EVALUATION OF LIPID-BASED DELIVERY SYSTEMS FOR INDUCTION OF MUCOSAL AND SYSTEMIC IMMUNE RESPONSES: EFFECT OF CpG MOTIFS

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ABSTRACT

CpG oligodeoxynucleotides (ODNs) are potent adjuvants that significantly enhance cellular and humoral responses to coadministered antigens when given parenterally or by mucosal routes. However, in vivo degradation of ODNs limits their uptake and their effectiveness as adjuvants.

The purpose of these studies was to investigate the ability of biphasic lipid vesicles (VTA) to enhance the adjuvant activity of CpG ODNs following systemic administration and to evaluate the feasibility of non-invasive methods for vaccine delivery such as mucosal and transcutaneous immunization.

Results showed that formulation of CpG ODN in VTA significantly enhanced its adjuvanticity and protected pigs against infection with *Actinobacillus pleuropneumoniae* without induction of the severe tissue damage seen with the commercial adjuvant VSA. The dose of CpG ODN required to induce protective immune responses in pigs when formulated in VTA was also established.

The present studies also showed that administration of antigen and CpG ODN in biphasic lipid vesicles resulted in induction of systemic and local antibody responses after immunization with a combined mucosal/systemic approach, while the protein either alone or with CpG ODN did not induce mucosal immune responses.

The mechanism by which these biphasic lipid vesicles may enhance the adjuvant effect of CpG ODN include: 1) increasing its availability by preventing dilution and degradation *in vivo*, 2) improving its uptake by antigen presenting cells (APCs) due to the particulate nature of lipid-based formulations. The least effective combinations induced mild or no inflammation, while formulations

containing both the antigen and CpG ODN induced cell infiltration 24 and 48 h after administration, suggesting that cellular infiltration may be essential for the induction of immune responses.

The ability of microneedles to facilitate the delivery of antigens after topical administration was also evaluated in these studies and the data presented clearly showed that only with previous application of microneedles, topical administration of CT induces systemic and mucosal immunity in pigs.

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who gave us her constant love and support, and taught us to be strong through out this journey that is life.

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LIST OF ABBREVIATIONS

APCs	antigen-presenting cells
App	*Actinobacillus pleuropneumoniae*
BALT	bronchial associated lymphoid tissue
CMIS	common mucosal immune system
CpG ODN	CpG oligodeoxynucleotides
CT	cholera toxin
CTL	cytotoxic T lymphocyte
DCs	dendritic cells
DDA	dimethyl dioctadecyl ammonium bromide
DLN	draining lymph nodes
DT	diphtheria toxin
GALT	gut-associated lymphoid tissue
HEV	high endothelial venules
ISCOMs	Immune-stimulating complexes
LALT	larynx-associated lymphoid tissue
LCs	Langerhans cells
LPS	lipopolysaccharides
LT	heat labile enterotoxin
LUV	large unilamellar vesicles
MALT	mucosa-associated lymphoid tissue
MDP	muramyl-dipeptide
MLV	multilamellar vesicles
MPCR	multiplex PCR
MPL	Monophosphoryl lipid A
MTP	muramyl-tripeptide
MVL	multivesicular liposomes

NALT	nasal associated lymphoid tissue\
NT	nasopharingeal tonsil
ODNs	oligodeoxynucleotides
OLV	oligolamellar vesicles
OmlA	outer membrane lipoprotein A
o/w	oil in water
PaT	palatine tonsil
PAMP	pathogen-associated microbial pattern
PGR	pharmacological grade reagents
PLG	poly(lactide-co-glycolide)
PMSF	phenylmethylsulfonyl fluoride
PT	*Bordetella pertussis* toxin
rhIL-2	recombinant human interleukin 2
S.I.	Stimulation index
SUV	small unilamellar vesicles
TCI	transcutaneous immunization
TLR	Toll-like receptors
TT	tetanus toxin
TuT	tubal tonsils
w/o	water in oil

1.0 INTRODUCTION

Since Jenner first successfully immunized individuals against smallpox over 200 years ago, vaccines have been one of the most effective tools in controlling or eradicating disease. Despite this success, there is still a great need to develop a new generation of safer vaccines that can be effectively administered by simple, economical, and practical immunization procedures. Most of the currently available vaccines are administered via parenteral routes. As a result, immunization requires trained medical personnel, is expensive, and may lead to injection site reactions, pain and in certain instances to infections by blood-borne pathogens because of the reuse of contaminated needles. Development of less-invasive and more readily administered vaccines has thus become a priority in prevention of human and animal diseases. 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. Furthermore, advances in the development of novel adjuvant and delivery systems have helped to identify vaccine formulations that are safe and effective. At the same time, the identification of immunomodulators that can direct the immune responses to humoral or cell mediated immunity and that can selectively induce type 1 or type 2 T cell responses has made possible the design of protective vaccines based on prior knowledge of the protective mechanism.

CpG oligonucleotides (ODNs) are nonspecific immune activators that can augment immune responses in an antigen-specific manner by synergizing with signals delivered through the B-cell receptor (Krieg, *et al.*, 1995) and by activating antigen presenting cells (APCs) including monocytes, macrophages and dendritic cells (DC) (Sparwasser, *et al.*, 1998; Sparwasser, *et al.*, 2000).

CpG ODNs have shown to be potent mucosal and systemic adjuvants, mostly in rodents and humans.

Biphasic lipid vesicles constitute a new class of delivery system into which antigens and adjuvants can be incorporated. The use of liposomes as antigen/adjuvant carriers for vaccines has several distinct advantages. These include: a) biocompatibility, biodegradability and low or lack of toxicity; b) the effective targeting of encapsulated antigens to APC; c) the slow release of the antigen; d) the possibility of co-entrapping several antigens and adjuvants; and e) induction of humoral and cellular responses.

Development of less-invasive and more readily administered vaccines has become a priority for public health agencies and is associated with an emergence of new technologies in the areas of vaccine formulation and delivery. Recently, there has been great interest in the potential use of non-invasive routes of vaccine administration through the skin and the nose. The advantages of these approaches include increased safety and acceptability, as well as induction of mucosal immunity.

2.0 LITERATURE REVIEW

2.1 Disease control through vaccination: implications for vaccine design

On a global scale, infectious diseases remain the number one cause of morbidity and mortality. Vaccination has had the greatest impact on human and animal health of any medical intervention. In livestock animals it constitutes the most cost-effective method to reduce economic loss and animal suffering from infectious diseases.

Implementation of effective immunization regimes requires a thorough understanding of the host responses, the pathogen and the immune responses involved in inducing protection. Advances in molecular biology and immunology have contributed greatly to the identification and characterization of protective antigens from pathogenic microorganisms. Antigens must be recognized as foreign for immune responses to occur. Immune mechanisms have evolved to recognize "danger" signals expressed on microorganisms.

Regardless of whether vaccines are used for animals or humans, injection site reactions are of major concern. These reactions can be due to the vaccine itself, to contaminating components such as lipopolysaccharides (LPS), or to the adjuvants required to improve the efficacy of the vaccine, as well as the method of delivery. It is imperative that better vaccines, adjuvants and delivery systems be developed to reduce unwanted side effects and improve the safety of vaccination. Depending on the disease, several requirements are necessary for vaccines to be effective: safety, prolonged protection, induction of protective antibody and cellular immunity. Manufacturing issues such as stability of the formulation, ability to produce large quantities and easy administration are also important parameters to consider.

New generation vaccines are being designed to elicit cellular immune responses, especially of the Th1 type. Such responses are not only important for targeting infectious diseases that may have an intracellular stage (e.g. HIV1, herpes viruses, hepatitis C, *Plasmodium falciparum*, *Mycobacterium tuberculosis*), but also for the development of therapeutic vaccines against cancer, autoimmune diseases or allergies (Moingeon, 2002a). New vaccines are also being developed to elicit mucosal immune responses, which involve the exploration of new routes of administration, new formulations, and new adjuvant systems.

2.1.1 Conventional and new generation vaccines

Many of the vaccines used routinely today are whole-organism vaccines, consisting of live attenuated, or killed whole bacteria or viruses. Live attenuated vaccines are often able to induce strong, long-lasting immunity. However, there exists a risk of reversion to virulence, which can lead to disease. Killed vaccines, on the other hand, cannot replicate and are therefore non-infectious, but are less powerful than live vaccines at inducing protective immunity.

Advances in molecular biology, antigen identification and expression have allowed implementation of vaccine strategies based on genetically engineered vaccines that are not only more effective at inducing the correct type of immune response, but also make the vaccine safer.

In order to induce effective immune responses, different types of vaccines require specific delivery methods as well as special formulations. For example, live vaccines require minimal formulation and can be delivered through different routes. On the other hand, recombinant subunit vaccines are less immunogenic than conventional vaccines and therefore generally they require formulation with stronger adjuvants or delivery systems to stimulate appropriate immune responses.

2.1.1.1 Inactivated vaccines

Inactivated vaccines are produced by treating the infectious agent so that it cannot replicate in the host. Traditionally, chemicals such as formalin are used in the deactivation process. However, studies have shown that most inactivating agents have an impact on the immunogenicity of the protective proteins, and a balance is therefore sought between inactivation and reduction in immunogenicity (Duque, *et al.*, 1989; Ferguson, *et al.*, 1993). On the other hand, incomplete inactivation may lead to disease. A major disadvantage of killed vaccines is that they are not very immunogenic; therefore, they need to be combined with strong adjuvants to improve their efficacy (Audibert and Lise, 1993). Although many adjuvants have been shown to be effective, only a few are licensed for human use (e.g. alum). One disadvantage of adjuvants such as alum is that the spectrum of immunity they induce is generally narrow (Gupta, 1998); immune responses after the administration of inactivated vaccines is typically characterized by induction of humoral responses, with limited cell-mediated or mucosal immunity. The inability of the vaccine to replicate in the host minimizes induction of cytotoxic cellular responses.

2.1.1.2 Live attenuated vaccines

Live vaccines consist of microorganisms that replicate in the host similarly to the natural microorganism, thereby eliciting an immune response similar to that elicited by the natural infection. Attenuation is achieved by passaging the agent *in vitro* either in the presence of mutagenizing agents or under various *in vitro* culturing conditions. Following a number of passages, the variants are selected for reduced virulence. Increased knowledge of the virulent genes of

many pathogens is allowing introduction of multiple directed mutations or deletions in specific genes in order to reduce virulence. Using such approaches is possible to develop more stable mutations and decrease the risk of reversion to virulence.

Viruses (e.g. vaccinia, adenovirus, poxvirus) or bacteria can also be used as carriers or vectors for genes from other pathogens. The strategy is to present the recombinant antigen in the context of a live virus infection so that the immune system responds to the vaccine antigen as it would in a natural infection where stimulation of humoral as well as cellular immune responses occurs. However, the immune response to the vector *per se* may limit the effectiveness of revaccination. The efficacy of vaccine vectors can be further increased by incorporation of cytokines or immunomodulatory genes into the vector.

Live vaccines induce a broader immune response (humoral and cellular) than killed vaccines, similar to that induced by natural infections. Furthermore, they can be delivered via the natural routes of infection, such as mucosal surfaces, thereby providing a broader range of immune responses and consequently better protection, especially at the site of entry of the pathogen. Another advantage of live vaccines is that since they replicate in the host, they generally induce a longer duration of immunity than killed vaccines. Although these vaccines are generally very effective, there has been concern about their potential safety.

2.1.1.3 Subunit vaccines

Subunit vaccines contain one or more pure or semipurified antigens, which are selected based on the understanding of the pathogenesis of the microorganism and the proteins involved in the induction of protective immunity.

Among the advantages of using subunit vaccines there is particularly their safety since there are no risks of reversion to virulence, also, they have lowered antigenic competition among irrelevant proteins, an ability to target the vaccine to a site where immunity is required, and the potential ability to differentiate vaccinated from infected animals.

2.1.1.4 DNA vaccines

DNA vaccines consist of plasmid DNA expression vectors, which encode the antigen or antigens of interest under the control of strong viral promoters recognized by the mammalian host. When the plasmid is administered to an animal, the antigen is expressed endogenously and therefore, all the posttranslational modifications are similar to those occurring during a natural infection.

DNA vaccines induce a broad range of immune responses. DNA expression plasmids have proven to be advantageous in eliciting cytotoxic T lymphocyte (CTL), and T helper (Th) responses, as well as humoral immunity to encoded antigens (reviewed by Babiuk, *et al.*, 1999).

The immune responses to DNA vaccines can be enhanced by the DNA acting as its own adjuvant, through the immunostimulatory properties of CpG motifs. Several strategies have been tested to manipulate the immune response generated upon DNA immunization, such as co-delivery of plasmid encoding co-stimulatory molecules (CD86) or cytokines (IL-12, GM-CSF) (Kim, *et al.*, 1997a; Kim, *et al.*, 1997b). The immune responses induced by DNA vaccines are influenced by the method of immunization, e.g. Th1 response by saline injection versus Th2 response using a gene gun (Feltquate, *et al.*, 1997). The Th1/Th2 balance may also be affected by the dose of antigen (Barry and Johnston, 1997) or by its cellular location (Lewis, *et al.*, 1996).

This vaccination method offers a number of advantages: simplicity of producing large quantities, applicability to various pathogens, ability to induce cellular immune responses through MHC class I presentation, and the potential to manipulate the immune response through the co-delivery of genes encoding immunologically relevant molecules. Another advantage is that DNA vaccines can induce immune responses in neonates even in the presence of passive antibody (Lewis, *et al.*, 1999; Van Drunen Littel-van den Hurk, *et al.*, 1999). One of the major problems with DNA vaccines is decreased gene expression due to the rapid degradation of plasmid in vivo. To overcome this problem, various delivery systems are being explored including: 1) injection by microneedles, pressure injection, or particle bombardment; 2) electroporation; and 3) liposomes, or encapsulation of DNA in various polymers.

A number of safety questions need also to be considered when delivering DNA vaccines to mammalian hosts, the DNA could integrate into the host genome or anti-DNA antibodies could be induced. However, none of these possible dangers have so far been observed in animal studies.

2.2 The role of adjuvants and delivery systems

The word 'adjuvant' is derived from the Latin word 'adjuvare', which means 'to help'. Adjuvants encompass a highly heterogeneous group of substances commonly used in vaccines to enhance or modulate immune responses. The use of adjuvants to enhance vaccine immunogenicity was introduced in the 1920s. The development of novel adjuvants over the past two decades has been critical for the advancement of recombinant subunit vaccines, which are poorly immunogenic (Newman and Powell, 1995).

A wide range of adjuvants and delivery systems have been designed and tested experimentally. They include mineral compounds (e.g. aluminum hydroxide or aluminum phosphate); w/o (e.g. incomplete Freund's adjuvant) or

o/w (e.g. MF59) emulsions; chemically or genetically detoxified bacterial toxins, such as cholera toxin (CT) or heat labile enterotoxin (LT); saponins (QuilA, QS21); muramyl di- or tripeptides and derivatives (MTP-PE); copolymers; ISCOMS; cytokines and CpG ODN.

Only few adjuvants are licensed for human or veterinary use. In animals, some adjuvants are associated with the induction of local inflammatory responses and strong tissue damage; these problems have hampered the use of some of these adjuvants. Therefore, improvements in vaccine formulations should balance the safety aspect with the ability of stimulate immune responses.

Recent advances in the understanding of immune mechanisms have led to a more 'rational' design of adjuvants and formulations, which should have the following properties:

- Attract antigen-presenting cells (APCs). A range of molecular stimuli providing 'danger signals' to the immune system has recently been identified; including double stranded (ds) RNA (Cella, *et al.*, 1999), lipopolysaccharides (LPS) (Rietschel, *et al.*, 1996), and unmethylated CpG motifs (Wagner, 1999). Most of these molecules appear to function as ligands for Toll-like receptors (TLR3, TLR4 and TLR9, respectively), and can rapidly stimulate immune cells to produce pro-inflammatory cytokines, including IL-1, IL-6, IL-12, IL-18, TNF-α and IFN-γ.
- Target APCs. Formulating antigens to better target APCs might be facilitated by the recent identification of a variety of surface receptors expressed preferentially by APCs such as the high affinity receptor for IgGs (FcγRI, CD64) (Fanger, *et al.*, 1997). There is evidence that targeting the antigen to such surface receptors allows antigen internalization, and presentation to T-cells in an MHC class I restricted manner (Raychaudhuri and Rock, 1998).

- Induce dendritic cell (DC) maturation by cross-linking CD40 molecules with CD40L or anti-CD40 antibodies, mimicking signals associated with T-cell help (Moingeon, *et al.*, 2001).

Adjuvants can influence many parameters of immune responses including the specificity, type, intensity and duration. Some may facilitate long-term persistence of the antigen at the injection site ('depot' effect); others may target APCs by presenting antigens in a particulate state, or may specifically elicit the production of a pattern of cytokines relevant to the induction of a Th1 or Th2 response (Schijns, 2000).

Proposed modes of action of adjuvants include: 1) depot effect at the site of injection, providing prolonged release and interaction of antigen with APCs; 2) enhanced uptake and transport of both antigen and adjuvant to draining lymph nodes by macrophages and DC; 3) provision of "danger signals" or a pathogen-associated microbial pattern (PAMP).

2.2.1 General overview of the different types of adjuvants used in vaccines

Attempts to organize adjuvants in categories have been difficult because of multiple and overlapping biological effects of many adjuvants; thus, many compounds can be included in more than one category. A practical categorization of different types of immunostimulators proposed by Edelman (Edelman and Tacket, 1990) includes: adjuvants *per se*, carriers, and vehicles. The adjuvant category includes aluminum salts, saponin, muramyl di- and tripeptides, monophosphoryl lipid A, *Bordetella pertussis* and cytokines. The carriers, which mainly provide T cell help, include bacterial toxins, fatty acids and living vectors. The vehicle category includes mineral oil emulsions, biodegradable oil emulsions, non-ionic block copolymer surfactants, liposomes, and biodegradable polymer microspheres. "Adjuvant formulations" are also proposed as a separate category defined as mixtures of the various categories

(Edelman and Tacket, 1990). Many adjuvants do not possess all the features required for optimal induction of immunity. Therefore, the most effective formulations have generally resulted from the combination of two or more adjuvants (Baca-Estrada, *et al.*, 1997; Thoelen, *et al.*, 1998; Weeratna, *et al.*, 2000).

Adjuvants can also be classified according to their mode of action: 1) immunomodulation (modification of cytokine networks); 2) targeting APCs; 3) enhancing antigen presentation; 4) CTL induction; and, 5) depot generation (Cox and Coulter, 1997; Singh and O'Hagan, 1999).

2.2.1.1 Aluminum Adjuvants

Aluminum hydroxide and aluminum phosphate are particulate adjuvants widely used in human and veterinary vaccines. Their biologic activity consists of:

- Formation of antigen depot in tissues to produce prolonged exposure.
- Production of particulate antigens to facilitate targeting to APC.
- Activation of complement and stimulation of macrophages to induce retention and activation of lymphocytes.

Aluminum adjuvants typically are effective in enhancing antibody responses to protein antigens. They induce Th2 type responses, but they are not effective for inducing cell-mediated immunity or for peptide antigens. In addition, aluminum salts promote the stimulation of IgE antibodies, potentially inducing hypersensitivity reactions (Mancino and Ovary, 1980; Mark, *et al.*, 1997). Therefore, despite the success of aluminum salts as vaccine adjuvants and their extensive use in licensed human and animal vaccines, there is a need to develop adjuvants that promote a wide range of immune responses with minimal side effects.

2.2.1.2 Synthetic adjuvants

A number of chemicals have been shown to stimulate or activate cells of the immune system, suggesting their potential as adjuvants in vaccine formulations. Avridine, a lipid amine, can induce the synthesis of interleukin 1 (IL-1) and interferon α (IFN-α) *in vitro* and, has been shown to enhance both cellular and humoral immunity to vaccines *in vivo*, especially if incorporated in oil emulsions (Hughes, *et al.*, 1991). Similarly, dimethyl-dioctadecyl ammonium bromide (DDA), another lipid amine, can enhance antibody responses and these responses can be augmented when coadministered with trehalose dimycolate (Dzata, *et al.*, 1991).

2.2.1.3 Oil emulsions

The most classical examples of oil emulsions are the incomplete and complete Freund's adjuvants. However, the use of both emulsions has been restricted due to their reactogenicity. Less toxic and equally effective alternative formulations are now commercially available; some examples are TiterMAx, Ribi, and Lipovant. MF59A is a w/o emulsion which has proven effective in a large number of experimental human vaccines (Ott, *et al.*, 1995; Heineman, *et al.*, 1999) and it is already incorporated in a commercial vaccine (Aguado, *et al.*, 1999).

The use of mineral oils and emulsions in veterinary vaccines creates concern regarding the cost of injection site reactions and residues induced by this type of adjuvant, due to the fact that mineral oils are not metabolized. Plant-derived oils are being investigated as replacements for mineral oils since they are metabolized. In addition to providing oil components for vaccine adjuvants, some plant components have immunomodulatory properties. A plant derivative from *Quillaja saponaria*, a saponin (QuilA), has demonstrated its adjuvant

activity when combined with T-dependent and T-independent antigens (Kensil, *et al.*, 1991). Although crude QuilA has toxic activity, different fractions have been shown to have reduced toxicity, retaining adjuvant activity (White, *et al.*, 1991). QS-21 is used in various vaccines and its effects can be further enhanced by combining it with other adjuvants (Wu, *et al.*, 1992).

2.2.1.4 Lipid-based delivery systems

Vehicles composed of particulate structures, such as immune-stimulating complexes, liposomes, virosomes, etc., may facilitate the capture and the entry of the antigen into APCs.

Immune-stimulating complexes (ISCOMs) are symmetrical colloidal particles with an open cage-like structure in the size range of 30-100 nm, composed of the saponin-adjuvant QuilA, cholesterol, phospholipids and the antigenic protein of interest. Thus, they act as delivery vehicles and also enhance the immune response to the antigen. ISCOMs have been used in conjunction with glycoproteins from enveloped viruses (Sjolander, *et al.*, 1997; Rimmelzwaan, *et al.*, 2001) and have been delivered by parenteral or mucosal routes (Hu, *et al.*, 2001; Mohamedi, *et al.*, 2001; Mowat, *et al.*, 2001). Generally, ISCOM-based vaccines induce both antibody and cellular responses (Mowat, *et al.*, 2001; Sambhara, *et al.*, 2001; Windon, *et al.*, 2001).

The adjuvant activity of liposomes was also established in a large number of studies (Gregoriadis, *et al.*, 1987; Gregoriadis and Panagiotidi, 1989; Alving, 1991). Liposomes are composed of phospholipid bilayers that enclose aqueous compartments. Many types of liposome structures were prepared over the last twenty years, e.g. multilamellar vesicles (MLV), large unilamellar vesicles (LUV), small unilamellar vesicles (SUV), oligolamellar vesicles (OLV) and multivesicular liposomes (MVL). They can also vary in their stability, fluidity, and permeability

depending on the ratio of phospholipid to cholesterol. Liposomes have been used since the late 1960's, initially as model membranes and drug carriers for studying cell membrane interactions *in vitro*.

In early studies, traditional liposomes were shown to have potential for the delivery of many small molecules, proteins, peptides and DNA in general (Allison and Gregoriadis, 1974; Heath, *et al.*, 1976; Shek and Sabiston, 1982; White, *et al.*, 1995; Gregoriadis, *et al.*, 1997). Since then, a wide variety of liposomal formulations have been evaluated both as adjuvants and as delivery systems for antigens and adjuvants, eliciting protective immune responses against bacterial, viral and parasitic infections (Fries, *et al.*, 1992; Reddin, *et al.*, 1998; Mader, *et al.*, 2000; Ninomiya, *et al.*, 2002).

The adjuvant effect of liposomes can be further enhanced by incorporation of immunomodulatory molecules together with vaccine antigens (Kersten and Crommelin, 1995; Babai, *et al.*, 1999; Childers, *et al.*, 2000; Joseph, *et al.*, 2002). In this way, the two molecules are delivered simultaneously to the same site. It has been shown in a number of models that this type of lipid vaccine formulation is more efficient at inducing immunity than simple mixtures of antigen and adjuvant alone (Abraham and Shah, 1992a; Baca-Estrada, *et al.*, 1997; Li, *et al.*, 2001; Li, *et al.*, 2003).

Improvements were made to liposomes to further enhance vaccine delivery; protein cochleate formulations, virosomes, transfersomes, and biphasic delivery systems have been developed and tested (Mannino and Gould-Fogerite, 1995; Ambrosch, *et al.*, 1997; Paul, *et al.*, 1998; Baca-Estrada, *et al.*, 2000b). Lipid based delivery systems also offer advantages in safety over conventional oil adjuvants. They can be made from biodegradable plant derived and synthetic lipids and have shown to be safe (Ben-Yehuda, *et al.*, 2003).

In addition to their safety, liposomes were shown to stimulate both humoral and cell-mediated immunity, including CTL responses (Reddy, *et al.*,

1992; Bacon, *et al.*, 2002; Bala, *et al.*, 2002; Li, *et al.*, 2003). Furthermore, liposomes can potentiate mucosal immune responses as demonstrated in a number of models, by both intranasal (Aramaki, *et al.*, 1994; el Guink, *et al.*, 1989; Baca-Estrada, *et al.*, 2000a; Sakaue, *et al.*, 2003), and oral (Fujii, *et al.*, 1993; Guzman, *et al.*, 1993; Perrie, *et al.*, 2002; Minato, *et al.*, 2003) routes. In addition, lipid based formulations were shown to be effective after transcutaneous delivery of vaccines in mice (Baca-Estrada, *et al.*, 2000b; Babiuk, *et al.*, 2002).

2.2.1.5 CpG ODN

The immunostimulatory properties of bacterial DNA were first discovered in studies of tumor regression (Tokunaga, *et al.*, 1988). The functional sequences of bacterial DNA were identified as CpG motifs flanked by two 5' purines and two 3' pyrimidines. CpG motifs are found at a much higher frequency in bacterial DNA than mammalian DNA (Krieg, 1999). In addition, these motifs are not methylated in bacterial DNA, whereas they are methylated in mammalian DNA. This difference in methylation is critical for CpG activity since methylation abolishes their immunostimulatory activity (Krieg, 1999).

The utility of CpG as a vaccine adjuvant was suggested by its strong activating effect on B cells and the ability to induce cytokine secretion (IL-6, IL-12, IFN-γ, IFN-α). These effects have been shown to have direct and indirect effects on B and T cells (Krieg, *et al.*, 1995; Jakob, *et al.*, 1998; Krieg, 1999). In addition, CpG ODNs induce maturation and activation of DC (Sparwasser, *et al.*, 1998; Sparwasser, *et al.*, 2000). The ability of specific CpG motifs to activate subsets of human DC supports the use of CpG ODNs for immunotherapy against cancer, allergy, and infectious diseases (Hartmann, *et al.*, 1999; Peng, *et al.*, 2001; Jahrsdorfer and Weiner, 2003).

CpG ODN must enter cells to elicit its effects (Hacker, *et al.*, 1998). Recently, a Toll-like receptor was found which recognizes bacterial DNA, suggesting that binding to this receptor is essential for CpG immunostimulatory activity (Hemmi, *et al.*, 2000). The adjuvanticity of CpG is associated with the induction of both pro-inflammatory and Th1-type cytokines and chemokines, and the induction of MHC and costimulatory molecules on APCs. The resulting immune responses in mice are Th1 biased with high levels of cytotoxic T lymphocytes (CTL), IFN-γ and IgG2a production (Krieg, 2000). *In vivo* and *in vitro* studies confirm that CpG ODN can also activate the immune system in a variety of domestic species including cattle, sheep, pigs, horses, dogs, cats, and chickens (reviewed by Mutwiri, *et al.*, 2003). Porcine peripheral blood mononuclear cells respond to CpG ODN by proliferating and secreting IL-6, IL-12 and TNF-α (Kamstrup, *et al.*, 2001), effects similar to those reported in mice and humans.

CpG motifs have been used as adjuvants with subunit as well as killed viral vaccines (Davis, *et al.*, 1998; Hancock, *et al.*, 2001; Hogarth, *et al.*, 2003). Synthetic CpG ODNs were shown to serve as potent adjuvants for the induction of mucosal immune responses (McCluskie and Davis, 1998; McCluskie, *et al.*, 2000a; McCluskie, *et al.*, 2000b; Jiang, *et al.*, 2003), and have synergistic action with other adjuvants (Weeratna, *et al.*, 2000; McCluskie, *et al.*, 2001; Ioannou, *et al.*, 2002b; Jiang, *et al.*, 2003).

Several reports have shown the importance of close physical association of CpG ODN with the antigen to enhance its adjuvant effect (Tighe, *et al.*, 2000; Li, *et al.*, 2003). In a study aimed to investigate the benefit of using liposomes to deliver CpG ODN as an adjuvant for T independent antigens Li *et al* showed that a hapten-specific response was obtained when CpG ODNs were encapsulated in biotinylated liposomes, a poorly immunogenic model, but not when co-administered (Li, *et al.*, 2001). The incorporation of CpG ODN and antigen into

liposomes provides long *in vivo* stability and results in the increased induction of cytokines, and enhanced interaction with APCs (Agrewala, *et al.*, 1996; Mui, *et al.*, 2001; Joseph, *et al.*, 2002).

2.2.2 Immunomodulation: a cytokine's world

Protection against infectious agents is determined, in part, by the pattern of cytokines secreted by the different subsets of T lymphocytes. Encounter of pathogen-derived or vaccine antigen will stimulate T helper cells to secrete cytokines and once an immune response is induced, it tends to get polarized to one subtype. Thus, it is important that the correct immune responses are generated following vaccination. The type and the magnitude of the immune response induced by a particular antigen are the result of multiple factors such as the biochemical properties of the molecule, the type of adjuvant used, and the method of delivery. Hence, a single adjuvant or delivery system may not be universally available.

The concept of polarization of immune responses following antigen stimulation is based on the existence of Th1 and Th2 regulatory subsets of Th cells, capable of driving the immune response preferably towards cellular immunity, or antibody production, respectively. This concept has been largely documented in murine systems [Abbas, 1996 #1; Carter, 1996 #32; Mosmann TR, 1996 #152]. Naïve CD4+ Th cells (Th0 cells) can differentiate in presence of the antigen towards either Th1 or Th2 cells, depending on the cytokine environment (Rengarajan, *et al.*, 2000). For example, intracellular pathogens will induce production of IL-12 or IL-18 by activated macrophages and dendritic cells, driving the differentiation of Th0 cells toward a Th1 phenotype producing IFN-γ, IL-2 and TNF-β. Th1-type responses are associated with class I-restricted CTL responses mediated by CD8+ T cells, delayed-type hypersensitivity, production of complement-fixing antibodies and IgG2a/IgG2b isotypes in mice.

Precursor Th cells are differentiated into Th2-type cells by dendritic cells matured in the presence of products from helminth pathogens and allergens under the influence of IL-4. Th2 responses are characterized by production of IL-4, IL-5, IL-6, IL-10 and IL-13, which support humoral immunity, with IgG1and IgE production (Figure 2.2.1). Antigen dose, strength of signals transduced through the T cell receptor, co-stimulation signals provided by different subsets of dendritic cells, all appear to play a role in Th1/Th2 polarization. Th0 cells can be induced to differentiate into the Th1 or Th2 lineage *in vitro* in presence of the antigen by the addition of exogenous cytokines (Rengarajan, *et al.*, 2000). In presence of IL-12 secreted by macrophages or dendritic cells, Th1 differentiation is initiated. Conversely, IL-4 stimulation of Th0 cells initiates Th2 differentiation. Inactivated pathogens, recombinant proteins or peptides appear to be rather inefficient in terms of inducing Th1 responses. Other vectors, in contrast, appear to elicit potent T cell responses. Such vectors express the antigen inside professional APCs such as macrophages and dendritic cells. In this regard, live attenuated pathogens, lipopeptides, recombinant viruses (poxvirus, alphavirus) have provided some encouraging results (Bonnet, *et al.*, 2000).

The ability of cytokines to influence not only the magnitude, but also the type of immune response induced when co-administered with a vaccine antigen has been demonstrated in several animal models (reviewed in Heath, 1995). Cytokines capable of influencing the magnitude and phenotype of the immune response offer the potential to selectively tailor vaccines capable of inducing protection against different diseases. However, the potential side effects need to be addressed before this technology can be employed.

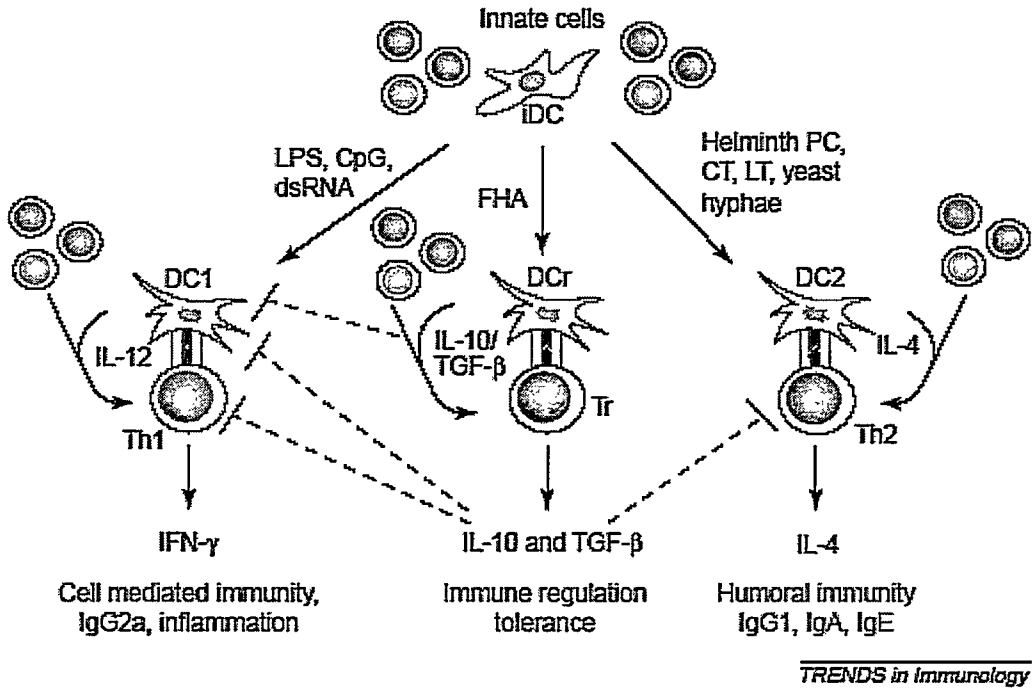

Figure 2.2.1 Proposed model for T-cell differentiation and function in immunity to infection.
Certain pathogen-derived immunomodulatory molecules bind to dendritic cells (DCs) or other innate cells, including macrophages, and stimulate maturation of immature DCs (iDCs) into DC1 and DC2, which direct the differentiation of Th1 and Th2 cells, respectively. Other pathogen-derived molecules might activate maturation of iDCs into DCs, which direct the induction of regulatory T (Tr) cells (designated DCr). The function of Tr cells is to suppress Th1, and in certain cases, Th2 responses, by the release of anti-inflammatory cytokines or contact-dependent mechanisms, acting directly on the T cell or the antigen presenting cell (APC). From McGuirk and Mills (2002) with permission from Elsevier Science.

2.3 Routes of immunization

Currently, the majority of vaccines are delivered parenterally, which does not induce mucosal immunity, and therefore one important component of the immune system is not primed. There are also several concerns regarding parenteral delivery of vaccines such as broken needles in animal carcasses, tissue damage, accidental needle sticks, and spread of disease from reusing needles. Finally, because of the pain associated with needles, compliance may be reduced. The use of non-invasive routes for vaccine delivery that allow efficient uptake of antigen by APCs is necessary to make administration simple, painless and economically practical. Therefore, the goal is to improve the efficacy of vaccines using noninvasive methods of administration such as mucosal and transcutaneous delivery.

2.3.1 Mucosal delivery of vaccines

Increased awareness of the fact that most infectious agents use mucosal membranes as frequent portal of entry has directed efforts to the development of vaccines and appropriate antigen delivery systems that can efficiently induce mucosal immunity. The importance of stimulating mucosal immune responses as a first line of defense against invading pathogens was recognized by a number of investigators (Haan, *et al.*, 2001). Mucosal immunization offers many benefits including reduced vaccine-associated side effects and the potential to overcome the known barriers of parenteral vaccination caused by either pre-existing systemic immunity from previous vaccination or in young animals from maternal antibodies (Capozzo, *et al.*, 2004).

Mucosal delivery of vaccines induces mucosal immunity more efficiently than parenteral immunization (McGhee, *et al.*, 1992; Liu, *et al.*, 1998; McCluskie, *et al.*, 2002; Goonetilleke, *et al.*, 2003). However, most vaccines licensed for use

in humans and animals are injected intramuscularly or subcutaneously and fail to generate mucosal immunity. Few of the current vaccines that are approved for human use are administered mucosally: the modified live oral polio vaccine, the oral killed whole-cell B subunit and live-attenuated cholera vaccines, an oral live-attenuated typhoid vaccine, and an oral adenovirus vaccine restricted for military use. Two additional mucosal vaccines, an oral live-attenuated vaccine against rotavirus diarrhea and a nasal enterotoxin-adjuvanted inactivated influenza vaccine, were withdrawn after a short time on the market because of potential serious adverse reactions, illustrating the complexity of mucosal vaccine development (Murphy, *et al.*, 2001). The ineffectiveness of simple delivery of soluble antigens to mucosal membranes for immunization has stimulated extensive studies of strategies for delivery systems that would: a) increase the antigen absorption; b) prevent its degradation; and, c) induce the desired type of immune response (B vs. T cell response; mucosal vs. systemic; protective vs. tolerance). Hence, current efforts to overcome obstacles to the development of effective mucosal vaccines are mainly directed towards finding a more efficient means of delivering appropriate antigens to the mucosal immune system, and towards discovering effective, safe mucosal adjuvants or immunoregulatory agents that provide protective immunity against infections.

2.3.1.1 The nose as a non-invasive site of immunization

The nasal mucosa is an important arm of the mucosal system since it is often the first point of contact for inhaled antigens. There are several reasons why the nose is an attractive route for immunization (Partidos, 2000):

- Easily accessible.
- Highly vascularized.

- Presence of numerous microvilli covering the nasal epithelium generates a large absorption surface.
- Mucosal and systemic immune responses can be induced.
- Immune response can be induced at distant mucosal sites through dissemination of effector immune cells in the common mucosal immune system.
- The nose can be used for the easy immunization of large population groups.
- Nasal immunization does not require needles and syringes, which are potential sources of infection.

A range of antigens with a variety of adjuvants or delivery systems have been shown to be immunogenic by the nasal route. Clinical trials using nasally delivered vaccines have been performed with influenza virus glycoproteins and LT (Gluck, *et al.*, 1999; Gluck, *et al.*, 2000), diphtheria and tetanus toxoids with polysorbate and glycerides (Aggerbeck, *et al.*, 1997), live attenuated respiratory syncytial virus (Gonzalez, *et al.*, 2000), group B meningococcal vesicles (Drabick, *et al.*, 1999), liposome-formulated antigen from *Streptococcus mutans* (Childers, *et al.*, 1999) and killed whole cell pertussis alone (Berstad, *et al.*, 2000), among others. Most of the trials have reported good immunogenicity and none have reported major adverse reactions. However, a recent study in mice, suggested that formulation of vaccines with CT might help to direct nasally delivered antigens to neural tissue via the olfactory bulb (van Ginkel, *et al.*, 2000a).

2.3.1.2 Mucosa-associated lymphoid tissue (MALT)

Mucosal defense against pathogens involves innate barriers, such as mucous, proteolytic enzymes, epithelium, and innate immune mechanisms, in addition to a highly specialized immune system.

The organized lymphoid tissue in the small and large intestine, rectum and appendix is collectively referred to as the gut-associated lymphoid tissue (GALT). Similar organized lymphoid structures are found in bronchial-associated lymphoid tissue (BALT), and nasal-associated lymphoid tissue (NALT), including salivary glands, and nasopharingeal (NT) and palatine tonsils (PaT). The subepithelial regions of the gastro-intestinal, respiratory and nasal mucosa contain a considerable amount of immunocompetent B and T lymphocytes and other cell populations including macrophages and DC.

The respiratory immune system can be divided into three parts (Sminia and Kraal, 1999):

- An epithelial compartment at the surface of the epithelium and the underlying connective tissue that contains immunocompetent cells.
- Lymphoid structures of the nose and bronchus-associated epithelium: NALT, larynx-associated lymphoid tissue (LALT), and BALT.
- Lymph nodes draining the respiratory system.

The epithelium in the nasal cavity is covered by numerous microvilli and hence a large surface area is available for absorption. Four different cell types may be found in the epithelial barrier, alveolar macrophages, DC, M-cells and intraepithelial lymphocytes. The subepithelial layer is highly vascularized and the venous blood from the nose passes directly into the systemic circulation. A mucus layer covers the respiratory epithelium, protecting the underlying tissue by acting as a barrier to the diffusion of solutes such as enzymes, antigens, and drugs and also by the mucociliary clearance mechanism to remove any inhaled molecules. Theoretically, the mucus layer is renewed every 10 to 15 min. Thus,

a major factor responsible for the difficulties in inducing immune responses after nasal application is competition between absorption and removal of molecules by the mucociliary clearance mechanism from the site of absorption.

NALT is a well-organized structure, consisting of B- and T-cell areas, which are covered by an epithelial layer containing microfold M-cells. NALT bears certain similarities to the Peyer's patches (PP), but the two differ markedly in morphology, lymphoid migration patterns and the binding properties of the high endothelial venules (HEV) (Kuper, *et al.*, 1992). In some species, including man, the tonsils represent an important part of the NALT and they are capable of a variety of complex immunological functions. The tonsils are organs of local immunity and the PaT and NT appear to be engaged continuously in a process of immune surveillance and protection.

2.3.1.3 Common mucosal immune system

Antigenic exposure at mucosal sites activates mucosal B and T lymphocytes to migrate from the inductive site and home to various mucosal effector sites. In this way, immune responses that originate in one mucosal site disseminate to other mucosally associated lymphoid sites. Thus, the common mucosal immune system (CMIS) involves homing of antigen-specific lymphocytes to mucosal effector sites other than the site where initial antigen exposure occurred. This migration of cells between mucosal sites forms the basis for intranasal or oral immunization strategies aimed at improving immunity at other mucosal sites where the response is necessary to protect against a particular invading microorganism. Hence, this functional integration of the mucosal immune system can be exploited for vaccine development but accumulating evidence suggests that regionalized homing mechanisms must be taken into account since immune responses at mucosal-effector sites are not

uniform (Russell and Wu, 1991; Wu and Russell, 1993; Moldoveanu, *et al.*, 1995). Homing from GALT appears to be determined mainly by an integrin on primed cells, interacting with a mucosal addressin cell adhesion molecule expressed on the microvascular endothelium in the intestinal lamina propria. Other adhesion molecules appear to be employed by immune cells primed in BALT and NALT. The urogenital tract might employ similar molecular homing mechanisms as those of the upper respiratory and digestive tracts and therefore, appears to receive primed cells from the NALT (Brandtzaeg, *et al.*, 1999a; Brandtzaeg, *et al.*, 1999b). Hence, proper stimulation of a mucosal-inductive site can induce immune responses at remote mucosal-effector sites. However, stimulation of different mucosal-inductive sites induces immune responses that are distributed unevenly at different effector sites (Figure 2.2.2). An explanation for the differences in generating non uniform responses by stimulating different mucosal sites is not clear, but it may be related to: anatomical location and physiological environment of the mucosal-inductive sites; differences in the dissemination of immune cells; and, limitations in the migration of immune memory cells to other sites after stimulation. Nasal vaccine delivery is superior to oral delivery in inducing specific immunoglobulin A (IgA) and immunoglobulin G (IgG) responses in the upper respiratory tract (Rudin, *et al.*, 1999). The first study to investigate this approach in humans found that nasal vaccination was a good way of inducing high titers of antibodies in vaginal secretions, but not in cervical secretions. However, nasal administration has emerged as the optimal vaccination route for the induction of genital antibody responses in rodents (Baldridge, *et al.*, 2000; Gallichan, *et al.*, 2001). Consequently, the rational design of nasal vaccines for clinical use depends on the availability of information about the inductive conditions and in particular, the migratory properties of mucosal B cells (Brandtzaeg, *et al.*, 1999b).

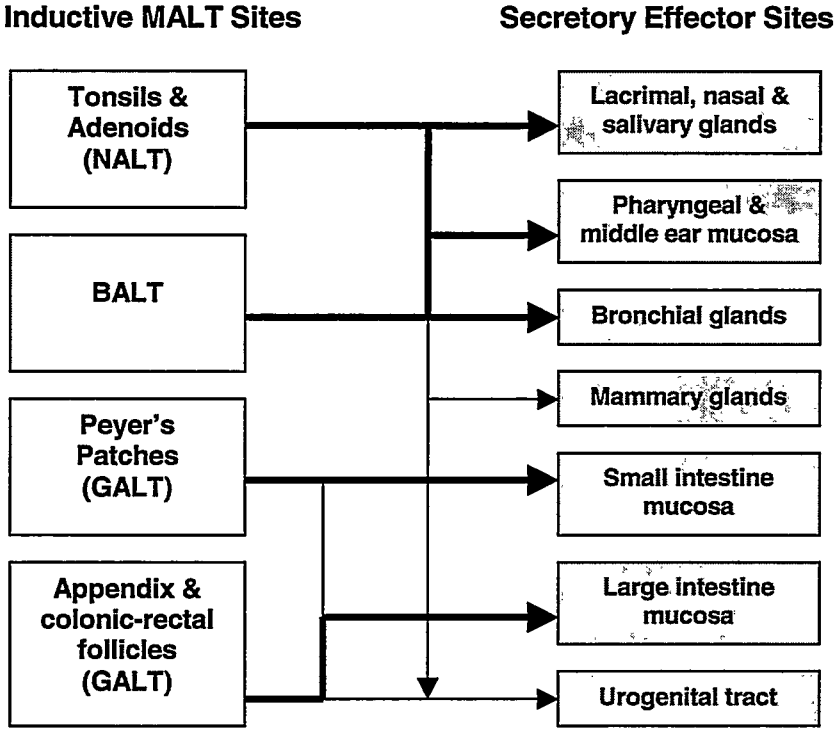

Figure 2.2.2 Homing of primed lymphoid cells.
Putative regionalization in communication between inductive and effector sites is indicated, the heavier arrows representing preferential B cell migration pathways. From Davis (2001) with permission from Elsevier Science.

2.3.1.4 The immune response following intranasal administration

The administration of an antigen to a mucosal surface can lead to different results. The balance between active immunity and tolerance is dependent on the nature of the antigen and its interaction with the mucosal inductive site (Partidos, 2000). Factors such as the dose, the use of an adjuvant, frequency of administration and genetic background of the host are contributing factors. Therefore, an understanding of the mechanisms that lead to a mucosal immune response is crucial in developing appropriate strategies to develop effective mucosal vaccines.

After intranasal immunization, the antigen is sampled and passed to underlying lymphoid cells in the submucosa where antigen processing and presentation take place. This results in the activation of T cells, which help B cells to develop into IgA plasma cells (Wu, *et al.*, 1997a; Wu and Russell, 1997b; Partidos, 2000). The NALT drains preferentially to the cervical lymph nodes (Kuper, *et al.*, 1992). The antigen can then elicit a local response or lead to tolerance (Sminia and Kraal, 1999). Thus, antigen specific B and T lymphocytes migrate from the NALT to regional lymph nodes and then into the circulation (Figure 2.2.3). These primed lymphoid cells can then home to distant effector sites.

The hallmark of the mucosal immune system is the production of secretory IgA (S-IgA). S-IgA may provide an early defense against invading pathogens, while serum IgG may be required for prevention of systemic infection, but also functions at mucosal surfaces (van Ginkel, *et al.*, 2000b). S-IgA is induced when antigens contact mucosal surfaces and are transported to mucosal lymphoid tissue. S-IgA may protect against pathogens that replicate on or enter via mucosal surfaces by blocking their attachment or colonization and

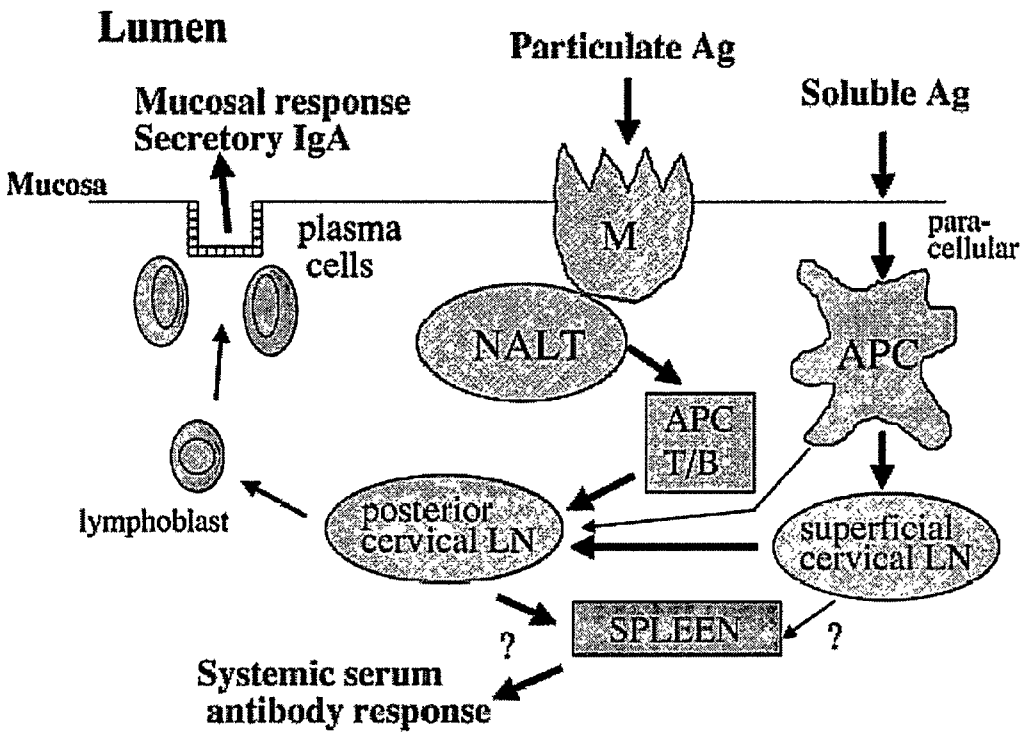

Figure 2.2.3 Hypothetical scheme of pathways eliciting a local mucosal response and a systemic response or tolerance, via NALT and nasal mucosa.

APC, antigen-presenting cell (macrophages, dendritic cells); M, microfold epithelial cell; NALT, nasal-associated lymphoid tissue; PCLN, posterior cervical lymph node; SCLN, superior cervical lymph node. From Davis (2001) with permission from Elsevier Science.

by neutralizing surface acting toxins. S-IgA results from transcytosis of polymeric IgA (pIgA) across the epithelium through binding to the polymeric immunoglobulin receptor (pIgR). Precursors of mucosal IgA plasma cells originate mainly in organized lymphoepithelial structures. These precursors that are committed to IgA synthesis mature in the regional lymph nodes and enter the circulation via the thoracic duct. They can then lodge in the lamina propria of distant mucosal sites where differentiation can occur. Hence, isotype switching occurs in mucosal inductive sites, while IgA production by plasma cells occurs in mucosal effector sites (McGhee, *et al.*, 1999). Each of these stages requires specific signals, such as co-stimulatory molecules, cytokines and Th cells, to give rise to antigen-specific S-IgA antibodies in mucosal effector sites. Either Th1 or Th2 cells or a combination of both may be important in supporting antigen-specific S-IgA responses. Th2-type responses have been shown to be important in the terminal differentiation of B cells (Mosmann and Sad, 1996), while the Th1 cytokine IFN-γ, has been implicated in the induction of the receptor required to transport S-IgA (Sollid, *et al.*, 1987). Studies by Hiroi et al. have suggested that NALT is characterized by a Th0 environment, which can derive into a Th1 and/or Th2 phenotype; in contrast, the nasal passage is considered to be a Th2 dominant site with some Th1 cells (Hiroi, *et al.*, 1998).

Evidence suggests that mucosal inductive sites contain specialized T cells or DC beneficial for the differentiation of B cells into IgA-producing cells. Studies have shown that activated T cells and DC from the PP are more effective in switching IgM^+ B cells to IgA-producing cells than T cells and DC from the spleen (Spalding, *et al.*, 1984).

2.3.1.5 Adjuvants and delivery systems for mucosal administration

It is clear that the performance of a nasal vaccine can be greatly influenced by the physical nature of the antigen and the chosen delivery system. Mucosal application of vaccines with an appropriate adjuvant can induce immune responses at both systemic and mucosal sites and as a consequence, may prevent not only infectious diseases but also colonization at mucosal surfaces (Wu and Russell, 1997b).

To elicit mucosal immunity, many approaches have exploited the immunomodulatory effects of soluble holotoxins, such as the holotoxin from *Vibrio cholerae* (CT), and its close relative *Escherichia coli* heat labile enterotoxin (LT), or *Bordetella pertussis* toxin (PT). CT and LT consist of two subunits: an enzymatically active A subunit with ADP-ribosylase activity that is responsible for the toxicity and B subunits that mediate binding to their receptors, the ganglioside GM1, on epithelial target cells. Although CT and LT have been shown to be effective mucosal adjuvants for the nasal delivery of numerous antigens (Rappuoli, *et al.*, 1999; Takahashi, *et al.*, 1996; Williams, *et al.*, 1999), their use may be restricted due to their toxicity. Therefore, attempts have been made to separate the toxic effects of these molecules from their adjuvant activity. Site-directed mutagenesis has permitted the generation of LT and CT mutants that have reduced toxicity but retain significant adjuvanticity when given to animals by the nasal route or even by the oral route (Pizza, *et al.*, 2001). Another approach is to link the enzymatically active A subunit to a cell-binding moiety such as the cell-binding domain of *Staphylococcus aureus* protein A (CTA1-DD). However, CTA1-DD functions when applied nasally but not when given orally (Agren, *et al.*, 1997). This problem has been addressed by the incorporation of CTA1-DD fused to a short peptide into ISCOMs. Oral vaccination with this complex induced systemic and mucosal responses, however, numerous immunizations were required, indicating that the procedure

needs further improvements (Mowat, *et al.*, 2001). To avoid toxicity, isolated CTB and LTB have been explored for their ability to augment immune responses against co-administered antigens, but their capacity as mucosal adjuvants has proven to be much less than that for the holotoxins (Pizza, *et al.*, 2001). Both CTB and LTB are poor adjuvants in animals when given together with non-coupled antigens by the oral route, although they display a more significant adjuvant activity when administered via the nasal route (Pizza, *et al.*, 2001). Mice vaccinated intranasally with an influenza virus HA vaccine together with LTB had higher levels of antiviral IgA and IgG, both in serum and in nasal and lung secretions compared with mice given the subunit vaccine alone, and were also protected against an intranasal viral challenge (Haan, *et al.*, 2001). Adjuvanticity of CTB or LTB is improved when antigens are coupled (George-Chandy, *et al.*, 2001).

The strong adjuvant action of CT and LT may be related to several effects, which include: a) increased permeability of the epithelium leading to enhanced uptake of co-administered antigen; b) enhanced antigen presentation by a variety of cell types; c) promotion of isotype differentiation in B cells leading to increased IgA formation; and, d) complex stimulatory as well as inhibitory effects on T-cell proliferation and cytokine production. CT has shown to increase antigen presentation by DC, macrophages and B cells and has also been found to make intestinal epithelial cells to become effective APCs *in vitro*. CT also up-regulates the expression of MHC and co-stimulatory molecules as well as chemokine receptors (Gagliardi, *et al.*, 2000). Furthermore, CT induces secretion of IL-1β from DC (Eriksson, *et al.*, 2003), which not only induces the maturation of DC, but is also by itself an efficient mucosal adjuvant when co-administered with protein antigens and might be responsible for a substantial part of CT activity (Staats and Ennis, 1999). CT primarily induces Th2 immune responses characterized by CD4+ T-cells producing IL-4, IL-5, IL-6 and IL-10,

and by the production of IgA, IgG1 and IgE antibodies (Marinaro, *et al.*, 1995). LT, on the other hand, has been reported to induce a mixed Th1 and Th2 immune response (Takahashi, *et al.*, 1996). However, some studies also indicate that CT induces mixed responses, in contrast to CTB, which appears to induce a more restricted Th2-type response (Eriksson, *et al.*, 2003). Several combinations of cytokines can replace CT as nasal adjuvant. IL-1 combined with Th1-inducing cytokines such as IL-12, IL-18 and GM-CSF can elicit mucosal and systemic responses with the same potency as CT. The combination of IL-1 and IL-12/IL-18/GM-CSF also induces a combined Th1 (CTL and IFN-γ) and Th2 (mucosal IgA) profile against synthetic peptides (Staats, *et al.*, 2001).

A variety of mucosal adjuvants and delivery systems have been developed, including ISCOMs, liposomes, biodegradable microspheres as well as different live-attenuated bacterial or viral vector systems. Chitosan, a cationic polysaccharide derived by deacetylation of chitin, has been shown to have potential as a delivery system for protein vaccines delivered by the nasal route. Chitosan appears to augment immune responses by enhancing the uptake of antigens across the nasal mucosa (McNeela, *et al.*, 2000).

Monophosphoryl lipid A (MPL), a derivative of LPS, activates APCs and elicits 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). While MPL delivered intranasally can augment humoral and cellular immune responses to foreign antigens (Baldridge, *et al.*, 2000), it has been found to be more effective when combined with other adjuvants or delivery systems. For example, MPL with QS-21 significantly enhanced Th1 responses to HIV gp120 recombinant glycoprotein (Moore, *et al.*, 1999). MPL has also shown to be effective as an adjuvant for augmentation of mucosal and systemic immune responses to intranasally delivered liposome-formulated *Streptococcus mutans* crude

glucosyltransferase (Childers, *et al.*, 2000). Another strategy is to use live bacteria and viruses as mucosal vaccines both against the corresponding disease, and as delivery system for heterologous diseases (Liljeqvist and Stahl, 1999). One major goal of intranasal vaccination with a replicating microorganism is to induce secretory and systemic immune responses that more closely resemble those provided by the natural infection.

An alternative approach is to combine mucosal delivery with agents that have intrinsic adjuvant activity. Microparticles prepared from the biodegradable polymer poly(lactide-co-glycolide) (PLG), have been tested extensively as vaccine delivery systems in laboratory animal models (Kersten and Gander, 1996). The potential of PLG microparticles as a delivery system relies on their capacity to release entrapped antigen over extended periods and on their ability to deliver antigen directly to phagocytic APCs. For induction of mucosal immunity, particulate antigen <10 μm in size can be delivered orally and taken up by specialized cells in the PP of the small intestine. Similarly, intranasal immunization with vaccine antigens incorporated into microspheres results in protection against various respiratory pathogens (Cahill, *et al.*, 1995; Eyles, *et al.*, 1998). However, different studies in mice using polymeric particles indicated variable immune responses. In some cases, the serum IgG responses were increased when an antigen was administered in a microparticle, but in other cases, a decreased or unchanged response was found. Importantly, only in a few instances were mucosal IgA responses increased through an encapsulation strategy as compared to the administered free antigen (reviewed by Lemoine, *et al.*, 1999). The size of the particles appears to affect the outcome of the immune response, with the larger microparticles more effective for inducing cellular immunity and smaller nanoparticles favouring the induction of antibody responses (Conway, *et al.*, 2001). ISCOMs have also shown to enhance local

and systemic antibody responses to nasally administered respiratory syncytial virus envelope antigens (Hu, *et al.*, 1998).

Many efforts have been made to develop an ideal mucosal antigen delivery vehicle that can satisfy the requirement of stability, targetability, and antigenicity. Liposomes can be made to be acid-sensitive, acid-resistant, or heat-sensitive by using different lipids or combinations. Hence, they vary in resistance to acids, bile and enzymes. It is important that liposomes used for mucosal delivery maintain integrity from the time of administration until they reach the MALT. An approach to improve the stability of liposomes is to cross-link the lipids in the liposome membrane so they are acid and bile resistant but can be digested within APCs. Liposomes have been used to induce mucosal immunity in several studies. They have induced increases in specific S-IgA production when administered via the oral (Khemka, *et al.*, 1998; Minato, *et al.*, 2003), nasal (Abraham and Shah, 1992a; Klavinskis, *et al.*, 1997), or intragastric (Clarke and Stokes, 1992) route. Antigen-specific IgG and local IgA responses were 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 S-IgA responses after intranasal delivery in humans (Childers, *et al.*, 1999). Antigens from influenza virus incorporated in the membrane of liposomes, administered nasally with LT as adjuvant, enhanced virus-specific IgA responses in human volunteers (Gluck, *et al.*, 1999). Formulation of antigen into liposomes may also induce antigen-specific cellular immune responses. Liposome-formulated *Yersinia pestis* vaccine induced strong IFN-γ responses in mice after intranasal administration, with no IL-4 detected (Baca-Estrada, *et al.*, 2000a).

The liposome itself may have intrinsic adjuvant activity, but it may not be potent enough for many weak antigens. Incorporating other adjuvant or immunomodulating molecules may be necessary to enhance systemic or local immune responses. A synthetic peptide vaccine encapsulated in liposomes together with an anti-CD40 antibody was shown to induce protective immunity against influenza A virus in mice (Ninomiya, *et al.*, 2002). Also, intranasally administered CpG ODNs encapsulated in liposomes were more effective than formulations containing un-encapsulated CpG ODN in inducing serum and mucosal antibodies, as well as cellular responses and protection in both influenza and hepatitis B models (Joseph, *et al.*, 2002).

Biphasic lipid vesicles are a novel type of lipid-based formulation suitable for the delivery of proteins, peptides and ODNs. These formulations, designed specifically for vaccine application, also called Vaccine-Targeting Adjuvants (VTA), have shown to enhance the adjuvant activity of CpG ODNs following intranasal administration in mice (Babiuk, *et al.*, 2004).

CpG ODNs have shown to be effective mucosal adjuvants. Nasal delivery of CpG ODN augmented humoral and cell-mediated systemic and mucosal responses to Hepatitis B surface antigen in mice (McCluskie and Davis, 1998). It was also found that CpG and CT act synergistically to provide potent antibody responses, which were stronger than when either adjuvant was used alone (McCluskie, *et al.*, 2000a). In another study, nasal vaccination with a purified envelope glycoprotein (gB) from HSV-1 induced strong vaginal IgA and systemic IgG2a responses together with the induction of both systemic and mucosal CTLs. Furthermore, the immune response obtained was cross-protective against the related HSV-2 (Gallichan, *et al.*, 2001). Oral delivery of CpG ODN together with purified protein antigens promotes a mucosal Th2 response with IgA formation and a systemic Th1 response (McCluskie, *et al.*, 2000b).

2.3.2 Transcutaneous vaccines

The skin presents a readily accessible surface area for absorption (1.2-2.3 m^2), which offers a distinct advantage of exploiting its immune system for delivering vaccines. The immunological competence of the skin as a site for vaccine delivery has been clearly demonstrated in systems using DNA-vaccines and CT (Leitner, *et al.*, 1997; Condon, *et al.*, 1996; Glenn, *et al.*, 1998a; Glenn, *et al.*, 1998b). The hydrophilic nature and large molecular size of protein antigens versus the lipophilic character of the stratum corneum is the major barrier in the use of the epidermis as a potential site for vaccine delivery.

Conventional delivery of vaccines to livestock by injection is associated with the risk of needle-related complications that may lead to abscess formation and an impairment of meat quality. Injecting vaccines also exposes animals to the risk of transmission of infectious diseases. On the other hand, the immunostimulation that results from parenteral, intranasal or oral administration of adjuvanted vaccines may be accompanied by adverse side effects such as local inflammation at the site of injection, rhinorrhea, or diarrhea. Recently, transcutaneous immunization (TCI) strategies have been introduced as an alternative non-invasive administration route. In this approach, the antigen is topically applied to intact skin, thereby targeting the antigen to Langerhans cells (LC), which will subsequently migrate through the skin into draining lymph nodes to initiate the immune response (Moingeon, *et al.*, 2002b).

2.3.2.1 General Structure of the Skin

The skin is the principal interface with the external environment, keeping water and nutrients in, and protecting the host from invading pathogens. For this purpose, the skin is equipped with immunocompetent cells, such as keratinocytes, LC, subsets of T lymphocytes and strategically located lymph

nodes. Keratinocytes, apart of being responsible for establishing the physical barrier of the skin and guaranteeing the structural integrity of the epidermis, produce a wide range of cytokines upon activation by various stimuli (Uchi, *et al.*, 2000). These cytokines shape the local microenvironment to help maintain the appropriate balance of skin immune responses, and stimulate the maturation and migration of LCs.

Human skin ranges from 0.05 to 2 mm thick and is composed of an epithelial layer, the epidermis, and a layer of connective tissue, the dermis. The junction of dermis and epidermis is irregular. Beneath the dermis lies the hypodermis or subcutaneous tissue, a loose connective tissue that may contain a pad of adipose cells. The external layer of the skin is relatively impermeable to water, which prevents water loss by evaporation (Junqueira, *et al.*, 1998).

The epidermis consists mainly of five layers of keratinocytes that form a stratified squamous keratinized epithelium, but also contains melanocytes, LCs, and Merkel's cells. The stratum corneum is the outermost layer and consists of non-nucleated keratinized cells that are constantly shed and renewed. Because of its large size, the skin has an impressive number of lymphocytes and APCs (i.e. LC). LCs are found regularly spaced throughout the epidermis, forming a semicontinuous network; they are defined by their dendritic morphology and the presence of a unique intracytoplasmic organelle, the Birbeck granule. LCs are the only population in normal epidermis to express MHC class II (Lappin, *et al.*, 1996).

The dermis is the connective tissue that supports the epidermis and binds it to the subcutaneous tissue. The surface of the dermis is very irregular and has many projections. A basal lamina is always found between the epidermis and the dermis. Underlying this basal lamina is a net of reticular fibers, the basement membrane. The dermis contains two layers, the papillary layer and the deeper reticular layer. The papillary layer is composed of loose connective tissue; and

presents fibroblasts, mast cells and macrophages. Extravasated leukocytes are also seen. The reticular layer is composed of irregular dense connective tissue, and therefore has more fibers and fewer cells than the papillary layer. The dermis has a rich network of blood and lymph vessels and also contains epidermal derivatives such as hair follicles, and sebaceous and sweat glands (Junqueira, *et al.*, 1998). Over 90% of the skin-associated T cells in normal skin are found in the dermis, while intraepidermal T cells represent a minor population; B cells are rarely observed (Debenedictis, *et al.*, 2001).

The subcutaneous tissue consists of loose connective tissue that binds the skin loosely to the subjacent organs. It contains fat cells that vary in number according to the area of the body.

For TCI to provide maximum efficacy, the vaccine must penetrate the surface lipid, the sebum, and the stratum corneum for sufficient uptake and processing by immune cells. The stratum corneum is highly hydrophobic, it has a complex structure composed of closely associated cellular and lipid components. Substantial variation occurs between species both in structural characteristics and in lipid composition of skin (Hammond, *et al.*, 2000).

2.3.2.2 Adjuvants and Delivery methods for vaccine administration through the skin

Since the skin is designed to protect the body from external insults, it is not surprising that uptake of vaccines transdermally is not very efficient. Delivery of adjuvant and antigen to the immune system by TCI requires hydrated skin. It is believed that hydration of the skin facilitates the absorption and penetration of antigen and adjuvant through the stratum corneum to reach the network of LC above the basal layer of keratinocytes in the epidermis (Roberts and Walker, 1993). In the absence of an effective delivery mechanism, topically applied

vaccines normally do not penetrate through the stratum corneum, which is considered the principal barrier to penetration by foreign substances and organisms. To overcome this impediment, a number of devices are being developed to break the skin barrier with minimal pain. Vaccines delivered by jet propulsion are formulated in liquid excipients, which are delivered under pressure through the stratum corneum. Depending on the size of the orifice of the injector and the velocity of the jet, the vaccine can be delivered intradermally, subcutaneously, or intramuscularly. A variation of the jet injector is the gene gun, which is used for delivery of plasmid-based vaccines (Pertmer, *et al.*, 1995) and more recently, protein antigens (Chen, *et al.*, 2001). Gold beads coated with DNA are "fired" through the skin into cells, where the DNA is released from the gold particles. This method of plasmid delivery is relatively efficient, although the amount of DNA that can be loaded onto gold beads is limited resulting in the need for multiple shots. Electroporation has been used successfully to increase gene expression *in vivo* following delivery of plasmids into the skin (Zhang, *et al.*, 2002). In this technique, cells are pulsed with an electrical field and the nucleus and plasmatic membranes are temporarily made permeable, allowing molecules to enter the cell.

Another alternative is the use of microneedles, which creates painless, superficial pathways through the stratum corneum that allow the penetration of macromolecules. Percutaneous administration employing a multiple puncture instrument has been widely used for vaccination of humans against tuberculosis (Cundall, *et al.*, 1988; Al Jarad, *et al.*, 1999). Such devices also enabled topical immunization of mice with naked DNA, inducing stronger and less variable immune responses than via needle-based injection. In fish, the efficacy of a streptococcus vaccine administered by a multiple puncture/immersion method was equivalent to that achieved by intra-peritoneal injection (Nakanishi, *et al.*, 2002).

TCI requires the use of an adjuvant co-administered with the antigen to induce both a humoral and cellular immune responses (Scharton-Kersten, *et al.*, 2000). Initial studies of TCI focused on the adjuvant activity of CT and LT. Application of these toxins to intact and hydrated skin induces potent immune responses without the systemic toxic side effects that are associated with their use via oral, nasal, and parenteral delivery (Glenn, *et al.*, 1998a; Glenn, *et al.*, 1998b; Glenn, *et al.*, 1999; Scharton-Kersten, *et al.*, 1999; Beignon, *et al.*, 2001). Thus, TCI may provide a valuable means for exploiting the superior adjuvant effects of toxins while avoiding their severe side effects. Application of either CT from *V. cholerae*, or the related protein LT from *E. coli* to mouse skin for 2 h resulted in a potent anti-toxin IgG response in the sera within 2 weeks of the immunization and the response was boosted with subsequent immunizations. Also, application of CT with other proteins including TT, DT, and BSA resulted in a systemic IgG response against the coadministered antigen (Glenn, *et al.*, 1999), indicating that this method has broad utility for the induction of host immunity through the skin. The immune response induced using CT as an immunogen or adjuvant for TCI appears to be physiologically relevant since vaccinated mice were protected from a lethal intranasal challenge with CT. Despite these advantages, both these toxins and in particular CT, induce predominantly a Th2 type of immune response which might have a detrimental effect in individuals sensitive to allergic reactions, or when a Th1 type response is needed for protection.

The use of liposomes for topical skin application was reported in the early 1980's (Mezei and Gulasekharam, 1980; Mezei and Gulasekharam, 1982). Since then, studies have demonstrated that liposomes can improve the penetration of entrapped as well as non-entrapped molecules (Verma, *et al.*, 2003a), and that the particle size of liposomes may influence dermal delivery of substances into skin (Verma, *et al.*, 2003b). Although, it was also reported that

transport of vesicular systems into skin did not depend on vesicle size (Sentjurc, *et al.*, 1999). Hence, the influence of liposome size on the penetration may depend on their lipid composition, the lipophilic nature of the molecule, and also the nature of the skin. Topical administration can also be enhanced by formulation with different lipid-based delivery systems containing permeation enhancers such as cadherin agonists to disrupt host cell tight junctions and the stratum corneum. Studies have indicated that enhanced dermal delivery of small and large therapeutic compounds can be achieved using biphasic lipid vesicles (Foldvari and Baca-Estrada, 1999a). Such vesicles were used to enhance IFN-α (Foldvari, *et al.*, 1999b) delivery into human skin and also, application of transdermal patches containing insulin in biphasic lipid vesicles induced a sustained decrease in blood glucose in diabetic rats (King, *et al.*, 2002).

2.4 The pig as a model to evaluate vaccines

The mouse has been utilized as a model to rapidly explore the efficacy and safety of vaccines with a variety of antigens and delivery vectors. However, direct application of the knowledge gained from murine studies to larger animals has not always been successful for many novel and experimental immunization devices or techniques. Comparisons between different adjuvants derived from *in vitro* studies, or from studies using adjuvants in rodents, are often not predictive for safety, adjuvant effects, or vaccine efficacy in more relevant animal species or humans. Thus, the physiological relevance of an animal model to an intended target species becomes a key factor, since this directly affects how efficiently a 'proof of concept' can be translated into a successful clinical treatment.

The implementation of effective nasal vaccines requires understanding the interaction of antigens with the different regions and structures of the upper and lower respiratory tract. Hence, differences among species need to be considered since there is variability in the nature of secondary lymphoid tissue.

Mammals have evolved organized lymphoid tissue (inductive sites) in the upper respiratory tract to facilitate antigen uptake and, processing and presentation for the induction of mucosal immune systems. The existence of lymphoid aggregates in the bronchial wall similar to PP in the intestine, has been described for several animal species, including rats, rabbits, mice, and guinea pigs. However, in other species such as cats, pigs and humans, there are few organized aggregates, which constitute a difference with species commonly used as experimental models (Bienenstock, *et al.*, 1999). In humans, there is a ring of lymphoid tissue (Waldeyer's ring) that comprises the nasopharingeal tonsil or adenoid, palatine tonsils, tubal tonsils and the linguinal tonsils. The tonsils contain aggregates of lymphoid cells and are similar to PP in the intestine. A Waldeyer's ring is also present in pigs, which makes this animal a good model for nasal vaccination studies.

An increasing number of experiments are being performed using the transcutaneous route of administration, and many of those experiments are being carried out in mice. However, there are marked differences in the structure and composition of skin between different species. The human and pig stratum corneum share very similar lipid types. Since the pig and human also contain very similar percentages of lipids, the information gained from research utilizing the pig model may be applicable to human trials, although the cornified layer thickens as pigs mature to a greater extent than that seen in humans (Hammond, *et al.*, 2000).

2.4.1 *Actinobacillus pleuropneumoniae* infection

Respiratory tract infections with bacteria like *Actinobacillus pleuropneumoniae* (*App*) are extremely common in pigs and are of major veterinary relevance. Infection with *App* is the main cause of pleuropneumonia in pigs. This pathogen causes a rapidly developing and highly contagious form

of bronchopneumonia with characteristic hemorrhagic lesions and fibrin deposition (Fenwick and Henry, 1994). *App* most likely infects the host via inhalation and by colonization of the upper and lower respiratory tracts (Liggett, *et al.*, 1987). In an acute infection, death can occur in less than 24 h after initial contact. A chronic infection has no serious symptoms but is still contagious, making detection and eradication of this pathogen difficult. Currently available porcine *App* vaccines afford only minimal protection by decreasing mortality but not morbidity (Fenwick and Henry, 1994). Pigs that survive infection can still be carriers, with the pathogen residing in their nasal cavities, in their tonsils, or within lung lesions (Sebunya and Saunders, 1983); hence, once infected, a herd remains infected.

An increased number of bacterial proteins have been identified that are highly immunogenic and protective vaccine components (Frey, *et al.*, 1996; Willson, *et al.*, 2001; Seah *et al.*, 2002). The outer membrane lipoprotein A (OmlA) of *App* has been shown to induce a strong immune response in pigs and to reduce mortality and clinical signs of disease after challenge (Gerlach, *et al.*, 1993; Bunka, *et al.*, 1995). Current vaccines against swine pleuropneumonia, which have been developed for parenteral administration primarily, induce a humoral antibody response (Hensel, *et al.*, 2000) but may also cause toxic side effects and local tissue damage (Rosendal, *et al.*, 1981; Straw, *et al.*, 1985). Although they may reduce mortality rates, they do not prevent the development of lung lesions and/or colonization by the bacteria (Fedorka-Cray, *et al.*, 1993; Madsen, *et al.*, 1995). On the other hand, natural infection with *App* results in protective immunity against challenge infection (Inzana, *et al.*, 1991), which might be related with the fact that the immune responses induced by intramuscular immunization with bacterins are very different from those induced by aerosol infection (Furesz, *et al.*, 1997).

3.0 HYPOTHESES, OVERALL OBJECTIVE AND SPECIFIC AIMS

3.1 Hypotheses

This research is based on two hypotheses: 1) Biphasic lipid vesicles [VTA (Vaccine Targeting Adjuvant)] are effective vaccine delivery systems for both antigens and CpG ODN when administered by systemic and mucosal routes; 2) Immune responses to protein antigens are induced by non-invasive approaches to immunization such as transcutaneous and intranasal administration.

3.2 Overall objective

The overall objective of this project was to assess the ability of biphasic lipid vehicles to enhance mucosal and systemic immune responses to bacterial antigens and to enhance the immunomodulatory activity of CpG ODN. An additional objective of this project was to understand the mechanisms by which this vaccination strategy mediates the induction of immune responses.

3.3 Specific aims

In order to examine the validity of the proposed hypotheses the following specific aims were established: (1) to determine the immune responses in mice immunized with biphasic vesicle formulations containing bacterial antigens alone or in combination with CpG ODN; (2) to assess the induction of antigen-specific immune responses in pigs after immunization with biphasic vesicle formulations containing OmlA from *Actinobacillus pleuropneumoniae* and CpG ODN; (3) to evaluate the immune responses induced by transcutaneous and intranasal

immunization of pigs; (4) to assess the local inflammatory response induced by different immunization approaches.

4.0 MATERIALS AND METHODS

4.1 Antigens

Recombinant streptococcal surface dehydrogenase (GapC) protein from *Streptococcus uberis* was obtained as previously described (Fontaine, *et al.*, 2002). Recombinant outer membrane lipoprotein A (OmlA) from *App* kindly provided by Dr P. Willson (V.I.D.O, University of Saskatchewan, Saskatoon, SK) was purified as previously described (Gerlach, *et al.*, 1993). Protein purity and endotoxin levels were determined by SDS-PAGE and Limulus amoebocyte lysate assay, respectively. The same antigen preparation was used for immunization and *in vitro* assays.

4.2 Adjuvants and delivery systems

Biphasix^{TM}-Vaccine Targeting Adjuvant (VTA) (PharmaDerm Laboratories Ltd. Saskatoon, SK) was prepared as described previously (Foldvari, 1998; Foldvari and Baca-Estrada, 1999a). Briefly, for intranasal immunization, biphasic vesicles (Biphasix^{TM} vaccine targeting adjuvant; intranasal VTA: Code VTAM1) were prepared by the mixing of a submicron emulsion (olive oil; polyoxyl 40 hydrogenated castor oil; methylparaben; propylparaben) and the phospholipid phase (soya phosphatidylcholine (Phospholipon 90H; Natterman GmbH, Germany); cholesterol; cetylpyridinium chloride and propylene glycol), to form lipid vesicles entrapping the emulsion droplets. The cetylpyridinium chloride used in this formulation was selected due to its mucosal compatibility.

For subcutaneous administration, VTA2 and VTA4 were used. The submicron emulsion in VTA2 contained caprylic/capric triglycerides (Gattefosse,

Mississauga, Ont.); glycerol monostearate; linoleamidopropyl-PG-dimonium chloride phosphate (Mona, Paterson, NJ); methylparaben; propylparaben; and the phospholipid phase contained soya phosphatidylcholine (Phospholipon 90H); and propylene glycol.

In VTA4 several ingredients of formula VTA2 were replaced to comply with the FDA pharmaceutically approvable list of excipients. The VTA4 formulation was as follows: the submicron emulsion contained cottonseed oil; polysorbate 80; benzalkonium chloride; methylparaben; propylparaben; and the phospholipid phase contained soya phosphatidylcholine (Phospholipon 100H); and propylene glycol.

VPE1 formulation was developed to further enhance the topical permeation of proteins. This formulation is similar to VTA4, however, an acylated amino acid derivative (compound code PDM3) was incorporated into the lipid phase of the vesicles.

All ingredients in VTA formulations were w/v, USP grade, from Spectrum (New Brunswick, NJ) unless specified otherwise. Antigen in endotoxin- free saline (Baxter Corporation, Toronto, ON) was mixed with VTA formulations using a ratio of 1 part antigen to 9 parts VTA.

CpG ODN 1826 (TCCATGACGTTCCTGACGTT) and 1982 (TCCAGGACTTCTCTCAGGTT) were used in mice experiments. CpG ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT) was chosen for pig experiments because its GTCGTT motif is optimal for stimulation of lymphocytes in several domestic species (Rankin, *et al.*, 2001). The non-CpG ODN 2041 (CTGGTCTTTCTGGTTTTTTTCTGG) was also used in these experiments. Both ODNs had a phosphorothioate backbone modification to increase resistance to nuclease degradation (QIAGEN GmbH, Hilden Germany). The commercial

adjuvants Emulsigen and VSA are oil in water emulsions (MVP Laboratories, Ralston, NE) and were used as a reference adjuvant.

4.3 Animals

Six to eight-week-old female BALB/c mice were purchased from the Animal Resource Center at the University of Saskatchewan. Four week-old male and female landrace pigs from an App free herd were purchased from the Prairie Swine Center Inc., SK. Canada. Animals were treated in compliance with the regulations of the Canadian Council for Animal Care under protocols approved by the University Committee on Animal Care and Supply.

4.4 Mouse model

4.4.1 Immunization

Mice were immunized by subcutaneous (s.c.) route twice, 10 or 14 days apart, with 100µl of the corresponding formulation containing 10 µg (OmlA or GapC) or 20 µg (GapC) of antigen alone or in combination with 10 µg of CpG ODN 1826. Naïve groups were immunized with saline to be used as negative controls. Antigen-specific antibody levels prior to immunization were determined by ELISA.

4.4.2 Detection of antigen-specific antibodies by ELISA

Ninety-six well plates (Immulon 2, Dynatech Laboratories Inc., Alexandria, VA) were coated with 100 µl of 1 µg/ml solution of antigen (GapC or OmlA) in 50mM sodium carbonate buffer (pH 9.6). After each incubation, six

washes were performed using PBS-Tween (PBS-T). After overnight (O.N.) incubation at 4°C different dilutions of serum in PBS-T 0.5% gelatin (PBS-TG) were added (100 µl/well) and incubated 2 hours at room temperature (R.T.). One hundred µl/well of affinity purified biotinylated goat-anti mouse IgG(H+L), IgG1 or IgG2a 1/10000 (Caltag, Toronto, ON) were added and incubated for 1h at R.T. followed by incubation with streptavidin-alkaline phosphatase 1/10000 (Jackson Immuno Research, Mississauga, ON). Di(Tris)-*p*-Nitrophenyl phosphate (PNPP) (Sigma, Oakville, ON) substrate was added and absorbance was measured at 405 nm.

4.4.3 Isolation of lymphocytes

Spleen and lymph nodes were removed from naïve and immunized mice and teased through a nylon mesh. Erythrocytes from spleens were lysed by 1 min incubation with Tris-buffered ammonium chloride. Cells were washed twice with minimal essential medium (MEM) and resuspended in AIM-V medium (Gibco Life Technologies, Burlington, ON) supplemented with 100 µM non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES (Gibco), and 50 µM 2-mercaptoethanol. Cell viability was determined by tripan blue exclusion.

4.4.4 Lymphocyte proliferation assay

Triplicate cultures with 2.5 x 10^5 cells/well were incubated in a 96 well-plate with the appropriate amount of antigen. The optimal concentration of antigen for assessing proliferative responses was determined in preliminary experiments (5 µg/ml GapC or OmlA). Concanavalin A (ConA, 0.5 µg/ml) (Sigma) was included as positive control and wells without antigen as negative

controls. Cultures were incubated for 72h with 0.4 μCi [methyl-^{3}H]thymidine added during the last 8h of culture. Plates were frozen and incorporation of [methyl-^{3}H]thymidine was determined using a beta scintillation counter. Proliferative responses were expressed as a stimulation index (counts per minute (cpm) in the presence of antigen /cpm in the absence of antigen).

4.4.5 Detection of cytokine-secreting cells by ELISPOT

ELISPOT assays for interferon (IFN)-γ and interleukin (IL)-4 were performed using cells from spleen and lymph nodes (axillary and inguinal) as previously described (Baca-Estrada, *et al.*, 1997). Briefly, 1x10^6 cells/well were incubated in 96-well culture plates in the presence or absence of antigen in AIM-V medium and incubated at 37°C and 5% CO_2 for 24 h. The optimal concentration of antigen to detect cytokine-secreting cells was determined in preliminary experiments (5 μg/ml). ConA (0.5 μg/ml) was included as positive control. After incubation, cells were resuspended in fresh medium and seeded on nitrocellulose plates (Millipore, Mississauga, ON) coated with either rat anti-mouse IFN-γ or IL-4 capture monoclonal antibody (2 μg/ml; Pharmingen, San Diego, CA). Biotinylated rat anti-mouse IFN-γ or IL-4 specific antibodies (2 μg/ml; Pharmingen) were used to detect secreted cytokines, followed by streptavidin-alkaline phosphatase 1/1000 (Jackson Immuno Research) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate tablets (Sigma). After 10-30 min plates were washed in distilled water and air-dried. Spots representing individual cytokine-secreting cells were enumerated with a dissecting microscope. Values are expressed as the number of spots per 1x10^6 cells.

4.5 Porcine model

4.5.1 Immunization

Subcutaneously immunized animals received 500 µl of the corresponding formulation twice in the same site, 3 weeks apart, containing 50µg of antigen alone or in combination with CpG ODN 2007. Naïve groups were immunized with saline to be used as negative controls.

Transcutaneously immunized pigs were administered different formulations after receiving multiple percutaneous punctures in the right ear with a specially designed device (Figure 4.1). All the animals were administered the respective formulations twice, 12 or 17 days apart. OmlA and CpG ODN doses were 50 µg, except for the last study in which 250 µg were used. Cholera Toxin (CT) (List Biological Laboratories Inc., Campbell, CA) dose was 60 µg. Antigen solutions were applied in 150 mg of methylcellulose gel or 500 µl of lipid-based formulations.

For the systemic/mucosal protocol, all animals were subcutaneously immunized with OmlA (50 µg) and CpG (50 µg) in VTA4 (500 µl). Three weeks later, pigs were divided into four groups and immunized intranasally (i.n.) with 1 ml per nostril of the different formulations. OmlA, CpG ODN 2007 and CT doses were 100 µg, 50 µg and 5 µg, respectively. A placebo group received CpG in VTA4 or VTAM1 for the s.c and i.n immunizations respectively, without antigen.

For the mucosal/systemic protocol, pigs received two intranasal (i.n.) immunizations on day 0 and 30, consisting of 1 ml per nostril of the different formulations. OmlA, CpG ODN 2007 and CT doses were 250 µg, 50 µg and 10 µg, respectively. On day 21, all the animals received a s.c. immunization,

consisting of 500 µl of OmlA (50 µg) and CpG ODN 2007 (50 µg) in 30% (v/v) Emulsigen (MVP Labs.). A control group received saline only.

Animals vaccinated with the commercial vaccine Pleuro-Star 4™ (Novartis, Mississauga, ON) received two i.m. immunizations three weeks apart.

4.5.2 Actinobacillus pleuropneumoniae challenge and clinical examination

Pigs were challenged by exposure to an aerosol generated from a suspension of 1.5 x 10^5 CFU/ml of *A. pleuropneumoniae* serotype 1 as previously described (Gerlach, *et al.*, 1993). Briefly, an aerosol of bacteria was generated with a Devilbis 65 nebulizer into a Plexiglass and steel chamber where pigs were randomly allocated and allowed to breathe the mist for ten minutes (Osborne, *et al.*, 1985). A veterinarian and an animal health technician evaluated daily clinical signs of disease in all pigs. The following ordinal scoring system was used: clinically normal (0), slight increase in respiratory rate and effort with slight depression (1), marked increase in respiratory rate and effort with marked depression (2), severe increase in respiratory rate and effort with severe depression, mouth breathing and/or cyanosis (3). Pigs with a clinical score of 3 were euthanized. Bacterial swabs were taken from lung lesions and tracheobronchial lymph node to determine the presence of *App.* Lung lesions were evaluated by using a scoring system based on the proportion of lung with pneumonic lesions, determined as the portion of dorsal and ventral surfaces of the lungs with gross lesions of pneumonia, with (0) being normal, (1) mild, (2) moderate, (3) moderate to severe, and (4) severe. In pigs with severe infection the examination and bacterial isolation were done at the time of euthanasia. On day 5 after challenge all remaining pigs were euthanized, and examined postmortem.

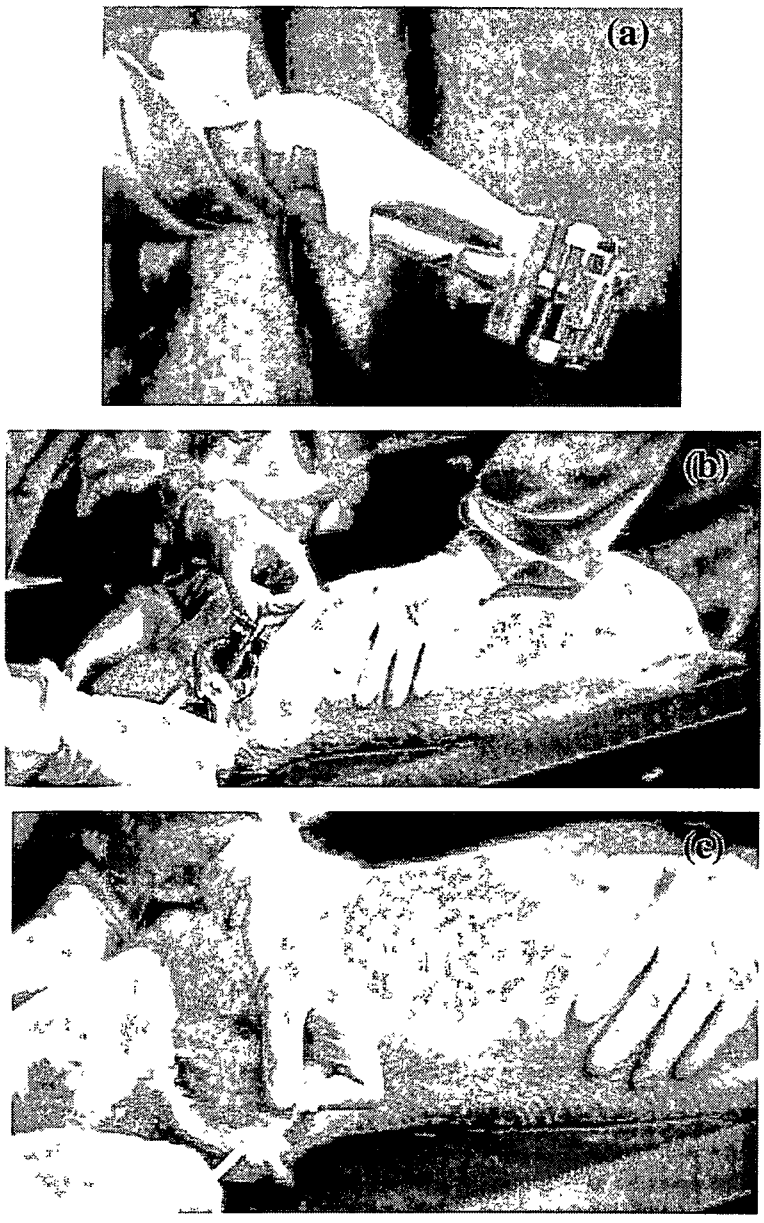

Figure 4.1 Microneedles device used for topical application.
A specially designed device (a) was used to make multiple percutaneous punctures in the right ear of pigs (b), followed by application of the different formulations (c).

4.5.3 Collection of nasal secretions

Nasal secretions were collected with two absorptive swabs (Merocel Inc., Jacksonville, ON) after spraying 150 µl of PBS into each nostril. The swabs were placed proximal to the external nares to absorb fluid without disrupting the nasal mucosa. Nasal swabs were placed in 1.5-ml Eppendorf tubes (Brinkmann Instruments, Mississauga, ON) and kept on ice. Tubes were pierced at the bottom, placed inside a second tube containing 10 µl of 0.1 M PMSF (Sigma), and centrifuged for 30 s at 15,850 x *g*.

4.5.4 Detection of OmlA-specific antibodies by ELISA

The concentration of OmlA- and CT-specific antibodies in serum and nasal secretions were determined by enzyme-linked immunosorbent assay. Immulon 2 plates (Dynatech Laboratories Inc.) were coated with 0.1 µg of antigen per well and incubated with serially diluted samples. Mouse anti-porcine IgA 1/250, IgG1 1/200 or IgG2 1/500 monoclonal antibody (Serotec, Oxford, UK), followed by biotinylated goat-anti mouse IgG(H+L) 1/5000 (Zymed, San Francisco, CA); or alkaline phosphatase goat anti-porcine IgG(H+L) 1/5000 (KPL, Gaithersburg, MD) were used as detecting antibodies. PNPP (Sigma) was used as the chromogenic substrate. A porcine immunoglobulin reference serum in which 1 Unit is equivalent to 1µg (Bethyl Laboratories Inc., Montgomery, TX) was used as a reference standard from 1000 to 1.95 ng/ml and 100 to 0.78 ng/ml, for serum and nasal secretions, respectively.

4.5.5 Isolation of lymphocytes

Porcine blood was collected in Vacutainer tubes containing citrate (Becton Dickinson, Franklin Lakes, NJ). Peripheral blood mononuclear cells (PBMCs) were isolated from the blood as follows. Blood was centrifuged at 2000 rpm for 30 minutes. The buffy coat was removed and resuspended in PBS-0.1 M EDTA and cells were overlayed on ficoll-paque (Pharmacia Biotech, Upssala, Sweden) and centrifuged at 2500 rpm for 45 minutes. The PBMCs were removed and washed three times in PBS-EDTA and resuspended in RPMI medium (Gibco Life Technologies).

4.5.6 OmlA-specific ELISPOT assay

To determine the number of OmlA-specific cytokine-secreting cells, a porcine cytokine ELISPOT assay was performed as described (Baca-Estrada, *et al.*, 1997) with the following changes: PBMCs ($0.5x10^6$ cells/well) were cultured for 24hs at 37°C and 5% CO_2 in 96-well culture plates in the presence of antigen (OmlA 10 μg/ml) and recombinant human IL-2 (rhIL-2; 40 U/ml; Boehringer Ingelheim, Laval, QC). Cells were resuspended in fresh medium and seeded on nitrocellulose plates coated with mouse anti- porcine IFN-γ- (3 μg/ml), IL-10- (5 μg/ml) or IL-4-specific (10 μg/ml) capture monoclonal antibody. Biotinylated rabbit anti porcine IFN-γ- (0.3 μg/ml), IL-10- (0.5 μg/ml) or IL-4-specific (1μg/ml) antibodies (BioSource Int., Camarillo, CA) were used to detect secreted cytokines, followed by streptavidin-alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (SIGMAFAST™; Sigma). Controls included cells cultured with rhIL-2 and no antigen, and cells cultured with the mitogen phytohaemoagglutinin (PHA, 10 μg/ml; Sigma).

4.5.7 IFN-γ ELISA

The concentration of IFN-γ in supernatants of cultured cells was determined by ELISA. Immulon 2 plates (Dynatech Laboratories Inc.) were coated with mouse anti-porcine IFN-γ monoclonal antibody (2μg/ml; BioSource Int.). Supernatants were added undiluted and incubated for 2h. Biotin mouse anti-porcine IFN-γ (0.3μg/ml; BioSource Int.) followed by streptavidin-alkaline phosphatase (BIO/CAN Scientific, Mississauga, ON) was used as the detecting antibody. Di(Tris) p-nitrophenyl phosphate (PNPP; Sigma) was used as the chromogenic substrate. Recombinant porcine IFN-γ (Genentech Inc., San Francisco, CA) was used as a standard to determine concentrations.

4.5.8 RNA extraction and reverse transcription

PBMC (10×10^6 cells/well) were incubated in 24 well microplates (Becton Dickinson) in the presence of antigen (OmlA 10 μg/ml) and rhIL-2 (40 U/ml; Boehringer Ingelheim) for 24 hrs. Controls included cells cultured with rhIL-2 and no antigen, and cells cultured with PHA (10 μg/ml; Sigma). Total RNA was extracted from cells using Trizol Reagent (Gibco Life Technologies) according to the manufacturer's instructions. Contaminating DNA was removed using Dnase I treatment (Gibco Life Technologies). Total RNA concentration was measured and 4 μg from each sample were reverse-transcribed using the Omniscript RT kit (QIAGEN, Mississauga, ON) in a total reaction volume of 40 μl. The RT reaction was carried out in the presence of oligo-dT and ribonuclease inhibitor (RNAseOUT; Gibco Life Technologies) at 37°C for 60 min, followed by heat inactivation at 93°C for 5 min and rapid cooling in ice.

4.5.9 Detection of cytokine expression by multiplex PCR (MPCR)

Cytokine gene expression was examined using PCR amplification with porcine cytokine-specific primers and cDNA template derived from cultured cells. Optimal stimulation time for cells was determined previously. IL-2, IL-4, IL-10 and IFN-γ expression were detected using a multiplex PCR (MPCR) as described elsewhere (Suradhat, *et al.*, 2003). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene to compare cytokine gene expression. PCR was performed in a total volume of 50 µl, consisting of 2 µl cDNA template, 1 µl of each primer, 200 µM dNTP mix, 2 U *Taq* DNA polymerase (Amersham, Piscataway, NJ) and 1x concentration of the PCR buffer provided with the enzyme. The final concentration of the primers was 0.05 µM for GAPDH, 0.2 µM for IL-10, and 0.6 µM for IFN-γ, IL-4 and IL-2. Cycling parameters were as follows: 1) start at 95°C for 5 min; 2) denaturing at 94°C for 30 sec; 3) annealing at 55°C for 45 sec; 4) extension at 72°C for 45 sec; and 5) final extension at 72°C for 5 min. The number of PCR cycles (40) was optimized to assure that none of the products reached a plateau phase during PCR amplification. Images of the MPCR products resolved in ethidium bromide-stained 2% agarose gels were visualized using an UV illuminator and digitally saved for further quantification of the bands by densitometry using the Alpha Analyzer software. The expression level of each product was determined by normalizing its expression against that of the housekeeping gene.

4.5.10 Histological evaluation of injection sites

To assess tissue damage, tissue samples were obtained from the injection sites following sedation for euthanasia, ten days after the last

immunization, fixed in 10% formalin buffer and embedded in paraffin. Five-µm-thick sections were stained with hematoxylin and eosin (H&E). Inflammation was assessed blindly by a pathologist and scored semiquantitatively as follows: 0 = no visible lesions; 1+ = focal, mild mononuclear cell aggregation; 2+ = focal, moderate mononuclear cell aggregation; 3+ = locally extensive, moderate to severe cellular aggregations with moderate fibrosis, necrosis and some giant cells; 4+ = diffuse, severe mononuclear cell aggregation with severe fibrosis, necrosis and moderate number of foci of giant cells and calcification.

To assess cellular infiltration after administration of formulations by different routes, tissue samples were obtained from the administration sites 24 and 48 h after immunization. Seven-µm-thick sections were obtained from frozen samples and stained with Giemsa. Cellular infiltration was assessed blindly by a pathologist as specified above.

4.6 Statistical analysis

Data was analyzed using the Graph Pad InStat™ program Version 3.0 (Graphpad Software, Inc., San Diego, CA). Differences among groups were analyzed using Kruskal-Wallis one-way analysis of variance (ANOVA) followed by Dunn's test, or Mann Whitney t test when comparing two groups. Comparisons of survival curves were done by the log rank test. Clinical scores were compared using repeated measures ANOVA and the Friedman test. p values of 0.05 or less were considered significant.

5.0 RESULTS

5.1 Effect of immunization of mice with biphasic formulations containing bacterial antigens alone or in combination with CpG ODN

A variety of lipid formulations can be designed to contain different characteristics including variations in the electrical charge, size, and structure of the lipid vesicles. These properties affect not only the stability but also the effectiveness of the formulation as vaccine delivery system. Biphasic lipid vesicles are novel formulations suitable for the delivery of proteins, peptides and oligo/polynucleotides (Foldvari and Baca-Estrada, 1999a; Foldvari, *et al.*, 1999b; Baca-Estrada, *et al.*, 2000b; Babiuk, *et al.*, 2002; King, *et al.*, 2002). In contrast to biphasic lipid formulations used in previous studies, the formulations employed in this study were designed specifically for vaccine application [Vaccine-Targeting Adjuvants (VTA)].

The use of a mouse model is important for the initial screening of formulations and for elucidation of their mechanisms of action. Hence, different formulations were tested for their ability to induce antigen specific humoral and cellular responses. These formulations were also tested to determine their efficacy as delivery systems for CpG ODN.

5.1.1 Determination of the ability of GapC to induce immune responses in mice

Streptococcal surface dehydrogenase (SDH or GapC), a 35,8-kDa protein, was recently identified as one of the major surface proteins of group A streptococci. Structurally and functionally it is a member of the glyceraldehide-3-

phosphate dehydrogenase (GAPDH) family. It also binds various mammalian proteins such as lysozyme, fibronectin, plasmin, and the cytoskeletal proteins actin and myosin (Pancholi and Fischetti, 1992). In addition to its GAPDH activity, it is an ADP-ribosylating enzyme (Pancholi and Fischetti, 1993). Because of its specific interaction with plasma or connective tissue proteins of the host, GapC is assumed to play an important role in the colonization and persistence of this pathogen. This, in conjunction with the conservation of the protein among the streptococcal groups, makes it a potential antigen for the development of vaccines against streptococcal diseases. The first studies were designed to establish and optimize the immune responses to GapC in mice.

In order to assess the ability of the streptococcal protein GapC to induce immune responses, groups of five mice were immunized subcutaneously (s.c.) with the protein (10 μg) formulated either in VSA (o/w emulsion), or in saline together with CpG ODN (10 μg). A naïve group received saline to be used as negative control. Animals received a boost 10 days after the primary immunization and samples were taken 3 weeks after the first immunization to assess induction of specific humoral and cellular immune responses. Different antigen concentrations and other variables were tested to determine the optimal conditions to be used in the different assays (data not shown). Antigen-specific antibodies were measured in serum by ELISA.

Subcutaneous administration of the protein in VSA induced higher ($p<0.01$) antibody titres than those in naive mice, whereas antibody titres obtained after immunization with the protein together with CpG ODN in saline increased slightly compared with naïve animals (Figure 5.1.1).

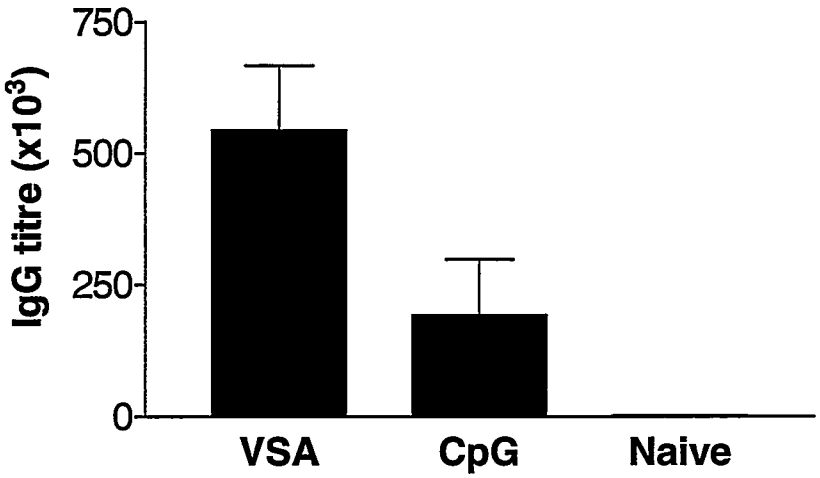

Figure 5.1.1 GapC-specific antibody responses in mouse serum.
Mice were immunized with the protein GapC in VSA or in saline with CpG ODN. Animals received a boost 10 days after the primary immunization and GapC-specific IgG was measured by ELISA 3 weeks after the first immunization. Titres are expressed as the mean titre ± SEM of five mice.

Antigen-specific cell mediated immunity was measured by proliferation assay and by detection of cytokine secreting cells by ELISPOT. Results similar to the antibody response were obtained for the cytokine-secreting cells and proliferation assays, in which the antigen formulated in VSA induced the highest frequencies of IL-4 ($p<0.01$ vs naïve; Figure 5.1.2 a), IFNγ-secreting cells ($p<0.05$ vs naïve; Figure 5.1.2 b) and stimulation index ($p<0.05$; Figure 5.1.3).

5.1.2 Screening of biphasic lipid formulations in the mouse model

Having demonstrated that it is possible to induce immune responses to GapC in mice using a strong adjuvant such as VSA, the next step was to assess the ability of lipid-based formulations to enhance the immune response to GapC and to determine the effect of CpG ODN when used in combination with these formulations, compared to Emulsigen, a commercial oil emusion. Mice were immunized twice s.c. 10 days apart with different formulations as described in Table 5.1.1.

5.1.2.1 Antibody responses induced by GapC and CpG ODNs formulated in VTA

Although serum samples from mice immunized twice with all the formulations contained significant IgG titres compared with non-immunized animals, a strong enhancement of antibody responses was seen in mice immunized with the lipid formulations containing the antigen and CpG ODN ($p<0.001$ for VTA2/CpG and $p<0.01$ for VTA1/CpG) (Figure 5.1.4). However, antigen specific induction of IL-4-secreting cells was not observed after *in vitro*

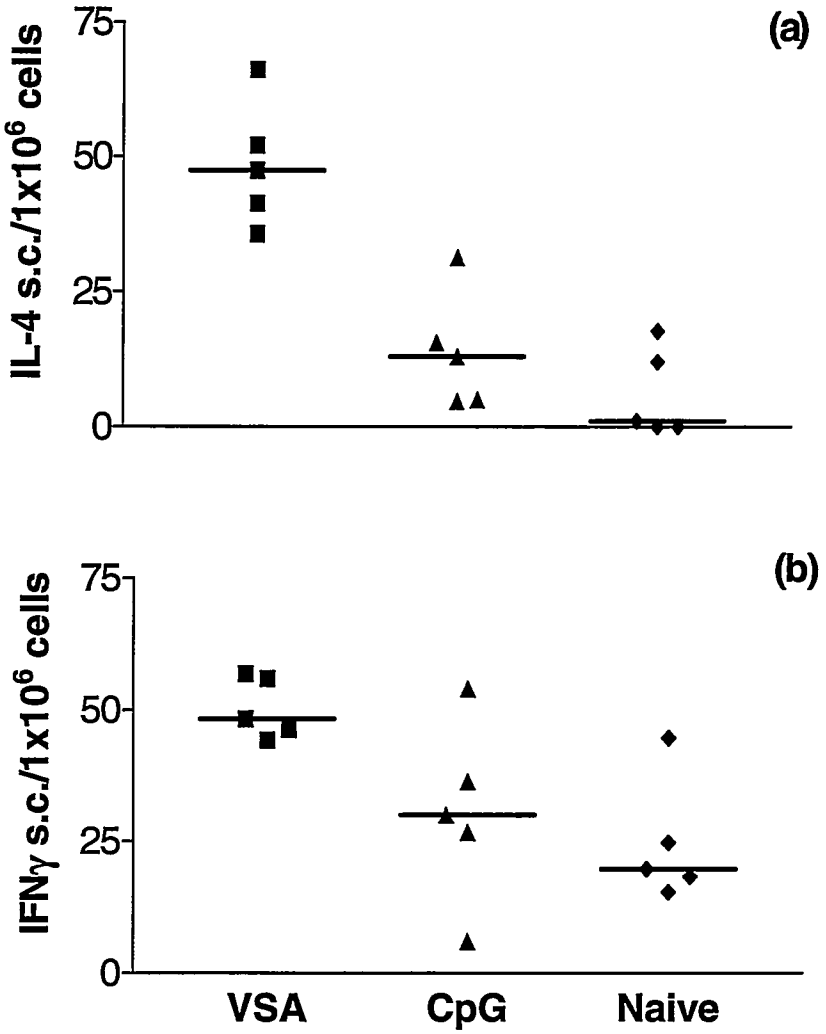

Figure 5.1.2 Frequency of GapC-specific cytokine-secreting cells in the spleen of mice.
Mice were immunized with the protein GapC in VSA or in saline with CpG ODN. Animals received a boost 10 days after the primary immunization and spleen lymphocytes were cultured in the presence of 5µg/ml GapC 3 weeks after the first immunization. The frequency of GapC-specific IL-4- (a) and IFN-γ- (b) secreting cells was determined by ELISPOT. Each point represents an animal and the median for each group is shown.

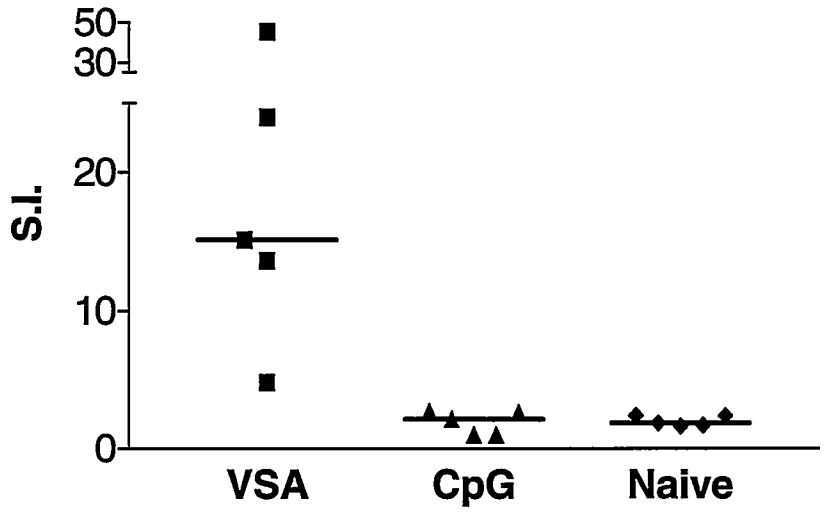

Figure 5.1.3 GapC-specific proliferative responses of spleen cells.
Mice were immunized with the protein GapC in VSA or in saline with CpG ODN. Animals received a boost 10 days after the primary immunization and spleen lymphocytes were cultured in the presence of 5μg/ml GapC 3 weeks after the first immunization. Proliferative responses are expressed as a stimulation index (counts per minute (cpm) in the presence of antigen /cpm in the absence of antigen). Each point represents an animal and the median for each group is shown.

Table 5.1.1 Groups design for the screening of biphasic lipid formulations in mice

Group [1]	Antigen [2]	Vehicle	Adjuvant [3]
1) Emuls.	GapC	30% Emulsigen	
2) CpG	GapC	Saline	CpG 1826
3) Em/CpG	GapC	30% Emulsigen	CpG 1826
4) VTA1	GapC	VTA1	
5) VTA2	GapC	VTA2	
6) VTA1/CpG	GapC	VTA1	CpG 1826
7) VTA2/CpG	GapC	VTA2	CpG 1826
8) Naive		Saline	

[1] Each group consisted of 5 mice.
[2] and [3] Antigen and adjuvant doses were 10 μg each, given s.c. twice at a 10 day interval.

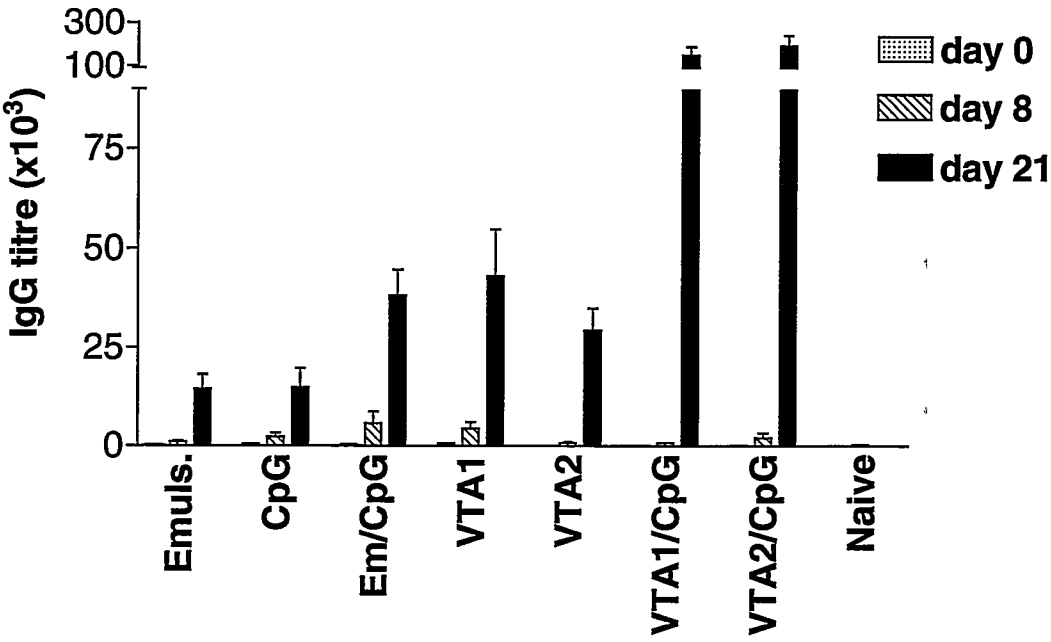

Figure 5.1.4 Effect of the incorporation of GapC and CpG ODN in biphasic lipid formulations on the antibody responses induced.
Mice were immunized s.c. with different formulations and received a boost 10 days after the primary immunization. GapC-specific IgG was measured by ELISA on day 0, 8 and 21 after the first immunization. Titres are expressed as the mean titre ± SEM of five mice.

stimulation with GapC, 3 weeks after the first immunization (data not shown), and only a variable and slight increase in the number of IFN-γ-secreting cells was observed (Figure 5.1.5 a). On the other hand, proliferative responses were only induced by immunization with the antigen formulated in VTA2 in the absence of CpG ODN ($p<0.05$) (Figure 5.1.5 b).

5.1.2.2 Characterization of cellular responses induced by biphasic lipid formulations

A subsequent experiment was performed to determine whether specific cell-mediated immune responses could be induced by immunizing mice with a higher amount of antigen. In addition, the adjuvant effect of CpG ODN was also evaluated in this study using the lipid formulation VTA2. VTA2 was chosen based on the results of previous experiments, which demonstrated that this formulation induced the highest antibody response in the presence of CpG ODN and a significantly higher proliferative response when used with GapC alone.

Animals were immunized s.c. twice, 14 days apart with GapC (20μg) formulated in the lipid formulation VTA2 with or without CpG ODN (10μg). Specific cellular immune responses were assessed by splenocyte proliferative responses and cytokine secreting cells.

The number of specific IL-4- (Figure 5.1.6 a) and IFN-γ-secreting cells (Figure 5.1.6 b) was significantly higher in immunized animals compared with naive mice ($p<0.01$), although CpG ODN enhanced slightly the frequency of IFNγ-secreting cells compared with VTA2 without CpG ODN, the difference was not statistically significant (Figure 5.1.6 b). Proliferative responses were also

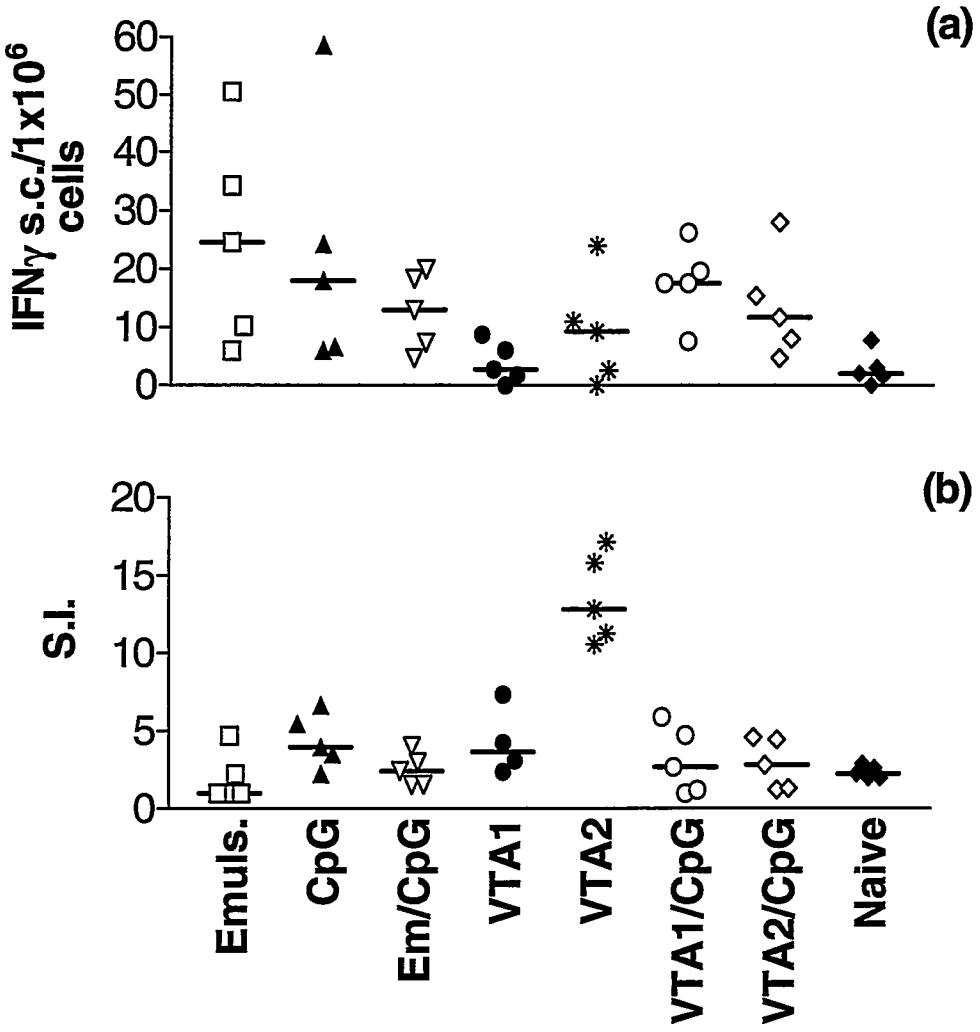

Figure 5.1.5 Antigen-specific cellular immune responses after immunization with GapC and CpG ODN in biphasic lipid formulations.
Mice were immunized twice s.c. 10 days apart, and the frequency of IFN-γ-secreting cells (a) and lymphocyte proliferation (b) were assessed following in vitro stimulation of cells with GapC 3 weeks after the first immunization. Proliferative responses are expressed as a stimulation index (counts per minute (cpm) in the presence of antigen /cpm in the absence of antigen). Each point represents an animal. The median of each group is shown.

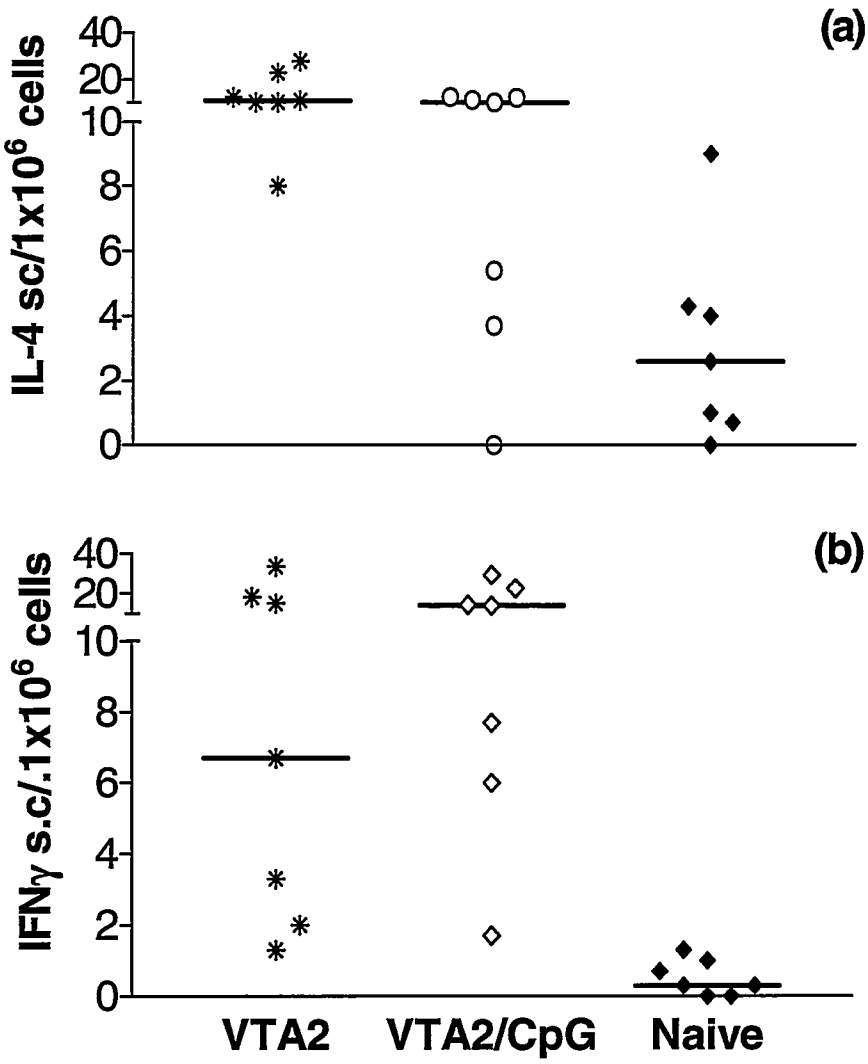

Figure 5.1.6 Frequency of GapC-specific cytokine-secreting cells following immunization with a higher dose of antigen.
Mice were immunized twice s.c., 14 days apart, and IL-4- (a) and IFN-γ- (b) secreting cells were assessed following in vitro stimulation of cells with GapC 3 weeks after the last immunization. Each point represents a mouse. The median of each group is shown.

higher in animals that received the antigen in VTA2 with or without CpG ODN, compared with naïve animals ($p<0.05$ and 0.01 respectively) (Figure 5.1.7).

In agreement with the previous experiment, antibody responses where higher in animals immunized with both protein and CpG ODN in VTA2 (data not shown).

5.1.2.3 Assessment of immunomodulatory effects of non-CpG ODN

It has been shown that synthetic non-CpG ODNs may exert adjuvant activity in some circumstances due to their nuclease-resistant phosphorothioate backbone (Monteith, *et al.*, 1997; Davis, *et al.*, 1998). To address whether the effects observed in previous experiments were specific to the CpG motifs, a new set of experiments were performed using a non-CpG ODN alone and formulated in VTA2 as a control. Mice were immunized twice s.c. 10 days apart with different formulations as described in Table 5.1.2.

The results of these experiments demonstrated that the adjuvant effect observed was not due to the backbone of the ODN used since immunization with non-CpG ODN did not enhance the GapC-specific antibody levels when used either alone or formulated in VTA2. Only sera from mice immunized with the antigen and CpG ODN in VTA2 contained significant IgG titres ($p<0.001$; Figure 5.1.8).

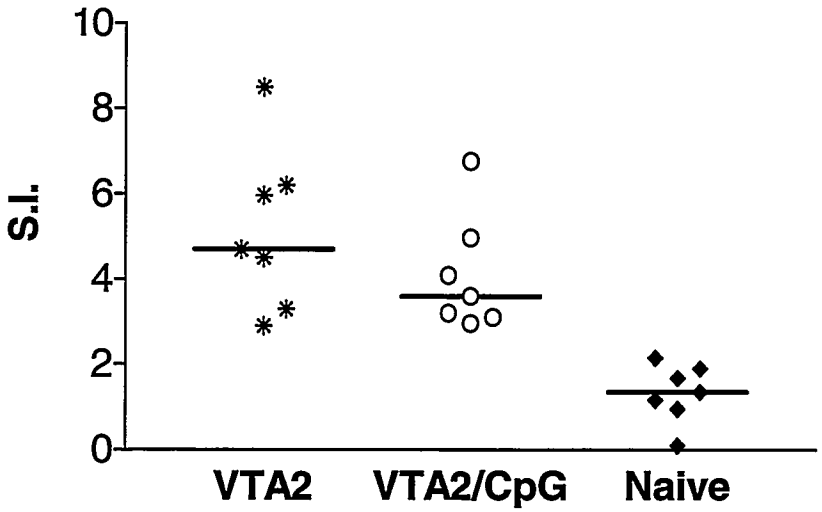

Figure 5.1.7 Proliferative responses following immunization with a higher dose of GapC.
Mice were immunized twice s.c., 14 days apart, and proliferative responses were assessed following in vitro stimulation of cells with GapC 3 weeks after the last immunization. Proliferative responses are expressed as a stimulation index (counts per minute (cpm) in the presence of antigen /cpm in the absence of antigen). Each point represents an animal. The median of each group is shown.

Table 5.1.2 Groups design for the evaluation of the backbone effect

Group [1]	Antigen [2]	Vehicle	Adjuvant [3]
1) GapC	GapC	Saline	
2) CpG	GapC	Saline	CpG 1826
3) non-CpG	GapC	Saline	CpG 1982
4) VTA2/CpG	GapC	VTA2	CpG 1826
5) VTA2/non-CpG	GapC	VTA2	CpG 1982
6) Naive		Saline	

[1] Each group consisted of 5 animals immunized s.c. twice, at a 10 day interval.
[2] Antigen dose was 20 μg.
[3] Adjuvant dose was 10 μg.

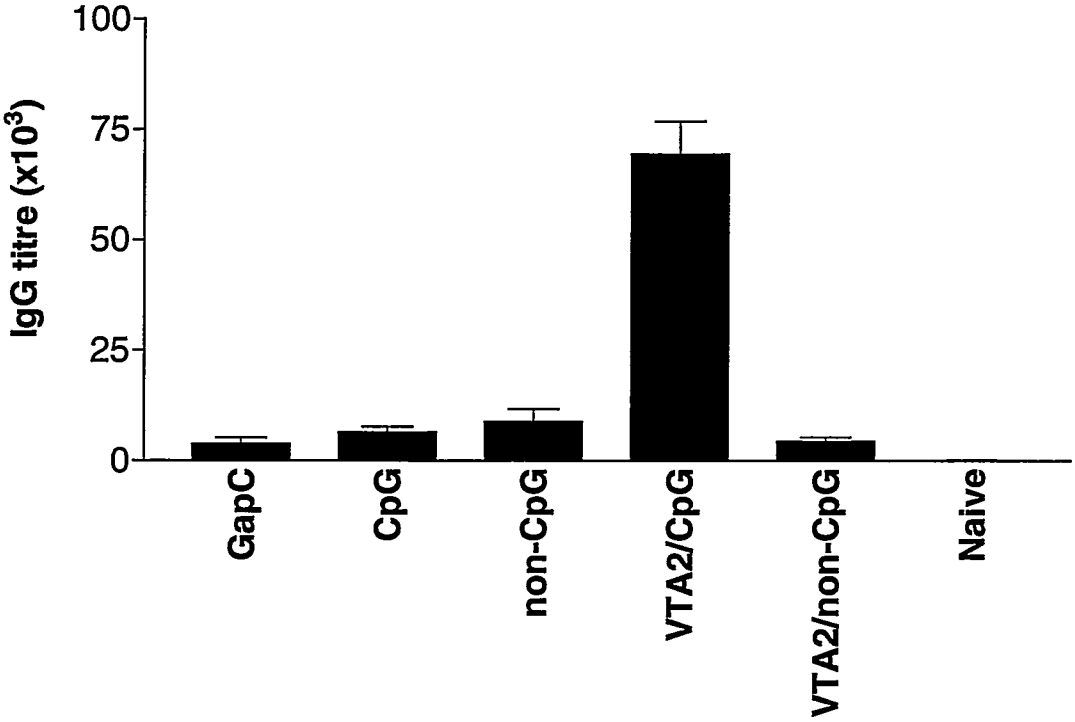

Figure 5.1.8 GapC-specific antibody responses induced by immunization with lipid-based formulations containing antigen together with CpG ODN or non-CpG ODN.
Mice were immunized s.c. twice, 10 days apart, and IgG titres were determined 18 days after the last immunization. Titres are expressed as the mean titre ± SEM of five mice.

5.1.3 Induction of specific immune responses in mice by VTA formulations containing OmlA and CpG ODN

Due to the fact that VTA2 was not prepared using pharmacological grade excipients, it was necessary to evaluate whether the use of pharmacological grade excipients did not compromise the effectiveness of this formulation as a vaccine delivery system. The VTA4 formulation was prepared using PGR and the protein OmlA from *Actinobacillus pleuropneumoniae* (*App*) was used as antigen to compare the ability of the two delivery systems (VTA2 and VTA4) to enhance the adjuvant effect of CpG ODN. The use of OmlA as antigen was important because this antigen has been shown to be effective in inducing protective immune responses in pigs (Gerlach, *et al.*, 1993; Bunka, *et al.*, 1995). In addition, the availability of a pneumonia challenge model in swine using *App* provides an opportunity to evaluate whether the immune responses induced with these formulations are protective (see section 5.2).

Groups of seven animals were immunized s.c. twice, 14 days apart with OmlA (10μg) and CpG ODN formulated either in VTA2 or VTA4.

Mice immunized with the antigen and CpG ODN in either VTA2 or VTA4 presented higher OmlA-specific IgG concentrations than naïve animals (Figure 5.1.9 a) ($p<0.001$ and 0.05 respectively) with production of both IgG1 and IgG2a, with IgG1 being predominant. VTA2 induced a more balanced response although not statistically different from VTA4 (Figure 5.1.9 b).

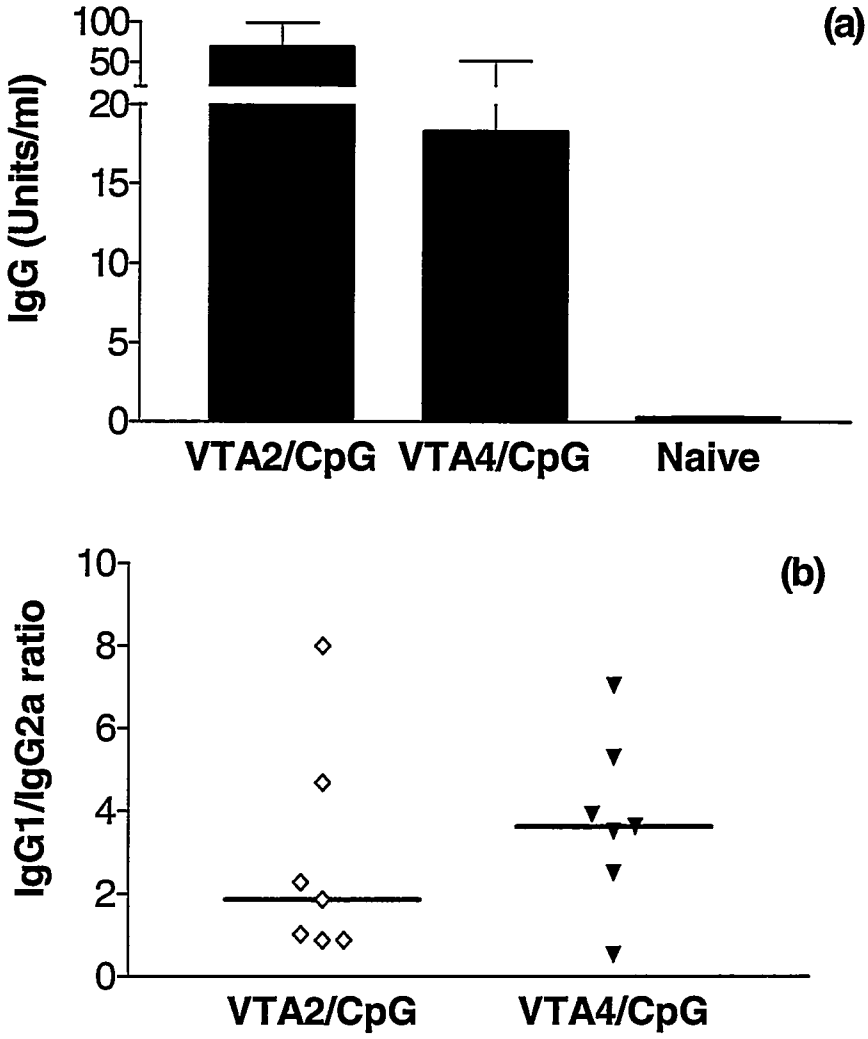

Figure 5.1.9 Induction of OmlA-specific antibodies in serum by two different delivery systems containing CpG ODN.
Mice were immunized s.c. twice, 14 days apart. Total IgG concentrations (a) and IgG1 and IgG2a ratios (b) were determined 10 days after the last immunization. (a) Results are expressed as the mean concentration ± SEM of seven mice; (b) Each point represents an animal with bars indicating the median for each group.

To further compare the two formulations, cellular immune responses were determined in spleen and draining lymph nodes from immunized animals 10 days after the last administration. Both formulations induced similar patterns of cytokine secretion, with a predominance of IFN-γ-secreting cells in spleen ($p<0.01$ for VTA2 and $p<0.05$ for VTA4 compared to naive). In addition, VTA2 induced a slight increase in IFN-γ-secreting cells in draining lymph nodes ($p<0.05$) (Figure 5.1.10 b), and IL-4 secretion in spleen ($p<0.05$) compared to naïve animals (Figure 5.1.10 a), although not statistically different from VTA4.

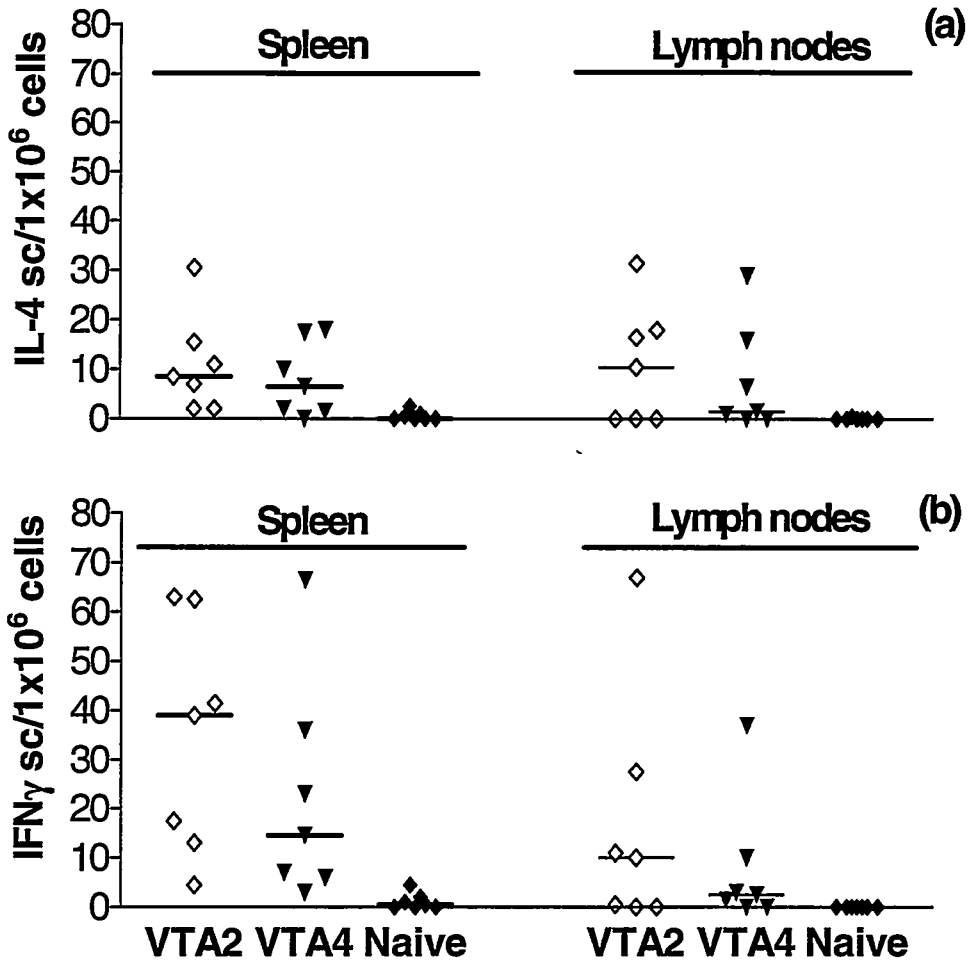

Figure 5.1.10 OmlA-specific cellular responses in spleen and lymph nodes from animals immunized with antigen and CpG ODN formulated in biphasic lipid formulations.

Mice were immunized s.c. twice, 14 days apart. Antigen-specific IL-4- (a) and IFN-γ-secreting cells (b) were determined 10 days after the last immunization. Each point represents an animal with bars indicating the median for each group.

5.1.4 Conclusions

Results in this section showed that the protein GapC induced specific immune responses in mice when combined with a strong adjuvant such as VSA. These responses were characterized by the induction of antigen-specific IgG in serum, IL-4- and IFN-γ-secreting cells and lymphocyte proliferation. Furthermore, 10 μg of CpG ODN did not enhance the immune responses to the GapC protein when given in saline.

In contrast, VTA lipid-based formulations were effective delivery systems for both antigen and CpG ODN.

All lipid formulations tested (VTA1, 2 and 4) enhanced the adjuvant activity of CpG ODN when assessed by the stimulation of antigen-specific antibody responses. Results also showed that the responses observed were due to the presence of CpG motifs in the ODNs.

The formulation VTA2 was chosen based on the stimulation of antigen-specific antibody and cellular immune responses. Cellular immune responses were characterized by the predominant stimulation of IFN-γ secreting cells.

Immune responses induced by VTA2 were similar to those induced by VTA4, indicating that the use of pharmacological grade reagents did not affect the effectiveness of the formulation as a delivery system. Responses were balanced in terms of induction of Th1/Th2, at both the cellular and the antibody level.

5.2 Induction of protective immune responses in pigs after immunization with biphasic formulations and CpG ODN

Although the stimulatory effects of CpG ODN on the immune system of mice and humans are well established, it was important to determine whether CpG is immunostimulatory in husbandry animals since it may be of value as vaccine adjuvant.

An increased number of bacterial proteins have been identified that are highly immunogenic and protective vaccine components (Gerlach, *et al.*, 1993; Frey, *et al.*, 1996). Vaccination with the outer membrane lipoprotein A (OmlA) of *App* has been shown to induce a strong immune response in pigs and to reduce mortality and clinical signs of disease after challenge (Gerlach, *et al.*, 1993; Bunka, *et al.*, 1995).

The aim of this study was to evaluate the effect of incorporation of CpG ODN in biphasic lipid formulations on the immune response to the OmlA protein and to assess the ability of these responses to confer protection against infection of pigs with *App*.

5.2.1 Characterization of humoral immune responses

In order to assess the ability of biphasic lipid formulations to enhance the adjuvant effect of CpG ODN in pigs, groups of eight animals were immunized with OmlA (50 μg) and CpG ODN 2007 (1 mg) in saline (CpG group) or formulated in VTA2 (VTA2/CpG group). To compare the immune responses induced by CpG ODN formulations with a commercial o/w adjuvant, a group of animals was immunized with OmlA in VSA (VSA group). A negative control group received saline (Naïve group). All the animals received two s.c.

immunizations 3 weeks apart and ten days after the last immunization OmlA-specific total IgG and IgG isotypes (IgG1 and IgG2) were measured in serum.

Only animals immunized with the antigen and CpG ODN in VTA2 or with the antigen in the commercial adjuvant showed higher OmlA-specific IgG concentration than naïve animals ($p<0.01$ and 0.001 respectively). The response induced by CpG ODN in VTA2 was similar to that induced by VSA (Figure 5.2.1 a). To further characterize the immune response observed in these animals, antibody isotype levels were determined. Both vaccine formulations induced a balanced response, with production of IgG1 and IgG2 (Figure 5.2.1 b).

5.2.2 Assessment of tissue damage

The level of tissue damage induced by VTA2 formulations and CpG ODN was determined by measuring the induction of local inflammatory response at the injection site. Biopsies from the immunization site were collected using a punch (8 mm) ten days after the boost.

Histological examination of the skin and underlying tissues revealed that while the commercial adjuvant VSA induced necrosis and severe infiltration consisting predominantly in mononuclear cells, VTA2 formulation containing CpG ODN and OmlA induced mild or no inflammation. These results demonstrate that the degree of inflammation induced by VTA2 formulation was significantly lower ($p<0.001$) than that induced by the commercial adjuvant VSA. (Fig. 5.2.2)

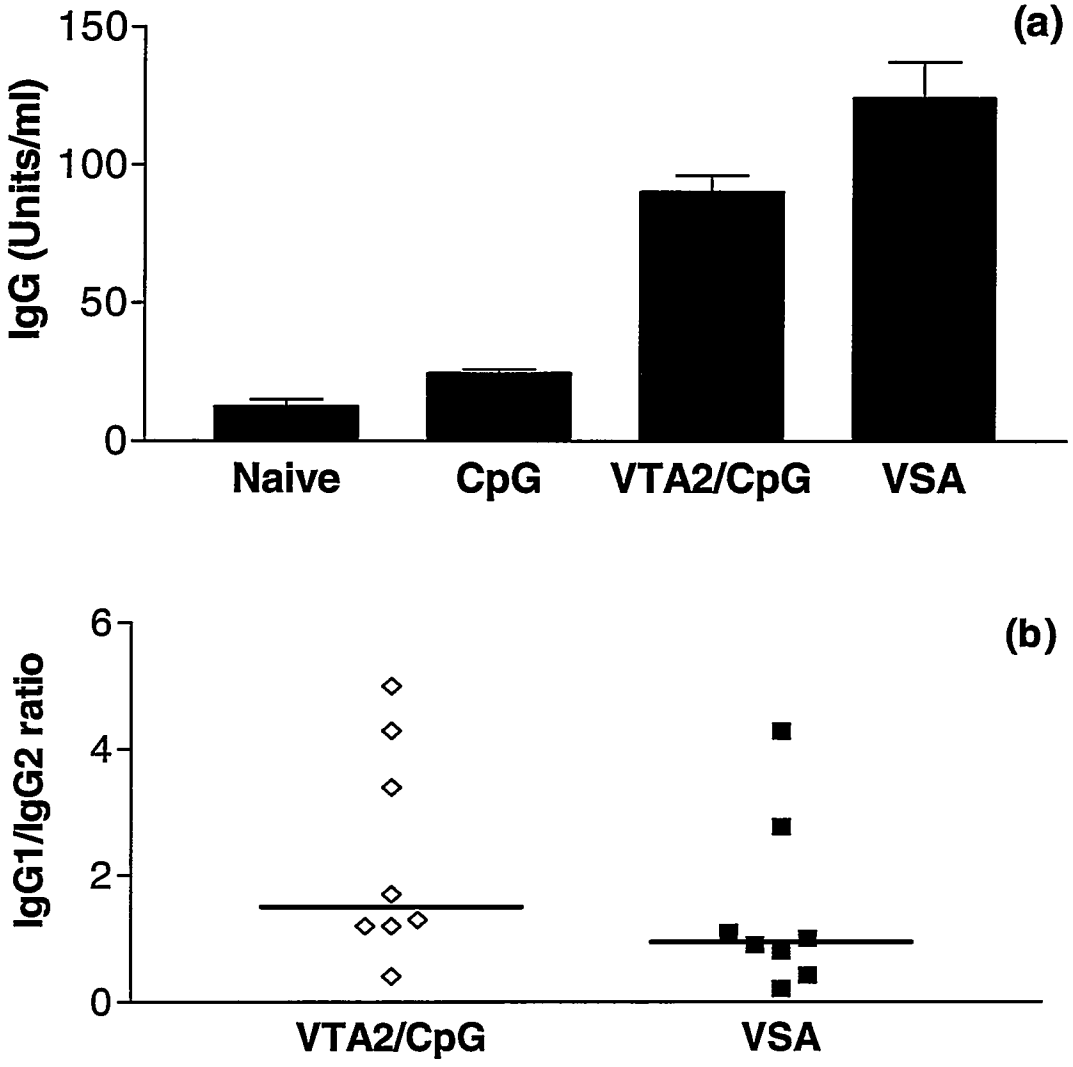

Figure 5.2.1 Induction of OmlA-specific antibody responses in pigs by lipid-based formulations containing CpG ODN.
Pigs were immunized s.c. twice, 21 days apart. Total antigen-specific IgG concentrations (a) and IgG1 and IgG2 ratios (b) were determined in serum 10 days after the last immunization. (a) Results are expressed as the mean concentration ± SEM of 8 pigs; (b) Each point represents an animal with bars indicating the median for each group.

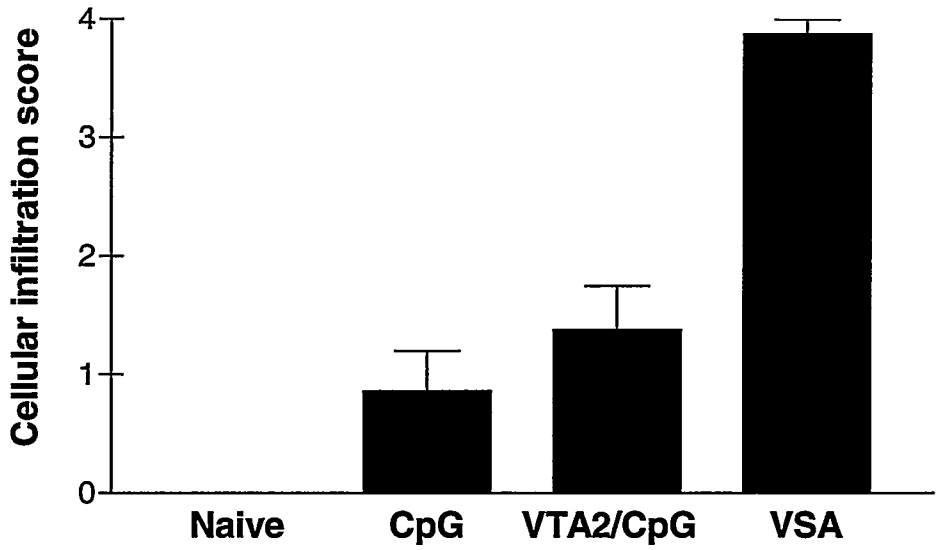

Figure 5.2.2 Tissue damage induced by VTA2 and CpG ODN.
Pigs were immunized s.c. twice, 21 days apart, and histological examination of the injection site was performed 10 days after the last immunization. Scores represent mean ± SEM of 8 pigs.

5.2.3 Protection against challenge

Having demonstrated that VTA2 formulations enhanced the immunoadjuvanticity of CpG ODN, the following studies were designed to confirm that the immunoadjuvant effect observed was due to the specific CpG motifs and also to assess the efficacy of these vaccine formulations to induce protection against a respiratory challenge with *App.* Pigs were immunized twice s.c. 3 weeks apart with OmlA (50 μg) in VTA2, combined with either CpG ODN 2007 (1 mg; VTA2/CpG group) or non-CpG ODN 2041 (1 mg; VTA2/non-CpG group). To evaluate the contribution of VTA2 to the enhancement of immune responses one group was immunized with OmlA in VTA2 alone (VTA2 group); a negative control group received saline (Naïve group). Immunization of pigs with VTA2 containing CpG ODN induced significantly higher OmlA-specific IgG than immunization with formulations containing non-CpG ODN or with VTA2 alone ($p<0.001$; Figure 5.2.3).

To assess whether the immune response induced by immunization with these formulations confers protection against disease, all animals were exposed to *App* by inhalation ten days after the last immunization. The severity of lung lesions was recorded at autopsy 5 days postchallenge. In pigs with severe infection, examination was done at the time of euthanasia. Pigs immunized with OmlA and CpG ODN in VTA2 showed lower mortality (Fig. 5.2.4 a) and fewer lung lesions (Fig. 5.2.4 b) than control animals ($p<0.05$). *App* was isolated from all lymph nodes although fewer bacteria were isolated from the lungs and lymph nodes of animals immunized with CpG ODN in VTA2 compared to the rest of the groups (Table 5.2.1).

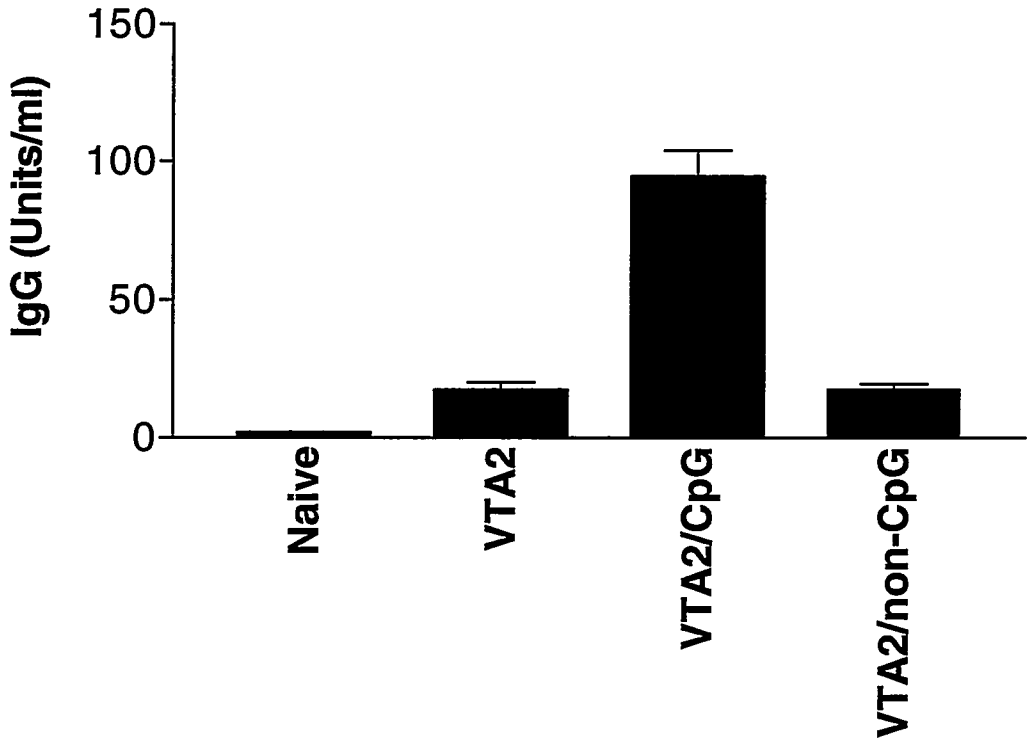

Figure 5.2.3 OmlA-specific antibody responses induced by CpG ODN or non-CpG ODN incorporated in lipid-based formulations.
Pigs were immunized s.c. twice, 21 days apart and anti-OmlA IgG concentrations in serum were determined 10 days after the last immunization. Results are expressed as the mean concentration ± SEM of 8 pigs.

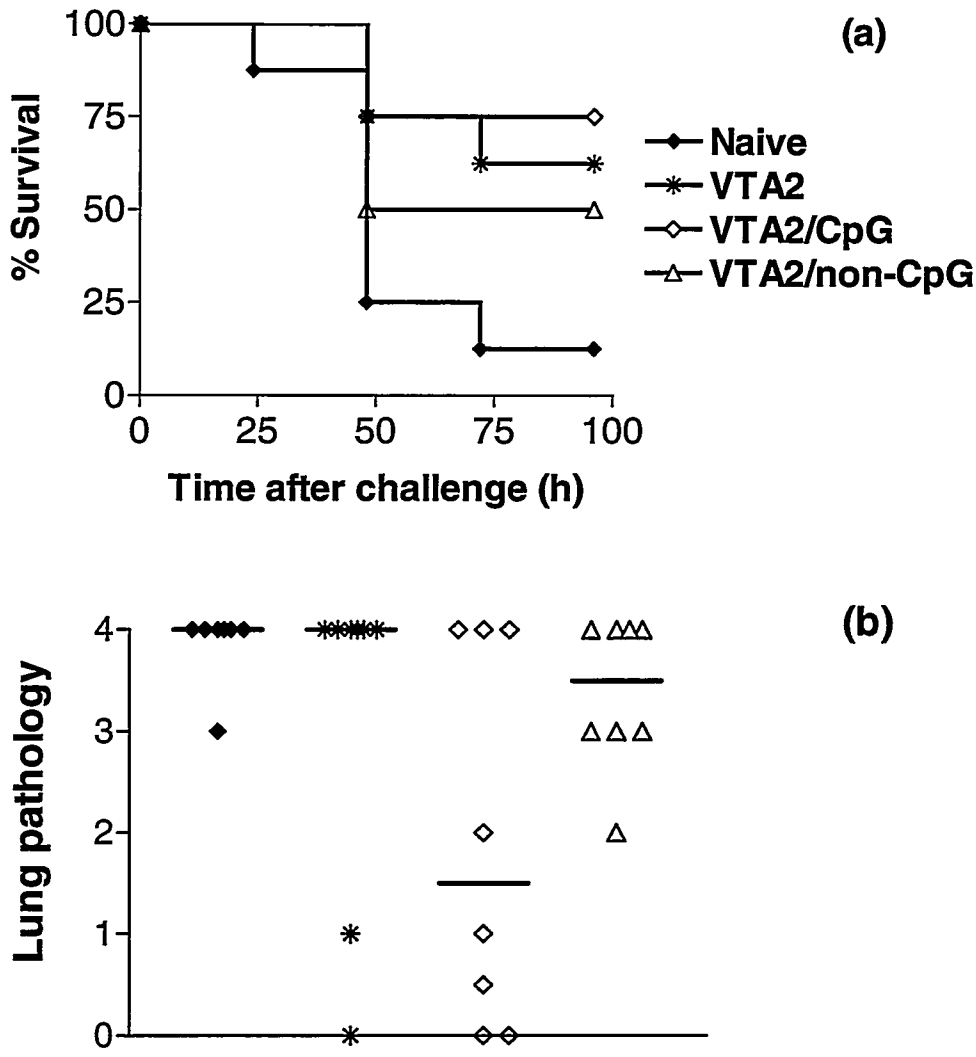

Figure 5.2.4 Protection against infection with *App*.
Pigs were immunized s.c. twice, 21 days apart. Animals were challenged ten days after the last immunization and survival (a) and the severity of lung lesions (b) were recorded. (b) Each point represents an animal and the median of each group is shown.

Table 5.2.1 Bacterial isolation after challenge

Group	Lymph node		Lung	
	Growth score	No. of animals	Growth score	No. of animals
Naïve	2+	7/7	3+	5/7
	1+	(100%)	1+	(71.4%)
	2+		3+	
	1+		0	
	1+		0	
	2+		3+	
	2+		3+	
VTA2	1+	7/7	1+	4/7
	1+	(100%)	0	(57.1%)
	3+		1+	
	2+		0	
	1+		0	
	1+		3+	
	2+		3+	
VTA2/CpG	2+	8/8	0	3/8
	2+	(100%)	1+	(37.5%)
	1+		0	
	1+		0	
	1+		0	
	1+		2+	
	1+		0	
	2+		2+	
VTA2/non-CpG	2+	8/8	0	4/8
	1+	(100%)	0	(50%)
	2+		0	
	1+		0	
	2+		2+	
	2+		3+	
	1+		3+	
	2+		2+	

5.2.4 CpG ODN dose titration

Incorporation of CpG ODN in VTA2 enhanced the adjuvant effect of CpG ODN, inducing protective immune responses. The following set of experiments was designed to determine the optimal dose of CpG ODN required for effective adjuvant activity. Groups of seven pigs were immunized s.c. twice 3 weeks apart with OmlA (50 μg) formulated with VTA2 containing either 1000, 50 or 5 μg of CpG ODN. A control group received saline only. Ten days after the last immunization, OmlA-specific IgG and IgA antibodies were determined in serum and nasal secretions and all pigs except those receiving 1000 μg of CpG ODN were challenged with *App*. Animals immunized with 50 μg of CpG ODN presented antibody levels comparable to 1000 μg/dose and were significantly different from control animals ($p<0.001$; Figure 5.2.5 a and b).

There was a significant effect of immunization on survival ($p<0.01$) following *App* infection. Pigs that received antigen with 50 μg/dose of CpG ODN had a median survival that was undefined (longer than the length of the experiment). The survival times of pigs immunized with antigen together with 5 μg of CpG ODN did not differ from those of naïve animals ($p = 0.08$; Figure 5.2.6 a). There was also a significant effect of treatment on the outcome of the disease. Animals that received antigen together with 50 μg of CpG ODN presented decreased signs of disease compared to control animals ($p<0.05$) (5.2.6 b).

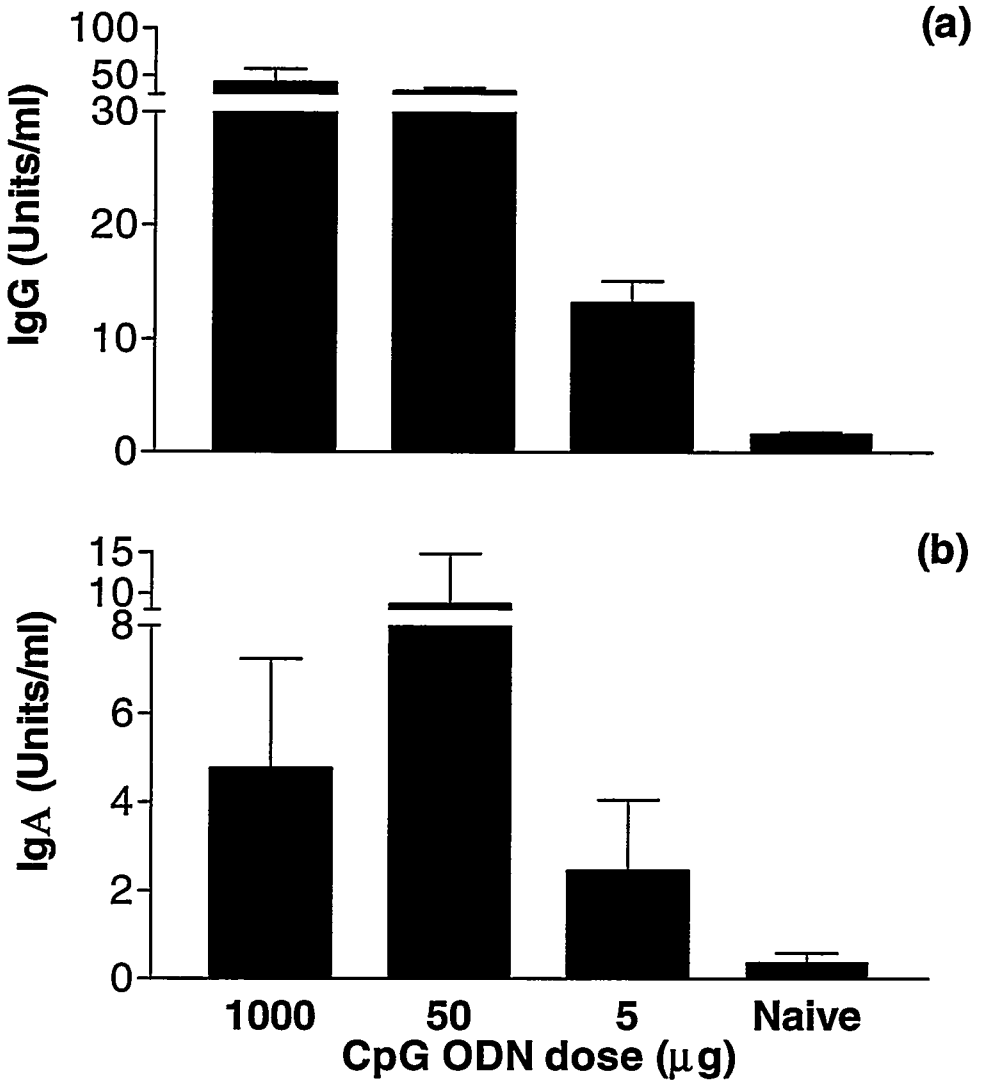

Figure 5.2.5 OmlA-specific serum antibodies induced by lipid-based formulations containing different amounts of CpG ODN.
Pigs were immunized s.c. twice, 21 days apart with OmlA in VTA2 containing either 1000, 50 or 5 μg of CpG ODN. A negative control group received saline only. Serum IgG (a) and IgA (b) were assessed 10 days after the last immunization. Results are expressed as the mean concentration ± SEM of 7 pigs.

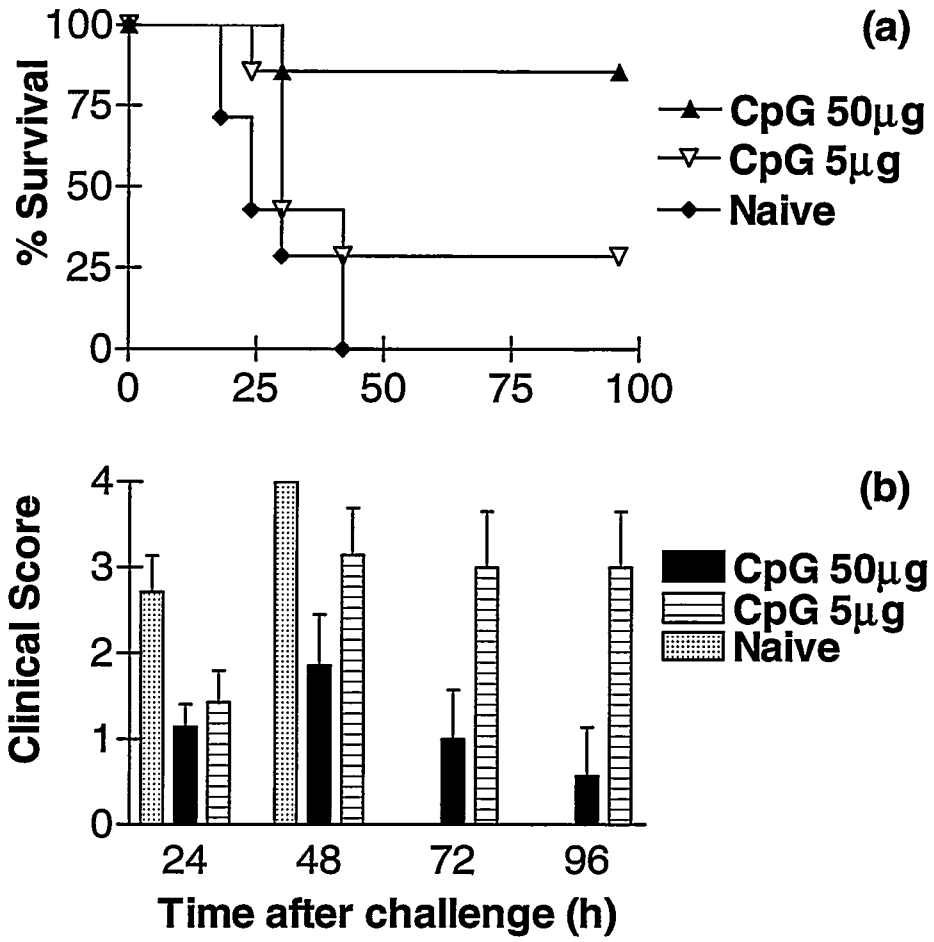

Figure 5.2.6 Protection against challenge with *App.*
Groups of seven pigs were immunized s.c. twice, 21 days apart with OmlA in VTA2 containing either 50 or 5 µg of CpG ODN. A negative control group received saline only. Ten days after the last immunization, pigs were challenged and observed daily for survival (a) and clinical signs of disease (b). Pigs with a clinical score of 3 were euthanized; on day 5 after challenge all remaining pigs were euthanized.

5.2.5 Conclusions

Results demonstrated that incorporation of CpG ODN in VTA2 enhanced the adjuvanticity of CpG ODN in pigs. Immunization of pigs with the *App* OmlA protein and CpG ODN formulated in the biphasic lipid delivery system VTA2 induced higher IgG antibodies than immunization with antigen and either CpG ODN or VTA2 alone or formulated non-CpG ODN. The responses elicited were balanced, with production of IgG1 and IgG2, and also were comparable to those induced by the standard commercial adjuvant VSA. Furthermore, following challenge, animals from the VTA2/CpG group presented reduced mortality and more pigs were protected from developing pneumonia. Additionally, histological assessment of the site of injection revealed that in contrast to the local severe cell infiltration induced by VSA, administration of VTA2/CpG induced mild or no inflammation.

It was also possible to decrease the amount of adjuvant required to stimulate protective immune responses since 50 μg of CpG ODN effectively induced specific antibody levels that were comparable to those induced by immunization with 1000 μg of CpG ODN. The responses induced by immunization with formulations containing 50 μg were sufficient to reduce mortality and prolong survival of pigs challenged with virulent *App*.

5.3 Alternative approaches to parenteral immunization

The use of non-invasive routes for vaccine delivery that allow efficient uptake of antigen by APCs is necessary to make administration simple, painless and economically practical. Several studies have provided evidence that the nose and the bare skin are potential routes for immunization (Partidos, *et al.*, 2001).

5.3.1 Ability of microneedles to facilitate delivery of antigens

The immunological competence of the skin as a site of vaccine delivery has been clearly demonstrated in systems using DNA vaccines and CT (Condon, *et al.*, 1996; Leitner, *et al.*, 1997; Glenn, *et al.*, 1998a; Glenn, *et al.*, 1998b). The hydrophilic nature and large molecular size of protein antigens versus the lipophilic character of the stratum corneum is the major barrier in the use of the epidermis as a potential site for vaccine delivery. In the absence of an effective delivery mechanism, topically applied vaccines cannot penetrate through the stratum corneum. One potential approach is the use of microneedles to create painless, superficial pathways through the stratum corneum of the skin that allow the penetration of macromolecules.

In order to determine whether the delivery of antigens and adjuvants can be facilitated by the use of microneedles, pigs were immunized by topical administration of OmlA and different adjuvants (CT, CpG ODN) after treatment of the skin with microneedles. This approach was also compared with intradermal delivery of the same formulations.

5.3.1.1 CT-specific immune responses after application of microneedles

Transcutaneous immunization of pigs with CT induced significantly higher CT-specific IgG in serum compared to naïve animals only when applied after treatment with microneedles. In order to determine whether transcutaneous immunization with the bacterial antigen OmlA could be achieved, a number of approaches were taken that combined the use of microneedles, adjuvants (CT and CpG) and lipid-based delivery systems. Both proteins were co-administered after pretreatment of the skin with microneedles. Groups of seven animals received OmlA (50 μg) and CT (60 μg) together or separate twice, 12 days apart. Control animals received both proteins without pretreatment with microneedles. This approach was also compared to topical application of OmlA and CT formulated in a lipid-based delivery system designed to help proteins to cross the skin and reach the immunocompetent cells (VPE1).

None of the immunized animals had OmlA-specific IgG, regardless the treatment (data not shown). On the other hand, animals that received CT and microneedles treatment showed higher CT-specific IgG than control animals ($p<0.05$; Figure 5.3.1a). Serum IgA levels in animals receiving CT and microneedles treatment were also higher than in control groups ($p<0.05$; Figure 5.3.1b). These results were consistent with those obtained previously. There were no CT-specific antibody responses in animals topically immunized with CT in VPE1.

Animals immunized by topical application of CT following microneedles treatment also showed a significant induction of CT-specific IgA in nasal secretions compared to control animals ($p<0.05$; Figure 5.3.2). IgA responses were not detected in saliva of immunized animals (data not shown).

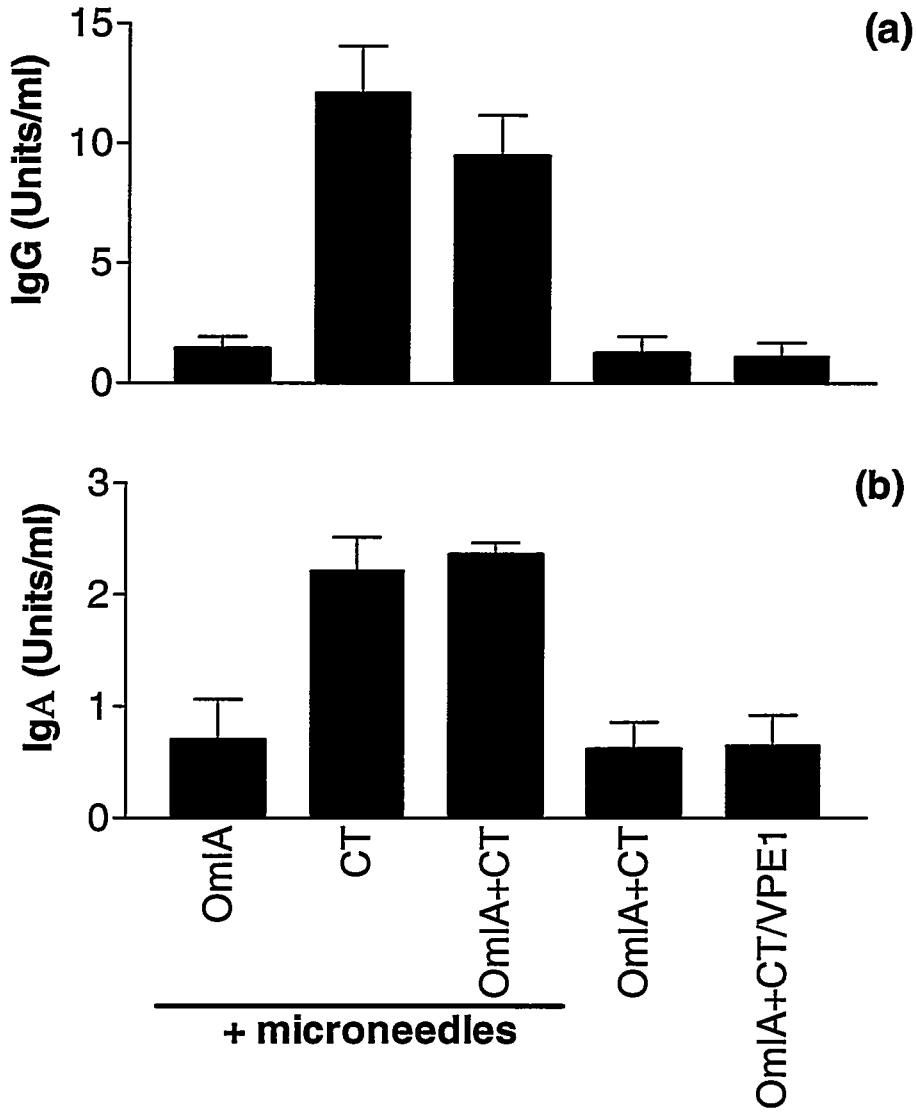

Figure 5.3.1 CT-specific serum antibodies after immunization of pigs following treatment with microneedles.
Animals were administered the respective formulations twice, 12 days apart. Anti-CT IgG (a), and IgA (b) concentrations in serum were determined 10 days after the last immunization. Results are expressed as the mean concentration ± SEM of 7 pigs.

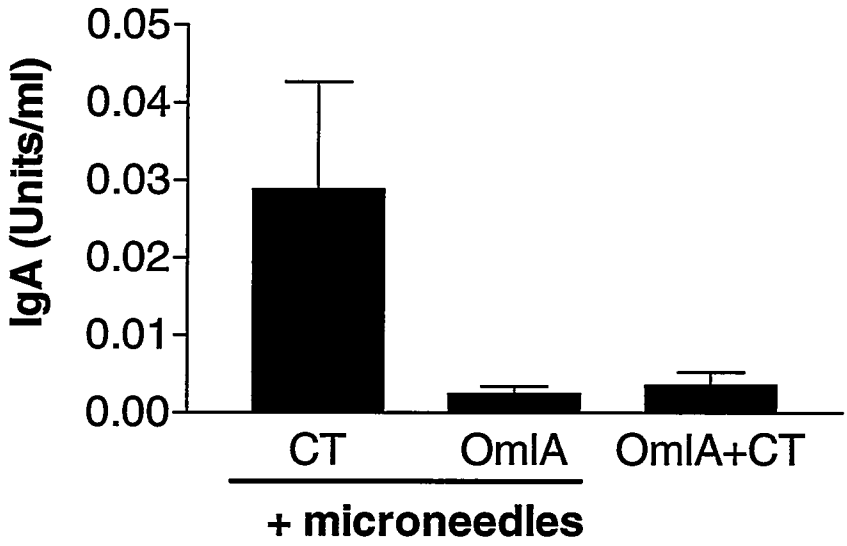

Figure 5.3.2 CT-specific IgA in nasal secretions after immunization of pigs following treatment with microneedles.
Animals were administered the respective formulations twice, 12 days apart. Anti- IgA concentrations in nasal secretions were determined 17 days after the last immunization. Results are expressed as the mean concentration ± SEM of 7 pigs.

5.3.1.2 Penetration of skin by microneedles

Disruption of the stratum corneum is the key to successful transdermal delivery. To assess the level of penetration and disruption of the stratum corneum caused by treatment of the skin with the microneedles device, an India ink solution was applied onto the ear before puncturing with the microneedles to test the pattern and length of skin penetration. The total thickness of the stratum corneum varies from 10 to 40 μm with an average thickness of 20 μm (Panchagnula, *et al.*, 1997). The average length of the microneedles is 2mm, and disruptions found in frozen sections of the ear caused by the microneedles ranged from 0.1mm to 0.8mm (Figure 5.3.3).

5.3.1.3 Transcutaneous immunization with biphasic lipid formulations and CpG ODN

From the results above, it is clear that direct application of antigen and adjuvant in VPE1 was not effective and that microneedles are necessary for the induction of antigen-specific immune responses. VTA formulations were effective delivery systems for CpG ODN when administered by the subcutaneous route. The next approach was to determine whether these vaccine formulations were effective when used for transcutaneous immunization. Transcutaneous immunization of pigs with OmlA and CpG ODN in VTA4 in combination with microneedles treatment did not induce OmlA-specific immune responses, even after a third immunization with the same dose used subcutaneously (data not shown).

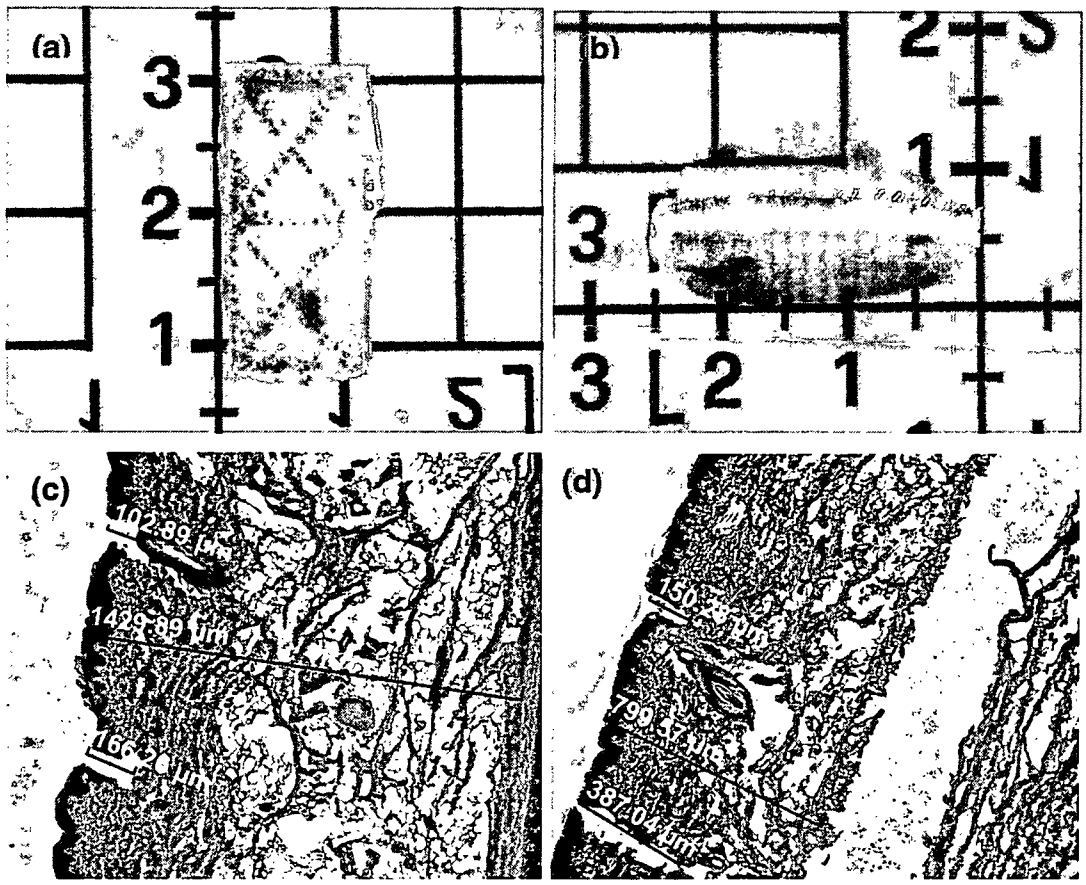

Figure 5.3.3 Penetration of skin by microneedles.
Section of porcine ear showing the site where the India ink and the microneedles were applied (a) and (b); cross-section of the ear showing disruptions to the skin by the microneedles (c) and (d).

Given the lack of response to OmlA in the above experiments using doses of OmlA and CpG ODN similar to those used subcutaneously, it was important to determine whether higher doses of antigen and adjuvant were needed for transcutaneous immunization. The following experiment was also carried out to compare the topical application after microneedles treatment with the intradermal administration for the induction of OmlA-specific immune responses. Since intradermal administration targets the same immunological milieu than transcutaneous immunization, it can provide important and relevant insights into the interaction between the lipid formulation and the skin immune system.

Groups of three animals were immunized topically or i.d twice, 17 days apart, with formulations containing 250 μg of both OmlA and CpG ODN in VTA2. Intradermal administration of the lipid formulation induced an increase in serum IgG after one immunization compared to naïve animals ($p<0.05$). IgG concentrations in serum were even higher after a second i.d. administration (Figure 5.3.4 a). On the other hand, an increase in antigen-specific IgA levels ($p<0.05$) was observed only after a second immunization (Figure 5.3.4 b). OmlA-specific IgG was also detected in nasal secretions of animals immunized twice with the lipid formulation intradermally ($p<0.05$; Figure 5.3.5), but no IgA was detected (data not shown).

When the same formulation was applied topically after treatment with microneedles, there was no induction of antigen-specific antibodies in either serum (Figure 5.3.4 a and b) or in nasal secretions (Figure 5.3.5).

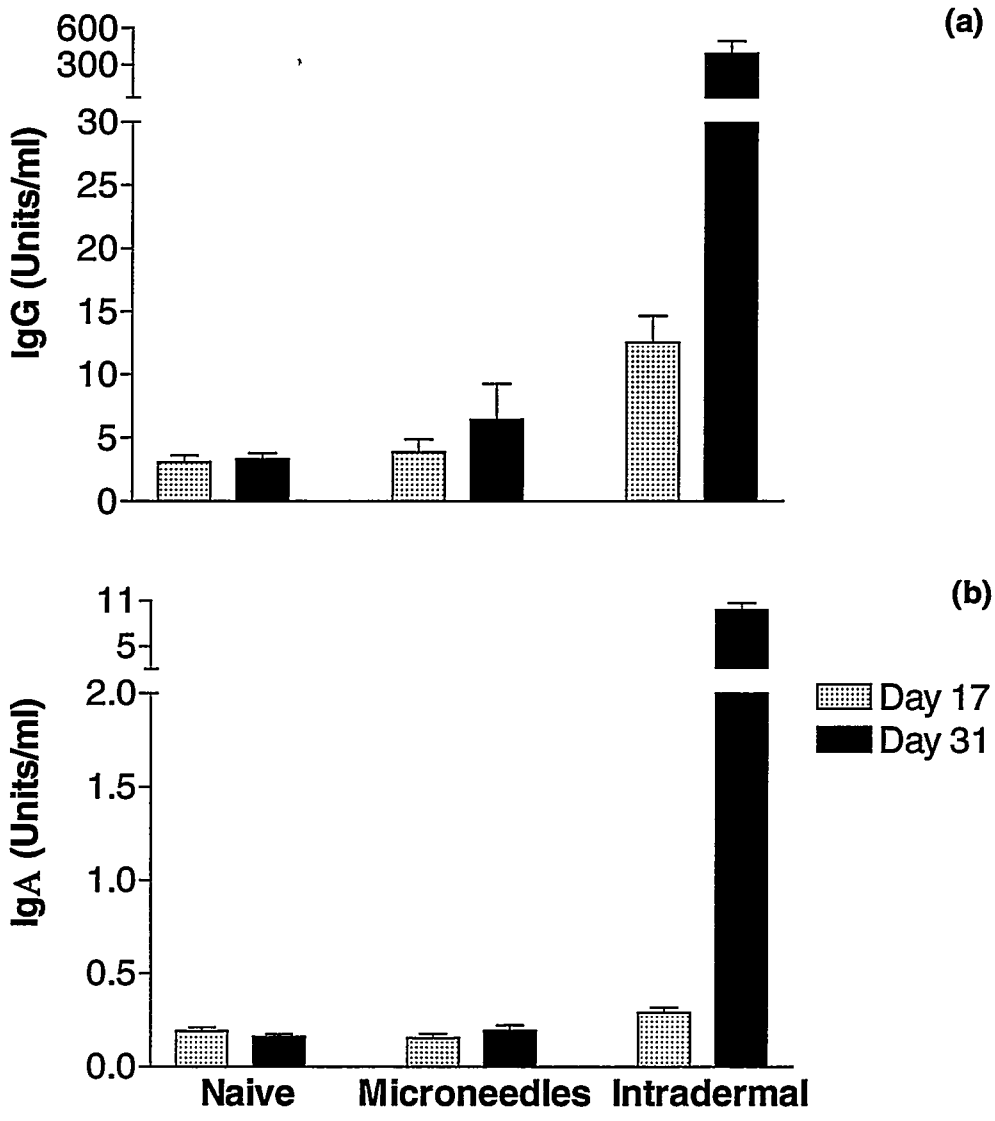

Figure 5.3.4 OmlA-specific antibody responses in serum after intradermal administration or transcutaneous immunization following treatment with microneedles.
Animals were administered OmlA and CpG ODN in VTA2 twice, 17 days apart. IgG (a), and IgA (b) in serum were determined 17 and 31 days after the first immunization. Results are expressed as the mean concentration ± SEM of 3 pigs.

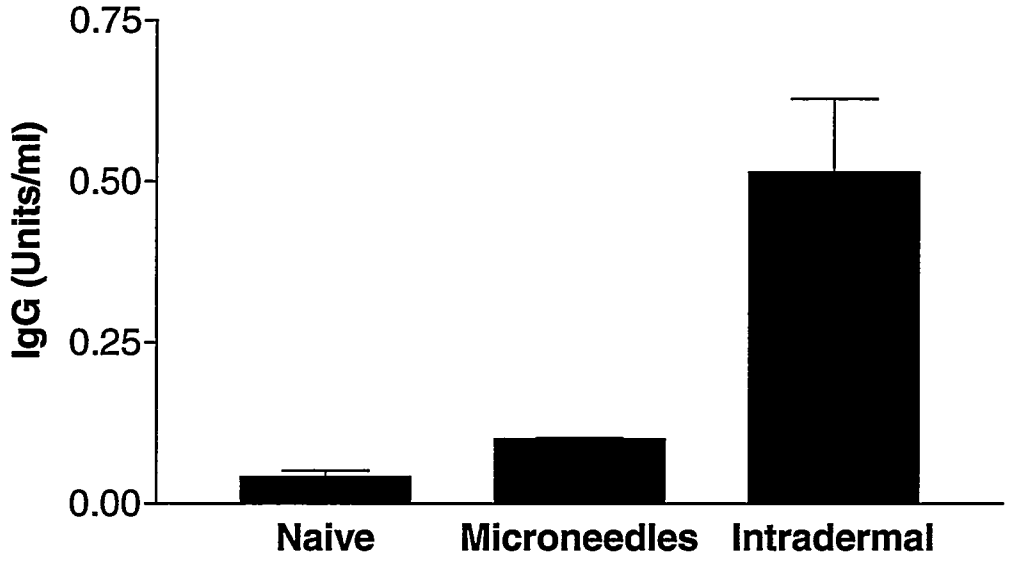

Figure 5.3.5 Induction of OmlA-specific IgG in nasal secretions after intradermal administration or transcutaneous immunization following treatment with microneedles.
Animals were administered OmlA and CpG ODN in VTA2 twice, 17 days apart. IgG concentrations in nasal secretions were determined 45 days after the first immunization. Results are expressed as the mean concentration ± SEM of 3 pigs.

5.3.2 Induction of mucosal immunity through intranasal delivery

The fact that most infectious agents use mucosal membranes as a frequent portal of entry into the host has lead to the development of vaccines and appropriate delivery systems that can efficiently induce mucosal immunity. Administration of vaccines through mucosal sites is more effective in inducing mucosal immunity than parenteral immunization (McGhee, *et al.*, 1992; Kaul and Ogra, 1998) but mucosal immunization protocols frequently use large doses of antigen, live organisms or bacterial toxins as adjuvants (Holmgren, *et al.*, 2003). Successful delivery of antigens via the intranasal route requires delivery systems that protect the antigen from degradation and facilitate its uptake and transport to the lymphoid tissue for presentation to immunocompetent cells. It has already been shown that lipid-based delivery systems enhance the adjuvant effect of CpG ODN and induce protective immune responses when administered subcutaneously. A different formulation was developed for intranasal delivery of CpG ODN (VTAM1). Combinations of different routes of delivery (systemic prime and mucosal boost or mucosal prime and systemic boost) were used to determine whether these immunization protocols induce mucosal as well as systemic immune responses against the OmlA protein. The main objective of these experiments was to assess the effectiveness of intranasal delivery; therefore, groups of pigs received different formulations through the intranasal route, but were immunized with the same formulation systemically.

5.3.2.1 Immune responses after systemic/mucosal immunization

Groups of seven animals received a s.c. immunization and an i.n. boost 3 weeks later as described in Table 5.3.1. CT was included as a positive control for mucosal immunization. Negative control animals received the adjuvant and delivery system without antigen.

After the i.n. boost, animals that received the antigen and CpG ODN or CT presented higher IgA responses in serum compared to placebo animals ($P<0.05$) but only a slight increase in IgG (Figure 5.3.6). Similarly, IgG responses were higher in nasal secretions of those same groups compared to placebo animals ($p<0.05$ for CpG and $p<0.01$ for CT; Figure 5.3.7 a) while an increase in local IgA was detected in only three out of seven animals immunized with CpG alone or in VTAM1, these responses were not statistically different (Figure 5.3.7 b). The combination of CpG/VTAM1 also elicited an increase in IgG in saliva compared to other groups ($p<0.05$; Figure 5.3.8).

5.3.2.2 Immune responses after mucosal/systemic immunization

Animals received two i.n. immunizations at day 0 and 30 and one s.c. at day 21 as described in Table 5.3.2. CT was included as a positive control for mucosal immunization. Negative control animals received saline. Serum and local antibody responses were assessed after each immunization and compared to responses induced by a biphasic lipid formulation containing antigen and CpG ODN after two s.c. immunizations.

Table 5.3.1 Groups design for the evaluation of immune responses after a systemic/mucosal protocol of immunization

Group [1]	s.c. immunization [2]	i.n. immunization [3]
1) Placebo	CpG/ VTA4	CpG/VTAM1
2) CT	OmlA+CpG VTA4 (9:1)	OmlA+CT (5 μg)
3) CpG	OmlA+CpG VTA4 (9:1)	OmlA+CpG (50 μg)
4) VTAM1	OmlA+CpG VTA4 (9:1)	OmlA VTAM1 (9:1)
5) VTAM1/CpG	OmlA+CpG VTA4 (9:1)	OmlA+CpG VTAM1 (9:1)

[1] Each group consisted of 7 animals.
[2] Animals received a s.c. immunization on day 0. OmlA and CpG doses were 50 μg each.
[3] Animals received an i.n. immunization on day 21. OmlA dose was 100 μg.

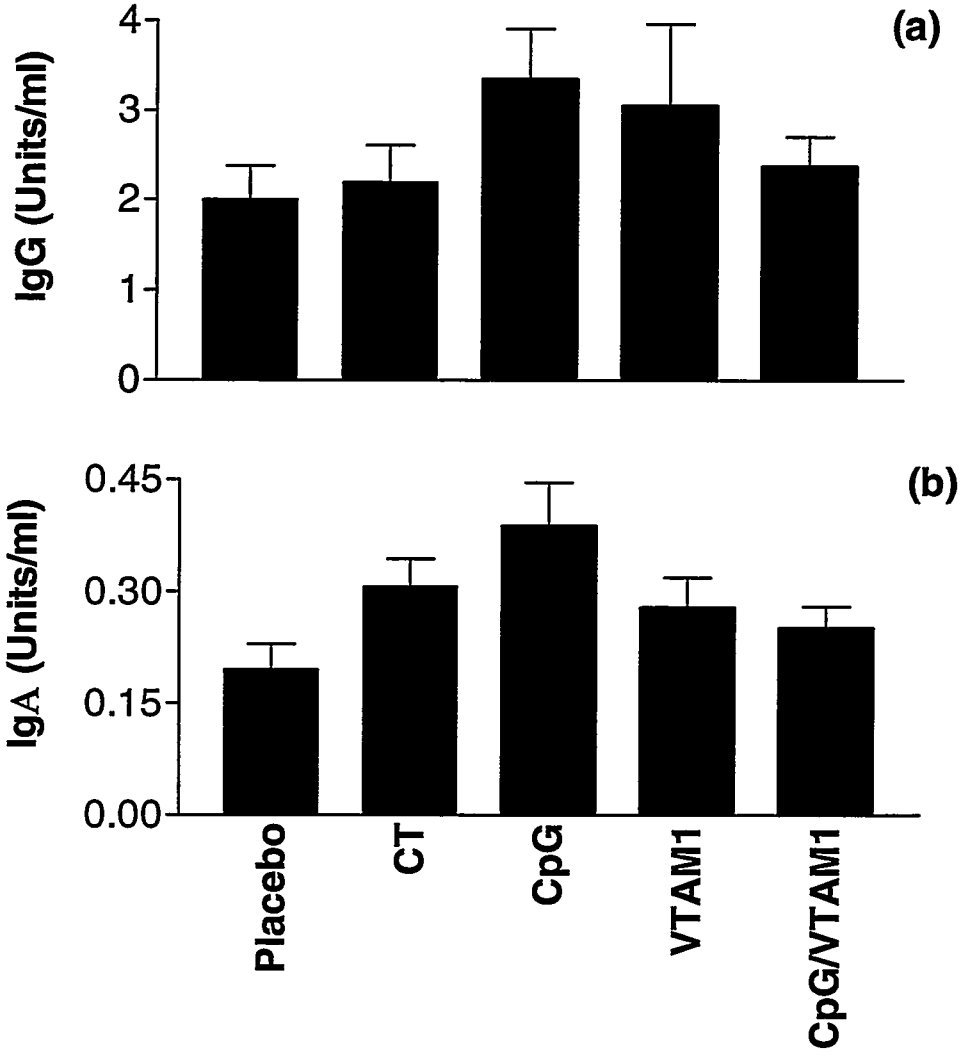

Figure 5.3.6 Antibody responses in serum after a systemic/mucosal immunization protocol.
Animals received a s.c. immunization with OmlA and CpG in VTA4, followed by an i.n administration of different formulations 3 weeks later. Anti-OmlA IgG (a), and IgA (b) concentrations in serum were determined 30 days after the first immunization. Results are expressed as the mean concentration ± SEM of 7 pigs.

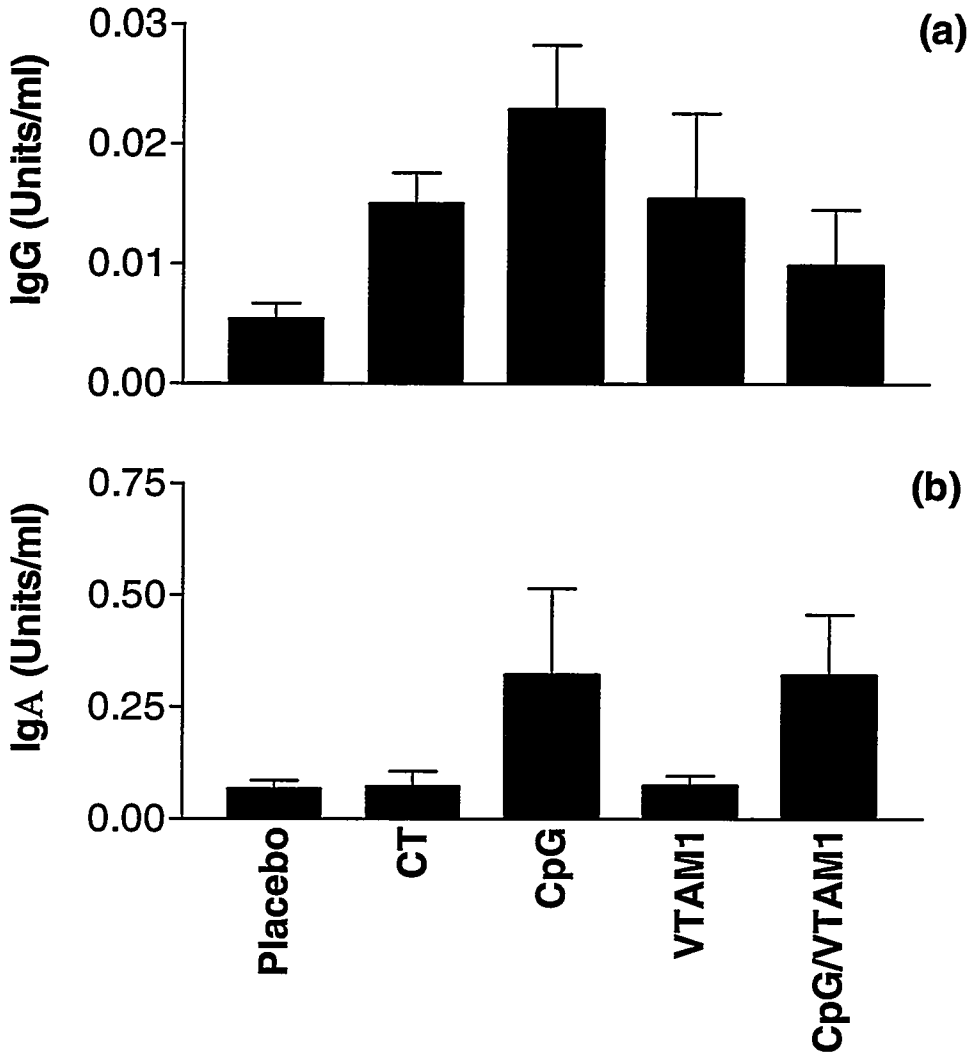

Figure 5.3.7 OmlA-specific antibodies in nasal secretions of pigs immunized by systemic/mucosal routes.
Animals received a s.c. immunization with OmlA and CpG in VTA4, followed by an i.n administration of different formulations 3 weeks later. Anti-OmlA IgG (a), and IgA (b) concentrations in nasal secretions were determined 30 days after the first immunization. Results are expressed as the mean concentration ± SEM of 7 pigs.

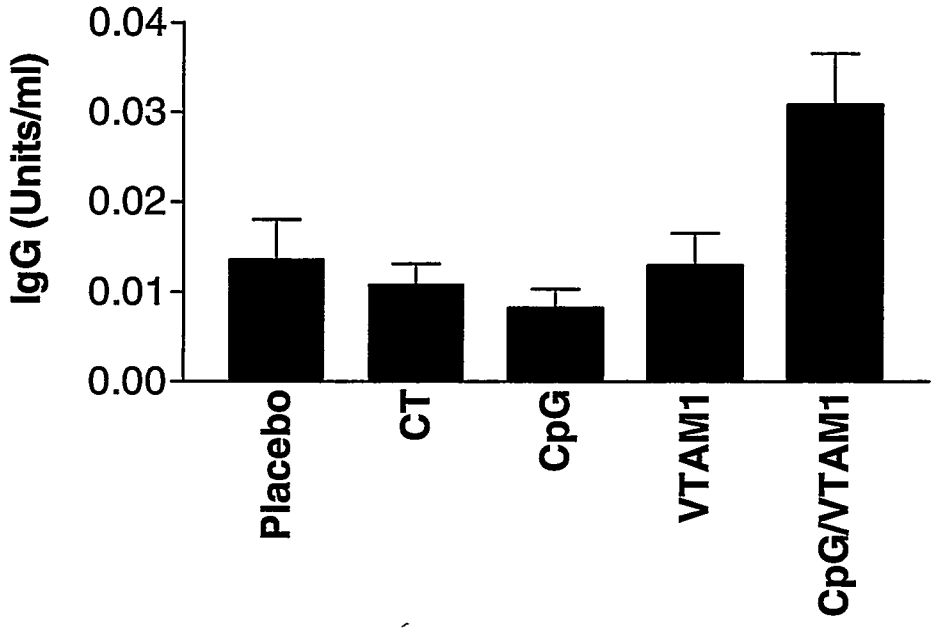

Figure 5.3.8 OmlA-specific IgG in saliva of pigs immunized by systemic/mucosal routes.
Animals received a s.c. immunization with OmlA and CpG in VTA4, followed by an i.n administration of different formulations 3 weeks later. Anti-OmlA IgG concentrations in saliva were determined 30 days after the first immunization. Results are expressed as the mean concentration ± SEM of 7 pigs.

Table 5.3.2 Groups design for the evaluation of immune responses after a mucosal/systemic protocol of immunization

Group [1]	i.n. immunization [2]	s.c. immunization [3]
1) Naive	Saline	Saline
2) OmlA	OmlA	OmlA+CpG 30% Emulsigen (v/v)
3) CT	OmlA+CT (10 μg)	OmlA+CpG 30% Emulsigen (v/v)
4) CpG	OmlA+CpG (250 μg)	OmlA+CpG 30% Emulsigen (v/v)
5) VTAM1/CpG	OmlA+CpG/VTAM1 (1:9)	OmlA+CpG 30% Emulsigen (v/v)
6) VTA/CpG		OmlA+CpG VTA2 (1:9)

[1] Each group consisted of 7 animals.
[2] All animals except group 3 received i.n. immunizations on day 0 and 30. OmlA dose was 250 μg.
[3] Animals received a s.c. immunization on day 21. Group 6 received two s.c. immunization 3 weeks apart (day 0 and 21). OmlA and CpG doses were 50 μg each.

Seven days after the s.c. boost, animals that received the antigen and CpG ODN formulated in VTAM1 presented higher IgG and IgA responses in serum compared to naïve animals ($P<0.01$ and 0.001 respectively; Figure 5.3.9). However, local immune responses were not detected, even in the group that received OmlA with CT (Figure 5.3.10).

In order to evaluate whether it was possible to induce mucosal immunity, animals received a second i.n. immunization and antibody responses were assessed one week later. There was an increase in serum IgG and IgA levels, particularly in animals vaccinated with the antigen and CT ($p<0.001$) or CpG ODN in VTAM1 ($p<0.01$), although animals receiving OmlA alone also showed increased serum antibodies ($p<0.05$; Figure 5.3.9). Only animals immunized with the antigen and CpG ODN in VTAM1 developed local immunity ($p<0.01$ for IgG and $p<0.001$ for IgA). IgA in nasal secretions was also higher in animals from the positive control compared with naïve animals ($p<0.05$; Figure 5.3.10). Antibody responses induced by CpG/VTAM1 after the combined protocol, were similar to those induced after two s.c. administrations both for serum (Figure 5.3.9) and nasal secretions (Figure 5.3.10).

To define the type of immune response generated, OmlA-specific IgG1 and IgG2 levels were determined. Immunization with CpG ODN alone induced a Th1-type response while the rest of the formulations induced a balanced response, with production of both IgG1 and IgG2 (Figure 5.3.11).

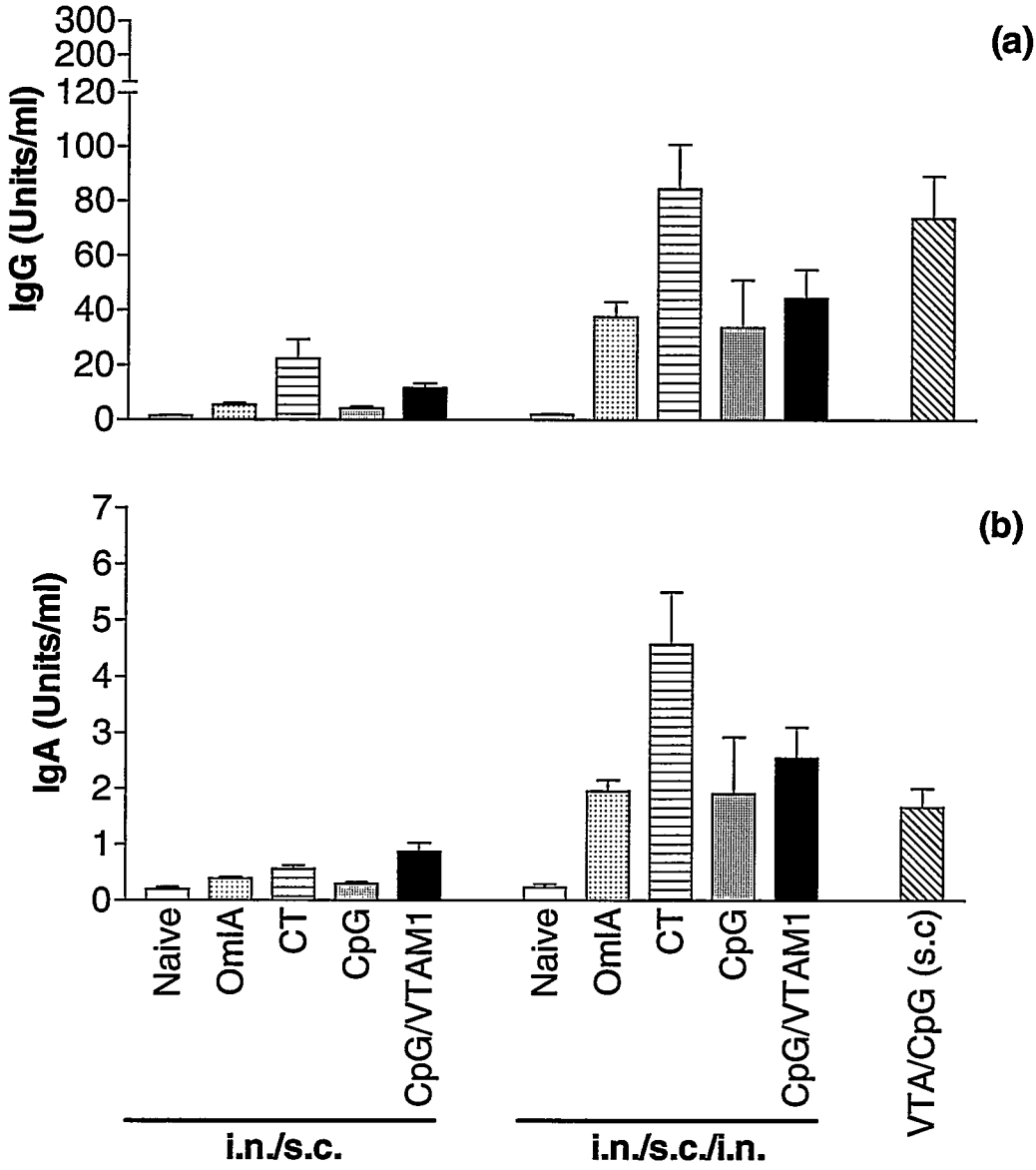

Figure 5.3.9 OmlA-specific serum antibodies after a mucosal/systemic immunization protocol.
Animals received two i.n. immunizations with different formulations at day 0 and 30 and one s.c. at day 21 with OmlA and CpG ODN in Emulsigen. Anti-OmlA IgG (a), and IgA (b) concentrations in serum were determined 7 days after each boost and compared with s.c. immunizations. Results are expressed as the mean concentration ± SEM of 7 pigs.

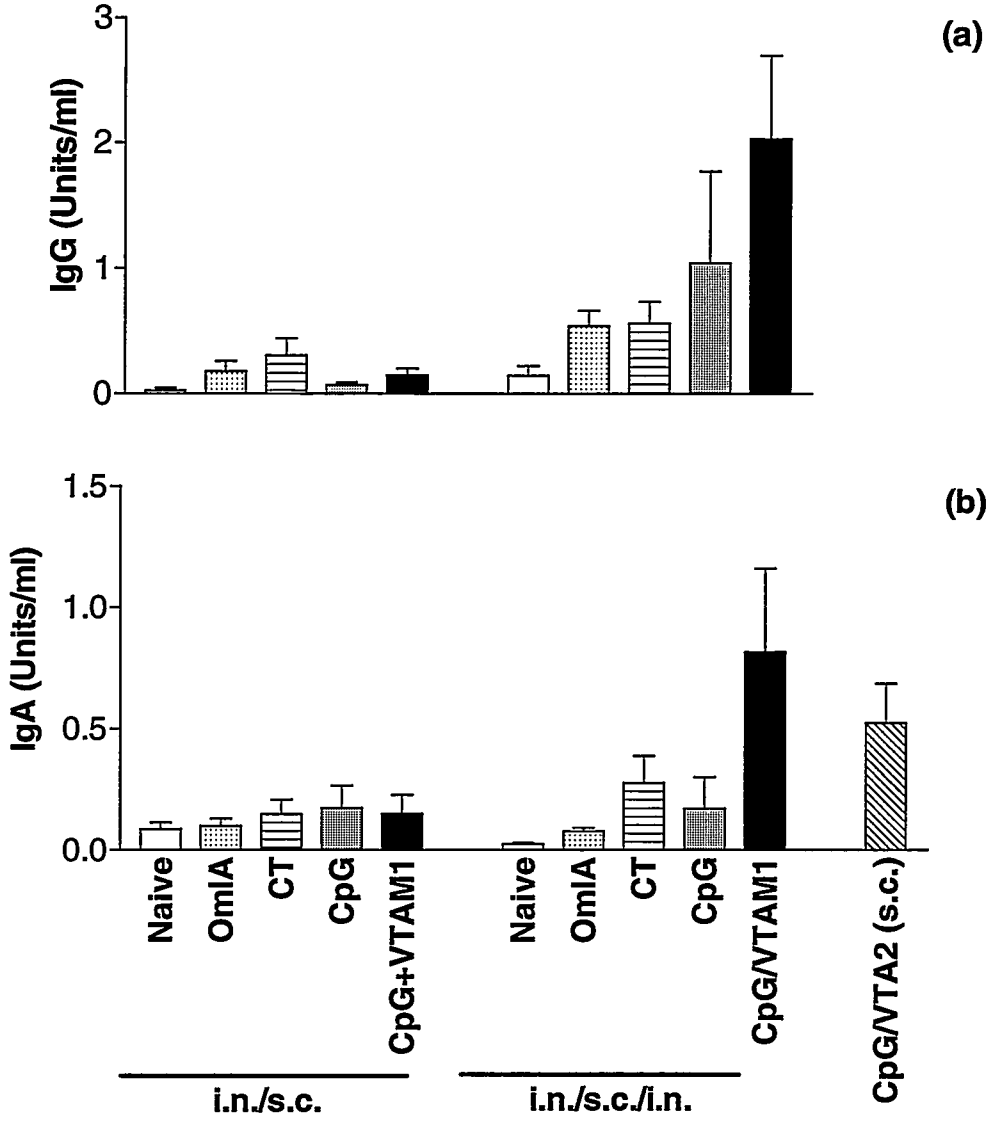

Figure 5.3.10 OmlA-specific antibody responses in nasal secretions after a mucosal/systemic immunization protocol.
Animals were immunized as explained in Figure 5.3.9. Anti-OmlA IgG (a), and IgA (b) concentrations in nasal secretions were determined 7 days after after each boost and compared with two s.c. immunizations. Results are expressed as the mean concentration ± SEM of 7 pigs.

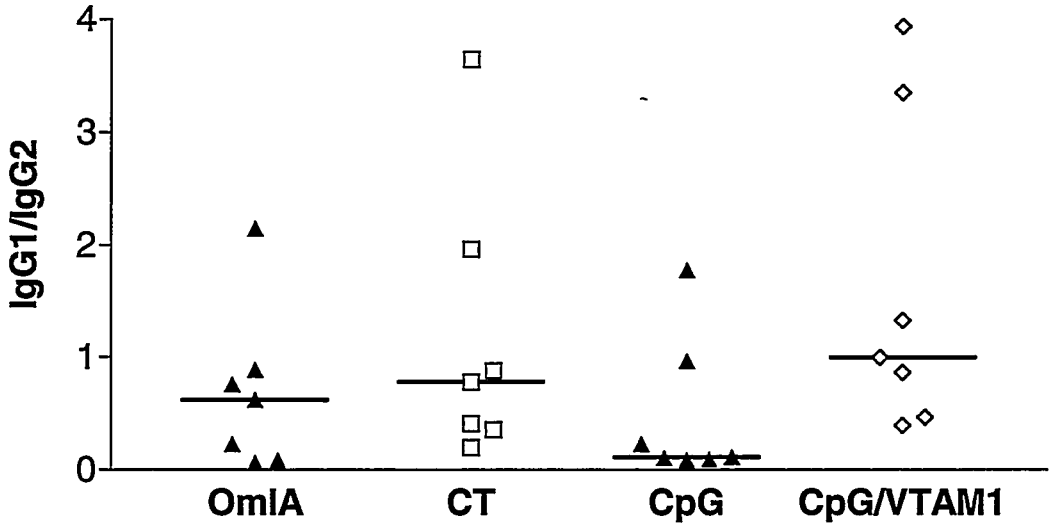

Figure 5.3.11 OmlA-specific IgG1 and IgG2 ratios.
Animals were immunized as explained in Figure 5.3.9. Anti-OmlA IgG1 and IgG2 titres in serum were determined 7 days after the last boost. Results show isotype ratios for individual animals, with bars indicating the median for each group.

5.3.3 Conclusions

Results showed that multiple punctures with microneedles facilitated the delivery of CT and the induction of CT-specific humoral and mucosal antibodies. On the other hand, none of the approaches used was effective in mediating the induction of immune responses to OmlA following transcutaneous immunization. In contrast, intradermal delivery of the antigen and CpG ODN in VTA2 induced strong IgA and IgG responses in serum, as well as IgG in nasal secretions.

The intranasal route was an effective needle-free vaccine delivery route in pigs, inducing both systemic and local immune responses, when combined with s.c. boost. Induction of mucosal immunity was achieved only when the antigen and CpG ODN were formulated in VTAM1.Antibody levels elicited in serum after a combined mucosal/systemic protocol were comparable to those obtained after two subcutaneous immunizations. However, formulations had to be administered at least twice through the intranasal route. The responses elicited were balanced, with production of IgG1 and IgG2.

5.4 Mechanisms of action by which the lipid-based formulations enhance the immunoadjuvant activity of CpG ODN

Understanding how lipid-based formulations potentiate the immunoadjuvant activity of CpG ODN is critical to the rational design of new effective vaccination strategies. A number of experiments were designed to assess whether changes in formulation and delivery impact the recruitment of cells to the site of inoculation or the type of cellular immune responses induced systemically. Several experiments were also carried out initially to develop and optimize the techniques needed to detect antigen-specific cytokine production in peripheral blood mononuclear cells (PBMCs) from immunized pigs.

5.4.1 Development of assays to determine porcine antigen-specific cytokine secretion in PBMCs

In preliminary studies, animals vaccinated twice i.m. 3 weeks apart with the commercial vaccine Pleuro-Star 4^{TM} were used to optimize the conditions for the detection of OmlA-specific cellular responses since this vaccine induces a very strong immune response. IFNγ-secreting cells were detected by ELISPOT and secreted IFNγ was assessed in supernatants from cultured cells by ELISA.

PBMCs from naive pigs were cultured with different mitogens (LPS, PHA, ConA) for different time periods to determine the optimal stimulation time. Results indicated that cytokine production peaked at 24 h, and this culture time was used in further experiments (data not shown).

To determine the optimal cell density and antigen concentration for *in vitro* culture, PBMCs were isolated from vaccinated and naïve animals and cultured at two different cell densities for 24h in the presence of 10, 5 or 2 μg/ml

of OmlA. Controls included no antigen and the mitogen phytohaemoagglutinin (PHA, 10 μg/ml). Cells cultured at a concentration of $0.5x10^6$ cells/well with 10μg/ml of OmlA showed the highest number of IFN-γ-secreting cells and IFNγ secreted, although this response was not statistically significant (Figure 5.4.1).

It has been shown that activated lymphocytes are preferentially localized in lymphoid and non-lymphoid tissue and very few may be found in peripheral blood (Mackay, 1992); to evaluate whether the poor responses obtained in PBMCs were due to the kinetics of circulation of antigen specific-cells, cellular responses were measured in splenocytes of vaccinated and naïve animals. A low but consistent IFN-γ response was observed by ELISPOT and ELISA (Figures 5.4.2 a and 5.4.3). IL-4-secreting cells were detected only after stimulation with mitogen, but not with the antigen (Figure 5.4.2 b).

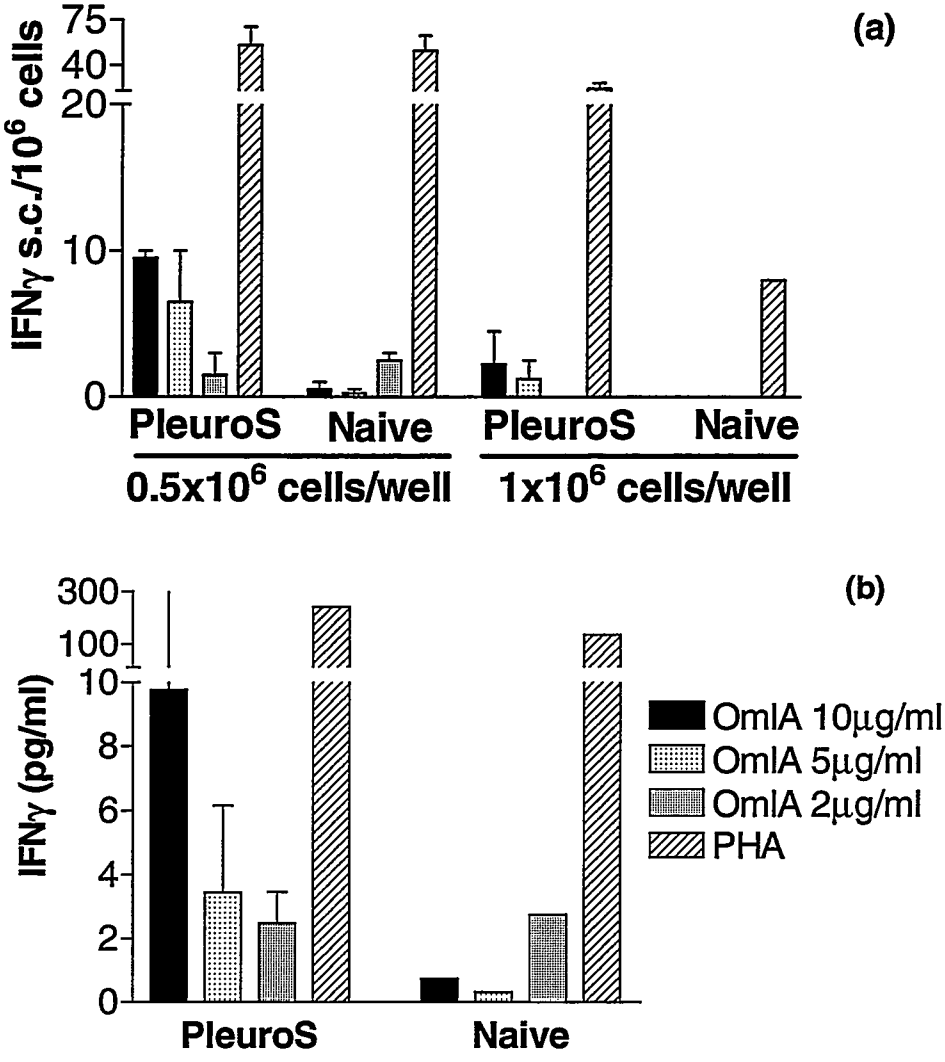

Figure 5.4.1 OmlA-specific IFNγ production.
PBMCs isolated from i.m. vaccinated and naïve animals were cultured at 0.5 and 1x10^6 cells/well with different concentrations of OmlA for 24 h. IFN-γ-secreting cells were detected by ELISPOT (a); IFNγ in supernatants of cells cultured at 0.5x10^6 cells/well with different concentrations of antigen was detected by ELISA (b). Results are expressed as means ± SEM of 2 pigs.

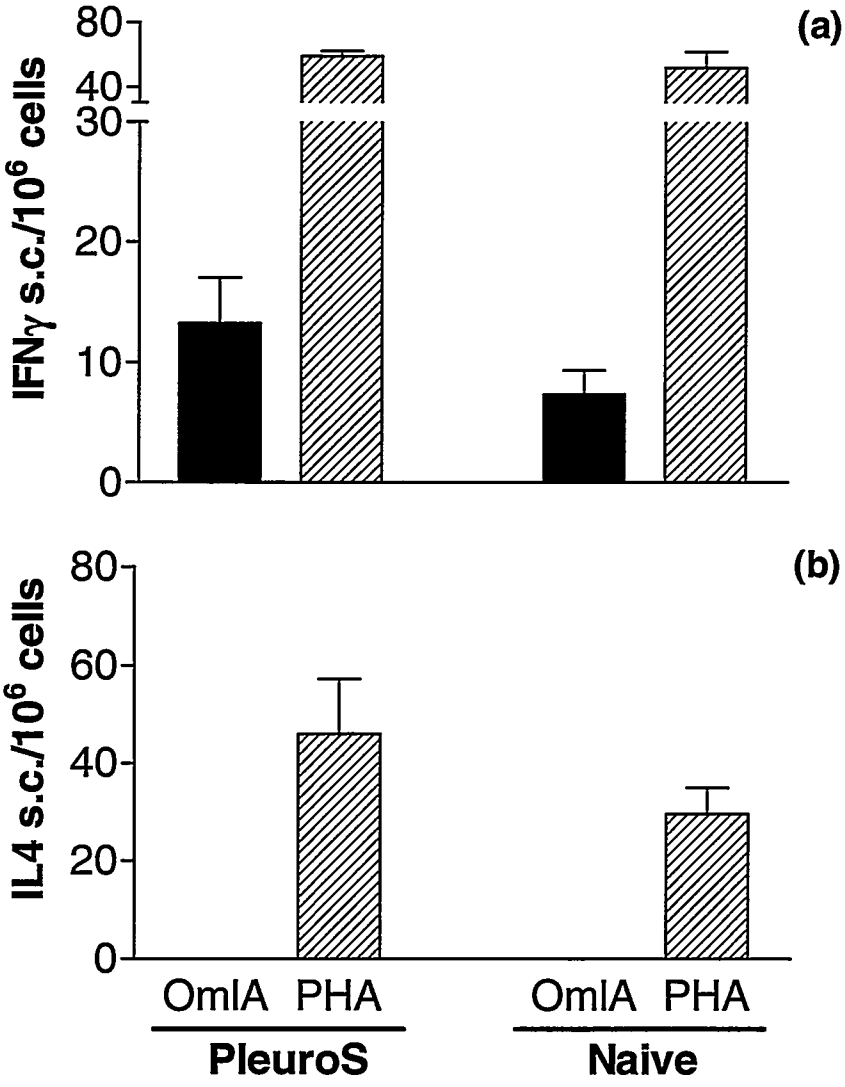

Figure 5.4.2 OmlA-specific cytokine production in spleen cells from vaccinated and naïve animals.
Pigs were vaccinated twice i.m. with the commercial vaccine Pleuro-Star 4^{TM}. Splenocytes were cultured at 0.5x10^6 cells/well with 10µg/ml of OmlA for 24 h. IFN-γ- (a) and IL-4-secreting cells (b) were determined by ELISPOT seven days after the last immunization. Results are expressed as means ± SEM of 8 pigs.

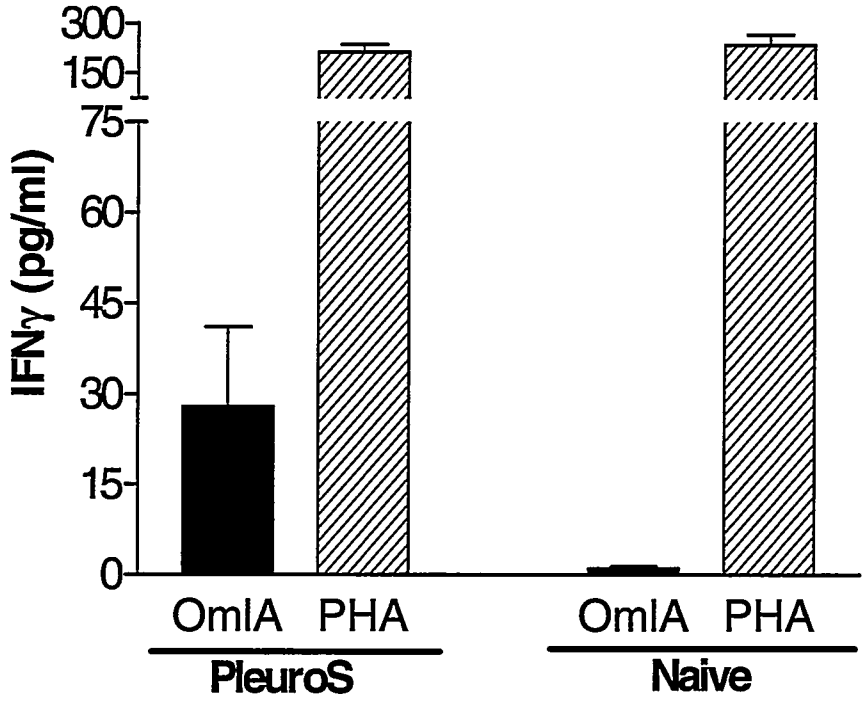

Figure 5.4.3 IFN-γ production after stimulation of splenocytes with OmlA.
Pigs were vaccinated twice i.m. with the commercial vaccine Pleuro-Star 4^{TM}. Splenocytes from vaccinated and naïve animals were cultured 24h at 0.5x10^{6} cells/well with 10μg/ml of OmlA, seven days after the last immunization. IFN-γ in supernatants was determined by ELISA. Results are expressed as means ± SEM of 8 pigs.

5.4.2 Effect of IL-2 in mediating *in vitro* antigen-specific stimulation

IL-2 is an important cytokine in the activation of cellular immunity (Mosmann and Sad, 1996), and induces proliferation of antigen-primed helper T cells (Mosmann and Sad, 1996; Murtaugh and Foss, 2002). Given the poor cytokine responses obtained following *in vitro* antigen stimulation of lymphocytes from immunized animals, IL-2 was used to supplement the media and provide better culture conditions for the stimulation of memory/effector T-cells and the secretion of cytokines. Pigs were vaccinated twice 3 weeks apart with the commercial vaccine Pleuro-Star 4^{TM}. PBMCs from naïve and vaccinated animals were cultured *in vitro* for 24 h with OmlA (10μg/m) and different amounts of rhIL2, 28 days after the last immunization. An increased induction of IFN-γ-secreting cells in the presence of rhIL2 was observed in a dose-dependent manner. Cells from vaccinated animals stimulated with the antigen and 40 IU/ml of rhIL-2 had the highest number of IFN-γ-secreting cells (Figure 5.4.4). To confirm the results obtained above, PBMCs from naïve and vaccinated animals were cultured again with OmlA (10μg/m) and rhIL-2 (40 IU/ml), two months after the second immunization. Vaccinated animals showed a significant induction of IFNγ-secreting cells after *in vitro* stimulation with the antigen, compared to naïve animals ($p<0.01$; Figure 5.4.5). Hence, the addition of rhIL-2 to the cultures improved the detection of antigen-specific cytokine-secreting cells after vaccination with the commercial vaccine Pleuro-Star 4^{TM}.

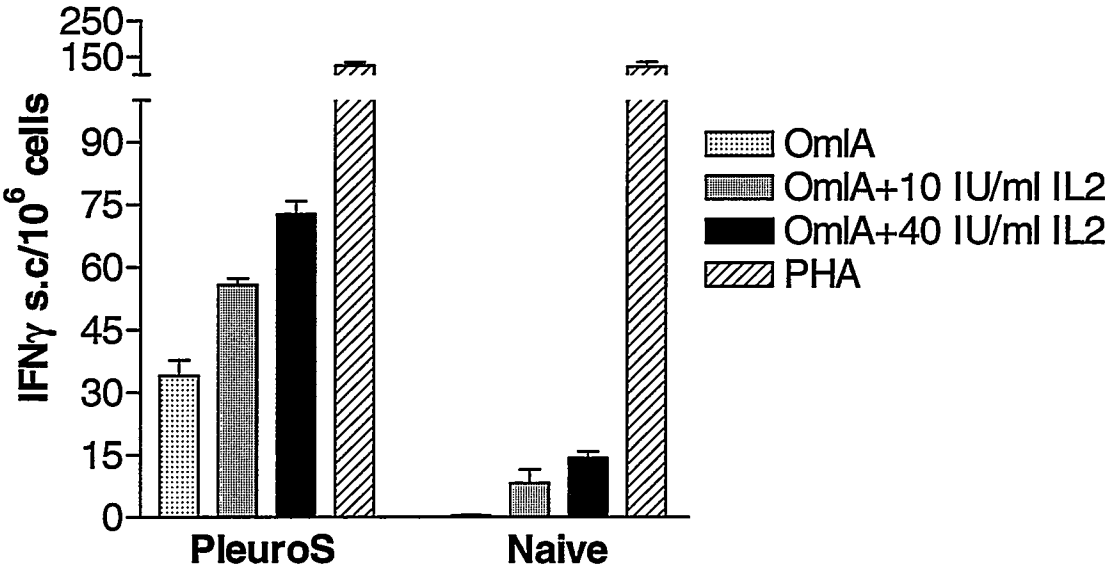

Figure 5.4.4 Induction of OmlA-specific IFN-γ-producing cells after incubation of PBMCs with antigen and different amounts of rhIL2.
Animals were immunized i.m. twice, 21 days apart, with the commercial vaccine Pleuro-Star 4™. PBMCs from vaccinated and naïve animals were cultured at $0.5x10^6$ cells/well with 10μg/ml of OmlA alone or in combination with different amounts of rhIL2, 28 days after the last immunization.

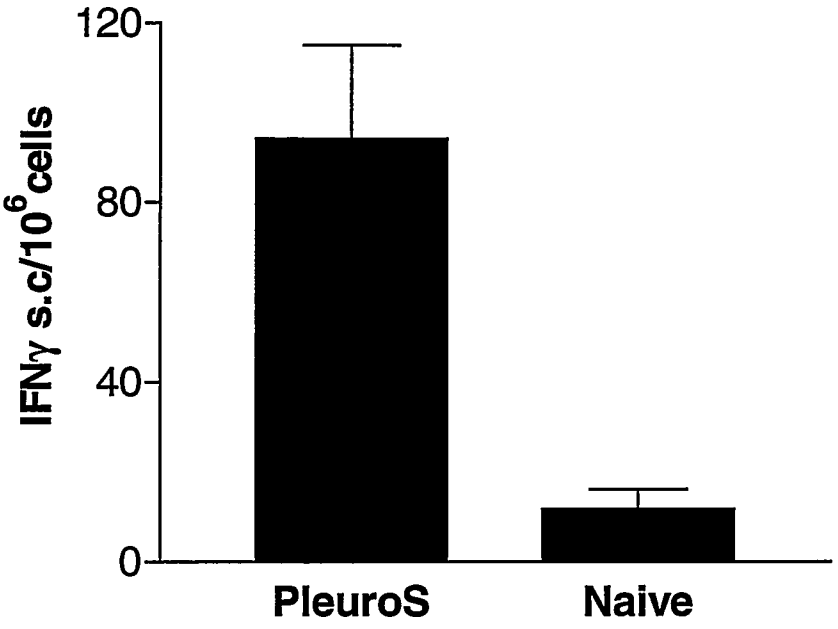

Figure 5.4.5 IFN-γ production after stimulation of PBMCs with OmlA and rhIL2.
Animals were immunized i.m twice, 21 days apart, with the commercial vaccine Pleuro-Star 4™. PBMCs from vaccinated and naïve animals were cultured at $0.5x10^6$ cells/well with 10μg/ml of OmlA in combination with rhIL2 (40IU/ml), 2 months after the last immunization.

5.4.3 Cellular responses induced by vaccination with biphasic lipid formulations

Having optimized and demonstrated that ELISPOT is a reliable technique to detect porcine cytokine producing cells, the following experiments were carried out to assess the type of cellular immunity induced by OmlA and CpG formulated in VTA2 at the mRNA and protein level.

PBMCs from animals immunized twice subcutaneously or intradermally with OmlA and CpG ODN (50 μg for s.c. and 250 μg for i.d.) formulated in VTA2 were isolated and cultured *in vitro* with the antigen for 24 h. Cytokine secreting cells were detected by ELISPOT and IFNγ was also assessed in supernatants from cultured cells by ELISA. Vaccinated animals presented a slight increase in IFNγ-secreting cells although not significantly different from naïve animals (Figure 5.4.6). Similar results were observed for secreted IFN-γ (Figure 5.4.7). Interestingly, animals that received the formulation intradermally showed a strong induction of IL-10-secreting cells compared to naïve animals ($p<0.05$). No significant induction of IL-4-secreting cells was detected, except when the mitogen PHA was used to stimulate cells (Figure 5.4.6).

To corroborate these results, cytokine gene expression after *in vitro* stimulation with the antigen was also assessed using a multiplex PCR (MPCR). RT-PCR is commonly used to study gene expression and has been shown to be a sensitive and effective method for measuring cytokine mRNA expression in porcine samples in response to various stimuli (Dozois, *et al.*, 1997; Reddy, *et al.*, 2000; Thanawongnuwech, *et al.*, 2001). The MPCR used here allowed analysis of IFNγ, IL-2, IL-4, IL-10 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression levels from porcine PBMCs in the

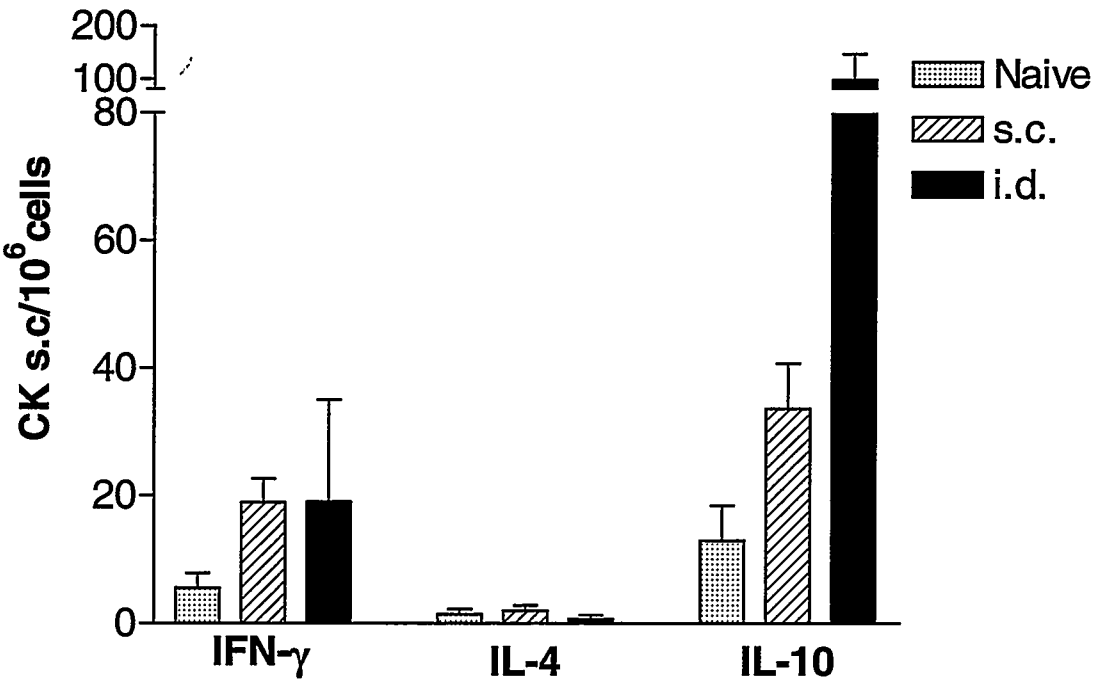

Figure 5.4.6 Induction of OmlA-specific cytokine-producing cells.
Pigs were immunized twice, 17 days apart, with OmlA and CpG ODN in VTA2 s.c or i.d. PBMCs from vaccinated and naïve animals were isolated four weeks after the last immunization and cytokine-secreting cells were determined by ELISPOT. Results are expressed as the mean ± SEM of 7 (naïve and s.c.) or 3 (i.d.) pigs.

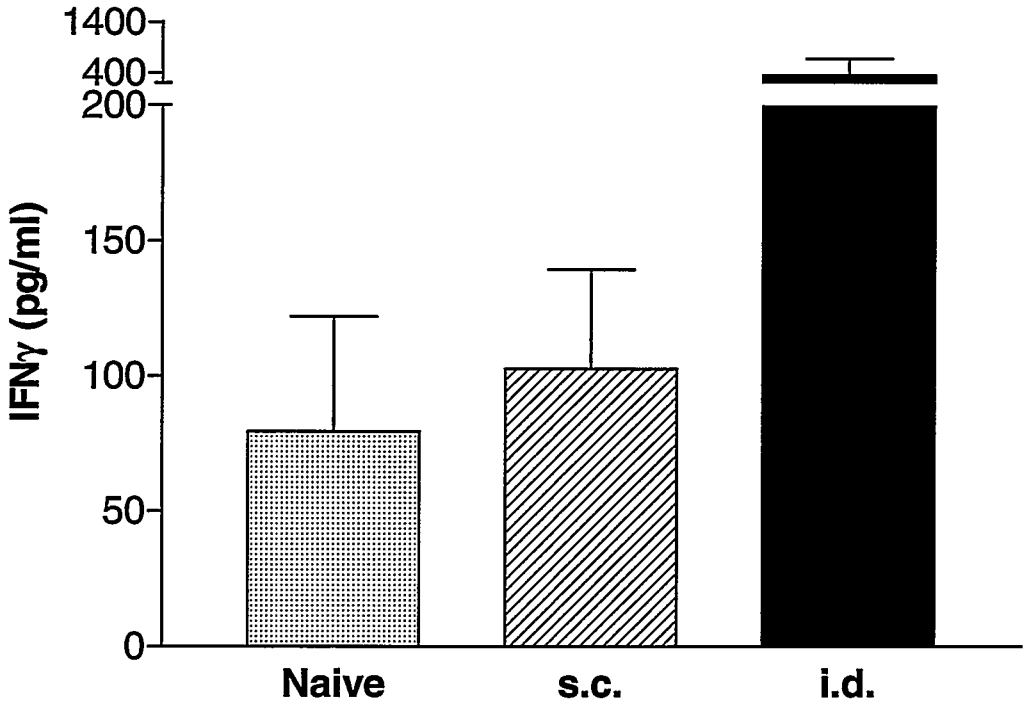

Figure 5.4.7 IFN-γ secretion after *in vitro* stimulation of PBMCs with OmlA.
Pigs were immunized s.c. or i.d. twice, 17 days apart, with OmlA and CpG ODN in VTA2. PBMCs from vaccinated and naïve animals were isolated four weeks after the last immunization and IFN-γ in supernatants was determined by ELISA. Results are expressed as the mean ± SEM of 7 (naïve and s.c.) or 3 (i.d.) pigs.

same reaction (Suradhat, *et al.*, 2003).

In vitro stimulation of cells from vaccinated animals induced cytokine mRNA for IL-10 predominantly and to a much lesser extent for IFN-γ and IL-2 relative to naïve animals (Figure 5.4.8). As expected, OmlA did not induce expression of IL-4.

5.4.4 Cellular infiltration in skin after administration of lipid formulations by different routes

In order to evaluate the effect of formulation and route of administration on the magnitude and type of cellular infiltration induced, pigs were inoculated subcutaneously, intradermally or topically following application of microneedles, with different formulations (antigen with CpG ODN, VTA2 or a combination of both). Skin biopsies were taken 24 or 48 h after administration and analysed for cellular infiltration by Giemsa staining.

An extensive and severe inflammation of the subcutaneous layer with edema was observed 24 h after s.c. administration of antigen and CpG ODN (50 μg each) in VTA2 (Table 5.4.1). The infiltration consisted of neutrophils and macrophages, many of which presented multiple vacuoles consistent with phagocytosis of lipid or oil (Figure 5.4.9 a). Numerous liposome-like structures were seen, which were surrounded by neutrophils and macrophages (Figure 5.4.9 b). Fourty-eight hours after administration, the inflammatory response was similar to 24 h (Table 5.4.1), with more cells being present, including lymphocytes. Few vessels with perivascular lymphocyte infiltration were also seen in one animal.

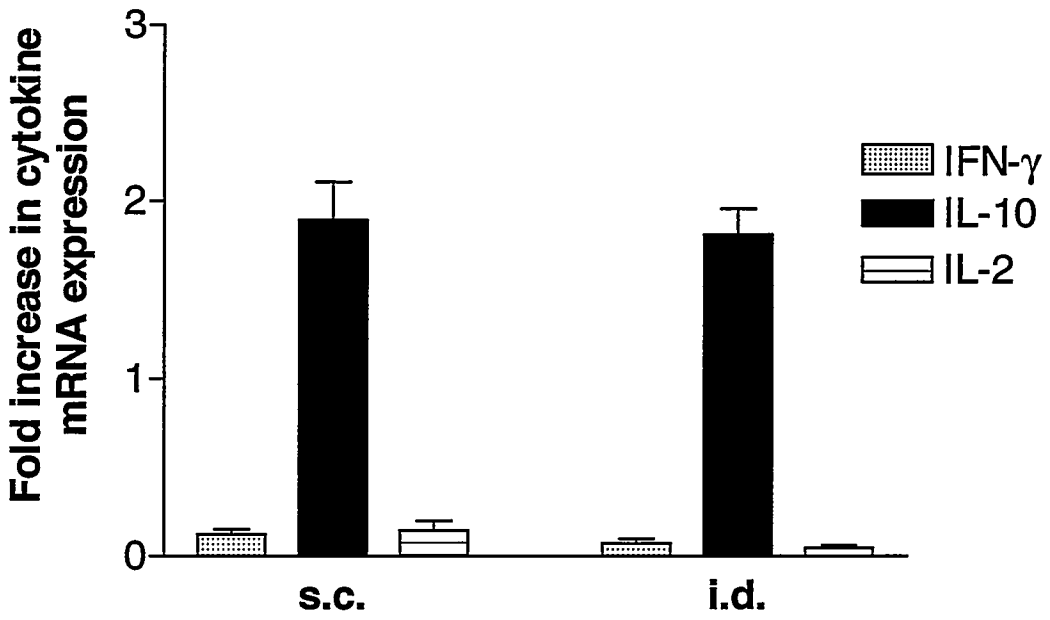

Figure 5.4.8 Cytokine mRNA expression in PBMCs from vaccinated animals.
Pigs were immunized s.c. or i.d. twice, 17 days apart, with OmlA and CpG ODN in VTA2. Cytokine mRNA expression after *in vitro* culture of cells with OmlA for 24 h was detected by MPCR. Results are expressed as the fold increase of cytokine expression relative to naïve animals and normalized using expression of the housekeeping gene GAPDH.

Table 5.4.1 Cellular infiltration after administration of lipid formulations by different routes

Group [1]	Route[2]	24 h [3]	48 h [4]
VTA2	s.c.	2+	3+
CpG	s.c.	1+	1+
VTA2/CpG	s.c.	3+	3+
Saline	s.c.	0	0
VTA2/CpG	i.d.	3+	3+
Saline	i.d.	0	0
VTA2/CpG	Microneedles	0	0
Saline	Microneedles	0	0

[1] Each group consisted of 3 animals. All groups received OmlA antigen except saline controls.
[2] OmlA and CpG ODN doses were 50 μg for s.c. and 250 μg for i.d. and microneedles applications.
[3] Skin samples were collected 24 h after administration.
[4] Skin samples were collected 48 h after administration.

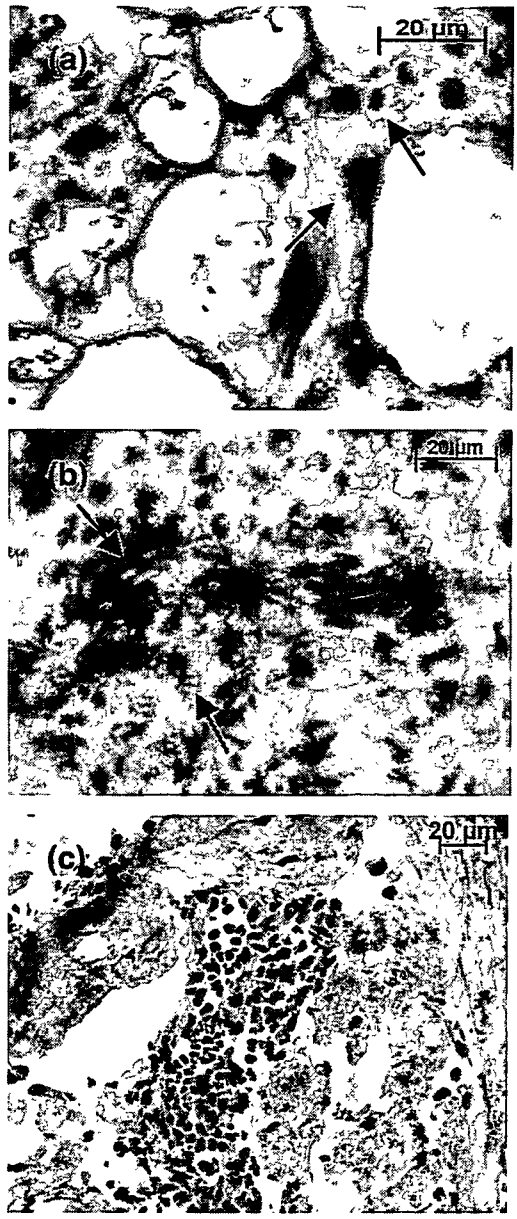

Figure 5.4.9 Effect of different vaccine formulations and immunization routes on cellular infiltration.
Skin biopsies were collected 24 after administration of formulations by different routes. Vacuolar macrophages present in subcutaneous tissue after s.c. administration of antigen and CpG ODN in VTA2 (a); adjuvant particles surrounded by neutrophils and macrophages (b); cells in the lumen of vessels in the dermis after i.d. administration of antigen and CpG ODN in VTA2 (c).

In animals that received antigen in VTA2 alone, the inflammatory response 24 h after s.c. administration varied from mild to moderate (Table 5.4.1), with infiltration of neutrophils, lymphocytes and macrophages, a few of which presented vacuoles in the cytoplasm. At 48 h, the cellular infiltration was severe (Table 5.4.1), except for one animal in which the cellular infiltration was absent, suggesting a resolution of the inflammatory response.

In animals that received antigen and CpG in saline, the inflammatory response 24 h and 48 h after s.c. administration ranged from absent to moderate (Table 5.4.1), with infiltration of some macrophages and lymphocytes in the septa of the subcutaneous tissue. At 48 h, lymphocytes were also seen around vessels.

Intradermal administration of antigen and CpG ODN (250 μg each) in VTA2 resulted in edema, fibrin deposition and a severe infiltration of cells 24 and 48 h after administration (Table 5.4.1), characterized by the presence of neutrophils, lymphocytes and macrophages with multiple vacuoles. Numerous cells were also observed in the lumen of vessels in the upper dermis 24 h after administration (Figure 5.4.9 c). On the other hand, topical administration of the same doses of antigen and CpG ODN in VTA2 did not induce cellular infiltration (Table 5.4.1).

5.4.5 Conclusions

Assessment of cytokine gene expression and secretion after vaccination with lipid vesicles containing antigen and CpG ODN showed a strong induction of IL-10 and a slight increase in IFN-γ production after s.c. and i.d. administration.

Analysis of cell recruitment at the site of administration showed an inflammatory response that was dependent on the vaccine composition and route. Following s.c. and i.d. immunization with antigen and CpG ODN in VTA2 the inflammatory response was characterized mainly by infiltration of macrophages and neutrophils. Mild inflammatory responses were induced by CpG ODN, while no cell recruitment was observed following transcutaneous immunization with antigen and adjuvant in lipid vesicles.

6.0 DISCUSSION

6.1 Induction of immunity by lipid-based formulations containing bacterial proteins and CpG ODN

The development of effective vaccines has greatly decreased the incidence of many infectious diseases worldwide. Adjuvants are commonly used in vaccines to enhance immune responses. In recent years, a large number of studies have demonstrated the immunostimulatory properties of CpG ODN. Responses have been especially dramatic in murine models (Chu, *et al.*, 1997; Davis, *et al.*, 1998; Corral and Petray, 2000; Gallichan, *et al.*, 2001).

One of the objectives of this project was to study the potential application of biphasic lipid vesicles as delivery systems for vaccines containing protein antigen and CpG ODNs. The results from this study demonstrated that VTA formulations are effective delivery systems for CpG ODNs. This effect was observed using two different bacterial proteins (GapC and OmlA), suggesting that these formulations may be used with a variety of antigens. Although many studies have shown that lipid-based formulations, including liposomes, are effective vaccine delivery systems (Babai, *et al.*, 1999; Chikh, *et al.*, 2001; Gursel, *et al.*, 2001) few studies have assessed the effectiveness of such formulations in animals other than rodents (Fries, *et al.*, 1992; Ben-Yehuda, *et al.*, 2003; Li, *et al.*, 2004; Neidhart, *et al.*, 2004). Furthermore, vaccines consisting of liposomes frequently require the incorporation of antigen and adjuvants when preparing the liposomes, which makes the evaluation of different antigens complex. On the other hand, vaccines consisting of VTA formulations are prepared by simple mixture of the lipid-based vesicles with a solution containing the antigen and adjuvant.

In a study where the effect of combining CpG ODN with a variety of adjuvants was assessed in mice using a viral protein as an antigen, Emulsigen, a mineral oil-based adjuvant, elicited the strongest immune responses, while other adjuvants made of metabolizable oils and non-oil-based adjuvants induced lower responses (Ioannou, *et al.*, 2002a). Therefore, VTA formulations constitute an effective alternative to other adjuvants in that they are made from metabolizable plant and synthetic lipids containing no animal derived products. In addition, VTA formulations induce immune responses that are comparable to commercially available adjuvants without the severe inflammatory responses.

Immunization of mice with biphasic lipid formulations induced lymphocyte proliferation and both IL-4 and IFN-γ secretion. Co-administration of CpG ODN increased antibodies in serum (Figure 5.1.4), and also IFN-γ secretion (Figure 5.1.6), an immunomodulatory effect of CpG ODNs that has been demonstrated in several studies (Chu, *et al.*, 1997; Brazolot Millan, *et al.*, 1998; Ioannou, *et al.*, 2002a). Cytokines polarize immune responses, and adjuvants have a strong influence on the quality of immune responses generated following immunization. Oil-based adjuvants such as Emulsigen and MF59 have been shown to induce Th2-like responses (Valensi, *et al.*, 1994), with production of IL-4, IL-5, IL-6 and IL-10 and strong antibody responses, which are more important for dealing with extracellular pathogens (Finkelman and Urban, 2001). On the other hand, as mentioned earlier, CpG ODNs induce Th1-like responses, including IFN-γ, IL-2 and cell-mediated immunity (Krieg, 2000), which are more effective for controlling infections with intracellular pathogens, including bacteria, parasites and virus (Ramshaw, *et al.*, 1997; Sin, *et al.*, 1999; Fischer, *et al.*, 2000). The ability of VTA formulations to induce balanced immune responses where both types of cytokines are produced is important because different diseases require different types of immunity to provide protection (Sjolander, *et al.*, 1998), since no single effector response can effectively deal with all forms of infection. For

example, control of infections caused by *Leishmania major*, an intracellular pathogen, relies on the induction of Th1 type responses (Reiner and Locksley, 1995); while resistance to infection with gastrointestinal worms such as *Trichinella spiralis* requires a Th2 cytokine response (Finkelman, *et al.*, 1997; Urban, *et al.*, 2000).

Phosphorothioate ODNs without CpG dinucleotides may have some stimulatory effects on CTL responses, B cell proliferation and co-stimulatory molecules expression, although generally lower than those seen with CpG ODNs (Monteith, *et al.*, 1997; Davis, *et al.*, 1998). Results here indicated that the synergistic effect between CpG ODN and VTA formulations was due to the CpG motif and not the ODN backbone, since non-CpG ODN did not induce an immune response either alone or combined with VTA (Figure 5.1.8), at the dose used in this study. The lack of biologic effects of non-CpG ODNs observed here may be related to the dose used; in the studies by Davis *et al.* the doses of non-CpG ODN used were between 10 and 50-fold higher than those used here.

It has been proposed that recombinant subunit antigens are weak immunogens compared to native subunit antigens (Matsuoka, *et al.*, 1994; Verdonck, *et al.*, Article in press). This might be due in part to the high level of purity and absence of contaminants such as LPS, which are normally found in conventional subunit vaccines and aid in the inflammatory process (Beuvery, *et al.*, 1983; Dertzbaugh, 1998; Del Giudice, *et al.*, 2001). In order to produce formulations on a large scale under Good Manufacturing Practice conditions, and to standardize and assure the quality of the product for human use, utilization of pharmacological grade reagents is important, but as with subunit vaccines, differences in the composition of VTA formulations due to the degree of purity of the reagents used, might compromise the effectiveness of the delivery system. Results showed that the use of pharmacological grade reagents in the preparation of the biphasic vesicles VTA4 did not affect the

ability of these formulations to enhance the immune responses when combined to the antigen and CpG ODN.

6.2 Induction of protective immunity in pigs after subcutaneous immunization with biphasic formulations and CpG ODN

The ability of CpG ODN to activate the immune system and its use as adjuvant has clearly been shown in mice and humans *in vivo* as well as *in vitro* (Krieg, *et al.*, 1995; McCluskie and Davis, 1998; Krieg, 2000). The identification of immunostimulatory motifs for additional species made possible the study of the adjuvant potential of these compounds in large animals. Although such motifs have now been identified for primates, cattle, sheep and pigs (Hartmann, *et al.*, 1999; Kamstrup, *et al.*, 2001; Rankin, *et al.*, 2001), only few studies examined the effect of CpG ODN in farm animals (Kamstrup, *et al.*, 2001; Rankin, *et al.*, 2001; Ioannou, *et al.*, 2002b). The outbred pig is an excellent model for testing efficacy of vaccines that may then be transferred to the human population because of the many similarities between humans and pigs regarding immune compartments (Bienenstock, *et al.*, 1999; Hammond, *et al.*, 2000).

Results presented here demonstrated that immunization of pigs with the protein OmlA from *App* and CpG ODN formulated in the lipid-based delivery system VTA2 induced higher antibody levels than immunization with the protein and CpG ODN in Saline (Figure 5.2.1), and provided protection against challenge with *App* (Figure 5.2.4 and Table 5.2.1). Furthermore, the antibody responses in these animals were comparable to the responses induced by the commercial mineral oil VSA (Figure 5.2.1). The observation that the immune response induced by immunization of the antigen in VTA2 alone was not different from that of naïve animals (Figure 5.2.3), suggests that VTA2 formulations are not on their own effective vaccine adjuvants.

Results regarding the stimulatory effects of non-CpG ODNs in pigs have been contradictory. Van der Stede *et al* showed that a non-CpG ODN also enhanced antigen-specific antibody responses in pigs after i.m. administration (Van der Stede, *et al.*, 2002). However, another study showed that the presence of the CpG dinucleotide was crucial for activating porcine PBMCs (Kamstrup, *et al.*, 2001). Results presented here showed that a non-CpG ODN either alone or formulated in VTA2 did not induce antibody responses to OmlA (Figure 5.2.3), confirming that in pigs, the immunostimulatory effects of ODNs are due to the presence of CpG motifs.

A potential advantage of the use of liposomes to formulate CpG ODN is the possibility to reduce the amount of ODN required to obtain a response. In order for CpG to be an economically viable adjuvant for veterinary use it must be inexpensive. Current CpG ODN doses used for immunization are still to high to be profitable. One possible way to decrease the functional dose of CpG ODN is to increase its efficacy through formulation with lipid-based vesicles. It was shown that when formulated in VTA2, the dose of CpG ODN can be reduced at least 20-fold without compromising the magnitude and effectiveness of the immune response (Figures 5.2.5 and 5.2.6). CpG ODN doses of 50μg administered in VTA2 induced protective immune responses while amounts as high as 1 mg of CpG ODN administered in PBS were not effective. This shows the importance of appropriate delivery to be able to induce immune responses and reduce the amount of CpG ODN required to obtain a biological response.

Clearance of CpG ODN *in vivo* occurs rapidly (Klimuk, *et al.*, 2000), limiting its uptake and subsequently its effectiveness. Pharmacokinetic studies in animals indicated that phosphorothioate antisense ODNs are rapidly cleared from plasma and distribute to various organs after parenteral delivery, with major sites of accumulation including liver, kidney, spleen, and bone marrow (Agrawal, *et al.*, 1991; Cossum, *et al.*, 1994); while ODNs encapsulated into liposomes

distribute primarily to the reticulo-endothelial system (Yu, *et al.*, 1999). It has also been shown that formulation of CpG ODNs in appropriate delivery systems potentiates their adjuvant effects by protecting them from degradation while increasing their uptake by cells of the immune system (Gursel, *et al.*, 1999). Cationic liposomes have been used to successfully deliver antisense oligonucleotides to inhibit protein expression in target tumor cells (Zhang, *et al.*, 2001) and anti-HIV therapy (Duzgunes, *et al.*, 2001), by facilitating cytoplasmic delivery and protecting them from nuclease digestion. It was also shown that liposomal delivery of FITC labeled immunostimulatory ODNs improves *in vitro* and *in vivo* uptake by mouse macrophages, B and dendritic cells (Gursel, *et al.*, 2001; Joseph, *et al.*, 2002). Hence, formulation of CpG ODN and antigen into biphasic lipid vesicles may provide long *in vivo* stability (De Oliveira, *et al.*, 2000) and enhance uptake and transport to the draining lymph nodes and spleen of both antigen and CpG ODNs by antigen presenting cells (Agrewala, *et al.*, 1996; Mui, *et al.*, 2001) that ultimately results in an increase in cytokine secretion (Mui, *et al.*, 2001). Sterically stabilized cationic liposomes (SSTC) containing positively charged and hydrophilic elements were shown to efficiently encapsulate CpG ODN and increase their uptake by cells of the immune system, resulting in an increase in the magnitude and duration of the response induced (Gursel, *et al.*, 2001).

Ultimately, VTA formulations may increase the amount of CpG ODNs that interact with Toll- like receptor 9 (TLR9), which is located on the inside of the endosomal membrane (Hemmi, *et al.*, 2000). Phagocytosed lipid-CpG ODNs complexes may be able to stimulate the TLR9 receptor more efficiency than free CpG ODNs, since there would be an increased uptake by APCs of CpG ODNs in lipid complexes compared to unformulated CpG ODNs (Gursel, *et al.*, 2001) and liposomal delivery of ODNs into cells is via an endocytotic pathway (Zelphati and Szoka, 1996; Islam, *et al.*, 2000).

The enhancement of immune responses by delivery with biphasic formulations could also be related to the structural features of the biphasic vesicles, where both the antigen and the CpG ODN can associate with the positively charged vesicles and distribute upon mixing with either the hydrophilic or hydrophobic compartments of the vesicles, providing also close physical association of CpG ODN with the antigen which has been shown to be important for the enhancement of the adjuvant effect of CpG ODN (Tighe, *et al.*, 2000).

Regarding induction of cellular immunity by biphasic vesicles containing antigen and CpG ODN, cytokine production displayed a high variation among animals within a group. Such heterogeneity has also been observed in other studies after *in vitro* (Kamstrup, *et al.*, 2001) and *in vivo* stimulation of porcine PBMCs (Van der Stede, *et al.*, 2002). Immunization of animals with antigen and CpG formulated in VTA2 induced mainly IL-10, and to a lesser extent IFN-γ (Figure 5.4.6 and Figure 5.4.7). Studies by Kamstrup *et al* showed that PBMCs from pigs stimulated *in vitro* with ODNs secrete pro-inflammatory (IL-6, TNF-α) and Th1-associated cytokines (IL-12) (Kamstrup, *et al.*, 2001). In another study, differences between groups immunized with or without CpG ODN were not found for IFN-γ, TGF-β and IL-10 expression, but animals that received CpG ODN presented a significant decrease in IL-6 expression and increased levels of IgA (Van der Stede, *et al.*, 2002), contrary to what would be expected from studies in mice and men (Beagley, *et al.*, 1989). However, the nature of the immune response elicited depends on the age of the animals, the route of antigen delivery (Brazolot Millan, *et al.*, 1998), and the nature of the antigen (Lee and Sung, 1998). Many microbial proteins, such as the major porin protein from *Neisseria meningitides* have potent immunostimulatory effects (Al-Bader, *et al.*, 2004), activating DCs and influencing the nature of the Th immune response induced. Hence, the type of response elicited by VTA formulations containing CpG ODN could be influenced both by the nature of the antigen and the route of

administration. For example, the same Lipid/ISS-ODN formulation that induced a Th1-dominant response against influenza antigens elicited a mixed Th1/Th2 response against HbsAg upon i.m. vaccination. Furthermore, even in the influenza model, a mixed response was seen after i.n. immunization (Joseph, *et al.*, 2002).

In the present study, administration of CpG ODN in VTA2 induced systemic IgG1, IgG2 and IgA as well as mucosal IgA, accompanied by high levels of IL-10, a regulatory cytokine (Wang, *et al.*, 1994) associated with antibody production (Reddy, *et al.*, 2000). The absence of IL-4 production (Figure 5.4.6) was not surprising, since similar findings have been reported in several studies in pigs (Reddy, *et al.*, 2000; Suradhat, *et al.*, 2003). It has been suggested that TNF-α may play a crucial role in antibody responses in pigs; analysis of this cytokine expression after vaccination with CpG ODN and VTA2 needs to be evaluated to determine if the antibody responses observed correlate with TNF-α production. Experiments in which several immunization regimens were evaluated suggested that immunization with plasmid alone, protein alone or prime-boost protocols, using CpG ODN and QuilA as adjuvants, results in a rather balanced immune response in piglets, while responses to plasmid and prime-boost immunization are Th1 biased in mice (Yu, *et al.*, 2004). However, few studies indicate the presence of Th1 and Th2 polarized T cell subsets in the pig, as described in mice. A Th1-like cytokine profile was detected in PBMCs from immunized pigs in response to the herpes virus Aujeszky's disease virus (ADV) *in vitro* (Fischer, *et al.*, 2000), and after vaccination with the virus (Zuckermann, *et al.*, 1998) and a DNA vaccine (Laval, *et al.*, 2002). In addition, mRNA for typical Th2 associated cytokines were found in liver and intestinal tissue from pigs infected with the parasite *Schistosoma japonicum* (Oswald, *et al.*, 2001). On the other hand, a typical Th1 type of immune response was observed in pigs after oral immunization with cholera toxin, resulting in cholera

toxin-specific delayed type hypersensitivity responses that were associated with increased secretion of IFN-γ (Foss and Murtaugh, 1999), while mucosal administration of CT induced primarily Th2 type responses in mice and humans (Marinaro, *et al.*, 1995; Gagliardi, *et al.*, 2000). Hence, while cytokines equivalent to those of rodents and humans exist in pigs, their association with ostensibly Th1/Th2 polarized stimuli is not clear yet and the Th1/Th2 dichotomy may not be as strict as that seen following immunization of mice and humans where CpG ODNs induce Th1-biased immune responses (Chu, *et al.*, 1997; Krieg, 2000). The degree of polarization of cytokine responses is also in part a reflection of the antigenic and environmental stimuli, and differences in the conditions to which pigs and inbred laboratory mice are exposed may account for differences in the type of response obtained after vaccination. Thus, immune responses developed in the mouse and pig model may not entirely correlate, which supports the need for testing vaccine strategies in outbred populations. Furthermore, several experimental observations in mice and humans appear difficult to rationalize with the dichotomy of a cross-regulatory Th1/Th2 paradigm (reviewed by Muraille and Leo, 1998) that seems to oversimplify the complexity of cytokine patterns.

6.3 Non-invasive approaches to immunization

The development of easier and safer means for delivery of vaccines is a high priority for both human and animal health. Parenteral vaccination via needles carries risks of broken needles, abscessation, accidental needle sticks and reduced compliance due to the pain associated with the use of needles. There are several different approaches to non-invasive needle-free delivery methods of immunization that have the potential to reduce the complications related to skin penetration by needle injection. Each of these approaches has

advantages and disadvantages. One major disadvantage is that the site, or route of administration is frequently hostile to molecules, which are subject to harsh conditions prior to absorption. The second problem is the absorption of sufficient amounts of antigen through the respective barrier layers after administration, which may be a significant factor in achieving a response. Two alternative non-invasive approaches to immunization were assessed in pigs in these studies, topical administration after microneedles application and mucosal delivery. The results are discussed below.

6.3.1 Induction of immune responses by topical administration of vaccine formulations.

The recognition that the skin is easily accessible, it has has an effective immune system, and its physical barrier is not so impermeable as previously thought made it an attractive route for non-invasive delivery of vaccines. Several studies demonstrated the potential of the skin as a non-invasive route to administer plasmid DNA (Fan, *et al.*, 1999), recombinant viruses (Tang, *et al.*, 1997), peptides (Beignon, *et al.*, 2002), or protein antigens (Glenn, *et al.*, 1998a; Glenn, *et al.*, 1999; Scharton-Kersten, *et al.*, 2000; Beignon, *et al.*, 2001).

Delivery of molecules through the skin requires efficient penetration of the stratum corneum, which is considered the main barrier to penetration of antigens. Various approaches are known to assist penetration through the stratum corneum. The common goal is to disrupt the stratum corneum structure in order to create pathways that are big enough for molecules to pass through. For example, hydration of the skin by occlusion is known to swell the stratum corneum and significantly disrupt the barrier function of the skin allowing passage of substances which normally are excluded. Preparing skin with alcohol

has also been found to augment the antibody response to CT delivered topically (Guerena-Burgueno, *et al.*, 2002).

The objective of this study was to determine whether the use of microneedles would facilitate the delivery of vaccines applied topically in pigs. Microneedles are comprised of arrays of micrometer sized needles that penetrate only into superficial layers of skin, thereby avoiding pain associated with standard hypodermic needles, providing a minimally invasive means to transport molecules into the skin.

For effective immunization through skin, the presence of an adjuvant might be critical. It is thought that adjuvants stimulate the antigen-loaded LC to migrate to draining lymph nodes (Guebre-Xabier, *et al.*, 2003). Because CT acts as both, antigen and at the same time adjuvant for coadministered proteins when applied to the skin (Glenn, *et al.*, 1999), it was used in the first studies in conjunction with the protein OmlA. CT binds to asialo-GM1 ganglioside expressed on a variety of epithelial and hematopoietic cell types and has ADP ribosylating activity. This could partially explain its ability to induce strong immune responses since binding to the receptor could potentially increase the uptake of antigen across the epithelium and lead to an enhanced presentation to the immune system, while the cAMP elevating activity and other signaling pathways induce cytokine production (Murtaugh and Foss, 2002). For CT in particular, it has been demonstrated that preincubation with GM1 gangliosides prior to its application onto bare skin, results in a significant reduction of systemic and mucosal anti-CT antibody responses (Beignon, *et al.*, 2001). CT also enhances antigen presentation by APCs (George-Chandy, *et al.*, 2001), and increases costimulatory molecules and inflammatory cytokines expression in porcine APCs (Murtaugh and Foss, 2002).

A significant enhancement of CT-specific immune responses following topical application was demonstrated only in animals receiving microneedles

treatment previous to immunization, indicating that the creation of superficial pathways in the skin was critical for effective immunization.

In addition to CT-specific serum antibodies, this approach also elicited IgA in nasal secretion (Figure 5.3.2). It was already shown that transcutaneous immunization stimulates both systemic and mucosal immune responses in other animal models. CT induced both serum and mucosal anti-CT IgG and IgA in response to topical application to the skin in a saline solution (Glenn, *et al.*, 1998b). The capacity of CT to induce immune responses after topical application in sheep was also reported (Cope and Colditz, 2000). Sheep immunized by TCI exhibited primary and secondary antibody responses to CT and in some animals, lymphocyte proliferative responses were also observed (Chen, *et al.*, 2000). Antibody responses to co-administered TT were also detected in sheep after TCI using CT as adjuvant (Chen, *et al.*, 2002).

The presence of CT-specific antibodies in the mucosa after TCI may be due to local production by antibody-secreting cells or through transudation of circulating immunoglobulin from the sera. Locally produced IgA is dimeric, while IgA transudated from serum is mostly monomeric (Mestecky, 1988). In general, mucosal IgG is thought to be transudative (Murphy, *et al.*, 1982; Meckelein, *et al.*, 2003), although Gockel *et al* showed local production of IgG after topical administration of TT to mice (Gockel, *et al.*, 2000). On the other hand, IgA detected at the mucosal level is usually presumed to be of local origin (Snider, 1995; Meckelein, *et al.*, 2003), but this remains to be determined for the anti-CT IgA detected here, since high levels of anti-CT IgA were also present in serum and the possibility of transudation cannot be discarded. The presence of CT-specific antibodies in mucosa may also be due to oral immunization occurred through grooming. Although the potential role of grooming was not evaluated here, studies by Glenn *et al* showed that the antibody response induced by oral administration of CT was significantly lower than that of animals given CT via

TCI. These low antibody responses suggest that CT does not tolerate the low gastric pH and that oral intake of the holotoxin does not play an important role in the antibodies induced by TCI in pigs. Furthermore, the actual amount of CT ingested after TCI was far below the dose required to induce anti-CT antibodies by oral delivery without bicarbonate (Glenn, *et al.*, 1999). Therefore, it seems unlikely that ingestion of the CT remaining in the skin would account for the antibody responses observed after topical application. This is supported also by the fact that control animals housed together with vaccinated animals remained seronegative. Similar results were obtained by Chen *et al.* immunizing sheep with CT via TCI (Chen, *et al.*, 2000).

Although topical administration after microneedles application was found to be an effective delivery method for inducing immune responses to CT, OmlA-specific immune responses were not elicited by this approach using CT as adjuvant. Similar results were observed by Hammond *et al.* using a recombinant flea salivary antigen to immunize dogs via TCI with CT as adjuvant, no antigen-specific antibody or proliferative responses were observed (Hammond, *et al.*, 2000).

A wide variety of adjuvants, including CpG motifs, LPS, muramyl dipeptide (MDP), alum, IL-2, and IL-12 have been shown to enhance the antibody titres to topically co-applied antigens. However, the responses were short lived and weaker than those induced in the presence of CT or LT (Scharton-Kersten, *et al.*, 2000). A synergistic adjuvant effect and shift of the immune response phenotype towards the Th1 type was demonstrated in a study where CT and CpG ODN were coadministered with a peptide representing a Th epitope from influenza virus haemagglutinin (Beignon, *et al.*, 2002). Considering that CpG ODNs have been shown to have adjuvant activity for protein antigens and peptides in the epidermis (Chen, *et al.*, 2001; Beignon, *et al.*, 2002) and that biphasic vesicles also were effective delivery systems for transcutaneous

immunization (Baca-Estrada, *et al.*, 2000b; Babiuk, *et al.*, 2002), it was decided to assess whether a combination of the microneedles approach with topical application of biphasic formulations containing OmlA and CpG ODN would induce OmlA-specific immune responses. At the same time transcutaneous administration was compared to intradermal delivery. OmlA-specific immune responses were not observed after topical application of these formulations, even though the doses of antigen and CpG used were 5 times higher than the ones used successfully for s.c. immunization. On the other hand, intradermal immunization induced strong OmlA-specific IgG and IgA levels in serum (Figure 5.3.4) as well as IgG in nasal secretions (Figure 5.3.5).

One explanation for the lack of OmlA-specific responses after topical administration could be that the microneedles were not disrupting the stratum corneum efficiently, while proteins with receptor binding activity, such as CT would still be able to penetrate and induce immune responses. However, results indicated that the insertion depth ranged from 0.1 to 0.8 mm, reaching the dermis (Figure 5.3.3). Similar devices, containing microfabricated silicon projections were shown to breach effectively the skin barrier in a human clinical trial, allowing direct access to the epidermis with minimal associated discomfort and skin irritation (Mikszta, *et al.*, 2002); and microneedle arrays containing microneedles 150 μm in length were shown to increase the permeability of human skin (Henry, *et al.*, 1998).

Another explanation comes from the fact that the diffusion of an antigen through the stratum corneum may dependent on its physicochemical properties and its molecular interactions with skin constituents. This could explain the differences in immunogenicity of several antigens after their application onto bare skin. In a recent study where the safety and immunogenicity of a prototype enterotoxigenic *E. coli* vaccine containing a recombinant colonization factor antigen with or without LT was assessed in adult volunteers after topical

application, only 68 and 53% were found to have antigen-specific serum IgG and IgA antibodies respectively, while all responded to LT, which acts as adjuvant but is also a potent immunogen (Guerena-Burgueno, *et al.*, 2002). Hence, antigens vary in their ability to induce immune response via TCI. These differences may be due to how the antigen interacts with the physical and structural components of the skin, and the success of TCI may therefore be in part dependant on the nature of the antigen being delivered (Hammond, *et al.*, 2000). Depletion of epidermal LC populations can readily be demonstrated after topical application of CT (Glenn, 2001); if LCs activated by CT migrate from skin before taking up enough amounts of OmlA, it is possible that a response to CT would be observed while no OmlA-specific immunity would be induced.

Another possibility is that the amount of OmlA reaching the layers of skin where antigen-presenting cells are present could not be sufficient to induce an immune response. In studies where rhTGFβ as a methylcellulose semi-solid gel was applied topically as a wound-healing agent in rats, it was found that approx. 35% of the applied dose was not absorbed and could be recovered after 24h. This indicated that a significant proportion of the applied protein may be rubbed off or simply not available for induction of a response (Zioncheck, *et al.*, 1994). Furthermore, a multiple puncture/immersion method was more effective in inducing an immune response and more antigen was incorporated into fish than by the immersion method alone, suggesting that the effectiveness of vaccination was primarily dependent on the amount of antigen taken up (Nakanishi, *et al.*, 2002). Nevertheless, delivery using microneedles has the advantage of reducing trauma, being essentially pain-free, and allowing control over the depth of penetration by varying the needle length.

6.3.2 Mucosal immunization in pigs

Mucosal surfaces are the most frequent portal of entry of infectious agents. Consequently, the induction of specific immune responses at mucosal surfaces is important to contain infection as a first line of defense (Haan, *et al.*, 2001). Mucosal immunization has generally been considered necessary for the induction of mucosal immune responses (McGhee, *et al.*, 1992; Klavinskis, *et al.*, 1997) and the nasal mucosa represents an attractive, noninvasive route for the delivery of antigens. The nasal epithelium and associated secretions present a physical and chemical barrier to molecules entering the respiratory tract. Consequently, the administration of protein antigens directly to mucosal surfaces results in little or no response. Therefore, mucosal immunization requires appropriate delivery systems and adjuvants are particularly important to enhance immune responses. Potent mucosal adjuvants often are toxins (Rappuoli, *et al.*, 1999). The majority of protein antigens that have been tested so far bind to cell surface receptors (i.e. CT, LT, and TT) and act as strong immunogens and adjuvants. To evaluate the feasibility of mucosal immunization in pigs and to investigate the efficiency of biphasic formulations as delivery systems for antigens and adjuvants for induction of systemic and mucosal immunity, combined systemic/mucosal or mucosal/systemic protocols were used. The combination of their binding activity and built-in adjuvanticity makes CT and LT powerful immunogens and adjuvants. As discussed earlier (Section 6.3.1), these proteins bind to a cell surface receptor and at the same time activate signaling pathways leading to cytokine production. By incorporating CpG ODNs into biphasic lipid vesicles, the same effect could be achieved, with the lipid vesicles targeting APCs and CpG ODNs providing the danger signal.

In a preliminary study, results showed a slight induction of systemic and mucosal antibodies after systemic priming followed by intranasal boost (Figure

5.3.6 and Figure 5.3.7). Results from studies that compared parenteral versus mucosal priming approaches in different models have been contradictory. Some researchers found that best results are obtained with parenteral priming followed by mucosal boost (Moldoveanu, *et al.*, 1993; McCluskie, *et al.*, 2002), while others observed the opposite (Lee, *et al.*, 1999; Eo, *et al.*, 2001; Vajdy, *et al.*, 2003). Therefore, in a subsequent experiment, mucosal priming followed by systemic boost was evaluated. Results presented here confirmed previous observations that CpG are effective mucosal adjuvants (McCluskie and Davis, 1999; McCluskie, *et al.*, 2000a; Gallichan, *et al.*, 2001; McCluskie, *et al.*, 2001) and further indicate that VTA formulations are suitable delivery systems to enhance the immunoadjuvant activity of CpG ODNs by the intranasal route. After i.n immunization, OmlA combined with CT induced OmlA-specific IgG and IgA responses in serum, but low responses in nasal secretions, while formulations containing the antigen and CpG ODN in VTAM1 elicited antibodies in serum (Figure 5.3.9) and nasal secretions as well (Figure 5.3.10), indicating that biphasic lipid formulations combined with CpG ODN were more effective at inducing a local immune response than a "classical" mucosal adjuvant (Rappuoli, *et al.*, 1999), at the administered dose. A low adjuvant activity of CT in pigs was also reported after oral administration (Foss and Murtaugh, 1999). Furthermore, immunization with VTA/CpG ODN formulations constitutes a safe alternative to the use of CT as mucosal adjuvant given the potential toxicity of CT for the central nervous system that may occur following intranasal administration (van Ginkel, *et al.*, 2000a; Fujihashi, *et al.*, 2002).

Results also demonstrated a stronger mucosal adjuvant activity of CpG ODN when formulated in VTAM1 as compared with that of CpG ODN alone (Figure 5.3.10), consistent with results obtained using the same biphasic formulations (Babiuk, *et al.*, 2004) and plasmid DNA-lipid complexes in mice (Klavinskis, *et al.*, 1997).

Remarkably, not only intranasal, but also subcutaneous immunization with biphasic formulations containing antigen and CpG ODN induced antibodies in nasal secretions. Similar results were observed in other studies (Haan, *et al.*, 2001; Van der Stede, *et al.*, 2002). However, mucosal immune responses induced by parenteral immunization are generally weaker, more variable and short-lived (Kaul and Ogra, 1998). Van der Stede *et al* found that ODNs can enhance the antigen specific IgA in mucosal secretions after i.m. immunization of pigs with OVA and ODNs in IFA. (Van der Stede, *et al.*, 2002). It is important to note that in both cases, the site for systemic administration was the neck. Systemic immunization on the neck may target the same lymph nodes as nasal administration (Saar and Getty, 1975). Hence, inducing an immune response in specific lymph nodes by systemic immunization could elicit a subsequent mucosal response, representing a crossroads between mucosal and systemic immune responses. To confirm this hypothesis, we determined which lymph nodes were draining the area after s.c. administration on the neck or after intranasal administration by tracking an Evans blue solution. We found that the superficial cervical, and the retropharyngeal lymph nodes were stained in both cases, indicating that these lymph nodes are most likely involved in the generation of immune responses after systemic immunization on the neck and intranasal delivery with the gun.

As mentioned earlier, detection of antibodies in nasal secretions could infer transudation from serum and further studies need to be done to exclude this possibility.

These results and those from others showed that the delivery system is a critical factor in mucosal immunization (Olszewska, *et al.*, 2000). Formulating antigen and CpG ODN in VTAM1 for nasal administration was found to be essential to elicit a specific local IgA response and also increased serum IgG and IgA levels. Although the mechanisms through which biphasic formulations

containing CpG ODN promoted the induction of immunity after intranasal delivery were not addressed in these studies, presumably, the mode of action is similar to that mediated by these formulations following parenteral administration. The effects seen here may be due to an increase in antigen presentation within the mucosal associated lymphoid tissue of the respiratory tract (NALT) and the draining lymph nodes. To efficiently induce immune responses after mucosal administration, the antigen must be transported across the epithelial barrier. The interaction between an antigen and the nasal mucosa and the NALT depends on a variety of factors, in particular, the physical nature of the antigen (soluble or particulate), the dose and the frequency of contact (Sminia and Kraal, 1999). Small soluble antigens are able to penetrate the nasal epithelium (and may be assisted by formulation additives such as penetration enhancers) and interact with DC, macrophages and lymphocytes (intraepithelial and subnasal). In contrast, particulate antigen is largely taken by M cells in the NALT. Electron microscopy studies showed that liposomes can be taken up by M cells and are seen in the transcytotic vesicles (Childers, *et al.*, 1990). Endocytosis of uncoated liposomes by M cells occurred when liposomes were injected into a rat intestinal loop containing a PP, while the enterocytes showed no evidence of liposome uptake. Preferential uptake of liposomes by M cells was also observed in murine intestinal loops (Zhou, *et al.*, 1995; Chen and Langer, 1998).

Uncoated liposomes may promote their own uptake because of their surface charge, particulate nature and relative hydrophobicity (Zhou, *et al.*, 1995; Chen and Langer, 1998). In this regard, lipid-based delivery systems may act as carriers for the antigen and adjuvant, targeting them to APCs, increasing their uptake and presentation to Th cells (Alving, 1991). Hence, VTAM1 may increase the uptake of antigen and CpG ODN by APCs at mucosal surfaces, which in turn migrate to the regional lymph nodes and effector cells disseminate

to different effector sites. However, there is evidence that compartmentalization may exist in the mucosal immune system, suggesting that the response seen at various mucosal sites may differ according to the route of mucosal immunization (Moldoveanu, *et al.*, 1995). Alternatively, from the highly vascularized tissue of the nasopharynx, antigen may directly enter the circulation and disseminate to the spleen, priming for the systemic immune response. Increased uptake of DNA and dissemination of the encoded antigen throughout the respiratory and gastrointestinal tracts, draining lymph nodes and spleen were demonstrated after intranasal immunization of mice with plasmid DNA-lipid complexes (Klavinskis, *et al.*, 1997). This distribution is reminiscent of the dissemination of phospholipid-based liposomes (Oku and Namba, 1994).

Results presented here showed the effectiveness of intranasal immunization with biphasic formulations in pigs. This is important because of the similarity between pigs and humans regarding organization of the NALT. Laboratory animals such as mice and rabbits present lymphoid aggregates along the respiratory tract, while pigs, as humans, have few organized aggregates. Instead, the tonsils are known to play an important role in the mucosal immune defense process since their position in the oropharynx provides an opportunity for close contact of the immune system with ingested and inhaled antigens. These similarities between pigs and humans make the former a better model to study immune responses after intranasal administration than mice.

6.4 Role of inflammation/cellular infiltration in induction of immune responses

Some adjuvants such as CpG mimic the signals caused by tissue damage without actually causing it (Ioannou, *et al.*, 2003), stimulating a cascade of signals including cytokines, chemokines, and co-stimulatory molecules that

induce cellular infiltration and activation of APCs. Immune responses to OmlA were enhanced following s.c and i.d administration of the antigen and CpG ODN formulated in biphasic vesicles, which at the same time induced a severe infiltration of cells 24 and 48 h after administration. Administration of antigen with CpG ODN alone on the other hand, did not induce immune responses and only a mild infiltration of cells was detected (Table 5.4.1), while administration of VTA alone induced cellular infiltration, but not immune responses. These data provide evidence of a role for inflammation/cellular infiltration and activation in inducing immune responses. VTA formulations induced cellular infiltration while CpG ODN activated the recruited cells. Therefore, combining CpG ODN with VTA formulations may allow more efficient presentation of antigen to APCs by VTA delivery while CpG ODN activates APCs through danger signals, causing them to move to the draining lymph nodes and present the antigen.

One of the major drawbacks to the development of novel vaccines has been the lack of safe yet effective adjuvants. Most adjuvants used in animal vaccines stimulate strong tissue reactions at the site of injection, which cost an estimated $9 per animal slaughtered in Canada (Van Donkersgoed, *et al.*, 1997). For example, the commercial *App* vaccine PleuroStar 4^{TM} induces protection after two i.m. administrations but it causes a considerable tissue damage (data not shown). Although recent studies showed enhancement of contact hypersensitivity and alteration of lymphoid organs after administration of CpG ODN in mice (Akiba, *et al.*, 2004; Heikenwalder, *et al.*, 2004), these immunotoxic effects might be due to the high amounts of ODN administered repeatedly for a minimun of 7 days. In other studies in humans, no significant local hypersensitivity reactions were reported after repeated immunizations with CpG ODN (Halperin, *et al.*, 2003).

The synergy of CpG ODN with VTA may be due to a depot effect, which could increase the period of time that both the antigen and the CpG ODN are

available to be presented to the immune system. Histological assessment of the site of injection ten days after the last immunization revealed that in contrast to the tissue damage induced by the commercial oil emulsion VSA, the inflammatory response induced by subcutaneous administration of CpG ODN in VTA2 was mild or absent 10 days after administration (Figure 5.2.2). Although no residual VTA formulation was found 10 days after inoculation, subcutaneous depot of VTA formulations could be found at shorter periods of time. This is in contrast to the longer deposition of mineral oil emulsion found at the site of immunization with VSA as well as with other oil-based adjuvants and aluminium salts (Lascelles, *et al.*, 1989; Gupta, 1998). These findings suggest that biphasic vesicles containing CpG ODN constitute a safer non-toxic alternative to mineral oils and are in agreement with studies that have shown that lipid-based delivery systems and CpG ODNs are safe (Fries, *et al.*, 1992; Weeratna, *et al.*, 2000; Ioannou, *et al.*, 2003).

Experiments presented here also demonstrated that microneedles are an effective delivery method for enhancing immune responses to CT, although not to OmlA under the conditions evaluated here. After topical administration of OmlA and CpG ODN in VTA, no cellular infiltration was detected (Table 5.4.1), in agreement with the hypothesis that cellular infiltration is crucial for the induction of immunity by these formulations. A similar effect on infiltration by topical administration of CT after microneedles application remains to be demonstrated.

6.5 General conclusions and future applications

It is clear that not only the antigen and route of immunization are important in inducing effective immune responses. The use of adjuvants and appropriate delivery systems was proven to be a key factor as well. In this project, it was found that immunization of both mice and pigs with only CpG ODNs and antigen was not as effective at inducing immune responses as formulation in VTA vesicles. Furthermore, CpG ODN delivered in these biphasic lipid formulations was a considerably more potent parenteral and mucosal adjuvant in pigs than the soluble form of CpG ODN. On the other hand, transcutaneous administration of these formulations was not successful in inducing a response to OmlA. However, a response to CT was demonstrated and this have established proof of principle, showing that transcutaneous immunization is feasible in pigs when combined with microneedles. For effective delivery of proteins that do not bind receptors on epithelial cells, several variables related to the nature of the antigen and characteristics of the skin barrier must first be overcome. Nonetheless, the effectiveness and the lack of apparent toxicity of VTA/CpG combinations make them promising adjuvants for future development of human vaccines.

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