DELIVERY OF POLYNUCLEOTIDES AND OLIGONUCLEOTIDES FOR IMPROVING IMMUNE RESPONSES TO VACCINES

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By

Shawn Babiuk

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Dr. Marianna Foldvari, D. Pharm. Sci., PhD.
Division of Pharmacy
College of Pharmacy and Nutrition

Dr. Maria Baca-Estrada, PhD.

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ABSTRACT

Vaccination is one of the major achievements of modern medicine. As a result of vaccination, diseases such as polio and measles have been controlled and small pox has been eliminated. However, despite these successes there are still many diseases of microbial origin that cause tremendous suffering because there are no vaccines or the vaccines available are inadequate for many diseases. The development of DNA based vaccines and immunostimulatory CpG oligonucleotides (ODNs) as adjuvants offers possibilities for developing new vaccines. However, even if vaccines were available for all infectious diseases there would be no guarantee that people would use them routinely. One of the major impediments to ensuring vaccine efficacy and compliance is that of delivery. Presently most vaccines are given by intramuscular administration. Unfortunately this is often traumatic, especially in infants. Thus if it were possible to replace intramuscular injection by mucosal (oral/intranasal) or transdermal delivery it might be possible to both enhance mucosal immunity and improve overall compliance rates.

The development of non-invasive methods for the delivery of vaccines through the skin will greatly improve the safety and the administration of human and veterinary vaccines for different species. This study examined the efficiency of topical delivery of plasmids by assessing the localization of gene expression using luciferase as a reporter gene and induction of immune responses using a plasmid encoding for the bovine herpesvirus type-1 glycoprotein D (pgD). Topical administration of plasmids in a lipid-

based delivery system (biphasic lipid vesicles [Biphasix™]) resulted in gene expression in the draining lymph nodes, whereas with intradermal injection, antigen expression was found in the skin. Following administration of plasmid by gene gun, antigen expression was observed in both the skin and the draining lymph nodes. Transcutaneous immunization with pgD formulated in biphasic lipid vesicles elicited gD-specific antibody responses and a Th2 type cellular response. In contrast, immunization by the intradermal route resulted in the stimulation of a Th1 type response. These findings have implications for both vaccine design and tailoring of specific immune responses.

Electroporation has been shown to increase the potency of DNA vaccines that have demonstrated significant potential in mice. However, there is a need to develop non-invasive or minimally invasive vaccination methods. In pigs, *in vivo* gene expression was assessed to compare intradermal needle injection to a needle-free dermal BioJect™ as a means of delivery of plasmids. Each administration method was further tested with and without surface electroporation. Experiments with plasmid DNA encoding luciferase demonstrated that needle-free administration results in higher expression levels than needle injection. Electroporation strongly enhanced gene expression for both intradermal delivery methods and immune responses to a DNA vaccine encoding hepatitis B surface antigen. Needle-free plasmid injection in combination with electroporation led to a more rapid induction of immune responses compared to other methods of plasmid administration. Priming and boosting with the DNA vaccine heightened the effectiveness of a subsequent protein boost, and electroporation after DNA injection augmented those benefits. It was concluded that

needle-free topical electroporation significantly enhances gene expression and immune response, possibly by improving cellular uptake of plasmid DNA.

It is generally recognized that DNA vaccines are often less effective in large animals than in mice. One possible reason for this reduced effectiveness may be transfection efficiency and the low level of expression elicited by plasmid vectors in large animals. A possible way to improve plasmid gene expression *in vivo* is electroporation. To determine whether immune responses in pigs could be enhanced by electroporation, plasmids encoding two different genes [bovine herpes virus glycoprotein D (gD) and hepatitis B surface antigen (HBsAg)] and two different electrodes, a single-needle electrode and a six-needle electrode, were used. Electroporation significantly enhanced immune responses to both antigens. Co-administration of two different plasmids (pgD and pHBsAg) did not result in significant interference between the plasmids. In addition, a DNA prime / protein boost strategy was used to show inhanced DNA priming with electroporation on the immune response to HBsAg following a protein boost.

One potential mechanism by which electroporation enhances immune responses to DNA vaccines is by increasing gene expression. However, the inflammation and accompanying cellular infiltration caused by electroporation may also be essential for enhancing immune responses to DNA vaccines. These parameters were investigated in pigs using different electroporation conditions that result in different levels of gene expression and cellular infiltration. Results indicated that the least effective strategy

was conventional intramuscular injection where there was low gene expression and low cellular infiltration. The most efficacious strategy was plasmid administration immediately followed by electroporation. This latter set of conditions elicited a combination of high gene expression and high cellular infiltration. This indicates that electroporation enhances immune responses to DNA vaccines by increasing gene expression and inflammatory cell infiltration.

CpG oligonucleotides (ODNs) are potent mucosal and systemic adjuvants. For practical applications, improvements in delivery need to be developed. It is possible to enhance the adjuvant properties of CpG ODNs by (1) physically coupling CpG ODNs to an antigen or alternatively (2) formulating the CpG ODNs in an appropriate delivery system. In this thesis, the ability of a novel type of biphasic lipid vesicles (Biphasix™), called Vaccine-Targeting Adjuvants (VTA), to enhance the immunoadjuvant activity of CpG ODNs following systemic or mucosal administration was assessed. Compared to gD alone VTA formulations in combination with CpG and gD were able to increase gD-specific IgG in serum and gave protection from a lethal HSV-1 challenge following subcutaneous immunization in mice. In addition, formulation of CpG ODNs in VTA enhanced IL-12 cytokine secretion following systemic administration. These results indicate that a suitable delivery system can increase the biological activity of CpG ODNs.

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LIST OF ABBREVIATIONS

APCs	antigen presenting cells
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BHV-1	Bovine herpes virus type 1
b.j.	BioJect
CEA	carcinoembryonic antigen
CMI	cell-mediated immunity
CMV	Cytomegalovirus
cpm	counts per minute
CT	cholera toxin
DC-Chol	N(N',N-dimethylaminoethane) carbamoyl) cholesterol
DDAB	dimethyldioctadecyl ammonium bromide
DLPE	dilauroylphosphatidylethanolamine
DMAEMA	2-(dimethylamino) ethyl methacrylate
DMPC	dimyristoylphosphatidylcholine
DMPE	dimyristoylphosphatidylethanolamine
DOPE	dioleoylphosphatidylethanolamine
DOTAP	N-(1-(2,3-dioleoxyloxy)propyl)-N,N,N,-trimethylammonium-methyl-sulfate
DOTMA	dioleyloxy-propyl- trimethylammonium chloride
DPPE	dipalmitoylphosphatidylethanolamine
DMRIE	dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium
gD	glycoprotein D full length form
g.g.	gene gun
EP	electroporation
GFP	green fluorescent protein
HBsAg	Hepatitis B surface antigen
HE	hematoxylin/eosin
HSV-1	Herpes simplex virus type 1
ICAMs	intercellular adhesion molecules
i.d.	intradermal
i.d.n.	intradermal needle
i.m.	intramuscular
i.m.n.	intramuscular needle
LPS	lipopolysaccharide
LU	light units
MP 35	micropatch round electrode
ODNs	oligonucleotides
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline-Tween
pgD	plasmid encoding bovine herpesvirus glycoprotein D
pGFP	plasmid encoding GFP
PHA	phytohemagglutinin
pHBsAg	plasmid encoding HBsAg

PINC	protective, interactive, non-condensing
PLA	D-poly L-lactate
pluc	plasmid encoding luciferase
PLGA	poly DL-lactic-co-glycolic acid
PNPP	*p*-nitrophenol phosphate
RLU	relative light units
SALP	stabilized antisense lipid particles
SD	standard deviation
SEM	standard error of the mean
SI	stimulation index
tgD	gD truncated form
TLR	Toll-like receptor
t.s.	tape stripped
VIDO	Veterinary Infectious Disease Organization
VLPs	virus like particles
vs	versus
VTA	Vaccine Targeting Adjuvant

1.0 LITERATURE REVIEW

1.1 Overview of traditional vaccination; identification of current and future trends including safety and delivery

Following the first reports by Jenner over 200 years ago, numerous attempts have been made to develop safer and more efficacious vaccines. Thus, approximately 100 years after Jenner described immunization with a live vaccine, Pasteur introduced the concept of using killed vaccines to control infectious diseases. For the next 100 years the great debate continued as to the advantages or disadvantages of the two different types of conventional vaccination strategies. For example, killed vaccines are better at inducing antibody responses as opposed to cell-mediated immune responses. In contrast, live vaccines generally induce a more balanced response; however, they may not be as safe due to the potential risk of reversion to virulence (Hooke, *et al*., 1985).

The developments in molecular biology in the 1970's heralded the era of recombinant DNA technology to produce a new generation of vaccines. Recombinant subunit vaccines are considered to be safer than conventional vaccines (Dertzbaugh, 1998). However, some challenge the use of the reductionist approach to vaccine development because they believe that a combination of proteins has the potential to induce a broader level of protection. Regardless of whether subunit or conventional killed vaccines are used, they need to be administered with adjuvants to elicit effective immune responses. Adjuvants can be categorized into two different types: non-specific immune stimulants such as lipid A, saponins (Gupta *et al*., 1993) or bacterial DNA (Klinman *et al*., 1996), and delivery vehicles such as mineral oil and liposomes

(Gregoriadis, 1990). Unfortunately many adjuvants cause tissue reactions and currently only alum and MF59 emulsion are is licensed for human use.

To increase the safety of conventional live vaccines, gene deleted vaccines are being developed by removing genes involved in pathogenicity of the microbe. Gene deleted vaccines are considered to be safer then conventional attenuated vaccines because they should not revert to virulence. This has been supported scientifically with a number of viruses (Mackett *et al*., 1996; Reddy *et al*., 1999) and bacteria (Kaper *et al*., 1984) that have been attenuated by removing virulence genes to create modified live vaccines. In parallel, gene deleted vaccines can also be used as "marker" vaccines to differentiate vaccinated individuals from non-vaccinated or potential carriers of the disease (Lubroth *et al*., 1996). This is especially important in the livestock industry in countries embarking on vaccination and eradication programs. Obviously, this has less relevance in human medicine. A further advantage of these gene deleted vaccines is that they can be used as vectors for carrying genes encoding foreign antigens, which under the proper conditions can elicit a protective immune response to the encoded antigen (Johnson, 1991). The best example of this is the use of vaccinia virus with bait to immunize wildlife against rabies virus (Rupprecht *et al*., 1986). This technology has greatly aided in reducing rabies virus spread from wildlife to domestic livestock and humans (Brochier *et al*., 1996).

Immunization of animals using plasmids encoding protective antigens is one of the most recent vaccination methods described. The basis behind DNA immunization is that an antigen encoded in a plasmid, with the proper regulatory sequences, transfects cells *in vivo* resulting in expression of the antigen and induction of immune response to

the expressed protein. Since the protein antigen is produced *in vivo* in a manner similar to what occurs in natural viral infections, the post-translational and intracellular processing of the protein are considered to be authentic and therefore both protective cellular and humoral immune responses are induced. The universality of this approach has been shown in various species and with numerous genes from different pathogens (Davis, 1998; Kuklin *et al*., 1997; Ulmer *et al*., 1998). Furthermore, DNA immunization has been shown to be effective in eliciting an immune response following intramuscular (Ulmer *et al*., 1993), intradermal (Tang, *et al*., 1992), intraperitoneal, intravenous (Yokoyama, *et al*., 1996), oral (Roy *et al*., 1999), intranasal (Okada *et al*., 1997a), and ocular (Degano, *et al*., 1998) administration. These results have encouraged many researchers and commercial companies to seriously investigate this vaccination approach in humans and animals. Indeed, DNA immunization has been referred to as the "third generation" of vaccines (Alarcon, *et al*., 1999).

1.1.1 The ideal vaccine of the future and challenges

Considering the wide variety of vaccine types available, it is critical to determine factors contributing to an ideal vaccine. The ideal vaccine would have the following characteristics. It would be safe, effective, inexpensive and would rapidly elicit lifelong immunity and delivered non-invasively in a single administration and protect against all relevant infectious diseases. In this section I will describe the ideal vaccine of the future and potential factors limiting each aspect of the ideal vaccine.

Safety of vaccines is the most important consideration and greatest concern because vaccines are administered in healthy people to prevent disease. Today's

vaccines are highly scrutinized for their safety and undesirable side effects such as tissue reactions. Since protection against microbial diseases requires that specific immune responses be mounted, it is essential that vaccines mediate the proper protective immune response, in order to be effective. This has become especially important when one considers that some inappropriate responses may actually enhance the disease, for example as seen with respiratory syncytial virus (Srikiatkhachorn and Braciale, 1997). A successful vaccine should not cause any vaccine related complications. But even the most successful vaccine, namely the small pox vaccine that eradicated the disease, caused significant medical complications in 1 out of 300,000 people. These vaccine complications were acceptable under the circumstances, since many more people were dying from the disease, and the risk of disease far outweighed the risk associated with vaccination. Today, since the risk of the disease caused by smallpox has virtually disappeared, the risks associated with smallpox vaccination are too high. Therefore, the general public is no longer vaccinated against smallpox. The risk to benefit ratio must be assessed for each vaccine to determine if it should be used. It is unlikely that a vaccine will ever be 100% risk free; however, the risk associated with a vaccine must be substantially lower than the risk of the disease. Injection site reactions are also an important safety issue with vaccines; however, the tissue damage caused at the injection site may be an important factor in the induction of immune responses. It may be possible to mimic the signals caused by tissue damage without causing an injection site reaction.

Although very few vaccines are 90-100% effective after a single immunization, most successful vaccines have efficacy rates of greater than 90% following secondary administration. It is critical that a vaccine be delivered appropriately to induce a

protective immune response. There are many factors to consider for optimal vaccination, such as route of delivery, targeting of immune cell compartments and stimulation of humoral and cellular immunity. Although there are several sites in which a vaccine can be administered, including muscle, mucosal surfaces and skin, the skin may be one of the best sites for vaccination for numerous reasons such as accessibility and immuno-competence.

Economics is also an important consideration for people who are given the vaccines as well as the companies that produce them. Vaccination is still the most cost effective way to prevent disease. Given this fact, the cost of vaccines is extremely low compared to the economic value to society. Unfortunately, the vast majority of individuals in the world cannot afford even the cheapest vaccines. This has impeded the development of vaccines against tropical diseases such as malaria. Vaccines are not simple drugs. They are complex biological products that require extensive manufacturing processes, especially for subunit vaccines. This along with the lower profitability of vaccines compared to pharmaceutical products has left only a few commercial companies remaining in the business of vaccine development, leading to a lower level of research and development in vaccines compared to that of pharmaceuticals.

Vaccination should also rapidly induce immunity; however, as long as immunity is elicited before a possible infection, the vaccine will be successful as illustrated by the current HBsAg vaccine. An ideal vaccine should rapidly elicit a lifelong immunity. Unfortunately very few vaccines, if any, can elicit lifelong immunity. Whether or not it is possible to induce lifelong immunity is not known. It would be possible to induce

lifelong immunity if memory lymphocytes were long lived. However, the survival requirements for memory lymphocytes, (whether or not they require antigen for survival) (Gray, 2002; Murali-Krishna *et al*., 1999; Swain, *et al*., 1999) as well as the lifespan of memory lymphocytes (Fearon, *et al*., 2001; Sprent and Tough, 2001) are currently unknown. Regardless of the answers to these questions, it is likely that re-vaccination will be required to maintain immunity. However, it may be possible to use controlled release delivery systems to slowly release antigen to boost immune responses (Cleland, 1999). The development of controlled release delivery systems may allow continued antigen stimulation allowing generation of effector cells from memory cells. This may be important in reducing the amount of time necessary for memory cells to become effector cells which are essential for protection against fast replicating pathogens.

The ideal vaccine would protect against all relevant infectious diseases. One of the potentially greatest leaps in vaccination technology would be the ability to vaccinate against all antigens simultaneously. Since vaccination is simply used to select specific B and T lymphocytes that can provide protection against specific pathogens, it is theoretically possible to select a specific network of cells to protect against many pathogens. For example, vaccination against one pathogen can protect against a different pathogen, potentially through cross-reactive epitopes (Chen *et al*., 2001b). Most vaccines currently used, protect against a single pathogen. However, there are also several combination vaccines, such as the measles, mumps and rubella, the diphtheria-tetanus-pertussis trivalent vaccine, and the Hepatitis A and B combination vaccine. Although it seems a simple concept to add all existing antigens into a single vaccine,

there are numerous regulatory, scientific and manufacturing issues that need to be addressed before more combination vaccines are available. The issue of combining different vaccine antigens was evaluated in this project by immunizing pigs with plasmids encoding two different antigens.

Finally, in order to increase compliance, and make the vaccine more economical, the ideal vaccine should work after a single immunization and be delivered by a needle-free method, either, for example, by the oral route or topical application onto the skin. Non-invasive administration would eliminate accidental needle sticks and spread of disease in poor countries, where reusing needles is tempting. Several different needle-free approaches are in various stages of development. Although some of these delivery systems such as BioJect (b.j.) are already used in clinical applications. Several others show promise in experimental systems; however, much more work is required before these delivery systems become clinically useful.

The following sections will describe DNA immunization, some of the potential benefits that make the skin attractive as a site of immunization. A subsequent section will describe delivery, adjuvants and will discuss some of the challenges regarding delivery of vaccines through the skin.

1.2 Plasmid DNA as a vaccine

The observation that administration of plasmid DNA into muscle results in expression of its encoded protein (Wolff *et al*., 1990) led to the idea of using a plasmid encoding an antigen to elicit an immune response to the antigen (Ulmer *et al*., 1993). For DNA immunization to be effective, the plasmid must enter the cell and the

nucleus and express the encoded gene. The mechanism of plasmid uptake remains unknown, but the efficiency is low due to the fact that most of the plasmid is degraded before gene expression occurs (see Figure 1.1).

Once the gene is expressed, in order to stimulate an immune response, the antigen must be presented to cells of the immune system (Figure 1.2). DNA vaccines generally induce efficient antigen presentation through class I MHC, compared to killed or subunit protein vaccines, and therefore can induce antigen specific CTLs. In addition, transfection by DNA vaccines may alter cellular function in the same manner as certain viral infections, enhancing antigen presentation.

The location of antigen expression can be altered through modification of the plasmid antigen constructs. These include, addition of signal sequences for secreting the antigen, addition or removal of membrane anchors (Lewis, *et al*., 1999) or targeting of the antigen to the proteasome through ubiquitination (Delogu *et al*., 2000; Rodriguez *et al*., 1998; Vidalin *et al*., 1999). These molecular targeting approaches have been able, in certain experimental systems, to enhance and/or modulate immune responses to DNA vaccines. However, these molecular targeting approaches are dependent on the antigen and there is no clear knowledge why in certain circumstances they are effective.

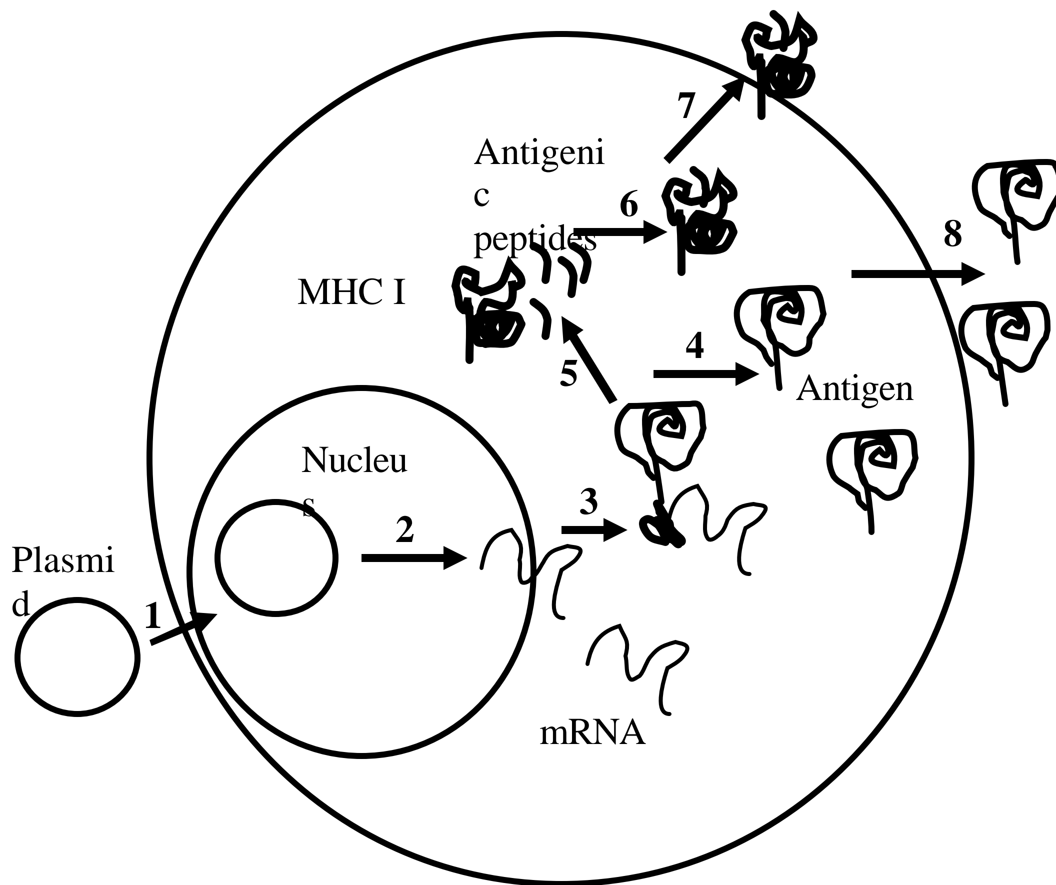

Figure 1.1 Production and presentation of antigen by plasmid DNA.

Plasmid DNA encoding an antigen enters a cell and the nucleus (1). The antigen is expressed by the host cell (2, 3 and 4). Once antigen is expressed it can be presented on class I MHC of the host cell (5, 6 and 7) or leave the cell (5).

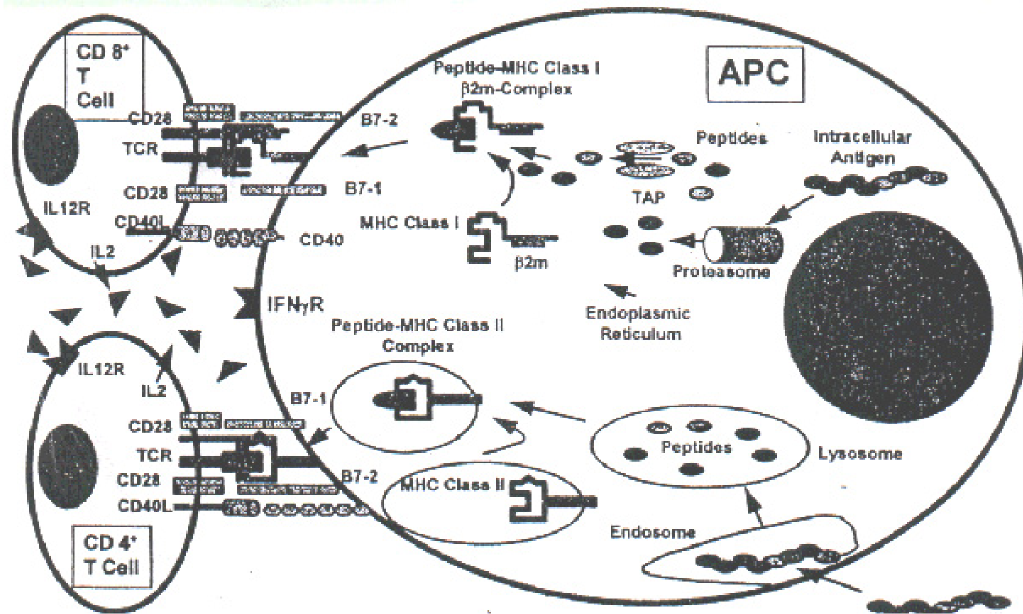

Figure 1.2 Antigen presentation to CD4+ and CD8+ T cells.

With classical antigen presentation intracellular antigens are processed and predominantly presented on Class I MHC, whereas extracellular antigens are processed and presented predominantly on Class II MHC. Figure from (Tuting, et al., 1998) with permission.

The inclusion of gene-encoded cytokines administered in separate plasmids, are an additional way to enhance or modulate immune responses to DNA vaccines. IL-12 and GM-CSF have been used to modulate and enhance immune responses elicited by DNA vaccines in mice (Iwasaki *et al*., 1997; Moore *et al*., 2002; Okada *et al*., 1997b). The cellular location of the cytokine and antigen production may play an important role: it was shown that a bicistronic construct (both antigen and cytokine gene on the same plasmid) encoding CM-CSF was more effective than co-immunizing with a separate GM-CSF plasmid (Barouch *et al*., 2002). However, if there was increased cytokine gene expression, it is likely that the effect of antigen cytokine localization would not be seen. The effectiveness of IL-12 as an encoding cytokine to enhance DNA vaccines in cats has recently been shown (Dunham *et al*., 2002).

One of the more innovative approaches of DNA vaccination is oral immunization of fetal lambs (Gerdts *et al*., 2000; Gerdts *et al*., 2002). This work demonstrated that fetuses are immunocompetent and protective immune responses can be induced by a single DNA immunization. This work was interesting for several reasons. Firstly, it contradicted the finding that some conventional vaccines are not as effective in newborns as in older subjects. Secondly, the dogma that fetuses are not immuno-competent was disproved. Thirdly, DNA vaccines administered orally in adult lambs do not induce immune responses, but it did in fetuses indicating that it is likely that fetal cells are much more permissive to transfection than fully developed cells (personal communication Dr. Philip Griebel, VIDO). *In utero* DNA vaccination may be clinically useful in the future for preventing mother-to-infant transmission of pathogens such as herpes, hepatitis C and HIV, once such vaccines have been developed.

DNA vaccines have several advantages over conventional immunization. These advantages include the ability to stimulate both humoral and cell-mediated immunity including cytotoxic T cells, without the use of conventional adjuvants (mineral oils, alum, etc.). The simplicity of this strategy is that it allows easier testing of antigens, by using the host cells as a bioreactor producing the antigen. Another advantage is that DNA vaccines are more stable and do not require cold storage if they are formulated in PBS, whereas conventional vaccines require refrigeration. Purification procedures for plasmid vaccines are also simpler and cheaper compared to protein-based vaccines.

Due to poor efficacy of DNA vaccines in humans and domestic animals improvements in delivery and vector design are very important. Another major hurdle for DNA immunization are some regulatory concerns with regards to safety. A primary safety concern with DNA immunization is the potential for antibodies being produced to the DNA. However, no significant amounts of DNA-specific antibodies are produced by DNA immunization (Mor *et al*., 1997), allowing for repeated administration of the vector. Another safety concern is the potential for integration of the plasmid into chromosomal DNA. Several studies have addressed this issue and have concluded there is a lack of evidence for integration, suggesting that integration is a very rare event if it does occur at all (Martin *et al*., 1999). The antibiotic resistance gene present in the plasmid is also another potential safety issue, however, other selectable markers can be used to replace the antibiotic resistance gene in the plasmid or can be removed from the plasmid after production using restriction enzymes and ligating the end strands together.

1.3 The skin as an immune compartment and a site for vaccine administration

It is essential to understand the structure and function of the skin in order to develop effective transdermal vaccination techniques. The skin is the outermost layer of the body. The function of the skin is to protect against water loss, ultraviolet light and as the first line of defense to prevent the entry of pathogens into the body. It is precisely because of these functions, that the skin presents a challenge to the topical delivery of both protein and DNA vaccines. This section will describe the structure as well as immune competence of the skin, to lay the foundation for the later section describing approaches for vaccine delivery through the skin.

The skin is composed of the epidermis, the dermis and the hypodermis (Figure 1.3). The hypodermis forms the deepest layer of the skin. It contains mostly adipocytes and fibroblasts. Above the hypodermis, is the dermis, which is a 2-3 mm thick (in human skin) consisting of connective tissue matrix and containing fibroblasts, macrophages and mast cells. Between the dermis and epidermis is the basal lamina, which is the basement membrane for the epithelial cells of the epidermis, the outermost layer of the skin. The viable (living) epidermis is composed of approximately 90% keratinocytes. Throughout the viable epidermis, immune competent dendritic cells, called Langerhans, present antigen. These Langerhans cells, despite only composing 1% of the cell population represent nearly 20% of the surface area of cells in the skin through their horizontal orientation and long protrusions which form a meshwork that allows them to take up antigens that cross the epidermal barrier (Bodey, *et al*., 1997). This network of Langerhans cells represents the second line of defense after the stratum corneum and initiate specific immune responses by presenting the processed antigens to

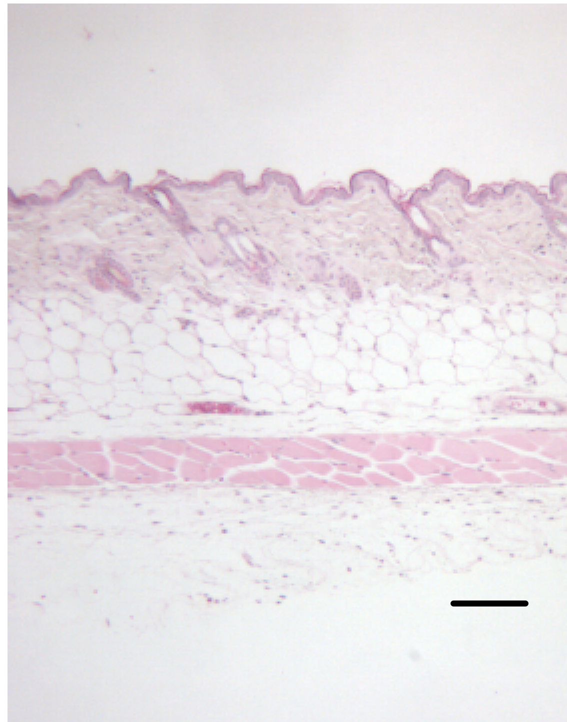

Figure 1.3 Histology of normal mouse skin

Normal mouse skin (HE stain). The stratum corneum is the outermost layer of skin in the epidermis. Under the epidermis is the dermis, is a layer of smooth muscle and the hypodermis. Bar is 100 µm.

other cells of the immune system. Keratinocytes in the epidermis undergo process of differentiation to form five different cell layers, namely the basal, spinous, granular, clear and horny layers (Eckert, 1989). The basal cell layer consists of undifferentiated keratinocytes and stem cells sitting on the basal lamina (Stanley *et al*., 1982).

Above the basal cell layer is the spinous layer (stratum spinosum) of cells, so-named because of the spine-like appearance created by the desmosomes. Next is the stratum granulosum. The transition zone or clear layer above the granular zone consists of both viable and dead cells. The cells in the transition zone undergo major continuous cellular changes, including remodeling of keratin filaments into more structurally stable bundles (Fuchs, 1995) and release of their lipid containing granules into the extracellular matrix. Above the transition zone lies the stratum corneum. The stratum corneum is 10-20 μm thick and consists of terminally differentiated keratinocytes called corneocytes. Corneocytes are flattened cells that contain intracellular cross-linked fibrillar bundles of keratin, giving the corneocyte a rigid structure. Desmosomes maintain corneocytes into the ordered structure of the stratum corneum (Chapman and Walsh, 1990). The stratum corneum also contains multilamellar arrays of lipids found extracellularly between the corneocytes. These multilamellar arrays are composed primarily of cholesterol, free fatty acids, and ceramides (Elias, 1983). The stratum corneum is constantly being sloughed and replaced. When this barrier layer is breached physically, chemically or biologically, many metabolic responses such as the synthesis of fatty acids, cholesterol, and ceramides occur, as well as secretion of preformed lamellar bodies to return the barrier to normal (Tsai *et al*., 1996). For these reasons the stratum corneum is not a static structure, but is continuously being altered in response to

the interaction with the environment, thus preventing prolonged disruption of the barrier. The remaining specialized cells include the pigment containing melanocytes, the function of which is to protect all of the lower cells from ultraviolet light, the epidermotropic lymphocytes and sensory Merkel cells.

There are differences in skin thickness and lipid composition among different animal species. Mouse skin has a much thinner stratum corneum and dermis compared to porcine skin (Hammond *et al*., 2000).

The skin acts as a barrier to environmental insults and maintains the viable cell layer in a state of homeostasis. When the skin is damaged, keratinocytes and Langerhans cells become activated. When activated, keratinocytes can synthesize a large number of cytokines involved in modulating the immune response (Luger and Schwarz, 1991). In addition, keratinocytes can express intercellular adhesion molecules (ICAMs) and other adhesion molecules for various immune cells (Lawley and Kubota, 1991). Keratinocytes are therefore immunologically active cells, in addition to providing structure to the skin. On activation, Langerhans cells increase phagocytic activity and move from the skin where they encounter antigens into draining lymph nodes where they present foreign antigens and initiate immune responses (Cumberbatch *et al*., 1996).

Numerous experiments using proteins (Romani *et al*., 1989) and peptides (Celluzzi and Falo, 1997) have established Langerhans cells as antigen presenting cells. Additional experiments using DNA have re-enforced the importance of Langerhans cells as APCs. The so called "van Gogh experiments" (Barry and Johnston, 1997) showed that removal of the vaccination site after DNA vaccination still resulted in specific immune responses to a DNA vaccine. It was demonstrated that Langerhans cells from

the skin, following administration of plasmid encoding green fluorescent protein (GFP) via gene gun, migrated to the local lymph nodes and expressed GFP (Condon *et al*., 1996). In addition, adoptive transfer of dendritic cells, transfected with antigen encoding plasmid *in vivo*, elicited antigen specific immune responses *in vivo* (Tuting *et al*., 1997). These studies all support the contention that dendritic cells are involved in inducing immunity to the foreign antigens they encounter in the skin.

Although dendritic cells are critical for the induction of immunity, they do not act alone. To investigate the role of other cells in immune induction and modulation, mice were immunized using a gene gun. After immunization, the skin at the site was removed sequentially at several time points, and grafted onto non-immunized mice. Antibody and cellular immune responses were measured and the results from these experiments showed that both dendritic and non-dendritic cells were important for (1) the initiation and (2) the magnitude of the response (Klinman *et al*., 1998). The specific role of CD4+ helper T cells was examined using DNA immunization encoding either a MHC class I restricted peptide or the class I restricted peptide plus a class II restricted peptide. These experiments demonstrated that a very weak immune response could be elicited from the single class I restricted peptide; however, strong immune responses were obtained when both class I and class II restricted peptides were used, clearly showing that help is essential for eliciting immune responses (Maecker *et al*., 1998). B cells are essential for the production of specific antibody and can act as APCs. B cell deficient mice were used to examine the role of the B cell in DNA immunization. B cell deficient mice produced cellular immune responses to the encoded antigen in the absence of an antibody response (Chen, *et al*., 1998). Taken together, these studies

support the concept that numerous cell types may be involved in DNA uptake, antigen expression and presentation and that the cells in the epidermal/dermal layers are excellent sites for vaccine administration.

1.4 Overview of delivery systems and devices currently used for vaccine delivery

In order to deliver vaccines and target immune cell compartments there is a wide variety of systems ranging from delivery devices to delivery vehicles. The ideal delivery vehicle should have several attributes, namely safe, non-toxic, non-immunogenic and economical, allowing widespread use. It would also be effective and induce reproducible immune responses to the delivered antigen. In addition, the delivery vehicle would be pharmaceutically acceptable, stable and biocompatible. As well, the ideal delivery vehicle would be non-invasive with the possibility of controlled release, thus increasing compliance.

1.4.1 Delivery devices

The skin is one of the most frequently used tissues for vaccination using delivery devices, due to the fact that it is easily accessible and monitored. Delivery devices can be classified as instruments that use physical brute force to deliver vaccine antigens through the stratum corneum into the skin. There are a wide range of delivery devices consisting of injection systems such as needle injection, jet stream injection and biolistics devices as well as membrane de-stabilizers such as electroporation,

ionophoration and sonophoration. Figure 1.4 compares efficiency and invasiveness of the different delivery systems.

Although injection is one of the most widely used delivery techniques, in vaccination it does have a number of drawbacks. These include pain associated with injection, risk of accidental needle sticks, spread of disease especially in developing countries due to reuse of needles, as well as expenses in biohazard disposal. Considering the trauma associated with needles, vaccination compliance may be improved if vaccines were to be administered less invasively. This has led to the development of new delivery systems.

Gene guns use DNA covered gold beads propelled through the skin by helium (Figure 1.5) (Fynan *et al*., 1993). Although the gene gun was first developed to transfer genes into plant cells, later refinements allowed its use in animals. The gene gun is essentially a miniature shotgun with gold beads acting as small projectiles to carry DNA through the skin into and around cells. The DNA is introduced into the cytoplasm and nuclei of cells facilitating expression of the plasmid-encoded gene. Since a gene gun delivers plasmids directly into cells, this increases the efficiency of gene transfer. One of the first reports of successful DNA immunization used a gene gun to administer plasmid (Tang, *et al*., 1992). Because of direct introduction of plasmid DNA into cells, immune responses are generated using nanograms of plasmid DNA (Pertmer *et al*., 1995). The disadvantage of the gene gun is that it is most suitable for DNA, although it can be used to deliver proteins with extensive formulation modifications (Chen *et al*., 2001a). It also requires extensive equipment and preparation of the vaccine, and only small amounts of DNA can be coated onto the gold particles without causing aggregation, requiring

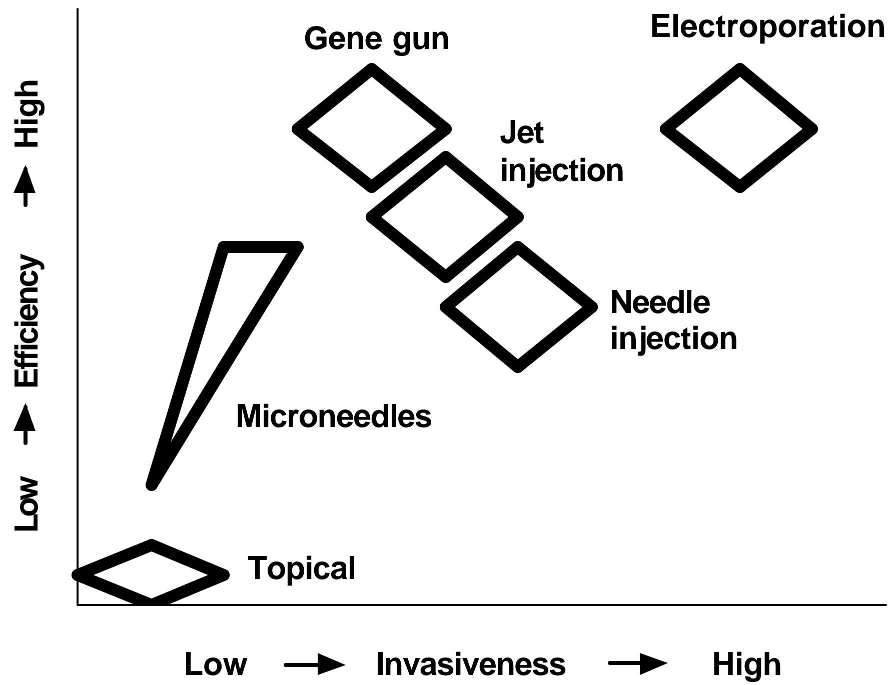

Figure 1.4 Current delivery systems for DNA vaccines: Comparison of efficiency and level of invasiveness.

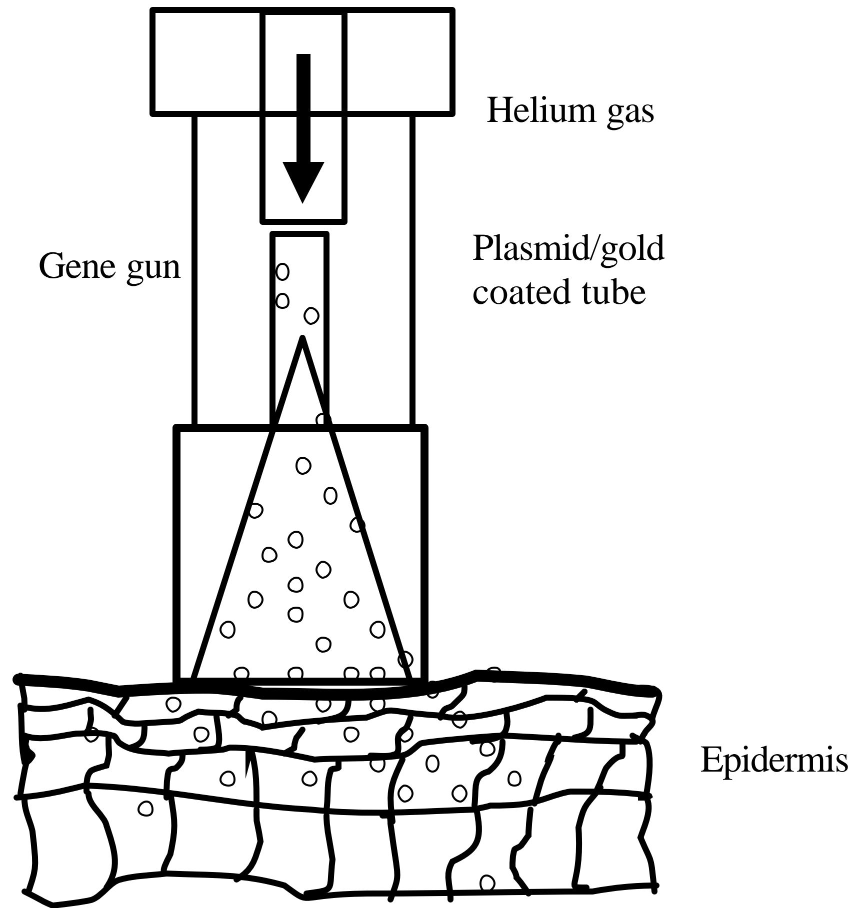

Figure 1.5 Delivery of plasmid using a gene gun to the epidermis.

Plasmid coated onto gold beads is propelled into the epidermis by helium using the gene gun.

numerous shots for animals other than mice. These inconveniences will probably preclude its extensive use unless they can be overcome.

Jet injection uses air pressure to force liquid through the skin and around cells (Figure 1.6). The liquid follows the path of least resistance; therefore tissue damage is reduced compared to conventional injection. Jet injectors can be used for intradermal, subcutaneous and intramuscular injections. This method was first used for the intramuscular delivery of BCG vaccines (Bleasdale, 1965) followed by HBsAg protein vaccines (Whittle, *et al*., 1987). Following successful use for conventional immunization, the jet injector was used for gene transfer experiments as well as DNA immunization (Degano, *et al*., 1998). The results showed the jet injector to be more efficient than conventional injection for delivery of plasmid, with the efficiency of DNA expression dependent on the force of the injection (Furth, *et al*., 1995). Varying the force varies the depth to which the protein or DNA is delivered. By using special spacers, it is possible to deliver most of the vaccine to the epidermis, a dendritic cell rich area of the skin. The delivery of both protein and DNA vaccines were tested in pigs using intradermal administration with the BioJect.

Recently, it was demonstrated that microfabricated microneedles approximately 150 µm long could be used to increase permeability of human skin (Henry *et al*., 1998). Arrays of these microneedles create conduits across the stratum corneum, potentially allowing transport of DNA and proteins into the epidermis. Since these microneedles only penetrate through the stratum corneum and into the viable epidermis, they do not reach nerve endings and applications are essentially painless. In addition, there are no visible skin reactions detected by light microscopy (Henry *et al*., 1998). These initial

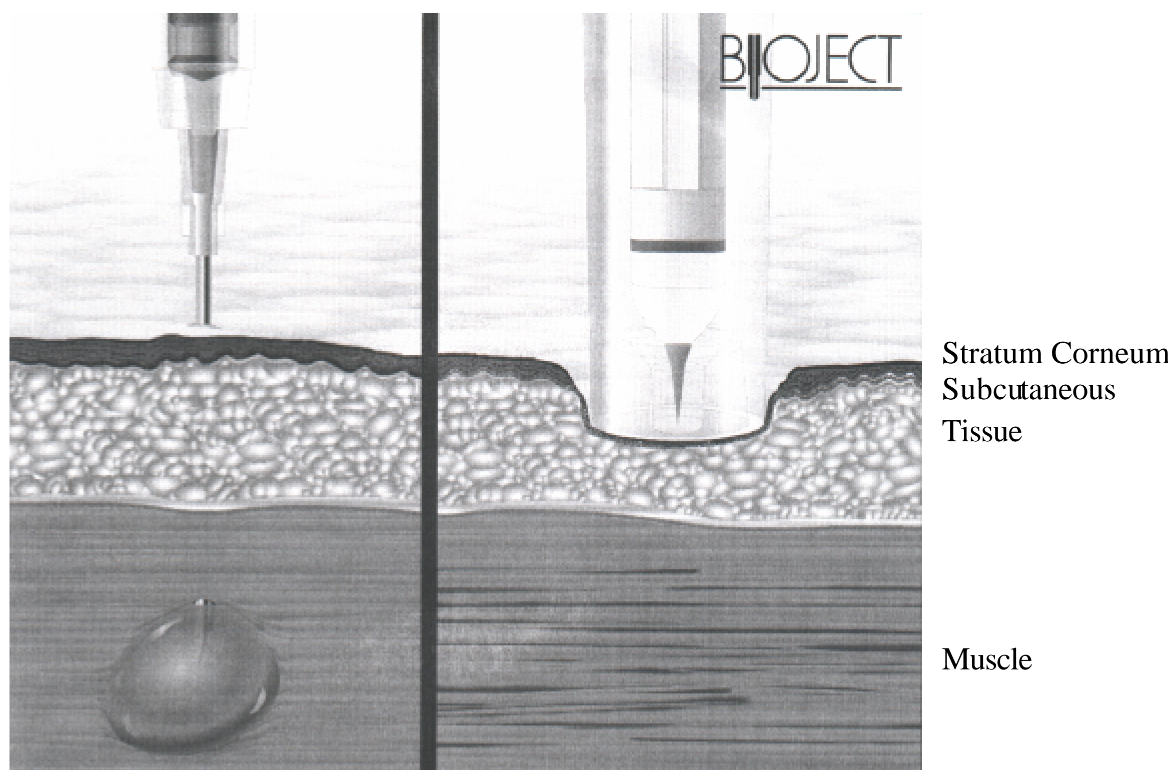

Figure 1.6 Comparison of intramuscular injection by conventional needle and using a BioJect jet injector.

Delivery by jet injection uses a high-pressure stream of liquid to penetrate the stratum corneum and delivery the liquid into the tissues. Figure from BioJect website, (www.bioject.com/tech/all3.html). Reprinted with permission.

studies are very encouraging and hopefully future experiments will determine the efficacy of microfabricated microneedles in delivering DNA and protein to the skin *in vivo.*

Electroporation is a technique that has been used for several years to deliver DNA into cells *in vitro*. Based on this success, it has recently been used *in vivo* (Zhang *et al*., 1996). The basis behind electroporation is that the plasma membrane of the cell, when pulsed with an electric field, becomes stretched and permeabilized so the DNA can enter the cell. Electroporation *in vivo* is accomplished by placing naked DNA on the skin, followed by pulsing the skin with electrical charges through calipers placed on the skin (Banga and Prausnitz, 1998). Recently electroporation was shown to improve DNA immunization, by increasing gene expression in mouse muscle and immune responses to the vaccine (Widera *et al*., 2000). There are several different variables in electroporation such as the strength of the electric field, pulse length and number of pulses. Increasing the electric field (voltage) and pulse length and electric field can all increase the effects of electroporation on cells. However, the relationship between how these variables interact with each other is currently unknown. To find the optimal balance between electroporation conditions that allow pore formation in cell membranes without causing cell death, as measured by gene expression; it was necessary to test these parameters *in vivo.* In addition, there are several different types of electrodes. For electroporation of deeper tissue, such as muscle, needle electrodes are used. There are also several different needle electrodes, for example, the single pair of needle electrode, which allows an electric field to be generated between the needles. Improvements to this method include arrangement of field array using more needles and switching schemes.

However, these arrays are extremely invasive and likely would only be used for severe circumstances like cancer (Hofmann *et al*., 1999b). A less invasive single-needle electrode was tested in this project for efficacy in DNA immunization. Other non-invasive electrodes such as the meander electrode (alternating cathodes and anodes placed on a flexible background material) and the MP 35 electrode (a flat disc electrode) that allows better skin contact and improved electroporation (personal communication, Georg Widera, Genetronics Inc.) were also tested for enhancing gene expression and immune responses to DNA vaccines.

Ionophoration (iontophoresis) is similar to electroporation; the main difference being that it uses weaker electric fields, for a longer period of time, than the strong electric fields used in electroporation (Oldenburg *et al*., 1995).

Sonophoration uses ultrasound waves to disorganize lipids of the stratum corneum, increasing the permeability of the skin to be increased (Mitragotri *et al*., 1995). Several proteins such as insulin, IFN-γ and erythropoietin have been delivered through the skin using this approach in animal models (Mitragotri *et al*., 1995). Sonophoration is a relatively new technique that may be used for delivering both DNA and protein based vaccines transdermally. Membrane destabilizing devices are relatively new but they offer great potential for DNA immunization in the future.

Photomechanical delivery uses a laser pulse to transiently increase the permeability of skin (Lee *et al*., 1998). High molecular weight dextrans were delivered through the skin using this technique, suggesting that the delivery of proteins and DNA possible with this method.

Combining delivery systems may be advantageous for enhancing immune responses to DNA vaccines, if the delivery systems are compatible. Since electroporation can increase plasmid uptake into cells, and delivery by BioJect increases the distribution of plasmid in tissue, the combination of the two would be a needle-free surface electroporation that could enhance gene expression and immune responses to a DNA vaccine.

1.4.2 Microencapsulation and polymeric complexes

Polycations have been used to complex DNA, allowing properties of size, charge and amphipathic nature to be controlled and have been used for plasmid delivery *in vitro*. It is believed or hoped that non-viral delivery systems can overcome problems such as safety and tissue tropism seen using viral delivery (Kabanov *et al*., 2002). In addition, certain polymers are not immunogenic permitting repeated use of the polymer, unlike viral vectors, which may only be used once due to elicitation of immune responses.

Microencapsulation is the physical entrapment of molecules within polymeric structures. In order for a polymer to be useful for microencapsulation it must be biodegradable, biocompatible (non-toxic) and non-immunogenic. Despite the large number of possible polymers only a few are biodegradable and therefore, a limited number have approval for clinical use in humans. Once a biological polymer is characterized for stability, biocompatibility, safety and the manufacturing conditions are optimized, it should be easy to manufacture. In addition, polymers can be designed to

include specific external ligands, which may enhance cell targeting. These properties make microspheres very attractive vaccine delivery systems.

Polymers can be categorized as either natural or synthetic. Natural polymers are various carbohydrates such as starch (Heritage *et al*., 1996; Heritage *et al*., 1998), alginate (Bowersock *et al*., 1999) and chitosan, as well as proteins such as albumin and gelatin (Truong-Le *et al*., 1998). Synthetic polymers with a proven track record in human trials are microspheres composed of D-poly L-lactate (PLA), poly (DL-lactic-co-glycolic acid) (PLGA) (Jain *et al*., 1998) or polycaprolactones (Jameela *et al*., 1997). Other synthetic polymers that may have potential use for vaccination are polyorthoesters, polyanhydrides (Chiba *et al*., 1997) and polyphosphazenes (Andrianov *et al*., 1998).

The advantage of natural polymers is that they do not require the harsh conditions of heat and/or organic solvents for encapsulation of antigen, as do some synthetic polymers. Gelatin and chitosan nanospheres allow DNA to be physically trapped in a solid matrix through the process of complex coacervation (Truong-Le *et al*., 1998; Truong-Le *et al*., 1999). *In vivo* injection of plasmid formulated in gelatin nanospheres to muscle showed a higher rate of gene expression as compared to naked DNA and DNA/lipofectamine complexes. Although nanospheres may be useful for intradermal delivery if injected, they are not effective for topical delivery. Other delivery vehicles such as alginate microspheres have been used extensively for delivery of protein antigens through mucosal surfaces (Bowersock *et al*., 1998; Moser *et al*., 1997). Based on this early success, alginate microspheres could potentially be used to encapsulate DNA for enhanced delivery especially to mucosal surfaces (Aggarwal *et al*.,

1999). Whether they can be designed in such a way as to deliver vaccines topically remains to be determined.

PLA and PLGA polymers can be adapted to improve the delivery of many types of vaccines such as proteins (Coombes *et al*., 1998), carbohydrates (Gupta *et al*., 1998) and DNA (Jones *et al*., 1998). These polymers are especially useful for delivery through mucosal surfaces of the gastrointestinal tract (Jones *et al*., 1997). These polymers protect the contents of the microsphere from degradation as well as allow controlled release of the contents. However, PLGA microspheres may not be suitable as transdermal delivery agents because they cannot penetrate the stratum corneum, and therefore would require injection.

Based on these findings, polymers may be useful for non-invasive vaccination at mucosal surfaces or possibly injection into the skin using novel delivery devices previously described. Even though microspheres would require injection for dermal vaccination, a major advantage of microspheres is the potential for controlled release of the antigen. Through the use of controlled release, it might be possible to imitate multiple boosts with a single immunization (Cleland, 1999). Even if microspheres have to be delivered by injection, they will require fewer administrations.

Polymers used to form polyelectrolyte complexes with DNA are synthetic peptides, dendrimers, polyethyleneimine and polyvinyl pyrrolidone. These polymers are relatively new, but have been shown to be effective in mediating transfection *in vitro* (Bielinska *et al*., 1996; Haensler and Szoka, 1993). Dendrimers are spherical structures of synthetic polymers that can form complexes with DNA. They are branched and contain a high surface charge due to the many amino groups they carry. Dendrimers

have been shown to deliver DNA to a variety of cell lines *in vitro* (Bielinska *et al.*, 1996). Another polymer, polyethyleneimine, which contains net positive charges and can condense DNA, has been shown to enhance DNA delivery *in vitro* to a variety of cell lines (Boussif *et al.*, 1995). 2-(dimethylamino) ethyl methacrylate (DMAEMA) homopolymers as well as the heteropolymers, DMAEMA methyl methacrylate co-polymers have been used to effectively introduce plasmid DNA into cells *in vitro* (van de Wetering *et al.*, 1998). Synthetic peptides such as poly-ornithine, poly-lysine, and poly-arginine as well as amphipathic peptides can increase transfection efficiency *in vitro* (Gottschalk *et al.*, 1996; Pouton *et al.*, 1998; Wadhwa *et al.*, 1997). A major concern with using synthetic peptides as delivery vehicles for vaccines is the possibility of inducing immune responses to the peptides. If an immune response to the peptide is generated, the delivery system may only be useful once due to blocking antibodies or in the worst-case scenario, to autoimmunity (Gaur *et al.*, 1992). Despite these risks, with the proper testing and design, peptides may be useful transdermal delivery systems because amphipathic peptides can mimic liposomes through their polar and non-polar regions. To improve these peptides, various components such as polyethylene glycol (Choi *et al.*, 1998), lipid derivatives such as stearyl-poly-lysine and low-density lipoprotein, can be added to form a terplex system (Kim *et al.*, 1998).

Protective, interactive, non-condensing (PINC) polymers made from polyvinyl pyrrolidone, polyvinyl alcohol or derivatives can form complexes with DNA, while able to diffuse throughout muscle, increasing the distribution of plasmid through out the tissue. Injection of PINC/plasmid complexes to muscle showed a ten-fold increase in reporter gene expression over naked plasmid. In addition, DNA immunization using

PINC/plasmid complexes administered by needle-free injection showed an increase in antibody titer compared to naked DNA (Anwer *et al*., 1999). The amphipathic nature of PINC polymers as well as the ability to diffuse through tissue gives these polymers potential to be useful for topical delivery of plasmids.

However, there has been little testing of these polymers *in vivo* for efficacy, safety, and biological effects such as adjuvant activity. Future *in vivo* experiments with these polymers will reveal their effectiveness. When designing vaccine formulations, it is critical that any adjuvant properties in the delivery system are compatible with the vaccine, since it is essential that the adjuvant induce the proper type of immunity necessary for protection.

1.4.3 Liposomes

The first practical use of liposomes was to enhance the efficacy of amphotericin B *in vivo* (Taylor *et al*., 1982). Several cationic lipids demonstrated no significant toxicity results in initial clinical trials (Nabel *et al*., 1994; Nabel *et al*., 1993). Following these successes, liposomes have been used to deliver various protein antigens for immunization studies (Baca-Estrada *et al*., 1997, Baca-Estrada *et al*., 1999, Griffiths *et al*., 1998, Kwak *et al*., 1998). These studies have shown the ability of liposomes to increase immune responses following either systemic or mucosal administration. In addition to enhancing the immune responses to antigens, liposomes can elicit cell-mediated responses (Walker *et al*., 1992). It is for these reasons, that researchers are excited about the potential of liposomes as immunization vehicles.

Liposomes are vesicles spontaneously formed when phospholipids and water mix (Bangham, 1995); however, to produce liposomes with different structures, energy from various manufacturing treatments such as sonication, homogenization, extrusion, dialysis and filtering are necessary. Liposomes/lipids can be made with a wide variety of characteristics. The net charge of liposomes can be cationic, anionic or neutral. Recent advances in manufacturing conditions have improved the homogeneity of the liposomes produced. Depending on manufacturing conditions, liposomes can be made to vary in size, number of lamellae, structure, and respective "payloads". Manufacturing conditions can also influence the different structures formed by the interactions between DNA and cationic lipids. These structures can be predominantly lamellar, the most stable, spaghetti-like and hexagonal, the least stable (May and Ben-Shaul, 1997). A recent report showed that the hexagonal phase structure was better at transfection compared to other structures due to its ability to rapidly fuse to membranes and release DNA (Koltover *et al*., 1998); however, this has only been shown to occur *in vitro* and may not be true *in vivo.*

The role of liposome charge in transdermal delivery is not well understood. The interaction of liposomes with skin *in vivo* showed that a negative charge as well as cholesterol inclusion, and the acyl chain length of the phospholipids did not appear to affect the penetration efficacy of the liposome into the stratum corneum (Kirjavainen *et al*., 1999). Although the charge of the liposome may not influence the penetration efficacy through the stratum corneum, it may influence the interaction with the protein/DNA thus influencing the penetration of proteins.

Liposomes can be created from a wide variety of lipids and cholesterol. When formulating vaccine antigens with liposomes, it is critical to determine the compatibility of the antigen with the liposomes. Different antigens can have markedly different chemical properties that influence their formulation; thus each vaccine must be formulated specifically for each antigen. The composition of lipids and cholesterol in the liposome give the liposome its properties. Penetration enhancers, or helper lipids such as dioleoylphosphatidylethanolamine (DOPE), allow greater uptake of the liposome and its contents through the skin (Kirjavainen *et al*., 1996), demonstrated, for example through experiments showing that liposomes containing DOPE penetrate deeper into the stratum corneum than liposomes without DOPE (Kirjavainen *et al*., 1999). Other helper lipids such as dilauroylphosphatidylethanolamine (DLPE), dimyristoylphosphatidylethanolamine (DMPE) and dipalmitoylphosphatidyl-ethanolamine (DPPE) are similar to DOPE, varying only in their lipid chain length and saturation.

A major concern with the use of liposomal delivery systems for vaccine antigens is toxicity to cells of the immune system. Cationic lipids are highly toxic to phagocytic cells such as macrophages (Filion and Phillips, 1997), perhaps due to destabilization of the lysosomal membranes by the cationic lipids (Wattiaux *et al*., 1997).

In addition to protein delivery for immunization, liposomes have been used to deliver plasmid DNA. The most commonly used lipids for delivery of plasmid DNA are the cationic lipids such as N-(1-(2,3-dioleoxyloxy)propyl)-N,N,N,-trimethylammonium-methyl-sulfate (DOTAP) (McLachlan *et al*., 1995), dioleyloxy-propyl-trimethylammonium chloride (DOTMA) (van der Woude *et al*., 1995),

dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium (DMRIE) (San *et al*., 1993), dimethyldioctadecyl ammonium bromide (DDAB) (Zhao *et al*., 1997) and (N(N',N-dimethylaminoethane) carbamoyl) cholesterol (DC-Chol). Liposomes for DNA immunization have been used for intranasal administration (Klavinskis *et al*., 1997; Okada *et al*., 1997a). These experiments demonstrated the induction of strong mucosal responses by this approach. There are also a few reports on the use of liposomes to deliver plasmids to other tissue sites. Enhancement of immune responses over those seen with direct injection into muscle was observed, when plasmid was incorporated into liposomes (Gregoriadis *et al*., 1997; Ishii *et al*., 1997).

1.4.4 Virus like particles

Virus like particles (VLPs) are the outer capsid viral proteins that spontaneously self assemble into particles. VLPs have been manufactured from numerous viruses such as papilloma virus (Kirnbauer *et al*., 1996), rotavirus (Conner *et al*., 1996), parvovirus (Sedlik *et al*., 1997), hepatitis B virus (Schirmbeck *et al*., 1996) and others. Their major advantage is that VLPs are more immunogenic than the individual protein subunits of which they are composed. This may be due to the repetitive nature of the proteins making up the VLPs (Bachmann and Zinkernagel, 1997) or to enhanced antigen presentation by APCs due to the particulate nature of VLPs (Kingsman and Kingsman, 1988). VLPs can be used without adjuvants to generate specific immune responses. Furthermore, peptide epitopes of a limited size can be directly incorporated into the viral protein without affecting assembly (Allsopp *et al*., 1996). VLPs can be used to encapsulate proteins (Adams *et al*., 1994) or DNA (Touze and Coursaget, 1998) to

facilitate vaccine delivery. Finally, at least some VLPs may be used repeatedly since they are not affected by prior exposure to the vector (Frenchick *et al*., 1992). VLPs made from viruses that generally enter through the skin, such as papilloma virus, may be especially attractive for transdermal delivery, although there is no reported evidence for their efficacy.

1.4.5 Viral vectors

The skin was first used as a site for vaccination by Jenner by rubbing infectious *Variola minor* on to scratched skin. Viruses such as papilloma and herpesvirus with a tropism for the skin may be good candidates as vectors for carrying antigens and eliciting immune responses. However, the stratum corneum must be breached to allow viruses to infect cells. For example, after mouse skin was treated using a depilatory reagent, an adenovirus vector encoding human carcinoembryonic antigen (CEA) placed on the skin during recovery from anaesthesia resulted in induction of CEA specific antibodies in 23 of 24 immunized mice (Tang *et al*., 1997).

Non-replicating viral vectors created through gene deletions resemble artificial non-viral vectors. Sophisticated non-viral vaccines can share more similarities with viruses by addition of viral replicons that allow the DNA vector to replicate in RNA form once inside a cell (Berglund *et al*., 1998). Non-viral based DNA vaccines delivered using lipids mimic very closely gene-deleted non-replicating viral vaccines with respect to intracellular expression of antigen and induction of immune response. However, viral based vaccines are still much more effective at transfecting cells *in vivo* and generally elicit stronger immune responses without adjuvants, compared to non-

viral vaccines. The advantages of using non-viral plasmid vaccines over viral vaccines are quality control issues as well as manufacturing issues. The manufacturing of plasmid vaccines is considerably easier than the production of viral vaccines due to the fact that plasmid vaccines are produced in a prokaryotic system as opposed to the complex cell culture system needed for production of viral vectors. In addition, non-viral based methods may be able to fulfill many product requirements such as a high safety profile, low cost, high reproducibility, and ability to elicit an effective immune response (Mumper *et al*., 1996).

1.5 Adjuvants

Adjuvants are components added to vaccines to increase their immunogenicity. Some killed vaccines such as HSV-1 (ultraviolet inactivated) (Schneweis *et al*., 1981) do not require addition of adjuvants to induce potent immune responses since many of their components have adjuvant activity. However, most vaccines (killed or subunit) require adjuvants to elicit protective immune responses. As vaccination technology advanced from rather crude killed vaccines that had many undefined components to highly purified protein subunit and peptide-based approaches, the immunogenicity of vaccines decreased dramatically.

To enhance immune responses, adjuvants are incorporated into vaccines. Adjuvants act either as a depot for the vaccine or by stimulating cells of the innate immune system to subsequently increase specific immune responses. The first adjuvant used was Freund's adjuvant. Freund's incomplete adjuvant is mineral oil that acts a depot and Freund's complete adjuvant is mineral oil containing killed mycobacterium.

Although both Freund's adjuvants are effective, they cause extensive tissue damage and are not licensed for commercial vaccines. Following Freund's adjuvant, the search for safer adjuvants led to the development of aluminum hydroxide (Alum) and MF59, currently the only licensed vaccine adjuvants for humans.

Today it is known that many microbial components such as lipopolysaccharide (LPS), flagella, cell wall components and bacterial DNA simulate the innate immune system through various receptors of the Toll-like receptor family (Schnare *et al.*, 2001). Microbial components that stimulate Toll-like receptors are potential adjuvants.

1.5.1 CpG motifs of bacterial DNA

1.5.1.1 Introduction

The immunogenic properties of bacterial DNA were first discovered in tumor regression in some mouse models (Tokunaga *et al.*, 1984). The functional sequences of bacterial DNA were identified as CpG sequence motifs flanked by two 5' purines and two 3' pyrimidines. These CpG motifs are found at a much higher frequency in bacterial DNA compared to mammalian DNA. In addition, these motifs are methylated differently in bacterial DNA with the cytosine residue being unmethylated, where it is methylated in mammalian DNA. These differences in methylation patterns are critical for CpG activity since methylation of CpG using mammalian methylases abolishes their immunostimulatory activity (Oumouna *et al.*, 2002). Thus, the immune system recognizes mammalian DNA as self and immunogenic bacterial DNA as foreign. These CpG motifs can be encoded in synthetic oligonucleotides allowing for a chemically defined molecule that displays multiple biological effects.

1.5.1.2 Cells which are stimulated by CpG

CpG motifs have a wide variety of activities, such as triggering B cell proliferation (Krieg *et al*., 1995), activation of macrophages (Stacey *et al*.1996) and activation of NK cells through stimulation of IL-12 (Zhan and Cheers, 1995). With the activation of these cells, a variety of cytokines such as IL-6, IL-12, and IFN-? are produced (Klinman *et al*., 1996). In addition, bacterial DNA is a potent stimulator of dendritic cells, promoting Th1 responses to antigens (Jakob *et al*., 1998). Although the exact mechanism by which CpG activates cells is currently unknown, CpG ODNs must enter cells to be biologically active. This was demonstrated by a lack of immune stimulation by immobilized CpG on a Sepharose beads that could not enter the cell (Manzel and Macfarlane, 1999). Recently, a Toll-like receptor (TLR9) was found which recognizes bacterial DNA. The possibility that binding to this receptor is essential for CpG immunostimulatory activity was suggested by the fact that TLR9 knockout mice fail to respond to CpG ODNs (Hemmi *et al*., 2000). TLR9 is expressed on B cells, macrophages and plasmacytoid dendritic cells in mice. In addition, transfection of the TLR9 gene into CpG non-responsive cells allows the cells to respond to CpG (Bauer *et al*., 2001; Chuang *et al*., 2002).

1.5.1.3 CpG ODNs as adjuvants and immunoprotective agents

CpG ODNs have been used as adjuvants with subunit (Davis *et al*., 1998) and killed viral vaccines (Moldoveanu *et al*., 1998). When CpG ODNs are used as adjuvants they promote Th1 type immune responses. This is extremely valuable since some pathogens such as *Leishmania* require induction of Th1 responses for protection (Soares

et al., 1994), whereas most conventional adjuvants such as alum promote Th2 type responses. CpG, in addition to being a systemic adjuvant, has also been shown to be a potent mucosal adjuvant (McCluskie and Davis, 1999; McCluskie and Davis, 2000; McCluskie *et al*., 2001).

Since CpG ODNs have potent immunostimulatory activity, they have been tested to assess their ability to protect mice against lethal challenge of *Listeria monocytogenes* (Krieg *et al*., 1998a), *Francisella tularensis* (Elkins *et al*., 1999), as well as *Leishmania major* (Zimmermann *et al*., 1998). CpG ODNs were able to immunoprotect mice against these diseases as well as other pathogens (personal communication, Arthur Krieg).

1.5.1.4 CpG motifs and DNA vaccines

It has been hypothesized that CpG motifs normally present in plasmid vectors are essential for DNA vaccines to be effective (Krieg *et al*., 1998c). There is some evidence that the presence of CpG motifs within the DNA backbone contributes significantly to the immune response (Klinman *et al*., 1997; Sato *et al*., 1996). Indeed, the propensity of DNA vaccines to induce Th1-type or mixed Th1/Th2 responses (Raz *et al*., 1996), appears to be at least partially due to the presence of CpG motifs in the plasmid backbone. If CpG motifs are methylated, immune responses are significantly reduced (Leclerc *et al*., 1997). Conversely, the removal of inhibitory CpG motifs (additional motifs which suppress the activity of CpG motifs) or the insertion of an optimal number of immunostimulatory CpG motifs can enhance immune responses (Krieg *et al*., 1998b).

It has been shown in mice that mixing CpG ODN with antigen-encoding plasmid DNA either only marginally increased the immune responses (Klinman *et al*., 1997), or resulted in a dose-dependent reduction of the immune response (Weeratna *et al*., 1998). A possible mechanism for reduction in immune responses is inhibition/blocking of entry of plasmid DNA into the cells resulting in decreased gene expression. However, there are several reports that show that addition of CpG motifs into plasmid vectors does not enhance antigen specific immune responses (Deml *et al*., 2001; Sun *et al*., 2002). From these studies it is clear that simply cloning additional CpG motif does not necessarily result in the enhancement of immune responses from DNA vaccines.

1.5.1.5 Enhancing biological effects of CpG

Depending on backbone chemistry, ODN can be degraded within minutes following *in vivo* administration (Semple *et al*., 2000, Semple *et al*., 2001). This rapid clearance may limit the uptake and subsequently the effectiveness of CpG ODN as an immunomodulating agent. Formulation of CpG ODN in appropriate delivery systems may potentiate its immunostimulatory effects, by either protecting CpG ODN from degradation, increasing uptake by immune cells (Gursel *et al*., 2001), or by prolonged stimulation of infiltrating cells such as macrophages at the site of injection. Liposomes provide a vehicle or a carrier system into which antigens and co-adjuvants can be incorporated (Gregoriadis, 1990) to induce protective immune responses against bacterial, viral and parasitic infections (Baca-Estrada *et al*., 2000; Childers *et al*., 1997). Cationic liposomes can protect antisense ODNs from nuclease digestion and have been used to successfully deliver them to the cytoplasm of target cells (Duzgunes *et al*., 2001;

Zhang *et al*., 2001). Encapsulation of CpG ODN in liposomes can prolong their stability *in vivo*, increase uptake by immune cells, prolong interaction with APCs and increase induction of cytokine responses (IL-12, IFN-γ and IL-6). Consequently, liposome formulated CpG increases the magnitude and duration of immune responses (Agrewala *et al*., 1996; Gursel *et al*., 2001; Mui *et al*., 2001). Liposome formulation of CpG ODN may also increase the dose of CpG ODN delivered to the draining lymph nodes, where many of the cells activated by CpG ODN are located. More efficient delivery of CpG ODN can effectively reduce the amount of CpG ODN required for a biological response.

In addition to liposomes, other vehicles have been used to enhance the activity of CpG ODN. Mice inoculated with CpG ODN mixed with stabilized antisense lipid particles (SALP) produced greatly increased levels of IL-12, IFN-γ, IL-6, monocyte chemoattractant protein-1 and TNF-α compared with mice given the same dose of free CpG ODN (Mui *et al*., 2001). The innate protection afforded by CpG DNA against pathogens in mice can be maintained for over five weeks if the CpG ODN is given in a formulation providing some sustained release, such as alum (Stacey and Blackwell, 1999).

1.5.2 Cholera toxin as an adjuvant for transcutaneous immunization

Several bacterial toxins such as cholera and tetanus toxins are some of the most immunogenic proteins known. Cholera toxin (CT) is a well-established mucosal adjuvant that has been shown to be a good adjuvant for transcutaneous immunization (Glenn *et al*., 1999, Scharton-Kersten *et al*., 1999; Scharton-Kersten *et al*., 2000). Furthermore, transcutaneous immunization with cholera toxin and whole inactivated

HSV-1 or cholera toxin and HSV-1 antigens elicited protective immune responses against HSV-1 (El-Ghorr *et al*., 2000). Recently, transcutaneous immunization has been shown to be feasible on humans using heat-labile enterotoxin, from *E. coli*, a toxin similar to CT (Glenn *et al*., 2000). CT was also able to induce immune responses to a peptide from influenza virus haemagglutinin when topically administered on the skin of mice (Beignon *et al*., 2002). In addition, co-delivery of plasmid encoding CT enhanced immune responses to several DNA vaccines administered to the skin by the gene gun (Arrington *et al*., 2002). CT enhances immune responses by increasing cellular infiltration (Jones *et al*., 2001), as well as by activating dendritic cells (Bagley *et al*., 2002). Adjuvants that stimulate dendritic cells are an ideal approach, since (1) dendritic cells are potent initiators of specific immune responses and (2) other adjuvants such as CpG ODNs can activate dendritic cells.

1.6 Advantages of dermal delivery of antigens

The pharmaceutical industry has developed successful transdermal delivery systems for a variety of small drugs such as nicotine, nitroglycerine, fentanyl and estrogen. Transdermal delivery (systemic delivery through the skin) is an alternative to more invasive administration routes and has the advantage of lower frequency of dosing, uniform blood levels and increased compliance. There has been extensive research on transdermal delivery of numerous low molecular weight drugs without the aid of physical disruption to the skin. However, there have been only a few reports on dermal and transdermal delivery of proteins or DNA either as therapeutic agents or vaccine antigens (Foldvari *et al*., 1998; M. Foldvari, 1999). Using a novel type of lipid-based

delivery system for leukotoxin (*Pasteurella haemolytica*) and hen egg lysozyme as model antigens, strong Th2 responses could be induced after topical application (Baca-Estrada *et al*., 2000). The use of transfersomes allowed induction of immune responses to a gap junction protein (Paul *et al*., 1998). When utilizing the skin as a route of entry for protein and DNA vaccine antigens, the main target sites are within the skin. Therefore 'dermal delivery' terminology will be used to describe the theory as well as the literature on the subject.

In order for vaccine antigens to be effective, it is critical that the vaccine is delivered to the epidermis where the APCs are localized. The stratum corneum is considered the greatest barrier to effective transdermal delivery (Scheuplein, 1976). The characteristics that make the stratum corneum ideal for protecting the body also provide an obstacle to delivery of proteins and polynucleotides. Comparison of the permeability of normal skin with tape stripped skin, where the stratum corneum is removed, revealed that tape stripping increases the permeability (Scheuplein, 1976), demonstrating that the stratum corneum is the rate-limiting barrier for delivery of many compounds to the body.

There are three possible pathways for the penetration of compounds through the skin, the transcorneocyte route, the intercellular pathway, and the transappendageal pathway. The specific pathway a vaccine antigen will take depends on the physiochemical characteristics of the antigen as well as the method of delivery used.

In the intercellular pathway, molecules travel through extracellular lipids that form continuous pathways surrounding the corneocytes (Muranishi, 1990). Experimental evidence, from penetration studies of certain molecules, reveal that

penetration is not correlated with the number of cell layers or the thickness but the lipid weight of the stratum corneum (Elias *et al*., 1981). Therefore lipid synthesis inhibitors can increase delivery of certain compounds due to the fact that the extracellular lipid barrier is reduced (Tsai *et al*., 1996). Compounds delivered via liposomes generally use the intercellular pathway for entry (Foldvari *et al*., 1990). Liposome/skin interaction studies using a fluorescent lipid bilayer marker and confocal laser scanning microscopy confirmed that liposomes penetrate through the extracellular lipids (Kirjavainen *et al*., 1996).

The transcorneocyte pathway of delivery occurs by passage through corneocytes (Zhang *et al*., 1996). Physical methods of delivery such as the gene gun and electroporation use the transcorneocyte pathway. Since corneocytes are dead cells, plasmids must pass through the corneocyte to enter the viable epidermis for gene expression to occur.

The transappendageal pathway is the route through the hair follicles and sweat glands (Lieb *et al*., 1997). The hair follicle openings are natural holes in the skin that are lined with epithelial cells. The transappendageal pathway avoids the stratum corneum by allowing the biomolecules to travel around it to the cells surrounding the hair follicle. The stratified barrier of epithelial cells lining the hair follicles is thinner than the stratum corneum, allowing increased penetration of molecules. The diameter of the hair follicles range from 50 to 100 μm and hair follicles range in density from 10% on areas such as the human scalp to 0.1% in areas with low follicle density (Lieb *et al*., 1997). The low percentage of surface area is an underestimation of the actual surface area available for dermal delivery of molecules since the hair follicles penetrate through

the skin surface. The transappendageal route may be more important for delivery in animals such as mice that have a much greater hair follicle density compared to humans. The transappendageal route is thought to be the major route by which large molecules such as oligonucleotides (Lieb *et al*., 1997) or liposome complexed DNA enters the skin (Li and Hoffman, 1995; Li *et al*., 1992). Topical application of a DNA vaccine administered in liposome complexes, elicited antigen specific immune responses in mice dependent on the presence of normal hair follicles (Fan *et al*., 1999).

Since the hydrophobic barrier of the stratum corneum is one of the major barriers to effective transdermal delivery of protein and DNA, transdermal delivery systems must be designed to penetrate this barrier. In addition, one must also consider the plasma and nuclear membranes as barriers for effective delivery of DNA. Lipid based delivery systems (liposomes and others) are suitable vehicles to overcome the plasma membrane barrier and are used extensively for transfection of cells *in vitro* (Felgner *et al*., 1994). Building on the *in vitro* observations, liposomes were also shown to be effective in transferring genes *in vivo* (Alexander and Akhurst, 1995; Niemiec *et al*., 1997). The efficacy of both *in vitro* and *in vivo* transfection can be modulated by the liposome composition (Yu *et al*., 1999). Unfortunately there is no correlation between the efficacy of liposome delivery *in vitro* and *in vivo* making development of liposome formulations more difficult.

1.7 Influence of formulation and delivery on immune responses elicited from vaccines

There is a need to increase the potency of DNA vaccines as well as CpG ODNs. The magnitude and type of immune responses elicited by polynucleotide vaccines can be influenced through formulation and method of delivery. One possible way to improve immune responses elicited by DNA vaccines is through delivery, by improving antigen presentation either by (1) targeting APCs or (2) increasing the amount of antigen available for presentation. In addition, the biological activity of CpG ODNs can also be enhanced through delivery to APCs.

2.0 HYPOTHESES, OVERALL OBJECTIVE AND SPECIFIC AIMS

2.1 Hypotheses

I hypothesized (1) that using delivery approaches such as i) a novel type of topical formulation biphasic lipid vehicles (Biphasix^{TM}), ii) gas-powered needle-free injection system (BioJect) and iii) electroporation, it might be possible to enhance immune responses elicited by DNA vaccines compared to conventional injection. In addition, (2) a novel type of injectable depot formulation [Biphasix^{TM}-VTA (Vaccine Targeting Adjuvant)] may be capable of enhancing the immunoadjuvant activity of CpG ODNs.

2.2 Rationale and overall objective

The rationale for this project is that current methods for the delivery of polynucleotides are inadequate for eliciting potent immune responses. The overall objective of this project was to investigate novel delivery approaches for polynucleotides and oligonucleotides to improve immune responses elicited by gene-based vaccines.

2.3 Specific aims

The specific aims in this thesis were: (1) Evaluation of topical delivery of a model plasmid formulated in biphasic lipid vehicles by analysis of gene expression and induction of immune responses in mice; (2) Assessment of the suitability of the electroporation method to improve gene expression in pig skin and increase immune responses after topical electroporation; (3) Assessment of the suitability of the

electroporation method to improve gene expression in pig muscle and increase immune responses following intramuscular administration; (4) Determination of the role of gene expression and cellular infiltration in enhancing immune responses elicited by a DNA vaccine delivery using electroporation; (5) Evaluation of CpG ODNs for enhancing immune responses elicited by DNA vaccines: (6) Evaluation of a novel injectable depot formulation a lipid-based delivery system [Biphasix^{TM}-Vaccine Targeting Adjuvant (VTA)] to enhance the immuno-adjuvant activity of CpG ODNs by systemic and mucosal routes.

3.0 TOPICAL DELIVERY OF PLASMID DNA USING BIPHASIC LIPID VESICLES (Biphasix^{TM}) IN MICE

(Manuscript in press)

3.1 Introduction

Delivery issues in vaccine development have been recognized as one of the important factors to achieve the desired efficacy. Most of the current vaccines are administered by injection, however, there is now evidence that transmucosal or transdermal delivery of antigens (Tang *et al*., 1997), including DNA (Cui and Mumper, 2001; Cui and Mumper, 2002), can elicit an immune response.

Similarly to the more traditional vaccines (single-shot subunit, therapeutic vaccines), genetic immunization also requires the presentation of the antigen in the most advantageous form at the required location. The success of DNA vaccines is dependent on the delivery of the DNA molecule through several membraneous barriers (i.e. the stratum corneum, cell and nuclear membranes) and expression of the antigen. Topical plasmid transfection to date could only be achieved by initially compromising the skin barrier properties such as by intense brushing with a toothbrush (Yu *et al*., 1999) or chemical depilation (Alexander and Akhurst, 1995; Shi *et al*., 1999a).

The epidermis is an ideal site for DNA immunization since the skin is equipped with a network of antigen presenting cells (APCs) that mediate the induction of immune responses. Immunization by gold particle bombardment of the skin using the gene gun has demonstrated that a small number of APCs are transfected (Condon *et al*., 1996). APCs directly transfected may be more effective in inducing immune responses as

compared to a large amount of antigen expressed by cells of non-lymphoid lineage (Fynan *et al*., 1993; Heiser *et al*., 2001; O'Hagan *et al*., 2001). Improving delivery of plasmid through the skin will likely enhance the efficacy of transcutaneous immunization with plasmid DNA.

The feasibility of a novel lipid-based delivery system (Biphasix^{TM}) was evaluated as a pharmaceutical delivery approach for the delivery of two model plasmids. Previously we have demonstrated that these formulations are effective in mediating the induction of immune responses to protein antigens following transcutaneous delivery (Baca-Estrada *et al*., 2000). Unlike conventional liposomes, the Biphasix^{TM} vesicles are multi-compartmental lipid vesicles consisting of multiple, concentric mixed-lipid bilayers entrapping lipophilic, micellar and aqueous sub-unit compartments. Our results demonstrated that topical delivery of plasmids formulated in biphasic vesicles resulted in gene expression in the draining lymph node and in the induction of cellular and humoral immune responses.

3.2 Materials and methods

3.2.1 Animals

Six-week-old female BALB/c mice purchased from the Animal Resource Centre at the University of Saskatchewan were used for all experiments. Animals were treated in compliance under the regulations of the Canadian Council for Animal Care.

3.2.2 Plasmids

Plasmid encoding the luciferase enzyme under the CMV promoter (pluc), was a gift from Dr. Heather Davis (University of Ottawa, ON) (Weeratna *et al*., 1998). A plasmid expressing bovine herpesvirus glycoprotein D (pgD) was constructed by inserting the gD gene into vector pCAN1 under the CMV promoter (Uwiera *et al*., 2001). Plasmids were purified using Qiagen (Mississauga, ON) endotoxin free plasmid kits.

3.2.3 Preparation of DNA delivery system

Plasmids were formulated in lipid-based delivery systems (biphasic vesicles) (Biphasix^{TM}) at a dose of 100 µg plasmid in 125 mg formulation. The three different formulation compositions were prepared based on soya phosphatidylcholine (Natterman GmBH, Germany): cholesterol (Sigma Chemical Co., St. Louis, MO) (10:4 weight ratio) and the following lipids: formula 1: cholesteryl 3ß-N-(dimethyl-aminoethyl) carbamate) (DC-chol, Sigma) at a 1:1 weight ratio; formula 2: dimyristoylphosphatidylcholine (DMPC, Sigma) at a 1:4 weight ratio and formula 3: dioleoylphosphatidylethanolamine (DOPE, Sigma) at a 1:4 weight ratio. Each lipid phase (vesicle forming lipids) was hydrated with a sub-micron emulsion aqueous phase, containing linoleamidopropyl-PG-dimonium chloride phosphate (Mona, Paterson, NJ), 4% and olive oil, 4% w/w, by mixing until a homogeneous cream was achieved (Foldvari, US Patent No. 5, 853, 755).

3.2.4 Immunization with gD encoding plasmid

For topical delivery, mice were carefully shaved with an electric razor 1 day prior to administration of plasmids to ascertain an intact skin surface (normal skin). The Biphasix formulation was placed on the back of the mouse for 48 hours using a patch construction as previously described (Baca-Estrada *et al*., 2000). Each patch contained 125 mg formulation with 100 μg DNA covering a surface area of approximately 1 cm^2. For topical immunization with DNA in solution ('naked' DNA), 100 μg of pgD in 30 μl of water was placed on the back of anesthetized mice until the solution appeared to absorb into the skin or evaporated. For intradermal administration, mice were injected in the back with 1.5 μg pgD in 10 μl PBS. For gene gun immunizations, gold beads (1.6 μm) were prepared according to the manufactures instructions for the Helios Gene Gun System (Bio-Rad, Hurcules, CA) as previously described using 0.05 μg of pgD +1.45 μg of non-coding vector (Loehr *et al*., 2000). For topical immunization all groups were boosted twice at two-week intervals. For intradermal and gene gun immunizations mice were boosted once two weeks after primary immunization. Cells were harvested from spleens and draining lymph nodes two weeks after the final immunization.

3.2.5 Determination of luciferase expression

The levels of gene expression were determined using a plasmid encoding the luciferase gene in the same manner as for pgD delivery. The doses of pluc were the same as those used for immunization with the different delivery methods. Testing of pluc delivery was carried out on both intact skin and tape stripped skin. An area of approximately 4 cm^2 was shaved. For removal of the stratum corneum, but not

Langerhans cells, was achieved using 20 times tape stripping with Scotch tape (3M) was used (Proksch *et al*., 1996). Treated skin samples (about 1 cm^2 area) as well as the axillary and inguinal draining lymph nodes were removed from mice and homogenized in 500 µL of lysis buffer (Promega, Madison, WI) with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) to produce protein extracts. Luciferase activity of the protein extracts was determined using Promega's luciferase assay system. Samples were counted for 30 seconds on a Packard Picolite Luminometer (Packard Instruments Canada LTD, Mississauga, ON). Tissues from untreated animals were used to calculate the background levels of light units (LU).

3.2.6 Characterization of humoral and cellular immune responses

BHV-1 gD-specific antibody responses were determined using ELISA as previously described (Baca-Estrada *et al*., 1996). Briefly, Immunlon 2 (DYNEX, Chantilly, VA) ELISA plates were coated with gD (1 µg/ml) or IgG standards (Serotec, Hornby, ON) overnight at 4°C. Glycoprotein D-specific IgG and IgG subclasses were determined by biotinylated antibodies specific to IgG and IgG subclasses (Caltag, Toronto, Ont.) followed by streptavidin-alkaline phosphatase staining (Jackson Immuno-research Labs, West Grove, PA). The alkaline phosphatase activity was determined by *p*-nitrophenol phosphate (PNPP) (Sigma). The absorbance was read after 15-20 minutes at 405 nm (Bio-Rad).

ELISPOT assays for IFN-γ and IL-4 were performed using cells isolated from spleens or draining lymph nodes (axillary and inguinal) as previously described (Baca-Estrada *et al*., 1997). Briefly, $1x10^6$ cells/well were placed in 96 well culture plates with

and without antigen (gD 0.4 μg/ml) in AIM-V media (Gibco, Life Technologies, Burlington, ON) and incubated at 37°C and 5% CO_2 for 24 hours. Cells were resuspended in fresh media and seeded on nitrocellulose plates (Millipore, Nepean, ON) coated with either IFN-γ or IL-4 specific mouse cytokine capture antibody 2 μg/ml (PharMingen, San Diego, CA). Biotinylated anti mouse IFN-γ or IL-4 specific antibodies a 2 μg/ml (PharMingen) followed by streptavidin-alkaline phosphatase (Jackson Immuno-Research Labs) and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate tablets (Sigma). Spots representing gD specific cytokine secreting cells were counted and expressed as number of cytokine secreting cells per $1x10^6$ cells. Con A stimulated cells were used as a positive control, with greater then 400 cytokine secreting cells per well.

3.2.7 Statistics

Differences in immune responses among vaccine groups were analyzed with Prism graphpad statistical software (GraphPad Software, Inc.) using a one-way ANOVA for the immunization experiments or an unpaired t-test for the gene expression experiments.

3.3 Results and Discussion

3.3.1 Evaluation of the delivery of antigen by measuring antigen-specific immune response

Plasmid delivery into the skin was evaluated by measuring the induced anti-gD specific antibodies following topical treatment with three different compositions of Biphasix™-pgD patches. All three formulations induced anti-gD IgG levels five times higher compared to either the 'naked' DNA or untreated animals (formulations#1 and #3 $p<0.05$) (Figure 3.1A). Topical delivery of plasmid in Biphasix™ system also induced cellular immune responses. Induction of gD-specific cellular response was only observed in spleen cells from mice immunized with formulation #3 (Figure 3.1B). This response was characterized by the predominant secretion of IL-4 by cells isolated from spleen and draining lymph nodes (Figure 3.1B and C). No IFN-γ secreting cells were observed in the spleen or draining lymph nodes (data not shown). Administration of Biphasic formulations without antigen (protein or plasmid), do not elicit any specific immune responses that are significantly different from naive. However, lipids in the formulation may be able to exert an adjuvant effect (in addition to the delivery effect) in combination with antigens.

In order to compare the magnitude of immune response elicited by topical immunization with other methods of delivery, mice were immunized with the gene gun or by intradermal injection with varying doses of gD encoding plasmid. Our results demonstrated that both of these methods of delivery resulted in the induction of similar gD-specific IgG1 antibody titers (Figure 3.2A), which were significantly different from the untreated animals ($p<0.01$ and $p<0.05$, respectively). The immune responses

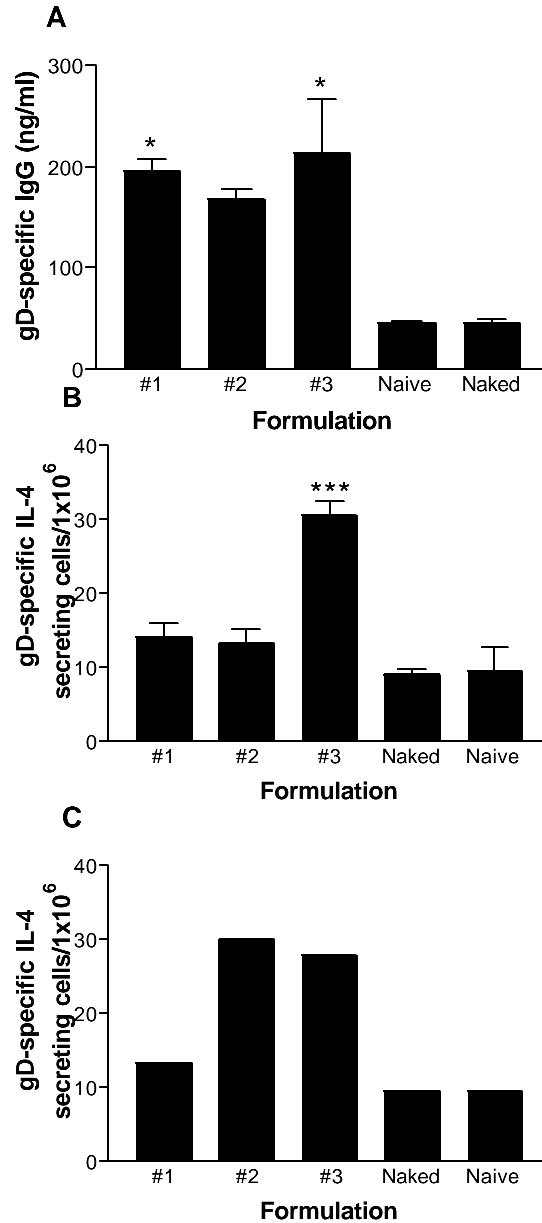

Figure 3.1 Immune responses following topical application of pgD in 3 different lipid-based delivery systems.

All groups (n = 3) were immunized three times at two weeks intervals with pgD (100 µg) in three different lipid-based formulations (#1-3) or naked pgD. At six weeks, two weeks following the final immunization gD-specific IgG was determined using ELISA (A) and gD-specific cellular immune responses were assessed using isolated cells from the spleen stimulated with gD antigen (B) or the draining lymph nodes stimulated with gD antigen (C). There are no error bars in C because this data was obtained using pooled samples (N=1). Error bars represent standard error of the mean, * $p<0.05$, *** $p<0.001$ vs naive.

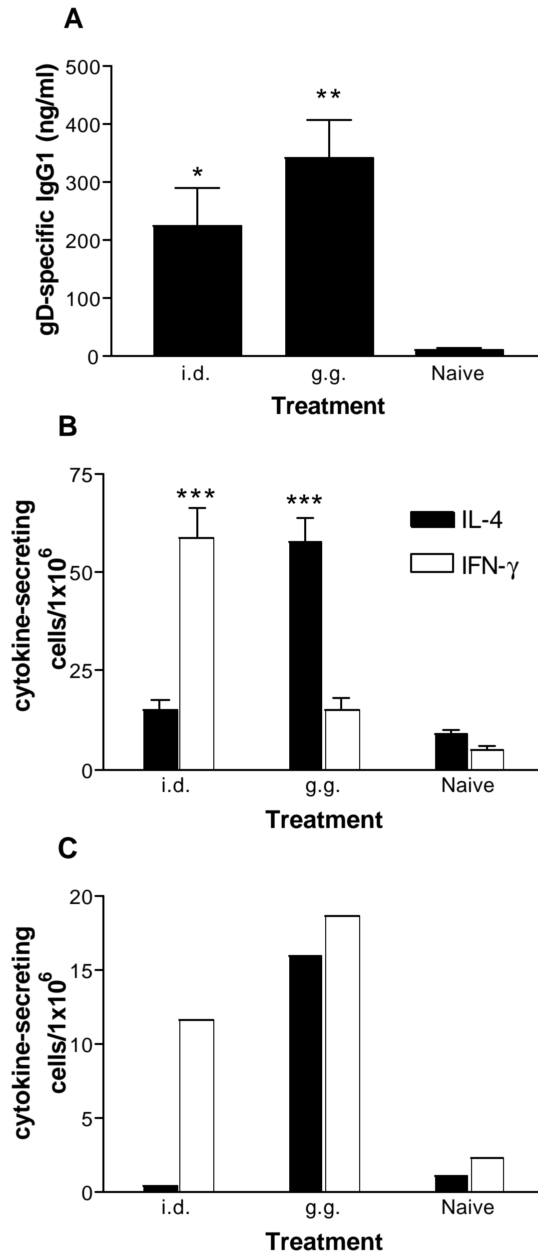

Figure 3.2 Immune responses induced by immunization with pgD either by intradermal injection (i.d.) or by particle bombardment using the gene gun (g.g.).

Groups of mice (n = 5) were immunized with 1.5 µg of pgD (i.d.) or with 0.05 µg of pgD administered by the gene gun (g.g.). At four weeks, two weeks following the final immunization gD-specific IgG1 was determined using ELISA (A) and ?gD-specific IFN–γ and IL-4 secreting cells in the spleen (B) and draining lymph nodes (C). There are no error bars in C because this data was obtained using pooled samples (N=1). Error bars represent standard error of the mean of five animals per group, * p<0.05, ** p<0.01, *** p<0.001 vs naive.

observed were similar to those achieved by topical administration of the Biphasix™-pgD/100µg patch at 6 weeks (i.e. about 200 ng/mL IgG by ELISA).

Further characterization of intradermal and gene gun mediated plasmid delivery indicated the absence of gD-specific IgG2a antibodies (data not shown). The cellular immune responses elicited by the gene gun were characterized by a Th2 type response in the spleen (Figure 3.2B) and mixed Th1/Th2 in the lymph nodes (Figure 3.2C) In addition, gene gun immunization with 1 µg pgD still elicited a predominant Th2 response (data not shown). In contrast, the cellular responses elicited by intradermal injection were predominantly Th1 in both spleen (Figure 3.2B) and lymph nodes (data not shown).

Although there was no difference between the gD-specific antibody isotypes induced following immunization by a number of delivery methods, the cytokine profile generated varied depending on the type of delivery method used. Therefore, the route of delivery influences the type of cellular immune response induced. Intradermal injection of pgD elicited a strong Th1 response, where as topical and gene gun administration elicited a predominant Th2. Antigen produced in the lymph node may result in increased antigen presentation by APCs compared to antigen produced in non-lymphoid tissue. These differences in antigen presentation may explain the differences in the immune responses elicited by the epidermal and intradermal routes.

Although the doses of pgD used in these immunizations were quite different, depending on the method of delivery, the total amount of plasmid was identical between the gene gun and intradermal immunizations suggesting that the differences in the type

of immune responses generated was not due to the amount of immunostimulatory molecules present in the plasmid.

The biphasic vehicles facilitate the penetration through the stratum corneum into the viable epidermis as well as hair follicles. Previous studies using liposomes suggest that hair follicles are important for transfection (Fan *et al*., 1999; Niemiec *et al*., 1997). However, it is not known which pathway(s) results in transfection from topical application of plasmid formulated in biphasic vehicles. From the three formulations tested in this study, formulation#3 containing PC/DOPE lipid combination, showed somewhat higher efficiency of transfection and immune response. This may indicate either a better skin interaction with vesicles containing unsaturated lipid or a better transfection ability with APCs, hence the expression in the lymph node.

3.3.2 Evaluation of luciferase expression after topical delivery of pluc

Plasmid delivery into the skin was evaluated by measuring the expression of luciferase activity 48 hours following topical, gene gun or intradermal administration. There was no increase in levels of luminescence in the skin after topical treatment with Biphasix™-pluc/100µg patch (formulation#3) (Figure 3.3A). Even when examined without the stratum corneum present (removed by tape stripping), expression of luciferase was not found in the skin following topical application of pluc (Figure 3.3A). However, there was an increase in luminescence in the lymph node homogenates after topical treatment on either intact or stripped skin. Topically applied naked pluc did not elicit any luciferase activity on normal or tape stripped skin (data not shown).

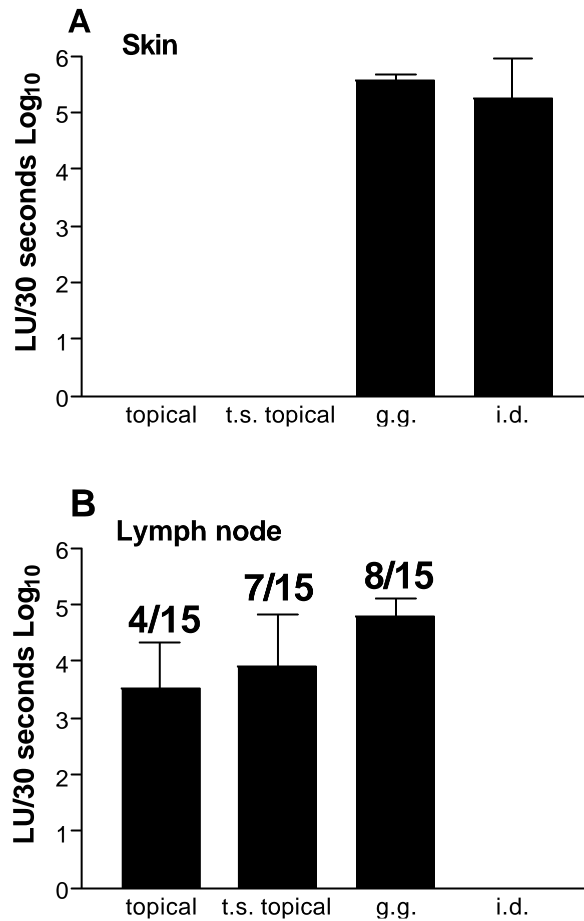

Figure 3.3 Gene expression following different delivery methods.

Luciferase activity was determined in the skin (A) and in the draining lymph nodes (B) 48 hours after topical administration of 100 μg of pluc formulated in lipid-based delivery system on normal and tape stripped (t.s.) skin, 0.05 μg of pluc administered by the gene gun (g.g.) or 1.5 μg pluc injected intradermally (i.d.). Luciferase activity was expressed in light units (LU) per 30 seconds. Numbers over bars represent the number of positive responses detected. Error bars represent standard deviation of the mean of 5-15 mice per group, * $p<0.05$ vs non-treated skin.

Intradermal injection (1.5µg) resulted in luciferase expression only in the skin, whereas administration of pluc with the gene gun (0.05µg/dose) resulted in gene expression in both skin and lymph nodes (Figure 3.3A and B).

3.4 Conclusions

Immunization by topical application of vaccine antigens is a new concept that has a great potential. Non-invasive strategies for vaccine administration onto the skin have recently been reported for protein (Glenn *et al*., 1998) and for plasmid DNA vaccines (Fan *et al*., 1999, Shi *et al*., 1999). In this study the use of biphasic lipid vesicles (Biphasix^{TM}) were assessed to enhance the delivery of plasmid DNA through the skin and subsequently the induction of immune responses to the encoded gene. Our results demonstrate that incorporating the plasmid in biphasic lipid vesicles enhances the immune responses induced following topical application. Furthermore, we extended previous studies by demonstrating that after topical administration, plasmid expression can be achieved in the draining lymph nodes. This observation may be important to our understanding of how such low doses of antigen, produced by DNA immunization, can elicit immune responses.

Collectively, this study demonstrated the feasibility of using biphasic vesicles to enhance topical delivery of plasmid DNA into the skin. Furthermore, these results also suggest that the type of immune responses induced by various methods of immunization into the skin may be influenced by the location of gene expression. It is unknown why different modes of application result in different types of immune responses, however,

dendritic cells may influence the type of immune response elicited (Rissoan *et al*., 1999). Our results indicated that gene expression in the draining lymph nodes likely results from directly transfected dendritic cells, which corresponded with induction of a Th2 response with the gene gun and topical administration. The ability to induce different types of immune responses is significant because different diseases require different types of immunity to provide protection (Sjolander *et al*., 1998). This information is relevant to the development of non-invasive vaccine delivery strategies.

4.0 NEEDLE-FREE TOPICAL ELECTROPORATION IMPROVES GENE EXPRESSION FROM PLASMIDS ADMINISTERED IN PORCINE SKIN

(Manuscript submitted)

4.1 Introduction

Vaccination is the most cost-effective way to prevent disease. However, there are still many diseases for which no vaccine exists or for which the currently available vaccines are inadequate. DNA immunization, which entails the administration of DNA encoding an antigen, may offer solutions in at least some of these cases. DNA vaccines use host cells as bioreactors for the production of proteins *in vivo* (Tang *et al*., 1992). By doing so, DNA vaccination mimics viral infection, with improved antigen presentation to the immune system relative to standard protein vaccines, and work more effectively at inducing cellular immunity as a result (Ulmer *et al*., 1993). Moreover, it offers these potential benefits without the safety and stability concerns associated with the administration of infectious agents.

DNA immunization has been effective in several mouse models (Donnelly *et al*., 1997). To achieve significant levels of immunity in humans and large animals with DNA delivery methods often requires very high doses of plasmids and multiple boosts (van Drunen Littel-van den Hurk *et al*., 2000). Clearly, there is a need to increase the potency of DNA vaccines in large mammals including humans (Gerdts *et al*., 1999). Even at high doses, DNA vaccines in humans still have failed to be efficacious (Le *et al*., 2000; MacGregor *et al*., 1998; Ugen *et al*., 1998; Wang *et al*., 1998). Therefore,

more potent forms of the DNA vaccines themselves and/or more effective means of delivery must be developed in order for the technology to realize its potential.

One reason for the inefficacy of DNA vaccines in humans could be inefficient cellular uptake of plasmid DNA and consequent poor gene expression. *In vivo* electroporation may allow increased gene expression by enhancing cellular uptake of plasmids by the application of short electrical pulses that transiently permeabilize cell membranes. Electroporation may enhance nuclear uptake as well. It has been clearly shown that DNA delivery to muscle tissue of mice, followed by electroporation, strongly increases gene expression and immunogenicity of DNA vaccines (Widera *et al*., 2000; Zucchelli *et al*., 2000). Furthermore, when used for DNA delivery in the skin, electroporation amplifies gene expression with both intradermal injection (Heller *et al*., 2001) and topical administration (Dujardin *et al*., 2001) of plasmids. Thus, it would follow that electroporation of skin should enhance DNA immunizations, and experimental results have confirmed that prediction in mice (Drabick *et al*., 2001).

One objective of this study was to explore whether the use of needle-free surface electroporation would significantly enhance gene expression and immune responses to a DNA vaccine in pigs. Another objective was to minimize the invasiveness of the immunization procedure. For this reason, needle-free injection of the DNA, using a BioJect™ device was compared with conventional injection by needle and syringe. An additional incentive for using fluid jet injection over needle and syringe was the previous findings that jet injection was more effective for DNA immunization in monkeys and sheep (Haensler *et al*., 1999; Jenkins *et al*., 1995) compared to intramuscular needle administration of plasmid. Both the fluid jet and needle methods

of DNA injection were assessed with respect to their ability to induce an immune response with and without needle-free surface electroporation.

Numerous studies have shown that the most powerful property of DNA vaccines may be their ability to prime the immune system for responses to other vaccines (Richmond *et al*., 1998; Robinson *et al*., 1999). The effects of electroporation were examined in the context of a DNA-prime/DNA-boost/protein-boost strategy to enhance immune responses in large animals, and compared the responses with the responses following standard protein vaccination. This strategy resembled DNA-prime/protein-boost strategies previously used in non-human primates, which yielded outstanding results for both malaria (Jones *et al*., 2001) and HIV vaccinations (Amara *et al*., 2001).

4.2 Materials and methods

4.2.1 Plasmids

The luciferase encoding plasmid (pluc), under the control of the CMV promoter in the pMAS backbone (Krieg *et al*., 1998b), was a gift from Dr. Heather Davis (University of Ottawa, ON) (Weeratna *et al*., 1998). The plasmid encoding green fluorescent protein (GFP) under the control of the CMV promoter was obtained through Quantum Biotechnologies (Montreal, QC). The hepatitis B surface Ag expression plasmid (pHBsAg), under the control of the human elongation factor 1a promoter with the first intron and the polyadenylation signal from human G-CSF cDNA in a pUC9 prokaryotic backbone, was previously described (Widera *et al*., 2000), and was custom-manufactured by Elim (San Francisco, CA). The other plasmids were purified using

Qiagen Endo Free plasmid kits (Qiagen, Mississauga, ON) according to the manufacturer's instructions.

4.2.2 Animals and Immunization

Four to six week old male and female outbred pigs weighing 20 to 40 pounds were purchased from the Prairie Swine Center (University of Saskatchewan, Saskatoon, SK). Animals were housed and treated the in compliance with the Canadian Council for Animal Care. Pigs were randomly assigned to six groups of five animals each. They were anesthetized with halothane prior to DNA injection and electroporation. Group 1 received 250 µg pHBsAg in 100 µl 0.1 M phosphate buffered saline (PBS) by a dermal BioJect B 2000 needle-free injection device (Bioject, Inc. Portland, OR) at each of two sites/animal on the abdomen, for a total of 500 µg pHBsAg. Group 2 was identical to group 1 except both sites were also treated with 70 V electroporation using a flat patch needle-free surface electrode, immediately following plasmid injection. Group 3 received 250 µg pHBsAg in 100 µl PBS by an intradermal injection at each of two sites/animal on the abdomen for a total of 500 µg pHBsAg. Group 4 was identical to group 3 except sites were treated with a 60 V electroporation, immediately following plasmid administration. Group 5 received 500 µl of the commercial hepatitis B protein subunit vaccine (Engerix-B, SmithKline Beecham Pharma, Oakville, ON) injected intradermally with the Bioject device in two 250 µl doses an the abdomen and Group 6 was immunized with Engerix-B by an intramuscular injection using a needle. Pigs were boosted with the same injection conditions after four weeks. All treatment groups were boosted at week eight with Engerix B vaccine by intramuscular needle injection for all

groups except group 5, which was injected intradermally with Engerix with the BioJect device as above (Table 4.1).

4.2.3 Electroporation

Electroporation was performed using the BTX ECM 830 Pulse Generator with the needle-free micropatch round electrode mounted on a handle (model MP 35) (Genetronics, San Diego, CA), and applying six square-wave pulses at 60, 70, or 80 V, respectively, with pulse duration of 60 msec, pulse interval of 200 msec, and reversal of polarity after three pulses.

4.2.4 Luciferase expression and assay

Plasmid DNA encoding luciferase was injected intradermally with the use of a 26-gauge needle or a BioJect device (Dermal BioJect^{TM}, BioJect, Inc., Portland, OR), followed by electroporation with various voltages. Intradermal needle injection was tested with no electroporation and with electroporation at voltages of 60V and 80V. Bioject delivery was tested with no electroporation and with electroporation at voltages of 60V, 70V, and 80V.

For each injection, a dose of 100 µg of pluc (Weeratna *et al.*, 1998) in 100 µl PBS was administered into the shaved abdomen skin of the animals. The luciferase-encoding plasmid was injected at eight standard sites, and electroporation was applied to four of those sites. Forty-eight hours after the administration of the plasmid, each injection site was sampled with an 8-mm diameter punch biopsy at a depth of approximately 8-mm.

Table 4.1 Experimental design for vaccine groups

	1st and 2nd immunizations[2]			3rd immunization[3] (Engerix-B)
Group[1]	Vaccine	Route/Method	Voltage (V)	Route/Method
1	pHBsAg	i.d./BioJect	-	i.m./needle
2	pHBsAg	i.d./BioJect	70 V	i.m./needle
3	pHBsAg	i.d./needle	-	i.m./needle
4	pHBsAg	i.d./needle	60 V	i.m./needle
5	Engerix-B	i.d./BioJect	-	i.d./BioJect
6	Engerix-B	i.m./needle	-	i.m./needle

[1]Each group consisted of five animals.
[2]First and second immunizations were administered on day zero and four weeks later.
[3]The third immunization was given eight weeks after the first immunization
i.d., intradermal; i.m., intramuscular.

Skin was homogenized in 500 µl lysis buffer (Promega, Madison, WI) with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) to produce protein extracts. Luciferase activity in the protein extracts was determined using a luciferase assay system (Promega). On a Packard Picolite Luminometer (Packard Instruments Canada LTD, Mississauga, ON), the bioluminescence of each 500 µl sample was counted for 30 seconds and recorded as relative light units (LUs). Untreated or PBS-injected tissues were used to determine the background luminescence levels.

4.2.5 Histological examination of skin

Forty-eight hours following intradermal injection by needle or BioJect of 100 µl PBS alone or 100 µg pluc in 100 µl PBS followed by electroporation with 60, 70, or 80 V, skin tissues were taken and fixed in 10% formalin. Tissues were processed routinely and embedded in paraffin wax, and 4-µm sections were stained with hematoxylin/eosin (HE).

4.2.6 GFP gene expression

To analyze the localization of gene expression, 100 µg of a plasmid encoding green fluorescent protein (GFP) in 100 µl PBS was administered by intradermal injection or Biojector, in combination with electroporation (60 V for intradermal injection and 70 V for Biojector administration). Twenty-four hours after administration the injection site was biopsied using an 8-mm punch. Skin samples were frozen in liquid nitrogen and stored at -70°C until they were sectioned. Skin samples were cut transversally with an IEC Minitome Microtome Cryostat (Damon, Needham, MS) into

7-µm sections. Sections were examined for GFP expressing cells and were photographed with an Olympus AH2-RFL microscope using blue fluorescent light.

4.2.7 Measurement of humoral responses

At various time points after immunization, serum was obtained by centrifugation of the blood collected from anesthetized animals. To measure if animals would have immune responses that would be considered to protect against the disease. Anti-HBsAg antibodies were quantitatively measured using the AUSAB™ EIA Clinical Diagnostic Kit, and quantification in milli-International Units/ml was performed in parallel with the AUSAB™ Quantification Panel, according to manufacturer instructions (Abbott Laboratories, North Chicago, IL). In humans, an antibody level >10 mIU/ml is considered to be protective for Hepatis B (West and Calandra, 1996).

Anti-hepatitis B IgG_1 and IgG_2 isotypes were also identified by ELISA as follows. Immunlon 2 ELISA plates (DYNEX, Chantilly, VA) were coated with HBsAg (BioDesign International, Saco, ME) (1 µg/ml in 20 mM Na_2CO_3) overnight at 4°C. Plates were washed with phosphate-buffered saline-Tween (PBST) (PBS, 0.05% Tween 20; Sigma Chemical Co., St. Louis, MO). Serum was diluted in diluent (PBST, 0.5% gelatin) (Sigma) 20-fold, followed by serial 4-fold dilutions, and incubated overnight at 4°C. Plates were washed six times in PBST. Porcine IgG_1 and IgG_2 isotypes were detected using specific mouse anti-porcine antibodies (Serotec, Hornby, ON). Following incubation at room temperature for one hour, plates were washed six times in PBST. Anti-mouse IgG_1 biotinylated antibodies (Caltag, Toronto, ON), diluted in diluent, were added and incubated for one hour at room temperature. Plates were

washed six times in PBST, and streptavidin-alkaline phosphatase (Jackson Immuno-Research Labs, West Grove, PA) was added to the plates and incubated for one hour. The alkaline phosphatase activity was measured by the conversion of *p*-nitrophenol phosphate (PNPP) (Sigma). The absorbance was read after 15 to 20 minutes at 405-nm wavelength on a plate reader (Bio-Rad, Hercules, CA).

4.2.8 Statistics

Differences between groups were analyzed using Prism graphpad statistical software (GraphPad Software, Inc.) using a one-way ANOVA, followed by Tukey's multiple comparison test.

4.3 Results

4.3.1 Gene expression and effect of electroporation on the skin.

To characterize gene expression of plasmid DNA delivered by intradermal needle or needle-free BioJect delivery, luciferase and GFP reporter genes were used. First, it was determined where the injected materials were localized in the skin by using Evans blue dye. Both the needle and the needle-free methods of injection delivered most of the dye to the dermis. Needle-free injection also deposited dye in the epidermis and the hypodermis, and thus, resulted in a broader distribution than that obtained by standard intradermal needle injection (data not shown).

To quantify the level of gene expression, the luciferase reporter gene was used. The luciferase assay results indicated that delivery by BioJect was significantly better than needle injections, with respect to the level of gene expression detected (Figure 4.1).

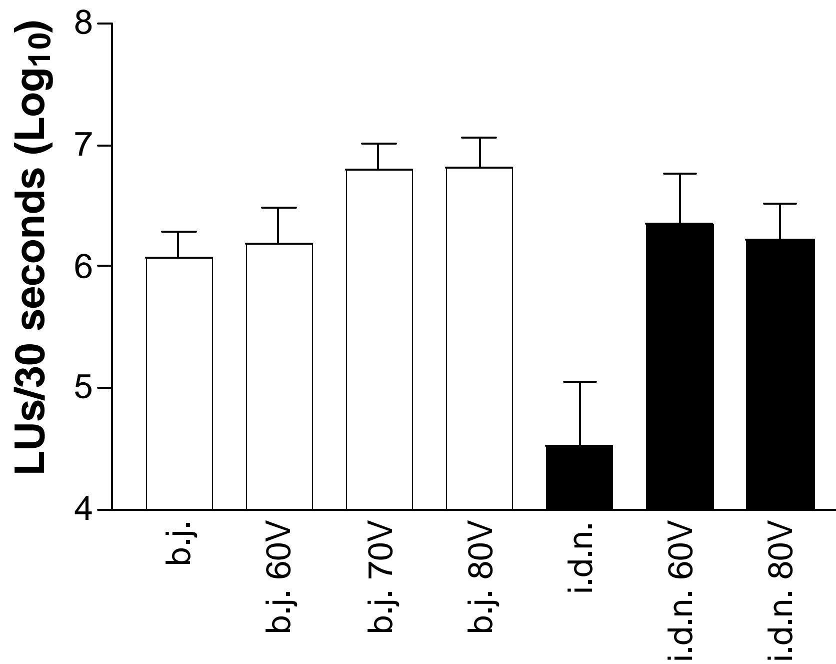

Figure 4.1 Electroporation improves gene expression levels, and intradermal jet injection is more effective than intradermal needle injection.

Luciferase encoding plasmid DNA was administered to abdominal porcine skin, and enzymatic activity was measured 48 hours following administration. Gene expression levels are shown for intradermal BioJect (b.j.) and needle (i.d.n.) injections, without or with electroporation, at the voltages indicated. Error bars represent SEM. Statistical differences were i.d.n vs b.j. ($p<0.05$) and i.d.n. vs i.d.n plus electroporation ($p<0.05$) by one-way ANOVA followed by Tukey's multiple comparison test.

Electroporation at 60V or 80V significantly enhanced the level of expression following intradermal needle injection (Figure 4.1). Similarly BioJect delivery of plasmids followed by electroporation enhanced gene expression following a 70V or 80V pulse, but did not following electroporation at 60V.

Examination of the location of gene expression using a plasmid encoding GFP supported the results of luciferase expression. With BioJect showing greater expression than intradermal needle injection and electroporation enhancing gene expression following both BioJect and intradermal needle injection (data not shown).

Following electroporation, there was superficial damage on the stratum corneum that increased with higher voltages. This superficial damage to the stratum corneum was transient and no longer detectable one week following electroporation. This observation supports the contention that topical electroporation is relatively safe and did not cause long-term scaring.

Histological examination of tissue sections was performed to determine how voltage could influence gene expression. The results of histological examination (Figure 2) showed that increasing the voltage increased the size of the lesion on the surface of the skin, as well as the increasing the level of cellular infiltration consisting of macrophages in the dermis and epidermis of the skin. The voltages chosen for the DNA immunizations were voltages that enhanced gene expression with the minimal amount of tissue damage. Those voltages were 60V with the intradermal needle injections and 70V with the BioJect deliveries.

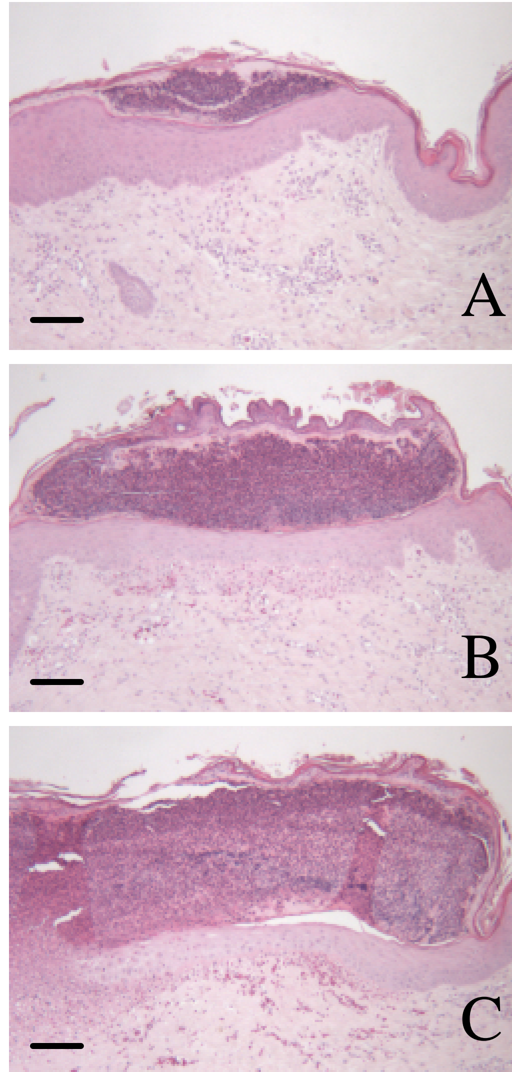

Figure 4.2 Electroporation causes tissue damage and inflammation in pig skin.

Skin tissues were collected 48 hours following electroporation at 60V (A), 70V (B) and 80V (C), (HE stain). Infiltration of neutrophils into the stratum corneum, forming small intracorneal pustules, was found (A). Moderate infiltration of neutrophils into the epidermis was found, forming superficial epidermal pustules (B). Severe epidermal pustule formation and as well subjacent dermal infiltration predominantly of neutrophils were observed (C). Bar is 100 µm.

4.3.2 Immune Responses in Immunized Pigs

Four weeks after the primary immunization, no animals in any of the groups, had any detectable immune responses to HBsAg. At 8 weeks following a second protein immunization, the two groups of animals that were immunized with a subunit vaccine injected either intramuscularly using a needle and syringe (the recommended route) or intradermally with the BioJect responded with antibody levels considered protective by the AUSAB (Table 4.2). Results indicated that the intramuscular injection of the conventional vaccine resulted in all animals 5/5 responding, whereas only 4/5 animals responded to intradermal injection of the conventional vaccine using the BioJect (Table 4.2). At 8 weeks, following a secondary plasmid vaccination, no animals immunized by intradermal injection with or without electroporation had any responding animals as detected by the AUSAB assay. Animals immunized with plasmid administered using the BioJect or BioJect with electroporation had 1/5 and 2/5 animals responding respectively with antibody levels considered protective by the AUSAB.

To assess the efficacy of DNA vaccination in priming the immune system, animals in all experimental groups were boosted with the HBsAg protein vaccine at week 8. Results in Table 4.2 demonstrate that even though immune responses were undetectable in most of the animals after two rounds of DNA immunization with respect to the AUSAB clinical assay, most animals responded rapidly to the protein boost with all immunized groups significantly different than the prebleed. The fact that nearly all DNA immunized animals responded within two weeks of the protein boost demonstrates an anamnestic response, since the protein vaccine alone did not induce detectable immune responses 4 weeks post immunization (Figure 4.3). There were no statistical

Table 4.2 Responses in Pigs to Various Hepatitis B Vaccination Protocols

	Number of Animals Responding[2]					
Vaccine Group[1]	**Week 4**		**Week 8**		**Week 10**	
	AUSAB	ELISA	AUSAB	ELISA	AUSAB	ELISA
(1) DNA, b.j.	0/5	0/5	1/5	1/5	4/5	3/5
(2) DNA, b.j. + EP	0/5	0/5	2/5	5/5	5/5	5/5
(3) DNA, i.d.n.	0/5	0/5	0/5	0/5	5/5	3/5
(4) DNA, i.d.n. + EP	0/5	0/5	0/5	1/5	5/5	5/5
(5) Engerix B, b.j.	0/5	0/5	4/5	4/5	5/5	5/5
(6) Engerix B, i.m.n.	0/5	0/5	5/5	5/5	4/4	4/4

[1]Hepatitis B vaccines were administered intradermally or intramuscularly, as indicated. Sera collected at four, eight, and ten weeks after the first immunization were analyzed by conventional ELISA or by using an AUSAB™ diagnostic kit.
[2]The number of animals that showed an immune response out of the total number of animals in a group is indicated. To be considered positive animals needed to have an antibody titer of at least 10 I.U. and an optical density of 2 SD over background for the AUSAB and ELISA respectively.
Groups 1-4 received plasmid at day 0 and week 4 and received the HBsAg protein vaccine after the week 8 bleed.
Groups 5 and 6 received the HBsAg protein vaccine at day 0, week 4 and week 8 administered by BioJect or intramuscular needle respectively.
b.j., BioJect; EP, electroporation; i.d.n. intradermal needle; i.m.n. intramuscular needle.

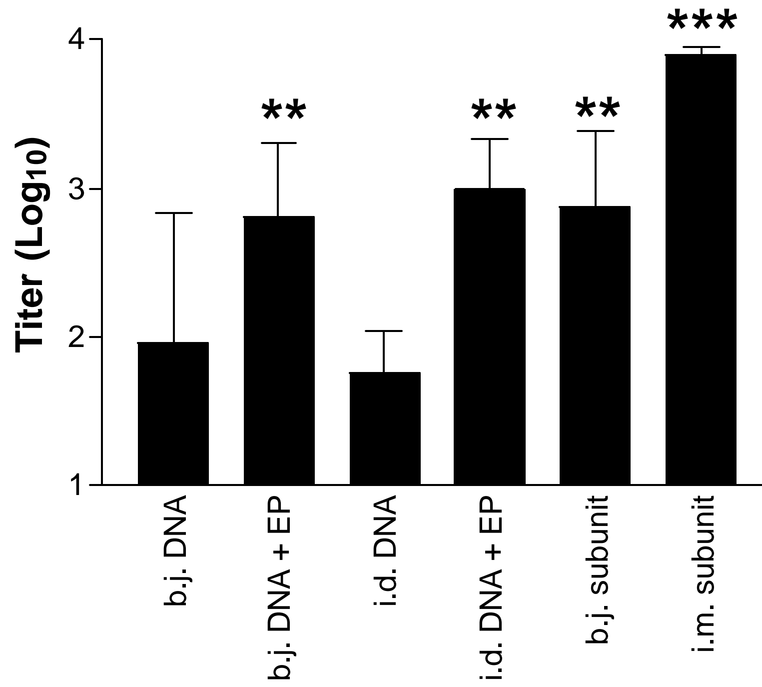

Figure 4.3 Immune responses to Hepatitis B after the different DNA prime/protein boost immunizations.

HBsAg antibody titers at 10 weeks (two weeks after the protein boost) were determined using the AUSAB^{TM} EIA, and are the geometric mean of 5 animals for all the groups except for the i.m. subunit group which had 4 animals. Error bars are SEM. Statistical differences were determined using a one-way ANOVA followed by Tukey's multiple comparison test **p<0.01, ***p<0.001 vs prebleed.

differences between the immunized groups at 10 weeks when immune responses were determined by the AUSAB clinical assay. However, only DNA vaccines administered with electroporation and the HBsAg protein vaccines were significantly different compared to prebleed serum.

To determine whether the experimental manipulations had an impact on the antibody isotypes generated, the sera were analyzed for the presence of anti-HBsAg IgG_1 and IgG_2 using an ELISA. Needle-free plasmid injection in combination with electroporation lead to a more rapid induction of immune responses compared to other methods of plasmid delivery (Figure 4.4). Figure 4.4 demonstrates that in those animals mounting an early response, most produced primarily IgG_1 at 8 weeks. However, after boosting with protein, a much more balanced response with approximately equivalent levels of IgG_1 and IgG_2 were evident. There were no significant differences between the groups at week 10. Although, the groups injected with DNA and treated with electroporation (2 and 4) produced antibodies similar in titer to those in the cohorts receiving multiple protein injections (5 and 6). In contrast, those groups that received DNA without electroporation (1 and 3) produced antibodies of lower titer, even after the protein boost.

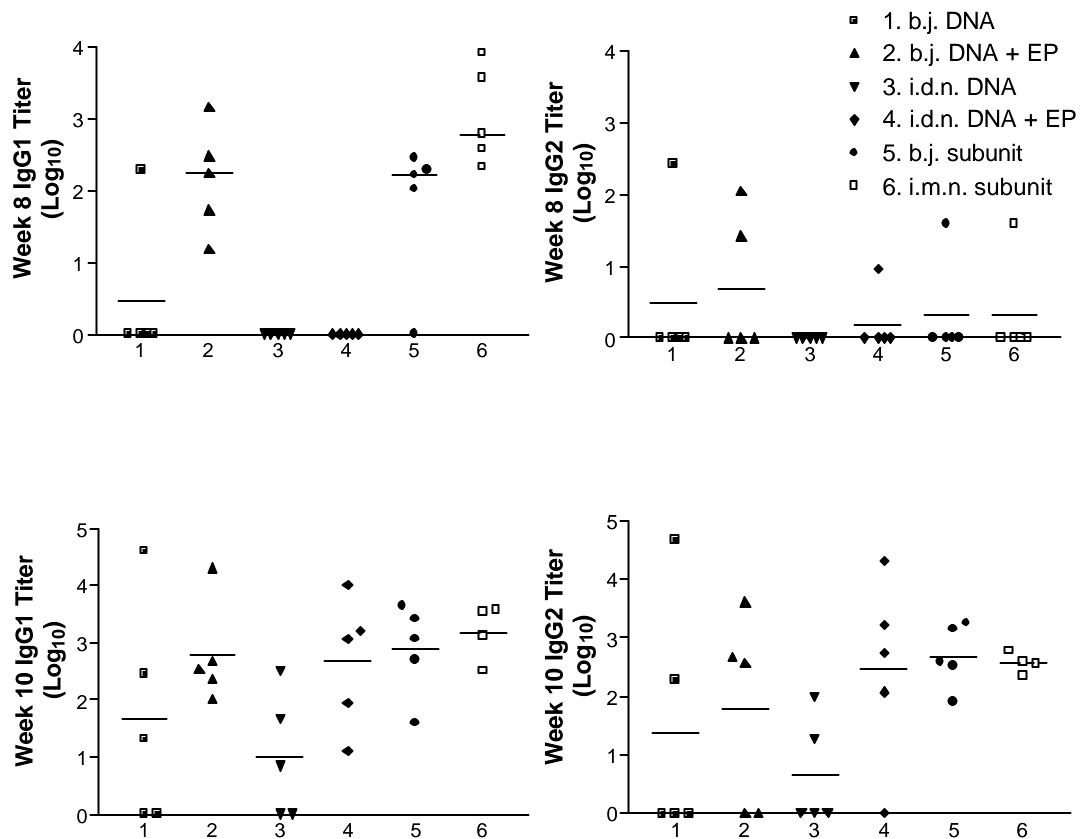

Figure 4.4 Antibody isotypes responses elicited by Hepatitis B immunizations.

Hepatitis B specific IgG1 and IgG2 titers at 8 and 10 weeks, data represent individual animals at 8 and 10 weeks and the bar is the geometric mean. Group 1 is BioJect DNA, Group 2 is BioJect DNA + EP, Group 3 intradermal injection DNA, Group 4 intradermal injection DNA + EP, Group 5 is BioJect administration of the subunit vaccine, and Group 6 is intramuscular injection of the subunit vaccine. At week 8 prior to boosting with protein the level of antibody (IgG1) in the BioJect DNA plus electroporation group vs i.d.n DNA plus electroporation ($p<0.001$) was statistically different by one-way ANOVA followed by Tukey's multiple comparison test. There was no statistical difference between the level of antibody (IgG1) in the BioJect DNA + electroporation and the Engerix-B groups at eight weeks by one-way ANOVA followed by Tukey's multiple comparison test. There was high variablity of immune responses elicited following immunization with DNA vaccines.

4.4 Discussion

There is a critical need to improve the potency of DNA vaccines if DNA vaccines are to be used routinely for vaccination. Recently, electroporation was shown to enhance the potency of DNA vaccines in mice and rabbits (Widera *et al*., 2000) as well as pigs (Babiuk *et al*., 2002). However, these intramuscular electroporations using 2- and 6-needle arrays were relatively invasive. Using a unique needle-free surface patch electrode, a significant enhancement of gene expression following intradermal plasmid administration was demonstrated. Furthermore, to eliminate the use of needles in the vaccine administration, a needle-free intradermal injection device combined with non-invasive electroporation was tested and it was found to be more effective than intradermal needle injection. It was shown that a totally needle-free delivery-electroporation system was the most effective way to administer DNA vaccines in the skin.

Luciferase gene expression experiments revealed that needle-free BioJect administration resulted in higher levels of gene expression than needle injection. This observation is in agreement with Sawamura, who showed that jet injections elicit higher gene expression levels than needle injections in rat and human skin (Sawamura *et al*., 1999). One possible reason for this result may be that administration with BioJect more efficiently distributes plasmid in the tissue, as determined by gross visualizations of injection patterns with Evans blue dye and GFP expression. This corroborates recent research finding that larger fluid injection volumes elicit enhanced gene expression in muscle tissues (Dupuis *et al*., 2000). Another possible reason for greater gene expression following delivery by the BioJect device could be the transient disruption of

membranes by the jet streams. Such disruption would increase the ability of the plasmid DNA to enter the cells and resemble the role of electroporation, which permeabilizes the cell membranes through the formation of pores as a result of applying high-intensity electrical fields. This would explain why electroporation enhanced needle injection to a greater extent than jet injection. However, any cellular DNA uptake resulting from membrane disruption by the jets appear to be much less significant than the permeability increase offered by electroporation, because substantial gains were still seen from coupling electroporation with jet injection, relative to the use of BioJect alone.

Topical electroporation sets up an electrical field that is greatest at the surface of the skin and diminishes in strength with increased distance from the electrodes. Although electroporation enhances gene expression with both needle injection and BioJect administration, the electroporation voltage needed was higher in the case of the jet injection. The difference in depth of plasmid distribution in the skin by the two delivery methods may explain this observation. The BioJect device delivered liquid more deeply into the skin than the purposely shallow intradermal needle injection. This deeper delivery may require a greater electroporation voltage because a sufficient electrical field is required to permeabilize cells in deeper layers of skin. However, electroporation at higher voltages causes greater tissue damage, which can reduce gene expression, especially in the epidermis, seen in the GFP studies (data not shown). Increased gene expression in the dermis offsets this effect. These conclusions concur with those of Glasspool-Malone *et al*, who used invasive electrodes and showed that electroporation of skin changes the distribution of gene expression and concentrates it in the dermis (Glasspool-Malone *et al*., 2000).

Although the mechanisms by which electroporation enhances immunogenicity of DNA vaccines are not yet completely understood, the increase in antigen production associated with electroporation is likely important. Studies in mice showed that reduction of antigen expression decreased immune responses (Weeratna *et al.*, 1998), and increases in antigen expression enhanced immune responses (zur Megede *et al.*, 2000). The results here support these suggestions since electroporation enhanced both gene expression and immune responses. Increasing the effectiveness of a DNA vaccine requires raising the levels of antigen production and/or increasing antigen uptake by APCs to heighten the immune response. Electroporation may serve to enhance the immune response not only by increasing antigen expression, but also by other effects on the electroporated tissue. The tissue damage caused by electroporation may trigger the release of cytokines that attract APCs to the exact site where increased levels of antigen are being produced. This explanation would resemble the mechanism of agents such as bupivacaine that damage muscle and thereby enhances the immune response when it is delivered prior to DNA immunization (Davis *et al.*, 1995). Thus, the minor damage caused by electroporation could be advantageous for eliciting immune responses.

The determination of antibody titers by both AUSAB™ and conventional ELISA generally yielded similar results for the various animal groups. However, there were discrepancies for individual animals. These individual differences are explainable by the different parameters measured by each assay. AUSAB™, in effect, measures levels of antibodies by sandwiching them between antigen and other antibodies. In contrast, the conventional ELISA less selectively measures levels of antibodies that bind at a certain affinity/avidity. Therefore, the observed discrepancy is expected.

The needle-free means of injection in combination with electroporation was more effective than needle injection at improving the kinetics of the immune response to DNA priming and boosting. These results are consistent with our luciferase assay results, which indicate that electroporation enhances gene expression, and needle-free delivery is more effective than needle delivery of plasmid DNA. Both delivery methods, each with electroporation, were about equally effective at producing an immune response after the protein boost. The conventional intramuscular route of administration of the HBsAg subunit vaccine was previously found to be superior to the intradermal route of administration (Henderson *et al.*, 2000). Antibody titers at ten weeks support that result, but also offer the perspective that needle-free intradermal delivery may be comparable to conventional intramuscular delivery, in terms of its elicitation of an immune response. That result is quite promising given the much less invasive nature of intradermal BioJect delivery.

In a previous experiment, when 1 mg of the identical HBsAg encoding plasmid was administered in muscle using a needle. Only 2/6 pigs responded by the AUSAB assay after two immunizations (data not shown), therefore there are no significant differences between DNA immunization with HBsAg plasmid in the skin and the muscle. However, electroporation with the 6-needle array in muscle resulted in all 6 pigs responding to the HBsAg DNA vaccine by the AUSAB assay. The likely reason why electroporation in muscle was more effective than needle-free electroporation in the skin was that electroporation in muscle resulted in a 10 fold greater level of gene expression compared BioJect in combination with needle-free electroporation as measured by luciferase.

In summary, these experiments demonstrate that the needle-free method of intradermal DNA-vaccine delivery offers advantages, including increased efficacy of gene expression and safety, over injection with needle and syringe. In addition, electroporation is quick and simple to apply, and is tolerated well by animals. These results are very encouraging for the development of needle-free vaccination methods, and the development of DNA vaccines.

5.0 ELECTROPORATION IMPROVES THE EFFICACY OF DNA VACCINES IN LARGE ANIMALS

(Manuscript in press)

5.1 Introduction

The vaccination of individuals with plasmid-based vaccines continues to attract attention as a novel approach to inducing immunity to a wide variety of infectious agents including viruses, bacteria and parasites [reviewed in (van Drunen Littel-van den Hurk *et al*., 2000), (Davis *et al*., 1996; Hoffman *et al*., 1994; Ulmer *et al*., 1998)]. The main attraction of this approach is that the immune response generated by DNA immunization closely resembles those induced by natural infection (Ulmer *et al*., 1993), due to the endogenous production of viral proteins and glycoproteins. This intracellular synthesis is especially important for inducing cell-mediated immunity (CMI) as well as antibody responses. Cell-mediated immunity by cytotoxic lymphocytes is often critical for helping clearance of the pathogen (Lehmann-Grube *et al*., 1993), which generally does not occur following immunization with killed, or sub-unit protein vaccines, which induce primarily a humoral immune response. Secondly, DNA vaccination is considered to be safer than using live attenuated vaccines, however, there are still perceived risks associated with DNA vaccines. More importantly does not require a cold chain to maintain the efficacy of the vaccine. This latter consideration is critical in developing countries where a cold chain is both expensive and often difficult to maintain.

However, for DNA vaccination to become routine, specific improvements are required to induce immune responses in humans and large animals. Thus, even with plasmid constructs that are very effective in inducing immune responses in mice, transferring these same plasmids to humans or large animals has generally resulted in disappointing results (MacGregor *et al*., 1998; Turnes *et al*., 1999; Ugen *et al*., 1998). In these human and large animal experiments, the responses are often extremely variable and require large and multiple doses of plasmid to develop detectable responses. DNA vaccination of humans and large animals is, however, often good at priming the immune response (Robinson *et al*., 1999). Thus, there is an increasing interest in using DNA vaccination for priming immune responses followed by boosting with proteins (Jones *et al*., 2001; Richmond *et al*., 1998). Although this is proving to be effective, having two different vaccine production and delivery systems is expensive and cumbersome to administer and monitor. Thus, if it were possible to enhance the efficacy of DNA vaccination it would benefit both the patients and the vaccine companies. Even if a prime boost regime were still required, having effective priming would be tremendously beneficial in vaccine development and delivery.

Although the reasons for reduced efficacy of DNA vaccination in humans and large animals are unknown, one factor is probably the amount of protein produced *in vivo*. One way of increasing protein expression is by using plasmids with strong promoters (van Drunen Littel-van den *et al*., 1998), or by reconstructing genes to improve codon biases (Kotsopoulou *et al*., 2000). However, even these modifications to the plasmid are not sufficient to induce effective levels of immunity in humans or large animals, possibly due to the fact that only a small percentage of the DNA enters and is

functional in cells. Thus, if it were possible to increase the level of transfection it might be possible to improve the efficacy of DNA vaccines. To address this issue electroporation was tested in parallel with intramuscular DNA delivery.

Electroporation has not only been shown to improve transfection efficiency *in vitro,* but recent studies have also shown enhanced gene expression in muscle (Widera *et al*., 2000; Zucchelli *et al*., 2000) and in skin (Glasspool-Malone *et al*., 2000; Heller *et al*., 2001). To further investigate the efficacy of *in vivo* electroporation, some cohorts used plasmids encoding two completely different antigens – the bovine herpesvirus glycoprotein gene gD – a membrane protein, and a plasmid expressing hepatitis B surface antigen (HBsAg) which assembles into a 22 nanometer particle. This allowed us to examine immune responses to membrane bound and particulate antigens in a single animal. Since these plasmids were delivered simultaneously into the same muscle, it also allowed the investigating of the compatibility of incorporating multiple plasmids into a single vaccine. This is a major consideration for protecting individuals against multiple diseases by a single vaccination. This approach would not only reduce costs of vaccination, but also improve compliance.

The pig was chosen as a model for both humans and large animals since it is significantly more difficult to induce immune responses in pigs (a large animal) than in mice (Turnes *et al*., 1999) and there appear to be many similarities in immune responses of pigs and humans. Thus, the outbred pig is an excellent model for testing efficacy of vaccines that may then be transferred to the outbred human population.

5.2 Materials and methods

5.2.1 Plasmids

The luciferase encoding plasmid (pluc) with the luciferase coding sequence under the control of the CMV promoter in the pMAS backbone was a gift from Dr. Heather Davis (University of Ottawa, ON) (Krieg *et al*., 1998b). The hepatitis B surface Ag expression plasmid (pHBsAg), with the HBsAg coding sequence under the control of the human elongation factor 1a promoter with the first intron and the polyadenylation signal from human G-CSF cDNA in a pUC9 backbone was previously described (Widera *et al*., 2000). Plasmid pgD expresses glycoprotein D (gD) of bovine herpes virus 1 (BHV-1). To generate this plasmid, the vector pCAN1 (Uwiera *et al*., 2001) was digested with *Bgl*II and the 1254 bp *Bgl*II fragment of pSLRV.Agd (Lewis *et al*., 1999) was inserted. Plasmids were purified using Qiagen Endo Free plasmid kits (Qiagen, Mississauga, Ont.) according to the manufacturers instructions.

5.2.2 Animals and immunization

Four to six week old male and female outbred pigs weighing 9-18 kg were purchased from the Prairie Swine Center (University of Saskatchewan, Saskatoon, SK). Animals were housed and treated in compliance with regulations of the Canadian Council for Animal Care. Animals were assembled randomly into six groups.

Pigs were anesthetized with halothane and shaved on the quadriceps of both hind legs, the site of vaccination. The experimental design is displayed in Table 5.1.

Table 5.1 Study Design: Immunization of Pigs With DNA and Protein Vaccines

	1st [a] and 2nd [b] immunizations			3rd [c] immunization (Engerix-B) + (tgD/VSA)
	Route/Method	Voltage	Vaccine	Route/method
Group 1	i.m./needle	-	pHBsAg	i.m./needle
Group 2	i.m./needle	-	pHBsAg + pgD	i.m./needle
Group 3	i.m./single-needle electrode	150 V	pHBsAg	i.m./needle
Group 4	i.m./single-needle electrode	150 V	pHBsAg + pgD	i.m./needle
Group 5	i.m./6-needle electrode	200 V	pHBsAg	i.m./needle
Group 6	i.m./6-needle electrode	200 V	pHBsAg + pgD	i.m./needle

[a] 1st immunization consisting of plasmid (pHBsAg only in groups 1, 3 and 5) or (pHBsAg + pgD in groups 2, 4, and 6) was administered at day 0.
[b] 2nd immunization consisting of plasmid (pHBsAg only in groups 1, 3 and 5) or (pHBsAg + pgD in groups 2, 4, and 6) was administered at four weeks.
[c] 3rd immunization consisting of (Engerix-B) + (tgD/VSA) was administered at six weeks to all the groups.

Groups were as follows: Group 1 received 1000 µg pHBsAg in 1000 µl 0.1 M phosphate buffered saline (PBS), delivered by two 500 µl intramuscular injections, one in each quadriceps muscle. Group 2 was co-immunized with 1000 µg of pHBsAg in 1000 µl PBS and 1000 µg pgD in 1000 µl PBS delivered by two adjacent 500 µl intramuscular injections into each quadriceps muscle. Group 3 received 1000 µg pHBsAg in 1000 µl PBS by intramuscular injections with the single-needle electroporation device into each quadriceps muscle as described above. Group 4 was co-immunized with 1000 µg of pHBsAg in 1000 µl PBS and 1000 µg pgD in 1000 µl PBS with the single-needle electroporation device into each quadriceps muscle as described above. Group 5 received 1000 µg pHBsAg in 1000 µl PBS by intramuscular injections into each quadriceps muscle, followed by electroporation with the six-needle electrode as described above. Group 6 was treated the same as group 5, except pigs were co-immunized with pHBsAg and pgD. Pigs were boosted in an identical fashion at four weeks. The third immunization at week six was delivered to all groups by intramuscular injection into the quadriceps muscles of both hind limbs, using the Engerix-B sub-unit vaccine (SmithKline Beecham Pharma, Oakville, Ont.), as well as 20µg tgD in 500 µl VIDO stimulatory adjuvant (VSA) (van Drunen Littel-van den Hurk *et al*., 1994) in two separate injections.

5.2.3 Electroporation and electrodes

Electroporation was performed using the BTX ECM 830 Pulse Generator with a 23 gauge hypodermic needle (insertion depth: 1 cm) placed in a novel single-needle electrode holder, or with the six-needle electrode (insertion depth: 1 cm) with auto-

switcher device (Genetronics, San Diego, CA.). The single-needle electrode uses the single needle as one arm of the electrode, which is in the center of the circular needle holder containing the second arm of the electrode, consisting of a 1 cm diameter ring that is placed on the surface of the skin. This single-needle electroporation set up results in a cone shaped electrical field. The six-needle electrode consists of six sharp needles (1 cm long) arranged equidistantly around a circle of 1 cm diameter, forming a regular hexagon resulting in a cylindrical shaped electrical field. Electroporation with the single needle electrode was performed with four pulses of 150 V and 20 msec duration each, at 5 Hz, and polarity was not switched.

The six-needle electroporation was performed with six pulses of 200 V and 20 msec duration each, at 5 Hz. The six pulses were delivered by firing two parallel needle pairs each, resulting in electrical fields rotating in a clockwise direction generated with the aid of an auto-switcher box allowing rotation of the electric field between opposing pairs of needles (Hofmann *et al*., 1999a). The polarity was reversed after each pulse using the auto-switcher box (Nanda *et al*., 1998).

The voltages selected were based on previous studies, which demonstrated a low level of tissue damage 24 hours after electroporation, but did indicate delivery of electricity by slight twitching of the muscle during electrical pulsing.

5.2.4 Luciferase assay

To determine the level of gene expression, a plasmid encoding the luciferase gene (pluc) (Weeratna *et al*., 1998) was used. A dose of 100 µg of luciferase encoding plasmid in 500 µl was administered by intramuscular injection into the quadriceps at five similar sites per pig in three additional pigs. Groups were as follows, no

electroporation, single-needle electroporation and six-needle electroporation, using the same electroporation conditions used for the immunizations. Twenty-four hours after administration of plasmid, the muscle tissue surrounding the injection site was sampled using an 8 mm biopsy punch. Muscle samples were homogenized and the luciferase activity was determined using Promega's (Madison, WI) luciferase assay system. Samples were counted for 30 seconds. Untreated muscle from each pig was used to measure the background level of light units (LU).

5.2.5 Measurement of antibody responses

At various time points blood was collected and serum was obtained following centrifugation. Anti-hepatitis B surface Abs were measured using the AUSAB™ EIA Diagnostic Kit, and quantification in milli-international Units/ml was done in parallel with the AUSAB™ Quantification Panel following instructions provided by the manufacturer (Abbott Laboratories, Abbott Park, IL).

Anti-hepatitis B IgG1 and IgG2 isotypes were determined by ELISA as follows. Immunlon 2 (DYNEX, Chantilly, VA) ELISA plates were coated with HBsAg (BioDesign International, Saco, Maine) (1 µg/ml) in 20 mM Na_2CO_3 overnight at 4°C. Plates were washed with phosphate buffered saline-Tween (PBST)(PBS, 0.05% Tween 20, Sigma, St. Louis, MO). Serum was diluted 1/20 in diluent (PBST, 0.5% gelatin, Sigma) followed by serial 4 fold dilutions and incubated overnight at 4°C. Plates were washed 6X in PBST and porcine IgG1 or IgG2 isotypes were determined using mouse anti-porcine IgG1 or mouse anti porcine IgG2 antibodies (Serotec, Hornby, ON), diluted in diluent and incubated at room temperature for 1 hour. Plates were washed 6X in PBST and anti-mouse IgG1 biotinylated antibodies (Caltag, Toronto, ON) diluted in

diluent were added, followed by a 1 hour incubation. Plates were washed 6X in PBST, streptavidin-alkaline phosphatase (Jackson Immuno-research Labs, West Grove, PA) was added, and plates were incubated for 1 hour. The alkaline phosphatase activity was determined using *p*-nitrophenyl phosphate (PNPP) (Sigma). The absorbance was read at 405 nm with a spectrophotometer (Bio-Rad, Hercules, CA) after 15-20 minutes.

The anti-gD IgG1 and IgG2 isotypes were determined by ELISA as above, except plates were coated with 1 µg/ml tgD.

Titers of anti-BHV-1 neutralizing antibody in sera were determined as previously described (van Drunen Littel-van den Hurk *et al.*, 1990). The titers were expressed as the highest dilution of serum that caused a 50% reduction in viral plaques compared to the virus control.

5.2.6 Measurement of cellular responses

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, Uppsala, 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, Burlington, ON).

Proliferation of gradient-purified cells were measured as previously described (Tikoo *et al.*, 1995). Briefly, 2.5 X 10^5 PBMCs in RPMI media (Gibco) were plated per well in 96 well plates either with no antigen, full length gD protein (1 µg/ml), or with

5μg/ml phytohemagglutinin (PHA) (Sigma) as a positive control. All assays were performed in quadruplicate. Cells were incubated at 37°C in 5% CO_2 /air for 4 days and then pulse-labeled with 0.4 μCi/well methyl [H^3] thymidine (Amersham Biosciences, Baie d'Urfe, QC) for 8 hours. Cells were harvested from wells using a semiautomatic cell harvester (Skatetron Inc, Sunnyvale, CA.) and ß emissions were measured as counts per minute (cpm) using a LS 1701 scintillation counter (Beckman Coulter, Mississauga, ON). Stimulation indexes (SI) represent mean cpm (stimulated cells)/ mean cpm (non-stimulated cells). The PHA stimulated control cells all had SIs greater than 100, indicating a high viability of cells.

To determine the number of gD-specific INF-? secreting cells, a porcine INF-? ELISPOT assay was performed as described (Baca-Estrada *et al*., 1996) with the following changes: $1x10^6$ cells/well were placed in 96 well culture plates with and without antigen (gD 1 μg/ml) in RPMI medium (Gibco) and incubated at 37°C and 5% CO_2 for 24 hours. Cells were resuspended in fresh medium and $5x10^5$ cells were seeded on nitrocellulose plates (Millipore, Napean, ON) coated with mouse monoclonal anti-porcine IFN-γ capture antibody, 5 μg/ml (BioSource International, Camarillo, CA.). Plates were washed in cold water followed by PBST. Rabbit polyclonal anti-porcine IFN-γ specific antibodies (BioSource International) at 5 μg/ml in diluent (PBST, 0.5% albumin) (Sigma) were added to the plates and incubated for 2 hours at room temperature. Plates were washed, biotinylated rat anti-rabbit IgG (Zymed, San Francisco, CA.) at 5 μg/ml in diluent was added, and the plates were incubated at room temperature for 2 hours. Plates were washed and streptavidin-alkaline phosphatase (Jackson Immuno-Research Labs) was added. Plates were developed using 5-bromo-4-

chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate tablets (Sigma). Spots representing gD specific cytokine secreting cells were counted and expressed as number of cytokine secreting cells per $5x10^5$ cells. PHA stimulated cells were used as a positive control, with greater than 20 cytokine secreting cells per well.

5.2.7 Histological examination of muscle tissue

Twenty-four hours after injection of 100 µg pMAS-luc in 500 µl PBS followed by no electroporation, single-needle electroporation or six-needle electroporation, muscle biopsies were taken and fixed in 10% formalin. Tissues were embedded in paraffin and 4 µm sections were stained with hematoxylin/eosin (HE).

5.2.8 Statistics

Differences between groups were analyzed using Prism graphpad statistical software (GraphPad Soffware, Inc.) using a one-way ANOVA followed by Tukey's multiple comparison test.

5.3 Results

5.3.1 Gene expression

A luciferase reporter gene was used to compare the amount of gene expression induced from plasmid administered without electroporation, and with single-needle and six-needle electroporation treatment. Figure 1 clearly shows that electroporation with the single-needle electrode enhances gene expression 100 times over no electroporation and electroporation with the six-needle array enhances gene expression an order of magnitude above the single-needle electrode.

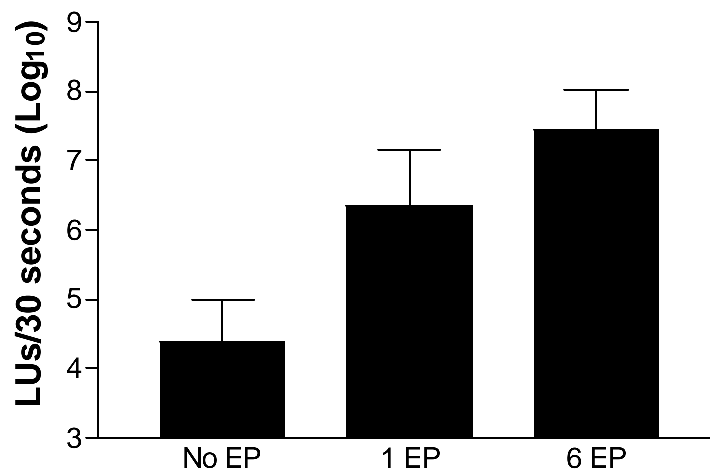

Figure 5.1 Increase in gene expression in the muscle by electroporation.

Porcine muscle was injected with 100 µg of pluc in 500 µl PBS at 5 different sites in the quadriceps muscle, followed by no electroporation, electroporation with a single-needle electrode (1 EP), or a six-needle array electrode (6 EP). Luciferase activity in the excised tissue was measured 24 hrs following administration of plasmid. No EP Vs 6 EP $p<0.05$ by one-way ANOVA followed by Tukey's multiple comparison test. There was no statistical difference between 1 EP Vs 6 EP. Error bars represent SEM.

5.3.2 Immune responses

No animals had detectable antibody responses to HBsAg at four weeks after the primary immunization. This was expected since vaccination with the commercial vaccine Engerix-B does not elicit antibody responses in pigs four weeks after primary immunization (data from previous chapter). However, by six weeks, immune responses to HBsAg elicited by a plasmid encoding the particular antigen (HBsAg) were evident if animals were treated with electroporation. Thus there was very little evidence of an immune response at six weeks post-vaccination in the absence of electroporation but excellent immune responses were detected in animals immunized with the six-needle electrode (Figure 5.2A). Indeed, animals immunized with a six-needle electrode had immune responses two orders of magnitude higher than those animals not experiencing electroporation and one order of magnitude higher than those immunized with a single-needle electrode. What was even more encouraging was that the number of animals responding increased following electroporation. Six weeks after immunization, all animals responded in the six-needle electroporated group vs. 66% in the group that received single-needle electroporation, and only 33% in the non-electroporated group. The immune response differences were maintained even after boosting with the HBsAg protein vaccine (Figure 5.2B). The six-needle electrode treated group had a ten-fold higher immune response when compared to the single-needle electrode group even after protein boost. The anti-HBsAg IgG1 and IgG2 response was also compared at 6 and 8 weeks. At 6 weeks, animals immunized with DNA without electroporation and the single-needle electroporated group were predominantly IgG1 whereas animals immunized with the six-needle electrode gave a more balanced response with both IgG1

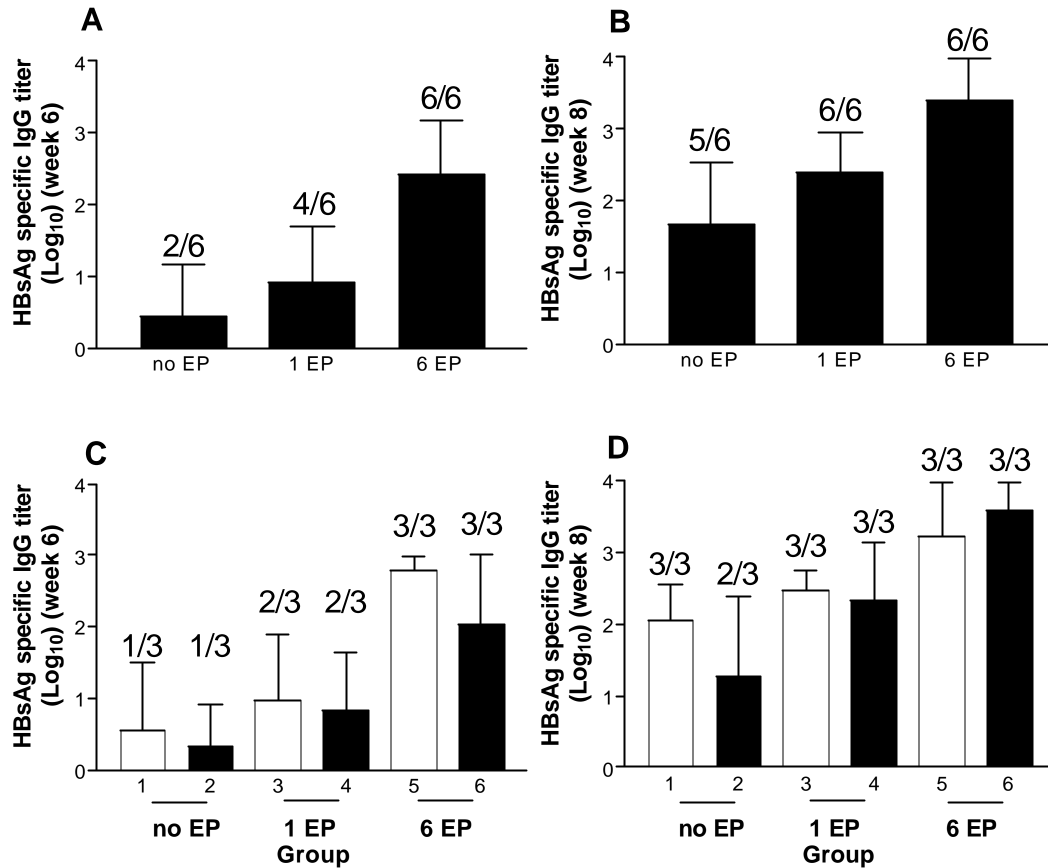

Figure 5.2 Effect of electroporation on anti-HBsAg titers in pigs at six and eight weeks after immunization.

Anti-HBsAg titers were determined using the Abbott kit. A: Anti-HBsAg titers are shown at six weeks after immunization with pHBsAg at 0 and 4 weeks with no electroporation (no EP), electroporation with a single-needle electrode (1 EP) or a six-needle electrode (6 EP), no EP Vs 6 EP $p<0.01$ and 1 EP Vs 6 EP $p<0.01$ by one-way ANOVA followed by Tukey's multiple comparison test. B: Anti-HBsAg titers at week 8 after HBsAg subunit boost at week 6, no EP Vs 6 EP $p<0.01$ by one-way ANOVA followed by Tukey's multiple comparison test. C and D: Data from Graphs A and B broken down into animals receiving pHBsAg only or pHBsAg and pgD. Groups 1 and 2 were immunized with no electroporation, Groups 3 and 4 were immunized with electroporation using the single-needle electrode and Groups 5 and 6 were immunized with electroporation using the six-needle electrode (see Table 1). For C and D: White bars represent pigs immunized only with pHBsAg and black bars are pigs co-immunized with pHBsAg and pgD. Numbers above the bars indicate the number of animals seroconverting per group; error bars represent SD.

and IgG2 isotypes (data not shown). At 8 weeks all groups had a more balanced response with both IgG1 and IgG2 isotypes present (data not shown).

To determine whether similar responses as to HBsAg could be generated to a membrane protein antigen, a plasmid encoding a bovine herpes virus glycoprotein (gD) was used. Figure 5.3 demonstrates that at 6 weeks after primary immunization with a gD encoding plasmid, followed by a boost at 4 weeks all animals exposed to electroporation developed more robust immune responses in both IgG1 and IgG2 isotypes compared to DNA immunized animals without electroporation and that the six-needle electrode was more effective than the single needle electrode. This response was further increased following a boost with purified recombinant protein (Figure 5.4). Furthermore the response in animals primed with a DNA vaccine was significantly higher than that in animals immunized with a protein alone, indicating that the DNA vaccine could effectively prime the immune response of pigs to a truncated (t)gD protein vaccine. The fact that DNA immunization induced neutralizing antibodies against bovine herpesvirus-1 (Figure 5.3C) confirms that the glycoprotein produced *in vivo* possessed the correct conformational epitopes, since gD conformation is critical for induction of neutralizing antibodies (van Drunen Littel-van den Hurk *et al.*, 1990).

Although there have been no detailed studies comparing murine and porcine isotypes reported, isotype analysis was performed in animals immunized in the presence or absence of electroporation to determine if electroporation could influence the balance of antibody responses (Figure 5.4). Immunization with gD protein alone produced a

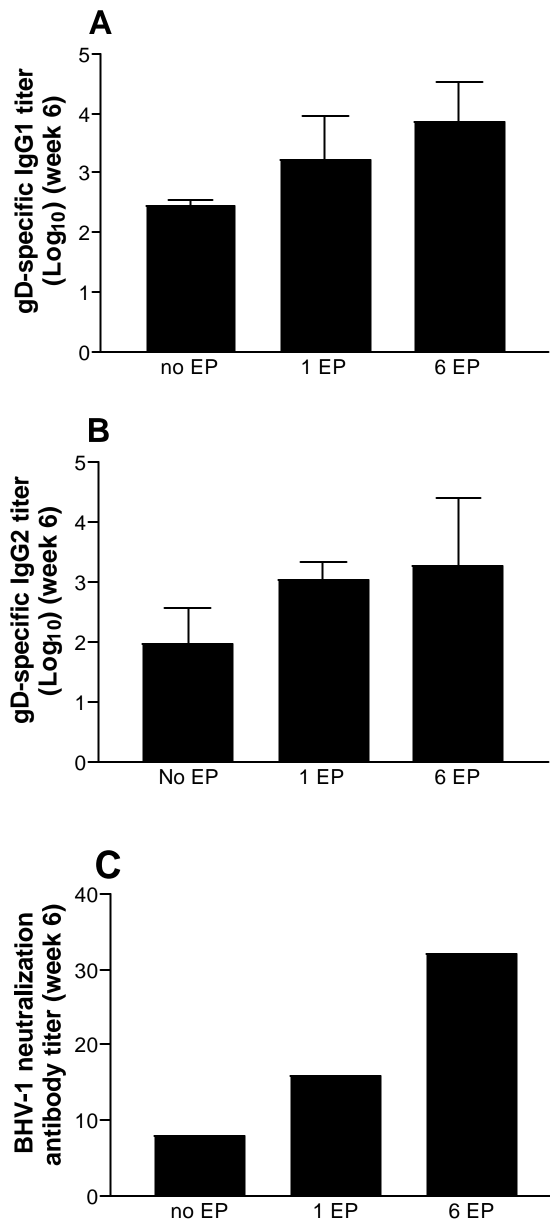

Figure 5.3 Increase in gD-specific IgG1 and IgG2 by electroporation.

Pigs were immunized with pgD and pHBsAg at 0 and 4 weeks with no electroporation (no EP), electroporation with a single-needle electrode (1 EP) or electroporation with a six-needle electrode (6 EP). Anti-gD titers were determined at 6 weeks using ELISA for IgG1 (A) and IgG2 (B). BHV-1 neutralizing antibodies from animals primed and boosted once at week 4 with pgD DNA (C). There are no significant differences beteen the groups in A and B. Error bars are not visible in C since all animals in each group had the same neutralization titer. Error bars represent SD and n = 3.

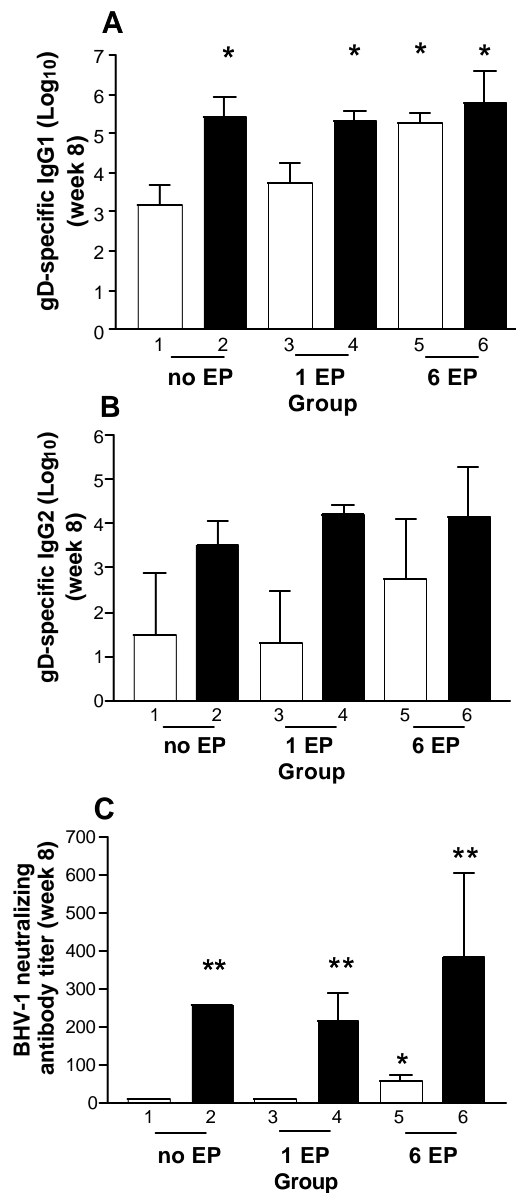

Figure 5.4 Effect of DNA prime and protein boost on gD-specific IgG1 and IgG2 titers.

Anti-gD IgG1 and IgG2 titers were determined by ELISA at week 8. Groups 1 and 2 were immunized with no electroporation, Groups 3 and 4 were immunized with electroporation using the single-needle electrode and Groups 5 and 6 were immunized with electroporation using the six-needle electrode (see Table 1). White bars represent groups not primed with pgD, while black bars are groups immunized twice with pgD (week 0 and 4) and then given a gD protein vaccine at 6 weeks *p<0.01 and **p<0.001 by one-way ANOVA followed by Tukey's multiple comparison test compared to group 1. Error bars represent SD and n = 3.

higher level of IgG1 (Figure 5.4), as expected for a soluble protein (Benne *et al*., 1997). However, immunization with a gD DNA vaccine produced a much more balanced response with higher levels of gD-specific IgG2 as compared to animals immunized first with a plasmid encoding HBsAg followed by gD protein immunization. This balanced response was maintained even after a boost with purified protein. It is of interest to note that if animals were first immunized with DNA and electroporated with a six-needle array and subsequently (two weeks later) immunized with glycoprotein at the same site, they developed high levels of gD specific IgG1 as well as IgG2 (Group 5).

To determine if Th1-like responses were obtained, it was investigated if lymphocytes from immunized pigs could produce interferon gamma – the true measure of Th1-like responses. Figure 5A demonstrates that DNA immunization stimulated significant numbers of interferon gamma secreting cells, whereas immunization with the glycoprotein generally did not. Thus, our studies support previous reports that DNA vaccination polarizes the response towards a Th1-like or balanced response (Martinez *et al*., 1997; Sjolander *et al*., 1998). It is of interest to note that electroporation with a six-needle array and subsequent (two weeks later) immunization with a purified glycoprotein in the same site resulted in, induction of high levels of interferon gamma secretion (Group 5). Indeed, these animals developed the same level of interferon-gamma secreting cells as animals first immunized with a gD-encoding DNA plasmid and then boosted with a glycoprotein (Figure 5.5A). Similarly, the lymphocytes from these animals had high levels of T-helper cell responses, as demonstrated by their ability to respond in a lympho-proliferative assay against purified gD glycoprotein *in vitro*

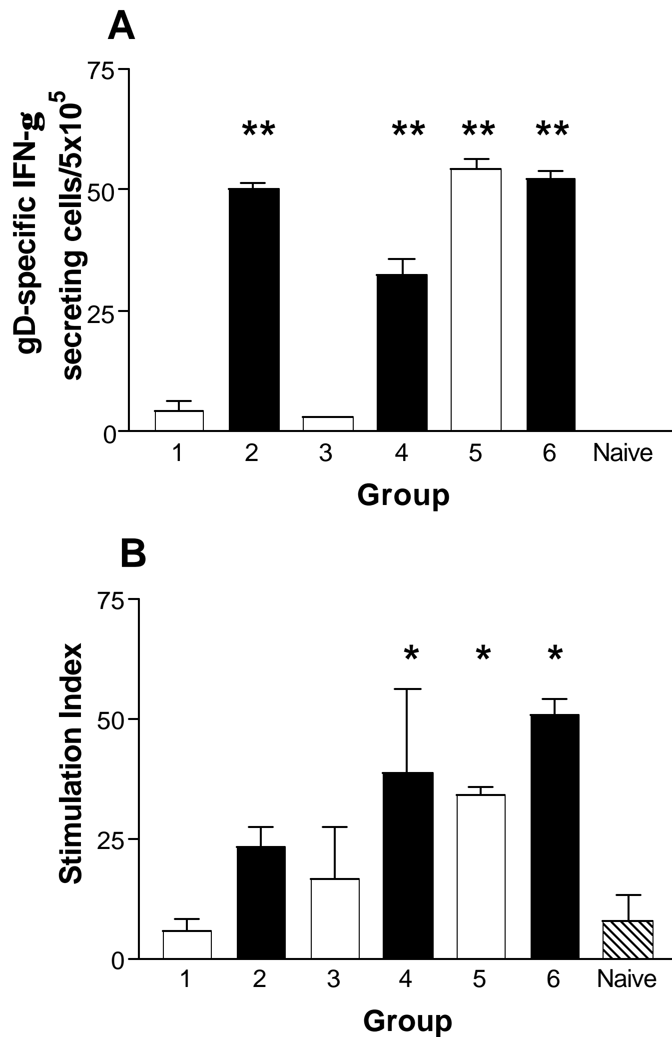

Figure 5.5 Cellular immune responses in gD immunized pigs.

The number of gD-specific IFN-? secreting lymphocytes was determined by ELISPOT (A) ** p<0.001 compared to groups 1 and 3 by one-way ANOVA followed by Tukey's multiple comparison test. Glycoprotein D-specific proliferative responses (B) were determined * p<0.01 compared to group 1. Groups 1 and 2 were immunized with no electroporation, Groups 3 and 4 were immunized with electroporation using the single-needle electrode and Groups 5 and 6 were immunized with electroporation using the six-needle electrode (Table 1). White bars represent groups not primed with pgD and black bars represent groups immunized twice with pgD. Naive pigs were age-matched controls housed with the experimental pigs. Error bars represent SD and n = 3.

(Figure 5.5B). The proliferation responses of animals exposed to the DNA vaccine without electroporation or to DNA immunization and electroporation with a single electrode followed by booster immunization with a glycoprotein further supported the suggestion that DNA vaccination primed the animal's immune response.

5.3.3 Plasmid compatibility

One of the major potential advantages of plasmid immunization is the compatibility of plasmids, allowing co-administration of vaccines directed against different pathogens. Incorporation of multiple antigens into a single vaccine should dramatically improve compliance and, thereby, vaccine coverage. Unfortunately, few studies have been done to date demonstrating plasmid compatibility in humans or large animals (Braun *et al*., 1998). To test this possibility, co-administration of plasmids encoding gD and HBsAg either with or without electroporation was evaluated. Figures 2C and 2D demonstrate that there was no significant interference in the level of antibody responses to HBsAg in either the number of responder animals or the magnitude of the immune responses following DNA administration alone (Figure 5.2C), or after a boost with protein (Figure 5.2D). Responses to tgD were also not altered when pgD was co-administered with pHBsAg since gD-specific antibody titers were detectable as early as two weeks following immunization in all animals (data not shown).

5.3.4 Histology of injection sites

To examine if electroporation would cause any tissue damage, histological examination of the electroporation site 24 hours after electroporation with the single-needle and six-needle electroporation was compared to no electroporation. There was an infiltration of neutrophils into the muscle as well as some minor muscle cell damage after electroporation with the six-needle array (Figure 5.6).

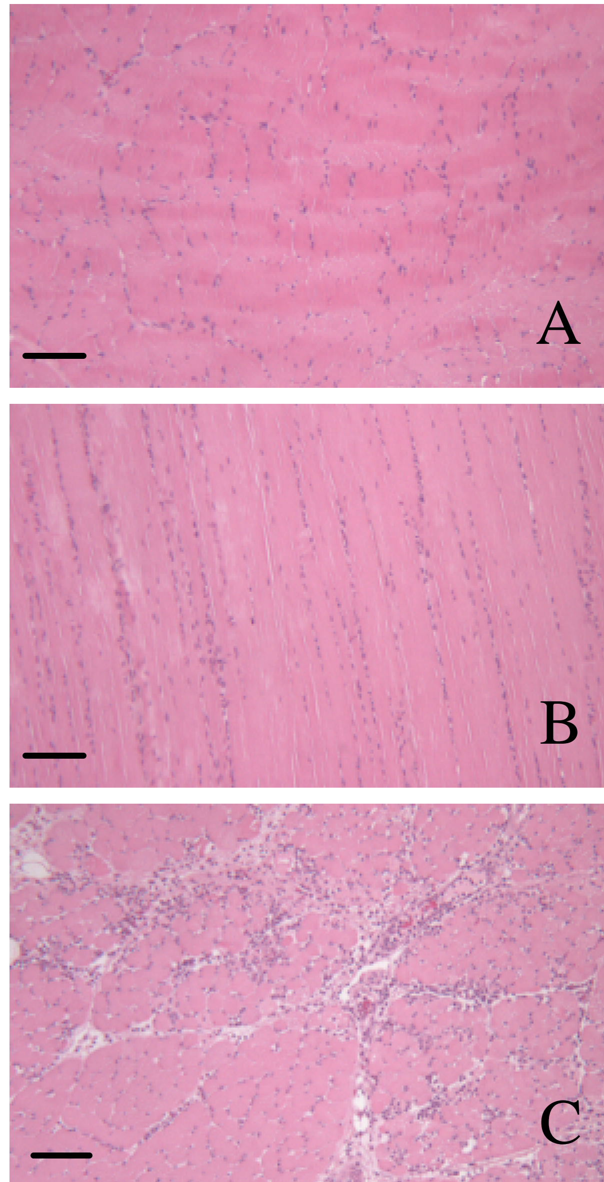

Figure 5.6 Effect of electroporation on muscle tissue.

Histological examination (HE stain) of muscle biopsies 24 hours after injection of plasmid (pMAS-luc) with no electroporation (A), single-needle electroporation (B) and six-needle electroporation (C). Mild infiltration of macrophages and neutrophils was observed in (A). Moderate infiltration was seen in (B) and severe infiltration was observed in (C). Bar is 100 µm.

5.4 Discussion

It was demonstrated that electroporation was effective in enhancing immune responses to two different antigens, the weakly immunogenic HBsAg, as well as the strongly immunogenic gD antigen. It is interesting that the kinetics of responses to these two antigens remains the same regardless whether administered by an electroporation enhanced DNA delivery approach or a protein subunit vaccine in an adjuvant: gD induces immune responses as early as two weeks after primary immunization and HBsAg induces immune responses only after a boost. In addition, it was shown that there was no interference with two different DNA vaccines. These results support the contention that injection of at least some combinations of multiple plasmids is possible without causing interference. However, different plasmid and antigen combinations must still be tested for compatibility on a case by case basis, because different antigen combinations may behave differently.

DNA immunization elicits IFN-? secreting cells a measure of Th1 immune responses whereas protein immunization generally do not induce IFN-? secreting cells. However, protein immunization with CpG in pigs was recently shown to promote Th1 IFN-? responses in pigs (Van der Stede *et al.*, 2002). These results are in agreement with this finding, however, it was also demonstrated that once the immune system is primed for a Th1 response, the Th1 response predominates Th2 promoting immunizations. In addition, electroporation prior to a gD protein immunization elicited a Th1 response indicating the possibility that electroporation may promote Th1 responses.

Inflammatory cytokines induced by electroporation as well as cellular infiltration may be possible mechanisms for the observed Th1 response.

Currently, it is assumed that the reason for the increased efficacy of DNA vaccines if delivered by electroporation is due to the increase in the transfection efficiency and concomitant increase in antigen expression *in vivo* (Widera *et al.*, 2000). It was shown that electroporation significantly enhances gene expression *in vivo* in large animals and that antigen expression correlated with the magnitude of elicited immune responses. Although the exact reason of observing increased gene expression as well as increased neutrophil infiltration and muscle damage with the 6-needle electrode compared to the single needle electrode is not currently understood, but is probable due mainly to differences in electrode configuration and firing patterns and less likely due to the different voltages used. It is not known whether electroporation favors expression of antigen in any specific cell population or whether all cells are equally affected. Furthermore, it is unclear whether the muscle cells in this case are damaged, thereby take up more plasmid and, therefore, release more antigen, or whether the damage results in enhanced inflammation in the vicinity of the plasmid, which creates a cytokine microenvironment more conducive to effective immune priming and expansion of immune cells. The suggestion that inflammation and cellular infiltration may play a role in enhancing the immune response was provided in animal group 5 as shown in Figures 4 and 5. DNA immunization with pHBsAg and electroporation of muscle tissue with the 6-needle array followed by administration of the tgD protein antigen two weeks later in the same location resulted in a very rapid response to tgD. Interestingly, this response was of similar magnitude as the one in pgD DNA primed animals, with or without

electroporation (animal groups 2 and 4). In contrast, the immune response after tgD administration in non-electroporated sites (animal group 1) and animals pretreated with the single-needle electrode (animal group 3) was weak. Histological examination of the electroporation site 24 hours after electroporation clearly demonstrated an infiltration of neutrophils into the muscle after electroporation with the six-needle array. This inflammatory response following electroporation with the 6-needle array lasts for at least two weeks (data not shown). The inflammatory responses as a result of electroporation are unlikely to cause long-term histological changes or pain, since systematic studies on electroporation effects on pig skin and muscle, albeit under different pulse conditions, did not show lasting side effects (Rabussay DP, 2002). Although pain elicitation on electroporation has been reported by a segment of patient population (Rodriguez-Cuevas S, 2001), experimental studies in animals examining the pain response to electroporation have not been investigated so far. Although previous studies have shown electroporation after protein delivery did not result in priming (Widera *et al*., 2000) this is not surprising since it is expected that the protein will have been cleared from the injection site by the time the peak of inflammatory response occurs following electroporation. It is possible these inflammatory responses also are important in enhancing immune responses to DNA vaccines, since antigen produced by plasmids would not be cleared from the site of inflammation as fast as conventional protein immunization. These issues are being addressed to determine the impact of gene expression and tissue damage on immune responses elicited by DNA immunization. These and additional studies will have to be performed before proceeding to clinical applications.

6.0 INCREASED GENE EXPRESSION AND INFLAMMATORY RESPONSES CAUSED BY ELECTROPORATION ARE BOTH IMPORTANT FOR IMPROVING THE EFFICACY OF DNA VACCINES

(Manuscript in preparation)

6.1 Introduction

DNA vaccines continue to generate considerable interest among researchers in the vaccine field as well as commercial companies for several reasons. DNA vaccines are 1) economical, 2) simple and 3) considered safe as compared to other vectors (Donnelly et al., 1997). While DNA vaccines are effective in mice, they are, however, less so in large animals and humans.

Although the reasons for reduced efficacy of DNA vaccination in humans and large animals are unknown, one potential factor is the low amount of protein produced *in vivo*. One way of increasing protein expression is by using plasmids with strong promoters (van Drunen Littel-van den Hurk et al., 1998); another is by reconstructing genes to improve codon biases (Kotsopoulou et al., 2000). Even these modifications to the plasmid, however, are not sufficient to induce effective levels of immunity in either humans or large animals, possibly due to the fact that only a low level of gene expression occurs from the small amount of intact plasmid entering cells. Electroporation, a delivery method which temporarily increases cell permeability, has been shown to increase the level of gene expression and significantly improve immune responses elicited to DNA vaccines in both small (Glasspool-Malone et al., 2000; Widera et al., 2000) and large animals (Babiuk et al., 2002).

Until recently, it was thought that the increase in gene expression caused by electroporation was the only mechanism responsible for increasing immune responses to DNA vaccines (Widera et al., 2000). Intramuscular administration of a protein vaccine in the same site previously treated with electroporation two weeks before, resulted in an enhancement of antigen-specific immune responses to the vaccine antigen, suggesting that muscle damage and ensuing inflammatory cell infiltration caused by electroporation may also be important mechanisms for enhancing immune responses to DNA vaccines (Babiuk et al., 2002).

In this study plasmids encoding two different antigens were used, namely the bovine herpesvirus type 1 glycoprotein gene gD, a membrane protein and highly immunogenic antigen, and a plasmid expressing hepatitis B surface antigen (HBsAg) which assembles into a 22 nanometer particle. This allowed a comparison of immune responses to membrane bound and particulate antigens in a single animal. Co-administration of vaccines is a major goal in the drive to protect individuals against multiple diseases by a single vaccination. This approach would not only reduce costs of vaccination, but also improve compliance if appropriate vaccines could be administered simultaneously in a single injection.

The pig was chosen as a model for both humans and large animals since there are many similarities between pigs and humans, such as they are both out bred populations and share common immune responses.

6.2 Materials and methods

6.2.1 Animals.

Four to six week old male and female out bred pigs weighing 9-18 kg were purchased from the Prairie Swine Centre Inc. (University of Saskatchewan, Saskatoon, SK). Animals were housed and treated in compliance with regulations of the Canadian Council for Animal Care.

6.2.2 Plasmids

The luciferase encoding plasmid (pluc) with the luciferase coding sequence under the control of the CMV promoter in the pMAS backbone was a gift from Dr. Heather Davis (University of Ottawa, ON) (Krieg et al., 1998). The hepatitis B surface Ag expression plasmid (pHBsAg), with the HBsAg coding sequence under the control of the human elongation factor 1a promoter, with the first intron and the polyadenylation signal from human G-CSF cDNA in a pUC9 backbone, was previously described (Widera et al., 2000). pgD expresses glycoprotein D (gD) of bovine herpes virus 1 (BHV-1). To generate this plasmid, the vector pCAN1 (Uwiera et al., 2001) was digested with *Bgl*II, and the 1254 bp *Bgl*II fragment of pSLRV.Agd (Lewis et al., 1999) was inserted. Plasmids were purified using Qiagen Endo Free plasmid kits (Qiagen, Mississauga, ON) according to the manufacturer's instructions.

6.2.3 Immunization

Animals were randomly assembled into six groups of six. Pigs were anesthetized with halothane and a square (3 cm) shaved over the quadriceps muscle (vastus lateralis) of the right hind leg to prepare the site for vaccination. The experimental design is

displayed in Table 6.1. All vaccine groups received a single intramuscular injection, consisting of 100 μg of pgD and 500 μg of pHBsAg in 500 μl of PBS, at that site. The dose of pgD used was lower than that of pHBsAg since in previous studies pgD elicited immune responses earlier than pHBsAg (Babiuk et al., 2002). Pigs were immunized on day 0 and received a second identical injection 4 weeks later contra-laterally (left leg) in the same muscle at the equivalent site. Vaccine treatments for groups were as follows: Group 1 received plasmid and no electroporation; Group 2 was treated with electroporation one hour prior to DNA immunization using the following parameters - electroporation with 6 rotating pulses of 20 msec at 200 V, with reversal of polarity between pulses; Group 3 was treated immediately following DNA immunization with 2 pulses of 60 msec at 100 V, without reversing the polarity; Group 4, immediately following DNA immunization, received 6 rotating pulses of 20 msec at 200 V, reversing the polarity between pulses; Group 5 was treated immediately following DNA immunization with 2 pulses of 60 msec at 200 V, without reversing the polarity. Naive pigs received no treatment.

Table 6.1 Experimental design: Vaccination parameters

Group	Electroporation (EP) conditions	Vaccine[a]	Number of Animals
1	No EP	100 µg pgD plus 500 µg pHBsAg	6 animals
2	200 V/20 msec/6 pulses 1 hour prior to DNA administration	100 µg pgD plus 500 µg pHBsAg	6 animals
3	100 V/60 msec/2 pulses	100 µg pgD plus 500 µg pHBsAg	6 animals
4	200 V/20 msec/6 pulses	100 µg pgD plus 500 µg pHBsAg	6 animals
5	200 V/60 msec/2 pulses	100 µg pgD plus 500 µg pHBsAg	6 animals
6	No treatment	No treatment	2 animals

[1] Plasmids were mixed together in 500 µl PBS and was administered in one intramuscular injection on days 0 and 28 on opposite sides.

6.2.4 Electroporation and electrodes

Electroporation was performed using the BTX ECM 830 Pulse Generator with a six-needle electrode (insertion depth: 1 cm) with an auto-switcher device (Genetronics, San Diego, CA.). The electrode consists of six sharp needles (1 cm long) arranged equidistantly around a circle of 1 cm diameter to form a regular hexagon, resulting in a cylindrical shaped electrical field.

For electroporation performed with six pulses, the pulses were delivered by firing two opposing parallel needle pairs around the injection site in a clockwise direction generated with the aid of an auto-switcher box allowing rotation of the electric field among opposing pairs of needles (Hofmann et al., 1999), and for reversal of the polarity after each pulse (Nanda et al., 1998). For electroporation parameters with 2 pulses, electrical charges were delivered by firing two opposing parallel needle pairs of the six-needle electrode without rotation of the field and without reversal of polarity, in effect using a four-needle array for pulse delivery.

The voltages were selected based on previous studies (Genetronics, unpublished data), which demonstrated differences in the level of tissue damage according to the voltage used for electroporation.

6.2.5 Luciferase assay

To determine the level of gene expression, a plasmid encoding the luciferase gene (pluc) (Weeratna et al., 1998) was used. Five additional pigs were used to determine luciferase gene expression following plasmid administration under the different electroporation parameters used for the vaccine trial (Table 6.1.) by

administering a dose of 100 μg of pluc in 500 μl by intramuscular injection into the quadriceps muscle on both hind legs at eight similar sites per pig for each parameter.

Forty-eight hours after administration of plasmid, the muscle tissue of the eight injection sites was sampled using an 8 mm biopsy punch. Muscle samples were homogenized in 1 ml of lysis buffer (Promega, Madison, WI) with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) to yield protein extracts. Luciferase activity of the protein extracts was determined using a luciferase assay system (Promega). Samples were counted for 30 seconds on a Packard Picolite Luminometer (Packard Instruments Canada LTD, Mississauga, ON). Untreated muscle samples were used to calculate the background levels of light units (LU).

6.2.6 Measurement of antibody responses

At various time points, blood was collected and serum was obtained following centrifugation. Anti-hepatitis B surface antibodies were measured using the AUSAB™ EIA Diagnostic Kit, and quantification in milli-international Units/ml was done in parallel with the AUSAB™ Quantification Panel, following the instructions provided by the manufacturer (Abbott Laboratories, Abbott Park, IL). In humans, the protective level is considered >10 mIU/ml (West and Calandra, 1996).

Titers of anti-BHV-1 neutralizing antibody in sera were determined as previously described (van Drunen Littel-van den Hurk et al., 1990). The titers were expressed as the highest dilution of serum that caused a 50% reduction in the number of viral plaques compared to the untreated virus control.

6.2.7 Measurement of cellular responses

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 900 g for 30 minutes. The buffy coat was removed and resuspended in PBS-0.1 M EDTA. The cells were overlayed on ficoll-paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 1400 g for 45 minutes. The PBMCs were removed, washed three times in PBS-EDTA and resuspended in RPMI medium (Gibco Life Technologies, Burlington, ON).

Proliferation of gradient-purified cells was measured as previously described (Tikoo et al., 1995). Briefly, 2.5 X 10^5 PBMCs in RPMI media (Gibco) per well were plated in 96 well plates either with no antigen, full length gD protein (1 µg/ml), or with 5 µg/ml phytohemagglutinin (PHA) (Sigma Chemical Co., St. Louis, MO) as a positive control. All assays were performed in quadruplicate. Cells were incubated at 37°C in 5% CO_2/air for 4 days and then pulse-labeled with 0.4 µCi/well of methyl [H^3] thymidine (Amersham Biosciences, Baie d'Urfe, QC) for 8 hours. Cells were harvested from wells using a semiautomatic cell harvester (Skatron Inc, Sunnyvale, CA.) and ß emissions were measured as counts per minute (cpm) using a LS 1701 scintillation counter (Beckman Coulter, Mississauga, ON). Stimulation index (SI) represent mean cpm (stimulated cells)/ mean cpm (non-stimulated cells). The PHA stimulated control cells all had SI greater than 100, indicating a high viability of cells.

To determine the number of gD-specific INF-? secreting cells, a porcine ELISPOT assay was performed as described (Baca-Estrada et al., 1996) with the following changes: 1x10^6 cells/well were placed in 96 well culture plates with and

without antigen (gD 1 µg/ml) in RPMI medium (Gibco) and incubated at 37°C and 5% CO_2 for 24 hours. Cells were resuspended in fresh medium and $5x10^5$ cells were seeded on nitrocellulose plates (Millipore, Napean, Ont.) coated with mouse monoclonal anti-porcine IFN-γ capture antibody, 5 µg/ml (BioSource International, Camarillo, CA.). Plates were washed in cold water followed by PBST. Rabbit polyclonal anti-porcine IFN-γ specific antibodies (BioSource International) at 5 µg/ml in diluent (PBST, 0.5% albumin) (Sigma) were added to the plates, which were incubated for 2 hours at room temperature. Plates were washed, biotinylated rat anti-rabbit IgG (Zymed, San Francisco, CA.) at 5 µg/ml in diluent was added, and the plates were incubated at room temperature for 2 hours. Plates were again washed, streptavidin-alkaline phosphatase (Jackson Immuno-Research Labs) was added, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate added (Sigma). Spots representing gD-specific cytokine secreting cells were counted and expressed as the number of cytokine secreting cells per $5x10^5$ cells. PHA stimulated cells were used as a positive control, with greater than 20 cytokine secreting cells per well.

6.2.8 Histological examination of muscle tissue

Muscle samples were obtained from all injection sites, using an 8 mm punch, immediately following euthanasia with an overdose of euthanol. Each of the muscle sites injected with pluc was sampled forty-eight hours post-injection for each of the plasmid administration conditions. From pigs immunized with gD and HBsAg DNA, the injection sites of both the primary immunization and the contra-lateral secondary immunization were sampled (at 6 and 2 weeks, respectively). Tissues were fixed in

10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Four µm sections were cut, and stained with hematoxylin and eosin (HE). With this routine stain, hematoxylin stains nuclei blue while cytoplasm/sarcoplasm is stained pink with eosin. Prior to analysis the identity of the slides were masked, so the treatment group of origin was unknown to the researcher. Determination of the total area of blue (nuclear) staining) is a crude method to measure inflammatory cell infiltration since the nuclear to cytoplasmic ratio is extremely low area in the cytoplasm (sarcoplasm)- rich muscle fibers, and is considerably higher for macrophages and neutrophils - thus an influx of inflammatory cells into muscle results in an increase in the area of blue staining roughly proportional to the severity of the response. Measurements of normal muscle from the control group of naïve pigs were used to determine the background level of nuclear staining. A standard area of 2.5 mm^2 from within the most severely affected area (selected subjectively by microscopic examination) on each tissue section, was captured and analyzed using Northern Eclipse computer software Version 6.0 (Empix Imaging Inc. Mississauga, ON) and the total area of blue staining was calculated.

6.2.9 Statistics

Differences between groups were analyzed using Prism GraphPad statistical software (GraphPad Software, Inc.) using a non-parametric Kruskal-Wallis one-way ANOVA for showing differences in gene expression and chi-squared test for showing differences between immune responses.

6.3 Results

6.3.1 Differences in the level of gene expression and cellular infiltration following different conditions of DNA administration

Before the DNA immunization experiments were conducted, gene expression and cellular infiltration were assessed in quadriceps muscle under the electroporation conditions described in Table. 6.1. Using the luciferase reporter gene, gene expression was determined for each treatment (Fig. 6.1 and Table 6.2). Pretreatment with electroporation (Group 2) did not significantly change gene expression compared to plasmid administered without electroporation (Group 1). In contrast, different electroporation parameters administered immediately following plasmid administration all increased gene expression similarly in all groups (Groups 3, 4 and 5) given electroporation.

Histological examination was carried out for each treatment on tissue from the injection sites sampled forty-eight hours following administration of plasmid. Plasmid administered without any electroporation (Group 1) caused a mild inflammatory response, assessed by the amount of blue (nuclear) staining, and consisted primarily of macrophages and neutrophils (Fig. 6.2 and Table 6.2). Electroporation conditions of 200 V/20 msec/6 pulses (Groups 2 and 4) and 200 V/60 msec/2 pulses (Group 5) caused muscle necrosis in addition to severe inflammation (marked influx of macrophages and neutrophils), whereas electroporation conditions of 100 V/20 msec/2 pulses (Group 3) resulted in muscle necrosis with moderate to severe infiltration of macrophages and neutrophils. In all groups treated with electroporation (Groups 2, 3, 4 and 5) there were scattered muscle fibers showing degeneration characterized by mildly increased

Table 6.2 Gene expression of luciferase encoding plasmid, and tissue damage 48 hours following administration determined by digital analysis of nuclear area.

Group	Electroporation conditions	Luciferase Gene expression	Severity of histological inflammatory reaction [1,2]
1	No EP	Low expression	Mild [3] (7% area [1])
2	(200 V)/20 msec./6 pulses 1 hour prior to DNA administration	Low expression	Severe (33% area [1])
3	(100 V)/60 msec./2 pulses	High expression	Moderate-Severe (24% area [1])
4	(200 V)/20 msec./6 pulses	High expression	Severe (33% area [1])
5	(200 V)/60 msec./2 pulses	High expression	Severe (29% area [1])
6	Naive	None	Normal (3% area area [1])

[1] The area of nuclear staining in a 2.5 mm^2 area was assessed on the most severely affected area determined subjectively prior to analysis.

[2] Nuclear area was used as a measurement of inflammatory cell infiltration.

[3] Mild < 5%, Moderate 5%-25% and Severe > 25%

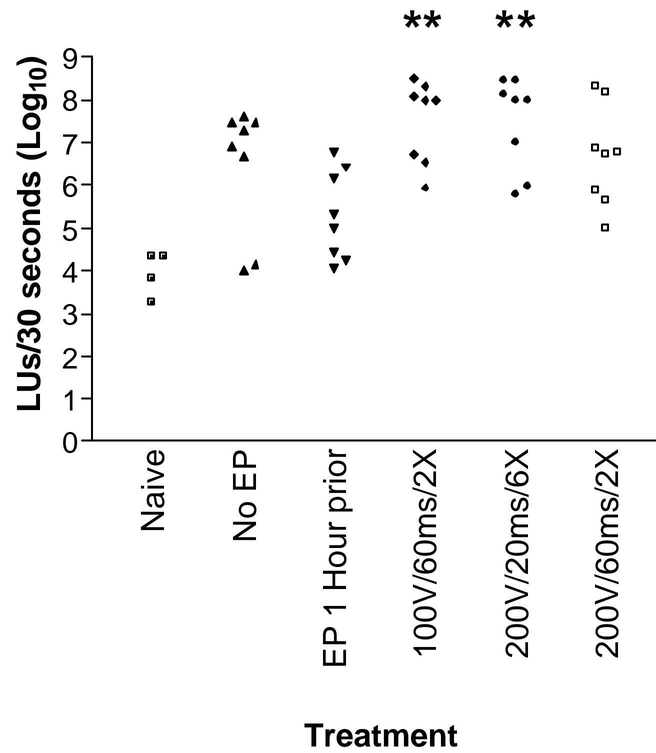

Figure 6.1 Luciferase gene expression in muscle 48 hours following administration of plasmid under different conditions.

Differences between groups, **p<0.01 100V/60ms/2X vs naive (no plasmid) and 200V/20ms/6X vs naive by one-way ANOVA Kruskal-Wallis test.

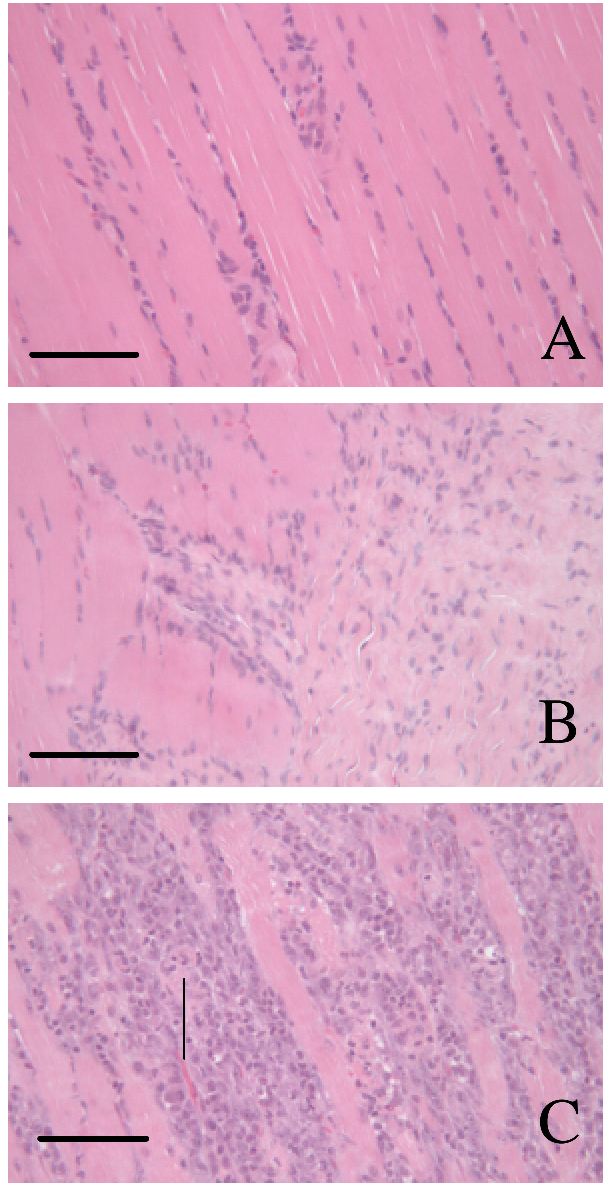

Figure 6.2 Histological examination of muscle (HE stain) 48 hours following plasmid administration.

(A) Muscle injected with plasmid, (B) muscle injected with plasmid followed by electroporation (2 Pulses 100V), (C) muscle injected with plasmid followed by electroporation (6 Pulses 200V) representative of (2 Pulses 100V). Mild infiltration of macrophages and neutrophils was observed in (A) and severe infiltration of macrophages and neutrophils was observed in (B) and (C) with scattered necrotic myofibers (arrow) were also noted. Bar is 100 µm.

eosinophilia.

6.3.2 Immune responses

Glycoprotein D-specific antibody responses were determined by BHV-1 neutralization assay (Fig. 6.3). A neutralization titer ≥ 32 can reduce viral shedding and clinical symptoms from BHV-1 challenge in cattle (van Drunen Littel-van den Hurk et al., 1998). Immunization with plasmid without electroporation (Group 1), conditions that give low gene expression and low cellular infiltration, elicited the lowest number, of animals, only 2/6, achieving a neutralization titer of ≥ 32. Animals treated with electroporation one hour prior to plasmid administration (Group 2), showed low gene expression with high cellular infiltration, with similar BHV-1 neutralization antibody responses to Group 2 (Fig. 6.3). Animals treated with electroporation immediately following immunization (Group 3, 4, and 5), conditions which gave high gene expression and high cellular infiltration (Table. 6.2), resulted in more animals achieving a neutralization titer of ≥ 32 compared to those treated with conventional plasmid immunization (Group 1) (Fig. 6.3).

Glycoprotein D-specific proliferation assays showed responses (Fig. 4) similar to the antibody responses (Fig. 6.3), with a trend of greater stimulation indexes in animals that received electroporation (Groups 2, 3, 4 and 5).

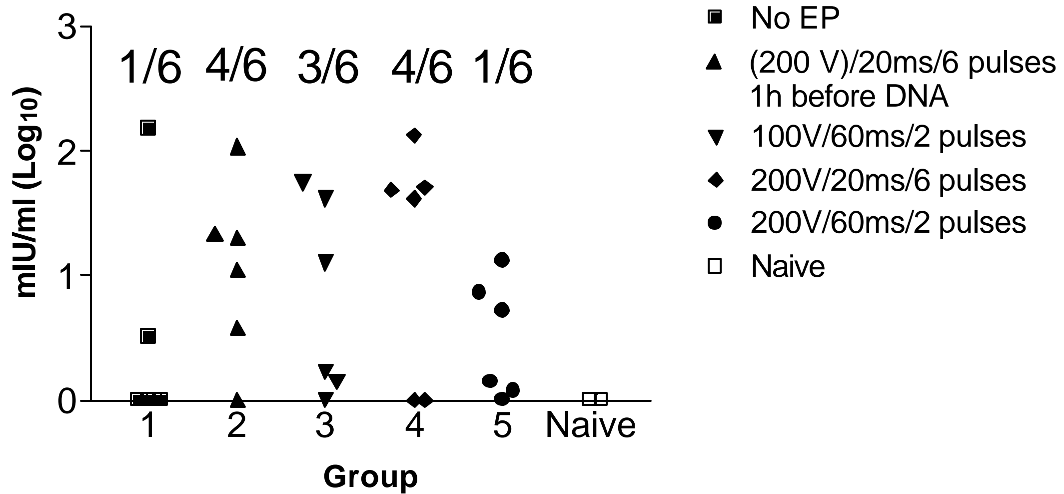

Figure 6.3 Glycoprotein D-specific antibody responses following electroporation.

Numbers above the groups indicate the number of animals that clinical signs would be expected reduced (>32 BHV-1 neutralization titer). Group 3, 4, and 5 vs prebleed $p<0.01$ by chi-squared test.

To determine if Th1-like responses were obtained, lymphocytes from immunized pigs were assessed for production of IFN-γ – the true measure of Th1-like responses. DNA immunization with a gD-encoding plasmid stimulated gD-specific interferon gamma secreting cells (Fig. 6.4), supporting previous reports that DNA vaccines polarize the response towards a Th1-like or balanced response (Martinez et al., 1997; Sjolander et al., 1998). However, all immunization conditions elicited similar numbers of gD-specific IFN-γ secreting cells.

Immune responses to HBsAg were determined using the Abbott clinical ELISA (Fig. 6.5). Animals immunized without electroporation (Group 1) had the weakest immune responses, with only two animals responding to the immunization and only 1/6 animals responding with a titer considered to be protective (>10 mIU/ml) (Fig. 6.5). In groups that received electroporation at the time of DNA immunization (Groups 3, 4 and 5), more animals responded and group 4, which received the strongest electroporation treatment, had the most animals considered protected (4/6). Animals which received electroporation one hour prior to DNA immunization (Group 2) had immune responses similar to animals which received an identical electroporation treatment at the time of immunization; with 4/6 animals considered protected despite the low level of antigen expression.

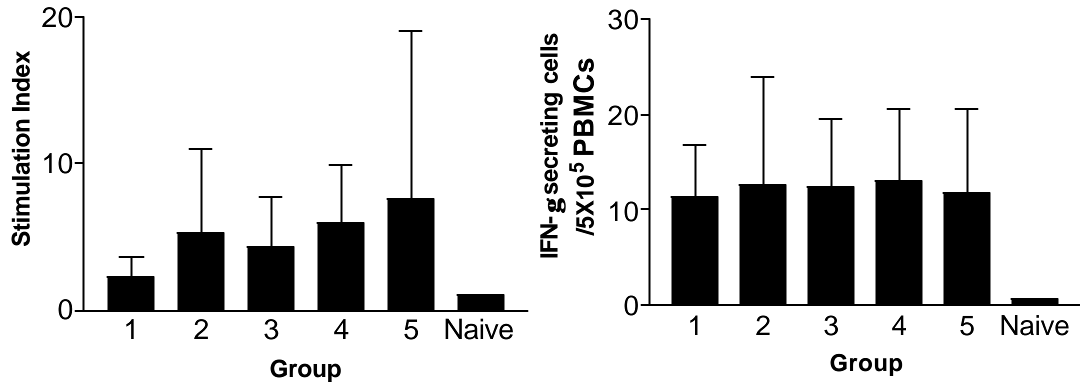

Figure 6.4 Cellular immune responses in gD immunized pigs assessed by proliferation and IFN-γ cytokine secreting cells.

Glycoprotein D-specific proliferative responses were determined at week 6. The number of gD-specific IFN-γ secreting lymphocytes was determined using ELISPOT at week six. There was no significant difference between the groups. Error bars represent SD and n = 6.

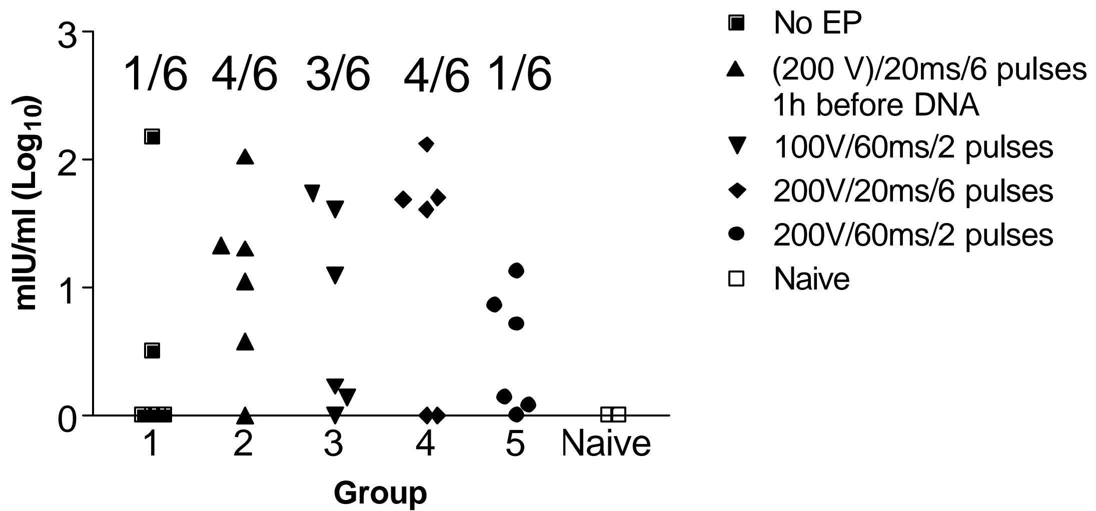

Figure 6.5 Effect of electroporation on anti-HBsAg titers in pigs six weeks after immunization.

Anti-HBsAg titers were determined using the Abbott clinical ELISA. Numbers above the groups are the number of animals considered protected (>10 mIU/ml). Groups 1 and 4 vs prebleed $p<0.01$ by chi-squared test.

6.3.3 Histological examination of muscle biopsies at two and six weeks following electroporation

Examination of muscle samples from animals two weeks following the second immunization carried out at day 28 in conjunction with electroporation (Groups 2, 3, 4 and 5) showed a greater degree of cellular infiltration than of those from animals that received no electroporation (Group 1) (Fig. 6.6). In animals treated with electroporation at the time of plasmid administration (Groups 3, 4, and 5), the cellular infiltration at two weeks following the second immunization consisted primarily of aggregates of lymphoblasts surrounding small vessels within the muscle, whereas in animals treated with electroporation prior to plasmid administration, the mild cellular infiltration consisted predominantly macrophages and neutrophils.

Muscle tissue examined six weeks after the primary immunization following electroporation conditions of 200V/20msec./6pulses (Groups 2 and 4) showed a mild cellular infiltration consisting predominantly of macrophages and neutrophils. Muscle tissue from all the other treatments (Groups 1, 3 and 4) was normal.

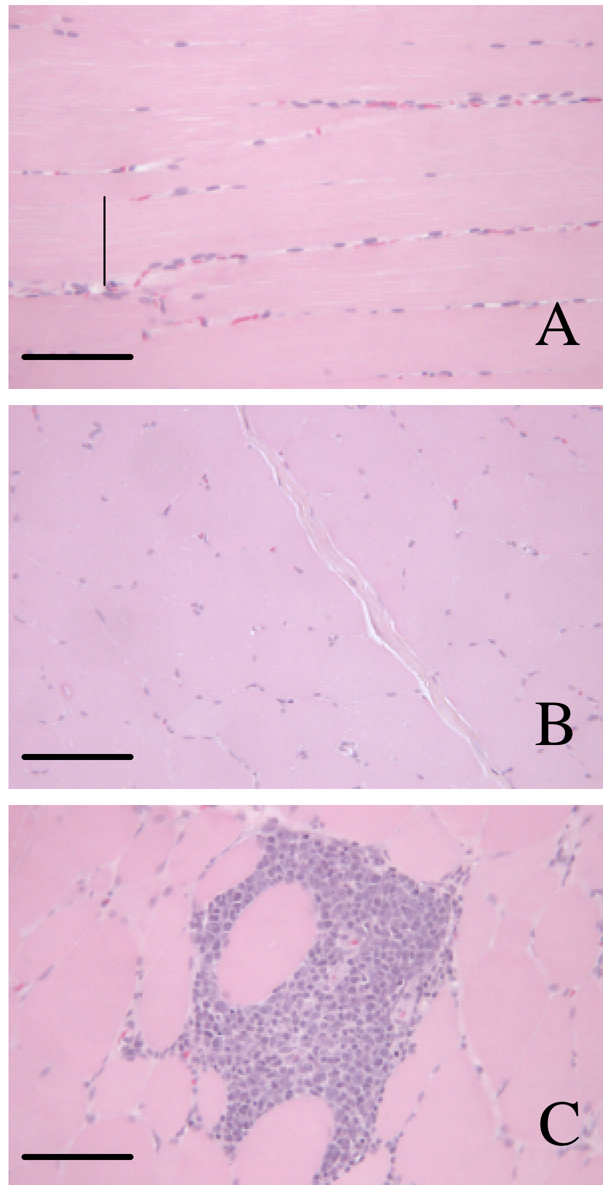

Figure 6.6 Histological examination of muscle (HE stain) 14 days following plasmid administration.

(A) Muscle treated with electroporation one hour prior to plasmid injection had a very minimal cellular infiltration of macrophages and neutrophils. (B) Muscle injected with plasmid and not treated with electroporation had no detectable cellular infiltration. (C) Muscle injected with plasmid followed by electroporation (200V/6P), (representative of groups 3, 4 and 5) showed moderate to severe multifocal lymphocytic infiltration. Bar is 100 µm.

6.4 Discussion

These data provide evidence of a role for inflammation/cellular infiltration, caused here by the use of electroporation, in enhancing immune responses on immune responses following DNA immunization. Although the effect of the inflammatory response was not as dramatic for gD compared to HBsAg (groups 2 and 4, respectively). The two antigens differ in their immunogenicity with gD being more immunogenic than HBsAg. The difference in immunogenicity between the two antigens may explain why the effect of cellular infiltration was less dramatic for gD than for HBsAg.

The inflammatory cell infiltration was demonstrated to be an important component for enhancing immune responses to DNA vaccines since prior treatment with electroporation enhanced immune responses to the HBsAg DNA vaccine but did not increase gene expression. However, the increase in gene expression caused by electroporation is absolutely critical for inducing protective immune responses as demonstrated using the gD DNA vaccine. The level of antigen produced is critical for inducing immune responses to DNA vaccines illustrated by the previous experiment where induction of antibody titers that would be considered protective in humans from hepatitis B could be achieved in 100% of animals, under electroporation conditions of 200V/20msec./6pulses and using two administration sites of 500 µg pHBsAg for the primary and secondary immunization (Babiuk et al., 2002). In the current study, with only one administration site of 500 µg pHBsAg for the primary and secondary immunization, the number of animals with titers considered protective was reduced to 66%. Thus, the mechanisms by which electroporation enhances immune responses to

DNA vaccines is a combination of increased gene expression and increased inflammation and cell infiltration. Previous studies using bupivacaine to enhance immune responses to DNA vaccines (Davis et al., 1995) suggested that muscle damage/inflammation was important for enhancing immune responses.

Improved antigen presentation is one possible mechanism by which increased inflammatory cell infiltrates may enhance immune responses to DNA vaccines delivered with electroporation. Infiltration of APCs such as macrophages or dendritic cells may result in increased antigen uptake, resulting in more efficient cross priming. It is not known whether electroporation affects other aspects of antigen presentation through leakage of antigen from injured muscle cells, or more efficient presentation of antigen to APCs when damaged muscle cells die. Electroporation may also activate APCs through danger signals allowing more efficient antigen presentation (Murtaugh and Foss, 2002).

Interestingly, lymphocytic infiltrations occurred only in muscle exposed to electroporation conditions that elicited high gene expression (Groups 3, 4 and 5). This can be explained as follows: antigen is the critical factor for the retention of lymphocytes, and if only a few cells are transfected, there will be very little antigen produced, so that the probability of finding lymphocytic infiltrates is very unlikely (Groups 1 and 2). Antigen specific lymphocytes may surround transfected cells 2 weeks after the secondary immunization since antigen is likely to still be present (Muramatsu et al., 2001). More supporting evidence for antigen specific lymphocytes in the muscle are gene therapy experiments where the rejection of transfected muscle fibers occurs within 3 weeks in the absence of immunosuppression (Vilquin et al., 2001).

The development of combination vaccines is a major goal in vaccine research. DNA vaccines encoding different antigens have been shown to be compatible (Braun et al., 1998). In previous studies, gD and HBsAg encoding plasmids did not cause interference when administered in the same muscle (Babiuk et al., 2002), although the plasmids were not mixed together. Here, different plasmids were mixed and administered in a single site and immune responses were induced to both antigens (gD and HBsAg); however, for development of combined DNA vaccines, different antigens will have to be tested for compatibility. Co-immunization with two different encoded antigens either in one plasmid or in different plasmids may actually improve immune responses to both antigens by providing additional T cell help through additional helper epitopes (Maecker et al., 1998). Further investigation of co-immunization of different antigen combinations will be necessary to determine the extent of this effect.

These experiments also demonstrate that electroporation is an effective delivery method for enhancing immune responses elicited by DNA vaccines. A similar effect on gene expression and infiltration could be achieved using the 6-needle array with 6 electrical pulses as with 2 electrical pulses. Muscle tissue examined histologically at six weeks following the two pulse electroporation parameters was indistinguishable from normal muscle, indicating that electroporation under these conditions does not cause any long-term effects. Reduction in the number of pulses may allow this electroporation procedure to be more useful clinically. Only partial sero-conversion to HBsAg plasmid immunization was achieved using electroporation under the experimental conditions of one administration site with a secondary immunization. It may be possible to increase the response and the number of responding animals by optimizing the plasmid promoter,

vector and gene and increasing the dose of plasmid used. The commercial HBsAg vaccine Engerix B requires three immunizations in humans to offer protection although recent data in humans suggest two immunizations may suffice (Iwarson, 2002). Immunization with Engerix B results in 100% of pigs responding after two immunizations (data not shown). Therefore, these results indicate the efficacy of DNA immunization administered with electroporation is approaching that of a conventional vaccine. Although efficacy in terms of immune response may be similar, long-term safety issues will need to be studied further. Modifications in the electroporation parameters such as voltage/time/number of pulses resulting in effective gene expression with less severe tissue damage are a step in that direction.

7.0 CPG ODNs DO NOT ENHANCE IMMUNE RESPONSES TO DNA VACCINES EVEN WHEN DELIVERED WITHOUT INTERFERING WITH ANTIGEN EXPRESSION

(unpublished)

7.1 Introduction

CpG ODNs are potent adjuvants for subunit vaccines (Davis *et al.*, 1998). In addition, CpG motifs in plasmid vectors may be essential for DNA vaccines to be effective (Krieg *et al.*, 1998c). However, when CpG ODNs are mixed with plasmids and administered by injection, immune responses are suppressed (Weeratna *et al.*, 1998). This suppression may be the result of decreased plasmid uptake by cells and consequently reduction of antigen expression (Weeratna *et al.*, 1998). The incompatibility of CpG ODNs and plasmid may be overcome by delivering plasmid directly into cells using the gene gun. The effect of co-administering CpG ODNs with plasmids was evaluated by comparing gene expression and immune responses following administration after intradermal injection and gene gun delivery.

7.2 Materials and methods

7.2.1 Plasmids and oligonucleotides

The following plasmids were used, a CMV promoter-driven luciferase expression vector pMAS-luc (pluc) (gift from Dr. Heather Davis, University of Ottawa, ON), and a full length gD gene from BHV-1 under a CMV promoter pCAN-gD (pgD) and pCAN-null (gifts from Dr. Reno Pontarollo, VIDO, Saskatoon, SK) were used in

this study. Plasmids were purified using Qiagen (Mississauga, ON) endotoxin free plasmid kits. Phosphorothioate backbone ODNs 1826 TCCATGACGTTCCTGACGTT containing two CpG motifs and 1982 TCCAGGACTTCTCTCAGGTT containing no CpG motifs (Qiagen) were used.

7.2.2 Immunization

Mice were injected intradermally in the back using a 27-gauge syringe with 1.5 μg pgD in 10 μl PBS alone or together with 10 μg of ODNs. For gene gun immunization gold beads were prepared according to the manufacturer's instructions for the Helios Gene Gun System (Bio-Rad, Hurcules, CA) using doses of 0.05 μg of pgD supplemented with 1.45 μg of pCAN-null (plasmid backbone) per shot. ODNs were coated on separate gold beads using doses of 1.5 μg ODNs per shot (Oehen *et al*., 2000) and administered by the gene gun immediately after plasmid was delivered with the gene gun. An additional group of mice were immunized using the gene gun under the identical conditions, with subsequent delivery of 10 μg of ODNs administered intradermally by injection under the subjacent area of skin.

7.2.3 Luciferase assay

Identical doses of plasmids used for immunization by the three delivery methods, were used to determine the level of gene expression. Forty-eight hours after plasmid administration, skin at the delivery site (1 cm^2) as well as the draining lymph nodes were removed from mice and homogenized. Luciferase activity was determined using a luciferase assay system (Promega, Madison, WI). Samples were counted for 30 seconds.

7.2.4 Characterization of humoral and cellular immune responses

Immunlon 2 (DYNEX, Chantilly, VA) ELISA plates were coated with tgD (1 μg/ml) or IgG standards (Serotec, Hornby, ON) in 20 mM Na_2CO_3 overnight at 4°C. gD-specific IgG and IgG_1 was determined using IgG or IgG_1 specific biotinylated antibodies diluted 1:10,000 (Caltag, Toronto, ON), followed by streptavidin-alkaline phosphatase diluted 1:10,000 (Jackson Immuno Research Labs, West Grove, PA), developed by *p*-nitrophenol phosphate (PNPP) (Sigma Chemical Co., St. Louis, MO). The absorbance was read after 15-20 minutes at 405 nm (Bio-Rad). ELISPOT assays for IFN-? and IL-4 were performed using cells from individual spleens or pooled lymph nodes as previously described (Baca-Estrada *et al.*, 1997). Briefly, $1x10^6$ cells/well were placed in 96 well culture plates with and without antigen (gD 0.4 μg/ml) in AIM-V (Gibco, Life Technologies, Burlington, ON) and incubated at 37°C and 5% CO_2 for 24 hours. Cells were resuspended in fresh media and seeded on Nitrocellulose plates (Millipore, Napean, ON) coated with either IFN-? or IL-4 specific mouse cytokine capture antibody 2 μg/ml (PharMingen, San Diego, CA). Biotinylated anti mouse IFN-? or IL-4 specific antibodies a 2 μg/ml (PharMingen) followed by streptavidin-alkaline phosphatase diluted at 1:1,000 (Jackson Labs) and developed using 5-bromo-4-chloro-3-indolyl phasphate/nitro blue tetrazolium (BCIP/NBT) substrate tablets (Sigma). Spots representing gD specific cytokine secreting cells were counted and expressed as number of cytokine secreting cells per $1x10^6$ cells.

7.3 Results

Luciferase gene expression in the skin and the lymph nodes was determined 48 hours following intradermal or gene gun administration of plasmid with or without ODNs (Figure 7.1A and D). Following intradermal administration of pluc, luciferase expression was detected in the skin but not in the draining lymph nodes (Figure 7.1A). When either CpG or null ODNs were mixed with pluc and administered intradermally there was a decrease in luciferase expression in skin. Following administration of pluc using the gene gun, luciferase expression was found in the skin and draining lymph nodes (Figure 7.1D). Luciferase gene expression in the skin and the lymph nodes was not influenced by co-administration of ODNs with pluc using the gene gun (Figure 7.1D).

Intradermal immunization with pgD alone elicited significant gD-specific IgG1 antibody responses but no gD-specific IgG2a responses, as well as gD-specific IFN-? secreting cells (Figure 7.1B and C). However, when ODNs were co-administered with pgD by intradermal injection, no significant gD-specific antibody or gD-specific IFN-? secreting cells were detected (Figure 7.1B and C).

Gene gun immunization with pgD alone elicited significant gD-specific IgG1 antibody responses but no gD-specific IgG2a responses, as well as gD-specific IL-4 secreting cells (Figure 7.1E and F). In contrast to intradermal administration, co-administration of ODNs with pgD using the gene gun did not influence the induction of humoral or cellular immune responses (Figure 7.1E and F). In addition, mice given CpG ODNs intradermally and immunized with the gene gun also did not generate enhanced immune responses (data not shown).

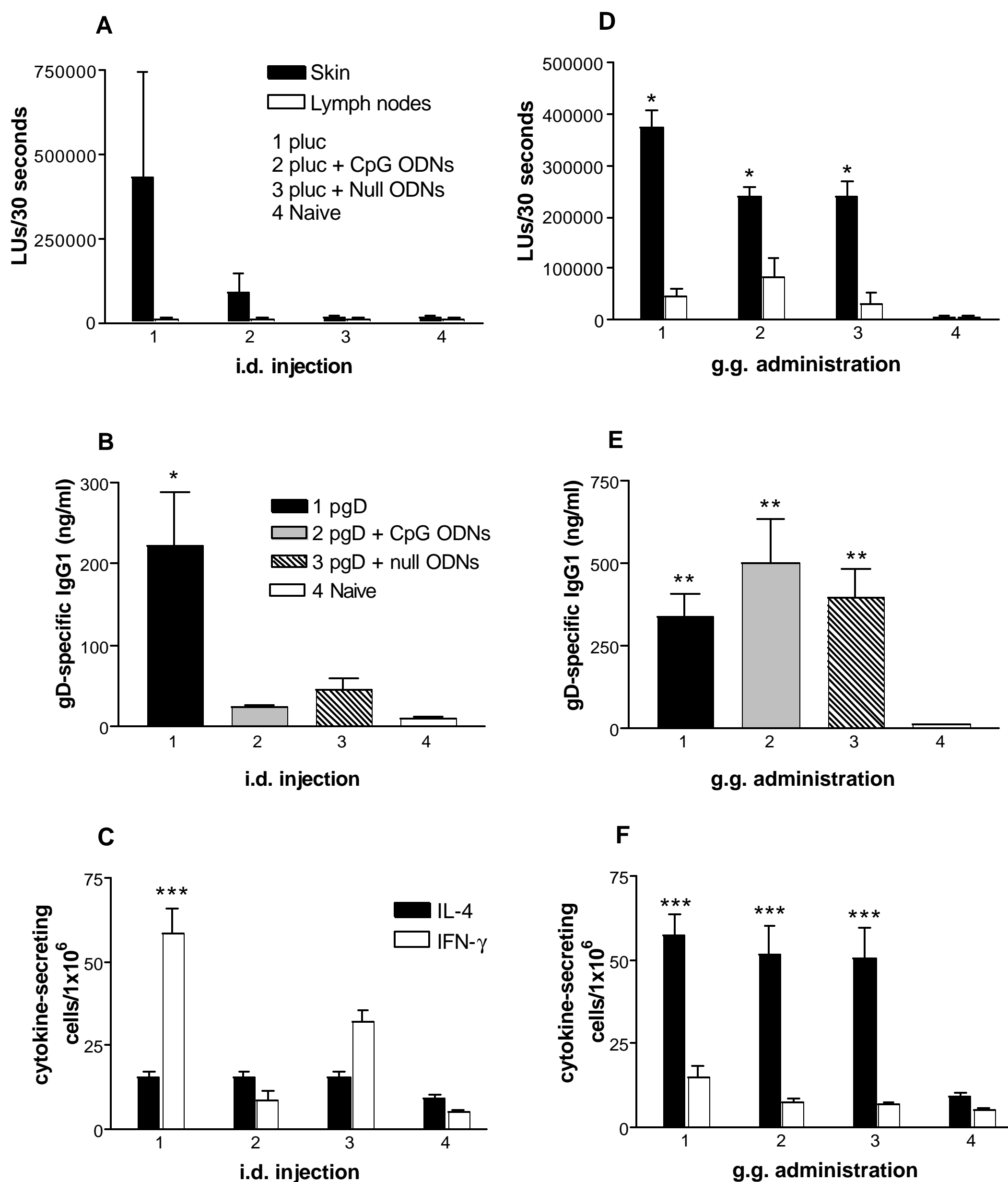

Figure 7.1 Effect of CpG ODNs on luciferase gene expression and immune responses induced by pgD.

Gene expression elicited in skin and lymph nodes by intradermal injection of pluc alone or together with CpG or null ODNs (A). Mice were immunized by intradermal injection with pgD alone or together with CpG or null ODNs. (B and C). Gene expression elicited in skin and lymph nodes by gene gun administration of pluc alone or together with CpG or null ODNs (D). Mice were immunized using the gene gun with pgD alone or together with CpG or null ODNs elicited antigen specific IL-4 and IFN-? secreting cells (E and F). Error bars represent standard error of the mean of five animals per group, * p<0.05, ** p<0.01, *** p<0.001 to naïve by ANOVA Tukey's multiple comparison test.

By comparing the two delivery methods, it was clear that intradermal injection elicited a Th1 response characterized by IFN-? secreting cells and that gene gun administration elicited a Th2 response characterized by IL-4 secreting cells. CpG ODNs were not able to modulate the Th2 responses elicited following gene gun immunization.

7.4 Discussion

Co-administration of ODNs with plasmid by intradermal injection causes a reduction in the magnitude of immune responses elicited compared to intradermal injection of the DNA vaccine without CpG ODNs. This effect is likely due to ODNs interfering with plasmid uptake into the cell (Weeratna *et al*., 1998). Since ODNs and plasmids both have similar properties such as a net negative charge, they will bind to similar receptors on the surface of a cell. There is a competition for receptors and there will be a lower level of receptor-mediated endocytosis with plasmids. As a consequence, fewer plasmids enter cells and gene expression by plasmid vectors is reduced. CpG ODNs did not interfere with the gene expression of plasmids when they were delivered using the gene gun, because in this case plasmid can be directly administered into the cell. CpG ODNs were not able to enhance or modulate the immune responses elicited by administration of plasmid using the gene gun, even though the doses of CpG used were suitable for adjuvanting protein vaccines in mice. In addition, CpG ODNs have been shown to be adjuvants for protein antigens following topical powder administration (Chen *et al*., 2001a), indicating that CpG ODNs have adjuvant activity in the epidermis. A higher dose of CpG ODNs likely would not be able to immunomodulate the Th2 responses elicited by plasmid administered by the

gene gun. One hypothesis for why gene gun administration elicits Th2 responses may be that transfection of Langerhans cells primes Th2 responses.

CpG motifs may not be critical for DNA vaccines to be effective. When CpG ODNs and plasmid are mixed together and administered with electroporation gene expression is not altered compared to plasmid administered by electroporation. However, even when CpG ODNs and plasmid are administered using electroporation, there was no adjuvant effect of CpG ODNs determined by comparing immune responses elicited by plasmid and CpG ODNs to plasmid alone (Dr. Jeffrey Ulmer, Chiron Corporation, personal communication). Even more convincing evidence that CpG motifs may not be critical for DNA vaccines to be effective are the observations that TLR9 knockout mice, which do not respond to CpG ODNs (Hemmi *et al*., 2000) develop immune responses to DNA vaccines (unpublished data, Shawn Babiuk).

8.0 BIPHASIC LIPID VESICLES (Biphasix™) ENHANCE THE ADJUVANTICITY OF CpG OLIGONUCLEOTIDES FOLLOWING SYSTEMIC AND MUCOSAL ADMINISTRATION

(Manuscript in preparation)

8.1. Introduction

Bacterial DNA has immunostimulatory activity in mammals. This effect is mediated by the ability of certain CpG motif patterns to activate a variety of cells of the immune system, such as B cells (Krieg *et al*., 1995), macrophages (Stacey *et al*., 1996) and dendritic cells (Jakob *et al*., 1998). Since CpG oligonucleotides (ODNs) activate antigen-presenting cells (APCs), they are potent systemic and mucosal adjuvants (McCluskie and Davis, 1998; Moldoveanu *et al*., 1998). In addition, CpG ODNs stimulate IL-12 cytokine secretion *in vivo* (Krieg *et al*., 1998a) and therefore are also potent stimulators of Th1 type immune responses.

In contrast to mice, protocols for immunizing humans and livestock require high doses of CpG ODNs to exert adjuvant activity. Enhanced immunostimulatory activity of CpG ODNs has been achieved by physically attaching the antigen to the CpG ODN (Cho *et al*., 2000) or by incorporating the vaccine antigen and the CpG ODNs into lipid or oil formulations (Gursel *et al*., 2001; Li *et al*., 2001; Mui *et al*., 2001).

Biphasic lipid vesicles (Biphasix™) are novel formulations suitable for the delivery of proteins, peptides and oligo/polynucleotides. The formulations employed in this study were designed specifically for vaccine application [Vaccine-Targeting Adjuvants (VTA)]. The objective of this study was to assess whether VTA formulations

were capable of enhancing the adjuvanticity of CpG ODNs when administered by systemic and mucosal routes. As well, potential adverse effects of VTA formulations were assessed using histology to determine their safety.

8.2 Materials and methods

8.2.1 Animals

Six-week-old female BALB/c mice, purchased from the Animal Resource Centre at the University of Saskatchewan, were used for all experiments. Animals were treated in compliance with the regulations of the Canadian Council for Animal Care.

8.2.2 Oligonucleotides

The CpG oligonucleotide 5'-TCCATGACGTTCCTGACGTT-3'(1826) and the non-CpG oligonucleotides 5'TCCAGGACTTCTCTCAGGTT-3'(1982) (Qiagen,) were used at 10 μg per subcutaneous immunization and 1 μg per mucosal immunization. Both these oligonucleotides contain a nuclease-resistant phosphorothioate backbone and have previously been used as systemic and mucosal adjuvants (McCluskie and Davis, 1998; Moldoveanu *et al*., 1998). The CpG oligonucleotide GGGGGACGATCGTCGGGGG (2116) contains a chimeric phosphodiester and phosphorothioate backbone.

8.2.3 Vaccine delivery system

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.

8.2.4 Immunizations

Subcutaneously immunized mice received 0.5 µg of recombinant glycoprotein D (truncated form)(tgD) from herpes simplex virus type 1 (HSV-1) (BioDesign International, Saco, ME) alone or in the presence of 10 µg of CpG ODNs or non-CpG ODNs. Vaccine groups were as follows: tgD in PBS (Group 1), tgD and non-CpG ODN in PBS (Group 2), tgD and CpG ODN in PBS (Group 3), tgD in VTA (Group 4), tgD and non-CpG ODN in VTA (Group 5), tgD and CpG ODN in VTA (Group 6), and tgD and CpG in 30% Emulsigen Plus (MVP Laboratories, Ralston, NE) (Group 7). Mice were immunized on day 1 and received a second immunization on day 15.

Intranasally immunized mice received 0.5 µg of tgD (BHV-1) with or without 10 µg of CpG ODNs in 20 µl (10 µl per nostril). Vaccine groups were as follows: tgD in PBS (Group 1), tgD and CpG ODNs in PBS (Group 2) and tgD and CpG in VTAM1 (Group 3). Mice were immunized on day 1 and received a second immunization on day 15.

8.2.5 HSV-1 challenge and clinical examination

Mice were challenged with HSV-1 using a skin scratch model two weeks following secondary immunization. The skin on the back was scratched with a lancet and HSV-1 was applied topically three times using a culturette swab with an approximate total volume of 35 µl of HSV-1. After infection, groups of mice were monitored without knowledge of the treatments they received. The viral challenge was assessed using a clinical score on a five-point scale: 0, no apparent infection; 1, minor redness; 2, small herpetic vesicles on the skin; 3, medium sized herpetic skin lesions; 4,

medium sized herpetic skin lesions and rough hair coat; 5, severe skin lesions with systemic dissemination of lesions and rough hair coat. Mice were euthanized if a clinical score of 5 was reached.

8.2.6 Characterization of antibody responses

Lung washes and nasal secretions were collected post mortem using 1.5 cm long tubes of Teflon TFE 24 (Cole-Parmer Instrument Company, Niles, IL) connected to a 23-gauge needle. For the lung washes the tube was inserted into the trachea and 500 µl of PBS was flushed in and out of the lungs twice. For the nasal washes, 300 µl of PBS was flushed cranial through the trachea and collected at the nares. Antibody responses specific for gD were determined using ELISA, as previously described (Baca-Estrada *et al*., 1997). Immunlon 2 (Dynex, Chantilly, VA) ELISA plates were coated either with tgD (HSV-1) (BioDesign International, Saco, ME) (0.1 µg/ml) or tgD (BHV-1) (1 µg/ml) in 20 mM Na_2CO_3 overnight at 4 °C. Biotinylated antibodies specific to IgA, IgG and IgG subclasses (Caltag, Toronto, ON) were used, followed by streptavidin-alkaline phosphatase (Jackson Immuno-Research Labs, West Grove, PA). Alkaline phosphatase activity was determined by p-nitrophenol phosphate (PNPP) (Sigma). The absorbance was read after 15-20 minutes at 405 nm (Bio-Rad).

8.2.7 Determination of IL-12 in plasma following CpG administration

Eight hours after mice were injected with 50 µg CpG ODNs (2116) in saline or formulated in VTA, plasma was collected in syringes containing heparin (HEPALIN 10 U.S.P. units)(Organon Teknika, Toronto, ON). Blood was centrifuged to separate the

plasma from the red blood cells and the plasma was collected and frozen at -20°C. Plasma concentrations for IL-12 was determined by ELISA as follows: Immunlon 2 (Dynex) ELISA plates were coated with anti-mouse IL-12 (p70) (2 µg/ml) (PharMingen, San Diego, CA) in 20 mM Na_2CO_3 overnight at 4°C. Plates were washed 4X with phosphate buffered saline 0.05% Tween 20 (Sigma) (PBST). Plasma samples, diluted with PBST-0.5% gelatin (Sigma) in serial dilutions, were placed on the plate with known amounts of recombinant mouse IL-12 (ranging from 5 ng/ml to 0.3 ng/ml) and incubated overnight at 4°C. The following day plates were washed 4X in PBST and biotin anti-mouse IL-12 (p40/70) (2 µg/ml in PBST-gelatin) (PharMingen) was added to the wells and incubated for 1 hour at room temperature. Plates were washed 4X in PBST and streptavidin-alkaline phosphatase (Jackson Immuno-Research Labs) was added to the wells. Plates were washed 8X in PBST and alkaline phosphatase activity was determined by *p*-nitrophenol phosphate (PNPP) (Sigma). The absorbance was read after 15-20 minutes at 405 nm (Bio-Rad).

8.2.8 Histological examination of skin following injection of CpG in various formulations

Skin tissue samples of the injection sites were taken at 1, 7, 14 and 29 days following subcutaneous administration of CpG ODNs in PBS, VTA or emulsigen as well as VTA alone. Tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Four µm sections were cut and stained with hematoxylin and eosin (HE). Prior to analysis, the identity of the slides was masked, so that the researcher was unaware of the treatment group of the individual tissues. The area of nuclear staining (blue with hematoxylin in HE stain) is an indirect measure of

cellular infiltration since cutis consists mostly of adipocytes that have a low nuclear to cytoplasmic ratio, infiltrating macrophages and neutrophils increase the area staining blue. Measurements of normal skin from the naive control group were used to determine background staining. The most severely affected area from each tissue section was determined subjectively prior to analysis. Within a standard area of 0.25 mm^2, the total area staining blue was calculated using Northern Eclipse computer software version 6.0 (Empix Imaging Inc. Mississauga, ON).

8.2.9 Statistics

Differences in immune responses among vaccine groups were analyzed with Prism statistical software (GraphPad Software, Inc.) using a one-way ANOVA followed by Tukey's multiple comparisons test.

8.3 Results

8.3.1 Immunization with CpG formulated in VTA increased serum antibody titers

Subcutaneous immunization with gD and CpG ODNs formulated in VTA induced significantly higher gD-specific IgG titers than immunization with gD in PBS. In contrast, the responses induced by immunization with gD and CpG ODNs (1826) were not statistically different from those induced by gD in PBS (Figure 8.1) In addition, the gD-specific IgG titers induced by immunization with gD and CpG (1826) in VTA were similar to those induced by gD and CpG (1826) in Emulsigen (Figure 8.1).

Since CpG ODNs are known to induce Th1 type immune responses, gD-specific IgG1 and IgG2a titers in serum were determined two weeks after the second immunization. Immunization with formulations that did not contain CpG ODNs induced a Th2 type response characterized by predominant gD-specific IgG1:IgG2a titer ratios (Figure 8.2). However, incorporation of CpG ODNs into the vaccine resulted in an increase in the titer of gD-specific IgG2a titers (Figure 8.2). VTA formulations significantly enhanced the gD-specific IgG1 titers when compared to the responses induced by gD in PBS.

8.3.2 Protection from HSV-1 infection

To evaluate the level of protection conferred by immunization with different formulations, mice were challenged with HSV-1 using a scratch model. All naïve and control (CpG alone) mice failed to control the HSV-1 infection and were euthanized (Table 8.1). Mice immunized with gD and non-CpG ODN (1982) developed severe infection and only 2 out of 5 mice survived the challenge (Table 8.1). Mice immunized with gD and CpG (1826) survived the challenge but developed moderate clinical scores; however, animals that received gD and CpG ODNs (1826) in VTA developed minimal signs of disease and therefore, were better protected against HSV-1 infection when compared to the other groups.

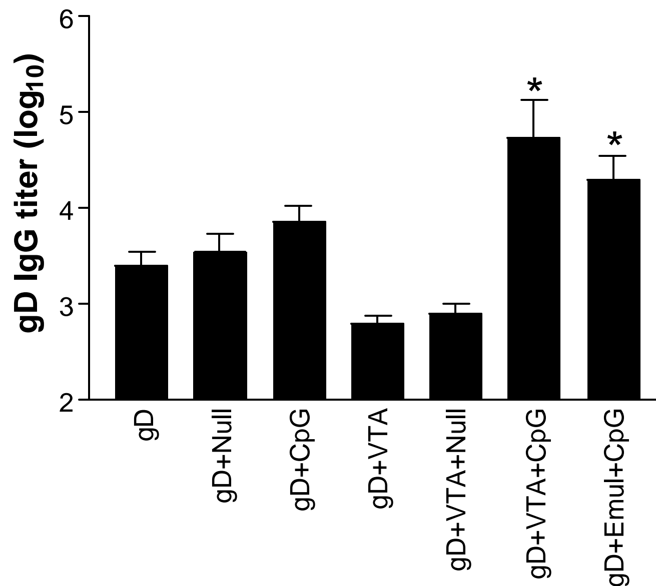

Figure 8.1 Effect of VTA formulation on gD-specific IgG responses following subcutaneous immunization.

All animals were immunized twice subcutaneously at two week intervals with 0.5 μg gD. Total gD-specific IgG was determined in serum two weeks after the boost by ELISA. Error bars represent SEM with 7 animals/group. gD+VTA+CpG is better than gD in PBS and gD+CpG in PBS, gD+Emul+CpG is better than gD in PBS, *$p<0.01$ by one-way ANOVA and Tukey's multiple comparison test.

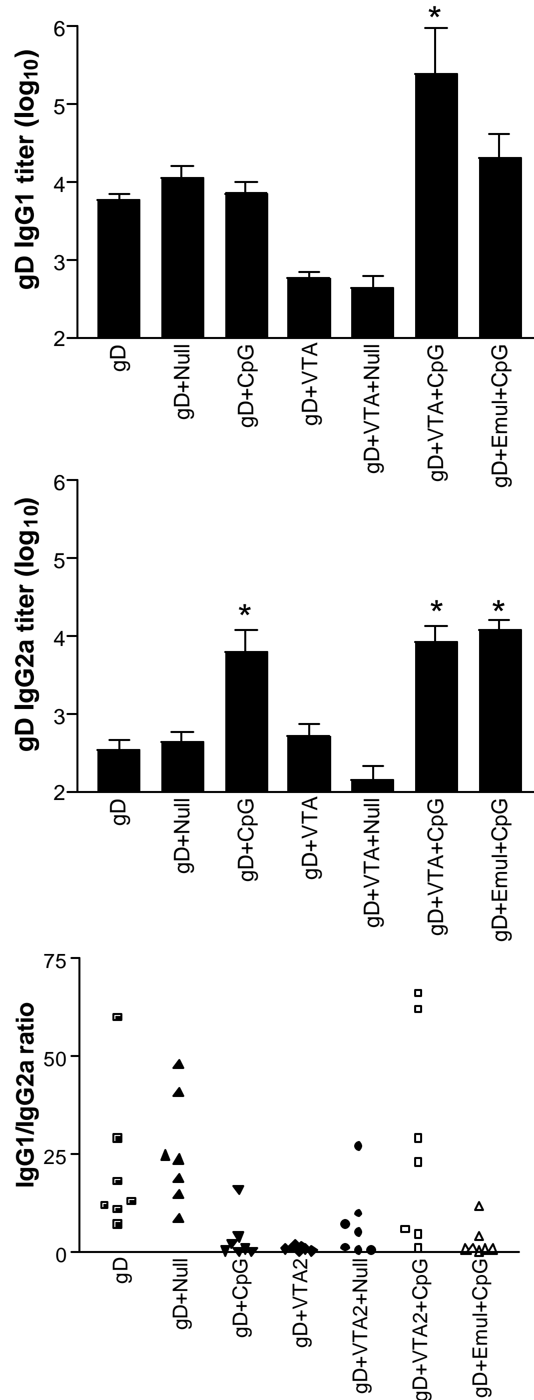

Figure 8.2 Effect of CpG ODNs on IgG1 and IgG2a antibody isotypes following subcutaneous immunization.

All animals were immunized twice subcutaneously at two week intervals with 0.5 μg gD. Total gD-specific IgG1, IgG2a titers and IgG1/IgG2a isotype ratios were determined from serum two weeks after the boost by ELISA. Error bars represent SEM with 7 animals/group, * $p<0.01$ compared to gD in PBS by one-way ANOVA and Tukey's multiple comparison test.

Table 8.1 Protection from HSV-1 scratch challenge

Group	Highest clinical score*	Survival
gD and non-CpG ODNs (PBS)	4	2/5
gD and CpG ODNs (PBS)	3	5/5
VTA gD and CpG ODNs	1	5/5
CpG alone	5	0/5
Naive	5	0/5

* Clinical scores increase with an increase in severity with higher numbers

In a separate experiment mice were immunized subcutaneously twice with gD in the formulations indicated above at two week intervals. Mice were challenged with HSV-1 two weeks after the boost.

8.3.3 Formulation of CpG ODNs in VTA enhances cytokine secretion

One possible mechanism by which VTA enhance the immuno-adjuvant activity of CpG ODNs is by increasing bioavailability and consequently biological effect. Therefore, it was assessed whether formulation of CpG ODNs in VTA resulted in an increase in IL-12 when administered *in vivo*. Mice were administered with CpG ODNs (2116) in saline or formulated in VTA by intraperitoneal injection and eight hours later the levels of IL-12 were determined in the plasma of treated animals, using an ELISA. The levels of IL-12 in plasma were significantly higher in animals that received CpG ODNs (2116) formulated in VTA when compared to those administered CpG ODNs (2116) in saline (Figure 8.3).

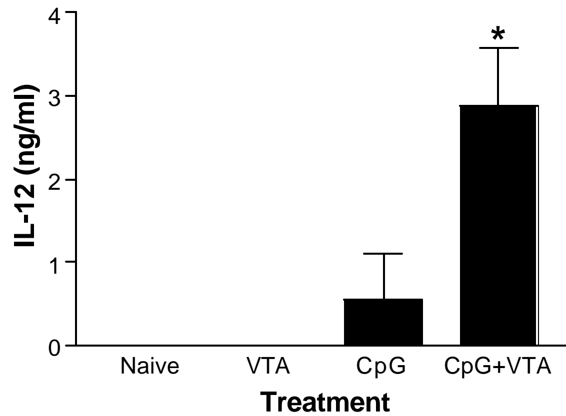

Figure 8.3 Effect of VTA formulation on IL-12 induction by CpG.

Mice were administered VTA alone, 50 μg CpG (2116) in PBS or 50 μg CpG (2116) formulated in VTA by intraperitoneal injection. Eight hours after administration of CpG, plasma was collected and the IL-12 concentration was determined. Error bars represent SD with 6 animals/group, * $p<0.01$ compared to CpG in PBS by one-way ANOVA and Tukey's multiple comparison test.

8.3.4 VTA formulations are safer than Emulsigen

To evaluate the safety of CpG ODNs and VTA formulations, skin tissue samples were obtained from the inoculation site 1, 7, 14 and 29 days following subcutaneous administration, and examined to characterize the type and the inflammatory cell response (Figure 8.4).

At one day after administration, CpG ODNs (1826) in saline caused a mild infiltration of macrophages into the hypodermis. Severe cellular infiltration of macrophages was observed in the hypodermis following subcutaneous inoculation with CpG (1826) in VTA or in Emulsigen, and VTA administered alone. The inflammatory response consisted of macrophages and neutrophils in the hypodermis. Administration of VTA with or without CpG and Emulsigen with CpG resulted in macrophages swollen with multiple vacuoles consistent with phagocytosis of lipid or oil.

At 7 and 14 days following administration, all treatments resulted in a severe subcutaneous cellular infiltration, predominantly of macrophages. Swollen vacuolated macrophages were observed in the groups that received VTA and Emulsigen. When CpG was delivered in Emulsigen, macrophages and neutrophils surrounded large clear vacuoles, interpreted to be oil droplets.

Twenty-nine days after the administration of CpG in saline, the skin appeared normal, whereas at the inoculation site of CpG in VTA there was a moderate cellular infiltration of lesser severity than at the previous time points, suggesting a resolution of the inflammatory response (Figure 8.5). However, in tissue samples obtained from mice treated with Emulsigen and CpG lesions were severe, characterized by the presence of

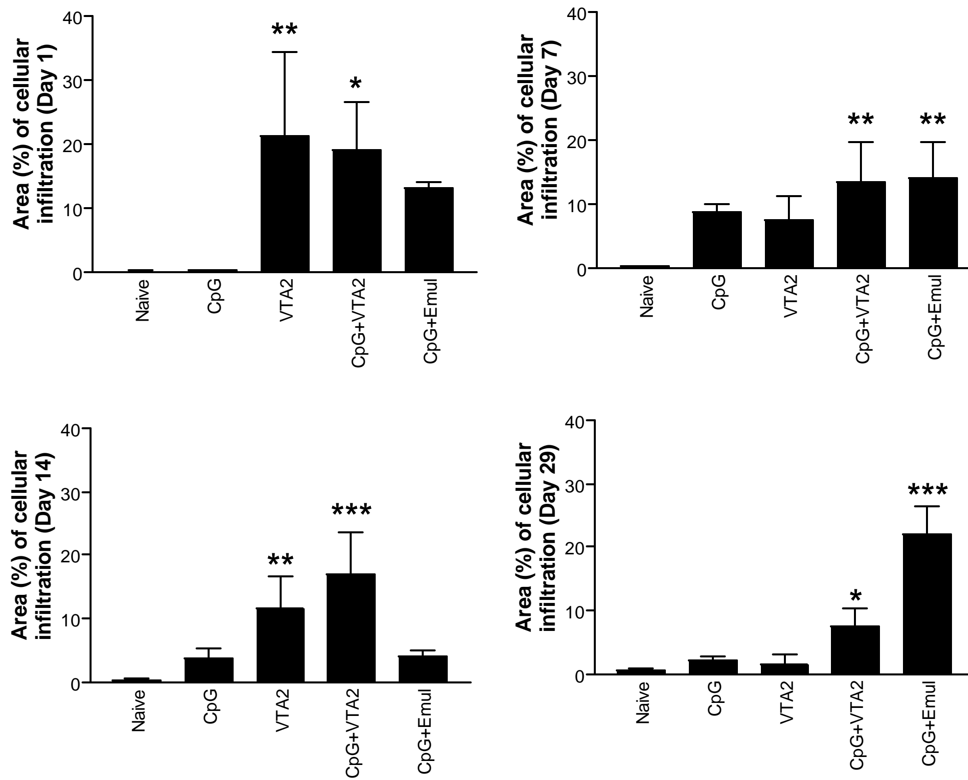

Figure 8.4 Cellular infiltration in skin following administration of CpG in different formulations.

Following administration of CpG in different formulations, skin was examined at 1, 7, 14 and 29 days after injection using HE sections. Cellular infiltration was determined by calculating the area of blue stain in the cutis as an indirect method to determine cellular infiltration. *P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA followed by Tukey's multiple comparison test compared to naive.

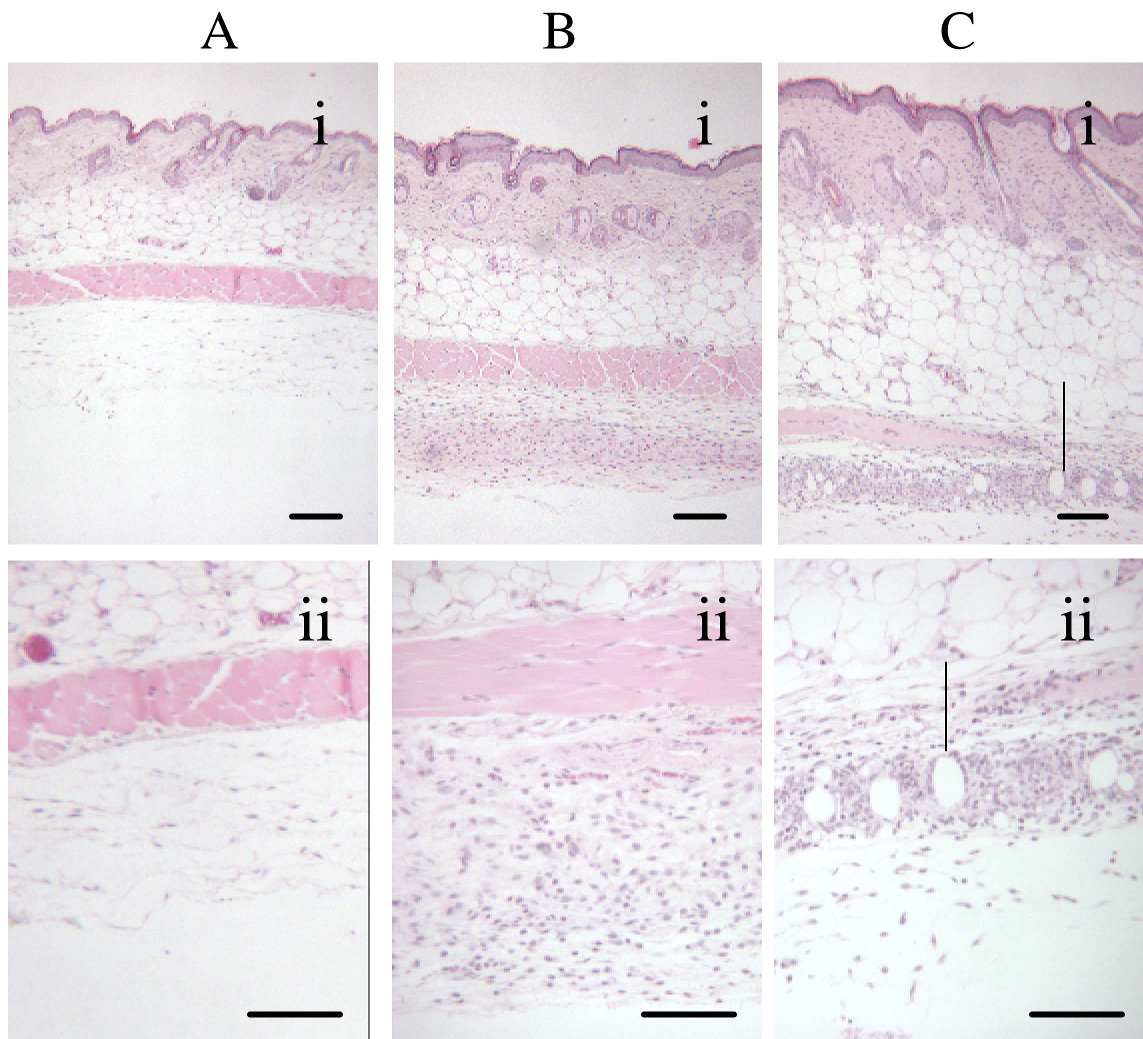

Figure 8.5 Effect of different vaccine formulations on mouse skin.

Mouse skin (HE stain) 29 days after administration of PBS (A), CpG in VTA (B), and CpG in Emulsigen (C). No change in cellularity was observed following PBS administration (A -i and ii). Infiltration of macrophages was observed in both treatments, mainly in the hypodermis (B and C -i and ii). Macrophages and neutrophils surrounded large clear vacuoles (presumed oil) indicated by arrow in (C -i and ii). Bar is 100 µm.

large numbers of macrophages and neutrophils surrounding large clear vacuoles (Figure 8.5).

8.3.5 VTA formulations enhance mucosal immune responses induced by CpG ODNs.

To evaluate the effect of VTA formulation in enhancing the immunoadjuvant effect of CpG ODNs (1826) following intranasal administration, gD-specific IgA in the lung and nasal secretions was determined. The gD-specific IgA responses in the lungs and nasal cavity induced by immunization with gD and CpG ODNs (1826) in VTA were significantly higher than those induced by gD and CpG (1826) (Figure 8.6).

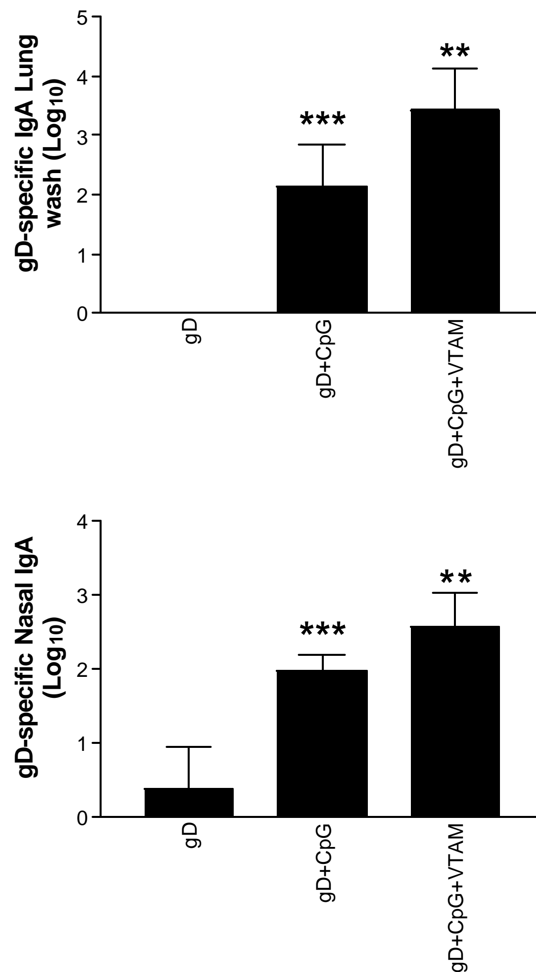

Figure 8.6 Effect of CpG and formulation on mucosal immune responses.

Mice were immunized and boosted intranasally at two-week intervals. Two weeks after the boost lung and nasal washes was characterized for gD-specific IgA. Error bars represent SD with 8 animals/group, gD vs gD + CpG (***p<0.001) and gD + CpG vs gD + CpG + VTAM (**p<0.01) for both lung and nasal IgA by one-way ANOVA and Tukey's multiple test.

8.4 Discussion

Numerous reports have demonstrated the immunostimulatory properties of CpG (Davis *et al*., 1998); however, the majority of these use CpG administered systemically in PBS. It has been previously reported that oils can improve the immuno-adjuvant activity of CpG ODNs (Jones *et al*., 1999; Sun *et al*., 1998). The results of this study demonstrated that formulation of CpG ODNs in Emulsigen enhance antibody responses. Also, VTA formulations enhanced the immuno-adjuvant activity of CpG and are safer than non-metabolizable oil adjuvants. Both VTA and Emulsigen cause severe cellular infiltration at early time points. However, 29 days following administration skin administered VTA formulations was healing, whereas skin injected with Emulsigen was not resolving. Formulation of CpG and gD in VTA increased IgG responses and protection from HSV-1 challenge.

The mechanism of enhancement of CpG immuno-adjuvant activity likely differs between Emulsigen and VTA. Emulsigen creates a depot effect of antigen and CpG ODNs. Although the exact mechanism of enhancement created by VTA formulations is currently unknown, it is likely that VTA formulations may: (1) increase antigen and CpG delivery to APCs or (2) protect CpG ODNs from degradation. These effects could 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. This is supported by the formulation of CpG in VTA increasing IL-12 cytokine production compared to CpG administered in saline, indicating that CpG ODNs in complexed lipid increase the number of CpG ODNs that stimulate APCs. In contrast, VTA formulation

alone did not induce IL-12 production indicating that VTA formulations are not intrinsically stimulatory. One possible mechanism by which VTA formulations enhance the immunoadjuvant effect of CpG ODNs is by increasing the amount of CpG ODNs that interact with Toll-like receptor 9 (TLR9) (Hemmi *et al*., 2000). Since TLR9 is located on the inside of the endosomal membrane, 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.

CpG ODNs have been previously shown to be potent inducers of Th1 type immune responses to numerous antigens (Chu *et al*., 1997; Stacey and Blackwell, 1999). Results here are in agreement with these studies. Using gD as an antigen, CpG ODNs significantly increased gD-specific IgG2a antibody titers following subcutaneous and mucosal immunization. Non-CpG ODNs administered subcutaneously with gD did not show any immuno-adjuvant effect.

There are a few reports have shown CpG ODNs as a mucosal adjuvant (Gallichan *et al*., 2001; McCluskie and Davis, 1999; McCluskie and Davis, 2000; McCluskie *et al*., 2001). However, there are no reports of CpG ODNs administered in lipids by the mucosal route. The results here, confirmed previous observations that CpG are effective mucosal adjuvants and further indicate that VTA formulations are suitable delivery systems to enhance the immunoadjuvant activity of CpG ODNs. VTA formulations may increase the uptake of CpG ODNs at mucosal surfaces and APCs in the mucosa. Thus VTA formulations may be useful for delivering CpG ODNs for immune modulation of allergies.

Histological examination of skin showed that VTA formulations do not cause long-term inflammation, whereas Emulsigen formulations cause severe tissue damage 29 days following injection. Since VTA formulations are made from metabolizable plant and synthetic lipids they are safer than Emulsigen (non-metabolizable oil).

Formulation of CpG ODNs may be critical for CpG to be an effective adjuvant. VTA formulations are as effective as, and safer than conventional oil adjuvants. These promising results with VTA formulations are currently being evaluated in large animals.

9.0 GENERAL DISCUSSION AND OVERALL CONCLUSIONS

9.1 DNA vaccination: an ideal concept with tremendous technical obstacles

The concept of DNA vaccination is simply to transfect host cells with plasmid vectors encoding vaccine antigens to induce an immune response. Since it results in antigen being produced by host cells, it mimics intracellular pathogens allowing antigen presentation on both Class I and Class II MHCs. The Achilles' heel for DNA vaccines is delivery of plasmid vectors in an effective way to increase the amount of protein produced *in vivo*. The efficacy of DNA vaccination is dependent on the quantity of antigen produced *in vivo*. Since the amount of antigen produced from plasmid vectors is significantly lower compared to subunit and viral approaches, it is not surprising that DNA vaccination is not as effective at inducing antibody responses. This was illustrated in Chapter 4 by a direct comparison of a DNA vaccine encoding HBsAg with the commercial HBsAg subunit vaccine, where the commercial protein vaccine was superior to the DNA vaccine. The reason for this result may be that the low amount of antigen is the critical limiting factor for DNA vaccines to elicit immune responses in large animals. For DNA vaccines a threshold level of antigen must be processed and presented by APCs. This can be achieved either (1) efficiently by direct transfection of APCs, or (2) inefficiently by production of antigen in non-lymphoid cells, followed by cross-presentation to APCs. These are the two different possibilities for enhancing antigen presentation with DNA vaccines.

9.2 Discussion of different delivery methods for DNA based vaccines and the critical role of gene expression for inducing immune responses

A plasmid is not antigen itself; rather it contains the instructions to produce an encoded antigen. Therefore, for DNA immunization, it is not the dose of plasmid that is important, but how much antigen is produced from the plasmid *in vivo.* Although DNA-based vaccines have been shown to be effective in several mouse models, the results in large animals have been disappointing. Immune responses to DNA vaccines were consistently achieved in mice using lower doses of plasmids (1 µg), whereas in pigs, immune responses were inconsistent, with only a fraction of the animals responding to orders of magnitude more plasmid (1 mg). One possible reason for this result may be a difference between the two species in susceptibility to transfection. It appears that mouse skin and muscle cells are much more permissive to transfection than the same tissues in larger animals. This was illustrated by the luciferase gene expression experiments. Luciferase levels of 10^6 RLU were obtained in mouse skin using 1 µg of plasmid by intradermal injection whereas luciferase gene expression levels of 10^5 RLU were measured in porcine skin using 100 µg of plasmid by intradermal injection. In addition, the volumes of diluent used to administer the plasmids in mice were lower than the volume used for pigs [increasing the volume of diluent, has been shown to increase the level of gene expression in mice (Dupuis *et al*., 2000)]. Even though there are differences in the size between biopsies from the different species compared, the size of the tissue biopsy is irrelevant as long as the entire injection site is sampled, since the level of reporter gene expression does not depend on the size of the tissue, only on the amount of reporter gene produced. Clearly, transfection and gene expression in skin is

more efficient in mice than large animals. The mechanism by which mouse skin is more easily transfected compared to porcine skin is unknown, just as the reason why different cell types are more easily transfected *in vitro* is also unknown. However, in general, the transfection of cells *in vivo* in all species is a rare event. In mice, high concentrations of plasmids are needed to transfect a small number of cells, and these events are even less frequent in large animals. If exogenous DNA could transfect cells efficiently, it would have a tremendous impact on evolution, especially if germ line cells were transfected. Therefore it is advantageous that transfection is a rare occurrence.

The low efficiency of transfection in all species has led to the development of different delivery devices for increasing gene expression and enhancing immune responses. These devices were directly compared to conventional needle injection with respect to gene expression and immune responses. Delivery of plasmid using BioJect as well as surface electroporation following intradermal injection enhanced gene expression by one logarithmic order of magnitude (10^6 RLU by BioJect and 10^6 RLU by surface electroporation following intradermal injection vs 10^5 RLU by conventional injection) in porcine skin. The combination of BioJect delivery and surface electroporation further enhanced gene expression (10^7 RLU) and increased the number of animals responding after the second boost to a HBsAg DNA vaccine (2/5 vs 0/5 with conventional injection of the DNA vaccine). Electroporation of muscle tissue using a six-needle array was more effective in increasing luciferase gene expression in muscle (10^8 RLU) compared to needle-free surface electroporation applied to the skin (10^7 RLU). In addition, electroporation using the six-needle array enhanced the immune responses to the HBsAg DNA vaccine in 6/6 animals assayed by the clinical Abbott

HBsAg ELISA. These studies together demonstrate the critical role of the level of gene expression for induction of immune responses to DNA vaccines.

Delivery devices such as BioJect and permeability enhancers such as electroporation possibly work by dispersing plasmid throughout the tissue more effectively than conventional injection. This may allow for increased gene expression due to plasmid uptake into more cells. In addition, it is also possible to disrupt membrane barriers by administering plasmids using the gene gun, jet injection or electroporation. Although these methods use different physical techniques and enhance gene expression by different magnitudes compared to conventional injection, the principles for improved delivery such as spreading plasmid throughout the tissue and disrupting cell membranes remain the same with these devices. In addition, the delivery devices may also influence the kinetics of gene expression. For example, jet injection may administer the plasmid deeper into the skin compared to the gene gun delivery. Transfected cells in the epidermis are sloughed off, whereas cells found in the dermis of the skin are not. However, even with electroporation, DNA based vaccines still produce less antigen than recombinant protein subunit vaccines. Thus if it were possible for DNA based vaccines to produce enough antigen, these vaccines could be at least as effective as intramuscular injection of subunit vaccines for eliciting antibody responses, and superior for inducing CTL responses.

Another approach to enhance the efficacy of DNA vaccines is to enhance antigen presentation by direct transfection of APCs. This can be achieved by delivering plasmids to the region of the skin where dendritic cells are residing and was illustrated

from topical delivery of plasmids formulated in Biphasix™ lipid vesicles. However, this approach did not elicit high gene expression in non-lymphoid cells in the skin.

There are several different approaches to administer DNA based vaccines. Each of these approaches has advantages and disadvantages with regards to efficacy, practicality and acceptability. Table 9.1 shows a comparative ranking of a number of important parameters for all the delivery methods tested in this thesis. Currently there is no delivery method that is effective, easily tolerated and painless. The simplest and most easily tolerated approach would be to apply the vaccine topically. Biojector and gene gun delivery of plasmids showed improvements in immune responses but there are still limitations related to the devices, such as the loading of DNA and the accuracy of dosing. Electroporation was found to be the most effective delivery method for inducing immune responses. However, electroporation is invasive and causes tissue damage, therefore the pursuit of a suitable delivery system for plasmids will still be required.

Table 9.1 Comparison of delivery methods for DNA vaccines in large animals

Method	Efficiency[1]	Pain level	Tissue damage	Comments
Electroporation	++++	severe	severe	Not very practical due to electroporation equipment and need for trained medical personnel
Gene gun	+++	moderate	moderate	Major formulation issues with coating DNA on beads
BioJect	+++	moderate	moderate	Simple; Jet injection device is already licensed
Conventional injection	++	moderate	moderate	Practical; Safety issues associated with accidental needle sticks
Topical administration	+	mild	mild	Simplest and safest method of administration

[1]Efficiency ranking:
+ inconsistent priming of immune responses
++ consistent priming of immune responses
+++ induction of protective immune responses in some animals
++++ induction of protective immune responses in most animals approaching the efficiency of some conventional vaccines

9.3 Epidermal immunization results in the induction of Th2 type immune responses

Different delivery methods to the skin result in different types of immune responses. Topical administration of proteins results in induction of Th2 responses (Baca-Estrada *et al*., 2000). Similarly, plasmid administration following either topical or gene gun administration resulted in elicitation of Th2 type responses, whereas intradermal injection produced Th1 responses. With topical or gene gun administration, plasmid is delivered to the epidermis, unlike intradermal plasmid administration into the dermis. It is likely that Langerhans cells in the epidermis were transfected directly following both topical and gene gun administration of plasmid since luciferase activity was detected in the draining lymph nodes, whereas Langerhans cells were not transfected following intradermal administration of plasmid. A likely hypothesis for why topical and gene gun administration results in Th2 responses following DNA immunization is that antigen presentation by Langerhans cells promoted Th2 responses. The induction of Th2 responses may enhance the disease for some pathogens such as *Leishmania major*. Therefore, it will be necessary to modulate immune responses towards Th1 responses to develop topical vaccines for diseases where Th1 type immune responses are required for protection.

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

When APCs are stimulated by numerous factors such as LPS, CpG ODNs, and danger signals, they become activated, expressing a variety of co-stimulatory molecules that improve antigen presentation to the specific immune system. The activation state of the APCs when they are presenting antigen is critical for the induction of immune responses. Cellular infiltration caused by electroporation and CpG motifs in plasmids likely activate APCs, causing APCs to move to the draining lymph nodes and present the antigen. Immune responses to a DNA vaccine were enhanced following electroporation treatment of muscle, and prior electroporation of muscle enhanced immune responses to a subunit vaccine. These results indicate that damage/danger signals are potent adjuvants.

Cellular infiltration and activation of macrophages by CpG ODNs are mechanisms by which immune responses are enhanced. The increased cellular infiltration caused by formulating CpG ODNs in VTA or Emulsigen is a likely mechanism of how formulation enhances the immuno-adjuvant effect of CpG ODNs.

Some adjuvants such as CpG mimic the signals caused by tissue damage without actually causing tissue damage. These adjuvants stimulate a cascade of signals including cytokines, chemokines, and co-stimulatory molecules, allowing cellular infiltration and activation of APCs.

9.5 Lipid-based formulation of CpG ODNs

Lipid-based formulations may enhance the biological activity of CpG ODNs by several possible mechanisms: (1) increasing the bioavailability of CpG ODNs by preventing dilution and degradation *in vivo*, (2) allowing improved delivery of CpG ODNs to APCs due to the particulate nature of lipid-based formulations, and (3) increasing the number of CpG ODNs that can stimulate TLR9 on a single cell level. Together these mechanisms result in a greater stimulation of TLR9 by CpG ODNs increasing the biological effects of CpG ODNs. It was shown that formulation of CpG ODNs in VTA formulations enhanced the biological activity of CpG ODNs as assessed by increased IL-12 cytokine secretion and increased antigen-specific antibody immune responses following delivery in VTA formulations compared to PBS.

9.6 Future of polynucleotide and oligonucleotides based vaccines

9.6.1 DNA vaccines

It was hoped that DNA-based vaccines would allow many new vaccines to be developed. However, DNA-based vaccines are not the panacea they were proposed to be. The hurdle of increasing the potency of DNA-based vaccines in a practical way is still a major problem with significant technical obstacles. Topical administration of plasmids further increases technical obstacles for gene expression. Currently, electroporation is the most effective way to administer DNA-based vaccines, as demonstrated by the results in Chapters 5 and 6. However, electroporation in its current form is quite invasive and would not be used for regular immunization in the general public, although it may have applications for use in cancer immunizations as well as

biodefence immunizations for military personnel since the benefit of immunization in these circumstances would outweigh the temporary pain from electroporation.

In the future, if electroporation could be developed to be minimally invasive, it could gain widespread acceptance. This could be achieved possibly by targeting the epidermis of the skin using microneedles or perhaps in combination with multiple microjet injection. However, currently there are not even prototypes of these devices, therefore they are likely years away from being developed and fully tested.

9.6.2 CpG ODNs as an adjuvant

Alum currently is the only approved adjuvant on the market for use in humans in North America, whereas MF59 is approved in Europe for a human influenza vaccine (Gasparini *et al*., 2001). CpG ODNs are now in clinical trials and preliminary results look promising; however, time will reveal if CpG ODNs will become a licensed product. In this project, it was found (see Chapter 8) that simply mixing CpG ODNs with the antigen is not as effective at inducing antibody immune responses as formulating CpG ODNs in VTA formulations. For CpG ODNs to become a successful adjuvant for multiple vaccine antigens a suitable delivery vehicle will be required.

9.7 Future research directions

There are several potential directions where future research can build on the work from this thesis. These include continuing the investigation of delivery of plasmids and further test new delivery methods such as magnetofection (Scherer *et al*., 2002), based on principles of distribution and membrane disruption. Currently,

increasing the distribution of plasmids as well as techniques that temporarily disrupt membranes are the most promising approaches to enhance the gene expression of plasmids *in vivo*. Physical methods of plasmid administration are likely to be more effective compared to molecular targeting approaches such as peptides, polymers or liposomes, that could theoretically enhance entry into the cell by receptor mediated endocytosis.

Another direction in research could be the molecular characterization of danger signals caused by electroporation. By comparing gene expression profiles between normal and electroporated muscle tissue using DNA microarray analysis, the genes involved in tissue damage/danger signals could be identified, which may lead to the understanding of how adjuvants work and how new adjuvants can be developed.

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