IN UTERO ORAL DNA IMMUNIZATION: INDUCTION OF SPECIFIC IMMUNITY IN THE SECOND TRIMESTER OVINE FETUS

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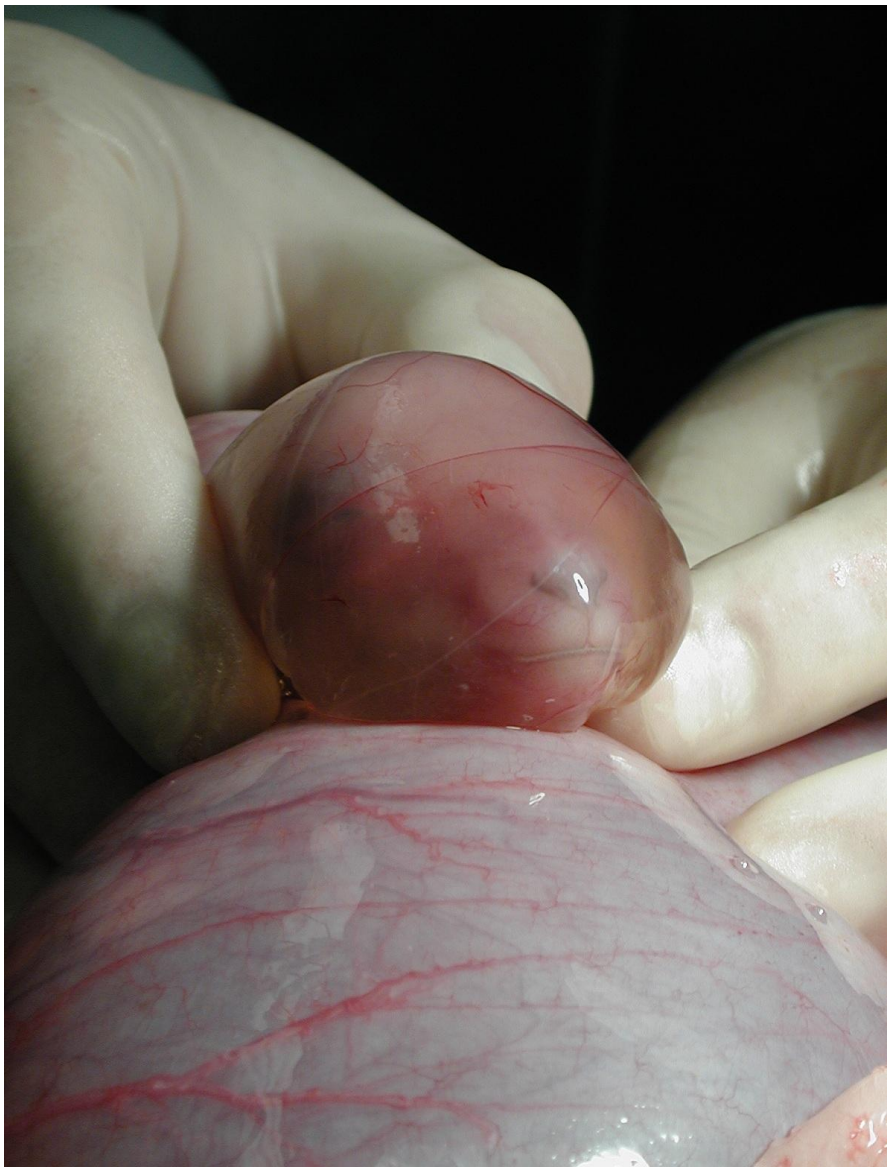

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ABSTRACT

Vaccination has proven a cost-effective method of managing infectious diseases, but attempts to develop an effective fetal vaccine have proven difficult due to the immaturity of the immune system and the propensity of the developing immune system to induce tolerance to immunizing antigens. This thesis is concerned with the induction of specific immunity in the second trimester ovine fetus using the oral DNA immunization method. *In utero* oral delivery of naked DNA plasmid was selected as the method of immunization due to previous successes in the third trimester ovine fetus and the immunostimulatory properties of the bacterial DNA backbone, which may help overcome developmental tolerance. Transfection and expression studies in the third trimester ovine fetus revealed the oral mucosal epithelium as the primary site of transgene expression and functionally active antigen was also localized to lymph nodes draining the oral cavity. Efficient transfection and expression of plasmid following oral delivery was specific to the fetus and correlated with a lesser degree of epithelial differentiation. Oral DNA delivery in the second trimester resulted in detection of transgene activity in 100% of treated fetuses and the level of transgene activity was greater than in fetuses treated in the mid-third trimester. Using a plasmid encoding the gene for bovine herpesvirus-1 truncated glycoprotein D (tgD), immunization studies were then conducted in the second trimester fetus. A new lower age limit for fetal immunization was established at 55-60 days gestation (gestation period is 148 days), which coincides with the appearance of lymphocytes in peripheral tissues. Antigen-specific antibody, interferon-γ responses and/or neonatal anamnestic responses were

detected in 66% of fetuses immunized between 55 and 84 days gestation. The duration of fetal primary immune responses was equivalent to that achieved in young lambs following optimized DNA vaccination, but the magnitude of fetal immune responses was limited. The persistence of immune memory from the second trimester to birth was consistent with experimental data which showed that the duration of immune memory had a stronger correlation to the duration, as compared to the magnitude, of the primary antibody response. Overall, the experiments within showed that oral DNA immunization of the early second trimester fetus is feasible and not associated with the induction of tolerance. These findings suggest that it may be possible to protect against mother-to-child transmission of infectious pathogens by targeting protection at the level of the fetus.

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LIST OF ABBREVIATIONS

AIRE	Autoimmune regulator
APC	Antigen-presenting cell
BCR	B cell receptor
BHV-1	Bovine herpesvirus-1
BSA	Bovine serum albumin
CD	Cellular differentiating factor
CDR	Complementarity-determining region
CFA	Complete Freund's adjuvant
CI	Confidence interval
CpG(s)	Phosphorylated-cytosine guanine motif(s)
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dg	Days gestation
DMEM	Dulbecco's modified Eagle's medium
DMRI/DOPE	(N-(1-(2,3-dimyristyloxypropyl)-N,N-dimethyl-(2-hydroxyethyl) ammonium bromide/dieoyl phosphatidylethanolamine
DNA	Deoxyribonucleic acid
dNFB	Dinitro-fluorbenzene
dpi	Days post-immunization/injection
DTH	Delayed-type hypersensitivity
EAU	Experimental autoimmune uveitis
EHV-1	Equine herpesvirus-1
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
fDC	Follicular dendritic cell
GALT	Gut-associated lymphoid tissue
gB	Glycoprotein B
GC	Germinal center
gD	Glycoprotein D
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBsAg	Hepatitis B surface antigen
HIV	Human immunodeficiency virus
ID	Intradermal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
LCMV	Lymphocytic choriomeningitis virus

LN(s)	Lymph node(s)
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
mCTL	Memory cytotoxic T lymphocyte
MDBK	Madin-Darby bovine kidney
MEM	Modified Eagle's medium
MHC	Major histocompatibility complex
MLV	Modified-live virus
MTEC	Medullary thymic epithelial cell
MuLV	Moloney's murine leukemia virus
ND	Not determined
NeoPT	Neomycin phosphotransferase
NS	Not specified
ODN	Oligodeoxyribonucleotide
PCR	Polymerase chain reaction
PP	Peyer's patch
RLP	Recirculating lymphocyte pool
RSV	Rous sarcoma virus
SCv	Superficial cervical
SD	Standard deviation(s)
sIg	Surface immunoglobulin
SIV	Simian immunodeficiency virus
TCR	T cell receptor
tgD	truncated glycoprotein D
Th	CD4+ T helper cell
Th1	Type-1 CD4+ T helper cell
Th2	Type-2 CD4+ T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
ULA	Units luciferase activity
V gene	Variable region gene
VIDO	Vaccine and Infectious Disease Organization
VLP	Virus-like particle
WHO	World Health Organization
wpi	Weeks post-immunization/injection

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Vaccination has proven to be a cost-effective method of managing infectious diseases; however, to date, advances in disease prevention and the development of vaccines have failed to decrease the rate of infection by vertically transmitted and neonatal pathogens, such as human immunodeficiency virus (HIV), hepatitis and *Chlamydia spp.*. In 2005, an estimated 700,000 infants worldwide became infected with HIV, primarily through mother-to-child transmission (UNAIDS/WHO 2005). In 2000, the mortality rate of children under the age of 5 in areas such as sub-Sahara Africa was estimated at 9.5%, with the majority of deaths occurring in the first year of life (Ventor 2002). The rapid rate of viral and bacterial replication in the immunologically naïve fetus or neonate may result in such an overwhelming pathogen load that vaccinating the infant after infection may result in the naïve immune system being unable to catch up with the rate of disease progression. An alternative strategy to infant vaccination or prophylaxis is the development of an effective fetal vaccine that would prevent *in utero* disease transmission and extend protection to the neonatal period. A number of obstacles must be overcome before an effective fetal vaccine can be developed. These include: (i) maternal antibodies, which interfere with subunit and killed vaccines before they can be

properly presented to the fetal immune system (Reynolds and Griffin 1990; van Maanen, Bruin et al. 1992; Blasco, Lambot et al. 2001); (ii) high cortisol levels (Munroe 1971; Berdusco, Hammond et al. 1993; Nathaniels 1994) and immaturity of the immune system, both of which can skew responses towards a type-2 response, which in many cases are ineffective and in some cases pathological (Bondada, Wu et al. 2001; Liu, Tu et al. 2001; Liu, Tu et al. 2001); (iii) low numbers of B cells and immature memory, requiring repeated boosting (Siegrist 2001); and (iv) the immaturity of the immune system, which is unable to deliver adequate co-stimulatory signals and, therefore, increases the risk of tolerance induction (Bondada, Wu et al. 2001; Tschernig, Debertin et al. 2001; Matzinger 2002). There is substantial data in the literature to suggest that it is possible to design an effective fetal immunization protocol, and induction of either tolerance or protective immunity is dependent upon a number of combined factors, including the route, dose and nature of the vaccinating antigen and the gestational age of the fetus at the time of immunization. As these factors appear to be species specific, an animal model that is relevant to the human developing immune system is also required.

This thesis is concerned with the induction of antigen-specific immune responses in the early ovine fetus. We specifically asked whether a vaccine could be delivered in such a manner as to avoid the induction of tolerance in the second trimester ovine fetus and whether the second trimester ovine fetus would respond to DNA immunization by developing an antigen-specific protective immune response that persisted into the neonate. The following is a review of the scientific literature pertaining to these questions. As induction of fetal immune responses has often been described as a break in self-tolerance, theories of immune induction with a focus on mechanisms of self/non-

self discrimination are discussed. This is followed by a review of immune memory: without immune memory, neither immune-mediated protection nor tolerance can persist into the neonate. A summary is provided of experiments which have demonstrated either the induction of tolerance or immune protection following fetal antigenic exposure, with particular focus on experiments conducted in the fetal lamb model. Lastly, there is a review of the literature on DNA vaccines, which appear to be a method of antigen delivery well suited to the induction of protective fetal immune responses.

1.2. Tolerance and Immune Protection During Development

There are four ways in which the immune system can respond to an antigen. It can (i) mount a protective immune response characterized by humoral and/or cell-mediated immunity (CMI), (ii) actively suppress humoral and/or CMI responses to an antigen via induction of tolerance, (iii) mount a non-protective immune reponse, or (iv) "ignore" the antigen by neither inducing a protective immune response nor inducing tolerance (Matzinger 2002). Induction of either protection or tolerance depends on whether the immune system perceives an antigen to be self or non-self. Therefore, the following is a short review of the literature pertaining to self/non-self discrimination and how it is regulated during development of the immune system.

1.2.1. Mechanisms of Self-Non-Self discrimination

One of the most widely accepted models of immune induction is the Bretscher-Cohn Two-Signal Theory of Lymphocyte Activation (1970). In addition to describing a mechanism of lymphocyte activation, it also provides a framework for tolerance

induction. According to the original hypothesis, activation of precursor lymphocytes requires the recognition of two linked antigenic epitopes: the first epitope is recognized by the precursor cell (signal 1) and the second epitope is recognized by a carrier antibody (signal 2). In its modern context, signal 1 is provided by either T cell receptor (TCR) recognition of an antigen presented in the context of a major histocompatibility complex (MHC) molecule or recognition of an epitope by surface-bound immunoglobulins (sIg), and signal 2 is delivered by an antigen-presenting cell (APC) usually in the form of a cytokine, such as interleukin (IL)-2, -4, -10 or interferon (IFN)-γ (Swain, McKenzie et al. 1988), or by cell-to-cell contact (e.g., CD28 interaction with B7, or CD40 interaction with CD154). Generation of signal 1 in the absence of signal 2 leads to inactivation of the lymphocyte and immunological tolerance (Bretscher and Cohn 1970). Since signal 2 for B cells is provided by T cells, the Two-Signal Theory also provided a mechanism by which induction of antigen-specific unresponsiveness in the peripheral B cell population could be entirely attributed to antigen-specific tolerance in the T cell compartment (Chiller, Habicht et al. 1971), i.e., that central tolerance could be achieved by T cell regulation alone.

Within the framework of the Two-Signal Model, it was hypothesized that CD4+ T helper (Th) cells played a large role in determining whether the immune response deviated to either a humoral or CMI response (Parish 1972; Zinkernagel and Doherty 1975). In an inflammatory environment, signal 2 includes IFNγ which results in the preferential induction of type-1 CD4+ T helper (Th1) cells and CMI. Alternately, the presence of IL-4 and -10 during antigen presentation results in the induction of type-2 CD4+ T helper (Th2) cells and humoral immunity. What is often perceived as tolerance

may actually be immune deviation (Oldstone and Dixon 1967). This is especially true for tolerance to many intracellular pathogens, where lack of CMI results from the induction of a non-protective antibody response. Deviation towards a Th2 response can also result in allogeneic graft acceptance, as demonstrated in miniature swine (Haller, Esnaola et al. 1999). One method of inducing antibody responses (and Th2 immune deviation) is the intravenous (IV) administration of high doses of an antigen in the absence of adjuvant, i.e., high dose tolerance (Power, Wei et al. 1998).

A modification of the Two-Signal Theory is Matzinger's Danger Hypothesis (2002). According to this model, antigens are regarded as either non-infectious self (tolerized) or infectious non-self (not tolerized). The latter category includes antigens that cause cell stress or lytic cell death. Often, for an antigen to be recognized as infectious non-self, a third signal is required that warns the immune system of imminent danger. This "danger signal" can be pathogen-derived or endogenously-expressed and is required for activation of signal 2, thus providing an alternative mechanism for self/non-self discrimination. In the absence of a danger signal, an antigen is perceived as non-infectious self and tolerized. Examples of this third signal include bacterial lipopolysaccharides (LPS), unmethylated cytosine-guanine motifs (CpGs), inflammatory mediators and defensins, all of which have been utilized as vaccine adjuvants.

There are many experimental examples of adjuvants breaking tolerance to self-antigens, e.g., (i) administration of myelin basic protein in complete Freund's adjuvant (CFA) induced experimental autoimmune encephalomyelitis in mice (Panitch and Ciccone 1981), and (ii) immunization with retinal S antigen in either CFA or pertussis toxin induced experimental autoimmune uveoretinitis (EAU) in mice (McAllister,

Vistica et al. 1986). In contrast, when retinal S antigen was delivered with incomplete Freund's adjuvant, mice were immunized against EAU induction. Each danger signal or adjuvant mediates its activity via a specific pathway. For example, APCs can be stimulated by activating toll-like receptors (TLRs), which are expressed in or on the surface of APCs, and this results in the delivery of signal 2. The Danger Model also bears striking resemblance to Cohen's Immunological Homunculus model (1992), according to which, protective immune responses are induced when antigens are presented to naïve T cells in the presence of pro-inflammatory mediators. In his model, Cohen paid particular attention to the role of endogenous heat shock proteins as inducers of co-stimulatory signals in infection and autoimmunity.

1.2.2. Developmental Tolerance

The concept that self versus non-self is established during ontogeny of the immune system was first proposed in the Natural Selection Theory of Antibody Formation (Jerne 1955). According to this theory, the random generation of natural antibodies results in a broad immunological repertoire that includes self-reactivity and to prevent autoimmunity, autoreactive antibodies are deleted during ontogeny while the immune system is immature. These postulates were expanded upon in the Clonal Theory of Selection, in which Burnet (1959) proposed that deletion of self-reactive antibody-forming cells occurs during ontogeny as a result of immature cells undergoing a period of hyper-responsiveness to antigen-induced suppression or deletion. As a result, exposure to an antigen sufficiently early in life results in a reduced ability to react to that antigen at a later period in life. As a corollary, a B cell can only mature in an

environment devoid of its corresponding antigen. This process was described by Burnet as “actively acquired tolerance”.

The notion of a developmental tolerogenic period has become immunological dogma on the basis of a few seminal experiments. One of the earliest reports of immunological tolerance was written by Traub (1938), who observed that *in utero* or neonatal infection of mice with lymphocytic choriomeningitis virus (LCMV) resulted in a percentage of mice becoming chronically infected. *In utero* infection also resulted in the lowest levels of LCMV-specific serum antibodies, the least immune-mediated pathology and the highest levels of viremia for the longest duration. In contrast, adult mice infected with LCMV experienced a subclinical infection and virus was cleared in approximately 3 weeks. Traub interpreted persistent infection as a “failure” or accommodation of the immune system. Similarly, in humans, hepatitis B virus infection in adults results in an acute clinical onset that persists for a few months. In contrast, infants born to infected mothers become chronically infected (WHO 1977) and this outcome is thought to reflect the immunological status of the fetus at the time of infection. Developmental induction of tolerance to MHC alloantigens has been described in dizygotic twin cattle (Owen 1945). Vascular anastomosis between placentas of twins results in erythrocytic chimeras with shared blood types composed of a 50/50 to 60/40 mixture of red blood cells from each of the twins. Chimerism is stable for the lifetime of the animal, thus showing that the erythrocytic precursors from one twin confers tolerance in the congeneic twin. Tolerance also extends to skin grafts from one twin to the other later in life (Rawles 1948). Chimerism between twins is not specific to cattle and has also been described in humans (Dunsford, Bowley et al. 1953).

In 1953, Billingham et al. conducted a series of tolerance studies in mice and chickens. Eleven-day embryonic chicks (incubation period is 21 days) were injected intravenously (IV) with allogeneic blood cells. Three of 5 chicks developed partial tolerance to postnatal skin grafts from the original donor, while the remaining two chicks developed complete tolerance. Similarly, grafting experiments were conducted between CBA and A strain mice. Injection of strain A donor cells into recipient CBA mice at 15-16 days gestation (dg) (gestation length is 20-21 days) resulted in the induction of tolerance to postnatal strain A skin grafts in 3 of 5 mice. Of the three tolerized mice, two were completely tolerant of the postnatal skin graft and the third mouse showed delayed rejection of the graft. In the two mice that failed to develop tolerance to postnatal skin grafts, there was no evidence of an *in utero* immune response to the priming dose of strain A cells. Billingham et al. (1953) referred to this period of fetal development, during which a foreign antigen either failed to induce a detectable immune response or induced specific tolerance, as an "immunological null period". In other experiments, the observation that non-inherited maternal antigens are tolerized by offspring also supports the concept of an immunological null period during fetal development. Exposure of fetal H-2b/b mice to the non-inherited maternal antigen H-2d resulted in graded postnatal tolerance to the H-2d antigen (Andrassy, Kusaka et al. 2003). Postnatal tolerance was demonstrated by the acceptance of H-2d/d heart transplants by 57% of mice exposed as fetuses to the non-inherited maternal antigen and tolerance encompassed $CD8^+$, Th1 (decreased IL-2, IFNγ and IgG2a) and Th2 (IL-5, -4 and IgG1) mediated immune responses.

1.2.3. Regulation of Developmental Tolerance

Tolerance is regulated at two levels (reviewed by Starr, Jameson et al. 2003). The deletion of autoreactive immature thymocytes in the thymus (clonal deletion or thymic deletion) is referred to as central tolerance; however, the continual generation of cells in the periphery, thymic involution and the presence of the blood-thymic barrier, which limits the entry of peripheral antigens into the thymus, necessitate a second tier of immune regulation referred to as peripheral tolerance. Fetal vaccination is concerned with avoiding central tolerance and breaking peripheral tolerance.

The current model suggests that clonal deletion of developing thymocytes occurs as a result of high affinity/avidity interaction of TCRs with (self) antigen-MHC complexes, which results in activation-induced apoptosis (Ashton, Rockardt et al. 1994). Some of the studies that support this model are described here. In one study, pregnant mice expressing the transgene for LCMV-specific TCR were infected with LCMV. Two days after infection, virus particles were detected in fetal thymuses and this was followed by the thymic deletion of both double positive CD4+CD8+ thymocytes and single positive CD8+ thymocytes (Pircher, Burki et al. 1989). Smith et al. (1989), then showed that clonal deletion could be mediated through high affinity binding of the CD3/TCR complex. The addition of anti-CD3 antibodies to organ cultures of fetal mouse thymocytes resulted in apoptosis, with double positive CD4+CD8+ thymocytes accounting for 83% of dying cells. Clonal deletion can also be mediated by engaging only the TCR. TCR-Vβ17a binds to the MHC class-II IE protein with high affinity. Expression of the TCR-Vβ17a transgene in IE mice resulted in the deletion of Vβ17a+ cells from the mature thymocyte and peripheral T cell pools, but not from the immature

thymocyte pool (Kappler, Roehm et al. 1987; Kappler, Wade et al. 1987). More recent studies have implicated several protein kinases in the apoptotic pathway, including a 21 kDa tyrosine kinase activity on the intracellular portion of the TCR, MINK serine-threonine kinase and Jun kinase (McCarty, Paust et al. 2005). Together, these signals lead to activation of the apoptotic pathway.

Clonal deletion can occur during either the double negative (CD8-CD4-) stage or as T cells progress to the single positive stage (Starr, Jameson et al. 2003). The stage during which deletion occurs depends on the location of antigen within the thymus. Double-negative thymocytes are located in the cortical region of the thymus, which is an antigen-privileged zone due to the presence of the thymic-blood barrier that functions to reduce the number of peripheral antigens entering the cortical parenchyma. As with all thymocytes, negative selection is mediated by high affinity binding of the TCR (Viret C, Sant'Angelo et al. 2001). Due to the presence of the blood-thymic barrier, there is little negative selection of double negative T cells specific for self-antigens. This is consistent with their role in the periphery, which is to suppress autoreactive T cells (Priatel, Utting et al. 2001).

In comparison, deletion of single positive (CD4+ or CD8+) thymocytes occurs mostly in the thymic medulla. Experimental evidence has suggested that self-antigens are presented on the surface of either medullary thymic epithelial cells (MTECs) or bone marrow-derived APCs, such as dendritic cells (DCs) and monocytes (Gallegos and Bevan 2004). Interaction with APCs is required for the deletion of CD4+ thymocytes and, to a lesser extent, CD8+ thymocytes. APCs can acquire peripheral antigens en route from the bone marrow (Zinkernagel, Ehl et al. 1997) or, in species with hemichorial

placentation, antigens might be delivered to the thymic medulla via maternal cells entering the fetal circulation (Andrassy, Kusaka et al. 2003). MTECs, which are primarily involved in the deletion of CD8+ thymocytes, express ectopic antigens under the control of the autoimmune regulator (AIRE) protein (Sospedra, Ferrer-Francesch et al. 1998). These tissue-specific antigens include anatomically privileged antigens, such as myelin basic protein and retinal S antigen, and antigens that are expressed only at certain stages of development, including pregnancy and lactation-associated antigens that are expressed after thymic involution. MTECs have a reduced ability to express co-stimulatory signals relative to other APCs (Anderson, Venanzi et al. 2005) and, therefore, are ideal for presentation of self-antigen for the purpose of clonal deletion. Deficiency in AIRE has been shown to result in increased numbers of autoreactive CD8+ and CD4+ T cells, as well as an increase in the incidence of multi-organ autoimmune disease in both mice and humans (Aaltonen, Bjorses et al. 1997; Anderson, Venanzi et al. 2002). Although clonal deletion has been formally demonstrated for anti-hapten, anti-viral and anti-MHC thymocytes (Kappler, Roehm et al. 1987; Smith, Williams et al. 1989), thymic exposure to antigen could also result in the induction of an anergic state or the positive selection of antigen-specific suppressor cells (Schönrich, Momburg et al. 1992; Jordan, Boesteanu et al. 2001).

Clonal deletion may also occur in the bone marrow of mice, resulting in B cell tolerance. Experiments in cytochrome c-transgenic mice have shown that the pre-B cell pool contains many clonal precursors specific for cytochrome c, the number being analogous to that for a comparable foreign peptide (Jemmerson, Minnerath et al. 1998). In contrast, there are far fewer cytochrome c-specific cells in the mature B cell pool, thus

suggesting that an *in vivo* purging of the self-reactive repertoire occurs during the pre-B to B transition. Exposure of pre-B cells to self-antigen in the presence of T cell help (signal 1 plus signal 2) can override the tolerogenic potential of signal 1 alone and result in antibody-producing clones (Linton, Rudie et al. 1991). The sensitivity of the B cell to down-regulation appears to depend upon the stage of cellular differentiation (Nossal 1983). In order of decreasing susceptibility, these are: cells in transition from the pre-B to B cell stage (self-antigens first interact with developing B cells at this stage), immature B cells, mature B cells and plasma cells. Despite mechanisms for clonal deletion of autoreactive B cells, B cell tolerance is achieved, for the most part, through tolerance in the T cell compartment (Chiller, Habicht et al. 1971).

Mechanisms other than clonal deletion may also limit fetal immune responses to antigen exposure. These include: (i) immaturity of the immune system and the low frequency of lymphocytes present during development, (ii) lack of immune memory resulting in a higher threshold for lymphocyte activation, (iii) interference by maternal antibodies, and (iv) general suppression of the immune system by periparturient increases in cortisol levels (Anderson, Flint et al. 1975). Corticosteroid-induced apoptosis of thymocytes may result in the elimination of immature thymocytes specific for new maternal and environmental antigens immediately before birth (Munroe 1971; Cahill, Kimpton et al. 1997).

Experiments with human cord blood-derived APCs have also shown a reduced capacity for antigen presentation in the fetus. Cord-blood derived B cells expressed lower levels of MHC class-II and CD86 on their surface, and demonstrated reduced antigen-specific proliferation relative to adult-derived B cells. Both of these deficits

could be overcome, however, by stimulation with CpGs (Tasker and Mashall-Clarke 2003); but, CpGs could not overcome homing defects in cord blood-derived B cells (Tasker and Mashall-Clarke 2003). Also, cord blood-derived DCs produced less IFNα upon stimulation with CpGs than adult-derived DCs (DeWit, Olislagers et al. 2004). The induction of peripheral tolerance mechanisms may also be involved in the lack of immune responses following fetal immunization.

1.2.4. Fetal Immunity

The concept of developmental tolerance was established by Billingham et al. (1953), who showed that injecting newborn mice with allogeneic bone marrow cells resulted in the induction of tolerance to future grafts from the same donor. Further studies in the area of fetal and neonatal tolerance suggested that the lack of CMI was due to a quantitative lack in the number of DCs (Liu, Tu et al. 2001; Tschernig, Debertin et al. 2001) and the inability of DCs to deliver appropriate co-stimulatory signals upon activation (Bondada, Wu et al. 2001; Liu, Tu et al. 2001). The limited ability to produce type-1 cytokines might also explain the frequently observed Th2 bias in neonatal immune responses (Adkins, Ghanei et al. 1994; Bondada, Wu et al. 2001).

Many of the studies that support the developmental tolerance dogma have been conducted in the mouse model, using traditional vaccines or transplantation antigens, and a narrow definition of "tolerance". These studies have overshadowed a body of research conducted in large animal, primate and human models which have demonstrated that fetal immune responses can be induced to specific antigens. Unfortunately, studies in these alternative animal models have been limited by

prohibitive costs, facilities and ethical considerations. Interpretation of data obtained from large animal experiments has also been complicated by the high degree of variability among individuals in an outbred species. The first laboratory experiments to demonstrate successful fetal immunization were conducted in the fetal calf (Fennestad and Borg-Petersen 1957) and fetal lamb models (Silverstein, Uhr et al. 1963; Silverstein and Prendergast 1964). Table 1.1 summarizes fetal immunization experiments in animal models other than sheep and a summary of immunization experiments in the fetal lamb model is presented in Section 1.3.

In 1996, Otsyula et al. (Table 1.1) reported that *in utero* immunization of rhesus macaques with attenuated simian immunodeficiency virus (SIV) resulted in the induction of SIV-specific fetal antibody titers and post-natal protection from challenge with pathogenic SIV. There are also studies showing the induction of fetal immunity in premature human infants (Uhr, Dancis et al. 1960; Uhr, Dancis et al. 1962). Post-mortem examination of a 5-month old human fetus that had succumbed to syphilis revealed inflammatory lesions with large numbers of plasma cells and cells with a phenotype consistent with DCs (Silverstein 1962). CD45RO+ memory T cells have also been detected in the cord blood of premature infants (23-29 weeks gestation) with brain injury (Duggan, Maalouf et al. 2001). The antigenic specificity of these memory cells was not determined but, their presence in the preterm infant suggested that priming by a specific antigen had previously occurred.

While there is evidence of antigen-specific fetal immune responsiveness, a review of the literature also suggests that the fetal immune system has a limited antigenic repertoire and minimum immunogenicity threshold to which it can respond.

Table 1.1. Fetal immunization experiments in species other than sheep

Species	*Time of fetal immunization[1] (gestational length in days)*	*Vaccine /route of delivery*	*Method of read-out*	*Response[2]*	*Reference*
Rhesus macaque	65 dg (160-170 d)	Attenuated simian immunodeficiency virus /NS	Protection from virulent SIV challenge at 17 mo of age	2° antibody titers (2/3) but no CTL responses	(Otsyula, Miller et al. 1996)
Rhesus macaque	70-114 dg (160-170 d)	Group 1: 100 mg BSA; Group 2: 1-8 mg BSA in adjuvant /NS	Response to neonatal boost	Group 1: 5/6 macaques were tolerized; Group 2: developed transient fetal antibodies	(Cotes, Hobbs et al. 1966)
Baboon	3 repeats: 90 + 120 + 150 dg (180 d)	Recombivax HB (commercial HBsAg subunit vaccine) /NS	Fetal IgG	6/8	(Watts, Stanley et al. 1999)
Pig	Group 1: 90 dg; Group 2: 104 dg (114 d)	HBsAg DNA vaccine /IM	Fetal antibody and neonatal memory	Group 1: Precolostrum titers (5/9); Group 2: no responses (7/7)	(Fazio, Ria et al. 2004)
Cow	214-223 dg (280 d)	*L. saxkoebing* in guinea pig liver suspension /intracotyledon	Fetal antibody	Precolostrum titers (3/3)	(Fennestad and Borg-Petersen 1957)
Mouse	Post-coitus (20-21 d)	Viral antigen DNA vaccine /transplacental	Post-birth anamnestic responses	Significant increase in anamnestic responses of offspring of immunized mice	(Okuda, Xin et al. 2001)
Human	Premature newborn	Phage ϕX174 /NS	Postnatal, primary antibody	Titers >0.3 (11/11); no controls were reported	(Uhr, Dancis et al. 1962)
Human	Premature newborn	dNFB /NS	dNFB patches at 4 w	DTH (3/10)	(Uhr, Dancis et al. 1960)

BSA = bovine serum albumin; dg = days gestation; dNFB = dinitro-fluorbenzene; DTH = delayed type hypersensitivity; HBsAg = Hepatitis B surface antigen; IM = intramuscular; NS = not specified.

[1] In many of the experiments, fetuses at different gestational ages were grouped together.

[2] Numbers refer to the number of responders relative to the total number of animals that were immunized.

In the studies by Watt et al. (1999), Fazio et al. (2004) and Gerdts et al. (2002) (Table 1.1), using hepatitis B surface antigen (HBsAg) as the model antigen resulted in detection of HBsAg-specific fetal immune responses. HBsAg self-assembles into a secreted, non-soluble, slow-degrading virus-like particle (VLP) (Petit, Maillard et al. 1986) that has superior immunogenicity when compared with monomeric or dimeric equivalents (Fernandez, Conner et al. 1998). It has been suggested that the immunogenicity of HBsAg is due to the highly repetitive nature of the VLP, which has been shown to facilitate T cell-independent B cell expansion (Fehr, Skrastina et al. 1998) and enhance presentation on MHC class-I molecules (Abbing, Blaschke et al. 2004). These results suggest that there may be a correlation between the degree of immunogenicity of the antigen and successful fetal immunization. Other model antigens with a lesser degree of immunogenicity may be less effective at overcoming the reduced immune competence of the fetal immune system.

Induction of fetal immune responses have also been demonstrated using DNA vaccines (Gerdts, Babiuk et al. 2000; Okuda, Xin et al. 2001; Gerdts, Snider et al. 2002; Fazio, Ria et al. 2004). The *in utero* oral DNA immunization studies by Gerdts et al. (2000; 2002) are discussed in Section 1.6. Okuda et al. (2001) (Table 1.1) injected pregnant mice with DNA plasmid encoding either β-galactosidase, HIV-1 antigen or influenza virus antigen. Reverse transcriptase-polymerase chain reaction (PCR) and X-gal staining were used to show transgene expression in fetal tissues and activation of the fetal immune system was shown indirectly by the detection of anamnestic responses following intranasal DNA immunization at 6 weeks of age.

Aside from those studies conducted in our laboratory, the only other study to examine the *in utero* delivery of naked plasmid in a large animal model is Fazio et al. (2004) (Table 1.1). In their study, 200 μg of HBsAg-plasmid was injected into the semimembranous muscle of fetal pigs in the third trimester of gestation (at either 90 or 104 dg; gestation length approximately 115 days). HBsAg-specific antibody titers were detected in precolostrum sera in 5 of 9 piglets immunized at 90 dg. The authors suggested that fetal antibody responses might have waned in three additional piglets based on the observation that boosting with plasmid at 1 week of age resulted in the induction of protective antibody titers in 8 of 9 piglets that had been immunized as fetuses, but not in control piglets. In a second set of experiments, seven piglets that had been immunized with plasmid at 104 dg did not possess detectable precolostrum antibody titers. The authors suggested that immune memory might have been detected in three of the pigs postnatally, but the lack of age-matched controls (for the 104 dg group) made data difficult to interpret. CMI responses were not examined in piglets immunized at either 90 or 104 dg. To date, two independent groups (Gerdts, Babiuk et al. 2000, 2002; Fazio, Ria et al. 2004) have reported the successful induction of fetal antibody responses and neonatal immune memory following immunization with naked DNA plasmid in the third trimester fetus.

Gerdts et al. (2000) hypothesized that fetal immune responses were induced as a result of the inherent immunogenicity of the bacterial DNA plasmid backbone which contains immunostimulatory CpG and polyG motifs. In brief, CpGs stimulate DCs to upregulate MHC class-II, co-stimulatory molecules and inflammatory cytokines (Yamamoto, Yamamoto et al. 1992; Yi, Chace et al. 1996; Yi, Klinman et al. 1996;

Roman, Martin-Orozco et al. 1997). Experiments on full-term human cord blood-derived B cells have shown that CpGs enhance expression of MHC class-II and CD86 despite high cortisol levels (Tasker and Mashall-Clarke 2003) and immunization studies in neonatal mice have shown that DNA vaccines can overcome developmental tolerance (Forsthuber, Yip et al. 1996; Sarzotti, Dean et al. 1997; Bot, Bot et al. 1998). There are many aspects of the developing immune system which are not influenced by CpGs: stimulation with CpGs did not stimulate full-term human cord blood-derived DCs to express IFNγ (DeWit, Olislagers et al. 2004); nor, could CpGs reverse the homing deficits in cord blood-derived B cells that prevent their entering peripheral lymph nodes (LNs) (Tasker and Mashall-Clarke 2003). There is one report of DNA immunization in neonatal mice with plasmid containing the gene for *Plasmodium yoelii* circumsporozoite protein that resulted in the induction of tolerance (Mor, Yamshchikov et al. 1996). Tolerance, however, was overcome by co-immunization with plasmid expressing granulocyte-macrophage colony-stimulating factor (GM-CSF) (Ishii, Weiss et al. 2000), therefore suggesting that certain DNA plasmids might lack appropriate co-stimulatory activity.

1.2.5. Split Tolerance: Immune Deviation in the Fetus

Immune deviation, which was first described by Parish (1972) and demonstrated by Zinkernagel and Doherty (1975), is characteristic of fetal and neonatal immune responses and has implications for fetal vaccination. A review of the literature also suggests that the concept of immune deviation was first observed in fetal and neonatal immunization studies and coined as "split tolerance" (Asherson 1967). "Split tolerance"

refers to the impairment of CTL and Th1-mediated immune responses, which are required for the resolution of intracellular infections, and may be a more accurate description of the fetal immune response to antigenic stimulation than "developmental tolerance".

In the original study of LCVM-infection in fetal mice (Traub 1938), infection failed to induce detectable complement-fixing or neutralizing antibodies and it was concluded that fetal mice became persistently infected as a result of developmental tolerance. In 1967, Oldstone and Dixon repeated Traub's experiment and LCMV-specific IgG were found in the kidneys of neonatally infected mice. Despite the presence of antibodies, 80% of these mice became chronically infected and some gave birth to persistently infected or "tolerized" offspring who also had LCMV-specific IgG deposits in glomeruli. This finding suggested that the fetal immune system was capable of responding to LCMV infection, albeit in a non-protective manner.

Split tolerance has also been reported in other studies. In the macaque study by Otsyula et al. (1996) (Table 1.1), fetal immunization with attenuated SIV resulted in detection of SIV-specific antibody titers, but not CTL responses. When the immunized macaques were challenged at 17 months of age, 2 of 3 animals mounted detectable SIV-specific CTL responses, suggesting that, with respect to SIV, an antibody-type response had not imprinted upon the the developing immune system. In contrast, inoculation of fetal mice with LCMV did result in these mice developing a permanent deviation towards humoral immunity with respect to future LCMV challenge (Oldstone and Dixon 1967) (Table 1.1).

Tolerance, like protective immunity, is actively acquired and as such, can be expected to diminish with time unless boosting occurs. In the case of the SIV-infected macaques, tolerance may have been lost as a result of the gradual loss of suppressor or anergic cells, or as a result of the lymphocyte pool being replenished with new, randomly generated precursors cells. In contrast, studies of immune memory duration have shown that LCMV-specific CD8+ memory cells are permissive and easily stimulated by other, non-related viruses (Selin, Nahill et al. 1994). The same may also be true of LCMV-specific memory suppressor cells.

Split tolerance appears to be conceptually synonymous with immune deviation in which the cytokine environment, antigen dose and method of antigen presentation have resulted in the preferential induction of a Th2 response; however, reference to the original fetal sheep studies by Silverstein et al. (1963; 1964) (Section 1.4) also raises the possibility that split tolerance occurs as a result of Th2 immune responses developing prior to Th1 and CTL responses. It was observed that antigen-specific humoral responses could be induced in the fetal lamb as early as 60-66 dg (Silverstein, Uhr et al. 1963). In contrast, delayed-type hypersensitivity (DTH) responses did not develop until 74-76 dg (Silverstein and Prendergast 1964). Therefore, split tolerance may occur if the fetal immune system is exposed to a specific antigen at a time when Th2 cells are functional, but Th1 and CD8+ T cells are not.

Split tolerance may also reflect the immunosuppressive nature of pregnancy, which prevents the dam from rejecting the conceptus (Hansen 1998). Given the data which support the association of pregnancy with a Th2 cytokine environment and the number of studies demonstrating split tolerance, it is interesting that a large number of

studies use CMI and DTH as measures for the immune status of the fetus. This is especially true of transplantation studies, where fetuses have been described as immunologically incompetent based on the long-term survival of a third-party allograft in the absence of immunosuppressive treatment.

1.3. Immune Memory

Immune memory is an important component of fetal vaccination. For a vaccine to be effective, immunity must endure until the time of exposure to the pathogen. Achieving long-term immune memory in the fetus is especially challenging since the placenta acts as a barrier against cross-reactive environmental antigens and low levels of specific antigen, which are otherwise capable of boosting the waning immune response. Due to the placental barrier, fetal immunization must induce a memory component capable of persisting into the perinatal period, during which there is the greatest risk of infection.

1.3.1. Models of Immune Memory Generation

There are four popular models of memory cell induction. In the classic model of B cell differentiation (Ahmed and Gray 1996), a single B cell precursor has the potential to give rise to either effector or memory cells. The type of progeny that is produced depends on the environment: large amounts of antigen in an inflammatory environment favor differentiation into plasma cells, whereas antigen plus costimulation in the absence of inflammation preferentially activates memory B cells. As a result, effector cells tend to be generated during the early stages of an infection while cells that encounter antigen

later in the infection process tend to develop into memory cells. This model has also been proposed for the generation of CD4+ memory T cells; specifically, naïve CD4+ T cells that enter the draining LNs at later times after immunization divide less and eventually give rise to progeny with the phenotype of central memory cells (Catron, Rusch et al. 2006).

According to the linear differentiation model, memory cells are derived from effector cells (Liu and Janeway 1990; Swain 1994). Swain established an adoptive transfer model in which CD4+ helper T cells activated *in vitro* and adoptively transferred into host mice gave rise to populations of memory cells with a defined Th1 or Th2 response as determined by the cytokine milieu at the time of *in vitro* activation. Following the initial expansion stage, over 95% of activated T cells underwent cell death while the remaining 5% of cells, characterized by higher affinity TCR and IL-2R^{lo} CD44^{+}CD45RB^{lo}, entered the memory cell population. According to this model, the induction of a primary immune response should always give rise to a minority population of memory cells. The efficacy of many vaccines has been evaluated with this model in mind, i.e., an effective vaccine is one with a large primary effector response.

The two less popular models are the Klinman lineage hypothesis (Klinman 1997) and the decreasing potential model (Renno, Hahne et al. 1995; Miethke, Vabulas et al. 1996). The Klinman lineage hypothesis was based on the identification of two precursor subpopulations in the spleens of naïve mice (Linton, Decker et al. 1989). The predominant Ia+ J11D^{hi} B cell population, upon exposure to antigen and T cell help, formed primary antibody producing cells with a short half-life. The remaining 10-15%

of B cells were Ia+ J11D^{lo}, had a longer half-life and possessed the ability to acquire somatic mutations, thus giving rise to memory cells with increased diversity.

According to the decreasing potential or terminal differentiation model, antigenic stimulation of naïve T cells results in their differentiation into memory cells. With each successive antigenic stimulation, the potential of the cell to undergo apoptosis increases (Renno, Hahne et al. 1995; Miethke, Vabulas et al. 1996). Based on this model, high doses of antigen over a prolonged period will lead to terminal differentiation of all clonal progeny and the lack of immune memory.

In each of the above four models of memory cell generation, the number of memory cells generated is proportional to the size of the primary lymphocyte burst. Accordingly, most vaccines have been designed to elicit as large a primary immune response as possible (Hou, Hyland et al. 1994; Kaech and Ahmed 2001; Kaech, Wherry et al. 2002; Campos and Godson 2003); however, the size of the primary burst is unlikely to influence the longevity of immune memory. Gray and Skarvall (1988) have suggested that the longevity of humoral memory is not dependent upon intrinsic properties of the B cell, but rather the constant activation and turnover of B cells in response to persisting antigen in the form of antigen reservoirs or periodic infection. Primary activation by antigen has been hypothesized to produce a subset of B cells with a very low stimulation threshold that can be chronically activated by either low levels of persistent antigen, cross-reactive environmental antigens or anti-idiotypic interactions (reviewed by Klaus, Humphrey et al. 1980).

Until recently, the majority of molecular signaling pathways required for activation of naïve T cells have been assumed relevant to both effector and memory cell

induction. Induction of memory T cells does require TCR-recognition of antigen in the context of MHC class-I or -II along with a simultaneous second signal, such as B7.1 (CD80) interacting with CD28 (Liu, Jones et al. 1992); however, the induction of a strong primary immune response is not always accompanied by the induction of immune memory. This suggests that induction of immune memory requires signals/mechanisms in addition to or different from those required for the induction of effector cells. Recent experiments have shown that induction of memory Th1 cells requires the transcriptional activator protein STAT-1 (Maecker, Varfolomeev et al. 2005). Many hypothetical requirements have been suggested for memory CD8+ T cell induction. These include IL-2, -7, -15 and type-1 IFN (Sprent, Zhang et al. 1999; Schluns and Lefrancois 2003). IFN-1 induces macrophages to synthesize IL-15 which binds to the IL-2β receptor expressed by CD8+, but not CD4+, cells (Sprent, Zhang et al. 1999). It has also been suggested that induction of memory CD8+ T cells requires that low levels of antigen be presented in the context of MHC class-1 molecules on the surface of follicular DCs (fDCs) (Belz, Wilson et al. 2006).

Induction of memory B cells also requires signals in addition to antigen and interaction of B7 with CD28 which is expressed on the surface of T cells. Differentiation of memory B cells *in vitro* was dependent on the presence of antigen-antibody complexes on fDCs (Phipps, Mandel et al. 1984). *In vivo* memory B cell differentiation occurred in germinal centers (GCs) and was dependent on CD40 interaction with CD154 (a.k.a. CD40 ligand) (Gray, Dullforce et al. 1994). CD40-CD154 interaction activates the transcription factor B-cell specific activator protein (a.k.a. B-cell activating factor) (Neurath, Stuber et al. 1995; Manser 2004). CD40-CD154 interaction also stabilizes

CFLIP, which inhibits the Fas-mediated apoptotic pathway (DiSanto, Bonnefoy et al. 1993; van Eijk, Medema et al. 2001). Within GCs, CFLIP inhibition of the apoptotic pathway is also sustained by B cell interaction with fDCs. A subpopulation of CD4+ T cells, named follicular B helper T cells, has also been identified and these cells secrete cytokines that are conducive to memory B cell survival (Vinuesa, Tangye et al. 2005). There is also a population of γδ+CD3+CD4+ T cells that can substitute for follicular B helper T cells in GC formation; however, B cells activated in this manner can only express IgM following primary and secondary immunization (Zheng, Marinova et al. 2003).

Overall, the literature pertaining to immune memory suggests that immune memory is not always induced during primary activation of the immune system. It may be possible to devise strategies to enhance both the quantity and quality of memory cells that are generated during primary immunization.

1.3.2. The Role of Antigen in Immune Memory

It is known that memory T cells are a stable population with the ability to avoid Fas-Fas ligand (CD95-CD178)-mediated apoptosis (Inaba, Kurasawa et al. 1999). Relative to naïve lymphocytes, they express a larger number and variety of adhesion molecules, including the higher-affinity IL-2 receptor and high affinity TCR, which allows them to better respond to low doses of antigen (Zheng, Xue et al. 1994). Currently, the debate exists as to whether memory T cells can survive for long periods in the absence of antigen or require repeated, low-levels of antigenic stimulation. It is

generally accepted that memory B cells can survive for long periods in the absence of continued antigenic stimulation

Chronic antigenic stimulus can result from incidental exposure to cross-reactive environmental antigens (Selin, Nahill et al. 1994), low-grade persistent infections (Sarkar, Mitra et al. 1973; Friedman, Phillip et al. 1989; Lievano, Papania et al. 2004) or long-term antigen deposits in the spleen and LNs. Antigen-antibody complexes are maintained on the surfaces of both B cells and fDCs in the spleen, but only fDCs in LNs (Tew, Mandel et al. 1979). Experiments with human serum albumin, which is easily catabolized, have shown that antigen is very stable when retained on the surface of fDCs (Tew, Mandel et al. 1979). These stable depots can theoretically stimulate the peripheral pool of memory cells as they periodically recirculate through the LNs. Interestingly, both splenic and LN antigen depots are susceptible to cortisol treatment (Tew, Mandel et al. 1979), suggesting that loss of antigen depots and a subsequent reduction in immune memory cells may contribute to the lower level of immune resistance associated with pregnancy and parturition.

Experimental evidence argues both for and against the need for persistent antigen. With regard to memory B cells, it was previously thought that survival depended on persistent antigen depots on fDCs since adoptive transfer of primed B cells into naïve rats resulted in the loss of immune memory after 6-12 weeks (Gray and Matzinger 1991). In 2000, (Maruyama, Lam et al.), designed an experiment in which an inducible recombinase transgene was used to "turn off" the antigenic specificity of primed B cells following adoptive transfer into naïve recipient mice. In doing so, the possible effects of residual antigen being carried over by donor T and B cells into naïve

recipients was negated. Using this experimental system, memory B cells persisted up to 15 weeks in the absence of antigenic stimulation. As a result of this finding, the role of fDCs in the maintenance of B cell memory has come under severe scrutiny and fDCs are now hypothesized to play a role in CD40-CD154 interaction (Hannum, Haberman et al. 2000; William, Euler et al. 2002).

The debate also exists as to whether or not survival of memory T cells is dependent upon chronic or repeated antigen exposure. Gray and Matzinger (1991) hypothesized that memory T cell survival was dependent upon continuous exposure to antigen. Upon transfer into naïve syngeneic hosts, primed CD4+ T cells had a half-life of 2-3 weeks. In comparison, cells were still viable after 7 months when adoptive transfer was accompanied by the simultaneous injection of antigen. On the other side of the debate, adoptive transfer of IL-2R^{lo} CD44+ CD45RB^{lo} CD4+ memory T cells into naïve mice in the absence of antigen was followed by survival of cells for up to 9 months (Swain 1994). The use of different antigens in the above experimental systems may have contributed to the difference in duration of survival of memory CD4+ T cells.

It is generally accepted that CTL-mediated immune memory persists for a longer duration than CD4+ T cell-mediated immune memory (Nanan, Rauch et al. 2000; Crotty, Felgner et al. 2003) and survival of memory mCTLs is less dependent on persistent antigen (Hou, Hyland et al. 1994; Lau, Jamieson et al. 1994). The exception to this generalization is a study by Oehen et al. (1992), which showed that survival of mCTLs was both antigen-dependent and short-lived (approximately 13 weeks in the presence of antigen). In this study, CTL responses were measured in donor mice that had received splenocytes from mice that had been primed 7 days earlier with major nuclear

protein antigen from LCMV (delivered in the form of live LCMV or using vaccinia virus as a vector). The short period between primary immunization and adoptive transfer suggests that the authors may have been observing a long-lived primary CTL response that was sustained by the continued presence of high levels of infectious virus and not a true population of mCTLs. In studies where there was an extended duration between primary immunization with a live virus and adoptive transfer into naïve hosts, CTL responses were detected after a much longer period. mCTLs specific for the H-Y antigen survived between 6-16 weeks following adoptive transfer into naïve female recipient mice (Gray and Matzinger 1991). At 28 weeks post-transfer, mCTL activity was still evident in 2 of 5 mice. Mullbacher (1994) reported that mCTLs in the spleen survived up to 25 weeks in the absence of persistent antigen and Sendai-virus specific mCTLs survived up to 35 weeks following adoptive transfer into naïve recipient mice (Hou, Hyland et al. 1994). Lastly, mCTLs isolated from mice infected with LCMV and sorted by a fluorescent-activated cell sorter (FACS) to remove any contaminating APCs, survived for 26 months following adoptive transfer into naïve recipients (Lau, Jamieson et al. 1994). Therefore, the bulk of the literature suggests that CTL memory can persist for a reasonable duration in the apparent absence of the specific immunizing antigen.

Among the studies presented above, the duration of mCTL survival ranged from 6 weeks to 26 months. The range in survival times may have been due to differences in specific antigen, the method of antigen delivery, strain-specific differences between mice used in different experiments or differences in experimental methods. The duration of memory may also depend upon the subpopulation of mCTLs being examined. Two subpopulations of mCTLs have recently been defined: (i) central mCTLs have a

$CD62L^{+}CCR7^{+}$ phenotype, a high rate of proliferation, a longer half-life and the ability to home to the spleen and LNs, and (ii) "effector-memory" CTLs express IFNγ, are present in high numbers following contraction of the primary burst, have a relatively shorter half-life and are limited to peripheral tissues (Lau, Jamieson et al. 1994; Crotty, Felgner et al. 2003; Combadiere, Boissonnas et al. 2004). Given that antigen depots in the spleen and LNs are susceptible to cortisol treatment (Tew, Mandel et al. 1979; Tew, Mandel et al. 1979), there may be a higher ratio of effector-memory:central-memory CTLs in the neonate.

The survival of CTL-mediated memory responses for years following certain viral infections, such as measles and vaccinia virus, is dependent on stimulation by an antigen, but not necessarily the immunizing antigen (Selin, Nahill et al. 1994; Nanan, Rauch et al. 2000; Crotty, Felgner et al. 2003). The stimulatory antigen could hypothetically exist in the form of an MHC-restricted self-antigen or heterologous virus. Support for the former comes from a study in which adoptive transfer of mCTLs into MHC class-I-deficient mice resulted in shortening of the survival time of cells by two-thirds relative to controls (Hou, Hyland et al. 1994); however, adoptively transferred $CD44^{hi}$ mCTLs have also been shown to survive indefinitely in the absence of MHC class-I (Murali-Krishna, Lau et al. 1999). The stimulation of mCTLs by heterologous virus has been implicated as a mechanism for survival independent of the immunizing antigen and is based on the observation that mCTLs from mice immunized with LCMV could be stimulated *in vivo* and *in vitro* with either Pichinde or vaccinia virus (Selin, Nahill et al. 1994). Similarly, *in vitro* stimulation of mCTLs, from Pichinde or vaccinia virus-immunized mice, with LCMV resulted in low levels of proliferation. The ability to

undergo repeated stimulation by cross-reactive antigens is likely due to the lower threshold of activation specific to memory cells. The localization of central mCTLs in the spleen, blood and LNs (Lau, Jamieson et al. 1994) is likely to enhance contact with cross-reactive antigens, thus ensuring the low level renewal of mCTLs.

Overall, reports in the literature suggest that the duration of humoral immune memory is limited by the survival of memory CD4+ T cells, which is antigen-dependent. Memory CTL responses are also antigen-dependent, but the permissiveness of the TCR may enable continuous stimulation by antigens with low levels of cross-reactivity. Based upon these conclusions, it may be possible to extend the duration of immune memory following vaccination by delivering antigen in such a manner as to be presented in the context of MHC-class I molecules. Alternately, if the goal is to induce long-lasting humoral immune memory, it may be advantageous to deliver antigen in a stable, persistent or self-renewing form.

1.4. The Fetal Lamb Model

There are a number of reasons for selecting the fetal lamb as our model for *in utero* immunization studies. Of particular importance is the fact that the development of the ovine immune system has been well characterized in numerous studies and specific characteristics of immune development are closely related to that in humans. Furthermore, established protocols for timed breeding allow for experimentation at specific stages of immune development and surgical manipulation throughout most of the 148 day gestation period is associated with a low risk of abortion, thus allowing for *in vivo* fetal studies (Hein and Griebel 2003). Lastly, the organization of the ovine

placenta excludes maternal antibodies as well as most environmental antigens, thus ensuring that the fetus is truly immunologically naïve. As a result, it is possible to study the induction of a fetal immune response to a specific antigen, without having to account for the effects of cross-environmental antigens or prior immune status.

1.4.1. Ovine Placentation

Immune ontogeny in the ovine fetus differs from that in mice or humans due to the nature of the syndesmochorial (or syndesmo-epitheliochorial) ovine placenta. Unlike the hemichorial placenta found in mice and humans, the ovine placenta effectively blocks both maternal antibodies and naturally occurring environmental antigens from accessing the amniotic cavity (Needham 1931; Wild 1979). Thus, the lamb is immunologically naïve at birth, except in the event of infection with pathogens capable of crossing the ovine placenta or in the event that the placenta is disrupted. Immunological naïvety is reflected in the lack of GC formation in lymphatic tissues in the normal ovine fetus (Pearson, Simpson-Morgan et al. 1976).

In the ovine placenta, six layers of tissue separate fetal from maternal blood. These are (i) the endothelium of the fetal capillaries, (ii) fetal connective tissue, (iii) fetal chorionic epithelium, (iv) maternal uterine epithelium, (v) maternal connective tissue, and (vi) maternal endothelium. From 86 dg onwards, degeneration of the maternal epithelium and phagocytosis by cells in the chorionic epithelium result in areas of the placentome consisting of only 5 tissue layers that are collectively referred to as the syndesmochorion (Stevens 1975). The multiple layers of the syndesmochorial placenta prevent the passive transmission of metabolites, molecules and infectious agents

(Needham 1931; Wild 1979). The ovine placenta also excludes maternal antibodies (Evans and Smith 1963; Richardson, Conner et al. 1971), thus ensuring that antibodies found in the fetal circulation are produced by the fetus. In addition, sheep are prone to multiple conception and fetuses develop in separate fetal membranes. The lack of vascular anastamoses between twin fetuses (Mellor 1969) enables surgical or immunological manipulation of only one twin while reserving the other as a control.

1.4.2. Immune Organ Development and Lymphopoiesis

Major events in ovine immune ontogeny are summarized in Table 1.2. Immune ontogeny in the fetal lamb is similar to that in humans in the sense that the thymus is functional and all of the cellular components of the peripheral immune system are present, albeit not necessarily functional, by the end of the first trimester (Bryden 1969; Griebel 1998). Functional maturation of the immune organs occurs primarily in the second and third trimesters of gestation and by parturition, the immune system is well developed. This contrasts with mice, where lymphoid organs, including the thymus, spleen and LNs undergo the majority of their development postnatally as a result of the short gestation period (21 days) (Abbas, Litchman et al. 1994).

In the fetal lamb, hematopoiesis occurs in the yolk sac between 19-27 dg (Table 1.2). The thymus reaches its definitive position at 31 dg and CD5+ immature thymocytes are present by 35 dg (reviewed in Cahill, Kimpton et al. 1999). By 50-55 dg, there is significant T cell emigration from the thymus to the spleen and LNs and, by 65 dg, the size of the T cell pool in the fetus is approximately 10% of that in the neonate (Hein,

Table 1.2. Immune development in the fetal lamb

Days gestation	*Organ/tissue/cell*	*Developmental event*	*Reference*
31	Thymus	Reaches definitive position	(Bryden 1969)
35-40	Thymus, T cells	Appearance of CD5+ thymocytes in the cervical thymus	(Bryden 1969; Cahill, Kimpton et al. 1999)
45-48	Spleen, B cells	B cells appear in spleen; detection of Igλ V gene diversification	(Press, Hein et al. 1993; Griebel 1998)
50-55	B cells	Appearance of sIg	(Symons and Binns 1975; Press, Hein et al. 1993)
50-55	T cells	Appearance of T cells in spleen and LNs	(Griebel 1998; Cahill, Kimpton et al. 1999)
55-60		End of the "preimmune" period	(Flake, Harrison et al. 1986)
65	Jejunal PP	Appearance of IgM+ cells	(Alitheen, McClure et al. 2003)
66	Humoral response	Natural antibodies are present in serum and earliest detection of antigen-specific antibody responses	(Silverstein, Thorbecke et al. 1963; Silverstein, Uhr et al. 1963; Hashiguchi, Nanba et al. 1979)
68-70	Ileal PP	Appearance of IgM+ cells	(Press, Hein et al. 1993; Alitheen, McClure et al. 2003)
75	Jejunal PP	Appearance of primary follicles	(Reynolds and Morris 1983)
77-85	CTLs	Earliest rejection of allogeneic tissue grafts (DTH) and virus-specific CD8 activity	(Silverstein and Prendergast 1964; Hashiguchi, Nanba et al. 1979)
78	Lymphatic network	Becomes fully functional	(Pearson, Simpson-Morgan et al. 1976)
92	Pharyngeal and palatine tonsils	Appearance of lymphoid cells	(Chen, Alley et al. 1990)
98-100	Ileal PP	Appearance of primary follicles	(Reynolds 1986)
120	Thymus	Reaches maximum size as % body weight	(Pearson, Simpson-Morgan et al. 1976)
130	Ileal PP	Becomes major lymphopoietic organ	(Reynolds 1986; Reynolds 1987)
130	LNs	Appearance of primary follicles in naïve fetus	(Pearson, Simpson-Morgan et al. 1976)
135	GALT	Appearance of gut-homing T cells	(Cunningham, Cahill et al. 1999)
148		Parturition	

DTH = delayed-type hypersensitivity; GALT = gut-associated lymphoid tissue; sIg = surface immunoglobulins; LNs = lymph nodes; PP = Peyer's patch.

Dudler et al. 1990). Between 65-75 dg, there is a significant expansion of the αβ-T cell pool, which is consistent with the time of onset of humoral responses and graft rejection (Silverstein, Uhr et al. 1963; Silverstein and Prendergast 1964). The increase in the size of the thymus, from 0.5 to 1.7 g at 85 dg, is concomitant with increased lymphopoiesis and by 120 dg, the thymus reaches its maximum size as a percentage of body weight (Pearson, Simpson-Morgan et al. 1976).

Two subpopulations of thymic emigrants have been identified in the fetal lamb. Lymphocytes that target the spleen are primarily short-lived CD8+ T cells, while those that target the peripheral LNs tend to be longer-lived and have a ratio of CD4:CD8:γδ equal to 6:2:1 (Cahill, Kimpton et al. 1999). With respect to fetal oral immunization, where the immunizing antigen is being targeted to LNs draining the oral cavity, the longevity of T cells potentially results in a relative increase in both the overall number of T cells and the frequency of T cells with a single antigenic specificity in the LNs at any time.

In the peripheral immune system, IgM+ immature B cells first appear in the spleen at 45-48 dg (reviewed by Griebel 1998; Yasuda, Jenne et al. 2006) consistent with the detection of λ-chain expression at 48 dg (Table 1.2). From 50 to 100 dg, the spleen is the primary organ of B cell development. Clusters of B cell lymphopoiesis are detectable by 60-80 dg and from the spleen, pre-B cells migrate to the LNs, bone marrow, liver, intestines and blood. IgM+ cells are first detected in the ileum at 68-70 dg; however, primary follicles do not appear in the ileal Peyer's patch (PP) until 97 dg.

Prior to development of the gut-associated lymphoid tissue (GALT), the majority of B cells emigrate from the spleen to LNs. Development of LNs and lymphatics are

closely related to one another. The lymphatics develop from mesenchymal cell clusters that are arranged in parallel to the veins, which are used as a scaffold for growth (Bailey and Weiss 1975). The LNs develop at lymphatic sacs where connective tissue invaginations form the framework of the LN. Infiltration of lymphocytes into spaces within the connective tissue results in the formation of early fetal LNs. The superficial cervical (SCv) LNs are the first to form in the fetus and are the largest individual nodes until 140 dg (Washington, Kimpton et al. 1992). In the normal, immunologically naïve fetus, cortico-medullary junctions appear in the SCv LNs at 75 dg and primary follicles are absent before 130 dg (Pearson, Simpson-Morgan et al. 1976; Cahill, Kimpton et al. 1999). Between 120 dg and 4 weeks of age, the size of the SCv LNs increases by 25-fold and the number of B cells in these nodes increases by 3.5-fold as a percentage of LN weight (Kimpton, Washington et al. 1990; Cunningham, Cahill et al. 1999).

Although the lymphatics are not fully formed until 78 dg, lymphocytes do recirculate through sections of the lymphatics as early as 65 dg (Pearson, Simpson-Morgan et al. 1976) (Table 1.2). In both LNs and spleen, approximately 90% of thymic immigrants become resident cells while the remaining 10% enter the recirculating lymphocyte pool (RLP) and migrate between the lymphatic system, secondary lymphoid organs, the blood and peripheral tissues (Cahill, Kimpton et al. 1999; Cunningham, Cahill et al. 1999). The lymphatics provide a mechanism by which soluble antigen introduced into the fetus can enter the LNs. Alternately, processed antigen could be delivered to fetal lymph nodes via antigen presenting cells (Kimpton, Washington et al. 1995).

Table 1.3. Lymphopoiesis in the fetal lamb

Age	*GALT-associated lymphocytes (cells/h)*	*Recirculating lymphocyte pool (cells/L)*	*Total T cells*	*Superficial cervical lymph nodes afferent output (cells/h)*
65-75 dg	ND	8×10^7	$<3 \times 10^6$	ND
77-100dg	ND	1.8×10^8	ND	ND
100-130dg	ND	5×10^8	2.6×10^{10}	2×10^7
130dg – birth	$40\text{-}80 \times 10^6$	1.2×10^{10}	ND	ND
2 weeks	30×10^7	2.5×10^{10}	2×10^{11}	1×10^8
3 months	$60\text{-}120 \times 10^7$	$70\text{-}80 \times 10^{10}$	ND	2.5×10^8

ND = not determined.

(Pearson, Simpson-Morgan et al. 1976; Kimpton, Washington et al. 1995; Cunningham, Cahill et al. 1999)

Between 70 to 100 dg, there are few changes to the fetal immune system other than an increase in the size of the lymphocyte pool (Table 1.3). At 100-105 dg, the ileal PPs replace the spleen as the primary organ of B cell lymphopoiesis - this differs significantly from the mouse model where the bone marrow is the primary B cell organ. Following development of the ileal PPs, there is a sudden increase in the percentage of B cells in the peripheral lymphocyte pool, an increase in the number of both T and B cells and an expansion in the immunological repertoire (Cahill, Kimpton et al. 1999; Yasuda, Jenne et al. 2006). From 70 dg to birth, both the rate and density of T cells flowing through the lymphatic system increases by 150-fold (Pearson, Simpson-Morgan et al. 1976) (Table 1.3). Viewed from another perspective, the RLP at 65-70 dg contains only 0.7% of the number of lymphocytes present at birth. This is significant with respect to limiting the number of antigen-specific clones, and therefore, the number of antigens recognized by the fetal immune system, as well as limiting the frequency of cells specific to any one antigen.

At 130 dg, the rate of lymphopoiesis (5.2% of cells per hour) in the ileal PP is 15 to 20-fold greater than in the thymus (Reynolds 1987). B cell lymphopoiesis in the fetal lamb is antigen-independent and only 5% of progeny survive while the rest undergo apoptosis (Reynolds 1986). Apoptosis occurs following cross-linking of surface Ig molecules (Mousavi, Rabbani et al. 1998) and cells that survive apoptosis emigrate from the ileals PPs to the peripheral lymphoid compartment, including the RLP and LNs (Reynolds 1986; Reynolds, Kennedy et al. 1991). Emigration of B cells from the ileal PPs is responsible for the significant increase in the size of the RLP between 120 dg and 3 months of age (Table 1.3) (Cunningham, Cahill et al. 1999) and surgical removal of

the ileal PPs from neonates was reported to cause a prolonged, systemic B cell deficiency (Gerber, Morris et al. 1986).

By the mid-third trimester fetus (120-145 dg), B cells and T cells constitute approximately 8% and 92% of the peripheral RLP, respectively. Of the latter compartment, approximately 23% are CD8+, less than 18% are γδ+ and the remainder are CD4+ (Cunningham, Kimpton et al. 2001). Fetal T cells have a relatively long lifespan with a half-life of approximately 20 days and a rate of recirculation through the peripheral tissues, including the skin and gut, that is 4.6-fold greater than in adult animals (Kimpton, Washington et al. 1995; Cahill, Kimpton et al. 1997). The lack of tissue-specific homing in the fetus, along with the high rate of recirculation, ensures that there is effective surveillance of the periphery despite the low number of lymphocytes and limited immunological repertoire relative to the adult. With respect to *in utero* oral immunization, the high level of peripheral T cell migration may also enhance local induction of antigen-specific T cells.

It has also been hypothesized that the longevity and high rate of peripheral recirculation of naïve T cells in the fetus is required for the induction of self-tolerance to antigens not expressed in the thymus (Cahill, Kimpton et al. 1999). Using the Danger Hypothesis as a model (Matzinger 2002), the absence of inflammation and pro-inflammatory cytokines in fetal tissues and LNs would ensure that lymphocytes are tolerized to peripheral self-antigens. One observation that supports this hypothesis is the 2-fold decrease in the ratio of CD4+ to CD8+ T cells in afferent lymph relative to high endothelial venule input, which suggests a potential deletion or down-regulation of self-reactive T cells in the periphery (Cahill, Kimpton et al. 1999). The Danger Hypothesis

does leave room for the potential induction of autoimmunity in the event of transplacental infection or events resulting in the upregulation of endogenous danger signals, such as stress proteins.

Within the first week of postnatal life to approximately 3 months of age, there is a second wave of thymic lymphopoiesis that replaces 80% of the fetal lymphocyte pool and increases the size of the RLP by 2 to 3-fold (Table 1.3). These "adult" T cells have a phenotype that is distinct from their fetal counterparts. They have a half-life of less than 8 days, only activated (i.e., effector or memory) cells can enter the afferent lymphatics and there is a distinct subpopulation of lymphocytes that circulate through the gut-associated lymphoid tissues (GALT) (Cunningham, Cahill et al. 1999; Cunningham, Kimpton et al. 2001). Activated lymphocytes from adult animals are also resistant to cortisol-induced cell death (Munroe 1971; Anderson, Flint et al. 1975; Inaba, Kurasawa et al. 1999). One can hypothesize that activated fetal lymphocytes are also capable of escaping the cortisol-induced apoptosis that accompanies birth; this hypothesis is consistent with observations that immune memory induced in the fetus can persist in the neonate (Otsyula, Miller et al. 1996; Gerdts, Snider et al. 2002).

1.4.3. Development of the Immune Repertoire

The size of the immune repertoire determines the diversity of antigens against which the immune system can react. The repertoire consists of variations in the MHC, TCR and immunoglobulin (Ig) molecules and is developed through Ig-chain combination, gene conversion, gene rearrangement (also known as somatic recombination or VDJ recombination) and somatic mutation. Very little information is

available about the size of the immune repertoire in the second trimester fetus; however the low numbers of lymphocytes, limited variable region (V) gene usage and low levels of somatic hypermutation all suggest that the fetal immune repertoire is severely restricted relative to the neonate.

The basic antibody molecule consists of two heavy chains and two light chains. The following heavy chains have been described in sheep: δ, μ1, μ2, γ1, γ2, ε and α. Each heavy chain has a constant, V, D and J region. In the sheep, there are nine germline heavy chain V gene segments (Defour and Nau 1997). All nine gene segments are closely related with an overall nucleotide homology of approximately 70%, six are expressed in the adult and three are pseudogenes. Pseudogenes are hypothesized to donate nucleotide sequences in a process referred to as gene conversion (Reynaud, Mackay et al. 1991; Gontier, Ayrault et al. 2005). The ovine heavy chain V gene segment contains two complementarity determining regions (CDRs): CDR1 (codons 27-34) and CDR2 (codons 56-62). CDR3 is formed by the VDJ rearrangement, is 21 codons in length and (unlike the mouse CDR3) has the least somatic variability. There are also six heavy chain J segments of which two are functional and four are pseudogenes.

Sheep, similar to mice and humans, have two different light chains: λ and κ. Light chains are composed of constant, V and J chains. λ is the major light chain isotype and is expressed by 75-90% of B cells in the adult sheep (Griebel and Ferrari 1994). The exact number of V gene segments is still under debate, but the large number λV gene segments that have been identified to date suggest that repertoire diversity in sheep is primarily due to λ chain variability. According to Reynaud's group (Reynaud, Mackay et al. 1991; Reynaud, Garcia et al. 1995) there are 60 to 90 Vλ genes that are organized

into 6 families of which there is preferential utilization of 20 segments. Due to the limited Vλ gene usage, Reynaud's group hypothesized that the majority of diversity in the repertoire resulted from antigen-independent somatic hypermutation. A more recent model of repertoire expansion has been proposed by Reynolds' group and is based on the identification of a minimum of 120 Vλ gene segments in sheep (Jenne, Kennedy et al. 2003). According to this model, combinatorial gene rearrangement is more important than antigen-independent somatic hypermutation in generation of the immune repertoire.

All data suggest that the immune repertoire is severely restricted in the fetus relative to the young lamb. In the 144 dg fetus, only four of the six heavy chain V gene segments are expressed and of these, two are used almost exclusively (Gontier, Ayrault et al. 2005). Between 61-146 dg, 12 distinct Vλ gene segments and six to ten Vκ gene segments are preferentially expressed (Hein and Dudler 1999). As a result of the small number and close relatedness of the available germline genes (Reynaud, Mackay et al. 1991), the combinatorial diversity of the fetal lamb is inherently low relative to the adult. In addition, there is no evidence in the literature as to whether gene conversion occurs in the fetal lamb.

Unlike combinatorial rearrangement, which remains constant with age, the frequency of somatic hypermutation increases with age. The age at which somatic hypermutation first occurs is uncertain. According to Gontier et al. (2005), there is little to no hypermutation in the fetal lamb. This conclusion was based on PCR analysis and gene sequencing of the rearranged heavy chain V gene in 3 lambs between 144 dg and 3-months of age. According to their study, there were no age-dependent changes in the rate of mutational variability between 144 dg and 3 months of age. The rate observed

was 2.8% (0 to 11%), which contrasted with 18.6% in the adult (>6 months of age). This agreed somewhat with a previous report by Hein and Dudler (1999), who characterized mRNA from B cells of fetal lambs between 61-146 dg and identified germline sequences in all heavy chain V transcripts. In this study, Vλ transcripts were also present as germline sequences; however, there was limited diversity in the Vκ segment. Overall, these data suggest that the diversity in the fetal immune repertoire is primarily dependent upon rearrangement of a limited number of Vλ and Vκ genes, and Ig chain recombination and, therefore, is fairly restricted when compared to the full repertoire in adult sheep. The size of the immune repertoire in the fetal lamb has implications with respect to the ability of the fetus to respond to a wide variety of antigens. In the study by Silverstein et al. (1963), fetal lambs between 60 and 130 dg did not respond to immunization with *Mycobacteria*, BCG, *Salmonella* or diphtheria toxoid, all of which are highly immunogenic.

The above studies were conducted in normal, immunologically naïve fetuses and there is no data available on somatic hypermutation in activated fetal B cells. Yasuda et al. (2006) mentioned that a study is soon to be published which describes activation-induced cytidine deaminase, which mediates somatic mutation, at different stages in fetal lamb development. The presence of activation-induced cytidine deaminase in the fetus may allow for affinity maturation in activated fetal B cells.

1.4.4. Development of Antigen-Specific Immune Responses

Since the end of the first trimester occurs at 50 dg, tolerance in the first trimester fetal lamb must be regulated at the level of the thymus, i.e., central tolerance. This

period of development, prior to migration of lymphocytes into the periphery, has been referred to as the "preimmune" period and may extend to 55-60 dg (Flake, Harrison et al. 1986). It has been hypothesized that the preimmune fetus is immunologically incompetent as a result of the immaturity of the immune system and antigens to which the fetus is exposed are perceived as self. Therefore, the preimmune fetus corresponds with Billingham's immunological null period (1953) and the fetal lamb does not acquire the ability to respond to peripheral antigens until the early second trimester. The end of the preimmune period should be concomitant with the population of the spleen and LNs by T and B cells (Hein, Dudler et al. 1990; Berek, Berger et al. 1991).

The components of the immune system become functional at different stages of ontogeny. Natural antibodies first appear in normal fetal lamb serum at approximately 66 dg (Silverstein, Thorbecke et al. 1963). GCs are not normally present in the naïve fetal lamb (Silverstein, Thorbecke et al. 1963; Silverstein, Uhr et al. 1963; Pearson, Simpson-Morgan et al. 1976) and do not form unless there is primary B cell activation and clonal expansion (Jacob, Kelsoe et al. 1991). Antigen-specific antibody responses have been induced as early as 60-66 dg (Silverstein, Uhr et al. 1963). In contrast, CTL and DTH responses were not detected prior to 77-85 dg (Silverstein and Prendergast 1964); however, this may be an artifact of using skin graft rejection as a read-out for CTL responses, as 77-85 dg is also the time when naïve T cells and DCs first appear in fetal skin (Kimpton, Washington et al. 1995). There have been reports of skin graft rejection as early as 53-55 dg, but rejection was mediated by innate immune mechanisms, specifically complement (McCullagh 1989; Chen, Shelton et al. 1995). Based on the available literature, the earliest reported time for detection of a fetal

immune response following immunization is 66 dg (Silverstein, Uhr et al. 1963). In studies in which fetal lambs were exposed to infectious virus prior to 60 dg (Table 1.4), virus-specific fetal antibodies were not detectable before 70 dg, suggesting that there may have been a period after infection during which the immune system was unable to respond to the virus. Therefore, the ability of the fetal lamb to respond to a specific antigen is limited by the age of the fetus, the low number of lymphocytes and a limited immunological repertoire (Sections 1.4.2 and 1.4.3).

In the seminal paper on the immunological competence of the fetal lamb (Silverstein, Uhr et al. 1963) (Table 1.4), lambs were administered a single intramuscular (IM) injection of a combined vaccine composed of ovalbumin, horse spleen ferritin, killed *Salmonella typhus*, phage ϕX174, live Bacillus Calmette-Guerin (BCG), diphtheria toxoid and *Mycobacteria* (in the form of CFA). The earliest elicited antibody response was to ϕX174, which appeared between 60-70 dg. A serum antibody titer of 1/50 was reported for the one fetus immunized at 60 dg, but there was no comparison to titers from age-matched controls and titers were significantly below that of naïve fetuses at 110 dg. Significant titers to ferritin and ovalbumin could only be detected starting from 70-80 dg and 120-125 dg, respectively. This ordered acquisition of specific reactivities over time was referred to as an "antigenic hierarchy". This antigenic hierarchy is likely a reflection of the low number of lymphocytes and restricted immune repertoire discussed in Sections 1.4.2 and 1.4.3. Characterization of the order of antigens within this hierarchy would significantly advance the field of fetal and neonatal immunization research.

Table 1.4. Fetal lamb immunization experiments

Age of fetus at time of immunization (dg)	*Immunizing agent*	*Route*	*Outcome*	*Reference*
14-28	Bovine viral diarrhea virus (pestivirus)	NS	Tolerance	(Swasdipan, McGowan et al. 2002)
37-70[1]	Akabane virus	Trans-placental	Virus neutralizing antibodies at 70 dg; LPR at 80 dg.	(Hashiguchi, Nanba et al. 1979)
45	*Neospora caninum*	NS	No response	(Buxton, Maley et al. 1998)
45-90[1]	*T. gondii*	Trans-placental	Fetal antibody titers detected at 35 dpi.	(Dubey, Emond et al. 1987)
55-60[2]	β-gal (MuLV vector)	IP	Postnatal tolerance (humoral and cytolytic responses)	(Tran, Porada et al. 2001)
55-62[2]	Neomycin phosphotransferase (MuLV vector)	IP	Potential tolerance[3]	(Porada, Tran et al. 1998)
58-65[2]	Bovine leukemia virus	Trans-placental	Precolostrum antibody titers	(Onuma, Baumgartener et al. 1977)
60-130dg	Combined vaccine: ovalbumin, ferritin (1 mg), Salmonella typhus, phage ϕX174 live BCG, diphtheria toxoid, Mycobacterium (CFA)	IM	Anti-phage titer: immunization at 60-66 dg (n=1) resulted in titers less than that in naïve 110-130 dg fetuses; anti-ferritin titers (70-80 dg)[2]; anti-ovalbumin titers (120-125 dg)[2]. No responses at any time to Salmonella, BCG, Mycobacterium (CFA) or diphtheria toxoid.	(Silverstein, Uhr et al. 1963)
65 or 90	*N. caninum*	NS	Fetal antibodies in 1/8 lambs immunized at 65 dg; 2/8 lambs immunized at 90 dg.	(Buxton, Maley et al. 1998)
70	*Chlamydia abortus*	Trans-placental	Antibodies in fetal serum and thoracic fluid. Also antigen-specific CD8+ and γδ-T cells.	(Kennedy, McCullough et al. 2001) (Buxton, Anderson et al. 2002)

(Continued…)

(Table 1.4 continued)

80-110[2]	β-gal (adenoviral vector)	Intratracheal	Potential tolerance (up to 38 dpi)[3]	(Peebles, Gregory et al. 2004)
85-100[2] or 115-125[2]	*Brucella abortus*	Intracardiac	Predominantly IgM (low IgG titers) peaked about 3 wpi. 6/9 immunized at 85-100dg. Memory persisted at least 40 d. 12/14 immunized at 115-125 dg.	(Richardson, Conner et al. 1971)
95-140[2]	*B. abortus*	Oral via amniotic fluid	Fetal agglutination titers (8/12)[4]	(Richardson and Connor 1972)
112-134[2]	β-gal (adenoviral vector)	Intra-tracheal	CMI	(Vincent, Trapnell et al. 1995)
120-125[2]	Bovine herpesvirus glycoprotein D (DNA vaccine)	Oral cavity	Fetal IgG1, neutralizing antibodies and proliferative responses. Neonatal immune memory. Activation of retropharyngeal, but not mesenteric LNs.	(Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002)
135-142[2]	Horse spleen ferritin, 500 mg	Amniotic fluid	Precolostrum serum IgM (2/5)[4]; intestinal IgA and IgM (4/5)[4]; plasma cells in lymphoid tissues (6/6)[4]. Bulk of antigen deposited in the jejunum.	(Husband and McDowell 1975)
140	Human Factor IX (adenoviral vector)	Umbilical vein	Antibodies to Factor IX and adenovirus starting at 7 days post-injection	(Themis, Schneider et al. 1999)

CFA = complete Freund's adjuvant; dg = days gestation; dpi = days post-immunization; IM = intramuscular; IP = intraperitoneal; LNs = lymph nodes; MuLV = Moloney murine leukemia retroviral vector for gene therapy; NS = not specified; wpi = weeks post-immunization.

[1] Pregnant dams were first exposed to virus at the earlier date. The infective source was removed at the latter date.

[2] Where a range is indicated, fetal lambs with differing gestational ages were grouped together.

[3] No tests for suppression of immune responses were conducted. Tolerance was inferred from the presence of antigen in thymic tissues and lack of inflammation in transduced tissues.

[4] Number of responders/total number of treated animals.

Studies of autoimmune thyroiditis in the fetal lamb model suggest that an antigenic hierarchy might also apply to the induction of tolerance to self-antigens. Thyroidectomy at 51-54 dg resulted in the rejection of autologous thyroid tissues upon reimplantation at 110-115 dg, thus suggesting that the immune system had not become tolerized to thyroid antigens during the first trimester and that reactivity to thyroid antigens was acquired at some point in the intervening 60 days (King, Hagan et al. 1998). Autologous thyroid tissues reintroduced between 82-93 dg were tolerized (Belin, Hollingsworth et al. 1976). Taken together, these studies suggest that there might be a critical period, between 93-110 dg, during which the TCR gene undergoes rearrangement to produce T cells specific for thyroid antigens.

It is now apparent that the lack of responsiveness to specific antigens is due to the limited immunological repertoire in the fetus, and the temporal acquisition of reactivity to specific antigens reflects the preferential usage of certain heavy and light chain gene segments at different stages of development. The antigenic hierarchy might also reflect the low number of lymphoctes in the fetus, which would restrict the number of clones with any specific TCR- or Ig-gene rearrangement. Without a minimum number of lymphocytes specific for a corresponding antigen, the chances of inducing an immune response are limited. As a result, the antigenic repertoire against which the immune system can respond should increase proportionally to the number of cells in the peripheral lymphocyte pool. In fact, there is a 5-fold increase in the number of recirculating peripheral T cells between 135 dg and 2 weeks of age (Cunningham, Cahill et al. 1999). An expansion in the repertoire at this time would allow the neonatal lamb to respond to the onslaught of new antigens that accompanies birth.

1.4.5. Self-Non-Self Discrimination in the Fetal Lamb

The fetal lamb is a desirable model for the study of fetal immunization because of its immunological naïvety and the absence of maternal antibodies. Studies of immune induction in the fetal lamb model are summarized in Table 1.4. A large number of fetal transplantation and gene replacement studies have also been conducted in the fetal lamb model and these are summarized in Table 1.5. Together, these studies give insight into mechanisms of induction of tolerance and immune protection in the fetal lamb model. It should be noted, however, that most studies reported a high degree of variability in responses within an experimental group. Therefore, the trends described below are based on the majority response.

A review of the transplantation studies shown in Table 1.5 suggests that a number of factors, including the type of graft, its location within the host, and the age of the fetus at the time of transplantation, may contribute to determining whether a graft is accepted or rejected. The Zanjani-Porada group hypothesized that tolerance to a graft could be achieved by introducing the graft while the immune system was immature (Flake, Harrison et al. 1986; Zanjani, Ascensao et al. 1992; Zanjani, Pallavicini et al. 1992; Rice, Flake et al. 1993; Zanjani and Anderson 1999; Almeida-Porada, Porada et al. 2004; Porada, Park et al. 2004). This hypothesis was based on the stable incorporation of allogeneic stem cells into preimmune fetal hosts. Other groups, however, have shown that tolerance to foreign stem cells can also be achieved when transplantion occurs after the preimmune period (i.e., at 65-79, 85 and 95 dg) (Miyasaka and McCullagh 1982; Liechty, MacKenzie et al. 2000; Sasaki, Nagao et al. 2005). The

Table 1.5. Fetal lamb transplantation studies

Age of fetus at time of transplant[1]	*Graft*	*Route/ Location*	*Outcome*	*Reference*
40-45 dg	Human CD3-depleted cord blood hematopoietic stem cells	IP	Tolerance (4/12)[2]	(Young, Holzgreve et al. 2003)
45-60 dg	Maternal cells	IP	No effect	(Miyasaka and McCullagh 1982)
48-54 dg	Human fetal liver hematopoietic stem cells	IP	Tolerance (13/33)	(Zanjani, Pallavicini et al. 1992)
48-64 dg	Ovine liver-derived mesenchymal cells	IP	Tolerance	(McCullagh 1989; Schoeberlein, Holzgreve et al. 2004; Sasaki, Nagao et al. 2005; Schoeberlein, Holzgreve et al. 2005)
53-55 dg	Fetal or adult ovine skin allografts	Skin graft	Fetal grafts were rejected by hyperacute mechanisms; adult grafts were tolerized	(McCullagh 1989)
55-73 dg	Monkey stem cells	Intrahepatic	Tolerance (4/4)	(Sasaki, Nagao et al. 2005)
64-67 dg	Fetal ovine skin	Skin graft	Tolerance	(Silverstein and Prendergast 1964)
65, 85 or 95 dg	Human mesenchymal stem cells	IP	Tolerance; detection of donor cells in the thymus.	(Liechty, MacKenzie et al. 2000)
77-139 dg	Ovine skin	Skin graft	Rejection (13/13)[2]	(Silverstein and Prendergast 1964)
110-133 dg	Maternal cells (non-inherited maternal antigen)	IP	Tolerance; suppression of neonatal CTL responses.	(Miyasaka and McCullagh 1982)
120-139 dg	Fetal ovine skin	Skin graft	Rejection	(Silverstein, Prendergast et al. 1963)

dg = days gestation; IP = intraperitoneal.

[1] Where a range is indicated, fetal lambs with differing gestational ages were grouped together.

[2] Number of responders/total number of treated animals.

importance of the age of the fetus at the time of transplantation may depend on the specific type of graft, e.g., skin grafts introduced prior to 77 dg (when the fetus becomes DTH-competent) were consistently tolerized, while skin grafts introduced after the onset of DTH-competence were rejected (Silverstein, Prendergast et al. 1963; Silverstein and Prendergast 1964; McCullagh 1989).

In all of the above studies in which stem cell grafts were accepted, cells were delivered via the intraperitoneal (IP) or intrahepatic routes, both of which result in the establishment of transplant-derived hematopoietic stem cell populations in the bone marrow. The percentage of chimerism obtained by intrahepatic injection of monkey stem cells was 1-2% of the hematopoietic bone marrow populaton in 4 of 4 lambs (Sasaki, Nagao et al. 2005). Hematopoietic stem cells in the fetus have the potential to differentiate into cells of any lineage, including granulocytes, lymphocytes and MTECs (Liechty, MacKenzie et al. 2000; Almeida-Porada, Porada et al. 2004). The migration of transplant-derived cells to the thymus would allow the foreign MHC to be regarded as self. In Tran et al. (2001) and Porada et al.(1998), IP delivery of either *lacZ* or *neoPT*, the gene for neomycin phophotransferase (NeoPT), in the context of retroviral vectors at 55-60 dg resulted in the expression of both β-galactosidase and NeoPT in hematopoietic cells and the thymus. The stable expression of NeoPT at 14 months after gene delivery suggested that tolerance had been induced. In a study by Peebles et al. (2004), delivery of *lacZ* to the tracheal epithelium of 80-110 dg fetuses using adenovirus vectors also resulted in the induction of specific tolerance. The authors hypothesized that tolerance was the result of transduction of stem cell populations in the immature tracheal epithelium at 80-110 dg. In contrast, when *lacZ* in the context of an adenoviral vector

was delivered to the tracheal epithelium at 112-134 dg (when stem cells are no longer present), CMI was induced (Vincent, Trapnell et al. 1995). Taken together, the above studies suggest that tolerance can be induced by targeting genes that encode specific antigens to stem cells, or by immunizing with multipotent stem cells expressing a specific antigen. In this context, the age of the fetus at the time of immunization does determine whether or not stem cells are present at specific anatomical locations and, by extension, the preferred route of delivery.

A review of the studies presented in Table 1.4 also suggested that the fetal lamb might be described as having three stages of immune responsiveness. (i) The pre-thymic stage occurs prior to 31 dg and introduction of a foreign antigen during this period is likely to result in the induction of tolerance. For example, infection with bovine pestivirus during this period resulted in chronic infection (Swasdipan, McGowan et al. 2002). (ii) The preimmmune stage, as described by the Zanjani-Porada group, occurs between 31 and 55-60 dg. Neither tolerance nor protective immunity appears to be induced to foreign antigens present in peripheral tissues for the duration of this period. (iii) The immune-competent stage starts at approximately 60-66 dg, which is the age when the humoral arm of the immune system becomes functional (Silverstein, Uhr et al. 1963). During the immune-competent stage, the immune system acquires the ability to respond to specific antigens according to an antigenic hierarchy. This is consistent with the findings in a number of studies (Silverstein, Uhr et al. 1963; Hashiguchi, Nanba et al. 1979; Dubey, Emond et al. 1987) in which the introduction of an antigen to the peripheral immune system prior to its being represented in the immune repertoire did not result in the induction of tolerance; however, once the antigen is included in the immune

repertoire, the induction of either tolerance or protective immunity depends upon the context in which the antigen is delivered (Section 1.2.1).

The fetal immune system can respond to an assortment of antigens, including live virus, purified protein, soluble protein, phage, bacteria, and antigens delivered in genetic form in the context of either bacterial plasmid or adenovirus. Fetal immune responses can be induced to small or large antigens as demonstrated by the induction of antibodies to both bovine herpesvirus-1 (BHV-1) truncated glycoprotein D (tgD) and HBsAg (Gerdts, Babiuk et al. 2000). TgD is a secreted, soluble 61-kDa glycoprotein (Tikoo, Campos et al. 1995) while HBsAg is a non-soluble VLP with a diameter of approximately 20nm (Petit, Maillard et al. 1986; Aberle, Aberle et al. 1999). Of the studies summarized in Table 1.4, fetal immune responses were only detected in those studies in which antigen was delivered accompanied by an adjuvant (e.g., bacterial DNA, CFA) or danger signal (e.g., infection). The exception is the study by Husband and McDowell (1975) in which fetal immune responses were detected following the injection of horse spleen ferritin into the amniotic cavity in the absence of any adjuvant; however, the induction of an immune response may have been influenced by the dose of antigen, which was 500-fold larger than the dose previously shown to induce ferritin-specific fetal antibodies (Silverstein, Uhr et al. 1963).

Overall, the literature supports the hypothesis that the fetal lamb is immune competent beyond 60 dg and can mount an antigen-specific protective immune response provided the antigen is not targeted to the thymus. Furthermore, the literature supports the existence of an immunological “null period” during which foreign antigens introduced to the thymus are regarded as self and antigens introduced into the periphery

do not appear to have any effect on the functionally immature immune system; however, this null period does not extend for the duration of prenatal development as was originally suggested by Billingham et al. (1953). The null period for a specific antigen ends when it is represented in the immune repertoire and can occur as early as 55-60dg. At this time, the antigen is either tolerogenic or immunogenic depending on the route of delivery and the presence or absence of co-stimulatory signals.

1.4.6. *In Utero* Gene Delivery in the Fetal Lamb Model

Successful methods of *in utero* gene delivery have been reported in the fetal lamb model and the gene expression data from these studies are summarized in Table 1.6. The immunological outcome of studies by Gerdts et al. (2000; 2002), Peebles et al. (2004), Tran et al. (2001), Porada et al. (1998) and Vincent et al. (1995) were summarized in Table 1.4. In most cases, the method of gene delivery resulted in the induction of tolerance and, therefore, is appropriate for gene therapy but not fetal vaccination.

In Tran et al. (2001) and Porada et al. (1998), IP delivery of genes in the context of the Moloney murine leukemia virus (MuLV) vector resulted in the transduction of hematopoietic stem cells and detection of plasmid-encoded protein in a range of tissues and cell types for a prolonged duration. This method of gene delivery is inappropriate for fetal immunization as genes delivered to the thymus consistently resulted in the induction of tolerance. Intratracheal injection of adenovirus-vectored genes resulted in consistently high levels of gene expression in the airway and respiratory epithelium. The induction of either tolerance or protective immune responses depended upon the time of

Table 1.6. *In utero* gene delivery in the fetal lamb

Vector	*Route of delivery*	*Reporter gene/ protein*	*Gene distribution/ localization of antigen*	*Duration of expression*	*Reference*
DNA plasmid	Oral cavity	BHV-1 tgD	NS	NS	(Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002)
Japan hemagglutin-ating retrovirus	Ductus arteriosus	CAT	Subendothelial and smooth muscle of ductus arteriosis	40 d	(Mason, Bigras et al. 1999)
MuLV	IP	NeoPT	Most tissues/organs including bone marrow, thymus (epithelial cells). Not germline cells.	5+ y	(Porada, Tran et al. 1998)
MuLV	IP	*lacZ*, NeoPT	Hematopoietic cells in blood and bone marrow, thymus. Not tested elsewhere	40 mo	(Tran, Porada et al. 2001)
Adenovirus (E1 and E3 vectors)	Umbilical vein	*lacZ*	Fetal liver, lung, gonads, adrenals, kidneys, brain, heart. Maternal liver and placenta.	NS	(Themis, Schneider et al. 1999)
Adenovirus (E1 and E3 vectors)	Umbilical vein	Factor IX	Inconsistent. Systemic in 2/5 fetuses.	Up to 15d	(Themis, Schneider et al. 1999)
Adenovirus (formulated with or without Na-caprate[1] and DEAE)	Intratracheal (ultrasound-guided)	*lacZ*	Tracheal epithelium and bronchi.	38 d	(Peebles, Gregory et al. 2004)

BHV-1 tgD = bovine herpes virus-1 truncated glycoprotein D; CAT = chloramphenicol acetyl transferase; IP = intraperitoneal; MuLV = Moloney's murine leukemia virus; NeoPT = neomycin phosphotransferase; NS = not specified.

[1] Na-caprate is a fatty acid that opens tight junctions to enhance viral access to the basolateral surface of respiratory epithelial cells; increases gene expression by 90-fold relative to adenovirus alone.

injection and tolerance was induced up to 110 dg (Vincent, Trapnell et al. 1995; Peebles, Gregory et al. 2004). Direct injection of a foreign gene in the context of a retroviral vector into the ductus arteriosus resulted in high levels of localized transfection and expression (Mason, Bigras et al. 1999). Unfortunately, the immunological outcome and potential negative reffects of direct retroviral injection were not reported as part of this study.

Injection of an adenovirus vector into the umbilical vein was the least effective method of *in utero* gene delivery in the fetal lamb (Themis, Schneider et al. 1999). Adenoviral vectors containing either *lacZ* or the gene for Factor IX were delivered to four and five fetuses, respectively. The small number of animals per experimental group, the range of dose, the utilization of more than one gene, the range of fetal age at the time of injection (102 – 140 dg) and assaying for gene expression at different times post-injection limited any conclusions with regards to the efficacy of umbilical vein injection as a route of gene delivery. Furthermore, adenovirus at a dose of 2.4×10^{12} was lethal, while doses between 1×10^{11} to 4.8×10^{11} gave inconsistent results ranging from no detectable gene delivery to widespread, non-specific transfection of most fetal tissues, the placenta and the maternal liver.

Of the different methods of *in utero* gene delivery described in the fetal lamb model, oral delivery of naked DNA plasmid (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002) appears to be best suited for the specific purpose of fetal immunization. The other gene delivery methods described in Table 1.6 rely upon delivery in the context of viral or retroviral vectors which have potential toxic effects, especially in the developing

fetus, and theoretically, can only be used once due to the potential induction of immune responses to the vector itself.

1.5. DNA Vaccination at Mucosal Surfaces

A fetal vaccine should be designed to maximize both systemic and mucosal immunity, since mucosal surfaces are the major site of entry for numerous neonatal pathogens. In general, most vaccines are administered via the IM or subcutaneous routes despite experiments which have shown that parenteral immunization is not an effective means of stimulating mucosal immunity (Brokstad, Eriksson et al. 2002; Cox, Brokstad et al. 2004). Induction of mucosal immunity requires that antigen be targeted to mucosal surfaces or, more specifically, to mucosal-associated lymphoid tissues (MALTs).

Unfortunately, there are a number of obstacles to mucosal vaccination. Low pH, proteases and other enzymes decrease the stability of traditional vaccines delivered via the vaginal surface or digestive tract. The mucociliary escalator that lines the trachea, bronchi and bronchioles impedes delivery of particulate matter to the respiratory epithelium and the potential induction of oral tolerance limits targeting of antigens to the PPs or mesenteric LNs. Furthermore, mucosal immunity is characterized by Th2-mediated responses (i.e., IgA) which may be less effective than CMI at protecting against intracellular pathogens, such as bovine respiratory syncytial virus (Mapletoft, Oumouna et al., 2008). DNA vaccination might overcome many of the obstacles to effective mucosal immunization; the continual expression of antigen could potentially counter degradation and clearance by innate immune mechanisms and the inherent

adjuvanticity of the bacterial DNA might stimulate a Th1-component within the mucosal immune response.

1.5.1. DNA Vaccines

DNA vaccines are the latest technology for inducing long-term immunity and an alternative to either virus or bacteria as a gene delivery vehicle. The primary component of a DNA vaccine is purified plasmid DNA containing a gene encoding a specific antigen under control of an eucaryotic promoter. Plasmid DNA is easy to produce in large quantities at a low cost and remains stable upon production. Furthermore, advances in cloning technology have allowed the easy adaptation of DNA vaccine technology to a number of different antigens/pathogens.

The ability to transfect cells *in vivo* with DNA plasmid was first demonstrated in 1990 (Wolff, Malone et al.) and led to the hypothesis that expression of a foreign gene *in vivo* would result in the induction of an immune response (Tang, DeVit et al. 1992; Ulmer, Donnelly et al. 1993). DNA vaccination studies have since been carried out in a number of different species, using genes encoding different antigens, with variable success. For example, a DNA vaccine containing the gene for equine herpesvirus-1 (EHV-1) glycoprotein D (gD) induced both antibody and lymphoproliferative responses in mice and protected mice against a subsequent challenge with EHV-1 (Ruitenberg, Walker et al. 1999). Cattle immunized with either gD- or tgD-plasmid delivered via the intradermal (ID), but not IM, route were protected against challenge with BHV-1 (van Drunen Littel-van den Hurk, Braun et al. 1998) and sheep immunized with tgD-plasmid delivered by gene gun induced long-term CMI and moderate antibody titers (Braun,

Babiuk et al. 1998). A number of DNA vaccine studies have also been carried out in the neonate. Among these studies, a plasmid encoding the circumsporozoite protein of *P. yoelii* was the only DNA vaccine found to induce immune tolerance (Ichino, Mor et al. 1999).

The effectiveness of DNA vaccination has been attributed to the inherent immunogenicity of the bacterial DNA backbone, which contains large numbers of CpG and polyG motifs. The former are hexameric sequences consisting of unmethylated cytosine and guanosine dimers flanked on either side by two purines (Krieg, Yi et al. 1995). This contrasts with vertebrate genomes, where CpG motifs are under-represented and heavily methylated (Cardon, Burge et al. 1994). As a result of these differences, the vertebrate immune system has evolved to recognize CpG motifs as pathogen-associated molecular patterns and generates strong innate and CMI responses when confronted with CpGs (Krieg, Yi et al. 1995; Yi, Klinman et al. 1996; Schnare, Barton et al. 2001).

The immunostimulatory activity of CpGs is mediated by TLR-9 which is expressed in endosomes of B cells, macrophages and DCs (reviewed by Mutwiri, Pontarollo et al. 2003). Upon binding of TLR-9, the MyD88/TRAF6 signaling pathway is activated, resulting in production of type-1 cytokines (IFNγ, IL-1, -6, -10 and -12), up-regulation of MHC class-II and B7 co-stimulatory molecules, cellular proliferation and inhibition of B cell apoptosis. Furthermore, the inherent adjuvanticity of bacterial DNA translates into a lowered risk of inflammatory damage and lesions when compared with the use of conventional adjuvants such as alum and CFA (Gupta, Relyveld et al. 1993).

In addition to their immunostimulatory effect, DNA vaccines may also enhance the duration of immune memory. Transfection of host cells and plasmid expression by

the host-cell machinery results in the presentation of antigen on MHC-class I molecules and the preferential induction of CD8+ T cell-mediated responses. The prolonged survival of mCTLs was reviewed in Section 1.2.1. Transfected host cells also become factories for the continual production of antigen and as long as these cells remain transcriptionally active, there is antigen available for the stimulation of memory lymphocytes. This differs from traditional, subunit or killed vaccines, which are rapidly degraded or cleared.

Although DNA vaccination studies in mice have been successful, that success has not translated into large animal models largely as a result of low rates of transfection and/or expression. Numerous methods for enhancing plasmid transfection have been reported. Electroporation (Babiuk, Baca-Estrada et al. 2004) and low frequency ultrasound (Kim, Greeleaf et al. 1996) enhanced transfection efficiency but resulted in significant inflammatory damage and cell lysis, respectively. In one simple, yet successful study, increasing the hydrostatic pressure at the time of IM injection, e.g., by injecting an equal volume of plasmid over a 5 second period as opposed to a 20 second period, increased gene expression by 1000-fold (Liang, Nishikawa et al. 2004).

The basic principle of using a lipid carrier to transfect cells *in vitro* has also been applied to DNA vaccination. E.g., formulation of plasmid with the cationic lipid carrier (N-(1-(2,3-dimyristyloxypropyl)-N,N-dimethyl-(2-hydroxyethyl) ammonium bromide/dieoyl phosphatidylethanolamine (DMRIE/DOPE) resulted in a 7-fold increase in plasmid expression relative to unformulated plasmid (Talsma, Babensee et al. 2006). Complexing with lipids has many effects. These include (i) enlarging the positive surface potential, which has been shown to directly correlate with increases in

transfection efficiency, and (ii) increasing the efficiency of plasmid translocation across the nuclear membrane. The conjugation of lipid carriers to sigma-1 attachment protein from recombinant reovirus type-3 resulted in a 7-fold increase in translocation of plasmid to the nucleus of fibroblasts *in vitro* and a 16-fold increase in transgene expression (Talsma, Babensee et al. 2006). Nuclear translocation was also enhanced by complexing plasmid to M9, which is a modified mRNA transporter derived from ribonucleoprotein-A1 (Byrnes, Nass et al. 2002), or by incorporation of the SV40 enhancer into the plasmid (Young, Benoit et al. 2003).

In the study by Gerdts et al. (2000), oral delivery of DNA plasmid to fetal lambs resulted in levels of antigen expression sufficient to induce an immune response. This indicated that high rates of transfection and expression were achieved in fetuses despite the absence of formulation or the use of mechanical means, such as electroporation, to increase transfection efficiency.

1.5.2. Induction of Immunity or Tolerance in the Mucosal Immune System

Oral delivery of an antigen is associated with the risk of inducing tolerance. Induction of a type-1 or type-2 response or oral tolerance depends on a number of factors, including the specific antigen and where the primary inductive event takes place. In mice, peroral administration of a soluble antigen, OVA, resulted in oral tolerance (Spahn, Weiner et al. 2002). Induction of tolerance required that OVA reach both the PPs and mesenteric LNs and was characterized by suppression of IFNγ and decreases in both Th1- and Th2-mediated responses. In contrast, peroral immunization with a soluble cholera vaccine in humans resulted in the induction IgG and IgA titers in both serum and

saliva (Czerkinsky, Svennerholm et al. 1991). These studies show that oral delivery does not necessarily equate with oral tolerance.

The term "oral delivery" in the context of vaccine delivery has been used to refer to feeding/ingestion, intubation, gastric lavage, injection into the amniotic fluid or application to the oral cavity (without swallowing). The induction of oral tolerance requires that antigen reach the GALT where it is taken into intestinal M cells and translocated to underlying lymphoid follicles, which contain large numbers of B cells that are committed to only producing IgA (Cebra, Gearhart et al. 1976). If induction occurs in both the mesenteric LNs and PPs, oral tolerance occurs. Alternately, if induction is restricted to the PPs, there is preferential induction of type-2 immune responses (Spahn, Weiner et al. 2002). Following induction, B cells can migrate to distal mucosal sites or enter the circulation. IgA-secreting B cells preferentially home to MALTs, including the salivary glands and tonsils (Cebra, Gearhart et al. 1976; Kubagawa, Bertoli et al. 1987).

The salivary glands are specialized and do not take part in primary immune induction. They do, however, contain large numbers of primed B cells. Environmental antigens that enter the oral cavity can directly stimulate B cells in the salivary glands to produce antibody upon secondary or subsequent exposure to antigen (Nair and Schroeder 1983). The sublingual and submandibular salivary glands contribute over 50% of total IgA in gastric juices, gastric mucus and feces. They also make a small, but significant, contribution to serum IgG (Shirai, Wakatsuki et al. 2000). The salivary glands also play a role in deviation towards Th2 immune responses. When *H. pylori* sonicate was administered to sialoadenectomized mice (i.e., mice from which sublingual

and submandibular salivary glands had been excised), Th2 responses decreased and Th1 responses increased (Shirai, Wakatsuki et al. 2000). The restoration of Th2 responses by the addition of salivary gland extract to the bacterial sonicate suggested that the salivary glands produce large amounts of type-2 cytokine (Mbow, Bleyenberg et al. 1998). Unlike in salivary glands, primary B cell activation does occur in the tonsils of the oral cavity (Brandtzaeg 1989; Inoue, Fukuizumi et al. 1999).

In utero oral DNA immunization studies showed that delivery of antigen (in the form of DNA plasmid) to the oral mucosal epithelium also induced CMI (Gerdts, Babiuk et al. 2000). Antigen delivered to epithelial surfaces may be presented by Langerhans cells, which preferentially activate CD8+ T cells, or delivered to draining LNs where the type of immune response is dictated by the nature of the antigen and the cytokine environment.

1.5.3. DNA Vaccine Delivery to the Oral Epithelium

There are advantages to delivering vaccines to the oral epithelium, such as targeting of antigen to MALT, avoiding degradation of antigen in the digestive tract, and an increased surface area relative to the nasal cavity. The oral cavity in humans is divided into three major areas with respect to drug delivery (Shojaei 1998). (i) The buccal cavity is lined by mucosal epithelium, has a rich blood supply, is relatively permeable and is the traditional, preferred site for drug delivery. (ii) The sublingual mucosa lines the bottom of the mouth and lower palate and also consists of mucosal epithelium. (iii) The tongue and lips are covered by cutaneous epithelium. The buccal, sublingual and cutaneous epithelium differ in their rates of epithelial cell turnover,

vascular supply, lymphocyte subpopulations and permeability. In humans, the buccal mucosa is stratified and made up of 40-50 layers of squamous cells. There are fewer cell layers in the sublingual mucosa and more in the cutaneous regions. The superficial-most layers of the tongue and lips are also keratinized. The main barrier to aqueous solutions is provided by the secretion of keratohyalin into the intercellular spaces in the upper one-third layers of the cutaneous epithelium. In the absence of keratohyalin, fibrous keratin by itself is not a significant barrier to aqueous solutions and keratinized and non-keratinized epithelium have similar permeabilities (Squier and Hall 1984).

In order of increasing permeability are: skin, cutaneous epithelium, palatal mucosa, buccal mucosa and sublingual mucosa (Galey, Lonsdale et al. 1976; Harris and Robinson 1992; Hengge, Pfutzner et al. 1998). Using beagles as an animal model, it was found that injection of equivalent doses of *lacZ*-plasmid into either the oral mucosa or epidermis resulted in 35-fold higher levels of β-galactosidase expression in the oral mucosa (Hengge, Pfutzner et al. 1998). Although the mucosal epithelium had a higher transfection efficiency, gene expression was more transient. At 3 hours post-injection, plasmid was present in the basal and supra-basal layers of the mucosal epithelium, but was only detectable in the superficial-most layer by 24 hours. Therefore, both the permeability and the rate of epithelial turnover must be considered for a specific surface as the maximum level of expression is related to the number of plasmid copies able to reach nucleated cells and plasmid is only expressed until the transfected cells are sloughed off. One method for prolonging the duration of DNA vaccines at mucosal surfaces is to deliver into the fetus, which has a lower rate of mucosal epithelial cell turnover (Smeaton and Simpson-Morgan 1985).

Epithelial cells in the cutaneous epithelium (e.g., the tongue and lips) can produce IL-1, -3 and -6, GM-CSF and TNF and, therefore, can augment local inflammation and lymphocyte activation. Human keratinocytes can also up-regulate MHC class-II *in vitro* upon stimulation with IFNγ (Wittmann, Purwar et al. 2005). Langerhans cells are present in cutaneous, but not mucosal, epithelium superficial to the basal cell layer (Bodde, DeVries et al. 1990; Ratzinger, Gaggers et al. 2005). Langerhans cells can express MHC class-I, class-II and B7, however, antigen presentation by Langerhans cells usually results in the induction of CTL responses. The mucosal epithelium contains low numbers of cells that stain for mucosal leukocyte antigen (a marker for homing to mucosal tissues), CD8 and γδTCR, but the majority of macrophages and lymphocytes are located in the lamina propria (Ratzinger, Gaggers et al. 2005).

A number of delivery systems and formulations have been reported to enhance drug delivery to the buccal mucosa. These include delivery via mucoadhesive hydrogels and buccal patches, and formulation with sodium glycocholate, sodium ethylenediamine tetraacetic acid (EDTA) or aprotinin (Veillard, Longer et al. 1987; Bodde, DeVries et al. 1990; Shojaei 1998). Normally, the oral mucosa is coated by saliva which contains mucus. At physiological pH, the mucus network carries a negative charge, which may repel naked DNA plasmid (Tabak, Levine et al. 1982). The efficiency of plasmid delivery to the oral mucosa may be enhanced in the fetus, in which these surfaces are bathed in amniotic fluid. The fetus also has a lower rate of epithelial cell turnover relative to the adult (Smeaton and Simpson-Morgan 1985) which may prolong the duration of antigen expression.

1.5.4. *In Utero* Oral DNA Immunization

In utero oral DNA immunization was originally described in Gerdts et al.(2000; 2002). In brief, DNA plasmid containing the gene for BHV-1 tgD was injected into the oral cavity of fetal lambs at 120-125 dg. The uterus was accessed via an abdominal incision and the uterus was manipulated such that the head of the fetus, still within the uterus, was exteriorized through the incision. A needle was then inserted through the uterine wall into the oral cavity and a DNA solution was deposited into the fetal oral cavity. To assess the efficacy of *in utero* immunization, antibody and CMI responses against authentic (i.e., virus-derived, non-truncated) BHV-1 gD were assessed in the preparturient fetus (140-145 dg). Oral immunization induced titers of gD-specific serum IgG that were 100- to 500-fold greater than in age-matched, naïve lambs. Ig-secreting B cells and gD-specific lymphoproliferative responses were also detected in the retropharyngeal LNs, suggesting that the bulk of the transfection and expression had occurred in either the oral cavity or LNs draining the oral cavity. The lack of detectable gD-specific responses in mesenteric LNs suggested that oral delivery did not result in plasmid or plasmid-encoded protein reaching the gut. The effectiveness of the *in utero* oral delivery method was further demonstrated by the induction of neutralizing antibodies to BHV-1, decreased viral shedding following intranasal challenge and tgD-specific anamnestic responses in the neonate with both systemic and mucosal elements. Similar results obtained with a DNA vaccine encoding HBsAg, demonstrated the relevance of the *in utero* oral DNA immunization method to a human pathogen.

This thesis also makes use of BHV-1 tgD as a model antigen. BHV-1 is an important pathogen in cattle that causes a number of clinical diseases, including upper

respiratory disease (shipping fever), infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, conjunctivitis and abortion (Gibbs and Rweyemamu 1977). BHV-1 encodes several surface glycoproteins, including gD and glycoprotein B (gB), which have been shown to consistently induce immune responses in both cattle and sheep when used as vaccinating antigens (van Drunen Littel-van den Hurk, Gifford et al. 1990; van Drunen Littel-van den Hurk, van Donkersgoed et al. 1994) (also see references in Table 3.1). Unfortunately, there is no reliable virus-challenge model available for BHV-1 infection in sheep.

1.6 Concluding remarks

The scientific literature supports the effective design of fetal vaccines, provided that factors such as the age of the fetus, the route and method of antigen delivery, the provision of co-stimulatory signals and the specific antigen for which an immune response is desired are taken into account. With recent advances in genetic research, it will be possible to identify antigens that are represented in the fetal immune repertoire, and use of these antigens may allow us to identify factors which determine whether the outcome of fetal immunization is tolerance or immune protection. Given the evidence in support of fetal immune competence, it is interesting that developmental tolerance remains an immunological dogma. Challenges to this dogma may result in the effective design of fetal vaccines against vertically transmitted pathogens.

CHAPTER 2

OBJECTIVES AND HYPOTHESES

A number of problems have been identified that limit the effectiveness of fetal and neonatal vaccination. These include (i) maternal antibodies, which interfere with subunit and killed vaccines before they can be properly presented to the fetal immune system (Reynolds and Griffin 1990; van Maanen, Bruin et al. 1992; Blasco, Lambot et al. 2001); (ii) immune deviation towards type-2 responses (Liu, Tu et al. 2001; Liu, Tu et al. 2001), which in some cases are ineffective and in many cases pathological; (iii) low numbers of B cells and immature memory, requiring repeated boosting (Siegrist 2001); (iv) immunosuppression due to high cortisol levels (Munroe 1971; Berdusco, Hammond et al. 1993); and (v) the induction of tolerance as a result of the immaturity of the immune system and its inability to deliver adequate co-stimulatory signals (Liu, Tu et al. 2001; Liu, Tu et al. 2001; Tschernig, Debertin et al. 2001). In 2000 and 2002, our laboratory reported a new method of generating specific immunity in the fetus and neonate - *in utero* oral DNA immunization - that appeared to circumvent many of the problems listed above (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002). Using the fetal lamb model, Gerdts et al. demonstrated that oral delivery of a DNA vaccine to the 125 dg fetus induced antigen-specific, long-term immunity with both systemic and

mucosal components. This thesis is a continuation of the study of oral DNA immunization in the fetal lamb model.

Two hypotheses were tested in this thesis:

1) **Hypothesis #1: The mucosal epithelium of the oral cavity is the primary site of plasmid transfection and expression following *in utero* oral delivery of DNA plasmid.** Injection of tgD-plasmid into the fetal oral cavity induced strong mucosal and systemic gD-specific immune responses in the fetus (Gerdts, Babiuk et al. 2000), thus confirming that transfection and expression of the plasmid had occurred. The detection of antigen-specific lymphocyte proliferation in the retropharyngeal LNs of immunized fetuses suggested that transfection likely occurred in the oral cavity or LNs draining the oral cavity. Localization of plasmid transfection to the oral mucosal epithelium has consequences with respect to tolerance induction and the ability to extend oral DNA delivery to younger or older developmental ages. The oral epithelium undergoes major histological changes during development. If plasmid transfection is restricted to the oral epithelium, increases in (i) the thickness of the epithelium, (ii) the rate of epithelial cell turnover, and (iii) effectiveness with respect to its barrier function that accompany suckling may limit the ability to extend oral DNA delivery to the neonatal period. On the other hand, the decreased rate of epithelium cell turnover and decreased barrier function in the younger fetus may allow for prolonged antigen expression and increased drainage of plasmid and/or

antigen to regional LNs, respectively. Failure to restrict plasmid transfection and expression to the oral epithelium increases the risk of systemic antigen distribution and induction of tolerance.

2) **Hypothesis #2: Oral DNA immunization in the second trimester (49-98 dg) is an effective method of inducing a fetal immune response with a memory component capable of persisting into the neonate.** The earliest report of immune competence in the fetal lamb is 60-66 dg following IM immunization with crystallized horse spleen ferritin in the presence of adjuvant (Silverstein, Uhr et al. 1963). Others have reported that injection of fetal lambs prior to 60-62 dg with either allogeneic stem cells (McCullagh 1989; Zanjani, Pallavicini et al. 1992; Schoeberlein, Holzgreve et al. 2004; Sasaki, Nagao et al. 2005; Schoeberlein, Holzgreve et al. 2005) or retroviral vectors expressing foreign antigens (Porada, Tran et al. 1998; Tran, Porada et al. 2001) induced long-term tolerance in fetal lambs. In contrast, oral delivery of DNA plasmid encoding a specific antigen should not result in the induction of tolerance if transfection is restricted to the oral cavity and does not reach the thymus. The presence of CpGs in the bacterial backbone should also stimulate local APCs to up-regulate MHC class II and pro-inflammatory cytokines, thus overcoming the inherent lymphopenia and inhibiting peripheral tolerance mechanisms. Furthermore, the low rate of epithelial cell turnover and the continued expression of antigen should ensure that antigen

persists and, therefore, is able to support the development and maintenance of immune memory.

The following six experimental objectives were defined in order to test the above hypotheses:

1) **Objective #1: To identify the location and duration of plasmid expression following oral DNA delivery.** The successful transfection and expression of plasmid DNA following *in utero* oral delivery has been indirectly shown by the induction of immune responses specific to the plasmid-encoded antigen (Gerdts, Babiuk et al. 2000). Using the same delivery protocol as in Gerdts et al. (2000), DNA plasmid containing the gene for soluble, non-secreted firefly luciferase was orally delivered into 125 dg fetal lambs. At specified times following plasmid delivery, fetal tissues were collected and examined for the presence of luciferase activity. Using this method, the location and duration of transgene expression were determined.

2) **Objective #2: To identify factors that might influence the level of plasmid transfection and expression.** Once the primary sites of plasmid transfection and expression were identified, those factors that might increase the efficiency of *in utero* plasmid transfection and/or expression were determined. Manipulation of these factors to increase the efficiency of oral

plasmid delivery in the second trimester fetal lamb ensured that failure to detect an immune response following second trimester immunization would not be the result of a failure in vaccine delivery. Information gained from this objective will also allow us to define the limitations of oral vaccine delivery and answer the question, "Is oral DNA vaccine delivery restricted to the fetus?"

3) **Objective #3: To determine the earliest possible time for induction of immune responses using a single oral DNA vaccination.** Increasing the duration of fetal immunity *in utero* might provide additional protection against infectious agents that are transmitted prior to or during birth. Intrinsic factors influencing the earliest potential age of immunization are maturity of the immune system, the potential induction of tolerance and the ability of the fetal immune system to process, present and recognize a specific antigen. Success might also be limited by toxic effects of the plasmid at earlier gestational ages. *In utero* oral DNA immunization studies were conducted using BHV-1 tgD, which has previously been shown to be represented in the ovine fetal immune repertoire (Gerdts, Babiuk et al. 2000). The age of the fetus at the time of immunization was gradually decreased from 125 dg (the age at which fetal responses to BHV-1 tgD were reported by Gerdts et al. (2000)) to 55 dg (when T and B lymphocytes first appear in peripheral lymphoid tissues).

4) **Objective #4: To determine if a single oral DNA vaccination in the 2nd trimester can induce detectable immune responses and immune memory that persist into the neonatal period.** According to the WHO (2003), the highest prevalence of vertical transmission of persistent viral pathogens, such as HIV, occurs during the neonatal period, which in humans is less than 28 days of age. Therefore, the immune response induced by fetal vaccination must be able to persist until the time of birth in order to provide adequate protection. Protection is most effective if both primary effector functions and immune memory are present at birth. The duration of immune memory is influenced by a number of factors including antigen persistence and exposure to cross-reactive environmental antigens, both of which can boost waning immune memory; however, the placenta acts as a barrier that prevents the fetal immune system from being exposed to numerous environmental antigens. Therefore, the duration of immune memory following *in utero* immunization is largely dependent upon the primary immunization event. Lambs that were immunized in the second trimester were examined at birth for the presence of primary antibody and CMI responses. To test for survival of immune memory into the neonatal period, *in utero*-immunized lambs were administered a second DNA immunization at 2 weeks of age. The presence of anamnestic responses was used as an indicator of survival of immune memory.

5) **Objective #5: To determine if failure to detect primary immune responses following second trimester oral DNA immunization might be due to the induction of tolerance.** Secondary immunization in the neonate also provided an opportunity to determine if lack of detectable primary immune responses might be due to the induction of tolerance. By decreasing the age of the fetus at the time of DNA immunization, the risk of inducing tolerance to the immunizing antigen is enhanced. Due to the individual variability that is expected when conducting experiments in an outbred population, it was expected that second trimester immunization might fail to induce detectable immune responses in some lambs; therefore, secondary immunization in the neonate was also used to determine if there was antigen-specific immune suppression in non-responders. In the event that tolerance was induced in some lambs, *in utero* oral DNA delivery might be proposed as a method for gene replacement therapy or treatment of autoimmunity, allergy or asthma.

6) **Objective #6: To evaluate the magnitude and duration of fetal immune responses and immune memory.** The ovine syndesmochorial placenta maintains the fetal lamb in a state of true immunological naïvety, and therefore, allows us to determine the duration of fetal primary immune responses and immune memory achieved by a single immunization. In order to assess the quality of immune responses achieved in the ovine fetus, a relevant comparison was required; therefore, a study (Chapter 3) was

conducted in which young lambs, aged 2-3 months, were immunized with the same BHV-1 tgD plasmid used for the fetal vaccination studies described in Objectives #3 through #5. Delivery of the DNA vaccine was optimized to obtain the maximal magnitude and duration of primary immune responses. The duration of immune memory following a single DNA immunization was also determined. The parameters obtained from this study were then used as an appropriate comparison to assess the magnitude and duration of immune responses obtained by second trimester oral DNA immunization. This study also examined the influence of the primary immune response on immune memory.

CHAPTER 3

A SINGLE DNA IMMUNIZATION IN COMBINATION WITH ELECTROPORATION PROLONGS THE PRIMARY IMMUNE RESPONSE AND MAINTAINS IMMUNE MEMORY FOR SIX MONTHS[1]

3.1. Abstract

Recombinant protein vaccines and vaccines using killed or inactivated pathogens frequently require multiple vaccinations to induce protective immune responses which may be of relatively short duration. There is also increasing concern regarding adverse local and systemic reactions to injected vaccines and this concern is driving the quest for vaccine formulations capable of inducing protective immunity following a single administration. Vaccine studies frequently evaluate immune responses and disease protection within a relatively short interval following primary and secondary immunizations and, therefore, fail to address the duration of immunological memory or protection. DNA vaccines offer a unique opportunity to enhance the duration of immune responses through their capacity for prolonged antigen expression. The route of DNA vaccination and the method of plasmid delivery are critical factors which can determine transfection efficiency and the duration of vaccine antigen production. Studies were completed which demonstrated that a single IM DNA vaccination, when combined with

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electroporation significantly enhanced the onset and duration, but not the magnitude, of the primary antibody response. A secondary protein vaccination was performed 6 months after the primary DNA immunization. A significant ($p \leq 0.0001$) correlation was observed between both the magnitude ($r^2 = 0.40$) and duration ($r^2 = 0.74$) of the primary antibody response and the occurrence of a secondary antibody response. Therefore, an effective primary DNA vaccination has the potential to significantly prolong the duration of an antibody response and possibly reduce the frequency of revaccination.

3.2. Introduction

Vaccines are an important tool in the prevention and control of infectious disease. Effective vaccines have been developed for a wide variety of viral and bacterial diseases and as new pathogens, such as West Nile virus, emerge there is an increasingly rapid development of vaccines (Chang, Kuno et al. 2004). Thus, humans and domestic animals are subjected to an increasing number and frequency of vaccinations. This has led to increased concern regarding the risks and adverse reactions associated with vaccination (Moylett and Hanson 2004) and renewed interest in developing vaccines that require fewer booster shots to induce and maintain protection against infectious disease. These objectives differ from the more traditional goals of vaccine development, which included the identification of immunogenic antigens and evaluation of immune-mediated protection. For example, numerous vaccine trials were performed to demonstrate that a truncated form of glycoprotein D (tgD) was a protective antigen for BHV-1. The basic design of these experimental trials involved primary, secondary and tertiary immunizations within 3-6 week intervals and viral challenge within 2-5 weeks after the

last immunization (Table 3.1). These trials did not, however, address critical questions such as the onset or duration of immune-mediated protection following single or multiple vaccinations.

A single vaccination with a modified-live BHV-1 vaccine can induce immune memory and reduce clinical disease when animals are infected 25 weeks after vaccination (Platt, Burdett et al. 2006); however, the use of modified live virus (MLV) vaccines is associated with a number of safety concerns, including reactivation and shedding of both latent and vaccine virus, and the possible reversion of the vaccinating strain to virulence (Strube, Abar et al. 1995). These safety concerns are not an issue with DNA vaccines; however, there is no evidence that a single DNA vaccination can induce long-term protective immunity despite reports that transfected genes can be expressed over a prolonged period (Muramatsu, Arakawa et al. 2001).

DNA vaccines remain an attractive vaccine technology due their safety, stability, and the relatively low cost of DNA vaccine production. Furthermore, the immunostimulatory properties of plasmid DNA (Uwiera, Gerdts et al. 2001; Uwiera, Rankin et al. 2001) eliminate the need to formulate DNA vaccines with adjuvants, which often induce a marked local inflammatory response and tissue pathology (Nichani, Kaushik et al. 2004). Unfortunately, commercial development of this technology has been limited by difficulties with achieving efficient DNA plasmid delivery and high levels of plasmid gene expression in target species (Babiuk, Pontarollo et al. 2003).

Numerous vaccination methods have been evaluated for their capacity to enhance DNA plasmid delivery and the induction of immune responses. These

Table 3.1. Vaccine trial protocols with recombinant tgD protein and DNA vaccines

Primary[1] (Species)	*Secondary[2] (Interval)*	*Tertiary[3] (Interval)*	*Challenge[4] (Interval)*	*Reference*
25 µg tgD[5] (Bovine)	25 µg tgD (3 weeks)	---	BHV-1 aerosol (4 weeks)	(Aberle, Aberle et al. 1999)
50 µg tgD (Bovine)	50 µg tgD (5.6 weeks)	---	BHV-1 aerosol (2 weeks)	(van Drunen Littel-van den Hurk, Braun et al. 1998)
500 µg pRSVgIV[6] (Bovine)	500 µg pRSVgIV (4 weeks)	500 µg pRSVgIV (8 weeks)	BHV-1 aerosol (4 weeks)	(Lewis, van Drunen Littel-van den Hurk et al. 1999)
500 µg pSLIAtgD (Bovine)	500µg pSLIAtgD (3 weeks)	---	BHV-1 aerosol (3 weeks)	(Gerdts, Snider et al. 2002)
500 µg pSLIAtgD (Ovine)	500 µg pSLIAtgD (15 weeks)	---	Immune response following secondary immunization	(Widera, Austin et al. 2000)
500 µg pMASIA-tgD (Bovine)	500 µg pMASIA-tgD (4 weeks)	---	BHV-1 aerosol (3 weeks)	(Braun, Babiuk et al. 1999)
500 µg pSLIAtgD (Ovine)	500 µg pSLIAtgD (7 weeks)	---	Antibody titers monitored for 8 weeks	(van Drunen Littel-van den Hurk, Tikoo et al. 1997)
500 µg pSLKIAtgD (Bovine)	500 µg pSLKIAtgD (5 weeks)	---	BHV-1 aerosol (3 weeks)	(Cox, Zamb et al. 1993)
500 µg pCItgD (Bovine)	500 µg pCItgD (4 weeks)	500 µg pCItgD ID (4 weeks)	Intranasal BHV-1 (4 weeks)	(Pontarollo, Babiuk et al. 2002)
250 µg pCIgD and pCItgD (Bovine)	250 µg pCIgD and pCItgD (4 weeks)	Killed BHV-1 vaccine (4 weeks)	Intranasal BHV-1 (5 weeks)	(Pontarollo, Babiuk et al. 2002)

[1] Vaccine antigen and species immunized in parenthesis.

[2] Vaccine antigen and interval between 1^{o} and 2^{o} immunization in parenthesis.

[3] Vaccine antigen and interval between 2^{o} and 3^{o} immunization in parenthesis.

[4] Method of BHV-1 infection and interval between final immunization and viral infection in parenthesis.

[5] Immunization with recombinant, BHV-1 tgD protein.

[6] Immunization with various DNA plasmid (p) constructs encoding BHV-1 glycoprotein D (tgD, gIV, or gD).

technologies include formulation with polymers and liposomes (Gregoriadis, Saffie et al. 1997), biolistic or gene gun delivery (Fynan, Webster et al. 1993), and electroporation (Dupuis, Denis-Mize et al. 2000). Currently, electroporation appears to be the most effective method of delivering plasmid DNA and has been reported to increase gene expression 10- to 100-fold in tissues such as skin and muscle (Mir, Moller et al. 2005). Electroporation enhances DNA transfection by creating temporary pores in cell membranes and increasing plasmid distribution throughout the tissues (Zaharoff, Barr et al. 2002). Electroporation may also enhance the immunogenicity of DNA vaccines through the induction of an acute, localized infiltration of immune cells (McMahon, Signori et al. 2001; Babiuk, Baca-Estrada et al. 2004) and the up-regulation of cytokines, heat shock proteins, and other co-stimulatory molecules (Pazmany, Murphy et al. 1995). The acute inflammatory response induced by electroporation probably works in concert with increased gene expression to enhance immune responses to plasmid-encoded antigens. Enhanced immune responses as a result of electroporation have been reported in mice (Widera, Austin et al. 2000), pigs (Babiuk, Baca-Estrada et al. 2002) and sheep (Scheerlinck, Karlis et al. 2004). Thus, electroporation appears to be the best available technology with which to analyze immune responses following a single DNA immunization.

The duration of immune memory following a single DNA immunization has rarely been investigated. We previously reported that a single ID immunization of newborn lambs with plasmid expressing tgD induced a detectable primary immune response and an anamnestic immune response was observed following secondary immunization 15 weeks later (van Drunen Littel-van den Hurk, Braun et al. 1999).

Furthermore, a single injection of DNA plasmid encoding tgD into the oral cavity of third trimester fetal lambs was able to support anamnestic responses in the neonate (Gerdts, Snider et al. 2002). Thus, there is evidence that a single DNA immunization, in the absence of formulations to enhance delivery or transfection efficiency, can induce protective immunity and immune memory which persist much longer than expected based upon previous investigations with recombinant protein vaccines (Table 3.1). Therefore, we hypothesized that enhancing DNA transfection and expression would significantly enhance the onset, magnitude and possibly the duration of a primary immune response. It has also been suggested that immune memory is dependent upon both the magnitude of the primary immune response (Ahmed and Gray 1996) and continued exposure to antigen (Gray and Skarvall 1988). Thus, increasing both the magnitude and duration of a primary immune response should not only prolong the duration of immune-mediated protection against infection but may also provide an approach to prolong immune memory and reduce the frequency of revaccination.

3.3 Materials and Methods

3.3.1. Animals

Experiments were performed with 2-3 month old, female and castrated male Suffolk-cross lambs housed in a single outdoor pen. Lambs were randomly assigned to six treatment groups (Table 3.2; n =7/group) and all experimental procedures were approved by the local animal care committee under the guidelines of the Canadian Council on Animal Care.

Table 3.2. Experimental design and vaccination protocol

Group	*n*[1]	*Primary Immunization (Day 0)*		*Secondary Immunization (Week 25)*	
		Route	*Vaccine*	*Route*	*Vaccine*
Control	6	-	No vaccine	SC	20µg recombinant tgD[2]
Protein	7	SC	20 µg recombinant tgD	SC	20µg recombinant tgD
DNA-ID	7	ID[3]	500 µg pSLIA-tgD[4]	SC	20µg recombinant tgD
DNA-IM	7	IM[5]	500 µg pSLIA-tgD	SC	20µg recombinant tgD
DNA+Electro	7	IM	500 µg pSLIA-tgD + electroporation	SC	20µg recombinant tgD
DNA(2X)+ Electro	6	IM	1 mg pSLIA-tgD + electroporation	SC	20µg recombinant tgD

SC = subcutaneous; ID = intradermal; IM = intramuscular

[1] Number of lambs per group at the time of primary immunization. Lambs were removed from the trial upon development of detectable gB-specific antibody titers.

[2] Purified recombinant truncated glycoprotein D (tgD) protein of BHV-1.

[3] Intradermal injection of DNA plasmid on the convex side of the left ear.

[4] DNA plasmid (pSLIA) encoding truncated glycoprotein D (tgD) protein of BHV-1.

[5] Intramuscular injection of DNA plasmid into the semimembranosus muscle.

The 42 lambs were screened for the presence of serum antibodies specific to or cross-reactive with BHV-1 glycoprotein B (gB) and gD prior to the first vaccination. Animals with gD and gB titers less than 100 were considered naïve and all animals were monitored at monthly intervals throughout the trial for possible development of gB-specific antibodies. Development of a gB-specific titer was used as evidence of possible exposure to a natural BHV-1 infection or infection with a cross-reactive ovine herpesvirus. One animal was removed from each of the Control and DNA(2X)+Electro groups (Table 3.2; n = 6) at the beginning of the trial due to the presence of both gD-specific titers (>40,000) and gB-specific titers (>970). Three other sheep developed detectable gB-specific titers at week 17 (one each from the Control, Protein, and DNA-IM groups) and two animals (one each from the Protein and DNA+Electro groups) developed detectable gB-specific titers at week 26. Only data up to and including the time that gB titers still tested negative were included in data analyses.

3.3.2. Preparation of the DNA Vaccine and tgD Protein Vaccine

The pSLIA-tgD plasmid encodes the gene for a secreted 61-kDa truncated form of glycoprotein D from BHV-1 (tgD) expressed under control of the human cytomegalovirus immediate early promoter/1A-region (van Drunen Littel-van den Hurk, Braun et al. 1998). Plasmid was grown in *E. coli* DH5α (New England Biolabs, Mississauga, ON) and purified using the EndoFree™ Plasmid Giga Kit (Qiagen, Mississauga, ON). Purified plasmid was resuspended at 2 mg/ml in endotoxin-free Dulbecco's PBS (Sigma-Aldrich, St. Louis, MO) and stored at -20 °C. The size and quality of the plasmid was confirmed by restriction enzyme digest. Endotoxin activity,

assessed using the QCL-1000 Limulus Amebocyte Lysate Kit (BioWhittaker Inc., Walkersville, MD), was <0.01 EU/ml, which is below the minimal threshold required to induce a mitogenic response in ovine leukocytes (Burrells and Wells 1977).

Each dose of the tgD-protein vaccine was injected subcutaneously and consisted of 20 μg of purified, recombinant BHV-1 tgD protein (van Drunen Littel-van den Hurk, Gifford et al. 1990) and 100 μg CpG ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT) formulated in an oil and water emulsion (30% Emulsigen Plus; MVP Laboratories, Ralston, NE) in a 2 ml volume. This specific vaccine formulation had previously been shown to induce primary and secondary antibody responses of greater magnitude than formulation of tgD with Emulsigen alone (Ioannou, Griebel et al. 2002b).

Detection of an anamnestic response following secondary immunization was used as an indicator that immue memory was present. Secondary immunization with a protein vaccine is the most effective method to elicit anamnestic responses following DNA immunization (Toussaint, Letellier et al. 2005). Therefore, all animals received a secondary immunization with recombinant tgD protein at 25 weeks following primary immunization (Table 3.2).

3.3.3. DNA Vaccination

Three different methods of DNA immunization were compared in the present study (Table 3.2). Previous experiments had demonstrated that ID DNA immunization with pSLIA-tgD plasmid was an effective method to induce both a primary antibody response and enhanced secondary antibody responses following revaccination 15 weeks later (van Drunen Littel-van den Hurk, Braun et al. 1999). Thus, animals in the DNA-ID

group provided a positive control for the DNA vaccine and received 500 μg of plasmid, which was divided into 100 μl aliquots and injected ID into five sites between the central ridges on the convex side of one ear. The second method of DNA vaccination was IM injection of 500 μg of tgD-plasmid in a 500 μl volume into a single site in the left semimembranosus muscle. The third method of DNA vaccination was to combine IM plasmid injection with electroporation. For this method of immunization, animals were sedated by intravenous injection of 12.5 mg/kg Pentothal (Abbott Labs, Montreal, PQ). The DNA vaccine was then injected into two adjacent sites in the left semimembranosus muscle. The DNA+Electro group received a total of 500 μg of tgD-plasmid (i.e., 250 μg of tgD-plasmid in a 500 μl volume per injection site). The DNA(2X)+Electro group received a total of 1 mg of tgD-plasmid (i.e., 500 μg of tgD-plasmid in a 500 μl volume per injection site). For each DNA injection, the needle was inserted to a depth of 1-cm in the muscle. Immediately following each DNA injection, an electrode with six 23-gauge needles arranged equidistant in a 1-cm diameter circle (BTX ECM 830 Pulse Generator; Genetronics, San Diego, CA) was placed directly over each injection site and inserted 1-cm into the muscle. Six pulses (200V/20 ms, 5 Hz per pulse) were applied to each injection site. Previous studies have shown that this electroporation protocol resulted in a significant increase in DNA plasmid gene expression relative to DNA immunization alone (Pazmany, Murphy et al. 1995; Mir, Moller et al. 2005).

3.3.4. Serum Collection and Analysis of Antibody Titers

Serum was collected from all lambs prior to vaccination, at 10 days following primary immunization, and then at weekly intervals for a period of 27 weeks. The last two serum collections (weeks 26 and 27) were at one and two weeks following the secondary vaccination. All serum samples were stored at -20 °C. Antibody titers for all samples were analyzed within the same assay in order to eliminate inter-assay variability.

To determine BHV-1 gB and tgD-specific antibody titers, sera were serially diluted in 0.1 M Tris-buffered saline containing 0.05% Tween-20 (pH 7.5) and added to triplicate wells in Immulon-2 96-well plates (Dynex Labs Inc, Franklin, MA) which had been coated with 0.5 μg/ml of recombinant BHV-1 gB or tgD as previously described (van Drunen Littel-van den Hurk, Braun et al. 1998). Bound antibody was detected using phosphatase-labeled rabbit anti-sheep IgG (H+L) (Kirkegaard-Perry Labs, Gaithersburg, MD) and 100 mg/ml p-nitrophenyl phosphate in 1% diethanolamine, 0.5 mM $MgCl_2$ (pH 9.8). Titers were expressed as the reciprocal of the highest serum dilution to give a positive reaction. To identify individual animals with positive tgD-titers, the cut-off was defined as the upper 99% confidence interval (CI) for time-matched serum samples collected from naïve animals (Table 3.2; Control group). The serum titer cut-off for an acceptable background level of gB-specific antibody was set at the upper 99% CI for the average of titers for all animals (n = 42) when using serum samples collected at the beginning of the experiment.

3.3.5. Statistical Analysis

Data for antibody titers were not normally distributed. To compare each treatment group against the Control group or to compare responses among selected treatment groups at selected time points, data were analyzed using the Kruskal-Wallis test for non-parametrically distributed samples followed by Dunn's comparison of selected data sets (GraphPad Prism 4.0, GraphPad Software, San Diego, CA). A positive tgD-specific antibody titer was defined as a titer exceeding the 95% CI of titers observed in the naïve Control group when measured within the same assay and at the same time point.

Regression analysis was performed to determine if a relationship existed between either the magnitude or the duration of the primary antibody response and the magnitude of the secondary antibody response at one week following the secondary immunization. To explore these possible relationships, each animal receiving a primary immunization was first ranked for magnitude of the primary antibody response. This ranking was based on the maximal antibody titer measured for an individual animal at any time following primary immunization (see Section 3.3.1, Fig. 3.1). Animals were then ranked according to the duration of a detectable primary antibody response. The duration of a primary antibody response was defined as the interval between primary immunization and the time when the tgD-specific antibody titer of each animal returned to a level comparable to the Control group. An increase in the magnitude of the secondary antibody response was used as one possible indicator of immune memory having persisted until the time of secondary immunization.

3.4. Results

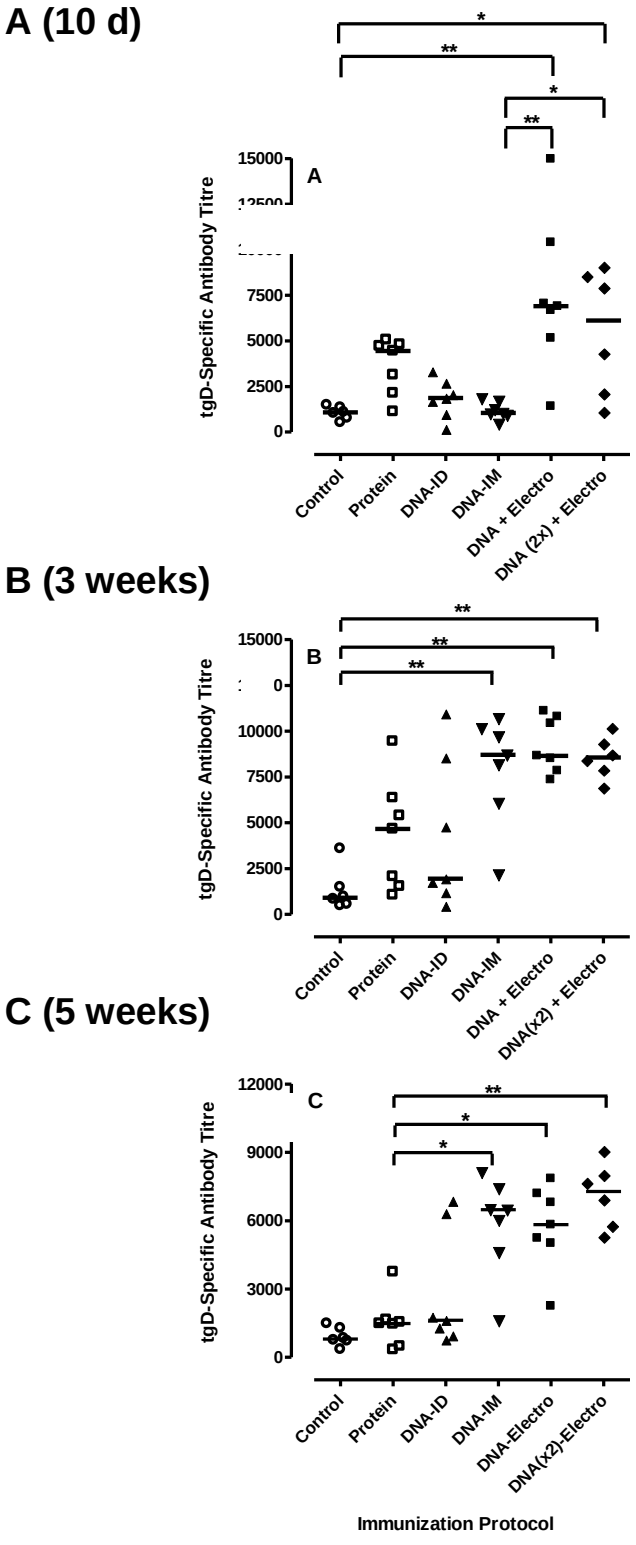
3.4.1. Effect of Electroporation and DNA Immunization on the Primary Antibody Response

A significant increase in tgD-specific antibody responses was first observed in the DNA immunization groups. At 10 days post-primary immunization, both groups which had received DNA immunization in combination with electroporation, had tgD-specific serum antibody titers significantly different ($p \leq 0.05$) from the Control group (Fig. 3.1A). Furthermore, these same two groups had tgD-specific antibody titers that were significantly different ($p \leq 0.05$) from the group receiving IM DNA immunization. There was not, however, a significant difference in antibody titers when comparing between the two groups that received DNA immunization in combination with electroporation, or when comparing these same two groups with the group that received recombinant tgD protein. Thus, electroporation enhanced the onset of a primary immune response induced by IM DNA vaccination, but doubling the dose of DNA vaccine did not significantly enhance the effectiveness of electroporation (Fig. 3.1A).

Three weeks after primary immunization, all immunized groups had achieved maximum median antibody titers (Fig. 3.1B). At this time, the tgD-specific antibody titers of all IM DNA immunized groups were significantly ($p \leq 0.01$) different from the Control group, but there were no significant differences among the immunized groups.

Figure 3.1. Primary tgD-specific antibody titers.

Comparison of primary antibody responses following immunization with either tgD protein or tgD-plasmid showed that DNA vaccination with electroporation resulted in the highest tgD-specific antibody titers. TgD-specific serum antibody titers 10 days (**A**), 3 weeks (**B**), and 5 weeks (**C**) following primary immunization. The immunization protocol for each group is as follows: Control (naïve lambs); Protein (lambs immunized with 20 μg recombinant tgD protein); DNA-ID (lambs injected intradermally with 500 μg tgD-plasmid); DNA-IM (lambs injected intramuscularly with 500 μg tgD-plasmid); DNA+Electro (intramuscular injection of 500 μg tgD-plasmid was followed by electroporation at the plasmid injection site); and DNA(X2)+Electro (IM injection of 1 mg tgD-plasmid was followed by electroporation at the plasmid injection site). The tgD-specific antibody titers were determined by ELISA and expressed as the reciprocal of the highest serum dilution to give a positive reaction. All samples were analyzed within a single assay and the cut-off for positive tgD titers was set at the 99% upper CI of the Control group. Data presented are values for individual animals with group medians indicated by the horizontal bars. *$p \leq 0.05$; **$p \leq 0.01$.


A (10 d)
B (3 weeks)
C (5 weeks)
tgD-Specific Antibody Titre
Control
Protein
DNA-ID
DNA-IM
DNA + Electro
DNA (2x) + Electro
DNA(x2) + Electro
DNA-Electro
DNA(x2)-Electro
Immunization Protocol

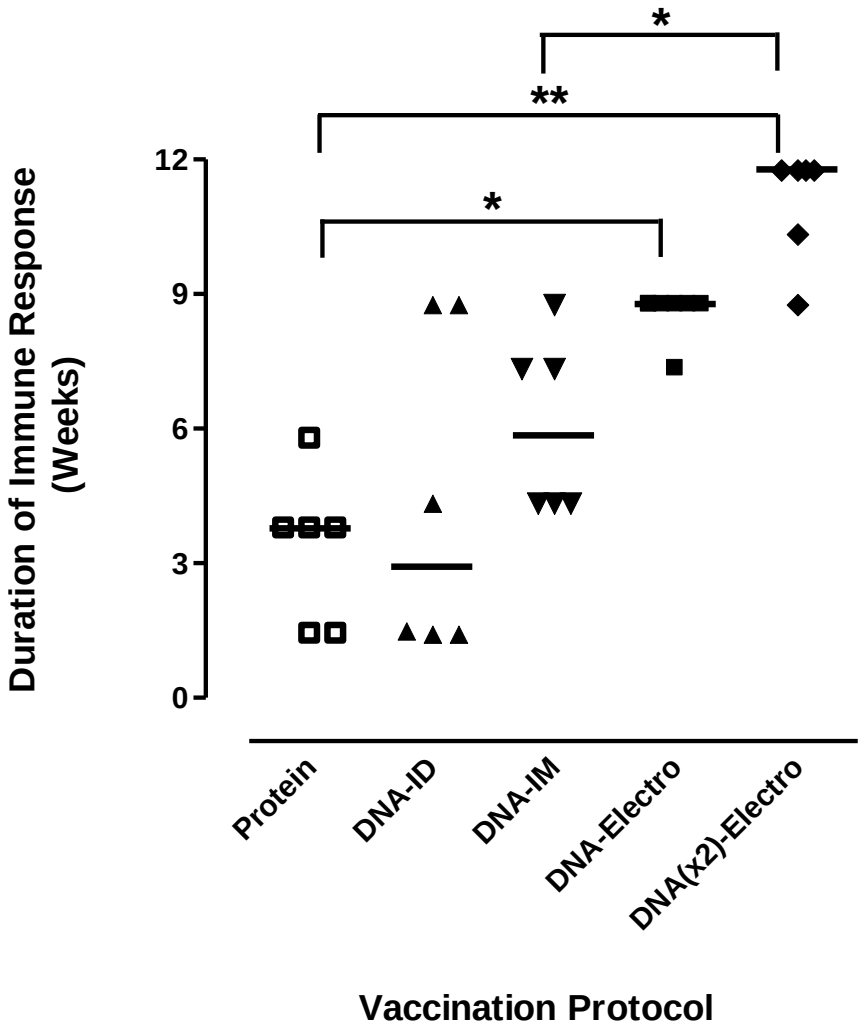
(Fig. 3.1.)

Thus, DNA immunization, in combination with electroporation, did not significantly increase peak serum antibody titers. Electroporation did, however, improve the consistency with which individual animals responded to vaccination; all 13 animals in the two DNA vaccine plus electroporation groups developed antibody titers greater than 7000 (Fig. 3.1B). In contrast, only 5 of 7 animals in the DNA-IM group and 2 of 7 animals in the DNA-ID group developed tgD-specific titers that exceeded 7000. The consistency in individual animal responses to DNA immunization plus electroporation was maintained at 5 weeks post-immunization (wpi) (12 of 13 animals had titers greater than 5000). Furthermore, at 5 wpi, there were significant ($p \leq 0.05$) differences between the tgD-specific antibody titers of the group immunized IM with DNA and the group receiving recombinant tgD protein (Fig. 3.1C). Thus, IM DNA immunization, with or without electroporation, induced significantly ($p \leq 0.05$) elevated antibody responses when compared with the Protein group at 5 wpi.

The duration of primary antibody responses in individual animals was then analyzed throughout the 25 week interval between primary and secondary immunization. The duration of a primary antibody response was defined as the interval between primary immunization and the time when the tgD-specific antibody titer for an individual animal returned to a level comparable to the Control group. This analysis revealed that IM DNA immunization, only when combined with electroporation, resulted in a significantly ($p \leq 0.05$) prolonged primary antibody response when compared to immunization with tgD protein (Fig. 3.2). Furthermore, when

Figure 3.2. Duration of tgD-specific serum antibody responses following primary immunization.

The longest duration of tgD-specific primary antibody titers was achieved in the group that received high-dose DNA vaccination plus electroporation. The immunization protocol for each group was as follows: Control (naïve lambs); Protein (lambs immunized with 20 μg recombinant tgD protein); DNA-ID (lambs injected intradermally with 500 μg DNA plasmid encoding tgD protein); DNA-IM (lambs injected intramuscularly with 500 μg DNA plasmid encoding tgD protein); DNA-Electro (intramuscular injection of 500 μg DNA plasmid encoding tgD protein was followed by electroporation at the plasmid injection site); and DNA(X2)-Electro (intramuscular injection of 1 mg DNA plasmid encoding tgD protein was followed by electroporation at the plasmid injection site). The duration of the primary immune response for each animal was defined as the interval between vaccine injection and the time point when their tgD-specific antibody titer was no longer positive. The cut-off for a positive tgD titer was set at the 99% upper CI of the Control group. TgD-specific antibody titers were determined at weekly intervals following primary immunization with all samples analyzed within a single assay. Data presented are values for individual animals with group medians indicated by the horizontal bars. *$p \leq 0.05$; **$p \leq 0.01$.


*
**
*
12
9
6
3
0
Duration of Immune Response (Weeks)
Protein
DNA-ID
DNA-IM
DNA-Electro
DNA(x2)-Electro
Vaccination Protocol

(Fig. 3.2)

electroporation was combined with a 2-fold increase in the dose of DNA vaccine, the duration of the tgD-specific antibody response was significantly ($p \leq 0.05$) prolonged relative to IM DNA immunization. A primary IM immunization with 1 mg of DNA plasmid, in combination with electroporation, induced a tgD-specific antibody response that was still detectable in 4 of 6 animals at 11 weeks post-immunization. In contrast, IM immunization with 20 μg of recombinant tgD protein induced a tgD-specific antibody response that was detectable for only 4 weeks post-immunization in 4 of 6 animals.

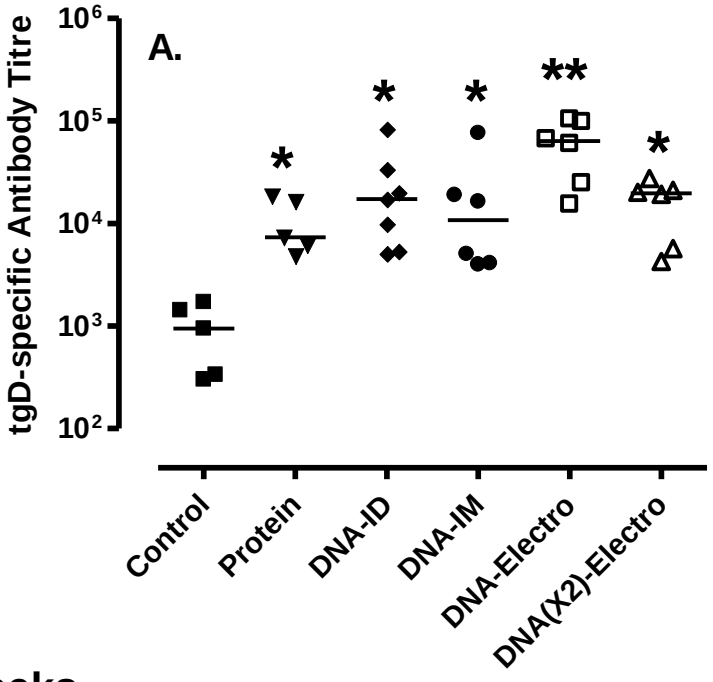
3.4.2 Effect of Electroporation and DNA Immunization on the Secondary Immune Response

An interval of 25 weeks between primary and secondary immunization was selected. This interval exceeded the 15 week interval used in a previous ID DNA immunization study in sheep (van Drunen Littel-van den Hurk, Braun et al. 1999), and was equivalent to the interval used to evaluate immune memory and protection against BHV-1 infection following a single immunization with a modified-live virus BHV-1 vaccine (Platt, Burdett et al. 2006). At one week following secondary immunization with tgD protein, it was apparent that all previously immunized groups had significantly greater tgD-specific antibody responses than the Control group, which received a primary immunization (Fig. 3.3A). The detection of anamnestic antibody responses provided evidence for the persistence of immune memory for at least 25 weeks following primary immunization. At two weeks following secondary immunization, only those groups that had received a primary IM DNA immunization, with or without

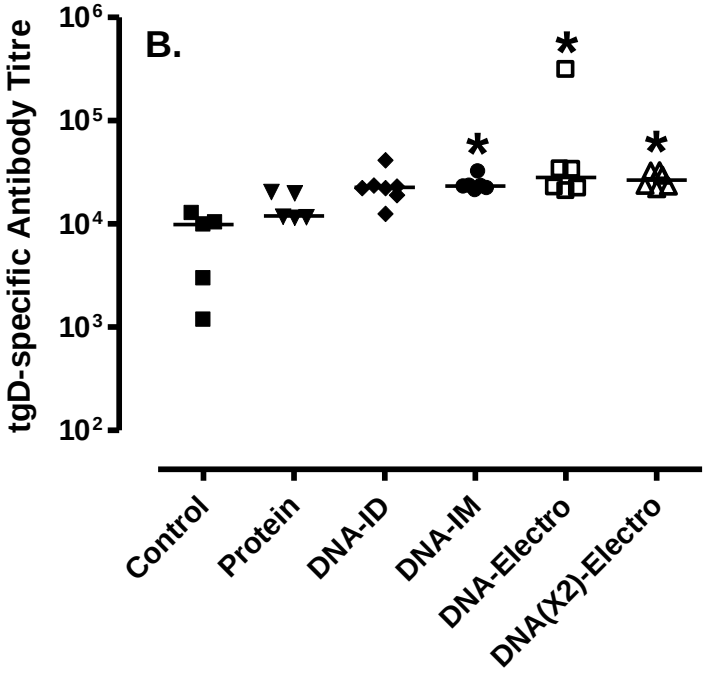
Figure 3.3. The tgD-specific serum antibody titers at one week (A**) and two weeks (**B**) following secondary immunization with recombinant tgD protein.**

Anamnestic antibody responses were detected in all groups that had been immunized 25 weeks previously. Delivery of DNA vaccine via the IM route , with or without electroporation, significantly increased the magnitude of secondary antibody titers at 2 weeks post-boost. Control (lambs receiving a primary immunization with 20 μg recombinant tgD protein while other groups received a secondary immunization with recombinant tgD protein); Protein (lambs were immunized with 20 μg recombinant tgD protein 25 weeks prior to secondary immunization); DNA-ID (lambs were injected intradermally with 500 μg DNA plasmid encoding tgD protein 25 weeks prior to secondary immunization); DNA-IM (lambs were injected intramuscularly with 500 μg DNA plasmid encoding tgD protein 25 weeks prior to secondary immunization); DNA-Electro (intramuscular injection of 500 μg DNA plasmid encoding tgD protein was followed by electroporation at the plasmid injection site 25 weeks prior to secondary immunization); and DNA(X2)-Electro (intramuscular injection of 1 mg DNA plasmid encoding tgD protein was followed by electroporation at the plasmid injection site 25 weeks prior to secondary immunization). Data presented are values for individual animals with group medians indicated by the horizontal bars. *$p \leq 0.05$; **$p \leq 0.01$.

A. 1 week



B. 2 weeks



(Fig. 3.3)

electroporation, had antibody responses that were significantly greater than the Control group (Fig. 3.3B). Thus, the persistence of memory T or B cells in the Protein or ID DNA immunized groups may have been sufficient to support a more prompt antibody response without significantly altering the magnitude of the secondary immune response.

The fixed interval between primary and secondary immunization resulted in marked differences among individual animals when comparing the interval between when primary antibody responses were no longer detectable and secondary immunization. For example, the interval during which there was no longer a detectable primary antibody response and secondary immunization for most animals in the Protein group was 21 weeks, but only 14 weeks for most animals in the DNA(2X)-Electro group (Fig. 3.2). Thus, it was possible to ask whether both the magnitude and the duration of the primary antibody response had a significant effect on the secondary antibody response. The responses of all animals that had received a primary immunization, regardless of antigen form or route of delivery, were included in this analysis. All animals were ranked based on the peak magnitude of their tgD-specific antibody titers during the primary response (without restricting the time required to achieve this maximal response). Each animal was then ranked according to the magnitude of the antibody response at one week following secondary immunization. This short time interval following secondary immunization was selected as the best indicator of a more rapid onset of antibody responses, which is one characteristic of an anamnestic response. Regression analysis of these two parameters revealed a significant ($p \leq 0.0001$)

correlation ($r^2 = 0.40$) between the magnitude of the primary and secondary immune responses (Fig. 3.4A).

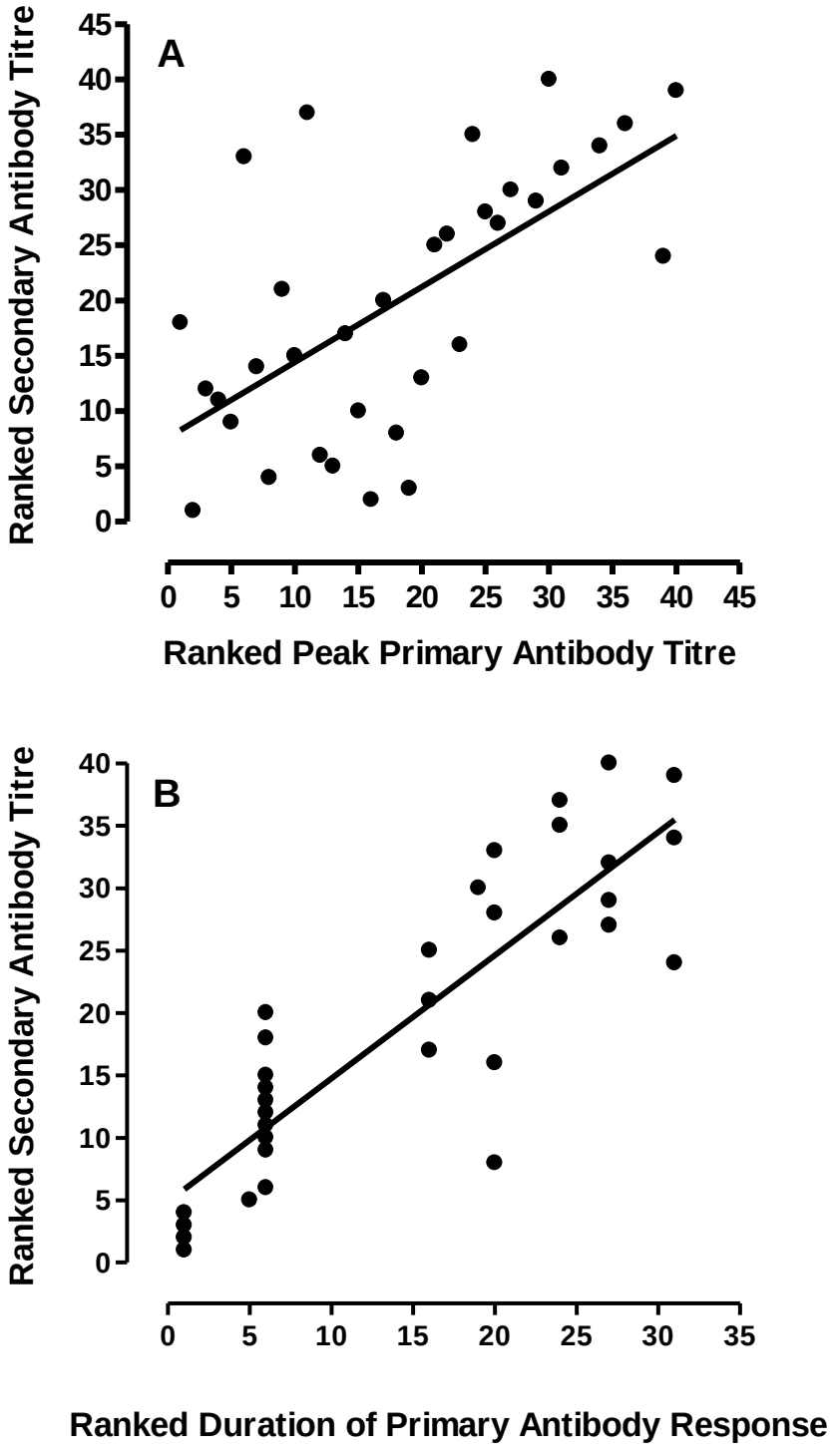
A second regression analysis was then performed to determine if a significant correlation existed between the duration of the primary immune response and the magnitude of the secondary immune response. All immunized animals were again included in this analysis and animals were ranked for duration of the primary immune response as shown in Figure 3.2. This analysis revealed a significant ($p < 0.0001$) and greater correlation ($r^2 = 0.74$) between duration of the primary immune response and magnitude of the secondary immune response (Fig. 3.4B). These regression analyses support the conclusion that factors which influence both the magnitude and the duration of a primary immune response may also influence immune memory.

3.5. Discussion

DNA vaccination held great promise as the third generation of vaccine technology capable of accelerating the development of new vaccines, simplifying vaccine formulation and delivery, and reducing the cost of vaccine production. Unfortunately, failure to achieve high levels of DNA transfection and gene expression has limited the transfer of this technology from the laboratory to clinical application (Babiuk, Pontarollo et al. 2003). Recent investigations have focused on the use of DNA vaccines within a prime-boost strategy, which relies on a secondary immunization with recombinant protein or vector-delivered antigen to enhance the relatively weak immune responses induced by single or multiple DNA vaccinations (Table 3.1). In contrast,

Figure 3.4. Correlation between peak magnitude (A**) and duration (**B**) of the primary antibody response and the magnitude of the secondary antibody response at one week post-immunization.**

Regression analysis revealed a significant correlation between the magnitude of primary and secondary antibody titers and a greater correlation between the duration of the primary and magnitude of secondary antibody titers. Peak magnitude of the primary antibody response for individual animals was determined between 3 to 5 weeks following primary immunization. Duration of the primary antibody response was determined as the interval between primary immunization and the time point when tgD-specific titers no longer exceeded the cut-off set at the 99% upper CI of the control group. A significant ($p \leq 0.0001$) correlation ($r^2 = 0.40$) was observed between amplitude of the primary and secondary immune responses. A significant ($p \leq 0.0001$) correlation ($r^2 = 0.74$) was also observed between duration of the primary immune response and amplitude of the secondary immune response. response equivalent in magnitude to that obtained with recombinant protein delivered with an optimized adjuvant formulation (Fig. 3.1). Furthermore, electroporation was able to significantly enhance both the onset of a primary antibody response and prolong the duration of this response approximately three-fold when compared to the recombinant protein vaccine (Fig. 3.2). Thus, a single DNA vaccination when combined with electroporation has the potential to provide rapid onset of immune-mediated disease protection and prolonged protection if antibody mediates this protection.


A
Ranked Secondary Antibody Titre
0
5
10
15
20
25
30
35
40
45
Ranked Peak Primary Antibody Titre
B
Ranked Secondary Antibody Titre
0
5
10
15
20
25
30
35
40
Ranked Duration of Primary Antibody Response

(Fig. 3.4)

the present investigation clearly demonstrates that a single DNA vaccination, when combined with electroporation, can induce an antibody response equivalent in magnitude to that obtained with recombinant protein delivered with an optimized adjuvant formulation (Fig. 3.1). Furthermore, electroporation was able to significantly enhance both the onset of a primary antibody response and prolong the duration of this response approximately 3-fold when compared to the recombinant protein vaccine (Fig. 3.2). Thus, a single DNA vaccination when combined with electroporation has the potential to provide rapid onset of immune-mediated disease protection and prolonged protection (if antibody mediates this protection).

The development of vaccines capable of inducing protective immune responses following a single immunization remains a challenge when using vaccine technologies other than MLV vaccines. This search for single-shot vaccines is driven by increasing concerns regarding vaccine safety (Moylett and Hanson 2004) and a lack of client compliance when multiple vaccinations are required to achieve protective immunity. These concerns have also been instrumental in raising questions regarding the duration of immune-mediated protection and immune memory following vaccination. The present investigation was designed to determine if a single DNA immunization, in combination with electroporation, could prolong the primary antibody response and support an anamnestic antibody response when the secondary immunization was performed 6 months later. A previous DNA vaccination study in newborn lambs indicated that a single ID DNA immunization could induce immune memory, detectable in the form of an anamnestic antibody response, up to 15 weeks after primary immunization (van Drunen Littel-van den Hurk, Braun et al. 1999). In this study, a

secondary protein immunization at 6 months after primary DNA immunization resulted in the induction of an anamnestic antibody response (Fig. 3.3). The secondary antibody response observed following DNA immunization, with or without electroporation, was not significantly different, however, from the secondary antibody response observed following a primary protein immunization. Regression analysis of ranked data for (i) the duration of the primary antibody response and (ii) the magnitude of the secondary antibody response revealed a relatively high correlation between these two parameters (Fig. 3.4B). Since DNA immunization with electroporation had the greatest impact on the duration of the primary antibody response (Fig. 3.2), it could be implied that this vaccination strategy may be a more effective approach to enhance the duration of immune memory. This conclusion would be consistent with reports that antigen persistence is a major factor in the maintenance of immune memory (Gray and Skarvall 1988). Therefore, achieving efficient DNA transfection and sustained antigen expression may be a key factor when attempting to reduce immunization frequency without compromising the duration of immune-mediated protection against an infectious disease.

The present data are also consistent with previous observations that a single injection of plasmid DNA into the oral cavity of fetal lambs induced both immune memory and protection against infection (Gerdts, Snider et al. 2002). More specifically, a single administration of plasmid encoding HBsAg into the oral cavity of fetal lambs induced protective levels of serum antibody that persisted for over 15 weeks. The long-term persistence of HBsAg-specific antibodies following a single DNA immunization may reflect, in part, the unique nature of this vaccine antigen. Recombinant HBsAg protein self-assembles into virus-like particles (VLP) that are very stable and the

prolonged immune response to HBsAg observed following a single DNA immunization is consistent with antigen persistence (Zi, Yao et al. 2006). These observations suggest another strategy that may be useful when attempting to design DNA vaccines with prolonged duration of primary immune responses and immune memory following a single vaccination: it may be possible to engineer recombinant proteins to enhance their stability and persistence through the incorporation of protein domains which support protein aggregation or assembly into stable multimeric complexes, such as VLPs (Aberle, Aberle et al. 1999). "Single-shot" vaccines which produce stable and persistent forms of antigen may, however, raise unique safety issues arising from the prolonged induction of an immune response (Zi, Yao et al. 2006).

Electroporation significantly enhanced the onset (Fig. 3.1) and duration (Fig. 3.2) of the primary antibody response in the present investigation. Previous studies have demonstrated that electroporation significantly enhances the level of DNA transfection (Mir, Moller et al. 2005) and may also enhance antigen presentation through recruitment of APCs to the site of immunization (Babiuk, Baca-Estrada et al. 2004). The rapid onset and prolonged duration of the primary antibody response observed in the present investigation are consistent with both enhanced antigen expression and antigen presentation. Of interest, however, was the observation that electroporation did not enhance the magnitude of the primary antibody response relative to either IM injection of plasmid alone or other forms of immunization (Fig. 3.1). This may reflect a paucity of lymphocyte trafficking through muscle. Previous investigations have also demonstrated that the form of gD expressed in transfected myocytes can significantly alter the onset and duration of an immune response (Lewis, van Drunen Littel-van den Hurk et al.

1999) and this may be related to the rate of tgD release. The prolonged duration of the tgD-specific primary antibody response, however, suggests that electroporation achieved a sufficient level of tgD expression to ensure prolonged antigenic stimulation. The duration of plasmid expression is a major factor limiting vaccine efficacy when DNA is targeted to skin where the high rate of cell turn-over rapidly eliminates transfected cells (Braun, Babiuk et al. 1999). The short duration of the primary antibody response and high inter-animal variation in antibody responses observed following ID DNA immunization (Fig. 3.1) supports the conclusion that IM delivery may be the optimal route for immunization when prolonged DNA transfection and expression is required.

The present investigation also revealed the importance of ensuring that animals are not exposed to cross-reactive or homologous antigen during the period between immunization and analysis of antibody responses. This is of particular concern when assessing the duration of immune responses to pathogens, such as BHV-1, which may be endemic and establish latent infections.The development of BHV-1 gB-specific antibody titers was used as a criterion to exclude animals which may have been exposed to either ovine or bovine herpesviruses. It was not possible, however, to directly monitor inadvertent exposure to antigens that may have re-stimulated gD-specific T or B cells. Therefore, controlling environmental exposure will be critical when conducting experiments to analyze the duration of immune responses following vaccination.

In conclusion, the present investigation clearly demonstrated that electroporation accelerated the onset and prolonged the duration of the primary antibody response induced by IM DNA vaccination. Similar effects may be achieved by the use of much larger quantities of protein antigen or improved adjuvants; however, these approaches to

changing protein vaccine formulation may be limited by cost and an increased risk of adverse reactions at the injection site. The use of electroporation remains an experimental procedure at this time and further development of the technology is required before it can become a practical method for DNA vaccine delivery. The present observations indicate, however, that increasing the efficiency of DNA vaccine delivery can have potential benefits for both the duration of a primary antibody response and possibly for the duration of immune memory following a single vaccination. Designing DNA vaccine trials to address these aspects of immune responses will be critical if we are to achieve the goal of a "single shot" vaccine that optimizes vaccine safety and minimizes the risk of adverse reactions while still providing both a rapid onset of protection against infection and the prolonged immune-mediated protection observed with a MLV vaccine (Platt, Burdett et al. 2006).

3.6. Acknowledgments

This work was supported by the Canadian Adaptation and Rural Development Fund, the Canada Institute of Health Research and the Canada Research Chair in Vaccinology, which is held by Dr. L.A. Babiuk. We also thank Dr. Hugh Townsend for assistance with data analysis and Dr. Don Wilson and the VIDO Animal Care staff for their invaluable assistance with vaccination and sample collection. This manuscript is published with the permission of the director of VIDO as manuscript no. 389.

CHAPTER 4

TRANSFECTION AND EXPRESSION FOLLOWING ORAL DELIVERY OF NAKED DNA PLASMID IN THE OVINE FETUS[1]

4.1. Abstract

We previously demonstrated that *in utero* oral DNA vaccination may be an effective strategy to prevent vertical disease transmission. The detection of antigen-specific immune responses in LNs draining the oral cavity of fetal lambs suggested that DNA plasmid had penetrated the oral mucosa. Experiments were performed to identify the sites of DNA transfection following oral injection of plasmid during the third trimester of gestation. Luciferase was used as a reporter gene and the analysis of luciferase activity revealed that oral injection of naked DNA plasmid resulted in a detectable level of protein expression in 14 of 15 fetuses (93%). The primary site of plasmid expression was the anterior aspect of the oral cavity, specifically the tongue and lips. At 11 dpi, the level of luciferase activity was still increasing and could be detected in all areas of the oral cavity, lymphoid tissues draining the oral cavity, as well as the liver and lungs of some fetuses. In contrast, oral delivery of plasmid DNA in newborn lambs did not induce a detectable immune response. Observed differences in the histology of the oral mucosa of fetuses at varying ages, supported the conclusion that

[1] Manuscript accepted with revisions. Tsang C, Kaushik RS, Mirakhur KK, Gerdts V, Babiuk LA, Griebel PJ. 2007. Dev Comp Immunol.

DNA transfection in the oral cavity of fetal lambs was due to a lack of mucosal epithelial cell differentiation. Therefore, the fetal oral cavity may provide a unique site where delivery of a naked DNA vaccine can achieve sufficient protein expression to induce an immune response.

4.2. Introduction

DNA vaccines have many potential applications, including induction of immune protection against pathogens, induction of tolerance to autoantigens and allergens, or replacement of a genetic deficiency. For a DNA vaccine to be of therapeutic value, it must transfect the appropriate cells and sufficient protein must be produced to induce an immune response. For example, enhancing DNA plasmid transfection efficiency and changing the site of DNA transfection significantly alters both the duration of a primary immune response and the persistence of immune memory (Tsang, Babiuk et al. 2007). Finding methods to deliver DNA vaccines to appropriate target cells, while obtaining high levels of transfection and expression, remain major barriers to the clinical application of DNA vaccine technology.

Current methods of DNA vaccine delivery can be divided into either vectored or non-vectored delivery systems. Viral vectors (Sharpe, Fooks et al. 2002; Xin, Ooki et al. 2002; Zhang, Mei et al. 2003; Pinto, Fitzgerald et al. 2004; Liu, Yang et al. 2005) and bacterial vectors (Paglia, Terrazzini et al. 2000; Bauer, Darji et al. 2005) have been used to target genes to specific cell types. Although these vectors provide efficient gene delivery, they can also induce inflammation, toxic responses, and disease (Lehrman 1999; Bessis, GarciaCozar et al. 2004). Viral vectors also carry a risk of integration into

the host genome (Miller, Rutledge et al. 2002) and the size of the transgene being packaged may be limited (Dong, Fan et al. 1996). Non-vectored delivery systems include the encapsulation of plasmid DNA in reagents, such as polyethylene glycol (Kaul and Amiji 2005) and cationic lipids (Clark, Stpoeck et al. 2000; Byrnes, Nass et al. 2002). Encapsulation enhances transfection efficiency and strategies have been reported for increasing the rate of plasmid transport across the nuclear membrane. Conjugation of a lipid carrier to a modified mRNA transporter (Byrnes, Nass et al. 2002) or sigma-1 attachment protein from recombinant reovirus (Talsma, Babensee et al. 2006) enhanced gene expression, while incorporation of the SV40 enhancer downstream of the transgene was shown to enhance nuclear translocation (Young, Benoit et al. 2003); however, disadvantages of encapsulation include cytotoxic effects (Kiefer, Clement et al. 2004) and failure to target appropriate cells.

Delivering therapeutic genes in the form of naked plasmid offers a number of important advantages. Plasmid can be administered repeatedly without risk of inducing specific immunity against the delivery vehicle. Purified plasmid can be produced in large quantities at a relatively low cost, is very stable and unlikely to have toxic effects. These potential advantages continue to drive the search for more efficient methods of DNA delivery. Electroporation (Babiuk, Baca-Estrada et al. 2004) and low frequency ultrasound (Talsma, Babensee et al. 2006) have been used to enhance plasmid transfection efficiency but induce significant inflammation and cell lysis, respectively. Although there is no limit to the size of the gene being inserted into the DNA plasmid, larger plasmids have a reduced transfection efficiency. This is especially true when compared to the relative high transfection efficiency of minicircles (Darquet, Cameron

et al. 1997). The presence of immunostimulatory CpGs in the bacterial DNA backbone may oppose stable gene expression, however, plasmids can be engineered such that immunostimulatory CpGs are removed or suppressed (Yamada, Gursel et al. 2002). Thus, genes delivered in the context of DNA plasmids have the potential to function as effective vaccines if an adequate level of plasmid transfection can be achieved.

Our laboratory has previously reported the successful induction of immune responses following injection of plasmid in saline solution into the oral cavity of third trimester fetal lambs (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002). Immune responses specific to the plasmid-encoded viral glycoprotein were detected in 19 of 23 (83%) injected fetuses. The induction of these responses occurred following a single plasmid injection and in the absence of any delivery vehicle or mechanical treatment to enhance transfection efficiency. The analysis of immune responses in fetal lambs indicated that the site of immune induction was restricted to LNs draining the oral cavity. Furthermore, incubation of plasmid DNA with fetal fluid revealed that the plasmid was rapidly degraded. These observations were interpreted as evidence that DNA plasmid either transfected the oral mucosal epithelium or penetrated the mucosal barrier and transfected cells in the draining LN. Plasmid transfection of mucosal epithelium would raise questions regarding the duration of gene expression, since previous studies determined that DNA transfection of skin and mucosal epithelium resulted in gene expression for less than 72 hours due to the rapid loss of epithelial cells (van Drunen Littel-van den Hurk, Braun et al. 1998; Loehr, Willson. et al. 2000). Furthermore, plasmid expression in epithelial cells was only observed in these studies following ballistic delivery of the DNA into epithelial cells.

In this study, we examined the site of plasmid transfection and the duration of gene expression following a single injection of luciferase-encoding plasmid into the oral cavity of 120-125 dg fetal lambs. Specifically, we examined the mucosal epithelium of the oral cavity for plasmid expression, since this was suggested to be the primary site of plasmid transfection (Gerdts, Babiuk et al. 2000). We also demonstrated proof of principle that fetal oral tissues can be transfected in the absence of either tissue manipulation or formulation of plasmid with transfecting agents. To determine if naked DNA transfection of the oral mucosa was unique to the fetus, a DNA vaccine was applied to the oral cavity of newborn lambs to determine if DNA transfection was sufficient to induce an immune response. Histological studies were also completed to determine if structural changes in the mucosal epithelium might be one factor limiting DNA transfection.

4.3. Materials and Methods

4.3.1. Animals

The estrus of Suffolk ewes were synchronized by insertion of vaginal sponges containing 60 mg medroxyprogesterone acetate (Veramix®, Pharmacia & Upjohn, Orangeville, ON). Upon removal of sponges, ewes were exposed to rams for a period of 5 days. Thus, the gestational age of fetuses was defined with a variance of $\pm$2.5 days and all pregnancies were confirmed by rectal ultrasound at 45 dg (gestational length in sheep is approximately 148 days). All experiments were conducted following the guidelines of the Canadian Council on Animal Care.

4.3.2. *In utero* and Post-Partum DNA Injection

Fetal surgeries and *in utero* oral injections were carried out as described in Gerdts et al. (2000). Briefly, ewes were placed under general anaesthetia and a ventral midline incision was made along the linea alba. The uterus was manipulated such that a portion containing the head of the fetus was exteriorized (Fig. 4.1A). The lips of the fetus were parted by applying pressure at the commissures of the mouth and a 22-gauge needle or 28-gauge catheter was placed into the oral cavity. Oral deliveries were initially performed with a 22-gauge needle, and later replaced with a 28-gauge catheter as a precaution against possible disruption of the oral mucosal epithelium. The following criteria were used to ensure that the oral epithelium was not penetrated: (i) following insertion of the catheter into the oral cavity, the plunger on the syringe was withdrawn to ensure aspiration of amniotic fluid and (ii) there was no resistance noted upon depression of the plunger. After injection, the uterus was returned to the abdominal cavity and the abdominal incision was closed. At specified times post-injection (pi), ewes were euthanized and fetal tissues collected. Age-matched control fetuses were either injected orally with endotoxin-free saline or did not undergo surgical manipulation.

For post-partum immunizations, four groups of newborn lambs (n = 5 per group), between 0-3 days of age, received either saline containing plasmid DNA or saline alone. Solutions were injected ID (5 x 100 μl aliquots) between the central ridges on the convex side of the ear or injected into the oral cavity.

All animals were randomly assigned to experimental groups.

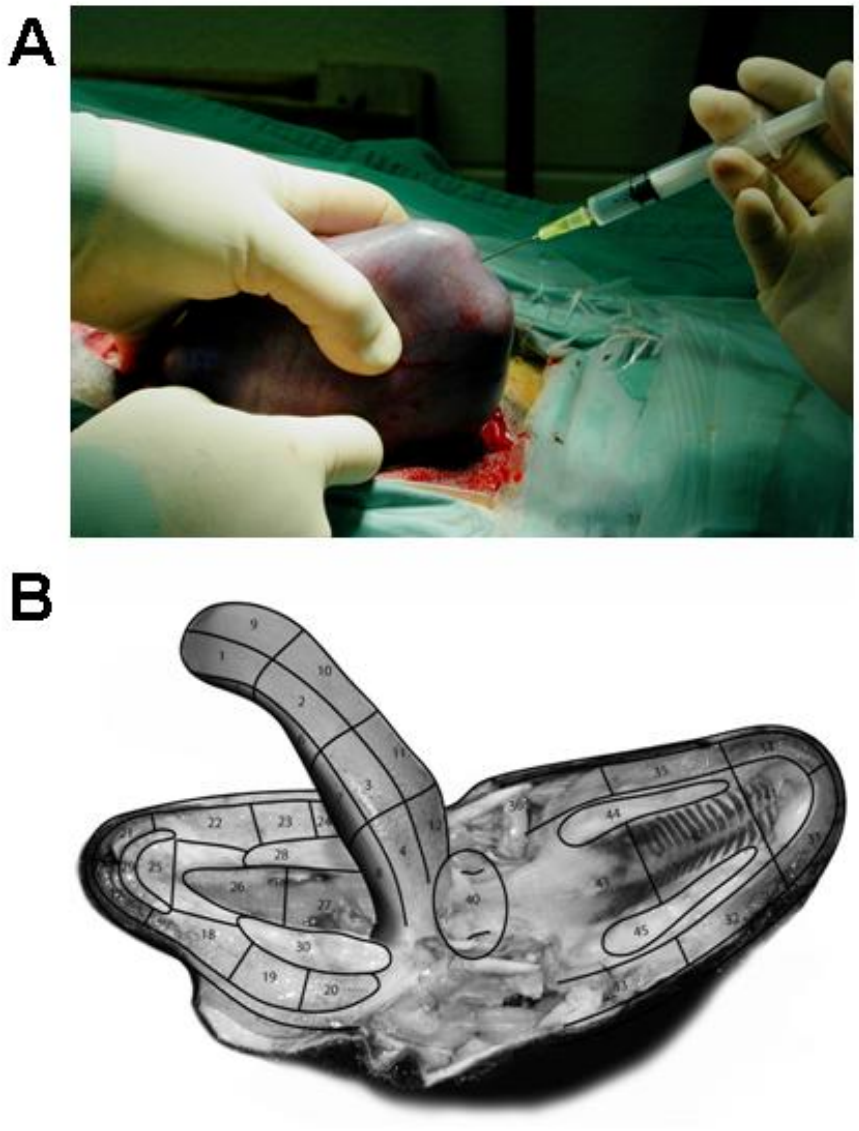


Figure 4.1. Delivery of plasmid at 125 days gestation and tissue collection.

(**A**) The head of the fetus within the uterus was exteriorized through an abdominal incision. DNA plasmid was injected through the uterine wall and into the opened oral cavity. (**B**) Anatomical map for the collection of mucosal epithelium from the fetal oral cavity. The mucosa and attached submucosa of the oral cavity, tongue and oropharynx were harvested in approximately 1-cm^2 sections. Samples 26 and 27 refer to tissue deep to the frenulum and sample 40 includes the palatine tonsils. The retropharyngeal and superficial cervical lymph nodes, lung, liver and tip of the left ear were also collected.

4.3.3. Plasmid Preparation

The plasmid pMASIA-luc contains the gene for firefly luciferase, which is a non-secreted, soluble protein (Uwiera, Rankin et al. 2001), and pSLIA-tgD contains the gene for BHV-1 tgD (Braun, Babiuk et al. 1997). Plasmids were amplified in *E. coli* DH5α (New England Biolabs, Mississauga, ON) and purified using the EndoFree™ Plasmid Giga Kit (Qiagen, Mississauga, ON). Purified plasmid was reconstituted at 0.5-2 mg/ml in endotoxin-free Dulbecco's PBS (Sigma-Aldrich, St. Louis, MO) and stored at -20 °C. DNA quality was confirmed by electrophoresis and sterility was confirmed by plating plasmid solution on 2x YT in the absence of antibiotic. Endotoxin levels were measured using the QCL-1000 Limulus Amebocyte Lysate Kit (BioWhittaker Inc., Walkersville, MD) and ranged between 0.01-0.7 EU/ml of plasmid solution. These levels were below the minimal threshold required to induce non-specific stimulation of ovine leukocytes (Messina, Gilkeson et al. 1991).

4.3.4. *In Vitro* Transfection Assay

All media and transfection reagents unless otherwise noted were obtained from InVitrogen (Burlington, ON). Madin-Darby bovine kidney (MDBK) cells (ATCC, Manassas, VA) were cultured in Modified Eagle's Medium (MEM) with 10% FBS and grown in Nunclon 6-well tissue culture plates (Nalge-Nunc, Rochester, NY). Upon reaching 80% confluence, triplicate cultures were transfected with Opti-MEM containing pMASIA-luc, Lipofectamine Plus™ and Lipofectamine™ at a ratio of

1:10:2.5 (w/v/v) according to manufacturer's directions. Cultures were harvested at 24 hour intervals, lysed and analysed for luciferase activity.

4.3.5. Luciferase Assay

Cultured cells were prepared according to manufacturer's directions (Luciferase Assay System, Promega, Madison, WI). The mucosal epithelium and submucosa were collected from the oral cavity and oropharynx in 1-cm^2 samples mapped to specific anatomical loci (Fig. 4.1B). Samples were snap frozen in liquid nitrogen and stored at -70 °C. Tissues were minced in liquid nitrogen, homogenized and then assayed for luciferase activity using the Luciferase Assay System. The lower detection limit of the assay using the Packard Picolite® Luminometer (United Technologies Packard, Palo Alto, CA) was 100 pg as determined from a standard curve constructed using QuantiLum® Recombinant Luciferase (Promega, Madison, WI). Luciferase activity was expressed as units of luciferase activity per mg of protein (ULA). Total protein per sample of homogenized tissue or cultured cells was determined using the Bradford assay (BioRad, Hercules, CA). The cut-off for non-specific luciferase activity (i.e., non-specific luminescence) in a specific tissue or tissue group was calculated as the upper 95% CI from age-matched, control fetuses.

4.3.6. *Ex Vivo* Transfection Assay

The dorsum of the tongue was removed from three fetal lambs between 129-134 dg and each tongue was cut into ten pieces with an approximate mucosal surface area of 7.5 mm^2 and tissue thickness of 3-5 mm. Sections were placed in individual wells of a

Nunclon 6-well tissue culture plate containing 37 °C AIM-V media (InVitrogen) and positioned such that the epithelial surface was above the meniscus of the media. The epithelial surface was then overlaid with 50 μg of pMASIA-luc in saline for 15, 30 or 60 minutes (n = 3 tissue samples per fetus per time point). Transfection was terminated by repeated flushing of each tissue fragment with sterile saline to remove non-adhered plasmid and explants were then submerged in fresh AIM-V with 2% FBS and antibiotic-antimycotic (Sigma-Aldrich) and incubated for 24 hours at 37 °C with 5% CO_2. Luciferase activity was measured as described previously. Control tissue sections (one per fetus) were treated with a 50 μg of an irrelevant plasmid (pCAN-GFP (Uwiera, Gerdts et al. 2001)). The cut-off for non-specific luciferase activity was calculated as the upper 99% CI from tissues transfected with an irrelevant plasmid.

4.3.7. BHV-1 tgD-Specific Serum IgG Titers

ELISAs were carried out as described previously (Gerdts, Babiuk et al. 2000). Immunolon-2 plates (Dynatech Laboratories, Gaithersburg, Maryland) were coated with 0.5 μg/ml purified BHV-1 tgD per well and incubated with serial dilutions of serum. Alkaline-phosphate-conjugated rabbit-anti-sheep IgG (H+L) (1:6000 dilution; Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used to detect sheep Igs captured by tgD protein. The reaction was visualized with 100 mg/ml p-nitrophenyl phosphate (Sigma-Aldrich). Antibody titers were expressed as the reciprocal of the highest serum dilution to give a positive reaction. A positive reaction was defined as an OD value which was two times greater than the average value for serum collected from a

naïve fetus. Each serum sample was analyzed in triplicate and the average value used to calculate serum antibody titers.

4.3.8. Histology

Tissue samples taken from the tongue and lips of fetuses and newborn lambs were fixed in 10% buffered formalin and submitted to Prairie Diagnostic Services (Saskatoon, SK) for hematoxylin and eosin staining and immunohistochemical staining for Ki67 nuclear antigen (clone MIB-1; InVitrogen).

4.3.9. Statistical Analysis

All analyses were carried out before logarithmic transformation of data. The upper 95 or 99% CI was used as the cut-off for non-specificity in all assays. The Kruskal-Wallis test, followed by Dunn's comparison, was used to compare data between groups. All statistical analyses were conducted using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

4.4. Results

4.4.1. Validation of Luciferase Plasmid

In vitro and *in vivo* experiments were conducted to verify the activity of the luciferase protein encoded in pMASIA-luc and to identify the interval between plasmid transfection and the expression of a detectable level of luciferase activity. MDBK cells were transfected *in vitro* with 0.1, 1, 2 or 5 μg of pMASIA-luc. At 24 and 48 hours, a

significant relationship between plasmid dose and luciferase activity was observed for 0.1 and 5 μg of plasmid ($p<0.05$) (Fig. 4.2A). Plasmid dose had no effect on either the time of peak luciferase expression or the duration of luciferase expression. Luciferase activity reached peak levels at 24 hours for all groups.

To validate the assay for detection of luciferase activity in tissue samples, 100 μg of pMASIA-luc in 100 μl saline was injected subcutaneously at each of two sites on either side of the midline of the dorsal and ventral lips, and at three sites along the midline of the tongue of a single, six-month old lamb. At 24 hours, each injection site (contained in a 1-cm^2 piece of tissue) was tested for luciferase activity. Luciferase activity was detected in each tongue sample that contained an injection site as well as both the left and right retropharyngeal LNs. No luciferase activity was detected in the palatine tonsils or samples of tongue taken from areas adjacent to the sites of injection (data not shown). Thus, the functional activity of the pMASIA-luc plasmid as well as the capacity of the luciferase assay to detect gene expression in tissues was confirmed.

4.4.2. *Ex vivo* transcutaneous transfection of fetal oral tissues with naked plasmid DNA

An *ex vivo* transfection experiment was conducted to determine if naked plasmid could transfect fetal oral mucosa. Fragments of fetal tongue (129-135 dg) were overlaid with 50 μg of pMASIA-luc in saline for 15, 30 or 60 minutes. At 24 hours post-transfection, luciferase activity was detected in 7 of 27 treated tongue fragments (Fig. 4.2B). Increasing the duration of plasmid contact did not increase the level of detectable

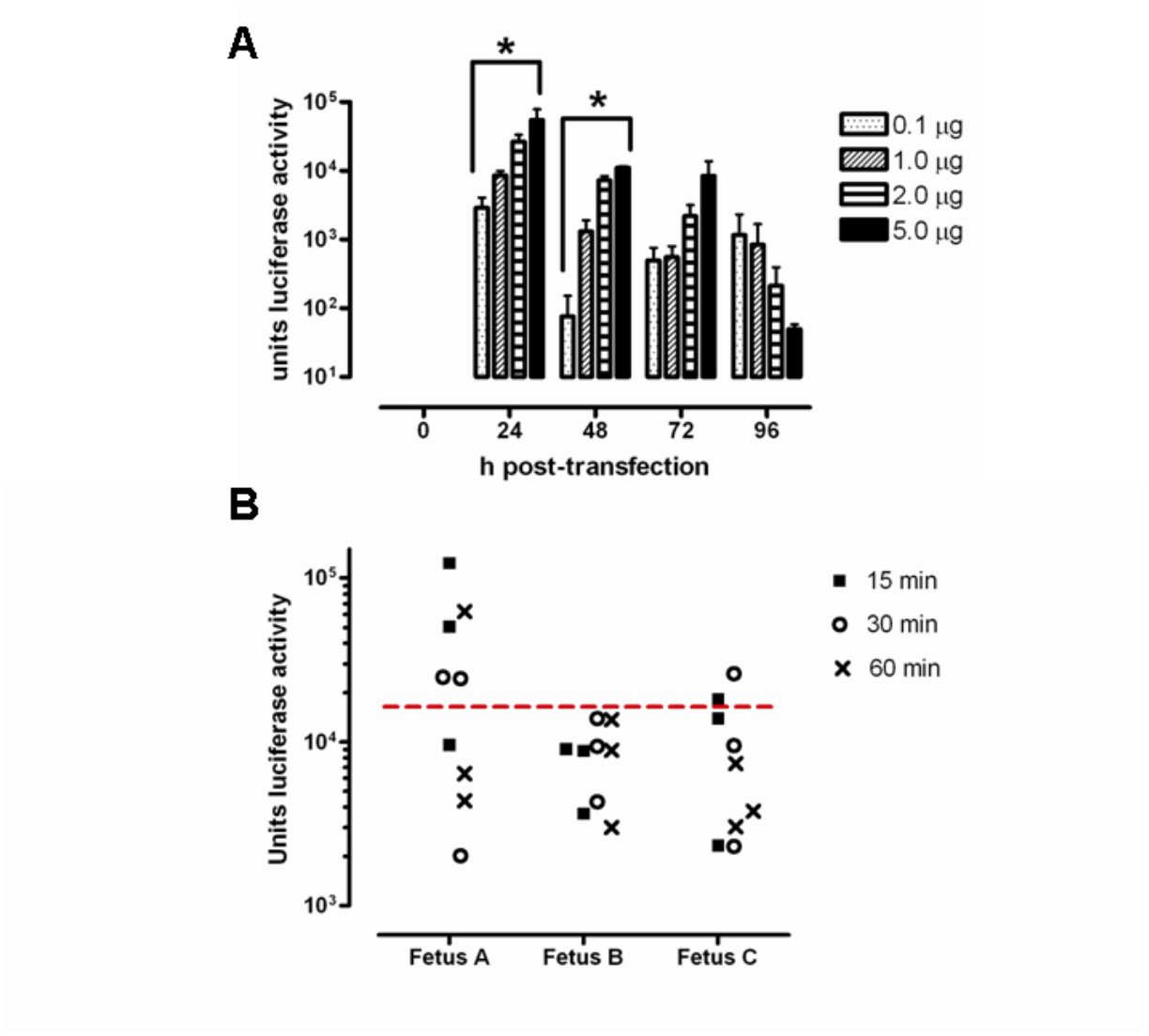


Figure 4.2. Expression of luciferase-plasmid following *in vitro* transfection of MDBK cells and *ex* vivo transfection of fetal tongue.

Experiments were conducted to validate the biological activity of the luciferase-plasmid and to demonstrate proof of principle that fetal mucosal epithelium can be transfected with DNA plasmid in the absence of either tissue manipulation or plasmid formulation. (**A**) Luciferase activity in transfected MDBK cells. Luciferase levels were compared using the Kruskal-Wallis test followed by Dunn's comparison. (**B**) Luciferase activity in 1-cm^2 sections of fetal tongue following overlay with 50 µg of luciferase-plasmid in saline. Plasmid was in contact with the epithelial surface for 15, 30 or 60 minutes. Luciferase activity was measured 24 hours after removal of plasmid. The cut-off (---) for luciferase activity was set at the 99% CI of three samples treated with control plasmid.

luciferase activity. Low amounts of transfected plasmid DNA may have limited the detection of positive samples. Regardless, this experiment provided evidence for transfection of mucosal epithelium within 15 minutes, which was consistent with previous evidence that DNA plasmid is rapidly degraded after *in utero* oral injection (Gerdts, Snider et al. 2002).

4.4.3. Optimizing Plasmid Dose for Oral Transfection

In previous oral injection studies, the arbitrary use of 0.5 mg of plasmid delivered in a 5 ml volume of saline resulted in gene expression in 80% of injected fetuses (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002). Therefore, we investigated the effect of plasmid dose on oral transfection efficiency. A dose response study was performed with oral injection of 0.5, 1 or 2 mg of pMASIA-luc in 1 ml of saline (n = 3 fetuses/group). At 72 hours post-injection (hpi), the fetal oral epithelium was harvested in 1-cm^2 sections mapped to specific anatomical loci (Fig. 4.1B) and luciferase activity was measured for each tissue section. Considerable non-specific luciferase activity was noted in all animals and all tissues. To differentiate between non-specific luciferase activity and luciferase activity resulting from expression of luciferase-plasmid, luciferase activity was measured in tissue samples from 4 age-matched control fetuses (Fig. 4.3A): fetuses 103a and 336a were non-surgical controls and 114a and 115b were orally injected with saline. Tissues related by function and anatomical loci were grouped and the cut-off value for each tissue group was set at the upper 95% CI of values established with samples collected from control animals. Only tissue samples

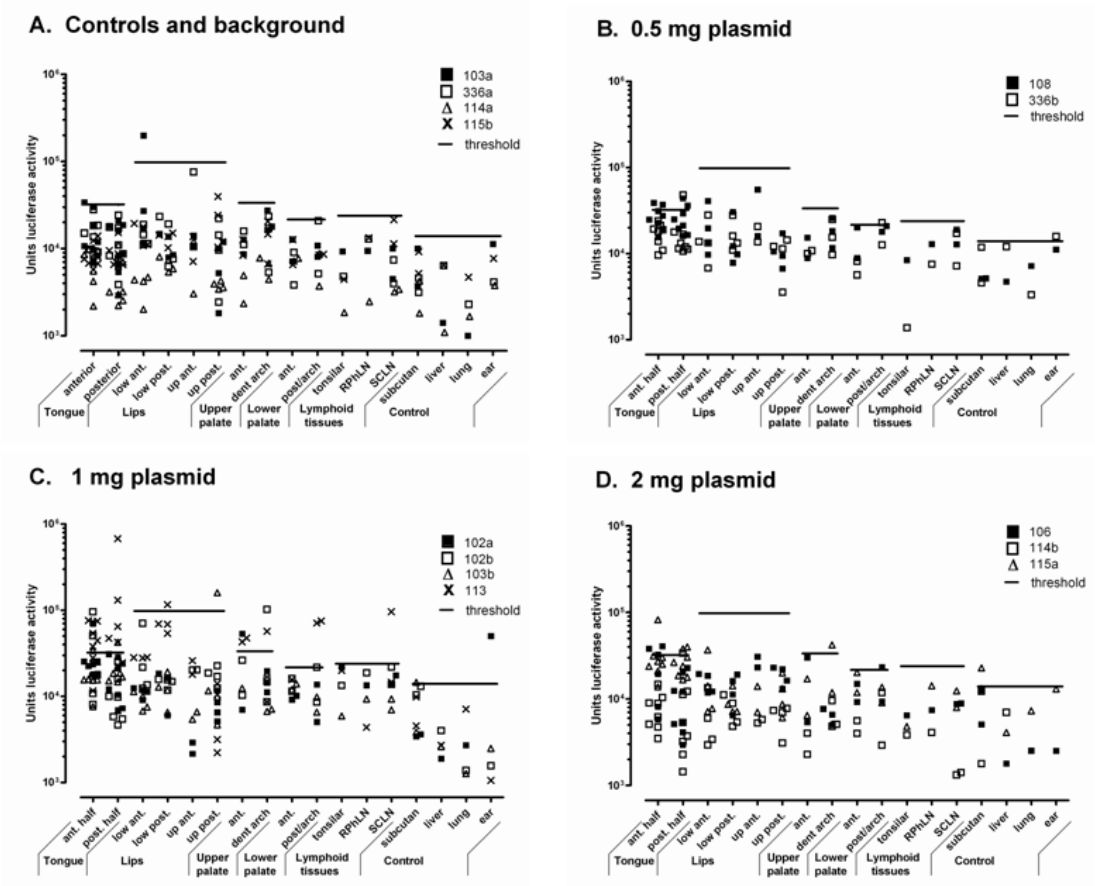


Figure 4.3. Dose titration of orally-injected luciferase plasmid.

A 1 mg dose of luciferase-plasmid resulted in the highest levels of luciferase activity. Considerable variation in transfection and expression efficiency was also noted between individual animals and between different tissue groups. Each symbol represents the luciferase activity in a 1-cm^2 tissue sample mapped to a specific anatomical location (Fig. 4.2B). The identity of individual fetuses is given and small case designations ("a" and "b") refer to twin fetuses. (**A**) The level of non-specific luciferase activity detected in tissues from control fetuses (n = 4) at 120-125 dg. The cut-off for non-specific luciferase activity in each related tissue grouping was defined as the upper 95% CI for grouped tissue samples (—). Fetuses (n = 3) between 120-125 dg were orally injected with 0.5 mg (**B**), 1 mg (**C**), or 2 mg (**D**) of luciferase-plasmid in a 1 ml volume of sterile saline. The oral mucosa was collected at 72 h post-injection and each sample was assayed for luciferase activity. Only those tissue samples with luciferase activity above the cut-off (—) were considered positive for the expression of luciferase protein.

with luciferase activity above the cut-off were considered positive. Due to the high threshold, the number of samples that tested positive provided a minimum estimate of transfection efficiency. Eight of the 9 orally-injected fetuses had between 1 and 5 tissue samples that tested positive for luciferase activity (Figs. 4.3B-D) and the dorsal surface of the tongue appeared to be the primary site of transfection. A 4-fold increase in plasmid dose from 0.5 to 2 mg did not significantly alter transfection efficiency or gene expression levels at 72 hpi. Therefore, 1 mg of pMASIA-luc in a 1 ml volume of saline was selected as the dose for subsequent transfection experiments.

4.4.4. Duration and Localization of Luciferase Expression

Studies were then conducted to determine the location and duration of plasmid expression following oral injection of 1 mg of pMASIA-luc into 120-125 dg fetuses. Tissues were harvested at 2, 3, 5, 7 and 11 dpi and tested for luciferase activity. Luciferase activity was detected on all days assayed (Table 4.1). To determine if there was an overall increase in luciferase expression with time, the total ULA was calculated for each fetus and expressed as a value relative to the mean total ULA of the 4 control fetuses: $\sum_{\text{injected fetus}} (ULA_1,\ldots,ULA_{48}) / \text{mean}_{n=4} [\sum_{\text{control \#1}}(ULA_1,...,ULA_{48}),\ldots,\sum_{\text{control \#4}}(ULA_1,\ldots,ULA_n)]$. At 2 and 3 dpi, there was a median 2.1-fold increase in luciferase activity relative to control fetuses (Fig. 4.4). This increased to 6.2-fold between 5 and 11 dpi. To determine if there was a significant increase in luciferase activity over time, a regression analysis was performed and a significant correlation ($p<0.05$, $r^2 = 0.44$) was observed between duration of transfection and luciferase levels. These observations support the conclusion that there was sustained luciferase expression following oral

Table 4.1. Localization of luciferase expression in fetal tissues following oral injection with 1 mg of luciferase-plasmid at 120-125 days gestation.

Days post-injection	Fetus[1]	Number of tissues sections[2] with luciferase activity > background[3]						
		Tongue (10)[4]	Lips (14)	Upper palate (5)	Lower palate (6)	Lymph tissues[5] (3)	Subcut. Oral (2)	Control[6] (3)
2	116	1	3	0	0	0	0	0
	117a	4	0	0	0	0	1	0
	117b	0	0	0	0	0	0	0
3	102a	1	0	1	0	0	0	E
	102b	1	1	1	0	0	0	0
	103b	0	1	0	0	0	0	0
	113	3	4	3	2	SCv	0	0
5	112a	10	6	2	2	SCv	2	Lv, E
	112b	2	2	3	2	RPh	1	0 [5]
7	118	2	4	3	1	T, SCv	1	Lg
	121	2	8	3	4	T, SCv	0	0
	122	5	6	3	3	T, SCv, RPh	1	Lv, Lg
11	119	4	5	4	1	T, SCv, RPh	1	Lv, Lg [5]
	120	3	8	2	2	-	0	Lv, Lg [5]
	123	9	13	5	1	RPh	0	Lv, Lg, E

[1] Fetuses with the same numerical designation followed by “a” or “b” refer to twins.

[2] Tissues were sampled in 1-cm^2 sections mapped to specific anatomical loci (Fig. 4.2B).

[3] Background was defined as luciferase activity below the upper 95% CI as determined by grouped tissues from age-matched control fetuses (n=4).

[4] The total number of 1-cm^2 sections comprising a tissue group.

[5] Lymphoid tissues with luciferase activity are indicated: retropharyngeal lymph nodes (RPh), superficial cervical lymph node (SCv), palatine tonsils (T).

[6] The presence of luciferase activity was tested for in tissues that were not specifically targeted by oral delivery: liver (Lv), lung (Lg), ear (E). Ear samples were tested from fetuses 112b, 119 and 120.

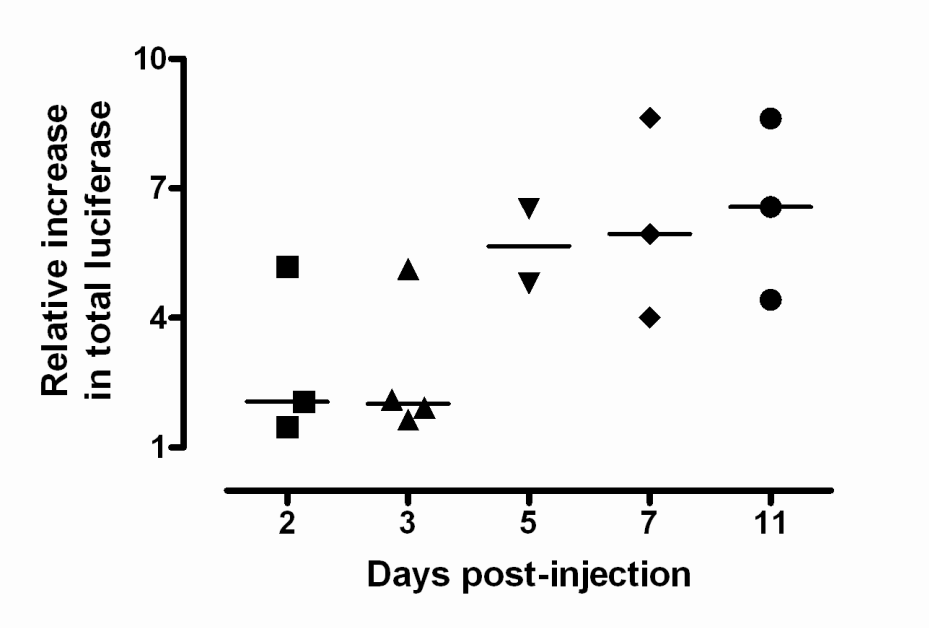


Figure 4.4. **Total luciferase activity in individual fetuses at various times following a single oral injection of plasmid.**

The total luciferase activity in an individual fetus increased with time for a minimum of 11 days. Fetuses were orally injected with 1 mg of luciferase-plasmid in 1 ml of saline at 120-125 dg. Data shown is the sum of luciferase activity in all tissue sections collected from individual fetuses expressed as a value relative to the mean total luciferase from four age-matched controls.

injection of plasmid; however, the number of samples falling outside the 95% CI (5/15) suggested a significant variation in transfection efficiency among individual fetuses.

Localization of plasmid expression was determined by assaying luciferase activity in individual tissue samples collected from the oral cavity and adjacent tissues. On 2 and 3 dpi, 27 of the 29 samples with detectable luciferase activity localized to the anterior aspects of the oral cavity, specifically the lower lip and anterior regions of the tongue (Table 4.1). These data were consistent with the previous data collected at 72 hpi (Fig. 4.3). Throughout all experiments, the tongue and lips were consistently the tissues with the highest levels of luciferase expression with respect to both total ULA per cm^2 section and the percentage of tissue samples testing positive. By 5 dpi, luciferase activity was detectable in all tissues and tissue groups tested, including the upper and lower palate, lymphoid tissues and liver or lung in some fetuses. At 7 dpi, luciferase activity was detected in the palatine tonsils (4/15 fetuses), which sample material in the oral cavity. Luciferase activity was also detected in the SCv (6/15) and medial retropharyngeal (4/15) LNs, which drain the oral cavity (Schwartz-Cornil, Epardaud et al. 2005), starting at 3 and 5 dpi, respectively. Luciferase activity in these LNs may reflect drainage of either plasmid or protein via lymphatics from the sites of transfection. Detection of luciferase activity in tissues not directly exposed to plasmid or lymph, such as the liver (5/15) and oral tissues deep to the frenulum (5/15), may be more consistent with a systemic distribution of luciferase protein.

A total of 14 of 15 fetuses (93%) had detectable levels of luciferase in sampled tissues. Only fetus 117b, which had the lowest level of total luciferase activity (1.5 times control; Fig. 4.4), lacked tissue samples with luciferase activity above threshold.

Possible explanations for this lack of detection include a failure in plasmid delivery or insufficient time between plasmid injection and tissue collection at 48 hours. During *in utero* oral injection studies, we observed through the amniotic membrane that fluid streamed out of the oral cavity in a dorso-caudal direction (data not shown). This dispersion of plasmid, immediately following delivery, may explain the apparent failure of delivery in fetus 117b and also the detection of luciferase activity in tissue collected from the ears of fetuses 102a, 112a and 123.

4.4.5. Oral Delivery of Plasmid in Newborn Lambs

The mucosal delivery of DNA vaccines or DNA plasmid has required the use of a variety of delivery vehicles (Roy, Mao et al. 1999; Loehr, Willson. et al. 2000; Chen, Yang et al. 2004). Therefore, the effective delivery of naked DNA plasmid to the fetal oral mucosa (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002) suggests that the fetus may represent a unique opportunity for the delivery of DNA vaccines. To test this hypothesis, newborn lambs were orally injected with naked DNA plasmid encoding the gene for BHV-1 tgD. This plasmid had previously been used to induce detectable immune responses following oral injection in fetuses (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002) and detectable immune responses and immune memory in newborn lambs following ID injection (van Drunen Littel-van den Hurk, Braun et al. 1999). Newborn lambs between 0-3 days of age (n=5 per group) were immunized as shown in Table 4.2.

Only lambs that were immunized ID had a significant increase in tgD-specific antibody titers on 21 dpi (Fig. 4.5A). On day 28, all lambs were administered a second

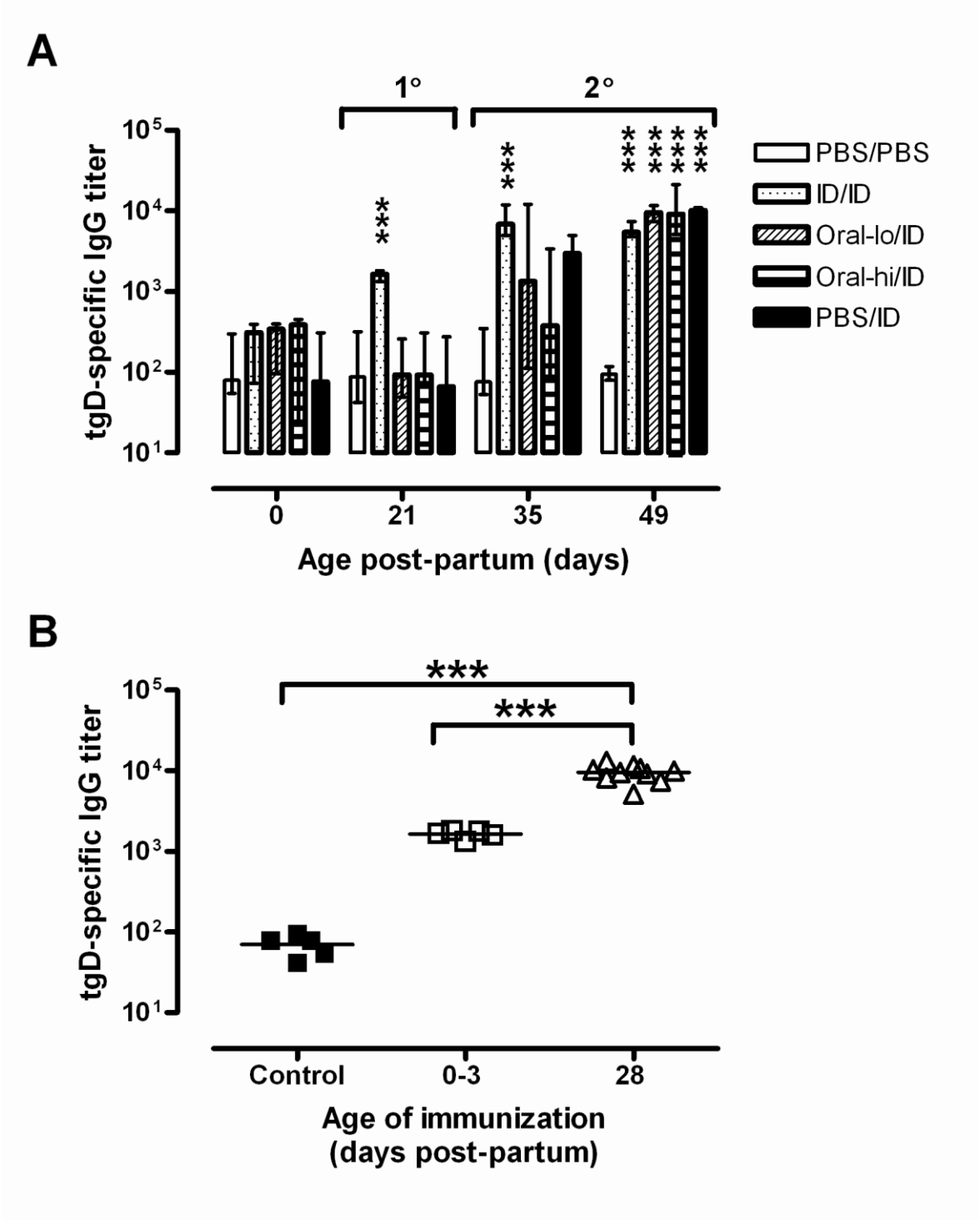
Table 4.2. Oral DNA injection in neonatal lambs aged 0-3 days.

Group	*n*	*Day 0*		*Day 28*	
		Treatment	*Route*	*Treatment*	*Route*
1 - negative control	5	Saline	ID[1]	Saline	ID[1]
2 – positive control	5	0.5 mg pSLIA-tgD	ID[1]	0.5 mg pSLIA-tgD	ID[1]
3	5	0.5 mg pSLIA-tgD	Oral	0.5 mg pSLIA-tgD	ID[1]
4	5	2.5 mg pSLIA-tgD	Oral	0.5 mg pSLIA-tgD	ID[1]
5 - negative control	5	Saline	ID[1]	0.5 mg pSLIA-tgD	ID[1]

[1] Intradermal injection was in the left ear.

Figure 4.5. Oral immunization of newborn lambs with tgD-plasmid.

Oral DNA immunization with tgD-plasmid did not result in the induction of detectable tgD-specific antibody titers, suggesting that plasmid transfection and expression may not have occurred. A positive correlation between age and magnitude of primary antibody titers was also noted. (**A**) Oral DNA immunization failed to induce a detectable immune response in newborn lambs. All lambs were immunized as shown in Table 4.2. Antibody titers for each group were compared with the age-matched PBS/PBS control group. (**B**) The primary tgD-specific antibody response induced by tgD-plasmid was significantly lower in newborn lambs (0-3 d of age) than lambs immunized at 28 d of age. Newborn (n = 5) and 28-day old lambs (n = 10) were immunized by injecting 0.5 mg of tgD-plasmid ID in the ear. Age-matched control lambs (n = 5) received an ID injection of 500 μl of saline. Peak tgD-specific serum antibody titers were detected at 3 weeks post-immunization. $p<0.001$ (***).


A
1°
2°
tgD-specific IgG titer
10^5
10^4
10^3
10^2
10^1
PBS/PBS
ID/ID
Oral-lo/ID
Oral-hi/ID
PBS/ID
0
21
35
49
Age post-partum (days)
B
***
***
tgD-specific IgG titer
Control
0-3
28
Age of immunization
(days post-partum)

(Fig. 4.5)

ID DNA immunization to determine if the lack of detectable antibody responses in the orally-immunized group was the result of tolerance to the antigen. All lambs that had received a primary oral-immunization (Groups 3 and 4) responded to the secondary ID immunization with a significant increase in tgD-specific antibody titers. The delayed kinetics of this response was characteristic of a primary antibody response, suggesting that primary oral immunization had induced neither immune tolerance nor sufficient immune memory to support an anamnestic response.

On days 35 and 49, there were no significant differences in tgD-specific titers between groups that had received either one or two ID immunizations (Fig. 4.5A). The apparent lack of anamnestic responses in the ID/ID group suggested that either immune memory was lacking, or that the magnitude of the primary immune response may increase with age. To differentiate between these possibilities, newborn (1-3 day old) and 28 day old lambs were administered identical primary ID DNA immunizations and tgD-specific immune responses were monitored weekly. Lambs immunized at 28 days of age achieved significantly greater tgD-specific IgG titers than lambs immunized at birth (Fig. 4.5B).

Overall, these data show that oral DNA delivery failed in the neonate. As these data also showed the capacity of newborn lambs to respond to DNA immunization, it is likely that transfection failed or that the level of protein expression in the newborn lamb was insufficient to induce a detectable immune response.

4.4.6. Structural Differences in Fetal Versus Newborn Oral Epithelium

The failure of oral DNA immunization in the neonate (Fig. 4.5A) suggested that oral transfection with naked DNA might be limited by developmental changes in the mucosal epithelium. The histological structure of the oral mucosal epithelium was examined in 100 dg fetuses, 140 dg fetuses and 3 day old lambs. All tissue sections were obtained from the dorso-rostral surface of the tongue, which corresponded to the region with the highest rate of DNA transfection *in utero* (Table 4.1). At 100 dg, the mucosal epithelium consisted of stratified squamous epithelium with nucleated cells present in the superficial-most layers (Fig. 4.6A). At 140 dg, there was an increase in keratinization and localized areas of cornification were noted (Fig. 4.6B). By 3 days post-partum, the stratum corneum was continuous, with sloughing of superficial layers and the presence of filiform papillae (Fig. 4.6C). Thus, there were marked structural changes in the mucosal epithelium following birth that might create a barrier between nucleated epithelial cells and the oral cavity.

The prolonged detection of luciferase activity in the oral mucosa (Fig. 4.4) also suggested that the rate of epithelial cell turnover might have a major impact on the duration of gene expression. Therefore, tissue sections from the dorso-rostral surface of the fetal and newborn tongue were stained for the Ki67 antigen as a measure of epithelial cell proliferation. Immunohistochemical staining revealed that fewer than 7% of basal epithelial cells had entered the S- to M-phase of the cell cycle in 100 dg (Fig. 4.6D) and 140 dg fetuses (Fig. 4.6E). Less than 1% of basal epithelial cells were visibly stained with Ki67 in newborn lambs (Fig. 4.6F). The low level of Ki67 staining was consistent with previous reports of a much lower rate of epithelial cell turnover in the

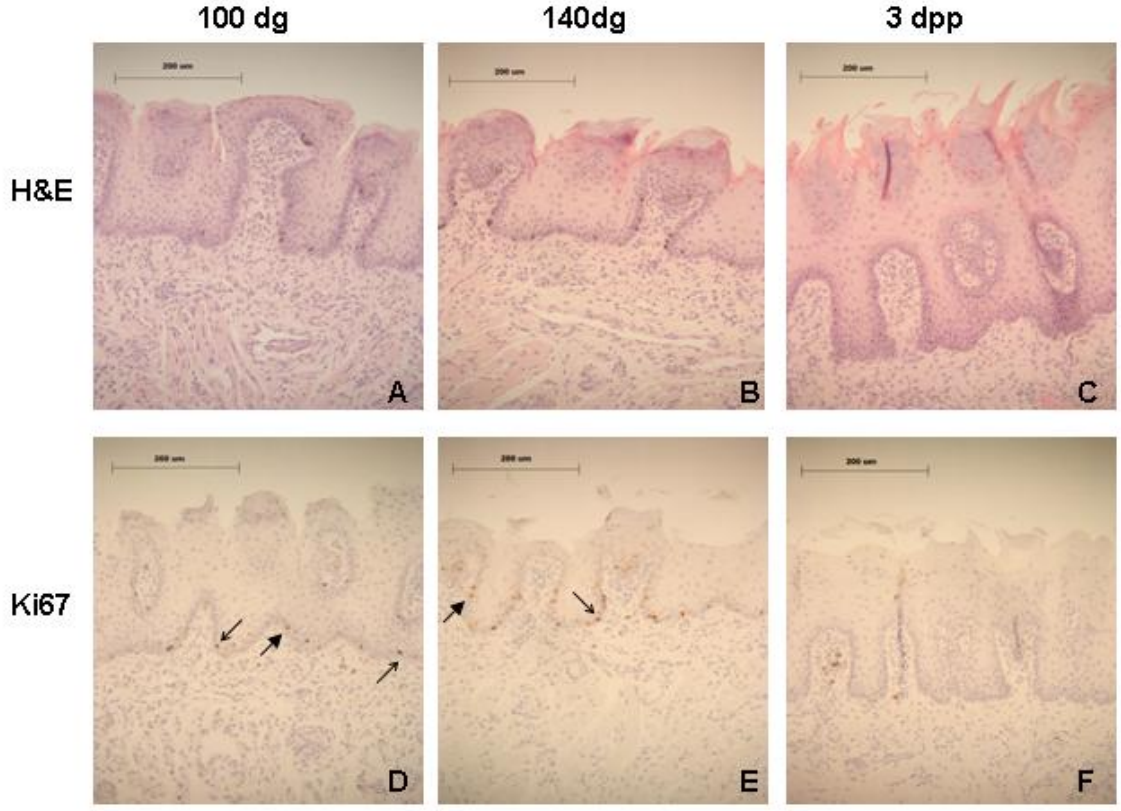


Figure 4.6. Histological comparison of oral mucosal epithelium in fetal and newborn lambs.

The oral mucosal epithelium at 100 dg is characterized by a large number of nucleated cells located in the upper-most strata, a lack of desquamation and moderate staining for nuclear proliferating antigen (Ki67). With increasing age, there is a decrease in the number of nucleated cell at the superficial most strata, an increase in desquamation and a decrease in cellular proliferation. The dorso-rostral surface of the tongue was collected from 100 dg fetuses (**A,D**), 140 dg fetuses (**B,E**) and 3 day old lambs (**C,F**). Tissues were formalin-fixed and stained with either hematoxylin and eosin (**A-C**) or monoclonal antibody specific for the Ki67 antigen (**D-F**). Examples of $Ki67^{+}$ cells (→) and melanocytes (➔) are indicated.

fetal lamb when compared to adult animals (Smeaton and Simpson-Morgan 1985) and was also consistent with the persistent expression of luciferase in mucosal epithelium collected from the surface of the tongue and lips (Table 4.1).

4.5 Discussion

Successful fetal vaccination has been reported following IM injection of naked DNA plasmid into porcine fetuses (Fazio, Ria et al. 2004) and oral delivery of naked DNA plasmid into fetal lambs (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002). There are also reports of naked plasmid delivery by a variety of methods to mucosal surfaces in adult animals, but in most cases plasmid delivery failed or resulted in low levels of transfection and/or expression and failure to induce detectable immune responses (Roy, Mao et al. 1999; Chen, Yang et al. 2004). In contrast, the present study confirmed that delivery of naked DNA plasmid to the fetal oral cavity resulted in transfection of the mucosal epithelium. Furthermore, recombinant protein could be detected in a wide variety of tissues that were not directly exposed to DNA plasmid (Table 4.1). This observation indicates that oral delivery of DNA plasmid may provide an effective method for both mucosal and systemic delivery of a recombinant protein. The current study indicates, however, that effective DNA transfection of the oral mucosa is restricted to the fetus.

Properties unique to the fetal oral mucosa appear to be critical for transfection by naked DNA plasmid. This was revealed by the *ex vivo* transfection of oral mucosa with naked luciferase-plasmid formulated in saline (Fig. 4.1) and the failure to induce a detectable immune response to the plasmid encoded protein following oral delivery of

naked DNA plasmid in newborn lambs (Fig. 4.5). Histological examination of the mucosa on the dorsum of the tongue revealed major structural changes during fetal maturation and immediately following birth (Fig. 4.6). The stratum corneum is the principal barrier of the oral mucosa (Smeaton and Simpson-Morgan 1985), and its thickness is inversely proportional to its permeability (Loehr, Willson. et al. 2000). Thus, we hypothesize that efficient DNA transfection was attributed to the presence of nucleated cells in the superficial layer of oral mucosal epithelium, lesser keratinization and a lower rate of epithelial cell turnover. The low frequency of Ki67-positive basal epithelial cells in the fetal oral mucosa (Fig. 4.6) is consistent with a low rate of epithelial cell turnover (Smeaton and Simpson-Morgan 1985), and this may be a critical factor contributing to the sustained expression of plasmid DNA in the fetus (Fig. 4.4). If the immature structure of the mucosal epithelium is critical for DNA transfection competence, then it may be possible to extend DNA delivery to much earlier periods of fetal development without compromising the efficiency of vaccine delivery.

We were unable to directly demonstrate DNA transfection of mucosal epithelial cells with either PCR or immunohistochemical staining for luciferase (data not shown). Other plasmid delivery studies, using either hypotonic shock (Lemoine, Farley et al. 2005) or gene gun-mediated delivery (Loehr, Willson. et al. 2000), have suggested that the basement membrane is an effective barrier to DNA plasmid and that transfection is limited to epithelial cells. Previous studies have also found that DNA plasmid is rapidly degraded in fetal fluid so there is a limited time for plasmid uptake in the oral cavity (Gerdts, Snider et al. 2002). Also, luciferase has a short half-life of 3-4 hours in mammalian cells (Leclerc, Boockfor et al. 2000). Thus, the persistent detection of

luciferase activity in the oral mucosa (Fig. 4.4) is consistent with sustained protein expression at this site. Lymphatic drainage from the mucosal epithelium may then account for the detection of luciferase activity in a wide variety of tissues, such as LNs and liver, which are not directly exposed to plasmid (Table 4.1). Future studies should also include a survey of the thymus, since the presence of a foreign protein in this tissue may have important implications for the possible induction of immune tolerance.

Although the luciferase assay had a relatively low sensitivity due to high tissue-specific thresholds, successful plasmid delivery was detected in 14 of 15 (93%) treated fetuses. This exceeded the 80% success rate that had been reported by Gerdts et al. (2000, 2002) in which immune responses were used to monitor plasmid delivery. The difference between luciferase expression and the induction of an immune response may be explained by a level of gene expression which is insufficient to induce a detectable immune response. Marked variation in transfection efficiency among individual fetuses was observed throughout *ex vivo* (Fig. 4.1) and *in vivo* experiments (Fig. 4.3). The absence of a detectable immune response following oral immunization of the neonate (Fig. 4.5) may, therefore, be explained by either a failure in plasmid transfection of the oral mucosa or limited plasmid expression due to increased shedding of mucosal epithelial cells (Fig. 4.6). The current study also demonstrated that the neonate had a reduced capacity to respond to DNA vaccination (Fig. 5B) and this reduced immune competence may also apply to the fetus. The risks associated with fetal oral DNA vaccination will dictate that very efficient and consistent delivery methodologies be identified before considering this procedure for clinical application.

4.6. Conclusions

Oral transfection and expression by naked plasmid DNA appears to be specific to the fetus. The majority of gene expression was localized to the oral mucosal epithelium but luciferase assays provided evidence that foreign protein could be detected in a wide variety of anatomical sites, including LNs draining the oral cavity. A comparison of fetal and neonatal oral mucosal histology supports the conclusion that prolonged plasmid expression may be dependent upon both a lack of epithelial cell differentiation and a low rate of basal cell proliferation in the fetus. Furthermore, a combination of high levels of transgene expression with prolonged plasmid expression may be critical to ensure the induction of an immune response by the immature fetal immune system.

4.7 Acknowledgements

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CHAPTER 5

ORAL DNA IMMUNIZATION IN THE SECOND TRIMESTER FETAL LAMB AND SECONDARY IMMUNE RESPONSES IN THE NEONATE[1]

5.1. Abstract

We previously demonstrated that oral DNA vaccination of the third trimester fetus may be an effective strategy to prevent vertical disease transmission despite an immature immune system and the possibility of developing tolerance. The present investigation examined oral DNA vaccine delivery and immunogenicity in the second trimester fetal lamb (50-100 dg). First, we examined the efficiency of oral delivery of DNA plasmid in the second trimester. Histological examination revealed the presence of nucleated cells in the superficial layers of the oral mucosa. Luciferase-encoding DNA plasmid was then injected into the oral cavity of 65-70 dg fetuses to monitor plasmid expression. Luciferase activity was detected in the oral mucosa of all fetuses, but the level of luciferase activity varied among individual fetuses. Luciferase activity was also detected within the tonsils and LNs draining the oral cavity. Oral DNA immunizations were then conducted in fetuses between 55 and 83 dg. Immunization with a tgD-encoding plasmid induced GCs in draining LNs and detectable tgD-specific serum antibody titers and/or IFNγ-secreting cell responses in 16 of 24 (67%) fetuses. TgD-

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specific antibody and IFNγ-responses persisted until birth in some fetuses but the magnitude of antibody titers in second trimester fetuses was low relative to antibody titers induced following oral DNA immunization in the third trimester. Lambs immunized during the second trimester of gestation responded to neonatal DNA immunization and anamnestic responses were detected in some lambs immunized as early as 67–72 dg. These observations confirmed that oral DNA immunization of the early second trimester fetus induced antigen-specific immune responses with no evidence of tolerance induction.

5.2. Introduction

Fetal vaccination is a potential method of preventing vertical transmission of diseases, such as HIV or hepatitis B, and the immune protection afforded by fetal immunization might be further enhanced if carried out at the earliest possible fetal age; however, advances in the field of fetal immunization have been hindered by the dogma that exposure of the fetal immune system to antigen results in the induction of immune tolerance (Billingham, Brent et al. 1953). This is despite studies in large animal models which document the induction of primary immune responses in fetuses following immunization or infection in the second (Silverstein, Uhr et al. 1963; Otsyula, Miller et al. 1996; Buxton, Maley et al. 1998; Kennedy, McCullough et al. 2001; Buxton, Anderson et al. 2002) and third trimesters of gestation (Fennestad and Borg-Petersen 1957; Richardson and Connor 1972; Watts, Stanley et al. 1999; Gerdts, Babiuk et al. 2000). Gerdts et al. (2000; 2002) confirmed that fetal lambs during the mid-third trimester of gestation could be orally immunized with a single injection of naked DNA

plasmid encoding BHV-1 tgD. This oral DNA immunization induced both humoral and CMI responses and immune memory which persisted in the neonate.

Many fetal immunization studies have been conducted using the fetal lamb model due to developmental similarities between the ovine and human immune systems. In both species, thymic colonization and T cell development occur in the first trimester of gestation and this is followed by T and B cell population of peripheral lymphoid tissues, such as the spleen and LNs (Ohama and Kaji 1974; Anderson, Bird et al. 1981; Griebel 1998; Riley 1998; Al Salami and Filippich 1999; Cahill, Kimpton et al. 1999; Hein and Griebel 2003). In a series of seminal studies on immune competence in the fetal lamb, 67-70 dg (gestational length is 148 days) was identified as the earliest time at which the fetus generated a specific immune response to a foreign antigen (Silverstein, Uhr et al. 1963; Silverstein and Prendergast 1964). The concept of a pre-immune period during fetal development is also supported by experiments which showed that allogeneic cells were tolerogenic in the fetal lamb prior to 60-65 dg (reviewed by Almeida-Porada, Porada et al. 2004). The pre-immune fetal period has been defined on the basis of studies conducted with allogeneic stem cells and antigens expressed by retroviral vectors that can infect the thymus (Porada, Tran et al. 1998; Tran, Porada et al. 2001). These studies may not, however, reflect immune responses induced by oral immunization, which delivers antigen to peripheral lymphoid tissue.

In this study, we hypothesized that *in utero* oral DNA immunization could induce an antigen-specific immune response as early as 55-60 dg. At this age, T and B cells have recently appeared in the spleen and peripheral LNs (Symons and Binns 1975; Griebel 1998). A previous study has also suggested that plasmid delivered to the fetus

per os results in transgene expression primarily in the oral cavity (Gerdts, Babiuk et al. 2000). Furthermore, the immunostimulatory effects of bacterial DNA in the plasmid backbone (reviewed in Mutwiri, Pontarollo et al. 2003) might overcome developmental tolerance. Since the ovine placenta effectively blocks transfer of maternal antibodies to the fetus (Needham 1931), there should be no pre-existing antibodies to interfere with the induction of an immune response. Thus, the duration and magnitude of the primary immune response and immune memory in the fetus should be influenced only by the primary immunization event.

5.3. Materials and Methods

5.3.1. Animals

Vaginal progesterone sponges (Veramix®, Pharmacia & Upjohn, Orangeville, ON) were used to synchronize estrus in Suffolk ewes. Rams were then introduced into the flock for a period of 5 days, thus allowing determination of the gestational age of the fetus within $\pm$2.5 days. Pregnancies were confirmed by assays for serum progesterone levels (Prairie Diagnostic Services, Saskatoon, SK) and rectal ultrasound at 42 dg. Blood was collected from ewes at the time of fetal surgery and also at parturition, and serum was tested for the presence of tgD-specific antibody titers. Ewes did not have tgD-specific serum antibody titers at any time. All animals were randomly assigned to experimental groups. All experiments were conducted in accordance to the Canadian Council on Animal Care Guidelines.

5.3.2. DNA plasmid preparation

The plasmid pSLIA-tgD contains the gene for BHV-1 tgD which is a 61-kDa, secreted glycoprotein (van Drunen Littel-van den Hurk, Braun et al. 1999). The empty pSLIA vector was used as a control plasmid for fetal immunization experiments. pMASIA-luc contains the gene for firefly luciferase (Uwiera, Rankin et al. 2001). The immediate early promoter/1A-region from human cytomegalovirus was located upstream of both the tgD and luciferase genes. Plasmids were grown in *E. coli* DH5α and purified using the Endo-Free™ Plasmid Giga Kit (Qiagen, Mississauga, ON). Purified plasmids were resuspended in endotoxin-free Dulbecco's phosphate buffered saline (Sigma-Aldrich, St. Louis, MO) and the endotoxin activity in each plasmid preparation was assessed using the QCL-1000 Limulus Amebocyte Lysate Kit (BioWhittaker Inc. Walkersville, MD). Endotoxin activity was <0.01 EU/ml in all samples, which is below the minimum threshold required to induce mitogenic stimulation of ovine leukocytes (Burrells and Wells 1977).

5.3.3. Fetal immunization

Fetal surgeries were performed at defined gestational periods during the second trimester. Briefly, ewes were pre-medicated with acepromazine and butorphanol and anaesthesia was induced using sodium Pentothal (Abbott Labs, Guelph, ON) and then maintained with isoflurane in oxygen. Ewes were maintained on IV fluid therapy and assisted ventilation throughout anesthesia and positioned in dorsal recumbency. Access to the fetus was achieved by exposing the uterus through a midline abdominal incision. The myometrium and chorion were incised, which made it possible to view the fetus

through the intact amniotic membrane. The head of the fetus was manipulated into the amniotic bubble (Fig. 5.1A), a 22-gauge catheter was inserted through the amniotic membrane into the oral cavity of the fetus (Fig. 5.1B), and sterile saline solution containing DNA plasmid was injected into the oral cavity. See Table 5.1 for fetal immunization groups. The uterine and abdominal incisions were closed and pregnancy allowed to proceed until the time of fetal tissue collection or birth.

5.3.4. Neonatal Immunization

IM DNA immunization was performed by injecting 325 μg of pSLIA-tgD plasmid into each semimembranous muscle. Where indicated, IM plasmid injection was followed immediately by electroporation which was performed as described previously (Babiuk, Baca-Estrada et al. 2002). Briefly, lambs were sedated with Pentothal, injected IM with plasmid, and then an electrode, with six 23-gauge needles arranged equidistant in a 1-cm diameter circle (Genetronics, San Diego, CA), was inserted to a 1-cm depth into the muscle such that the electrodes surrounded the DNA injection site. Electroporation was performed by delivering 6 pulses of 200 V/ms (5 Hz per pulse).

5.3.5. Antibody Detection

Fetal blood was collected by intracardiac puncture and postnatal blood was collected from the jugular vein. Blood was collected from newborn lambs prior to suckling to avoid possible transfer of maternal antibodies through the colostrum. Blood was collected into SST Vacutainer tubes (BD, Oakville, ON) and tubes were centrifuged

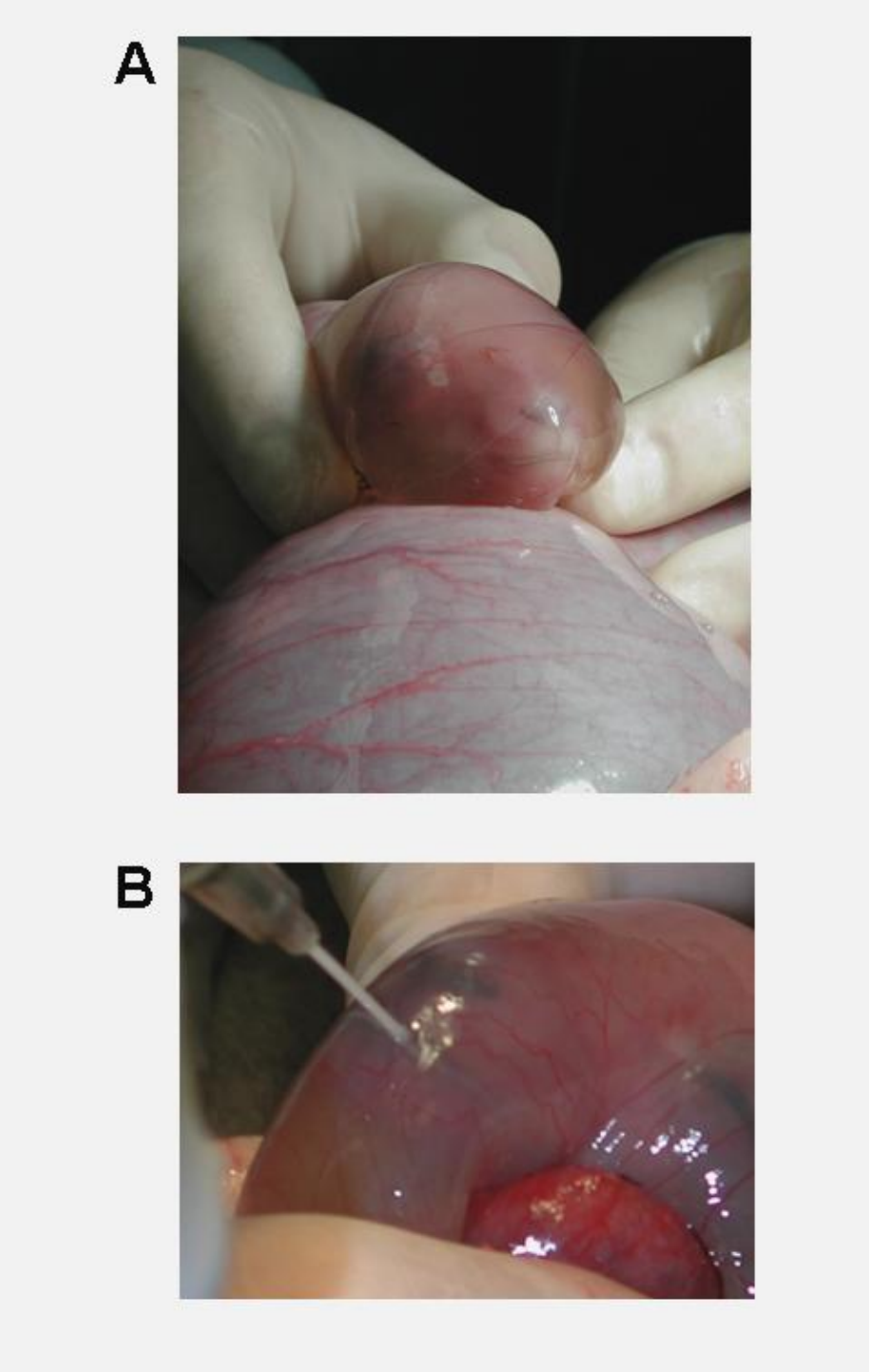


Figure 5.1. ***In utero*** **oral DNA immunization in the second trimester fetal lamb at 55-60 days gestation.**

Head of the fetus visualized within the amniotic bubble (**A**). Insertion of a catheter through the amniotic membrane, into the oral cavity (**B**).

Table 5.1. Immunization groups.

Exp	*Age of fetus at time of* in utero *immunization*	*Group*	*n*	*Fetal immunization*	*Neonatal immunization (2 weeks of age)*
1	65-70 dg	Immunized	10	0.5 mg pMASIA-luc	–
		Control	3	No treatment	–
2	55-60 dg	Immunized	13	1 mg pSLIA-tgD	–
		Control	8	1 mg pSLIA-null	–
	120-125 dg	Immunized	2	1 mg pSLIA-tgD	–
		Control	3	1 mg pSLIA-null	–
3	67-72 dg	Immunized	4	1 mg pSLIA-tgD	650 µg pSLIA-tgD (IM) + electroporation
		Control	4	No treatment	650 µg pSLIA-tgD (IM) + electroporation
	78-83 dg	In-Utero	7	1 mg pSLIA-tgD	1 mg pSLIA-tgD (IM)
		Primary	5	1 mg pSLIA-null	1 mg pSLIA-tgD (IM)
		Naïve	4	No treatment	No treatment

to isolate serum. The ELISA for the detection of tgD-specific antibodies has been previously described (van Drunen Littel-van den Hurk, Gifford et al. 1990). Briefly, serum was diluted using a 2-fold dilution series, starting with neat serum, and applied to Immulon-2 96-well plates (Dynex Labs Inc, Franklin, MA) that had been coated with 0.5 μg/ml of purified recombinant tgD. Bound antibodies were detected using phosphatase-labeled rabbit anti-sheep IgG (H+L) (Kirkegaard-Perry Labs, Gaithersburg, MD) and 100 mg/ml p-nitrophenyl phosphate in 1% diethanolamine, 0.5 mM $MgCl_2$ (pH 9.8). All titers were expressed as the reciprocal of the highest serum dilution to give a positive reaction. The threshold for a positive response was defined as the mean titer plus 2 SD of age-matched control fetuses. The biological relevance of this cut-off with respect to BHV-1 protection has been previously established (van Drunen Littel-van den Hurk, Gifford et al. 1990). All tgD-specific antibody titers were determined within the same assay with the exception of data presented in Figure 5.3A, which was obtained using a different lot of recombinant tgD protein to coat wells in the ELISA plates.

5.3.6. Detection of Interferon (IFN)-γ-responses

Peripheral blood mononuclear cells and splenocytes were purified as previously described (Ioannou, Griebel et al. 2002b). The number of tgD-specific IFNγ-secreting cells was assayed using a modified enzyme-linked immunospot assay. Microtiter nitrocellulose filtration plates (Polyfiltronics, Rockland, MS) were coated with 2.5 μg/ml of anti-bovine IFNγ monoclonal antibody (clone 2.2.1A; IgG1 isotype). One million cells were added to triplicate wells in a final volume of 200 μl of media alone (AIM-V media plus 2% heat inactivated FBS (InVitrogen, Burlington, ON)), media with

5 μg/ml tgD or media with 20 μg/ml concanavalin A (Sigma-Aldrich, Oakville, ON). Captured IFNγ was visualized with rabbit anti-bovine IFNγ antisera and biotinylated-goat anti-rabbit IgG (H+L) (Zymed Laboratories, San Francisco, CA). The number of tgD-specific IFNγ-secreting cells was expressed as the difference between the mean number of spots per 10^6 cells from triplicate tgD-stimulated cultures minus the mean number of spots per 10^6 cells from triplicate cultures in medium alone. The threshold for determining tgD-specific IFNγ-responses was calculated as the mean number of spots plus 2 SD for age-matched naïve animals.

5.3.7. Luciferase Assay

The mucosal epithelium and submucosa were collected from the oral cavity and oropharynx in 1-cm^2 samples mapped to specific anatomical loci. Samples were also collected from the liver, lungs, ears and LNs draining the oral cavity. Frozen tissues were minced in liquid nitrogen, homogenized, and then assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI). The lower detection limit of the assay using the Packard Picolite® Luminometer (United Technologies Packard, Palo Alto, CA) was 100 pg as determined from a standard curve constructed using QuantiLum® Recombinant Luciferase (Promega). The luciferase activity in each 1-cm^2 tissue sample was expressed as units of luciferase activity per mg of protein (ULA). Total protein per sample was determined using the Bradford assay (BioRad, Hercules, CA). The cut-off for non-specific luciferase activity in a specific tissue or tissue group was calculated as the upper 95% CI for the corresponding tissue samples collected from three age-matched, control fetuses.

5.3.8. Histology

Tissues were fixed in 10% buffered formalin and submitted to Prairie Diagnostic Services for staining with hematoxylin and eosin and immunohistochemical staining with monoclonal antibody for the detection of Ki67 nuclear antigen (clone MIB-1; InVitrogen).

5.4. Results

5.4.1. Plasmid Transfection and Expression in the 65-70 dg Ovine Fetus

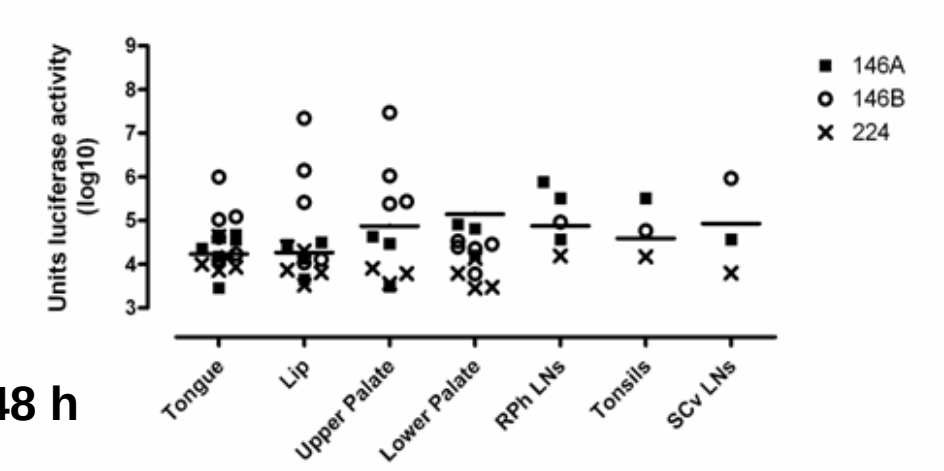
Transfection studies were conducted to confirm that oral injection would be an effective method for delivering naked DNA plasmid in the second trimester fetus. Luciferase-plasmid was injected into the oral cavity of fetuses between 65-70 dg and the sites of plasmid expression and expression levels were determined. This age of fetal gestation was selected since it has been reported that this is the interval during which the ovine fetus acquires immunological competence (Silverstein, Uhr et al. 1963). Luciferase plasmid was injected into the oral cavities of 10 fetuses. The cut-off for non-specific luciferase activity in the fetal oral cavity was determined from 3 untreated, age-matched fetuses.

At 24, 48 and 72 hours post-injection (hpi), luciferase activity was detected in the oral cavity of fetuses injected with pMASIA-luc (Fig. 5.2). There was considerable variation among individual fetuses with respect to the number of tissue samples with positive luciferase activity and the level of luciferase activity at specific anatomical locations. The tongue was, however, the primary site of luciferase expression with the

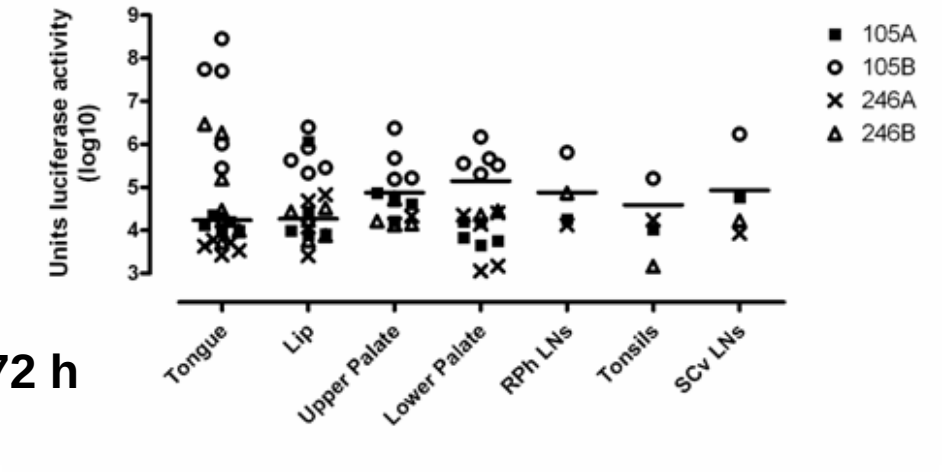
Figure 5.2. Luciferase expression in the oral cavity of the second trimester fetus.

Transgene activity was detected in all fetuses and localized primarily to the mucosal epithelium of the tongue and rostral-most aspects of the oral cavity. Fetuses (65-70 dg; n=10) were orally-injected with 0.5 mg of luciferase-plasmid. Tissues were then collected in 1 cm^2 samples, with each sample corresponding to a specific anatomical locus, and the luciferase activity measured. The distribution and level of luciferase activity in the oral cavities and lymph nodes of injected fetuses at 24 (n=3; **A**), 48 (n=4; **B**) and 72 (n=3; **C**) hours post-injection. Each data point represents the luciferase activity/mg of total protein in an individual 1 cm^2 tissue sample. The identities of fetuses are shown in the legends. The horizontal bar (—) indicates the upper 95% CI of luciferase activity in all samples comprising the same anatomical grouping in 3 age-matched control fetuses. Abbreviations: retropharyngeal lymph nodes (RPh LNs), superficial cervical lymph nodes (SCv LNs).

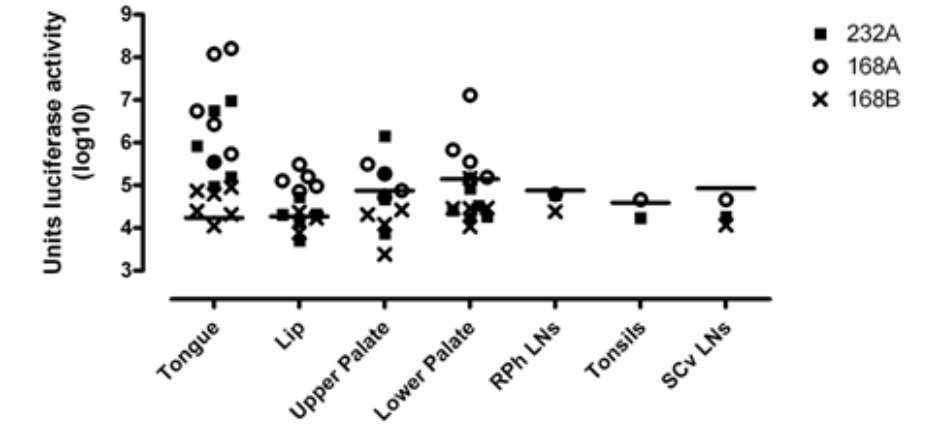
A. 24h



B. 48 h



C. 72 h



(Fig. 5.2)

highest frequency of positive samples and the highest levels of luciferase activity within individual samples. Furthermore, a significant level of luciferase activity was detected in a minimum of one tissue sample/tongue from all injected fetuses. The total luciferase activity in the tongue of each fetus was determined and linear regression analysis was used to determine if there was a change in the amount of luciferase expression over time. Due to variation among individual animals within each group, there was no significant increase or decrease in luciferase activity between 24 to 72 hpi.

Luciferase activity was also detected in the lips, upper palate and lower palate. The detection of luciferase activity in samples of chin (6/10 fetuses) and ear (3/10 fetuses) was consistent with the observation that fluid frequently streamed out of the oral cavity immediately following plasmid injection. Luciferase activity was also detected in the tonsils, LNs draining the oral cavity and liver of four fetuses.

At all times examined, there was considerable variation among fetuses in the number of oral mucosal samples with detectable luciferase activity, and the level of luciferase activity per tissue sample. When the total luciferase activity (i.e., sum of luciferase activity in 29 tissues samples) was calculated for each injected fetus (n=10) and expressed as an increase in total luciferase activity relative to control fetuses (n=3), there was a 2.5- to 100-fold increase in luciferase activity in three fetuses, and a 100- to 450-fold increase in luciferase activity in four fetuses. In the remaining 3 of 10 fetuses, luciferase expression was restricted to very few sites and did not contribute to a significant overall increase in total luciferase activity.

For transfection to occur, the plasmid must transfect the oral mucosal epithelium or bypass this barrier by being ingested or inhaled. Histological examination of the oral

mucosa at 65-70 dg revealed that the mucosal epithelium on the dorsal aspect of the tongue consisted of 2-3 layers of nucleated cells with a developing stratum corneum (Fig. 5.3A). Neither desquamation nor papillae were observed. In contrast, the mucosal epithelium on the tongue of a 120-125 dg fetus had differentiated with a thick, anuclear stratum corneum and desquamation of epithelial cells (Fig. 5.3B). Thus, histology revealed that nucleated cells were available for transfection at the surface of the oral mucosa of 65-70 dg fetuses.

Overall, these experiments confirmed that injection of 0.5 mg of naked DNA plasmid into the oral cavity resulted in the expression of luciferase within the oral mucosa and protein was also detected in LNs draining the oral cavity.

5.4.2. Fetal immune responses following oral DNA immunization in the early second trimester

Low levels of plasmid transfection and expression have been identified as major factors limiting the efficacy of DNA vaccination in humans and many other species. The detection of luciferase activity in 100% of fetuses that received an oral DNA injection on 65-70 dg supported the hypothesis that oral DNA immunization might be extended to the second trimester (55-60 dg) of gestation when the spleen and peripheral LNs are being populated with lymphocytes (Symons and Binns 1975; Griebel 1998). Fetuses were immunized between 55-60 dg (Table 5.1) and tgD-specific serum antibody titers were measured at 14, 30 and 50 dpi. A tgD-specific antibody response was detected in 9 of 13 (69%) immunized fetuses (Table 5.2).

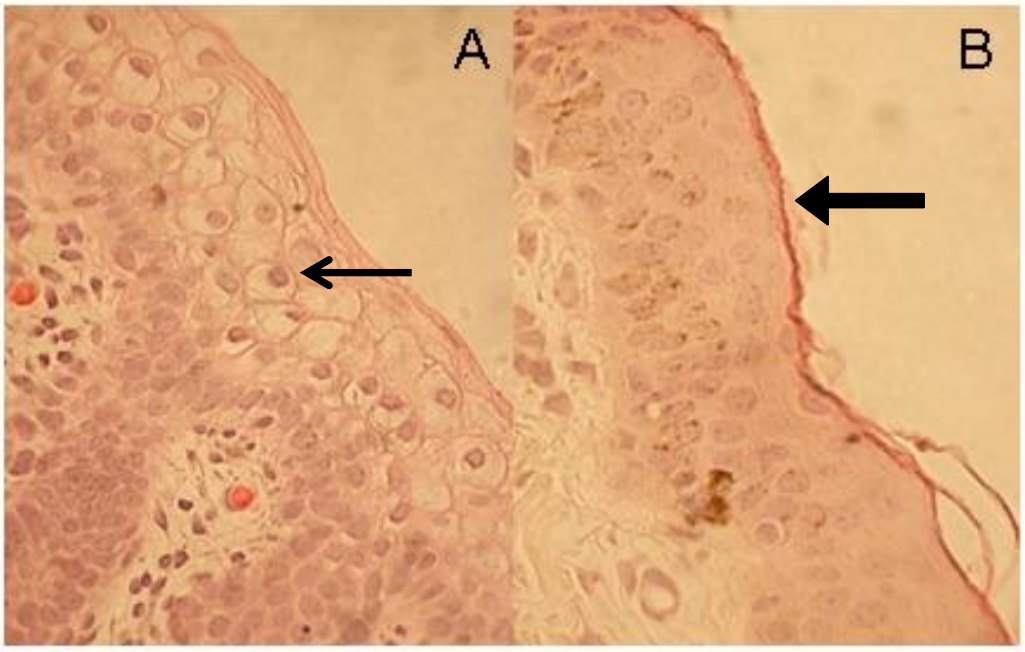


Figure 5.3. Mucosal epithelium from the dorsal aspect of the tongue.

In the second trimester fetus, there are nucleated cells (→) in the superficial-most layer of the mucosal epithelium. In the third trimester, the epithelium is more differentiated and there is a thick stratum corneum (➡). Fetuses at 65-70 dg (**A**) and 120-125 dg (**B**). Hematoxylin and eosin (x 40).

Table 5.2. Induction of fetal immune responses following oral immunization with tgD-plasmid in the second trimester of gestation

Age of fetus at immunization[1] (days gestation)	*Sample/assay time (days post-immunization)*	*No. of age-matched controls*	*No. of responders / no. immunized*		
			Antibody responses[2]	*IFNγ-secreting responses[2]*	*Total no. of responders*
55-60	14 d	4[3]	1/1	ND[4]	1/1 (100%)
55-60	30 d	4	3/5	1/5	3/5 (60%)
55-60	50 d	4	5/7	1/7	5/7 (71%)
67-72	Postnatal[5] (>78 d)	4	2/4[6]	ND	2/4 (50%)
78-84	Postnatal (>65 d)	5	3/7	3/6[7]	5/7 (71%)

[1] All fetuses were orally-immunized with 1 mg of pSLIA-tgD in a 1 ml volume of saline with gestation age defined within an accuracy of ±2.5 days.

[2] A responder was defined as any animal with a tgD-specific antibody titer or IFNγ response that exceeded the mean plus 2 SD of responses in relevant, age-matched control animals.

[3] The tgD-specific antibody titer was significantly elevated relative to titers in fetuses immunized with control plasmid at 55-60 dg and assessed 30 days later (n=4).

[4] Assay was not done (ND).

[5] For postnatal assessment, fetal serum antibodies and/or IFNγ responses were measured at birth, prior to suckling, and anamnestic responses were measured after a secondary DNA immunization at 13 days of age.

[6] Both precolostrum and secondary tgD-specific serum antibodies were detected in one lamb. Only secondary tgD-specific antibody titers were detected in the second responder.

[7] One lamb that had been immunized *in utero* died within 24 hours of birth and, therefore, was unavailable for IFNγ testing.

The fetus examined at 14 dpi had a tgD-specific serum antibody titer of 27, which exceeded the threshold determined from sera collected from 30 dpi control fetuses. Examination of the SCv and retropharyngeal LNs from this fetus revealed the LNs to be small and gelatinous and there was no demarcation of the cortex from medulla. Staining of serial tissue sections for Ki67 nuclear proliferating cell antigen revealed individual cells and small clusters of stained cells randomly distributed throughout the LNs (data not shown).

A tgD-specific antibody response was detected in 3 of 5 fetuses at 30 dpi (Fig. 5.4A) and 5 of 7 fetuses at 50 dpi (Fig. 5.4B). Ki67-staining of serial sections revealed large foci of proliferating cells in the cortex of at least one of the parotid, submandibular, retropharyngeal or SCv LNs in all fetuses with elevated antibody titers (Fig. 5.5A). In contrast, no proliferating cell foci were detected in LNs collected from control fetuses (Fig. 5.5B). Examination of hematoxylin and eosin stained serial sections from the same LNs revealed that the $Ki67^{+}$ staining foci were lymphoid follicles with an architecture similar to GCs (data not shown). For 7 of the 8 responders (Fig. 5.4), the magnitude of the tgD-specific antibody titers was relatively low (i.e., <150) when compared within the same ELISA to the serum antibody responses of fetuses orally immunized in the third trimester (titers of 561 and 597) or tgD-specific antibody responses following DNA immunization in the neonate (see Figs. 5.6C, E). Fetus #54 was an exception with an antibody titer of 2461 and tgD-specific IFNγ-secreting cells following *in vitro* re-stimulation of peripheral blood mononuclear cells (data not shown). Also, $Ki67^{+}$ GCs were detected in all LNs draining the oral cavity (retropharyngeal, mandibular, parotid and SCv LNs) of fetus #54 and these LNs were at least 5-fold larger than the

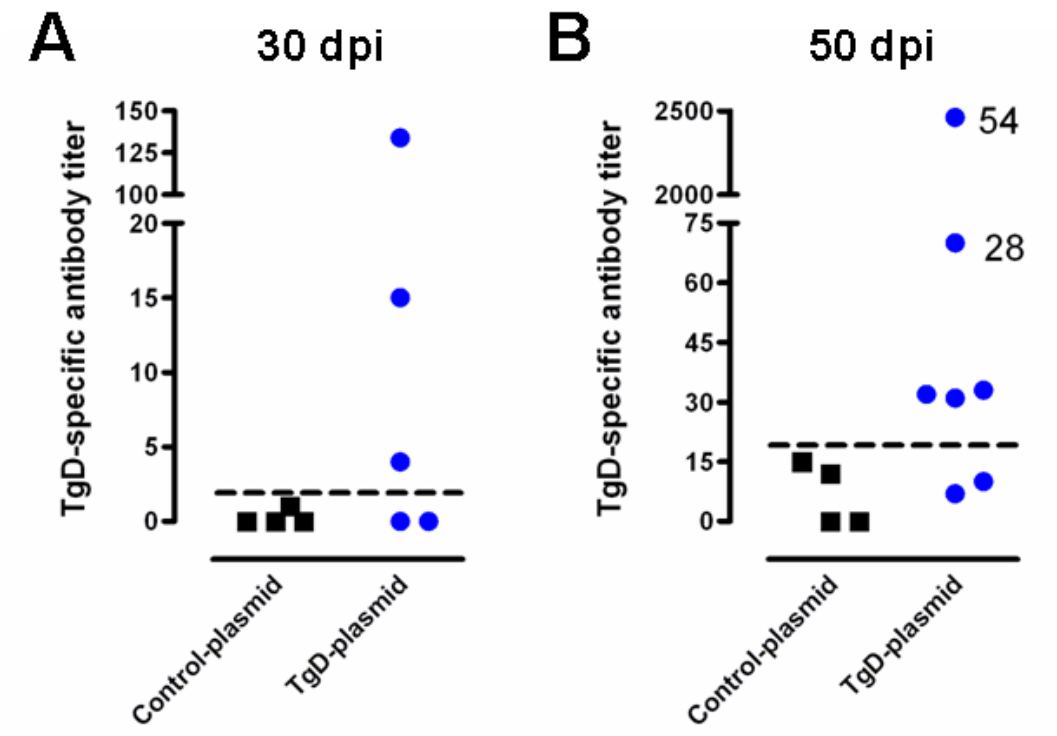


Figure 5.4. TgD-specific serum antibody responses following oral DNA immunization in the early second trimester.

Oral tgD-plasmid immunization at 55-60 dg resulted in significant tgD-specific antibody titers in 3 of 5 and 5 of 7 immunized fetuses at 30 dpi and 50 dpi, respectively. There was considerable variation in the magnitude of antibody titers and fetus #54 had a markedly elevated titer. Fetuses (55-60 dg) were orally immunized with either 1 mg of control- or tgD-plasmid. Total tgD-specific serum antibody titers at 30 dpi (**A**) and 50 dpi (**B**) were measured in two separate ELISAs. The horizontal line (—) represents the mean plus 2 SD of cross-reactive antibody titers from 4 age-matched control fetuses and indicates the cut-off for tgD-specific fetal antibody responses ($p<0.05$).

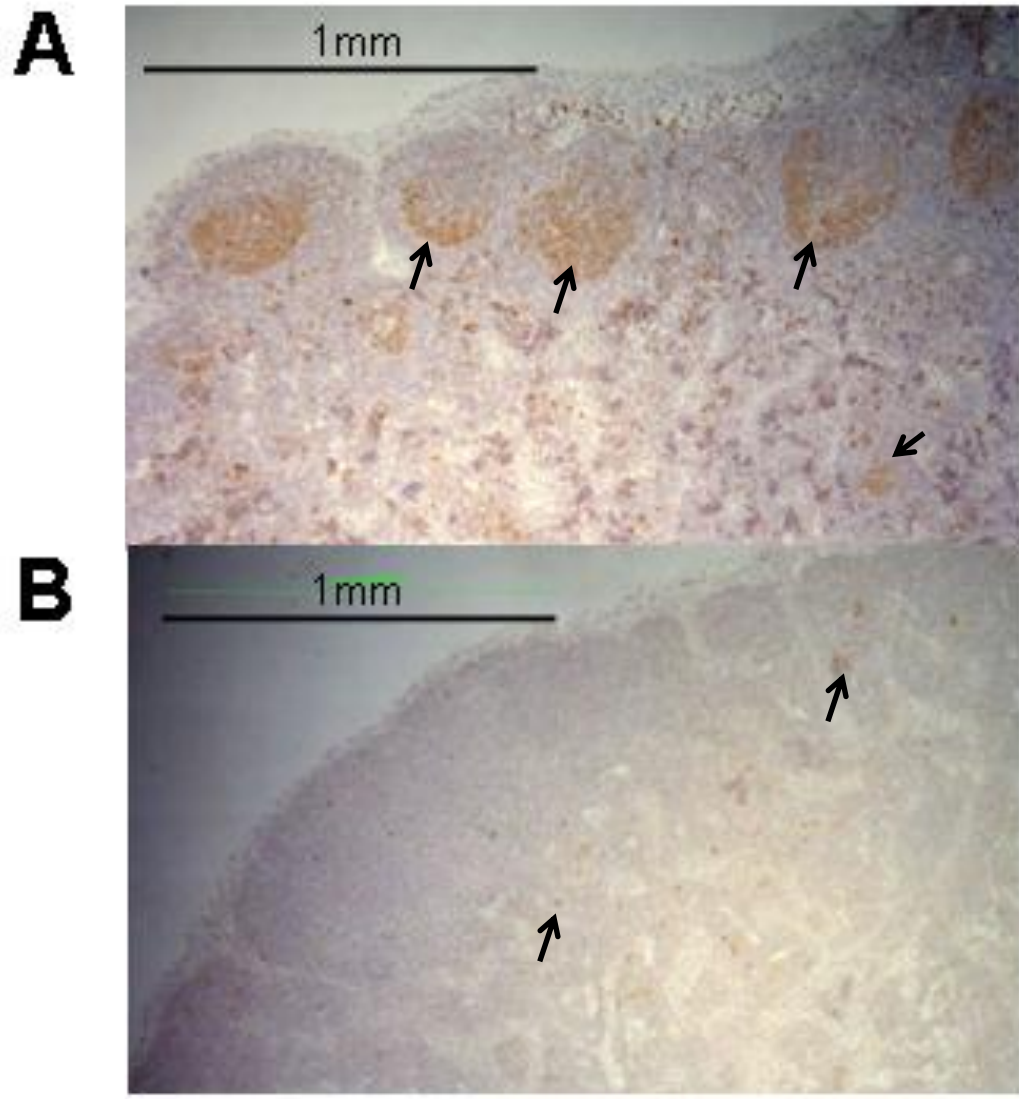
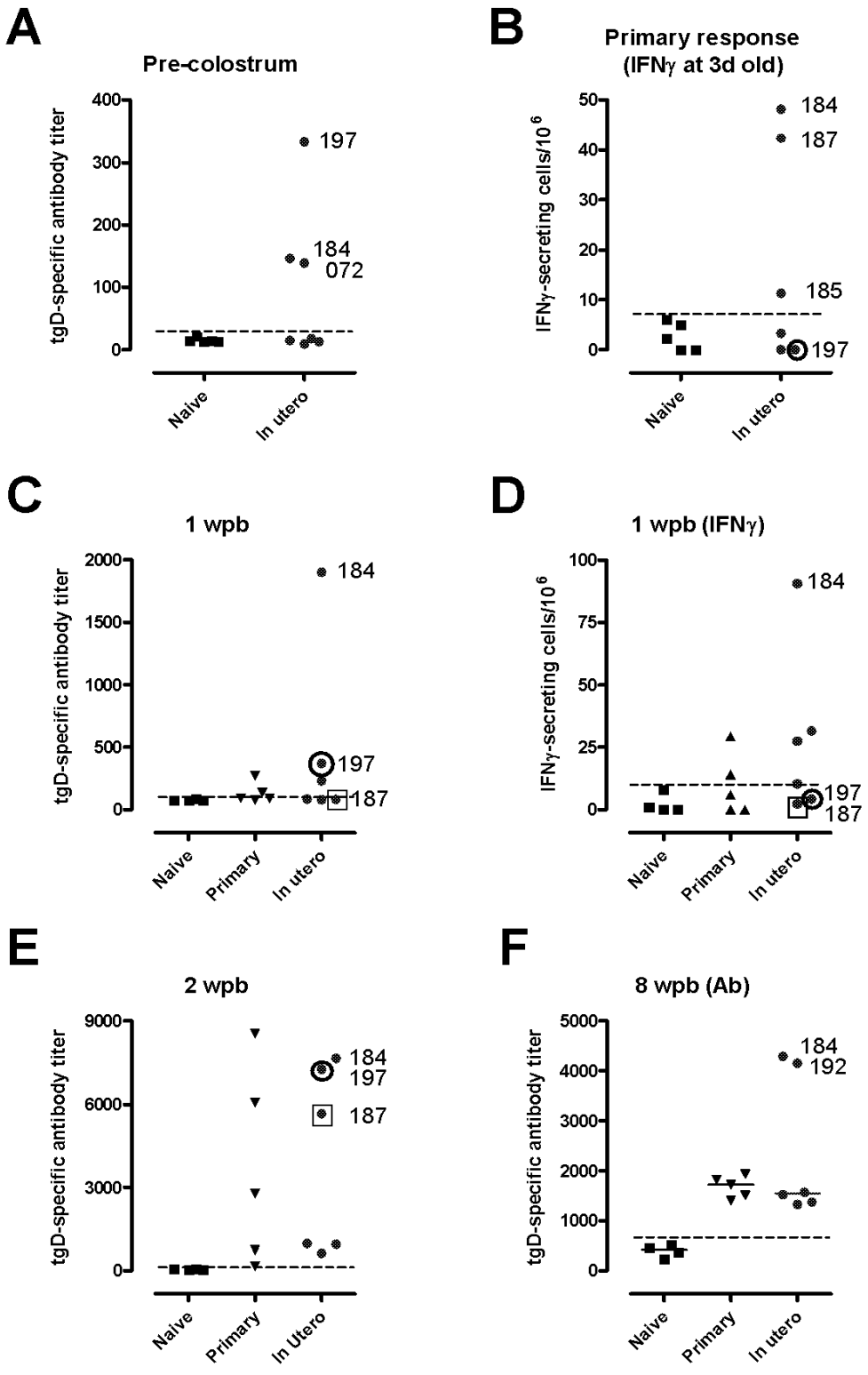


Figure 5.5. Retropharyngeal lymph nodes from 105-110 dg fetuses stained for Ki67 nuclear proliferating antigen.

Oral immunization with tgD-plasmid resulted in the development of germinal centers in lymph nodes draining the oral cavity. Sections of retropharyngeal lymph node from a fetus immunized with tgD-plasmid at 55-60 dg (**A**) and an age-matched naïve fetus (**B**). Section were stained with with antibody specific for Ki67 nuclear proliferating antigen (→).

Figure 5.6. Immune responses in newborn lambs following oral DNA immunization at 78-84 days gestation.

Primary tgD-specific antibody titers were detected at birth in 3 of 7 lambs. Primary IFNγ responses were also detected in 3 of 6 newborn lambs. Based on earlier onset and greater magnitude of antibody responses, anamnestic responses were present in 3 fetuses (#184, 197 and 192). Fetal lambs were orally immunized at 78-84 dg with 1 mg of tgD-plasmid (n = 7). TgD-specific fetal serum antibody titers were measured in newborn lambs prior to suckling (**A**) and tgD-specific IFNγ responses were measured in peripheral blood mononuclear cells isolated from 3 day old lambs (**B**). One lamb in the in utero-immunized group died between birth and 3 days of age. The horizontal line (—) in **A** and **B** represent the mean tgD-specific response plus 2 SD from 5 age-matched naïve lambs. The identities of lambs that responded to fetal immunization are given. At 2 weeks of age, lambs that had been orally immunized at 78-84 dg (In-Utero; n = 6) were boosted with an intramuscular injection of 650 μg of tgD-plasmid. Age-matched lambs were either immunized with the same protocol (Primary; n=5) or did not receive any treatment (Naïve; n = 4). TgD-specific immune responses were assayed weekly and the following data are shown: serum antibody titers at 1 wpb (**C**), IFNγ-responses at 1 wpb (**D**), serum antibody titers at 2 wpb (**E**) and 8 wpb (**F**). The horizontal line (—) in **C** through **F** represents the mean response plus 2 SD for the Naïve group.


A
Pre-colostrum
tgD-specific antibody titer
400
300
200
100
0
197
184
072
Naive
In utero
B
Primary response
(IFNγ at 3d old)
IFNγ-secreting cells/10^6
50
40
30
20
10
0
184
187
185
197
Naive
In utero
C
1 wpb
tgD-specific antibody titer
2000
1500
1000
500
0
184
197
187
Naive
Primary
In utero
D
1 wpb (IFNγ)
IFNγ-secreting cells/10^6
100
75
50
25
0
184
197
187
Naive
Primary
In utero
E
2 wpb
tgD-specific antibody titer
9000
6000
3000
0
184
197
187
Naive
Primary
In Utero
F
8 wpb (Ab)
tgD-specific antibody titer
5000
4000
3000
2000
1000
0
184
192
Naive
Primary
In utero

(Fig. 5.6)

corresponding LNs collected from the other 4 fetuses with detectable antibody responses at 50 dpi. A medial longitudinal cross-section of the retropharyngeal LN from fetus #54 contained 43 $Ki67^+$ foci but only two $Ki67^+$ foci were observed in a medial longitudinal cross-section of the retropharyngeal LN collected from fetus #28, which had a tgD-specific antibody titer of 70.

Overall, 69% of fetal lambs responded to oral DNA immunization at 55-60 dg. These responses included tgD-specific serum antibody responses and foci of $Ki67^+$ cells within LNs draining the oral cavity. In one fetus, an IFNγ-secreting cell response was also detected.

5.4.3. Secondary tgD-Specific Immune Responses in the Neonate Following Oral DNA Immunization During the Second Trimester

To optimize immune protection against vertically transmitted diseases, immune effector and memory functions must persist until birth when there is the greatest risk of infection. We therefore examined the duration of primary immune responses and immune memory following second trimester oral DNA immunization. Fetuses were orally immunized with tgD-plasmid at 67-72 dg (Table 5.1), which resulted in a 12 week interval between primary immunization and birth. This interval is equivalent to the longest duration of a primary antibody response reported following a single DNA immunization of young lambs with tgD-plasmid (Tsang, Babiuk et al. 2007). A positive tgD-specific serum antibody titer was detected in 1 of 4 newborn lambs that had been immunized at 67-72 dg (lamb #253, Table 5.2). At 2 weeks of age, lambs received a secondary tgD-plasmid immunization and lambs #253 and #249 displayed tgD-specific

antibody titers significantly higher ($p < 0.05$) than titers detected in age-matched controls. One interpretation of these data is that tgD-specific antibody responses and immune memory may have waned prior to birth.

The interval between primary fetal immunization and birth was reduced to determine if waning of immune responses was a significant factor contributing to the presence of detectable antibody titers at birth. When fetuses were immunized at 78-83 dg (Table 5.1), which shortened the interval between immunization and birth to 9.5 weeks, tgD-specific immune responses were detected in 5 of 7 (71%) newborn lambs; significant tgD-specific antibody titers were detected in 3 of 7 lambs (Fig. 5.6A) and IFNγ-secreting responses were detected in 3 of 6 lambs (Fig. 5.6B). Lamb #072 died within 24 hours of birth and was not tested for an IFNγ-secreting cell response. Only one newborn lamb (#184) had both tgD-specific antibody and IFNγ -secreting responses.

A secondary IM tgD-plasmid vaccination was then performed at 2 weeks of age (Table 5.1) to determine if anamnestic responses or antigen-specific immune tolerance was present in newborn lambs. At the time of DNA boost, there was no significant difference in tgD-specific antibody titers among the Naïve, Primary or In-Utero groups (data not shown). The response to neonatal DNA immunization was evaluated by comparing the serum antibody titers of individual immunized lambs against the mean plus 2 SD of serum antibody titers from the Naïve group ($p < 0.5$). Immunization with tgD-plasmid induced tgD-specific antibody and IFNγ responses in all immunized lambs (Fig. 5.6C-F).

To determine if anamnestic responses were present, immune responses of individual lambs in the In-Utero group were compared to the mean plus 2 SD of

responses in the Primary group (n=5). Immune responses exceeding this value were significant ($p < 0.05$) and considered to be an anamnestic response. At 1 week post-boost (wpb), anamnestic responses were detected in 2 of 6 In-Utero lambs: lamb #197 had a significantly increased tgD-specific antibody titer (Fig. 5.6C) and lamb #184 had both significantly increased antibody and IFNγ responses (Fig. 5.6C, D). Between 2 and 7 wpb, tgD-specific antibody titers in the In-Utero group were indistinguishable from titers in the Primary group. At 8 wpi, tgD-specific antibody titers were significantly higher in lambs #184 and 192 (Fig. 5.6F). Collectively, these data suggested that an anamnestic response had occurred in 3 of 6 (50%) lambs orally immunized *in utero* during the second trimester. In only one lamb (#184) was there evidence of both humoral and cellular anamnestic responses. In addition, all lambs in the In-Utero group responded to neonatal DNA immunization with induction of both tgD-specific antibody and IFNγ responses. This indicated that immunization during the second trimester did not induce immune tolerance.

5.5. Discussion

In this study, oral DNA immunization was extended from the third to second trimester of fetal gestation. The feasibility of second trimester oral DNA immunization is dependent upon successful delivery of plasmid to the oral mucosa, transfer of plasmid-encoded protein to the draining LNs, the presence of a functional immune system, and the induction of a protective immune response rather than immune tolerance. In the first part of this study, reporter gene studies were completed which confirmed that oral delivery of DNA plasmid could overcome the variety of physical and

chemical barriers that reduce transfection efficiency at mucosal surfaces (reviewed by Hobson, Barnfield et al. 2003). Oral delivery of luciferase-plasmid to second trimester fetuses resulted in the consistent detection of luciferase activity in the oral mucosa and protein was detected in the LNs draining the oral cavity (Fig. 5.2). Therefore, oral delivery of DNA plasmid appeared to be an effective route of delivery to target protein to the peripheral immune system.

There was marked variation in the level of luciferase activity detected within the oral mucosa of individual fetuses, which indicated that the level of plasmid transfection varied among fetuses. Plasmid transfection efficiency may vary as a result of either inconsistent oral delivery or differences in the degree of epidermopoeisis or keratinization of the mucosal epithelium (Fig. 5.3). The detection of luciferase activity in LNs draining the oral cavity is consistent with reports that the peripheral lymphatic system becomes functional between 60 and 65 dg (Cahill, Kimpton et al. 1999). Furthermore, the presence of GC reactions within the draining LNs and detectable serum antibody titers following immunization with tgD-plasmid provided direct evidence for protein uptake from the oral cavity. The variable level of luciferase activity in the oral mucosa may be correlated with the variation observed for both serum antibody responses and the numbers of GCs observed within individual LNs. The percentage of fetuses with greater than 2.5-fold increases in total luciferase activity (70%) was similar to the frequency of fetuses with detectable tgD-specific antibody responses (67%). This observation supports the conclusion that increasing the efficiency of oral plasmid delivery may be an effective strategy to improve the consistency of immune responses during the second trimester of gestation.

To determine the earliest possible age for fetal DNA vaccination, fetuses were immunized starting at 55-60 dg when T and B cells first appear in the periphery (Symons and Binns 1975; Press, Hein et al. 1993; Griebel 1998). A tgD-specific antibody response was first detected within 14 dpi, which is consistent with the earliest reported age of antigen-specific antibody responses in the fetal lamb (Silverstein, Uhr et al. 1963). Visible GC reactions were not detected in the retropharyngeal or SCv LNs until 30 dpi. The presence of GCs at 30 and 50 dpi, in conjunction with tgD-specific antibody titers, confirmed that tgD-specific responses had occurred despite the low magnitude of serum antibody responses. These results contrast with other studies where delivery of a foreign gene at 55-60 dg, in the context of retroviral vectors, resulted in protein expression within the thymus and the induction of antigen-specific immune suppression/tolerance (Porada, Tran et al. 1998; Tran, Porada et al. 2001). We hypothesize that developmental tolerance in 55-60 dg fetuses was overcome by delivering a foreign protein via the oral DNA immunization method, which combines a peripheral route of antigen delivery with the immune-stimulatory effects of the bacterial DNA backbone (Mutwiri, Pontarollo et al. 2003). The bacterial DNA may function as a potent danger signal (Matzinger 2002) or adjuvant within an otherwise sterile fetal environment.

Oral DNA immunization in the second trimester also induced antigen-specific IFNγ-secreting cell responses in 4 of 24 (17%) lambs despite the reported suppression of IFNγ responses in the ovine fetus (Buxton, Anderson et al. 2002). The presence of both antigen-specific humoral and IFNγ responses in two fetuses that were assayed pre-parturtion and in all lambs that received a secondary neonatal DNA immunization

indicated that immune deviation had not occurred following fetal immunization (Ahmed 1991; Otsyula, Miller et al. 1996). The lack of detectable IFNγ-secreting responses in the majority of fetuses at 69-74 dg or 87-92 dg may reflect a delayed development of cell-mediated immune responses relative to humoral responses in the ovine fetus (Silverstein, Prendergast et al. 1963; Silverstein, Uhr et al. 1963).

The duration of fetal immune responses was also examined in this study. Data obtained in another study (manuscript submitted) suggests that oral DNA delivery in the third trimester fetus resulted in prolonged protein expression which may correlate with a relatively low rate of mucosal epithelial cell renewal. Prolonged expression of a foreign protein should theoretically sustain and possibly amplify fetal immune responses in the absence of cross-reactive environmental antigens. DNA immunization in the second trimester did not enhance the magnitude of fetal immune responses but may, instead, have influenced the duration of immune responses. GCs were clearly visible at 7 wpi, which exceeded the expected 3-5 week period reported for GCs following a single protein exposure (Kesmir and De Boer 1999). Also, fetal antibody titers were still detectable at 10 wpi (78-84 dg to birth) and possibly up to 11.5 wpi (67 dg to birth), which is comparable to the maximum duration of primary tgD-specific antibody responses following a single DNA immunization in young lambs (Tsang, Babiuk et al. 2007).

In postnatal animals, the survival of immune memory cells may be aided by exposure to cross-reactive environmental antigens (Gray and Matzinger 1991; Selin, Nahill et al. 1994); however, the ovine placenta prevents fetal exposure to most environmental antigens (Needham 1931). The exclusion of foreign antigen in control

fetuses was confirmed by the absence of tgD-specific serum antibodies and lack of GC development within the LNs. Despite the absence of environmental antigens or commensal microflora in the intestine, a single oral DNA immunization in the second trimester induced immune memory that persisted throughout the 11.5 week interval between immunization at 67-72 dg to birth. Thus, the duration of fetal immune responses and immune memory was similar to that reported following a single IM immunization of newborn lambs with the same tgD-plasmid (Tsang, Babiuk et al. 2007). The prolonged immune memory observed following IM DNA immunization was attributed to sustained antigen expression within myocytes. Therefore, we hypothesize that the persistence of immune memory in immunized fetuses may be a consequence of prolonged antigen expression in transfected oral mucosal epithelial cells and the effective uptake of this protein by the immune system.

The low magnitude of fetal immune responses and the absence of detectable immune responses in many neonatnal lambs raises the question of whether fetal immune responses can prevent vertical disease transmission. The magnitude of immune responses during the second trimester may have been limited by the limited diversity of the fetal immune repertoire (Hein, Dudler et al. 1990; Reynaud, Mackay et al. 1991), as well as the low number of lymphocytes present in the fetus (Pearson, Simpson-Morgan et al. 1976; Hein, Dudler et al. 1990), which restricts the frequency of antigen-specific lymphocytes at the time of immunization and limits the magnitude of immune responses despite prolonged antigen expression by transfected cells. The occasional fetus responded to second trimester DNA immunization with a relatively high serum antibody

response (Fig. 5.4B) which suggests that genetic variability may be an important factor to consider during *in utero* immunization.

In conclusion, this study demonstrated that oral DNA immunization during the second trimester of gestation resulted in the induction of antigen-specific humoral and CMI responses with no evidence for the induction of immune tolerance. Further studies will be required, however, to determine if vaccine-induced fetal immune responses have the ability to confer immune-mediated protection against vertical disease transmission.

5.6. Acknowledgements

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

This thesis addressed the feasibility of DNA vaccination in the second trimester fetus. Specifically, the hypotheses being tested were that a DNA vaccine introduced into the "preimmune" fetus could induce an immune response and that oral DNA injection is an effective method to deliver a foreign antigen into the second trimester fetus. Delivery of a DNA vaccine as early as possible in gestation would increase the period of time during which the developing immune system is exposed to the antigen. This would potentially result in the continued induction of fetal immune responses for as long as the antigen persisted, thus increasing both the magnitude and duration of the primary immune response. The presence of a primary immune response in the fetus may have implications for protection from infectious pathogens that are transmitted to the fetus during the pre- or periparturient period. Similarly, exposing the fetal immune system to an antigen for a prolonged period might enhance immune memory, which is also a hallmark of an effective vaccine, thus enhancing neonatal immune protection.

In order to test the main hypothesis – that it is possible to immunize the second trimester fetus - it was necessary to select a method of antigen delivery that would be consistent, that would not induce tolerance, and would not be detrimental to the fetus. In Section 1.2.1, mechanisms of self/non-self discrimination were reviewed and the

requirement for a pro-inflammatory component, danger signal or adjuvant to induce a protective immune response and circumvent tolerance induction, was discussed. The use of a traditional killed or subunit vaccine was negated due to studies in other models which have demonstrated inactivation by maternal antibodies (van Maanen, Bruin et al. 1992; Blasco, Lambot et al. 2001), thus preventing its ability to be translated to the human model. It is also inadvisable to increase the immunogenicity of traditional or subunit vaccines with the addition of potent adjuvants, such as alum or CFA, due to the increased risk of abortion. While MLV vaccines are inherently immunogenic and very effective at inducing CMI in the older animal (Platt, Burdett et al. 2006; Reber, Tanner et al. 2006), the lack of cross-reactive or well-developed innate immune responses in the fetus limit the ability to control viral replication, thus increasing the risk of abortion. Retroviral vectors, which are a very effective method of antigen delivery in the fetus, preferentially induce tolerance (Table 1.5). We selected oral DNA vaccination as our method of antigen delivery for two primary reasons: (i) the immunostimulatory properties of the bacterial DNA backbone should overcome the lack of danger signals in the fetus; and (ii) previous experiments have shown that *in utero* oral DNA immunization is an effective method of immunizing the third trimester ovine fetus (Gerdts, Babiuk et al. 2000; Gerdts, Snider et al. 2002).

The mechanism of action of DNA vaccines was reviewed in Section 1.5. In order for DNA vaccination to be effective in the fetus, host cells must be transfected and expression of the plasmid-encoded protein must be at a level exceeding the minimal threshold for activation of immune cells (MacGregor, Boyer et al. 1998; Ugen, Nyland et al. 1998; van Drunen Littel-van den Hurk, Gerdts et al. 2000; Babiuk, Pontarollo et al.

2003). Furthermore, fetal immune cells must express TLR-9, which mediates the immunostimulatory activity of CpGs motifs endogenous to the bacterial DNA backbone. The ovine fetus undergoes significant developmental changes between the second and third trimesters of gestation; and developmental differences in the mucosal epithelium and immune surveillance system, specifically the rate of leukocyte trafficking and lymphatic drainage of the oral cavity, were identified as potential factors limiting the ability to extend oral DNA immunization from the third to second trimester of gestation. Prior to testing the immune competence of the second trimester fetus, it was necessary to demonstrate the consistency of the oral delivery protocol and ensure that adequate levels of plasmid transfection and expression could be achieved in the second trimester fetus.

Initial plasmid dose and transfection/expression studies were conducted in the third trimester ovine fetus between 120-125 dg, i.e., the age of fetus used by Gerdts et al. (2000; 2002) to conduct immunization experiments. A simple dose titration study conducted with luciferase-plasmid (Fig. 4.3) confirmed that injection of 1 mg of plasmid resulted in higher levels of transgene expression in the oral cavity relative to a 500 μg dose of plasmid, which was the arbitrary dose used in the original studies by Gerdts et al.. As other studies have shown a correlation between high levels of transgene expression and successful DNA vaccination, 1 mg was selected as the preferred dose for subsequent transfection/expression and immunization experiments.

In vitro experiments conducted with tissue explants collected from 120-125 dg fetuses provided proof of principle that transfection of fetal mucosal epithelium can occur in the absence of either tissue manipulation or DNA plasmid formulation and evidence to suggest that transfection of mucosal epithelium with naked DNA plasmid

occurs within 15 minutes of contacting the mucosal epithelium (Section 4.4.2). This latter finding was consistent with studies showing that circular plasmid was degraded within 4-8 hours in amniotic fluid (Gerdts, Snider et al. 2002). Within 24 hours of oral delivery, transgene expression was located primarily in the tongue and lips. Between 24 and 72 hours, the level of luciferase activity increased slightly and from 5 dpi onward, there was a significant increase in both the level and distribution of luciferase activity. When compared with the duration of luciferase expression in cultured MDBK cells (72 hours) and the reported half-life of luciferase in mammalian cells (3-4 hours), the continual increase in levels of luciferase activity up to 11 dpi suggested that plasmid expression was ongoing in transfected cells. Therefore, *in utero* oral injection of DNA plasmid is followed by persistent transfection of cells and prolonged expression of plasmid in the recipient fetus. The oral DNA vaccination method was selected for second trimester fetal immunization studies for its potential to provide long-term antigenic stimulation to the naïve fetal immune system, as well as to provide long-term antigenic support to activated memory cells.

Based on the detection of luciferase activity in fetal tissues, oral injection of DNA between 120-125 dg resulted in successful transgene expression in 93% of fetuses. This corresponded with a greater that 83% immunization rate in fetuses, which was obtained using a lower dose of orally delivered DNA plasmid (Gerdts, Babiuk et al. 2000; 2002). Oral DNA delivery experiments were also conducted in both newborn and second trimester fetal lambs. Delivery of plasmid solution to the oral mucosal epithelium of newborn lambs (less than 24 hours of age; n=3) did not result in detectable levels of luciferase activity in tissues collected at 24, 48 or 72 hours post-injection (1 lamb per

collection time; data not shown). This absence of detectable luciferase activity was consistent with failure to immunize newborn lambs with tgD-plasmid using the oral delivery method (Section 4.4.5). In contrast, oral delivery of luciferase-plasmid to fetuses between the ages of 65-70 dg resulted in luciferase detection in a larger percentage area of tissues in the oral cavity and higher levels of luciferase activity per sample area relative to fetuses treated at 120-125 dg (Fig. A.1). Furthermore, oral delivery of luciferase-plasmid in the second trimester resulted in detectable levels of luciferase activity in 100% of treated fetuses (Fig. 5.2). We hypothesized that the enhanced transfection and expression of DNA plasmid in the second trimester fetus might translate into a higher rate of immune induction following oral DNA immunization, provided that the immune system in the second trimester was competent at the time of immunization. Significant variation in transfection efficiency was noted between individual animals in all transfection/expression experiments and low levels of transgene expression might limit the efficiency of *in utero* oral DNA immunization in some fetuses.

Variation in levels of transgene expression among individual fetuses may be caused by a number of factors, including escape of plasmid solution from the oral cavity and differences in transfection competence. The oral delivery protocol was modified for all second trimester injection/immunization studies, such that the fetus was clearly visible within an amniotic bubble (Fig. 5.1). This modification of the surgical procedure had no effect on fetal survival rates, but did allow visualization of the injection process and further assurance that oral tissues were neither directly injected nor damaged during plasmid delivery. We noted fluid flowing out of the oral cavity from the commissures of

the lips during oral injection of some, but not all, fetuses. This loss of plasmid from the oral cavity likely contributed to detection of transgene activity in external body tissues, such as the ear, as well as lower levels of transfection in certain fetuses. Plasmid solution might also have been lost from the oral cavity as a result of diffusion into the upper respiratory tract or swallowing. Unfortunately, neither intestinal tissues nor mesenteric LNs were analyzed for transgene activity.

In Chapter 2 (p. 68), we hypothesized that transfection occurred primarily in the mucosal epithelium of the oral cavity. This hypothesis was based on the prior detection of tgD-specific lymphocyte proliferative responses in LNs draining the oral cavity, but not elsewhere in the body (Gerdts, Babiuk et al. 2000). Luciferase experiments in second and third trimester fetuses gave similar localization results; specifically, that transgene activity was present at the earliest times examined post-injection and at the highest levels in the mucosal epithelium of the oral cavity. We then refined our hypothesis to state that transfection and expression occurred primarily in nucleated cells in or superficial to the stratum basale as a result of plasmid being unable to permeate the basement membrane (Loehr, Willson. et al. 2000; Lemoine, Farley et al. 2005). Studies in human fetuses have shown that the basement membrane of skin has developed by the first trimester (Hentula, Peltonen et al. 2001), but no equivalent studies have been conducted in sheep. Unsuccessful attempts were made to identify the type and location of cell(s) preferentially transfected with plasmid using either immunohistochemical staining for luciferase protein or fluorescence microscopy (following oral delivery of green-fluorescent protein (GFP)-encoding plasmid). The inability to detect individual

cells expressing either luciferase or GFP may reflect a very low frequency of cell transfection in the oral mucosa.

Our hypothesis that transfection occurred in the epithelial layer was consistent with the findings that: (i) oral delivery was not successful in newborn lambs (Section 4.4.5), and (ii) the level of luciferase activity was higher in fetuses that were orally injected at a younger gestational age (Fig. A.1). The different types of epithelium present in the oral cavity were discussed in Section 1.5.3. Increased cornification and keratinization of cutaneous surfaces, such as the tongue and lips, which were identified as primary sites of plasmid transfection and expression, might restrict plasmid uptake and transfection in the neonate relative to in the fetus. In addition, the higher rate of epithelial cell turnover in neonatal lambs would result in a more rapid loss of transfected cells, thus limiting the duration and level of transgene expression. Without adequate levels of antigen expression, the minimum threshold for immune activation may not be reached. In contrast, reduced cornification and keratinization, the lower rate of epithelial cell turnover as shown by low levels of Ki67 staining, and the presence of nucleated cells at the superficial surface are all consistent with increased levels and duration of transgene activity in fetuses injected at 65-70 dg as compared to fetuses injected at 120-125 dg. Another factor that may also influence the age-dependence of transfection efficiency is the total surface area of the oral cavity at the time of plasmid delivery. Between 65 and 120 dg, the surface area of the oral cavity increased by 240% (based on dissection of the oral mucosa into 1-cm^2 tissue sections). Studies have shown that increasing the volume of an injection of naked DNA plasmid relative to the surface area

(i.e., increasing the hydrostatic pressure at the contact surface) resulted in higher transfection efficiency (Hiraoka, Koike et al. 2003).

The induction of an immune response requires effective antigen presentation to naïve fetal lymphocytes. DNA vaccine studies in adult animals have shown that the basement membrane forms an effective barrier against non-specific transport of plasmid and protein between the mucosal epithelium and underlying submucosa (Loehr, Willson. et al. 2000; Lemoine, Farley et al. 2005); however, the effectiveness of the fetal basement membrane at preventing diffusion of plasmid or plasmid-encoded protein to the lamina propria has not been determined. According to Bodde et al. (1990), the mucosal epithelium does not support Langerhans cells; yet, immunohistochemistry revealed the presence of low numbers of MHC class II^+ cells with a dendritic morphology interspersed among epithelial cells in the stratum basale of the oral mucosal epithelium in third trimester fetuses (Fig. A.2). Large numbers of cells expressing MHC class II were also detected in the fetal lamina propria, which is consistent with reports that there is a high rate of lymphocyte recirculation through fetal peripheral tissues (Kimpton, Washington et al. 1995; Cahill, Kimpton et al. 1997). Despite the presence of MHC class II^+ cells in the fetal oral mucosa, the detection of functional luciferase activity in the retropharyngeal, mandibular, parotid and SCv LNs strongly suggests that protein (and possibly some plasmid) was well distributed throughout tissues of the head and neck and arrived at the draining LNs via the lymphatics (Fig 6.1). The use of plasmid isolation, followed by either PCR amplification or transformation of bacteria, did not result in detection of plasmid in draining LNs (data not shown). The development of germinal centers in LNs draining

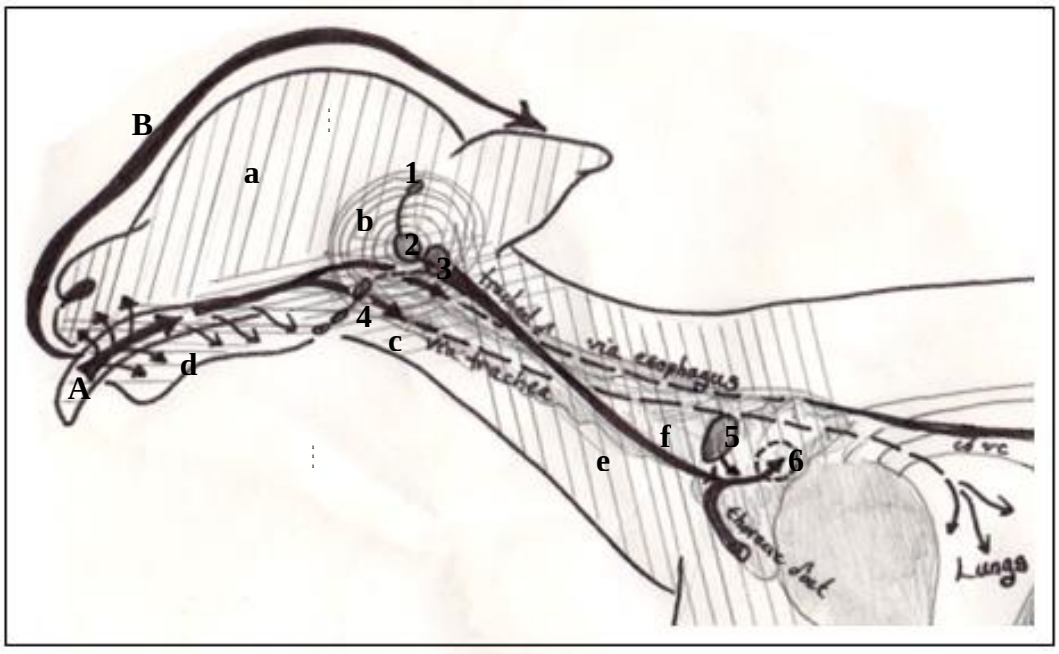


Figure 6.1. Flow of plasmid solution following oral injection and lymphatic drainage of the head and neck.

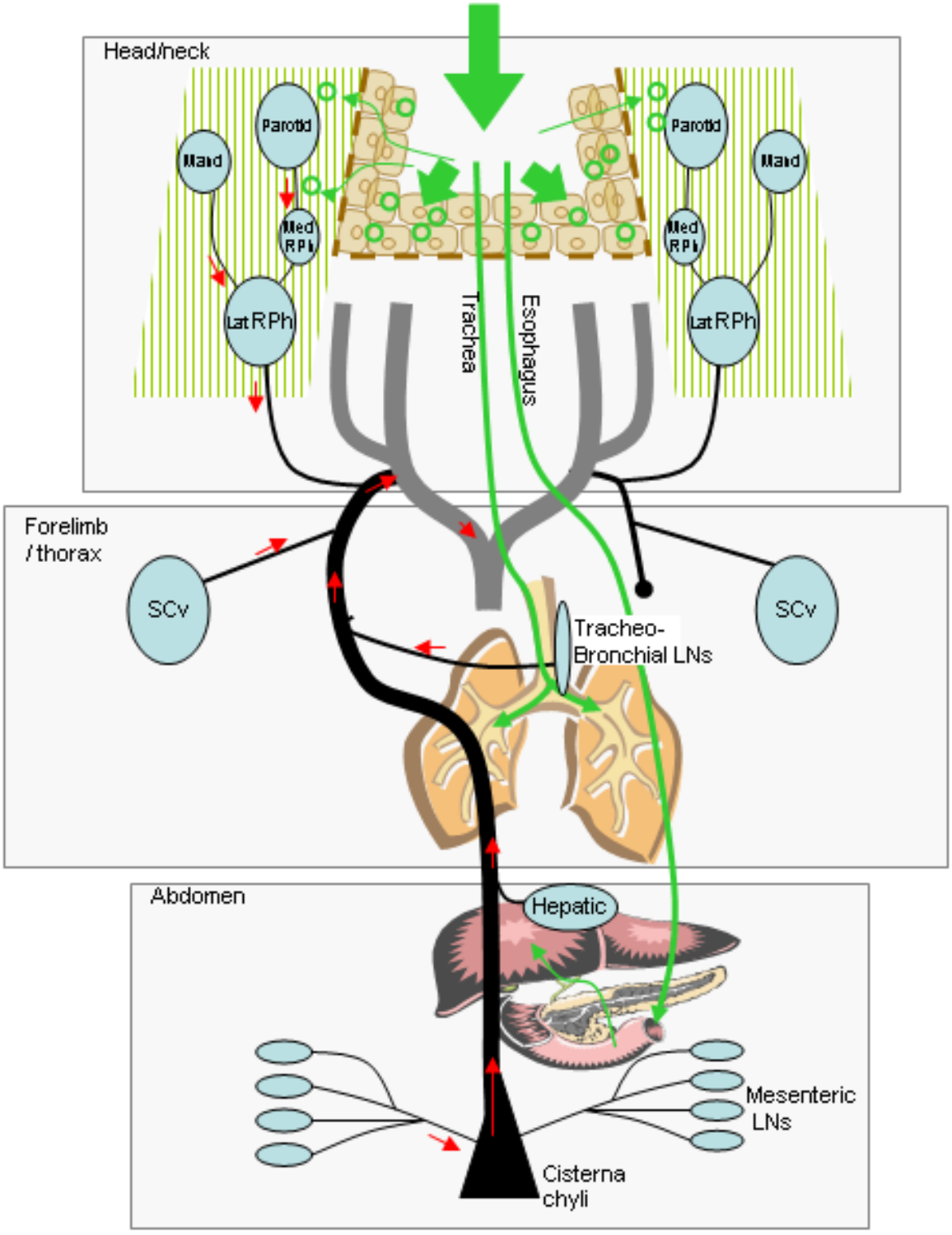
Plasmid solution injected into the oral cavity could permeate the mucosal epithelium, be swallowed, enter the respiratory tract or escape the oral cavity. Lymph from the head and anterior cervical region eventually collect at the lateral retropharyngeal (RPh) lymph nodes (LNs). Lymph from the pharynx, neck and anterior-most portions of the thorax collect at the superficial cervical (SCv) LNs. Both the SCv and RPh LNs drain into the jugular vein via the thoracic duct. **1**. Parotid LN; **2**. medial RPh LN; **3**.lateral RPh LN, **4**. mandibular LN, **5**. SCv LN; **6**. entry of the thoracic duct into the jugular vein; **a**. drainage area corresponding to the parotid LN; **b**. drainage area corresponding to the medial RPh LN; **c**. drainage area corresponding to the lateral RPh LN; **d**. drainage area corresponding to the mandibular LNs; **e**. drainage area corresponding to the SCvLN; **f**. arrow showing direction of lymph flow in the tracheal duct, i.e., from the head region to the thoracic duct; **A**. arrow indicating primary flow pattern of plasmid solution upon oral injection; **B**. arrow indicating flow of plasmid solution from the oral cavity to the external ear. (Sisson and Grossman 1953; Dyce, Sack et al. 2002).

the oral cavity does, however, support the conclusion that protein and/or plasmid was transported via the lymphatics. According to the literature, the lymphatic system becomes fully functional in the fetal lamb at 78 dg (Pearson, Simpson-Morgan et al. 1976). Our data suggest that portions of the lymphatic network, including those in the head and neck, are functional as early as 69 dg. The localization of plasmid-encoded protein to the SCv LNs may be a significant factor contributing to the success of *in utero* oral DNA immunization as these are the dominant LNs in the ovine fetus up to 140 dg (Bryden 1969; Washington, Kimpton et al. 1992). The significant increase in size, cellularity and organization of LNs in fetuses that responded to tgD-plasmid immunization compared with either control (untreated or null-plasmid-treated) or non-responding fetuses suggests that LN development may be driven by antigenic stimulation. Accelerated LN development may also increase the ability of the fetus to respond to other antigenic challenge.

In addition to the oral mucosa and draining LNs, luciferase activity was also detected in the liver and/or lungs in a total of 7 fetuses from 5 dpi, onwards. There are many potential pathways by which luciferase activity may have arrived in either the lungs or liver (Fig. 6.2). Fluid containing plasmid may have entered the trachea during or immediately after oral injection. Diffusion of plasmid within the respiratory tract may have resulted in low levels of respiratory epithelial cell transfection. Similarly, plasmid solution may have been swallowed, resulting in either transfection of epithelial cells lining the gastrointestinal tract or non-specific absorption of plasmid through the intestinal mucosa, and subsequent transport of plasmid and/or protein to the liver via the

Figure 6.2. Potential pathways for distribution of plasmid or transgene product following *in utero* oral DNA immunization.

Oral delivery of a naked DNA plasmid solution resulted in detection of transgene activity in the following tissues and organs: the oral mucosa; the retropharyngeal (RPh), parotid, mandibular (Man) LNs, which drain the head and neck; the superficial cervical LNs (SCv), which drain portions of the neck, anterior portions of the thorax and forelimbs; lung parenchyma; and liver parenchyma. The majority of transgene activity was located in the oral mucosa in the epithelium and/or lamina propria. The level of keratohyalin expression and the integrity of the basement membrane in the fetal oral epithelium are factors that likely determine the amount of plasmid and transgene product present in the lamina propria (depicted as green hatching). Drainage of lymph from the oral cavity and elsewhere in the head would result in transport of protein and/or plasmid to regional LNs. Lymph collected from the head and neck eventually enters the lateral RPh LNs, from where it enters the tracheal ducts. Lymph from the left tracheal duct joins the venous return via the thoracic duct, while the right tracheal duct enters directly into the right jugular vein. Diffusion of plasmid and/or transgene product to cervical tissues would result in drainage to the SCv LNs. Once plasmid and.or protein enters the blood, it is readily distributed elsewhere in the body, including the lungs and liver. Alteratively, plasmid may reach the lungs via the trachea. Either plasmid or protein (secreted by transfected cells in the oral epithelium) may be swallowed. Swallowed plasmid may transfect epithelial cells lining the gastrointestinal tract. Either plasmid or protein may enter the bloodstream as a result of non-specific intestinal absorption in the fetus. Examination of the mesenteric or tracheobronchial LNs for germinal center development might give some indication of the levels of transgenic protein present outside the oral cavity. (Sisson and Grossman 1953; Dyce, Sack et al. 2002).


Head/neck
Parotid
Parotid
Mand
Mand
Med RPh
Med RPh
Lat R Ph
Lat R Ph
Trachea
Esophagus
Forelimb
/ thorax
SCv
SCv
Tracheo-
Bronchial LNs
Abdomen
Hepatic
Mesenteric
LNs
Cisterna
chyli

(Fig. 6.2)

portal vein. Alternately, transgenic protein may have been secreted into the oral cavity and ingested, resulting in absorption through the gut. In Gerdts et al. (2000), oral delivery of tgD-plasmid at 120-125 dg failed to induce tgD-specific proliferative responses in the mesenteric LNs, suggesting that levels of plasmid/antigen that potentially reached the intestines were well below the threshold required to induce an immune response. The observation that luciferase activity in the lungs, liver and LNs reached detectable levels at approximately the same time, points towards the gradual accumulation of transgene product in the blood of orally-injected fetuses. Future experiments should include analysis of serum and gastrointestinal mucosa for the presence of the transgenic protein.

As discussed earlier, the presence of functional luciferase in LNs strongly suggests that antigen processing and presentation occurs in the LNs. The induction of a protective immune response in a fetal environment otherwise lacking in danger signals suggests that portions of the bacterial DNA backbone must also be transported to the LNs. Despite the possible introduction of plasmid and/or protein into the circulation, the lack of induction of tolerance in orally immunized fetuses indicates that neither plasmid nor functional protein was able to cross the blood-thymic barrier.

In fetuses that were injected at 120-125 dg, luciferase activity was still present in the oral mucosa at 11 dpi and luciferase levels were still rising. Due to the short half-life of luciferase in mammalian cells (3-4 hours; Leclerc, Boockfor et al. 2000) and the short half-life of DNA plasmid in amniotic fluid (2-4 hours) (Gerdts, Snider et al. 2002), it can be surmised that prolonged antigen presence was due to the continued expression of plasmid by transfected epithelial cells. The persistence of transfected cells and the

number of cells that are transfected are critical factors when determining the efficacy of *in utero* oral DNA immunization and the duration of both the primary immune response and immune memory. We hypothesize that the overall efficiency of oral delivery increases as the age of the fetus decreases. A number of factors may contribute to this age-dependence: (i) the younger fetus has a lower rate of epithelial cell turnover, thus resulting in the prolonged survival of transfected cells; (ii) there is an increase in the number of nucleated cells at the epithelial surface at younger gestational age, thus, increasing plasmid-to-cell contact; and (iii) the epithelium appears to be more permeable in the younger fetus allowing for transfection of cells closer to the basement membrane; (iv) greater permeability of the basement membrane may allow more protein and/or plasmid to enter the lamina propria; (v) the smaller oral cavity in the younger fetus translates into relative increases in both the plasmid concentration and injection volume: surface area ratio; and (vi) the faster rate of epithelial cell turnover in older animals result in more rapid sloughing of transfected cells. This inverse correlation between transfection efficiency and the age of the fetus at the time of oral injection is also consistent with failure to detect transgene activity following oral DNA delivery in the neonate.

Based on data obtained from transfection and expression studies, we hypothesized that oral DNA immunization could be extended to the second trimester as early as 50 to 70 dg, i.e., when the ovine fetus acquires immune competence. The emergence of immune competence coincides with the appearance of T and B cells in peripheral lymphoid tissues between 50-55 dg (Symons and Binns 1975; Press, Hein et al. 1993; Griebel 1998; Cahill, Kimpton et al. 1999) and Silverstein et al. (1963)

reported that antigen-specific antibodies were detectable in fetal serum as early as 66-70 dg following immunization with phage ϕX174 and horse spleen ferritin. We reasoned that the higher level of transgene expression and the persistence of transfected cells - which may or may not exceed, but should be equal to, the duration of transfected cells in the third trimester fetus - would compensate for lower numbers of lympocytes in the second trimester fetus. In particular, the continued delivery of antigen to the draining LNs, specifically the SCv LNs, would allow for the continued activation/stimulation of naïve fetal lymphocytes so long as transgene expression persisted, thus allowing for amplification of the primary immune response. Furthermore, as immune ontogeny progresses, the antigenic repertoire might expand to include additional epitopes represented on the immunizing antigen. The continued presence of antigen might also prolong the survival of memory cells, thus enhancing the potential for post-natal protective immunity.

Second trimester oral DNA immunization experiments were conducted with fetuses aged 55-60 dg and 78-84 dg (Chapter 5). A total of 24 fetuses were orally immunized with plasmid encoding BHV-1 tgD and a 66% success rate (16/24) was observed based on detectable tgD-specific antibody titers, IFNγ secretion responses and/or anamnestic responses in newborn lambs. IFNγ secretion is a hallmark of Th1-type immune responses and detection of IFNγ responses indicated that some Th1 activation did occur despite the reported impairment of Th1 responses in the fetus and newborn (Section 1.2.5). The 66% successful immunization rate was consistent with data obtained from transfection/expression studies showing high levels of transgene expression in 70% of orally injected fetuses (Section 5.4.1.). Therefore, it may be

possible to increase the efficiency of immune induction by further optimizing the rate of plasmid transfection and expression; however, differences in tgD-reactivity or the rate of immune development might also have influenced the ability of individual animals in an outbred population to respond to second trimester DNA immunization.

The earliest fetal age that was examined for tgD-specific immune responses was 69-74 dg (i.e., 14 dpi). In the single fetus that was examined at this age, both tgD-specific antibody responses and germinal centers were observed. This is consistent with the earliest time reported by Silverstein et al. (1963) for the detection of antigen-specific humoral responses. In the study by Silverstein et al., fetuses were immunized from 60 dg onwards, whereas in our study, fetuses were immunized as early as 55 dg (i.e., during the preimmune period). Due to the persistence of transgene expression following oral DNA immunization, it is entirely possible that a lag period (of no more than a few days) occurred between oral immunization and the first inductive event. In the event that such a lag period did occur, and despite having immunized during the preimmune period, it is significant to note that tolerance was not induced and this may be attributable to the immuno-stimulatory properties of the bacterial DNA backbone.

At 14 dpi, the amplitude of the tgD-specific antibody response was very low but statistically significant (Section 5.4.2). When the period between oral immunization and detection of immune responses was extended to 30 dpi and 50 dpi, there was a noted increase in the amplitude of primary antibody titers. We were unable to conduct a time course study to determine the time of peak fetal antibody titers due to the limited availability of fetuses and prohibitive costs; however, the observed increase in serum antibody titers between 14 dpi and 50 dpi supported our prior hypothesis that persistent

antigen might result in the continued induction of naïve lymphocytes, thus amplifying the primary immune response.

Immune induction in second trimester fetuses was also evident as histological changes within LNs draining the oral cavity. LNs develop from mesenchymal cell clusters arising from the lymphatics. In naïve 70 dg fetuses, the parotid, retropharyngeal and submandibular LNs were difficult to isolate due to their small size and lack of cellular organization. Oral DNA immunization appeared to enhance LN development. At 14 dpi (approximately 70 dg), there was clear demarcation between the cortex and medulla and medullary cords were visible. By 30 dpi (approximately 86 dg), primary follicles and germinal centers were clearly present in a number of immunized fetuses. In fetuses that were orally injected with the pSLIA-null control plasmid, LN enlargement and primary follicles were also observed, albeit to a lesser extent. In contrast, neither primary follicles nor germinal centers were detected in the retropharyngeal, SCv, parotid or submandibular lymph nodes of naïve fetuses examined up to 105 dg. Based on these observations, it would appear that induction of humoral responses does not require the prior development of primary follicles and stimulation with plasmid DNA drives LN development. Injection of circular plasmid containing CpG motifs has previously been reported to increase the recruitment of lymphocytes to a regional lymph node and GC development within that LN (Uwiera, Gerdts et al. 2001).

Despite the prolonged expression of the plasmid, the amplitude of fetal antibody titers induced by oral DNA immunization in the second trimester was relatively low compared with titers previously induced in third trimester fetuses (Gerdts, Babiuk et al. 2000). Oral immunization at 55-60 dg resulted in low titers (<135) in 15 of 16

responding fetuses. The only exception was fetus no. 54, which had a tgD-specific titer of 2400. Antibody titers in precolostrum sera of newborn lambs immunized at 67-72 and 78-84 dg, were similarly low (<150) with the exception of lamb no. 197 (titer = 333). Gerdts et al. (2002) had reported mean antibody titers of 5589 $\pm$ 3650 (Experiment I) and 1083 $\pm$ 352 (Experiment II) in precolostrum sera of lambs immunized at 125-130 dg. To determine if the disparity in antibody titers was physiological or the result of experimental artifact, sera from lambs immunized at 55-60 dg were reanalyzed and compared within the same ELISA to precolostrum sera obtained from two lambs in Experiment I of Gerdts et al.. These two reference sera had the highest gD-specific antibody titers of that experiment and had been stored at -70 °C with PMSF to inhibit protease activity. Our ELISA revealed that titers in serum from fetuses immunized at 55-60 dg remained unchanged, but antibody titers for the two reference sera were 561 and 597. We concluded that the 2- to 10-fold decrease in ELISA sensitivity was the result of the specific batch of recombinant tgD that had been used to coat ELISA plates. Although the sensitivity of our ELISA was much lower than that reported in Gerdts et al., our ELISA did have a higher specificity. At no time during our experimentation did tgD-specific titers in naïve or control-plasmid immunized fetal or newborn (precolostrum) sera exceed a titer of 20. In contrast, the mean background titers reported for Experiments I and II by Gerdts et al. were 112 $\pm$ 57 and 168 $\pm$ 143, respectively. Based on these data, the fetal responses observed in our experiments appeared to be real despite the low amplitude of observed titers.

While the low amplitude of titers in the *in utero* immunization studies may be due to the specific batch of recombinant tgD used for ELISAs, this could not explain the

small increase in antibody titers relative to background in 10 of 16 responders; in these 10 fetuses and newborn lambs, positive antibody titers did not exceed 50. In Chapter 5, we hypothesized that the low magnitude responses reflected the relative lymphopenia in the ovine fetus prior to 130 dg. We did not conduct FACs analysis of peripheral blood lymphocytes at 55-60 dg, however, the total number of lymphocytes[1] in the peripheral blood of naïve fetuses aged 83-88 dg was $1.8 \pm 0.5 \times 10^5$ cell/ml and there was no significant increase in the number of lymphocytes in the blood of immunized fetuses. At birth, i.e., when lambs were assessed for precolostrum antibody titers and persistence of fetal IFNγ responses, the total peripheral blood lymphocyte count was approximately $1.2 \pm 0.5 \times 10^6$ cells/ml. According to the literature the peripheral RLP undergoes significant expansion from 2-5 days of age onward, i.e. after parturition-associated cortisol levels have subsided (Taylor 2000). The low number of lymphocytes observed in the peripheral blood of second trimester fetuses might also explain the limited success in quantifying tgD-specific proliferative responses in cultures of peripheral blood mononuclear cells following oral DNA immunization (Fig. A.3). TgD-specific lymphocyte proliferation was detected in the peripheral blood of >50% of immunized fetuses at 30 and 50 dpi; however, the stimulation indices were well below that obtained in young lambs following immunization with tgD-plasmid or recombinant tgD (data not shown). Results obtained in FACS and antigen-specific lymphoproliferative assays are consistent with the low magnitude of tgD-specific antibody titers in responders.

The low frequency of tgD-specific lymphocytes observed in fetuses orally-immunized in the second trimester suggests that the expansion of the lymphocyte pool in the fetus may be tightly regulated by the same homeostatic mechanisms which function

[1] The total lymphocyte number was determined from CD5+ and CD72+ cells using FACS.

in the adult to prevent uncontrolled lymphocyte accumulation and increase the diversity of the immune repertoire (Freitas, Agenes et al. 1996). If this is the case, the majority of lymphocyte expansion in the fetus is antigen-independent and the selective amplification of a single or limited number of antigen-specific clones in an immunologically naïve animal, such as the fetal lamb, should not influence the ability of the immune system to respond to other antigens. Experiments in mice have shown that even in the event of severe lymphopenia, the percentage of T cells with a monoclonal specificity is restricted to 10% of the total number of T cells in the RLP (Rocha, Dautigny et al. 1989).

Despite the low amplitude of tgD-specific antibody titers, titers persisted for a relatively long period (i.e., up to 11.5 weeks, from the time of immunization at 67-72 dg until birth). Similarly, tgD-specific IFNγ responses were still detectable at 10-11 weeks post-immunization. Therefore, the duration of antigen-specific antibody and cellular responses were comparable to that reported for a primary immune response achieved after optimized delivery of a DNA vaccine to young lambs (Section 3.3.2). In Chapter 3, different routes and methods of DNA vaccine delivery were compared and the duration of primary antibody responses were found to be enhanced by (i) delivery of plasmid to muscle, which has a lower rate of turnover than epithelial cells in the young lamb, and (ii) the use of electroporation, which increases transfection efficiency. Intramuscular delivery of a high dose of tgD-plasmid, followed by electroporation, induced primary antibody responses that persisted for an average of 11 weeks in young lambs. In contrast, IM injection of tgD-plasmid alone resulted in primary antibody responses that persisted for an average of 6 weeks only. The mechanism(s) behind the long duration of fetal immune responses was not examined. It is known that the ovine fetus supports a

population of long-lived CD4+ thymic emigrants with a half-life of approximately 20 days that preferentially target the peripheral LNs (Cahill, Kimpton et al. 1999) and these CD4+ T cells may have a similarly long-lived phenotype when activated. Currently, little is known about the characteristics of activated fetal lymphocytes. The prolonged duration of primary fetal responses might also result from the simultaneous turnover of activated lymphocytes and induction of naïve lymphocytes, with both occurring at a similar rate. In this scenario, primary immune responses would persist for the duration that antigen is present in quantities exceeding the threshold for activation of naïve lymphocytes. This mechanism would be more consistent with findings from our electroporation studies in young lambs. The persistence of antigen as a property of *in utero* oral DNA immunization was discussed above. Fetal immunization studies with antigen delivered in different forms (e.g., as both a subunit protein and DNA vaccine) or as an inducible gene may allow determination of the mechanism(s) behind the longevity of the primary fetal immune response.

Of the 16 (of 24) fetuses that did respond to second trimester immunization, only two fetuses had detectable levels of both IFNγ-secretion and antibody responses. Thus, the possibility existed of having induced either tolerance in the apparent non-responders or deviation towards Th2-type responses in some responders. Secondary DNA immunization in the neonate was used to test for the possible induction of tolerance or immune deviation. In addition, secondary immunization allowed testing for the survival of immune memory.

The survival of immune memory is the keystone of an effective vaccine and this is especially true of a fetal vaccine where there is a lack of natural boosting by cross-

reactive environmental antigens from the time of immunization to birth. We had originally hypothesized that immune memory induced in the second trimester fetus would be capable of surviving until birth based on continued antigen expression by transfected host cells. Indeed, anamnestic responses were present in 2 of 4 neonatal lambs immunized at 67-72 dg, and 3 of 6 neonatal lambs immunized at 78-84 dg. Therefore, immune memory persisted throughout the remaining 11-12 weeks of fetal development, plus an additional 2 weeks after birth. The classical characteristics of an anamnestic response are earlier onset, increased amplitude and prolonged duration of effector functions. In lambs that had been immunized at 67-72 dg and boosted by IM injection plus electroporation, immune memory was present in the form of an earlier onset of a detectable increase in antibody titers, as well as a 2-fold increase in peak antibody titers following neonatal DNA immunization relative to controls. In lambs that had been immunized at 78-84 dg and boosted with IM injection of tgD-plasmid without electroporation, immune memory was evident in the earlier onset and prolonged duration of secondary antibody titers, as well as an increase in the magnitude of peak antibody titers.

The increase in magnitude of anamnestic responses was not dramatic and, overall, the induction of immune memory by oral immunization in the second trimester did not appear to provide a significant immune advantage to the neonate given the risks of the *in utero* surgical procedure. This contrasted with the finding by Gerdts et al. (2002), that third trimester oral DNA immunization was able to induce memory cells that significantly enhanced neonatal immune responsiveness to tgD. The low amplitude of secondary antibody titers may have been due to a combination of the low sensitivity

of the antibody detection method as a result of the specific preparation of recombinant tgD used to coat ELISA plates and the inherent reduction in the amplitude of antibody responses characteristic of young lambs less than 21 days of age (Section 4.4.5). We also propose that the number of memory cells generated *in utero* in the second trimester was restricted by the size of the peripheral lymphocyte pool and limited immune repertoire. Therefore, despite the ability of activated fetal effector and memory cells to survive gestation and the cortisol-induced apoptosis that accompanies birth, memory cells may not be present in numbers sufficient to enhance neonatal immune protection.

The duration of immune memory achieved by *in utero* oral DNA immunization was surprising given the low amplitude of fetal immune responses. Furthermore, the duration of fetal immune memory was comparable to the duration of immune memory reported for neonatal lambs following a single ID immunization with tgD-plasmid (van Drunen Littel-van den Hurk, Braun et al. 1998). This similarity in duration of immune memory was observed despite differences in lymphocyte numbers and exposure of memory cells in the neonate, but not the fetus, to environmental antigens. In Chapter 3, we found that the correlation between the duration of a primary immune response and immune memory ($r^2 = 0.74$) was greater than that between the amplitude of the primary response and immune memory ($r^2 = 0.40$). Due to practical limitations, e.g., the availability of fetuses and high cost of experimentation, the duration and peak magnitude of fetal immune responses were never properly explored. Of the 5 lambs with evidence of immune memory at birth, three lambs (nos. 253, 184 and 197) also had detectable fetal immune responses at birth. This observation agrees with our previous conclusion

that a correlation exists between duration of the primary immune response and immune memory.

With respect to the primary immune response, the magnitude of the response appears to be largely a function of the number of activated lymphocytes, whereas duration appears to be heavily influenced by the persistence of antigen. The duration of immune memory, particularly that mediated by CD4+ T cells, may also depend on the continued presence of antigen (Gray and Skarvall 1988). To properly determine if the duration of primary immune responses and immune memory in the fetal lamb was related to the prolonged expression of antigen by transfected host cells, it will be necessary to compare immune responses in fetuses that have been immunized with tgD-plasmid versus soluble, recombinant tgD protein. The lengthy duration of both the primary immune response and immune memory in the fetus may also support the existence of a subpopulation of long-lived fetal lymphocytes.

In conclusion, this thesis showed that the second trimester fetal lamb is immune competent and oral DNA immunization can be successfully carried out as early as 55-60 dg, which coincides with the appearance of lymphocytes in peripheral lymphoid tissues and is a period that has previously been described as “preimmune”. Furthermore, the level of oral DNA transfection increased as the age of the fetus decreased at the time of immunization. The analysis of luciferase expression within 72 hours of oral plasmid injection revealed transgene expression in 100% of fetuses; however, transfection did not necessarily result in the effective induction of fetal immune responses. Low levels of transgene expression may account for failure of immunization in up to 30% of fetuses. In the 16 of 24 (66%) fetuses that did respond to oral DNA immunization in the second

trimester between 55 and 84 dg, only 2 fetuses (8.3%) had primary immune responses of sufficient amplitude to possibly provide immune protection in the event of an infection and only 1 of 6 fetuses (16%) had clear evidence of both humoral and cell-mediated immune memory. In the remaining responders, the amplitude of immune responses was limited. These data contrast with results reported by Gerdts et al. (2000; 2002) that *in utero* immunization induced significant primary immune responses and secondary immune responses were sufficient to afford protection to the neonate against viral challenge.

We propose that the major limitation to second trimester oral DNA immunization is the low number of lymphocytes present at this time. According to the literature, the fetal immune system undergoes two major expansions in the size of the lymphocyte pool. The first expansion occurs at approximately 100-110 dg with emigration of B cells from the spleen to the ileal PPs and is moderate (Cahill, Kimpton et al. 1999; Yasuda, Jenne et al. 2006). The second expansion occurs between 130 dg and birth when the ileal PPs undergo massive lymphopoiesis in order to fully populate the peripheral lymphoid tissues (Reynolds 1987). The high magnitude of antibody titers and lymphoproliferative responses reported in Gerdts et al. (2000; 2002), may be the result of having immunized at or immediately prior to expansion of the peripheral lymphocyte pool in the fetus. When these data are considered in relation to the data and conclusions arrived at in Chapter 3, there appears to be an optimal time for *in utero* oral DNA immunization that occurs around 130 dg (Fig. 6.3). This period is defined as the interval following major expansion of the fetal lymphocyte pool, i.e., after 110 dg when the ileal PPs becomes the

primary site of lymphopoeisis, but prior to maturation of the mucosal epithelium and the onset of parturition-associated cortisolemia.

The work in this thesis ties together much of the existing research in the fields of vaccine development, immune modulation, developmental immunity and neonatal infection. Major factors influencing the needle-free or oral delivery of DNA vaccines were identified. We also confirmed the ability of DNA vaccination to overcome the developmental tendency towards tolerance induction and deviation towards Th2 immune responses. These findings have a practical significance given the low cost of DNA vaccine production and its ready adaptability to different pathogens, including emerging diseases. A new lower age limit for fetal immunization was defined in this thesis that challenges existing theories regarding fetal immune competence and mechanisms of developmental tolerance. As an experimental model, second trimester oral DNA immunization in the ovine fetus may be invaluable for testing theories such as the Danger Hypothesis and the antigen-dependence of immune memory.

Of the animal species that are currently used for research purposes, immune ontogeny in the sheep is the most similar to that in humans and the findings in this thesis suggest that it may be possible to vaccine the fetus against vertically transmitted pathogens, such as HIV. Unfortunately, despite the fact that second trimester oral DNA immunization appeared to have no negative physiological effects on the fetus, our research also suggests that the inherent low amplitude of fetal immune responses in the majority of an out bred population, weighed against the risks associated with fetal surgery, current;y precludes the clinical application of this technology in protecting against vertically transmitted diseases. With ongoing advances in surgical techniques,

such as incorporation of advanced imaging and development of new endoscopic tools, it may be possible to limit surgical risks to a degree that vaccination in the second trimester would be considered despite its success rate of between 8 to 67%.

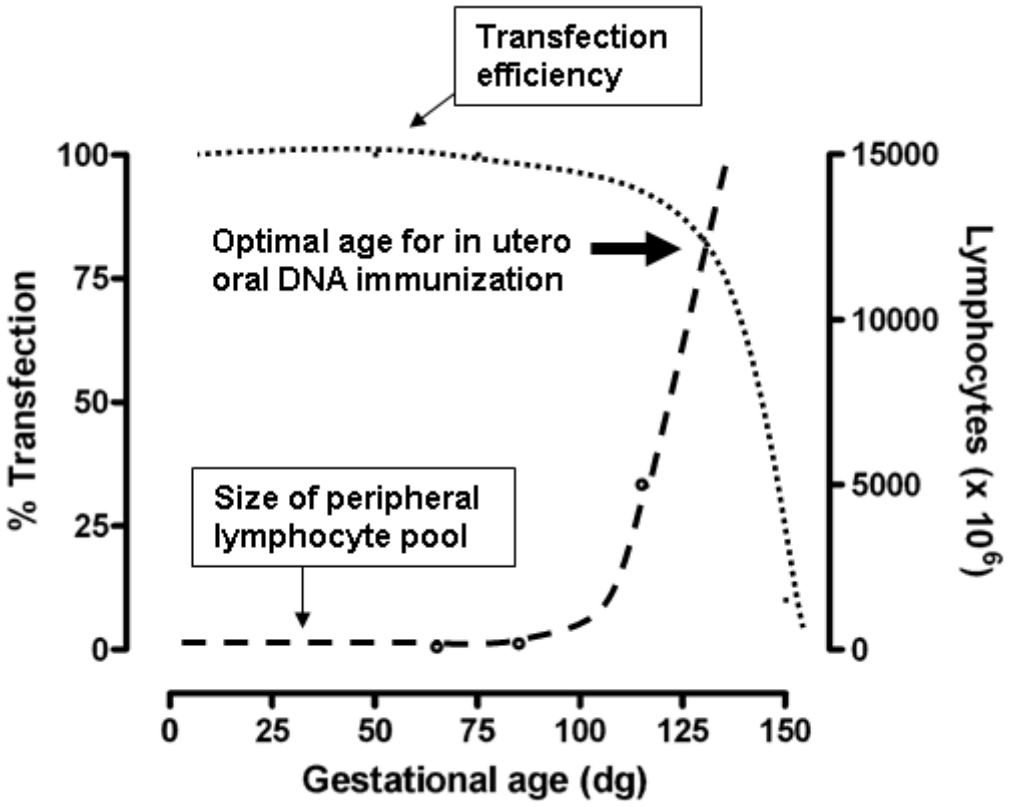


Figure 6.3. Factors influencing the optimal age for *in utero* oral DNA immunization

The efficiency of plasmid transfection and expression may be inversely correlated to the age of the fetus and the degree of maturation of the mucosal epithelium. Also, the size of peripheral lymphocyte pool is severely limited in the second trimester ovine fetus and major expansion does not occur until approximately 100-125 days gestation. Factoring in the rise in cortisol-induced lymphocyte apoptosis that accompanies birth, the optimal age for *in utero* oral DNA immunization is proposed to occur between approximately 125-135 days gestation.

APPENDIX A

ADDITIONAL FIGURES NOT INCLUDED IN MANUSCRIPTS

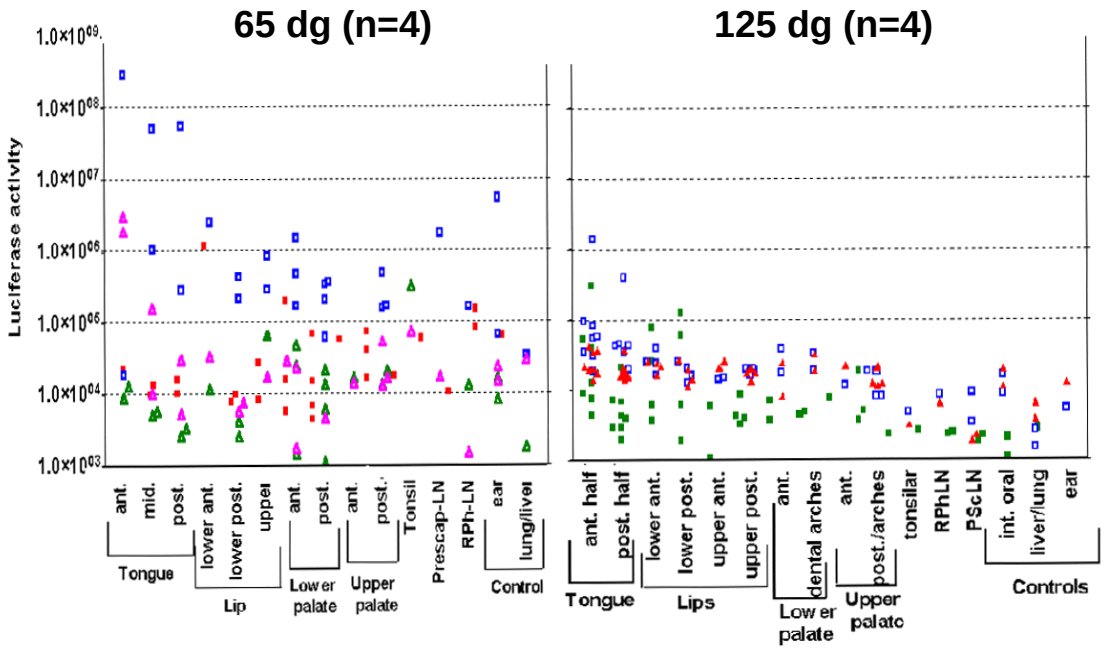


Figure A.1. Comparison of transgene expression following oral injection of luciferase plasmid in second and third trimester ovine fetuses.

Higher levels of luciferase activity were achieved in tissues from fetuses that had been treated at 65 dg. Tissue samples were collected 2 days after 1 mg of luciferase-plasmid was delivered to the oral cavity of each fetus. Each data point represents the level of luciferase activity in a single 1-cm^2 section of mucosal epithelium collected from the oral cavity at 48 hours post-injection. Levels of luciferase expression was also higher in 65 dg fetuses (n=3) when samples were analyzed at 3 and 5 days after oral delivery of luciferase plasmid. (Refer to Sections 4.3 and 5.3 for Material and Methods.)

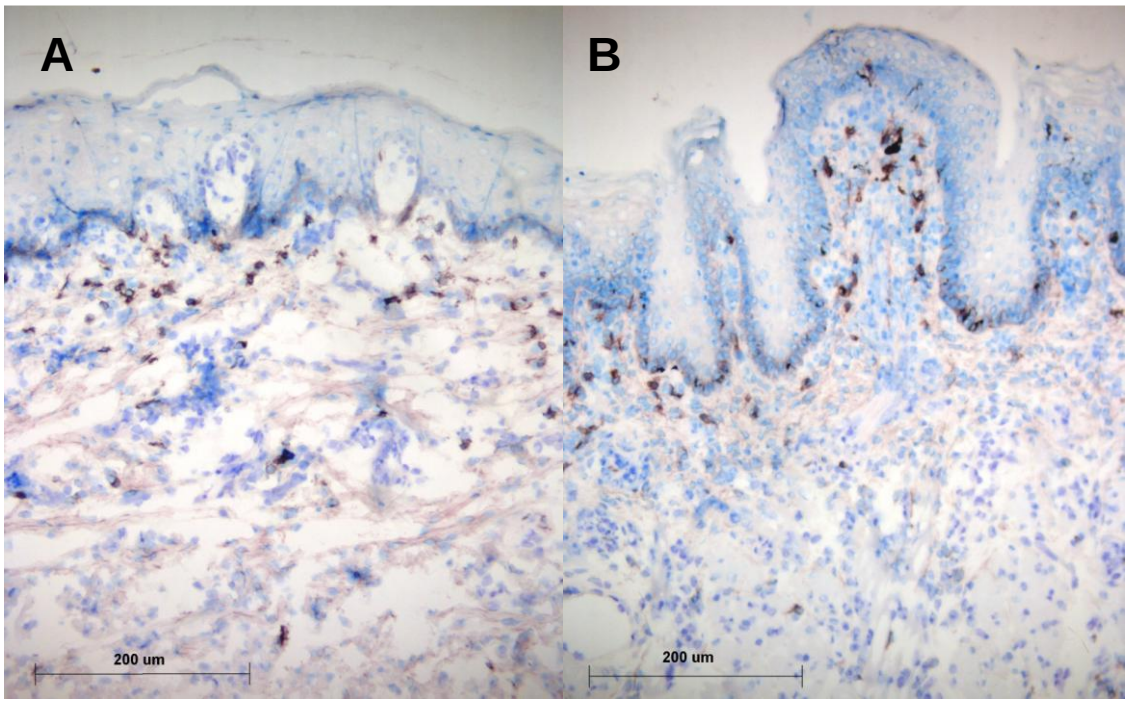


Figure A.2. Immunohistochemical staining for MHC class II in oral mucosa from a 120 dg ovine fetus.

MHC class II is a marker of T and B lymphocytes and professional antigen-presenting cells. Their presence in the basal layer of the mucosal epithelium and oral submucosa provides a mechanism by which antigen delivered to the oral cavity can be sampled and transported to the draining lymph nodes. Tissues collected from the upper lip (**A**) and dorso-rostral surface of the tongue (**B**) of 120 dg fetuses were stained with monoclonal antibody for MHC class II. Frozen tissues were cut into 6 μm sections, mounted onto Colorfrost Plus Slides (Fischer Scientific, Pittsburgh, PA) and fixed with acetone. Tissue sections were blocked in three stages: tissues were first blocked with 0.1 mg/ml goat serum in PBS, followed by Avidin B Blocking Solution (Vector Laboratories, Burlingame, CA) and then with Biotin Blocking Solution (Vector Laboratories). Tissue sections were incubated with TH14B monoclonal antibody, specific for bovine MHC class II (HLA-DRα) (1:1000 dilution; VMRD, Inc., Pullman, WA), and then with biotinylated goat-anti-mouse IgG2a (1:1000 dilution; Caltag/Invitrogen, Burlington, ON). Antibody labeling was visualized using the Vector Elite Vectastain® ABC kit (Vector Laboratories). Slides were counterstained in 0.5M $CuSO_4$ in 0.9% NaCl, followed by a 5% Giemsa solution.

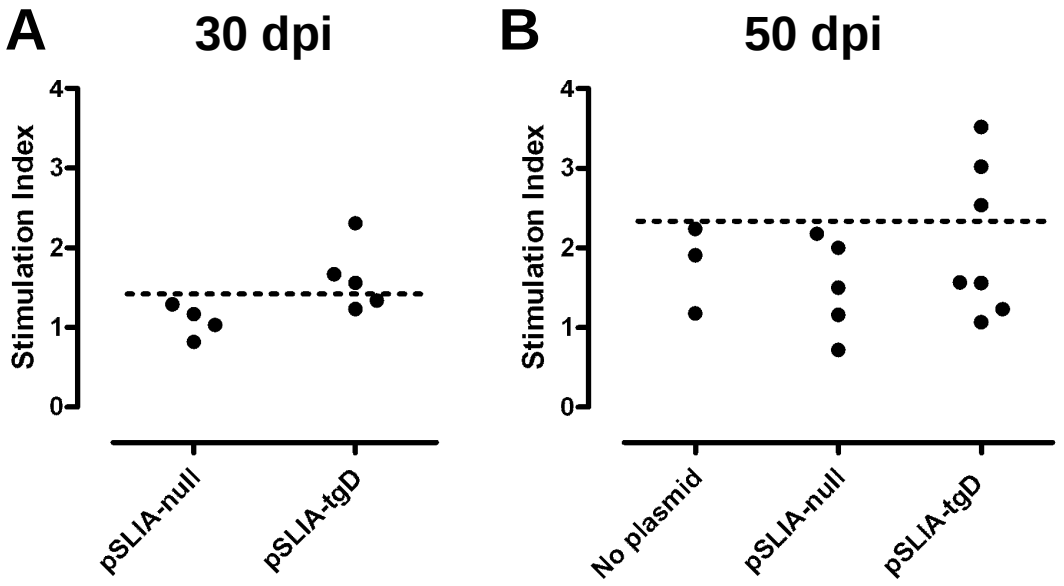


Figure A.3. TgD-specific peripheral blood lymphoproliferative responses at 30 and 50 days following *in utero* oral DNA immunization.

Oral DNA immunization with tgD-plasmid at 55-60 dg resulted in the induction of tgD-specific lymphoproliferative responses in a total of 6 of 12 (50%) fetuses. In all fetuses, the stimulation index was low relative to control fetuses.
Fetuses were immunized between 55-60 dg with control plasmid (pSLIA-null) or tgD-plasmid and peripheral blood mononuclear cells were isolated at 30 dpi (**A**) and 50 dpi (**B**). Triplicate cultures of 1 x 10^6 cells were incubated for a period of 6 days in media alone or media containing 5 μg/ml recombinant tgD. ^{3}H-thymidine was added to media for the last 16 hours of culture. The stimulation index was calculated as the mean counts per minute (cpm) of tgD-stimulated cells divided by the mean cpm of cells in media alone. The dotted line represents the upper 95% confidence interval for the group treated with control plasmid. Using the upper 95% CI as the threshold for antigen-specific proliferative responses, tgD-specific proliferative responses were observed in 50% of fetuses ($p<0.5$); however, this conclusion was deemed questionable based on experiences with tgD-immunization experiments in adult sheep in which stimulation indices were at least 10-fold greater. The low stimulation indices are likely a reflection of the low number of peripheral blood lymphocytes present in the second trimester ovine fetus.

APPENDIX B

LIST OF MANUSCRIPTS AND ABSTRACTS

Manuscripts

Tsang C, Kaushik RS, Mirakhur KK, Gerdts V, Babiuk LA, Griebel PJ. 2007. Transfection and expression following oral delivery of naked DNA plasmid in the ovine fetus. Dev Comp Immunol: (*Accepted with revisions*).[1]

Tsang C, Mirakhur KK, Babiuk LA, Griebel PJ. 2007. Oral DNA immunization in the second trimester fetal lamb and secondary immune responses in the neonate. Vaccine **25**: 8469-8479.[2]

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Gerdts V, Tsang C, Griebel PJ, Babiuk LA. 2004. DNA vaccination *in utero*: a new approach to induce protective immunity in the newborn. Vaccine **22**: 1717-1727.

Mutwiri G, Pontarollo R, Babiuk S, Griebel P, van Drunen Littel-van den Hurk S, Mena A, Tsang C, et al.. 2003. Biological activity of immunostimulatory CpG DNA motifs in domestic animals. Vet Immunol Immunopathol **91**: 89-103.

[1] Chapter 4.
[2] Chapter 5.
[3] Chapter 3.
[4] Shared first authorship.

Conference Proceedings/Abstracts:

Tsang C, Mirakhur K, Babiuk LA, Griebel PJ. *In utero* oral DNA immunization in the 2nd trimester ovine fetus: Induction of fetal immune responses. ImmunNet Retreat (U. of Alberta). June 2005. Lilylake Resort, AB, Canada.

Tsang C, Gerdts V, Babiuk LA, Griebel PJ. *In utero* oral DNA immunization during the second trimester: Induction of responses in pre-immune lambs. Keystone Symposia: Mechanisms of Immunological Tolerance and its Breakdown. Jan 2003. Snowbird, UT, USA.[1]

[1] Awarded Keystone Symposia Scholarship, 2003.

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