1996, Giuliani *et al.*, 1998), were kindly provided by Chiron Corporation, Siena, Italy. Cholera toxin (CT) was obtained from Sigma.

2.10 Preparation of alum-adsorbed antigens

An alum-adsorbed DTPa vaccine was prepared by mixing FHA (2.5 μg), PT-9K/129G (5 μg), PRN (2.5 μg), TT (10 μg) and CRM_{197} (10 μg) with alhydrogel (Alum, Superfos, Biosector, Denmark, 300 μg) and incubating the mixture overnight at 4°C with constant agitation.

2.11 Immunizations

Mice were immunized either by intra-peritoneal (i.p.) injection with a volume of 300 μl, subcutaneous (s.c.) injection into the footpad with a total volume of 50 μl or intranasally (i.n.) by resuspending the vaccine in a maximum volume of 25 μl and applying to the external nares using a pipette or given into the lungs or pulmonary route by dropping a maximum volume of 50 μl vaccine onto the nares of mice under light halothane anesthesia (Sigma). Mice were immunized i.n., s.c., or i.p. with various combinations of antigens (CRM_{197}, ORF1, ORF40, DTPa, KLH) and adjuvants (LT, LTR72, LTK63, chitosan) and antigen-specific immune responses assessed.

2.12 Detection of immune responses

Immunized BALB/c mice were sacrificed by cervical dislocation usually one or two weeks after one, two or three immunizations. Sera were collected from immunized and naïve animals by cardiac puncture and analysed for antigen-specific total IgG or IgG1 and IgG2a subclasses. In certain experiments the sera were also analysed for diphtheria toxin specific neutralizing antibodies. Lungs were also removed and the concentrations of antigen-specific IgA determined. Whole spleens and lymph nodes from BALB/c mice were removed and cell suspensions prepared for the detection of cellular immune responses.

2.12.1 Detection of murine T cell responses *in vitro*

Whole spleens or lymph nodes were homogenized on sterile grids, and cells washed and resuspended in fresh RPMI-1640 medium supplemented with 8% FCS. Spleen mononuclear cells (2×10^6) or lymph node cells (1×10^6) were cultured in triplicate wells of 96-well microtitre plates at 37°C and 5% CO_2 with various concentrations of antigen, or with the mitogens PMA (Sigma) and purified anti-mouse CD3 (PharMingen, SanDiego, USA) or medium only as positive and negative controls

respectively. Supernatants were removed after 72 hours to test for IL-4, IL-5, IFN-γ and IL-10. Cells were cultured for a further 24 hours and pulsed with 0.5 μCi of [^{3}H]-thymidine (Amersham Pharmacia Biotech) in 25 μl of complete RPMI for the final 4-6 hours of culture. After 4-6 hours of incubation, cells were harvested onto glass fiber filters (Wallac) with an automatic cell harvester. The filters were then dried using an infrared lamp and sealed in plastic sample bags with 5 ml of non-aqueous scintillation fluid (BetaScint, Wallac). [^{3}H]-thymidine incorporation was assessed using a Beta-plate scintillation counter (Wallac). Results are expressed as mean counts per minute (cpm) of [^{3}H]-thymidine incorporation for triplicate cultures of spleen cells or lymph node cells.

2.12.2 Assessment of cytokine concentrations by ELISA

The concentrations of IL-4, IL-5, IL-10 and IFN-γ were measured by enzyme linked immunosorbent assay (ELISA) using pairs of antibodies as described in Table 2.1. Maxi-sorb immunoassay plates (Nunc) were coated with 50 μl/well of purified rat anti-mouse IL-5, IL-4 and IFN-γ at 1 μg/ml and incubated overnight at 4°C. The plates were washed 3-4 times with wash buffer (PBS/0.05% Tween 20) and then incubated with 200 μl/well of blocking solution (PBS supplemented with 10% w/v milk powder) at room temperature for 2 hours to prevent non-specific binding. After washing, plates were incubated overnight at 4°C with 50 μl/well of test supernatant or a serially diluted recombinant cytokine standard (Table 2.2). Plates were then washed and incubated for 1 hour at room temperature with 50 μl/well of biotinylated anti-mouse IL-4, IL-5 or IFN-γ at 1 μg/ml. Cytokine concentrations in test supernatants and diluted standard concentrations were detected by incubating plates for 20 mins at room temperature with 50 μl/well of alkaline phosphatase conjugated ExtrAvidin (Sigma, 1:1000 in PBS) followed by 100 μl/well of p-nitrophenyl phosphate (p-NPP) substrate (Sigma) dissolved in diethalonamine buffer, pH 9.8 (1 mg/ml). O.D. values were measured at 405 nm using a microtiter plate reader (Molecular Devices, VERSAmax tunable microplate reader). Concentrations of cytokines in the test supernatants were determined with reference to a standard curve prepared for recombinant cytokines of known concentration. The limits of the assay were 0.5 ng/ml for IFN-γ and 20 pg/ml for IL-4 and IL-5. IL-10 concentrations in test supernatants were evaluated as described in section 2.18.2.

2.12.3 IgG ELISA

Antigen-specific IgG antibodies in the sera of immunized and naive mice were determined by ELISA. 96-well immunoassay plates were coated overnight with 50 µl/well of antigen (1 µg/ml). Plates were washed 3-4 times in wash buffer (PBS/0.05% Tween 20) to remove excess antigen and non-specific binding sites blocked by incubating plates with 200 µl/well of PBS supplemented with 10% (w/v) milk powder for 2 hours at room temperature. After washing, serial twofold or threefold dilutions of serum in PBS (50 µl/well) were added to the plates, which were then incubated overnight at 4°C or for two hours at room temperature. Plates were washed and bound IgG antibodies detected by incubation for 1 hour at room temperature with 50 µl/well of alkaline phosphatase conjugated, rat anti-mouse IgG monoclonal antibody (1/1000 in PBS/10% FCS), followed by 100 µl/well of pNPP substrate (Sigma) in diethanolamine buffer (1 µg/ml). Absorbance was read at 405 nm and results are expressed as $\log_{10}$ endpoint antibody titres calculated by extrapolation of the linear portion of a curve of OD versus reciprocal serum dilution to a cutoff point two standard deviations above control values obtained for serum from naïve mice.

2.12.4 IgG subclass ELISA

Antigen-specific IgG1 and IgG2a antibodies in the sera of immunized mice were determined by ELISA. Plates were coated, blocked and serum samples added as for IgG (Section 2.12.3). Bound IgG1 or IgG2a antibodies were detected by incubating the plates with 50 µl/well of biotinylated antibody specific for IgG1 or IgG2a (Table 2.1, 1:10,000 in PBS) for 1 hour at 37°C. The plates were then washed and incubated with 100 µl/well of horseradish-peroxidase (HRP) conjugated streptavidin (1:200 in PBS/1% BSA) at 37°C for 30 mins. Finally, after washing 100 µl/well of the substrate solution TetraMethylBenzidine (TMB, Sigma) was added to the plates. The colour reaction was allowed to develop for approximately 20 mins after which time 50 µl/well of 1 M H_2SO_4 was added to stop the enzymatic reaction. The OD values were determined by measuring absorbance at 450 nm using a microtiter plate reader and IgG1 or IgG2a end-point titres were determined as for IgG (Section 2.12.3).

2.12.5 IgA ELISA

Lungs were homogenized in RPMI containing 8% FCS and 1 μg/ml of the protease inhibitor phenylmethyl sulfonyl fluoride (PMSF, Sigma). Cellular debris was pelleted by centrifugation (15,000g for 5 mins). Antigen-specific IgA levels in the supernatants were determined by ELISA using the same method as for IgG. Bound IgA antibodies were detected using sheep anti-mouse IgA antibody (1/3000 in PBS, 50 μl/well) followed by alkaline phosphatase conjugated donkey anti-sheep IgG antibody (1/3000 in PBS, 50 μl/well). Results are expressed as end-point IgA titres calculated by regression of a curve of OD versus reciprocal lung homegenate dilution to a cutoff point two standard deviations above control values obtained for lung homogenates of naïve mice.

2.12.6 Diphtheria toxin neutralizing antibodies

The following solutions were prepared prior to the assay

Fixing solution

2.5 g Sodium chloride (NaCl)
50 ml 40% Formaldehyde
500 ml Ethanol
450 ml ddH2O

Staining solution

5.8 g Crystal violet
3 g Sodium chloride (NaCl)
400 ml Ethanol
600 ml ddH2O

Eluent solution

500 ml Ethanol
500 ml ddH2O

Diphtheria toxin-specific neutralizing antibody concentrations in the sera of immunized and naïve mice were assessed by vero cell assays using a method similar to Miyamura *et al.* (1974). Serial twofold or threefold dilutions of serum or standard

antitoxin for diphtheria (equine anti-sera from CBER, FDA, Bethesda, USA supplied by Chiron Corporation, Siena as a 6 IU/ml solution, diluted 1/200 prior to assay) in M199 medium without FCS were added to the wells of 96-well tissue culture plates (20 µl/well). Plates were incubated at 37°C for three hours with 20 µl/well of diphtheria toxin (Chiron Corporation, Siena, 80 Lf/ml diluted $1:10^5$ prior to the assay). Following incubation, 200 µl of M199 medium, supplemented with 8% (v/v) FCS, containing 10^4 Vero cells was added to the wells. The neutralizing effect of antibodies versus diphtheria toxin was evaluated by analysis of the growth of Vero cells after 3 days incubation at 37°C. Supernatants from each well were removed and viable cells, which remained adherent to the plates, were fixed with 100 µl/well of fixing solution and stained with 50 µl/well of crystal violet staining solution. Following solubilization of the dye by the addition of 100 µl/well of eluent solution, cell density was detected by measurement of the absorbance at 540 nm using a microtiter plate reader (Molecular Devices). The levels of neutralizing antibodies in serum samples were expressed as IU/ml with reference to a standard curve prepared using standard antitoxin.

2.13 Generation of CRM_{197}-specific T cell lines and clones

T cell lines were established from the spleens of individual mice 10 weeks after immunization (0, 4 and 8 weeks) with CRM_{197} (Chiron Corporation, Siena) and chitosan (West Pharmaceutical Services, UK). Spleen cells were isolated from immunized mice as described in Section 2.12.1 and cultured *in vitro* at 2×10^6 cells/ml with native CRM_{197} antigen (5 µg/ml) in RPMI-1640 medium supplemented with 2% (v/v) normal mouse serum (Biological Labs, Ballina, Co. Mayo). After 7 days incubation, autologous irradiated spleen cells (APC, 2×10^6/ml) were added and 5 days later the cells were washed, centrifuged (1200 rpm for 5 mins) and resuspended at 1×10^5/ml with native CRM_{197} (5 µg/ml) and irradiated APC (2×10^6 cells/ml) in RPMI-1640 medium supplemented with 8% (v/v) FCS. T cell lines were established by maintaining the cells at 2×10^5/ml with splenic APC and antigen for 4-5 days followed by 7-8 days incubation with APC alone. After 2 rounds of antigen stimulation, the T cell lines were cloned by limiting dilution at 10, 5 and 1 cell(s)/well in 100 µl volumes of RPMI-1640 containing APC (2×10^6/ml), CRM_{197} antigen (5 µg/ml) and recombinant murine IL-2 (5 U/ml) in 96-well plates. Following 4-5 days

incubation, rIL-2 was added to the cells to give a final concentration of 5 U/ml, and 7-8 days later actively-growing T cell clones were transferred in 1-2 ml volumes to 24-well plates (Costar) and restimulated with APC and antigen. T cell clones were maintained by restimulation at the initial concentration of 1 x 10^5/ml with antigen and APC every 11-12 days, with rIL-2 added 4-5 days after stimulation, and were progressively expanded to 25 cm^3 tissue culture flasks. The specificity of the T cell clones was tested against CRM_{197} antigen using proliferation and cytokine assays.

2.13.1 Preparation of APC

Whole spleens from naïve BALB/c (H-2^d) mice were placed in vials containing ice-cold RPMI-1640 medium/8% FCS. These vials were inserted into the irradiation chamber of a Nordian Gammcell 3000 Elan irradiator and irradiated at a dose of 30 Gy over 2 mins, which prevents subsequent cell proliferation. The irradiated spleens were homogenized in fresh RPMI-1640 medium supplemented with 8% FCS. The cell suspension was then washed twice, resuspended in fresh medium and cell viability assessed.

2.14 Guinea pigs

Guinea pigs were maintained and immunized at West Pharmaceutical Services, UK. Sera samples from naïve and immunized guinea pigs were couriered to Ireland where they were analysed for their levels of diphtheria toxin specific neutralizing antibodies (Section 2.12.6).

2.15 Human clinical trial

A phase I clinical trial was conducted between March and August 2001 at St. George's Hospital Medical School, London and blood and serum samples from the subjects were sent to Ireland where they were analysed for antigen-specific IgG responses and specific diphtheria toxin neutralizing antibodies. In addition, CRM_{197}-specific T cell responses were assessed from peripheral blood mononuclear cells (PBMCs) isolated from human blood by density gradient centrifugation.

2.15.1 Vaccines

A non-toxic mutant of diphtheria toxin, CRM_{197} (Porro *et al.*, 1980), was prepared and treated with 0.18% formaldehyde for stabilization by Chiron Vaccines, Siena, Italy.

CRM_{197} (50 μg/dose) was then formulated with phosphate salts (0.5 mg/dose) and mannitol (2.5 or 9.5 mg/dose), with or without chitosan glutamate (7 mg/dose) as powder solutions at West Pharmaceutical Services, Nottingham, UK. The powders were packaged in Valois® monopowder nasal devices and stored at 4°C in a dessicator prior to use. A commercial aluminium hydroxide adsorbed diphtheria vaccine (Chiron, 7.5 μg DT/dose) was used as the parenteral control vaccine.

2.15.2 Immunizations

25 subjects were enrolled in the study and were randomised to receive a powder formulation containing CRM_{197}, with or without chitosan, intranasally or conventional diphtheria vaccine intramuscularly. 10 volunteers received CRM_{197} (50 μg/dose) as a powder formulation, with chitosan (7 mg/dose) and mannitol (2.5 mg/dose). 10 volunteers received a powder formulation of CRM_{197} (50 μg/dose) with mannitol alone (9.5 mg/dose). Vaccines were administered to a single nostril on days 0 and 28. In addition, 5 volunteers were immunized intramuscularly once with conventional diphtheria vaccine (7.5 μg DT/dose), as a parenteral reference group.

2.15.3 Isolation and *in vitro* stimulation of PBMC

Fresh venous blood samples (12-15 ml) were drawn into preservative-free heparinized tubes and sent by courier to Ireland where they were processed within 24 hours. Peripheral blood mononuclear cells (PBMC) were prepared on Ficoll gradient using the method described by Ryan *et al.* (1997). Blood was diluted 1:2 in FCS-free RPMI-1640 medium (Gibco BRL), layered onto a Ficoll gradient (Sigma, Histopaque 1077, St. Louis, MO, USA) and centrifuged at 1500 rpm for 30 mins (at room temperature). After centrifugation, PBMC were removed from the gradient, washed twice and the final pellet was resuspended in RPMI-1640 medium supplemented with 8% FCS. Cells were cultured at 2 x 10^6 cells/ml in triplicate wells of 96-well microtitre plates (200 μl/well) with native CRM_{197} antigen (1-15 μg/ml, Chiron) or chemically detoxified diphtheria toxin (DT, 5 μg/ml, Chiron) for 3 days at 37°C in a humidified CO_2 incubator. Control stimuli included medium alone, phytohemagglutinin (PHA, 2.0 μg/ml), or anti-CD3 (UCHT1, 1.0 μg/ml) and phorbal myristate acetate (PMA, 25 ng/ml, Sigma). Supernatants were removed after 72 hours and the levels of IFN-γ and IL-5 measured by specific immunoassays using the method described by Barnard *et al.*

(1996), using pairs of monoclonal antibodies purchased from PharMingen, San Diego, CA (Table 2.1). The plasma obtained from the isolation of PBMC by density gradient centrifugation was frozen at –80°C until it was required for the analysis of IgG antibodies (Section 2.15.5) or diphtheria toxin neutralizing antibodies (Section 2.12.6)

2.15.4 Assessment of IL-5 and IFN-γ concentrations by ELISA

96-well microtiter plates were coated overnight at 4°C with 50 µl/well of purified IL-5 or IFN-γ coating antibody (1 µg/ml in PBS). After washing, non-specific binding sites were blocked by incubating plates for 2 hours at room temperature with 200 µl/well of blocking solution (PBS/10% milk powder). Plates were washed and incubated overnight at 4°C with 50 µl/well of test supernatant or serially diluted IL-5 (0-1000 pg/ml in RPMI medium) or IFN-γ (0-5000 pg/ml in RPMI medium) standard (Table 2.2). After washing, 50 µl/well of biotinylated detection antibody (1 µg/ml in PBS) was added and plates incubated at room temperature for 1 hour. Finally, plates were washed and cytokine levels in test supernatants and diluted standards were detected by incubating plates for 20 mins at room temperature with 100 µl/well of alkaline phosphatase conjugated ExtrAvidin (Sigma) followed by 100 µl/well of p-nitrophenyl phosphate (p-NPP) substrate (Sigma, 1:1000 in PBS) dissolved in diethalonamine buffer, pH 9.8 (1 mg/ml). Absorbance was read at 405 nm using a microtiter plate reader (Molecular Devices) and cytokine concentrations in the test supernatants were derived from standard curves prepared for recombinant human cytokines of known concentration. Specific cytokine production in response to antigen was calculated by subtracting concentrations measured in un-stimulated cultures with medium alone from those observed in antigen-stimulated cultures. The limits of sensitivity of the assays were 15 pg/ml.

2.15.5 IgG ELISA

Anti-diphtheria toxoid (DT) specific IgG levels were measured by enzyme-linked immunosorbent assay (ELISA) on plasma prepared from blood samples. ELISA plates were coated overnight with DT (1 µg/ml) at 4°C. After washing, non-specific binding sites were blocked by adding 200 µl/well of blocking solution (PBS/10% milk powder) and incubating plates for 2 hours at room temperature. The plates were washed and incubated overnight at 4°C with 50 µl/well of serially diluted plasma samples. After

washing, bound IgG antibodies were detected by incubating plates for 1 hour at room temperature with 50 µl/well of alkaline-phosphatase conjugated antibody specific for human IgG (Sigma, 1:2000 in PBS/10% FCS), followed by 100 µl/well of pNPP substrate in diethalonamine buffer, pH 9.8 (1 mg/ml). Absorbance was read at 405 nm and IgG end-point titres were calculated by regression of a curve of OD versus reciprocal plasma dilution to a cut-off point two standard deviations above a control value obtained from plasma of an individual who had never been immunized against diphtheria.

2.15.6 Diphtheria toxin neutralizing vero cell assays

Human plasma or serum samples were tested for specific anti-diphtheria toxin neutralizing antibodies using the method described in Section 2.12.6.

2.16 Generation of murine bone marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDC) were generated from BALB/c (H-2^d), C3H/HeN or H3H/HeJ (H-2^k) mice using a method similar to Lutz *et al.* (1999). Mice were euthanized and their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with RPMI medium and cell aggregates were disintegrated using a syringe. The resulting cells were washed, pelleted by centrifugation (1200 rpm for 5 mins at 4 °C) and resuspended in fresh RPMI medium. Cell viability was assessed and immature BMDC were prepared by culturing cells at 1 x 10^6 per tissue culture flask in RPMI-1640 and 10% FCS supplemented with 5-10% of a supernatant from a GM-CSF expressing cell line (provided by Nathalie Winter, Institute Pasteur, Paris, France). The cells were maintained for a period of 9 days by washing, pelleting by centrifugation (1200 rpm for 5 mins) and reculturing with fresh RPMI/10% FCS containing 10% GM-CSF cell supernatant every 3 days. On day 10, the viability of the BMDC was assessed and the cells were then ready for use.

2.17 Molecules for inducing DC maturation

E. coli LPS (serotype 127:B8) was supplied by Sigma-Aldrich. Phosphorothioate-stabilized oligodeoxynucleotide-containing CpG motifs (CpG-ODN) (5'GCTAGACGTTAGCGT) were custom synthesized by Sigma-Aldrich.

2.18 Analysis of BMDC maturation and cytokine/chemokine secretion

BMDC (10^6/ml) were cultured in 24-well tissue culture Nunc plates at 37°C for 24 hours with 1 or 10 μg/ml of LT, CT, LTK63, LTR72 or with *E. coli* LPS (1 μg/ml) and murine IFN-γ (20 ng/ml) or CpG-ODN (10 μg/ml) as positive controls or medium only as a negative control. Alternatively, BMDC (10^6/ml) were cultured at 37°C for 2 hours in the presence or absence of 1 or 10 μg/ml of CT, LT, LTR72 or LTK63 before stimulation with LPS (1 μg/ml) and murine IFN-γ (20 ng/ml). Supernatants were removed after 24 hours for analysis of cytokine and chemokine concentrations. IL-12p40, IL-12p70, IL-10, IL-6, TNF-α, CCL3 (MIP-1α), CCL4 (MIP-1β) and MIP-2 concentrations were measured by immunoassay using pairs of antibodies (Table 2.1) as described in the following sections. In some cases, the cells were gently removed from the base of the plates, washed and used for immunofluorescence staining (Section 2.18.5).

2.18.1 IL-12 immunoassay

IL-12 was detected by immunoassay, using commercially available antibodies (Genzyme) specific for the IL-12 p40 or p35 (detects bioactive p70) subunits. 96-well microtiter plates were coated overnight at 4°C with 50 μl/well of a purified anti-IL-12p40 or anti-IL-12p70 monoclonal antibody. After washing, non-specific binding sites were blocked by adding 200 μl/well of blocking solution (PBS/10% milk powder) and incubating plates at room temperature for 2 hours. Plates were washed and 50 μl/well of test supernatant or serially diluted IL-12 standard (Table 2.2, 0-5000 pg/ml) was added and plates were incubated overnight at 4°C. The plates were then washed and IL-12 was detected using a two-step antibody detection system. Firstly, plates were incubated with 50 μl/well of rat anti-IL-12p40 (2 μg/ml in PBS) for 1 hour at room temperature followed by 50 μl/well of anti-rat alkaline phosphatase conjugated IgG2a antibody (PharMingen, 1:1000 dilution) for 2 hours at room temperature. Finally plates were washed and incubated with 100 μl/well of pNPP substrate in diethalonamine buffer (1 mg/ml). Absorbance was read at 405 nm and cytokine concentrations in the test supernatants were determined with reference to a standard curve prepared using murine recombinant IL-12 standard of known concentration.

2.18.2 IL-10 and TNF-α immunoassay

Murine IL-10 and TNF-α concentrations were determined by specific immunoassay using matched pairs of antibodies (Table 2.1) as recommended by the manufacturers. 96-well microtiter plates were coated overnight at 4°C with 50 µl/well of rat anti-mouse IL-10 capture antibody (2 µg/ml in PBS) or purified anti-mouse TNF-α coating antibody (1.25 µg/ml in sodium carbonate/sodium hydrogen carbonate buffer). After washing, non-specific binding sites were blocked by adding 300 µl/well of blocking solution (1% BSA, 5% sucrose in PBS for IL-10, PBS/0.5% BSA for TNF-α) and incubating plates for 2 hours at room temperature. Plates were washed and 100 µl/well of test supernatant (diluted 1:2 in PBS/1% BSA for IL-10 or PBS/0.1% Tween 20/0.5% BSA for TNF-α) or serially diluted IL-10 standard (0-2000 pg/ml in PBS/1% BSA) or TNF-α standard (0-1000 pg/ml in PBS/0.1% Tween 20/0.5% BSA) was added and plates were incubated overnight at 4°C. After washing, plates were incubated with 50 µl/well of biotinylated goat anti-mouse IL-10 detection antibody (400 ng/ml in PBS/1% BSA) or biotinylated TNF-α detection antibody (0.05 µg/ml in PBS/0.1 % Tween 20/0.5 % BSA) for 2 hours at room temperature. Plates were washed and IL-10 or TNF-α were detected by incubating plates in the dark for 20 mins with 100 µl/well of horseradish-peroxidase (HRP) conjugated streptavidin (1:200 in PBS/1% BSA) followed by washing and adding 100 µl/well of the substrate solution TetraMethylBenzidine (TMB, Sigma) to the plates. The colour reaction was allowed to develop for approximately 20 mins after which time 50 µl/well of 1 M H_2SO_4 was added to stop the enzymatic reaction. The OD values were determined by measuring absorbance at 450 nm using a microtiter plate reader and cytokine concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse IL-10 or TNF-α of known concentration and potency.

2.18.3 IL-6 immunoassay

Murine IL-6 was detected by specific immunoassay using matched pairs of monoclonal antibodies (PharMingen, Table 2.1). ELISA plates were coated overnight at 4°C with 50 µl/well of purified rat anti-mouse IL-6 (1 µg/ml in sodium hydrogen carbonate buffer). After washing, non-specific binding was prevented by incubating the plates for 2 hours at room temperature with 200 µl/well of blocking solution (PBS/3 % BSA). The plates were then washed and incubated overnight at 4°C with 50

µl/well of test supernatant or serially diluted IL-6 standard (0-5000 pg/ml in RPMI-1640 medium). After washing, 50 µl/well of biotinylated IL-6 detection antibody (1 µg/ml in PBS/0.05 % Tween 20/10 % FCS) was added and plates were incubated for 1 hour at room temperature. IL-6 was detected by incubating plates for 30 mins at room temperature with 100 µl/well of Streptavidin-HRP (1:1000 in PBS/0.05 % Tween 20/1 % BSA) followed by 100 µl/well of TMB, as for IL-10 (Section 2.18.2). Absorbance was read at 450 nm and concentrations of IL-6 in test supernatants were evaluated from a standard curve prepared using recombinant murine IL-6 of known concentration and potency.

2.18.4 Chemokine immunoassays

The chemokines CCL3 (MIP-1α), CCL4 (MIP-1β), MIP-2 and RANTES were detected by immunoassay using commercially available antibodies (R&D Systems, Table 2.1) as recommended by the manufacturer. ELISA plates were coated overnight at 4°C with 100 µl/well of capture antibody (1 µg/ml in PBS) specific for the chemokine in question. After washing, plates were incubated for 2 hours at room temperature with 200 µl/well of blocking solution (1% BSA in PBS), to prevent non-specific binding. The plates were then washed and 50 µl/well of the test supernatant or serially diluted chemokine standard (0-5000 ng/ml in RPMI-1640 medium) was added and plates were incubated overnight at 4°C. After washing, the plates were incubated with 100 µl/well of biotinylated detection antibody (0.2 µg/ml in TBS) for 1 hour at 37°C. The plates were washed and incubated for 15 mins at 37°C with 100 µl/well of Streptavidin-HRP (Sigma, 1:1000 in PBS/0.05% Tween 20/1% BSA). Finally after washing, chemokine concentrations were evaluated by incubating the plates with 100 µl/well of TMB substrate, as for IL-10 (Section 2.18.2). Absorbance was read at 450 nm and chemokine concentrations for test supernatants were determined by reference to a standard curve prepared for recombinant chemokines of known concentration and potency. The sensitivity limit of the assays was 31 pg/ml.

2.18.5 Flow cytometry analysis

BMDC surface marker expression was analysed by flow cytometry using a FACScan™ flow cytometer (Becton-Dickinson, San Jose, CA). The FACScan was calibrated using the Autocomp software in conjunction with commercially prepared

fluorescent beads (Calibrate beads, Becton-Dickinson). BMDC to be analysed were washed, resuspended in PBA and transferred to FACS tubes (Falcon). Non-specific binding was prevented by incubating the cells with 2% normal mouse serum (Biological Labs, Ballina, Co. Mayo) for 30 mins on ice. The cells were then stained with biotin-conjugated anti-CD11c antibody (PharMingen) for 30 mins on ice, washed three times in PBA and stained with CD80-PE, CD86-FITC, CD40-FITC, CCR5-PE or MHC Class II-PE and streptavidin-PerCP (PharMingen) for 30 mins on ice. Cells incubated with an isotype matched directly conjugated antibody with irrelevant specificity acted as a control. Stained cells were then fixed with 4% formaldehyde solution and immunofluorescence analysis was performed on a FACScan. The results were analysed using CELLQuest™ software. 10,000 cells were analysed per sample.

2.19 *IN VITRO* J774 MACROPHAGE STIMULATION

J774 cells (1 x 10^6/ml) were cultured at 37°C with complete medium or LPS (1 μg/ml) alone or with 0.1 or 1 μg/ml of LT, LTR72 or LTK63. Supernatants were removed after 24 hours and analysed for MIP-1α, MIP-1β and MIP-2 by specific immunoassay (Section 2.18.4).

2.20 ADOPTIVE TRANSFER OF DC INTO NAÏVE MICE

BMDC from BALB/c mice were isolated and cultured as described in Section 2.16. BMDC (10^6/ml) were cultured in 24-well tissue culture Nunc plates at 37°C for 24 hours with medium alone as a negative control or with KLH (5 μg/ml) alone or with CT (1 μg/ml) or CpG (1 μg/ml). After 24 hours the cells were gently removed from the base of the wells and washed five times in RPMI medium to remove all traces of KLH, CT or CpG. Naïve BALB/c mice were injected i.v. with unstimulated or stimulated DC (10^5 cells/mouse) in a total volume of 300 μl. After 7 days, mice that had received stimulated DC were injected i.v. with soluble KLH (5 μg/dose). Cellular responses were assessed from spleen cells stimulated *in vitro* with KLH (1-100 μg/ml) and cytokine concentrations were determined as described in Sections 2.12.1 and 2.12.2.

2.21 STATISTICAL ANALYSES

Statistical analyses were performed using the computer-based mathematical package GraphPad InStat®. Statistical differences in mean antibody, cytokine or chemokine

values between two experimental groups were determined by the Student's *t* test for normal distributions or Mann-Whitney U test for non-parametric distributions and statistical differences in values between more than two experimental groups were determined by the Tukey-Kramer ANOVA test for normal distributions or Dunn's test for non-parametric distributions as described in the figure legends. P values of less than 0.05 are considered significant.

TABLE 2.1 ORIGIN AND SPECIFICITES OF ANTIBODIES FOR ELISA

Antibody Specificity	Clone	ELISA[a]	Purified/ Conjugate	Supplier	Neutralising ability[b]
Mouse IFN-γ	RA-6A2	c IFN-γ	Purified	PharMingen	Yes
Mouse IFN-γ	XMG1.2	d IFN-γ	Biotin	"	Yes
Mouse IL-4	11-B-11	c IL-4	Purified	NIH, USA	Yes
Mouse IL-4	BVD6-24G2	d IL-4	Biotin	PharMingen	No
Mouse IL-5	TRFK5	c IL-5	Purified	"	Yes
Mouse IL-5	TRFK4	d IL-5	Biotin	"	Yes
Mouse IL-6	MP5-20F3	c IL-6	Purified	"	Yes
Mouse IL-6	MP5-32C11	d IL-6	Biotin	"	No
Mouse IL-12p40	C17.8	c IL-12p40	Purified	"	Yes
Mouse IL-12p70	9A5	c IL-12p70	Purified	"	nd
Mouse IL-12	C15.6	d IL-12	Purified	"	nd
Rat IgG2a	R19-15	d IL-12	AKP	"	nd
Mouse IL-10	-	c IL-10	Purified	R&D	nd
Mouse IL-10	-	d IL-10	Biotin	"	nd
Mouse MIP-1α	-	c MIP-1α	Purified	"	Yes
Mouse MIP-1α	-	d MIP-1α	Biotin	"	nd
Mouse MIP-1β	-	c MIP-1β	Purified	"	Yes
Mouse MIP-1β	-	d MIP-1β	Biotin	"	nd
Mouse MIP-2	-	c MIP-2	Purified	"	Yes
Mouse MIP-2	-	d MIP-2	Biotin	"	nd
Mouse RANTES	-	c RANTES	Purified	"	Yes
Mouse RANTES	-	d RANTES	Biotin	"	nd
Mouse TNF-α	-	c TNF-α	Purified	Biosource	nd
Mouse TNF-α	-	d TNF-α	Biotin	"	nd
Mouse IgG1	A85-1	d IgG1	Biotin	PharMingen	nd
Mouse IgG2a	R11-89	d IgG2a	Biotin	"	nd
Human IFN-γ	NIB42	c IFN-γ	Purified	"	Yes
Human IFN-γ	4S.B3	d IFN-γ	Biotin	"	Yes
Human IL-5	JES1-39D10	c IL-5	Purified	"	Yes
Human IL-5	JES1-5A10	d IL-5	Biotin	"	Yes

[a] c = coating, d = detection

[b] nd = not determined

TABLE 2.2 ORIGIN OF RECOMBINANT CYTOKINES FOR USE AS ELISA STANDARDS

Cytokine	Source
Murine IFN-γ	NIBSC, Herts, UK
Murine IL-4	"
Murine IL-6	"
Murine IL-5	PharMingen, USA
Murine IL-12	Stanley Wolf, Genetics Institute Inc., USA
Murine IL-10	R&D Systems. UK
Murine MIP-1α	"
Murine MIP-1β	"
Murine MIP-2	"
Murine RANTES	"
Murine TNF-α	Biosource International Inc.
Human IFN-γ	NIBSC, Herts, UK
Human IL-5	NIBSC, Herts, UK

CHAPTER 3

ADJUVANT ACTIVITY OF CHITOSAN FOR NASAL VACCINE DELIVERY IN MICE

3.1 Introduction

Diphtheria is a rapidly developing acute bacterial disease, involving both local and systemic pathology, which is caused by *Corynebacterium diphtheriae*. Most of the clinical symptoms of the disease are due to the release of the potent diphtheria toxin from lysogenized strains of the bacteria. Therefore, disease can be prevented by toxin neutralizing antibodies (antitoxin) induced through active immunization with non-toxic forms of the toxin. Current diphtheria vaccines are prepared by converting diphtheria toxin to its non-toxic, but antigenic, toxoid by formaldehyde treatment, and are mostly combined with tetanus toxoid and whole-cell or acellular pertussis vaccines for infant immunization (Rappuoli, 1997).

Routine immunization against diphtheria, introduced in the 40's and 50's, led to the almost complete eradication of this disease from developed countries by the 1970's. However, a high proportion of the European population has been shown to have diphtheria antitoxin titres below the putative protective level of 0.01 IU/ml (Kjeldsen *et al.*, 1985, Rappuoli *et al.*, 1988, Garcia-Corbeira *et al.*, 1999, Maple *et al.*, 2000). As a result there has been a resurgence of diphtheria in several European countries in the 80's and 90's (Rappuoli *et al.*, 1988, Galazka and Robertson, 1996). In most countries it is now recommended that booster doses of diphtheria vaccine be administered every ten years. Although the current vaccines against diphtheria have been very successful, they may be associated with some adverse effects, due to the presence of accessory antigens in partially purified diphtheria toxoid (DT) preparations (Pappenheimer *et al.*, 1950) or due to the inclusion of aluminium compounds as adjuvants (Mark *et al.*, 1995). It would therefore be beneficial to develop a new, more refined vaccine against diphtheria.

Diphtheria toxin is composed of two disulfide-linked fragments, an enzymatically active A fragment, and a B fragment, that is responsible for binding and entry of the toxin into sensitive eukaryotic cells. Protein synthesis in these cells is inhibited by inactivation of the ribosomal elongation factor 2, caused by the specific ADP ribosylating activity of fragment A, resulting in cell death (Ratti *et al.*, 1983). Several non-toxic or partially toxic mutants of diphtheria toxin that cross-react immunologically with it, and hence are called cross-reacting materials (CRM), have been isolated. One such mutant, CRM_{197}, contains a glycine to glutamic acid mutation at position 52 in the A subunit, rendering it enzymatically inactive and therefore non-toxic (Rappuoli, 1997). Since CRM_{197} is naturally non-toxic it does not require

formaldehyde detoxification and can be obtained at close to 100% purity, avoiding the cross-linking to accessory antigens that occurs during formaldehyde treatment of diphtheria toxin. Studies have shown that CRM_{197} is less immunogenic than conventional diphtheria toxoid, but that its immunogenicity for antibody responses can be increased after stabilization with formaldehyde (Porro *et al.*, 1980, Gupta *et al.*, 1997).

Traditionally, vaccines such as diphtheria, tetanus and pertussis have been administered parenterally by intramuscular injection. However, mucosal immunization has the advantage over conventional parenteral immunization of stimulating both systemic and mucosal immunity. In addition, mucosal delivery of vaccines minimizes adverse effects and allows for easier administration, making vaccination in the home a possibility (Walker, 1994, O'Hagan, 1998). Mucosal immunization would therefore be of particular benefit where frequent boosting is required, as is the case for diphtheria. However, soluble antigens tend to elicit poor immune responses following mucosal administration and require the use of delivery vehicles, immunostimulants or adjuvants to increase their immunogenicity.

Chitosan is a cationic polysaccharide, comprising copolymers of glucosamine and N-acetylglucosamine, derived by partial deacetylation of chitin, a natural material found in the exoskeletons of various crustaceans. Chitosan is insoluble at alkaline and neutral pH, but forms salts with inorganic and organic acids. In solution the amino functions of chitosan are protonated and the resultant soluble polysaccharide carries a positive charge, allowing it to bind strongly to negatively charged sites on cell surfaces and mucus. Cationic chitosan salts have been shown to greatly improve the absorption of drugs from the nasal cavity (Schipper *et al.*, 1996, Illum, 1998, Thanou *et al.*, 2001). Chitin derivatives have been found to have many other beneficial effects, including wound-healing properties (Biagini *et al.*, 1991) and candidacidal (Suzuki *et al.*, 1987) and anti-viral activities (Iida *et al.*, 1987). It has been shown that 70% deacetylated chitin could activate peritoneal macrophages (Nishimura *et al.*, 1984) and natural killer cells (Nishimura *et al.*, 1985) and induce various cytokines in mice (Nishimura *et al.*, 1986), and in addition could non-specifically confer protection against Sendai virus and *E. coli* infections (Iida *et al.*, 1987). Chitosan is non-toxic in humans and is widely used in the food industry as a food additive and a weight-loss product (Hirano, 1996). Its safety, low production costs and possible adjuvant activity make chitosan an ideal candidate for the mucosal delivery of vaccines.

It has previously been demonstrated that nasal delivery of filamentous haemagglutinin from *Bordetella pertussis* with chitosan can stimulate local and systemic antibody responses (Jabbal-Gill *et al.*, 1998). Chitosan-based nano- or microparticles have also been used to enhance the immunogenicity of various antigens, such as Tat toxoid (Le Buanec *et al.*, 2001), beta-human chorionic gonadotropin (Seferian and Martinez, 2000), influenza DNA (Bacon *et al.*, 2000) and peanut allergen DNA (Roy *et al.*, 1999) following oral or intranasal administration.

In this study the T cell and antibody responses in mice were examined following intranasal (i.n.) and parenteral immunization with CRM_{197} alone or formulated with chitosan. Previous reports have shown that both native and formaldehyde-treated CRM_{197} induce similar anti-DT antibody responses in mice (Gupta *et al.*, 1997). However, there is little information available on the effect of formaldehyde treatment on T cell responses. Here the effects of formaldehyde treatment of CRM_{197} on the induction of both T cell and antibody responses for intranasal or parenteral immunization were evaluated. In addition, immune responses generated by priming parenterally with DT and boosting intranasally with CRM_{197} with or without chitosan were assessed in mice. The results provide evidence that a nasally delivered vaccine may be effective against diphtheria, especially when used for boosting individuals primed with the conventional diphtheria vaccine.

3.2 Results

3.2.1 Parenteral immunization in mice with CRM_{197} adsorbed to alum induces immune responses similar to those obtained with diphtheria toxoid

Parenteral (i.p.) immunization with alum adsorbed CRM_{197} induced antigen-specific IgG responses, which were equal to, or marginally greater than, those generated with a similar dose of DT in a conventional diphtheria-tetanus vaccine. Anti-DT IgG concentrations were higher, but not significantly, in sera of mice immunized with CRM_{197} treated with higher doses of formaldehyde (0.18% and 0.6%) compared with sera from mice immunized with native CRM_{197} (Fig. 3.1).

Analysis of cellular responses demonstrated that i.p. immunization with both DT and alum adsorbed CRM_{197} induced splenic T cells that produced IL-5 and IFN-γ in response to specific antigen stimulation *in vitro*. However, progressive treatment of CRM_{197} with formaldehyde reduced this mixed Th1/Th2 response; the high formaldehyde treated (0.6%) preparation only induced very weak T cell responses. DT induced similar IFN-γ levels, but lower levels of IL-5, to those induced by native or low dose-formaldehyde treated CRM_{197} (Fig. 3.2).

3.2.2 Formulation of CRM_{197} with chitosan enhances T cell and antibody responses for intranasal immunization in mice

In contrast to parenteral immunization, CRM_{197} preparations were found to be poorly immunogenic when administered in solution by the i.n. route. However, when formulated with chitosan, CRM_{197} generated modest cellular immune responses, as assessed by antigen-specific T cell proliferation and cytokine production. Three intranasal immunizations with CRM_{197} with or without chitosan induced T cells that secreted IL-5, but no detectable IFN-γ, following antigen stimulation of spleen cells *in vitro*. This predominant Th2 response was stronger, although not significantly, in mice immunized with CRM_{197} formulated with chitosan (Fig. 3.3).

Chitosan was also found to enhance local and systemic antibody responses following intranasal immunization with CRM_{197}. Analysis of anti-diphtheria toxoid IgG and IgA levels in the sera and lung homogenates demonstrated that mice immunized with CRM_{197} formulated with chitosan generated higher antibody titres than the same antigen without chitosan. This finding was consistent for doses of 5, 10 and 15 μg of CRM_{197} administered intranasally, with 10 μg of CRM_{197} and chitosan

generating significantly higher antibody titres than the same dose of antigen alone. Furthermore, 10 μg of CRM_{197} formulated with chitosan generated stronger IgG and IgA antibody responses than doses of 5 or 15 μg of CRM_{197} (Fig. 3.4). Mice immunized with this dose of CRM_{197}, formulated with chitosan, also generated low levels of anti-DT neutralizing antibodies (0.01 to 0.05 IU/ml; Table 3.1). In contrast, neutralizing antibodies could not be detected in the sera of mice immunized with 10 μg CRM_{197} without chitosan.

3.2.3 T cell subtypes induced following intranasal immunization with CRM_{197} and chitosan

To confirm the induction of a predominant Th2-type response following intranasal immunization with CRM_{197} and chitosan, diphtheria-specific T cell clones were established from individual mice immunized intranasally with CRM_{197} and chitosan. Analysis of cytokine production by two established T cell clones demonstrated potent production of IL-4 and IL-5, but no detectable IFN-γ, following stimulation with native CRM_{197} antigen in the presence of autologous APC, indicating that the diphtheria-specific T cell clones were of a Th2 phenotype (Fig. 3.5).

3.2.4 Mild formaldehyde treatment of CRM_{197} enhances immune responses induced following intranasal immunization in mice

It was found that formaldehyde treatment of CRM_{197} reduced T cell responses, but had little effect on antibody levels, in parenterally immunized mice (Fig. 3.1 and 3.2). An examination of the effect of formaldehyde treatment on the induction of immune responses following i.n. immunization demonstrated that the immunogenicity of CRM_{197} was affected by formaldehyde treatment for both T cell and antibody responses. The strongest and most consistent levels of IL-5 were produced by spleen cells from mice immunized with mildly formaldehyde treated (0.18%) CRM_{197} formulated with chitosan. Serum IgG and local IgA responses were also significantly higher in these mice. In contrast, native CRM_{197} and low dose (0.06%) formaldehyde-treated CRM_{197} only induced very weak anti-DT IgG and IgA antibody responses (Fig. 3.6).

3.2.5 Priming parenterally with DT in alum and boosting intranasally with CRM_{197} induces potent DT-specific immune responses

The development of a mucosally delivered diphtheria vaccine could be utilized for boosting individuals who had previously been immunized parenterally with DT, as part of the DTP vaccine. Therefore, the effects of priming mice parenterally with one immunization with diphtheria and tetanus vaccine in alum (0.17 of a human dose) and boosting intranasally 4 weeks later with CRM_{197} with or without chitosan were examined. The levels of serum IgG observed in mice primed i.p. with DT in alum and boosted i.n. with CRM_{197} with or without chitosan were similar to those from mice immunized i.p. twice with DT adsorbed to alum. Boosting i.n. with CRM_{197} with or without chitosan also enhanced local IgA responses. Interestingly, IgA antibody titres were higher in mice boosted with CRM_{197} without chitosan (Fig. 3.7). Analysis of neutralizing antibodies in murine sera demonstrated similar levels of anti-DT antibodies from mice primed parenterally with DT and boosted mucosally with CRM_{197} (with or without chitosan) or primed and boosted parenterally with DT in alum. However, anti-DT neutralizing antibodies were found to be more consistent from mice boosted with CRM_{197} formulated with chitosan (Table 3.1).

Analysis of cytokine responses of spleen cells demonstrated production of IL-5 and low levels of IFN-γ from spleen cells of all mice immunized i.p. with DT in alum, indicating the priming of both Th1 and Th2 populations of T cells *in vivo*. In contrast to priming parenterally with DT in alum, two i.n. immunizations with CRM_{197} and chitosan failed to generate moderate levels of IL-5 or IFN-γ by antigen-stimulated spleen cells *in vitro*. However, T cells from these mice did respond to the polyclonal activators, PMA and anti-CD3, which indicated that these cells were capable of responding *in vitro* (Fig. 3.8). In addition, mice immunized i.n. twice with CRM_{197} formulated with chitosan only generated very weak anti-DT IgG and undetectable levels of IgA (Fig. 3.7) or neutralizing antibodies (Table 3.1). This finding was consistent with previous results where it was found that a minimum of three i.n. immunizations with CRM_{197} plus chitosan were required to generate potent T cell and antibody responses. In contrast, a single booster immunization by the i.n. route generated potent immune responses in parenterally primed mice.

3.2.6 Influence of delayed antigen administration, following chitosan, on the induction of immune responses.

Having established that chitosan can enhance immune responses to i.n. delivered antigen, we sought to determine its mechanism of action. Illum *et al.* (1994) reported that medium molecular weight chitosan could successfully enhance the absorption of polypeptides nasally in sheep and rat models (Illum *et al.*, 1994). Intranasal immunization with CRM_{197} and chitosan generated stronger, although not significantly, antigen-specific IL-5 responses and anti-DT IgG responses when administered together rather than one day later (Fig. 3.9). In addition, anti-DT IgA responses (Fig. 3.9) and neutralizing antibody responses (0.03 IU/ml; Table 3.1) could only be detected in mice immunized intranasally with CRM_{197} and chitosan together. The enhancement of immune responses following intranasal immunization with CRM_{197} formulated with chitosan may therefore be due to the ability of chitosan to increase the residence of the antigen in the nasal cavity and to open tight junctions between the cells thereby promoting absorption into the lymphoid tissue.

3.2.7 Chitosan enhances immune responses to parenterally delivered CRM_{197} antigen in mice

In order to determine if the enhancing action of chitosan extended beyond antigen uptake at mucosal surfaces, its adjuvant effect by the i.p. route was examined. In comparison with antigen alone, formulation of CRM_{197} with chitosan enhanced IL-5 production by antigen-specific spleen cells *in vitro*. However IFN-γ production was similar to that observed from mice immunized with CRM_{197} without chitosan (Fig. 3.10). Therefore, chitosan would appear to selectively enhance the induction of Th2 cells *in vivo* following parenteral immunization with CRM_{197} antigen.

Parenteral immunization with CRM_{197} formulated with chitosan also generated significantly higher IgG antibody titres than the same antigen without chitosan. Although chitosan enhanced both primary and secondary responses, the difference was particularly striking for primary immunizations, with antibody titres in the range 3.5-3.75 (log_{10}) for mice immunized with CRM_{197} and chitosan compared to 0-1.25 (log_{10}) for mice immunized with the same antigen without chitosan (Fig. 3.11). Analysis of the neutralizing ability of these antibodies demonstrated that formulation of CRM_{197} with chitosan, for parenteral administration, induced neutralizing anti-DT antibodies in the range 0.17 to 0.39 IU/ml. In contrast, neutralizing antibodies were undetectable

following i.p. immunization with CRM_{197} without chitosan, demonstrating that chitosan is capable of enhancing the immunogenicity of parenterally delivered antigen (Table 3.1).

3.3 Discussion

New approaches to vaccination against diphtheria must be capable of generating immune responses that equal or surpass those induced with the conventional vaccine with an increased safety profile. In this study it was shown that a highly purified non-toxic mutant of diphtheria toxin, CRM_{197}, delivered parenterally is capable of inducing cellular and humoral immunity comparable to that induced with conventional diphtheria toxoid.

Although it has been demonstrated that certain soluble antigens, such as filamentous haemagglutinin from *Bordetella pertussis* (Cahill *et al.*, 1995), can induce immune responses by the nasal route, most antigens delivered mucosally are poorly immunogenic and may induce immunological tolerance (Lowrey *et al.*, 1998). In this study it was found that nasal administration of CRM_{197} induced very weak immune responses. In contrast, formulation of the same antigens with chitosan considerably enhanced their immunogenicity for intranasal immunization in mice. However, three immunizations and a dose of 10 μg CRM_{197} were necessary to induce relatively modest T cell and antibody responses in mice, which were still weaker than those observed with conventional DT administered parenterally. Nevertheless, boosting i.n. with CRM_{197} plus chitosan, after parenteral priming with DT, induced immune responses comparable to those observed following two parenteral immunizations with DT.

The exact mechanism by which chitosan enhances the immunogenicity of CRM_{197} following i.n. immunization is not clear. It has been reported that chitosan is capable of enhancing the nasal absorption of polypeptides, such as insulin in rats, thought to be due to the transient opening of tight junctions in the nasal membranes. Immunohistological studies have shown that chitosan can open the tight junctions between cells through an effect on F-actin filaments (Borchard *et al.*, 1996, Schipper *et al.*, 1997, Dodane *et al.*, 1999). In a similar manner, chitosan may also increase the absorption of antigens for nasal immunizations thereby allowing the antigen to gain rapid access to the nasal associated lymphoid tissue (NALT). However, in this study it was found that chitosan is also capable of enhancing immune responses for parenteral immunization with CRM_{197} antigen. These results suggest that the adjuvant effect of chitosan given nasally may extend beyond enhancement of antigen uptake at mucosal surfaces. Chitosan has been reported to bind to CD14 (Otterlei *et al.*, 1994), an LPS receptor, and may therefore be involved in activation of innate immune responses.

Expression of macrophage markers, such as MHC class I and II and Fc receptors, has reportedly been induced after treatment of murine peritoneal macrophages with chitosan. IL-8 has been induced in the supernatants of rat primary cultured dermal fibroblasts stimulated with chitin and its derivatives (Ueno *et al.*, 2001). Chitosan may also stimulate innate immunity through interaction of its N-acetyl-glucosamine subunits with mannose binding protein, an acute phase protein, which can activate complement (Stahl and Ezekowitz, 1998).

This study also provides significant new information on the induction of T cell subtypes with conventional DT adsorbed to alum and with a new nasally delivered antigen, CRM_{197}. Intranasal immunization with CRM_{197} with or without chitosan selectively induced Th2 cells *in vivo*. Chitosan would appear to preferentially enhance Th2-type responses induced following intranasal or parenteral immunization with CRM_{197}. Similarly, Seferian *et al.* (2000) found predominant IgG1 responses, indicative of Th2-type responses, in mice immunized intranasally with beta human chorionic gonadotropin and chitosan-based particles. However, mixed IgG1, IgG2a and IgG2b responses were observed following immunization with the same antigen and a chitosan-containing emulsion (Seferian and Martinez, 2000). In contrast to intranasal immunizations, parenteral immunizations with DT adsorbed to alum or with CRM_{197} preparations induced a mixed Th1/Th2 response. It has been shown that immunization with *Haemophilus influenzae* type b-CRM_{197} conjugate vaccine elicits a CRM_{197}-specific mixed Th1/Th2 type response in humans following systemic immunization (Kamboj *et al.*, 2001).

Formaldehyde treatment is routinely used to inactivate bacterial toxins and viruses for their use in vaccines. In addition, mild formaldehyde treatment may be used for antigen stabilization, as has been shown with a non-toxic mutant of pertussis toxin, PT-9K/129G, which in its native form is less stable than chemically detoxified PT upon storage and heating (Petre *et al.*, 1996). However, it is important that the immunogenicity of antigens, such as CRM_{197}, be retained after formaldehyde treatment. It has already been reported that there may be differences in the immunogenicity of native or formaldehyde-treated CRM_{197} and diphtheria toxoid following parenteral immunizations (Porro *et al.*, 1980, Gupta *et al.*, 1997). However, there is little information on the effects of formaldehyde treatment on T cell responses or on responses induced by mucosal immunization. In this study differential effects of formaldehyde treatment of CRM_{197} on T cell and antibody responses were observed

following parenteral and intranasal immunizations. The effect on antibody responses was more dramatic after nasal delivery. Gupta *et al.* (1997) found little variation in antibody levels from mice immunized with either native or formaldehyde-treated CRM_{197} after one or two subcutaneous immunizations (Gupta *et al.*, 1997). In this study it was found that i.p. immunization with CRM_{197} treated with 0.18% or 0.6% formaldehyde induced similar antibody responses to that generated with DT. Porro *et al.* (1980) examined the immunogenicity of formaldehyde-treated CRM_{197} and diphtheria toxoid in guinea pigs and found mildly formaldehyde-treated CRM_{197} to be less immunogenic than conventional DT after one immunization, but found no differences in immunogenicity after two doses (Porro *et al.*, 1980). In the present study, an examination of antibody responses after nasal delivery showed that formaldehyde treatment of CRM_{197} increased its immunogenicity significantly. Porro *et al.* (1980) found by tandem-crossed immunoelectrophoresis that formaldehyde-treated CRM_{197} was more resistant to proteolytic degradation by trypsin than native CRM_{197} (Porro *et al.*, 1980). The significantly lower antibody responses observed in this study from mice immunized with low dose formaldehyde treated (0.06%) and native CRM_{197} may therefore be related to the lower stability of these molecules against proteolysis when administered intranasally.

In contrast to the beneficial effects of mild formaldehyde treatment of CRM_{197} on antibody responses, especially for nasal delivery, T cell responses (both IL-5 and IFN-γ concentrations) to parenterally delivered antigen were progressively reduced as CRM_{197} was treated with increasing concentrations of formaldehyde. Formaldehyde treatment may result in the alteration or loss of T cell epitopes, thereby interfering with T cell receptor recognition of antigen peptides bound to major histocompatability complex (MHC) molecules. It has been reported that both human and murine T cells specific for pertussis toxin, and other *B. pertussis* antigens, proliferate optimally in response to untreated rather than glutaraldehyde and formaldehyde treated proteins *in vitro* (di Tommaso *et al.*, 1994). It appears that detoxification may limit the generation of some T cell epitopes by restricting antigen processing.

It has previously been demonstrated that a single s.c. immunization of mice or guinea pigs with alum-adsorbed DT or formalinized CRM_{197} generated modest ELISA and neutralizing antibody responses, which were considerably enhanced by a booster immunization by the same route (Gupta *et al.*, 1997). A significant finding of this study was that priming parenterally with DT and boosting mucosally with CRM_{197} was

a highly effective method of vaccination. Mice immunized in this way generated similar IgG and neutralizing antibody titres to mice parenterally immunized twice with conventional DT, but in addition generated high levels of local IgA. These results suggest that intranasal boosting with CRM_{197} antigen may be very effective in individuals already immunized with conventional parenterally delivered DT or DTP vaccines. The development of alternative mucosally delivered diphtheria booster vaccines, such as the highly purified CRM_{197} with chitosan, may eliminate some of the side-effects associated with the conventional vaccine and may also be more acceptable for frequent boosting against diphtheria.

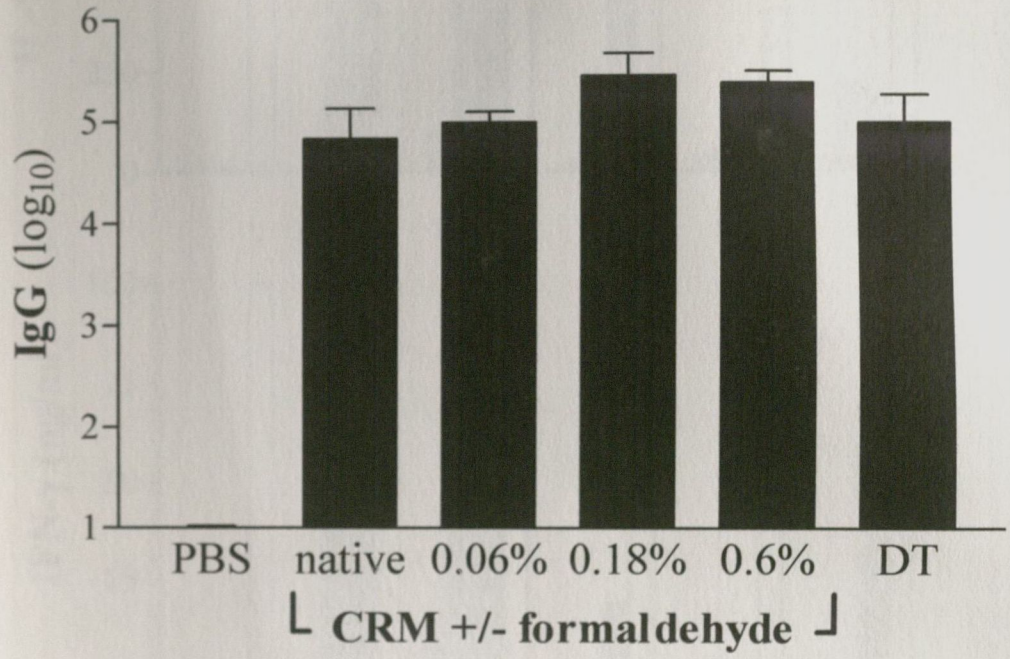


Figure 3.1. Parenteral immunization with CRM_{197} induces IgG antibody responses similar to those observed with conventional diphtheria toxoid. Mice were immunized i.p. at 0 and 4 weeks with alum adsorbed CRM_{197} (5 μg), that had been untreated (native) or pre-treated with 0.06%, 0.18% or 0.6% formaldehyde, with DT (5 IU) adsorbed to alum or with PBS only as a control. Anti-DT IgG antibody responses were assessed by ELISA two weeks after the second immunization. Results are expressed as mean (+/- S.E.) IgG antibody end-point titres for five mice per experimental group tested individually in triplicate.

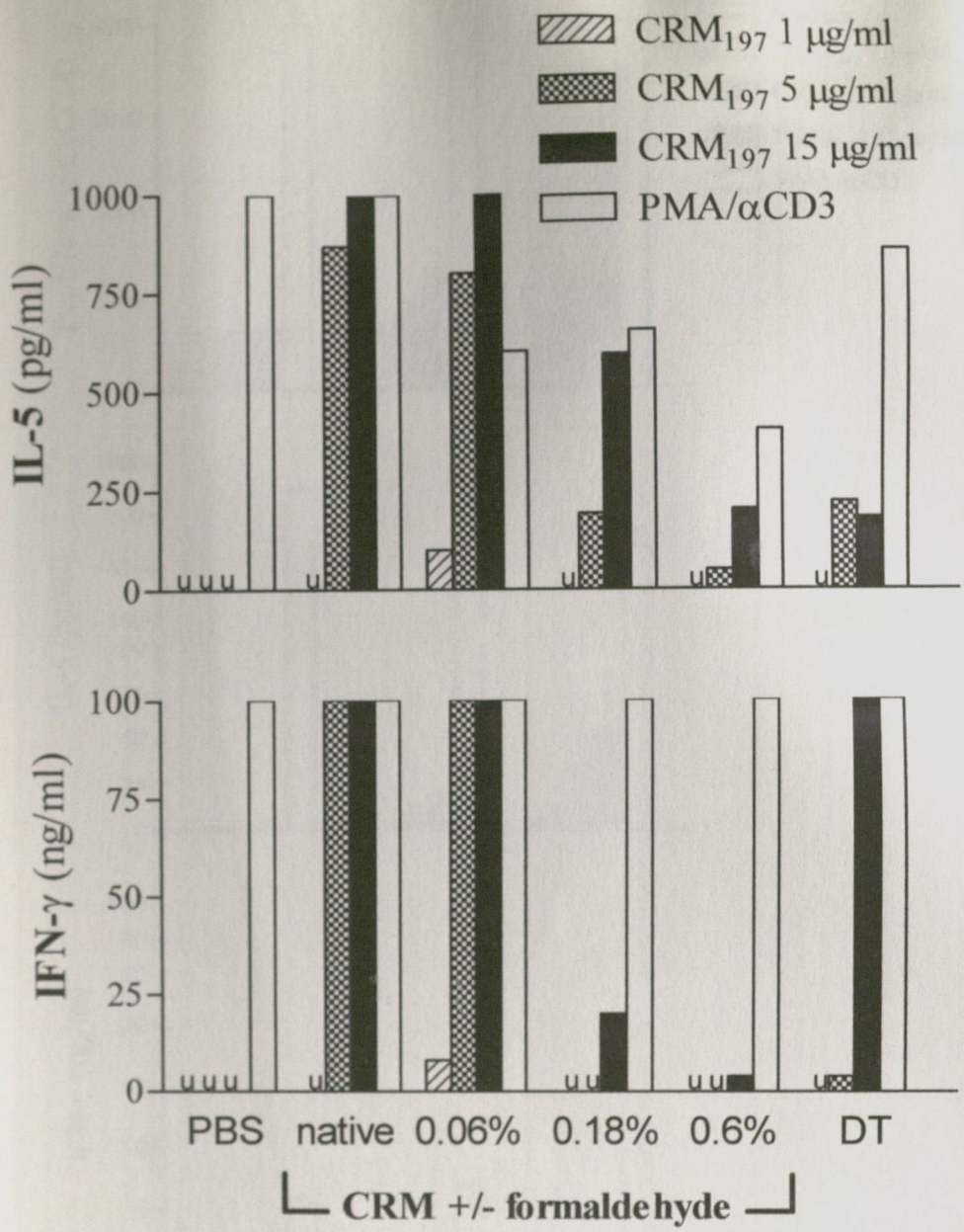


Figure 3.2. Effect of formaldehyde treatment of CRM_{197} on T cell responses to parenterally delivered antigen. Mice were immunized as described in the legend to Fig. 3.1. Cellular immune responses were assessed at week 6 by stimulating spleen cells *in vitro* with native CRM_{197} antigen (1-15 μg/ml) or with PMA and anti-CD3 as a positive control. Results are mean IL-5 or IFN-γ concentrations in the supernatants from five mice per experimental group tested in triplicate. The upper limits of the assays were 1000 pg/ml for IL-5 and 100 ng/ml for IFN-γ. u = undetectable concentrations of cytokine.

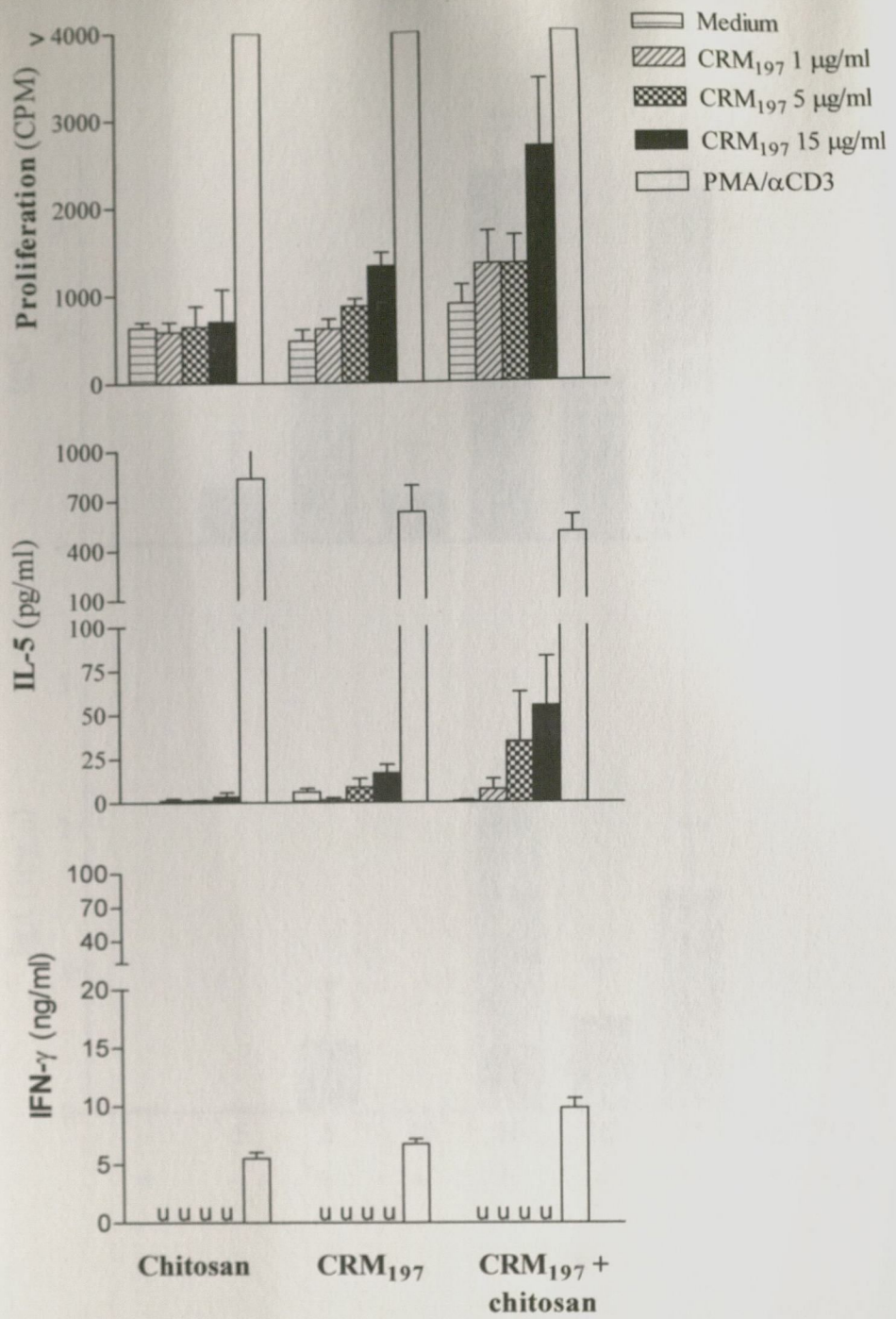


Figure 3.3. Chitosan enhances T cell responses induced by intranasal immunization with CRM_{197}. Mice were immunized at 0, 4 and 8 weeks with 0.18% formaldehyde-treated CRM_{197} (10 μg) with or without chitosan (100 μg) or with chitosan only as a control. Spleen cells were stimulated as described in the legend to Fig. 3.2 two weeks after the third immunization. Proliferative responses were assessed by [^{3}H]-thymidine incorporation and IL-5 and IFN-γ concentrations were assessed by specific immunoassay. Results are mean (+/- S.E.) responses for four mice per experimental group, tested individually in triplicate. u = undetectable concentrations of cytokine.

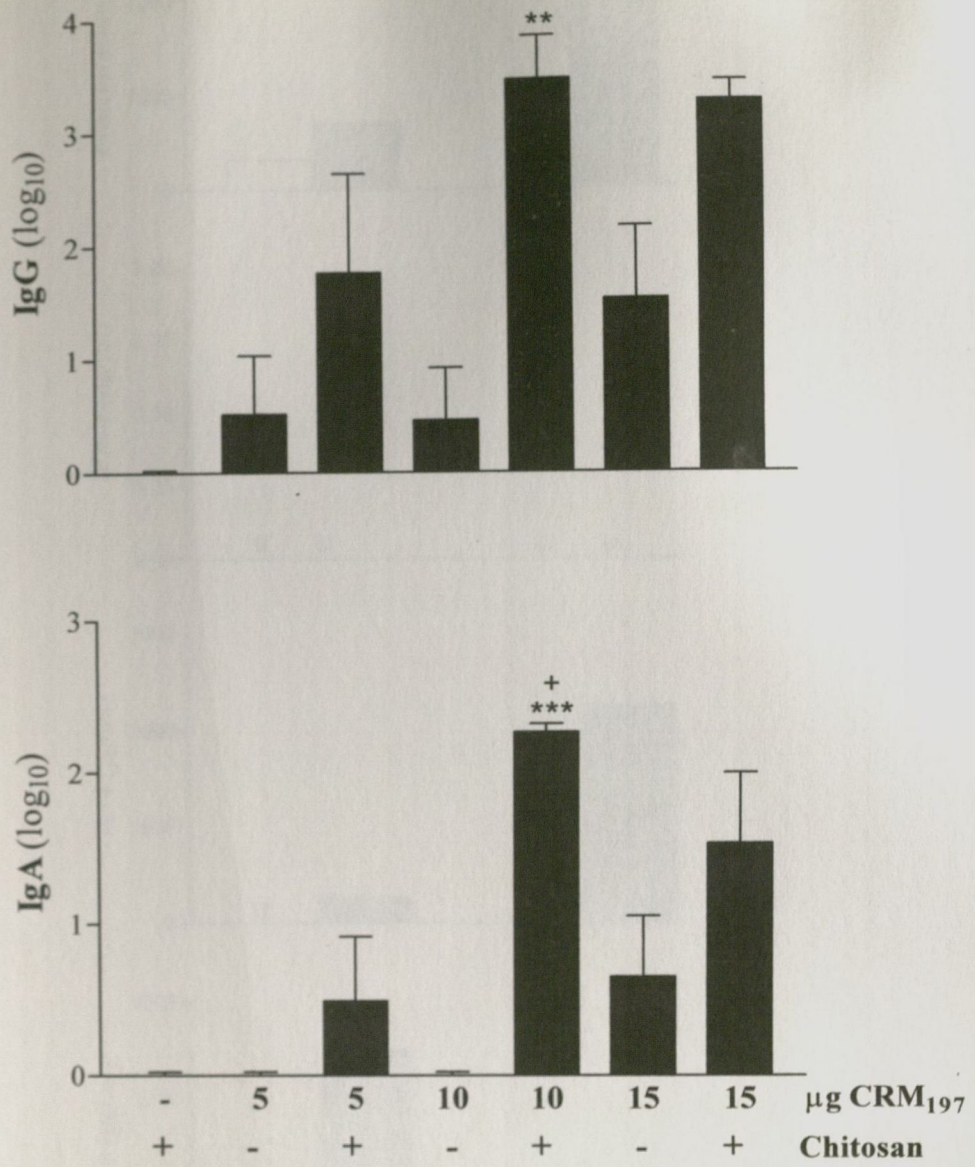


Figure 3.4. Intranasal delivery of CRM_{197} formulated with chitosan enhances anti-DT antibody titres. Mice were immunized i.n. at 0, 4 and 8 weeks with 5, 10 or 15 µg of 0.18% formaldehyde-treated CRM_{197} with or without chitosan (100 µg), or with chitosan only as a control. Anti-DT IgG and IgA titres were determined by ELISA on serum and lung homogenate samples recovered two weeks after the third immunization. Results are mean (+/- S.E.) endpoint titres for five mice per experimental group, tested individually in triplicate. ** and ***, $P < 0.01$ and $P < 0.001$ respectively versus CRM_{197} alone, +, $P < 0.05$ versus 5 µg CRM_{197} and chitosan (Tukey-Kramer test).

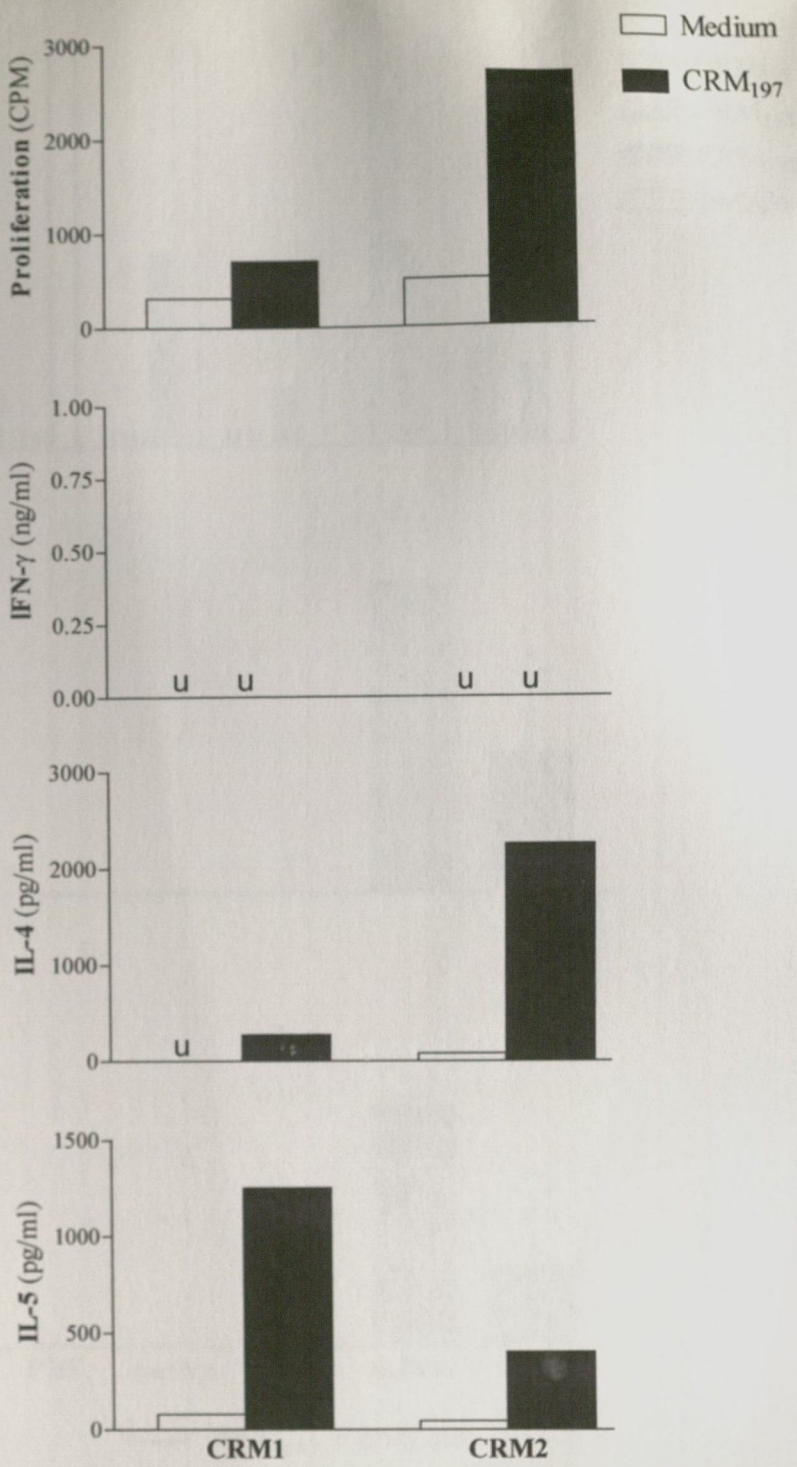


Figure 3.5. Cytokine profile of diphtheria-specific T cell clones generated from spleens of mice immunized i.n. with CRM_{197} and chitosan. Antigen-specific T cell clones were stimulated *in vitro* with medium only or with native CRM_{197} (1 μ g/ml) in the presence of autologous APC and recombinant IL-2 (5 IU/ml). Proliferative responses were assessed after 96 hours as described in the legend to Fig. 3.3 and cytokine levels in the supernatants were measured after 72 hours by specific immunoassays. u = undetectable concentrations of cytokine.

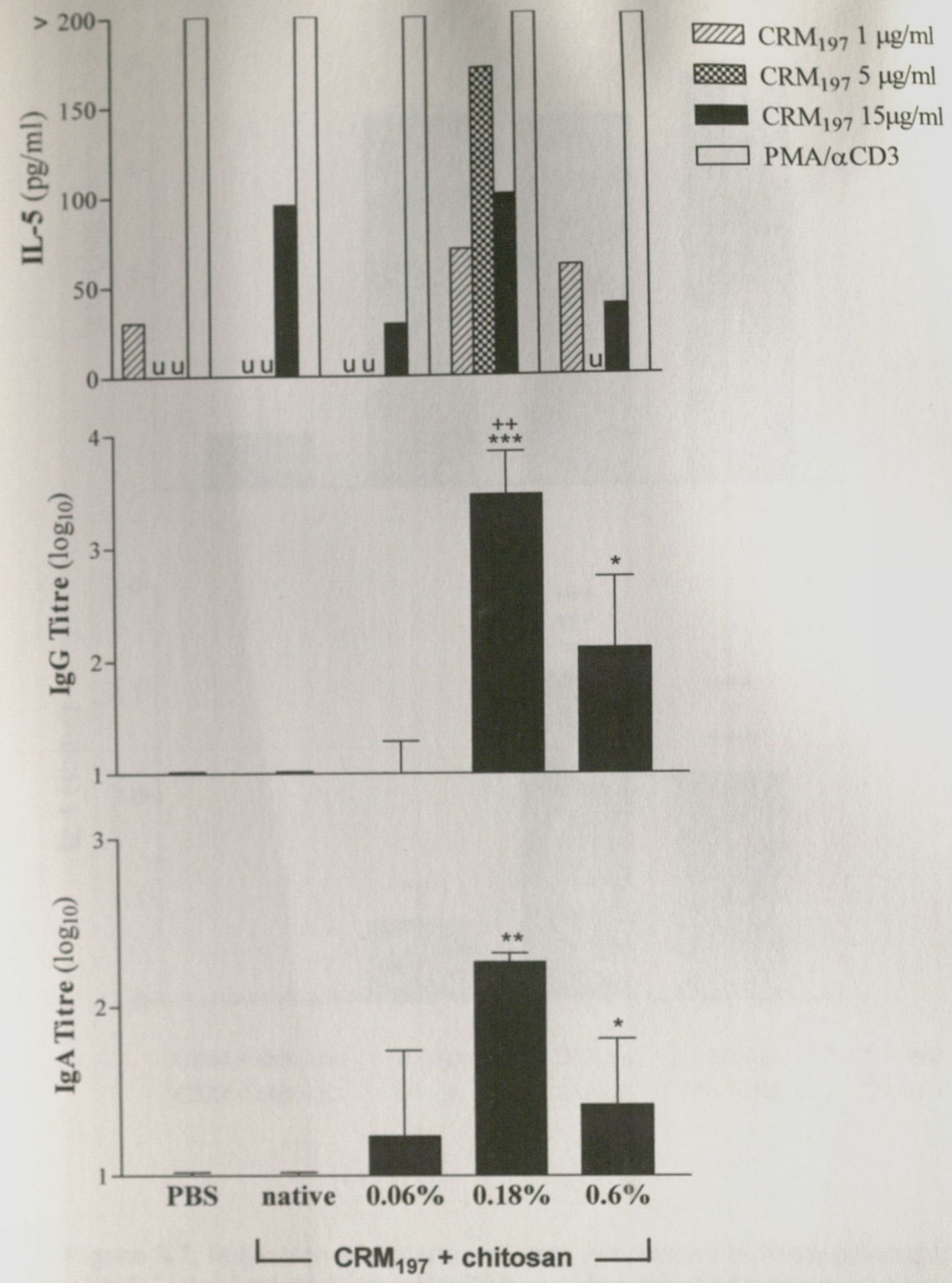


Figure 3.6. Mild formaldehyde treatment (0.18%) of CRM_{197} enhances immune responses following intranasal immunization. Mice were immunized at 0, 4 and 8 weeks with native or 0.06%, 0.18% or 0.6% formaldehyde-treated CRM_{197} (10 µg) formulated with chitosan (100 µg) or with PBS only as a control. IL-5, IgG and IgA responses were determined by ELISA as described in the legends to figures 3.2 and 3.4. Results are mean concentrations for IL-5 or endpoint titres (+/- S.E.) for IgG and IgA for five mice per experimental group. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus native CRM_{197}, ++, $P < 0.01$ versus 0.06% formaldehyde-treated CRM_{197} (Tukey-Kramer test). u = undetectable concentrations of cytokine.

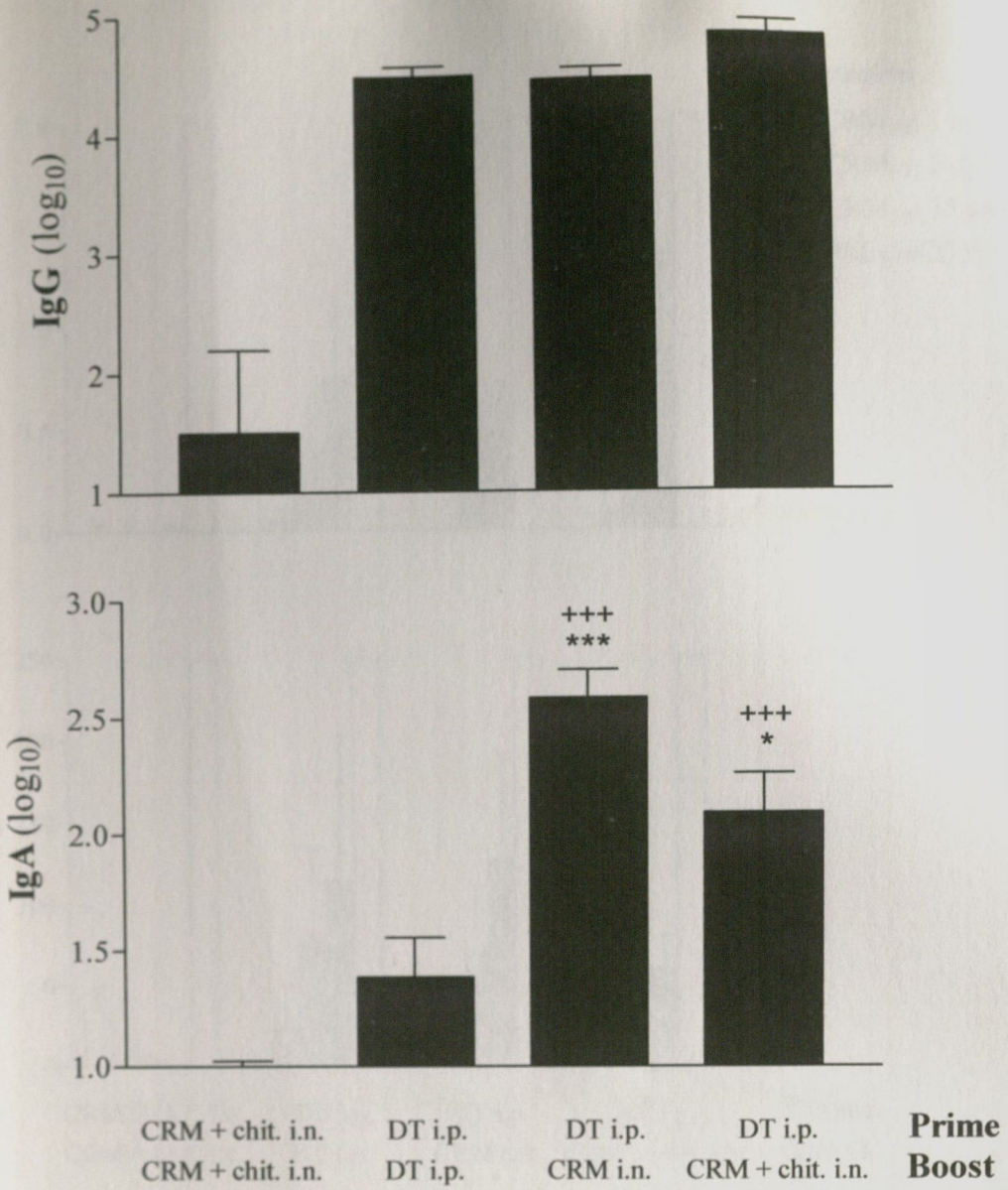


Figure 3.7. Induction of potent antibody responses in mice primed i.p. with DT and boosted i.n. with CRM_{197}. Mice were primed i.p. at week 0 with diphtheria and tetanus vaccine (5 IU of DT per mouse) and were boosted at week four either i.p. with diphtheria and tetanus vaccine or i.n. with 0.18% formaldehyde-treated CRM_{197} (10 μg) with or without chitosan (100 μg). One group of mice was immunized i.n. at 0 and 4 weeks with CRM_{197} plus chitosan. IgG and IgA titres were determined by ELISA and are expressed as the mean (+/- S.E.) endpoint titre for five mice per group, tested individually in triplicate. * and ***, $P < 0.05$ and $P < 0.001$ respectively versus two i.p. immunizations with DT, +++, $P < 0.001$ versus two i.n. immunizations with CRM_{197} and chitosan (Tukey-Kramer test).

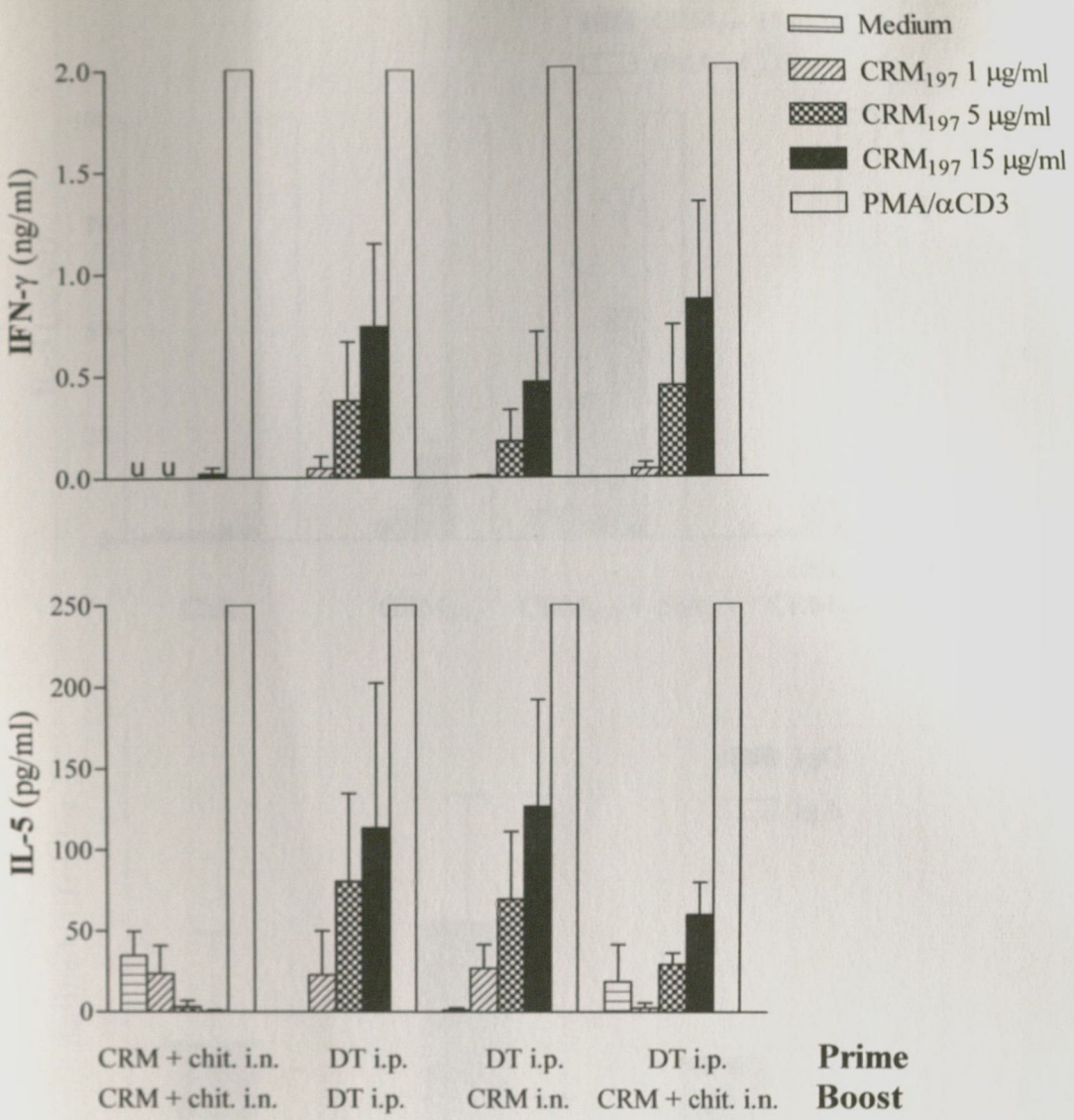


Figure 3.8. Priming parenterally with DT and boosting intranasally with CRM_{197} induces strong T cell responses. Mice were immunized as described in the legend to Fig. 3.7. IL-5 and IFN-γ responses were assessed, two weeks after the second immunization, as described in the legend to Fig. 3.2 and represent the mean (+/- S.E.) cytokine concentration for five mice per group. u = undetectable concentrations of cytokine.

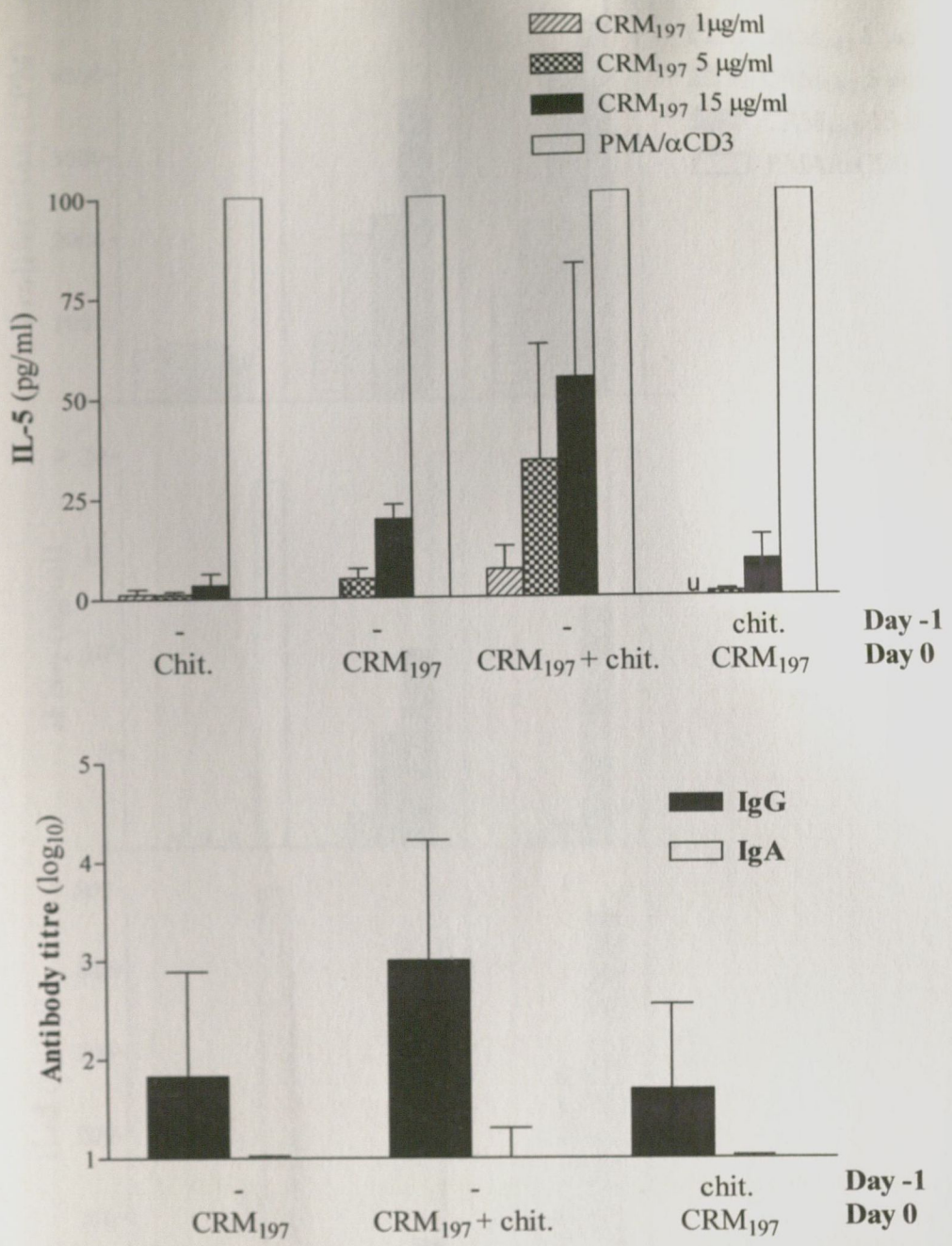


Figure 3.9. Influence of delayed antigen administration, following chitosan, on the induction of immune responses. Mice were immunized i.n. at 0, 4 and 8 weeks with 0.18% formaldehyde-treated CRM_{197} (10 μg) alone or with chitosan (100 μg), administered either together the same day or one day later than chitosan, or with chitosan only as a control. Spleen cells were stimulated, two weeks after the final immunization, as described in the legend to Fig. 3.2. IL-5 production was assessed by specific immunoassay and serum IgG and lung homogenate IgA titres were determined by ELISA. Results are expressed as mean (+/- S.E.) IL-5 concentrations or antibody titres for four mice per experimental group tested individually in triplicate. u = undetectable concentrations of cytokine.

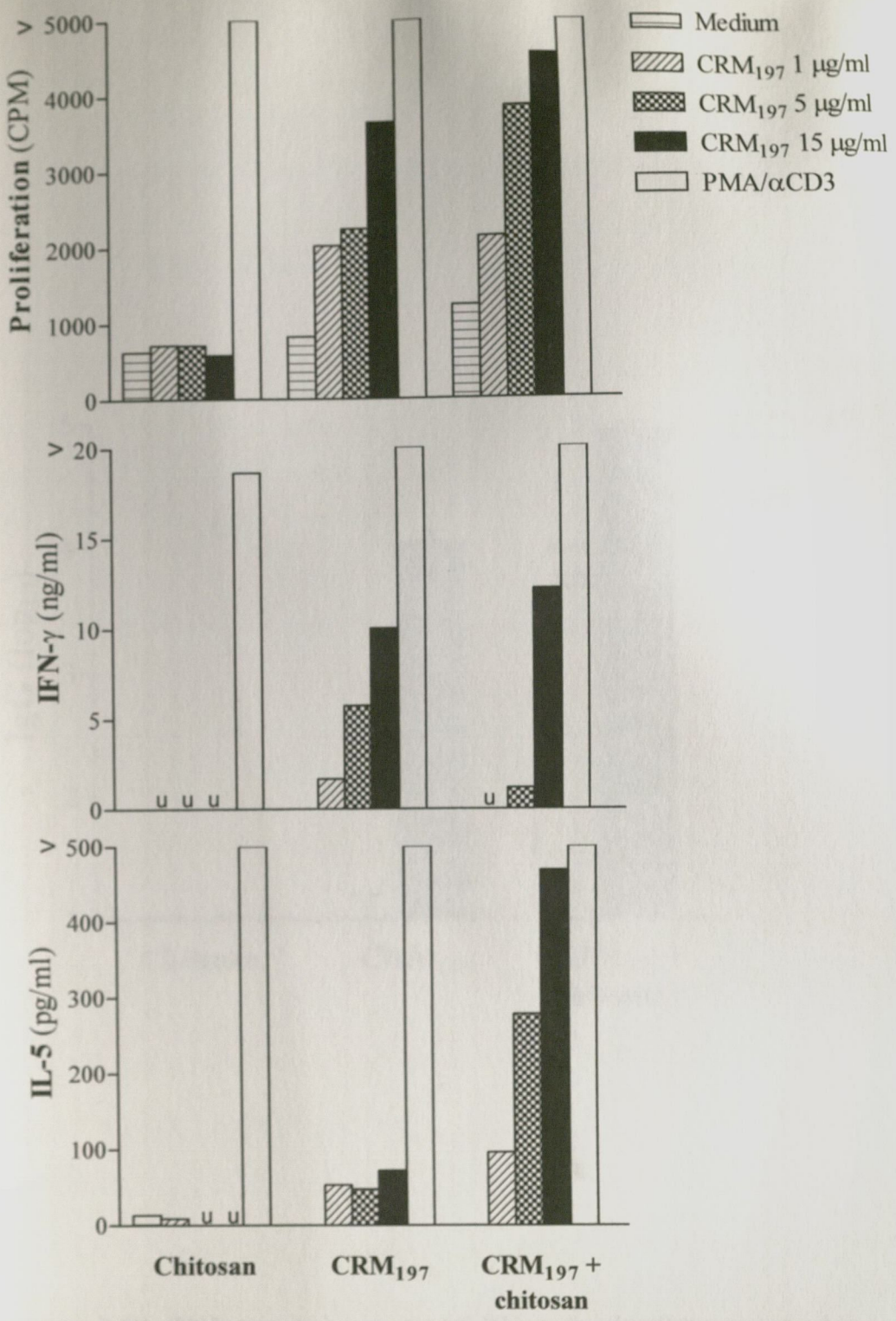


Figure 3.10. Parenteral immunization with CRM_{197} antigen formulated with chitosan enhances cellular immune responses. Mice were immunized i.p. at 0 and 4 weeks with 0.18% formaldehyde-treated CRM_{197} (10 μg) with or without chitosan (100 μg) or with chitosan only as a control. Proliferative and cytokine responses were assessed, two weeks after the second immunization, as described in the legend to Fig. 3.2 and Fig. 3.3. Results are mean proliferative responses or cytokine concentrations for four mice per experimental group. u = undetectable concentrations of cytokine.

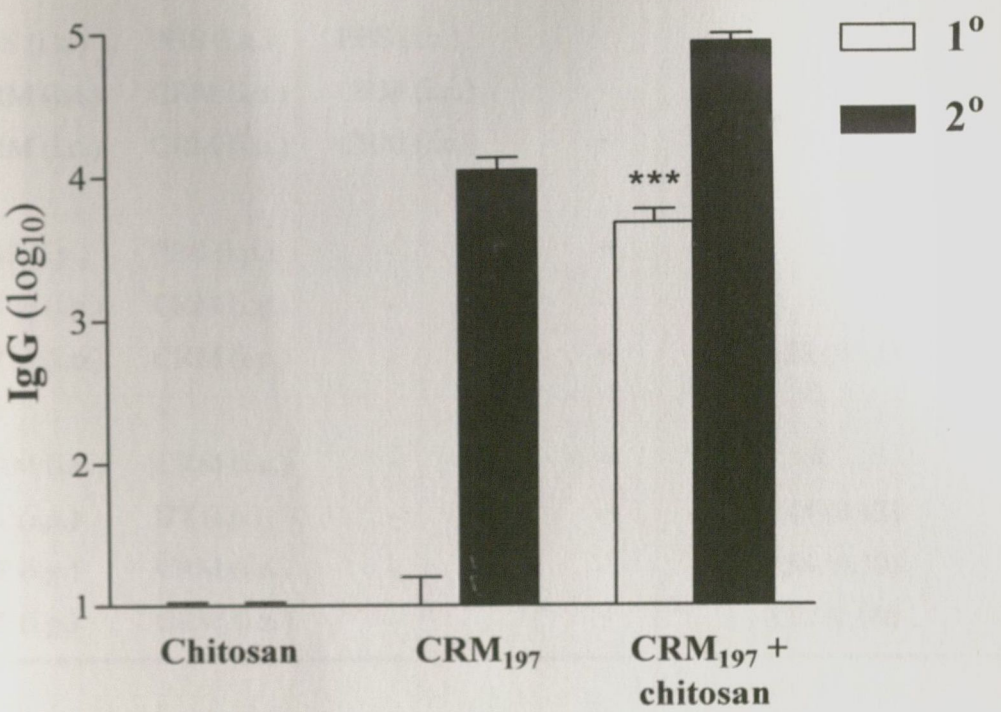


Figure 3.11. Chitosan enhances anti-DT antibody titres induced by parenteral immunization with CRM_{197}. Mice were immunized as described in the legend to Fig. 3.10. Anti-DT IgG titres were determined by ELISA, two weeks after the first (1^o) or the second (2^o) immunization. Results are mean (+/- S.E.) IgG endpoint titres for four mice per group, tested individually in triplicate. ***, $P < 0.001$ versus CRM_{197} alone (Tukey-Kramer test).

Table 3.1. Induction of toxin neutralizing antibodies following intranasal or parenteral immunization with CRM_{197} formulated with chitosan.

Immunization[a]				Neut Ab
1°	2°	3°	Chitosan	(IU/ml)
PBS (i.n.)	PBS (i.n.)	PBS (i.n.)	+	<
CRM (i.n.)	CRM (i.n.)	CRM (i.n.)	-	<
CRM (i.n.)	CRM (i.n.)	CRM (i.n.)	+	0.03 (0.02)
PBS (i.p.)	PBS (i.p.)	-	+	<
CRM (i.p.)	CRM (i.p.)	-	-	<
CRM (i.p.)	CRM (i.p.)	-	+	0.28 (0.11)
CRM (i.n.)	CRM (i.n.)	-	+	<
DT (i.p.)	DT (i.p.)	-	-	0.44 (0.13)
DT (i.p.)	CRM (i.n.)	-	-	0.54 (0.30)
DT (i.p.)	CRM (i.n.)	-	+	0.50 (0.09)

[a]Mice were immunized either i.n. 3 times (0, 4 and 8 weeks) with 10 μg CRM_{197} (treated with 0.18% formaldehyde) with or without chitosan, i.p. twice with CRM_{197}, with or without chitosan, or were primed with DT (as part of the diphtheria and tetanus vaccine adsorbed to alum) and boosted 4 weeks later with the same preparation or with CRM_{197} with or without chitosan. The concentrations of specific anti-diphtheria toxin neutralizing antibodies were measured by vero cell assays in serum samples taken 2 weeks after the final immunization. Results are mean (± S.E.) neutralizing antibodies (Neut Ab) expressed as IU/ml for four or five mice per experimental group tested individually in triplicate.

CHAPTER 4

PRE-CLINICAL AND CLINICAL STUDIES USING CHITOSAN AS AN ADJUVANT FOR THE NASAL DELIVERY OF CRM_{197}

4.1 INTRODUCTION

New approaches to vaccination must be capable of generating immune responses that equal or surpass those induced with conventional vaccines, while reducing adverse reactions that may be associated with traditional vaccines. Since the majority of invading human pathogens initially infect through the mucosal tissues, mucosal delivery of vaccines may facilitate the prevention of certain infectious diseases, through the induction of local immune responses. In addition, mucosal immunization abrogates the requirement for injections and may minimize certain of the adverse effects observed with parenteral immunization (Walker, 1994, O'Hagan, 1998). However, soluble antigens administered mucosally tend to elicit poor immune responses and require the use of adjuvants or delivery systems to augment the response.

In the previous chapter it was shown that chitosan, a cationic polysaccharide derived from chitin, was capable of enhancing immune responses to a mucosally delivered diphtheria vaccine in mice. Intranasal immunization with CRM_{197}, a highly purified mutant of diphtheria toxin, formulated with chitosan, enhanced anti-diphtheria toxin neutralizing antibodies and Th2 responses in mice. It was also found that priming parenterally with DT in alum and boosting intranasally with CRM_{197} and chitosan was a very effective method of immunization in mice, capable of inducing high concentrations of neutralizing antibodies in the serum and secretory IgA in the respiratory tract. These results suggested that boosting intranasally with CRM_{197} and chitosan might be very effective in individuals who have previously been immunized parenterally with a conventional diphtheria toxoid vaccine.

In many industrialized countries, serological data have demonstrated that a large proportion of the population, particularly older subjects, have low immunity to diphtheria, making them susceptible to the disease (Rappuoli *et al.*, 1988, Garcia-Corbeira *et al.*, 1999, Maple *et al.*, 2000). Disease outbreaks can most often be ascribed to waned immunity, because periodic boosters were not administered due to adverse reactions. This was the case recently in Russia, where concerns over untoward reactions, associated with DT vaccines, resulted in a switch to a lower dose vaccine for primary vaccination of children and a concomitant resurgence in the disease (Hardy *et al.*, 1996, Galazka, 2000). There is therefore a requirement for booster immunizations to be administered with safe, purified vaccines.

The development of alternative mucosally delivered diphtheria booster vaccines, such as CRM_{197} with chitosan, may eliminate some of the side effects associated with the conventional vaccine and may also be more acceptable for frequent boosting against diphtheria. CRM_{197} is naturally non-toxic and may be obtained at close to 100% purity (Rappuoli, 1997). Adverse reactions, resulting from the presence of accessory antigens in crude or partially purified DT preparations, should therefore be reduced following immunization with this mutant molecule. CRM_{197} has been shown to be safe in humans and is used as a carrier protein for the capsular polysaccharide of *Haemophilus influenzae* type b (Rothstein *et al.*, 1991), and as a conjugate for pneumococcal (Shelly *et al.*, 1997) and group C meningococcal polysaccharides (Rennels *et al.*, 2001). Chitosan is non-toxic in humans and may actually be administered orally to reduce dietary fat absorption (Ylitalo *et al.*, 2002). Studies have shown that when administered nasally only transient decreases in mucociliary rates are detected *in vitro*, leaving no permanent damage to this defence system (Aspden *et al.*, 1997). Its safety, proven ability to deliver drugs across the nasal cavity and potential adjuvant activity, make it an ideal candidate for the nasal delivery of vaccines such as diphtheria.

This study evaluated the immune responses induced following intranasal immunization, in both guinea pigs and humans, with CRM_{197} formulated with chitosan. Although mice are the most useful models for fundamental studies on immunity, they are resistant to the lethal effects of DT and the toxin may not bind to receptors on murine cells. In contrast, the toxin is lethal in guinea pigs and these, therefore, provide a more realistic model for assessing vaccine efficacy. Furthermore, guinea pigs are more suitable for nasal delivery of the vaccine in a powder formulation, an approach that may be favoured for nasal delivery of vaccines in humans, since it is not possible to administer bioadhesive powder formulations to animals as small as mice. Pre-clinical studies were therefore performed in guinea pigs to assess the efficacy of CRM_{197} powder formulations for primary and booster immunizations.

Diphtheria toxoid has been delivered intranasally to experimental animals using a wide range of adjuvants and delivery systems (Gizurarson *et al.*, 1995, Isaka *et al.*, 1999, Schroder and Svenson, 1999). However, there has been little testing in larger animal models and humans to ascertain if the promising results obtained in rodents can be reciprocated without compromising safety. This study demonstrates the potent

immunogenicity of a nasally delivered diphtheria vaccine formulated with chitosan in human volunteers.

4.2 RESULTS

4.2.1 Intranasal immunization with CRM_{197} and chitosan induces potent neutralizing antibody responses in guinea pigs.

Nasal administration of CRM_{197} and chitosan powder formulations generated high concentrations of anti-diphtheria toxin neutralizing antibodies in the sera of guinea pigs. Two weeks after three immunizations, the neutralizing antibody concentrations were similar to, or slightly greater than, those generated from guinea pigs immunized i.p. three times with CRM_{197} adsorbed to alum (Fig. 4.1).

This study was also designed to investigate the relevance of the ratio of CRM_{197} to chitosan in nasal powder formulations on the induction of neutralizing antibodies. Nasal immunization with CRM_{197} and a low dose of chitosan (CRM:chitosan; 1:35) induced antibody responses similar to those observed following i.n. immunization with CRM_{197} alone. However, with increasing CRM_{197} to chitosan ratio the protective antibody concentration in the sera increased. A powder formulation containing a ratio of CRM:chitosan of 1:140 generated very high concentrations of neutralizing antibodies (> 2 IU/ml) following three i.n. immunizations (Fig. 4.1).

4.2.2 Induction of neutralizing antibodies following an intranasal boost with CRM_{197} and chitosan in DT-primed guinea pigs.

The majority of the population in developed countries have been vaccinated against diphtheria as infants. Therefore it was important to evaluate the immunogenicity of nasally delivered CRM_{197} in primed animals with low antibody titres. Guinea pigs were primed s.c. with a low dose of DT and after 112 days, when neutralizing antibody levels were undetectable, the animals were boosted i.n. with CRM_{197} and chitosan. Evaluation of neutralizing antibody titres demonstrated that a single nasal booster vaccination, of DT-primed animals, with CRM_{197} formulated with chitosan induced antibody concentrations similar to, or greater than, those induced by s.c. boosting with CRM_{197} adsorbed to alum. All guinea pigs tested had fully protective concentrations of antibodies (> 0.1 IU/ml) following an i.n. boost with CRM_{197} formulated with chitosan as a solution, or with the same antigen combined with chitosan in a powder formulation. Furthermore, these protective responses were maintained for at least 28 days following booster vaccination (Fig. 4.2).

4.2.3 Persistence of immunity to diphtheria

Serological studies throughout Europe have shown that a considerable proportion of the population, particularly older subjects, may be susceptible to diphtheria due to a decline in the immune response over time. This study evaluated the immune response to diphtheria in 6 adults, ranging from 20-35 years of age, who had not received a booster immunization since early childhood. The persistence of cell-mediated immunity to diphtheria was evaluated from PBMCs isolated from these volunteers. Cytokine analysis demonstrated low levels of antigen-specific IFN-γ from PBMCs isolated from 5 of the subjects and moderate levels of this cytokine from one individual. PBMCs from two subjects secreted very low levels of IL-5 in response to antigen-stimulation *in vitro*. These results indicate the persistence of low levels of diphtheria-specific cell-mediated immune responses, with type 1 or mixed Th1/Th2 type responses in all subjects (Fig. 4.3).

The concentrations of neutralizing antibodies in the plasma were also assessed in these subjects. Anti-diphtheria toxin neutralizing antibody titres may be classified, according to established criteria, into three categories; concentrations of less than 0.01 IU/ml indicate susceptibility, concentrations of 0.01 to 0.1 IU/ml provide protection against the toxic manifestations of diphtheria, and concentrations greater than 0.1 IU/ml offer full protection (Gupta *et al.*, 1996). The neutralizing antibody titres in two of five plasma samples were below the protective concentration of 0.01 IU/ml, while only one of the subjects examined had antitoxin titres above the concentration of 0.1 IU/ml, considered to offer full protection (Fig. 4.4).

4.2.4 Effect of conventional booster immunization on the immune response to diphtheria

Since to date there is little information on the subtype of T cells induced by vaccination with conventional DT in humans, a preliminary study was performed to evaluate the type of T cell responses induced with a systemic diphtheria booster vaccination in humans. This study, which was carried out on three healthy lab workers who had been boosted i.m. with DT vaccine prior to commencing work using diphtheria toxin, allowed the development of assays for assessment of DT-specific cell-mediated immunity in humans. It was shown that PBMCs isolated from two of the adults secreted IFN-γ and IL-5 in response to antigen stimulation *in vitro*, indicating a

mixed Th1/Th2 type response. PBMCs from one subject secreted very low levels of antigen-specific IFN-γ and no IL-5 (Fig. 4.3).

The concentrations of antitoxin in the plasma of the three subjects were above the protective concentration of 0.01 IU/ml one year after conventional booster immunization. Neutralizing antibody concentration was shown to be greater than 0.1 IU/ml in one of the adults, indicating full protection against the disease (Fig. 4.4).

4.2.5 Antibody responses following nasal booster with CRM_{197} with or without chitosan in human volunteers

A phase I clinical trial was undertaken to assess the immunogenicity of a nasally delivered CRM_{197} and chitosan vaccine in human volunteers. The study comprised three parallel groups of male and female volunteers, 18-40 years of age. Subjects were chosen who had not received a DT booster vaccination or a CRM-conjugated vaccine in the 5 years prior to entry into the study, and should therefore have low levels of protective immunity to diphtheria. Volunteers were boosted intranasally at days 0 and 28 with CRM_{197} powder formulations with (n=10) or without chitosan (n=10) or intramuscularly on day 0 with conventional DT (n=5). Blood was taken for cytokine and antibody analysis on days -7, 27, 42 and 84.

Antibody analysis demonstrated induction of potent IgG antibody responses following intranasal boosting with CRM_{197} alone (Fig. 4.5A) or formulated with chitosan (Fig. 4.5B). Anti-DT IgG antibody responses induced following a single nasal booster immunization with CRM_{197} (Fig. 4.5 A,B) were similar to those generated with a booster immunization with conventional DT by the intramuscular route (Fig. 4.5C). In comparison to intranasal immunization with CRM_{197} alone, formulation of CRM_{197} with chitosan significantly enhanced anti-DT IgG antibody responses following two nasal booster immunizations (Fig. 4.6). These superior antibody responses were observed two weeks after a second nasal booster with CRM_{197} formulated with chitosan, and were maintained up to eight weeks post this vaccination.

Analysis of neutralizing antibody concentrations in human plasma and sera demonstrated induction of potent antitoxin responses following intranasal boosting with CRM_{197} alone or with chitosan. Analysis of anti-diphtheria toxin neutralizing antibodies in plasma samples following a single i.n. immunization demonstrated that the number of individuals with antitoxin titres greater than the level considered to be fully protective, 0.1 IU/ml, increased from 7/10 to 9/10 with CRM_{197} alone and from

4/10 to 9/10 with CRM_{197} formulated with chitosan. Comparable antitoxin concentrations were observed following a booster immunization with parenterally-delivered DT. Immunization with DT induced fully protective levels of neutralizing antibodies in each of the 5 volunteers. Following a second intranasal booster with CRM_{197} with or without chitosan, the plasma neutralizing antibody concentrations were found to be greater than 0.1 IU/ml for all volunteers (Fig. 4.7). The concentrations of anti-diphtheria toxin neutralizing antibodies in the sera of immunized individuals were observed to be greater than the corresponding plasma antitoxin concentrations. However, it was found that the sera anti-diphtheria toxin neutralizing antibodies also dramatically increased following intranasal boosting with CRM_{197} with or without chitosan. All volunteers were found to have sera antitoxin concentrations above the fully protective concentration of 0.1 IU/ml following an intranasal boost with CRM_{197} with or without chitosan or intramuscular boost with conventional DT (Fig. 4.8). In addition, it was observed that two intranasal immunizations with CRM_{197} and chitosan induced significantly higher levels of neutralizing antibodies in the sera and plasma of volunteers than immunization with CRM_{197} antigen alone. These potent anti-diphtheria-toxin neutralizing antibody responses were maintained for at least 8 weeks post the second booster immunization with CRM_{197} and chitosan (Fig. 4.9).

4.2.6 Th1/Th2 profile following nasal boosting with CRM_{197} and chitosan in human volunteers

DT-specific T cell responses were evaluated using PBMCs isolated from blood samples taken pre- and post-booster immunizations with CRM_{197} alone or formulated with chitosan, or with conventional DT. PBMCs from volunteers immunized twice, days 0 and 28, with CRM_{197} and chitosan secreted significantly higher concentrations of DT-specific IL-5, compared with PBMCs from volunteers immunized with CRM_{197} alone. The concentration of IL-5 secreted by PBMCs had decreased by 8 weeks post booster immunization (day 84) (Fig. 4.10A). IFN-γ production also increased, although not significantly, post immunization. However, chitosan had no significant effect on the concentrations of antigen-specific IFN-γ induced by intranasal immunization with CRM_{197} (Fig. 4.10B).

Intranasal vaccination with CRM_{197}, with or without chitosan, induced PBMCs that secreted both antigen-specific IL-5 and IFN-γ following one or two booster immunizations, indicating the priming of mixed Th1/Th2 type cells *in vivo*. Similar,

but slightly weaker, T cell responses were observed following intramuscular boosting with conventional DT (Fig. 4.10 A, B).

4.3 DISCUSSION

Traditional vaccines have been designed using an empirical approach, with little knowledge of the protective antigens or correlates of immunity. Nevertheless, this approach was highly successful and killed and attenuated vaccines have been responsible for preventing millions of deaths each year from infectious diseases. However, increases in antibiotic-resistant bacteria, the emergence of new pathogens and concerns over the safety of killed and attenuated vaccines and crude preparations of vaccines, such as diphtheria, have highlighted the requirement for new vaccines and for the refinement of existing vaccines to render them safer and more effective. Recent developments in genetic engineering together with significant advances in our understanding of protective immune responses have facilitated a more rational approach to vaccine design, based on recombinant protein or nucleic acid approaches. Furthermore, advances in the development of novel adjuvant and delivery systems have helped to identify vaccine formulations that are safe and effective when administered mucosally.

Most of the clinical symptoms of diphtheria are mediated by diphtheria toxin and, therefore, protection may be obtained by toxin neutralizing antibodies (antitoxin) induced through active immunization. Therefore, a new generation vaccine against diphtheria should be possible using genetically detoxified recombinant toxins delivered parenterally or mucosally with a delivery system or adjuvant, such as chitosan, that can enhance neutralizing antibodies and thereby offer protection against disease.

Several studies have shown that after primary immunization against diphtheria, in the absence of exposure to circulating toxigenic strains of *Corynebacterium diphtheriae*, the antitoxin concentration begins to decline steadily after 15 years (Scheibel *et al.*, 1966, Kjeldsen *et al.*, 1985). A study performed in the British population in 1996 showed that less than 20% of those aged 25 years and above had fully protective diphtheria antitoxin levels (> 0.1 IU/ml), while less than 70% had levels of 0.01 IU/ml or greater (Maple *et al.*, 2000). Similar antitoxin levels have been reported for other European countries (Kjeldsen *et al.*, 1988, Rappuoli *et al.*, 1993, Garcia-Corbeira *et al.*, 1999). The lowest seroprotection rates in European countries have been reported in the 20-50 (Galazka and Kardymowicz, 1989, Maple *et al.*, 1995) or 30-60 (Cellesi *et al.*, 1989, Rappuoli *et al.*, 1993) age groups. In a study involving 6 healthy adults, that had not been immunized with DT since early childhood, it was

found that one subject had neutralizing antibody titres below a protective level of 0.01 IU/ml, while only one third of those examined had antitoxin levels greater than 0.1 IU/ml. Although the limited sample size precludes any statistical correlations, the results of this study are in agreement with those obtained from serological studies throughout Europe, and demonstrate the requirement for frequent boosting against diphtheria.

Compliance of the population to booster immunizations is influenced by the rate of adverse effects associated with vaccination. Untoward reactions to DT have been reported, particularly following adult vaccination. The reactions observed may be of the immediate, anaphylactic type, mediated by IgE (Mark *et al.*, 1995) as well as the delayed type, mediated by T cell immunity (Pappenheimer, 1955). Arthus type reactions have also been reported following frequent booster vaccinations, due to the high levels of circulating specific antibodies (Pappenheimer, 1984). Most adverse reactions to DT vaccination are thought to be associated with the presence of accessory antigens in crude or partially purified DT preparations (Pappenheimer *et al.*, 1950, Rappuoli, 1997) or with the use of aluminium phosphate as an adjuvant in conventional vaccines (Blennow *et al.*, 1994, Mark *et al.*, 1995). Improved diphtheria booster coverage may well result from the development of vaccines with similar efficacy to conventional DT vaccines, but with less reactogenicity. An alternative mucosally delivered vaccine, such as the highly purified CRM_{197} with chitosan, may eliminate some of the adverse effects associated with conventional DT adsorbed to alum. This study evaluated the immunogenicity of this novel vaccine, for intranasal immunization, in pre-clinical studies in guinea pigs and in a phase I clinical trial in humans.

In the previous chapter it was found that chitosan enhanced the immunogenicity of CRM_{197} for nasal delivery to mice. Intranasal boosting with CRM_{197}, with or without chitosan, was found to be a particularly effective method of immunization, in terms of both local and systemic immunity induced. However, guinea pigs provide a more suitable model for assessing the efficacy of diphtheria-based vaccines, as they, unlike mice, are susceptible to the lethal effects of the toxin. It has been shown that substantial differences may be obtained in *in vitro* neutralization assays performed with sera from mice and guinea pigs. Gupta and collaborators (1997) described significantly lower concentrations of antitoxin titres for mouse sera compared to guinea pig, following immunization with CRM_{197} (Gupta *et al.*, 1997).

Quality control of diphtheria vaccines is normally performed with sera from immunized guinea pigs, and the concentration of antitoxin required for vaccine certification is 0.5 IU/ml after a single immunization. It has previously been found that intranasal immunization with CRM_{197} and chitosan is capable of generating high concentrations of toxin neutralizing antibodies, similar to those observed with conventional DT vaccine, in a guinea pig passive challenge model (McNeela *et al.*, 2000). Pre-clinical studies were therefore undertaken in guinea pigs to investigate the immunogenicity of various CRM_{197} and chitosan formulations following intranasal delivery. The results demonstrated that i.n. immunization with CRM_{197} powder formulations induced potent concentrations of neutralizing antibodies, particularly when CRM_{197} was formulated with higher doses of chitosan. In addition, a single nasal booster of CRM_{197} formulated with chitosan, administered as a powder or a solution, induced protective antitoxin responses similar to, or greater than, those induced by s.c. boosting with CRM_{197} adsorbed to alum. It has previously been shown that the effect of chitosan, in terms of promoting the transport of drugs across the nasal membrane, may be enhanced by the use of chitosan powder formulations as compared to solution formulations (Illum, 1998). The results of the present study demonstrated that CRM_{197} chitosan powder formulations might be very effective for intranasal boosting of individuals previously immunized systemically with conventional DT.

Diphtheria toxoid has been administered mucosally to experimental animals using a number of adjuvants and delivery systems. Enhanced immunogenicity in mice has been reported following intranasal immunization with DT together with recombinant cholera toxin B subunit (Isaka *et al.*, 1999), a mutant of cholera toxin E112K (Ohmura *et al.*, 2001), various non-ionic excipients having different lipophilic characteristics (Gizurarson *et al.*, 1995) and with monoglyceride/fatty acid lipid suspensions (Schroder and Svenson, 1999). However, there has been little testing in larger animal models and humans with nasally delivered vaccines. To date, clinical trials using nasally delivered vaccines have been performed with subunit influenza vaccines with LT (Glück *et al.*, 1999, Glück *et al.*, 2000) or MF59 (Podda, 2001) as adjuvant, live attenuated influenza virus (Boyce *et al.*, 1999, Belshe *et al.*, 2000) and respiratory syncytial virus (Gonzalez *et al.*, 2000) vaccines, Group B meningococcal vesicles (Haneberg *et al.*, 1998, Drabick *et al.*, 1999), *Shigella flexneri* LPS complexed to meningococcal outer membrane proteosomes (Fries *et al.*, 2001), CRM_{197}-conjugated pneumococcal oligosaccharide (Shelly *et al.*, 1997), liposome-formulated

antigen from *Streptococcus mutans* (Childers *et al.*, 1999), group A Streptococcal M protein (D'Alessandri *et al.*, 1978), grass pollen extract with MPL as adjuvant (Drachenberg *et al.*, 2001), killed whole cell pertussis alone (Berstad *et al.*, 2000), *Pseudomonas aeruginosa* outer membrane protein (Larbig *et al.*, 2001) and diphtheria and tetanus toxoids with polysorbate and glycerides (Aggerbeck *et al.*, 1997). Most of the trials have reported good immunogenicity and none have reported major adverse effects. This study reports the first trial conducted in humans of an intranasally delivered diphtheria vaccine adjuvanted with chitosan.

It was found that intranasal boosting with CRM_{197} and chitosan induced significantly higher levels of IgG antibodies and neutralizing antitoxin in humans compared to boosting with CRM_{197} alone. In addition, a single booster immunization with CRM_{197} induced immune responses comparable to those obtained following an intramuscular booster immunization with conventional diphtheria toxoid. After one or two intranasal immunizations with CRM_{197} with or without chitosan the sera or plasma, respectively, of all volunteers tested were found to have antitoxin levels above 0.1 IU/ml. The concentrations of anti-diphtheria toxin neutralizing antibodies were generally found to be greater in sera rather than plasma samples, which may reflect the time delay in obtaining plasma from whole blood or the specific action of proteases not present in the sera samples. Aggerbeck *et al.* (1997) reported enhanced immunogenicity in clinical trials of diphtheria and tetanus toxoids using enhancer mixtures of polysorbate and caprylic/capric. However, although only two volunteers, compared to 51 pre-immunization, failed to attain protective antibody concentrations (> 0.01 IU/ml), four weeks after intranasal vaccination, all the intranasal formulations showed lower immunogenicity than conventional DT in man, as well as in pre-clinical guinea pig studies (Aggerbeck *et al.*, 1997). In contrast, in the present study it was found that i.n. boosting with CRM_{197} with or without chitosan was as effective as i.m. boosting with conventional DT for the generation of anti-diphtheria toxin neutralizing antibodies.

There is relatively little information available in the literature on the types of cellular immune responses induced by diphtheria vaccines. In this study it was found that chitosan appeared to selectively enhance the induction of Th2 type cells *in vivo* following nasal immunization with CRM_{197} antigen. Similar responses have been observed in mice, where it was shown that chitosan enhanced Th2 responses induced by intranasal immunization with CRM_{197}. In addition, chitosan enhanced IL-5

responses, but had no effect on the concentrations of antigen-specific IFN-γ, following parenteral immunization in mice (previous chapter). It has previously been reported, for various antigens, that the choice of adjuvant may influence the type of cellular response induced. Entrapment of antigen in PLG allowed the CD4+ T cell responses induced with soluble protein antigens to switch from a predominant Th2 to a Th1 subtype (Moore *et al.*, 1995, Conway *et al.*, 2001). MPL, a derivative of lipopolysaccharide, has been shown to induce predominant Th1 responses following mucosal delivery with a number of antigens (Moore *et al.*, 1999, Baldridge *et al.*, 2000). It has been demonstrated in a number of studies that cholera toxin, or mutants of the toxin, may enhance Th2 responses to mucosally co-administered antigens (Munoz *et al.*, 1990, Xu-Amano *et al.*, 1994, Yamamoto *et al.*, 1997b).

Overall, it was found that intranasal boosting with CRM_{197} with or without chitosan, or with conventional diphtheria toxoid, induced mixed Th1/Th2 type responses in humans. A mixed Th1/Th2 profile has also been observed in PBMC isolated from children immunized with acellular pertussis vaccines (Ryan *et al.*, 1998), suggesting that a polarized Th2 response is not a pre-requisite for protective immunity to toxin-mediated diseases. In addition, in this study it was shown that low levels of diphtheria-specific cellular immunity may persist in the population for many years following primary immunization.

In conclusion, therefore, the findings of this study show that a nasally delivered diphtheria vaccine formulated with chitosan induced potent immunogenicity, in terms of neutralizing antibodies, in both pre-clinical studies in guinea pigs and in human volunteers. Vaccination with CRM_{197} and chitosan may eliminate some of the side effects associated with conventional DT vaccines because this vaccine employs a highly purified mutant of DT as antigen and precludes the use of alum as an adjuvant.

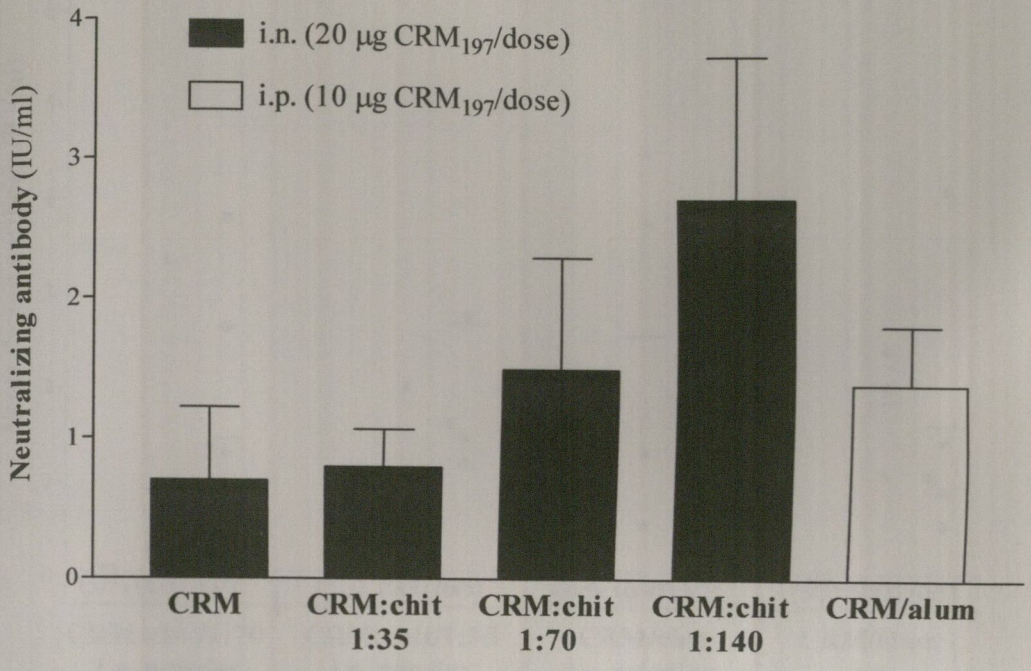


Figure 4.1. Induction of toxin neutralizing antibodies in guinea pigs following intranasal immunization with CRM_{197} formulated with various doses of chitosan. Guinea pigs were immunized i.n. at 0, 4 and 8 weeks with CRM_{197} (20 μg) in combination with chitosan and mannitol from various powder formulations (CRM:chitosan; 1:35, 1:70, 1:140) or with CRM_{197} (20 μg) and mannitol alone. For comparison, one group of guinea pigs was immunized i.p. with CRM_{197} (10 μg) adsorbed to alum. Neutralizing antibody levels were assessed by vero cell assays in serum samples taken two weeks after the third immunization. Results are mean (+/- S.E) neutralizing antibodies expressed as IU/ml for five guinea pigs per experimental group, tested individually in triplicate.

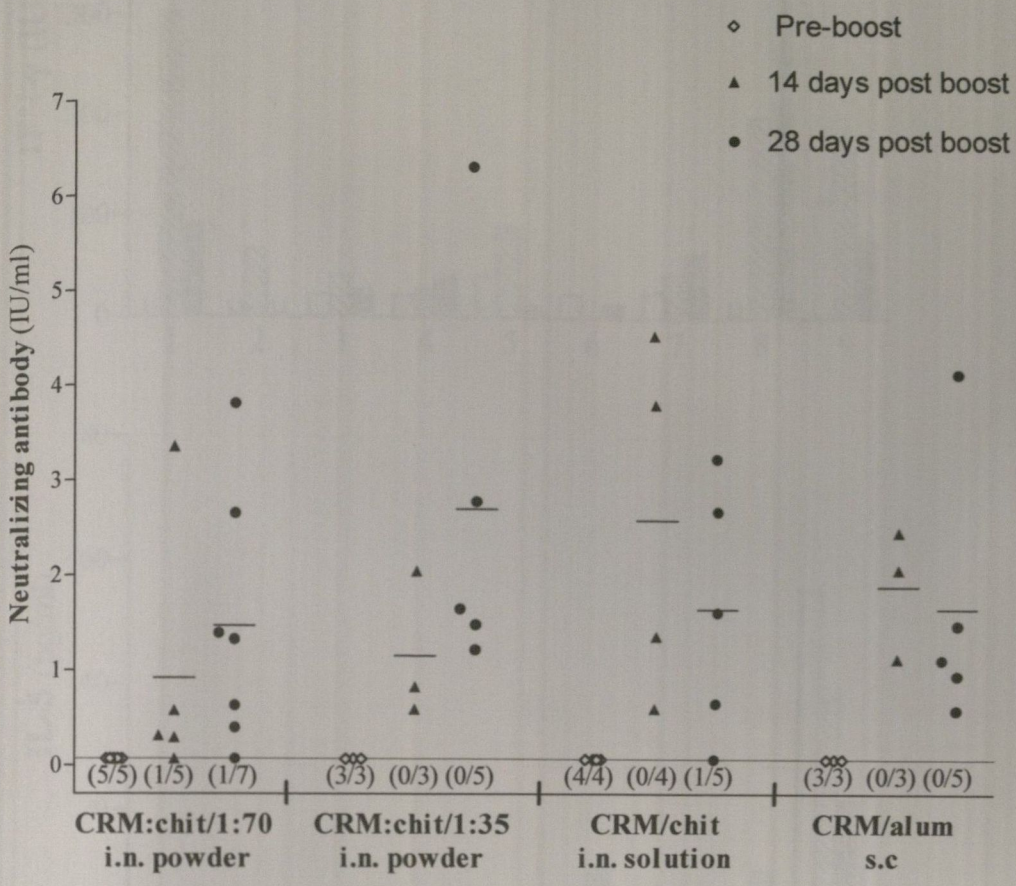


Figure 4.2. Neutralizing serum antibody levels following nasal administration of CRM_{197} and chitosan in DT primed guinea pigs. Naïve guinea pigs were primed s.c. with a low dose of DT (0.3 LF IU) and left for 112 days until the antibody titres were sufficiently low. Guinea pigs were then boosted either i.n. with CRM_{197} (20 μg) in combination with chitosan from powder or solution formulations or s.c. with CRM_{197} (10 μg) adsorbed to alum. The concentrations of specific anti-diphtheria toxin neutralizing antibodies were measured by vero cell assays in serum samples taken pre- and 14 and 28 days post booster immunizations. Results are expressed as neutralizing antibody levels (IU/ml) for individual guinea pigs tested in triplicate. Bars indicate mean values for each group. Values in parentheses at the origin represent the number of samples in each group with undetectable neutralizing antibody levels.

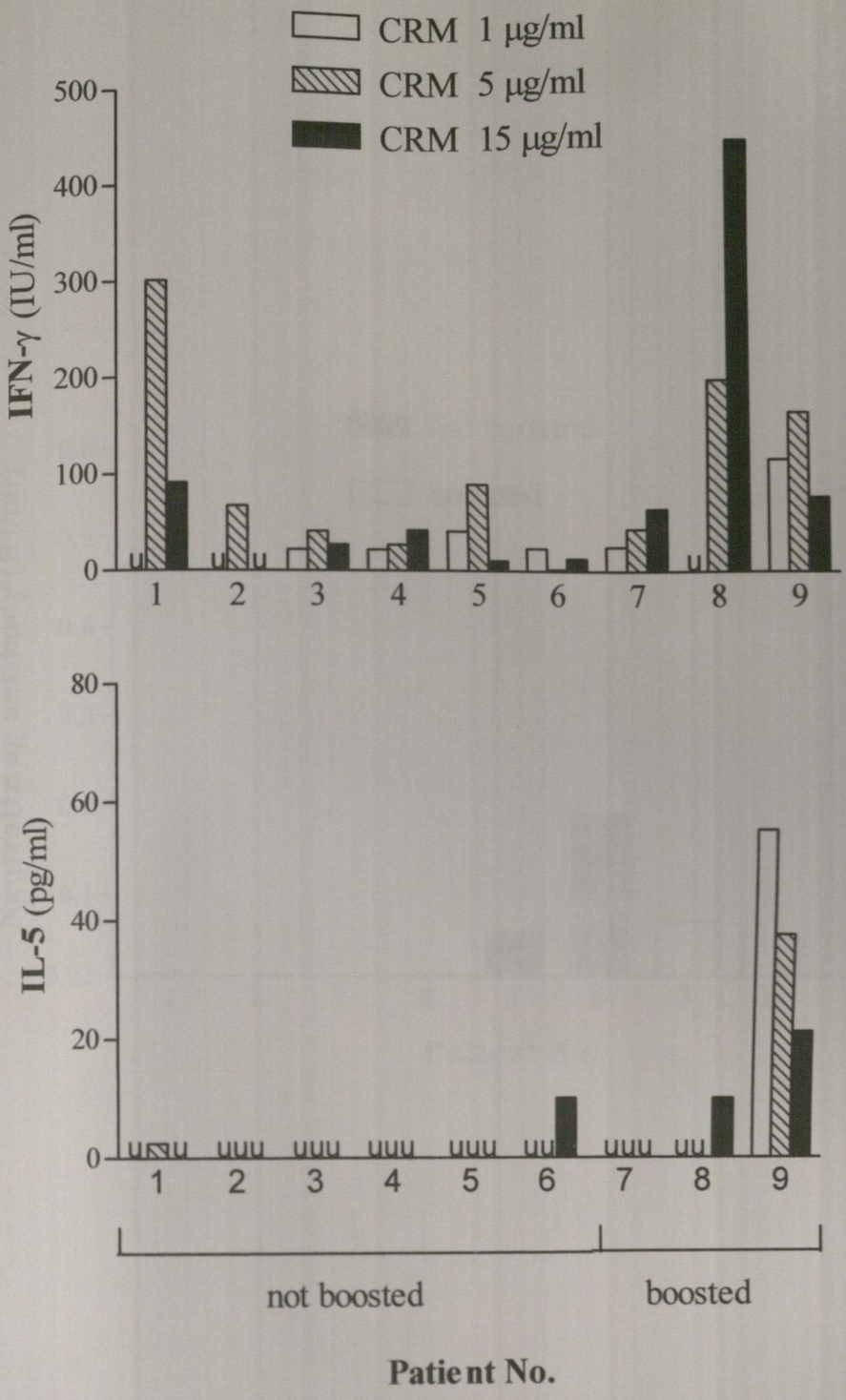


Figure 4.3. Persistence of cell-mediated immunity to diphtheria and the effect of booster immunization on antigen-specific Th1/Th2 profile. Blood samples were taken from 6 individuals up to 35 years post vaccination with DTP as infants (not boosted), and from three individuals one year post diphtheria booster vaccination (boosted). PBMC were isolated by density gradient centrifugation and stimulated *in vitro* with medium alone or with native CRM_{197} antigen (1-15 μg/ml). Supernatants were removed after 72 hours, and the concentrations of IL-5 and IFN-γ were assessed by specific immunoassay. Results are expressed as the mean cytokine concentrations for triplicate PBMC cultures for each individual. Background responses with medium alone, which were negligible in most cases, were subtracted from antigen-specific cytokine responses. u = undetectable concentrations of cytokine.

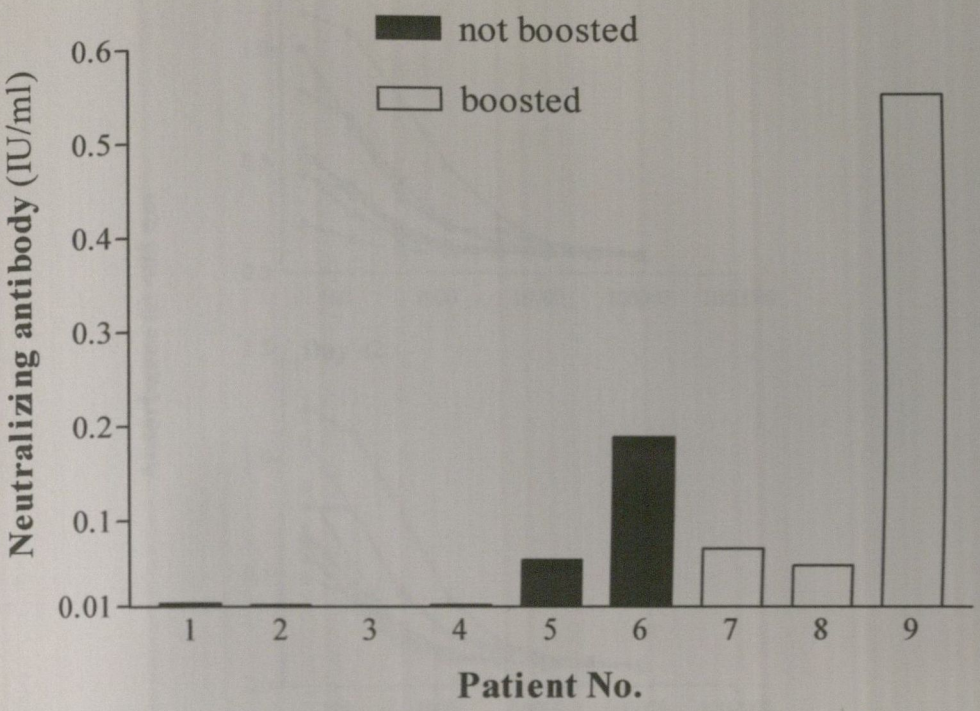


Figure 4.4. Prevalence of diphtheria toxin neutralizing antibodies in human plasma. Blood samples were obtained from nine individuals as described in the legend to figure 4.3. Anti-diphtheria toxin neutralizing antibody levels in plasma samples were assessed by vero cell assays. Results are expressed as the mean neutralizing antibody concentration (IU/ml) for each individual tested in triplicate.

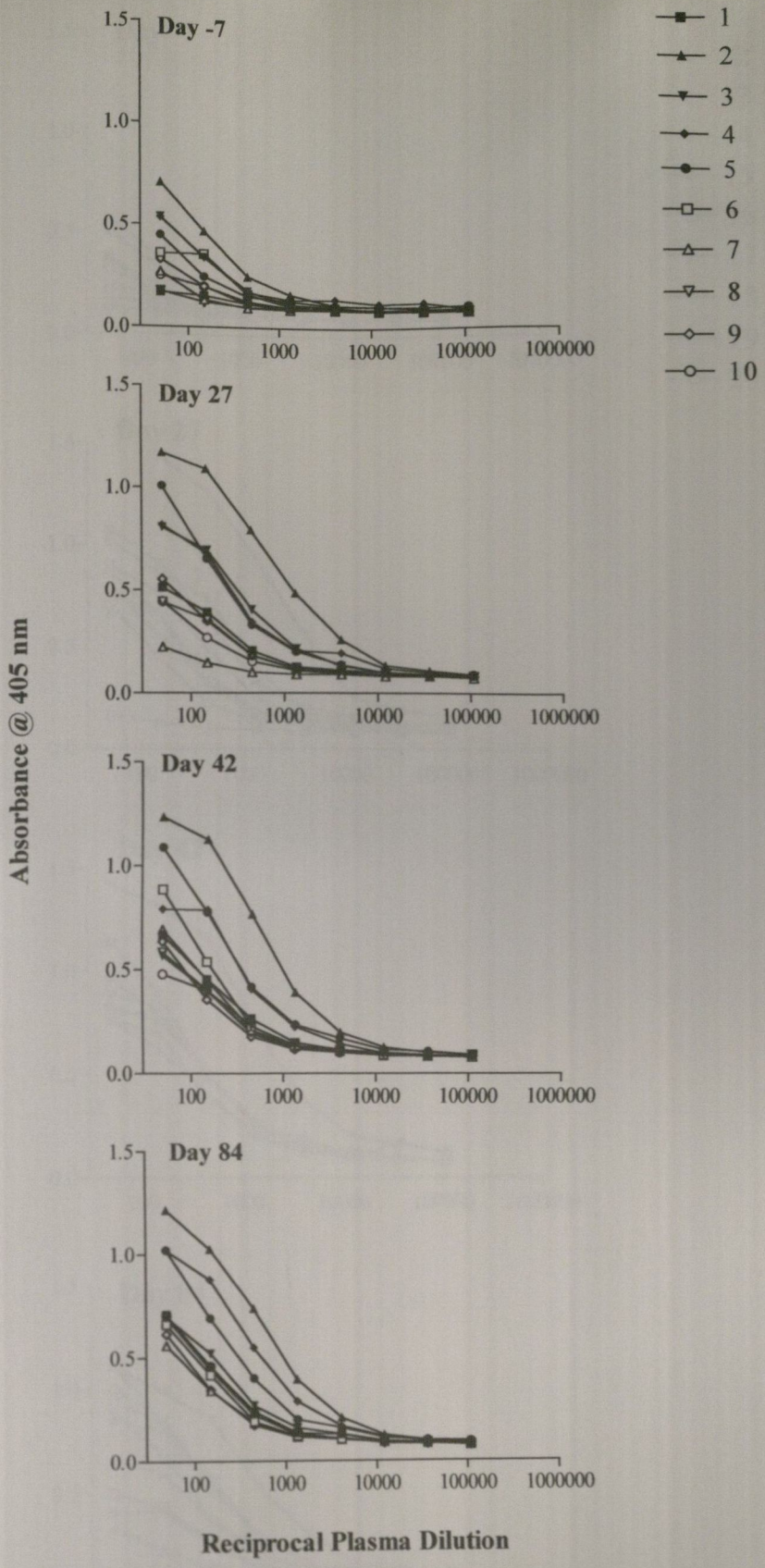


Figure 4.5(A). Human plasma IgG antibody responses pre- and post- nasal booster with CRM_{197}. Volunteers were boosted either i.n. at days 0 and 28 with a powder formulation containing CRM_{197} and mannitol (n=10, 1-10) or with the same formulation with chitosan (n=10, 11-20), or were boosted i.m. at day 0 with conventional diphtheria vaccine (n=5, 21-25). Blood samples were obtained 7 days pre- and 27, 42 and 84 days post- the first booster immunization. Each titration curve represents pre-immunization or post-immunization individual IgG antibody response as determined by ELISA.

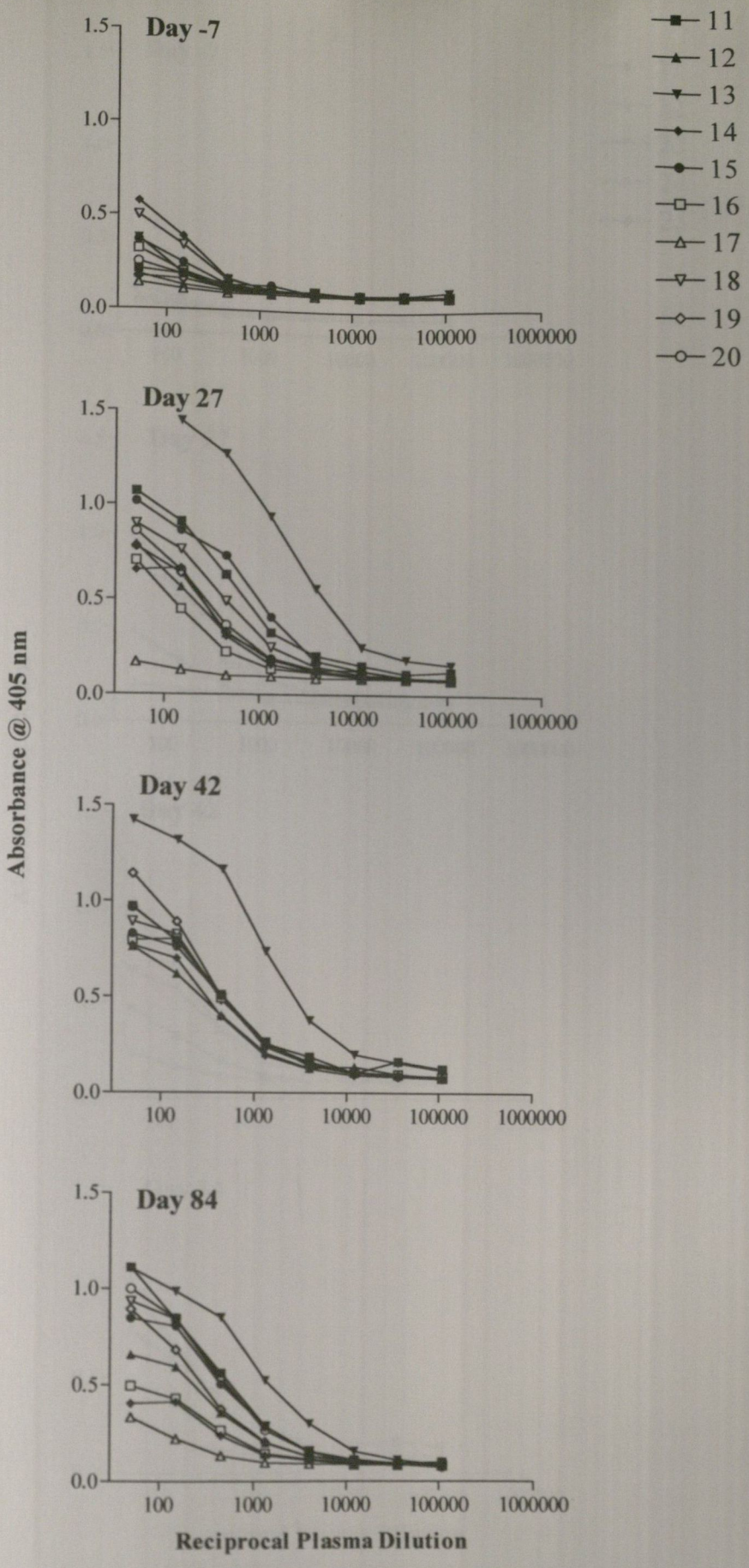


Figure 4.5(B). Human plasma IgG antibody responses pre- and post-nasal booster with CRM_{197} and chitosan.

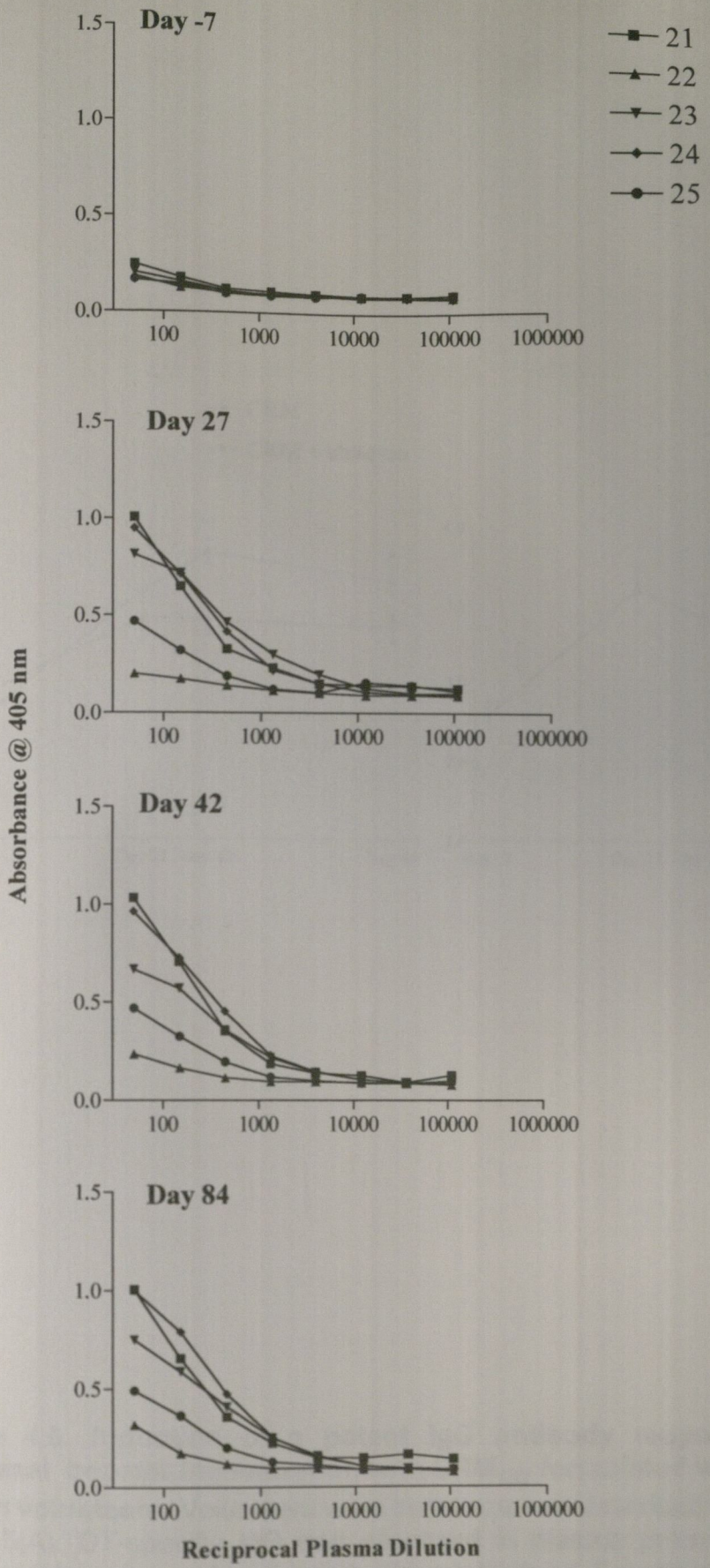


Figure 4.5(C). Human plasma IgG antibody responses pre- and post-intramuscular booster with conventional DT.

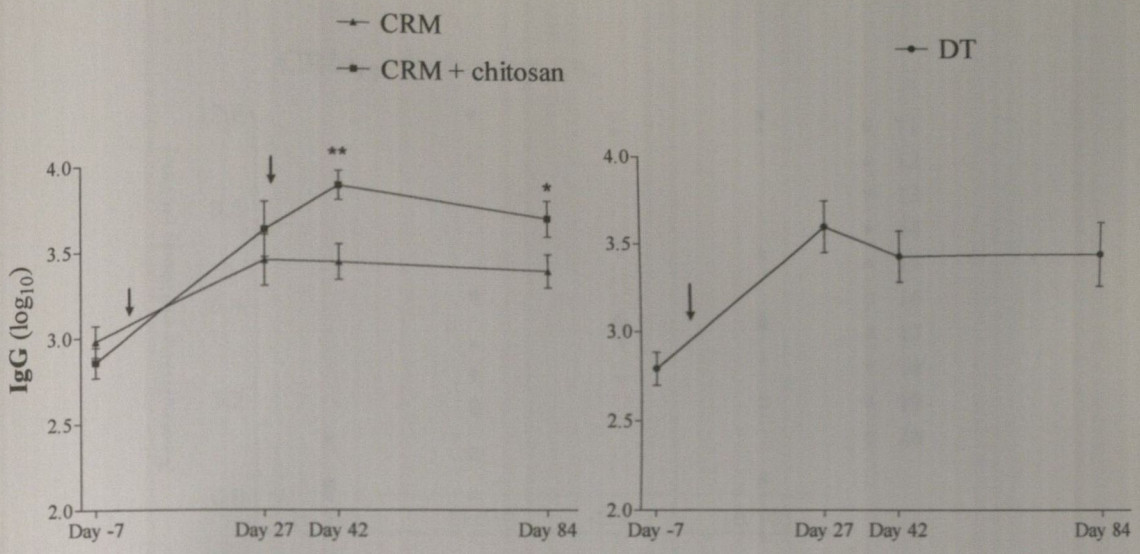


Figure 4.6. Induction of a potent IgG antibody response following intranasal booster immunization with CRM_{197} formulated with chitosan in human volunteers. Volunteers were immunized as described in the legend to Fig. 4.5(A). DT-specific IgG was assessed in plasma prepared from blood samples taken pre- and post-booster immunizations. Results are presented as mean IgG antibody end-point titres (+/- S.E.) for 5 or 10 volunteers per group. Booster immunizations are represented by arrows. * and **, $P < 0.05$ and $P < 0.01$ respectively versus CRM_{197} alone (Student's *t* test).

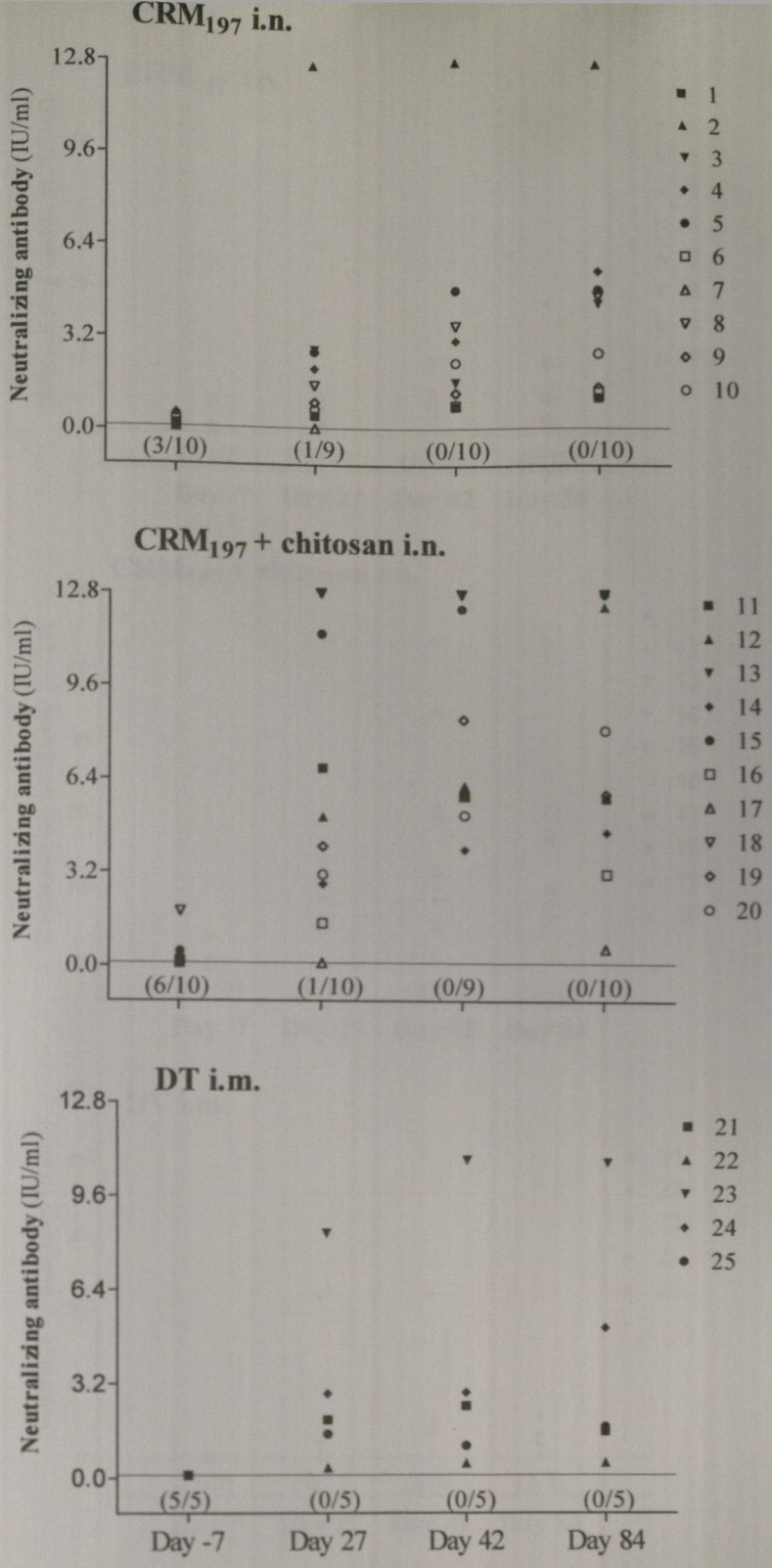


Figure 4.7. Plasma anti-diphtheria toxin neutralizing antibody concentrations pre- and post- intranasal booster immunization with CRM_{197} with or without chitosan. Volunteers were immunized as described in the legend to Fig. 4.5 (A). Neutralizing antibody concentrations were assessed by vero cell assays in plasma prepared from blood samples taken pre- and post-booster immunizations. Results are expressed as neutralizing antibody levels (IU/ml) for 25 individual volunteers tested in triplicate at each time-point. Values in parentheses at the origin represnt the number of volunteers in each group with neutralizing antibody concentrations below the detection level of 0.1 IU/ml for the assay.

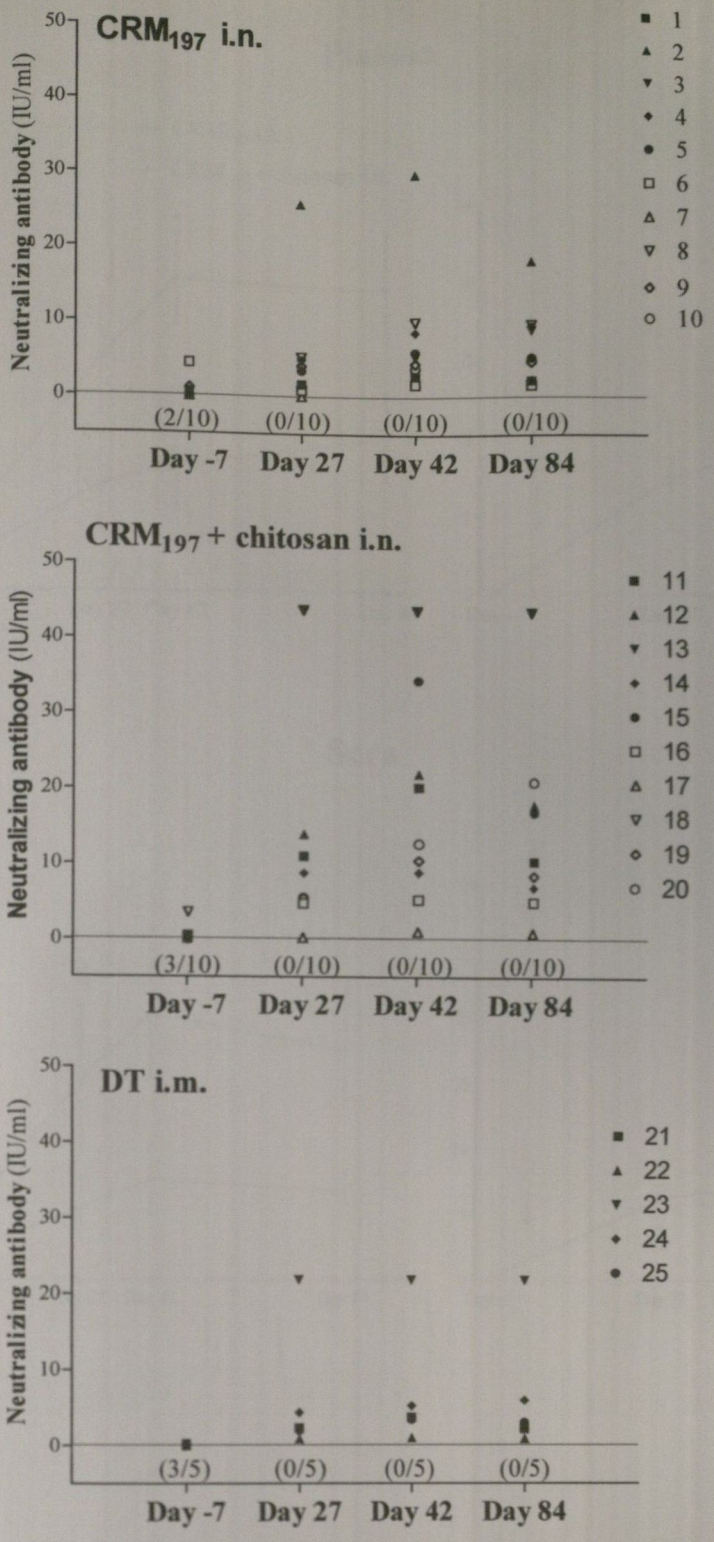


Figure 4.8. Sera anti-diphtheria toxin neutralizing antibody concentrations pre- and post- intranasal booster immunization with CRM_{197} with or without chitosan. Volunteers were immunized as described in the legend to Fig. 4.5(A). Neutralizing antibody concentrations were assessed in sera samples obtained pre- and post-booster immunizations as described in the legend to Fig. 4.7. Results are expressed as neutralizing antibody concentrations (IU/ml) for 25 individual volunteers tested in triplicate at each time-point. Values in parentheses at the origin represnt the number of volunteers in each group with neutralizing antibody concentrations below the detection level of 0.1 IU/ml for the assay.

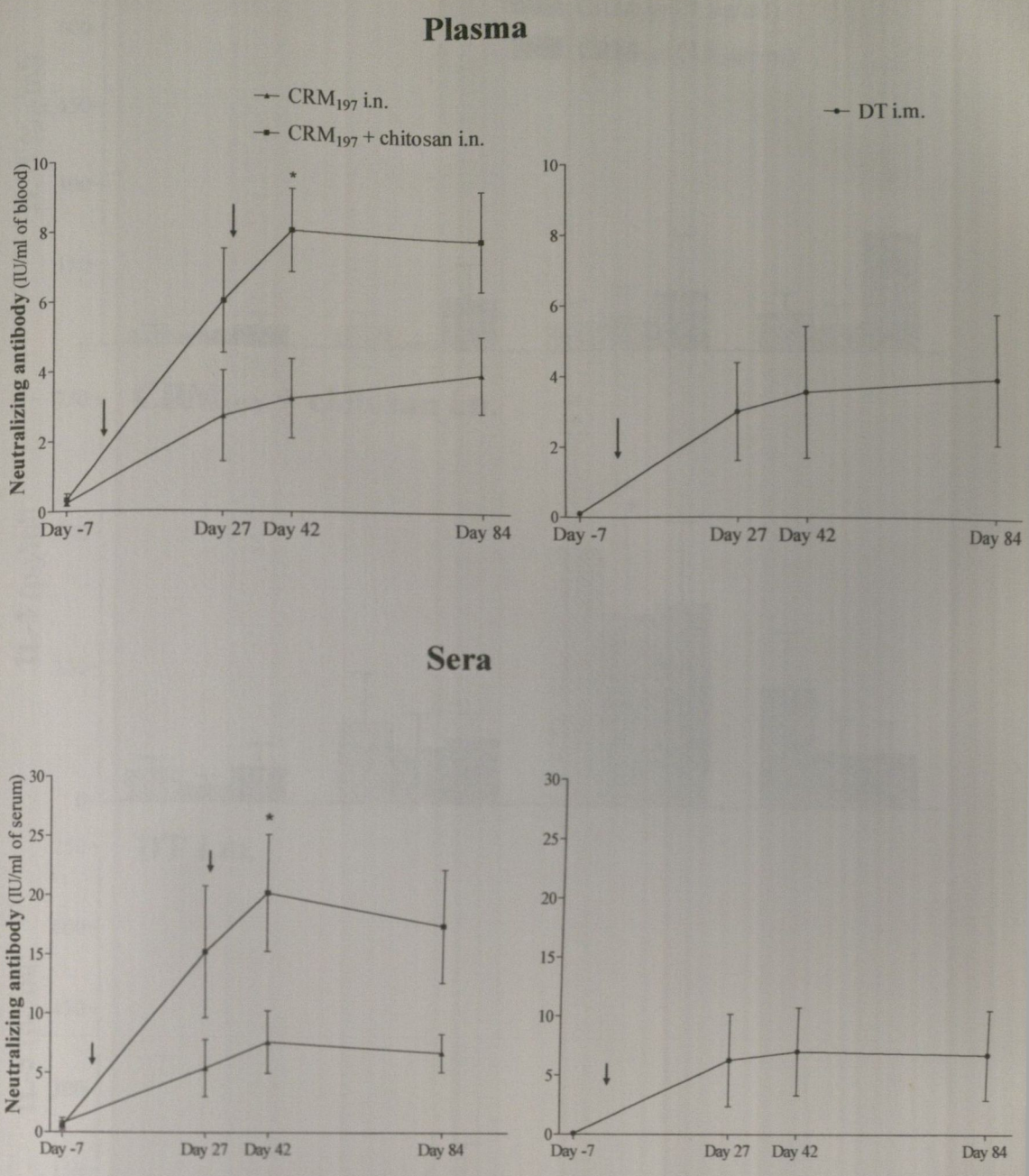


Figure. 4.9. Intranasal booster immunization with CRM_{197} formulated with chitosan significantly enhances anti-diphtheria toxin neutralizing antibody concentrations. Volunteers were immunized as described in the legend to Fig. 4.5(A). Neutralizing antibody concentrations in human plasma and sera samples were determined as described in the legend to Fig. 4.7. The upper limits of the assay were 12.8 IU/ml for plasma samples and 44 IU/ml for serum samples. Results are presented as estimated mean neutralizing antibody concentrations (+/- S.E) for 5 or 10 volunteers per group. Booster immunizations are represented by arrows. *, $P < 0.05$ versus CRM_{197} alone (Mann Whitney U test).

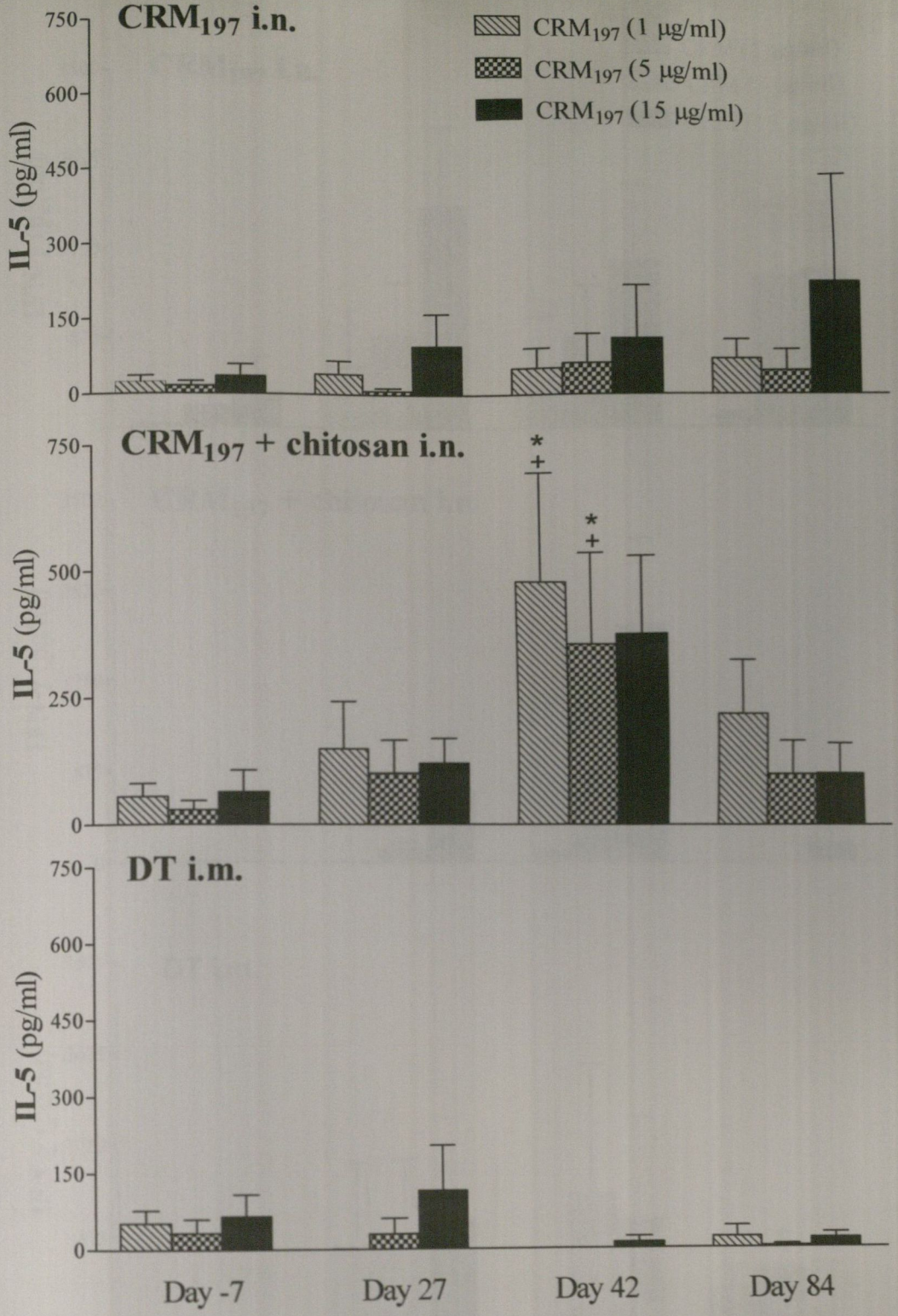


Figure 4.10(A) Th1/Th2 profile following nasal booster immunization against diphtheria in human volunteers. Volunteers were immunized as described in the legend to figure 4.5(A). PBMC were isolated by density gradient centrifugation of blood samples taken pre- and post- booster immunization and stimulated *in vitro* with native CRM_{197} antigen (1-15 μg/ml). Supernatants were removed after 72 hours and the concentrations of antigen-specific IL-5 and IFN-γ were assessed by specific immunoassay. Results are expressed as the mean IL-5 concentrations (pg/ml) for triplicate PBMC cultures for each individual. *, $P < 0.05$ versus CRM_{197} alone, +, $P < 0.05$ versus Day -7 (Dunn's test).

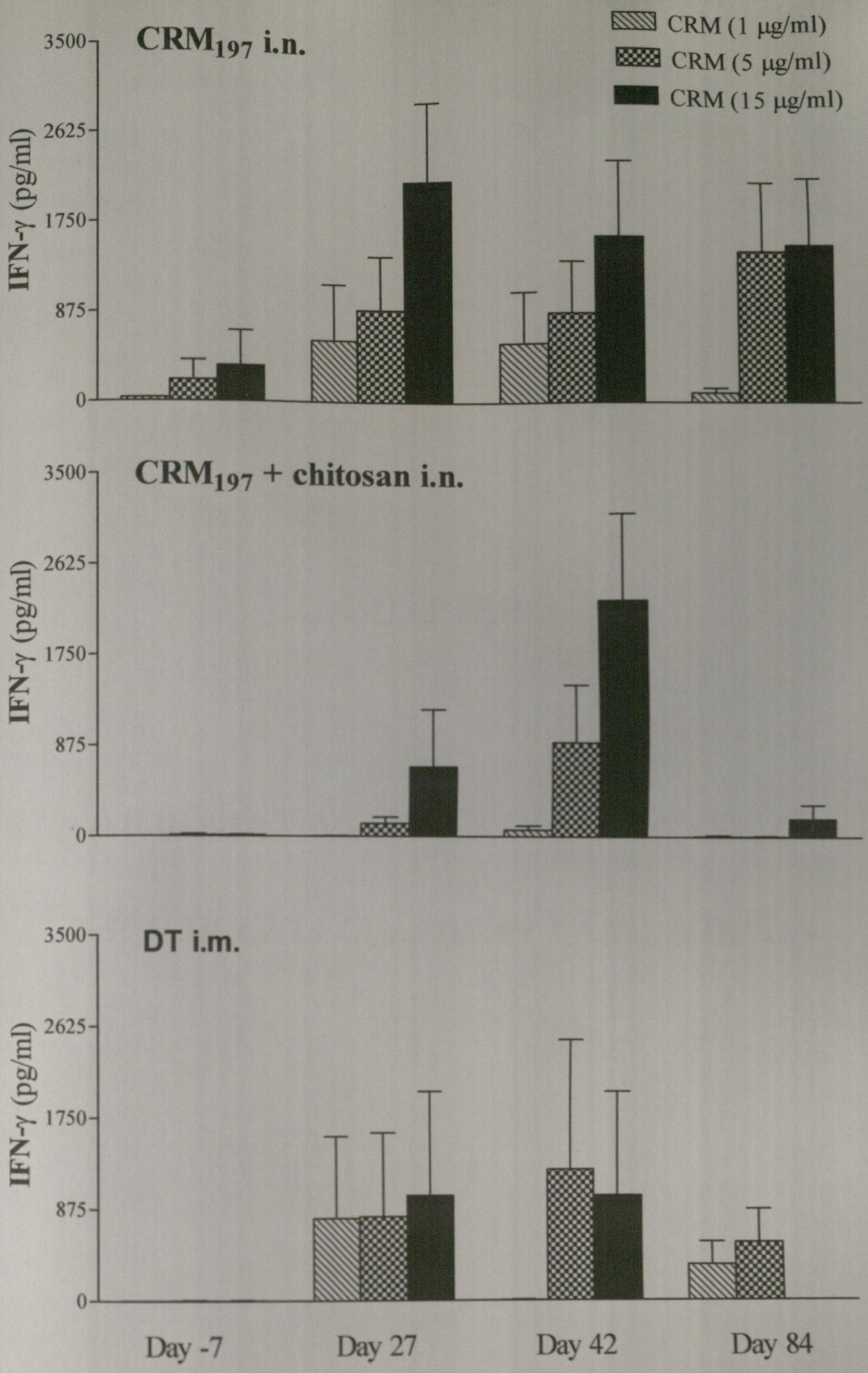


Figure 4.10(B). Th1/Th2 profile following nasal booster immunization against diphtheria in human volunteers. Results are expressed as the mean IFN-γ concentrations (pg/ml) for triplicate PBMC cultures for each individual.

CHAPTER 5

ADJUVANT ACTIVITY OF MUTANTS OF *ESCHERICHIA COLI* HEAT LABILE TOXIN

5.1 INTRODUCTION

It was shown in the previous chapters that chitosan is capable of enhancing immune responses to intranasally delivered CRM_{197} antigen, particularly in parenterally primed animals or individuals who had previously been immunized systemically with conventional diphtheria vaccine. One of the aims of this chapter was to compare chitosan with other molecules known to act as adjuvants for mucosally delivered antigens. The most powerful mucosal adjuvants described to date are cholera toxin (CT) and *Escherichia coli* heat labile toxin (LT). The mucosal immune system is able to recognize these molecules as a threat and shortly after they make contact with a mucosal surface a powerful immune response is generated against them. This anti-toxin response is so potent that immune responses may also be stimulated against foreign bystander molecules that are present simultaneously at mucosal surfaces. This property of the molecules has been extensively exploited for the mucosal delivery of a variety of antigens in animal models (Rappuoli *et al.*, 1999).

CT and LT are composed of two subunits: an enzymatically active A subunit that ADP-ribosylates GTP-binding proteins and is responsible for the toxicity, and a B subunit that mediates binding of the molecules to receptors on the surface of mammalian cells (Spangler, 1992, Zhang *et al.*, 1995). Although LT and CT have been shown to be effective adjuvants for the mucosal delivery of numerous antigens their clinical use may be restricted due to their toxicity. Therefore a number of successful attempts have been made to reduce the toxicity of these molecules without affecting their adjuvanticity. Single amino acid substitutions have been introduced by site-directed mutagenesis in the enzymatically active cleft of the A subunit to generate mutants such as the completely non-toxic molecule, LTK63, and the partially toxic mutant, LTR72.

LTK63 and LTR72 have been evaluated as mucosal adjuvants in different studies using a broad range of antigens. Although both molecules have been shown to elicit immune responses to co-administered bacterial and viral antigens and synthetic peptides (Giuliani *et al.*, 1998, Barackman *et al.*, 1999, Barchfeld *et al.*, 1999, Douce *et al.*, 1999, Partidos *et al.*, 1999, Ryan *et al.*, 1999), it has generally been found that LTR72 retains the strongest adjuvant activity of the two mutant toxins (Giuliani *et al.*, 1998, Barackman *et al.*, 2001). Indeed in several studies, LTR72 has been reported to induce comparable immune responses to wild-type LT (Del Giudice and Rappuoli, 1999, Olszewska *et al.*, 2000, Barackman *et al.*, 2001). However, stronger immune

responses may be induced with LTK63 as adjuvant by altering the toxin dose (Giuliani *et al.*, 1998) or route of immunization (Park *et al.*, 2000, Neidleman *et al.*, 2000).

It would appear that CT, LT and mutant toxins differentially regulate the induction of T cell subtype responses to mucosally delivered antigens. In most studies, CT has been shown to induce predominant Th2 type responses to co-administered antigens (Xu-Amano *et al.*, 1993, Marinaro *et al.*, 1995, Yamamoto *et al.*, 1997a,b). Furthermore, polarization of immune responses towards Th2 has been maintained using enzymatically inactive mutants of CT such as CTK112 (Yamamoto *et al.*, 1997b, Ohmura *et al.*, 2001). Reports on the induction of T cell subtypes with native LT, or mutants of LT, are less consistent. In several reports LT has been shown to induce mixed Th1/Th2 type responses to mucosally delivered antigens (Takahashi *et al.*, 1996, Cheng *et al.*, 1999, Douce *et al.*, 1999). However, the subtype of T cells induced with certain LT mutants has been shown to differ from that of the wild-type toxin. Nasal delivery of the non-toxic mutant, LTK63, has been shown to enhance both Th1 and Th2 type responses to co-administered antigens. In contrast, the partially toxic mutant, LTR72, specifically enhanced antigen-specific Th2 type responses at low doses of adjuvant, but augmented both Th1 and Th2 type responses at higher toxin doses (Ryan *et al.*, 1999, Ryan *et al.*, 2000). It was therefore suggested that the type of T cell responses induced following mucosal delivery of antigens may be influenced by the enzyme activity of the mutant toxins. However, selective augmentation of Th2 type responses has been reported with the enzymatically inactive B subunit of LT (Richards *et al.*, 2001) and LTR72 has also been reported to preferentially enhance IgG2a titres following intranasal immunization with influenza antigen (Barackman *et al.*, 1999), indicating that other factors are likely involved in the regulation of T cell responses *in vivo*. The toxin dose, form and purity as well as the route of delivery and the nature, dose and purity of the co-administered antigen may all potentially influence the enhancement or suppression of specific T cell responses *in vivo*. This study seeks to examine the influence of some of these factors, including route of immunization, toxin dose and nature of co-administered antigen, on the induction of specific T cell and antibody responses.

In this study, the effectiveness of LTK63 and LTR72 is examined for the intranasal delivery of CRM_{197} to mice. Furthermore, new information is presented on the adjuvanticity of LTK63 and LTR72 for the intranasal delivery of two meningococcal group B antigens. *Neisseria meningitidis* is the causative agent of the

life-threatening bacterial infections meningitis and septicaemia, which particularly affect young adults and infants. There are five pathogenic *N. meningitidis* serogroups: A, B, C, Y and W135 (Gotschlich *et al.*, 1969a, Gotschlich *et al.*, 1969b). Recently, a conjugated meningococcal vaccine has been introduced into the U.K. and Ireland that is very effective against serogroup C strains of *N. meningitidis*. However, group B strains still remain a problem, especially in Europe and the United States, as there is currently no effective vaccine available. The complete genomic sequence of a meningococcal B strain has been sequenced, allowing the identification of putative antigenic genes termed open reading frames (ORFs). These DNA sequences were amplified, cloned and expressed in *E. coli* in order to obtain recombinant proteins, such as ORF1 and ORF40 employed in this study, which may be used to immunize mice (Pizza *et al.*, 2000, Tettelin *et al.*, 2000).

5.2 RESULTS

5.2.1 LT mutants enhance T cell and antibody responses to intranasally delivered CRM_{197}

It was shown in chapter 3 that three doses of CRM_{197} and chitosan were necessary to generate modest T cell and antibody responses following intranasal immunization in mice. The present study was designed to compare the immune responses induced using mutants of LT and chitosan as adjuvants following two intranasal immunizations with CRM_{197} as antigen. Both the non-toxic mutant of LT, LTK63, and the partially toxic mutant, LTR72, enhanced systemic cellular immune responses to intranasally delivered CRM_{197}, as assessed by antigen-stimulated spleen cells *in vitro*. CRM_{197}-specific T cell-proliferative responses were significantly enhanced following formulation of the antigen with 10 μg of LTK63 or LTR72 as adjuvant. In addition, LTR72 significantly augmented the levels of IFN-γ induced following intranasal immunization with CRM_{197}. However, the toxins had no enhancing effect on antigen-specific IL-5 production, indicating a selective augmentation of Th1 type responses. In contrast, spleen cells from mice immunized intraperitoneally with conventional diphtheria toxoid (DT) adsorbed to alum secreted moderate levels of IFN-γ and significant concentrations of IL-5 and upon antigen stimulation *in vitro*. Interestingly, intranasal immunization with CRM_{197} and LTK63 or LTR72 induced stronger T cell responses than intranasal delivery of the same antigen formulated with chitosan (Fig. 5.1).

LTK63 and LTR72 mutant toxins also enhanced local and systemic antibody titres following intranasal immunization with CRM_{197}. Both serum IgG and lung homogenate IgA titres were higher, although not significantly, in mice immunized with CRM_{197} and LTK63 or LTR72 compared to mice immunized with CRM_{197} alone or formulated with chitosan. The antibody responses generated following intranasal immunization with CRM_{197} alone, or the same antigen formulated with chitosan, were found to be quite variable between mice. However, formulation of CRM_{197} with LTK63 or LTR72 resulted in consistently stronger antigen-specific local and systemic antibody responses. Two intraperitoneal immunizations with conventional DT adsorbed to alum induced significantly higher levels of IgG antibodies compared to intranasal immunization with CRM_{197}. However, in contrast to intranasal immunization with CRM_{197} and LTK63 or LTR72, intraperitoneal delivery of DT failed to induce consistently high levels of IgA antibodies in the lung (Fig. 5.2).

5.2.2 Effect of adjuvant dose on T cell subtype responses to intranasally delivered CRM_{197}

There is evidence to suggest that the dose of toxin used for mucosal immunizations may have profound effects not only on the strength of the immune response induced, but also on the type of T cell and antibody responses generated. Therefore, the effects of formulating CRM_{197} with two different doses, 1 or 10 μg, of either LTK63 or LTR72 on the induction of antigen-specific immune responses were evaluated. LTK63, at a dose of 1 μg, had no significant effect on cytokine secretion by antigen-stimulated spleen cells. Intranasal immunization with 1 μg of LTR72 and CRM_{197}, on the other hand, resulted in significant IL-4 production, indicating a selective augmentation of Th2 type responses. However, increasing the dose of both LTK63 and LTR72 to 10 μg induced stronger secretion of IFN-γ and IL-4, although these levels were not significant in the case of LTK63, suggesting that at higher doses both mutant toxins are capable of stimulating the differentiation of Th1 and Th2 type cytokines. In addition, 1 or 10 μg of LTR72 enhanced IL-5 production, although not significantly, following intranasal immunization with CRM_{197} (Fig. 5.3).

LTK63, at the higher dose of 10 μg, and 1 μg of LTR72 enhanced total IgG titres, although not significantly, to nasally delivered CRM_{197} antigen. In addition, analysis of IgG subclass antibody production from the sera of mice immunized with CRM_{197} and 10 μg of LTK63, or 1 and 10 μg of LTR72, as adjuvant demonstrated production of both IgG1 and IgG2a subclasses (Fig. 5.4), confirming the induction of mixed Th1/Th2 type responses by spleen cells *in vitro* (Fig. 5.3).

5.2.3 Effect of immunization route on cellular and humoral immune responses to CRM_{197}

The ability of LT mutants to augment immune responses to nasally co-administered antigens may result from their effects in enhancing vaccine uptake in the nasal cavity and/or from the ability of these molecules to non-specifically stimulate immune responses. Therefore, a study was designed to examine the adjuvanticity of LT, LTR72 and LTK63 for nasal versus parenteral immunization. Intranasal immunization with CRM_{197} alone or formulated with 1 μg of LTK63 induced very low levels of cytokine production, as assessed by spleen (Fig. 5.5) or lymph node (Fig. 5.6)

cells stimulated with antigen *in vitro*. However, formulation of CRM_{197} with 1 μg of LTR72 or LT significantly enhanced antigen-specific IFN-γ and IL-4 or IL-5 production following intranasal immunization, indicating the augmentation of both Th1 and Th2 type cytokines (Fig. 5.5). Furthermore, enhancement of local Th1 and Th2 type responses was observed by antigen-stimulated lymph node cells from mice immunized intranasally with CRM_{197} and 1 μg of LTR72 or LT as adjuvant (Fig. 5.6).

In contrast to intranasal immunization, intraperitoneal administration of CRM_{197} stimulated relatively high levels of systemically produced cytokines, in particular IL-4 and IL-5. Intraperitoneal immunization with 1 μg of LTK63 and CRM_{197} induced spleen cells that secreted strong, but not significant, levels of IL-4 following antigen stimulation *in vitro*. In contrast, 1 μg of LTR72 or LT significantly enhanced both Th1 and Th2 type cytokines following intraperitoneal immunization with CRM_{197} (Fig. 5.5).

Significant enhancement of CRM_{197}-specific IgG antibodies was only observed following intraperitoneal immunization using LTK63, LTR72 or LT as adjuvant. In contrast, intranasal immunizations with CRM_{197} with or without the toxins only induced very weak levels of antigen-specific IgG responses (Fig. 5.7). However, intranasal immunization did have the advantage over conventional parenterally delivered antigen in being able to stimulate high levels of local cellular immune responses (Fig. 5.6).

5.2.4 Intranasal immunization with DTPa vaccine formulated with LTK63 induces comparable immune responses to conventional parenterally delivered vaccine

Diphtheria vaccines are routinely administered to infants as parenterally delivered diphtheria, tetanus and pertussis (DTP) combination vaccines adsorbed to alum. Traditionally, these vaccines were prepared using inactivated *Bordetella pertussis* bacteria (whole-cell pertussis vaccines). However, more highly purified acellular pertussis (Pa) vaccines, composed of specific antigenic components of *B. pertussis*, have been developed and are now routinely used in many countries. Therefore, a study was designed to examine the effectiveness of LTK63 as an adjuvant for nasal delivery of DTPa vaccine to parenterally primed animals. The pertussis components of the DTPa vaccine included recombinant pertussis toxin (rPT), pertactin

(PRN) and filamentous haemagglutinin (FHA), while tetanus toxoid (TT) and CRM_{197} were used as the tetanus and diphtheria components respectively.

Cellular immune responses to pertussis and diphtheria antigens were weak from all mice primed parenterally with DTPa adsorbed to alum (Fig. 5.8). However, two intranasal immunizations with DTPa and LTK63 did induce spleen cells that secreted significant levels of IL-5 following stimulation with FHA or CRM_{197} *in vitro*. Intranasal or intramuscular immunization with DTPa induced relatively strong TT-specific cellular immune responses. Two intranasal immunizations with DTPa and LTK63, or intranasal boosting of parenterally primed mice with the same formulation, induced TT-specific T cell proliferative responses similar to those generated following two intramuscular immunizations with conventional DTPa adsorbed to alum. In addition, analysis of cytokine production by spleen cells from mice boosted intranasally with DTPa and LTK63 demonstrated significantly higher levels of TT-specific IL-4 and IL-5 compared to mice boosted intramuscularly with DTPa. It was also observed that boosting intranasally with DTPa and LTK63 was more effective than boosting with DTPa alone for the generation of T cell responses (Fig. 5.8).

LTK63 was also capable of acting as an effective adjuvant for the induction of local and systemic antibody responses to intranasally delivered DTPa. The levels of serum IgG specific for PRN, TT and CRM_{197} after two intranasal immunizations, or intranasal boosting with DTPa and LTK63, were found to be similar to those generated after two parenteral immunizations with conventional DTPa adsorbed to alum. However, FHA- and PT-specific IgG titres were significantly lower from mice immunized with DTPa intranasally. In contrast to parenteral immunization, mucosal delivery also had the advantage of significantly enhancing local IgA responses to all the antigens tested (Fig. 5.9).

5.2.5 Differential immune responses to two meningococcal group B antigens using mutants of LT as adjuvant

At present there is no vaccine available to prevent meningococcal group B infections. Therefore, the effectiveness of LTK63 and LTR72 as adjuvants for the intranasal delivery of two novel meningococcal B antigens, ORF1 and ORF40, was examined in mice. Spleen cells (Fig. 5.10) and lymph node cells (Fig. 5.11) from mice immunized with ORF1 or ORF40 secreted very low levels of antigen-specific IFN-γ and IL-5. However, formulation of the same antigens with LTK63 or LTR72

enhanced local and systemic cellular immune responses, as assessed by antigen-specific cytokine secretion. Furthermore, it was found that the co-administered ORF antigens influenced the type of T cell responses induced by the mutant toxins (Fig. 5.10 and Fig. 5.11).

LTK63, at a dose of 1 μg, enhanced IFN-γ secretion, although not significantly, following co-administration with ORF40, but had no augmentative effect on ORF1-induced cytokine secretion (Fig. 5.10). However, formulation of both ORF1 and ORF40 antigens with 1 μg of LTK63 did increase antigen-specific local IL-5 production by cervical lymph node cells *in vitro* (Fig. 5.11). Furthermore, increasing the dose of LTK63 to 10 μg resulted in a significant increase in antigen-specific IFN-γ and IL-5 following intranasal immunization with ORF40. 1 μg of LTR72 significantly enhanced ORF1-specific IFN-γ production, but had no significant effect on IL-5 secretion, by antigen-stimulated spleen cells *in vitro* (Fig. 5.10). In contrast, cervical lymph node cells from mice immunized with the same formulation secreted high levels of both IFN-γ and IL-5 *in vitro* (Fig. 5.11). Therefore, LTR72 would appear to enhance both Th1 and Th2 type responses locally, but to selectively augment Th1 responses systemically, following intranasal immunization with ORF1. On the other hand, formulation of LTR72 with ORF40 significantly enhanced both IFN-γ and IL-5 production by antigen-stimulated spleen cells (Fig. 5.10) and also augmented local Th1 and Th2 type responses (Fig. 5.11).

The co-administered antigens also influenced the antibody responses induced using LTK63 or LTR72 as adjuvant (Fig. 5.12). Intranasal immunization with ORF1 and ORF40 induced relatively low levels of total IgG, IgG1 and IgG2a. LTK63 and LTR72, at a dose of 1 μg, had no significant effect on ORF1-induced IgG antibody responses. In contrast, formulation of the same toxins with ORF40 significantly enhanced both total IgG and IgG subclass antibody responses following intranasal delivery. Analysis of IgG subclass production revealed selective enhancement of IgG1 subclasses using 1 μg of LTK63 as adjuvant. In contrast, 10 μg of LTK63 or 1 μg of LTR72 significantly enhanced both IgG1 and IgG2a subclasses following intranasal immunization with ORF40, suggesting augmentation of both Th1 and Th2 type responses and therefore confirming the mixed type responses observed from antigen-stimulated spleen cells *in vitro* (Fig. 5.10).

5.2.6 Local and systemic T cell subtype responses induced following injection of LTK63, LTR72 or CT with KLH.

There are numerous conflicting reports on the type of T cell responses induced following mucosal delivery of LT and its mutant toxins with various antigens. However, in many studies endotoxin contamination of the antigen is not considered in the regulation of Th1 and Th2 type responses by mucosally delivered LT molecules. In the present study the effect of LTK63 and LTR72 on the induction of Th1 and Th2 type responses was examined using endotoxin-free KLH as antigen. BALB/c mice were immunized into the footpad with KLH with or without 10 μg of LTK63, LTR72 or CT as adjuvant and cellular immune responses were assessed 7 days later from spleen cells and local lymph node cells stimulated with antigen *in vitro*.

Lymph node (Fig. 5.13) or spleen cells (Fig. 5.14) from mice that received KLH alone produced very low or undetectable levels of cytokines following antigen stimulation *in vitro*. However, formulation of KLH with 10 μg of LTK63, LTR72 or CT enhanced both Th1 and Th2 type cytokines. LTK63 and CT induced comparable antigen-specific T cell responses following s.c. injection with KLH. However, spleen cells from mice immunized with KLH and LTR72 induced significantly higher levels of antigen-specific IL-5 and IL-10, in comparison to mice immunized with KLH and LTK63, indicating a greater enhancement of systemic Th2 or T regulatory type responses using LTR72 as adjuvant (Fig. 5.14). In contrast, antigen-stimulated lymph node cells from these mice secreted higher levels of IFN-γ and IL-4, although not significantly, compared to mice injected with KLH and LTK63, suggesting a stronger increase in local Th1 and Th2 type cytokines (Fig. 5.13).

Both LTK63 and CT also enhanced antigen-specific IgG antibody responses following s.c. delivery with KLH (Fig. 5.15). Injection of LTK63 and KLH induced significantly higher levels of IgG2a subclass responses, compared to s.c. delivery of KLH alone, indicating the enhancement of Th1 type responses. In contrast, the ratios of IgG1:IgG2a were similar from mice injected with KLH and LTR72 or CT, and may be indicative of the induction of both Th1 and Th2 type responses *in vivo*.

5.3 DISCUSSION

This study demonstrates that LTK63 and LTR72 can act as effective adjuvants for the nasal administration of CRM_{197} and two novel meningococcal type B antigens, ORF1 and ORF40. Furthermore, the results suggest that the nature of the antigen, the route of administration and the toxin dose all affect the induction of T cell subtype and antibody responses using LTK63 and LTR72 as adjuvants.

Both LTK63 and LTR72 were shown to enhance immune responses to intranasally delivered CRM_{197}. Indeed the mutant toxins stimulated superior local and systemic immune responses to those generated using chitosan as an adjuvant, following two intranasal immunizations with CRM_{197}. Therefore, it would appear that while three intranasal immunizations are necessary to generate modest T cell and antibody responses to CRM_{197} using chitosan as an adjuvant (Chapter 3), only two intranasal immunizations with LTK63 or LTR72 and CRM_{197} are sufficient to enhance antigen-specific immune responses. Furthermore, it was shown that the non-toxic mutant, LTK63, may augment T cell and antibody responses induced by an intranasally delivered diphtheria, tetanus and acellular pertussis (DTPa) vaccine to levels comparable to those obtained following two parenteral immunizations with DTPa adsorbed to alum. In particular, this type of vaccine induced potent immune responses in mice primed parenterally with DTPa adsorbed to alum, suggesting that it may be very effective for mucosal boosting of individuals who had been immunized systemically with conventional DTPa as infants.

Initially it was thought that ADP-ribosylation and the accumulation of cAMP were necessary for the adjuvant activity of LT (Lycke *et al.*, 1992). However, mutants lacking ADP-ribosyltransferase activity (Dickinson and Clements, 1995, de Haan *et al.*, 1998a, Giuliani *et al.*, 1998, Douce *et al.*, 1999, Ryan *et al.*, 1999) and the A and B subunits of LT or CT alone (Ågren *et al.*, 1997, de Haan *et al.*, 1998a, de Haan *et al.*, 1998b, Isaka *et al.*, 1998, Kozuka *et al.*, 2000, Mowat *et al.*, 2001) have been shown to retain the ability to act as mucosal adjuvants, indicating that the adjuvanticity may be derived from independent contributions of the A and B subunits, or the enzymatic activity and the non-toxic AB complex. While it is now generally accepted that ADP-ribosylase activity is not a pre-requisite for adjuvanticity, several studies have shown that LT derivatives with higher enzymatic activity exert stronger adjuvant effects compared to those with little or no enzymatic activity (Giuliani *et al.*, 1998, Cheng *et al.*, 1999, McCluskie *et al.*, 2001b), especially following oral administration. However,

increasing the dose of toxins with weaker, or no, enzymatic activity may induce significantly stronger immune responses, as has been shown in studies using LTK63 as adjuvant (Giuliani *et al.*, 1998, Barackman *et al.*, 2001). LTK63, at a dose of 100 μg, induced immune responses similar to those generated with 10 μg of LTR72 or wild-type LT following intragastric administration of influenza haemagluttinin (Barackman *et al.*, 2001). Furthermore, Komase *et al.* (1998) examined the mucosal adjuvanticity of a panel of LT mutants and showed that while the least toxic mutant, LTK7, retained high levels of adjuvant activity this adjuvanticity was lost rapidly with a decrease in the toxin dose. Therefore, relatively high amounts of the mutant toxin, compared to wild-type toxin, were necessary to enhance immune responses to nasally delivered influenza vaccine (Komase *et al.*, 1998).

In this study it was found that the non-toxic mutant, LTK63, at a dose of 1 μg was generally a weaker adjuvant for the enhancement of immune responses to nasally delivered CRM_{197} compared to the partially toxic mutant LTR72, which retains about 1% of the wild-type enzyme activity. However, increasing the dose of LTK63 to 10 μg resulted in a concomitant enhancement of cellular and humoral immune responses to co-administered CRM_{197}. Moreover, 10 μg of LTK63 was capable of augmenting CRM_{197}-specific antibody responses to levels similar to those observed with the same dose of LTR72 administered by the nasal route.

In addition to influencing the strength of immune responses, altering the toxin dose may also affect the type of T cell responses induced following nasal immunization with unrelated antigens. Ryan *et al.* (1999) showed that lower doses of LTR72 (1 μg) selectively augmented Th2 type responses whereas higher doses (10 μg) enhanced both Th1 and Th2 type responses following intranasal immunization with an acellular pertussis vaccine. In contrast, the enzymatically inactive mutant, LTK63, enhanced both Th1 and Th2 responses at both higher and lower doses (Ryan *et al.*, 1999). Similarly, in this study it was found that 1 μg of LTR72 selectively enhanced systemic Th2 type responses to intranasally delivered CRM_{197}, whereas, 10 μg of LTK63 or LTR72 augmented both Th1 and Th2 type responses. However, the type of T cell responses induced using mutants of LT as mucosal adjuvant are likely to be influenced by other factors such as the antigen employed in the study. Indeed, 1 μg of LTR72 was shown to significantly enhance systemic IFN-γ production when co-administered with ORF1 antigen.

A number of hypotheses have been put forward to explain the differential effects of LT and its mutants on the induction of Th1 and Th2 type responses. Bowman and Clements (2001) constructed hybrid toxins consisting of the A subunit of either LT or CT in conjunction with the B subunit of the alternative toxin and evaluated their adjuvant properties for intranasal immunization of tetanus toxoid. Studies using these hybrid toxins suggested that the ability of LT to enhance Th1 type responses was a property of the A subunit of LT, as LT-A~CT-B, but not CT-A~LT-B, was capable of augmenting antigen-specific IFN-γ responses to levels comparable to those induced using native LT as adjuvant (Bowman and Clements, 2001). However, the B subunit of LT has been shown to selectively enhance IgG2a antibody titres to co-administered *Helicobacter pylori* antigens (Weltzin *et al.*, 2000), suggesting that the ability of LT to augment Th1 responses is not solely a property of the A subunit. The B subunit of LT may induce NF-κB activation (Bone *et al.*, 2000), possibly through binding to GM1 or some other pattern recognition receptor, which may lead to the transcription of several immune response genes, such as those that regulate IL-12 production (Baldwin, 1996, Ballou *et al.*, 1996). Alternatively, the ADP-ribosylase activity of the A subunit leads to the accumulation of cAMP (Cheng *et al.*, 1999, Ryan *et al.*, 2000), a potent immunomodulator that is capable of inhibiting the development of Th1 type responses but has enhancing effects on Th2 cells (Zidek, 1999). Therefore, it has been suggested that the immunodulatory effect of LT on T cell responses may be derived from independent contributions of the enzymatic activity and the non-toxic AB complex (Ryan *et al.*, 2000). An alternative, but not mutally exclusive, explanation for the effects of LT, CT and mutant toxins on T cell polarization is their ability to affect APC function, in particular DC maturation (discussed further in chapter 6).

In many studies using LT and its mutants as adjuvants the possibility of LPS contamination, which may have its own adjuvant effect, has not been examined. Indeed, LPS may be capable of influencing the type of T cell responses induced through its ability to bind to TLR4 on host cells and activate a pathway leading to the production of cytokines such as IL-12 (Hoshino *et al.*, 1999, Akira *et al.*, 2001). Therefore, the effects of CT, LTK63 and LTR72 on the induction of immune responses were evaluated using endotoxin-free KLH as antigen. All the toxins enhanced both cellular and humoral immune responses to injected KLH, with the induction of both Th1 and Th2 type responses. However, LTR72 did induce significantly stronger splenic Th2 type responses in comparison to LTK63, and analysis of IgG subclasses

revealed a higher ratio of IgG2a:IgG1 from mice injected with LTK63 and KLH.

Although LT and its mutants have mainly been used for the mucosal delivery of antigens, several studies have also shown that they may enhance the immunogenicity of antigens following parenteral immunization (Scharton-Kersten *et al.*, 2000, Weltzin *et al.*, 2000). However, there is little information available on the effect of parenteral administration of LT on the induction of Th1 and Th2 responses. Therefore, the immune responses induced using LTK63, LTR72 and LT as adjuvants for CRM_{197} were directly compared for intranasally and parenterally immunized mice. Both LTR72 and LT enhanced lymph node and/or splenic Th1 and Th2 responses following intranasal or parenteral delivery. However, LTK63 had no significant effect on T cell responses when delivered nasally with antigen but did slightly enhance antigen-specific IL-4 production following parenteral delivery. It was also observed that the route of delivery influenced the antibody responses induced to CRM_{197}. LT, LTK63 and LTR72 significantly enhanced CRM_{197}-specific IgG titres following parenteral immunization, whereas the same toxins only induced very weak IgG responses when delivered nasally. In contrast, an earlier study showed the induction of potent antibody responses following intranasal immunization of mice with CRM_{197} and LTK63 or LTR72 as adjuvant (Fig. 5.2). However, in this study anaesthetic and a high delivery volume (40 µl) was used for the nasal delivery of CRM_{197}, which may favour antigen delivery to the lungs. It has been shown that following intranasal immunization of anaesthetized mice with tetanus toxoid, a smaller delivery volume of 10 µl resulted in most of the vaccine formulation being retained in the nasal passages. In contrast, if the vaccine delivery volume was increased to 50 µl, almost half the vaccine penetrated into the lungs. Furthermore, antigen-specific IgG and IgA antibodies were only detected using the larger delivery volume of 50 µl (Eyles *et al.*, 1999).

In addition to enhancing immune responses to intranasally delivered CRM_{197}, LTK63 and LTR72 were also shown to act as effective adjuvants for the intranasal delivery of two novel meningococcal serogroup B antigens, ORF1 and ORF40. However, the co-administered antigen influenced the type of T cell and antibody responses induced with LTK63 or LTR72. LTR72 selectively enhanced systemic Th1 type responses but had no effect on IgG antibody production following intranasal immunization with ORF1. In contrast, formulation of LTR72 with ORF40 significantly enhanced antigen-specific Th1 and Th2 type responses and IgG antibody levels following intranasal delivery. Furthermore, LTK63 at a dose of 1 µg had no

augmentative effect on ORF1 induced cytokine secretion or IgG antibody production, but enhanced antigen-specific IFN-γ secretion and IgG responses when administered with ORF40. It has previously been shown that the nature of the antigen may profoundly affect the type of immune responses induced *in vivo*. Th2 type responses may be preferentially activated by soluble antigens, that lack immunomodulatory activity, whereas, particulate antigens have been shown to induce Th1 type responses through the secretion of IL-12 (Yang *et al.*, 1993, Fulton *et al.*, 1996, D'Orazio and Niederkorn, 1998). Interestingly in this study, two related antigens, which were both derived from the same strain of *N. meningitidis* and prepared in a similar manner, were capable of inducing distinct immune responses following nasal delivery with LT mutants. Intranasal immunization with ORF1 and LTR72 resulted in a more cellular type of response with the absence of detectable antibody-mediated immunity, whereas ORF40 and LTK63 or LTR72 induced mixed Th1/Th2 type responses and potent IgG responses.

In summary, this study shows the effectiveness of LTK63 and LTR72 for the intranasal delivery of a variety of antigens, including CRM_{197} and two recently characterised meningococcal group B antigens. However, the antigen and toxin employed, the dose of the toxin and the route of immunization were all shown to influence the strength and type of immune response induced. Identification of antigens, adjuvants and routes of delivery that differentially influence the type of immune response induced should make it possible to design effective vaccines that provide protection against specific pathogens based on prior knowledge of the protective mechanism. For example, more effective acellular vaccines against pertussis may be possible by the identification of combinations of antigens and adjuvants that stimulate Th1 responses, which function together with antibody in the rapid elimination of bacteria from the lungs.

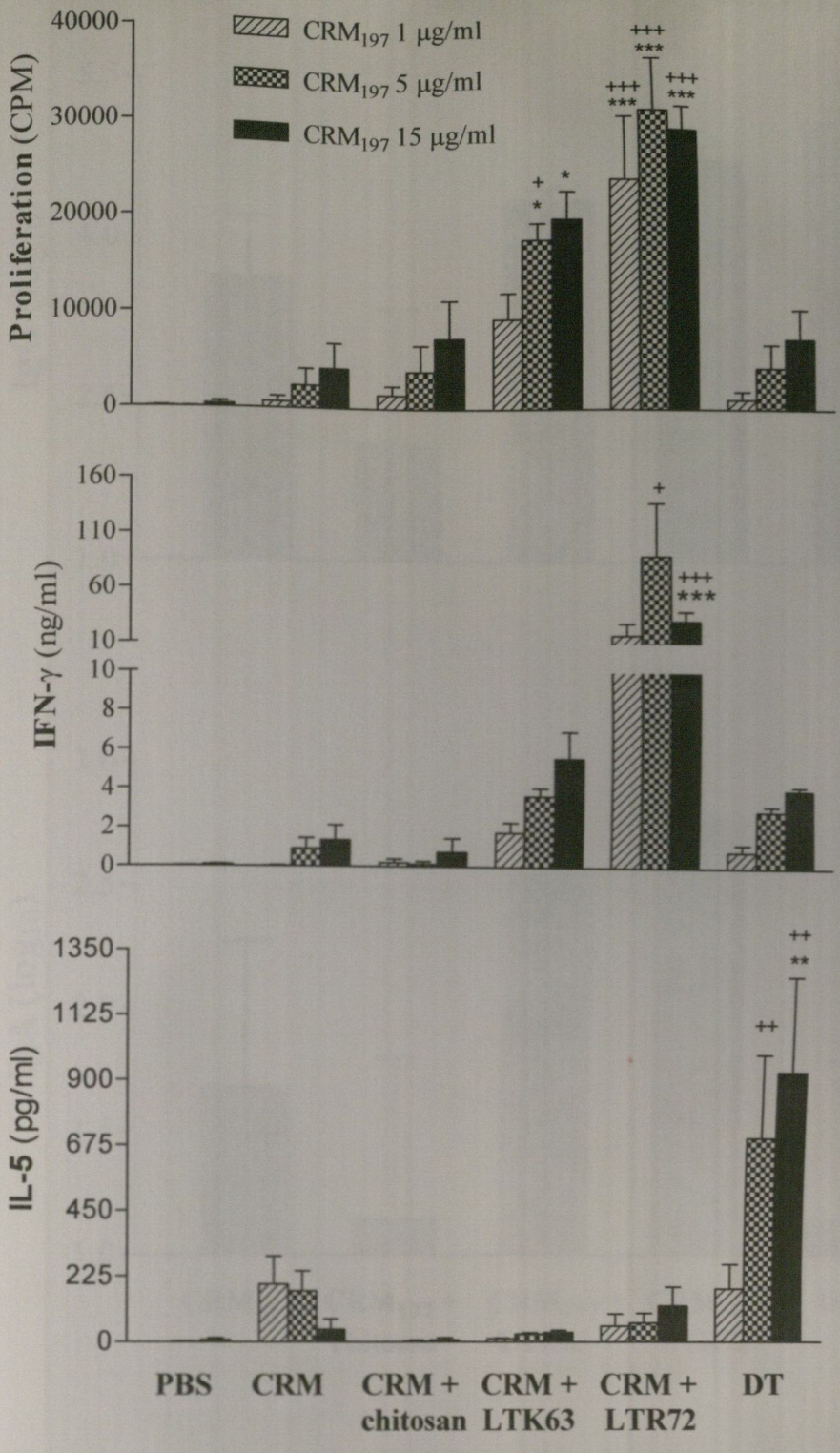


Figure 5.1. Effect of LTK63 and LTR72 on cellular immune responses to intranasally delivered CRM_{197}. Mice were immunized i.n. at 0 and 4 weeks under light anesthesia with 0.18% formaldehyde-treated CRM_{197} (10 µg) alone, or with either chitosan (100 µg), LTK63 (10 µg) or LTR72 (10 µg) as adjuvant in a total volume of 40 µl, or with PBS only as a control. For comparison one group of mice was immunized i.p. at 0 and 4 weeks with DT (10 µg) adsorbed to alum. Cellular responses were assessed at week 6 from spleen cells stimulated *in vitro* with native CRM_{197} (1-15 µg/ml). Results are mean (+/- S.E.) proliferative or cytokine responses for four mice per group, tested individually in triplicate. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus CRM_{197} alone, +, ++ and +++, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus CRM_{197} and chitosan (Tukey-Kramer test).

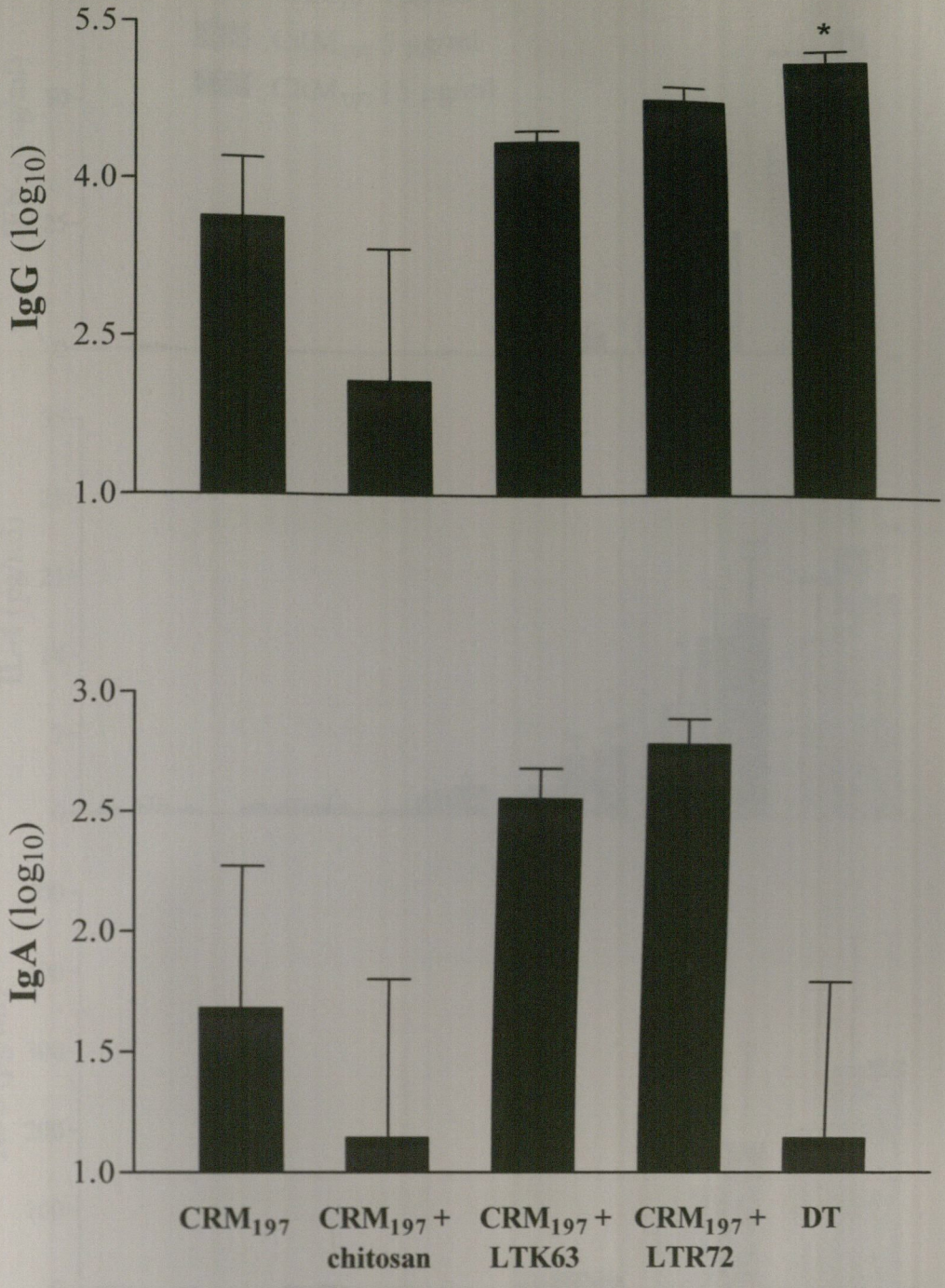


Figure 5.2. LTK63 and LTR72 enhance anti-DT local and systemic antibody responses to nasally delivered CRM_{197}. Mice were immunized as described in the legend to Fig. 5.1. Anti-DT IgG and IgA titres, in serum and lung homogenates recovered two weeks after immunization, were determined by ELISA. Results are expressed as mean (+/- S.E.) endpoint titres for four mice per group. *, $P < 0.05$ versus CRM_{197} i.n. (Tukey-Kramer test).

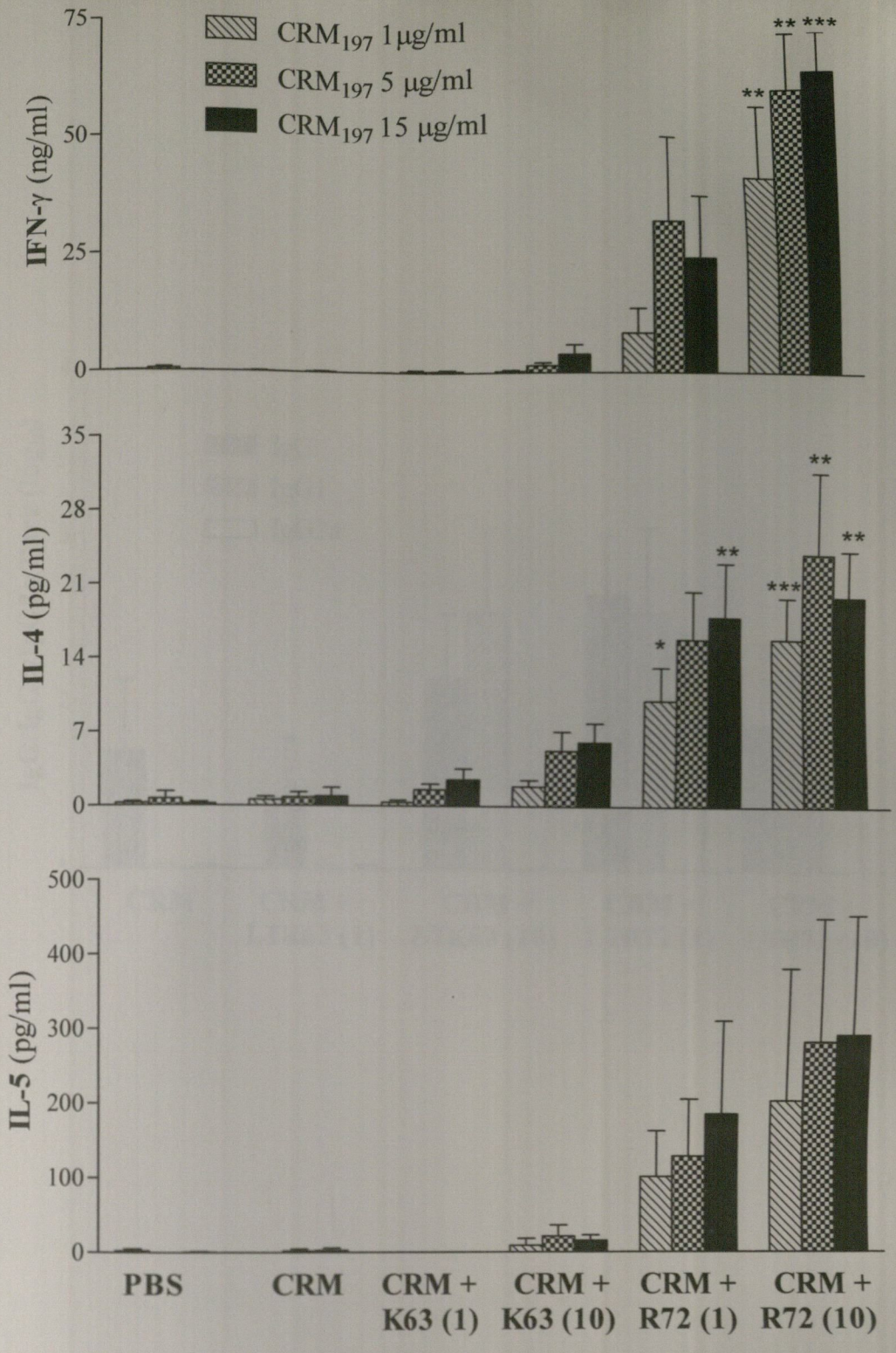


Figure 5.3 Effect of toxin dose on systemic cellular immune responses to intranasally delivered CRM_{197}. Mice were immunized i.n. at 0 and 4 weeks with PBS only as a control or with CRM_{197} (10 µg) alone or with 1 or 10 µg of LTK63 or LTR72 as adjuvant. Cellular responses were assessed at week 6 from spleen cells stimulated *in vitro* with native CRM_{197} (1-15 µg/ml). IFN-γ, IL-4 and IL-5 levels were determined by ELISA. Results are presented as mean cytokine concentrations (+/-S.E.) for four mice per experimental group tested individually in triplicate. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus CRM_{197} alone (Tukey-Kramer test).

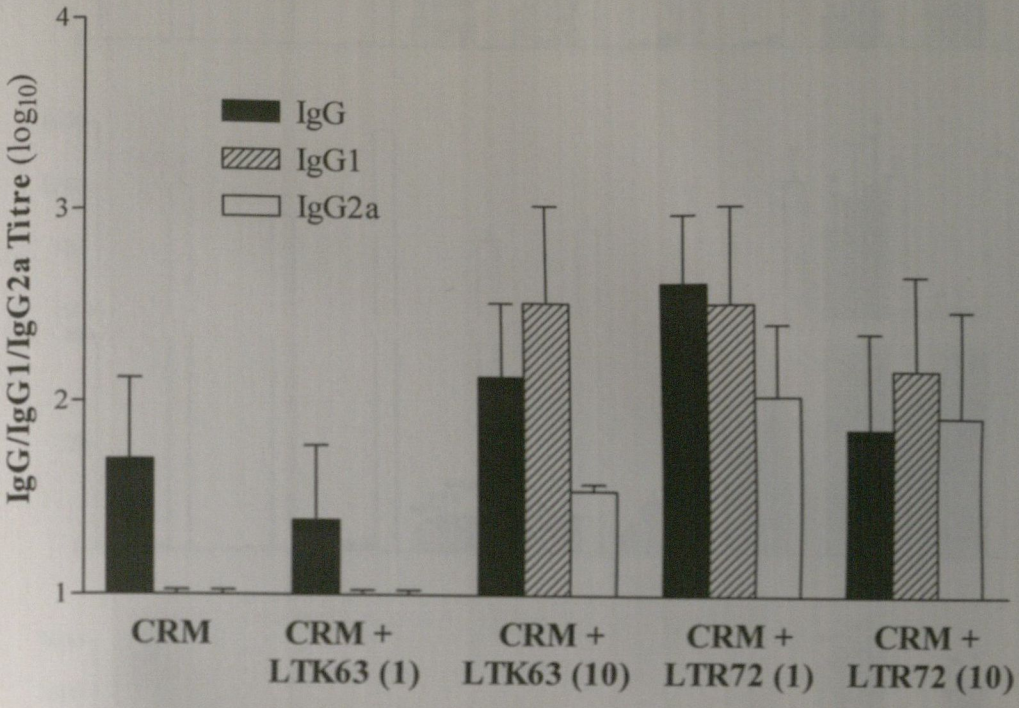


Figure 5.4. Effect of toxin dose on IgG antibody responses to intranasally delivered CRM_{197}. Mice were immunized as described in the legend to Fig. 5.3. Total IgG, IgG1 and IgG2a levels were determined by ELISA on serum samples recovered two weeks after the second immunization. Results are presented as mean antibody endpoint titres (+/- S.E.) for four mice per experimental group.

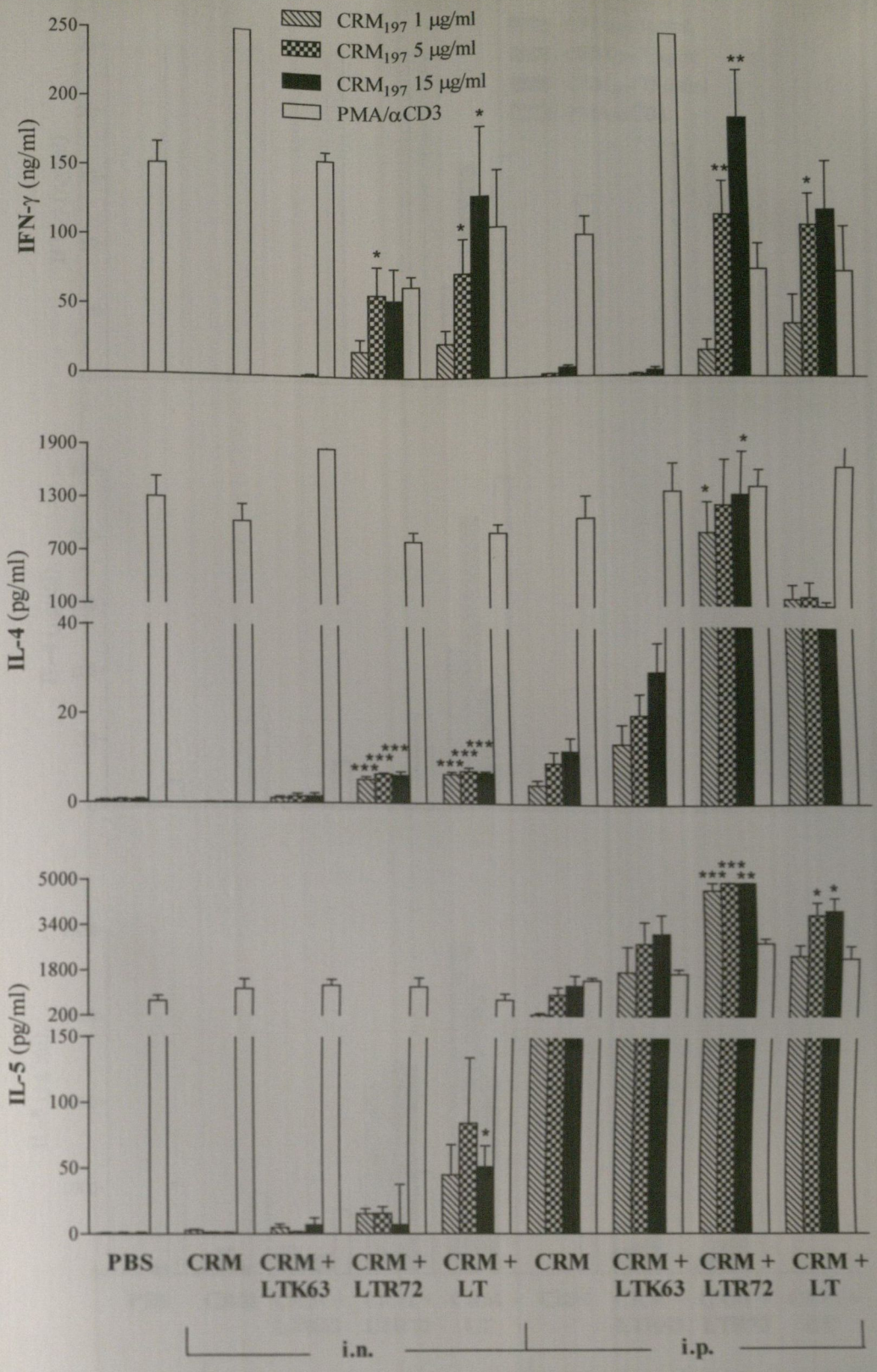


Figure 5.5. Splenic T cell responses induced following intranasal or intraperitoneal immunization with CRM_{197} and LTK63 or LTR72 as adjuvant. Mice were immunized i.n. or i.p. at 0 and 4 weeks with CRM_{197} (10 µg) alone or formulated with LTK63 (1 µg), LTR72 (1 µg) or LT (1 µg) as adjuvant. Cellular immune responses were assessed as described in the legend to Fig. 5.3. Results are presented as mean IFN-γ, IL-4 or IL-5 concentrations (+/- S.E.) for four mice per group tested individually in triplicate. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus CRM_{197} alone (Tukey-Kramer test).

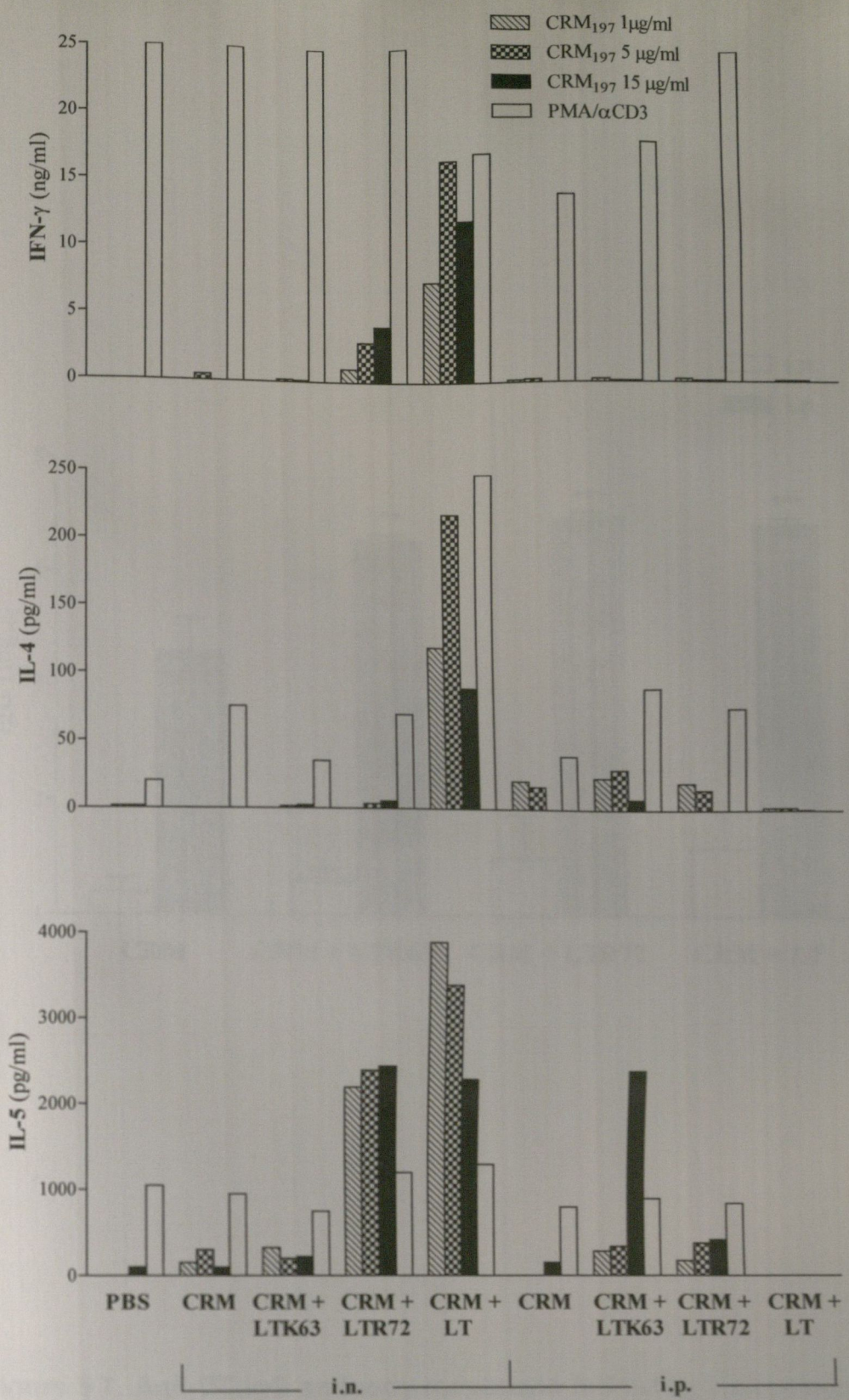


Figure 5.6. Cervical lymph node T cell responses induced following intranasal immunization with CRM_{197} and LTK63 or LTR72 as adjuvant. Mice were immunized as described in the legend to Fig. 5.5. Cellular immune responses were assessed at week 6 from cervical lymph node cells stimulated *in vitro* with CRM_{197} (1-15 µg/ml) or with PMA and anti-CD3 as a positive control. Results are presented as mean IFN-γ, IL-4 or IL-5 concentrations from triplicate cultures of pooled cervical lymph node cells from each experimental group.

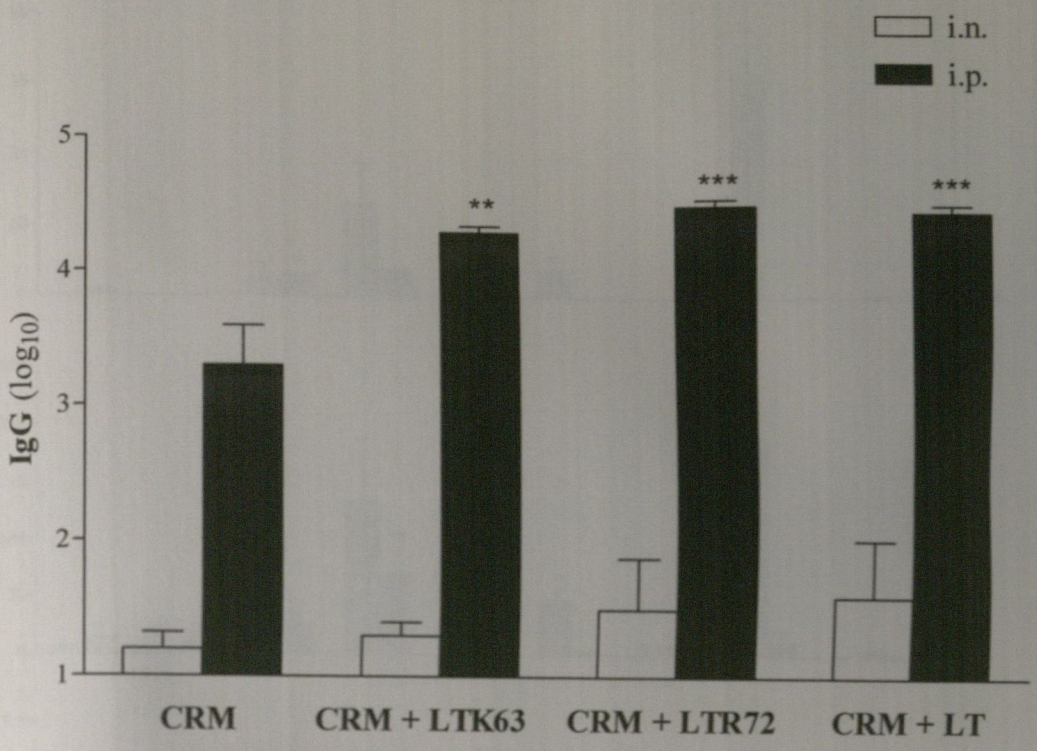


Figure 5.7. Anti-DT IgG antibody responses following intranasal or intraperitoneal immunuization with CRM_{197} and LTK63, LTR72 or wild-type LT as adjuvant. Mice were immunized as described in the legend to Fig. 5.5. Anti-DT IgG levels were determined by ELISA on serum samples taken two weeks after the second immunization. Results are presented as mean IgG end-point titres (+/- S.E.) for four mice per experimental group. ** and ***, $P < 0.01$ and $P < 0.001$ respectively versus CRM_{197} alone (Tukey-Kramer test).

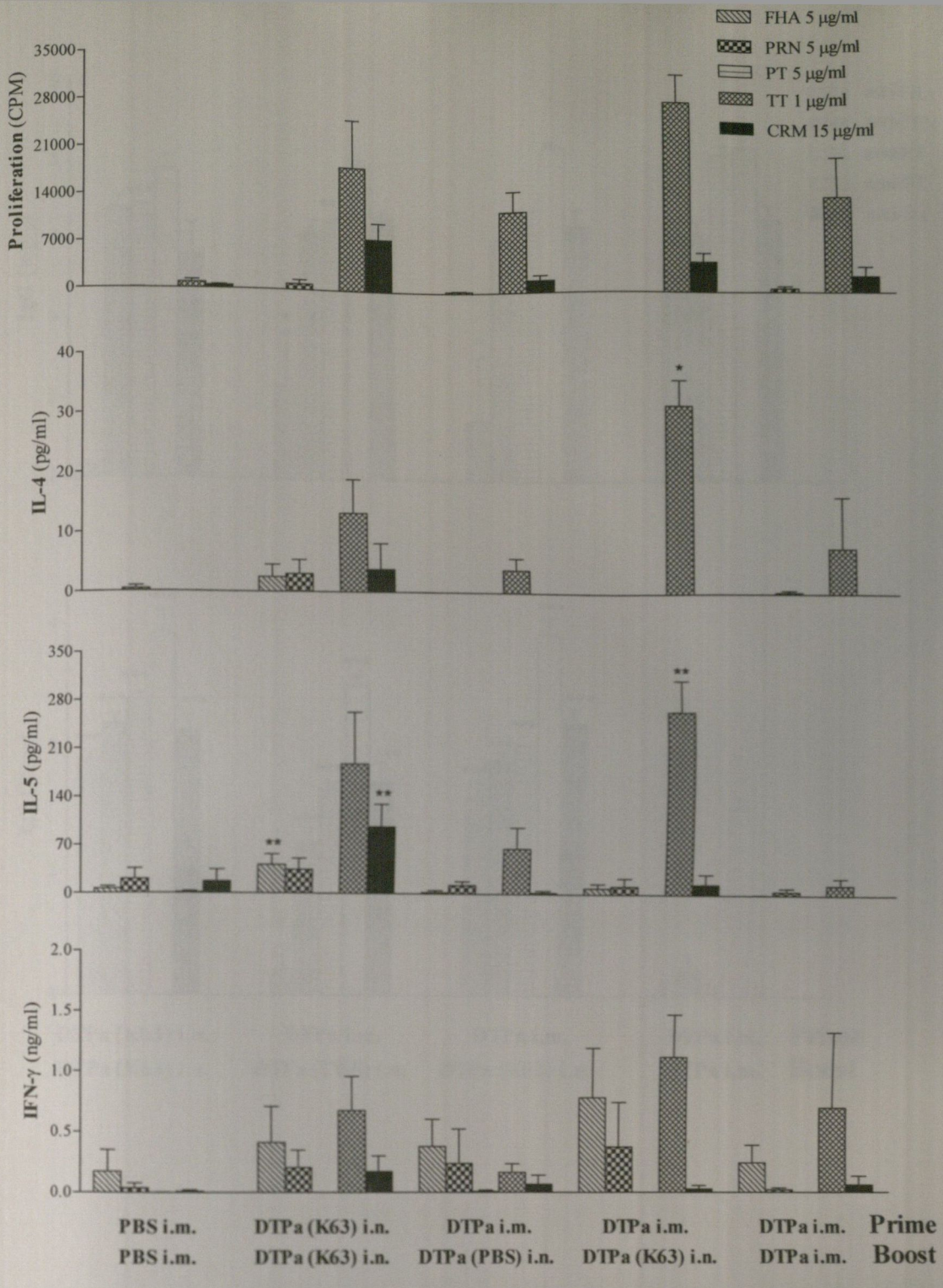


Figure 5.8. Induction of potent T-cell responses following nasal delivery of DTPa and LTK63. Mice were immunized at 0 and 4 weeks i.n. under light anesthesia with DTPa, containing rPT (5 µg), FHA (2.5 µg), PRN (2.5 µg), TT (10 µg) and CRM_{197} (10 µg), and LTK63 (10 µg) as adjuvant or with the same DTPa vaccine adsorbed to alum and administered i.m.. Two additional groups of mice were immunized i.m. with DTPa adsorbed to alum and boosted 4 weeks later with DTPa with or without LTK63 as adjuvant. Cellular responses were assessed at week 6 from spleen cells stimlated *in vitro* with FHA, PRN, PT, TT and CRM_{197}. Results are presented as mean proliferative or cytokine responses (+/- S.E.) for 5 mice per group tested individually in triplicate. * and **, $P < 0.05$ and $P < 0.01$ respectively versus two i.m. immunizations with DTPa (Tukey-Kramer test).

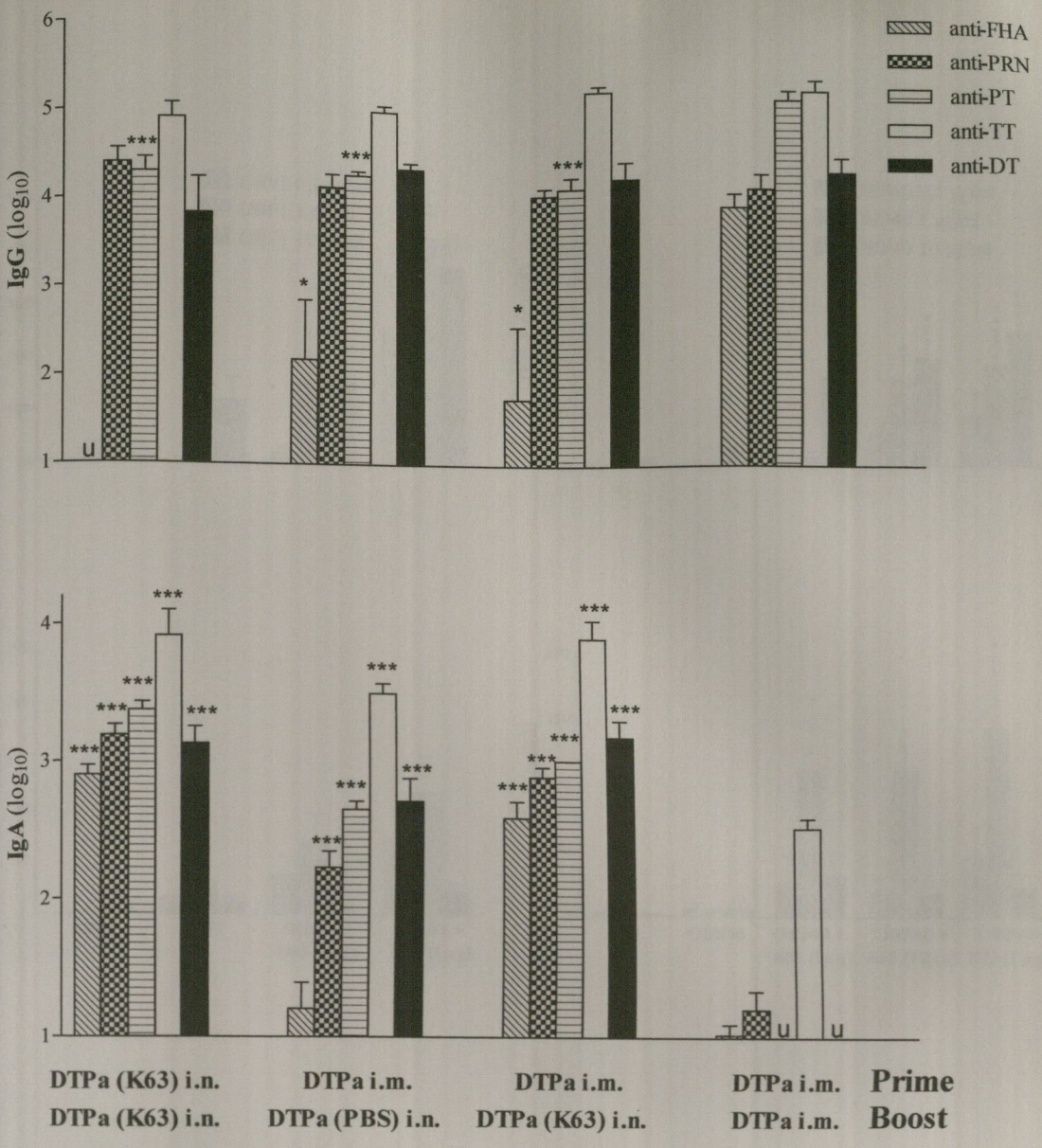


Figure 5.9. Induction of potent IgG and IgA antibody responses against diphtheria, tetanus and pertussis antigens following intranasal delivery of DTPa with LTK63. Mice were immunized as described in the legend to Fig. 5.8. Serum IgG and lung homogenate IgA responses against FHA, PRN, PT, TT and CRM_{197} were assessed by ELISA two weeks after the second immunization. Results are expressed as mean (+/- S.E.) endpoint titres for 5 mice per experimental group. * and ***, $P < 0.05$ and $P < 0.001$ respectively versus two i.m. immunizations with DTPa (Tukey-Kramer test). u = undetectable antibody titres.

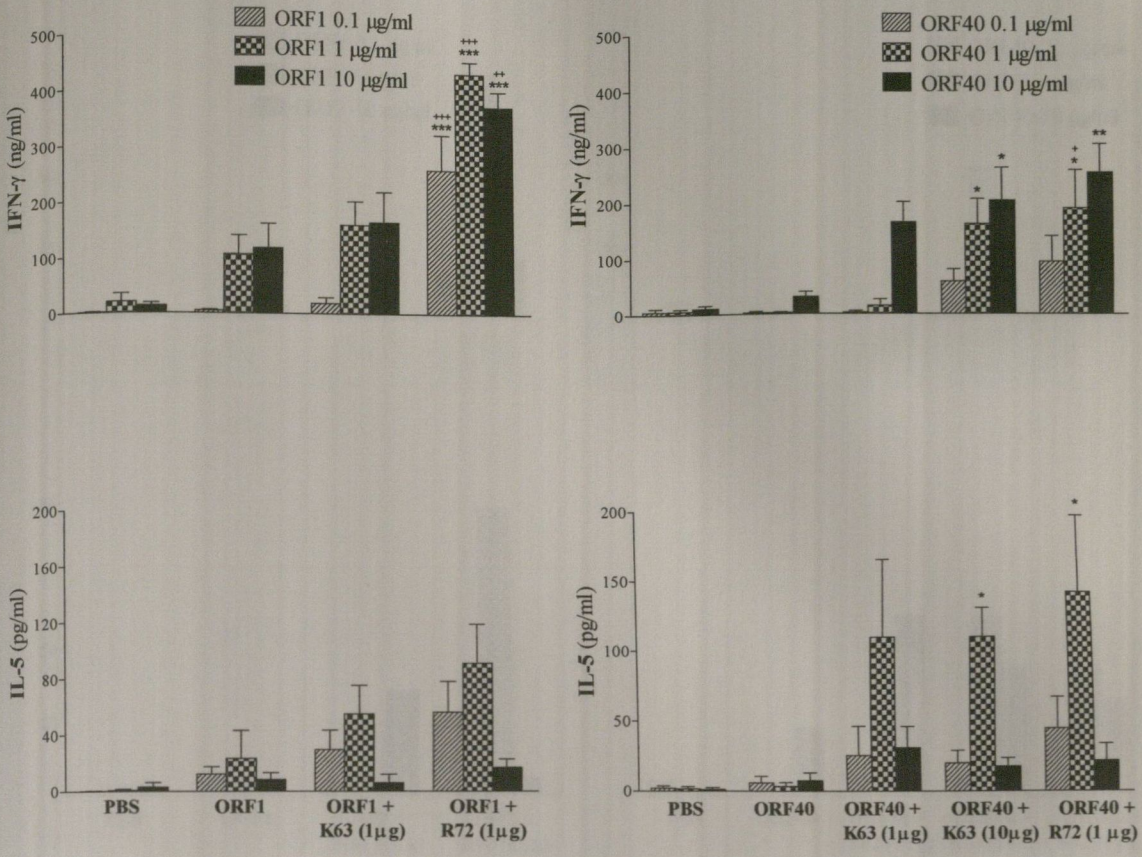


Figure 5.10. Enhancement of splenic cellular immune responses to meningococcal group B antigens following intranasal immunization with LTK63 and LTR72 as adjuvant. Mice were immunized i.n. at 0 and 4 weeks under light anesthesia with ORF1 (5 µg) or ORF40 (5 µg) alone, or with either LTK63 (1 or 10 µg) or LTR72 (1 µg) as adjuvant, or with PBS only as a control. Cellular responses were assessed at week 6 from spleen cells stimulated *in vitro* with ORF1 or ORF40 antigens (0.1-10 µg/ml). Results are expressed as mean IFN-γ or IL-5 responses (+/- S.E.) for five mice per group tested individually in triplicate. *, **, *** $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus ORF1 or ORF40 alone, +, ++, +++, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus ORF1/ORF40 and LTK63 (1 µg/dose) (Tukey-Kramer test).

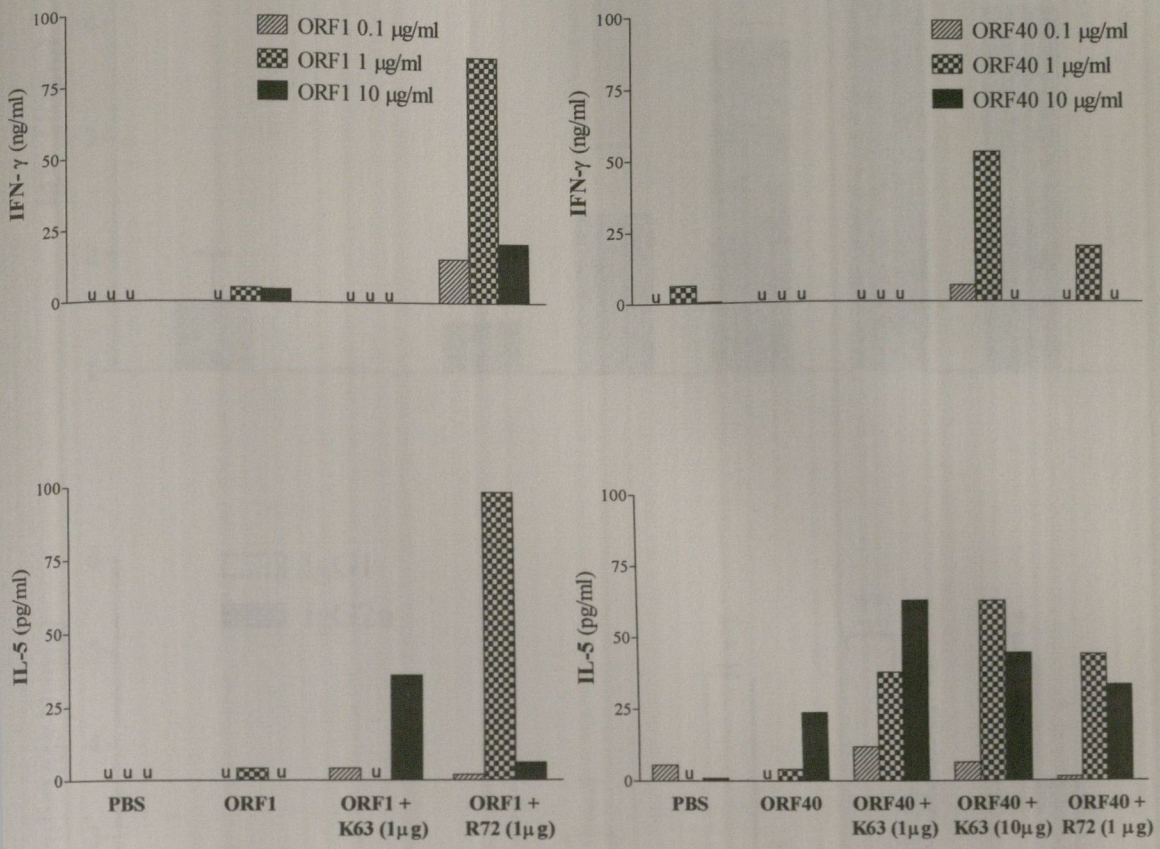


Figure 5.11. Enhancement of cervical node cellular immune responses to meningococcal group B antigens following intranasal immunization with LTK63 and LTR72 as adjuvant. Mice were immunized as described in the legend to Fig. 5.10. Two weeks after the second immunization, cells were isolated from the superior cervical nodes of immunized mice and were stimulated with ORF antigens *in vitro*. Results are presented as mean IFN-γ or IL-5 responses from triplicate cultures of pooled lymph node cells from each experimental group. u = undetectable concentrations of cytokine.

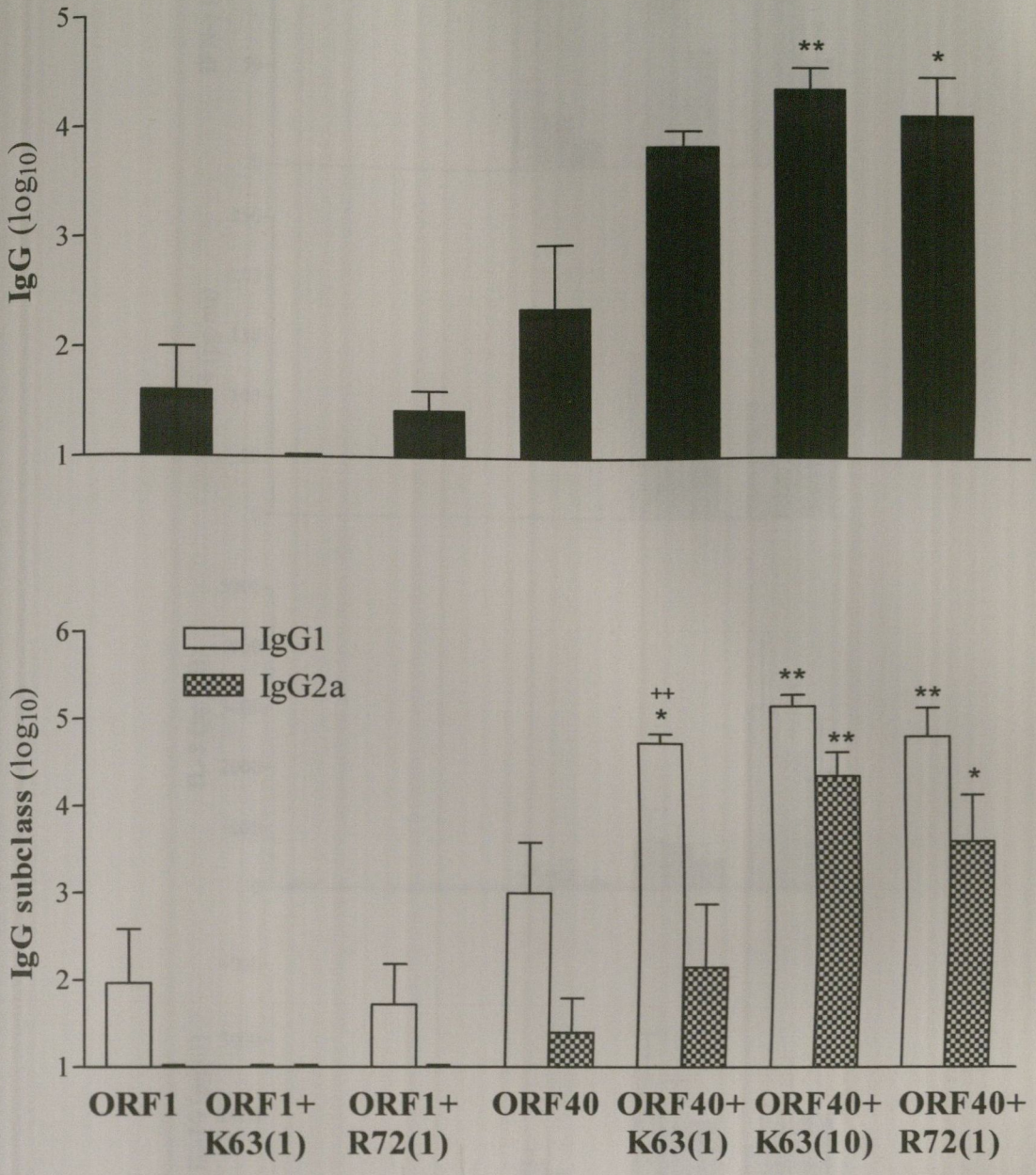


Figure 5.12. Enhancement of antibody responses following intranasal immunization with ORF40 and LTK63 or LTR72 as adjuvant. Mice were immunized as described in the legend to Fig. 5.10. Anti-ORF1/ORF40 total IgG, IgG1 and IgG2a titres were determined by ELISA on serum samples taken two weeks after the second immunization. Results are presented as mean antibody end-point titres (+/- S.E.) for five mice per experimental group tested individually in triplicate. * and **, $P < 0.05$ and $P < 0.01$ respectively versus ORF40 alone, ++, $P < 0.01$ versus IgG2a (Tukey-Kramer test).

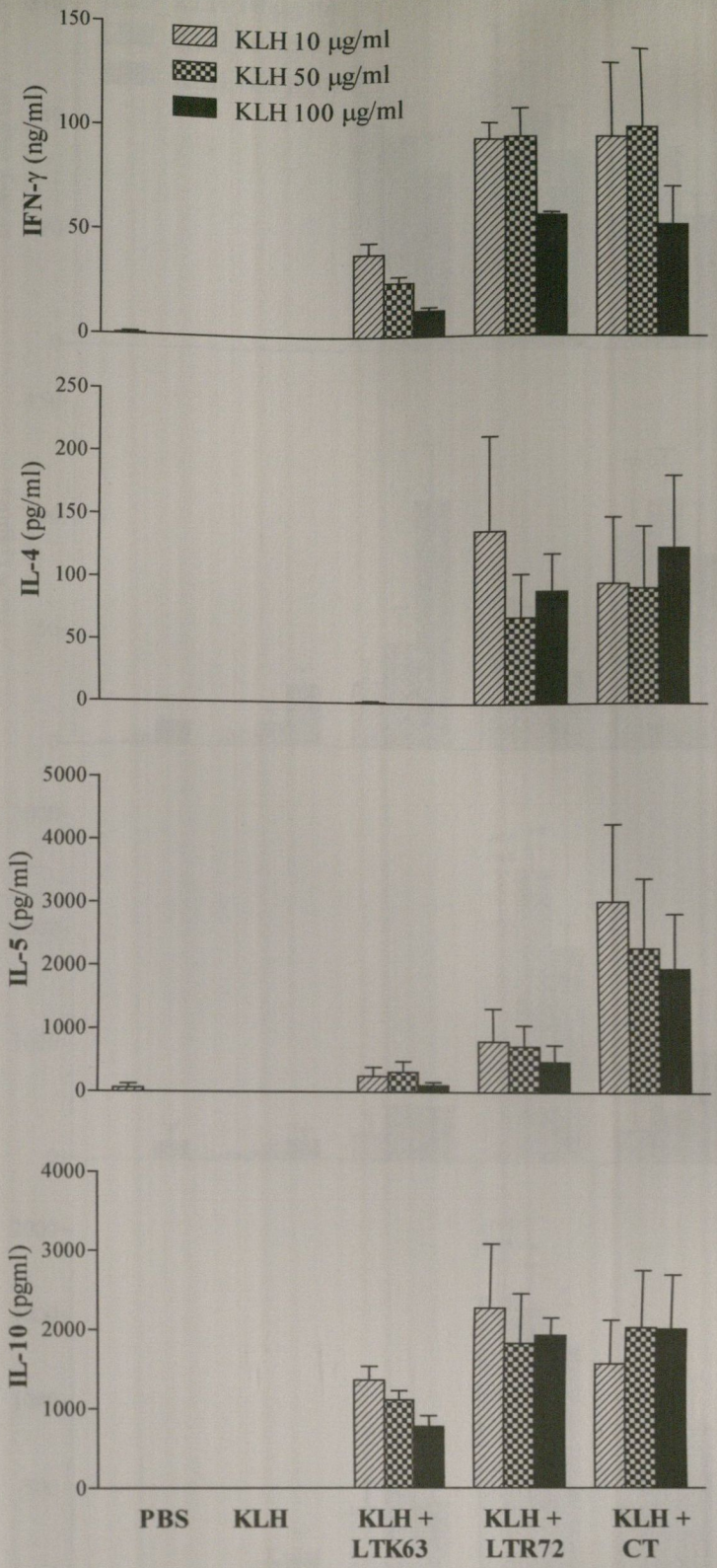


Figure 5.13. Effect of LTK63 and LTR72 on the induction of popliteal node T cell responses to injected KLH *in vivo*. Mice were immunized into each hind footpad with 25 μl of either PBS, KLH (5 μg) alone or formulated with LTK63 (10 μg), LTR72 (10 μg) or CT (10 μg) as adjuvant. Popliteal lymph node cells were isolated 7 days later and stimulated *in vitro* with KLH (10-100 μg/ml). IFN-γ, IL-4, IL-5 and IL-10 concentrations were measured by ELISA on supernatants removed after 72 hours. Results are presented as mean cytokine concentrations (+/- S.E.) for 4 mice per group tested individually in triplicate.

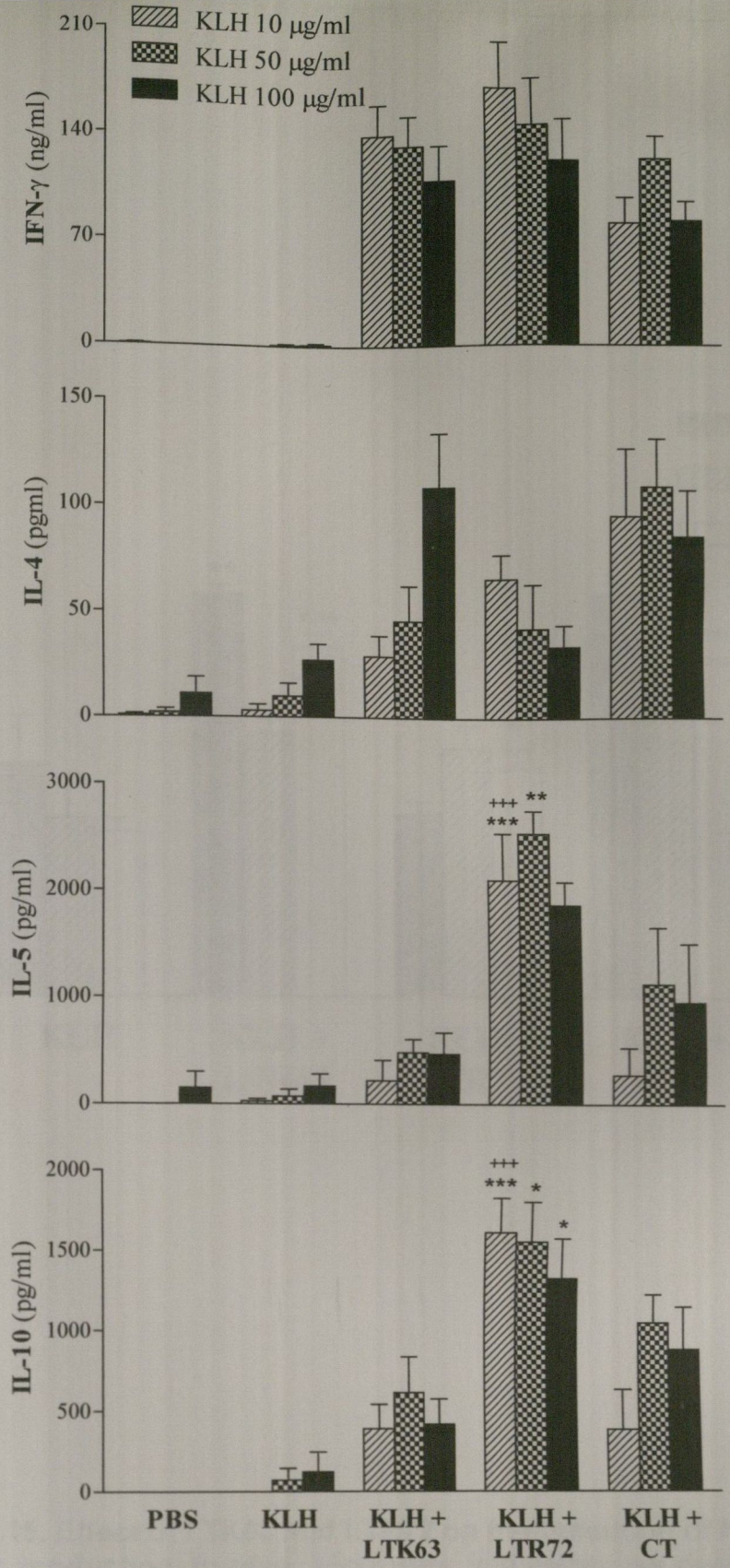


Figure 5.14. Effect of LTK63 and LTR72 on the induction of splenic T cell responses to injected KLH *in vivo*. Mice were immunized as described in the legend to Fig. 5.13. Cytokine concentrations were assessed as described in the legend to Fig. 5.13 from spleen cells stimulated *in vitro* with KLH (10-100 µg/ml). Results are presented as mean cytokine concentrations (+/- S.E.) for four mice per experimental group tested individually in triplicate. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus KLH + LTK63, +++, $P < 0.001$ versus KLH + CT (Tukey-Kramer test).

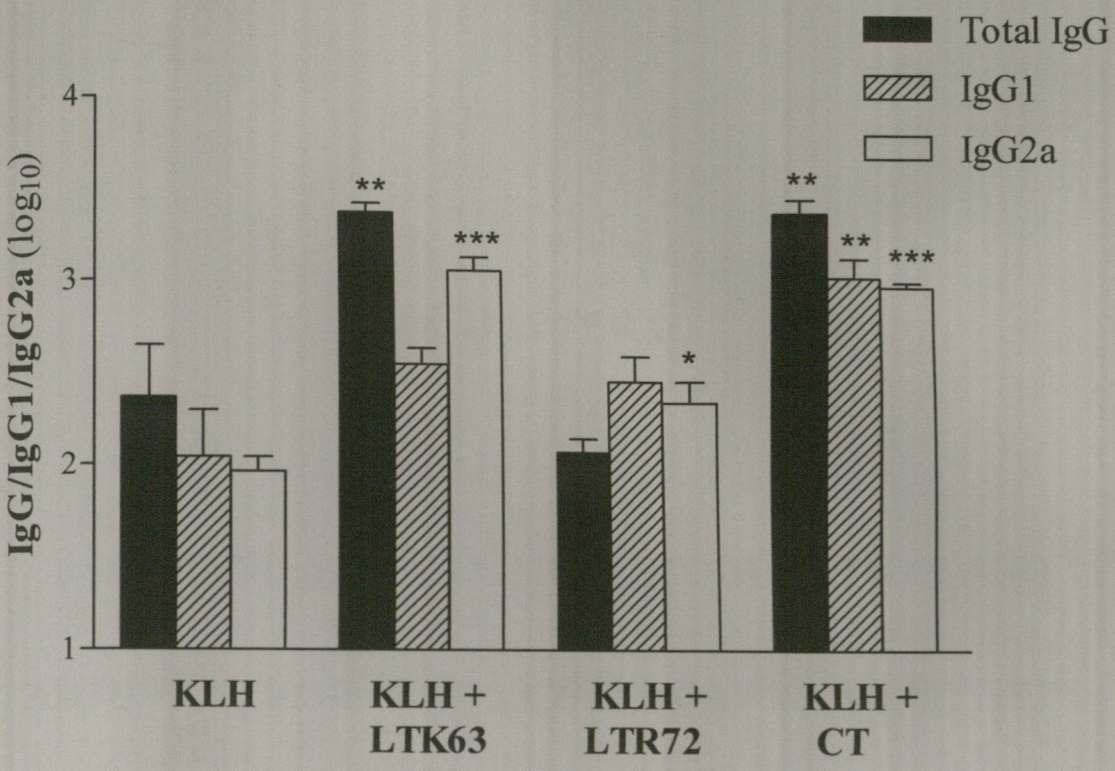


Figure 5.15. Effect of LTK63 and LTR72 on the induction of KLH-specific antibody production *in vivo*. Mice were immunized as described in the legend to figure 5.13. Total IgG, IgG1 and IgG2a levels were determined by ELISA on serum samples taken two weeks after the second immunization. Results are presented as mean antibody endpoint titres (+/- S.E.) for four mice per group. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus KLH alone (Tukey-Kramer test).

CHAPTER 6

MECHANISMS OF IMMUNOMODULATION AND ADJUVANTICITY OF *E. COLI* HEAT LABILE TOXIN

6.1 INTRODUCTION

The heat labile toxin of *Escherichia coli* (LT) and cholera toxin (CT) are highly immunogenic molecules and possess powerful mucosal adjuvanticity for the delivery of a broad range of antigens. In the previous chapter it was shown that LT and the partially toxic mutant, LTR72, or the completely non-toxic mutant, LTK63, of LT retained adjuvant activity for the intranasal delivery of several antigens, including CRM_{197} and meningococcal B antigens. However, LTR72 was generally a stronger adjuvant than LTK63 for the induction of antigen-specific cellular and antibody-mediated immunity, especially when lower doses of the toxins were employed. In addition, LTR72 and LTK63 were shown to differentially regulate the induction of Th1 and Th2 type responses, especially at a lower dose of 1 μg/ml, although the types of immune responses generated were influenced by the nature of the co-administered antigen and route of delivery. The present study was designed to investigate some of the properties of CT, LT and LT mutants on immune cells *in vitro*, which may partially explain the potent adjuvanticity of these molecules *in vivo*. In addition, the immunomodulatory properties of CT, LT, LTR72 and LTK63 on LPS-induced cytokine and chemokine production by murine dendritic cells and macrophages *in vitro* were examined, which may be associated with the ability of these molecules to differentially regulate Th1 and Th2 type responses *in vivo*.

In many studies CT has been shown to induce a Th2 type response to both itself and co-delivered antigen, resulting in an increase in the secretion of IL-4, IL-5 and IL-10 cytokines, production of IgG predominately of the IgG1 subclass and induction of antigen-specific IgE responses (Xu-Amano *et al.*, 1993, Marinaro *et al.*, 1995, Yamamoto *et al.*, 1997a, b). Furthermore, CT-induced responses are markedly reduced in IL-$4^{-/-}$ mice (Vajdy *et al.*, 1995). LT, on the other hand, has been shown to induce more mixed Th1/Th2 type responses following mucosal delivery (Takahashi *et al.*, 1996, Cheng *et al.*, 1999, Douce *et al.*, 1999). It has been demonstrated that the partially toxic mutant, LTR72, selectively enhances antigen-specific Th2 type responses at lower doses but both LTR72 and LTK63, which is completely devoid of enzymatic activity, augment Th1 and Th2 type responses at higher doses of the toxins following intranasal delivery with CRM_{197} or pertussis antigens (Chapter 5, Ryan *et al.*, 1999, Ryan *et al.*, 2000). However, the type of T cell response induced using mutants of LT as adjuvants was also shown to depend on the nature of the co-administered antigen, as intranasal delivery of a lower dose of LTR72 (1 μg) with a

meningococcal type B antigen selectively augmented systemic Th1 type responses (Chapter 5).

CT and LT are composed of two subunits: a monomeric A subunit with ADP-ribosyltransferase activity and a pentameric B subunit that mediates toxin binding with high affinity to the GM-1 ganglioside receptor, expressed ubiquitously on the surface of eukaryotic cells. Once the toxin is internalised by the cell, the A subunit ADP-ribosylates the α subunit of Gs protein, a GTP-binding protein which regulates the activity of adenylate cyclase. ADP-ribosylation of this Gs protein results in the permanent activation of adenylate cyclase and intracellular accumulation of cAMP (Holmgren, 1981, Field *et al.*, 1989, Gill and Woolkalis, 1991). cAMP is a potent immunomodulator and may strongly inhibit the production of pro-inflammatory cytokines such as IL-12 *in vivo* (Zidek, 1999). However, LT may also induce the activation of NF-κB (Ryan *et al.*, 2000) (Bone *et al.*, 2000), possibly through binding of the B subunit to GM1 or alternatively through binding of the toxins to a pathogen recognition receptor, such as a Toll-like receptor, expressed on mammalian cells. Activation of NF-κB results in the transcription of target genes including those encoding pro-inflammatory cytokines and chemokines and adhesion molecules (Baldwin, 1996, Ghosh *et al.*, 1998). It is therefore possible that the adjuvant activity of CT, LT and LT mutants on T cell responses may be due to a balance in signalling by cAMP and NF-κB in cells of the immune system.

Potentiation of APC function may partially explain the potent immunogenicity and adjuvanticity of CT, LT and mutant toxins. It has been demonstrated that CT and LT enhance antigen-presentation by macrophages to T cells *in vitro*, but only after the generation of MHC Class II-peptide complexes (Matousek *et al.*, 1996, Matousek *et al.*, 1998). Furthermore, CT has been shown to induce IL-1 secretion by macrophages *in vitro* (Bromander *et al.*, 1991) and CT or LT up-regulate the expression of the co-stimulatory molecules, CD80 and/or CD86, on murine or human macrophages *in vitro* (Cong *et al.*, 1997, Ryan *et al.*, 2000, Yamamoto *et al.*, 2000, Yamamoto *et al.*, 2001). Dendritic cells (DC) are the most potent types of APC specialized in antigen capture and T cell priming and are present in several tissues including mucosal epithelia (Iwasaki and Kelsall, 1999). CT and LT once they have crossed the mucosal barrier may therefore come into direct contact with immature DC and any interactions with these cells may significantly influence the types of immune responses generated.

Immature DC reside in unperturbed non-lymphoid organs where they specialize in the uptake and processing of foreign antigen. However, in response to certain stimuli, such as LPS or IL-1 and TNF-α, DC lose the ability to acquire and process antigen and become professional APC due to enhanced surface expression of molecules such as B7.1, B7.2, MHC class II and adhesion molecules (Banchereau *et al.*, 2000). Furthermore, this transformation or maturation is accompanied by a down-regulation in the expression of inflammatory chemokine receptors, such as CCR5 and CCR6, and an upregulation of the expression of constitutive chemokine receptors, such as CCR7 and CXCR4. This enables homeostatic chemokines such as CCL19 and CCL21 to recruit mature DC to the draining lymph nodes where they may prime naïve T cells and drive their polarization towards a particular Th subtype (Dieu *et al.*, 1998, Sallusto *et al.*, 1998a, Sozzani *et al.*, 1998, Sozzani *et al.*, 2000). CT, either alone or in combination with TNF-α and IL-1β, has been shown to induce the phenotypic and functional maturation of human DC and these CT-matured DC drive the polarization of naïve T cells towards a Th2 phenotype *in vitro* (Gagliardi *et al.*, 2000, de Jong *et al.*, 2002). However, the effects of LT and mutants of the toxin, such as LTK63 and LTR72, on DC have not been investigated. Therefore, one of the aims of this study was to investigate the effects of both CT and LT on DC maturation and cytokine or chemokine secretion by murine DC. Furthermore, the enzymatically active mutant, LTR72, and the non-toxic mutant, LTK63, provided powerful tools with which to investigate the roles of the enzyme activity and the non-toxic AB complex of LT on DC maturation and immunomodulation *in vitro*.

6.2 RESULTS

6.2.1 Effect of LT and LT mutants on cytokine and chemokine production by BMDC *in vitro*

LT and LT mutants were examined for their ability to stimulate inflammatory cytokine and chemokine production from murine BMDC *in vitro*. However, it is possible that LT, LTR72 and LTK63 may contain small quantities of contaminating LPS, which may also be capable of inducing inflammatory responses by immune cells *in vitro*. The levels of endotoxin contamination in these toxins were therefore determined by a specific *Limulus amebocyte* lysate (LAL) test. It was found that LTK63, LTR72 and LT preparations contained 74, 25 and 5 EU of endotoxin per microgram of protein respectively. Although these levels of LPS contamination are low, they may nonetheless be sufficient to induce the production of cytokines and chemokines by BMDC *in vitro*. Immature BMDC from both C3H/HeN and C3H/HeJ strains of mice were therefore stimulated *in vitro* with LTK63 (1 or 10 μg/ml), LTR72 (1 or 10 μg/ml) or LT (1 μg/ml), with medium only as a negative control or with LPS and IFN-γ or CpG as positive controls. C3H/HeJ mice are characterised by a point mutation within the signalling domain of TLR4, the receptor to which LPS binds and initiates a signalling cascade. Therefore, C3H/HeJ mice, unlike the C3H/HeN strain, display a hyporesponsiveness to LPS and macrophages from these mice are unable to induce inflammatory cytokines, including TNF-α, IL-1 and IL-6, in response to LPS stimulation *in vitro* (Morrison and Ryan, 1979). BMDC from both C3H/HeN and C3H/HeJ mice were therefore employed in this study to discriminate between toxin-induced and contaminating LPS-induced cytokine or chemokine responses.

LTK63, at a dose of either 1 or 10 μg/ml, induced significant production of IL-12p70 and TNF-α by BMDC isolated from C3H/HeN mice. However, stimulation of BMDC from C3H/HeJ mice with the same mutant toxin failed to result in significant IL-12p70 production and TNF-α was only induced at the higher dose of LTK63 (10 μg/ml). In addition, LTK63 stimulated significant secretion of IL-12p40 and IL-10 at a dose of 10 μg/ml but failed to induce the production of these cytokines by DC from the LPS-hyporesponsive strain. LTR72 stimulated significant production of TNF-α and IL-12p40 at the higher concentration of toxin (10 μg/ml). Low levels of IL-12p40 and TNF-α, but nonetheless significant, were also produced following stimulation of BMDC from C3H/HeJ mice with 10 μg/ml of LTR72 (Fig. 6.1).

Analysis of chemokine production by the same cells revealed significant production of MIP-1α, MIP-1β and RANTES by DC from C3H/HeN mice stimulated *in vitro* with either LTK63 (1 or 10 μg/ml) or LTR72 (10 μg/ml). However, both mutant toxins failed to induce significant levels of the same chemokines by DC from the LPS-tolerant strain. LT, at a dose of 1 μg/ml, induced significant secretion of RANTES by BMDC from C3H/HeN, but not C3H/HeJ, mice. Interestingly, all of the toxins tested stimulated significant levels of MIP-2 production by DC isolated from either C3H/HeN or C3H/HeJ mice (Fig. 6.2).

6.2.2 CT, LT and LT mutants up-regulate membrane expression of co-stimulatory molecules on BMDC

To investigate whether CT, LT and LT mutants induce maturation of murine DC, bone marrow-derived DC were cultured for 24 hours with the toxins and then analysed for the surface expression of the co-stimulatory molecules, CD80 and CD86, by FACS. LPS was included in the study as a positive control for DC maturation. It was found that CT, LT, LTR72 and to a lesser extent, LTK63, induced the up-regulation of both CD80 (B7.1) and CD86 (B7.2) on the surface of BMDC after 24 hours of stimulation (Fig. 6.3).

6.2.3 CT, LT and LT mutants down-regulate expression of the chemokine receptor, CCR5, on BMDC

The chemokine receptor CCR5 binds inflammatory chemokines and generally maturation of DC is accompanied by a decrease in the surface expression of this receptor. Therefore, in order to evaluate further the effects of the bacterial toxins on DC maturation, CCR5 expression on toxin-treated BMDC was analysed by FACS. CT, LT, LTR72, LTK63 and LPS all down-regulated the expression of CCR5 on BMDC from C3H/HeN mice following 24 hours of stimulation. However, minute amounts of contaminating LPS may also induce phenotypic maturation of DC and therefore the study was repeated using BMDCs from C3H/HeJ mice. Down-regulation of CCR5 expression was observed when BMDCs from C3H/HeJ mice were stimulated for 24 hours with CT, LT and LT mutants, but not LPS, indicating that the ability of these bacterial toxins to induce DC maturation was not due to contaminating endotoxin (Fig. 6.4).

6.2.4 Immunomodulation by CT, LT and LTR72 on LPS-induced pro-inflammatory cytokines and chemokines

The modulatory effects of CT, LT and LT mutants on pro-inflammatory responses were examined by assessing their effects on LPS-induced cytokine and chemokine production by BMDC or J774 macrophages *in vitro*. CT (10 μg/ml) significantly decreased IL-12p70, TNF-α, IL-10 and MIP-1α production by LPS-stimulated BMDC *in vitro*. However, the toxin had no suppressive effect on LPS-induced IL-6 and MIP-2 secretion by the same cells, but in contrast high levels of MIP-2 were secreted by BMDC stimulated with CT alone (Fig. 6.5). LT and LTR72 also inhibited pro-inflammatory cytokine and/or chemokine secretion by LPS-stimulated J774 macrophages (Fig. 6.6) or murine BMDC (Fig. 6.7) *in vitro*. LPS induced potent production of MIP-1α and MIP-1β by J774 macrophages *in vitro*. Addition of 1 μg/ml of LTK63 did down-regulate LPS-stimulated MIP-α production, but not to the same extent as LTR72 and LT which completely blocked LPS-induced MIP-1α production. In addition, LTR72 and LT (0.1 or 1 μg/ml) significantly decreased MIP-1β production by J774 macrophages in response to LPS. In contrast, LTK63 (1 μg/ml) and LTR72 (0.1 or 1 μg/ml) significantly enhanced LPS-induced MIP-2 production by these murine macrophage cell lines *in vitro* (Fig. 6.6). LT and LTR72 significantly suppressed LPS-induced IL-12p70, TNF-α and MIP-1α production by BMDC *in vitro* but had no effect on LPS-stimulated IL-10, IL-6 and MIP-2 secretion. LTK63 also down-regulated TNF-α production but this inhibition was not as significant as that observed by LT or LTR72 (Fig. 6.7).

6.2.5 CT, LT and LTR72 suppress LPS-induced CD40 expression on BMDC

It has been demonstrated that CD40-CD40L signalling may be important for the development and enhancement of Th1 type responses (Campbell *et al.*, 1996, Kamanaka *et al.*, 1996). Furthermore, DC maturation may be strengthened during interactions with T cells via CD40-CD40L interactions (Caux *et al.*, 1994, Kitajima *et al.*, 1996). Therefore, the effects of CT, LT, LTR72 and LTK63 on CD40 expression and LPS-induced expression of this surface marker by BMDC were evaluated. None of the toxins were capable of enhancing CD40 expression by BMDC following 24 hours of stimulation (Lavelle and Mills, unpublished observations). However, it was found that CT, LT and LTR72 significantly down-regulated LPS-stimulated CD40

expression by murine BMDC. In contrast, the enzymatically inactive mutant, LTK63, had little or no effect on LPS-induced CD40 expression (Fig. 6.8).

6.2.6 Effect of adoptive transfer of CT-stimulated DC into naïve mice on the induction of immune responses *in vivo*

It has previously been shown that CT may induce phenotypic and functional maturation of DC and enable the DC to induce Th2 polarization of naïve T cells *in vitro* (Gagliardi *et al.*, 2000). However, the ability of these DC to induce similar types of responses *in vivo* has not been assessed. Therefore, murine BMDC were incubated overnight with KLH with or without CT or CpG and adoptively transferred into naïve mice. These mice were then given soluble KLH antigen 7 days later and cytokine responses were assessed from spleen cells stimulated *in vitro* with KLH. CpG was included in the study as a control, as it has been shown to induce the potent production of Th1-associated cytokines, including IL-12 and IFN-γ (McCluskie *et al.*, 2001a, Davis *et al.*, 1998). It was found that spleen cells from mice that received DC stimulated with medium alone only produced weak or moderate cytokine responses following antigen stimulation *in vitro*. In contrast, spleen cells from mice that were injected with KLH-treated DC followed by soluble KLH 7 days later produced significantly higher levels of antigen-specific cytokines. Interestingly, treatment of DC with CT in combination with KLH did not induce any additional enhancement of antigen-specific cytokine responses, compared to DC treated with KLH alone. In contrast, these DC induced slightly lower levels of antigen-specific IFN-γ and IL-4 compared to DC stimulated with KLH without CT. Furthermore, spleen cells from mice that received DC treated with KLH and CpG produced significantly lower levels of antigen-specific cytokines, in particular IL-5 and IL-10, compared to mice that were injected with DC stimulated with KLH alone (Fig. 6.9). Analysis of IgG subclass production revealed that both IgG1 and IgG2a antibody titres were higher, although not significantly, from mice that received DC stimulated with KLH in combination with CT or CpG compared to mice that received DC stimulated with KLH alone (Fig. 6.10).

6.2.7 LTK63 and LTR72 enhance cell recruitment to the local lymph node following s.c. injection with KLH

It was shown in chapter 5 that s.c. injection of KLH with CT, LTK63 or LTR72 as adjuvant resulted in the induction of potent T cell and antibody responses. Transient recruitment of effector cells to the site of immunization following administration of CT or LT may partially explain the potent immunogenicity and adjuvanticity of these molecules *in vivo*. To examine this further, cell recruitment into the local lymph nodes was evaluated following s.c. injection of KLH alone or with LT, CT, LTR72 or LTK63. The local draining popliteal lymph nodes were removed 7 days after injection and the cell number assessed. A dramatic increase in cell number was observed from lymph nodes of mice injected with KLH and either LTK63 or LTR72 (10 μg/dose). In association with this increase, swelling of the footpad was observed following s.c. injection of KLH and LTR72. Interestingly even though swelling of the footpad was also noted following injection of CT or LT, this swelling did not correlate with an increase in cell number (Fig. 6.11).

6.3 Discussion

In the previous chapter it was shown that the non-toxic mutant, LTK63, and the partially toxic mutant, LTR72, of LT were effective adjuvants for the nasal delivery of diphtheria and meningococcal B antigens. This study seeks to examine some of the properties of these mutant toxins, which may contribute to their potent mucosal adjuvanticity *in vivo*. It was found that both LTK63 and LTR72 were capable of inducing the phenotypic maturation of murine bone marrow-derived DC *in vitro*. However, these mutant toxins differentially regulated LPS-induced cytokine and chemokine secretion and CD40 expression by murine DC, which may partially explain their ability to induce different types of T cell responses following mucosal delivery with antigens, such as CRM_{197} or pertussis antigens.

The ability of LT or its mutants to induce DC maturation or regulate the production of cytokines or chemokines by these cells may confer adjuvant properties to these molecules *in vivo*. However, the toxins may also contain small quantities of contaminating LPS, a molecule capable of inducing potent inflammatory responses by APC *in vitro*. Therefore, LT, LTR72 and LTK63 were examined for their ability to induce cytokine and chemokine secretion by DC from both C3H/HeN and C3H/HeJ mice. C3H/HeJ mice, unlike the C3H/HeN strain, carry a point mutation in the *tlr*4 gene locus and consequently display a hyporesponsiveness to LPS (Hoshino *et al.*, 1999). It was found that both LTK63 and LTR72, particularly at a higher dose of 10 μg/ml, induced the secretion of a variety of cytokines or chemokines by BMDC from C3H/HeN mice *in vitro*. However, incubation of the same toxins with BMDC from the LPS-hyporesponsive strain, C3H/HeJ, resulted in the secretion of substantially lower levels of cytokines or chemokines, which in many cases were not significantly different from those produced by control cells incubated with medium alone. Furthermore, wild-type LT was unable to stimulate significant production of IL-12, TNF-α, IL-10, MIP-1α, MIP-1β or RANTES by BMDC from C3H/HeJ mice. Similarly, it has been demonstrated that CT does not induce the production of these cytokines or chemokines by human DC *in vitro* (Gagliardi *et al.*, 2000). The results of the present study suggest that LTK63 and LTR72 may contain endotoxin-contamination, which warrants careful consideration in any *in vitro* studies with these toxins. It is also possible that LT signals through TLR4, which may explain the significantly lower concentrations of cytokines and chemokines secreted by BMDC from C3H/HeJ mice in response to stimulation by LT and its mutants *in vitro*.

However, in another study it was found that stimulation of BMDC from BALB/c or C3H/HeN mice with LT, LTR72 or LTK63 in combination with polymixin B, an inhibitor of LPS, resulted in the secretion of significantly lower concentrations of cytokines or chemokines compared to stimulation with LT or its mutants alone, suggesting that these toxins are indeed contaminated with LPS (Lavelle and Mills, unpublished observations). Nonetheless, in the present study it was found that all of the toxins induced significant production of MIP-2 chemokine by C3H/HeJ-derived DC *in vitro* and LTR72 at a dose of 10 μg/ml was capable of inducing small but significant levels of TNF-α and IL-12p40 by murine C3H/HeJ-derived DC *in vitro*. Furthermore, LTK63 and LTR72 were capable of augmenting LPS-induced MIP-2 production by J774 cells *in vitro*. MIP-2 is considered to be the main neutrophil attracting and activating chemokine in mice (Lee *et al.*, 1995) and may be involved in the induction of innate immune responses to the toxins.

In contrast to LT or its mutants, incubation of DC from either C3H/HeN or C3H/HeJ mice with CpG induced potent secretion of all cytokines or chemokines tested. CpG signals through TLR9 (Hemmi *et al.*, 2000) and therefore a mutation in TLR4 should not interfere with the ability of this molecule to induce inflammatory responses by DC *in vitro*. It has previously been shown that LPS-induced responses are severely impaired in C3H/HeJ-derived BMDC (Akira *et al.*, 2001). In contrast, in this study LPS (1 μg/ml) was still capable of inducing significant cytokine or chemokine production by DC from C3H/HeJ mice. It is possible that the LPS itself contained small quantities of contaminants that are recognized by TLR2 (Hirschfeld *et al.*, 2000), and may therefore be still capable of initiating inflammatory responses by DC from C3H/HeJ mice.

In this study it was found that CT, LT and the enzymatically active mutant of LT, LTR72, strongly up-regulated the surface expression of both CD80 and CD86 on murine BMDC. Similarly, it has been shown that CT enhances the expression of CD80 and CD86 on murine Flt3L-expanded DC (Williamson *et al.*, 1999) and on human DC (Gagliardi *et al.*, 2000). Furthermore, both native CT and a fusion protein with an intact A1 subunit enhanced CD80 and CD86 expression on B cells (Ågren *et al.*, 1997). In contrast, other studies have shown that CT or LT preferentially enhance CD86 or B7.2 expression. CT preferentially augmented CD86 expression by bone marrow-derived macrophages (Cong *et al.*, 1997) and incubation with both LT and CT

upregulated the surface expression of CD86 on macrophages and B cells obtained from the Peyer's patches (Yamamoto *et al.*, 2000, Yamamoto *et al.*, 2001).

It has been suggested that CT- or LT-induced up-regulation of co-stimulatory molecule expression by APC may be due to the specific ADP-ribosylase activity of the A subunit. CTB failed to enhance CD86 expression on B cells and macrophages (Yamamoto *et al.*, 1999, Yamamoto *et al.*, 2001) and had no effect on CD80 or CD86 expression by human DC (Gagliardi *et al.*, 2000). However, in this study it was found that the non-toxic mutant, LTK63, which is completely devoid of enzymatic activity, was also capable of enhancing CD80 and CD86 expression on BMDC, although not to the same extent as LT or LTR72. A CT chimeric protein that is deficient in ADP-ribosylase activity has been shown to significantly enhance CD86 expression on B220+ cells of NALT following i.n. administration (Martin *et al.*, 2001a). Furthermore, pre-treatment of CT with GM1 blocked CD86 expression (Yamamoto *et al.*, 1999), indicating that binding of the B subunit to APC via a specific receptor, in addition to the enzymatic activity of the A subunit, may be important for the enhancement of co-stimulatory molecule expression by these toxins.

The ability of CT, LT and mutants of LT to up-regulate CD80 or CD86 expression on APC and the resulting enhancement in co-stimulatory activity of these cells may be important for the adjuvanticity of these toxins *in vivo*. Both CT- and LT-treated APC enhance proliferative responses in anti-CD3 stimulated T cells. However, these responses may be suppressed by the addition of anti-CD86 monoclonal antibody, indicating that the enhancement of CD86 expression by CT or LT may play a critical role in the augmentation of CD4+ T cell responses *in vivo* (Cong *et al.*, 1997, Yamamoto *et al.*, 1999, Yamamoto *et al.*, 2000). Furthermore, administration of anti-CD86 monoclonal antibody has been shown to suppress antigen-specific serum and mucosal antibody responses in mice immunized orally with KLH and CT (Cong *et al.*, 1997). Initially it was thought that CD80 and CD86 differentially enhanced Th1 and Th2 type responses, as antibodies against CD80 or CD86 have been shown to selectively inhibit the development of Th1 or Th2 type responses respectively (Freeman *et al.*, 1995, Kuchroo *et al.*, 1995). Consequently, it was hypothesised that the ability of CT to selectively up-regulate CD86, as has been shown in several studies, may be associated with the selective enhancement of Th2 type responses by this toxin *in vivo* (Cong *et al.*, 1997, Yamamoto *et al.*, 1999). However, it would now appear that CD80 and CD86 both contribute to the magnitude of T cell activation, rather than the

selective augmentation of Th1 or Th2 type responses (Elloso and Scott, 1999). The ability of CT, LT and LT mutants to up-regulate both CD80 and CD86 may therefore be partly responsible for the potent enhancement of T cell responses, observed following intranasal immunization of these toxins with a range of antigens (Chapter 5), (Ryan *et al.*, 1999, Yamamoto *et al.*, 1997a).

In addition to enhancing CD80 and CD86 expression by BMDC, CT, LT and LT mutants also down-regulated expression of the inflammatory chemokine receptor, CCR5, by these cells, thereby confirming their ability to induce DC maturation *in vitro*. Furthermore, it was shown that down-regulation of CCR5 expression was not due to endotoxin contamination, as expression of this surface marker was also down-regulated by BMDC from C3H/HeJ mice stimulated with CT, LT and mutant toxins. In agreement with the present study, it has been demonstrated that CT is capable of inducing the phenotypic maturation of human DC, as assessed by enhancement of MHC expression and co-stimulatory molecule upregulation. However, human DC stimulated with CT failed to down-regulate the expression of CCR1 and CCR5 at early time points after stimulation, and any decrease in CCR5 or CCR6 expression at later time points was ascribed to the maturation program activated in DC by that time (Gagliardi *et al.*, 2000).

Having established that CT, LT and mutants of LT may induce DC maturation *in vitro*, the ability of CT-stimulated DC to regulate the differentiation of T cell responses *in vivo* was established by the adoptive transfer of KLH and CT-treated DC into naïve mice. Previously, it has been shown that human DC2, matured either by stimulation with CT alone or CT in combination with TNF-α and IL-1β, may drive the polarization of naïve T cells towards a Th2 phenotype *in vitro* (Gagliardi *et al.*, 2000, de Jong *et al.*, 2002). However, the effects of these cells on T cell differentiation *in vivo* were not determined. In the present study it was found that spleen cells from mice that received KLH and CT-stimulated DC induced similar or lower levels of antigen-specific cytokine responses, compared to mice that received DC stimulated with KLH alone. It is possible that an enhancement or polarization of T cell subtype responses may have been observed following adoptive transfer of CT-stimulated DC if the immune responses were assessed at an earlier time point or if lower quantities of soluble KLH were administered following the adoptive transfer of DC.

It has been shown that CT and LT possess potent anti-inflammatory properties *in vitro*. CT has been shown to inhibit the secretion of IL-12 and TNF-α by human

monocytes and DC in response to a variety of stimuli (Braun *et al.*, 1999, Gagliardi *et al.*, 2000). In addition, CT suppressed the production of inflammatory chemokines, such as MIP-1α, MIP-1β and RANTES by LPS- or CD40L-stimulated human DC (Gagliardi *et al.*, 2000). In agreement with these findings, in the present study it was found that CT down-regulated the production of IL-12p70, TNF-α and MIP-1α by LPS-stimulated murine BMDC. LT and the enzymatically active mutant, LTR72, also significantly inhibited MIP-1α and MIP-1β secretion by LPS-stimulated J774 macrophages and IL-12p70, TNF-α and MIP-1α production by murine BMDC in response to LPS stimulation *in vitro*. Similarly, LT has been shown to suppress IL-12p70 production by human monocytes in response to stimuli (Braun *et al.*, 1999) and both LT and the enzymatically active mutant, LTR72, inhibited the production of IL-12 and TNF-α in LPS-stimulated murine macrophages (Ryan *et al.*, 2000).

In contrast to LTR72 or LT, the enzymatically inactive mutant, LTK63, had only minimal effect on LPS-induced pro-inflammatory cytokine or chemokine secretion by murine BMDC or J774 macrophages. It has previously been shown that purified CTB and LTK63, which are both devoid of enzymatic activity, have little or no effect on IL-12p70 production by mature human DC (Braun *et al.*, 1999) or on pro-inflammatory cytokine production by LPS-stimulated murine macrophages (Ryan *et al.*, 2000) respectively. In addition, forskolin, an agent that enhances intracellular cAMP levels, is capable of inhibiting pro-inflammatory cytokine and chemokine secretion by mature DC (Gagliardi *et al.*, 2000). Other factors that increase cAMP levels such as β2-agonists (Panina-Bordignon *et al.*, 1997) or PGE_2 (Kalinski *et al.*, 1997) have also been shown to inhibit IL-12 production by human DC. These findings suggest that the ability of CT, LT and LTR72 to inhibit LPS-induced pro-inflammatory cytokine or chemokine production may be ascribed to the enzymatic ADP-ribosyltransferase activity of the A subunit. However, more recently CTB has been found to suppress the production of LPS-induced TNF-α and IL-12p70 by macrophage cell lines and human PBMC *in vitro* (Burkart *et al.*, 2002), indicting that cAMP may not be the only factor involved in the suppression of inflammatory responses by CT and LT toxins.

In the previous chapter it was found that low doses of LTR72 selectively enhanced systemic Th2 type responses to intranasally delivered CRM_{197}, whereas, higher doses of LTK63 or LTR72 augmented both Th1 and Th2 type responses.

Similar effects of these mutant toxins on the polarization of T cell responses have been observed following intranasal delivery with acellular pertussis vaccines (Ryan *et al.*, 1999). Furthermore, in several studies CT has been shown to induce predominant Th2 type responses to co-administered antigen (Xu-Amano *et al.*, 1993, Marinaro *et al.*, 1995). It is therefore possible that the augmentation of Th2 type responses by these molecules may be due in part to their ability to inhibit LPS-induced inflammatory responses or to other events mediated by the cAMP activity of the A subunit. CT inhibited the production of IFN-γ by anti-CD3 stimulated human PBMC, an effect mediated in part by the ability of the toxin to suppress LPS-induced IL-12 production by APC, but also due to CT-mediated inhibition of the β1 and β2 chains of the IL-12 receptor on activated T cells (Braun *et al.*, 1999). Elevation of cAMP has been shown to be associated with the inhibition of Th1 cytokine production but augmentation of IL-5 gene expression in Th2 cells (Gajewski *et al.*, 1990, Munoz *et al.*, 1990, Betz and Fox, 1991, Zidek, 1999). Classically, cAMP exerts its effects by activating the cAMP-dependent protein kinase A (PKA), which subsequently phosphorylates downstream effector proteins such as CREB (Daniel *et al.*, 1998, Sassone-Corsi, 1998). It has been suggested that the PKA pathway regulates cAMP-induced IL-5 promoter activation in the thymoma cell line EL-4 (Lee *et al.*, 1993). However, cAMP may also induce IL-5 gene expression in untransformed Th2 cells in a PKA-independent fashion (Staples *et al.*, 2001). The transcription factor GATA-3 is reportedly important for Th2 cell differentiation and has been shown to directly interact with a critical regulatory element of the IL-5 gene promoter, thereby regulating Th2-specific IL-5 gene expression (Zhang *et al.*, 1997, Lee *et al.*, 1998). It has been demonstrated that GATA-3 may induce the secretion of Th2 type cytokines in committed Th1 cells and a Th1 clone. This effect may be synergistically augmented by cAMP, although the molecule on its own was unable to induce Th2 cytokine production in polarized Th2 cells (Lee *et al.*, 2000). GATA-3 is a substrate of p38 kinase and cAMP has been shown to potently activate p38 MAPK in Th2, but not Th1, cells (Chen *et al.*, 2000).

An alternative, but not mutually exclusive, explanation for the inhibition of pro-inflammatory responses by CT or LT may be provided by the ability of these toxins to induce the production of the regulatory cytokine, IL-10, *in vitro* and *in vivo*. Agents that elevate intracellular cAMP levels, such as PGE_2, inhibited TNF-α release and suppressed antigen presentation by DC *in vitro*, which was found to be associated with enhanced IL-10 production and lower MHC class II expression. Furthermore, the

inhibition of antigen presentation and MHC class II expression could be reversed by treatment of the DC with a neutralizing IL-10 antibody (Kambayashi *et al.*, 2001). LT and LTR72 have been shown to significantly enhance LPS-induced IL-10 production by murine macrophages *in vitro*, and a neutralizing antibody to IL-10 partially attenuated the inhibitory effects of these toxins on LPS-induced IL-12 production by the same cells (Ryan *et al.*, 2000). Furthermore, it has been shown that CT inhibition of IL-12 and TNF-α by LPS-stimulated macrophages was mediated by increased IL-10 production, as an anti-IL-10 monoclonal antibody abrogated the inhibition (Cong *et al.*, 2001). In the present study the enzymatically active toxins, CT, LT and LTR72 failed to induce IL-10 production by BMDC or augment LPS-induced secretion of this cytokine *in vitro*. Indeed, CT slightly inhibited IL-10 production by murine BMDC in response to LPS stimulation *in vitro*. However, the kinetics of IL-10 production may be important for its detection. More recent studies performed in the lab suggest that CT may synergise with LPS to induce high concentrations of IL-10 by murine BMDC at early time-points after stimulation (Leavy and Mills, unpublished data). In this study, IL-10 concentrations were measured after 24 hours of stimulation, which may not have been optimal for detection of this cytokine. Peak levels of IL-10 were observed in CTB-pretreated monocytes after 5 hours of LPS stimulation, but declined rapidly thereafter (Burkart *et al.*, 2002).

In addition to inhibiting LPS-induced pro-inflammatory cytokine and chemokine responses, CT, LT and LTR72 also down-regulated LPS-stimulated CD40 expression by BMDC. However, the enzymatically inactive mutant, LTK63, had little or no effect on LPS-induced CD40 expression. CT has been shown to significantly suppress the expression of CD40L on anti-CD3 stimulated $CD4^+$ T cells. In addition, CT-treated $CD4^+$ T cells only induced low levels of CD40-dependent cytokines, such as IL-12p70, IL-2 and TNF-α, in monocytes and monocyte-derived DC. However, LT-IIa and LT-IIb did not suppress CD40L expression and induced significantly greater production of IL-12p70, IL-2 and TNF-α (Martin *et al.*, 2001b).

It is possible that the ability of CT, LT and LTR72 to down-regulate LPS-induced CD40 expression by murine DC may correlate with their suppression of inflammatory responses *in vitro* and the augmentation of Th2 type responses *in vivo*. Exposure of DC to pro-inflammatory Th1-inducing molecules has been shown to enhance CD40 expression on these cells and lead to an increase in their ability to secrete IL-12 in response to CD40 engagement (Verhasselt *et al.*, 1997, Schulz *et al.*,

2000, MacDonald *et al.*, 2001). Furthermore, the ability of DC to secrete IL-12 in response to certain pathogens and subsequently during communications with T cells via CD40-CD40L interactions allows for the augmentation of the developing Th1 response (Caux *et al.*, 1994, Cella *et al.*, 1996). In agreement with this, several studies have demonstrated the importance of CD40-CD40L interactions in the development of Th1 responses during microbial infections. Mice deficient in either CD40 or CD40L are unable to generate a Th1 type response necessary to control active leishmanial infection (Campbell *et al.*, 1996, Kamanaka *et al.*, 1996), which is related to their inability to induce the production of IL-12 from macrophages (Campbell *et al.*, 1996). However, more recently it has been shown that CD40-CD40L interactions may also play an important role in the induction of Th2 type responses (MacDonald *et al.*, 2002). Furthermore, oral immunization of CD40 deficient mice with CT or LT as adjuvant failed to induce mucosal or systemic antibody responses to co-administered antigen (Yamamoto *et al.*, 2000), suggesting that although CT, LT and LTR72 may downregulate CD40 expression by LPS-stimulated DC some degree of CD40-CD40L signalling is essential for the mucosal adjuvanticity of these toxins.

It was also found in this study that s.c. injection of LTK63 or LTR72 with KLH resulted in a massive recruitment of cells to the local popliteal lymph nodes. Receptor binding by LTB *in vitro* appears to result in the expansion and activation of B cells, as assessed from lymphocyte cultures treated with LTB or the receptor-binding mutant, LTB(G33D) (Nashar *et al.*, 1996a). In addition, the ability of LTK63 and LTR72 to downregulate the expression of the inflammatory chemokine receptor, CCR5, by murine DC may enable homeostatic chemokines such as CCL19 and CCL21 to recruit mature DC to draining afferent lymphatics and subsequently to T cell areas of the lymph nodes (Dieu *et al.*, 1998, Sozzani *et al.*, 1998, Sozzani *et al.*, 2000). In contrast to LTK63 and LTR72, CT and LT did not induce cell recruitment to the local lymph node following injection with KLH. However, these toxins did induce swelling of the footpad after injection with KLH, suggesting that they also exhibit some inflammatory properties *in vivo,* which may contribute to the enhancement of immune responses following co-administration with unrelated antigens.

DC are localized in the mucosae of the respiratory and intestinal tracts where they play a pivotal role in protection against foreign antigen (Steinman, 1991, Iwasaki and Kelsall, 1999). CT, LT and LT mutants, once they cross the mucosal barrier, may come into direct contact with DC and therefore any interactions between these

molecules and DC may be of critical importance in determining the subsequent types of immune responses generated. It has been shown in this study that interaction of the toxins with immature DC may result in DC maturation, with upregulation of co-stimulatory molecule expression and downregulation of CCR5 expression, which may partly explain their potent adjuvanticity for the delivery of mucosal vaccines. Furthermore, it was shown that toxins with enzymatic activity (CT, LT, LTR72), in contrast to the enzymatically inactive molecule, LTK63, inhibited the production of pro-inflammatory cytokines and chemokines and suppressed CD40 expression by LPS-stimulated murine DC *in vitro*. These effects may partially explain the ability of CT, LT and LTR72 to induce Th2 type responses in certain studies *in vivo*. However, the types of T cell responses induced *in vivo* following administration of CT, LT, LTR72 or LTK63 may also depend on a host of other factors not considered by *in vitro* studies, including the nature and dose of the co-administered antigen, the route of immunization and interactions between other cells of the immune system.

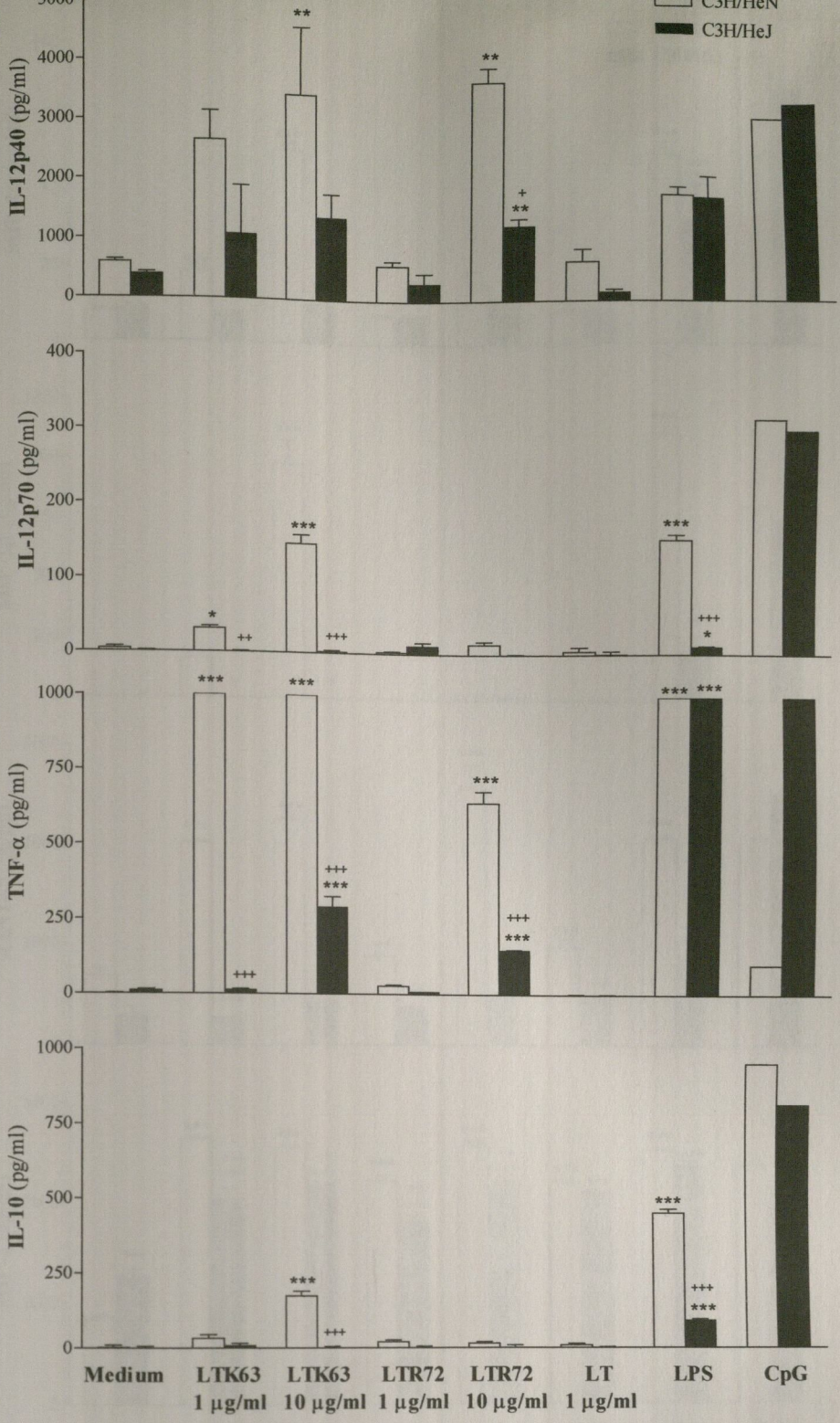


Figure 6.1. The ability of LT and LT mutants to induce cytokine secretion by BMDC is affected by TLR4 expression. Immature BMDC (1 x 10^6 cells/ml) from C3H/HeN or C3H/HeJ mice were cultured for 24 hours with either medium alone, LTK63 (1 or 10 μg/ml), LTR72 (1 or 10 μg/ml), LT (1 μg/ml), LPS (1 μg/ml) or CpG (10 μg/ml). After 24 hours incubation, the supernatants were removed and the levels of IL-12p40, IL-12p70, TNF-α and IL-10 were assessed by specific immunoassay using pairs of commercially available antibodies. The upper limit of the TNF-α immunoassay was 1000 pg/ml. Results are expressed as mean (+/- S.E.) cytokine concentrations for triplicate cultures. *, **, ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus unstimulated cells (Tukey-Kramer test). +, ++ and +++, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus cells from C3H/HeN mice (Tukey-Kramer test).

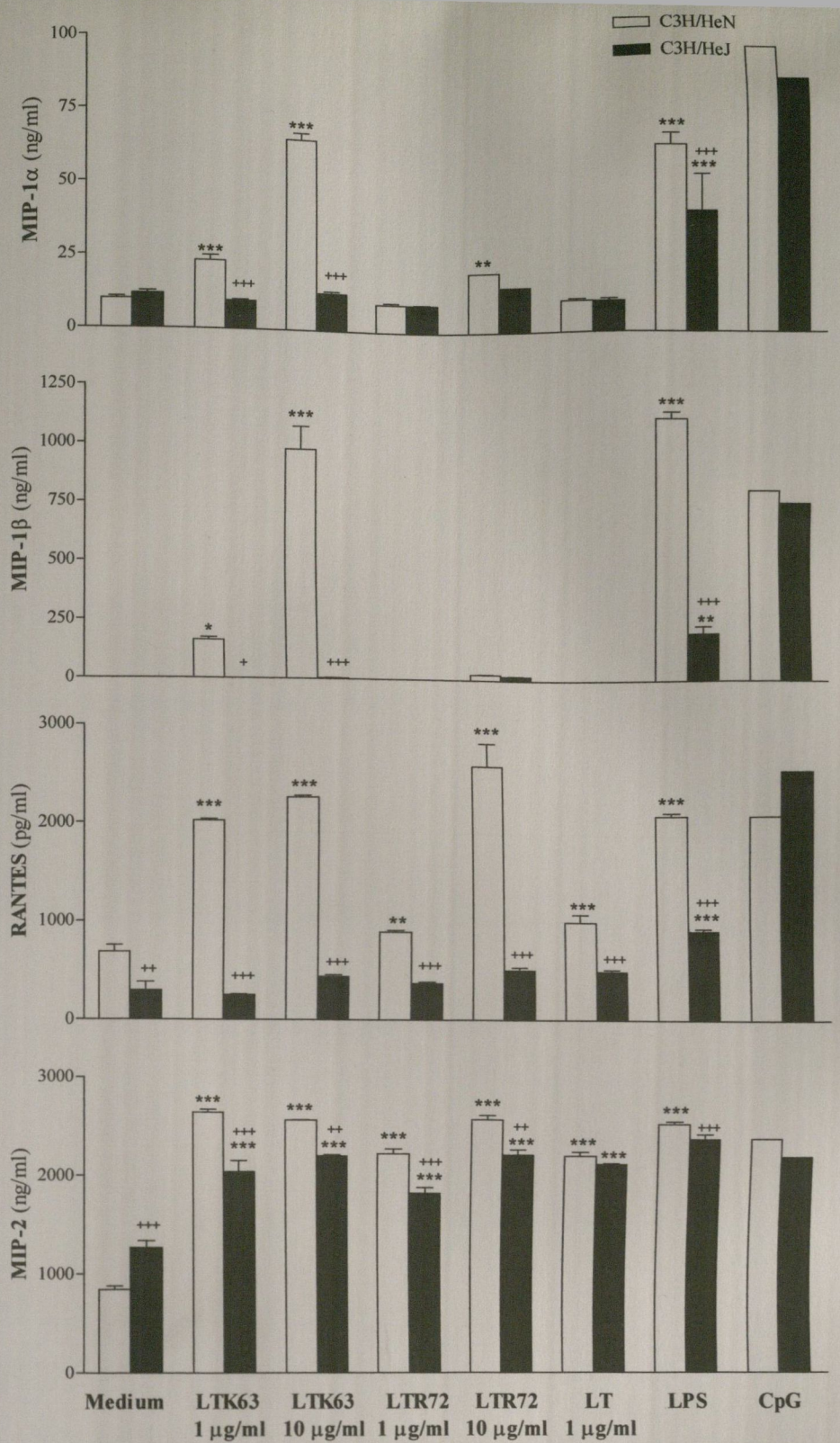


Figure 6.2. The ability of LT and LT mutants to induce chemokine secretion by BMDC is affected by TLR4 expression. Immature BMDC (1 x 10^6 cells/ml) from C3H/HeN or C3H/HeJ mice were cultured as described in the legend to Fig. 6.1. After 24 hours incubation, the supernatants were removed and the levels of MIP-1α, MIP-1β, RANTES and MIP-2 were tested by specific immunoassay using pairs of commercially available antibodies. Results are expressed as mean (+/- S.E.) chemokine concentrations for triplicate cultures. *, **, ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus unstimulated cells (Tukey-Kramer test). +, ++ and +++, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus cells from C3H/HeN mice (Tukey-Kramer test).

Figure 6.3. CT, LT and LT mutants enhance the expression of the co-stimulatory molecules, CD80 and CD86, on BMDC. Immature BMDC (1 x 10^6 cells/ml) from BALB/c mice were cultured for 24 hours with medium alone, with 10 μg/ml of CT, LT, LTR72 or LTK63 or with 1 μg/ml of LPS as a positive control. After 24 hours incubation, cells were recovered, washed and stained with anti-CD80-PE and anti-CD86-FITC followed by anti-CD11c-PerCP and analysed on a FACScan. Results are presented as mean fluorescence intensity of CD80 (blue) or CD86 (red) expression for treated versus untreated (control, black) CD11c+ cells.

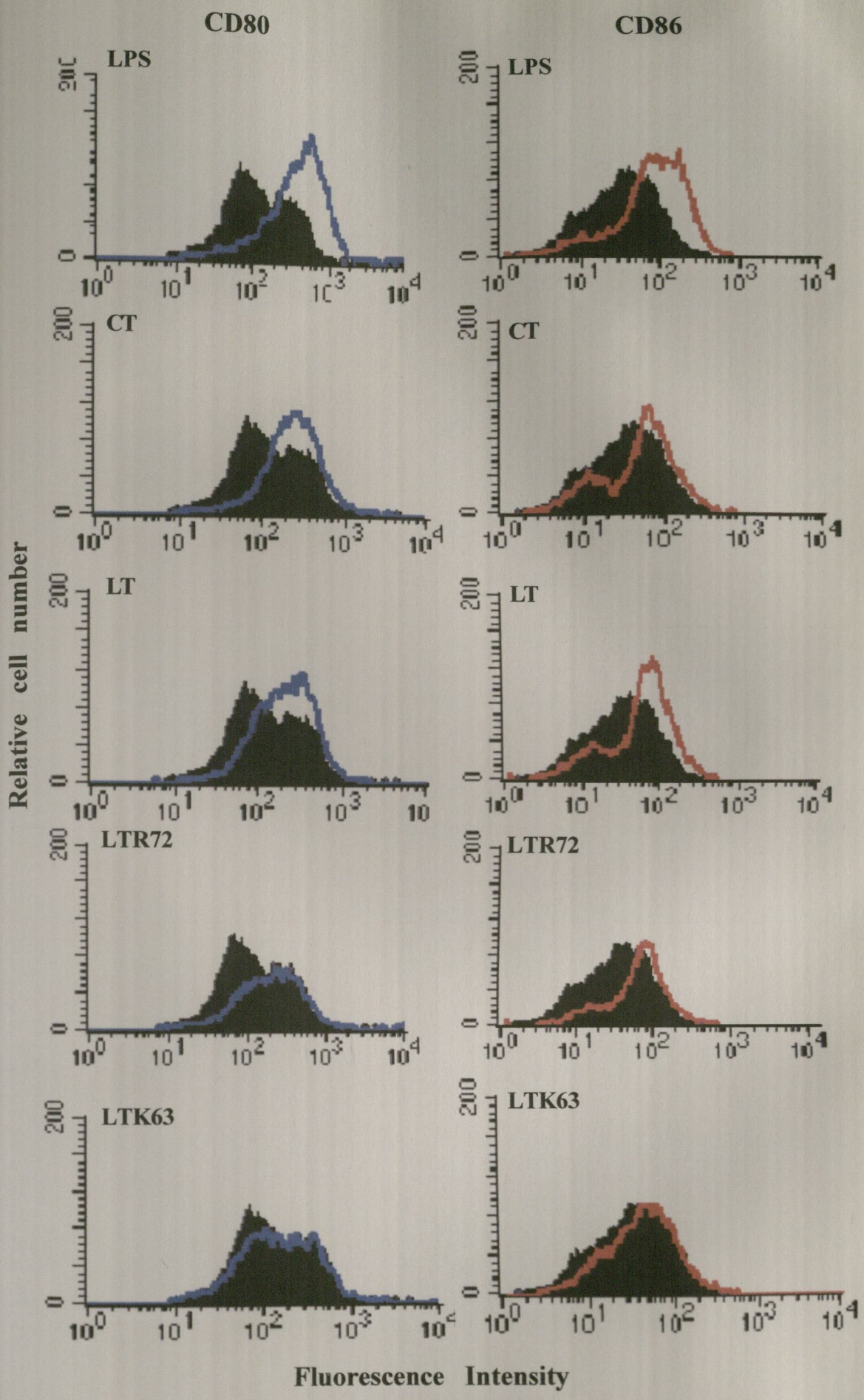
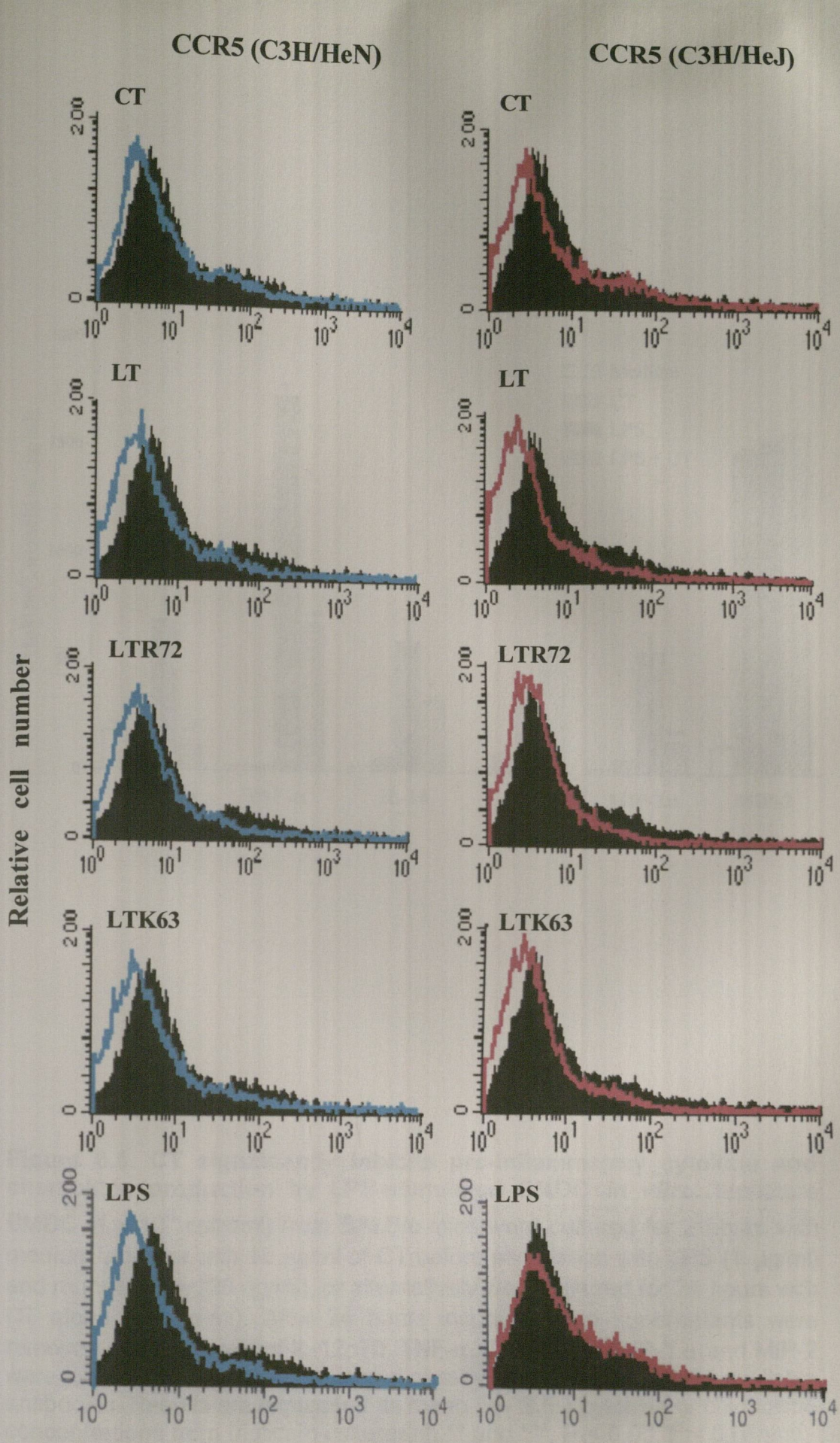

CD80
CD86
LPS
LPS
CT
CT
LT
LT
LTR72
LTR72
LTK63
LTK63
Relative cell number
Fluorescence Intensity

Figure 6.4. Down-regulation of the chemokine receptor, CCR5, on BMDC cultured with CT, LT or LT mutants is not affected by TLR4 expression. Immature BMDC (1×10^6 cells/ml) from both C3H/HeN and C3H/HeJ strains of mice were cultured for 24 hours with medium alone, with 10 μg/ml of CT, LT, LTR72 or LTK63 or with 1 μg/ml of LPS. After 24 hours incubation, cells were recovered, washed and stained with anti-CCR5-PE followed by anti-CD11c-PercP and analysed on a FACScan. Results are presented as mean fluorescence intensity of CCR5 expression for toxin-treated versus untreated (control, black) CD11c+ cells from C3H/HeN (blue) or C3H/HeJ (pink) mice.


CCR5 (C3H/HeN)
CCR5 (C3H/HeJ)
CT
LT
LTR72
LTK63
LPS
Relative cell number
Fluorescence Intensity

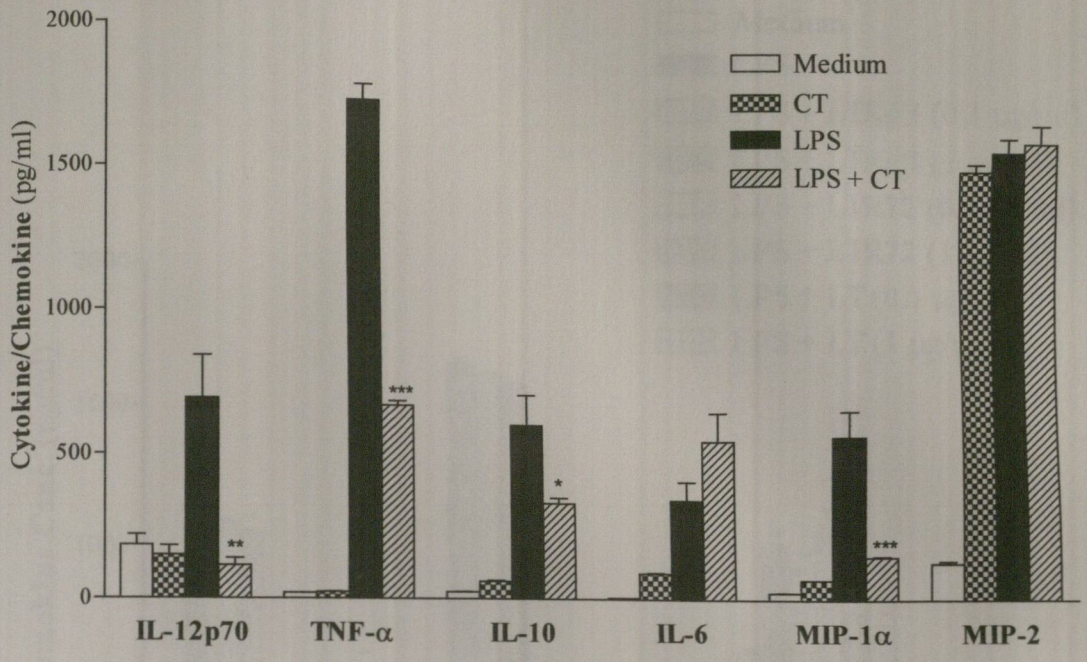


Figure 6.5. CT significantly inhibits pro-inflammatory cytokine and chemokine production by LPS-stimulated BMDC *in vitro*. Immature BMDC (1 x 10^6 cells/ml) from BALB/c mice were cultured for 2 hours with medium alone or with 10 μg/ml of CT before stimulation with LPS (1 μg/ml) and murine IFN-γ (20 ng/ml), or alternatively were cultured for 24 hours with CT alone (10 μg/ml). After 24 hours incubation the supernatants were removed and the levels of IL-12p70, TNF-α, IL-10, IL-6, MIP-1 α and MIP-2 were determined by specific immunoassay using commercially available antibodies. Results are expressed as mean (+/- S.E.) cytokine or chemokine concentrations from triplicate cultures. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus LPS alone (Tukey-Kramer test).

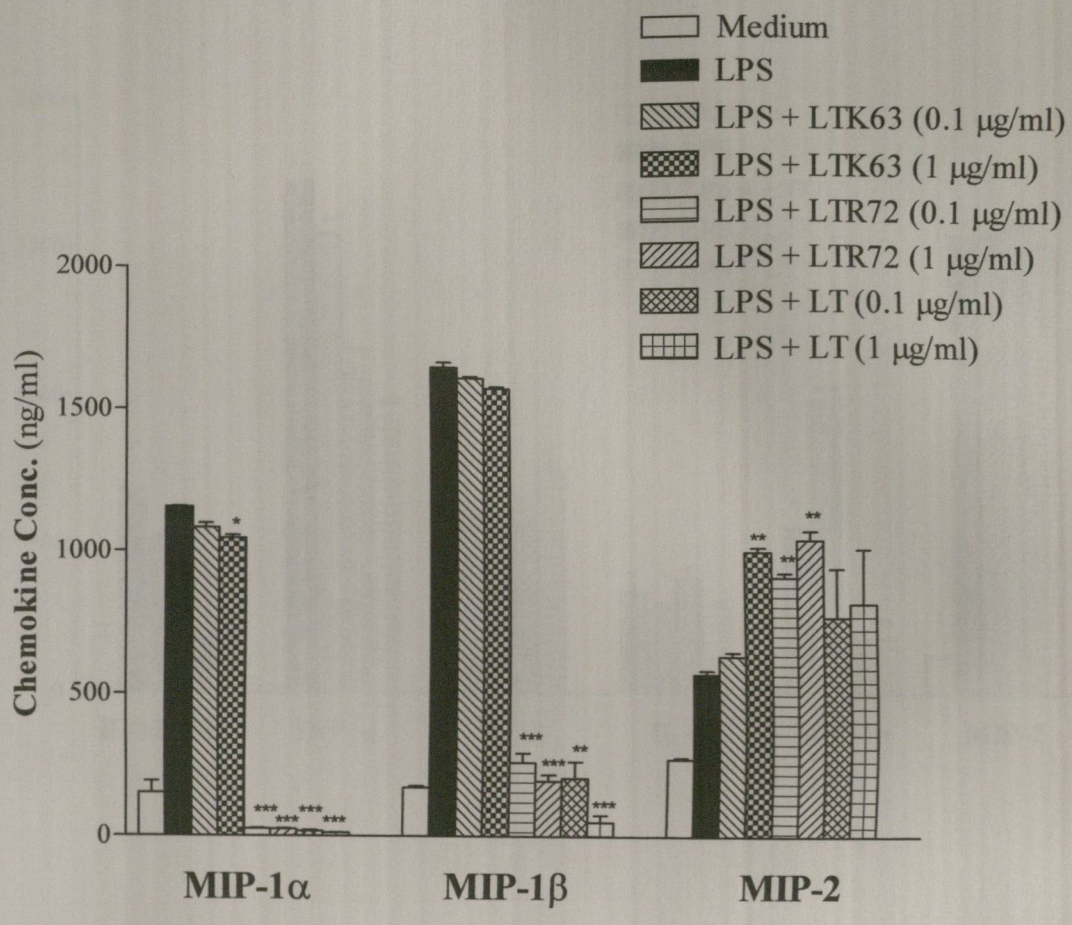


Figure 6.6. LTR72 and LT, but not LTK63, significantly inhibit MIP-1α and MIP-1β production by LPS-stimulated J774 macrophages *in vitro*. J774 cells (1 x 10^6 cells/ml) were stimulated *in vitro* with medium alone, with LPS (1 μg/ml) only as a control or with LPS and either LTK63, LTR72, or LT (0.1 or 1 μg/ml). After 24 hours incubation the supernatants were removed and the levels of MIP-1α, MIP-1β and MIP-2 were determined by specific immunoassay using commercially available antibodies. Results are expressed as mean (+/- S.E.) chemokine concentrations from triplicate cultutres. *, **, ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus LPS alone (Tukey-Kramer test).

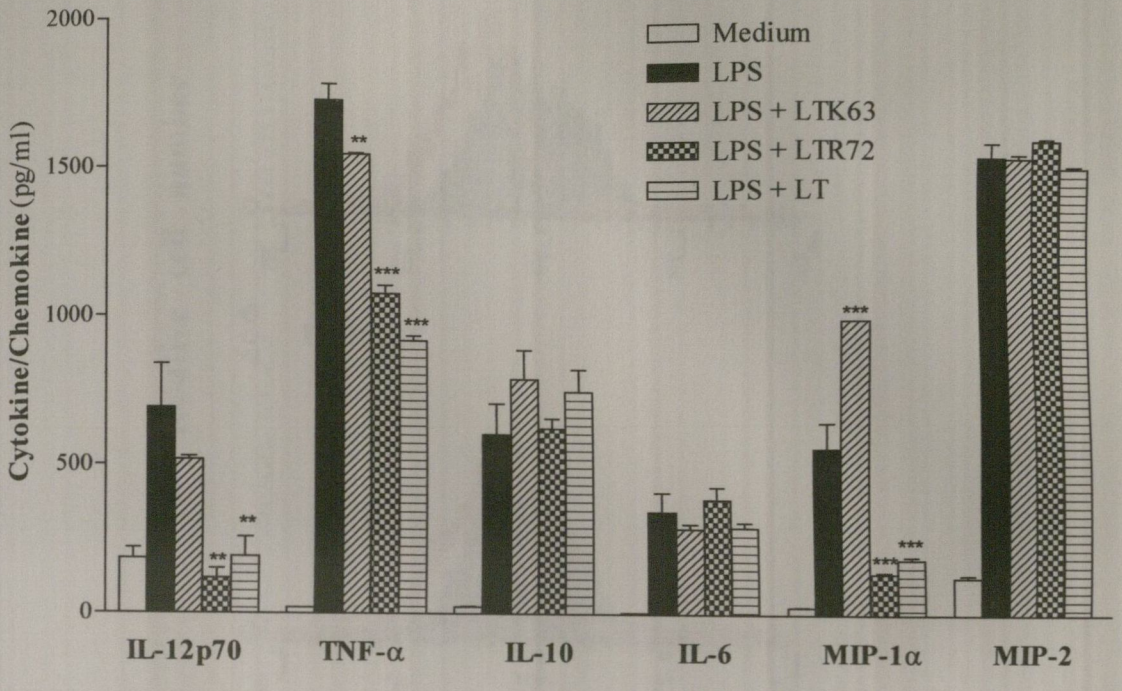


Figure 6.7. LT and LTR72 significantly inhibit IL-12p70, TNF-α and MIP-1α production by LPS-stimulated BMDC *in vitro*. Immature BMDC (1 x 10^6 cells/ml) from BALB/c mice were cultured for 2 hours with medium alone or with 10 μg/ml of LTK63, LTR72 or LT before stimulation with LPS (1 μg/ml) and murine IFN-γ (20 ng/ml). After 24 hours incubation the supernatants were removed and the levels of IL-12p70, TNF-α, IL-10, IL-6, MIP-1α and MIP-2 were determined by specific immunoassay using commercially available antibodies. Results are expressed as mean (+/- S.E.) cytokine or chemokine concentrations from triplicate cultures. ** and ***, $P < 0.01$ and $P < 0.001$ respectively versus LPS alone (Tukey-Kramer test).

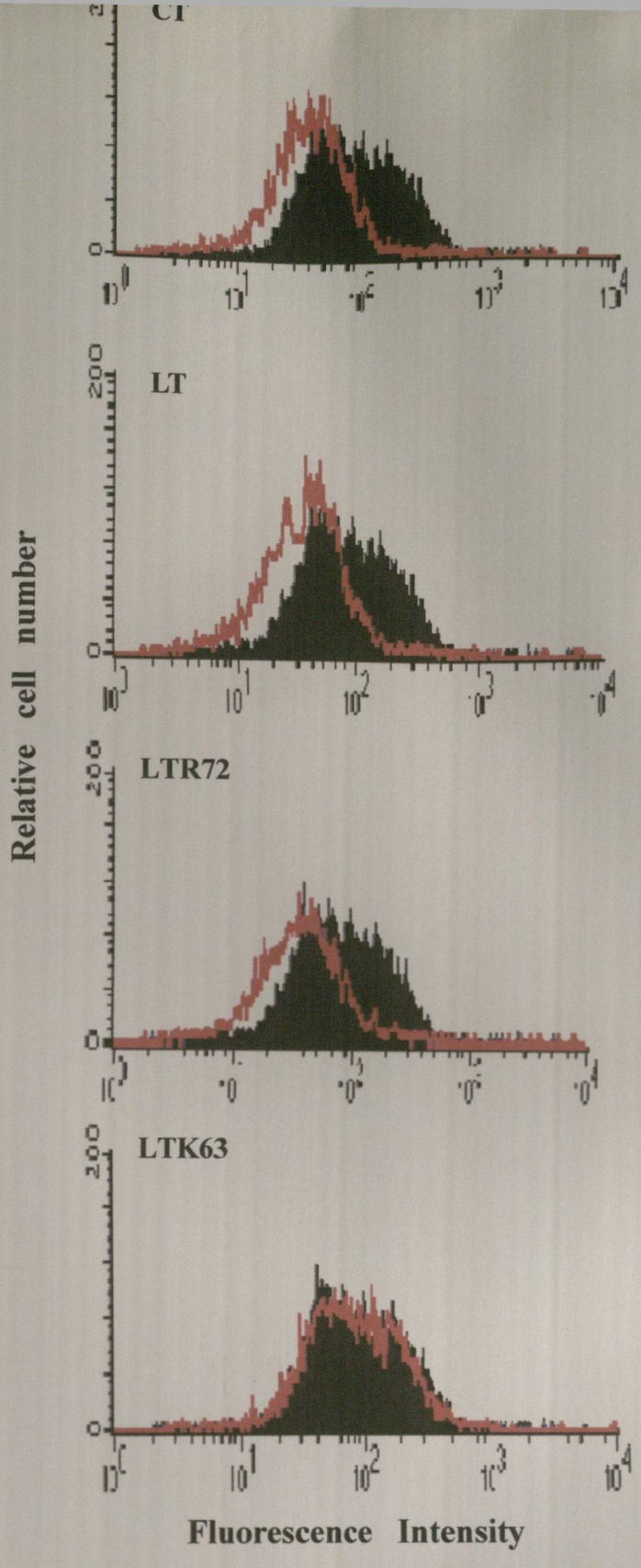
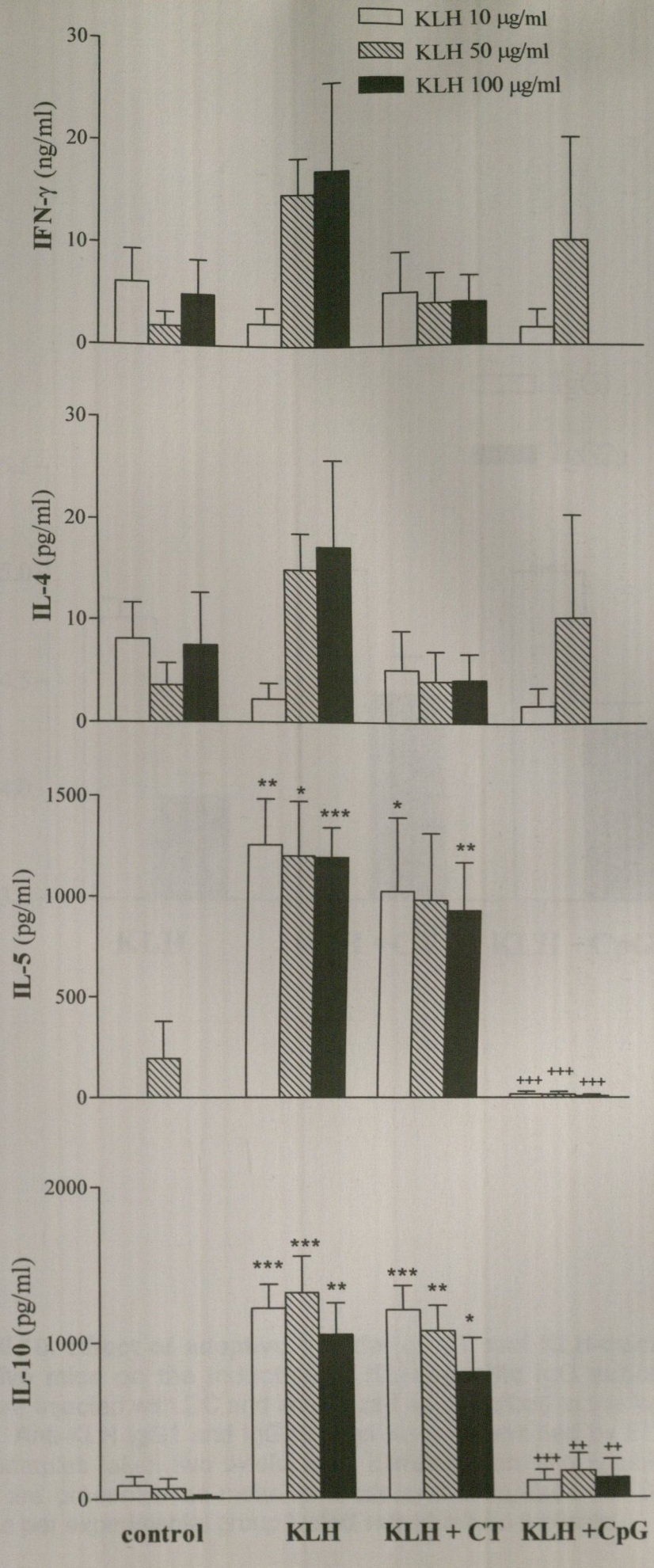


Figure 6.8. CT, LT and LTR72, but not LTK63, down-regulate the expression of LPS-induced CD40 on BMDC. Immature BMDC (1 x 10^6 cells/ml) from BALB/c mice were cultured for 2 hours with medium alone or with 10 μg/ml of CT, LT, LTR72 or LTK63 before stimulation with LPS (1 μg/ml) and murine IFN-γ (20 ng/ml). After 24 hours incubation, cells were recovered, washed and stained with anti-CD40-FITC followed by anti-CD11c-PerCP and analysed on a FACScan. Results are presented as the mean fluorescence intensity of CD40 expression (red) from toxin-treated versus untreated (control, black) CD11c+ cells.

Figure 6.9. Effect of adoptive transfer of CT and KLH-stimulated DC into naïve mice on KLH-specific T cell responses. Immature BMDC (1 x 10^6 cells/ml) from BALB/c mice were cultured for 24 hours with medium alone as a negative control or with KLH (5 μg/ml) alone or with either CT (1 μg/ml) or CpG (1 μg/ml). After 24 hours incubation, the cells were recovered, washed 5 times to remove all traces of KLH, CT or CpG and injected i.v. into naïve mice (10^5 cells/mouse). Mice that received KLH-, KLH and CT- or KLH and CpG-stimulated DC were immunized i.v. with soluble KLH (5 μg/dose) 7 days later. Cellular responses were assessed 2 weeks after immunization from spleen cells stimulated *in vitro* with KLH (10-100 μg/ml) and cytokine concentrations were determined by specific immunoassay on supernatants recovered after 72 hours incubation. Results are presented as mean IFN-γ, IL-5, IL-4 or IL-10 concentrations (+/- S.E.) for four mice per group tested individually in triplicate. *, ** and ***, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively versus control mice that received DC stimulated with medium alone, ++ and +++, $P < 0.01$ and $P < 0.001$ respectively versus mice that received DC stimulated with KLH alone (Tukey-Kramer test).


KLH 10 μg/ml
KLH 50 μg/ml
KLH 100 μg/ml
IFN-γ (ng/ml)
30
20
10
0
IL-4 (pg/ml)
30
20
10
0
IL-5 (pg/ml)
1500
1000
500
0
**
*
***
*
**
+++
+++
+++
IL-10 (pg/ml)
2000
1000
0
***
***
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*
+++
++
++
control
KLH
KLH + CT
KLH +CpG

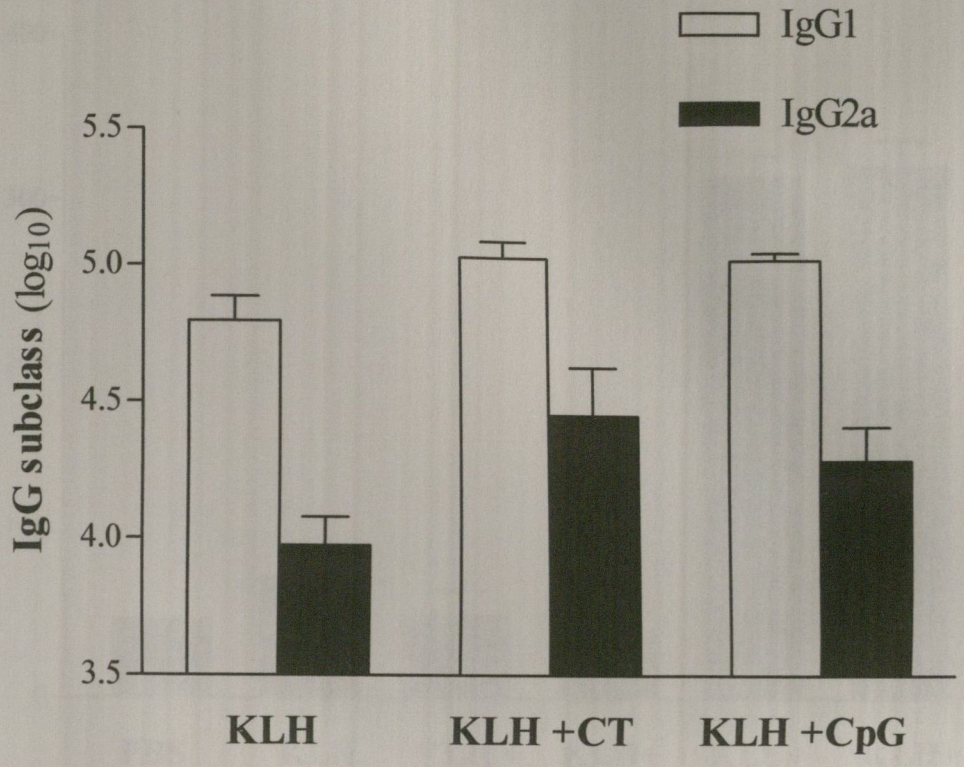

Figure 6.10. Effect of adoptive transfer of CT and KLH-treated DC into naïve mice on the induction of KLH-specific IgG subclasses. Mice were injected with DC and immunized as described in the legend to Fig. 6.9. Anti-KLH IgG1 and IgG2a titres were determined by ELISA on serum samples taken two weeks after immunization with soluble KLH. Results are presented as mean antibody end-point titres (+/- S.E.) for four mice per experimental group tested individually in triplicate.

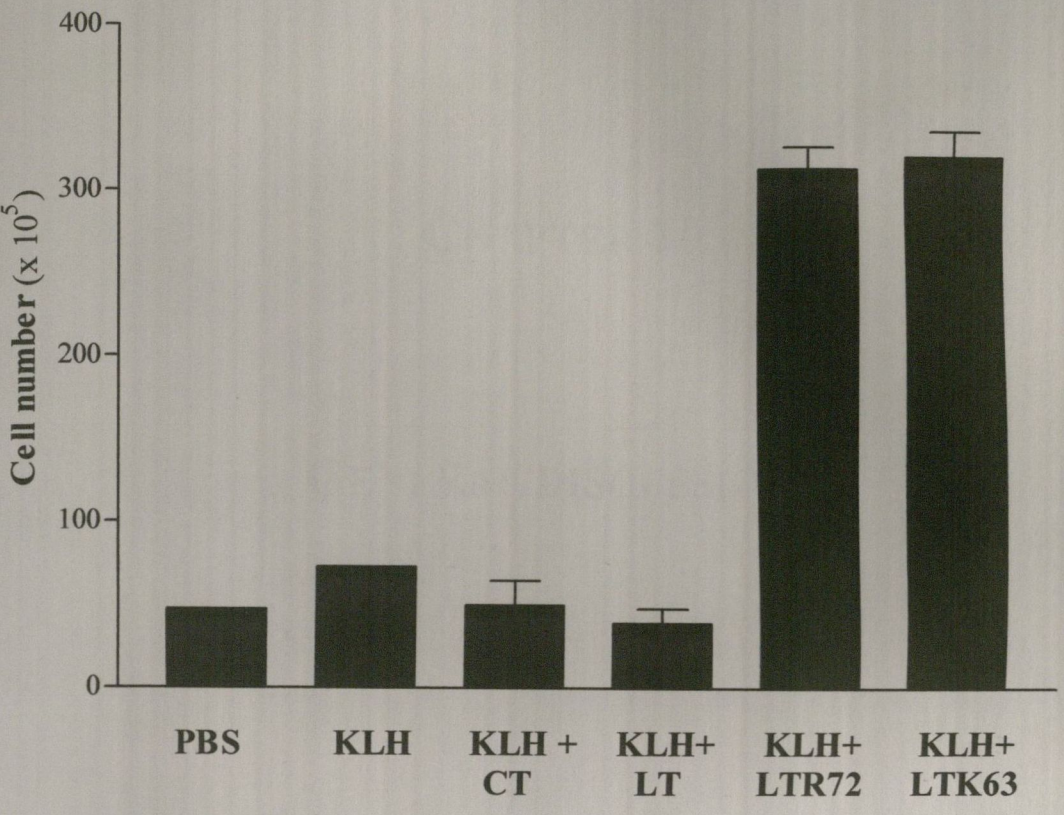

Figure 6.11. LTR72 or LTK63 enhance the recruitment of cells to the local lymph node following s.c. injection with KLH. BALB/c mice were immunized into each hind footpad with 25 μl of either PBS, KLH (5 μg) alone or formulated with LTK63 (10 μg), LTR72 (10 μg) or CT (10 μg). Popliteal lymph node cells were isolated 7 days later, homogenized and a cell viability count assessed. Results are expressed as mean (+/- S.E.) cell numbers from four mice per group.

CHAPTER 7

GENERAL DISCUSSION

7.0 General Discussion

Current licensed vaccines have been very effective in eradicating or dramatically decreasing the occurrence of diseases such as polio, smallpox and measles during the last century. However, increases in antibiotic-resistant bacteria, the emergence of new pathogens, or concerns over the safety of traditional killed and attenuated vaccines or partially purified toxoid vaccines have highlighted the requirement for new vaccines or for the refinement of existing vaccines to render them safer and more effective. Recent developments in biotechnology have enabled the development of safer subunit vaccines, such as acellular pertussis and recombinant hepatitis B vaccines, consisting of specific components of the pathogen. Furthermore, alternative routes of vaccine delivery may increase patient compliance and enhance safety.

Mucosal delivery of vaccines may facilitate the eradication of pathogens that invade through mucosal surfaces, due to the induction of both local and systemic immunity. Administration of vaccines by mucosal routes may also be associated with reduced adverse effects, higher acceptance by the public, easier administration and the potential for frequent boosting (Walker, 1994, O'Hagan, 1998). Mucosal vaccines may therefore be particularly beneficial for immunization against diseases such as diphtheria, where booster doses of the vaccine are generally recommended every 10 years. However, soluble protein antigens are often poorly immunogenic when delivered mucosally and may induce tolerance (Lowrey *et al.*, 1998). Therefore, safe and effective adjuvants or delivery systems are required to enhance the immunogenicity of these antigens.

The AB toxins, cholera toxin (CT) and *E. coli* heat labile toxin (LT), are the most powerful mucosal adjuvants described to date, yet their clinical use is restricted due to their toxicity (Rappuoli *et al.*, 1999, Williams *et al.*, 1999). Site-directed mutants of LT have been generated by site-directed mutagenesis with reduced or no enzymatic activity (Douce *et al.*, 1995, Giuliani *et al.*, 1998, Komase *et al.*, 1998, Douce *et al.*, 1999). The immunogenicity and adjuvanticity of two active-site mutants of LT were examined in this study, LTK63, a non-toxic mutant that is completely devoid of enzymatic activity but retains the binding activity of the B subunit, and LTR72, a partially toxic mutant that retains about 1% of the wild-type enzyme activity. Chitosan, a cationic polysaccharide derived by deacetylation of chitin, has also previously been shown to enhance the immunogenicity of several antigens following

mucosal immunization (Jabbal-Gill *et al.*, 1998, Roy *et al.*, 1999, Bacon *et al.*, 2000, Le Buanec *et al.*, 2001). One of the aims of this study was to examine the immune responses induced in mice, guinea pigs and humans using chitosan as an adjuvant for the intranasal delivery of diphtheria vaccines.

Diphtheria is an acute and often fatal bacterial disease, which may be prevented by toxin-neutralizing antibodies (anti-toxin) induced through active immunization. Currently, diphtheria vaccines are prepared by converting diphtheria toxin to its non-toxic, but antigenic, toxoid by formaldehyde treatment and are mostly combined with tetanus and pertussis vaccines for routine infant immunizations (Rappuoli, 1997). Although traditional vaccines against diphtheria have been very effective, they may be associated with adverse effects due to low purity (Pappenheimer *et al.*, 1950, Rappuoli, 1997). Furthermore, several serological studies throughout Europe have shown that a large proportion of the adult population lack protective immunity to diphtheria (Rappuoli *et al.*, 1993, Maple *et al.*, 1995, Garcia-Corbeira *et al.*, 1999, Maple *et al.*, 2000). In support of these findings, in the present study it was found that only 33% of adults tested had antitoxin titres above the fully protective level of 0.1 IU/ml. There is therefore a requirement for boosting of individuals with safe and effective vaccines against diphtheria.

CRM_{197}, a naturally non-toxic mutant of diphtheria toxin, may be obtained at close to 100% purity and may therefore be a safer alternative to DT for immunization against diphtheria (Rappuoli, 1997). However, it has been reported that this molecule is more susceptible to protease degradation than DT and therefore displays lower immunogenicity *in vivo* (Pappenheimer *et al.*, 1972), although its immunogenicity may be enhanced by stabilization with formaldehyde (Porro *et al.*, 1980). In this study it was found that either native or formaldehyde treated CRM_{197} induced similar antibody responses to conventional DT following parenteral administration, although T cell responses were progressively reduced as CRM_{197} was treated with increasing concentrations of formaldehyde. In contrast, the immunogenicty of this antigen was considerably lower following intranasal immunization and therefore the antigen was formulated with either chitosan, LTK63 or LTR72 as adjuvant.

It was found that chitosan enhanced the immunogenicity of CRM_{197} following nasal immunization in mice. However, three intranasal immunizations with CRM_{197} and chitosan were necessary to generate modest T cell and antibody responses, which were still weaker than those observed following parenteral delivery of conventional

diphtheria toxoid. In contrast, the mutants of LT, LTR72 and LTK63, were capable of inducing potent T cell and antibody responses following two intranasal immunizations with CRM_{197}. Furthermore, mice primed parenterally with a diphtheria, tetanus and acellular pertussis vaccine (DTPa) adsorbed to alum and boosted intranasally with the same vaccine formulated with LTK63 generated potent antigen-specific T cell and antibody responses. In particular, this method of immunization induced similar IgG antibody responses in mice to two parenteral immunizations with DTPa adsorbed to alum, but boosting intranasally with DTPa and LTK63 also had the advantage of significantly enhancing local IgA antibody responses to all of the vaccine components. It has previously been shown that nasal administration of DT with an active site mutant of CT, CTE112K, induced potent DT-specific neutralizing antibody responses in mice (Ohmura *et al.*, 2001).

Although chitosan was not as effective an adjuvant as LTK63 or LTR72 for enhancing the immunogenicity of intranasally delivered CRM_{197} in naïve mice, it was found that intranasal boosting of parenterally primed mice with CRM_{197} and chitosan induced potent T cell and antibody responses. Furthermore, this method of immunization was found to be highly effective for the generation of strong anti-diphtheria toxin neutralizing antibody responses in guinea pigs. Guinea pigs, unlike mice, are sensitive to diphtheria toxin and they may therefore provide a more suitable model for assessing the potency of diphtheria-based vaccines *in vivo*. Having shown that CRM_{197} and chitosan were capable of inducing potent neutralizing antibody responses following intranasal immunization in guinea pigs, it was decided to perform a Phase I clinical trial to assess the immunogenicity of this vaccine in human volunteers. A significant finding of this study was that intranasal boosting with CRM_{197} and chitosan induced potent IgG and neutralizing antibody responses in humans, which were significantly higher than those observed following immunization with CRM_{197} alone. Furthermore, a single intranasal booster of CRM_{197} and chitosan induced comparable neutralizing antibody responses to those generated following an i.m. booster with conventional DT. It has previously been shown that intranasal immunization of humans with diphtheria and tetanus toxoids, administered with enhancer mixtures of polysorbate and caprylic/capric, significantly enhanced anti-diphtheria toxin neutralizing antibody concentrations. However, in contrast to the results presented here, all of the intranasal formulations showed lower immunogenicity than conventional parenterally delivered DT (Aggerbeck *et al.*, 1997). The present

clinical trial therefore provides the first evidence that a nasally delivered diphtheria vaccine may be a safer alternative to the traditional DT vaccine, without compromising the potent immunogenicity of this vaccine.

There is currently no effective vaccine available against meningococcus strain B, which is a major cause of meningococcal disease in Europe and the United States. Recently, the entire genome sequence of a virulent meningococcal serogroup B strain was determined, in order to identify potential vaccine candidates (Pizza *et al.*, 2000, Tettelin *et al.*, 2000). Open reading frames (ORFs) were identified that potentially encoded novel surface-exposed or exported proteins that may have antigenic properties *in vivo*. In addition to enhancing the immunogenicity of nasally co-administered CRM_{197}, LTR72 and LTK63 were also evaluated as adjuvants for the nasal delivery of two of these novel meningococcal type B antigens, ORF1 and ORF40. Both LTK63 and LTR72 were found to significantly enhance T cell and antibody responses to co-administered ORF40. Furthermore, LTR72 significantly enhanced systemic Th1 type responses to intranasally delivered ORF1. However, it remains to be seen if nasal administration of meningococcal type B antigens, such as ORF1 and ORF40, with mutants of LT as adjuvant are capable of inducing bactericidal antibodies, which correlate with protection against meningococcal disease in humans (Goldschneider *et al.*, 1969).

Having established that LTK63 and LTR72 were effective adjuvants for the nasal delivery of antigens such as CRM_{197} or the meningococcal type B antigen ORF40, some of the properties of these molecules that may contribute to their adjuvanticity *in vivo* were examined. It was initially thought that the enzymaytic ADP-ribosyltransferase activity of the A subunit of LT was required for its adjuvanticity (Lycke *et al.*, 1992). More recently, the identification of non-toxic mutants of LT or CT that retain adjuvant activity following mucosal delivery (Douce *et al.*, 1995, Yamamoto *et al.*, 1997b, Ryan *et al.*, 1999) or the use of the B subunits of these toxins as adjuvant (Isaka *et al.*, 1998, Kozuka *et al.*, 2000, Richards *et al.*, 2001) suggest that enzymatic activity is not essential for effective mucosally delivered vaccines. Nonetheless, it has been shown in several studies that mutants of CT or LT with higher levels of enzymatic activity retain the highest adjuvant activity *in vivo* (Giuliani *et al.*, 1998, Cheng *et al*, 1999, McCluskie *et al.*, 2001b). In agreement with these findings, in the present study it was found that the partially toxic mutant of LT, LTR72, was generally a more effective adjuvant for the nasal delivery of antigens, such as CRM_{197},

compared to the enzymatically inactive mutant, LTK63. However, increasing the dose of LTK63 did result in an enhancement in immune responses. Similarly, Giuliani *et al.* (1998) reported an enhancement in the adjuvanticity of LTK63 with an increase in toxin dose (Giuliani *et al.*, 1998).

Adjuvants or delivery systems may firstly increase the absorption of the co-administered antigen from the nasal cavity or the gut following mucosal delivery. It has been reported that chitosan enhances the nasal absorption of drugs, possibly due to the transient opening of tight junctions between cells of the nasal membrane (Borchard *et al.*, 1996, Schipper *et al.*, 1997, Dodane *et al.*, 1999). In the present study it was found that nasal administration of chitosan and CRM_{197} together induced stronger immune responses in mice, compared to administration of chitosan one day earlier than the antigen. These results suggest that chitosan may act as an adjuvant by enhancing the absorption of CRM_{197} from the nasal cavity, allowing the antigen to gain rapid access to and stimulate cells of the nasal associated lymphoid tissue. However, chitosan also enhanced immune responses to parenterally delivered CRM_{197}, suggesting that the adjuvant properties of this molecule may extend beyond antigen uptake at mucosal surfaces. CT has previously been shown to increase the gut permeability for dextran following oral delivery (Lycke *et al.*, 1991). In addition, the ability of CT, LT and mutants of LT to preferentially target GM1 may enhance the immunogenicity of these toxins *in vivo*. Studies have demonstrated a GM1-dependent preferential localization of CTB to M cells of the GALT, over other adjacent mucosal cells (Owen *et al.*, 1986). Furthermore, Lian *et al.* (1999) found that formulation of an HIV envelope protein in CTB-associated GM1 vesicles elicited significantly stronger immune responses than the same antigen and CTB formulated in GM1-free vesicles (Lian *et al.*, 1999). Adjuvants may also increase cellular infiltration to the site of administration, ensuring more cells are present to acquire and present antigen. In this study it was shown that LTR72 and LTK63 dramatically enhanced cellular recruitment to the local lymph node following s.c. administration with KLH.

In addition to facilitating antigen uptake, adjuvants may also directly activate cells of the innate immune system. Chitosan has been reported to stimulate the production of cytokines by murine macrophages and to enhance the expression of activation markers, such as MHC class I, by these cells *in vitro* (Nishimura *et al.*, 1986, Ueno *et al.*, 2001). CT or LT have been shown to enhance the expression of the co-stimulatory molecules, CD80 and CD86, on B cells (Ågren *et al.*, 1997) and

macrophages (Ryan *et al.*, 2000). Furthermore, LTK63 and LTR72 enhanced CD80 expression by murine macrophages *in vitro* (Ryan *et al.*, 2000). The most important APC involved in antigen capture and T cell priming are dendritic cells (DC), which in their immature state circulate through peripheral tissues and readily acquire antigen. Following uptake of antigen and cell activation, DC undergo maturation and migrate to the lymph nodes where they have the unique ability to present antigen to naïve T cells (Banchereau *et al.*, 2000). CT, either alone or in combination with TNF-α or IL-1β, has been shown to induce human DC maturation, and enable these mature DC to polarize naïve T cells towards a Th2 phenotype *in vitro* (Gagliardi *et al.*, 2000, de Jong *et al.*, 2002). In the present study it was shown that CT, LT, and the mutants of LT, LTR72 and LTK63, induced murine DC maturation *in vitro*, as assessed by an enhancement in co-stimulatory molecule expression and downregulation of the inflammatory chemokine receptor, CCR5, on these cells. It is therefore possible that these toxins may also induce DC maturation *in vivo*, resulting in the subsequent initiation of primary T cell responses and the polarization of particular T cell subtypes.

Although LTK63 and LTR72 were capable of inducing murine DC maturation *in vitro*, these two mutant toxins differentially regulated LPS-induced cytokine or chemokine production by these cells. Both LT and the enzymatically active mutant of LT, LTR72, significantly inhibited LPS-induced pro-inflammatory cytokine and chemokine production by DC *in vitro*. In contrast, the enzymatically inactive mutant of LT, LTK63, had little or no effect on LPS-induced cytokine or chemokine responses. Furthermore, CT, LT and LTR72, but not LTK63, down-regulated the expression of CD40 by LPS-stimulated DC *in vitro*.

It would appear that highly purified LT and mutant toxins, which are completely devoid of LPS, are unable to induce the secretion of inflammatory cytokines or chemokines by DC. Incubation of BMDC from C3H/HeJ mice, which are hyporesponsive to LPS, with the toxins *in vitro* failed to stimulate the production of significant concentrations of a range of cytokines or chemokines. However, all of the toxins tested did induce MIP-2 secretion by BMDC from C3H/HeJ mice or J774 cells *in vitro*, indicating that this chemokine may contribute to the potent immunogenicity of these molecules *in vivo*.

It was found in this study that a low dose of LTR72 selectively enhanced systemic Th2 type responses to intranasally delivered CRM_{197}, whereas higher doses of both LTK63 and LTR72 augmented both Th1 and Th2 type responses. Similar T

cell responses have been observed following intranasal delivery of these mutants of LT with pertussis antigens (Ryan *et al.*, 1999). Furthermore, in several studies CT has been shown to induce predominant Th2 type responses to co-administered antigen following mucosal delivery (Xu-Amano *et al.*, 1993, Marinaro *et al.*, 1995). It is therefore possible that the ability of toxins with enzymatic activity to inhibit LPS-induced cytokine or chemokine production by DC may be related to the augmentation of Th2 type responses by these molecules *in vivo*. The ADP-ribosylation activity of the A subunit may lead to the intracellular accumulation of the potent immunomodulator, cAMP (Cheng *et al.*, 1999, Ryan *et al.*, 2000). Upregulation of cAMP has been shown in several studies to induce Th2 cytokine production but to inhibit the release of pro-inflammatory cytokines (Gajewski *et al.*, 1990, Munoz *et al.*, 1990, Betz and Fox, 1991, Zidek, 1999). The ability of cAMP to induce Th2 cytokines may be partly related to its ability to induce phosphorylation of the transcription factor required for Th2 cell differentiation, GATA3, and to stimulate GATA-3 dependent promoter activity (Chen *et al.*, 2000). In contrast, the B subunit of LT may induce NF-κB activation (Bone *et al.*, 2000, Ryan *et al.*, 2000), possibly through binding to GM1 or to a pathogen recognition receptor expressed on innate cells. Activation of NF-κB may subsequently lead to the transcription of target genes including those encoding inflammatory cytokines and chemokines and adhesion molecules (Baldwin, 1996, Ghosh *et al.*, 1998). Furthermore, it has been reported that cAMP-elevating agents may repress NF-κB-dependent transcription by a variety of different mechanisms (Ye, 2000). It has therefore been suggested that the outcome of the immune response generated by CT, LT or mutants of the toxins may be dependent on a balance between these two distinct biochemical signalling pathways for cAMP and NF-κB (Ryan *et al.*, 2000). However, LTR72 has also been shown to induce predominant Th1 type responses to co-administered antigen (Barackman *et al.*, 1999) and one group have reported enhanced Th2 type cytokines and increased IgG1 over IgG2a using the B subunit of LT alone (LTB) as an adjuvant (Richards *et al.*, 2001). These studies suggest that the types of immune responses generated using AB toxins as mucosal adjuvants may also depend on a number of other factors.

In the present study it was found that the types of immune responses induced using LTR72 or LTK63 as adjuvant varied depending on the nature of the co-administered antigen and the route of delivery. It was shown that nasal administration of LTR72 with ORF1 induced predominant Th1 type responses in the absence of

detectable IgG induction. In contrast, formulation of the same adjuvant with another meningococcal type B antigen, ORF40, induced more mixed Th1/Th2 type responses and potent IgG responses following nasal delivery. The immune responses induced using chitosan as an adjuvant also varied depending on the nature or dose of the antigen. It was found that mild formaldehyde treatment of CRM_{197} enhanced both cellular and humoral immune responses following nasal delivery with chitosan as adjuvant. Furthermore, a dose of 10 μg of CRM_{197} with chitosan induced the strongest and most consistent local and systemic antibody responses following nasal immunization.

The route of delivery may also influence the strength and type of immune response generated. It was generally found in this study that intranasal immunization of mice with CRM_{197} with chitosan, LTK63 or LTR72 as adjuvant induced weaker levels of antigen-specific IgG responses compared to parenteral delivery of the same vaccine formulations or parenteral immunization with CRM_{197} adsorbed to alum. Nonetheless, intranasal immunization did have the advantage over conventional parenterally delivered antigen of being able to stimulate high levels of cellular or humoral immune responses in the lymph nodes or lungs of immunized mice respectively. The route of vaccine administration also influenced the type of T cell responses induced. Intranasal immunization of mice with CRM_{197} and chitosan induced predominant Th2 type responses, with the absence of detectable IFN-γ. In contrast, parenteral immunization with CRM_{197} and chitosan or intranasal boosting of parenterally primed mice with the same vaccine formulation induced more mixed Th1/Th2 type responses. Similarly, intranasal boosting with CRM_{197} with or without chitosan or parenteral immunization with conventional diphtheria toxoid induced mixed Th1/Th2 type responses in humans, as assessed by antigen-stimulated PBMCs *in vitro*.

The results of the present study demonstrate the superior immunogenicity of a CRM_{197} and chitosan vaccine for the induction of specific immunity against diphtheria, following intranasal delivery to adults previously immunized with conventional diphtheria vaccine. Furthermore, the mutants of LT, LTK63 and LTR72, were found to be effective adjuvants for the nasal delivery of CRM_{197} or novel meningococcal type B antigens to mice. The adjuvanticity of molecules such as chitosan and AB toxins may be related to their ability to enhance antigen uptake at mucosal surfaces but additionally to activate cells of the innate immune system. In particular, LTK63 and

LTR72 were capable of inducing murine DC maturation *in vitro*, which may be associated with the potent adjuvanticity these toxins possess *in vivo*. However, the safety of adjuvants such as LTR72 and LTK63 needs to be carefully considered if these adjuvants are to be used routinely in mucosal vaccines for human use, in particular the potential risk of neurological side effects. LTK63 is currently being evaluated in a clinical trial for nasally delivered influenza, which may provide more information on the safety of this toxin *in vivo*. The balance between protective immunity and controlled inflammation and reactogenicity will need to be finely monitored in the development of new and improved vaccines against infectious diseases based on nasal administration.

CHAPTER 8

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