CHARACTERISATION OF ANTIBODY RESPONSES TO HERPESVIRUS VACCINES

THE CHARACTERISATION OF ANTIBODY RESPONSES TO DIFFERENT HERPESVIRUS VACCINE VECTOR STRATEGIES

By

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ABSTRACT

Herpes simplex virus is man's oldest viral enemy. Infections result in symptoms ranging from mild skin lesions to deadly herpes simplex encephalitis, making HSV one of the most costly of viral diseases to treat. Thus the development of a vaccine is imperative. To this end, several vaccine strategies have been utilized to generate immunity to HSV in rodents. These include the use of recombinant DNA, recombinant adenoviruses, and dendritic cells transduced with either of the former and re-introduced to the host to induce immunity. In this study, different aspects of these vaccine types were examined. Antibody and cytotoxic T-cell (CTL) responses to a DNA vaccine encoding gB of HSV-1 (gB-DNA) were evaluated. This resulted in variable long lived antibody responses to a wide range of dosages and CTL responses which followed dose-response relationships. An adenovirus expressing gB of HSV-1 (AdgB) which is able to generate IgA responses (Gallichan *et al.*, 1993) was utilized to determine the best method of mucosal administration to optimize these responses. It was suggested in a previous report that nasal associated lymphoid tissue (NALT) was the desired target for inducing IgA responses (Heritage *et al.*, 1997). Accordingly, the hypothesis was formulated that NALT can produce IgA responses similar to those produced from a combination of inductive sites. To test this hypothesis, mice were immunized either awake or asleep with AdgB assuming that awake delivery restricts induction to the NALT, whereas asleep

administration disseminates AdgB throughout the respiratory system. The results demonstrate participation of lower airways in the induction of immunity is desirable for generating IgA responses. Lastly, dendritic cells transduced with AdgB were assessed for their ability to generate systemic and mucosal antibody responses, resulting in the inability to generate IgA, but the ability to generate systemic antibody responses.

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ATP	adenosine tri-phosphate
ADCC	antibody-dependent cell-mediated cytotoxicity
(ab')	antibody fragment
AFC	antibody forming cell
APCs	antigen presenting cells
ASN	asparagine
bp	base pair
B-gal	beta-galactosidase
b2M	beta-two-microglobulin
BM	bone marrow
BALT	bronchial associated lymphoid tissue
CAV-2	canine adenovirus type-2
CSF	cerebral spinal fluid
CD8+ CTL	cluster of differentiation marker 8 positive cytotoxic T-cell
CMV	cytomegalovirus
Tc cells	cytotoxic T-cells
pCTL	cytotoxic T-cell precursor frequency
CTL	cytotoxic T lymphocyte
DTH	delayed type hypersensitivity
DC	dendritic cells
DCs	dendritic cells
DC-INL	dendritic cells administered intranasally
DC-IV	dendritic cells administered intravenously
DNA	deoxyribonucleic acid
DMSO	dimethlysulfoxide
ddH_20	distilled de-ionized water
ER	endoplasmic reticulum
E. coli	*Escherichia coli* sp.
FCS	fetal calf serum
gB	glycoprotein B
gD	glycoprotein D
GPI	glycosyl phosphatidylinositol
g	grams
GM-CSF	granulocyte macrophage colony stimulating factor

HSP	heat shock protein
Th	helper T-cell
ThLp	helper T-cell precursor frequency
HbsAg	hepatitis B surface antigen
HSE	herpes simplex encephalitis
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
h	hours
HIV-1	human immuno-deficiency virus type 1
ICP 27	immediate early cell protein 27
ISS	immuno-stimulatory sequences
IFNg	interferon gamma
ID	intra-dermal
IP	intra-peritoneal
IT	intra-tracheal
IM	intramuscular
INL	intranasal
IV	intravenous
kb	kilobase
LC	Langerhan cells
LAT	latency associated transcripts
LPS	lipo-polysaccharide
l	litre
LB	Luria-Bertani medium
LN	lymph node
LCMV	lymphocytic choriomeningitis virus
$H\text{-}2^b$	major histocompatibility complex haplotype 2 b
MC57	methylcholranthrene transformed C57BL/6 fibroblasts
µg	micrograms
µl	microlitre
µM	micromolar
mg	miligrams
ml	mililitre
mM	milimolar
min	minutes
M	molar
mAb	monoclonal antibody
MOI	multiplicity of infection
HEPES	N-2-Hyeroxyethylpiperazine-N-2-Ethane sulfonic acid

ng	nanograms
nm	nanometre
nM	nanomolar
NALT	nasal associated lymphoid tissue
NGF	neural growth factor
OD	optical density
OVA	ovalbumin
p-IgA	polymeric immunoglobulin "A" receptor
pIgR	polymeric immunoglobulin receptor
PBS	phosphate buffered saline
AdgB	recombinant adenovirus containing glycoprotein B of HSV
r-IL-4	recombinant interleukin 4
RNA	ribonucleic acid
RSV	Rouse sarcoma virus
S-C	secretory component
SIV	Simian immuno-deficiency virus
SVBalb	Simian virus 40 transformed Balb/c fibroblasts
SC	sub-cutaneous
TAP	transporters associated with antigen presentation
TBS	tris-buffered saline
TBE	tris, boric acid and edta buffer
VSV	vesicular stomatitis virus

I. INTRODUCTION

1.1. Herpes Simplex Virus

1.1.1. General Introduction

> "O'er ladies' lips, who straight on kisses dream. Which oft the angry Mab with blisters plagues"

Although Shakespeare's Romeo and Juliet gives us a clue as to the history of Herpes Simplex Virus (HSV) infections, the story of herpes virus predates his days'. In fact, herpes simplex virus infections can be traced to the beginnings of recorded history (Nahmias and Dowdle, 1968). Descriptions of herpes infections can be found by physicians in the times of Ancient Greece, in the writings of Hippocrates. Genetically, modern techniques estimate the age of the herpesvirus to be more than 2-3 million years. The first histopathological studies were done by Unna in 1896, but for modern man, the infectious nature of herpesvirus was not elucidated until the early 1920's by Gruter (1920) and by Lowenstein (1919). The immunobiology of herpes was revealed by Andrews and Carmichael (1930), who demonstrated neutralizing antibodies in patients with recurring lesions. Although first postulated by Lipschitz (1921), and later by Plummer *et al.* (1968) it was not until the late 1960's that it was demonstrated that two distinct serotypes of herpes existed; herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2

(HSV-2), collectively referred to as herpes simplex viruses (HSV) (Nahmias and Dowdle, 1968). HSV-1 is primarily associated with oral lesions, and HSV-2 with genital lesions, but the viruses are also responsible for more serious diseases such as keratoconjuctivitis, neonatal herpes, meningo-encephalitis, and devastating disseminated herpes virus infections.

1.1.2. Biology and pathology of Herpesviruses

1.1.2.1. Biology

There is significant homology between HSV-1 and HSV-2, with more than half of their DNA sequences conserved, leaving restriction enzyme digestion as the only reliable method of determining the actual viral type (Roizman and Furlong, 1974). HSV are composed of double stranded positive sense DNA molecules encoding for more than 60 gene products (Roizman, 1979). There are three classes of HSV genes, named according to the time they are expressed; class I - alpha genes (early genes), which have no requirement for viral, or viral DNA synthesis; class II - beta genes (intermediate genes), which are required for the synthesis of alpha proteins but not replication of viral DNA; and finally gamma genes (late genes), which encode the structural proteins (Roizman and Furlong, 1974). The double stranded viral DNA of HSV is wrapped around a protein scaffold by a multisubunit assembly moiety in an adenosine tri-phosphate (ATP) dependent manner (Healy *et al.*, 1997). This protein scaffold-DNA complex interacts

with a major capsid protein and a maturational protease to form a partial capsid, then forms an unstable capsid intermediate (procapsid) and finally a closed icosahedral capsid found in the mature virion (Newcomb *et al.*, 1996). The capsid contains 162 capsomers and is approximately 15 nm thick and 125 nm in diameter (Newcomb *et al.*, 1996). The virus then buds through the nuclear membrane and picks up an envelope, which contains several glycoproteins which are essential for the infectivity and structural stability of the virion (Browne *et al.*, 1996). The respective roles of the glycoproteins in the envelope of HSV vary greatly, from viral entry, cell to cell spread and tissue tropism to immune evasion. There have been 12 viral glycoproteins identified to date, however as there are many precursor forms there may be many more as yet unidentified.

The HSV-1 and HSV-2 glycoproteins share several antigenic and structural similarities. They range in molecular weight from 59, 000 (gD) to 120, 000 (gB). Similarities include the conservation of signal sequences involved in membrane anchoring (McGeoch, 1985), ASN (asparagine) residues which have n and o-linked oligosaccharides (Johnson and Spear, 1983) and the incorporation of inorganic sulfates (Hope and Marsden, 1983; Hope *et al.*, 1982). Oligosaccharides play a role in complement binding via gC (Eisenberg *et al.*, 1987; Hung *et al.*, 1992), and are implicated in antigen masking (Olofsson *et al.*, 1991). The glycoproteins are thought to be members of the glycosyl phosphatidylinositol (GPI) anchored protein family, which contain an upstream peptide required for direct glycolipid addition to the COOH-terminal

region (Beghdadi-Rais *et al.*, 1993). This feature is associated with the biology of the neurotropic alphaherpesviruses (Edson, 1993). One dissimilarity between glycoproteins involves the presence of fatty acid acylation on gE-1 (Johnson and Spear, 1983), which may be involved with modifying the hydrophobic properties of the virus (Schmidt and Burns, 1991).

1.1.2.2. Pathology

HSV can cause a variety of diseases, the most severe and debilitating of which is herpes encephalitis (HSE), caused by HSV travelling up the trigeminal ganglia to the brain (Whitley, 1991). Afflicted individuals may present fever, altered consciousness, bizarre behaviour, disordered mentation, localized neurological findings and even coma (Whitley, 1985; Whitley *et al.*, 1989). Fatality from HSE can be as high as 70%, however use of diagnostic PCR (Lakeman and Whitley, 1995; Rowley *et al.*, 1990) and treatment with acyclovir has reduced mortality to less than 20% (Whitley, 1993; Whitley and Gnann, 1992). HSV infection of infants is referred to as neonatal herpes, which may manifest as benign or severe skin lesions involving the eyes, skin or mouth or as a deadly disseminated infection affecting the liver, central nervous system (CNS), adrenals and brain, with fatality often reaching 80% (Whitley, 1991). The prophylactic treatment of mothers infected with genital herpes with acyclovir near time of delivery has reduced incidence and mortality (Whitley, 1991).

The general course of HSV infection following direct infection of the mucosa or abraded skin often results in lesions at the infection site. Ultimately, sensory or autonomic nerve endings are infected and the virus is transported up the axons to the cell bodies in the ganglia (Hill, 1985), from where it can spread to the skin via peripheral sensory nerves causing infections at distant sites (Baines and Roizman, 1991). This is a quick phenomena. In mice, HSV can be recovered from nervous ganglia two days following vaginal or dermal inoculation (Geiger *et al.*, 1979; Klein *et al.*, 1979; Stanberry *et al.*, 1983). Once the virus reaches the sensory ganglia, it can lapse into a lifelong latent, or quiescent state with frequent active periods (Fraser *et al.*, 1992; Stevens, 1989).

1.1.3. Latency of HSV

The ability of herpesvirus to establish latency was suspected by Cushing in the 1900's (Cushing, 1905). It was demonstrated that once in the nervous ganglia HSV undergoes structural changes and is maintained in an episomal or concatemeric like state (Efstathiou *et al.*, 1986; Mellerick and Fraser, 1987; Puga *et al.*, 1984; Rock and Fraser, 1983). Once in this state, the 75 genes which are normally active in a normal HSV infection are suppressed (Fawl and Roizman, 1994; Stevens, 1989). The role of latency-associated transcripts (LAT) which remain active is not clear, but they are thought to reduce viral gene expression (Garber *et al.*, 1997). Regardless of the exact molecular mechanisms of latency, reactivation of HSV can result from many factors, ranging from psychological to

host immune status, in humans (Biondi and Zannino, 1997; Oakley *et al.*, 1997), and UV light, immunosuppression or trauma to skin or nervous ganglia in animals (Stevens, 1978). This suggests that there must be a life-long monitoring of HSV by the immune system to prevent recurrent disease and disseminated infection.

1.1.4. Immunity to Herpesviruses

Both innate and adaptive immunity are critical in the protection of the organism against herpesvirus infection. Multiple cell populations including NK cells, macrophages, B cells, and T lymphocytes, as well as the cytokines generated by these cells, play a part in the host's defences against HSV infections (Cunningham and Merigan, 1983; Notkins, 1974; Yasukawa and Zarling, 1984). The innate immune response is important in controlling the degree and severity of the initial herpes virus infection, but it is only the beginning of a response to herpes virus infections.

1.1.4.1 Humoral Immunity:

1.1.4.1. 1. Role of antibody and antibody reactions.

In humans, neutralizing antibodies titres are detectable 2 weeks following the appearance of clinical lesions, and rise until 3-4 weeks after clinical symptoms subside (Zweerink and Corey, 1982). The majority of studies performed involving human subjects involve the characterization of antibody titres in different populations (Hashido

and Kawana, 1997; Perkins *et al.*, 1996). Animal studies have been important in determining the function of antibody in herpes infection, and in attempting to identify the mechanism of protection as being direct neutralization or antibody-dependent-cell-mediated-cytotoxicity (ADCC). The majority of the animal studies rely on the passive transfer whole or $F(ab')_2$ antibody fragments. Using whole IgG specific for HSV in a mouse footpad challenge model, McKendall (1985) reported faster clearance of virus from the epidermis, a significant reduction in the amount of virus in the nerve ganglia near the injection site and reduced incidence of viral spread to the opposite limb. Using passive transfer of $F(ab')_2$ antibody fragments, there was a slower clearance of virus from the footpad, a less significant reduction in the amount of virus in the ganglia near the injection site and no reduction of the virus spreading to the opposite limb (McKendall, 1985). Another group performed similar experiments using an ocular model of challenge (Oakes and Lausch, 1981). Passive transfer of whole IgG in the ocular model resulted in complete protection at early and late time points post infection, but the passive transfer of $F(ab')_2$ fragments only provided protection at early time points after infection. Passive transfer of whole IgG in the zosteriform spread model in nude mice resulted in reduction of zosteriform spread and prolonged survival (Hayashida *et al.*, 1982; Kohl *et al.*, 1984). The passive transfer of $F(ab')_2$ fragments in the same mice had no effect (Hayashida *et al.*, 1982). The results of these studies were consistent with the suggestion that ADCC played a significant role in protecting mice from herpesvirus infections and neutralization

to a lesser degree. To further clarify whether the protective mechanism was ADCC, or due to the alternative complement pathway, several groups used complement knockout mice in their studies, all finding that complement activation contributed little in the protection conferred by whole IgG (Hayashida *et al.*, 1982; McKendall, 1985; Oakes and Lausch, 1981). NK cell involvement in ADCC has also been implicated in the ocular challenge model for HSV-1 in which NK cell reconstituted SCID mice showed clear associations with NK cells and reduced pathology and severity of infection (Bouley *et al.*, 1996).

Antibody reactions are also involved in the maintenance of latency, as proposed by Stevens and Cook (1974), who found that the reactivation of herpes from infected trigeminal ganglia implanted in spleens or peritoneal cavities of uninfected mice could be prevented by the passive transfer of HSV specific IgG. A different mechanism by which antibody may be involved in maintaining latency in humans has been suggested by Russell and Saertre (1976), who examined antibodies in cerebral spinal fluid (CSF) of patients and found correlations between antibody levels in CSF and viral latency. Dicou *et al.*, (1991) further speculated that antibodies against nerve growth factor (NGF), not only promote HSV latency (Clements and Kennedy, 1989), but that anti-NGF antibodies modulate the cytokine function of NGF and therefore play a role in maintaining latency *in vivo*. Recently, Hill *et al.* (1997) lent support to Dicou's hypothesis by reporting that the transfer of anti-NGF mAb induced a latent infection to become active in the rabbit ocular

model.

1.1.4.1.2. The Role of Herpes Glycoproteins in Humoral Immunity

It was known from a very early time point that the predominant antibody response to HSV in humans was directed at HSV glycoproteins (Glorioso *et al.*, 1984; Para *et al.*, 1985; Vestergaard and Grauballe, 1979). Passive transfer of antibody against gB-1 in athymic nude mice prevented the spread of skin lesions and reduced latent infections, but were not protective against lethal challenge (Yamamoto *et al.*, 1986). Passive transfer of anti-gD-1 mAb given intra-peritoneally (IP) 24 h post-infection strongly protected mice depleted of CD4+ cells against stromal keratitis (Staats *et al.*, 1991). Monoclonal antibodies to glycoproteins B, C, D, and E of HSV-1 protected the mice against encephalitis and prevented the development of necrotizing stromal keratitis that leads to permanent corneal scarring and blindness (Metcalf *et al.*, 1988). Mester and colleagues (1991), using mAbs against gB, gC and gD of HSV-1 in the murine zosteriform spread model found all of the anti-gC and anti-gD mAbs, and one of four anti-gB mAbs were protective (Mester *et al.*, 1991). The seeming confusion regarding which mAbs were protective was clarified by Eis-Hubinger *et al.* (1991), who showed that protection depended on the epitope specificity of the monoclonal antibodies. These findings were expanded on by Bystricka *et al.* (1997), who found that any antigenic site on gC and gB contains epitopes responsible for protective immunity, but individual monoclonals to

different epitopes of the same antigenic site showed extreme variability in their protective ability. Although important in the protection of the host from HSV at early and intermediate time points and possibly in latent infections the humoral response is only one part of the tapestry which confers complete protection against HSV.

1.1.4.2. Cell Mediated Immunity:

The importance of T cell-mediated immunity has been demonstrated in animal models by utilizing adoptive transfer. Using this strategy T cell subsets have been isolated and their respective roles in protection defined (Kapoor *et al.*, 1982). The principal effector cells involved in herpes immunity are CD8+ and CD4+ T cells, which may effect cell-mediated cytotoxicity, delayed type hypersensitivity (DTH) reactions and cytokine release.

1.1.4.2.1. Cytotoxic T cell responses

Following the immunization of mice by intravenous (IV), IP, sub-cutaneous (SC), or mucosal routes, herpes-specific cytotoxic T cells (Tc cells) can be detected in the draining lymph nodes after 4 days (Pfizenmaier *et al.*, 1977), peak after 6-7 days and are undetectable after 14 days (Nash *et al.*, 1980). During this period, CTL precursor frequencies (pCTL) specific for HSV are initially near undetectable (1 in 250, 000) but climb to significant levels (1 in 3, 500 to 1 in 15, 000) (Rouse *et al.*, 1983). These herpes

specific CTL are MHC-I restricted (Jennings *et al.*, 1984; Sethi *et al.*, 1983) and protect against lethal challenge with HSV (Larsen *et al.*, 1983). The successful generation of CTL requires the use of live virus preparations rather than killed or attenuated virus (Rouse *et al.*, 1983). After the induction of CTL *in vivo*, the majority of CTL recognize both HSV-1 and 2 infected syngeneic target cells (Carter *et al.*, 1982; Eberle *et al.*, 1981; Nash and Ashford, 1982) but this requires *in vitro* culture for 2-3 days (Pfizenmaier *et al.*, 1977). High levels of interferon gamma (IFN-γ) are generated after this culture period and are present in adoptively transferred CD8's (Sethi *et al.*, 1983), which may enhance NK and macrophage activity or non-lytic suppression of virus (Lodmell and Notkins, 1974).

Glycoproteins are involved as important targets in generating CTL as demonstrated by utilizing inhibitors of glycoprotein synthesis (Carter *et al.*, 1981; Lawman *et al.*, 1980). The defined glycoprotein targets vary from group to group and depend on the method of immunization and genetic background of the animal. For instance, Eberle *et al.* (1981), suggest that the majority of the Tc responses are directed against gC (Eberle *et al.*, 1981), a finding which was confirmed in C57BL/6 mice (Glorioso *et al.*, 1985) and later in the CBA mouse model where gC, but not gB, gD or gE were recognized by HSV specific CTL's (Rosenthal *et al.*, 1987). Martin *et al.* (1993) determined that induction of CTL against gC varied between individual mice and was dependent on the form of immunization used. In addition, Martin *et al.* (1993) concluded that mice generated high

levels of Tc only if they expressed the H-2 K^b MHC allele. Indeed these findings explained several discrepancies in the literature. For example, Martin *et al.* (1988a), report that they were unable to generate CTL against gB or gD, whereas McLaughlin *et al.* (1988), using similar constructs were able to generate strong anti-gB responses. Witmer *et al.* (1990) also reported that H-2^b and H-2^d, but not H-2^k-restricted syngeneic target cells infected with AdgB2 were efficiently lysed by primary HSV-specific CTL. They also reported that HSV-specific CTL do not recognize gC, whereas a significant proportion of anti-viral CTL recognize gB presented in some but not all murine haplotypes (Witmer *et al.*, 1990). Further work demonstrated that gB is an important glycoprotein with the ability to generate high levels of HSV specific CTL precursors (Witmer *et al.*, 1990). Collectively these findings demonstrate that many factors must be considered when applying one CTL model to another strain of animal or species.

Glycoproteins are not the only targets for anti-HSV CTL. In fact, 30% of the CTL are specific for immediate-early proteins, whereas a minority of cytotoxic T cells recognize antigens from the infecting virus (Martin *et al.*, 1988b). The recognition of other viral antigens also seems to be haplotype specific. Martin *et al.* (1990), showed that HSV-1-specific CTL, restricted to class I MHC genes of the H-2^k haplotype but not the H-2^d or H-2^b haplotypes, lysed autologous cells expressing ICP4, the intracellular protein 4 of HSV. Further, the CTL response directed against ICP4 represented approximately one third of the total HSV-1-specific CTL responses (Martin *et al.*, 1990). Banks *et al.*

(1991), demonstrated that one quarter of the total HSV-1-specific CTL response was directed against the HSV immediate early protein ICP27. However, this was observed only in H-2^d mice (Banks *et al.*, 1991). Although HSV-1 specific CTLp were high, and the H-2^d mice survived a lethal IP challenge of HSV-1, neither antibody nor delayed-type hypersensitivity responses were induced and the mice were not able to clear local infections of HSV-1 (Banks *et al.*, 1991). Thus ICP27 alone is not able to provide complete protection (Banks *et al.*, 1991).

In humans, various glycoproteins can serve as targets for anti-HSV CTL. To date, two groups have determined that gD is a target antigen for human CTL (Tigges *et al.*, 1992; Zarling *et al.*, 1986). Yasukawa and Zarling (1985) characterized several HSV-1 and HSV-2 specific CTL and found clones specific for gB-1, gD-1&2, gE-1&2 and gG-2. These findings provide direct evidence that several HSV glycoproteins are recognized by human HSV-specific CTL (Yasukawa and Zarling, 1985). The diversity of the CD8+ response from a single individual indicates that several different antigens are recognized when presented in the context of a variety of class I HLA alleles (Tigges *et al.*, 1992). Posavad et al (1996), demonstrated the importance of CD8+ CTL by characterizing the pCTL in humans, finding significant CTLp frequencies in seropositive donors.

1.1.4.2.2. T helper cell responses:

Schmid and Rouse (1983) determined that heat-inactivated or UV-inactivated HSV-1

preparations were unable to induce anti-HSV CTL responses because HSV-1-specific subsets of helper T (Th) lymphocytes were unable to recognize the denatured forms of the antigen. They further reported that helper Th responses are required *in vitro* to generate CTL (Schmid and Rouse, 1983). Horohov *et al.* (1985) later confirmed the involvement of HSV-specific Lyt-1+ helper cells.

The role of Th cells in antibody production were initially demonstrated by Erlich *et al.* (1989) utilizing antibody-knockout experiments involving anti-CD4 in an acute neural infection. The treated mice were severely immuno-compromised but had little difference in pathology compared to control animals with normal antibody levels, the proposed explanation of this result was an increase in natural killer cell activity (Erlich *et al.*, 1989). These experiments do suffer from a lack of clarity due to limitations inherent in antibody-mediated knockout of T cells (Erlich *et al.*, 1989). The role of Th cells became easier to assess with the generation of cloned Th cell lines. Adoptive cell transfer of cloned Th cells helped to prime B cells to produce anti-herpes antibodies in an antigen-specific and dose-dependent manner (Leung *et al.*, 1984). The same clones also help unprimed T cells in the generation of effector DTH cells *in vitro* in an antigen-specific and dose-dependent fashion, which were able to protect against lethal challenge of HSV (Leung and Nash, 1986). The protective activity of the clones was associated with detection of interferon-gamma (IFN-γ) in the clone supernatant (Seid *et al.*, 1987), and the concurrent activation of macrophages (Seid *et al.*, 1986).

Although the above experiments clearly demonstrated a role for Th cells in mice, it was still unknown how many of these cells are involved in a typical immune response to HSV. Prymowicz et al (1985) estimated the frequency of HSV specific Th cells (ThLp) in suspensions of local lymph node (LN) cells 5 days after *in vivo* infection to be between 1 in 2, 470 to 1 in 5, 800, whereas the frequencies in cells from uninfected mice were below 1 in 100,000. In addition the removal of the Lyt-2+ (CD8+ CTL) cells from responder LN cells before culture increased the ThLp frequency two- to three-fold, suggesting suppression by the CD8+ T cells in unseparated cultures (Prymowicz *et al.*, 1985).

While Th cells provide help in generating humoral and CTL responses, they may also act as CTL (Yasukawa and Zarling, 1984). Doymaz *et al.* (1991), reported a population of HSV-specific CTL that were of the CD4+ phenotype and were MHC-II restricted. This finding was confirmed using beta 2-microglobulin (b2M) deficient mice, which generated CTL capable of only lysing MHC class II infected target cells (Niemialtowski *et al.*, 1994). When the authors compared the frequencies of CD4+ CTLp and CD8+ CTLp between b2M mice and normal mice, they found that the frequencies were similar (Niemialtowski *et al.*, 1994). In humans, it has now been demonstrated that HLA-DR-restricted CD4+ CTL (Koelle *et al.*, 1994a; Torpey *et al.*, 1989) are present in high precursor frequencies (Yasukawa *et al.*, 1989) and are the predominant killer cell phenotype against HSV (Schmid and Mawle, 1991).

Although it is clear that there is a strong involvement of Th cells in both humans and mice, less work has focussed on the antigen specificity of the Th cells than CD8+ CTL. In mice, Chan *et al.* (1985), demonstrated that gB was capable of activating Th cells which provided strong protective immunity. Vasilakos and Michael (1993) reported that the phenotype of CTL involved in protection of mice was CD8+ and H-2^d restricted. Depletion of CD4+ cells *in vivo*, did not prevent CTL generation suggesting that gB activated CD8+ CTL *in vivo* in a manner independent of CD4+ T cells (Vasilakos and Michael, 1993). In humans, the antigen specificity of CD4+ lymphocytes was determined by using 47 HSV-specific CD4+ CTL clones recovered from the HSV-2 lesions of five patients (Koelle et al., 1994b). Several clones had proliferative responses to gB-2 , gD-2 or gC-2, but represented a minority of the total, most being gC-2 and gD-2 specific, and one was specific for VP16 (Koelle *et al.*, 1994b).

1.1.4.3. Herpes and Immuno-evasion

The ability of herpes virus may remain latent suggests the virus itself must be capable of evading the hosts' immune response. In fact, HSV utilizes several mechanisms to effect this evasion. For example, HSV defends itself against the innate immune system and limits antigen presentation by directly infecting monocytes, thus interfering with their function (Hayward *et al.*, 1993). Via gC, HSV can bind and neutralize complement component C3b (Bielefeldt-Ohmann *et al.*, 1988; Eisenberg *et al.*, 1987; Friedman *et al.*,

1984; Huemer *et al.*, 1993). Further, HSV mutated in gC generates markedly reduced titres after exposure to immune and non-immune serum (Friedman *et al.*, 1996). Once an adaptive immune response has been initiated, HSV employs further defence mechanisms. HSV 1 and 2 have glycoproteins that act as receptors for the Fc domain of IgG (Baucke and Spear, 1979; Dubin *et al.*, 1991; Johnson and Feenstra, 1987), formed by glycoproteins E and I (Dubin *et al.*, 1994). Binding of the Fc region interferes with effector functions including ADCC, binding of complement component C1q and antibody-mediated neutralization of virus (Dubin *et al.*, 1992; Dubin *et al.*, 1991; Frank and Friedman, 1989). HSV can also directly infect NK and CTL from target cells, thus disabling them (York and Johnson, 1993). Further, HSV-infected human dermal fibroblasts are poor targets for cytotoxic T cells *in vitro,* a phenomena related to low MHC class I presentation (Koelle *et al.*, 1993; Posavad *et al.*, 1993). There is also reduced CTL function against these targets *in vivo* (Confer *et al.*, 1990). This latter finding may be of importance *in vivo*, as dermal keratinocytes and epithelial cells are the principal sites of HSV replication in the skin, and dermal fibroblasts are productively infected *in vivo* (Cunningham *et al.*, 1985). Normally, viral proteins are broken down by proteases and organized into proteasomes which include components encoded in the MHC locus (Monaco, 1992). These are then transported into the endoplasmic reticulum by ATP-dependent peptide transporters (TAP) which contains MHC I (Androlewicz *et al.*, 1993). Once this occurs, peptides and MHC I form complexes and viral peptides

presented in the context of MHC class I are expressed on the surface of the cell allowing effector cells to lyse the virus infected cells. However, the HSV immediate early protein ICP47 blocks the TAP system, preventing association of HSV peptides with MHC class I molecules and thus inhibiting lysis of infected cells by cytotoxic CD8+ T cells (York *et al.*, 1994; Fruh *et al.*, 1995; Hidaka *et al.*, 1991). However, IFN γ can assist CD8+ CTL by countering the effect of class I MHC reduction (Tigges *et al.*, 1992; Tigges *et al.*, 1996).

1.1.5. Mucosal Immunity and Herpes Simplex Virus

1.1.5.1. Background:

The mucosal surface encompasses more than 400 m^2 of epithelium (McGhee and Kiyono, 1993), and since one metre of intestine contains more than 10^{10} lymphocytes it is not surprising that the mucosal immune system contains more lymphocytes than anywhere else in the body (McGhee and Mestecky, 1990). The mucosal surface is constantly bathed in antigens and pathogens. Thus the mucosal immune system is the first line of defence against infection with a mucosal pathogen such as HSV. Functionally, the mucosal immune system can be divided into inductive areas and effector areas (McGhee *et al.*, 1992). Cells generated in the inductive areas can exit to the systemic circulation and subsequently enter distant mucosal tissues where they mature and differentiate into mucosal effector cells (Bienenstock *et al.*, 1983; Hanson *et al.*,

1988). This aspect of the mucosal immune system is often referred to as the "common mucosal immune system"(McGhee *et al.*, 1994).

The nasal associated lymphoid tissue (NALT) and bronchial associated lymphoid tissues (BALT), which are the primary lymphoid tissues of the upper respiratory tract, have been used successfully as inductive sites for generating anti-HSV mucosal immunity (Wu *et al.*, 1997). The NALT has been characterised in rats, and has been compared to be the human equivalent of Waldeyer's ring (Kuper *et al.*, 1992). The NALT lies on the cranial surface of the hard palate, on either side of the nasal septum, is oblong in shape and covered with M cells (Kuper *et al.*, 1992). While the NALT has some of the characteristics of a Peyer's patch, however, it has been reported that the cell populations making up the NALT place it somewhere functionally between a regional lymph node and Peyer's patch (Heritage *et al.*, 1997; Wu and Russell, 1993). The BALT, which has been studied in rats, rabbits and mice (reviewed in Sminia *et al.* (1989)), has many similarities with Peyer's patches (Bienenstock *et al.*, 1982). Presumably, the NALT and BALT jointly act as sites of induction for mice immunized intranasally (INL) with adenovirus vectors, the strategy generally utilized to generate mucosal responses to HSV (Gallichan *et al.*, 1995a; Gallichan and Rosenthal, 1995b). Indeed, it has been demonstrated that following INL immunization in mice, the NALT is involved in the generation of antibody secreting cells and also harbours T cells and memory T cells (Wu and Russell, 1993). As compared to a Peyer's patch, the NALT expresses more IL-4 and

IL-5, when cultured, which may assist in the development of IgA responses in mice (Kramer *et al.*, 1995; Lebman and Coffman, 1988; Murray *et al.*, 1987).

1.1.5.2. Effector Responses of the Mucosal Immune System

The major mediator of protection at mucosal surfaces is secretory IgA (S-IgA) (Liew *et al.*, 1984; McGhee *et al.*, 1994; Mestecky *et al.*, 1994). The source of secretory IgA found on mucosal surfaces is from B cells which have been induced to produce polymeric IgA (p-IgA). The polymeric immunoglobulin receptor (pIgR), which is located on the basolateral surface of epithelial cells in the mucosa, binds the p-IgA which is then internalized and the pIgR-pIgA complex is then transcytosed across the epithelial cell. Proteolytic cleavage of the secretory component (SC - the soluble extracellular domain of the pIgR) results in the release of SC-IgA (Mostov, 1994). Functionally, the secreted IgA is then able to bind to viruses, interfering with their ability to adhere to epithelial cells (Goldblum, 1990). Secretory IgA may also pick up antigen at the basal surface of the cell, transport it across the cell and subsequently release it at the apical surface, or alternatively as IgA is being transduced across the cell, it may interact with viral proteins during assembly and thus interfere with viral reproduction and release (Lamm, 1997).

There are several factors affecting the production of S-IgA. The most notable is the cytokine regulation of IgA responses. The production of p-IgA requires two steps, the first involves isotype switching to IgA, and the next step involves the stimulation of IgA

blasts to secrete IgA. TGF-β has been identified as the cytokine responsible for inducing IgM+ B cells to switch to S-IgA expression (Coffman *et al.*, 1989; Sonoda *et al.*, 1989), whereas IL-5 and IL-6 have been shown to promote IgA synthesis (Beagley *et al.*, 1988; Beagley *et al.*, 1989; Eckmann *et al.*, 1992; Kjerrulf *et al.*, 1997; Kramer *et al.*, 1995; Whittle *et al.*, 1997). Various cell types, including CD4+ T cells (Jackson *et al.*, 1996), gamma delta T-cells (Fujihashi *et al.*, 1996) and dendritic cells (Fayette *et al.*, 1997) also are involved in the induction and regulation of IgA production.

Another effector of mucosal immunity, which is of particular importance in the protection afforded by mucosal vaccines against HSV, is the cytotoxic T cell (CTL). Indeed, adoptive transfer of CTL has demonstrated their role in clearing viruses from mucosal surfaces and their ability to limit pathology (Mackenzie *et al.*, 1989; McDermott *et al.*, 1989a). T cells are also able to selectively localize to mucosal surfaces, and this selective localization is profound when there is re-exposure to the same pathogen (McDermott *et al.*, 1989a; Offit *et al.*, 1991). The role of T cells in the protection against HSV can best be demonstrated by the results obtained by McDermott *et al.* (1989b) who used the adoptive transfer of T cells to demonstrate that naive mice could be protected from a lethal challenge of HSV-2.

1.1.5.3. Mucosal Immunity and Herpes Simplex Viruses

Although attenuated herpesvirus vaccines are able to generate protective immunity at

mucosal surfaces (McDermott *et al.*, 1984), adenovirus vectors have been the predominant vaccine utilized to generate mucosal immune responses to HSV (Gallichan *et al.*, 1995a; Gallichan and Rosenthal, 1995b). Briefly, Ad vectors containing glycoprotein B of HSV-1 (AdgB) have successfully generated gB specific IgA, IgG and CTL responses at mucosal surfaces (Gallichan *et al.*, 1993; Gallichan *et al.*, 1995a; Gallichan and Rosenthal, 1995b; Gallichan and Rosenthal, 1996b). The specifics of these experiments will be discussed in detail in the following section, however these studies clearly demonstrate the importance of mucosal immunity in the protection against HSV in mice. In humans, following HSV infection, there is a strong mucosal response as measured by HSV-specific IgA . Most of the studies involving measurements of IgA at mucosal surfaces have examined the vaginal mucosa in infected populations and discuss the importance of IgA in preventing vertical transmission of HSV (Boggess *et al.*, 1997; Persson *et al.*, 1988).

1.2. Strategies Used to Generate Anti-Herpes Immunity:

1.2.1. DNA Vaccines

1.2.1.1. Background

In early studies of DNA immunization purified DNA transferred from one animal to another was reported to remain pathogenic, as measured by tumour formation and the elicitation of antibody responses. It was later shown that non-replicating plasmids

delivered *in vivo* could express their gene products. "DNA vaccination" has been shown to induce both humoral and cell-mediated responses to influenza A virus (Ulmer *et al.*, 1993), Hepatitis B (*Davis et al.*, 1994) and human immunodeficiency virus-1 (HIV-1) (Wang *et al.*, 1993). DNA vaccination against herpesvirus has only been utilized by a small number of researchers (Bourne *et al.*, 1996a). Some of the benefits of DNA immunization over traditional approaches are summarised in table 1.

DNA immunization relies on the use of highly purified preparations of bacterial plasmids which contain a gene against which an immune response can be generated. In short, a DNA vaccine has four obligate components, a plasmid, a promoter, insert and poly A sequence. The plasmids used in DNA vaccines are generally pUC or pBR322 derived, and thus do not replicate in mammalian cells at a detectable level (Wolff *et al.*, 1990). Thus a strong mammalian promoter is required for expression of the gene in mammalian cells. The most widely used is the promiscuous cytomegalovirus (CMV) immediate early gene promoter (Xiang and Ertl, 1995). The choice of the insert used is dependent upon the objectives of the vaccine. DNA vaccine strategies have included: using sequences coding for specific epitopes of a protein (Ciernik *et al.*, 1996), attachment of a leader sequence resulting in the secretion of the gene product (Barry *et al.*, 1995; Lu *et al.*, 1996), targeting MHC I presentation (Ciernik *et al.*, 1996), using multiple epitopes (Suhrbier, 1997) or the use of cytokines and costimulatory molecules as adjuvants (Pasquini *et al.*, 1997).

1.2.1.2. Administration of DNA Vaccines

DNA vaccines have the additional benefit of multiple delivery strategies. One of the most common delivery methods for plasmids has been via intramuscular (IM) injection. Davis *et al.* (1993a), after direct comparison found that injection of naked DNA into skeletal muscle resulted in better expression than that obtained with some adenovirus vectors. The mechanism of DNA uptake in skeletal muscle is not well understood, but the T-tubules and caveolae of skeletal muscle have been implicated (Wolff *et al.*, 1992). It is estimated that only picogram quantities of DNA are successfully taken up by the muscle fibres resulting in less than 100 muscle fibres (1-2% of total, or 10% at the site of injection) affected at the injection site (Davis *et al.*, 1995a). It is thought that the physical architecture of the muscle interferes with plasmids coming into direct contact with the skeletal muscle (Davis *et al.*, 1993). This theory is supported by the observation that the diaphragm muscle in a mouse, which is lacking the same connective tissue barriers as skeletal muscle, can be easily transfected with naked DNA (Davis and Jasmin, 1993b). Several groups have investigated formulations which may enhance the efficiency of transfection of muscle cells. Davis *et al.* (1993a), reported that regenerating muscle takes up naked DNA with greater efficiency than resting muscle. Muscle regeneration can be induced by the use of agents such as snake toxins or local anaesthetics (Whalen *et al.*, 1990). Transfection appears to be ten times more efficient in regenerating muscle as compared to resting muscle (Davis *et al.*, 1993a) or as high as 40 times with the use of

the local anaesthetic bupivacaine (Danko *et al.*, 1994). It is however very important to note that even without additional agents, protective immune responses can be obtained using only saline as a delivery vehicle (Cox *et al.*, 1993; Donnelly *et al.*, 1996; Ghiasi *et al.*, 1995; Lagging *et al.*, 1995; Manickan *et al.*, 1995a; Montgomery *et al.*, 1993; Rhodes *et al.*, 1994; Ulmer *et al.*, 1993; Xiang *et al.*, 1994; Yokoyama *et al.*, 1995)

An alternate method of immunization which has only been recently applied to DNA vaccines is intranasal (INL) immunization. Naked DNA was first administered by this route in 1993 (Fynan *et al.*, 1993b). Subsequently other groups began to apply this technically simpler methodology (Barnfield *et al.*, 1997; Gao *et al.*, 1997; Hinkula *et al.*, 1997; Wang *et al.*, 1997). In initial studies performed by Fynan *et al.* (1993a), INL immunization with naked DNA alone did result in protection against influenza virus in mice, but with moderate to severe disease and barely detectable levels of antibody, which was likely due to the poor level of gene transfer obtained using naked DNA. When Barnfield *et al.* (1997) used cationic lipids as an adjuvant the efficiency of gene transfer was increased 30 fold over naked DNA resulting in the elicitation of cellular and humoral immune responses. In fact since the initial study by Fynan *et al.*, (1993b), only one other group has successfully used INL administration of naked DNA to generate immune responses, however the responses obtained were weak (Asakura *et al.*, 1997). Most groups have used some form of liposome (Okada *et al.*, 1997; Sasaki *et al.*, 1998; Vadolas *et al.*, 1995), cholera toxin (Kuklin *et al.*, 1997), particle carrier (Alpar *et al.*,

1997) or co-administration with a cytokine producing vector (Okada *et al.*, 1997) as adjuvants to improve immune responses to INL immunization.

DNA may also be delivered via a Gene Gun. This technique uses DNA-coated particles propelled at high velocity into the cells of the skin, where the DNA is released from the gold particles and expressed (Eisenbraun *et al.*, 1993; Pertmer *et al.*, 1995). This method is by far the most efficient method of delivering DNA, where as little as 16 ng of plasmid DNA may be immunogenic (Pertmer *et al.*, 1995). Contrary to expression in muscle cells induced by DNA administered IM (Wolff *et al.*, 1990), DNA delivered by gene gun results in gene expression in keratinocytes (Raz *et al.*, 1994). Gene gun delivery has been successfully used to generate responses against lymphocytic choriomeningitis virus (LCMV) (Zarozinski *et al.*, 1995), simian immunodeficiency virus (SIV) (Lu *et al.*, 1996), influenza (Fynan *et al.*, 1993b; Justewicz *et al.*, 1995), mycoplasma (Lai *et al.*, 1995), central European encephalitis virus (Schmaljohn *et al.*, 1997), hepatitis C (Nakano *et al.*, 1997) and HIV-1 (Barnett *et al.*, 1997; Prayaga *et al.*, 1997).

It has become clear that IM immunization generated Th1 type responses while gene gun immunization generally generated Th2 type responses, as summarized in table 2 (Robinson and Torres, 1997). The Th balance with respect to INL immunization appears to be in favour of a Th2 response (Kuklin *et al.*, 1997; Okada *et al.*, 1997), although Th1 responses have also been observed (Gao *et al.*, 1997). Further study is required to

determine whether the DNA or the adjuvants used are responsible for the Th profile obtained in INL immunization (Gao *et al.*, 1997; Kuklin *et al.*, 1997).

1.2.1.3. Immunogenicity of DNA Vaccines

Following the initial reports of naked DNA transfer (Wolff *et al.*, 1990) research focussed on whether specific regions of the DNA were responsible for immunogenicity of the vectors. Indeed, actual base sequences within the intervening regions of plasmid DNA were found to be immunogenic and could be linked to direct B-cell activation in an antigen-specific manner, resulting in antibody production (Krieg *et al.*, 1995). Furthermore, these sequences, which contained a CpG dinucleotide, were required for effective intradermal (ID) immunization (Sato *et al.*, 1996) and the generation of NK activity (Ballas *et al.*, 1996). The adjuvant-like activity of the CpG dinucleotide was attributed to hypomethylation of cytosine residues in bacterial DNA (Bird, 1986; Pisetsky, 1996). Several studies have attributed the cytokine profiles generated following immunization to the CpG motifs in the bacterial plasmid backbone (Klinman *et al.*, 1997; Sato *et al.*, 1996). Tighe *et al.* (1998) suggest that the CpG motifs or "immunostimulatory sequences" (ISS) may activate the cytokine network required to produce IFN-γ in an antigen-independent fashion, which in the context of a protein antigen may promote the differentiation of naive CD4+ T cells into Th1 cells. These authors further suggest that this production of Th1 T-cells may result in a second burst of IFN-γ production in an antigen-dependent fashion, thus down-regulating the Th2

responses in favour of Th1 responses (Tighe *et al.*, 1998). Tighe *et al.* (Tighe *et al.*, 1998) further point out that the Th2 type responses observed with gene gun application are most likely due to the minute amount of plasmid used in the delivery system, which have been shown to favour Th2-type responses (Feltquate *et al.*, 1997). Thus for gene gun application to be successful, adequate expression of the gene insert is essential to ensure antigen specific immune response to the vector (Krieg *et al.*, 1995).

Wolff *et al.* (1990) reported that following IM vaccine administration, very few muscle fibres were transfected. Antibody responses to IM administration could be rationalized as being generated by secreted proteins from the transfected cells, but the induction of cellular immunity was more difficult to explain. Initial studies examining skeletal muscle for expression of MHC class I (Engel and Hohlfeld, 1994) reported that immature muscle fibres express more MHC I than mature fibres. However, this did not explain how a cellular response was generated since skeletal muscles lack the appropriate co-stimulatory molecules necessary for T cell activation (Schwartz, 1992) which in other systems is known to induce tolerance (Jenkins and Schwartz, 1987; Lamb *et al.*, 1983; Mueller *et al.*, 1989; Quill and Schwartz, 1987). Another possible explanation is that professional antigen presenting cells (APCs) may be recruited to the site of injection due to inflammation (Hohlfeld and Engel, 1994) to provide the necessary co-stimulation however PCR demonstrated that there is little or no expression of APC DNA in the tissue near the site of injection (Nichols *et al.*, 1995). The mystery of antigen presentation was

resolved using bone marrow (BM) chimeric mice, which revealed that BM derived cells provide the MHC class I restriction and co-stimulation required for the induction of CTL responses in IM injection of plasmid DNA (Corr *et al.*, 1996; Doe *et al.*, 1996b). This may seem unlikely, as current dogma holds that only endogenous peptide synthesis can result in the presentation of peptides in the context of MHC I, although a number of new studies demonstrate that proteins from an exogenous source are able to prime a MHC class I-restricted CTL responses (Kovacsovics-Bankowski and Rock, 1995; Martinez-Kinader *et al.*, 1995; Reis and Germain, 1995). Several studies outline how exogenous proteins may be transferred to MHC I: transfer of peptides to MHC I is facilitated by heat shock protein (HSP) chaperones (Suto and Srivastava, 1995), exogenous peptides may be endocytosed by macrophages or dendritic cells and presented in a MHC I pathway (Condon *et al.*, 1996b; Martinez-Kinader *et al.*, 1995), external loading of MHC I from self phagosomes or neighbouring cells (Bohm *et al.*, 1995; Martinez-Kinader *et al.*, 1995) or internal loading of MHC I may occur via a phagosome to cytoplasm shuttle that feeds exogenous peptides into the conventional pathway (Kovacsovics-Bankowski and Rock, 1995). Regardless of the precise mechanisms involved the absolute requirement of the TAP complex by BM derived APCs has been demonstrated (Huang *et al.*, 1996).

The explanation of the immune response to DNA vaccines delivered cutaneously or mucosally is much simpler. ID injection of plasmid DNA leads to expression in

keratinocytes, fibroblasts and cells with the morphology of macrophages and dendritic cells in the dermis (Condon *et al.*, 1996b; Raz *et al.*, 1994), the former being observed in lymph nodes draining the vaccination site after gene gun administration. Exogenous peptide is also released from transfected cells and can be endocytosed and presented in the context of MHC class II, thus MHC II-mediated responses are not dependent upon uptake of the DNA by an APC (Corr *et al.*, 1996; Engel and Hohlfeld, 1994).

1.2.1.4. Herpes Immune Responses to DNA Vaccination

DNA immunization against HSV has concentrated primarily on the glycoproteins B and D. Manickan *et al.* (1995a) used a plasmid containing HSV-1 gB in mice in an immunization regimen consisting of two IM administrations of 90 μg of DNA, one at day 0 and one on day 7. As an indicator of protection *in vivo*, they used the zosteriform spread model (Simmons and Nash, 1984). High antibody titres with a predominant IgG2a response and antigen-specific proliferation of lymphocytes resulted. Further, using adoptive transfer, the protection in this model was shown to be mediated by CD4+ lymphocytes (Manickan *et al.*, 1995a). Bourne *et al.* (1996b), using similar amounts of the DNA vaccine (50 and 100 μg), induced herpes immunity in guinea pigs. This study used several immunization protocols spread out over several days, immunizing on days 0, 21 and 42. In the guinea pig model, there were poor humoral responses, but the authors did demonstrate reductions in the severity of disease upon challenge, and a lessening in

recurrent disease (Bourne *et al.*, 1996b). Using a mouse model, Kriesel *et al.* (1996) used a single IM injection of a plasmid containing HSV-2 gD and subsequently challenged with HSV-2 intravaginally at 6 weeks post immunization. Unlike the guinea pig model, reasonable antibody titres were obtained for a primary immune response and a more dramatic demonstration of protection from intravaginal challenge, especially when vitamin D3 was used as an adjuvant (Kriesel *et al.*, 1996). Interestingly, IgA was not detected in vaginal washes (Kriesel *et al.*, 1996). Shortly after this publication Bourne *et al.* (1996a) confirmed the vaginal protection and serological aspects of the findings in a short study. The most noticeable difference between the studies was the use of four times more DNA by Bourne's group, and an immunization protocol that saw three administrations spaced three weeks apart (Bourne *et al.*, 1996a).

Kuklin and colleagues (1997) used cholera toxin as an adjuvant in INL immunization with a DNA vaccine encoding for gB of HSV-1. In this ambitious report, the authors examined the distribution of a marker plasmid following INL immunization. The distribution of plasmid was found primarily in the alveolar epithelial cells and the bronchi of the lung and in the cortical regions of the cervical lymph nodes of the recipient mice which were deeply anaesthetized. The resulting serology showed that INL immunization resulted in significantly lower titres of IgG than obtained using attenuated HSV or DNA administered IM, but the responses were respectable nevertheless. Cholera toxin generated a Th2 type response as measured by IgG isotype, whereas IM administration or

INL immunization without cholera toxin showed more of a Th1 type profile. Of importance was the detection of IgA in vaginal washes and faecal samples of all of the DNA immunized mice (Kuklin *et al.*, 1997). This is in contrast to the findings of Kriesel *et al.* (1996), who could not detect IgA after IM injection. There are two possible explanations to this discrepancy, one being that gD, the immunogen used by Kriesel's group is not able to illicit an IgA response in the DNA system, or a more plausible alternative is that the IgA detected after IM injection in Kuklin's experiments is an artifact. The second explanation is more feasible since high variability in the number of IgA secreting cells was found. Survival experiments showed that IM immunization was protective against high-dose viral challenge of HSV-2, whereas INL immunization was not (Kuklin *et al.*, 1997). INL immunization is however protective against low viral challenge doses (Kuklin *et al.*, 1997).

In summary, DNA immunization is a highly promising new technology for generating anti-herpes immune responses. Although still in it's infancy it is has already been demonstrated that it is capable of generating protection, high antibody titres, good levels of CTL activity, CD4+ T cell mediated protection against spread of HSV, and lastly mucosal immunity.

1.2.2. Recombinant Adenovirus Vectors

1.2.2.1. Background

Adenovirus is a non-enveloped virus with icosahedral symmetry ranging in size from 65-80 nm in diameter. The viral capsid is composed of 240 hexons and 12 penton bases. Adenoviruses generally cause benign disease in humans including: respiratory and gastrointestinal diseases, ranging from mild to severe (Horwitz, 1990; Klinger *et al.*, 1998; Simsir *et al.*, 1998), disease of the eye (Bobo *et al.*, 1997) , urinary bladder, and disseminated adenoviraemia, which in immunocopromised individuals often results in death(Hierholzer, 1992; McGrath *et al.*, 1998). A new serotype, adenovirus 7i, which has recently been discovered has been implicated in several fatalities (Mistchenko *et al.*, 1998). Adenoviruses were first isolated in the 1950's by Rowe *et al.* (1953). Since then 49 different serotypes (Ad1-Ad49) have been characterized, forming six groups (A-F) based on morphology, DNA homology and antigenic properties (Green *et al.*, 1979; Hierholzer *et al.*, 1991; Schnurr and Dondero, 1993; Wadell *et al.*, 1986). Adenoviruses infect epithelial cells by binding to an unknown cell surface receptor, possibly integrins (Croyle *et al.*, 1998; Goldman and Wilson, 1995; Roelvink *et al.*, 1996), via fiber proteins which are anchored to the penton bases in the viral coat. The virus then enters the cell in clathrin coated vesicles which fuse with endosomes, which in turn fuse with lysosomes (Greber *et al.*, 1993). Fusion of the viral envelope with the lysosomal membrane results in the loss of capsid proteins. The viral DNA enters the cytoplasm and subsequently

enters the nucleus through nuclear pores (Greber *et al.*, 1997) where it is transcribed.

The adenovirus genome consists of approximately 35 kb of DNA, including late genes, early genes, and the immediate early gene, E1A, which is a multi-functional transcription regulator required to activate other genes (Horwitz, 1990). If the E1A region of the virus is active, transcription of early genes occurs in the nucleus followed by DNA replication, late gene expression, virus particle assembly and cell lysis (D'Halluin, 1995). In the absence of E1A activity, the other viral genes are expressed at very low levels, resulting in limited viral replication (Lusky *et al.*, 1998). Proteins encoded by the E3 region play an important role in evasion of host cell-mediated immune responses *in vivo*. The gp19K protein encoded by E3 noncovalently associates with the heavy chain of MHC class I molecules and blocks transport of class I antigens to the cell surface (Cox *et al.*, 1991; Kvist *et al.*, 1978; Peterson *et al.*, 1986; Sester and Burgert, 1994; Sparer *et al.*, 1996) . The E3 region is not required for growth *in vitro*, but is maintained in natural isolates presumably due to its role in immuno-evasion (Sparer *et al.*, 1996). One of the most important advances in the understanding and application of adenoviruses was the discovery by Graham and colleagues (1992), that foreign genes could be inserted into the E1 and E3 regions of the adenovirus, and that these genes could subsequently be expressed *in vivo* (Berkner, 1992). This lead to the use of adenoviruses as recombinant vaccine vectors (Graham and Prevec, 1992; Imler, 1995) as vectors for use in gene therapy (Amalfitano *et al.*, 1998; Li *et al.*, 1998; Mack *et al.*, 1998) and anti-cancer

therapy (Emtage *et al.*, 1998; Marr *et al.*, 1998; Prince *et al.*, 1998). Most adenovirus based vaccines have been constructed using first generation replication competent, or E3 deleted, recombinant adenoviruses. These constructs have been used to induce immunity to: vesicular stomatitis virus (VSV) (Prevec *et al.*, 1989), rabies virus (Charlton *et al.*, 1992; Prevec *et al.*, 1990), hepatitis B and C viruses (Levrero *et al.*, 1991; Lieber *et al.*, 1996; Morin *et al.*, 1987), measles virus (Fooks *et al.*, 1995), HIV (Natuk *et al.*, 1992; Natuk *et al.*, 1993; Prevec *et al.*, 1991) and to HSV (McDermott *et al.*, 1989b; Zheng *et al.*, 1993). These vaccine constructs are capable of generating both cell-mediated and humoral immune responses to the expressed antigens and are able to protect against lethal challenge (Berkner, 1992; Imler, 1995). Although second generation replication incompetent, or E1 deleted recombinant adenoviruses are capable of generating immunity (Fooks *et al.*, 1995; Jacobs *et al.*, 1992), these vectors have generally been applied to gene therapy (for review see Bramson *et al.*, (1995)). Some of the benefits of adenoviruses over traditional vaccine approaches are summarized in table 1. In short, adenoviruses are well understood and are quickly becoming a viral work-horse for the application of gene transfer and as a preferred delivery vehicle for recombinant vaccines.

1.2.2.2. Administration of Adenovirus Vectors

Like DNA vaccines, adenoviruses can be delivered in many ways and the choice of delivery method is generally dependent upon the tissue which one is trying to transduce.

Adenoviruses were first administered orally. This strategy was used to immunize Canadian and U.S. military recruits to prevent adenovirus-induced acute respiratory disease (Chaloner-Larsson *et al.*, 1986; Top, 1975; Top *et al.*, 1971). Since then, oral delivery of adenovirus vectors has been used to prevent enteric infections in pigs (Tuboly *et al.*, 1993), for the treatment of gastric and colon carcinoma (Crystal *et al.*, 1997; Tanaka *et al.*, 1997), hepatitis B in monkeys (Lubeck *et al.*, 1989) and in the induction of herd immunity in wild animals to canine adenovirus type-2 (CAV-2) (Sumner *et al.*, 1988), the latter of which led to the development of a bait fed rabies vaccine (Lutze-Wallace *et al.*, 1995). The most recent use of oral adenovirus delivery has been to induce oral tolerance to adenovirus vectors, thus assisting in the long term expression of recombinant genes expressed by other adenovirus vectors (Ilan *et al.*, 1997; Kagami *et al.*, 1998).

INL administration has been the most widely used form of adenovirus delivery and has been successfully employed in several gene therapy applications (Draghia *et al.*, 1995; Glickman and Appel, 1981; Kahn *et al.*, 1996), the most notable of which is as a potential candidate for cystic fibrosis therapy (Bellon *et al.*, 1997; Brody *et al.*, 1994; Grubb *et al.*, 1994; Sene *et al.*, 1995). INL application of recombinant adenovirus vectors has been used in vaccination against tracheobronchitis (kennel cough) in canines (Glickman and Appel, 1981), coronaviruses (Baca-Estrada *et al.*, 1995), respiratory syncytial virus (RSV) (Collins *et al.*, 1990), HIV-1 (Natuk *et al.*, 1993) and HSV

(Gallichan *et al.*, 1993; Gallichan *et al.*, 1995a; Gallichan and Rosenthal, 1995b; Gallichan and Rosenthal, 1996a). Significant differences in the type and duration of immune responses results from the administration of adenovirus vectors INL versus IP delivery. For example, in direct comparisons with IP administration, INL immunization resulted in a longer duration of protection against INL challenge with HSV-2, than IP delivery of the same vector (Gallichan *et al.*, 1993).

INL and oral administration of adenovirus vectors have by far been the most popular of the delivery strategies, however other methods of administration have been used with varying degrees of success. Some of the more unique strategies used have included intraocular delivery (Stevenson *et al.*, 1995), intramarrow injection (IO) (Foley *et al.*, 1997) and ID injection (Lu *et al.*, 1997). IM injection of adenovirus has been used to correct hematopoietic deficiencies (Descamps *et al.*, 1994; Tripathy *et al.*, 1994), and to introduce neurotrophic factors (Ghadge *et al.*, 1995; Gimenez *et al.*, 1997). IM delivery of a recombinant adenovirus vaccine expressing pseudorabies virus gD in mice induced protection comparable to, but not better than the Nyvac poxvirus vaccines in use at that time (Gonin *et al.*, 1996). Incorporation of the CMV promoter in adenoviral vectors results in greater levels of expression of the inserted gene (Ambriovic *et al.*, 1997). This resulted in complete protection of mice from pseudorabies virus and better performance than that obtained with DNA vaccine constructs using the same gene inserts (Ambriovic *et al.*, 1997).

Although most of the above studies were performed using replication deficient viruses for gene therapy, they further illustrate differences in immune responses that can be generated by different methods of viral administration. For example, based upon the work of Descamps and colleagues (1994) it was found that IM injection is not as effective as IV injection in mice for adenovirus delivery (Descamps *et al.*, 1994), although IM injection is still capable of generating dose-dependent effects, long term gene expression and localized effect at the site of injection (Tripathy *et al.*, 1994). Furthermore, results obtained by Huard *et al.* (1995) suggest that the route of administration of adenovirus vectors has a significant impact on the viruses ability to infect various tissues. Huard and colleagues (1995), who examined levels of luciferase transgene expression after various routes of administration, found that, heart, diaphragm, intercostal muscles and thymus show high levels of transduction after intra-arterial injection of rats, whereas the liver exhibits high levels of transduction after IV injection (Huard *et al.*, 1995). This study also demonstrated that a substantial viremia develops within 2h of gastric-rectal, intraperitoneal or intracardiac administration of the recombinant luciferase adenovirus vector (Huard *et al.*, 1995). Only one study has directly compared INL immunization and IM immunization, concluding that in the pseudorabies virus mouse model, INL immunization is not as efficient as IM injection (Gonin *et al.*, 1996), but was still capable of generating the same protective responses at a higher dose. It is important to note though that findings from these studies should not be extrapolated to replication-

competent adenovirus vectors, or even vectors with different gene inserts, as the insert itself may influence the type of immune response obtained.

1.2.2.3. Immunogenicity of Adenovirus Vectors.

Much of what has been learned about the immunogenicity of adenoviruses has evolved from studies which have strived to overcome their limitations for gene therapy (Gahery-Segard *et al.*, 1998; Lusky *et al.*, 1998; Yang *et al.*, 1994; Yang *et al.*, 1995; Yang *et al.*, 1994; Yang *et al.*, 1996). These studies have illustrated that the predominant immune responses to adenovirus vectors are CD8+ T cell-mediated (Yang *et al.*, 1994; Yang *et al.*, 1995; Yang *et al.*, 1996), showing a Tc bias favouring E1A and E1B proteins with some involvement of E2 proteins (Mullbacher *et al.*, 1989). In mice following INL delivery, antigen presentation by MHC II of exogenous viral antigens activate CD4+ T cells of the TH1 subset, and to a lesser extent the TH2 subset, however, CD8+ T cell involvement is required to clear the vector (Yang *et al.*, 1995). TH2 responses in INL immunization, which have been linked to the formation of antigen specific IgA responses in C57BL/6 mice, can be abrogated with the co-administration of an adenovirus containing interferon gamma or interleukin-12, which activate TH1 cells to secrete IFN-gamma (Yang *et al.*, 1995). Indeed, the co-administration of these constructs reduced the activation of TH2 cells and the formation of IgA, allowing for efficient re-administration of recombinant virus (Yang *et al.*, 1995).

Humoral responses to adenovirus infection are mediated by the viral structural proteins (Flomenberg *et al.*, 1995; Gahery-Segard *et al.*, 1998), specifically the fiber and penton-base proteins. Gahery-Segard *et al.* (1997) demonstrated that mice immunized IP recognized only hexon proteins and preferentially switched to the production of the IgG2a subclass, whereas mice immunized IV gave a more complex response and displayed a higher neutralizing antibody activity than the serum obtained from IP immunized mice (Gahery-Segard *et al.*, 1997). The observed differences in neutralizing activity can be explained by the demonstration that for neutralization to occur, antibodies must be produced against hexon, fiber and penton base proteins (Flomenberg *et al.*, 1995; Gahery-Segard *et al.*, 1998).

Fortunately, the immune responses generated against adenovirus vectors which are of concern regarding their application to gene therapy, can be advantageous to their application as a vaccine. For example, the adenovirus gene region E3 codes for a protein, E3 19K, which can bind selectively to certain MHC class I molecules (Kvist *et al.*, 1978). The bound MHC I molecule can then be anchored to the endoplasmic reticulum (ER), preventing the migration of the MHC-I-peptide complex to the cell surface (Cox *et al.*, 1991) and thus disturbing the anti-adenovirus CTL (Hermiston *et al.*, 1993; Rawle *et al.*, 1989). This effect is most prominent in $H2^b$ mice (Mullbacher *et al.*, 1989; Rawle *et al.*, 1989). Although the E3 region assists second generation vectors in evading immune responses (Lee *et al.*, 1995; Zhang *et al.*, 1991), the absence of this region significantly

enhances CTL responses against first generation recombinant vectors and consequently their gene inserts (Mullbacher *et al.*, 1989; Rawle *et al.*, 1989). Aside from the E3 19K protein, the adenovirus encodes several different proteins which assist it in evading the immune system (for review see Hayder and Mullbacher, (1996). These immune evasion strategies include resistance to the action of cytokines and inhibition of apoptotic pathways (Hayder and Mullbacher, 1996). These evasion strategies may be beneficial for the immunogenicity of the gene inserts, as there may be fewer adenovirus antigens available to compete with the gene insert for antigen presentation. Unlike DNA vaccines which have the ability to act as an adjuvant directly, adenoviruses, especially replication competent adenoviruses cause inflammation upon administration (Brody *et al.*, 1994; Bruder and Kovesdi, 1997; Yang *et al.*, 1996), which assists in the recruitment of antigen presenting cells allowing the full immunogenic potential of the gene insert to be realised.

1.2.2.4. Herpes Immune Responses to Adenoviral Vectors

Adenovirus vectors containing herpes glycoproteins have been instrumental in defining the functions of specific herpes glycoproteins (Brunetti *et al.*, 1998; Hanke *et al.*, 1990; Hutchinson *et al.*, 1992; Hutchinson *et al.*, 1993; Hutchinson *et al.*, 1995), and for defining immuno-dominant epitopes involved in anti-HSV CTL (Hanke *et al.*, 1991; Witmer *et al.*, 1990). However, only the recombinant adenovirus constructs containing gD (Zheng *et al.*, 1993) and gB (Johnson *et al.*, 1988) have been used as vaccines.

Adenovirus vaccines containing gB were first administered by IP injection and elicited protection in mice from lethal systemic challenge with HSV-2 (McDermott *et al.*, 1989b) using AdgB2, a replication competent adenovirus containing sequences from Ad type 2 and type 5 (Johnson *et al.*, 1988). Gallichan *et al.* (1993) expanded on these studies by including INL immunization and by using AdgB8, a replication competent vector containing the wild-type gB of HSV-1 and sequences from only Ad type 5 (Hutchinson *et al.*, 1993). Interestingly, Gallichan *et al.* (1993) demonstrated that INL immunization induced both anti-HSV gB IgG in sera and IgA in lung and nasal washes, whereas IP immunization did not elicit anti-HSV gB IgA. In addition, anti-HSV CTL were generated from spleens of INL and IP immunized mice, but there was a time dependent decrease in the CTL activity from spleens of INL immunized mice (Gallichan et al., 1993). Furthermore, mice immunized INL with AdgB8 were protected against INL challenge with HSV-2 and this protection persisted longer than the protection mediated in IP immunized mice (Gallichan *et al.*, 1993). Therefore, INL immunization with AdgB8 can induce mucosal and systemic immune responses and long-term protection against HSV-2. This study also illustrated the significant differences in immune responses which can be generated by altering the immunization method. Expanding on their initial findings Gallichan and Rosenthal (1995b) also demonstrated that intravaginal immunization with AdgB8 resulted in little to no anti-HSV gB IgA, and only low levels of gB specific IgG in vaginal washes, wheras INL immunization did result in the

production of IgA in the female mouse genital tract. Gallichan and Rosenthal (1996a), again using AdgB8 demonstrated that the absolute titers of anti-HSVgB IgG and IgA in vaginal washes vary inversely with each other, with anti-gB IgA levels high at estrus, and anti-gB IgG levels being high at diestrus. In addition, this study showed that mice were susceptible to intravaginal infection with HSV-2 during diestrus (Gallichan and Rosenthal, 1996a). From the earlier observation that systemically immunizing mice resulted in short-term protection from mucosal HSV-2 challenge, but not long term protection even though HSV-specific CTL were detectable in the spleen (Gallichan *et al.*, 1993), Gallichan and Rosenthal (1996b) examined CTL memory responses in splenic and genital associated lymphoid tissue (iliac lymph nodes). Following either IP or INL routes of administration of AdgB8, mice were able to mount anti HSV-2 CTL responses in iliac nodes but after 18 months, anti HSV-2 CTL responses in the iliac lymph nodes could only be generated from INL immunized mice (Gallichan and Rosenthal, 1996b). In contrast, anti-HSV-2 CTL responses in the spleen could only be generated from mice immunized IP (Gallichan and Rosenthal, 1996b). Of importance is the fact that CTL precursor frequencies at 18 months post immunization were comparable to those observed shortly after immunization (Gallichan and Rosenthal, 1996b). These findings were extended to include the distribution of antibody secreting cells (ASC) which resulted in a similar phenomena when it was discovered that ASC were present in the genital tissue of only INL immunized mice rather than IP immunized mice suggesting

that only INL immunization provided long-term protection (Gallichan and Rosenthal, 1998).

Unlike the thorough characterization of immune responses induced by AdgB constructs, only one group has reported the use of other HSV glycoprotein adenovirus recombinants as vaccines. Zheng *et al.* (1993) examined the immunogenicity of an adenovirus type 5 recombinant with one to four tandem repeats of a linear neutralizing epitope of HSV-1 gD fused with a beta-galactosidase protein. Mice immunized by IP injection developed anti gD antibodies as measured by ELISA and HSV-1 neutralization assays. Interestingly, the antibody titre induced by a single administration increased with the number of gD repeats in the recombinant. Animals were also protected from a lethal challenge of HSV- 2 delivered by IP injection. It was observed that vectors carrying four repeats of the HSV epitope were as effective as the entire HSV gD protein in generating an antibody response or eliciting protection against HSV-2 challenge. These findings suggest that adenoviral constructs containing appropriate tandem repeats may be useful in the development of vaccines against HSV infection (Zheng *et al.*, 1993)

In summary, adenovirus based vaccines are a safe and effective delivery vehicle for foreign genes for gene therapy, immunotherapy and as vaccines. They can induce humoral, cellular and mucosal responses against herpes and other pathogens and can be delivered by non-parenteral administration. In short, recombinant adenovirus vectors have important roles in modern medicine and immunology.

1.2.3. Dendritic Cell Based Vaccines

1.2.3.1. Background

Dendritic cells (DCs) were first discovered in 1973 by Steinman and Cohn, who described them as unique cells displaying a veiled morphology. Since then, they have been found to be instrumental for the stimulation of B (Tew *et al.*, 1997) and T lymphocytes (Steinman *et al.*, 1988) and in immune surveillance (Neutra *et al.*, 1996; Sedgwick, 1995). Until recently, it has been difficult to study DCs because of a lack of specific cell markers (Aager *et al.*, 1990) and technical difficulties involved with their isolation (Howard *et al.*, 1995; Shortman *et al.*, 1995). New, more efficient methods are now in place for the isolation and expansion of DCs *in vitro* from DC progenitors (Caux *et al.*, 1992; Inaba *et al.*, 1992; Romani *et al.*, 1994; Romani *et al.*, 1996; Szabolcs *et al.*, 1995), generally utilizing GM-CSF and IL-4 as culture supplements (Bender *et al.*, 1997; Sallusto and Lanzavecchia, 1994), to induce differentiation. It has been due to these advances that a massive increase in the use of DCs as a "natural adjuvant" has occurred. DCs combined with immunogens have been used to treat a variety of cancers (Mayordomo *et al.*, 1997), and more recently using virally transduced (Brossart *et al.*, 1997) or transfected (Condon *et al.*, 1996a) DCs as adjuvants for viral or DNA-based vaccines, respectively.

1.2.3.2. Dendritic Cells an Overview

DCs are capable of performing different functions dependent upon their stage of

maturity (Chapuis *et al.*, 1997). Naive DCs specialize in capturing and processing antigens, presenting them in the context of MHC (for review, see Steinman and Swanson, (1995)). This is achieved by several mechanisms including phagocytosis (Inaba *et al.*, 1993; Reis *et al.*, 1993; Svensson *et al.*, 1997), macropinocytosis (Sallusto *et al.*, 1995) and receptor-mediated endocytosis facilitated by the macrophage mannose receptor (Sallusto *et al.*, 1995), the DEC-205 receptor (Jiang *et al.*, 1995), and the Fcγ and Fcε receptors (Sallusto *et al.*, 1995). Mature DCs, as mentioned, are capable of stimulating B or naive T cells. There are no distinguishing markers for mature DCs, but rather the quantity of surface markers, such as MHC and MHC complexes, are higher on mature versus immature DCs (Inaba *et al.*, 1997). DCs can be generated from blood monocytes if cultured in the appropriate cytokine environment, but the original haematopoietic progenitor is located in the bone marrow. These CD34+ bone marrow progenitors give rise to two DC populations, epidermal Langerhan cells (LC) or interstitial type DCs (Caux *et al.*, 1996; Strunk *et al.*, 1997). Functionally, only the interstitial DC is capable of stimulating naive B cells to generate antibodies (Caux *et al.*, 1997). Another recently characterized subset of DCs lacks some specific myeloid markers (for review see Saunders *et al.*, (1996)) and has been implicated in the induction of tolerance (Suss and Shortman, 1996). The signals governing the selective localization of DCs are not known, however they are influenced by both cytokines and chemokines, and can be induced to localize *in vivo* after treatment with lipopolysaccharide (LPS) (MacPherson *et al.*, 1995;

Roake *et al.*, 1995). Following activation by a pathogen, DCs travel to secondary lymphoid organs where they mature (Kitajima *et al.*, 1996). Here the DCs may stimulate lymphocyte proliferation or chemo-attract B or T cells (Adema *et al.*, 1997).

1.2.3.3. Preparation, Delivery and Immunogenicity of Dendritic Cell Based Vaccines

The first empirical evidence that DCs would be superior to the adjuvants currently in use with traditional peptide based vaccines came from Porgador and Bilboa (1995), who found that immunizing mice with DCs pulsed with ovalbumin (OVA) induced a potent CD8+ immune response which was superior to the response generated with OVA and incomplete Freund's adjuvant. More recently the strategy of peptide pulsed DCs have been used as anti-cancer therapy (McArthur and Mulligan, 1998; Nestle *et al.*, 1998), and as a potential vaccine candidate for HIV (Sandberg *et al.*, 1998).

Another method of generating DC based vaccines, relies on the delivery of *in vitro* transduced DCs (Bronte *et al.*, 1997; Cherrie *et al.*, 1992; Smith *et al.*, 1996). In general, after the isolation and preparation of a source of DCs, the DCs are infected with a virus, usually an adenovirus-based vector (Brossart *et al.*, 1997), with multiplicity of infections (MOI) ranging from 1 (Brossart *et al.*, 1997) to 100 (Lee *et al.*, 1997). The viral transduction of DCs has been applied to generate OVA responses in mice (Brossart *et al.*, 1997), antitumor immunity (Gong *et al.*, 1997; Lee *et al.*, 1997; Ribas *et al.*, 1997; Wan *et al.*, 1997), and to generate anti-adenovirus CTL in mice (Smith *et al.*, 1996).

The last method of generating DC-based vaccines utilizes transfection of DCs with plasmid DNA. The combination of DNA-based vaccines and DCs is a logical one considering the role of the DCs in the antigen presentation of DNA vaccines (Casares *et al.*, 1997; Condon *et al.*, 1996a; Doe *et al.*, 1996a; Iwasaki *et al.*, 1997). Briefly, DCs are expanded from PBMCs and then transfected *in vitro* with plasmid DNA, generally with the use of a liposomal complex, and delivered to the vaccine recipient (Manickan *et al.*, 1997). Using this strategy, effective immune responses have been generated against herpes virus (Manickan *et al.*, 1997; Morhenn, 1997). In addition, this approach has been successful as an anti-tumour therapy (Sharma *et al.*, 1997; Tuting *et al.*, 1997).

All DC based vaccines to date have been administered parenterally, however the route of administration varies. Groups have used IM (Sharma *et al.*, 1997; Tuting *et al.*, 1997), IP (Paglia *et al.*, 1996), SC (Brossart *et al.*, 1997; Sharma *et al.*, 1997) or IV (Mayordomo *et al.*, 1995) injections to deliver DCs. There has not been a study to date comparing the different routes of immunization or their resulting effects on immune responses.

The immunogenicity of DC based vaccines rely heavily on the professional antigen presenting characteristics of DCs. In the case of peptides, the dendritic cell can capture exogenous antigens for processing through the MHC class II pathway to MHC class II-rich compartments (MIICs) (Sallusto and Lanzavecchia, 1994; Winzler *et al.*, 1997). Viral or DNA-mediated delivery of antigens utilizes the classical MHC I or the cytosolic-independent "alternate" MHC class I pathways (Lanzavecchia, 1996) to directly stimulate

naive T cells (Bender *et al.*, 1995; Bhardwaj *et al.*, 1994; Steinman *et al.*, 1988). *Ex vivo* prepared DC behaviour after administration has only been characterized for intratracheal (IT) administration (Havenith *et al.*, 1993) and IV administration (Austyn, 1996). The former mode of administration results in DCs localizing in draining lymph nodes of the lung of mice (Havenith *et al.*, 1993), whereas IV administration results in early distribution to the marginal areas of the spleen, and eventually T cell areas of mice (Austyn, 1996). Previous studies suggest that DCs administered by SC or IM routes will localize to the draining lymph nodes via the afferent lymphatics (Austyn, 1989; Kupiec-Weglinski *et al.*, 1988). Regardless of the mode of administration, DCs are capable of sensitizing T cells (Steinman *et al.*, 1988) and B cell responses (Tew *et al.*, 1997).

1.2.3.4. Generation of Herpes Immunity with Dendritic Cell Based Vaccines

To date, only two studies have examined the ability of DCs to induce anti-HSV immunity and both have used DNA transfection to generate their DC-based vaccine (Manickan *et al.*, 1997; Rouse *et al.*, 1994). Rouse and colleagues (1994), in an attempt to optimize results obtained by direct DNA immunization, used DCs or macrophages transfected with plasmid DNA containing the immediate-early protein (ICP27) of HSV-1. The transfected DCs administered IM generated CTL much more efficiently than transfected monocytes and were primarily of the CD8+ phenotype (Rouse *et al.*, 1994). Furthermore, ICP27 transfected DCs alone were capable of stimulating CTL *in vitro* at

low effector to target ratios, whereas ICP27 DNA alone was only effective at higher effector to target ratios. Manickan and colleagues (1997) expanded on their earlier findings by including the use of HSV-1 gB and ICP27 expression plasmids. Although DNA vaccines containing gB or ICP27 gene inserts were able to provide protection against low dose challenge with HSV, only transfected DCs matched the levels of protection generated with an attenuated herpes vaccine at low and even moderate challenge doses (Manickan *et al.*, 1997). In addition, the peak expression of gB from the transfected DCs occurred 24 hours post-transfection as determined by RT-PCR (Manickan *et al.*, 1997). While the DC-gB vaccine generated neutralizing antibody at levels 10-fold greater than that obtained with gB-DNA alone, this represented only half the level of neutralizing antibody generated with the attenuated HSV vaccine (Manickan *et al.*, 1997). While all of the vaccines generated similar levels of IFN-γ and IL-2 production, lymphoproliferation and delayed type hypersensitivity reactions, only the attenuated HSV vaccine generated detectable levels of IL-4 from spleen cells in culture re-stimulated with HSV-1 (Manickan *et al.*, 1997). The authors concluded, based on their findings, that the enhanced immune responses generated with DC-delivered DNA versus direct DNA vaccine administration appear to be associated with an increased Th1 CD4+ T cell response (Manickan *et al.*, 1997).

In summary, with improved culture techniques to isolate and expand DCs from progenitor cells *in vitro*, DCs have valuable application to vaccine technology. Of further

interest, especially with regard to development of HSV vaccines, is the finding that only DCs can mediate immunoglobulin class-switching of T cell-activated B cells to produce dimeric IgA (Fayette *et al.*, 1997). This fact, taken together with the high efficiency of adenovirus vectors in gene transfer to DCs (Brossart *et al.*, 1997) and the ability of adenovirus to generate IgA responses to HSV (Gallichan *et al.*, 1993; Gallichan and Rosenthal, 1995b), suggests that a marriage of the two technologies may provide the best vaccine strategy to date in the search for a HSV vaccine.

1.3. Outline and Purpose of this Study

The initial objectives of this study were to examine the ability of DNA immunization to induce a mucosal immune response following mucosal delivery, to compare the responses generated from the DNA immunization to the mucosal responses generated with AdgB, and to examine the effect of combined immunization with AdgB and gB-DNA on mucosal immune responses. Upon investigation, it became apparent that gB-DNA alone was unable to generate mucosal antibody responses, consistent with the findings of Bourne *et al.* (1996a). Indeed, it was later demonstrated by Kuklin *et al.* (1997) that it was necessary to utilize cholera toxin as an adjuvant to generate mucosal immunity with a gB-DNA based DNA vaccine which was still highly variable and generally poor. The objectives of the study were therefore redefined to assess the variability and degree of antibody responses in mice to different dosages of a gB-DNA

vaccine delivered systemically. During early course of the comparative studies it was also necessary to identify the optimal method of generating mucosal antibody responses to AdgB. Since the initial studies examining intranasal immunization of mice with AdgB (Gallichan *et al.*, 1993), the NALT of mice had been characterized in mice (Asanuma *et al.*, 1995). Furthermore, it had been reported that upon INL immunization, IgA specific antibody forming cells (AFCs) were detectable in the NALT, suggesting that IgA was produced locally and was best obtainable by the direct local stimulation of NALT cells (Heritage *et al.*, 1997). Based upon these reports the hypothesis was generated that direct stimulation of NALT cells with AdgB should be sufficient to generate potent systemic and mucosal antibody responses which should be equivalent to responses induced with the same dosage distributed between multiple inductive sites. As previous studies which utilized INL administration of AdgB were performed on asleep animals (Gallichan *et al.*, 1993), it is possible that virus was distributed to the upper and lower airways, thus clouding the issue of whether the NALT was the sole inductive site in their report versus combined sites of induction such as the NALT, BALT and lungs of mice. To test this hypothesis, mice were immunized awake or asleep with the same dosages of AdgB and systemic and mucosal antibody responses were measured. The difference in the method of viral administration was based upon the theory that mice immunized awake limit virus distribution to the upper respiratory tract, as a result of intact gag reflexes and the ability to swallow fluid. In contrast, mice immunized asleep do not have an active gag reflex

which may result in mice inhaling fluid into the lower respiratory tract. Lastly, a collaborative opportunity arose to examine the role of AdgB transduced DCs in the induction of systemic and mucosal immune responses via airway mucosa and systemic transfer via lateral tail vein injection.

Table 1.2.1. Properties of DNA immunization vectors.

1. Plasmid vectors can be constructed and tested rapidly.
2. Rapid and large-scale manufacturing procedures are available.
3. DNA is more temperature stable than live vaccine preparations.
4. Microgram quantities of expression vector can induce immune responses.
5. DNA vaccines can induce humoral and cellular responses.
6. Studies demonstrate that protection can be achieved in large primate models of human infection.
7. Multiple vectors encoding several antigens can be delivered in a single administration.

Modified from (Chattergoon *et al.*, 1997)

Table 1.2.2. T-cell help associated with different methods of DNA delivery and forms of DNA-expressed antigens

	Form of DNA-expressed antigen		
Method of Delivery	Intracellular	Plasma Membrane	Secreted
Gene Gun	Th2(Pertmer *et al.*, 1996)	Th2 (Feltquate *et al.*, 1997)	Th2(Robinson and Torres, 1997)
i.m. saline injection	Th1 (Babiuk *et al.*, 1995; Hsu *et al.*, 1996; Pertmer *et al.*, 1996)	Th1 (Cardoso *et al.*, 1996; Feltquate *et al.*, 1997; Lekutis *et al.*, 1997; Manickan *et al.*, 1995a; Xiang *et al.*, 1994)	Th2 to Th1 (Klinman *et al.*, 1995), Th2 (Cardoso *et al.*, 1996; Waisman *et al.*, 1996)

Th1, T-Helper 1 biassed response; Th2, T-Helper 2 biassed response. The type of the T-helper response has been assigned based on the Ig subclasses of the specific antibody (studies in mice) or on lymphokine production by antigen-restimulated splenocytes (studies in mice and macaques). With the exception of responses raised by i.m. injections of DNA-expressed secreted antigens, the type of the raised T-cell help appears to be stable with time. I.d. injections of DNA in saline have raised more variable patterns of T-cell help. Taken from Robinson and Torres (Robinson and Torres, 1997).

Table 1.3.1. Properties of adenovirus vaccine vectors.

1. Adenoviral vectors can be constructed and tested rapidly.
2. Adenoviruses can be purified in large quantities and highly concentrated.
3. Adenovirus vaccines can induce humoral and cellular responses.
4. Protection can be achieved in large primate models of human infection.
5. Using multiple site inserts up to 8.3 kb of foreign DNA may be inserted.
6. Multiple vectors encoding several antigens can be delivered in a single administration.
7. Adenovirus vaccines are not particularly pathogenic in humans.
8. There is a history of safety in human applications.
9. Adenoviral vectors are capable of generating mucosal immunity and protection.

II. MATERIALS AND METHODS

2.1 Production of Plasmids

2.1.1. Bacterial culture growth

Recombinant plasmids were maintained in *Escherichia coli* (*E. Coli*) strain DH5a (Woodcock *et al.*, 1989) or HB101 (Bolivar and Backman, 1979; Boyer and Roulland-Dussoix, 1969). Bacteria were grown in Luria-Bertani medium (LB) (.5% w/v yeast extract, 1.0% NaCl, pH 7.0) (GIBCO Laboratories, Burlington ON) and plated on LB agar (LB containing 1.5% w/v Bacto agar) (GIBCO) for isolation of individual colonies. Ampicillin was used for plasmid selection at a concentration of 100 μg /ml in LB and LB agar.

2.1.2. Transformation of *E. coli* by Plasmid DNA

0.005-0.01 μl of plasmid DNA was added to 0.1 ml of competent bacteria in a 1.5 ml eppendorf tube. The mixture was incubated on ice for 15-30 minutes, transferred to a 37°C water bath for 1 minute and then incubated on ice again for 5 minutes. 1 ml of LB was added to the tube and the mixture was incubated with shacking at 37°C for 1 hour. Bacteria were then pelleted by brief centrifugation and the supernatant decanted. Bacteria were resuspended in the remaining liquid and spread onto LB agar plates containing the appropriate antibiotic. Plates were incubated overnight at 37°C and examined 12-20

hours later for resistant colonies.

2.1.3. Qiagen large scale DNA preparation

Qiagen DNA preparations were performed using a Qiagen kit (Qiagen, Valencia, CA) following the manufacturers' instructions. Briefly, a Qiagen-tip 500 DNA isolation column was equilibrated with 10 ml of buffer QBT (50 mM MOPS, pH 7.0, 750 mM NaCl, 15% EtOH, 0.15% Triton X-100). Following growth, bacteria were spun down and the pellet was resuspended in solution P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, 100 ug/ml RNaseA) and solution P2 (200 mM NaOH, 1% SDS) incubated at room temp 5 min and then solution P3 is added (3.0 M potassium acetate, pH 5.5) to prepare bacterial lysate. Bacterial lysate was added to the pre-equilibrated column and the column eluate was discarded. The column was then washed with 30 ml of buffer QC (50 mM MOPS, pH 7.0, 1.0 M NaCl, 15% EtOH). Plasmid DNA was eluted via the addition of 15 ml QF (50 mM Tris, pH 8.5, 1.25M NaCl, 15% EtOH). The eluted DNA was precipitated by the addition of 12 ml isopropanol, vortexing and centrifugation at 10, 000 rpm for 15 min at 4°C. The pellet was washed with 70% EtOH, centrifuged at 100000 rpm for 5 min and the supernatant was discarded. The pellet was air dried for 10 min. The pellet was then resuspended in TE or appropriate buffer for quantification.

2.1.4. Quantification and Quality of DNA

Plasmid preparations used for cloning were quantitated by measuring absorbance at 260 nm ($1OD_{260}$ = 50 mg/ml double stranded DNA). Purity of the plasmid was checked by determination of OD_{260}/OD_{280} and by gel electrophoresis. Briefly, 10 X DNA loading dye (50% glycerol, 0.4% xylene cyanol, 0.4% bromophenol blue, 1 mM EDTA, 1 ug/ml ethidium bromide) was added to DNA to a final concentration of 1 X and loaded onto 1% agarose TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) gels. Gels were run at 10 V/cm in 1 X TBE buffer. Upon completion of the gel run, gels were visualized by placing them on a UV transilluminator (Bio-Can, Mississauga, ON) and photographed.

2.2 Animals and cell cultures.

Female C57Bl/6 (H-2^b) mice were used in all studies and were 6 to 8 weeks of age during primary immunization. Mice were obtained from Charles River Laboratories, Constant, Quebec, Canada. Mouse colonies were maintained on a 12 hour light/dark cycle. All animal handling was performed in accordance with the national research guide for the care and handling of laboratory animals.

Vero, MC57 and SVBALBcells were grown in a-MEM (GIBCO), supplemented with 10% fetal calf serum (FCS; GIBCO), 100 ug/ml penicillin, 100 ug/ml streptomycin, 100 ug/ml L-glutamine (GIBCO) and 1% HEPES (GIBCO). 293 cells were grown in MEM-F11 (GIBCO) and supplemented as above. Cells were passaged three times per week at

confluence in 75 cm^2 tissue culture flasks. When confluent, growth media was removed by aspiration and the monolayer was washed with phosphate-buffered saline (PBS) (0.88 mM KH_2PO_4, 6.41 mM Na_2HPO_4, 137 mM NaCl, 2.68 mM Kcl, pH 7.3). Monolayers were removed by the addition of trypsin-EDTA (GIBCO) at 37°C for 2-5 min. Cultures were tapped to dislodge remaining cells from the surface and the cells were resuspended in the appropriate growth media supplemented as described above. Cells were quantitated by trypan blue exclusion.

293 N3S cells are a non-adherent cell line derived from 293 cells and were grown in spinner flasks with Joklit's media supplemented as described above. 293 N3S cells were maintained at a density of $1x10^5$ cells/ml. Dendritic cell (DC) preparations were kindly provided by Dr. Yonghong Wan (Department of Pathology, McMaster University). Briefly DCs were isolated from bone marrow from the femur of mice aged 6-8 weeks and grown in medium containing 1 ng/ml of murine granulocyte-macrophage colony stimulating factor (GM-CSF) and 1 ng/ml murine recombinant interleukin-4 (rIL-4) (R&D Systems, Minneapolis, MN) in 24 well plates at 1-2 x 10^6 cells / well. DCs were selected by removing non-adherent cells without dislodging clusters of developing DCs that attached loosely to a monolayer of adhered macrophages. Previously reported flow cytometric analysis of DCs released from the clusters at day 6 following this type of culture indicated that the DCs expressed CD45, CD80, CD86 and CD11c (mouse DC-restricted marker) and class I and class II MHC antigens (Wan *et al.*, 1997). DCs were

used for studies following 6 days of these culture conditions.

2.3. Virus strains

The constructs of the replication-competent recombinant adenovirus type 5 vector (AdgB8) and the AdE3- control virus, have been described previously (Haj-Ahmad and Graham, 1986; *Hutchinson et al.*, 1993). Briefly, AdgB8 contains the gB gene from HSV-1 coupled to the simian virus 40 (SV40) promoter and is inserted into the E3 region of human adenovirus type 5. The construct of recombinant adenovirus type 5 luciferase vector (AdLUC) vector has also been described elsewhere (Addison *et al.*, 1997). Briefly, the firefly luciferase gene coupled to the SV40 promoter was inserted into the E1 region of human adenovirus type 5 genome. Recombinant adenoviruses were grown by infecting 293-N3S cells at a density of $2x10^5$ cells/ml with an multiplicity of infection (MOI) of 20-50. The cell pellet was obtained by centrifugation, disrupted using two cycles of freeze-thaw and resuspended in 20 ml of solution 1 (10 mM Tris, 0.5% sodium deoxycholate, 20 mM $MgCl_2$ and .5 mg DNAse). Saturated CsCl solution was added to a final density of 1.34 g/ml. Virus was obtained from this solution by ultra-centrifugation and the viral band was collected. Virus was purified twice on CsCl gradients, dialyzed in TRIS buffer to remove CsCl, and titred on 293 cells. The vaccinia vector expressing gB of HSV-1 (VacgB11) was kindly provided by B. Moss (NIH, Bethesda, MD) and its construction has been previously described (Cantin *et al.*, 1987). VacgB11 was grown on

KB cells, isolated by three cycles of freeze-thaw followed by ultrasonification and centrifugation, and subsequently titered on KB cells.

2.4 Immunization of Mice

For AdgB8 immunizations performed asleep, mice were anesthetized using isoflourane (International Medication System Ltd., Bethlehem, PA), inverted and inoculated INL with indicated doses of AdgB8 by means of a micropipet, and placed in dorsally recumbent position for recovery. For INL immunization awake, mice were physically restrained by the scruff of their neck, held vertically, and inoculated with the indicated dose of AdgB8 with a micropipet. AdGB8 was introduced in a volume of 0.02 ml of phosphate buffered saline (PBS, pH 7.4) distributed equally between each nares.

For DNA immunization with pSH210-gB (gB-DNA), mice were lightly anaesthetized with isoflourane and their lower legs were shaved with a Wahl hair clipper (Wahl, Burlington, ON). gB-DNA was administered by means of an insulin syringe in the tibialis anterior muscle at a depth of approximately 2 mm. Injection depth was maintained by using a needle-gaurd constructed with polyethelene tubing (VWR, Mississauga, ON).

In vitro infected DCs were administered via lateral tail vein injection. Briefly, mice were restrained and their tails swabbed with alcohol and dipped in sterile warm water to promote vasodilation. DCs were introduced using insulin syringes in a volume of 0.3 ml

(Beckson-Dickenson).

2.5. Collection of fluids

For blood collection, mice were anaesthetized using isoflourane (International Medication Systems Ltd.) and a Natelson heperanized blood collecting tube was used to puncture the medial orbital plexus of the eye (GIBCO). Blood was spun in a microcentrifuge tube and plasma was collected and stored short term at -20°C, or long-term at -70°C. Nasal lavages were obtained by sacrificing the animal and washing the nasal cavity by revealing the trachea, making a small incision and inserting PE-20 tubing (VWR, Mississauga, ON) attached to a syringe containing 0.3 ml of PBS (pH 7.4). The PE tubing was gently guided into the nasal cavity, and care was exercised to obtain a pooled sample from both nares. Recovery of PBS ranged from 0.25-0.29 ml of PBS. Samples were collected individually, spun in a microcentrifuge, the supernatant collected and stored at -20°C until analysis. Lung lavages were similarly obtained by inserting the PE tubing in the trachea toward the lung. 0.3 ml of PBS was gently pushed into the lungs and slowly extracted. Recovery of PBS ranged from 0.1 to 0.25 ml. Samples which by visual examination were contaminated with blood were discarded in an attempt to obtain lavage fluid from mucosal surfaces only. Samples were spun in a microcentrifuge, the supernatants collected and stored at -20°C for analysis. Gut washes were performed by dissecting out the small intestine, washing the outside surface of the intestine and

inverting the intestine on forceps. The section of intestine was then incubated with 0.5 ml of a gut wash solution (PBS + 100 mM PMSF, 0.5 M EDTA, 0.5 U Aprotenin). Tissue weights were recorded, intestinal sections were removed post-incubation and the supernatant was collected following two rounds of centrifugation.

2.6 Antibody Determination

HSV gB-specific antibody titres were determined by ELISAs performed in flat-bottomed microtiter plates (Costar, Cambridge, MA). Plates were precoated with 2.5 ug/ml of recombinant HSV-2 gB (kindly provided by R.L. Burke, Chiron, Emeryville, CA) in borate-buffered saline (BBS) (0.15M NaCl, 0.02% Azide, 0.05M Boric Acid, 4 mM Borax solution, pH 8.5), and stored overnight at 4°C. A TRIS-buffered saline (TBS) solution (0.01 M Tris, 0.15 M NaCl, 0.02% Azide, pH 7.4) containing 10 mg/ml bovine serum albumin, was used to block any plastic not precoated with gB protein. Serially diluted samples of either test or control sera were added and the plates were incubated overnight at 4°C. This was followed by repeated washing with a 0.05% Tween 20-TBS (Sigma, St. Louis, MI) solution. Biotin-labelled goat anti-mouse IgG or IgA antibody (Southern Biotechnology Associates, Birmingham, AL) was added and the plates were incubated at room temperature for 4 hours. Plates were once again washed with TBS - tween 20 solution. 0.1 ml of alkaline phosphate (ExtrAvidin; Sigma, St. Louis, MI) was added to each well as the labelling reagent according to manufacturer's instructions at a

1:70 000 dilution in 1 mg/ml BSA-TBS. Plates were incubated with labelling reagent for 2 hours, then washed repeatedly with tween-20-TBS solution. The substrate, p-nitrophenyl phosphate (1 mg/ml)(Sigma, St. Louis) was then added and the plates were incubated for 30 min. The ELISAs were then read using a Titretek Multiscan Plus plate reader (Titretek, AL) at 405 nm and optical densities were recorded (OD). Antibody titres represent the inverse dilution of the sample at which 2 times the background absorbance of control fluid was reached.

2.7 Cytotoxic T-lymphocyte (CTL) assays

Spleen effector cells were prepared by teasing the tissues through a stainless steel grid. Splenic CTL were examined following secondary *in vitro* stimulation as follows; isolated spleen cells were incubated for 6 days with gamma-irradiated (5000 rad), AdgB8-infected, syngeneic MC57 cells, at an effector:stimulator ratio of 166:1 in RPMI 1640 medium (supplemented as above). Following stimulation, splenic effector cells were incubated with Vac gB11 infected (MOI - 10, 16 h infection period), syngeneic (MC57) or allogeneic (SVBalb) targets at effector to target ratios of 80:1, 40:1, 20:1, and 10:1 in a 6h ^{51}Cr release assay. Data are expressed as percent specific lysis - 100 x [(experimental cpm - spontaneous cpm) / (maximum release cpm - spontaneous release cpm)].

2.8. Luciferase Assays

Levels of luciferase expression were obtained by using a Promega luciferase assay kit (Promega, Madison, WI). Nasal tissue was isolated from the mice by dissecting the area of the nose from the mouse as previously described, but with modifications (Asanuma *et al.*, 1997). Briefly, the skin was reflected from the head of the mouse, the nose was removed including the incisors with scalpel and forceps. Using scissors, the rostral portion of the nasal cavity, including the hard palate was removed forward from a transverse line demarcated by the eyes and soft palate. Any lymphoid tissue which remained in the sinus bodies under the eyes was removed with forceps and included in the tissue. Trachea and bronchial associated lymphoid tissue was removed by careful dissection of the trachea, from the larynx to the bifurcation of the trachea at the lung where bronchial associated lymphoid tissue was recovered. Lungs were removed by carefully dissecting out the heart and pulmonary arteries, severing and removal of the esophagus and collection of each lobe of the lung. All tissues were collected in PBS (pH 7.3). Tissues were then weighed and transferred into 2ml of assay buffer (45 mM potassium phosphate buffer, 4.5 mM DTT, .5 mg/ml Bovine serum albumin, 5mM Tris, 0.5 mM EDTA, pH 7.8). Tissues were then homogenized using a tissuemeizer (IKA Labortechnik, Staufer, West Germany). After samples were spun at 1200 rpm for 15 min. 0.02 ml of sample supernatant was transferred to a luminometer tube (Sarstedt) containing 0.18 ml of assay solution (25mM glycyl glycine, pH 7.8, 15 mM $MgSO_4$, 5

mM adenosine tri-phosphate (ATP)), to which 0.1 ml of luciferin solution was added (0.19 mM luciferin in 25 mM glycly glycine, pH 7.8). Luciferase activity was measured using a luminometer (Lumat LB 9501 Luminometer, Berthold Systems Inc., Pittsburgh, PA). Measurements are expressed in relative light units.

2.9. Statistical Analysis

Data were analyzed using the GraphPAD InStat program (Graph PAD Software, San Diego, CA). For comparisons between 2 groups, data were analyzed by Student's t test of Fisher's exact test as appropriate. Comparison among the means of multiple groups was carried out using analysis of variance (ANOVA). Bonferroni's P value was used as the test statistic to adjust for multiple comparisons between groups in some ANOVA.

III. RESULTS

3.1. Characterization of antibody and CTL responses to a DNA based vaccine encoding for the gB protein of HSV-1 delivered by IM or IN routes.

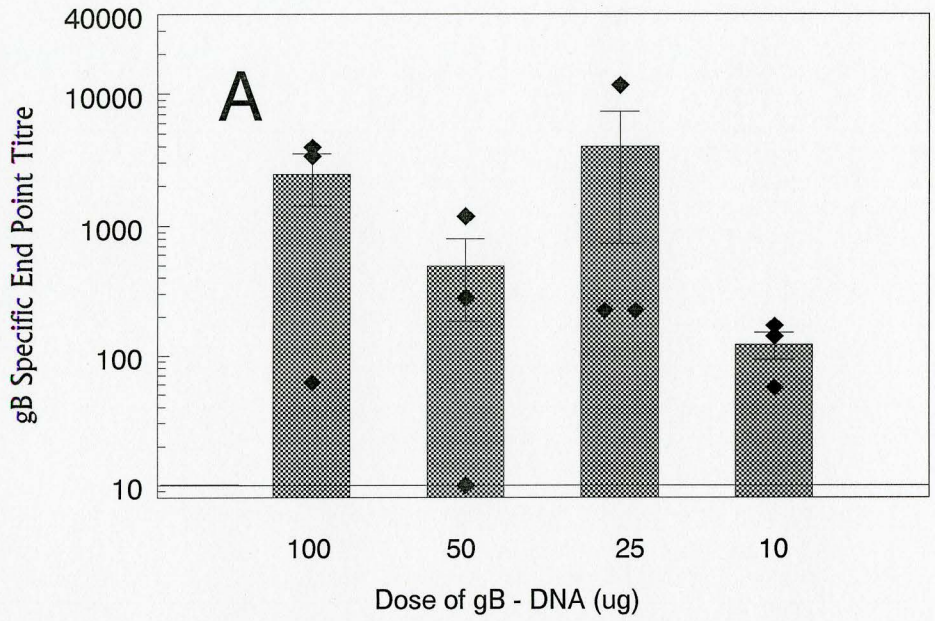
3.1.1. *Determination of the dose-dependent antibody response to plasmid DNA encoding gB of HSV-1 in mice, delivered by IM injection.*

It was previously demonstrated that plasmid DNA encoding HSV-1 gB (gB DNA) could generate antibody responses in mice (Manickan *et al.*, 1995a), however dose response relationships to a single administration of gB DNA have to date not been reported. Mice were immunized once by IM injection and the levels of gB IgG were determined in plasma two (Figure 3.1.1. A) and four (Figure 3.1.1. B) weeks post-immunization. From the results, it was apparent that gB DNA elicited antibody responses over a wide range of doses, however the responses were not dose-dependent. Although the lowest IgG responses were seen with 10 μg of gB DNA. Over the initial time period (Figure 3.1.1. A), antibody levels were detectable in all but one mouse at the 50 μg dose level. By four weeks (Figure 3.1.1. B), all animals had developed antibody responses.

3.1.2. *A second test of dose-dependent antibody response to gB in gB-DNA immunized mice up to six weeks, post immunization.*

To determine if a clearer dose response relationship existed after the four week time point a second set of mice were immunized and antibody responses in plasma were measured up to six weeks post immunization (Figure 3.1.2). Groups of five mice were

Figure 3.1.1. Antibody response to gB-DNA following IM administration. Bleeds were taken two (FIG 3.1.1. A) or four (FIG 3.1.1. B) weeks post immunization. Groups of three C57BL/6 mice were immunized once IM with gB-DNA in dosages ranging from 10-100 micrograms in the tibialis anterior muscle. 14 or 28 days later mice were bleed and plasma was obtained. gB specific IgG titres were determined by ELISA. Titres are expressed as the inverse of the minimum dilution at which absorbance reached two times that obtained for plasma for mice immunized with a control plasmid. Diamonds represent titres of individual mice. Thatched bars represent the mean end point titre, and error bars show standard error of the mean. The horizontal line in figure 3.1.1. A shows the minimum dilution performed in the experiment. Animals whose titres fell below this line were assigned a titre of the lowest dilution for calculation of the mean.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gB Specific End Point Titre
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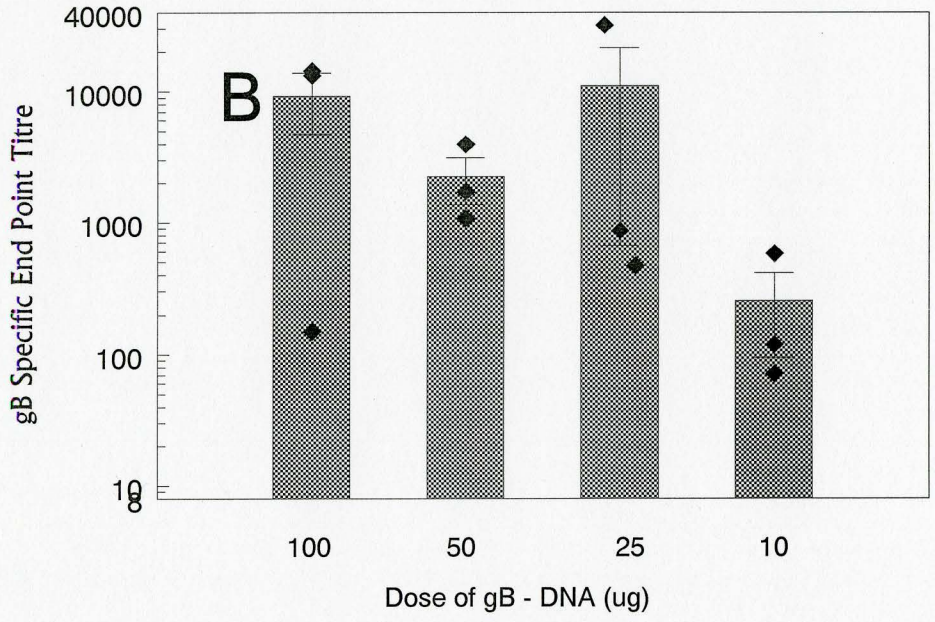
Dose of gB - DNA (ug)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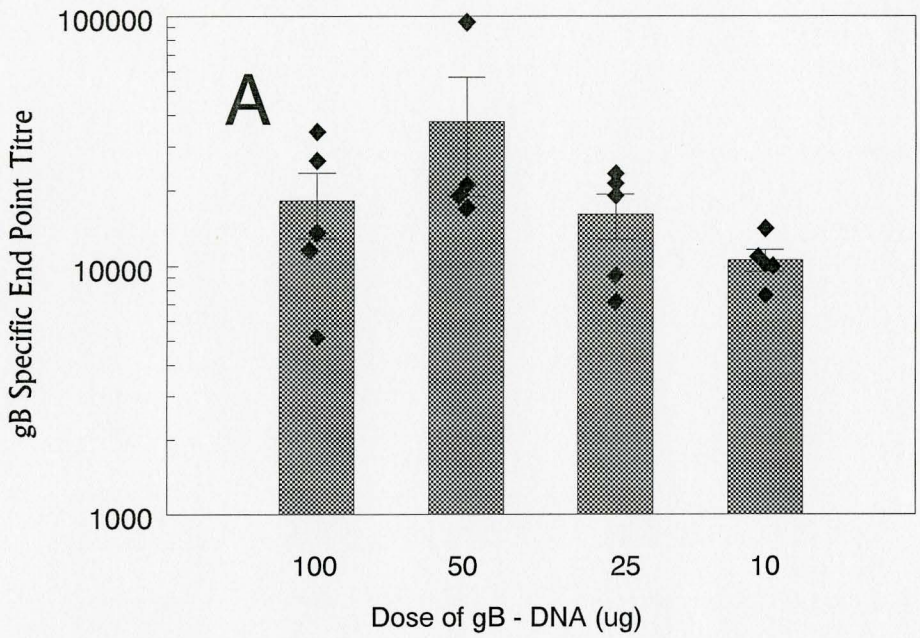
Dose of gB - DNA (ug)

Figure 3.1.2. Antibody response to gB-DNA at four (FIG 2A) or six (FIG 2B) weeks post immunization. Groups of five C57BL/6 mice were immunized as described in figure 3.1.1. and bled 28 or 42 days later. gB specific IgG titres were measured by ELISA and are expressed as described in figure 1. Diamonds represent titres from individual mice. Thatched bars represent the mean end point titre, and error bars are expressed as the standard error of the mean.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gB Specific End Point Titre
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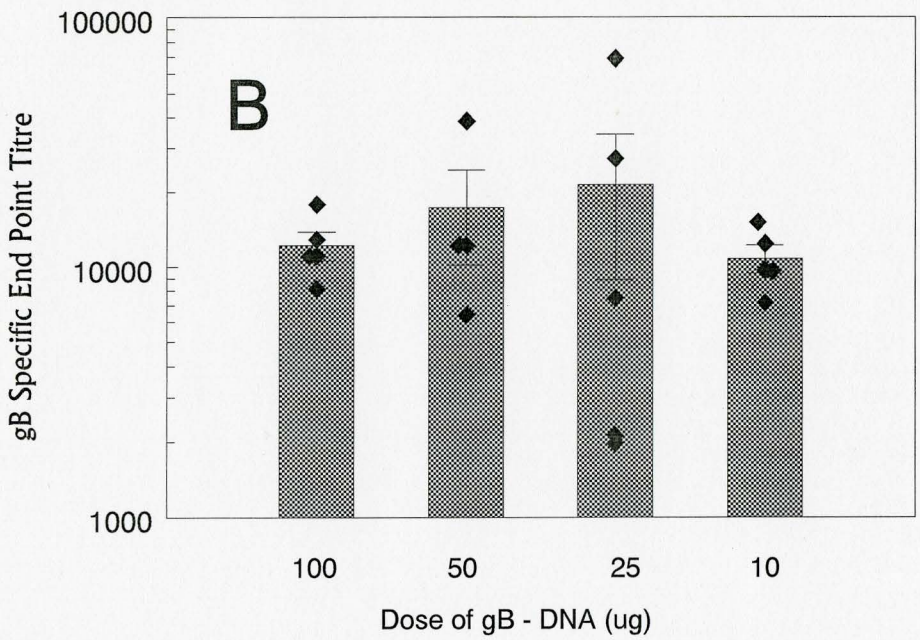
Dose of gB - DNA (ug)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gB Specific End Point Titre
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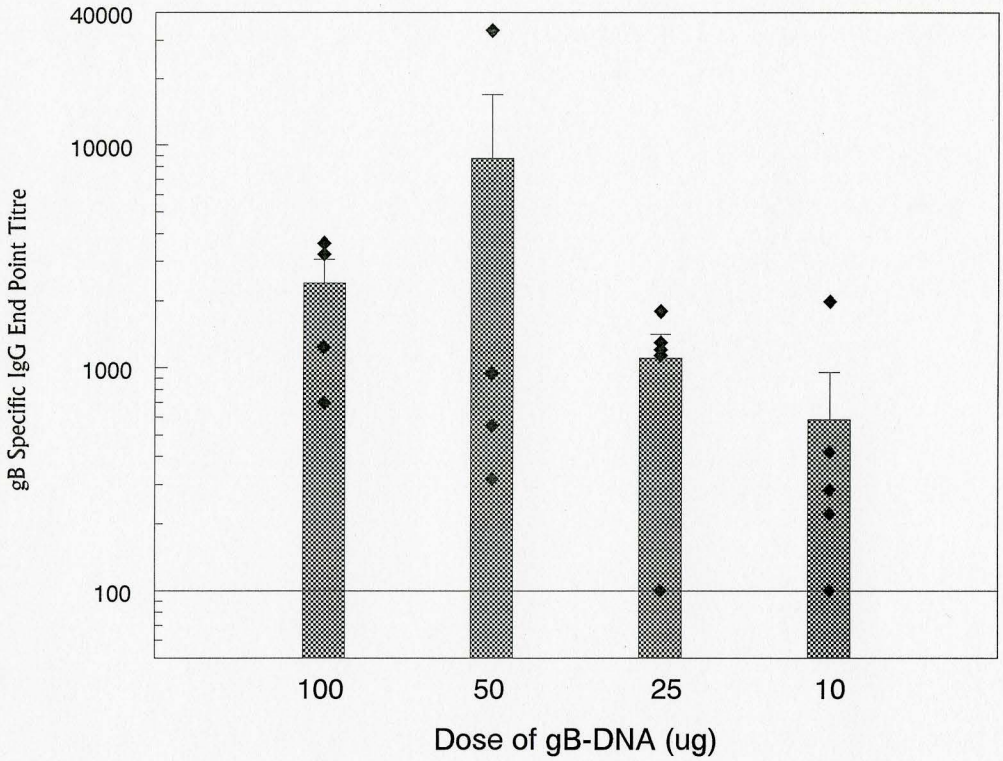
Dose of gB - DNA (ug)

immunized so as to increase the number of data points and more clearly demonstrate a dose response relationship. Consistent with the data in Figure 3.1.1., gB DNA produced antibody responses over an identical range of doses, but there were no significant differences in titres over that range. There was a trend to a dose response relationship at the four week time point as illustrated by the mean antibody titres of doses ranging from 50 μg to 10 μg (Figure 3.1.2. A), however, as before there was significant variability in responses as measured by ANOVA. Any suggestion of a dose response relationship was lost at the six week time point (Figure 3.1.2. B). The variability in antibody titres rose dramatically after six weeks, especially at the 50 and 25 μg dose levels (Figure 3.1.2. B). Collectively these results suggest that there is significant variability in antibody responses to gB DNA and that all doses tested resulted in similar amounts of antibody without an evident dose response relationship.

3.1.3. DNA vaccines are able to generate long-lived antibody responses following a single administration IM.

To determine if mice immunized with a DNA vaccine were able to maintain long-term antibody responses, and how long periods of time affected antibody levels, the same mice reported in Figure 3.1.2. were maintained for 11 months and their gB specific antibody levels were once again measured. Figure 3.1.3. demonstrates that all but 2 animals maintained antibody titres above 100. Of interest is the observation that animals at the 25 μg dosage level which initially demonstrated high variability in antibody

Figure 3.1.3. Antibody responses to gB-DNA following IM administration 11 months post immunization. Groups of five C57BL/6 mice were immunized as described in figure 3.1.2. 11 months later mice were bled and plasma was obtained. gB specific IgG titres were determined by ELISA and are expressed as before. Diamonds represent titres of individual mice. Thatched bars represent the mean end point titre, and error bars are express the standard error of the mean. The horizontal line represents the minimum dilution performed in the experiment. Animals whose titres fell below this line were assigned a titre of the minimum dilution for calculation of the mean.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gB Specific IgG End Point Titre
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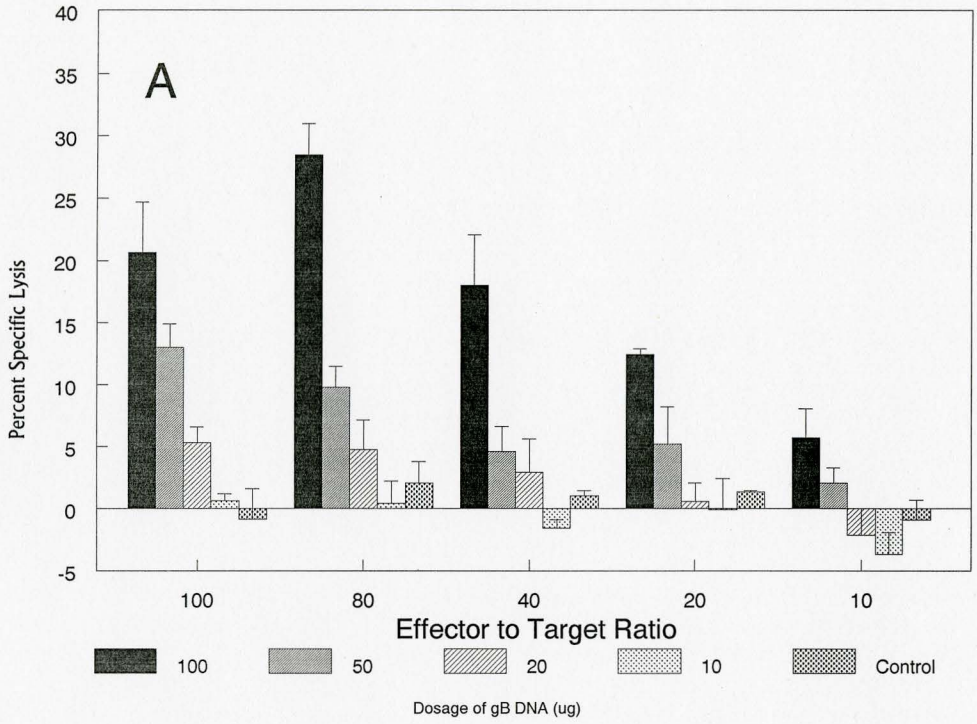
10
Dose of gB-DNA (ug)

responses (Figure 3.1.2. B), demonstrated a tight clustering of antibody levels after an extended period of time (Figure 3.1.3.). Again, no clear dose response relationship existed between antibody levels and dosage of DNA even after an extended period of time. However antibody titres decreased over time.

3.1.4. *DNA vaccination elicits a dose-dependent cell-mediated cytotoxicity response, which is specific and mediated by MHC I restricted effector cells.*

To examine whether a clear dose response existed following DNA vaccine administration, groups of three mice were immunized once by IM injection in the tibialis anterior muscle with doses of gB DNA ranging from 10 to 100 μg. Four weeks post immunization spleens were removed from each group and splenic lymphocytes were isolated and pooled (see methods). After six days in culture with syngeneic AdgB infected stimulator cells, splenic lymphocytes were then examined for their ability to lyse Vac gB expressing target cells, in a standard 6h ^{51}Cr release assay (see methods). Figure 3.1.4. A demonstrates that a clear dose response relationship exists between CTL and the dosage of DNA vaccine for effector to target rations from 80 to 10:1, particularly at the 100 μg of DNA dose. This relationship is not as clear at dosage levels less than 50 μg, as the levels of CTL approach those obtained in the non-specific lysis of uninfected MC57 syngeneic target cells (Figure 3.1.4. B). It is evident from examining the low levels of non-specific lysis of MC57 target cells (Figure 3.1.4. B) that killing in this assay (Figure 3.1.4. A) is specific for gB. Additionally, since in Figure 3.1.4. C target cells which were

Figure 3.1.4. Systemic CTL responses specific for HSV-1 gB. Groups of 3 C57Bl/6 mice were immunized by IM injection with gB DNA in dosages ranging from 100 to 10 μg. Four weeks post-immunization, spleens were removed and placed in culture for 6 days with irradiated MC57 ($H\text{-}2^b$) cells infected with AdgB as stimulators. Following stimulation splenocytes were examined for CTL activity against Vac gB infected MC57 targets (A), uninfected MC57 targets (B) or Vac gB SVBALB (H2-d) targets (C). Error bars show the standard error of the mean for three replicate wells.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Effector to Target Ratio
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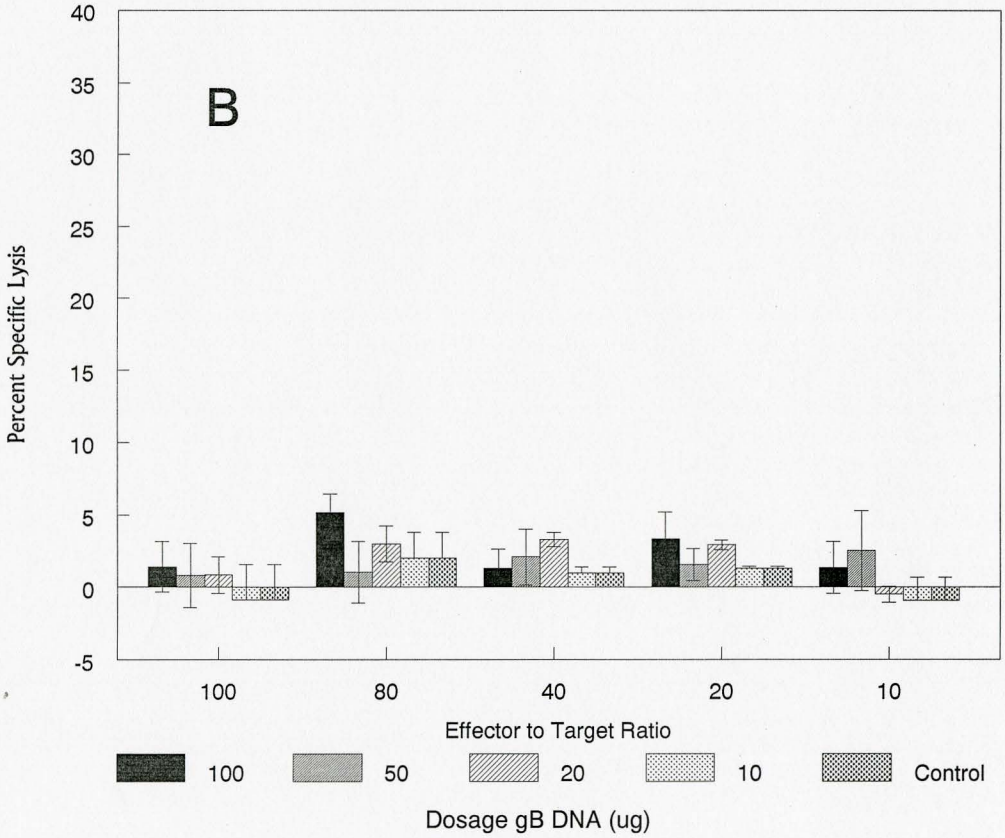
Control
Dosage of gB DNA (ug)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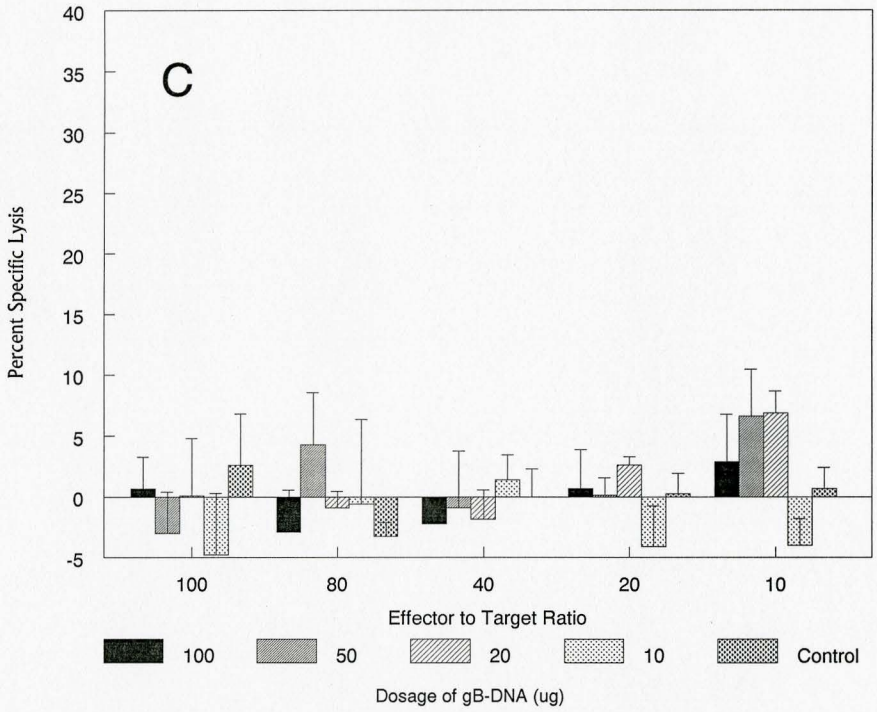
Control
Dosage gB DNA (ug)


C
Percent Specific Lysis
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Effector to Target Ratio
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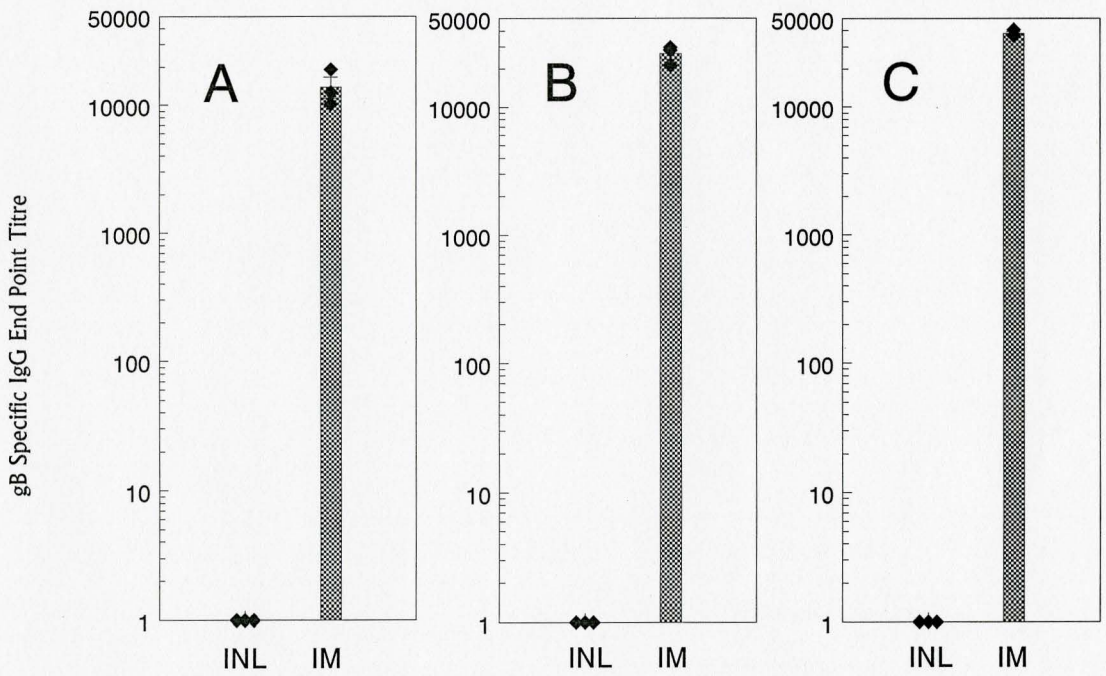
Control
Dosage of gB-DNA (ug)

from an allogeneic mouse source (H-2^d) and were not lysed, it is evident that killing is MHC I-restricted.

3.1.5. *A single intranasal immunization with gB-DNA is not able to generate antibody responses in C57BL/6 mice.*

To determine if an alternate method of delivery could generate IgG antibody responses, intranasal administration with 50 μg of gB DNA was performed. The same dosage was delivered IM as a positive control group for the assay, and gB specific end point titres were obtained by ELISA. Antibody levels were determined in plasma at two (Figure 3.1.5. A), four (Figure 3.1.5. B) or six (Figure 3.1.5. C) weeks post-immunization. Animals which were immunized IM generated high antibody titres which persisted throughout the six week period, whereas animals which were immunized INL failed to generate measurable antibody levels during the same period. These results show that a single INL immunization with naked plasmid DNA was unable to generate humoral immune responses as measured by IgG in the plasma. In the event that a local antibody response was generated that was not measurable in the plasma, mice were sacrificed at seven weeks and nasal lavages were performed to measure for gB specific IgA. No IgA antibody was detected in the nasal washes of any mice (data not shown).

Figure 3.1.5. Failure of gB DNA to generated gB specific antibody responses following a single administration of gB DNA intranasally. Mice were immunized either intranasally (INL) with 50 μg of gB DNA in 20 μl of PBS, or by intramuscular (IM) injection in 50 μl of PBS. At 2 weeks (A), 4 weeks (B) and 6 weeks (C) post immunization, mice were bled and gB specific IgG levels in plasma were obtained by ELISA. Bars represent the mean antibody titre, whereas diamonds represent individual animal responses. Error bars represent the standard error of the mean. Titres are expressed as the inverse of the minimum dilution at which two times the absorbance of plasma from mice receiving a control plasmid was reached. The minimum dilution used in this experiment was neat. Titres which fell below this level were assigned a titre of the minimum dilution for the calcuation of the mean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3.1.6. *Combination of gB-DNA delivered IM and a recombinant adenovirus expressing gB of HSV-1 (AdgB8) delivered INL (asleep), results in higher antibody titres than obtained by DNA immunization alone when either is used to induce the primary antibody response and the other is used to boost the antibody response.*

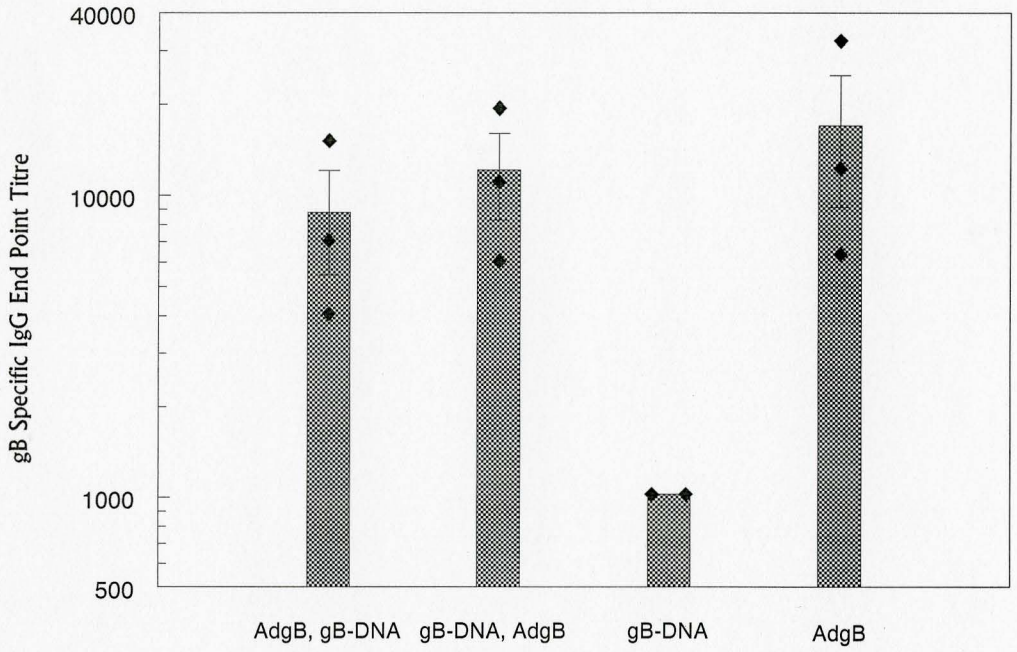
To determine if gB DNA or AdgB vaccines, which encode the same insert were able to boost and enhance primary antibody responses generated by one another, mice were either immunized with 50 μg of gB-DNA IM, or $1x10^8$ pfu of AdgB8 INL under anaesthetic, and then reciprocally boosted 2 weeks after primary immunization. A single administration of 50 μg of gB-DNA or $1x10^8$ of AdgB was used for comparison to a primary immune response. The results indicate that either combination vaccination protocol elicits antibody responses which are higher than those generated using the gB-DNA construct alone, however, the vaccine combination generates antibody levels which are the same, or lower than those obtained using AdgB on its own. Due to the high antibody responses generated by AdgB alone (Figure 3.1.6.), the results suggest that boosting with gB DNA does not enhance antibody responses over those generated with a single immunization with AdgB.

3.2. Characterization of antibody responses to AdgB delivered by intranasal immunization to animals either awake, or asleep.

3.2.1 AdgB administered either awake or asleep generates IgG antibody responses in a dose dependent manner which is generally similar, but differs significantly at different time points and dosages.

Intranasal immunization with recombinant AdgB has been well characterized

Figure 3.1.6. Effect of combining adenovirus based and DNA based vaccines. Mice were immunized at day 0 with either $1x10^8$ pfu of AdgB INL or 50 μg of gB-DNA IM. After 14 days, groups one and two were boosted with either 50 μg of gB-DNA or $1x10^8$ pfu of AdgB respectively. Four weeks following initial immunization, mice were bled, and gB specific IgG levels were obtained by ELISA. Thatched bars represent the mean titre, whereas diamonds represent titres obtained for individual mice. Error bars represent the standard error of the mean. Titres are expressed as the inverse of the minimum dilution at which two times the absorbance of plasma from unimmunized mice were reached.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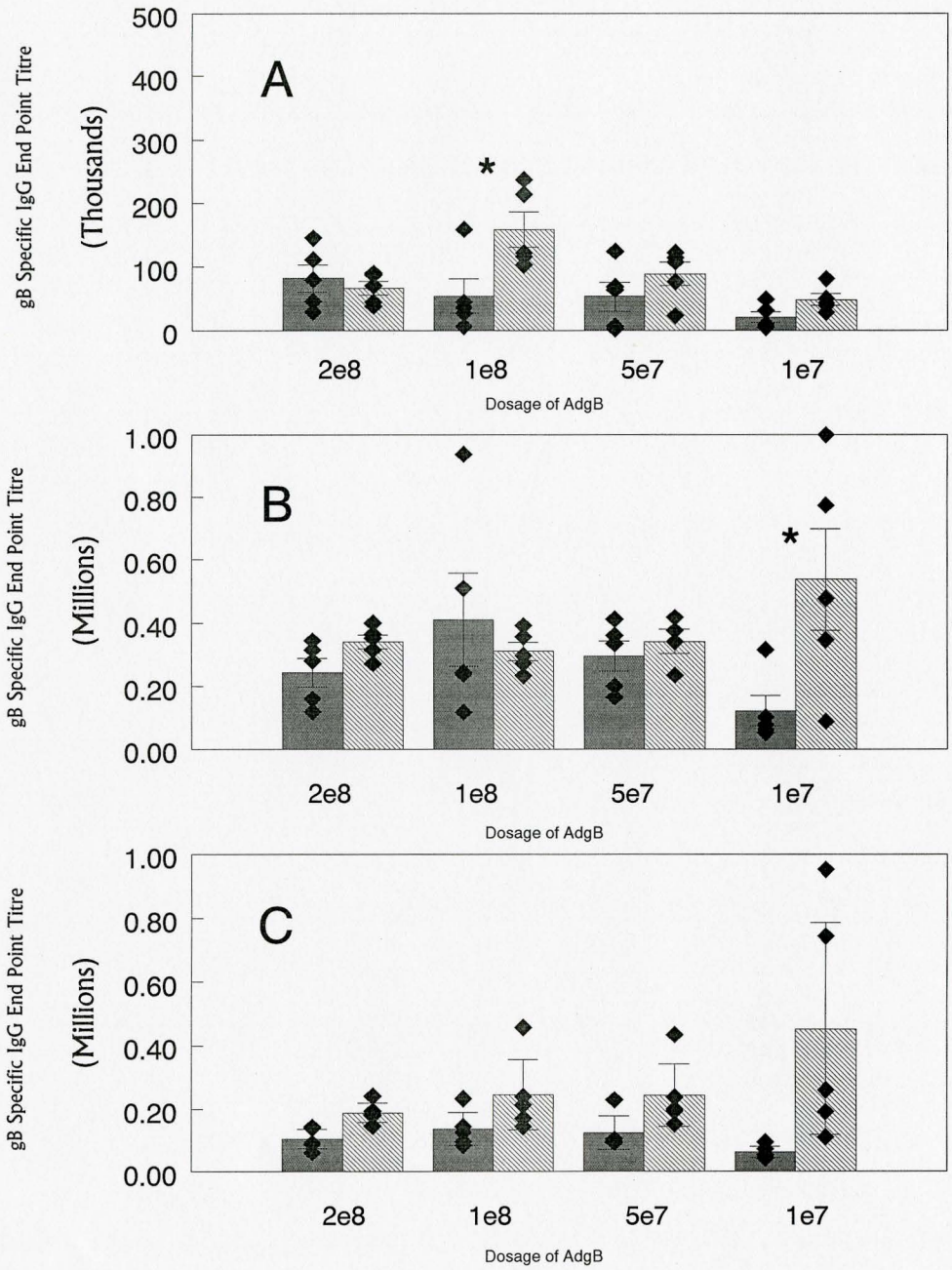
gB Specific IgG End Point Titre
AdgB, gB-DNA
gB-DNA, AdgB
gB-DNA
AdgB

(Gallichan et. al, 1993), however only by intranasal delivery to animals which have been anaesthetized. To determine if administration to awake animals resulted in significant differences in serum IgG antibody levels, mice were given dosages ranging from $5x10^7$ pfu to $2x10^8$ pfu of AdgB awake or asleep and their antibody levels were measured over a six week period (Figures 3.2.1. A-C). Figure 3.2.1.A demonstrates a clear dose response relationship between AdgB delivered asleep between the dosages of $1x10^8$ pfu and $1x10^7$ pfu. At the $2x10^8$ dosage level at this time point (Figure 3.2.1. A), there appears to be suppression of gB specific IgG responses, perhaps as a result of tolerance induction. Antibody responses to AdgB delivered awake did not generate a clear dose response at this time point (Figure 3.2.1. A). In fact, several animals failed to generate antibody levels greater than 100 (the minimum dilution used in this experiment). Despite apparent differences in mean antibody levels between the awake and asleep groups, the only significant difference between groups resulted at the $1x10^8$ dosage level ($p<0.05$) as measured by the student's t-test. Four weeks post-immunization (Figure 3.2.1. B), a similar antibody response pattern is observed in the awake mice as observed in Figure 3.2.1. A for the asleep mice. Again in awake mice, mean antibody titres suggest a dose response relationship between mice receiving adenovirus between the $1x10^8$ and $1x10^7$ dosage levels, whereas at the $2x10^8$ pfu dose of AdgB, antibody responses are suppressed (Figure 3.2.1. B). At the four week time point, the mice receiving adenovirus asleep lost the dose response relationship which was evident at two weeks, as antibody titres more than doubled, and variability significantly increased over the two week time point

Figure 3.2.1. gB Specific IgG responses in plasma to varying dosages of AdgB administered intranasally, either awake (solid bars) or asleep (striped bars). Groups of five C57Bl/6 mice were given doses of AdgB ranging from $5x10^7$ pfu. to $2x10^8$ pfu. Two (A), four (B) or six (C) weeks following immunization, mice were bled and plasma was obtained. gB specific IgG levels were determined by ELISA. Dark bars represent the mean end point titre of mice receiving AdgB while awake, whereas striped bars represent the mean end point titre of mice receiving AdgB while asleep. Error bars are expressed as the standard error of the mean. Diamonds represent the end point titres of individual mice. The minimum dilution used in these experiments was 1:100. Mice with end point titres below this level were assigned a titre of zero for calculation of the mean. Titres are expressed as the inverse of the minimum dilution at which two times the absorbance of plasma from mice receiving a control virus (AdE3-) were reached.


gB Specific IgG End Point Titre
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Dosage of AdgB
gB Specific IgG End Point Titre
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Dosage of AdgB

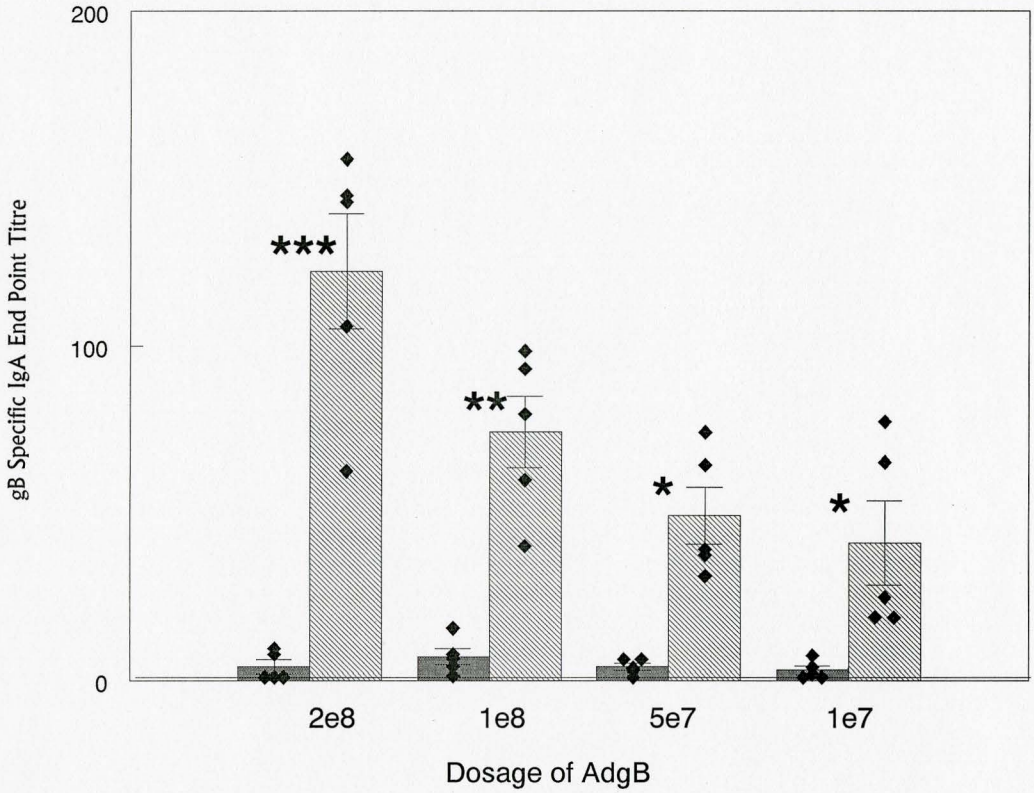
(Figure 3.2.1. A and 3.2.1. B). After four weeks, the only significant differences with respect to antibody titres between awake and asleep mice lies at the $1x10^7$ dose level ($p<0.05$). After six weeks (Figure 3.2.1. C), antibody titres in both groups levelled off, and no dose response relationships were evident, although the variability of individual responses had decreased at all dosage levels except $1x10^7$ pfu of AdgB in the asleep mice. Additionally, no significant differences in antibody responses exist between any groups at any dosage level after six weeks, despite the magnitude of responses in the asleep mice receiving $1x10^7$ pfu of AdgB (Figure 3.2.1. C). Collectively these results suggest that there is no significant differences with respect to total IgG responses in mice receiving AdgB awake or asleep after six weeks. Secondly, the results suggest that there may be a delayed kinetic response in the generation of antibody responses which follow a dose response relationship, as is illustrated in figures 3.2.1. A and 3.2.1. B respectively. Furthermore, in both awake and asleep mice, antibody responses appear to be suppressed at the $2x10^8$ pfu dose of AdgB.

3.2.2. AdgB administered either awake or asleep generates specific IgA responses in a dose dependent manner in asleep mice which are significantly different from IgA levels in awake mice at all doses.

To determine if any significant differences existed between awake and asleep administration of AdgB in the production of IgA antibody responses at a mucosal surface, mice from figure 3.2.1. were sacrificed after seven weeks, and nasal lavages were

performed. From figure 3.2.2., it is clear that awake administration of adenovirus limits the ability of the animal to produce IgA locally when compared to asleep administration. The most significant observation, as measured by the Bonferroni P value, is at the $2x10^8$ dose of AdgB ($p<0.001$), followed by the $1x10^8$ dose level ($p<0.01$), and finally the $5x10^7$ and $1x10^7$ dose levels ($p<0.05$). All mice receiving AdgB asleep generated measurable IgA antibody responses to gB, whereas several mice at various dosages failed to generate gB-specific IgA responses when AdgB was delivered awake. It is important to note that the mice which failed to generate IgA responses did strongly respond to the AdgB as measured by gB specific IgG responses in figures 3.2.1. A through to 3.2.1. C, and the failure to respond is not simply due to a technical failure or vaccine failure. Furthermore, the gB specific IgA responses in the asleep animals, as shown in figure 3.2.2., follow a dose response relationship from $2x10^8$ pfu of AdgB to $1x10^7$ pfu of AdgB. This dose response appears to follow an exponential, rather than linear distribution (co-efficient of determination = 92.6%). Mice which received AdgB while awake, failed to generate dose responses and resulted in very low gB specific IgA responses (Figure 3.2.2.). These results suggest that asleep delivery of AdgB is effective at generating IgA responses which obey a dose response relationship and which are significantly higher than IgA response generated from mice receiving AdgB awake.

Figure 3.2.2. gB Specific IgA responses in nasal lavage fluid to varying dosages of AdgB administered intranasally, either awake (solid bars) or asleep (striped bars). Seven weeks post immunization, mice from figure 1 were sacrificed and nasal lavages were obtained. gB specific IgA levels were determined by ELISA for doses of AdgB ranging from $2x10^8$ pfu to $1x10^7$ pfu delivered awake or asleep. Bars represent the mean end point titre of mice, error bars are expressed as the standard error of the mean. Diamonds represent the end point titres of individual mice. The horizontal line represents the minimum dilution used in this experiment (1:2). Mice which end point titres fell below this dilution were assigned a titre of the minimum dilution for an end point titre to calculate the mean. Asterisks represent significant differences between awake and asleep administration (*** - $p<0.001$, ** - $p<0.01$ * - $p<0.05$) as measured by Bonferroni's test statistic. Titres are expressed as the inverse of the minimum dilution at which two times the absorbance of nasal lavage fluid from mice receiving a control virus (AdE3-) were reached.


200
100
0
gB Specific IgA End Point Titre
***
**
*
*
2e8
1e8
5e7
1e7
Dosage of AdgB

3.2.3. *gB specific IgG levels in plasma of mice immunized with AdgB awake or asleep two weeks post immunization are consistent with earlier findings.*

To determine if findings in earlier experiments were reproducible, mice were immunized awake or asleep with $1x10^8$ pfu of AdgB and bled after two weeks (Figure 3.2.3.). This dosage and time point previously demonstrated significant differences in IgG levels between awake and asleep mice (Figure 3.2.1. A). Mice were then bled and gB specific IgG responses were obtained by ELISA. Although the differences between mean antibody titres in awake or asleep mice are similar to those observed earlier at the same time point and dosage (Figure 3.2.1. A), the results are not significantly different. These results suggest that although antibody responses generated with AdgB are reproducible, the IgG antibody responses between awake and asleep mice are not significantly different.

3.2.4. *gB specific IgA levels in mucosal tissues does not significantly differ five days following boosting of primary immunization with AdgB in mice when delivered awake or asleep.*

To determine if earlier observations could be extended to different mucosal tissues, AdgB was utilized in an immunization protocol which has previously been shown to generate gB specific IgA responses in various mucosal tissues when delivered to animals which are asleep (Gallichan *et al.*, 1993). Following the protocol described by Gallichan *et al.* (1993), animals generated mucosal responses in all tissues when AdgB was delivered to animals which were asleep (Figures 3.2.4. A, 3.2.4. B and 3.2.4. C).

Figure 3.2.3. gB specific IgG in plasma two weeks following delivery of AdgB either awake (solid bars) or asleep (striped bars). Two groups of five mice were immunized either awake or asleep with $1x10^8$ pfu of AdgB. Two weeks post-immunization, mice were bled and plasma was obtained. gB specific IgG titres were measured by ELISA. Titres are expressed as the inverse of the minimum dilution at which two times the absorbance of plasma from mice receiving a control virus (AdE3-) were reached.

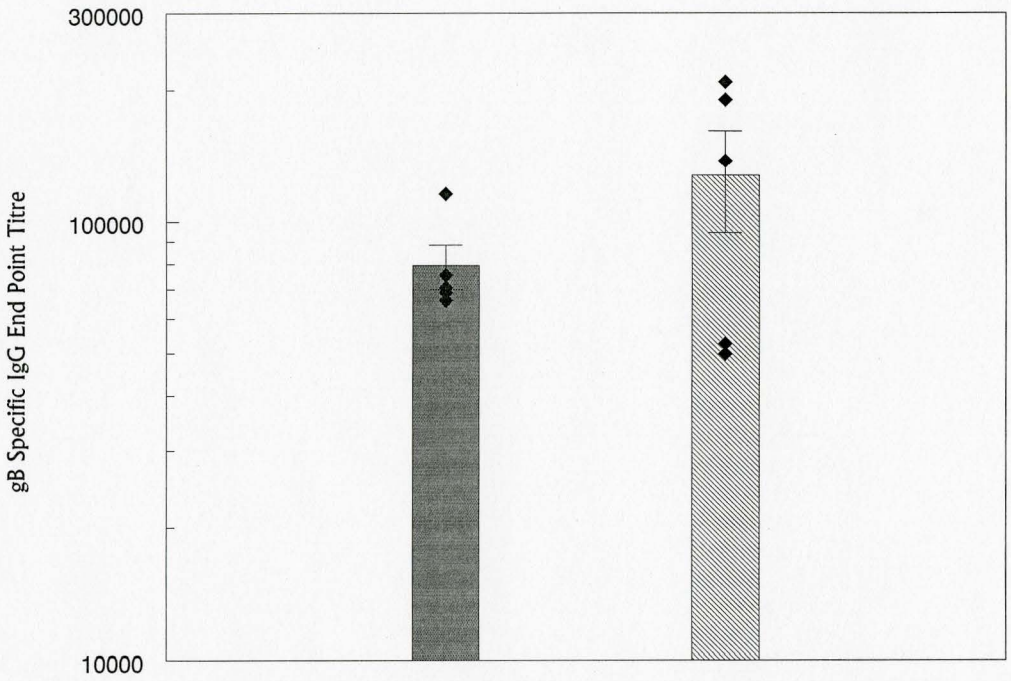
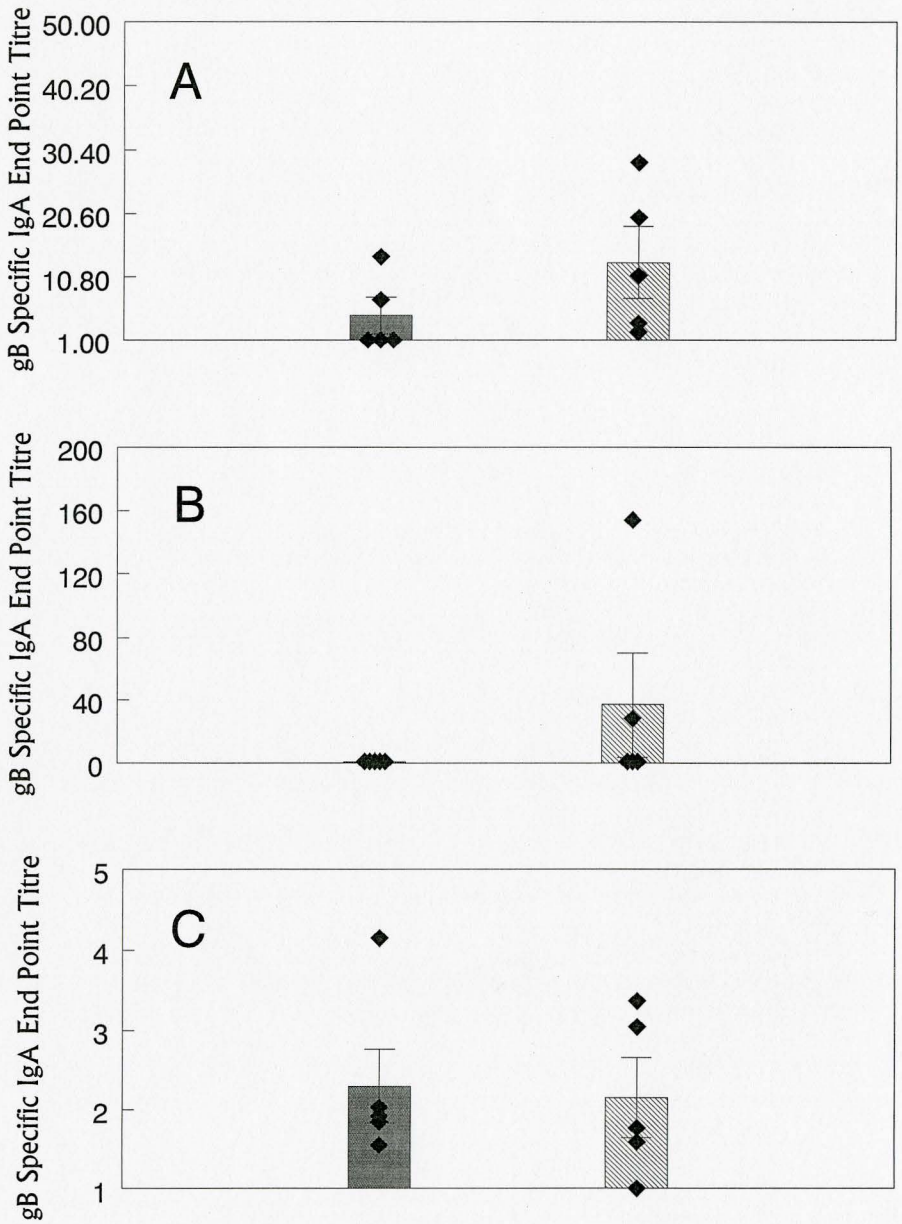

300000
100000
10000
gB Specific IgG End Point Titre

Figure 3.2.4. Comparison of gB specific IgA responses between mice immunized with AdgB either awake or asleep at various mucosal sites. Groups of 5 mice were immunized either awake (solid bars) or asleep (striped bars) with $1x10^8$ pfu of AdgB and boosted in the same manner four weeks post-immunization. Five days following boost, mice were sacrificed and gB specific IgA responses in nasal lavages (A), lung lavages (B) or in gut washes (C) were determined by ELISA. Bars represent the mean antibody responses, diamonds represent individual antibody titres and error bars represent the standard error of the mean. The minimum dilution used in this experiment was neat. Animals which did not generate a measurable titre at this level were assigned a titre of one for calculation of the mean. Titres represent the inverse of the minimum dilution at which twice the absorbance levels of control fluid from mice immunized with AdE3- were obtained.

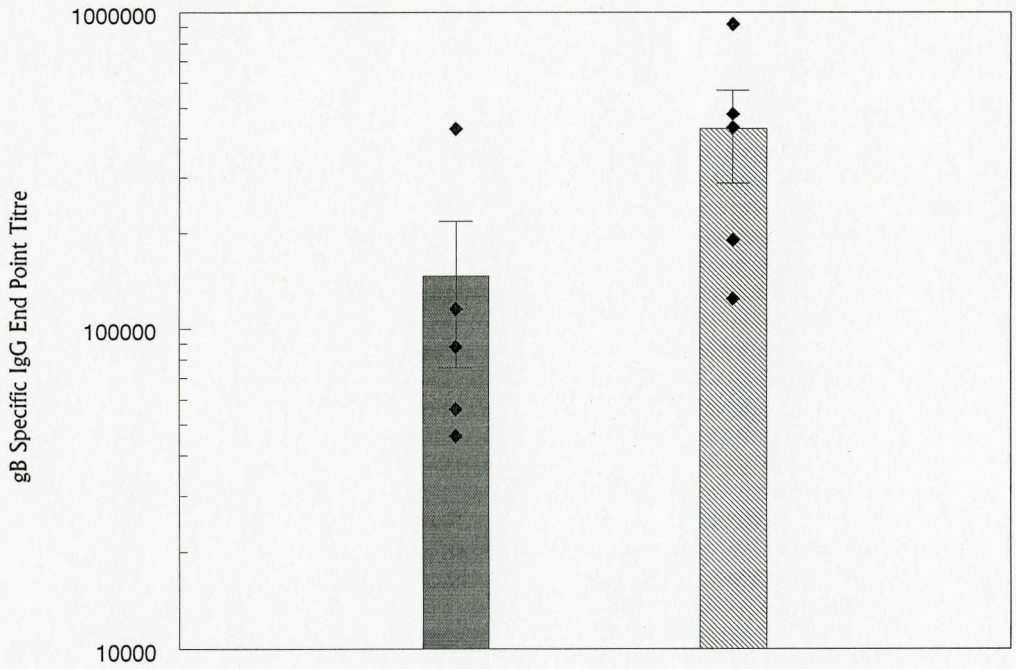

gB Specific IgA End Point Titre
50.00
40.20
30.40
20.60
10.80
1.00
A
gB Specific IgA End Point Titre
200
160
120
80
40
0
B
gB Specific IgA End Point Titre
5
4
3
2
1
C

However, when AdgB was delivered to animals which were awake, it failed to generate IgA responses in the lungs of mice (Figure 3.2.4. B). Similar levels of IgA were demonstrated in the nasal lavage fluid of boosted animals (Figure 3.2.4. A) as compared to animals receiving a single immunization (Figure 3.2.2.), although none of the responses were significantly different between groups at this time point. Additionally, this time point responses in gut lavages (Figure 3.2.4. C) were virtually identical.

3.2.5. *AdgB immunized mice awake or asleep, boosted four weeks post immunization have gB specific IgG levels in plasma which are similar to animals which have only had a single administration of AdgB.*

To determine if boosting animals significantly altered antibody responses between awake and asleep mice, mice were immunized awake or anaesthetized and boosted after four weeks. Two weeks post boost, mice were bled and gB specific IgG responses were measured by ELISA. The results obtained show similarities to antibody responses in mice immunized with a single dose of AdgB either awake or asleep at later time points (Figure 3.2.1. C). The most notable difference between the results obtained following boosting (Figure 3.2.5.) and those following single immunization (Figure 3.2.1. C) is that the mean antibody titre of animals receiving AdgB asleep, is more than double of those obtained by awake administration (Figure 3.2.1.), a result only obtained by single immunization at early time points (Figure 3.2.1. A) or low dosages (Figure 3.2.1.). These results demonstrate that boosting does not significantly alter the IgG antibody responses or trends observed by a single immunization with AdgB.

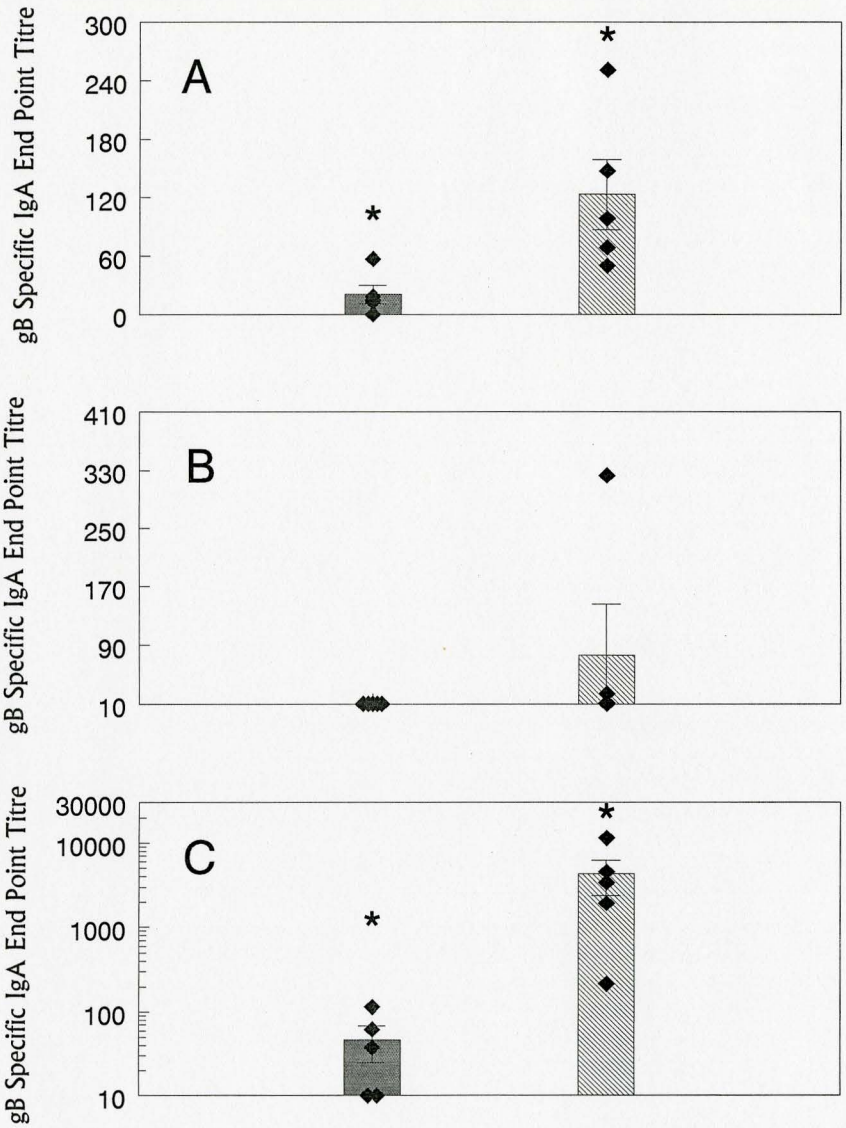
Figure 3.2.5. gB specific IgG in plasma two weeks following boost of primary immunization with AdgB either awake (solid bars) or asleep (striped bars). Two groups of five mice were immunized either awake or asleep with $1x10^8$ pfu of AdgB. Four weeks post-immunization, mice were boosted either awake (solid bars) or asleep (striped bars) with $1x10^8$ pfu of AdgB. Two weeks following boosting, mice were bled and plasma was obtained. gB specific IgG titres were obtained by ELISA. Titres are expressed as the inverse of the minimum dilution at which two times the absorbance of plasma from mice receiving a control virus (AdE3-) were reached.


1000000
100000
10000
gB Specific IgG End Point Titre

3.2.6. *gB specific IgA levels in mucosal tissues significantly differs two weeks following boosting of primary immunization with AdgB in mice when delivered awake or asleep.*

To determine if the failure to observe differences between IgA levels in awake or asleep immunized mice the immunization protocol described in figure 3.2.4. was used with the exception that mice were sacrificed two weeks following boosting (figure 3.2.6. A to C). Briefly, mice were administered $1x10^8$ pfu of AdgB awake or asleep and boosted in the same manner after four weeks. Two weeks post-boost, mice were sacrificed and gB specific IgA levels were obtained from nasal and lung lavages and gut washes by ELISA. As before, all animals receiving adenovirus asleep generated mucosal responses in all tissues, (Figures 3.2.6. A, 3.2.6. B and 3.2.6. C). Again mice immunized awake with AdgB failed to generate IgA responses in the lung (Figure 3.2.6. B). At this time point, not only was the trend similar between IgA levels in nasal lavage fluid of boosted animals (Figure 3.2.6. B) as compared to animals receiving a single immunization (Figure 3.2.2.), the responses were significantly different between groups of awake and asleep mice. Additionally, mean IgA titres in gut lavages from asleep mice were over 100 times greater than mean IgA titres of awake mice and were significantly different as measured by Bonferroni's P value ($p<0.05$) (Figure 3.2.6. C). This result contrasts with the five day time point (figure 3.2.4. A) in which the responses were virtually identical between awake and asleep mice. These results suggest that animals which receive AdgB awake are unable to generate IgA responses in the lung, and

Figure 3.2.6. Comparison of gB specific IgA responses between mice immunized with AdgB either awake or asleep at various mucosal sites. Groups of 5 mice were immunized either awake (solid bars) or asleep (striped bars) with $1x10^8$ pfu of AdgB and boosted in the same manner four weeks post-immunization. Two weeks following boost, mice were sacrificed and gB specific IgA responses in nasal lavages (A), lung lavages (B) or in gut washes (C) were determined by ELISA. Bars represent the mean antibody responses, diamonds represent individual antibody titres and error bars represent the standard error of the mean. Titres represent the inverse of the minimum dilution at which twice the absorbance levels of control fluid from mice immunized with AdE3- were obtained. The minimum dilutions used in this experiment were neat (A), 1:10 (B) and 1:10 (C). Animals whose responses were determined to be less than the minimum dilution were assigned a titre of the minimum dilution for determination of the mean.

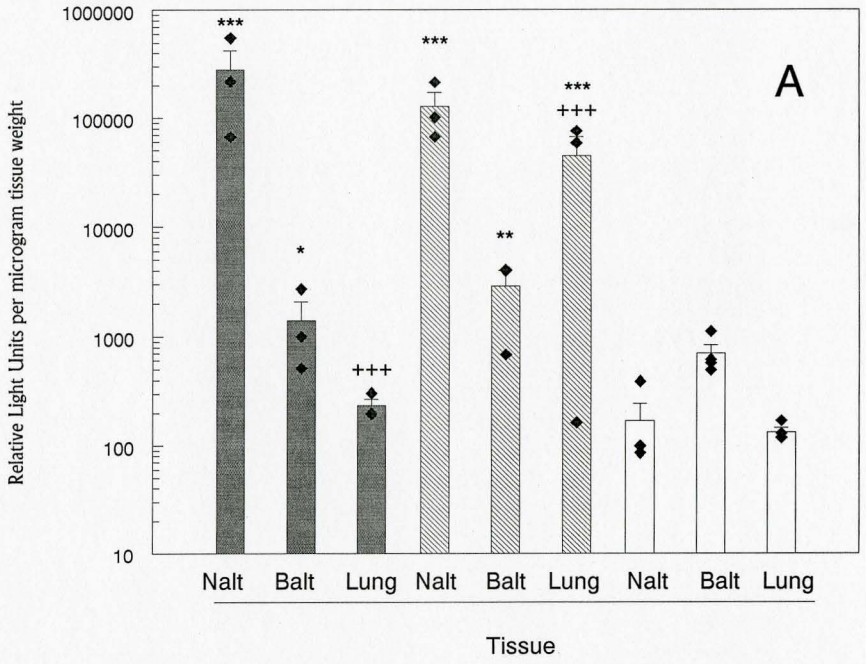

gB Specific IgA End Point Titre
300
240
180
120
60
0
A
*
*
gB Specific IgA End Point Titre
410
330
250
170
90
10
B
gB Specific IgA End Point Titre
30000
10000
1000
100
10
C
*
*

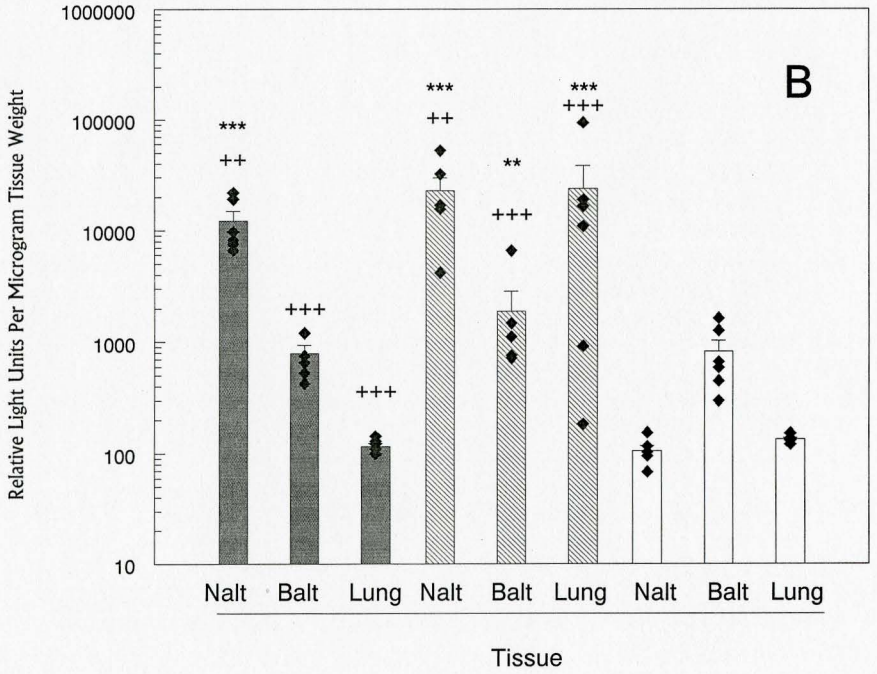
although capable of generating them locally (Figure 3.2.6. A) or at a distant mucosal site (Figure 3.2.6. C), they do not generate responses as effectively as mice receiving AdgB asleep.

3.2.7. *AdLUC delivered to mice awake is expressed only in the nasal passage, rarely in the trachea and not in the lung, whereas AdLUC delivered to mice asleep is expresses in the nasal passage, trachea and in the lung at levels significantly higher than in the awake mice.*

To determine if awake and asleep immunization with an adenovirus vector resulted in a different physical distribution of virus, groups of three (Figure 3.2.7. A) or six (Figure 3.2.7. B) mice were immunized with AdLUC, a replication deficient virus expressing the firefly luciferase gene. 48 hours post-immunization, tissues were removed, weighed, homogenized and assayed immediately for luciferase expression. Luciferase was measured in lung, nasal-associated lymphoid tissue and nasal tissue (NALT), and in the trachea and bronchial associated lymphoid tissue (BALT). NALT was significantly brighter than background absorbance measured from control tissue as measured by the F-test (light bars, Figure 3.2.7. A) ($P<0.001$). The levels between awake and asleep administration in this experiment for the NALT were not significantly different. In the second experiment (Figure 3.2.7. B), the levels again were significantly higher than background levels ($P<0.001$), but also were significantly different between awake and asleep administration ($P<0.01$). Mice generated mean luciferase expression in BALT which was significantly different from background levels, whereas in the second

Figure 3.2.7. Luciferase expression in various tissues following intranasal delivery of a replication deficient adenovirus expressing the firefly luciferase gene (AdLUC) to mice which were either awake (dark bars) or asleep (striped bars). $1x10^8$ pfu of AdLUC was delivered to groups of 3 (Figure 3.2.7. A) or groups of six (Figure 3.2.7. B) C57Bl/6 mice either awake or asleep. $1x10^8$ pfu of AdE1- was used as a control virus delivered to asleep animals (open bars). 48 hours post-immunization, mice were sacrificed and the following tissues were collected (see materials and methods): the nasal cavity and the associated lymphoid tissue (NALT), the trachea including the bronchial associated lymphoid tissue (BALT) and the lungs (LUNG). Tissues were homogenized and assayed for luciferase expression. It is important to note that for all groups of mice, luciferase has been corrected for tissue weights and sample volumes. Bars represent the mean of luciferase expression in the tissue indicated, error bars represent the standard error of the mean and diamonds represent luciferase expression in the homogenate of individual mice. Asterisks represent significance between mean luciferase expression in tissue indicated and control tissue as measured by the F-test statistic (*** = $P<0.001$, ** = $P<0.01$, * = $P<0.05$). Crosses represent significance between mean luciferase expression between awake and asleep administration in the same tissue as measured by the F-test statisitic (+++ = $P<0.001$, ++ = $P<0.01$).


1000000
100000
10000
1000
100
10
Relative Light Units per microgram tissue weight
A
***
*
+++
***
**
***
+++
Nalt
Balt
Lung
Nalt
Balt
Lung
Nalt
Balt
Lung
Tissue


1000000
100000
10000
1000
100
10
Relative Light Units Per Microgram Tissue Weight
B
***
++
+++
+++
***
++
**
+++
***
+++
Nalt
Balt
Lung
Nalt
Balt
Lung
Nalt
Balt
Lung
Tissue

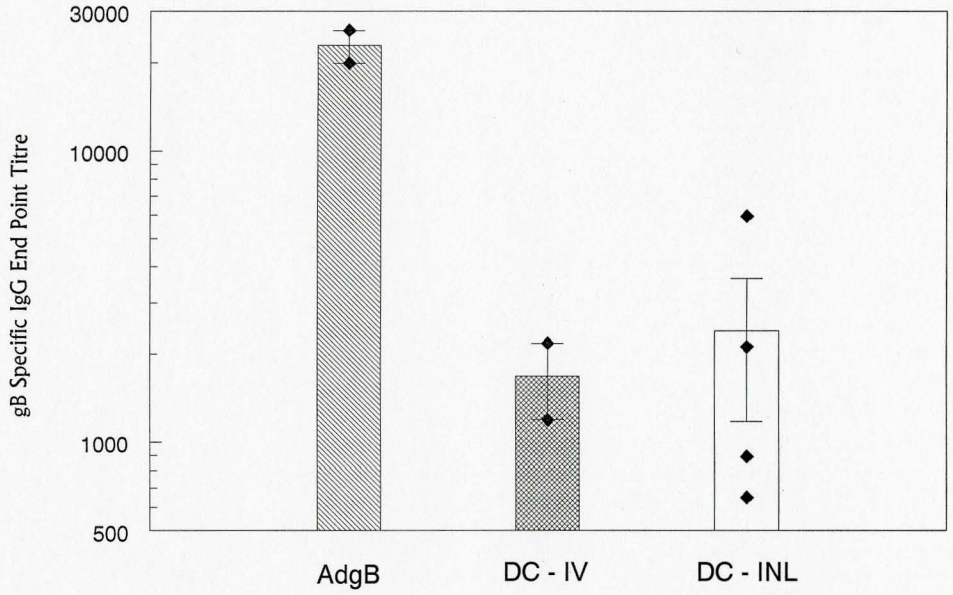
experiment using a greater number of animals, there was no significant difference observed. In both experiments there was a significant difference between background luciferase expression in the BALT in asleep mice ($P<0.01$). In addition, in the second experiment (Figure 3.2.7. B) there was a highly significant difference between luciferase expression in the BALT of awake and asleep mice ($P<0.001$). Lastly, and the most revealing about the physical distribution patterns of adenovirus delivered awake or asleep comes from examining luciferase expression in the lungs of immunized animals. Luciferase levels in the lungs of animals which received adenovirus awake in both experiments demonstrate no significant difference between background levels, yet are highly significant from the levels of luciferase expression in animals which have received luciferase asleep ($P<0.001$). Further, it is important to note that luciferase expression in the lungs of animals receiving AdLUC asleep is similar to levels detected in the NALT. Collectively these findings suggest that adenovirus which has been delivered awake passes through the NALT may rarely enter the BALT, but does not enter the lungs of immunized animals, wheras adenovirus which has been delivered to animals which are asleep passes through the NALT, enters the BALT and frequently (7/9 times) enters the lung where it is subsequently expressed. This suggests that awake administration of a replication deficient adenovirus restricts viral activity to the upper respiratory system, whereas asleep delivery distributes expression between the upper and lower respiratory systems.

3.3. Characterization of Antibody Responses to Dendritic Cells Transduced with AdgB Delivered Intranasally, Intravenously and Sub-Cutaneously.

3.3.1. *Dendritic cells transduced with AdgB are able to elicit gB specific antibody responses when delivered by either IV injection or intranasal immunization in a high volume.*

To determine if dendritic cells transduced with AdgB were able to generate gB specific antibody responses, mice were immunized with $1x10^6$ DCs transduced with AdgB and delivered IV (DC IV) or INL (DC INL) to anaesthetized mice. Figure 3.3.1. demonstrates that mice which received DCs IV were able to generate gB specific antibody responses. Additionally, mice which received DCs intranasally were also able to generate IgG responses which were similar to those obtained by intravenous administration. Due to the concentration of DC preparation, it was necessary to immunize mice INL with DCs in 80 μl of PBS, which is a four times higher volume than generally used for intranasal immunization. The same AdgB which was used to transduce the DCs was used as a positive control (delivered to mice INL asleep) in this experiment to demonstrate that any failures of DCs to generate immune responses were not due to a poor viral stock. These results demonstrate that the epithelial barriers of the lung do not prevent the entry of cells when in a relative high volume of fluid, and that sufficient DCs cross these epithelial barriers to generate antibody responses which are similar to those obtained by directly infusing DCs into the bloodstream.

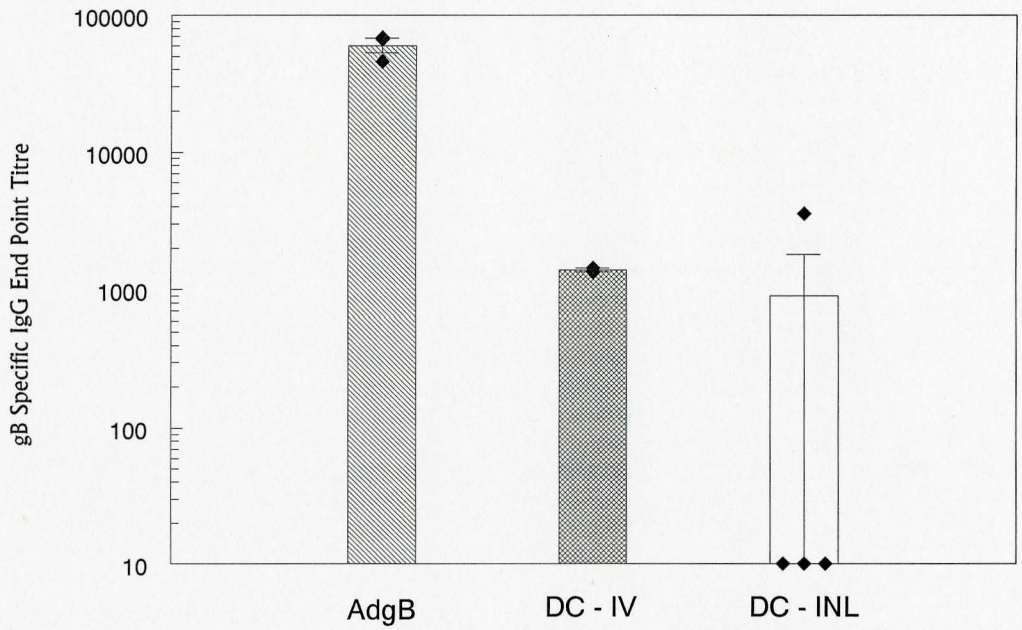
Figure 3.3.1. gB Specific IgG responses to dendritic cells transduced with AdgB and delivered by intravenous (DC - IV) (thatched bars) or intranasal (DC - INL) (open bars) administration. Groups of two (DC - IV) or four (DC - INL) mice were administered $1x10^6$ dendritic cells transduced with AdgB at an MOI of 100 for 48 hours. DC-IV mice received DCs in 300 μl of PBS, and DC - INL mice received DCs in 80 μl of PBS while anaesthetised. AdgB was administered to mice asleep at a dose of $1x10^8$ pfu/mouse in 20 μl of PBS INL as a positive control (striped bars). 4 weeks post immunization, mice were bled and plasma was obtained. gB specific IgG responses were determined by ELISA. Bars represent the mean antibody titre, diamonds individual antibody titres and error bars represent the standard error of the mean. Titres represent the inverse of the dilution at which twice the absorbance values of mice receiving a control virus (AdE3-) were obtained. The minimum dilution used in this experiment was 1:10.


gB Specific IgG End Point Titre
30000
10000
1000
500
AdgB
DC - IV
DC - INL

3.3.2. *Dendritic cells transduced with AdgB are able to elicit gB specific antibody responses when delivered by either IV injection and occasionally by intranasal immunization in a low volume.*

To confirm earlier findings, mice were again immunized with $1x10^6$ DCs transduced with AdgB, either IV (DC IV) or INL (DC INL). Figure 3.3.2. again demonstrated that mice which received DCs IV were able to generate gB specific antibody responses. However, in this experiment, from the mice which received DCs intranasally, only one was able to generate IgG responses which were similar to those obtained by intravenous administration, whereas the other mice in the group failed to generate responses. The same AdgB which was used to transduce the DCs was used as a positive control in this experiment to demonstrate that any failures of DCs to generate immune responses were not due to a poor viral stock. The apparent discrepancies in results between the initial experiment and the subsequent experiment may be explained by the volume used in the intranasal immunization procedure. In experiment 1 (Figure 3.3.1.), due to the concentration of cells it was necessary to administer two volumes of 40 μl of PBS containing the transduced DCs, a significant volume for mice. In this experiment, DCs were prepared in a concentration that would allow for administration in 20 μl of PBS, the volume generally desired for intranasal immunization. The results therefore suggest that a smaller volume of fluid rather than a larger volume of fluid is not capable of delivering DCs to areas where they are able to cross epithelial barriers. It is however of importance to note that there are no significant differences between mean antibody titres in mice

Figure 3.3.2. gB Specific IgG responses to dendritic cells transduced with AdgB and delivered by intravenous (DC - IV) (thatched bars) or intranasal (DC - INL) (open bars) administration. Groups of two (DC - IV) or four (DC - INL) mice were given $1x10^6$ dendritic cells transduced with AdgB at an MOI of 100 for 48 hours. AdgB was administered to mice asleep at a dose of $1x10^8$ pfu/mouse in 20 μl of PBS as a positive control (striped bars). 4 weeks post immunization, mice were bled and plasma was obtained. gB specific IgG responses were determined by ELISA. Bars represent the mean antibody titre, diamonds individual antibody titres and error bars represent the standard error of the mean. Titres represent the inverse of the dilution at which twice the absorbance values of mice receiving a control virus (AdE3-) were obtained. The minimum dilution used in this experiment was 1:10. Mice whose titres fell below this level were assigned a titre of the minimum dilution for determination of the mean.


gB Specific IgG End Point Titre
100000
10000
1000
100
10
AdgB
DC - IV
DC - INL

receiving DCs IV versus INL, nor have any direct comparisons been made between INL application of DCs in high and low volumes so this observation may merely be due to chance.

3.3.3. *Dendritic cells transduced with AdgB and delivered either intranasally or intravenously fail to generate gB specific IgA responses.*

To determine if DCs transduced with AdgB were capable of eliciting IgA responses, mice from figure 3.3.1. were sacrificed after five weeks and nasal lavages were performed and analysed for gB specific IgA by ELISA. As is evident from figure 3.3.3., neither mice who received DCs IV nor mice who received DCs INL generated any gB specific IgA. One mouse used as a positive control in this experiment failed to generate a specific IgA response, however all mice did generate gB specific IgG responses as demonstrated in Figure 3.3.1.. These results suggest that DCs, although able to generate IgG responses, fail to generate IgA responses when transduced with a vector which on its own is able to generate IgA responses.

3.3.4. DCs transduced with AdgB and delivered SC fail to generate gB specific IgG responses.

To determine if transduced DCs were able to generate gB specific IgG responses, fourteen mice were immunized SC with AdgB transduced DCs. From figure 3.3.4., it is evident that only one animal out of fourteen generated a measurable antibody titre. The lot of adenovirus used in this experiment was the same as used in figures 3.3.1. to 3.3.3.,

Figure 3.3.3. gB Specific IgA responses to dendritic cells transduced with AdgB and delivered by intravenous (DC - IV) or intranasal (DC - INL) administration. Groups of two (DC - IV) or four (DC - INL) mice were given $1x10^6$ dendritic cells transduced with AdgB at an MOI of 100 for 48 hours. DC-IV mice received DCs in 300 µl of PBS, and DC - INL mice received DCs in 80 µl of PBS. AdgB was administered to mice asleep at a dose of $1x10^8$ pfu/mouse in 20 µl of PBS INL as a positive control (striped bars). 5 weeks post immunization, mice were sacrificed and nasal lavage fluid was obtained. gB specific IgA responses were determined by ELISA. Bars represent the mean antibody titre, diamonds individual antibody titres and error bars represent the standard error of the mean. Titres represent the inverse of the dilution at which twice the absorbance values of mice receiving a control virus (AdE3-) were obtained. The minimum dilution used in this experiment was 1:1. Mice whose antibody levels fell below this level were assigned a titre of the minimum dilution for calculation of the mean.

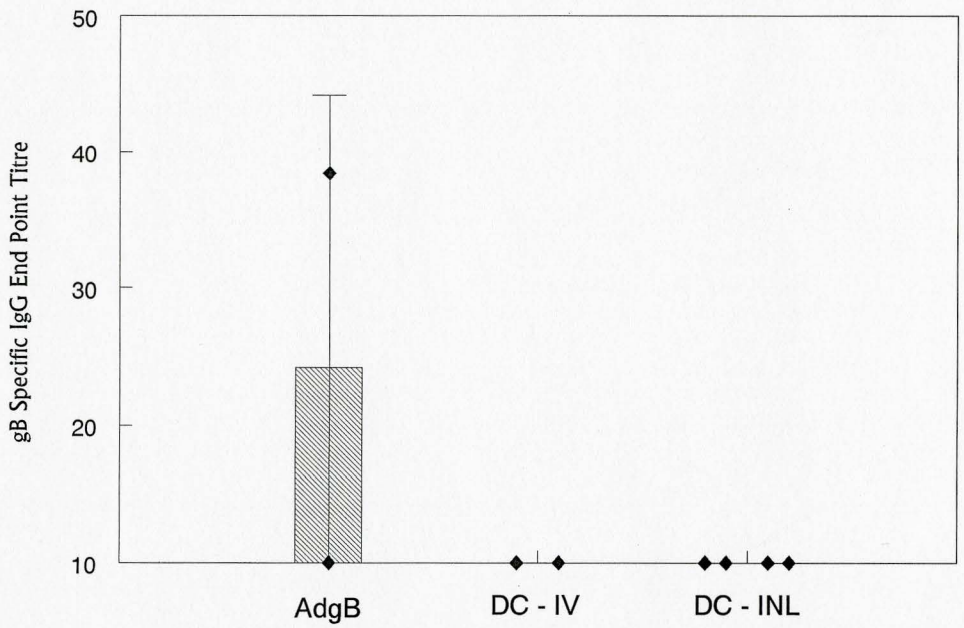
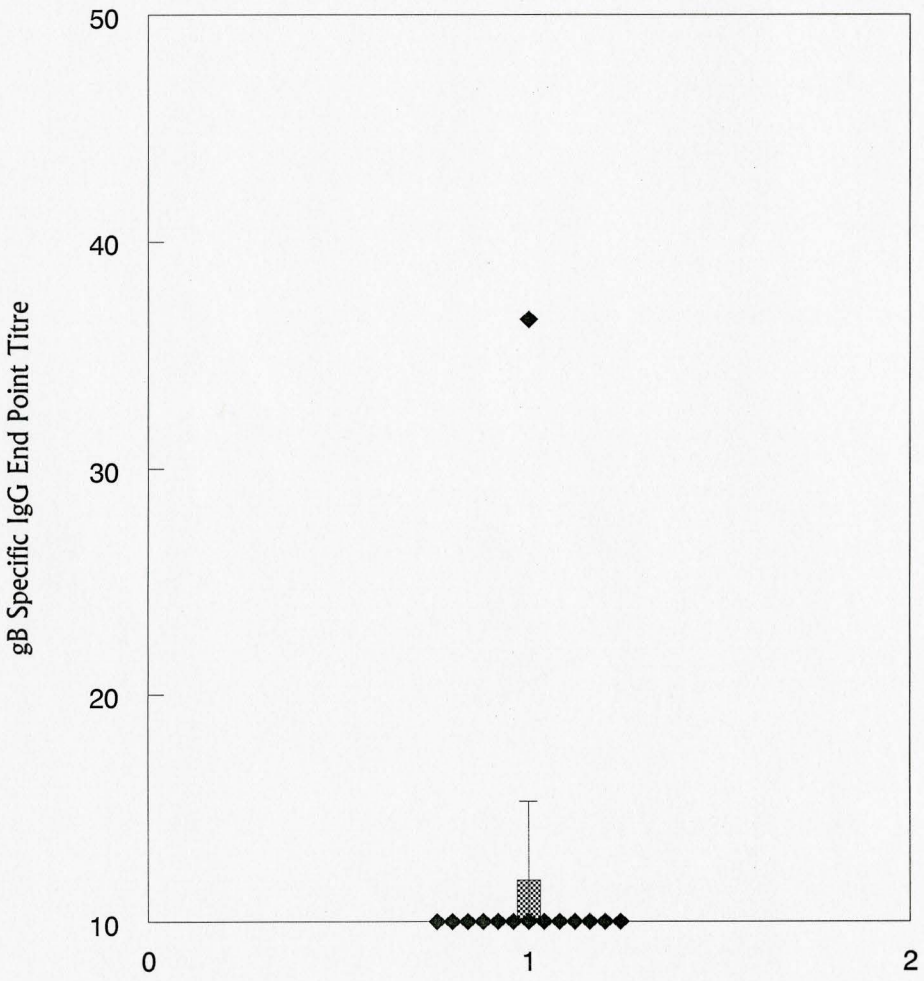

gB Specific IgG End Point Titre
50
40
30
20
10
AdgB
DC - IV
DC - INL

Figure 3.3.4. gB specific IgG responses in mice receiving DCs transduced with AdgB and administered sub-cutaneously (SC). Fourteen mice were given $1x10^6$ dendritic cells transduced with AdgB at an MOI of 100 for 48 hours SC. 4 weeks post immunization, mice were bled and plasma was obtained. gB specific IgG responses were determined by ELISA. Titres represent the inverse of the dilution at which twice the absorbance values of mice receiving DCs transduced with a control virus (AdE3-) were obtained. The minimum dilution used in this experiment was 1:10. Animals which titres fell below this level were assigned a value of the minimum dilution for calculation of the mean.


gB Specific IgG End Point Titre
50
40
30
20
10
0
1
2

so these results are likely not due to vaccine failure. These results demonstrate that sub-cutaneous administration is not a desirable method to generate antibody responses within the time period measured.

IV. Discussion

4.1. gB-DNA Based Vaccine Vector

4.1.1. Antibody responses to gB-DNA

DNA vaccines designed to induce immunity to herpes simplex virus have existed for more than three years. However, studies examining antibody responses to different dosages of these vaccines are lacking. Most of the reported literature in this area involves the use of protection studies (Kriesel *et al.*, 1996) or pooled sera to examine antibody responses in animals (Manickan *et al.*, 1995a). Although indicative of an immune response in a group of animals, these studies fail to provide some valuable information required for the potential application of these vaccines. For instance, the use of pooled sera gives us no indication of the variability in response between immunized animals. To address this issue, the current studies were undertaken to provide information on the variability on the amount of antibody produced by individual mice to different dosages of a DNA vaccine encoding gB of HSV.

To avoid complications associated with the effect of multiple immunizations on antibody responses, a method of immunization previously shown to generate antibody responses following a single IM administration was selected (Manickan *et al.*, 1995a). The results obtained in these experiments are consistent with a picture of highly variable responses that are highest at four weeks post immunization. Although a study of this kind has not been performed in the HSV model or others, smaller studies involving different

antigens support these observations (Ulmer *et al.* 1994; Bourne *et al.* 1996b; Nakano *et al.* 1997). In this study, it was suggestive that at the four-week time point a dose response relationship was evident in the majority of animals. It should be noted though that this relationship was not supported by statistical analysis, but merely by inspection. Similar results have been described using DNA vaccination in other models. Support for this type of trend may be present in a report by Ulmer *et al.* (1994) who reported that three IM administrations of a plasmid encoding influenza antigens, generated antibody responses to wide doses of vaccine which followed an apparent dose response as long as nine weeks post immunization. However, the authors presented only the mean antibody responses in groups containing 15 mice. Since no presentation of variability was made, (and the authors make no claim to a dose response) it could be suggested that the apparent dose response was merely a trend. The only other study which has reported data in a format which gave some measure of variability was performed by Bourne *et al.* (1996b) who measured antibody responses at one time point in animals to a range of doses of plasmid DNA encoding for gB of HSV following multiple IM administrations. This study was performed on guinea pigs and used a plasmid containing the antibiotic resistance gene for kanamycin and resulted in antibody dose responses to the vaccine construct which was highly variable. These results may be explained by the fact that kanamycin resistance genes do not encode immunostimulatory sequences (ISS) (Chu *et al.*, 1997), so it is possible that variability could be reduced by utilising a gD vaccine which does encode ISS's. Nakano *et al.* (1997) also demonstrated that DNA immunization generated highly

variable antibody responses which persisted until 18 weeks post immunization. Variability with respect to antibody responses is not a unique observation in DNA vaccination. Reports by groups using gene gun-based technology have reported high degrees of variability in antibody responses between mice. The clearest demonstration of variability in antibody responses was made by Pertmer and colleagues (1995) who reported high variability in antibody responses to a DNA vaccine designed to generate immune responses to human growth hormone when delivered by gene gun. More recently, Prayaga *et al.* (1997) demonstrated high variability to antibody responses generated against HIV proteins using a DNA vaccine delivered by gene gun.

Similarities to other studies are also observed with respect to early antibody response (Figure 3.1.1. A), where some mice failed to generate measurable antibody responses two weeks following immunization. This type of phenomenon was reported following IM administration of a DNA vaccine encoding hepatitis C antigens (Tedeschi *et al.*, 1997). In this study 10/12 animals developed antibody responses two weeks post immunization (Tedeschi *et al.*, 1997). In another study utilizing multiple IM administrations of a DNA vaccine encoding gD of HSV-2 with an adjuvant, Kriesel *et al.* (1996) reported antibody titres which rose after two weeks, however the authors used only one dose of vaccine and two time points. Furthermore, in the hepatitis C model, it has been demonstrated that antibody titres decline over time following DNA vaccination (Tedeschi *et al.* 1997). Nakano *et al.* (1997) reported similar observations to those reported in this study, with respect to the duration of the antibody response to gB (Figure 3.1.3.). The authors

reported that antibody titres were present up to 18 weeks post immunization, but following this period antibody titres in some mice were not detectable (Nakano *et al.*, 1997). A similar slow decline in antibody levels has been reported following IM administration of a DNA vaccine against HIV antigens (Okada *et al.*, 1997). Boosting may overcome this problem by ensuring a secondary antibody response. However, it has been reported that multiple immunizations may actually reduce antibody responses (Nakano *et al.*, 1997), contrary to observations utilizing protein antigens and adjuvants which suggests that antibody titres will continue to rise following repeated immunizations (Lane, 1996).

Another aspect of humoral responses in which there is a paucity of data, is the duration of antibody responses in organisms receiving a DNA vaccine. However, this is of more importance with vaccines designed to generate protective antibody responses. Of the studies performed in mice, most deal with multiple immunizations of a DNA vaccine and measurement of resulting antibody responses, with the exception of Davis *et al.* (1996), who followed antibody responses to a DNA vaccine encoding hepatitis B surface antigen (HbsAg) to 74 weeks post immunization. Their results showed that a single administration of a DNA vaccine by the IM route induced antibody responses that persisted nearly undiminished as long as 74 weeks and were stronger and longer lasting than responses induced by protein administration alone (Davis *et al.*, 1996). Antibody responses in higher primates and humans have been reported to persist as long as six months post immunization (Bassily *et al.*, 1997; Shiver *et al.*, 1996). In humans it was

noted that three IM vaccinations with a hepatitis B DNA vaccine induced antibody responses which could be measured in only a few children up to five years post immunization. However, boosting at this time caused a substantial secondary antibody response in all of the children, which the authors suggest means that boosting later is not required even though antibody responses may not be detectable (Da *et al.*, 1997). In mice, Nakano *et al.* (1997) used IM administration of plasmid encoding hepatitis C antigens at three time intervals (0, 3 and 9 weeks) and measured antibody responses at 6, 12 & 18 weeks post immunization. The results of this study demonstrated a steady decline in antibody responses over time (Nakano *et al.*, 1997), which exhibits similarity to the observations in Figure 3.1.3. which measured gB-specific antibody responses 11 months post immunization.

The similarities that exist between our observations and previous reports do not provide an explanation for the high degree of variability in antibody responses following IM administration of DNA vaccines. One possible explanation lies in the complexity of parenteral administration of a DNA vaccine due to the precision required to make a successful IM injection in the muscle desired. Yokoyama *et al.* (1997) report that IM administration to the tibialis anterior muscle is the best inductive site for immunity as compared to other muscles. It is possible that in this experiment and others that the amount of plasmid successfully administered to the tibialis anterior muscle may vary considerably. Without robotic precision and methods of measuring the physical quantities of vaccine entering the muscle, this possibility exists. Another possible

explanation to the variable responses encountered may lie in the nature of antigen presentation involved. The generation of antibody responses following immunization depends on the capture of soluble antigens by B cells and interaction with CD4+ T cells and follicular dendritic cells. In this scenario, it is important to consider the report of Wolf et al (1990) who reported that very few muscle fibres were transfected following IM delivery of plasmid DNA. Therefore, if by chance the number of muscle cells transfected increases, then the amount of a secreted gene product may increase proportionately due to the efficient nature of the promoters used in the vaccine construct. Thus, variability in the physical amount of a secreted gene product may account for some of the variability observed in antibody responses. Another possibility arises following a similar argument. Tighe *et al.* (1998) have demonstrated that ISS present in bacterial DNA may promote a burst of IFNγ which has been shown to induce the expression of MHC II in muscle cells and recruit antigen presenting cells (Hohlfeld and Engel, 1994). Taken together, variability introduced by injection and variability in the numbers of actual muscle fibres transfected may lead to a cascade of errors which may generate the significant variability in antibody responses observed in individual mice responding to the gB-DNA vaccine.

Further investigation in this area should concentrate on the administration schedule. Indeed, HIV specific antibody responses induced by DNA vaccination can be increased more than 10 fold by merely reducing the number of initial vaccinations and spreading out the vaccination schedule in rhesus monkeys (Fuller *et al.*, 1997a). Nakano *et al.* (1997) have also observed a reduction in overall antibody responses when multiple IM

administration was used to generate antibody responses against hepatitis B in mice. Furthermore, studies, when possible, should use a single administration of DNA vaccine before initiating studies using multiple immunizations. Results presented here demonstrate good antibody responses over a wide range of dosages following a single administration of gB-DNA vaccine, which should also respond to boosting. A single IM administration of a DNA vaccine encoding hepatitis B antigens in C57BL/6 mice was shown to generate persistent and nearly unchanged antibody responses for 74 weeks (Davis *et al.*, 1996). Following this period, antibody responses could be boosted with another administration of the DNA vaccine, or hepatitis B surface antigen protein, resulting in a further increase of antibody titres by 10-200 fold (Davis *et al.*, 1996).

The current study could be improved by modifying the ELISA system used to include a positive control to compare antibody responses between different studies and different time points, which would have allowed for statistical comparisons of kinetic responses between experiments. Although responses are always compared to a group of mice which have received a control plasmid, variability between experiments limits the usefulness of comparing titres between experiments, although it does not affect comparisons within experiments. A positive control was not included in these experiments as the primary interest was to compare responses within groups of responding animals and to present the variability of antibody responses. To include a positive control would have substantially increased both the raw materials used and the assay size required. Furthermore, the control plasmid used in this experiment is not ideally matched to the vaccine plasmid.

The plasmid was generously provided by Ray Lynne Burke (Chiron, Emeryville CA) and a matched control plasmid could not be found. Instead a plasmid which was matched for molecular weight of DNA and for antibiotic resistance was used. All experimental data were compared to this control which was preferable to the use of control sera of mice which had received PBS alone.

4.1.2. CTL responses to gB-DNA

CTL responses are recognized as one of the most fundamental immune responses to a DNA vaccine (reviewed in Donnelly *et al.*, (1997)). Many groups have reported CTL responses to various antigens when administered in a DNA vaccine. Many of these studies again fail to demonstrate CTL responses to a single administration of a DNA vaccine at various doses. Two studies have examined the kinetics of a typical CTL response to DNA vaccines following a single IM administration of vaccine. Davis *et al.* (1995b) performed a time course on CTL responses and determined that CTL was not only evident by day six post immunization, but persisted up to 120 days post immunization. The level of CTL activity against Vac gB infected target cells in this study was similar to those observed by Davis *et al.* (1995b) at lower effector to target ratios. Kuhober *et al.* (1996) also reported very similar CTL activity in splenocytes isolated from mice following a single administration of a DNA vaccine encoding the hepatitis B surface antigen in H-2^b mice. Strong CD8+ mediated CTL responses have also been found following a single immunization with a DNA vaccine encoding HIV proteins in mice

(Wagner *et al.*, 1996). This result supports the results presented here, as killing in this assay was H-2^b restricted as determined by an assay similar to that used by Zinkernagel and Doherty (1979). Previous studies using HSV protein-based DNA vaccines have also demonstrated CTL mediated killing by CD8+ effector cells. Initial investigations of *in vitro* CTL responses to gB of HSV demonstrated the involvement of CD8+ T cells (Rouse *et al.*, 1994). *In vivo* generated CTL were later isolated from Balb/c mice, but the role of CD8+ T cells was equivocal (Manickan *et al.*, 1995a). Since gB is an important CTL target in C57BL/6 mice (Gallichan and Rosenthal, 1996b) and since most of the CTL responses in H-2^d mice are directed to ICP27 (Banks *et al.*, 1991), these findings were expected. Indeed, it was further shown that in H-2^d mice receiving a DNA vaccine encoding ICP27, the majority of CD8+ T cell responses were directed against this epitope and that adoptive transfer of CD4+ T cells was responsible for protection in this model (Manickan *et al.*, 1995b). In the current study using C57Bl/6 mice, it appears that killing is mediated by CD8+ MHC class I restricted CTL, as only H-2^b expressing targets are lysed in the presence of effector cells.

In a study examining CTL dose responses induced by a DNA vaccine, Ulmer *et al.* (1994) used two separate doses of a DNA vaccine encoding flu antigens administered 3x IM. While only two dosages were used the authors demonstrated increased CTL activity with increased dosages of vaccine (Ulmer *et al.*, 1994). We have shown dose response relationships are apparent in the CTL activity measured responses. These responses were most evident at higher dosages of gB-DNA, but were also obtained at

most effector to target ratios and dosages used.

Future experiments should also examine the immunization regimen which may generate optimal CTL responses. It is possible that the CTL responses in this experiment are not optimal as no time course was performed. Furthermore, it is possible that CTL responses may have been higher in draining lymph nodes shortly after administration. However, most research has utilized splenocytes as a source of CTL (Etchart *et al.*, 1997; Sasaki *et al.*, 1998; Ulmer *et al.*, 1993). Further improvements to this study would include the use of an anti-CD8+ mAb to identify the effector population responsible for the gB specific killing in this assay.

In an attempt to determine if the INL route of vaccine administration could induce improved antibody responses or responses with reduced variability, mice were immunized with 50 μg of gB DNA vaccine in 20ul of PBS. It has been previously demonstrated using DNA vaccines encoding for flu antigens that it is possible to generate IgG and IgA responses following a single INL immunization with DNA in saline (Fynan *et al.*, 1993b). The current study followed a similar protocol used by Fynan *et al.* (1993b), but failed to generate measurable antibody responses in plasma up to six weeks post immunization. Failure in this experiment was probably related to the number of immunizations of gB DNA and the nature of the antigen. Following these experiments, Kuklin *et al.* (1997) were able to measure IgG responses in mice to gB of HSV following three INL administrations of DNA. The gB specific IgG responses were very low, and were only slightly raised with the combined use of cholera toxin as an adjuvant (Kuklin *et al.*,

1997). Following initial reports of successful INL application, most groups began using three separate INL administrations of their DNA vaccines. Using this methodology, antibody and CTL responses have been generated to HIV (Hinkula *et al.*, 1997; Okada *et al.*, 1997). The requirement of multiple immunizations is probably related to the fact that vaccines must be able to overcome mucosal barriers such as enzymes and mucous to successfully transfect cells and generate immune responses. Indeed, studies investigating the expression of luciferase plasmids in the nasal cavities of mice following a single intranasal administration found that expression levels of the luciferase vectors increased more than 30 times when a cationic lipid transfection agent was utilised (Barnfield *et al.*, 1997). Furthermore, Klavinskis *et al.* (1997) reported that luciferase specific IgG and IgA responses were only detectable in mice immunized with DNA-liposome complexes, which also induced CTL. Etchart *et al.* (1997) using a single INL administration of a DNA vaccine encoding measles virus haemagglutinin were able to generate CTL responses using naked DNA, however the responses were stronger when the vaccine was concurrently administered with a cationic lipid or cholera toxin as an adjuvant. Antibody responses were not reported (Etchart *et al.*, 1997). Based upon these reports, the initial success by Fynan *et al.* (1993b) were likely related to the highly antigenic nature of influenza.

Given the information now available in the literature, it is highly probable that gB specific antibody and CTL responses could be generated following INL administered DNA vaccines, which are comparable to those responses generated by IM administration

of the same vaccine given the use of the proper adjuvant. The most promising adjuvant candidate to date is a monophosphyrl lipid A adjuvant, which has been used to demonstrate that the INL route of administration is as effective as IM administration in the generation of HIV specific IgA and IgG (Sasaki *et al.*, 1998). This example illustrates that the type of antigen has a great deal to do with the generation of immune responses, as IgA responses using gB DNA vaccines were able to generate only low levels of IgA regardless of the method of vaccine delivery or the adjuvant used. The most recent report utilising a gB DNA vaccine found that even three INL immunizations with gB DNA and an adjuvant were required to generate antibody responses, which were less than 1/10 of those generated by IM administration, although reasonable levels of gB specific IgA were obtained in vaginal washes and faecal matter (Kuklin *et al.*, 1997). One caveat regarding multiple INL immunizations with a DNA vaccine comes from observations from Olsen *et al.* (1997) who demonstrated that INL immunizations with a DNA vaccine designed to prevent influenza in animals when immunized 3 weeks apart demonstrated no protection to lethal challenge, yet when administered 9 weeks apart, 90% protection was observed. Therefore, as with IM administration, it appears that future investigation must also consider the vaccination schedule when trying to elicit immune responses when the method of delivery is via INL immunization.

One potential application of DNA vaccines which has only recently been examined is the combined use of DNA vaccines with more traditional vaccines as either the primary inducer of immunity, or a booster vaccine. As previously mentioned, Nakano *et*

al. (1997) demonstrated that multiple immunizations with a DNA vaccine encoding hepatitis C antigens resulted in lowered antibody responses, thus an alternate vector may be desirable for boosting antibody responses induced by DNA immunization. In the current work, a gB-DNA vaccine was used in combination with an adenovirus-based gB vaccine. Although the results do not demonstrate antibody responses which are higher than those induced with adenovirus alone, animals which were primed with a DNA vaccine alone generated a secondary antibody response which was 10 times greater than primary antibody responses generated with a DNA vaccine alone (Figure 3.1.6.). Using this strategy, several groups have demonstrated that it is desirable to combine DNA vaccines with other vaccine vectors to generate immune responses which are greater than those generated by either vaccine on its own. The most striking evidence of this phenomenon has been observed using combinations of DNA with a peptide or viral-based vaccine vectors. Barnett *et al.* (1997) demonstrated a clear benefit of combining DNA vaccination with a peptide-based vaccine and an oil water adjuvant, whereas a DNA vaccine alone failed to generate a strong humoral response. Okuda *et al.* (1997) further demonstrated that this combination generated stronger anti-HIV immunity by inducing longer lasting humoral and cell mediated immunity than could be obtained by boosting with either vaccine alone. Ramsay *et al.* (1997) using a combination of a fowl pox vaccine and DNA vaccine have demonstrated protective cell-mediated and sustained systemic and mucosal antibody responses to HIV. Furthermore, in rhesus macaques, combinations of a DNA vaccine and vaccinia-based anti-SIV vaccines used by Fuller *et*

al. (1997b) demonstrated enhancement of antiviral immunity to SIV, and to HIV in rabbits (Richmond *et al.*, 1997). More pertinent to the current study are findings by Rothel *et al.* (1997) who used a combination of DNA vaccine and adenovirus-based vaccine to *Taenia ovis*. The authors demonstrated that sheep initially immunized with the DNA vaccine and boosted with the recombinant adenovirus generated IgG responses greater than 65-fold higher than those induced with either vaccine alone (Rothel *et al.*, 1997). It is possible that in the current experiment, the dosage of AdgB chosen was too high and masked any potential combination effects of immunization, as antibody responses induced by adenovirus alone in this experiment may already be optimal, as demonstrated in results section B figure 1B.

4.2 Adenovirus-gB Based Vaccine Vector

Intranasal immunization has continued to gain popularity as a non-invasive method of administering various types of vaccines. It has been used to successfully generate immunity to various pathogens, but many of these studies fail to discuss or identify what the potential inductive sites for the observed immune responses are. Inductive sites in the upper respiratory tract include the NALT, BALT, trachea, and lung (Kuper *et al.*, 1992). The NALT has been well characterized in mice (Asanuma *et al.*, 1995), and it has been reported that the NALT shares some characteristics of the spleen having lower B cell frequencies than PP and no significant frequency of IgA-switched cells (Heritage *et al.*, 1997). However, it has been reported that upon INL immunization, IgA specific antibody

forming cells (AFCs) were detectable in the NALT, suggesting that IgA is produced locally and is best obtained by local stimulation of NALT cells (Heritage *et al.*, 1997). Comparisons of the NALT to the PP and other secondary lymphoid structures were also performed by Asanuma *et al.* (1995) using stimulation with an influenza virus. Their characterization of the NALT is in agreement with the report by Heritage *et al.* (1997) which described the NALT as a phenotype which is between the extremes of the spleen and PPs following antigenic stimulation (Asanuma *et al.*, 1995) . Although a consensus is not obtained with respect to the functional differences between the NALT and other mucosal lymphoid structures, the literature is in agreement that the NALT is significantly different from other mucosal lymphoid organs, and that the NALT is more of a T-cell organ, while PPs are more of a B cell organ (Kuper *et al.*, 1992). In short, the NALT is present earlier in development than PPs or BALT (Van der Ven and Sminia, 1993), and is significantly different from any other secondary lymphoid structure with respect to its cellular composition.

Based upon these reports the hypothesis was generated that direct stimulation of NALT cells with AdgB should be sufficient to generate potent systemic and mucosal antibody responses. It had been previously reported that INL administration of AdgB was sufficient to generate good systemic and mucosal antibody responses in addition to long-lived CTL responses (Gallichan and Rosenthal, 1996b). However, as the mice in the study by Gallichan and Rosenthal (1996b) were immunized while asleep, it is possible that virus was distributed to the upper and lower airways, thus clouding the issue of

whether the NALT was the sole inductive site in their report versus combined sites of induction such as the NALT, BALT and lungs of mice. To test this hypothesis, mice were immunized awake or asleep with the same dosages of AdgB and systemic and mucosal antibody responses were measured. The difference in the method of viral administration was based upon the theory that mice immunized awake limit virus distribution to the upper respiratory tract, as a result of intact gag reflexes and the ability to swallow fluid. In contrast, mice immunized asleep do not have an active gag reflex which may result in mice inhaling fluid into the lower respiratory tract.

This is the first study which directly compared mucosal and systemic antibody responses following INL administration to mice either awake or asleep. The significance of this study is based upon the theory that adenoviral administration to awake animals restricts the inductive site to the NALT, whereas administration to asleep animals results in the combination of the NALT, BALT and lungs as potential inductive sites. The only study which is similar attempted to compare immune responses in mice generated following intra-tracheal (IT) or intranasal administration of an adenoviral vector containing B-gal (Van-Ginkel *et al.*, 1995), which could have clearly compared and contrasted immunity induced by the NALT only versus the BALT and lung collectively. However, the INL administration was performed on anaesthetized animals (Van-Ginkel *et al.*, 1995). The findings by Van Ginkel *et al.* (1995) are nevertheless important as they suggest that INL immunization of adenovirus to asleep mice rendered very similar immune responses compared to intra-tracheal (IT) immunization, including IgA

production. Studies based upon INL immunization to awake mice are generally limited to experiments which have utilized protein antigens coupled to the cholera toxin B subunit (Wu *et al.*, 1996; Wu and Russell, 1993). In these studies, direct comparisons of immune responses between NALT cells and cells from PP were compared. The authors found similar immune responses with respect to systemic and mucosal antibody responses induced by either oral or INL administration, but they noted that the NALT contained a higher frequency of T cells and a lower frequency of sIgA producing cells than were found in the PP (Wu *et al.*, 1996). Of interest and of possible relevance to the findings in this study, was the observation of low IgA responses in the tracheal washes of the INL immunized mice which were awake by Wu and Russell (1993). However since this was a completely different antigen system which was additionally combined with an adjuvant, it is difficult to draw any direct comparisons.

Intranasal immunization to animals which are asleep has become the standard method of immunization by most research groups. This method provides the advantage of ease of handling of animals while administering vaccines. In addition, this method of administration is frequently used to generate good systemic IgG and mucosal IgA levels to protein antigens generally in combination with a mucosal adjuvant (Aramaki *et al.*, 1994; Gizurarson *et al.*, 1995) or to recombinant viruses encoding antigenic proteins in small volumes of PBS (Gallichan *et al.*, 1993; Lubeck *et al.*, 1994; Takao *et al.*, 1997; Tamura *et al.*, 1989). More recently, intranasal immunization has been applied to DNA vaccination. The only studies to date which have attempted to identify cells involved in

the generation of an immune response following INL immunization utilized DNA vaccines encoding reporter genes. Two studies utilizing the firefly luciferase reporter gene found luciferase expression in the epithelium of the nasal tissue, the trachea, lungs, cervical and mesenteric lymph nodes, and the gastrointestinal tract following asleep administration (Barnfield *et al.*, 1997; Klavinskis *et al.*, 1997). In another study utilizing the beta-galactosidase (B-gal) reporter gene in a plasmid, B-gal expression was also found in the nasal tissues, lungs and cervical lymph nodes of mice following INL administration (Kuklin *et al.*, 1997). These results are in general agreement with the results obtained from this study which demonstrate that adenovirus which has been delivered awake passes through the NALT, rarely enters the trachea and does not enter the lungs of immunized animals. In contrast, adenovirus which has been delivered to animals which are asleep passes through the NALT, enters the trachea and frequently (7/9 times) enters the lung where it is subsequently expressed. This suggests that awake administration of a replication deficient adenovirus restricts viral activity to the upper respiratory system and limits immune induction to the NALT, whereas asleep delivery distributes expression between the upper and lower respiratory system allowing the participation of the NALT, BALT, trachea and lung as sites for immune induction. The fact that this study utilized a virus delivery vehicle for the reporter gene is a fundamental difference between this study and other studies reporting on the expression of reporter genes in plasmids following INL administration asleep. Viruses must follow receptor mediated uptake in tissues, so their distribution is likely to be more precise than expression from a plasmid which harbours

no tropism for different tissues. There have not been any reports in the literature to compare our results on virus distribution following INL administration of AdLUC to awake animals. Physical distribution of adenovirus is a key element of this study. If AdgB follows the same distribution pattern as the AdLUC expression virus, then many of the observed differences in immune responses may be explained by the fact that awake and asleep administration targets different sites for the induction of immune responses.

From observing systemic antibody responses at various dosages and at various time points, it is evident that awake administration which presumably limits induction to the NALT, is capable of generating systemic antibody responses which are not significantly different from those obtained by administering AdgB to mice which are asleep, which presumably allows the participation of the BALT and lungs in addition to the NALT for immune induction. This suggests that regardless of the site of induction similar numbers of antigen specific B cells are generated which then produce similar amounts of antibody. Similar findings were reported by Gallichan *et al.* (1993), who found similar systemic antibody responses in mice which had been immunized by IP or INL routes with AdgB. Similar antibody responses in awake or asleep recipient mice also suggest that the possible loss of virus to the gastrointestinal system in awake mice does not appreciably affect the ability of these mice to generate antibody responses illustrating that the differences observed later with regard to mucosal responses are not merely due to different dosages of virus.

The results of this study also expand on earlier findings by Gallichan *et al.* (1993) by

extending the examination of the antibody responses to various dosages of AdgB and the resulting variability. The results demonstrated dose response relationships at the two-week time point in mice immunized asleep and the four-week time point in mice immunized awake. A possible explanation of this delayed response in mice receiving AdgB awake may be in part due to different kinetics of antibody responses generated at one site, particularly the NALT, versus a combination of inductive sites, particularly the NALT, BALT and lung.

The test of the second part of the hypothesis, that intranasal immunization awake should generate mucosal antibody responses which are similar to those obtained by asleep administration resulted in the rejection of the hypothesis. Awake administration of AdgB failed to generate mucosal antibody responses which are similar to those generated following asleep administration of AdgB. Heritage *et al.* (1997) examined IgA specific AFC to cholera toxin, following administration to awake mice and suggested that the NALT is a desirable site for inducing local antibody production. However results obtained in the current study suggest that although the NALT may be a desirable site for inducing local antibody responses to a protein antigen, it is not a desirable site for inducing local antibody responses to an recombinant adenoviral vector, since at any dosage, gB specific IgA responses were significantly lower than those observed in animals which received AdgB asleep resulting in interactions with deeper tissues such as the BALT and lungs. These results suggest that while the NALT may be sufficient to generate IgA responses to adenoviral vectors following awake administration, it may not

be the ideal inductive site, and that the involvement of lymphoid structures like the BALT and other tissues such as the trachea and lung may be desirable for generating optimal mucosal antibody production, as is the case following asleep administration of adenovirus.

The only studies which have compared the delivery of viral vaccines to the upper and lower airways have been reported in humans receiving live and inactivated influenza vaccines (Treanor *et al.*, 1992; Waldman *et al.*, 1970). In the initial studies utilizing an inactivated influenza vaccine, Waldman *et al.* (1970) reported that small particles capable of entering the lungs of volunteers generated the best systemic antibody responses, whereas large particles restricted to the nasal passage generated only local antibody responses. Intranasal immunization in humans most recently has involved the administration of nasal droplets to individuals, resulting in only moderate numbers of individuals producing a local IgA response (Hashigucci *et al.*, 1996). This information, combined with the results obtained previously by Waldman *et al.* (1970) suggest that better immune responses to influenza would be obtained by a delivery method which would target the lower airways of individuals. These results collectively give indirect evidence that the significant differences observed between mucosal IgA production in this study are due to differences in where the site of induction is located. The most direct evidence to support the limited ability of the NALT to generate IgA is derived from a recent study by Heritage *et al.* (1998), who reported that the NALT is capable of generating systemic antibody responses with protein antigens entrapped in microparticles,

but not capable of generating local or systemic IgA responses. All of the animals used in their study were immunized awake (P.L. Heritage, personal communication).

Another possible explanation for the results of our study may be related to the high numbers of DCs present in the epithelium of the upper airway in mice and the high tropism which adenovirus have for DCs (Holt *et al.*, 1994; Holt *et al.*, 1995), many of which present antigen in an MHC II-dependent fashion (McMenamin *et al.*, 1994; McWilliam *et al.*, 1995). Airway DCs are present constitutively in the epithelium of conducting airways of mice, but also increase rapidly in number following infection with adenovirus and inflammation (McWilliam *et al.*, 1996; McWilliam *et al.*, 1994; Nelson *et al.*, 1995; Thepen *et al.*, 1994), a phenomena which is now recognized as a hallmark of the mucosal immune response. It may be possible that the distribution of adenovirus to the lower airways of animals following asleep administration takes advantage of this DC network to induce heightened antibody responses. Although the equivalent systemic antibody responses between awake and asleep mice do not support this theory, DCs may partially contribute to the significantly different IgA responses observed between awake and asleep mice. Support for this comes from the demonstration that a Th1/Th2 switch is involved in IgA production in response to inhaled antigens (Holt *et al.*, 1997).

The observed differences between mucosal antibody production, in light of the phenotypic differences between the NALT and BALT, suggest that the NALT may only contribute in a limited manner to the common mucosal immune system. The common mucosal immune theory proposes that immune responses generated at one mucosal site

generates mucosal antibody responses which are common to all mucosal secretions (McDermott and Bienenstock, 1979). A more modern definition of this theory may be that "true" mucosal inductive sites generate cells that can seed other mucosal sites allowing for the production of IgA at those sites. This study suggests that the NALT is not a true mucosal inductive site, or may be only partially effective at mucosal induction. For instance, when gB specific IgA was measured at various mucosal sites, regardless of the time point, IgA was not detectable in the lungs of immunized mice even though potent immune responses were generated as measured by systemic IgG antibody production. Furthermore, gB specific IgA responses were consistently higher in all mucosal tissues in mice immunized asleep. Assuming that the inductive site in this study following awake administration is indeed the NALT alone, the data suggests that the NALT may not be as desirable an inductive site as the BALT and lungs for inducing common mucosal secretions. The well-documented participation of the BALT in the common mucosal immune network (Bienenstock *et al.*, 1982) may actually explain why there are higher IgA levels in the nasal secretions of mice immunized while asleep. Given that following INL immunization asleep, there is participation of the BALT in the induction of immunity, then the BALT may act as a reservoir of IgA secreting cells which in turn may migrate to the NALT and as a result increase local IgA production above and beyond what was induced at the NALT alone. Furthermore Gallichan and Rosenthal (1998) reported that only INL immunization with AdgB to asleep mice maintained ASC in the genital tracts of immunized animals, whereas systemic immunization with AdgB failed to

do so, observations which may give indirect support to this theory.

In summary, the results of this study begin to suggest that there are important differences in the type of immune responses which may be generated from different inductive sites, or at the minimum, from awake or asleep administration in mice. To give further support to this study, immunohistochemistry was attempted to identify the physical distribution patterns of gB following administration of AdgB, but due to technical difficulties it was not successful. All suggestions involving physical distribution of AdgB are merely speculative and are based upon the fact that AdgB and Ad-LUC share the same receptors for their uptake. It is possible though that the properties of each protein may influence their expression at different sites. Further experiments that could be attempted in the future include the *in vitro* culture of cells from inductive sites with AdgB followed by a measurement of cytokine profiles to determine the involvement of different cytokine milieus in the different mucosal tissues. Furthermore, the addition of an IT recipient group in these experiments could help identify the individual involvement of the NALT and BALT and lung respectively. This study also begins to suggest that there may be important considerations with respect to current vaccine administration. For instance it is now generally accepted that the NALT serves as an important model in mice for the equivalent lymphoid structure (Waldeyer's ring) in humans (Heritage *et al.*, 1998). Assuming this model holds, and given the above reports of immune responses to influenza vaccines, this study suggests that future vaccine delivery to the airway mucosa should be formulated to involve deep lung responses if

mucosal responses are desirable. Furthermore, as Gallichan and Rosenthal (1996b) have demonstrated long lived CTL memory responses following mucosal immunization to asleep mice, modifying vaccine delivery to include lower airway involvement may significantly enhance the efficacy and longevity of not only systemic and mucosal antibody responses, but also cellular immune responses to vaccines against various pathogens.

4.3 Dendritic Cell-AdgB Based Vaccine Vector

Studies utilizing *in vitro* AdgB transduced DCs were used to determine to what degree DCs are involved in the generation of humoral and mucosal antibody responses in AdgB immunized mice and to characterize their abilities to generate humoral and mucosal antibody responses upon systemic or mucosal delivery to syngeneic mice. The early infiltration of DCs is characteristic of a mucosal immune response (McWilliam *et al.*, 1996), however their role in generating humoral and mucosal antibody responses following direct infection with AdgB *in vitro* had not been ascertained. It has been reported that DCs transfected *in vitro* with gB DNA were able to significantly enhance CD4+ T cell mediated protection (Manickan *et al.*, 1997) and generate CTL responses (Rouse *et al.*, 1994) following systemic transfer to syngeneic recipient mice, however the authors failed to report on their ability to induce or enhance antibody responses. Since the role of DCs in the stimulation of T cells is well understood (Schuler *et al.*, 1997), as is the interplay between naive B cells and CD4+ T cells (Kosco and Gray, 1992), it was

logical to examine whether DCs could generate comparable systemic and mucosal antibody responses to gB of HSV on their own following *in vitro* transduction with AdgB.

The results from this study suggest that DCs transduced *in vitro* with AdgB are not capable of generating comparable systemic antibody responses to gB and are not at all capable of generating mucosal antibody responses. These results are similar to results obtained by Brossart *et al.* (1997), who found that although direct immunization with a recombinant adenovirus vector expressing ovalbumin antigens generated high titres of antibodies, repeated injections of virus infected DCs only induced low titres of neutralizing antibody which were specific to ovalbumin. The failure of DCs to generate similar antibody responses to AdgB was unlikely due to a lack of infectivity *in vivo*, as it has been recently demonstrated that this method results in more than a 90% efficiency in infection (Dietz and Vuk-Pavlovic, 1998). The failure, paradoxically, is more likely related to this high efficiency of infection of DCs by adenovirus, as antigen presentation is shifted to MHC class I pathways following infection. Indeed, Brossart *et al.* (1997) also reported that although transduced DCs failed to generate comparable antibody responses to those induced with the vector alone, the DCs did significantly amplify CTL responses which increased after each subsequent introduction of the transduced DCs. These observations may demonstrate that the DCs used in this study, and the method of *in vitro* transduction may not be ideal for the generation of antibody responses. Furthermore, it has been reported that some DC subsets are more able to generate B cell responses

following direct stimulation (Caux *et al.*, 1996; Dubois *et al.*, 1997). As there was no characterization of the DCs used in these experiments, it is impossible to determine the predominant phenotype of the transferred DCs. Furthermore, it may have been desirable to include a source of follicular dendritic cells in these experiments to further stimulate B cell proliferation, as FDCs are not bone marrow derived (Matsumoto *et al.*, 1997) and thus would not be present in the DC population used in this study. Although DCs have been identified as important cells in the generation of mucosal antibody responses (Fayette *et al.*, 1997; Holt *et al.*, 1997), in this study transduced DCs failed to generate any mucosal antibody responses. Again, this failure may be related to the source and phenotype of DCs used in this experiment and the *in vitro* transfection method. To alleviate this problem, future experiments could include gB peptide to ensure presentation via the MHC class II antigen presentation pathway.

One interesting observation in this study is that mucosal delivery of DCs was able to generate antibody responses. These responses were more consistent when the volume of administration was high, however even when the volume was low, animals which did generate antibody responses generated responses which were as high, or higher than those obtained by intravenous administration. As expected, even though the DCs were transferred to a mucosal site, they still failed to generate mucosal antibody responses. A possible future experiment would be the co-administration of transduced DCs with AdgB. This may provide the necessary cytokine environment for the generation of mucosal responses that may be lacking when DCs are administered alone. This is the first report

to show successful generation of immune responses with transduced DCs following mucosal administration, and illustrates that not only are DCs able to cross external epithelial barriers (Havenith *et al.*, 1993), but are also able to generate immune responses. Further experiments should be performed to identify whether this is a unidirectional crossing, that is whether during infection in the lower airways of the lung DCs are able to enter the lumen, sample antigens and cross back to migrate to regional lymph nodes to induce immune responses. It is also of interest that the high volume administration of DCs was more consistent at generating antibody responses. It is probable that not only the state of wakefulness of animals, but the volume of administration may affect the distribution of fluid in recipient animals.

In summary, although adenoviral transduced DCs are capable of inducing CTL, and are used successfully for anti-tumour therapy, they are not as efficient as adenovirus vectors administered alone in the generation of systemic and mucosal antibody responses. This still does not rule out their usefulness where previous immunity to adenovirus exists, but limits their use as a primary therapeutic or vaccine for these purposes. This study does demonstrate that DCs are incredibly versatile cells which are able to cross mucosal barriers when delivered to a mucosal surface and still able to generate antibody responses which are as high or higher than responses generated from parenteral administration. Furthermore, it is possible that different preparations of DCs may still be able to generate systemic and mucosal antibody responses which are similar to those generated by direct application of vaccines alone.

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