EVALUATION OF IMMUNE RESPONSES AND PROTECTION IN MUCOSAL TISSUES FOLLOWING INTRANASAL IMMUNIZATION WITH A RECOMBINANT ADENOVIRUS VECTOR EXPRESSING GLYCOPROTEIN B OF HERPES SIMPLEX VIRUS

By

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EVALUATION OF MUCOSAL IMMUNITY FOLLOWING INTRANASAL IMMUNIZATION

To my parents, Neil and Argyle,
and my wife Carolyn

DOCTOR OF PHILOSOPHY (1996)
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TITLE: Evaluation of Immune Responses and Protection in Mucosal Tissues Following Intranasal Immunization With a Recombinant Adenovirus Vector Expressing Glycoprotein B of Herpes Simplex Virus

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ABSTRACT

In recent years there has been an increased interest in the design of vaccines capable of inducing immune responses in mucosal tissues and of providing protection against pathogens that invade through mucosal surfaces. Much of this interest has developed as a direct result of our greater understanding of the unique immune functions present in mucosal tissues. Numerous vaccine strategies have been developed to induce immunity in mucosal tissues and provide protection. The studies outlined in this thesis have utilized a recombinant adenovirus vector encoding the herpes simplex virus glycoprotein B (gB) gene (AdgB8) as a mucosal vaccine vehicle. The induction of gB-specific mucosal humoral and cellular immune responses was evaluated following intranasal and systemic routes of immunization. Immunological memory was also examined as was protection against mucosal infections with herpes simplex virus type-2 (HSV-2).

Previous work has shown that adenovirus vectors expressing glycoprotein B of HSV-1 are capable of inducing systemic antibodies and protection against systemic HSV infection. Evaluation of mucosal immune responses following intranasal AdgB8 immunization demonstrated IgG and IgA antibodies in mucosal secretions of the respiratory and genital tract. In contrast, systemic immunization only resulted in IgG antibodies in the genital tract. A thorough examination of antibody levels in the female genital tract revealed an isotype

dependency on the stages of the estrous cycle. These observations become important when considering the maintenance of immunity throughout the estrous cycle.

Currently, our understanding of the homing and recirculation patterns of memory lymphocytes suggests that immunological memory may depend on the tissues in which immune induction occurred. In addition, lymphocytes originating in mucosal tissues selectively migrate to local and distal mucosal tissues. To examine these phenomena functionally, CTL memory responses were evaluated in mice immunized with AdgB8. Functionally, short- and long-term CTL memory was found to depend on the route of immunization. These results are consistent with the recent literature and expand our current understanding of immunological memory on a functional level.

Protection studies were then performed to examine the role of intranasal and systemic immunization in resistance to HSV-2 infections of the respiratory and genital tracts. From these studies it was clear that intranasal immunization provided optimal long-term protection. Taken together, the results presented here will have important implications in terms of developing successful mucosal vaccines designed to provide protection against sexually transmitted pathogens.

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PREFACE

This dissertation consists of seven chapters. Chapter one, the introduction, describes the basic concepts and advances in the field that the thesis deals with and the objectives of the study. Chapters two to six, the body of the thesis, are composed of five individual scientific papers, of which the first two have been published and the last three submitted. Preceeding each paper is a background section which briefly outlines the purpose and experimental approach of the study. Following each chapter there is a section listing my specific contributions and a synopsis of the main findings. The final chapter, chapter seven, consists of a summary where the main results drawn from the preceding chapters are discussed and conclusions and implications arrived at. The references cited in the introduction and summary chapters, as well as the sections surrounding the papers, are listed in the references section at the end of the thesis.

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LIST OF ABBREVIATIONS

ASC	antibody secreting cells
APC	antigen presenting cell
BALT	bronchus associated lymphoid tissue
CAF	$CD8^+$ T-cell antiviral factor
CLA	cutaneous lymphocyte antigen
CTL	cytotoxic T lymphocyte
CTLp	cytotoxic T lymphocyte precursors
ELISA	enzyme-linked immunosorbant assay
ELISPOT	enzyme-linked spot forming assay
ER	endoplasmic reticulum
GALT	gut associated lymphoid tissue
gB	glycoprotein B
gD	glycoprotein D
HBV	hepatitis B virus
HEV	high endothelial venules
HIV	human immunodeficiency virus
HSE	herpes simplex encephalitis
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IFN-γ	interferon gamma
IEL	intraepithelial lymphocytes
IPV	inactivated poliovirus vaccine
LPL	lamina propria lymphocytes
LPS	lipopolysaccharide
LCMV	lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLN	mediastinal lymph node
MLR	mixed lymphocyte reaction
NK	natural killer
OPV	oral poliovirus vaccine
PBMC	peripheral blood mononuclear cells
pIgR	poly immunoglobulin receptor
pIgA	polymeric immunoglobulin A
SC	secretory component
SCID	severely combined immunodeficient

SIgA	secretory immunoglobulin A
SIV	simian immunodeficiency virus
TNF-α	tumour necrosis factor-alpha
VSV	vesicular stomatitis virus

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CHAPTER ONE

INTRODUCTION

A. THE MUCOSAL IMMUNE SYSTEM

1. General Background

The concept of a mucosal immune system was first developed in 1963 by Chodirker and Tomasi following quantitative examination of mucosal fluids for the presence of various immunoglobulin isotypes. These studies found that external fluids that bathe mucosal surfaces contained predominantly IgA. Subsequent to these findings Tomasi *et al.* (1965) showed that IgA in saliva was present in a polymeric form and contained an additional component which was later termed secretory component (SC). Secretory component has since been shown to be produced by mucosal epithelial cells and is responsible for transport of polymeric IgA (pIgA) onto the luminal surface of mucosal tissues (reviewed in Underdown and Mestecky, 1994) (see Figure 1). The immunological implications of the selective transport of pIgA but not of monomeric IgA or IgG forms the framework upon which the concept of the mucosal immune system is built.

2. Secretory IgA

Secretory IgA (SIgA) constitutes the most recognized effector mechanism of the

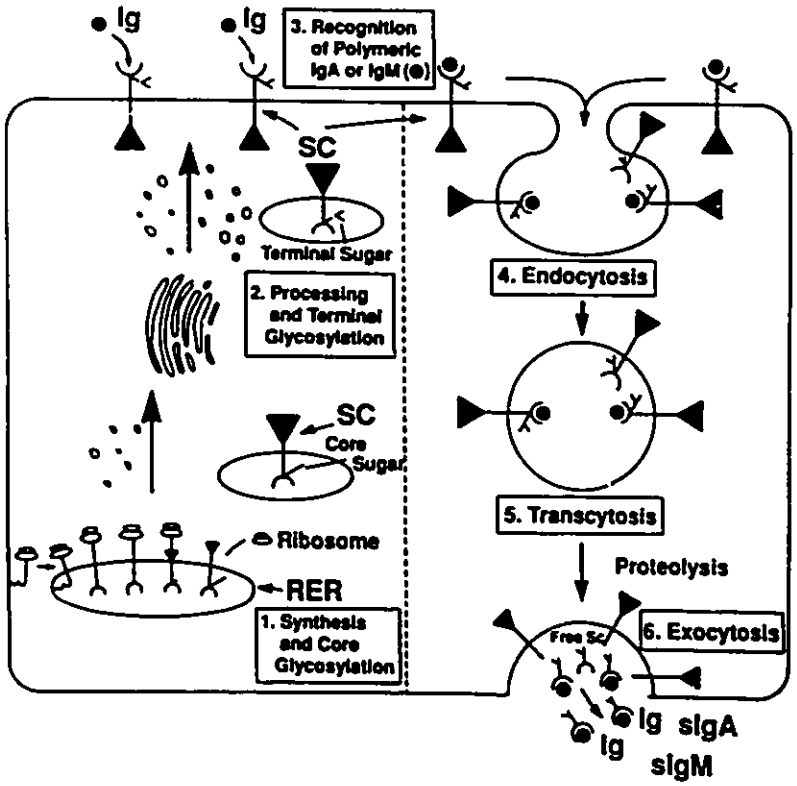
mucosal immune system and forms the first specific immune barrier against the penetration of mucosal pathogens. While most species contain a single class of IgA antibodies, human IgA antibodies are divided into two subclasses, IgA1 and IgA2. These subclasses differ mainly in the absence of a 13-amino-acid region in the hinge of IgA2. The presence of this extended hinge region of IgA1 molecules is thought to confer greater segmental flexibility, but at the same time renders the antibodies susceptible to proteases produced by bacterial pathogens at mucosal surfaces (Plaut, 1983). The absence of this hinge region on the IgA2 subclass is therefore advantageous for IgA2 molecules at mucosal surfaces. Interestingly, murine IgA appears to be similar to the IgA2 isotype of humans since it does not generally bind the lectin jacalin (Skea *et al.*, 1988), nor does it possess the hinge region characteristic of the IgA1 subclass (Underdown and Mestecky, 1994).

Comparative studies of polymeric immunoglobulins (Ig), including SIgA, with that of monomeric IgG immunoglobulins demonstrated that the monomers in SIgA are linked through disulfide bonds to a polypeptide chain, termed the J chain (Inman and Mestecky, 1974). Complexing of J chain with the monomeric units of Ig to form polymers occurs just prior to secretion (Parkhouse and Della Corte, 1973), and the conformation that the J chain lends to both IgA and IgM is required for complexing with SC (Hauptman and Tomasi, 1975). SC is the ligand for the poly Ig receptor (pIgR) that binds polymeric immunoglobulins at the basolateral surface of mucosal epithelial cells, internalizes it via receptor-mediated endocytosis, and transports it to the apical or luminal surface (see Figure 1) (Mostov *et al.*, 1984). Polymeric IgA found in mucosal secretions following active transport by pIgR is

Figure 1: Model for transport of IgA and IgM across the mucosal epithelium.

Secretory component is synthesized and core glycosylated on polysomes on the rough endoplasmic reticulum (RER) and inserted cotranslationally into the RER. SC then becomes integrated into the basal (and lateral) plasma membranes of the epithelium. The ectoplasmic domain of SC acts as the external receptor for J chain-containing polymeric Igs such as IgA and IgM. Endocytosis of the complex, as well as free SC, occurs in vesicles (endosomes) that carry the Ig-receptor complex to the luminal surface. Proteolysis occurs at an unknown stage, removing the cytoplasmic and membrane-spanning portions, but leaving SC attached.

(Reproduced from Tomasi, 1994)


Ig
Ig
3. Recognition of Polymeric IgA or IgM (●)
SC
Terminal Sugar
2. Processing and Terminal Glycosylation
SC
Core Sugar
Ribosome
RER
1. Synthesis and Core Glycosylation
4. Endocytosis
5. Transcytosis
Proteolysis
Free Sc
6. Exocytosis
Ig
Ig
sIgA
sIgM

associated with SC and is termed SIgA. Secretory IgA is a highly stable molecule and this stability is partially attributable to SC which renders SIgA less susceptible to attack by microbial enzymes. In addition, SIgA can bind trypsin and chymotrypsin in an antibody-independent manner that inactivates the enzymes (Shim *et al.*, 1969). Together, these attributes impart upon SIgA the ability to survive and function effectively at mucosal surfaces.

Although SIgA is recognized as the major antibody in external secretions, other isotypes are also present in mucosal secretions (Table 1). IgM is found in external secretions and is also associated with SC (Figure 1). Following transport by the pIgR, however, concentrations of IgM are somewhat lower than that of SIgA (Brandtzaeg, 1985) (Table 1). This is likely due, in part, to the size restriction in SC-dependent transport (Schiff *et al.*, 1983), as well as, to the lower proportion of IgM-producing cells in mucosal tissues (Table 1). Interestingly, SC-dependent transport of IgM may not occur in some species such as rodents and rabbits (Underdown *et al.*, 1992). The concentration of IgG in mucosal secretions is similar to that of IgM and is thought to enter nonspecifically via pericellular transport or fluid phase endocytosis (Underdown and Mestecky, 1994).

Secretory IgA found in external secretions is thought to be mainly derived from locally producing B cells (Lawton and Mage, 1969; Bienenstock and Strauss, 1970; Brandtzaeg, 1985; Mestecky *et al.*, 1991; Jonard *et al.*, 1984). In support of this is the high incidence of cells containing IgA found in the lamina propria in close anatomical relationship with the mucosal surface. The data in Table 1 indicates that Ig producing cells distributed in mucosal tissues are mainly of the IgA isotype. However, in the absence of SIgA, as occurs in

agammaglobulinemic patients, the administration of large amounts of IgA results in the appearance of SIgA in secretions (South *et al.*, 1966). Renegar and Small (1991) also demonstrated that the administration of pIgA but not monomeric IgA or IgG1 anti-influenza antibodies protected nonimmune mice against intranasal influenza challenge. These results suggest that serum-derived polymeric but not monomeric IgA is capable of being transported into external secretions. Indeed, serum pIgA likely competes with locally produced pIgA for available pIgR on cells and this is supported by the observation that fluids of the rat mammary gland and bile contain serum derived pIgA (Halsey *et al.*, 1980; Fisher *et al.*, 1979). In humans, serum pIgA is transported into saliva but only accounts for about 2% of the total SIgA (Delacroix *et al.*, 1982). Thus, SIgA likely originates from local plasma cells but serum-derived pIg may also be a contributing source depending on the concentration of antibodies supplied by local plasma cells and the availability of pIgR on epithelial cells.

3. Humoral Protection

The large amount of evidence demonstrating that the humoral components of the mucosal immune system are responsible for protection against mucosal pathogens has been based on correlations between specific SIgA levels and protection (reviewed in Mestecky *et al.*, 1994). Most of these studies are based on animal models involving antigen exposure and, thus, cannot rule out the participation of other immune functions such as T cells. However, several studies have explicitly demonstrated the role of IgA in protection of mucosal surfaces. Monoclonal IgA antibodies specific for Sendai or respiratory syncytial viruses passively

administered to the mouse respiratory tract conferred protection against subsequent challenge (Mazanec *et al.*, 1987; Weltzin *et al.*, 1994). Intranasal administration of SIgA specific to M protein protected mice against nasal inoculation with group A streptococci, whereas locally applied serum opsonizing antibodies were not protective (Bessen and Fischetti, 1988). Several studies have also shown that the production or administration of pIgA can provide the host with protection at the mucosal surface. Winner *et al.*, (1991) used "backpack" tumors of mouse hybridoma cells that produce pIgA antibody to *Vibrio cholerae* lipopolysaccharide (LPS) to protect against oral challenge with *V. cholerae*. As mentioned, when Renegar and Small (1991), gave mice pIgA against influenza virus hemagglutinin, it was transported into nasal secretions of mice and protected against nasal challenge.

There are several molecular mechanisms by which SIgA may provide protection at mucosal surfaces. First, inhibition of adherence of microorganisms to the mucosal surface may occur by agglutination (Williams and Gibbons, 1972; Abraham and Beachey, 1985). Whereas any antibody may be capable of inhibiting adherence, SIgA is unique in that the Fc-SC portion of the antibody is hydrophilic and negatively charged (Kilian *et al.*, 1988). This may allow SIgA to surround a microbe with a hydrophilic shell that repels attachment to mucosal surfaces. As indicated, SIgA is well suited also to the hostile mucosal environment through its greater ability to resist proteases produced by microbes as compared to IgG or monomeric IgA (Shuster, 1971; Underdown and Dorrington, 1974).

Secretory IgA antibodies can also function through neutralization of viruses and toxins at mucosal surfaces. Neutralization likely results in inhibition of viral binding to cellular

Table 1: Isotype distribution of immunoglobulin and cells in human fluids and tissue. (Reproduced from Underdown and Mestecky, 1994).

Fluid	Immunoglobulin concentration (mg/ml)			Tissue	Distribution of Ig+ cells[b]		
	IgG	IgA	IgM		IgG	IgA	IgM
Serum	12.0	3.0	1.5	Bone marrow	55	30	15
Milk	0.1	1.5	0.4	Mammary gland	4	86	10
Parotid saliva	0.004	0.04	0.006	Parotid gland	5	87	6
Jejunal fluid[c]	0.005	0.05	0.002	Jejúnum	3	79	18
Hepatic bile	0.09	0.07	0.02	Lacrimal gland	6	77	7
Tears	0.007	0.19	0.006				

[a] Data are from Brown *et al.*, 1975; Delacroix *et al.*, 1982,1985; Brandtzaeg, 1983a,b; Jonard *et al.*, 1984; Allansmith *et al.*, 1985; Kett *et al.*, 1986.

[b] Determined by immunofluorescence.

[c] The concentration is influenced by the amount of perfusate fluid added to the secretion. In some studies, IgG concentration ~IgA.

receptors, although other mechanisms may occur also depending on the concentration of antibody, the virus and cells involved. Armstrong and Dimmock (1992) demonstrated that while high concentrations of SIgA antibodies to influenza virus hemagglutinin inhibited cellular attachment, IgG or lower concentrations of pIgA permitted attachment but inhibited internalization or intracellular replication. More recently, pIgA has been shown to act intracellularly on viruses. Mazanec *et al.*, (1992 and 1995) demonstrated that pIgA antibodies specific for Sendai or influenza virus neutralized virus intracellularly during SC-mediated transit through epithelial cells. *In vivo* studies by Burns *et al.*, (1996) revealed that non-neutralizing IgA antibodies were able to protect animals from rotavirus as a direct result of intracellular inactivation . These results suggest that in addition to one of the commonly recognized functions of SIgA (ie. prevention of viral infection of mucosal epithelium), pIgA antibodies can also promote clearance or inactivation of virus following intracellular infection.

4. Cytotoxic Lymphocytes

Cytotoxic lymphocytes can be classified into two groups based on their specificity for antigen. Antigen non-specific cytotoxic lymphocytes form the first group and include several different cell types which are non-MHC restricted. Perhaps the lymphocytes best characteristic of this group are the natural killer (NK) cells. These cells usually arise during the early stages of a viral infection (Walker *et al.*, 1984; Welsh, 1978), and then tend to disappear with the onset of specific immunity. The implications of this are observed best in humans with an inherited deficiency of NK cells which leads to the development of more

severe herpesvirus infections when compared to normal individuals (Biron *et al.*, 1989). Infected individuals eventually do clear the virus in a manner similar to that of immunocompetent hosts, thus suggesting that NK activity plays an important early role in controlling the extent of mucosal viral infections.

The second group of cytotoxic lymphocytes consist of antigen-specific and MHC-restricted cytotoxic T lymphocytes (CTL). They are made up of $CD3^+$ T lymphocytes which are either $CD8^+$ or $CD4^+$. The $CD8^+$ T lymphocytes appear to be the major CTL effector with antiviral activity (Yap *et al.*, 1978; Kast *et al.*, 1986; Taylor and Askonas, 1986) although cytolytic $CD4^+$ T cells have been observed also and are discussed later. The T cell receptor (TCR) on the surface of $CD8^+$ T cells recognizes a short peptide derived from endogenously processed viral proteins in the context of the MHC Class I molecule expressed on the surface of infected cells (Madden, 1991). With the notable exception of neurons, MHC Class I restriction elements are expressed on nucleated cell types. This implies that $CD8^+$ CTL are able to exert their antiviral effects against most infected cells of mucosal surfaces. Since presentation of viral antigens in MHC Class I molecules requires cells to be actively infected, the role of $CD8^+$ CTL lies in prevention of viral spread and termination of infection but generally not in prevention of initial infection.

T lymphocytes bearing the $CD4^+$ marker are restricted to the presentation of antigen in the context of the Class II MHC. Class II MHC molecules are for the most part expressed on the surface of antigen presenting cells (APCs) such as Langerhans, dendritic, and B cells. In addition, Class II MHC determinants have been visualized by immunocytochemistry

intracellularily and on the surface of intestinal cells (Mayrhofer and Spargo, 1989, 1990). The role of $CD4^+$ T cells is generally considered to involve helper functions responsible for controlling humoral, as well as many cellular effector responses. Cytokines derived from $CD4^+$ T cells may also exert antiviral effects directly on infected cells not bearing Class II. In addition to these functions, $CD4^+$ T cells can mediate class II-restricted cytotoxicity, and have been extensively demonstrated in the herpesvirus system (Doymaz *et al.*, 1991; Manickan *et al.*, 1995; Schmid, 1988; Yasukawa and Zarling, 1984).

5. Lytic Mechanisms of Cytotoxic T Cells

Recent advances in the characterization of cytotoxic T cells have revealed that their anti-viral functions depend on several independent mechanisms. First, those using preformed proteins including perforin and granzymes, and second, ligand-induced triggering of surface receptors such as the Fas or tumour necrosis factor (TNF) receptor. Perforin induces the lysis of target cells without DNA fragmentation by binding to target-cell membranes and forming pores of up to 16 nm in inner diameter. These pores then allow the passage of ions and proteins (granzymes) from effector cells into the target cell (Tschopp and Hofmann, 1996). Mice with a defective perforin-encoding gene have CTLs and NK cells with severely impaired cytolytic activity (Lowin *et al.*, 1994; Kagi *et al.*, 1994a; Walsh *et al.*, 1994a). The serine protease granzymes (A and B) are released with perforin and are believed to be responsible for the apoptotic activity of the cytoplasmic granules of CTL and NK cells (Shi *et al.*, 1992; Shiver *et al.*, 1992).

In the absence of perforin, CTLs are still able to lyse target cells in a calcium independent manner and this residual activity has been shown to be due to the activity of Fas (Apo-1 or CD95) (Lowin *et al.*, 1994; Kagi *et al.*, 1994a). Fas ligand is expressed on activated CTLs and can cross link Fas on the surface of targets causing apoptotic cell death within hours (Nagata and Golstein, 1995; Krammer *et al.*, 1994). Even when perforin-granzyme and Fas pathways are deleted, CTLs still have considerable cytolytic activity directed towards target cells that are susceptible to TNF-α-induced lysis (Braun *et al.*, 1996). Activated T cells have been shown to express TNF-α and this mechanism is likely responsible for mediating this cytotoxicity *in vivo* (Ware *et al.*, 1995). More recently, an additional molecule has been isolated, NK-lysin, that has potent antibacterial and antifungal activity (Andersson *et al.*, 1995). NK-lysin is synthesized in activated $CD4^+$ and $CD8^+$ T cells as well as NK cells, and is found in cytoplasmic granules along with perforin and granzymes (Tschopp and Hofmann, 1996).

Traditionally, $CD8^+$ T cells are believed to mediate antiviral effects by causing lysis of infected cells by a number of possible lytic mechanisms as outlined earlier. Recently, results by Kagi *et al.*, (1994a) have confirmed this view. Perforin -/- mice that do not make a substantial virus-specific CTL response were unable to clear lymphocytic choriomeningitis virus (LCMV). New evidence, however, shows that perforin -/- $CD8^+$ T cells are able to clear vaccinia virus, vesicular stomatitis virus (VSV), and influenza virus (Kagi *et al.*, 1995). The difference might reflect the fact that these viruses are cytolytic, while LCMV is nonlytic and continues proliferation in cells in the perforin -/- mice. Thus, CTL must eliminate LCMV

infected cells in order to terminate the infection, whereas in lytic type infections, the cells inevitably die and release virus. Thus, the protective effect of cell-mediated immunity against lytic viruses may operate by interrupting dissemination, perhaps due to locally secreted cytokines or more recently recognized antiviral mechanisms (discussed in next paragraph). Therefore, the view that CTL control viral spread by lytic mechanisms is proving to be incomplete.

$CD8^+$ T cells have, in fact, been found to control virus infections nonlytically. In 1986, Walker *et al.* reported that $CD8^+$ T cells could nonlytically control the *in vitro* replication of human immunodeficiency virus (HIV) in peripheral blood mononuclear cells (PBMCs) of infected individuals. It was shown in other laboratories that a soluble factor(s) was responsible (Mackewicz and Levy, 1992; Gomez et al., 1994). The factor has been termed the $CD8^+$ T-cell antiviral factor (CAF) and lacks identity to known cytokines (Mackewicz *et al.*, 1994, Levy *et al.*, 1996). The CAF is produced by activated $CD8^+$ T cells and its activity appears to involve inhibition of virus transcription (Levy *et al.*, 1996; Mackewicz *et al.*, 1995; Copeland *et al.*, 1995). Second, two recent reports have also described factors which nonlytically control HIV production and these factors appear to be unique from the CAF. The first study identified three β-chemokines; RANTES, macrophage inflammatory protein 1α (MIP-1α) and MIP-1β (Cocchi *et al.*, 1995). The second study reported that IL-16, another chemotactic cytokine, also can control HIV replication (Baler *et al.*, 1995). All four of these molecules are produced by $CD8^+$ T cells and appear to inhibit replication but not transcription. Third, a recent model incorporating hepatitis B virus (HBV) transgenic mice

has shown that cytokines from $CD8^+$ T cells can mediate the degradation of HBV envelope transcripts expressed in hepatocytes (Guilhot *et al.*, 1993). Both TNF-α and IFN-γ were found to be responsible for this postranscriptional mechanism and it appears likely that a cytokine-mediated signal to the hepatocyte induces or activates specific cellular gene products which either inhibit the synthesis, destabilize, or actively degrade the viral gene products. This process results in the nonlytic inhibition of viral replication as shown *in vivo* (Guidotti *et al.*, 1994, 1995). These reports highlight the fact that $CD8^+$ T cells can impose their antiviral effects not only lytically, but also by several possible nonlytic mechanism which are more far reaching, suggesting a much larger role for $CD8^+$ T cells in host immunity than previously recognized.

6. Cytotoxic T Lymphocytes in Mucosal Tissues

Cytotoxic T lymphocytes have been found to play a large role in mucosal immunity and consequently have been identified in several mucosal immune compartments. Kagnoff and Campbell (1974) were the first to demonstrate that T lymphocytes from the Peyer's patches express allogeneic specific CTL activity. Additional studies by Kagnoff (1978) showed that feeding of allogeneic MHC protein increased the level of allogeneic cytotoxicity developed in secondary MLR cultures, suggesting that Peyer's patches were sites for the generation of CTL. Allogeneic specific CTLs from IELs and lamina propria lymphocytes (LPL) were also identified following application of allogeneic tumor cells (Davies and Parrott, 1980, 1981; Parrott *et al.*, 1983). The mesenteric lymph nodes which drain intestinal tissues, including

Peyer's patches, have been shown to contain vaccinia-specific CTL and T helper cell responses following enteric injection (Issekutz, 1984). Specific CTLs were also found in Peyer's patches and mesenteric lymph nodes following intraduodenal application of reovirus (London *et al.*, 1986). Since lymphocytes with CTL activity were isolated in the Peyer's patches prior to being observed in mesenteric lymph nodes, these results suggest a migration from Peyer's patches to mesenteric lymph nodes.

In addition to being observed within mucosal compartments, CTL have been shown to protect mucosal surfaces from viral infection. The transfer of reovirus specific $CD8^+$ CTL isolated from Peyer's patches or IELs restricted viral dissemination as well as protected against initial reovirus infection (Cuff *et al.*, 1991). In a similar manner, rotavirus infection of the gut mucosa results in the detection of effector CTLs in Peyer's patch, mesenteric lymph nodes, lamina propria, spleen, and interepithelial lymphocyte populations (Offit and Duddzik, 1989; Offit *et al.*, 1991). Isolation of $CD8^+$ immune lymphocytes from the spleen or IELs provided protection against rotavirus infection of severely combined immunodeficient (SCID) mice (Dharakul *et al.*, 1990, 1991).

The identification and role of virus-specific CTL in other mucosal tissues following infection has been extensively studied. For example, influenza virus-specific precursor and effector CTLs are found in the lung following intratracheal infection (Bennink *et al.*, 1978; Ennis *et al.*, 1978; Yap *et al.*, 1978; Allan *et al.*, 1990). The ability of these virus-specific CTLs to control influenza replication has been extensively proven (Yap *et al.*, 1978; Lukacher *et al.*, 1984; Taylor and Askonas, 1986). The restriction of replication of influenza as well

as other viruses in mucosal epithelial cells has also been shown following the passive transfer of CTL clones (Cannon *et al.*, 1988; McDermott *et al.*, 1987; Mackenzie *et al.*, 1989; Munoz *et al.*, 1991). Protection from disease in these mucosal tissues is mediated also by $CD8^+$ CTL (Mackenzie *et al.*, 1989; Munoz *et al.*, 1991; McDermott *et al.*, 1987), and McDermott *et al.* (1989) demonstrated that the adoptive transfer of herpes-specific genital lymph node lymphocytes with CTL activity protected mice from an intravaginal HSV-2 infection. Antiviral CTL have also been isolated directly from the vaginal epithelium of rhesus macaque monkeys infected intravaginally with simian immunodeficiency virus (SIV) (Lohman, 1995). The development of specific CTL in lymphoid tissues and mucosal populations following infection or immunization, and the fact that these lymphocytes can mediate protection upon adoptive transfer, underscores the vital importance of inducing these cells in vaccine formulations designed to protect mucosal surfaces against viral infection. Although the role of CTL appears prominent in controlling mucosal viral infections, specific CTL can also enhance the disease process by causing excessive tissue damage (Cannon *et al.*, 1988).

As previously indicated, $CD4^+$ cytotoxic T cells have been observed following herpesvirus infection of mice (Doymaz *et al.*, 1991). The generation of $CD4^+$ cytotoxic T cells also occurs in mice lacking the ability to generate $CD8^+$ T cells (ie. β2-microglobulin -/- mice), likely as a compensational mechanism (Yang and Wilson, 1995; Eichelberger *et al.*, 1991; Hou, 1992; Muller *et al.*, 1992). $CD4^+$ T cells are also necessary in eliciting a fully competent primary CTL response (Yang and Wilson, 1995). This occurs, in part, through activation of $CD4^+$ helper T cells of the Th1 subset, likely as a result of cytokine (IFN-γ, IL-

2) secretion (Mosmann and Sad, 1996). Additionally, the antiviral competence of Th1 $CD4^+$ T cells has been shown following the adoptive transfer of Th1 cell lines. These $CD4^+$ lines produced IFN-γ, IL-2, and TNF and protected mice from lethal challenge with influenza (Scherle *et al.*, 1992) and herpesviruses (Leung *et al.*, 1984). In fact, the $CD4^+$ T cell mediated clearance of virus can occur in the absence of interaction with infected epithelium, suggesting that cytokines and/or antibody are responsible (Topham *et al.*, 1996). Interestingly, $CD4^+$ T cells can terminate viral infections caused by influenza (Bender *et al.*, 1992; Eichelberger *et al.*, 1991), Sendai (Hou *et al.*, 1992), and vaccinia (Spriggs *et al.*, 1992) viruses, but not the nonlytic lymphocytic choriomeningitis virus (LCMV) that is dealt with by $CD8^+$ CTL quite effectively (Doherty *et al.*, 1993; Jung-Lueng *et al.*, 1991; Lehmann-Grube *et al.*, 1993). As important mediators of protection against herpesvirus infections, Manickan and Rouse (1995) and Smith *et al.* (1995) demonstrated that IFN-γ produced by $CD4^+$ T cells was responsible for resistance to HSV infection. The $CD4^+$ T cells mediating resistance to HSV are of the Th1 subclass (Manickan et al, 1995). Therefore, although $CD4^+$ helper T cells (especially Th1) are clearly important in controlling infections, $CD4^+$ cytotoxic T cells do not appear to play a large role in viral infections, especially in the presence of a functional immune system. This subject will be discussed further in Section F.

B. THE COMMON MUCOSAL IMMUNE SYSTEM

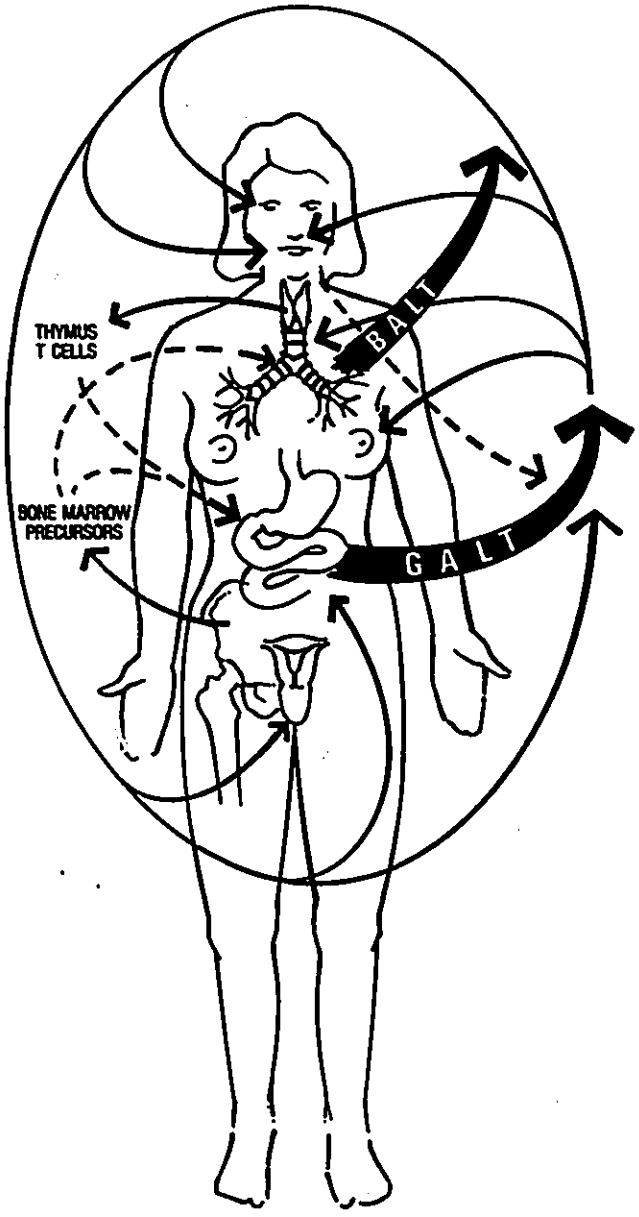
1. Distribution of Humoral Immunity in Mucosal Tissues

As discussed in Section A, SIgA is recognized as the main antibody isotype responsible

for immunity within mucosal tissues and is induced following mucosal infection or local application of antigen (Underdown and Mestecky, 1994). In 1974, Montgomery *et al.* generated great interest when they demonstrated that oral administration of dinitrophenylated pneumococcus to lactating rabbits led to the appearance of specific antibodies in milk. Numerous studies since then have confirmed the presence of specific antibodies in secretions of other glands following oral administration of antigens (Michalek *et al.*, 1976; Weisz-Carrington *et al.*, 1979; De Buysscher *et al.*, 1978). Experiments in Montgomery's laboratory excluded the possibility that the antigen travelled by the lymph or blood-stream to remote secretory glands where a local response was initiated (Montgomery *et al.*, 1974). Thus, SIgA antibodies induced in one mucosal tissue are capable of being expressed distally.

Examination of mucosal tissues has revealed that the majority of all Ig-producing cells in these sites are producing IgA (Tomasi and Bienenstock, 1968). Craig and Cebra in 1971 demonstrated that the Peyer's patches contained a rich source of precursors for IgA-producing cells capable of populating the intestinal lamina propria. The Peyer's patches are part of the gut-associated lymphoid tissue (GALT) and similar structures are found in the respiratory tract and nose and were termed bronchus-associated lymphoid tissue (BALT) (Bienenstock *et al.*, 1973) and nasal-associated lymphoid tissue (NALT), respectively. Lymphocytes from the BALT are capable of also migrating to local mucosal sites, resulting in IgA-containing cells in bronchial tissues (Rudzik *et al.*, 1975). Interestingly, Rudzik *et al.* showed that the BALT and GALT were also sources of cells capable of repopulating either the respiratory or intestinal tracts. Mesenteric lymph node lymphoblasts precommitted to IgA

Figure 2. Inductive sites and migrational pathways in the common mucosal immune system. Lymphoid elements in the GALT and BALT enter the mucosal through specific high endothelial venules to occupy the different comparments. Lymphocytes stimulated by antigen can proliferate and drain via afferent lymphatics to lymph nodes and undergo further expansion, then drain via efferent lymphatics to the thoracic duct to recirculate via the blood. These cells can repopulate various mucosa-associated lymphoid tissues. (Reproduced from Croitoru and Bienenstock, 1994).


THYMUS
T CELLS
BONE MARROW
PRECURSORS
BALT
GALT

synthesis were shown to home to the small intestine (McWilliams *et al.*, 1975, 1977), as well as the cervix and vagina, uterus, mammary glands and mesenteric lymph nodes (McDermott and Bienenstock, 1979; Roux *et al.*, 1977; Montgomery *et al.*, 1983; Jackson *et al.*, 1981; Husband and Gowans, 1978) (see Figure 2). Interestingly, BALT and GALT associated lymphocytes require up to 6 days to seed mucosal tissues, whereas lymphoblasts derived from lymph nodes draining mucosal tissues home to mucosal sites, including MALT, within 24 hours (McWilliams *et al.*, 1975; Rudzik *et al.*, 1975; McDermott and Bienenstock, 1979; Craig and Cebra, 1971). These observations highlight the importance of draining lymph nodes in the development of lymphocytes and our understanding of the migrational route of lymphocytes from Peyer's patch to mesenteric lymph node, to spleen, and then seeding to the lamina propria of the intestine (and other mucosal tissues) (Bienenstock and Befus, 1984). Peripheral lymph node immunoblasts however, return predominantly to their sites of origin and not mucosal tissues (McWilliams *et al.*, 1975; Rudzik *et al.*, 1975; McDermott and Bienenstock, 1979; Craig and Cebra, 1971).

Although IgA-precursor cells and immunoblasts are capable of migrating to several mucosal tissues, there appears to be a regional preference. Mesenteric lymph node cells localize much better in the small intestine than in the lungs and the reverse is true of cells from the mediastinal lymph nodes (McDermott and Bienenstock, 1979). Labelled thoracic duct cells taken after intraduodenal immunization and injected intravenously lodge preferentially in the jejunum, whereas those taken after intracolonic immunization lodge preferentially in the colon (Pierce and Cray, 1982). Despite a regionalized preference for localization, Robertson

and Cebra (1976) confirmed that antigen-sensitized precursors of IgA plasma cells originating from Peyer's patch lymphocytes do disseminate to other exocrine tissues.

However, there is a dependence of lymphocyte migration on regional blood flow since local inflammation can increase localization (Rose, *et al.*, 1976; Ottaway *et al.*, 1983). Local infection can also increase the accumulation of mucosally stimulated lymphocytes (McDermott *et al.*, 1989; Husband and Gowans, 1978; Ottaway *et al.*, 1983; Rose, *et al.*, 1976). However, in the absence of inflammation, localization is initially independent of local antigen. Several groups have demonstrated that lymphoblasts do not migrate toward their specific antigen when it is sequestered in the lamina propria (Guy-Grand *et al.*, 1974; Parrott and Ferguson, 1974; Hall *et al.*, 1977; Husband, 1982). Nevertheless, antigen can influence the outcome of migration. Antigen specific IgA precursor cells which enter tissues devoid of antigen tend not to congregate, whereas in the presence of antigen they remain (Husband, 1982; Husband and Dunkley, 1985) and even proliferate (Husband, 1982; Mayrhofer and Fisher, 1979).

Taken together, these results indicated that a common connection exists between mucosal surfaces in which secretory immune responses initiated at one site are shared among other mucosal tissues. As a direct consequence, the concept of the common mucosal immune system, initially proposed by Bienenstock in 1969, was developed (Rudzik et al., 1975; McDermott and Bienenstock, 1979; Weisz-Carrington *et al.*, 1979; Bienenstock *et al.*, 1983; Cebra *et al.*, 1976; Cooper *et al.*, 1974; Mestecky *et al.*, 1980; Lamm, 1976). Simply stated, antigenic stimulation of mucosal-associated lymphoid tissues (MALT), including the BALT

and GALT and likely the NALT, can led to the expression of secretory immune responses in mucosal tissues distant from the sight of antigen application. The migration of plasmacytes, originating in mucosal tissues, to local and distant mucosal sites indicates that the antibodies found in mucosal secretions are at least partially locally produced.

2. Evidence in Humans

Although our early understanding of the common mucosal immune system is based on studies performed in animals, there is ample evidence to support the existence of this system in humans. Specific SIgA antibodies were found in tears, parotid or whole saliva, milk, and nasopharyngeal secretions following oral ingestion of bacterial and viral antigens. Ingestion of lyophilized formalin-killed *S. mutans* resulted in the parallel induction of SIgA antibodies in tears, parotid saliva, whole saliva, and milk (Mestecky *et al.*, 1978; Gregory *et al.*, 1985; Czerkinsky *et al.*, 1987). Lactating women that became infected intragastrically with *E. coli* developed IgA antibodies in milk (Goldblum *et al.*, 1975). Enterically coated capsules containing influenza virus given orally resulted in specific IgA responses in tears, saliva, and nasal washes but not serum (Waldman *et al.*, 1986; Bergmann *et al.*, 1986). The lack of serum IgA also provides support for the concept that antigen-specific B cells induced in mucosal tissues migrate selectively to mucosal tissues and secrete antibodies that are expressed in mucosal secretions. Oral administration of adenoviruses results also in immunoglobulins in the respiratory tract and protection against respiratory disease and is discussed further in Section E.

In demonstrating that specific IgA producing lymphocytes are able to disseminate to distant mucosal surfaces in humans, IgA-committed precursor cells located in the peripheral circulation were examined during the time period following mucosal induction but prior to migration into distant secretory glands. Peroral inoculation of volunteers with *S. typhi* resulted in the transient presence of IgA-secreting plasma cells in the peripheral blood while secretory and serum IgA antibodies were absent (Kentele *et al.*, 1978). In a similar study, oral immunization with a combined cholera B-subunit/whole-cell vaccine resulted in peripheral blood lymphocytes that secreted specific IgA antibodies into tissue culture supernatants (Lycke *et al.*, 1985). Examination of volunteers orally immunized with enterically coated capsules containing *S. mutans* revealed that peripheral blood lymphocytes produced specific IgA prior to the appearance of specific SIgA antibodies in external secretions that included saliva and tears (Czerkinsky *et al.*, 1987). Together, these observations support the existence of the common mucosal immune system in humans.

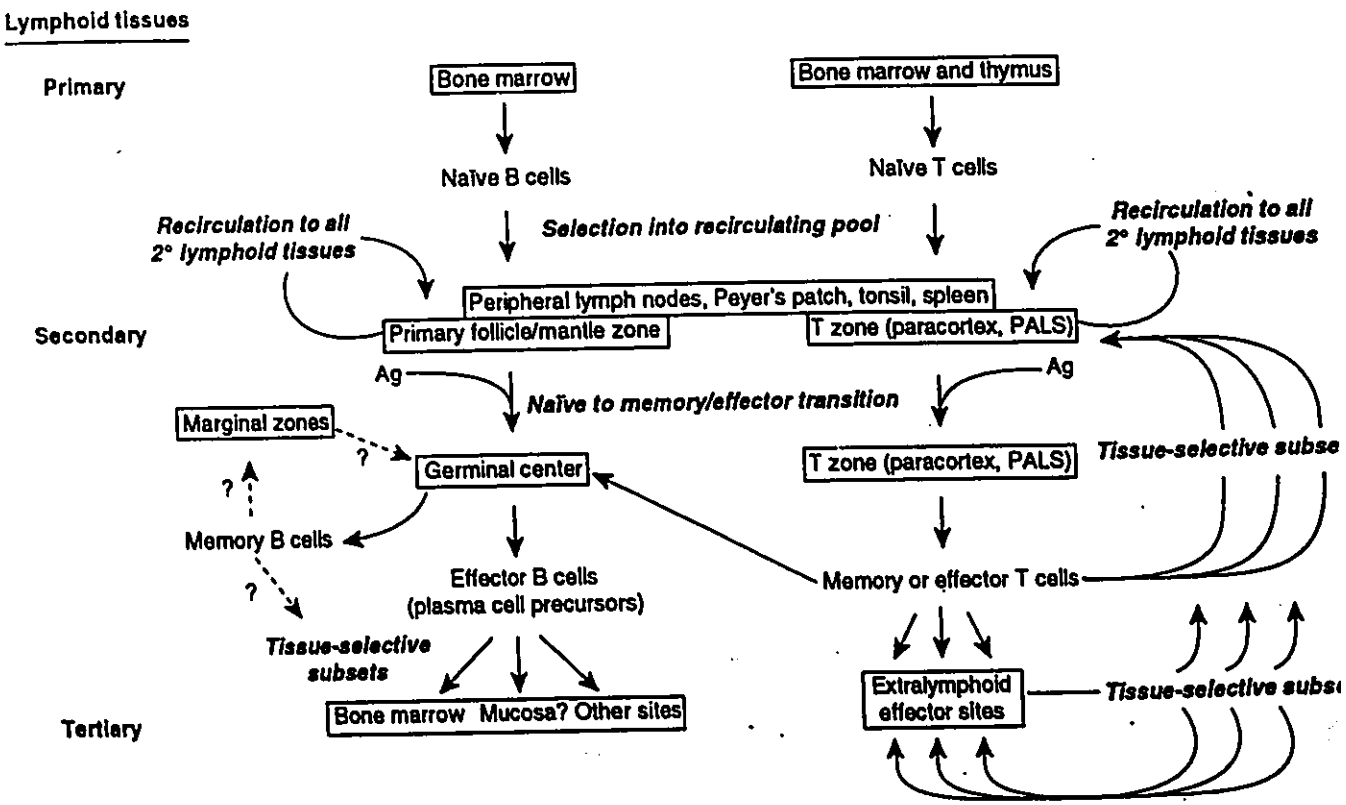
3. T Cell Participation

The participation of T cells within the common mucosal immune system has also been examined. T cells are found in mucosal sites, including the lamina propria (McDermott and Bienenstock, 1979; Rudzik *et al.*, 1975) and epithelium (Befus and Bienenstock, 1985), and mesenteric T lymphoblasts (activated cells) have been shown to localize to these mucosal sites in the gut following adoptive transfer (McDermott *et al.*, 1986; McDermott and Bienenstock, 1979). Other groups have also clearly demonstrated that T cell blasts home in a tissue specific

manner (Griscelli *et al.*, 1969; Butcher *et al.*, 1980). Further evidence is based on the migrational patterns of naive and memory or antigen induced T cells to mucosal tissues. Whereas naive T lymphocytes tend to recirculate through lymphoid tissues and not through tissues, it appears that different lymphoid tissues imprint distinct homing preferences on lymphocytes during the inductive stage of an immune response (reviewed in Butcher and Picker, 1996). This has been attributable to the different microenvironments, which influence the phenotype of cells during activation (Butcher and Picker, 1996). It is also possible that as memory cells traffic through a tissue they are exposed to a microenvironment that further affects their phenotype. For example, the cutaneous lymphocyte associated antigen (CLA) is up-regulated on T cells that are undergoing activation in skin-associated lymphoid tissue (Picker *et al.*, 1993). The lymphocyte homing receptor $\alpha 4\beta 7$ is present on mucosal memory T cells and binds to the mucosal vascular addressin MAdCAM-1, ensuring a distinct migrational pattern to mucosal tissues and MALT (Schweighoffer *et al.*, 1993; Berlin *et al.*, 1993). The selective homing of mucosally derived T-cell lines to mucosal, synovial or lymph node high endothelial venules (HEVs), functionally demonstrates this idea (Salmi *et al.*, 1992). Activated T cells derived from the lung were also shown to migrate differently compared with blood or spleen blast cells (Binns *et al.*, 1992). These studies indicate that activated or memory T cells originating in mucosal sites have tissue selective homing patterns that ensure the recirculation of these populations in local and distant mucosal tissues and MALT. Interestingly, naive T lymphocytes have migrational pathways distinct from activated or memory T cells. Naive T cells appear to show no preference in their migration to the

Figure 3. Recirculation pathways of naive and effector/memory cells.

Naive lymphocytes home to specific microenvironments within secondary lymphoid tissues and recirculate through these sites until they either die or encounter their specific antigen. Memory and effector T and probably B cells can efficiently extravasate in tertiary or extralymphoid tissues. Subsets of T cells display targeted trafficking to particular sites, including sites of inflammation, inflamed skin, intestinal mucosal, pulmonary tissues, and joints. (Reproduced from Butcher and Picker, 1996).


Lymphoid tissues
Primary
Secondary
Tertiary
Bone marrow
Bone marrow and thymus
Naïve B cells
Naïve T cells
Recirculation to all 2° lymphoid tissues
Selection into recirculating pool
Recirculation to all 2° lymphoid tissues
Peripheral lymph nodes, Peyer's patch, tonsil, spleen
Primary follicle/mantle zone
T zone (paracortex, PALS)
Ag
Naïve to memory/effector transition
Ag
Marginal zones
Germinal center
T zone (paracortex, PALS)
Tissue-selective subse
Memory B cells
Effector B cells
(plasma cell precursors)
Memory or effector T cells
Tissue-selective subsets
Extralymphoid effector sites
Tissue-selective subs
Bone marrow Mucosa? Other sites

different lymph nodes or Peyer's patches, and unlike memory/activated T cells, they tend not to enter tissues (Mackay, 1992).

4. Homing Mechanisms

Lymphocyte migration serves to bring the receptors of T and B cells into contact with antigen. It also serves to disseminate immune responses to other regions of the body, thus ensuring systemic immunity. The mechanisms responsible for the migration of lymphocytes involves active interactions between lymphocytes and endothelial cells in peripheral tissues (ie. flat endothelium) or secondary lymphoid tissues (ie. HEVs) (Mackay, 1993; Butcher *et al.*, 1980). Subsequent to these interactions, diapedesis of the lymphocytes across the vascular wall results in lymphocyte movement from the blood (extravasation) (Butcher, 1986; Woodruff *et al.*, 1987; Picker and Butcher, 1992). As discussed, the selective migration or homing of blast cells (effectors) to mucosal tissues provided the first evidence that lymphocytes migrate non-randomly. Indeed, in addition to homing to specific tissues, lymphocytes are able to recirculate from blood to tissues and back to blood. Recirculating cells that enter tissues eventually accumulate in the afferent lymphatic ducts, drain to the local lymph nodes, and return to the blood via the efferent lymphatic ducts and the thoracic duct. There are distinct recirculation patterns for naive and memory or effector lymphocytes as indicated previously (see Figure 3) (Butcher, 1986; Mackay, 1993; Smith *et al.*, 1980; Picker and Butcher, 1992). Naive lymphocytes are programmed to recirculate through secondary lymphoid tissues including spleen, tonsils, lymph nodes, and Peyer's patches. In these tissues

naive T and B cells are presented with antigen in the context of lymphoid microenvironments that drive their antigen-induced differentiation and proliferation (Butcher and Picker, 1996).

The recirculation tropism of effector or memory cells is imparted upon them during their maturation within these specialized microenvironments. It is the expression of homing receptors such as L-selectin or $\alpha 4\beta 7$ that become up or down-regulated during memory/effector differentiation that is responsible (Horgan *et al.*, 1992; Picker *et al.*, 1993a, 1993b). By varying the expression of these highly specialized homing receptors on lymphocytes as well as counter receptors on endothelial cells, the immune system can control the many specific homing pathways by permitting emigration of lymphoblasts from the vasculature at several locations (Butcher and Picker, 1996). It is possible that the maintenance of recirculation pathways within the memory cell compartment will be responsible for maintaining the ability to mount memory responses within those tissues (Mackay, 1992).

5. Mucosal Vaccines

Although there are a number of mucosal vaccines being investigated for human pathogens such as HIV, herpes simplex virus (HSV), influenza virus, rabies virus, hepatitis virus, respiratory syncytial virus, rotavirus, and cytomegalovirus, the only current mucosal vaccines in humans contain the live adenovirus or poliovirus. The oral (Sabin) poliovirus vaccine (OPV) was introduced in 1963 and along with the inactivated (Salk) polio vaccine (IPV) has resulted since 1979 in the elimination of endogenously transmitted paralytic disease caused by wild poliovirus in many parts of the world (Ogra and Faden, 1986). OPV has been

shown to elicit development of SIgA antibodies to poliovirus in the nasopharynx and intestine by 3 weeks post immunization, and that persist over a period of as long as 5 to 6 years (Ogra, 1984; Mestecky, *et al.*, 1994). In contrast, the IPV provides very little alimentary immunity, but does result in circulating antibody responses similar to those following orally administered OPV (Ogra *et al.*, 1980).

Enteric-coated live adenovirus preparations consisting of several serotypes (types 4, 7, and 21) have been used orally in military recruits for over 20 years in successfully restricting acute respiratory diseases caused by these viruses (Chaloner-Larsson *et al.*, 1986; Top, 1975; Top *et al.*, 1971a, 1971b; Dudding *et al.*, 1973). The immune mechanisms responsible are unclear and may involve cell-mediated as well as secretory immunity. After oral immunization with these enteric coated vaccines, SIgA antibody is observed in fecal samples, however, adenovirus-specific SIgA antibody activity in nasal secretions was not very strong (Ogra *et al.*, 1980). In contrast, natural infection results in significant SIgA and frequently IgG antibody responses in nasal secretions (Ogra *et al.*, 1980). Nevertheless, orally induced immunity to respiratory infections highlights the possibilities of the common mucosal immune system in the development of mucosal vaccines.

C. IMMUNITY IN THE FEMALE GENITAL TRACT

1. Background

A consensus on what immune functions within the female reproductive tract are active in providing protection against sexually transmitted diseases, and the best approach to

inducing these parameters is being investigated. This is due mainly to the complex environment in the genital tract that must be capable of reacting against microorganisms while at the same time ignoring other antigens such as the early embryo. In addition, the physiology and immunology of the reproductive tract in humans and other mammals undergoes many changes during the stages of the reproductive cycle. In humans, menses or the period of menstruation is followed by the proliferative or follicular phase, ovulation, and then the secretory or luteal phase. In mice, there is no overt menstruation, but the corresponding period occurs during metestrus, which is followed by diestrus, proestrus, and finally estrus, the period in which ovulation occurs. In mice, each estrous cycle occurs over a period of four to six days. During the estrous cycle, the anatomy of the vaginal epithelium is known to undergo changes from a thin (3 to 7 layers) stratified epithelium at diestrus to a thick (12 to 13 layers) keratinized epithelium with a clearly defined basement membrane at estrus (Allen, 1922; Parr, 1994).

2. Influences of the Reproductive Cycle on Specific Immunity

In terms of protection at mucosal surfaces, SIgA is an important component and this also applies to the genital tract (Brandtzaeg, 1994; Parr and Parr, 1994). The levels of total IgA and IgG antibodies in uterine fluids of rats fluctuates based on the stage of the estrous cycle. The sex hormones estradiol and progesterone have been implicated in controlling these fluctuations. Estradiol treatment of rats resulted in increased levels of IgA and IgG in uterine secretions (Wira and Sandoe, 1980), but lowered levels in cervicovaginal secretions (Wira and

Sullivan, 1985). Progesterone, on the other hand, inhibits cervicovaginal levels of IgA and IgG. Secretory IgA is transported into secretions at mucosal surfaces by SC, and estradiol treatment was found to increase levels of SC in uterine fluids and decrease levels in cervicovaginal secretions, in agreement with levels of SIgA (Wira and Sullivan, 1985). During the course of the estrous cycle, IgA and IgG levels in uterine washes were highest during proestrus, but unlike the IgG levels which sharply decreased during estrus, IgA levels remained high (Wira and Sandoe, 1977, 1980). In humans, a similar IgG antibody response is observed with levels of IgG being highest following menstruation, declining during the ovulatory phase and remaining low during the luteal phase (Usala *et al.*, 1989). Examination of uterine tissues of the rat revealed that IgA levels increased slightly during the progression from diestrus to estrus (Steele and Wira, 1989). Wira and collaborators also investigated levels of antigen-specific antibodies in rat uterine secretions (Wira and Sandoe, 1989). Anti-SRBC antibodies were induced in uterine fluids following intrauterine or Peyer's patch immunization and estradiol treatment was found to increase levels of specific IgA.

Both cellular immunity and antigen presentation are influenced by the estrous cycle. T and B lymphocytes, in addition to macrophages are present in high numbers in the rat uterus during proestrus, the stage when estradiol levels are known to be at their highest. In contrast, cell numbers were reduced at estrus and were either low or not detectable during diestrus (Wira *et al.*, 1994). An increase in the migration of plasma cells to the genital tract has also been observed during proestrus and estrus in the mouse (McDermott, 1980; Rachman, 1983). Antigen presentation by uterine epithelial cells was also influenced by the estrous cycle with

maximal presentation occurring during proestrus (Wira, *et al.*, 1994). These observations highlight the importance of the stages of the reproductive cycle and the influence that sex hormones have on local immunity, and suggest, that during the time of mating (ie. ovulation), immune functions in the female genital are at their peak of activity.

3. Induction of Immunity

All of the effector components of the mucosal immune system are reportably present in the female reproductive tract (Parr and Parr, 1994; Kutteh and Mestecky, 1994). Induction of these immune components has focussed on the administration of antigen by one or a combination of routes. Systemic, local mucosal, and distant mucosal immunization have all provided some level of immunity in the genital tract. Local mucosal immunization with protein antigens or killed microorganisms has resulted in antibody production in a few cases (Ogra and Ogra, 1973; Wira and Sandoe, 1989), but not in many others (Parr and Parr, 1994, 1992). Generally, there is a lack of protective immunity via this method (Parr and Parr, 1994), and the predominant antibodies are usually IgG and not SIgA. The induction of local immunity following immunization with live vaccines has met with greater success. McDermott and colleagues (McDermott *et al.*, 1984, 1987, 1989a, 1989b, 1990; Parr *et al.*, 1994) have demonstrated that the intravaginal administration of an attenuated strain of HSV-2 induced protective immunity to lethal challenge with wild-type virus and the presence of humoral immunity consisting mainly of IgG. Milligan and Bernstein (1995) found that intravaginal inoculation of mice with the same attenuated strain of HSV-2 did result in IgA in vaginal

washes, however, although titres were low and delayed in appearance, levels increased significantly following challenge with wild type HSV-2. Interestingly, adoptive transfer of lymphocyte subsets from iliac lymph nodes draining the site of immunization revealed that protection correlated with T lymphocytes (McDermott *et al.*, 1989). Other groups have, however, shown that following passive transfer or direct application of antibodies, mice were protected from an intravaginal HSV-2 infection (Whaley *et al.*, 1994; Eis-Hubinger *et al.*, 1993). Immunity to HSV-2 therefore appears to be mediated by either humoral and/or cellular immune functions.

Systemic immunization has been extensively examined and proposed by several groups as a route for vaccination against sexually transmitted agents. Systemic vaccination of guinea pigs with recombinant HSV-2 glycoproteins prevented virus-induced pathology after intravaginal challenge and this was attributed to the transudation of serum IgG anti-viral antibodies into genital secretions (Stanberry *et al.*, 1987; Berman *et al.*, 1995, 1988; Lasky *et al.*, 1984). Subcutaneous administration of glycoprotein D (gD) in an adjuvant formulation elicited cellular and humoral immune responses in the serum of guinea pigs, and resulted in fewer and less severe vaginal lesions, and decreased ganglionic latency compared to controls, however, mucosal responses were not reported (Byars *et al.*, 1994). Genetic vaccines incorporating DNA encoding gD administered intramuscularly to guinea pigs also elicited serum antibodies and protected against vaginal HSV-2 infection but mucosal responses were not reported (Bourne *et al.*, 1996). Several other groups using various formulations of glycoproteins or mutant HSV viruses have successfully protected animals from intravaginal

infection following parenteral vaccination (McLean *et al.*, 1994; Nakao *et al.*, 1994; Burke *et al.*, 1994; Heineman *et al.*, 1995); however, no report on mucosal immunity was provided.

In humans, IgG but not IgA antibodies were observed in the vaginal washes of women parenterally vaccinated with tetanus toxoid (Bouvet *et al.*, 1994). In human trials of HSV vaccines, subjects given a glycoprotein subunit formulation developed neutralizing serum antibodies (with glycoproteins B and D specificities), lymphoproliferative responses to HSV antigens, and CTL responses in some individuals (Mertz *et al.*, 1984; Zarling *et al.*, 1988). However, this vaccine failed to provide protection from acquisition of genital HSV infection (Mertz *et al.*, 1990). In another human vaccine trial, subjects who experienced recurrent genital HSV episodes received gD in alum (Straus *et al.*, 1994). The vaccine boosted neutralizing antibodies to HSV-2 and subjects experienced fewer virologically confirmed recurrences per month, and had a lower median number of recurrences for the study year. Taken together, these studies indicate that systemic immunization can provide protection against primary or secondary HSV-2 infections of the female genital tract. These studies did not appear to induce or enhance secretory immunity to HSV.

In contrast to most studies, systemic immunization has been shown to elicit local IgA responses in tears, saliva and bile following intramuscular (McChesney *et al.*, 1982) and subcutaneous (Levernson *et al.*, 1988) administration. Although systemic immunizations generally do not induce SIgA in the genital tract, immunization by this route can prime the mucosal immune system such that a second immunization of mucosal tissue results in enhanced mucosal antibody responses (Pierce *et al.*, 1977; Svennerhoom *et al.*, 1980). Drew

et al., (1992) used intraperitoneal priming followed by an intragastric boost with a conjugate of cholera toxin and gD peptide to induce IgG and IgA vaginal wash antibodies and protection against intravaginal HSV-2 infection. However, when Wu and Russell, (1994), compared mucosal to systemic priming, they found that the greatest antibody responses in the gut were achieved when an oral boost is preceded by priming orally rather than systemically.

The successful induction of immunity in the female genital tract by immunization at distant mucosal surfaces has recently been exploited. Following oral inoculation of mice with viable *Chlamydia psittaci*, intravaginal resistance was found to involve anti-*Chlamydia* SIgA antibodies (Nichols *et al.*, 1978; Ogra *et al.*, 1989). Oral administration of the strong mucosal immunogen, cholera toxin, alone or as an adjuvant for other antigens, has resulted in mucosal IgG and IgA antibody responses in the female genital tract (Haneberg, *et al.*, 1994; Wu and Russell, 1993; Dertzbaugh and Elson, 1993). Intranasal application of cholera toxin plus antigen also resulted in vaginal wash IgG and IgA antibodies, and when compared to oral immunization, titres of IgA were higher (Wu and Russell, 1993). Anti-HIV-1 IgA and IgG antibody responses were also present in vaginal washes following intranasal application of C4/V3 peptide derived from HIV plus cholera toxin (Staats *et al.*, 1996). Liposomes containing viral subunit antigen also induced antigen-specific SIgA responses in vaginal washes following immunization of the respiratory tract (Haan *et al.*, 1995). Furr *et al.*, (1993), showed that oropharynx infection with *Mycoplasma pulmonis*, following intranasal administration, protected against subsequent vaginal colonization with the same pathogen. Lubeck *et al.*, (1994), demonstrated that several intranasal immunizations with recombinant

adenovirus vectors expressing HIV proteins, followed by systemic subunit administrations, resulted in anti-HIV IgG and IgA antibody responses in genital secretions of chimpanzees.

The intranasal administration of live bacterial vaccines also has successfully induced local immunity in the female genital tract. The intranasal administration of pneumonitis biovar of *Chlamydia trachomatis* resulted in no *C. trachomatis* shedding from the vagina following challenge and subsequent fertility rates were as high as in mock infected animals (Thomas *et al.*, 1994). Hepatitis-specific SIgA was present in the vaginal secretions of mice immunized intranasally with *Salmonella typhimurium* carrying a plasmid encoding a hybrid form of the hepatitis B virus core antigen (Hopkins *et al.*, 1995). Live influenza virus vaccine capable of expressing a peptide of the pg41 protein of HIV was given intranasally and resulted in peptide specific IgA antibody responses in vaginal secretions for longer than one year (Muster *et al.*, 1995). This body of work suggests that distant mucosal immunization can successfully induce humoral immune responses in the female genital tract.

Combinations of systemic and mucosal routes of vaccination have recently been carried out in non-human primates in an attempt to provide protection against intravaginal SIV infection. Lehner *et al.*, (1992) used virus-like particles carrying the SIV gag protein p27, to immunize macaques by a vaginal, followed by an oral, vaccine regimen. Gag protein p27 specific SIgA and IgG antibodies were detected in vaginal fluid and $CD4^+$ T cell proliferation and helper function in B cell p27-specific IgA synthesis occurred in the genital lymph nodes. Another group (Marx *et al.*, 1993) used formalin-treated SIV in biodegradable microspheres by the intramuscular plus oral or plus intratracheal routes to induce IgA and IgG antibodies

in the vaginal tract. In this study, five of six macaques were protected against a first vaginal challenge and three of four following a second challenge. These results provide some optimism for the successful development of human vaccines directed against sexually-transmitted viruses.

These results, in addition to work by others, suggest that the successful induction of secretory immune responses within the genital tract may depend on the development of responses within the lymph nodes that drain mucosal tissues. This is made evident by a recent vaccine strategy involving parenteral immunization into anatomical regions that drain into mucosal tissues. Targeted lymph node immunization by intrapelvic injection with horse ferritin (Thapar *et al.*, 1990) or p27 antigen from SIV (Lehner *et al.*, 1994) resulted in an antigen-specific IgA immune response in vaginal secretions. These results suggest that the induction and development of mucosal specific and secretory immune responses can occur within lymph nodes associated with mucosal tissues. Certainly, immune induction within Peyer's patches or other MALTs results in lymphocyte migration through draining lymph nodes and bach to local and distant tissues. The fact that transfered Peyer's patch lymphocytes require 6 days to appear in mucosal tissues (and are found immediatly after transfer in mucosal-associated lymph nodes), whereas, mesenteric lymphocytes home immediatly to mucosal tissues, indicates that precursors are localized to MALT and require further development in lymph nodes following antigen exposure (discussed previously). Whether initiation of immune responses in draining lymph nodes of mucosal tissues (bypassing MALT) results in migrational patterns to all mucosal tissues has yet to be determined. Nevertheless, these

results demonstrate the central role of draining lymph nodes in the development of immune responses and the subsequent migration of effectors.

D. T CELL MEMORY

1. Background

Immunological memory, similar to cognitive memory, imparts upon an individual the ability to respond to a second exposure of the same or related antigen selectively and rapidly. The development of memory T and B cells following antigen exposure increases the likelihood that a subsequent infection will be adequately controlled and little pathology or disease will be incurred. Alternatively, memory responses may result in inappropriate responses causing tissue damage. In terms of the development of T cell memory there are three distinct phases that occur following antigen exposure (Ahmed and Gray, 1996). The first involves activation of antigen-specific lymphocytes followed by proliferation and expansion. During this stage a large pool of activated effectors is generated and antigen is dealt with. The second phase involves the death of the effectors by apoptosis as the amount of antigen declines. This stage can last for several weeks and as many as 95% of the antigen specific T cells disappear. The final phase is characterized by the maintenance of a pool of memory T cells that can persist for many years.

2. Recirculation of Memory T Cells

Memory cells maintain a blanket of surveillance by recirculating throughout the tissues

of the body. Mature lymphocytes can recirculate continuously, often several times a day, from blood to tissue and back to blood (Ford and Gowans, 1969). Naive lymphocytes typically recirculate through secondary lymphoid tissue such as lymph nodes, Peyer's patches, tonsils, and spleen (Butcher, 1986; Picker and Butcher, 1992; Mackay, 1993). Recirculation pattern affords lymphocytes the ability to interact with antigen collected in these organs and in an environment suitable for the generation and proliferation of an immune response (Butcher, 1986; Picker and Butcher, 1992; Mackay, 1993; Smith *et al.*, 1980; Butcher and Picker, 1996). As discussed, the recirculation patterns of effector and memory T cells are heterogeneous and very different from that of naive T cells. This heterogeneity is dependent on the distinct subsets that develop within unique tissue environments during initial immune development (Butcher, 1986; Picker and Butcher, 1992; Mackay, 1993; Smith *et al.*, 1980; Butcher and Picker, 1996). As discussed previously, the homing tropism of mucosal-associated effector or memory T cells is imparted during maturation within the specialized mucosal microenvironments (mucosal-associated lymphoid tissues) (Butcher and Picker, 1996). As indicated, migration is dependent on the expression of homing receptors such as L-selectin or $\alpha 4\beta 7$ that become up or down-regulated during memory/effector differentiation (Horgan *et al.*, 1992; Picker, 1993; Picker *et al.*, 1993). Unique counter receptors exist for these adhesion molecules within various tissue environments. The $\alpha 4\beta 7$ integrin mediates T cell binding to the mucosal vascular addressin MAdCAM-1 (Berlin *et al.*, 1993). By varying the expression of these highly specialized homing receptors on lymphocytes as well as counter-receptors on endothelial cells, the immune system can control the many specific

recirculation pathways of memory or effector T cells (Butcher and Picker, 1996). This has been demonstrated by *in situ* studies of mouse intestine where naive lymphocytes are targeted to the Peyer's patches, while memory or effector cells, but not naive cells, are targeted to specific extralymphoid effector sites in the lamina propria (Bargatze *et al.*, 1995; Berlin *et al.*, 1995).

As described, the development of an immune response progresses through three stages. During the early stages, effector cells predominate within the circulation. Migrational studies suggest that effectors, like memory cells predominantly recirculate within tissues similar to those in which they were induced. However, there is evidence that while this may be generally true, a period exists following viral infection in which effector cells are found throughout the lymphoid system, regardless of the route of inoculation. Offit *et al.*, (1991) found rotavirus-specific CTL precursors (CTLp) in several mucosal and systemic lymphoid tissues 21 days following oral or footpad inoculation. Initially however, specific CTLp were isolated mainly from lymphoid tissues related to the site of infection (Offit *et al.*, 1991). The period of nondiscreet lymphocyte recirculation in these experiments corresponds to the early phase of the immune response (Ahmed and Gray, 1996) during which effectors predominate, suggesting that perhaps activated effector lymphocytes may not be completely restricted in their pattern of circulation. This may be important biologically in terms of dealing with disseminated virus, whereas memory lymphocytes in the third phase need only recirculate within tissues that a second infection will occur in. Whether recirculation or access of memory lymphocytes is exclusively restricted to tissues in which immune induction occurred, has yet

to be functionally demonstrated.

3. Mechanisms of T Cell Memory

The mechanisms responsible for the maintenance of T cell memory have been extensively studied and currently there are two main views. The first dictates that memory T cells are dependent on the presence of persistent antigen, and the second implies that specific antigen stimulation is not required for maintenance of long-lived T cells (Beverley, 1990; Swain *et al.*, 1991; Vitetta *et al.*, 1991; Mackay *et al.*, 1993; Gray, 1993; Sprent, 1994; Ahmed, 1994; Ahmed and Gray, 1996; Lau *et al.*, 1994). There are many exquisite *in vivo* examples of both these views suggesting that the mechanisms responsible for maintaining long-lived memory T cells may be heterogeneous and, depending on the system, rely on one mechanism more than another. In addition, it has been argued that memory T cells may be inherently long-lived with specific and non-specific stimulus maintaining a state of readiness (Ahmed and Gray, 1996). Whether memory T cells are long-lived or not, T cell populations bearing "memory phenotypes" have been shown to divide with regularity (Tough and Sprent, 1995; Razvi *et al.*, 1995), suggesting that memory T cells may be long-lived and/or maintained at a population level through cycling. The stimuli driving this may be specific or nonspecific. Indeed, there are several possible mechanism that have been shown to nonspecifically stimulate memory T cells including cross-reactive environmental or self antigens (Beverley, 1990; von Boehmer and Hafen, 1993; Selin *et al.*, 1994; Matzinger, 1994; Allen, 1994), adhesion and costimulatory molecules (Beverley, 1990; Swain *et al.*, 1991; Vitetta *et al.*,

1991; Mackay, 1993; Gray, 1993; Sprent, 1994; Ahmed, 1994; Sprent and Tough, 1994), or hyperresponsiveness to cytokines (Unutmaz *et al.*, 1994).

Interestingly, there is little work examining the influence of recirculation on the maintenance of T cell memory. Clearly, while memory T cells migrate to distinct tissues, the local microenvironment is likely to influence their phenotype and therefore maintenance as memory cells. Microenvironments in local tissues likely are responsible for maintaining memory T cells which cycle through them by providing constant stimulation as a result of antigen persistence (Gray and Matzinger, 1991; Oehen *et al.*, 1992), or non-specific stimuli as discussed above. Recent evidence also suggests that memory T cells migrating to their unique tissue environments may be maintained by cytokines or engagement of integrins that can deliver potent apoptosis-inhibiting signals during recruitment, retention, and recirculation (Freedman *et al.*, 1990; Koopman *et al.*, 1994; Hynes, 1992). These results suggest that the local tissues may have a large impact on the maintenance of memory T cells during recirculation. Further, and perhaps not as obvious, the recirculation and retention of memory T cells and their subsequent maintenance, (ie. stimulation) may also impart a restriction on the location or tissues in which immunological memory is observed.

Typically, the examination of immunological T cell memory involves an estimation of the number of antigen-specific T cells present within the circulation or spleen. A quantitative increase in the number of specific T cells implies that memory exists. (Beverley, 1990; Swain *et al.*, 1991; Vitetta *et al.*, 1991; Mackay *et al.*, 1993; Gray, 1993; Sprent, 1994; Ahmed, 1994; Ahmed and Gray, 1996). Quantitation is best achieved in a limiting dilution assay

involving antigen expansion of specific splenocytes or PBMCs (Lefkovits and Waldmann, 1979). Expansion under limiting conditions allows an individual antigen-specific T cell to proliferate and be functionally evaluated, for example in a CTL assay. Examination of dilutions of lymphocytes in this way results in precursor frequencies which are indicative of the number of specific lymphocytes present per total number of lymphocytes in the spleen or circulation.

In addition to an increase in the numbers of antigen-specific lymphocytes, immunological memory is also characterized by the ability of memory T cells to become activated more readily and to lower doses of antigen than naive T cells (Kearney *et al.*, 1994; Beverley, 1990; Swain *et al.*, 1991; Vitetta *et al.*, 1991; Mackay *et al.*, 1993; Gray, 1993; Sprent, 1994; Ahmed, 1994; Ahmed and Gray, 1996). Several factors may contribute to this greater capacity to respond to antigen. Clonal selection or somatic hypermutation of TCR genes may result in a pool of memory T cells with higher affinity TCR (McHeyzer-Williams and Davis, 1995; Zheng, *et al.*, 1994). Memory T cells may also express the higher affinity IL-2 receptor which makes them hyperresponsive to IL-2 (Ahmed and Gray, 1996). In addition, increased levels of adhesion and costimulatory molecules may not only provide chronic stimuli, as discussed, but also arm memory T cells with the capacity to rapidly respond to specific antigen, and perhaps with a decreased threshold. These attributes, in addition to increased numbers of precursor lymphocytes, permit memory T cells to rapidly respond and expand following antigenic challenge and this can be functionally viewed by observing the more rapid development of a recall response *in vivo* (Ahmed, 1992; Doherty *et al.*, 1977; Mullbacher,

1994; Effros *et al.*, 1978; Hill *et al.*, 1992; Walker *et al.*, 1984; Nugent *et al.*, 1994, 1995; Nash *et al.*, 1980). Functional evaluation of the recall response can involve monitoring the magnitude of killing in a CTL assay, or enumeration of specific CTL in a limiting dilution assay. The *in vivo* detection of recall responses can be observed in the tissues, spleen or lymph nodes draining the site of infection. Recall responses following intravenous or intraperitoneal challenge can be observed in the spleen (Hill *et al.*, 1992; Mullbacher, 1994). Those following tissue challenge can be observed in locally draining lymph nodes (Walker *et al.*, 1984; Nugent *et al.*, 1994, 1995; Nash *et al.*, 1980) as well as in the tissues themselves.

E. ADENOVIRUS VECTORS

1. Background

Adenoviruses are double-stranded DNA viruses belonging to the family Adenoviridae, which contains two genera, *Mastadenoviruses* which infect mammalian species and *Aviadenoviruses* which infect mainly birds. The adenovirus virion is a nonenveloped icosahedron about 70 nm in diameter containing protein and linear DNA. Over 100 different serotypes have been isolated from various species, some 47 of them from humans (Straus, 1984; Hierholzer *et al.*, 1988). The human serotypes are organized into six subgenera (A-F) based on several criteria including hemagglutination properties of erythrocytes, DNA homology, restriction enzyme cleavage patterns, percentage G+C content and oncogenicity (Straus, 1984; Horwitz, 1990a). The serotypes best characterized include Ad5 and Ad2 of subgroup C, Ad7 of subgroup B and Ad12 of subgroup A. Although Ad12 has been reported

to have tumour inducing properties in newborn rodents (Trentin *et al.*, 1962; Huebner *et al.*, 1962), no adenoviruses have ever been linked to naturally occurring malignancies in any animal and investigations of human tumours have failed to find any association (Gilden *et al.*, 1970; McAllister *et al.*, 1972; Mackey *et al.*, 1976; Graham, 1984).

Human infections with adenoviruses are not usually associated with disease, and often are subclinical. Typically adenoviruses infect and replicate in mucosal tissues such as the upper respiratory tract, gastrointestinal tract, the eye and the urinary bladder, depending on the serotype (Straus, 1984; Horwitz, 1990b). Infection involves sequential interaction of the virus with two separate host cell receptors, the second involving αv integrins, found on epithelial cells of mucosal surfaces (Nemerow *et al.*, 1994; Wickham *et al.*, 1993; Mathias *et al.*, 1994). Infection usually results in mild disease with symptoms ranging from rhinorrhea, nasal congestion, and sneezing to fever, sore throat, and conjunctivitis (Horwitz, 1990b).

2. Adenovirus Vectors as Vaccines

Many studies have led to an extensive understanding of the genomic organization and molecular biology of adenoviruses which has resulted in the development of recombinant vectors capable of expressing foreign proteins in mammalian cells (Graham and Prevec, 1991; Berkner, 1992). These viruses have been used successfully as gene transfer vectors for gene therapy as well as vaccine vehicles. As vaccines, adenovirus vectors have induced sufficient expression of recombinant antigens *in vivo* in either abortive or productive infections to generate specific and protective immune responses to the recombinant antigens. Adenovirus

vectors expressing whole gB of HSV (Johnson *et al.*, 1988) or truncated forms (Hanke *et al.*, 1991), have been shown to induce anti-HSV neutralizing antibodies (McDermott *et al.*, 1989) and H-2^b-restricted cytotoxic T lymphocytes (Witmer *et al.*, 1990; Hanke *et al.*, 1991) and protect against lethal challenge with HSV (McDermott *et al.*, 1989). Recombinants expressing immunodominant gene products of vesicular stomatitis virus (VSV), cytomegalovirus (CMV), or rabies virus have also been shown to induce protective neutralizing and cellular immune responses (Prevec *et al.*, 1989; Marshall *et al.*, 1990; Prevec *et al.*, 1990; Johnson, 1991;).

Adenoviruses have long been recognized as safe and competent mucosal vaccines. For over 20 years military recruits have received live adenoviruses types 4, 7 and 21 by the oral route (Top *et al.*, 1971a). These enteric-coated live adenovirus preparations have been highly effective against acute respiratory diseases caused by adenoviruses (Chaloner-Larsson *et al.*, 1986; Top, 1975; Top *et al.*, 1971b), thus supporting the concept of the common mucosal immune system (McDermott and Bienenstock, 1979). More recently, intranasal and/or oral immunization with recombinant adenovirus vectors has proven successful in animal disease models. Oral or intranasal instillation of adenovirus vectors containing hepatitis B virus surface antigen, envelope glycoprotein (gp120) or gag of HIV, and fusion glycoproteins F and G of respiratory syncytial virus induced serum (Morin *et al.*, 1987; Lubeck *et al.*, 1989; Hsu *et al.*, 1992; Lubeck *et al.*, 1994; Natuk *et al.*, 1993) and mucosal (Lubeck *et al.*, 1994; Natuk *et al.*, 1993) antibody responses, and cellular immune responses (Lubeck *et al.*, 1994; Natuk *et al.*, 1993) that were shown in one case to protect from mucosal challenge (Hsu *et*

al., 1992).

These results demonstrate that adenovirus vectors are capable of inducing mucosal immunity and they provide an attractive vaccine vehicle for inducing protection against sexually transmitted diseases as well as mucosal pathogens.

F. HERPES SIMPLEX VIRUS

1. Background

The infectious nature of herpes simplex virus (HSV) was first observed following the passage of the virus from lip and genital lesions of humans to either the cornea or the scarified skin of the rabbit (Gruter, 1924). Goodpasture in 1925 demonstrated that herpes simplex encephalitis (HSE) was produced by inoculation of vesicular fluid from the lesions of herpes labialis onto scarified cornea of rabbits. Soon there after, HSV was shown responsible for encephalitis in newborns (Smith *et al.*, 1941) and adults (Zarafonetis et a., 1944) following isolation of virus from intranuclear inclusions in brain tissue. Since then, two antigenic types of HSV have been typed, HSV-1 which is primarily responsible of herpes infections "above the belt", and HSV-2 which causes herpes infections "below the belt" (Nahmias and Dowdle, 1968).

2. Biology of Herpes Simplex Viruses

The virion of HSV consists of a core, an icosadeltahedral capsid surrounding the core, an amorphous tegument surrounding the capsid, and an outer envelope exhibiting spikes on

its surface (Roizman and Sears, 1990). The genome of HSV is a linear, double-stranded DNA molecule encoding 60 to 70 or more gene products (Roizman and Sears, 1990). The virion contains upwards of 33 proteins designated as virion polypeptides (VPs), eight which are on the surface of the virion and are glycosylated. These glycoproteins are gB, C, D, E, G, H, and I. Expression of virion genes is highly regulated and is broken down into three classes based on the timing and requirements of their expression (Nahmias and Roizman, 1973). Following infection of cells, alpha genes are expressed first, followed by beta and finally gamma genes which encode most of the structural proteins. During HSV infection of neurons a latent state can occur in which the viral genome is present as circular episomes or concatemeres or is integrated into the cell genome (Rock and Fraser, 1983; Puga *et al.*, 1984).

Infection by HSV can occur at mucosal surfaces or abraded skin with replication in cells of the epidermis and dermis. Whether or not clinically apparent lesions develop, sufficient viral replication may occur allowing infection of nerve endings with intra-axonal transport to the nerve-cell bodies in ganglia. After resolution of the primary disease, infectious virus is generally not recoverable in the ganglia. Reactivation of latent virus is usually attributable to local stimuli such as injury to tissue innervated by neurons harboring latent virus or systemic stimuli such as physical or emotional stress, and menstruation.

Both primary and recurrent HSV infections can cause disease of the central nervous system including encephalitis which is estimated to occur in approximately one in a quarter to half a million individuals per year (Whitley, 1982). Mortality exceeds seventy percent and less than ten percent of surviving patients return to normal function (Longson, 1980; Whitley

et al., 1977, 1981). Generally, manifestations of HSV are associated with mucosal surfaces and genital infections have significant morbidity in adults, but rarely cause death in the immunologically normal host. In contrast, neonatal HSV infection can result in high mortality and frequency of subsequent neurological sequelae in survivors (Whitley, 1990). Individuals immunocomprimised, as in AIDS patients, also suffer more severe manifestations of mucosal surfaces.

3. Immunity to Herpes Simplex Virus

The host response to HSV infection in which both natural resistance and specific immunological responses are involved is very complex. Natural resistance mechanisms, mediated by macrophages, NK cells and cytokines, represent an early barrier to the virus but the eventual resolution of the infectious process and the establishment of long-lasting immunity is the property of specific responses. Both humoral and cell-mediated responses are involved in viral control, however, it is clear that the action of T cells is central for recovery from primary infection and in the control of recrudescent lesions. This was demonstrated early on when immunocompromised patients with defects in cell-mediated immunity experienced more severe and more extensive HSV infections than those with deficits in humoral immunity, such as agammaglobulinemia (Pass *et al.*, 1979; Naragi *et al.*, 1977; Meyers *et al.*, 1980). Similar results were observed in mice where experimental ablation of lymphocytes indicated that T cells have a major role in preventing lethal disseminated disease, although antibodies help reduce virus titers in neural tissue (Kapoor *et al.*, 1982).

Antibodies specific for the viral glycoproteins B, C, D, E and G have conferred protection against subsequent neurologic disease or ganglionic latency demonstrating that antibodies can be an integral part of the a first line of defence against infection (Balachandran *et al.*, 1982; Eisenberg *et al.*, 1985; Dix *et al.*, 1981; Rector *et al.*, 1982). Protection was correlated in some instances with viral neutralization (Balachandran *et al.*, 1982). Antibodies have also been shown to be very important in protection against corneal infection (Laucsch, *et al.*, 1989) where immune T cells responding to HSV infection play a critical role in the initiation of tissue damage (Metcalf *et al.*, 1979). In human studies, the role of SIgA in preventing primary, or controlling recrudescent episodes is unclear, although serum IgA antibody levels have been shown to rise with positive virus isolation (Persson *et al.*, 1988) and specific SIgA antibodies have been detected in the absence of serum antibodies (Guglielmino *et al.*, 1989). These results suggest that IgA may be involved in control of mucosal HSV infections.

The passive transfer of HSV primed lymphocytes demonstrated that subsets of T lymphocytes are responsible for conferring protection from subsequent challenge (Nash *et al.*, 1985). However, maximum protection is considered to require a coordination of these subsets since the presence of either $CD4^+$ or $CD8^+$ T cells alone results in a decreased level of protection (Nash *et al.*, 1985; Smith *et al.*, 1994).

Class I MHC-restricted cytotoxic responses are involved in containing virus and it is generally accepted that recovery from acute viral infection is mediated by virus-specific $CD8^+$ CTL (Zinkernagel and Doherty, 1979). Anti-HSV CTL are observed in mice following

infection by a number of routes and first appear in the draining lymph nodes by three to four days (Pfizenmaier *et al.*, 1977). Peak activity is usually reached by day six beyond which the killer response declines. Precursor frequencies increase from one in a quarter of a million up to as high as one in twenty one hundred (Rouse *et al.*, 1983; Nugent *et al.*, 1995). The passive transfer of $CD8^+$ HSV-specific CTL enhances viral clearance in vivo if the donor lymphocytes are present prior to or immediately following infection (Bonneau and Jennings, 1989, 1990; Larsen *et al.*, 1983; Sethi *et al.*, 1983). These CTL were also responsible for reducing infectious virus recovered from the dorsal root ganglia (Bonneau and Jennings, 1990, 1989), implementing them as mediators in the control of acute and latent infection. There is currently a controversy surrounding the dependence of anti-HSV $CD8^+$ CTL development on the presence of $CD4^+$ T cells. For example, the generation of HSV-specific CTL in CBA and C3H mice is independent of functional HSV-specific $CD4^+$ T cells (Nash *et al.*, 1987; Mercadal *et al.*, 1991), while there is an absolute requirement for $CD4^+$ T cells in the development of HSV-specific CTL in C57BL/6 (Jennings *et al.*, 1991) and BALB/c (Mercadal *et al.*, 1991) mice. This discrepancy in mouse strains has been suggested to be due to decreased levels of IFN-γ production by $CD8^+$ CTL, and recent work by Smith *et al.*, (1994) supports the idea that a lack of IFN-γ can result in decreased generation of anti-HSV CTL. Clearly, $CD4^+$ T cells can provide help in this capacity by producing IFN-γ in response to infection. Interestingly, there does not seem to be the same dependence on $CD4^+$ T cells for secondary anti-HSV $CD8^+$ CTL responses (Jennings *et al.*, 1991).

A feature of CTL arising in the lymph nodes of herpes infected mice is a requirement for

two to three days of *in vitro* culture (without antigen) before becoming functional killer cells. It is unclear why this culture period is necessary, however, it has been noted that pretreatment of mice with cyclophosphamide abolishes this requirement (Pfizenmaier *et al.*, 1977). Presumably, suppressor activity is lost *in vitro* and CTL can develop following a stimulus from the culture medium or factors derived from other cells present. Directly lytic anti-HSV CTL have also been observed in mice administered monoclonal antibodies against gC or gD just after infection with HSV (Sethi, 1983). The mechanism behind this effect are unclear.

Protection from HSV infection can also be conferred by Class II MHC-restricted helper T cells. Adoptive transfer of a $CD4^+$ clone, able to synthesize IFN-γ and mediate antigen specific delayed-type hypersensitivity, was protective *in vivo* (Leung *et al.*, 1984). In agreement with this, $CD4^+$ T cells from recently infected or immunized mice were able to mediate recovery upon adoptive transfer (Smith *et al.*, 1994; Manickan *et al.*, 1995a), and in the absence of $CD8^+$ T cells, $CD4^+$ T cells alone have been shown capable of mediating resistance (Manickan and Rouse, 1995). As discussed above, the production of Th1 type cytokines, especially IFN-γ, are likely responsible for mediating the $CD4^+$ T cell dependent mechanisms of protection (Smith *et al.*, 1994; Niemialtowski and Rouse, 1992).

Several groups have reported the presence of anti-HSV $CD4^+$ T cells with cytotoxic functions in humans (Yasukawa and Zarling, 1984; Schmid and Rouse, 1992). Although $CD8^+$ CTL are also observed following appropriate in vitro stimulation, there appears to be a disproportionate number of cytolytic $CD4^+$ T cells related with HSV infections (Schmid and Rouse, 1992). This may be related to the unusual finding that human fibroblasts infected by

HSV are not recognized by HSV-specific $CD8^+$ CTL (Posavad and Rosenthal, 1992). This resistance to killing by $CD8^+$ CTL occurs within a few hours after infection and prior to the virus-induced inhibition of host protein synthesis which affects MHC Class I expression. Interestingly, the addition of exogenous HSV peptides, which bypasses antigen presentation pathways, renders HSV-infected fibroblasts sensitive to killing by $CD8^+$ CTL (Koelle *et al.*, 1993). Recently, York *et al.*, (1994), demonstrated that an HSV protein, ICP47, is responsible for retaining MHC class I protein complexes in the endoplasmic reticulum (ER) of human fibroblasts. The inhibition of peptide loading by ICP47 appears to be responsible for this phenomena. The lack of MHC Class I presentation of viral peptides may be responsible for the decreased presence of $CD8^+$ CTL observed in humans and therefore for the skewed development of $CD4^+$ CTL, is akin to the development of $CD4^+$ CTL in environments lacking $CD8^+$ CTL as discussed previously. The $CD8^+$ CTL that are observed in humans appear to predominantly recognize viral structural proteins rather than the products of viral protein synthesis (Tigges *et al.*, 1992). Viral structural proteins would be the first to enter antigen processing pathways and likely would be able to escape the early blockade imposed by ICP47. It is important to note that ICP47 does not have the same dramatic effects in the mouse since HSV infected murine fibroblasts are readily killed by $CD8^+$ CTL, although HSV-2 infected murine target are not as readily lysed as HSV-1 infected ones (York *et al.*, 1994; Grunhaus *et al.*, 1994).

4. Glycoprotein B

Glycoprotein B (gB) is a membrane protein that is required to initiate the fusion of the viral envelope with the plasma membrane of the host cell. gB is an immunodominant antigen for both cellular and humoral immune responses and has been extensively studied as a putative immunogen for vaccines. In humans, antibodies to gB along with gD, gC/gE, and VP5 are detected in most patients by three weeks following the onset of primary symptoms (Ashley *et al.*, 1994). In addition, anti-gB antibodies are generally associated with HSV seropositive individuals (Goade *et al.*, 1996). In animal models, gB is found to induce high levels of neutralizing antibodies and gB specific monoclonal antibodies have been shown to protect against HSV-2 infection (Balanchandran *et al.*, 1982). In studying class I MHC restricted responses in C57BL/6 mice, gB-specific CTL account for as much as nine to twenty five percent of the overall HSV-specific CTL following primary infection (Nugent *et al.*, 1994; Witmer *et al.*, 1990). gB-specific CTL lines can also limit the extent of acute and latent viral infection on adoptive transfer (Bonneau *et al.*, 1989; Bonneau *et al.*, 1990). The epitope recognized by these CTL has been mapped to aa residues 498 to 505 of gB (Hanke *et al.*, 1991). In addition, gB-specific CTL constitute a substantial component of the memory CTL population present in the spleens of mice that have recovered from initial infection. Taken together, it appears that gB is an important immune target in protection against HSV infection.

G. PURPOSE OF THE STUDY

In work previously conducted in the laboratories of Drs. Mark McDermott, Frank Graham, Kenneth Rosenthal, and David Johnson, using the recombinant adenovirus vector expressing the glycoprotein B of HSV, AdgB2 (Johnson *et al.*, 1988), it was demonstrated that immunization resulted in systemic immune responses and protection against subsequent HSV infection (McDermott *et al.*, 1989; Hanke *et al.*, 1991; Witmer *et al.*, 1990). Immunization induced strong serum neutralizing antibody responses, as well as cellular immune responses in which the immunodominant H-2K^b restricted CTL epitope of gB was characterized (Hanke *et al.*, 1991; Witmer *et al.*, 1990). gB-specific CTL can also limit the extent of viral infection on adoptive transfer (Bonneau *et al.*, 1989; Bonneau *et al.*, 1993). Furthermore, in a murine model developed by McDermott *et al.*, (1984, 1987, 1989), mice were protected against intravaginal HSV-2 infection following intravaginal inoculation with an attenuated strain of HSV-2 (TK$^-$). Protection was observed after adoptive transfer of immune T cells from the genital lymph nodes of TK$^-$ inoculated mice. CTL activity was also observed in the genital lymph nodes and previous work (Bonneau and Jennings, 1989, 1990; Larsen *et al.*, 1983; Sethi *et al.*, 1983) has demonstrated that CTL are largely responsible for providing protection against HSV infection. In addition, antibodies have also been shown to play a role in protection from infections with HSV (Balachandran *et al.*, 1982; Eisenberg *et al.*, 1985; Dix *et al.*, 1981; Rector *et al.*, 1982), implementing both arms of the immune system in mediating resistance to herpesviruses.

To examine the hypothesis that intranasal administration of AdgB8 induces mucosal

specific immune responses and protection against HSV-2 infection, locally and distally in the female genital tract, the following points were investigated:

1. To analyze the development of gB-specific humoral immune responses in mucosal and systemic tissues following intranasal and systemic immunization with AdgB8.

2. To examine B cell and CTL memory in mucosal and system immune compartments following AdgB8 immunization with the proposition that the maintenance in mucosal tissues may depend on mucosal induction.

3. To evaluate the level of protection against mucosal infections with HSV-2 following intranasal and systemic immunization with AdgB8.

Secretory IgA and cellular immune responses induced within mucosal tissues can disseminate and provide protection, both locally and at distant mucosal sites (see sections A and B). In humans, infections with herpesviruses typically involve the oral or genital tissues. Recombinant adenoviruses expressing glycoprotein B of HSV administered systemically induce serum antibodies and splenic CTL and protect mice from a systemic HSV-2 challenge (McDermott *et al.*, 1989; Witmer *et al.*, 1990). Since adenoviruses are naturally tropic for the respiratory tract, mucosal immune responses initiated following intranasal administration of AdgB8 were investigated, and the results are presented in chapters two and three, respectively. In addition, mucosal responses were compared to those initiated after systemic immunization.

Traditionally, immunization strategies directed at providing protection against HSV

infections have focused on systemic administration of various antigenic formulations (see sections C and F). However, optimal protection against mucosal pathogens may well occur in the presence of mucosal specific immune responses (see sections A, B and C). In chapter four, protection against intravaginal HSV infection is investigated in mice immunized intranasally with AdgB8, and in chapters two and five, the level of protection in mucosal tissues is compared following systemic or mucosal immunization with AdgB8.

Expression of humoral immunity in the female genital tract has been shown to depend on sex hormones, namely, estrogen and progesterone. Total antibody levels have also been reported to vary with the stage of the estrous or menstrual cycle, and one report on the levels of specific antibodies in genital secretions suggests a degree of dependence on the stage of the estrous cycle (Wira, *et al.*, 1994). During the course of evaluating HSVgB-specific antibody levels in vaginal washes of mice immunized intranasally with AdgB8, IgG and IgA levels were found to vary. The work presented in Chapter 4 examines the dependence of gB-specific antibody levels on the stages of the estrous cycle and the sex hormone progesterone.

Immunological memory forms the foundation on which immunity is based. T cell memory has been examined extensively in several viral and antigen models and there is controversy over whether T cells are inherently long-lived or require persistent nonspecific or specific antigen stimulation (see section D). Regardless of the mechanism(s) responsible for maintaining memory T cells, T cell memory has been shown to persist for years systemically or in the circulatory system. Interestingly, memory lymphocytes are known to have recirculation patterns that are heterogenous and distinct for the tissues in which they

originated. In our early work it became apparent that although systemic immunization with AdgB8 induced long-lasting CTL in the spleen, mice immunized intranasally did not maintain the same level of splenic CTL (chapter 2). To investigate the hypothesis that memory T cells are maintained in tissue compartments associated with those in which they originated, T cell memory was examined in several immune compartments following intranasal or systemic immunization with AdgB8. The results from these investigations are presented in chapter six. In addition, in chapters 4 and 5, B cell memory responses in mucosal tissues were examined in mice immunized systemically or mucosally with AdgB8.

CHAPTER TWO

COMPARISON OF SYSTEMIC AND MUCOSAL IMMUNITY FOLLOWING INTRAPERITONEAL OR INTRANASAL IMMUNIZATION

Mucosal Immunity and Protection after Intranasal Immunization with Recombinant Adenovirus Expressing Herpes Simplex Virus Glycoprotein B (Gallichan *et al.*, 1993, J. Infect. Dis., 168:622-629).

1. Background

The work leading up to this report involved the utilization of the recombinant adenovirus vector, AdgB8, expressing the glycoprotein B gene of herpes simplex virus, to induce mucosal immune responses (McDermott *et al.*, 1989; Witmer *et al.*, 1990; Hanke *et al.*, 1991). Investigation of various routes of mucosal immunization with AdgB8 initially revealed that a single intranasal immunization resulted in a high degree of splenic CTL specific for herpes simplex virus. Thus the purpose of this study was to further explore the development of humoral and cellular immune responses following intranasal AdgB8 immunization and compare this to a systemic route of immunization (ie. intraperitoneal). Although mucosal immunity was the primary focus of this work, the induction of immunity in both systemic and

mucosal immune compartments was examined. In addition, protection from herpes simplex virus type 2 infection of the upper respiratory tract was evaluated.

Mucosal Immunity and Protection after Intranasal Immunization with Recombinant Adenovirus Expressing Herpes Simplex Virus Glycoprotein B

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A recombinant adenovirus (Ad) expressing glycoprotein B (gB) of herpes simplex virus (HSV) type 1 (AdgB8) was evaluated as a mucosal vaccine candidate. When administered intranasally (inl) to C57B1/6 mice, AdgB8 induced levels of serum anti–HSV gB IgG antibodies similar to those of mice immunized intraperitoneally (ip), which neutralized both HSV-1 and -2. Mice immunized inl with AdgB8 produced secretory IgA specific for HSV gB, but mice immunized ip did not. Splenic anti-HSV cytotoxic T lymphocytes (CTL) were observed after inl and ip immunization; however, there was a time-dependent decrease in the anti-HSV CTL activity from spleens of inl immunized mice. Anti-HSV CTL were also present in the mediastinal lymph nodes after inl but not ip AdgB8 immunization. Furthermore, mice immunized inl with AdgB8 were protected against heterologous inl challenge with HSV-2, and this protection lasted longer than in ip-immunized mice. These results indicate that mucosal immunization with a recombinant adenovirus can induce mucosal and systemic immune responses and provide long-term protection from mucosally or sexually transmitted viruses.

The high prevalence of herpes simplex virus (HSV) type 1 and 2 infections in humans underlies the need to develop a safe and effective vaccine [1]. Primary HSV infection usually occurs at oral or genital mucosal surfaces and can result, in the absence of viremia, in infection of neurons and the establishment of latency [2]. Recurrent disease is believed to occur through the activation of latent virus persisting in infected ganglia [2]. To achieve effective immunity against mucosally or sexually transmitted viruses, such as HSV or human immunodeficiency virus (HIV), it may be necessary to immunize at mucosal surfaces [3–6].

Recombinant viral vectors have been used successfully as mucosal immunogens. Intranasal (inl) immunization of mice with recombinant vaccinia vectors containing the hemagglutinin (H1) gene of influenza induced higher titers of mucosal IgA antibody and provided more complete protection of the upper respiratory tract than intraperitoneal (ip) administration of inactivated vaccine [7]. Rooney et al. [8] demonstrated that inl immunization with a recombinant vaccinia vector capable of expressing glycoprotein D of HSV-1 provided protection against development of a latent trigeminal ganglionic infection when mice were challenged with a sublethal dose of HSV by the lip or nasal route.

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Adenoviruses have also proven to be excellent mucosal vaccine vectors. Unattenuated adenovirus type 4 and 7 vaccines have been orally administered in enteric-coated capsules to millions of US military recruits and have proven effective in preventing adenovirus-induced acute respiratory disease without evidence of adverse reactions [9, 10]. Recombinant adenoviruses capable of expressing antigens from vesicular stomatitis virus [11], rabies virus [12], respiratory syncytial virus [13], hepatitis B virus [14, 15], and HIV-1 [16] have induced systemic humoral immune responses or provided protection after mucosal administration. Evidence that recombinant adenovirus vectors target mucosal tissues was recently obtained by the transfer of normal copies of both the human cystic fibrosis transmembrane conductance regulator gene and the α1-antitrypsin gene to airway epithelium of cotton rats [17, 18].

Previously, McDermott et al. [19] demonstrated that a single ip inoculation with a recombinant adenovirus vector capable of expressing glycoprotein B (gB) of HSV-1 protected mice from a lethal systemic (ip) challenge with HSV-2 [19]. Further, this recombinant vector was used to show that gB is a major antigen recognized by H-2^b-restricted anti-HSV cytotoxic T lymphocytes (CTL) [20, 21]. Since parenteral immunization with an adenovirus vector expressing gB of HSV-1 provided protection from systemic heterologous challenge, we sought to determine whether mucosal immunization would stimulate mucosal immune responses and provide protection at the mucosal surface.

Materials and Methods

Animals. Inbred, female C57B1/ 6 mice (purchased from Charles River Canada, St. Constant) were used for these studies.

Virus strains and cell cultures. The construction of the adeno-

virus recombinant vector AdgB8 was similar to that reported by Johnson et al. [22]. In short, the gB gene from HSV-1 was coupled to the SV40 promoter and inserted into the E3 region of an adenovirus vector. The orientation of the chimeric gB-SV40 gene was left to right and, unlike the construction of the previously reported AdgB2 vector that contains both Ad2 and Ad5 sequences [22], AdgB8 contains only Ad5 sequences and the gB gene of HSV-1 (Hutchinson L, unpublished data). A type 5 adenovirus containing an E3 deletion ($AdE3^-$) was used as a control virus [23]. The recombinant adenoviruses were grown in KB cells, purified twice on CsCl gradients [12], and titered on 293 cells. HSV-1 F and wild-type HSV-2 were grown and titered on Vero cells. MC57, a murine H-2^b target cell line, and 293 and Vero cells were grown in α-MEM (GIBCO Laboratories, Burlington, Canada), supplemented with 10% fetal calf serum (FCS; GIBCO), and 1% penicillin-streptomycin and L-glutamine (GIBCO). KB cells were grown in α-MEM F11 and supplemented as above.

Immunizations. Mice were immunized inl with 10^8 pfu of AdgB8 or $AdE3^-$ in 20 μL of PBS or ip with 10^8 pfu of AdgB8 in 200 μL of PBS. Animals immunized inl were ether anesthetized and inverted and 20 μL of virus was placed in their nares for inhalation.

Lavages and homogenates. To quantitate mucosal anti-HSV gB IgA, mice were inoculated inl and boosted 2–5 weeks later, and then 3–5 days later lavages of the lung and nose were done and tissue homogenates were prepared. Nasal lavage was done by surgically revealing the trachea, inserting a syringe, and washing 0.5 mL of PBS down the trachea. Fluid was collected from the nose of the inverted animal. Lavages of the lungs were done by inserting a 0.58-mm polyethylene tube into the trachea, tying it off, and drawing 0.5 mL of PBS in and out of the lungs through a syringe attached to the tube. Tissue homogenates of the nose, small intestine, and lungs were prepared; the lungs and 12 cm of small intestine were separately placed in 0.5 mL of PBS, and lysates were prepared with a tissue homogenizer; the nasal area was isolated and placed in 1 mL of PBS and ground with a tissue homogenizer. The cell-free supernatant from each tissue homogenate or lavage was frozen at −20°C.

ELISA. HSV-1 gB-specific ELISAs were done in flat-bottomed microtiter plates (Costar, Cambridge, MA). Plates were precoated with 0.25 μg of recombinant HSV-2 gB (provided by R. L. Burke, Chiron, Emeryville, CA) in borate-buffered saline (BBS), pH 8.5, and kept overnight at 4°C. A TRIS-buffered saline solution containing 10 mg/mL bovine serum albumin, pH 7.4, was used to block any plastic not precoated with HSV gB. Duplicate, serially diluted samples of either hyperimmune control, test sera, or sample supernatants were added, followed by either biotin-labeled goat anti-mouse IgG or IgA antibody (Southern Biotechnology Associates, Birmingham, AL). The labeling reagent was alkaline phosphatase (ExtrAvidin; Sigma, St. Louis); *p*-nitrophenyl phosphate (Sigma) was used as a substrate in the IgG ELISA, and the ELISA Amplification System (GIBCO) was used as a substrate for the IgA ELISA. Color was allowed to develop for 45 and 10 min for the IgG and IgA ELISAs, respectively. Arbitrary units (AU) were calculated from a standard curve using hyperimmune control, where undiluted hyperimmune sera = 1000 AU for IgG, and undiluted hyperimmune lavage solution = 100 AU for IgA.

CTL assay. Spleen and mediastinal lymph node cells were prepared by teasing the tissues through a stainless steel grid. Primary CTL were generated by the protocol of Pfizenmaier et al. [24] with modifications [25]. Briefly, in the primary CTL assay, lymph node cells were incubated for 3 days in RPMI 1640 medium with 10% FCS, 50 μM 2-mercaptoethanol, 1% L-glutamine, penicillin, and streptomycin. The effectors were then incubated with uninfected and HSV-1–infected syngeneic and allogeneic targets at effector-to-target ratios of 40:1, 20:1, and 10:1 in a 5-h ^{51}Cr release assay. The secondary CTL assay was done as follows: Isolated spleen or mediastinal lymph node cells were incubated for 6 days with γ-irradiated, AdgB8-infected, syngeneic MC57 cells at a stimulator-to-responder ratio of 1:166 in RPMI 1640 medium (supplemented as above). Effector cells were then incubated with target cells in a ^{51}Cr release assay.

The cytotoxic activity was calculated using the following formula:

$$\% \text{ specific lysis} = \frac{\text{counts min}^{-1}\text{ (test)} - \text{counts min}^{-1}\text{ (spontaneous)}}{\text{counts min}^{-1}\text{ (max)} - \text{counts min}^{-1}\text{ (spontaneous)}}$$

Neutralization assay. The neutralizing activity of pooled immune sera was assayed by a plaque-reduction assay in the presence of rabbit complement. Twofold dilutions of 100 μL of sera were mixed with 100 μL of viral suspension containing 100 pfu of HSV-1 F or HSV-2 and 10 μL of rabbit complement (Cederlane Laboratories, Hornby, Canada). The neutralization reaction proceeded for 1.5 h at 37°C; then virus-serum mixtures were added in duplicate to six-well plates containing monolayers of Vero cells. After the virus-serum mixture and Vero cells were incubated for 1 h at 37°C, medium containing human gamma globulin (HGG) was added. The plates were then incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO_2. Results are expressed as the reciprocal of the serum dilution causing 50% plaque reduction.

HSV-2 challenge. Groups of mice at 8 and 48 weeks after immunization were challenged inl with a 100% lethal dose ($LD_{100} = 5 \times 10^5$ pfu) of HSV-2. Animals that were moribund were sacrificed according to animal care regulations. The percentage of protection was expressed as the number of mice surviving, divided by the total number of mice challenged, multiplied by 100.

Statistics. Data were analyzed using the GraphPAD InStat program (Graph PAD Software, San Diego). The unpaired Student's *t* test was used to determine *P* values and significance between groups.

Results

Serum antibody. The level of specific anti-HSV gB IgG antibodies in serum was determined 4 and 62 weeks after a single inl or ip immunization with recombinant AdgB8. Figure 1 shows that at 4 weeks after immunization, the level of anti-HSV gB IgG in the serum of mice given AdgB8 ip (85 ± 28 AU) was significantly greater than that of mice immunized inl (54 ± 9 AU; $P < .05$) (figure 1). The high level of

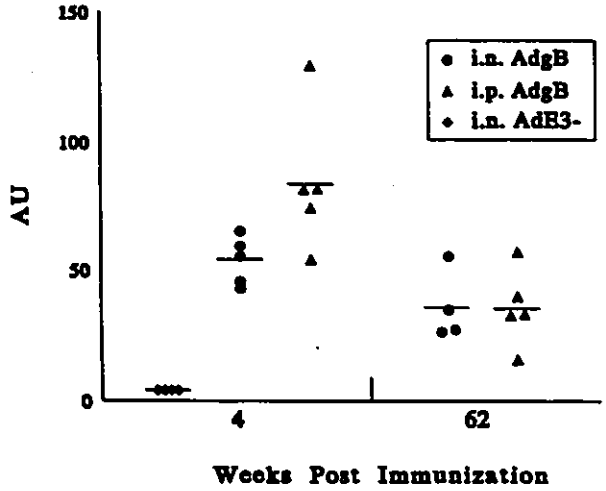

Figure 1. Serum anti-HSV glycoprotein B (gB) IgG antibodies in mice after intraperitoneal (i.p.) or intranasal (i.n.) immunization with adenovirus (Ad) gB at 4 and 62 weeks after immunization. All mice were given 10^8 pfu. AdgB = AdgB8. AdE3$^-$, Ad with deletion in E3 region. Horizontal bars, mean of each group.

anti-HSV gB IgG in the serum of ip mice immunized with AdgB8 was confirmed in other experiments, but was not always significantly greater than that from inl-immunized mice (data not shown). Control animals, immunized inl with AdE3$^-$, did not generate significant serum levels of anti-HSV gB IgG (figure 1). At 62 weeks after immunization, the serum anti-HSV gB IgG levels in mice immunized inl or ip with AdgB8 were similar at 36 ± 14 and 36 ± 15 AU, respectively (figure 1). There was a significant decrease over time in the total serum anti-HSV gB IgG in mice immunized ip ($P < .01$), while, on average, mice immunized inl maintained their serum anti-HSV gB IgG antibody levels over the observation period.

Mucosal antibody. To determine whether inl administration of AdgB8 induced a specific mucosal humoral immune response to HSV-1, homogenates of mucosal tissues were examined for anti-HSV gB IgA. To observe anti-HSV gB IgA in AdgB8-immunized mice, it was necessary to boost the primary response. For tissue homogenates, mice were immunized with AdgB8 and boosted with AdgB8 5 days before tissue examination. Homogenates of lungs from animals immunized inl contained significant levels (142 ± 96 AU; $P < .05$) of anti-HSV gB IgA; animals immunized ip had only background levels of anti-HSV gB IgA (figure 2). Nasal homogenates from mice immunized inl also contained significantly more anti-HSV gB IgA than did mice immunized ip (233 ± 48 and 29 ± 8, respectively, $P < .0001$). Control mice (immunized inl with AdE3$^-$) had only background levels of anti-HSV gB IgA in homogenates of the lungs and nose. Thus, anti-HSV gB IgA was present in the lungs and nasal tissues of mice immunized inl but not ip with AdgB8. Interestingly, the small intestines of animals receiving AdgB8 inl or ip contained 73 ± 45 and 59 ± 37 AU of anti-HSV gB IgA, respectively. The amount of anti-HSV gB IgA found in the small intestine after inoculation by either route was significantly greater ($P < .05$) than that of control mice receiving AdE3$^-$ inl. These results indicate that both routes of immunization stimulated intestinal IgA production.

Tissue lavages were done to determine whether the IgA observed in the tissue homogenates was secretory. To detect secretory anti-HSV gB IgA, it was necessary to boost all animals inl with HSV-1 3 days before lavage. Lung and nasal lavages from mice immunized inl with AdgB8 contained specific anti-HSV gB IgA; 10 ± 6 AU for the lungs and 14 ± 6 AU for the nasal lavages (figure 3). Secretory IgA was observed only in mice immunized inl and was significantly greater than in mice immunized ip with AdgB8 ($P < .05$ lung, $P < .005$ nasal; figure 3).

Cellular immunity. Cell-mediated immunity against HSV is important in preventing viral spread and the establishment of latency [26]. We compared anti-HSV CTL from mice immunized ip or mucosally (inl) with AdgB8. The initial levels of specific anti-HSV CTL induced in the spleen after either immunization with AdgB8 were similar (table 1). From 2 to 5 weeks (separate experiments) after immunization, both mucosal and systemic administration of AdgB8 produced spleen effector cells that markedly lysed HSV-infected syngeneic target cells. Lysis mediated by these splenic effector cells was T cell receptor-dependent, since incubation with anti-CD3 blocked lysis (table 1). Mice immunized with AdE3$^-$ did not generate anti-HSV CTL (table 1). Further, the low level of specific lysis of uninfected syngeneic

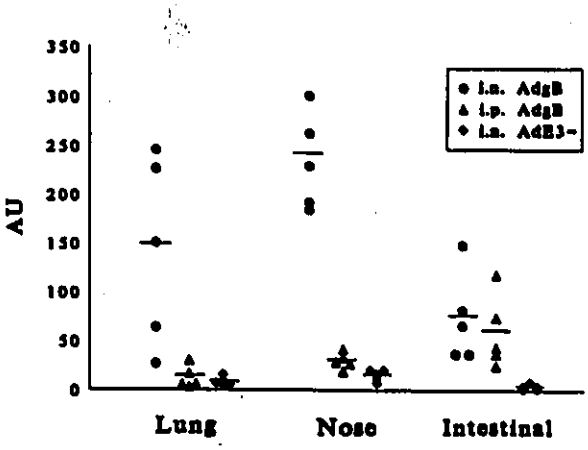

Figure 2. Mucosal anti-HSV glycoprotein B (gB) IgA antibodies after adenovirus (Ad) gB8 intranasal (i.n.) or intraperitoneal (i.p.) immunization of mice. Mice were boosted with AdgB8 (AdgB) 5 weeks after primary immunizations; samples of lung, nose, and intestine were obtained 5 days later. All mice received 10^8 pfu. AdE3$^-$, Ad with deletion in E3 region. Horizontal bars, mean of each group.

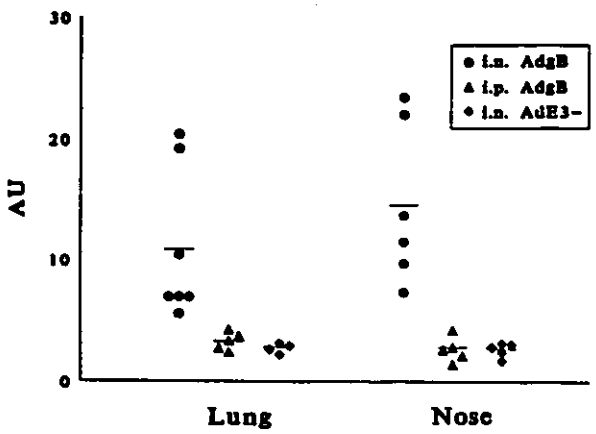

Figure 3. Secretory anti-HSV glycoprotein B (gB) IgA antibodies after adenovirus (Ad) gB8 intranasal (i.n.) or intraperitoneal (i.p.) immunization of mice. Mice were boosted i.n. with HSV-1 2 weeks after primary immunization; 3 days later lung and nasal lavages were done. All mice were given 10^8 pfu. AdgB = AdgB8. AdE3$^-$, Ad with deletion in E3 region. Horizontal bars, mean of each group.

targets as well as that of infected and uninfected allotargets (data not shown) indicates that the killer splenocytes were major histocompatibility complex (MHC)–restricted HSV-specific CTL. In inl-immunized animals, the systemic (splenic) memory response had disappeared by 9 weeks after immunization. At 9, 56, and 58 weeks after immunization with AdgB8, anti-HSV CTL activity was not detectable in spleen cells from these animals. In contrast, mice immunized ip with AdgB8 maintained significant memory anti-HSV CTL responses for up to 58 weeks after infection (table 1).

To determine whether inl administration of AdgB8 elicits a local cellular immune response, the draining lymph nodes of the respiratory tract were examined for anti-herpes CTL after immunization with AdgB8. In all experiments, mice immunized inl had specific anti–HSV-1 CTL in the mediastinal lymph nodes (table 2); little or no anti–HSV-1 CTL were observed in the mediastinal lymph nodes of mice immunized ip. In addition, there was no apparent anti–HSV-1 CTL in the mediastinal lymph nodes after inl administration of AdE3$^-$. The mediastinal lymph node effectors were MHC-restricted and HSV-specific CTL, as indicated by the low level of specific lysis of uninfected syngeneic targets and by the infected and uninfected allotargets (data not shown).

Neutralization. Serum from mice immunized and then rested for 2 or 48 weeks was examined for anti HSV-1 or -2 neutralizing antibodies. At 2 weeks, the HSV-1 neutralizing antibody titers from mice immunized once inl or ip with AdgB8 were 800 and 400, respectively (table 3). Also, HSV-2 neutralizing antibody titers after one immunization were identical to those of HSV-1. Mice immunized twice with AdgB8 (5 weeks between immunizations), inl or ip, and examined 5 days after secondary immunization had neutralizing antibody titers of 3200 and 1600 for HSV-1 and HSV-2, respectively. Of importance was the maintenance of the neutralization of HSV-1 and -2 for 48 weeks. At 48 weeks after immunization, AdgB8-immunized mice (either inl or ip) had HSV-1 and -2 neutralizing antibody titers similar to those observed after 2 weeks (table 3).

Protection against HSV-2 challenge. We observed that C57Bl/6 (H-2^b) mice are relatively resistant to inl HSV-1 but susceptible to inl infection with HSV-2 (data not shown). Further, HSV-1 gB, which is subcloned in AdgB8, is 85% homologous with HSV-2 gB [27]. The LD_{100} for C57Bl/6 mice infected inl with HSV-2 was 5×10^5 pfu/mouse (data not shown). Previously, McDermott et al. [19] established that ip immunization with AdgB protected mice from ip HSV-2 challenge. Since HSV infects at various mucosal surfaces of the body, mucosal vaccination and the generation of specific mucosal immune responses may be essential for protection. Therefore, we immunized mice with AdgB8 by inl and ip routes and then challenged the mice with HSV-2. Eight weeks after immunization, 12 of 13 inl-immunized

Table 1. Detection of splenic cytotoxic T lymphocytes (CTL) specific for HSV after immunization with adenovirus recombinant expressing HSV glycoprotein B (AdgB8).

	% specific lysis of infected targets by effector-target ratio										
	AdgB8 inl				AdgB8 ip				AdE3$^-$ inl		
Weeks after immunization	40:1	20:1	10:1	40:1 + αCD3	40:1	20:1	10:1	40:1 + αCD3	40:1	20:1	10:1
2	87	92	77	ND	100	100	84	ND	6	5	1
5	78	66	45	22	78	62	29	14	0	2	3
9	6	11	2	5	56	37	22	9	3	5	3
56	2	6	4	7	20	11	7	0	ND	ND	ND
58	6	7	7	6	81	63	47	3	ND	ND	ND

NOTE. Mice were immunized once with 10^8 pfu of virus. Effector cells were stimulated in vitro with γ-irradiated, AdgB8-infected MC57 cells. Anti-CD3 was incubated with effectors for 1 h before incubation with targets (syngeneic MC57 cells). Cytotoxicity was examined in separate experiments for each time period after immunization except for 5 and 9 weeks, which were examined together; inl, intranasally; ip, intraperitoneally; ND, not done.

Table 2. Detection of mediastinal cytotoxic T lymphocytes specific for glycoprotein B (gB) of HSV virus after immunization with recombinant adenovirus expressing HSV gB (AdgB8).

Immunization, effector type	% specific lysis of infected targets by effector-target ratio								
	AdgB8 inl			AdgB8 ip			AdE3⁻ inl		
	40:1	20:1	10:1	40:1	20:1	10:1	40:1	20:1	10:1
Primary in vivo*	46	23	14	8	6	2	ND	ND	ND
Secondary in vivo†	19	11	3	4	2	0	3	3	0
Secondary in vitro‡	46	36	21	ND	ND	ND	1	9	4

NOTE. Syngeneic MC57 cells infected with HSV-1 served as targets. inl, intranasally; ip, intraperitoneally; ND, not done.

* Mice immunized once with 5×10^5 pfu of AdgB8; mediastinal lymph node effector cells isolated 5 days later and cultured for 3 days in vitro.

† Primary immunization with 10^5 pfu of AdgB8 followed 5 weeks later with second immunization; mediastinal lymph node effector cells isolated 5 days later and cultured for 3 days in vitro.

‡ Primary immunization with 10^5 pfu of AdgB8; 2 weeks later mediastinal lymph node effector cells were stimulated in vitro for 6 days with γ-irradiated, AdgB8-infected MC57 cells.

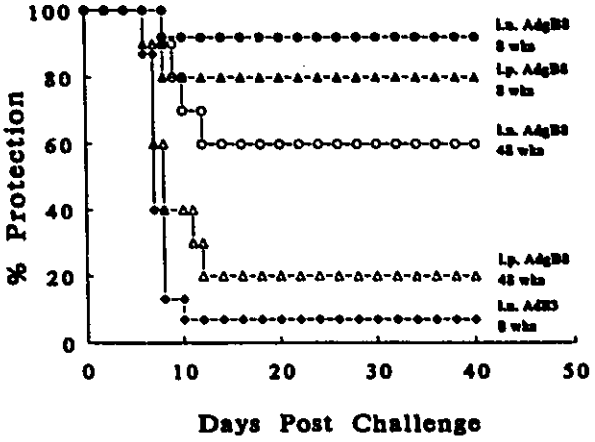

Figure 4. Protection of mice immunized intranasally (i.n.) or intraperitoneally (i.p.) with recombinant adenovirus expressing HSV IgB (AdgB8) against HSV-2 challenge. wks, weeks after immunization before i.n. challenge with 100% lethal dose (5×10^3 pfu) of HSV-2. All mice were given 10^8 pfu of AdgB8 or AdE3⁻, Ad with deletion in E3 region.

mice survived a lethal inl HSV-2 challenge (figure 4). Similarly, 8 of 10 mice immunized ip survived. The protection in mice 48 weeks after primary immunization was also examined. In animals that were immunized inl with AdgB8, 6 of 10 were resistant, whereas only 2 of 10 animals immunized ip survived. When control mice were immunized inl with AdE3⁻ and challenged at 8 weeks with HSV-2, only 1 of 15 survived. Thus, a high proportion of mice immunized inl with AdgB8 survived heterologous inl challenge with HSV-2. In addition, the protection conferred by inl immunization with AdgB8 lasted longer than that conferred by ip immunization with the same vector.

Table 3. Neutralizing antibody titers against HSV-1 and -2.

Glycoprotein, route and no. of immunizations	Weeks after immunization	Neutralizing antibody titer	
		HSV-1	HSV-2
AdgB8, inl			
Twice	2	3200	1600
Once	2	800	800
Once	48	800	800
AdgB8, ip			
Twice	2	3200	1600
Once	2	400	400
Once	48	800	800
AdE3⁻, inl, twice	2	<10	<10

NOTE. Sera were from mice immunized with 10^8 pfu of virus at 2 or 48 weeks after primary or 5 days after secondary immunization. Mice immunized twice were rested 5 weeks between immunizations. AdgB8, recombinant adenovirus expressing HSV-1 glycoprotein B; AdE3⁻, adenovirus with deletion in E3 region; inl, intranasally; ip, intraperitoneally.

Discussion

Our findings demonstrate that inl immunization of mice with a recombinant adenovirus expressing HSV-1 gB was able to generate mucosal and systemic humoral and cell-mediated immune responses. Further, mice immunized inl with AdgB8 were protected for a prolonged period against a lethal mucosal challenge with heterologous HSV-2.

HSV gB is a major component of the virion envelope and of HSV-infected cell membranes. It is essential for virus entry into cells [28–30] and an important target of the host immune response [20, 21, 31, 32]. The HSV-1 and HSV-2 gB genes are 85% homologous [27].

Previously we demonstrated [22] that a recombinant adenovirus vector that produces high-level expression of HSV-1 gB is a major target recognized by murine H-2^b–restricted anti-HSV CTL [20, 21]. Further, a single ip inoculation of mice with recombinant adenovirus containing HSV-1 gB protected mice from a lethal systemic HSV-2 challenge [19]. Since a major advantage of adenovirus-based vaccines is their utility as mucosal immunogens and in light of our previous results, we set out to examine mucosal immunity using AdgB8 and to determine its ability to protect against a mucosal HSV infection.

We demonstrated a significant level of anti-HSV gB IgG in the serum of mice 4 weeks after inl immunization with AdgB8. Mice immunized ip had significantly more anti-HSV gB IgG than did inl-immunized mice. In contrast, mice immunized inl with a control adenovirus containing a deletion of the E3 region (AdE3⁻) generated no significant anti-HSV gB IgG. At 62 weeks after immunization, anti-HSV gB IgG levels in the serum of mice immunized inl and ip had

decreased, especially in mice immunized ip, but remained significant. These results clearly show that mucosal immunization with AdgB8 generated significant levels of anti-HSV gB IgG in serum that persisted for 48 weeks.

After a single immunization with AdgB8, serum neutralizing antibodies were induced that could neutralize both HSV-1 and -2. The serum neutralizing antibody titers against HSV-1 and -2 were similar after inl or ip AdgB8 immunization and were maintained for 48 weeks. These results support previous observations by McDermott et al. [19], who showed cross-neutralization of HSV-1 and -2 after ip immunization with AdgB2.

An important component of the mucosal immune system is the presence of immunoglobulin, predominantly IgA, in external secretions [6, 33–36]. In several animal models, the presence of specific IgA after mucosal immunization has correlated with protection against mucosal viral challenge [4, 7, 37]. Our initial experiments demonstrated anti-HSV gB IgA in the lung lavages of mice immunized inl with AdgB8 (unpublished data). Subsequent boosting of the primary response induced significant levels of specific IgA in the lavages and homogenates of the nose and lungs of mice immunized inl. We observed anti-HSV gB IgA in the intestinal homogenates, but not in lavages, of mice immunized inl and ip. The presence of intestinal IgA specific for HSV in mice immunized ip is most likely due to local induction by AdgB8; however, in mice immunized inl with AdgB8, the intestinal IgA may be due to lymphocytes originating from the site of AdgB8 infection in bronchus-associated lymphoid tissue [35, 36]. These results indicate that inl immunization with a recombinant adenovirus vector induces significant mucosal anti-HSV gB IgA antibody.

CTL play a central role in controlling the spread and severity of HSV infection [38–42] and may contribute significantly to local protection and clearance of virus. Recombinant adenoviruses have been used to demonstrate that gB is a major target antigen for anti-HSV CTL [20, 21]. Since AdgB2 elicited a strong anti-HSV CTL response in C57Bl/6 mice after parenteral infection [19], we felt it important to determine whether anti-HSV CTL would be elicited after mucosal immunization with AdgB8. Splenocytes from mice immunized inl with AdgB8 generated an anti-HSV CTL response that was similar in magnitude to that generated in mice immunized ip. This anti-HSV CTL activity was H-2–restricted, virus-specific, and T cell receptor–dependent. Although initial levels of splenic anti-HSV CTL (≤5 weeks after immunization) were generally equivalent, with time the splenic anti-HSV CTL activity in inl-immunized mice had decreased compared to ip-immunized mice. This may be a result of locally stimulated lymphocytes migrating back to mucosal tissues and being undetectable in the spleen [33, 36, 42]. Nevertheless, it appears that mucosal immunization with a recombinant adenovirus containing a major CTL target antigen induces a systemic cell-mediated immune response similar in magnitude to parenteral immunization; however, the splenic memory response after mucosal immunization was comparatively short.

It is possible that local CTL specific for HSV may give the host an immune advantage when challenged mucosally by invading virus. Indeed, once HSV has infected the mucosa it is critical to prevent early ganglionic infection and establishment of latency. McDermott et al. [42] demonstrated that upon adoptive transfer, murine genital lymph node CTL, generated against an attenuated strain of HSV-2, preferentially migrated into genital tissue and provided resistance against genital infection with HSV-2. These results emphasize the importance of local mucosal T cell responses in protection against HSV infections. In our study, anti-HSV CTL were observed in the mediastinal lymph nodes of mice immunized inl after primary or secondary in vivo AdgB8 immunization or in vitro stimulation. In contrast, mice immunized with AdgB8 ip or with AdE3$^-$ inl showed insignificant levels of anti-HSV CTL activity in all of the lymph nodes examined. Thus, unlike ip immunization, mucosal immunization with AdgB8 induced primary anti-HSV CTL in the draining lymph nodes of the respiratory tract.

Since HSV is a mucosal pathogen and there was induction of mucosal immune functions after inl immunization with AdgB8, it was of interest to determine whether mice were protected from lethal mucosal challenge with HSV. After inl infection C57Bl/6 mice are relatively resistant to HSV-1 but are susceptible to HSV-2 (unpublished data). Therefore, we decided to determine whether mucosal immunization with AdgB8 would protect mice from a lethal mucosal challenge with HSV-2. Eight weeks after AdgB8 immunization, mice immunized inl or ip were highly resistant to a lethal mucosal challenge with HSV-2. At 48 weeks after primary immunization with AdgB8, inl-immunized mice were still resistant, while ip-immunized mice were more susceptible to inl HSV-2 challenge. These results indicate that mucosal immunization with AdgB8 provides a high level of prophylaxis against HSV and that this protection outlasts that provided by ip AdgB8 immunization.

Although we showed that mucosal immunization with a live recombinant adenovirus vector induces mucosal humoral and cell-mediated immune responses to HSV, we have yet to determine which immune response(s) is critical for protection. Nevertheless, our results demonstrate that mucosal immunization with recombinant adenoviruses may serve to provide maximum protection at the site of initial infection with HSV or other mucosally or sexually transmitted viruses.

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2. Contributions to Gallichan *et al.* (1993)

(A) Growth and purification of all recombinant adenovirus vectors used in this study. The construction and characterization of these viruses was carried out in Drs. Frank Graham's and David Johnson's laboratories and we thank them for provision of these essential tools.

(B) Immunization of mice and collection of all samples and tissues.

(C) Developed the ELISA system and performed the evaluation of gB-specific antibody levels in sera and mucosal washes. The recombinant HSV-2 gB protein was provided by R. L. Burke of the Chiron Corporation, CA.

(D) Analysis of CTL activity in the spleens and lymph nodes of immunized animals in primary and secondary cytotoxic T lymphocyte assays.

(E) Assessment of neutralizing capacity of serum by plaque-reduction assay.

3. Summary

In Gallichan *et al.* (1993) it was found that both intranasal and intraperitoneal AdgB8 immunized mice contained gB-specific serum antibodies and a similar capacity to neutralize HSV-2 for as long as one year. Evaluation of IgA levels revealed that only mice immunized intranasally with AdgB8 were capable of providing gB-specific IgA in mucosal tissues. The presence of IgA in mucosal lavages of intranasally immunized mice was also consistent with the secretory nature of IgA. Examination of splenic CTL demonstrated that while mice immunized intraperitoneally or intranasally with AdgB8 initially contained lymphocytes capable of lysing HSV-1 infected targets, mice immunized intranasally lost this capacity as

early as 9 weeks post immunization. Interestingly, only mice immunized intranasally with AdgB8 developed specific anti-HSV CTL in the mediastinal lymph nodes that drain the respiratory tract.

These observations suggested that in addition to the unique induction of mucosal IgA, intranasal immunization appeared to provide humoral and cellular immune responses in the systemic immune system that were similar to those occurring in intraperitoneally immunized mice. The lack of detectable splenic CTL several month following intranasal immunization was however intriguing. Nevertheless, subsequent assessment of protection from an intranasal HSV-2 challenge demonstrated that mice immunized intranasally were optimally protected and this protection lasted for as long as 48 weeks post immunization.

This study demonstrated for the first time that recombinant adenoviruses are capable of inducing cellular and humoral immune responses in both the systemic as well as local mucosal immune compartments following intranasal administration. In addition, when compared to systemic administration, intranasal AdgB8 immunization provided optimal protection from a local intranasal HSV-2 challenge. Since herpes simplex virus is a sexually transmitted pathogen, further studies on protection of the upper respiratory tract were not investigated, instead, we turned our focus on the evaluation of immunity and protection in the female reproductive tract.

CHAPTER THREE

EVALUATION OF HUMORAL IMMUNITY IN THE FEMALE REPRODUCTIVE TRACT FOLLOWING AdgB8 IMMUNIZATION

Specific secretory immune responses in the female genital tract following intranasal immunization with a recombinant adenovirus expressing glycoprotein B of herpes simplex virus (Gallichan, W.S., and Rosenthal, K.L., 1995, Vaccine, 13:1589-1595).

1. Background

As described in the introduction, a number of strategies have been employed for the induction of humoral immunity in the female genital tract. Although several of these systems have been successful in providing humoral immunity, few have resulted in SIgA induction. Those strategies that have proven most successful in providing IgA have followed local or distant mucosal administration of antigen, or alternatively, local lymph node immunization. Since our previous work demonstrated that recombinant adenoviruses are good inducers of local mucosal immune responses, we examined to what extent humoral immunity occurred at distant mucosal sites. In particular, our primarily interest has been the examination of immunity to the sexually transmitted herpesvirus and therefore, we investigated the female genital tract for the development of specific humoral immunity.

The approach that we undertook involved immunizing mice twice either intranasally, intraperitoneally, or intravaginally with AdgB8 and over time comparing the levels of gB-specific antibodies in the vaginal washes and sera. In addition, we examined similar groups of mice following two intravaginal boostings with AdgB8. Finally, to determine the secretory nature of these antibodies we examined the ratios of specific and total IgG and IgA antibodies in vaginal washes and sera.

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Specific secretory immune responses in the female genital tract following intranasal immunization with a recombinant adenovirus expressing glycoprotein B of herpes simplex virus

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Previously, we demonstrated that intranasal (i.n.) but not intraperitoneal (i.p.) immunization with a recombinant adenovirus vector expressing glycoprotein B (gB) of herpes simplex virus type 1 (HSV-1) induced mucosal immune responses and conveyed long-term protection to mice against an i.n. challenge with heterologous HSV-2. We now show that i.n. immunization of female mice with this same vector, AdgB8, provides secretory and serum-derived humoral immune responses in the genital tract. Intranasal immunization induced anti-HSVgB IgA and IgG in vaginal washes of mice, whereas i.p. immunization only induced IgG, which appeared to be serum-derived. Interestingly, intravaginal (ivag) immunization with AdgB8 resulted in little or no anti-HSVgB IgA and only low levels of specific IgG in vaginal washes. All three routes of inoculation induced gB-specific serum IgG and IgA; however, i.n. immunized mice demonstrated the highest level of serum anti-HSVgB IgA. Additionally, ivag boosting with AdgB8 did not significantly alter the serum or vaginal wash antibody responses in i.n. or i.p. immunized mice. The IgG to IgA ratios of gB-specific and total antibody titres in the serum and vaginal washes of i.n. immunized mice indicated that the IgA in the vaginal washes was likely to be secretory. Furthermore, the titres of anti-HSVgB IgA relative to total IgA were higher in vaginal washes than sera, suggesting that the gB-specific vaginal wash IgA present in i.n. immunized mice was locally produced.

Keywords: Mucosal immunity; herpes simplex virus; genital tract sIgA; recombinant adenovirus; immunity to STDs; intranasal immunization

Protection of mucosal surfaces against re-infection by pathogens is largely dependent on secretory IgA, specifically polymeric IgA (pIgA), originating primarily from plasma cells located within the lamina propria[1]. In rodents there may also be a contribution of pIgA from the circulation; circulatory pIgA is transported across the epithelium by secretory component (SC) in much the same manner as that produced locally by plasma cells[2]. Mice given an intravenous injection of anti-influenza virus antibodies demonstrate specific transfer of pIgA into nasal secretions, but lack transfer of monomeric IgA[2]. Furthermore, the titre of specific mucosal antibody is influenced by the route of immunization, with application of antigen to the mucosa being the most effective in stimulating specific IgA secretion at the site of immunization[3–8]. Despite this, the induction of significant secretory immunity in the female genital tract by the use of local or systemic vaccinations has proven difficult and requires the use of adjuvants, high doses of vaccine and/or numerous applications[8–14]. Also, it is well documented that the induction of specific immune responses at one mucosal site may result in these responses appearing at distant mucosal surfaces, including the genital tract[15–20]. We have utilized this knowledge in our approach to providing herpes simplex virus specific humoral immune responses in the female genital tract.

Herpes simplex virus type-2 (HSV-2) is a sexually transmitted agent that attaches, penetrates and undergoes infectious cycles of replication in the epithelium of the genital tract[21]. Previous studies have demonstrated that humoral immunity plays an important role in limiting or blocking HSV infection of the murine genital tract epithelium. The passive transfer of antibodies

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directed against glycoproteins of HSV either prevented vaginal HSV infection or rapidly cleared the virus thereby limiting inflammation and lethality[22,23].

We previously demonstrated that intranasal (i.n.) immunization with an adenovirus vector containing the glycoprotein B (gB) gene of HSV-1 (AdgB8) induced systemic as well as local immune responses in the respiratory tract[24]. The responses were both humoral and cellular, as evidenced by secretory anti-HSVgB IgA in the respiratory tract and HSV-specific CTL in the mediastinal lymph nodes draining the respiratory tract. These responses were also sufficient to provide long-term protection against subsequent i.n. challenge with heterologous HSV-2. In contrast, mice immunized i.p. did not generate significant levels of mucosal IgA and protection from i.n. challenge was short-lived[24].

Since HSV-2 is a sexually transmitted virus, we evaluated adenovirus vectors as potential mucosal vaccines by examining the ability of AdgB8 to induce IgA in the genital tracts of mice following local (ivag), distant (i.n.), or systemic (i.p.) immunization.

MATERIALS AND METHODS

Animals and cell cultures

Inbred female C57BL/6 mice (purchased from Charles River Canada, St. Constant, Quebec, Canada) were used for these studies. Two hundred and ninety-three cells were grown in α-MEM (Gibco Laboratories, Burlington, Canada), supplemented with 10% fetal calf serum (FCS; Gibco), and 1% penicillin-streptomycin and L-glutamine (Gibco).

Virus strains and immunization

The construction of the replication competent recombinant adenovirus type 5 vectors, AdgB8 and AdE3$^-$, are reported elsewhere[25]. In short, the vector AdgB8 contains the gB gene from HSV-1. The E3 deletion virus, AdE3$^-$, does not contain any HSV genes and was used as a control. The recombinant adenoviruses were grown in 293 cells, purified twice on CsCl gradients, and titered on 293 cells. Mice in each group were immunized with 10^8 p.f.u. of AdgB8 or AdE3$^-$ in the indicated volumes of buffer. Mice immunized intranasally (i.n.) were ether anesthetized and virus in 10–20 μl of phosphate-buffered saline pH 7.4 (PBS) was introduced into the nares by means of a micropipette[24]. Intraperitoneal (i.p.) immunization was performed by injection of virus in 0.2 ml PBS. Mice immunized by intravaginal (ivag) inoculation were anesthetized and maintained using halothane for the period of inoculation. While under halothane anesthesia, mice were ivag washed with PBS and swabbed with a cotton applicator before virus in 10–20 μl of PBS was instilled ivag for 1 h. Mice in six groups of 9–10 mice per group were immunized with AdgB8 or AdE3$^-$. Mice immunized i.n. or i.p. received two doses of vaccine with a two-week interval. Mice immunized i.n./ivag or i.p./ivag received two doses of vaccine as before (i.e. i.n. or i.p.); however, they were then immunized twice ivag at two-week intervals. Mice immunized ivag received four doses of vaccine at two-week intervals. Control mice were immunized twice i.n. with 10^8 p.f.u. of AdE3$^-$ with a two-week interval.

Collection of fluids

Blood samples were obtained from the mice by retro-orbital bleeding. Vaginal fluid was collected by pipetting 30 μl of PBS into and out of the vagina several times. The staging of the estrous cycle for each mouse was based on a smear from these washings[26]. Diff-Quik (Baxter Scientific Products, Miami, FL) was used to stain the smears. The vaginal washings were then centrifuged to remove particulate matter and the supernatants were stored at −20°C.

ELISA

HSV-1 gB-specific ELISAs were done in flat-bottomed microtiter plates (Costar, Cambridge, MA). Plates were precoated with 2.5 μg ml^{-1} of recombinant HSV-2 gB (provided by R.L. Burke, Chiron, Emeryville, CA) in borate-buffered saline (BBS), pH 8.5, and kept overnight at 4°C. A Tris-buffered saline (TBS) solution containing 10 mg ml^{-1} bovine serum albumin, pH 7.4, was used to block any plastic not precoated with HSV-gB. Serially diluted samples of either hyperimmune control, test sera, or sample supernatants were added, followed by either biotin-labelled goat anti-mouse IgG or IgA antibody (Southern Biotechnology Associates, Birmingham, AL). The labelling reagent was alkaline phosphate (ExtrAvidin; Sigma, St. Louis); *p*-nitrophenyl phosphate (npp) (Sigma) was used as a substrate in the serum IgG ELISA, and the ELISA Amplification System (EAS) (Gibco, Burlington, Ont.) was used as a substrate for the serum IgA and lavage IgA and IgG ELISAs. The ELISAs were read at 405 and 492 nm for the substrates npp and EAS, respectively. Antibody titres represent the inverse dilution of the sample at which twice the background absorbance of serum or lavage fluid from uninfected mice was reached.

Statistical analysis

Data were analyzed using the GraphPAD InStat program (Graph PAD Software, San Diego, CA). The paired and unpaired Student's *t*-test were used to determine *P* values and significance between groups. Comparison among the means of the groups was carried out using analysis of variance (ANOVA).

RESULTS

Comparison of different routes of immunization for the generation of HSVgB specific vaginal wash IgA and IgG

Groups of mice were immunized intranasally (i.n.), intraperitoneally (i.p.) or intravaginally (ivag) with two doses of recombinant adenovirus capable of expressing HSV-1 glycoprotein B (AdgB8). Subsequently, subgroups of mice immunized by these routes were further boosted twice with AdgB8 intravaginally. A control group consisted of mice immunized twice i.n. with recombinant adenovirus containing a deletion of the E3 region (AdE3$^-$). Significant levels of HSVgB-specific IgA were observed in vaginal washes of mice immunized i.n. with AdgB8 when compared to control or i.p. immunized mice (*Figure 1*). In contrast, mice immunized i.p. showed low or undetectable levels of specific vaginal wash IgA (*Figure 1*). Intravaginal boosting of i.n. or i.p.

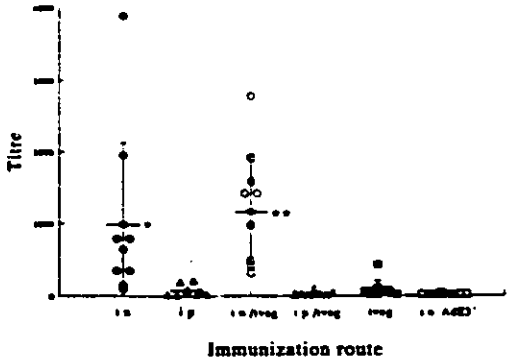

Figure 1 Anti-HSVgB IgA titres from the vaginal washes of mice immunized with AdgB8. Mice in each group were immunized at two-week intervals as follows: twice intranasally (i.n.); twice intraperitoneally (i.p.); twice intranasally followed by twice intravaginally (i.n./ivag); twice intraperitoneally followed by twice intravaginally (i.p./ivag); four times intravaginally (ivag); and twice intranasally with AdE3⁻ (i.n. AdE3⁻). Fifty-seven days post primary immunization vaginal washes were collected from each mouse and the titre of anti-HSVgB IgA determined by ELISA. Error bars indicate standard deviation. Differences between i.n. or i.n./ivag immunized groups and all other groups was determined by ANOVA: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$

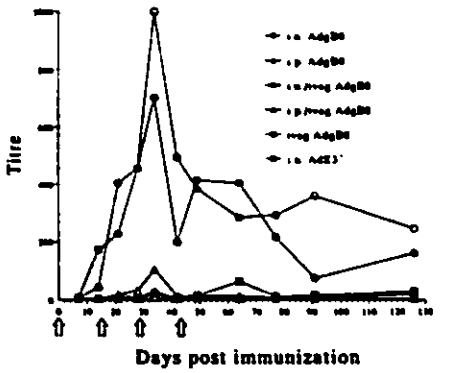

Figure 2 Time course of anti-HSVgB IgA titres in pooled vaginal washes of mice immunized with AdgB8. Mice in each group were immunized at two-week intervals as follows: twice intranasally (i.n.); twice intraperitoneally (i.p.); twice intranasally followed by twice intravaginally (i.n./ivag); twice intraperitoneally followed by twice intravaginally (i.p./ivag); four times intravaginally (ivag); and twice intranasally with AdE3⁻ (i.n. AdE3⁻). The arrows indicate the days on which mice were immunized. Vaginal washes from individual mice within each group were pooled and the IgA titres determined by ELISA

immunized mice with AdgB8 did not significantly increase these levels (*Figure 1*). Mice that were immunized four times ivag displayed little detectable vaginal wash anti-HSVgB IgA (*Figure 1*). Finally, mice immunized with AdE3⁻ displayed no significant vaginal wash anti-HSVgB IgA.

Vaginal wash samples from within groups of mice immunized by various routes were pooled and analyzed at various time points for specific anti-HSVgB IgA (*Figure 2*). Only mice immunized i.n. with AdgB8 demonstrated titres of anti-HSVgB IgA which persisted over the 126 days evaluated. Mice immunized i.n. with and without ivag AdgB8 boosting demonstrated titres of vaginal anti-HSVgB IgA that peaked 34 days post primary immunization. Intravaginal boosting with AdgB8 did not appear to affect these antibody levels

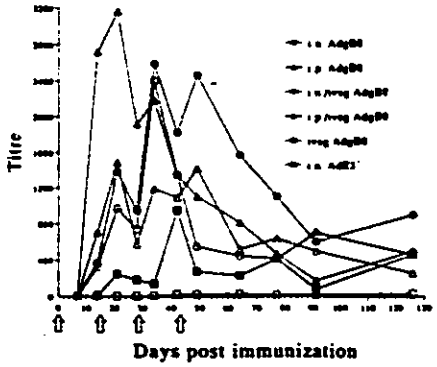

Figure 3 Time course of anti-HSVgB IgG titres in pooled vaginal washes of mice immunized with AdgB8. Mice in each group were immunized at two-week intervals as follows: twice intranasally (i.n.); twice intraperitoneally (i.p.); twice intranasally followed by twice intravaginally (i.n./ivag); twice intraperitoneally followed by twice intravaginally (i.p./ivag); four times intravaginally (ivag); and twice intranasally with AdE3⁻ (i.n. AdE3⁻). The arrows indicate the days on which mice were immunized. Vaginal washes from individual mice within each group were pooled and the IgG titres determined by ELISA

since titres generally decreased over the ivag boosting period. By two months following the second i.n. immunization, levels of anti-HSVgB IgA stabilized, especially in the ivag boosted group. Mice immunized i.p. or ivag with AdgB8 failed to demonstrate consistent levels of anti-HSVgB IgA in vaginal washes (*Figure 2*).

Mice immunized i.n. or i.p. with AdgB8 generated vaginal wash anti-HSVgB IgG within two weeks, peaking by 3–5 weeks (*Figure 3*). The levels of specific IgG varied considerably over time; however, mice immunized i.n. generated titres of anti-HSVgB IgG similar to that of i.p. immunized mice. When individual vaginal washes were analyzed, there was a broad range of responses in all groups (data not shown), similar to that observed for anti-HSVgB IgA. Intravaginal boosting on days 28 and 42 did not seem to significantly influence these levels. However, immunization of mice ivag four times did generate anti-HSVgB IgG in the vaginal wash which reached a peak titer at day 42. By 90 days the levels of specific IgG in the vaginal washes had decreased, but thereafter remained stable beyond 126 days (*Figure 3*). However, by 450 days post immunization, vaginal wash anti-HSVgB IgG and IgA levels had further decreased in all groups, and in a few cases was undetectable in individual mice (data not shown).

Comparison of different routes of immunization for the generation of specific serum IgA and IgG

Serum from within groups of mice was pooled at each time point investigated. Pooled serum samples from mice immunized i.p. with AdgB8 demonstrated high titres of anti-HSVgB IgG as seen in *Figure 4*, and indeed, antibody was still present over a year later (data not shown). Mice immunized i.n. developed similar levels of serum anti-HSVgB IgG (*Figure 4*) which were also present one year post immunization. Boosting of the i.n. and i.p. immunized groups by ivag inoculation did not increase the levels of specific serum IgG (*Figure 4*). Mice immunized i.p. displayed a peak titre of serum anti-HSVgB IgG on day 21 which corresponded to a peak

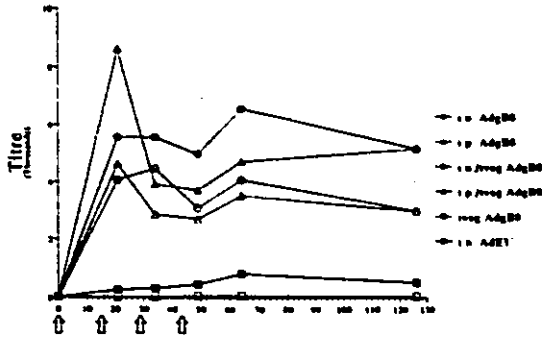

Figure 4 Time course of anti-HSVgB IgG titres in pooled sera of mice immunized with AdgB8. Mice in each group were immunized at two-week intervals as follows: twice intranasally (i.n.); twice intraperitoneally (i.p.); twice intranasally followed by twice intravaginally (i.n./ivag); twice intraperitoneally followed by twice intravaginally (i.p./ivag); four times intravaginally (ivag); and twice intranasally with AdE3⁻ (i.n. AdE3⁻). The arrows indicate the days on which mice were immunized. Serum from individual mice within each group was pooled and the IgG titres determined by ELISA

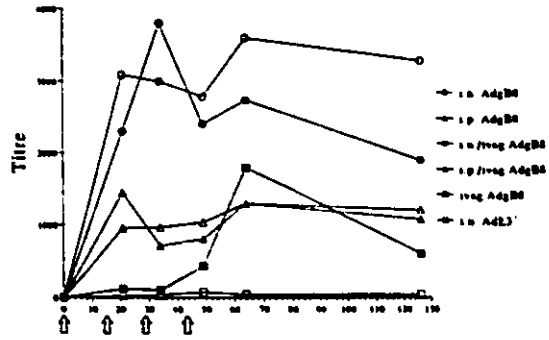

Figure 5 Time course of anti-HSVgB IgA titres in pooled sera of mice immunized with AdgB8. Mice in each group were immunized at two-week intervals as follows: twice intranasally (i.n.); twice intraperitoneally (i.p.); twice intranasally followed by twice intravaginally (i.n./ivag); twice intraperitoneally followed by twice intravaginally (i.p./ivag); four times intravaginally (ivag); and twice intranasally with AdE3⁻ (i.n. AdE3⁻). The arrows indicate the days on which mice were immunized. Serum from individual mice within each group was pooled and the IgA titres determined by ELISA

titre of anti-HSVgB IgG in the vaginal wash on day 21. Mice immunized ivag with AdgB8 demonstrated only a modest serum anti-HSVgB IgG response following four inoculations (*Figure 4*). When mice were examined individually, all mice immunized i.n. or i.p. demonstrated serum anti-HSVgB IgG, whereas not all mice immunized ivag exhibited specific antibody (data not shown). Control mice immunized with AdE3⁻ had no specific anti-HSVgB IgG (*Figure 4*).

Mice immunized i.n. with AdgB8 demonstrated serum anti-HSVgB IgA titres that were 2–4 times higher than mice immunized i.p. (*Figure 5*). Intravaginal boosting did not appear to have a marked effect on anti-HSVgB IgA serum levels in i.n. or i.p. immunized mice (*Figure 5*). Although four ivag immunizations with recombinant AdgB8 induced relatively low levels of serum anti-HSVgB IgG, the titres of serum anti-HSVgB IgA rose to levels comparable to that in i.p. immunized mice (*Figure 5*). These results indicate that i.n. AdgB8 immunization induced higher levels of specific serum IgA, but comparable levels of specific serum IgG, relative to i.p. immunization.

Comparison of anti-HSVgB IgG to IgA ratios in the serum and vaginal washes

In order to begin to address the source of the vaginal wash anti-HSVgB IgA, we determined the IgG to IgA ratios of specific and total antibodies in the serum and vaginal wash fluids of i.n. AdgB8 immunized mice. Four weeks post i.n. immunization, serum and vaginal wash anti-HSVgB and total IgG and IgA titres were determined for each mouse at estrus or on a daily basis over two estrus cycles. The ratio of IgG to IgA in the vaginal washes was found to be significantly lower than in the serum ($P<0.05$; paired Student's t-test) in all instances (*Table 1*). This was true for specific anti-HSVgB and total IgG to IgA ratios for all three groups, and in fact, in every mouse we have examined (data not shown). A significantly lower IgG to IgA ratio in the vaginal washes relative to the sera indicates an increase in the titre of IgA relative to IgG in the vaginal wash. Therefore, the IgA in the vaginal fluids is higher than would be expected if it was only present due to serum transudation (i.e. IgG in the vaginal fluids originates from serum transudation through the vaginal epithelium)[27]. Furthermore, the ratio of specific anti-HSVgB IgA to total IgA was, in all cases, higher in vaginal washes as compared to serum (*Table 1*). This demonstrates that the specific IgA present in the vaginal fluids of i.n. immunized mice was higher than in the serum, when examined as a ratio of the total IgA. Although these results suggest local production, the presence of specific anti-HSVgB IgA producing lymphocytes in the genital tract remains to be determined.

DISCUSSION

Previously, we demonstrated that intranasal (i.n.) AdgB8 immunization induced secretory IgA specific for gB of HSV-1 in lung and nasal washes, whereas intraperitoneal (i.p.) immunization did not induce local IgA[24]. Here we extend these results by demonstrating that i.n. AdgB8 immunization induced anti-HSVgB IgG and IgA antibodies in the genital tracts of mice. In contrast, no anti-HSVgB IgA appeared in vaginal washes following i.p. or ivag AdgB8 immunization. These results indicate that i.n. immunization of mice with a recombinant adenovirus is an effective method for inducing specific immune responses at local and distant mucosal surfaces. The genital tract may therefore serve as an effector site for immune responses generated within other tissues that make up the common mucosal immune system[1,16,17]. In support of this, Natuk *et al.* demonstrated anti-HIV antibody responses in the vaginal fluids of chimpanzees following oral or i.n. immunization with adenovirus type 4-, 5-, and 7-vectored vaccines expressing either the HIV *env* or *gag*-protease genes[28]. In particular, intranasal immunization appeared to induce the highest antibody responses. Others have also observed that following i.n. immunization with bacteria–CTB conjugates[29] or HSV-1 subunits[30], specific

Table 1 Comparison between the ratios of vaginal wash and serum antibodies in mice immunized intranasally with AdgB8

Group[a]	Anti-gB IgG/IgA ratios		Total IgG/IgA ratios		Anti-gB IgA/total IgA	
	Wash[b]	Serum[c]	Wash	Serum	Wash	Serum
1	0.23 ± 0.14	16.6 ± 15.3*	0.35 ± 0.43	16.1 ± 8.79***	0.091 ± 0.110	0.019 ± 0.013
2	7.46 ± 8.58	40.8 ± 7.59***	1.18 ± 1.79	6.68 ± 5.30*	0.047 ± 0.024	0.030 ± 0.013*
3	1.60 ± 1.39	45.5 ± 21.9***	0.57 ± 0.51	3.42 ± 1.78***	0.035 ± 0.019	0.011 ± 0.002**

[a]Samples taken 4 weeks post intranasal immunization with 10^6 p.f.u. of AdgB8. Group 1 represents nine mice sampled during estrus, and groups 2 and 3 represent the means of samples from individual mice sampled over two estrous cycles, i.e. 8 days. [b]Expressed as a ratio of titres ± standard deviation. [c]Significant difference between vaginal washes and serums by the paired Student's *t*-test: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.005$

vaginal wash antibodies were present; however, in the latter study no specific-IgA was detected. Furthermore, vaginal wash antibodies appeared following oral administration of sperm[31], bacteria[32], viruses[33], or conjugates of antigen with cholera toxin B subunit (CTB)[8,14]. These studies demonstrate that the murine genital tract can serve as an effector site for mucosal immune responses, and in particular, intranasal immunization with recombinant adenoviruses is an effective vaccination strategy for inducing secretory IgA in the genital tract.

Although humoral immune responses were evident in the genital tract following i.n. immunization with AdgB8, ivag immunization did not induce secretory anti-HSVgB IgA in vaginal washes. Indeed, little to no anti-HSVgB IgA was detected even after four ivag immunizations. In addition, while others have shown increased immune responses in the genital tract following mucosal boosting[8,14,34–36], we did not observe an increase in secretory IgA following ivag boosting of i.n. or i.p. immunized mice. These findings suggest that the murine genital tract is not a good inductive site for the generation of mucosal immune responses following recombinant adenovirus immunization. This may reflect the lack of functional mucosal-associated lymphoid tissue[27] (MALT) able to initiate mucosal immune responses in the vagina, or the inability of recombinant adenoviruses to infect and express in the murine genital tract. Although virus titres were not determined, ivag immunization did induce serum anti-HSVgB IgA and IgG, suggesting that recombinant adenoviruses were able to infect the genital tract and induce systemic immune responses in the absence of secretory IgA. Interestingly, immune responses in the genital tract are present following local administration of various antigens; however, adjuvants or the use of sutures to tie off the uterus were generally involved in these animal models[7,8,27,37]. In contrast to protein antigens, invasive microorganisms may penetrate the epithelium and induce local immune responses[38]. McDermott and colleagues observed IgG in the genital tract following ivag inoculation of mice with an attenuated strain of HSV-2; however, no specific IgA was detected[12]. These studies and our results imply that direct vaccination of the vagina may not be as effective at generating secretory IgA in the genital tract as immunization of other mucosal tissues.

The presence of IgG antibodies can also be important for protection of mucosal surfaces and, in fact, IgG antibodies specific for HSV or HSVgB can protect the genital tract from HSV challenge even in the absence of T-cells[22,23]. Our results demonstrate the presence of vaginal wash anti-HSVgB IgG following all three routes of AdgB8 immunization that persist for at least 128 days. Furthermore, in agreement with our previous results and those of others[24,39] serum IgG antibodies specific for HSVgB were also detected following i.n. and i.p. immunization. Additionally, serum anti-HSVgB IgG was observed following four ivag immunizations and while low compared to either of the other routes, seemed to remain constant. Interestingly, the peak pooled serum levels of specific IgG in i.p. immunized mice occurred on day 21 which corresponded with the peak vaginal wash anti-HSVgB IgG suggesting that the vaginal wash IgG was derived from serum transudation. This is consistent with other studies demonstrating that IgG transudates from the serum into the vaginal lumen[27].

Previous studies in mice have identified that specific IgA in vaginal fluids likely originates from the uterus since hysterectomized mice have only 5% of the vaginal wash IgA observed in controls[9]. Furthermore, secretory component (SC) is present in the uterine epithelium[27] and polymeric but not monomeric IgA appears on the surface of murine mucosal tissues[5]. In addition, IgA plasma cells are found in the body and horns of the uterus[40,41] and can migrate from other mucosal tissues[16], demonstrating that the genital tract is part of common mucosal immune system[1]. These studies suggest that the vaginal wash IgA observed in i.n. AdgB8 immunized mice may be secretory in nature, originating from the uterus due to serum transudation or local antibody producing B cells. In order to examine these issues we compared the antibody ratios in the serum and vaginal washes of i.n. immunized mice. Our results demonstrate that the specific and total IgG to IgA antibody ratios in the vaginal washes were significantly lower than in the serum, indicating that there was more IgA relative to IgG in the lavages than in the serum. This would imply that the majority of both total and specific IgA present in the vaginal washes is secretory and derived from active transport and not serum transudation as in IgG. In order to begin to determine the origin of the specific IgA, we examined the ratio of specific anti-HSVgB IgA to total IgA titres between the serum and vaginal washes. If there was local production of specific IgA due to B cells in the uterine mucosa, one would expect the ratio to be higher in the lavages than in the serum. In our studies we found several times more specific to total IgA in the vaginal washes, indicating that perhaps there was a local B-cell component to the production of the HSVgB-specific IgA in i.n. immunized mice. Interestingly i.p. and ivag immunizations did result in serum but not vaginal wash IgA. This may be due to the induction of monomeric IgA or

alternatively to the lack of HSVgB-specific B cells in the genital tract.

Our work demonstrates that intranasal administration of AdgB8 induces not only antigen-specific systemic antibodies but pronounced mucosal IgA responses in the genital tract. This indicates that mucosal or more specifically i.n. administration of adenovirus vectors expressing immunogenic antigens should serve as excellent vaccine candidates for STDs such as HSV or HIV. Indeed, scientists and policy makers have recently generated criteria for an "ideal" AIDS vaccine[42]. One key property includes a vaccine candidate that will induce local immunity in the genital tract. Additionally, several studies have recently demonstrated that systemic vaccination with glycoprotein D (gD) of HSV results in a reduction in the recurrence of genital HSV lesions[43–45]. These results suggest that mucosal administration of recombinant adenovirus vectors which induce both systemic and mucosal responses may also serve as immunotherapeutic agents for recurrent HSV lesions.

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2. Contributions to Gallichan, W.S., and Rosenthal, K.L. (1995)

(A) Growth and purification of all recombinant adenovirus vectors used in this study.

(B) Immunization of mice and collection of all samples.

(C) Evaluation of the gB-specific antibody levels in sera and vaginal washes by ELISA.

3. Summary

In Gallichan *et al.*, (1995) the evaluation of antibodies specific for gB of HSV were evaluated in the female genital tract following various routes of AdgB8 immunization. From these studies it is clear that distant intranasal immunization successfully induced gB-specific SIgA and serum derived IgG antibodies in vaginal washes. In contrast, intraperitoneal or intravaginal immunization only resulted in serum derived IgG antibodies in the vaginal tract. Boosting by intravaginal immunization with AdgB8 did not appear to influence the levels of gB-specific antibodies in vaginal washes or sera. These results demonstrate that intranasal immunization with a recombinant adenovirus vector is an excellent strategy for inducing both IgG and IgA antibodies in the female genital tract. Furthermore, these results support the view that the induction of SIgA in the female genital tract is dependent on the presentation of antigen to mucosal tissues. However, the genital tract itself is not considered a good inductive site and our results using AdgB8 administration are consistent with this.

These results may have important implications for the design of vaccines or immunotherapeutics for sexually transmitted pathogens since intranasal but not systemic or local intravaginal immunization provides both IgA and IgG antibodies in the female genital

tract. Interestingly, the levels of gB-specific IgA antibodies in vaginal washes were found to vary significantly from day to day as well as between mice. Despite this, over all antibody levels were found to persist for as long as one year following immunization. Finally, since intranasal AdgB8 immunization also provided serum antibodies at levels similar to those following systemic immunization, humoral immunity is not restricted to mucosal tissues following intranasal immunization.

CHAPTER FOUR

PROTECTION AGAINST INTRAVAGINAL HSV-2 INFECTION AND THE INFLUENCE OF THE ESTROUS CYCLE ON THE EXPRESSION OF HUMORAL IMMUNITY IN THE FEMALE GENITAL TRACT FOLLOWING INTRANASAL ADGB8 IMMUNIZATION

Humoral Immunity and Protection Against Herpes Simplex Virus Type 2 Infection in the Genital Tract of Intranasally Immunized Female Mice (Gallichan, W.S., and Rosenthal, K.L., 1996a, Virology, In Press)

1. Background

During the evaluation of vaginal wash antibodies in mice immunized intranasally with AdgB8 we observed that although the average gB-specific antibody titres remained relatively steady within a group of mice, levels in individual mice changed dramatically. As we discussed in the introduction, total antibody levels have been shown to be directly influenced by sex hormones. Therefore, we investigated specific IgG and IgA antibody levels in the vaginal washes of mice immunized intranasally and correlated this to the stage of the estrous cycle in individual mice.

Previous reports have shown that sex hormones also influence the susceptibility of mice

to intravaginal infection with HSV-2. In particular, mice treated with progesterone are readily infected with HSV-2. In this report we examined the influence of the estrous cycle on susceptibility of unimmunized mice to an intravaginal HSV-2 infection. In addition, we addressed the issue of protection against an intravaginal HSV-2 infection in mice immunized intranasally with AdgB8.

ABSTRACT

This study demonstrates that the levels of gB-specific IgG and IgA in vaginal washes of mice immunized intranasally (i.n.) with a recombinant adenovirus vector expressing herpes simplex virus (HSV) glycoprotein B (AdgB8) vary inversely with each other and are dependent on the stage of the estrous cycle. Anti-gB IgA titres in vaginal washes were significantly higher during estrus than diestrus or proestrus, whereas, specific IgG titers were significantly higher during diestrus than estrus. This was further demonstrated in hormone-treated mice, where progesterone administration induced a diestrus-like state that resulted in elevated specific IgG-to-IgA ratios. Interestingly, unimmunized mice were only susceptible to intravaginal (ivag) infection with HSV-2 during diestrus. Mice immunized i.n. with AdgB8 and given progesterone were protected from a lethal intravaginal HSV-2 challenge, despite the fact that virus replication was present for 4 days post challenge. Further, high numbers of gB-specific IgA and IgG antibody secreting cells (ASC) were present in both the genital tracts and draining iliac lymph nodes of i.n. immunized, but not unimmunized, mice 6 days following ivag HSV-2 challenge. These results demonstrate that the levels of specific antibodies in the female genital tract are dependent on the stage of the estrous cycle. Furthermore, i.n. AdgB8 immunization provided a significant level of protection and specific IgA and IgG antibody secreting cells in the genital tissues during resolution of an ivag infection with HSV-2.

INTRODUCTION

Antibodies in the mucosal secretions of the genital tract represent the first specific immune barrier against the penetration of sexually transmitted pathogens (Brandtzaeg et al., 1994; Mestecky, 1987; Mestecky et al., 1994; Mogens and Russell, 1994). Antibodies of both IgG and IgA isotypes are present in genital secretions and both have been shown to be important in this protection (Brunham et al., 1983; Eis-Hubinger et al., 1993; Merriman et al., 1984; Mogens and Russell, 1994; Whaley et al., 1994). Secretory IgA (SIgA) is particularly well suited for mucosal surfaces due to its greater avidity and resistance to proteolytic cleavage (Magnusson and Stjernstrom, 1982; Mogens and Russell, 1994). Although the primary function of IgA has been regarded as the ability to neutralize pathogens at the mucosal surface (Mogens and Russell, 1994), more recently, in vitro (Mazanec et al., 1992, 1995) and in vivo (Burns et al., 1996) models have demonstrated that secretory IgA is capable of forming intracellular complexes with viruses and inhibiting virus replication and subsequently preventing primary or resolving chronic infections.

The presence of secretory IgA at the mucosal surface of the murine genital tract is mainly a result of active transport by secretory component (SC) through the uterine epithelium, whereas IgG found in cervicovaginal secretions is generally due to transudation through the vaginal epithelium (Brandtzaeg et al., 1994; Mestecky, 1987, Mestecky et al., 1994; Parr and Parr, 1990; Parr and Parr, 1994a; Underdown and Mestecky, 1994). The levels of SC and total immunoglobulins in the female genital tract have been reported to change over the course of the estrous cycle (Sullivan and Wira, 1983a; Wira and Sandoe, 1977, 1980; Wira

et al., 1994). In rodents, SC is present at its highest levels in uterine fluids during proestrus and estrus (Sullivan and Wira, 1983a). Since SC is responsible for delivering IgA to the mucosal surface it is not surprising that total IgA levels in rat uterine secretions were significantly higher during estrus and proestrus when compared to diestrus (Wira and Sandoe, 1977,1980; Wira et al., 1994). The levels of total IgG have also been shown to change with the estrous cycle with the highest levels occurring during proestrus (Wira and Sandoe, 1977, 1980; Wira et al., 1994). The migration of lymphocytes into the genital tract is also influenced by the estrous cycle since increased numbers of IgA plasma cells are observed in genital tissues during proestrus and estrus (Canning and Billington, 1983; McDermott et al., 1980; Rachman et al., 1983). These cyclic changes in SC and total antibody levels are also observed in humans (Schumacher, 1980; Sullivan et al., 1984; Suzuki et al., 1984; Usala et al., 1989), suggesting that the hormones that control the reproductive cycle are intimately involved in these changes. Indeed, progesterone and estrogen have been shown to be directly responsible for influencing the levels of SC and antibodies, as well as, the numbers of B cells in the genital tract (Parr and Parr, 1994a; Sullivan et al., 1983b; Wang et al., 1996; Wira and Sandoe, 1977, 1980; Wira et al., 1994).

As mentioned, the induction of specific antibodies in genital secretions is considered necessary for protection against infection with sexually transmitted pathogens such as herpes simplex virus (HSV) or HIV (Brandtzaeg et al., 1994; Mestecky, 1987; Mestecky et al., 1994; Mogens and Russell, 1994). In examining the induction of humoral immunity in the female genital tract, immunization by systemic routes (sc, im, and ip) has been shown to induce

specific antibodies in several species including humans (Bouvet et al., 1994; Gallichan and Rosenthal, 1995; Miller et al., 1992; Nakao et al., 1994; Ogra and Ogra, 1973; Parr and Parr, 1990; Thapar et al., 1990a, 1990b). Although high titres of specific IgG antibodies were induced in these studies, the levels of specific IgA were generally low or not reported. As a mucosal route of immunization, intravaginal (ivag) or intrauterine inoculation has been shown by some groups to induce both specific IgG and IgA antibodies in the genital tract (Milligan and Bernstein, 1995;Ogra and Ogra, 1973; Wira et al., 1994). However, in many other studies the titres were low or consisted mainly of IgG antibodies (Gallichan and Rosenthal, 1995; Lehner et al., 1992; McDermott et al., 1990; Miller et al., 1992; Parr and Parr, 1994a; Parr et al., 1988; Thapar et al., 1990a, 1990b), perhaps attributable to the generalized lack of secondary lymphoid nodules in the genital tract (Parr and Parr, 1994a). In contrast, immunization at other mucosal sites (i.g. or i.n.) has been successful in generating specific IgG and IgA antibodies in the genital tract (Gallichan and Rosenthal, 1995; Lubeck et al., 1994; Muster et al., 1995; Wu and Russell, 1993). Few of the studies concerned with specific antibodies in the genital tract took into account the stage of the reproductive cycle during sampling and evaluation of antibody titres.

Herpes simplex virus type-2 is a sexually transmitted agent that attaches, penetrates and undergoes infectious cycles of replication in the epithelium of the genital tract. Most studies concerned with protection against genital HSV-2 infection have focused on systemic immunization (Bernstein et al., 1990; Burke et al., 1994; Byars et al., 1994; Heineman et al., 1995; Nakao et al., 1994; Straus et al., 1994), despite the evidence supporting mucosal

immunization as the optimum route for the induction of both IgA and IgG antibodies in the genital tract. The exception to this involves intravaginal (ivag) immunization with attenuated strains of HSV-2 that protected mice against ivag HSV-2 challenge (McDermott et al., 1987; Mclean et al., 1994; Milligan and Bernstein, 1995; Parr et al., 1994b). Recently we showed that intranasal (i.n.) immunization with a recombinant adenovirus capable of expressing gB of HSV-1 (AdgB8) induced both gB-specific IgA and IgG in vaginal washes of mice (Gallichan and Rosenthal, 1995). In this study we examined the influence of the estrous cycle on the levels of HSV-gB specific IgA and IgG antibodies in the female genital tract following i.n. immunization with AdgB8 (Gallichan and Rosenthal, 1995). In addition, mice immunized intranasally with AdgB8 were challenged intravaginally with HSV-2 and the subsequent pathology, viral replication, and B cell responses in the genital tissues were evaluated.

MATERIALS AND METHODS

Animals and cell cultures. Female C57Bl/6 (H-2^b) mice used in immunization and challenge studies and in the evaluation of vaginal wash antibody levels were 6 to 8 weeks of age during primary immunization and were obtained from Charles River Laboratories, Constant, Quebec, Canada. Mouse colonies were maintained on a 12 hour light/dark cycle. Vero and 293 cells were grown in α-MEM (GIBCO Laboratories, Burlington, Canada), supplemented with 10% fetal calf serum (FCS; GIBCO), and 1% penicillin-streptomycin and L-glutamine (GIBCO). 293-N2S cells are a non-adherent cell line derived from 293 cells and were grown in spinner flasks with Joklik's media supplemented as above.

Virus strains and inoculations. The construction of the replication-competent recombinant adenovirus type 5 vector, AdgB8 was reported elsewhere (Hutchinson et al., 1993). Briefly, AdgB8 contains the gB gene from HSV-1 coupled to the SV40 promoter and inserted into the E3 region of human adenovirus type 5. Recombinant adenoviruses were grown in 293-N2S cells, purified twice on CsCl gradients, and titered on 293 cells. HSV type 2 (HSV-2) strain 333 was propagated and titered on Vero cells.

For AdgB8 immunization, mice were ether anesthetized, inverted, and inoculated intranasally (i.n.) with 10^8 p.f.u. of AdgB8 by introducing virus in 10µl of phosphate buffered saline (PBS, pH 7.4) directly into the nares by means of a micropipet. For intravaginal (ivag) HSV-2 challenge, mice were first injected subcutaneously with 2 mg of progesterone/mouse (Depo-Provera, Upjohn, Don Mills, Ontario), five days later mice were anesthetized using halothane, swabbed intravaginally (ivag) with a cotton applicator, placed on their backs and

infected intravaginally for 1 hour with 10µl of HSV-2 while being maintained under anesthetic.

Collection of fluids and estrus staging. Vaginal fluid for estrous staging and antibody determinations was collected by pipetting 30µl of PBS into and out of the vagina several times. The staging of the estrous cycle for each mouse was based on a smear from these washings (Allen, 1922). Smears were stained with Diff-Quik (Baxter Scientific Products, Miami, FL). By examining the cells present in the smears we were able to determine whether the animal was in estrus (E), metestrus (M), diestrus (D), or proestrus (P). The vaginal washings were then centrifuged to remove particulate matter and the supernatants were stored at -20°C for subsequent antibody determination.

Viral replication and pathology in the reproductive tract. Following ivag HSV-2 inoculation, mice were sampled daily for 6 days by pipetting 30µl of PBS into and out of the vagina followed by a swabbing with a cotton applicator. Both the wash and applicator were combined with 0.97ml of PBS and frozen at -70°C. Viral titres were determined by plaque assay on Vero cell monolayers. The dilution of each vaginal wash supernatant was considered to be 10^{-2}.

Genital pathology was monitored daily following HSV-2 challenge and scoring was performed blinded. Pathology was scored on a 5 point scale; 0, no apparent infection; 1, slight redness of external vagina; 2, redness and swelling of external vagina; 3, severe redness and swelling of external vaginal and surrounding tissue; 4, genital ulceration with severe redness, swelling and hair loss of genital and surrounding tissue; 5, severe genital ulceration extending

to surrounding tissue. Mice were sacrificed upon reaching stage 5.

Antibody Determination. Total and HSV-gB-specific antibody titres were determined by ELISAs performed in flat-bottomed microtiter plates (Costar, Cambridge, MA). Plates were precoated with 2.5µg/ml of recombinant HSV-2 gB (kindly provided by R.L. Burke, Chiron, Emeryville, CA) for gB-specific antibody titres and 1/1000 dilution of goat anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL) for total antibody titres, in borate-buffered saline (BBS), pH 8.5, and kept overnight at 4°C. A TRIS-buffered saline (TBS) solution containing 10mg/ml bovine serum albumin, pH 7.4, was used to block any plastic not precoated with antibody or gB protein. Serially diluted samples of either hyperimmune control, test sera, or sample supernatants were added, followed by either biotin-labelled goat anti-mouse IgG or IgA antibody (Southern Biotechnology Associates, Birmingham, AL). The labelling reagent was alkaline phosphate (ExtrAvidin; Sigma, St. Louis) and the ELISA Amplification System (EAS) (GIBCO, Burlington, Ont.) was used as a substrate. Antibody titres represent the inverse dilution of the sample at which 2 times the background absorbance of control or lavage fluid from uninfected mice was reached. Antibody levels were examined individually, as ratios of IgG divided by IgA, or as grouped percents of maximum. Grouped percents of maximum were determined by first converting the titres of anti-gB IgG and IgA into a percent of maximum by dividing the titre determined for each day by the maximum that we observed over the course of the two cycles for individual mice. In this way we were able to pool the percent of maximums for each estrus stage from among several mice.

Lymphocyte isolation. Single cell suspensions from lymph nodes were prepared by smashing on a grid etched on plastic plates with an 18-gauge needle. To obtain lymphocytes from the genital tract, the vagina, cervix, and uterine horns were removed from groups of 5 mice, minced with scissors, and digested with an enzyme solution containing collagenase (0.5 U/ml, Boehringer Mannheim), Dispase II (1.2 U/ml, Boehringer Mannheim), and DNAse (5 U/ml, Calbiochem). Two successive digests were performed for 1 hour at 37°C. Lymphocytes were enriched by passing the cell fractions over Ficoll gradients and then culturing for 2 hr to remove adherent epithelial and fibroblast cells.

Antibody secreting cell (ASC) enumeration by ELISPOT assay. Ninety-six-well filtration plates backed with nitrocellulose membrane (Millipore Corp., Bedford, MA) were coated with 10 μg/ml of recombinant HSV-2 gB (provided by R.L. Burke, Chiron, Emeryville, CA) or 1/500 dilution of goat anti-mouse IgG or IgA (Southern Biotechnology Associates, Birmingham, AL) in phosphate-buffered saline (PBS), and kept overnight at 4°C. A PBS solution containing 10mg/ml bovine serum albumin pH 7.4, was used to block any nitrocellulose not precoated with antibody or gB protein. Serially diluted single cell suspensions of iliac lymph nodes or genital tract digests plus supplemented RPMI media (10% FCS) were plated at 37°C for 16 hours. The number of plasma cells in each preparation secreting total or HSVgB-specific IgA, or IgG antibody was determined by washing each well with PBS-Tween 20 to remove cells and developing the plate by the addition of biotinylated goat anti-mouse IgA or anti-IgG (Southern Biotechnology Associates, Birmingham, AL), followed by avidin-peroxidase. Spots representing individual antibody secreting cells were

visualized by developing with peroxidase substrate containing H_2O_2 and 3-amino-9 ethylcarboazole in acetate buffer. Spots were enumerated by digitized image analysis and discrimination from background was based on gray density and then size (program written by Dr. L. Arsenault, Microscopy Group, McMaster University, Hamilton, Ont.). Counts were visually confirmed in case of overlapping spots. Results are expressed as the mean number of antibody-secreting cells (ASC) per million mononuclear cells for each tissue.

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 or Fisher's exact test as appropriate. Comparison among the means of multiple groups was carried out using analysis of variance (ANOVA).

RESULTS

Variation in specific vaginal wash antibody levels during the estrous cycle. In our previous studies we observed the presence of specific antibodies to gB of HSV-1 in the vaginal washes of mice immunized i.n. with AdgB8 (Gallichan and Rosenthal, 1995). The levels of these antibodies tended to vary greatly between mice and within individual mice over time. Since immunoglobulin levels are influenced by the estrous cycle, we determined the titres of HSVgB-specific IgG and IgA in vaginal washes on a daily basis and correlated them with the stage of the estrous cycle. Figure 1A shows data from a representative mouse immunized six weeks previously with AdgB8 and sampled daily over 2 estrous cycles. The absolute titres of gB-specific antibodies in this mouse demonstrated that, indeed, there was a large variation from day-to-day. Moreover, there appeared to be a cyclic fluctuation in the titres of gB-specific IgG and IgA that varied with the stage of the estrous cycle. Anti-gB IgA titres were generally highest during estrus and conversely, anti-gB IgG titres tended to be lowest during estrus and higher during other periods of the cycle. To examine these fluctuations in a group of mice, titres from the vaginal washes of 4 mice immunized i.n. with AdgB8 were pooled over two estrous cycles and expressed as percent of maximums to accommodate the varying magnitudes of specific antibodies observed. In Figure 2A it is clear that the levels of anti-gB IgA and IgG fluctuated with the estrous cycle. In fact, the levels of anti-gB IgA during estrus were significantly higher than those observed during diestrus or proestrus ($p \leq 0.05$). In contrast, the levels of anti-gB IgG observed during diestrus were significantly higher than during estrus ($p \leq 0.001$) (Fig. 2A). This inverse relationship between

isotype expression indicates that over the course of the estrous cycle specific IgA levels are highest during periods of estrus and lowest during diestrus, whereas specific IgG levels are highest during periods of diestrus and lowest during estrus. The levels of specific IgA and IgG during metestrus and proestrus lie in between.

Ratios of specific IgG-to-IgA antibodies in vaginal washes. The relationship between the levels of specific IgG relative to IgA was examined at each stage of the estrous cycle by determining the ratios of gB-specific IgG-to-IgA. Figure 1B demonstrates that the gB-specific IgG-to-IgA ratios from the vaginal washes of the mouse in Figure 1A followed a cyclic pattern and were highest during diestrus and lowest during estrus. In addition, there was as much as a 93-fold difference in ratios between estrus and diestrus. These cyclic trends were similar in all mice that we examined. To examine a group of mice, the ratios of anti-gB IgG-to-IgA from 4 mice were pooled (over two cycles each) from each of the 4 estrous stages by first normalizing the ratios in each mouse to a scale of 1.0. The ratios in figure 2B demonstrate that the relative levels of gB-specific IgG compared to IgA were significantly higher during diestrus when compared to all other stages ($p \leq 0.001$). In addition, the ratio at diestrus was 15 times greater than during estrus, and conversely, during estrus the levels of specific IgA were relatively much higher than IgG. The inverse relationship between levels of gB-specific IgG and IgA at each stage of estrous was also observed for total antibodies in vaginal washes (data not shown).

Influence of the estrous cycle on susceptibility to HSV-2 infection. To examine the influence of the estrous cycle on susceptibility to ivag HSV-2 infection, unimmunized mice

were infected at various estrous stages and monitored for virus replication by examining vaginal fluids daily and tissues on day 6 after infection. Table 1 demonstrates that over the 6 days examined, virus replication was only observed in the vaginal washes of mice infected during diestrus or late metestrus. There was no virus replication observed in mice infected at estrus. The results presented in Table 1 are representative of several experiments examining a number of animals at each stage. Interestingly, the dose of HSV-2 used for infection represents more than a 1000-fold lethal dose (unpublished data). Thus, mice appeared to be highly resistant to intravaginal HSV-2 infection at estrus.The stages of the estrous cycle occurring on days subsequent to infection did not seem to influence the amount of virus recovered in vaginal washes (data not shown). By day 6 the mice that were infected during diestrus or late metestrus had developed moderate to severe pathology of the exterior genital tissues. In contrast, mice infected during estrus displayed no genital pathology.

To examine the site and extent of tissues infected, the uterus, vagina (including the cervix), and nerves innervating the genital tissues were isolated. Only the tissues from mice infected during diestrus or late metestrus contained virus (Table 1). The level of infection in vaginal tissues was several fold higher than in the uterus of all mice we examined indicating that the primary site of HSV-2 infection following intravaginal inoculation is in the vagina. Virus was also observed in the nerves of severely infected animals. These results indicate that mice are primarily susceptible to HSV-2 infection during early to late periods of diestrus and that the primary site of infection is in the vagina, with further infection of the uterus and ultimately the nerves occurring in severely infected animals.

Influence of progesterone on specific antibody ratios in vaginal washes. Administration of progesterone induces mice into a diestrus-like state and is known to make mice highly susceptible to intravaginal infection with HSV (Parr et al., 1994b). To determine the influence of this treatment on specific antibody levels in the genital tract we examined the ratios of vaginal wash anti-gB IgG-to-IgA in 6 mice that were immunized intranasally with AdgB8. By day 3 the ratios had increased dramatically and were significantly greater than the preceding days ($p<0.05$) (Fig. 3). In addition, by day 3 post hormone treatment all 6 mice had entered a diestrus-like state which was maintained for more than a week. During this diestrus-like state the titres of gB-specific IgA antibodies were extremely low or undetectable.

Intranasal immunization protects against intravaginal HSV-2 infection. Next, we determined whether mice immunized intranasally with AdgB8 were protected from intravaginal (ivag) HSV-2 infection during a progesterone-induced, diestrus-like state. Five days following progesterone treatment control (unimmunized) and immunized mice were inoculated ivag with a lethal dose of HSV-2 (Table 2). Subsequent daily evaluation of genital pathology, survival and virus replication in vaginal washes of control and immunized animals was determined in a blinded fashion. All of the control animals were infected with virus and demonstrated genital pathology by day 4, eventually succumbing to infection on day 6 (having reached a genital pathology score of 5). In contrast, mice immunized i.n. with AdgB8 demonstrated a significant level of protection against HSV-2 infection. Although virus replication was observed in the genital tracts of all the immunized mice for the first 4 days post infection, by day 5, virus was isolated from only 8 of the immunized mice ($p\leq0.01$). In

addition, by day 5 there was a significant decrease in viral replication in the 6 remaining immunized mice ($p<0.0001$). The severity of infection or genital pathology was also significantly reduced in the i.n. immunized mice over the 6 days ($p<0.0001$). Of the 14 AdgB8 immunized mice, 5 were completely protected from any discernable genital pathology. Interestingly, these 5 mice were among the mice that had cleared any signs of viral replication by day 5. The remaining 9 mice all developed genital pathology, however, 4 of these mice displayed only minor symptoms (pathology score ≤ 3), and subsequently cleared the infection by day 7 and any signs of pathology by day 12. Therefore, a significant number of immunized mice (9 of the 14) survived past acute infection ($p<0.005$).

Detection of specific antibody secreting cells in the genital tract. The presence of antibody secreting cells (ASC) specific for gB of HSV-2 was examined in the genital tracts and iliac lymph nodes (ILN) of immunized and control mice following an ivag HSV-2 challenge. High numbers of gB-specific IgA and IgG ASC were present in both the genital tracts and the draining iliac lymph node (ILN) of i.n. immunized mice 6 days following ivag HSV-2 challenge (Table 3). The gB-specific ASC observed in the ILN represented 65 and 35 percent of the total IgA and IgG ASC present, respectively. In the genital tracts they represented 8 and 92 percent of the total IgA and IgG ASC present, respectively. This is in contrast to control animals in which no gB-specific ASC were observed in the genital tissues and only a small number of IgG ASC were observed in the ILN. The IgG ASC present in the ILN of control animals likely represents the development of a primary response and consisted of only 2 percent of the total IgG ASC present (and was 19 times fewer than that observed

in the ILN of immunized mice). These results were confirmed in 2 separate experiments.

DISCUSSION

The capability of the female genital tract to maintain a high level of sterility and at the same time provide an environment suitable for conception requires the coordination of a large number of specific and nonspecific factors. The main specific factors that contribute to protection in the genital tract include the effector functions of humoral and cell mediated immunity (Brandtzaeg et al., 1994; Mestecky et al., 1994; Parr and Parr, 1994a; Tristram and Ogra, 1994; Underdown and Mestecky, 1994). Indeed, both arms of the immune system have been shown to be important for resistance to various pathogens that infect the female genital tract (Brunham et al., 1983; Eis-Hubinger et al., 1993; McDermott et al., 1987, 1989, 1990; Merriman et al., 1984; Parr and Parr, 1994a; Whaley et al., 1994). Arguably, humoral immunity or antibodies are the first specific barrier encountered by sexually transmitted pathogens (Brandtzaeg et al., 1994; Mestecky, 1987; Mestecky et al., 1994; Underdown and Mestecky, 1994). Both IgA and IgG are present in secretions from the murine genital tract and anatomically, IgA originates mainly from the uterus/endocervix and IgG from the vagina (Mestecky, 1987; Parr and Parr, 1990, 1994a; Wira et al., 1994). In our previous investigations, we demonstrated that i.n immunization with a recombinant adenovirus vector capable of expressing gB of HSV-1 (AdgB8) induced both IgA and IgG specific for gB in the genital tract (Gallichan and Rosenthal, 1995). The current study demonstrates that the levels of specific IgG and IgA antibodies in the genital tract of intranasally immunized mice vary inversely with each other and are dependent on the stage of the estrous cycle. Indeed, specific IgA titres were higher during estrus than diestrus and specific IgG titres were higher during

diestrus than estrus. This was further demonstrated in hormone-treated mice where progesterone administration induced a diestrus-like state that resulted in elevated specific IgG-to-IgA ratios. In fact, while specific IgG titres reached their highest levels, specific IgA titres were extremely low to undetectable. The fluctuations in levels of specific antibodies that we detected are similar to those reported for total (Parr, 1994; Sullivan et al., 1984; Wang et al., 1996; Wira et al., 1994) and specific (Wira and Sandoe, 1980) antibodies during the various stages of the estrous cycle or under the influence of sex hormones .

This inverse relationship in specific antibody levels likely reflects the changes that occur in the female reproductive tract during the course of the estrous cycle. During estrus, or the time of mating, the female genital tract is subjected to numerous pathogens (Parr and Parr, 1994a; Profet, 1993; Tristram and Ogra, 1994). In fact, sperm has been shown to be a vector for bacteria, whereby the bacteria attaches to the tails of the sperm as it moves up the reproductive tract (Profet, 1993). In terms of protection at mucosal surfaces secretory IgA is an important component (Brandtzaeg et al., 1994; Magnusson and Stjernstrom, 1982; Mestecky, 1987; Mestecky et al., 1994; Mogens and Russell, 1994; Underdown and Mestecky, 1994) and therefore, it follows that there be high levels of IgA present during this period to deal with the increased pathogen load associated with mating. Indeed, antibodies can be found in the uterine lumen bound to bacteria shortly after mating (Parr and Parr, 1985). There may be two hormonally controlled factors that contribute to increased IgA levels during estrus. Firstly, there is an increased migration of plasma cells to the genital tract resulting in a greater number of IgA plasma cells during proestrus and estrus (McDermott et

al., 1980; Rachman et al., 1983). Secondly, there is an increase in production of secretory component (SC) in the uterine epithelium that fluctuates with the estrous cycle and is associated with estradiol administration (Sullivan et al., 1983b; Wira et al., 1994). Interestingly, exogenous estradiol and progesterone influence the levels of total IgA or IgG in the genital tract as well as both of the above phenomena, suggesting that the increased IgA levels observed during estrus are indirectly a result of the fluctuating hormone levels that occur over the course of the estrous cycle (Wang et al., 1996; Wira et al., 1994; Wira and Sandoe, 1980).

The factors contributing to the relative decrease in IgG during estrus are likely due in part to architecture changes in the epithelium of the vagina. IgG originates mainly from serum transudation through the vagina and the vaginal epithelium is known to undergo changes from a thin (3 to 7 layers) stratified epithelium at diestrus to a thick (12 to 13 layers) keratinized epithelium with a clearly defined basement membrane at estrus (Allen, 1922; Parr and Parr, 1994a). These changes are also influenced by hormones with estrogen-dominated and progesterone-dominated vaginal epithelium being similar to that of estrus and diestrus, respectively (Parr et al., 1994b). The physical structure of the vaginal epithelium, which is thinner during diestrus, also explains the increase in specific IgG that we observed during this stage and which is likely due to increased serum transudation. In fact, proteins can penetrate the vaginal epithelium during diestrus and IgG has been localized in the intercellular spaces during this period (Parr and Parr, 1994a). It would thus appear that the results presented here reflect the ability of the genital tract to compensate for the decrease in protective IgG during

estrus by actively recruiting IgA plasma cells and transporting IgA via SC into the uterine lumen.

The physical changes that occur in the vagina during the transition from diestrus to estrus are likely in preparation for mating and the increased pathogen load associated with this activity. Our data clearly shows that unimmunized mice were susceptible to intravaginal HSV-2 infection during diestrus but not during estrus. Indeed, even at 1000-fold lethal dose mice were resistant to ivag infection during estrus. Thus our findings confirm those originally made by Tepee et al. (1990) and Parr et al. (1994b). Following infection at diestrus, the stage of the estrous cycle did not affect the level of virus recovered in vaginal washes. In other words, once infection was established subsequent stages of the estrous cycle did not appear to influence virus replication. The difference in thickness and permeability of the vaginal epithelium, as well as availability of viral receptors, during these periods may be responsible (Allen, 1922; Parr and Parr, 1994a).

To examine immunity in the genital tract within the environment of increased viral susceptibility (but decreased IgA levels) animals were induced into a diestrus-like state by progesterone treatment prior to intravaginal HSV-2 challenge. Our results demonstrate that while none of the control animals survived infection, a significant number of mice immunized intranasally with AdgB8 did survive (Table 2). Subsequent studies showed that intranasally immunized mice infected with a lower but still lethal dose of virus were completely protected from mortality as well as genital pathology (Gallichan and Rosenthal, manuscript in preparation). Of interest was the fact that although gB-specific antibodies (mainly IgG) were

present at the time of infection in immunized animals, sterile immunity was not observed and the viral replication that occurred over the first 4 days post infection was similar to that occurring in control mice. In addition, while some immunized mice developed genital pathology, several of these completely cleared the primary infection and lacked any genital morbidity by day 12. These results suggest that the induction of specific anamnestic responses and not neutralization by mucosal IgG antibodies present during initial infection were responsible for subsequent protection. In examining the immune mechanisms present during the period of viral clearance, we observed that the draining iliac lymph nodes (ILN) and the genital tissues of immunized but not control mice contained a large percentage of antibody secreting cells (ASC) specific for gB of HSV-2. Both IgG and IgA secreting plasma cells were present demonstrating that intranasal immunization induced memory lymphocytes of both isotypes that were capable of responding to infection and entering the genital tract. Interestingly, the gB-specific ASC in both the ILN and genital tissues represented a large percentage of the total ASC present, however, the gB-specific IgA ASC in the genital tissues were not as highly represented (Table 3). This may be due to the progesterone dominated environment since it has been observed that the circulation of IgA plasma cells into the genital tissues decreases during diestrus (McDermott et al., 1980). Nevertheless, gB-specific IgA ASC were present in the genital tissues during the resolution phase of infection and were more numerous than the HSV-2 specific ASC reported in similar experiments where mice were protected from a lethal HSV-2 challenge following intravaginal immunization with an attenuated HSV-2 virus (Milligan and Bernstein, 1995). Interestingly, Eis-Hubinger et al.

(1993) demonstrated that administration of gB specific monoclonal antibodies resulted in the rapid clearance of virus from the genital tracts of mice that had an established HSV-1 infection. Moreover, recent studies have revealed that in addition to neutralization at the mucosal surface, secretory IgA is capable of resolving established viral infections in mucosal tissues through intracellular neutralization of virus. Therefore, although we have not demonstrated that specific antibodies were responsible for protection, our results clearly demonstrate that intranasal immunization with AdgB8 provided a memory B cell response in the genital tract consisting of both IgA and IgG specific antibody secreting cells during the same period in which viral clearance was observed in protected animals. It is important to note that we recently observed that mice immunized intranasally with AdgB8 and challenged ivag with HSV-2 contained strong CTL recall responses in their ILNs (Gallichan and Rosenthal, manuscript in preparation). Further, in light of the work by McDermott et al. (1989) which demonstrated that HSV-2 specific T cells from the ILNs protected mice from ivag HSV-2 challenge, it is unclear whether cellular or humoral immunity or both are responsible for protection in our model.

The changes in specific antibody levels and susceptibility to HSV-2 infection that occur over the course of the normal estrous cycle have important implications for the development and analysis of mucosal vaccines designed against sexually transmitted pathogens. In evaluating humoral immunity, isotype expression and the stage of the estrous or menstrual cycle should be taken into account. Indeed, there is evidence in humans that a similar dependence exists between the presence of each isotype and the menstrual cycle

(Schumacher, 1980; Sullivan et al., 1984; Suzuki et al., 1984; Usala et al., 1989). Furthermore, in light of our observations it is clear that the induction of specific antibodies of both IgG and IgA isotypes in mucosal secretions is a requirement that vaccines will have to meet in order to maintain a blanket of humoral immunity in the female genital tract over the course of the reproductive cycle, thus increasing the likelihood of sterile immunity. Alternatively, despite the narrow window of susceptibility of mice to ivag HSV-2 infection during diestrus, the period dominated by IgG, mice immunized intranasally displayed a rapid recall response of both IgG and IgA antibody secreting cells and were protected. Finally, these results demonstrate for the first time that intranasal immunization provides a significant level of protection in the genital tract against an intravaginal HSV-2 infection and a B cell memory response capable of providing both IgA and IgG ASC in the genital tissues during resolution of a primary HSV-2 infection.

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FIG. 1. The effect of the estrous cycle on gB-specific antibody levels in the vaginal washes of a mouse immunized intranasally with AdgB8. The stage of the estrous cycle was determined daily by cytology from a smear of the vaginal washes of a representative mouse over a 16 day period: Estrus (E); Metestrus (M); Diestrus (D); Proestrus (P). (A) The titres of HSVgB-specific IgA (hatched bars) and IgG (solid bars) were determined daily using a gB-specific ELISA. (B) The ratios of HSVgB-specific IgG-to-IgA over the 16 days for the same mouse in Fig. 1A.

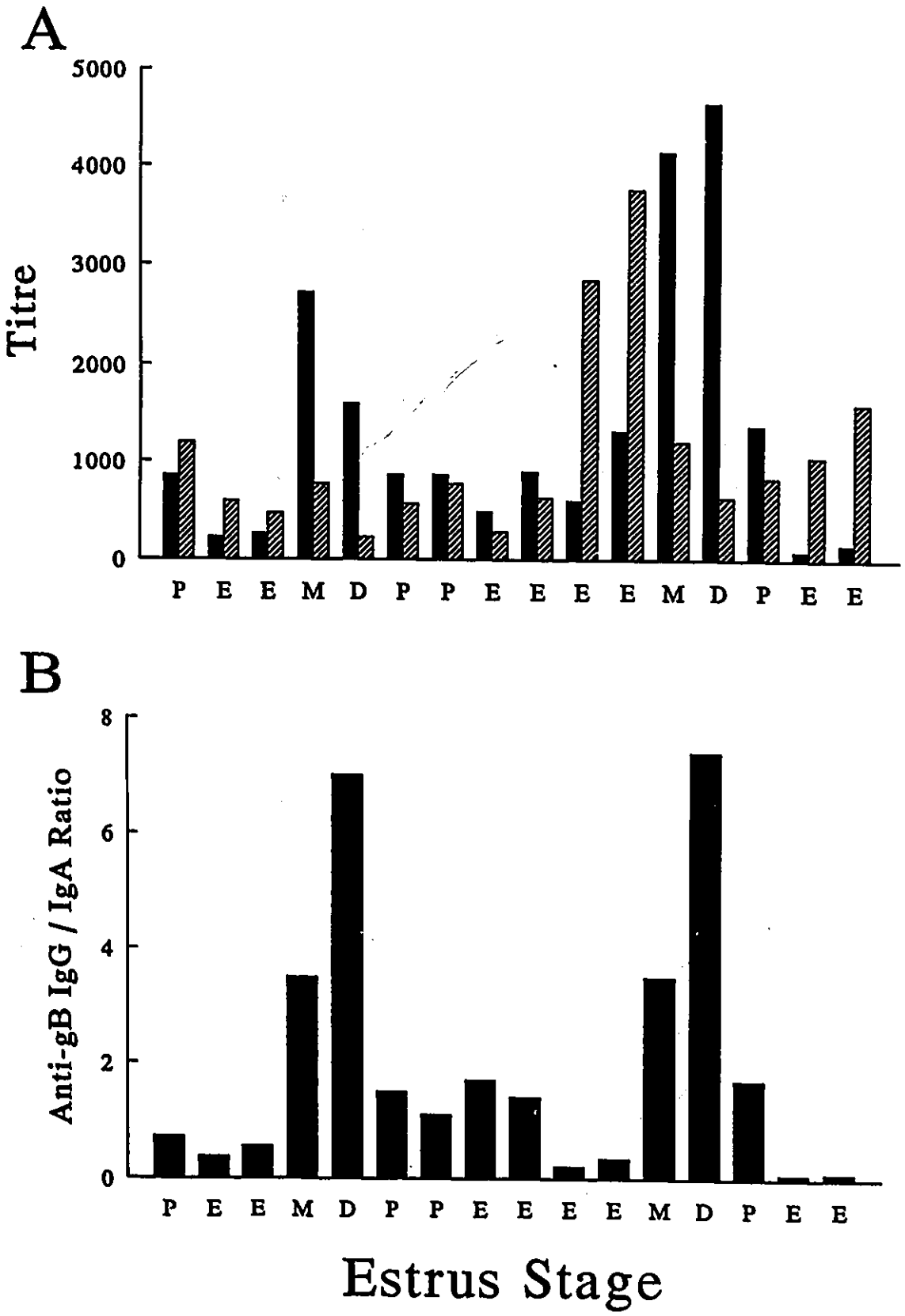
A
Titre
5000
4000
3000
2000
1000
0
P E E M D P P E E E E M D P E E
B
Anti-gB IgG / IgA Ratio
8
6
4
2
0
P E E M D P P E E E E M D P E E
Estrus Stage

FIG. 2. The relative changes in total HSVgB-specific IgG and IgA antibody levels during the estrous cycle in a group of mice immunized intranasally with AdgB8. The stages of the estrous cycle were determined daily by cytology from a smear of the vaginal washes of a group of 4 mice each examined daily over 2 estrous cycles. Estrus (E), n=16; Metestrus (M), n=9; Diestrus (D), n=8; Proestrus (P), n=5. (A) The titres of gB-specific IgA (hatched bars) and IgG (solid bars) in each mouse were expressed as a percentage of maximum observed over the two cycles and then grouped between and within mice according to the stage of the estrous cycle. HSVgB-specific IgG during diestrus was significantly greater than during estrus, $p \leq 0.001$; HSVgB-specific IgA during estrus was significantly greater than during diestrus or proestrus, $p \leq 0.05$. (B) The ratios of HSVgB-specific IgG-to-IgA for each day over two estrous cycles for each of the 4 mice in Fig. 2A were normalized to a scale of 1 and then grouped together. Anti-gB IgG-to-IgA during diestrus was significantly greater than during the other three stages, $p \leq 0.001$.

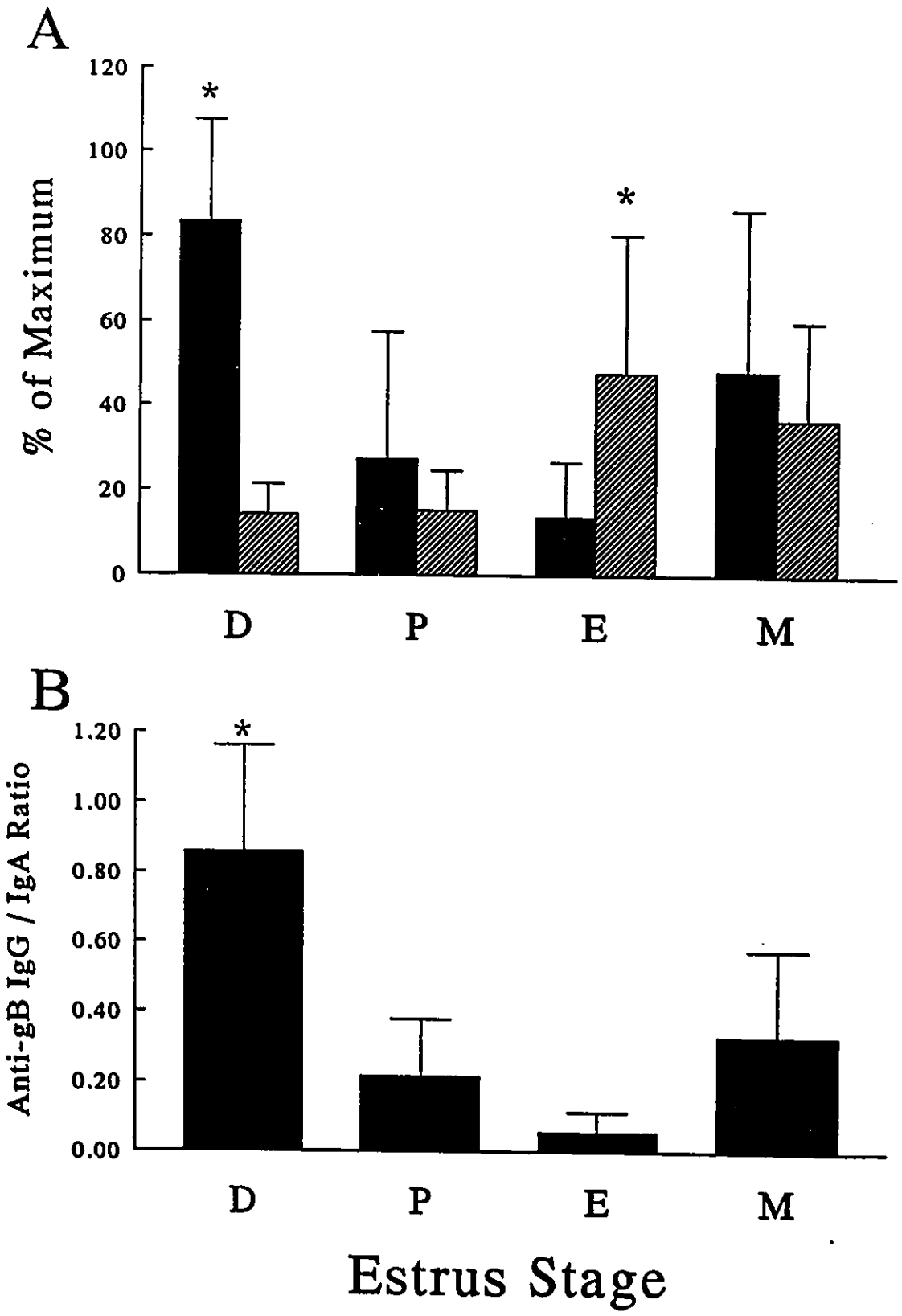
A
120
100
80
60
40
20
0
% of Maximum
*
*
D
P
E
M
B
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Anti-gB IgG / IgA Ratio
*
D
P
E
M
Estrus Stage

FIG. 3. The effect of progesterone treatment on the ratios of HSVgB-specific IgG-to-IgA in the vaginal washes of mice immunized intranasally with AdgB8. The vaginal washes of a group of 6 mice immunized 3 weeks previously were sampled daily following s.c. injection of progesterone and the stage of the estrous cycle was determined by cytology. The ratio of HSVgB-specific IgG to IgA was determined for each vaginal wash and then normalized to a scale of 1 for each mouse prior to grouping. Day 3 was significantly greater than day 0, $p \leq 0.05$ (*).

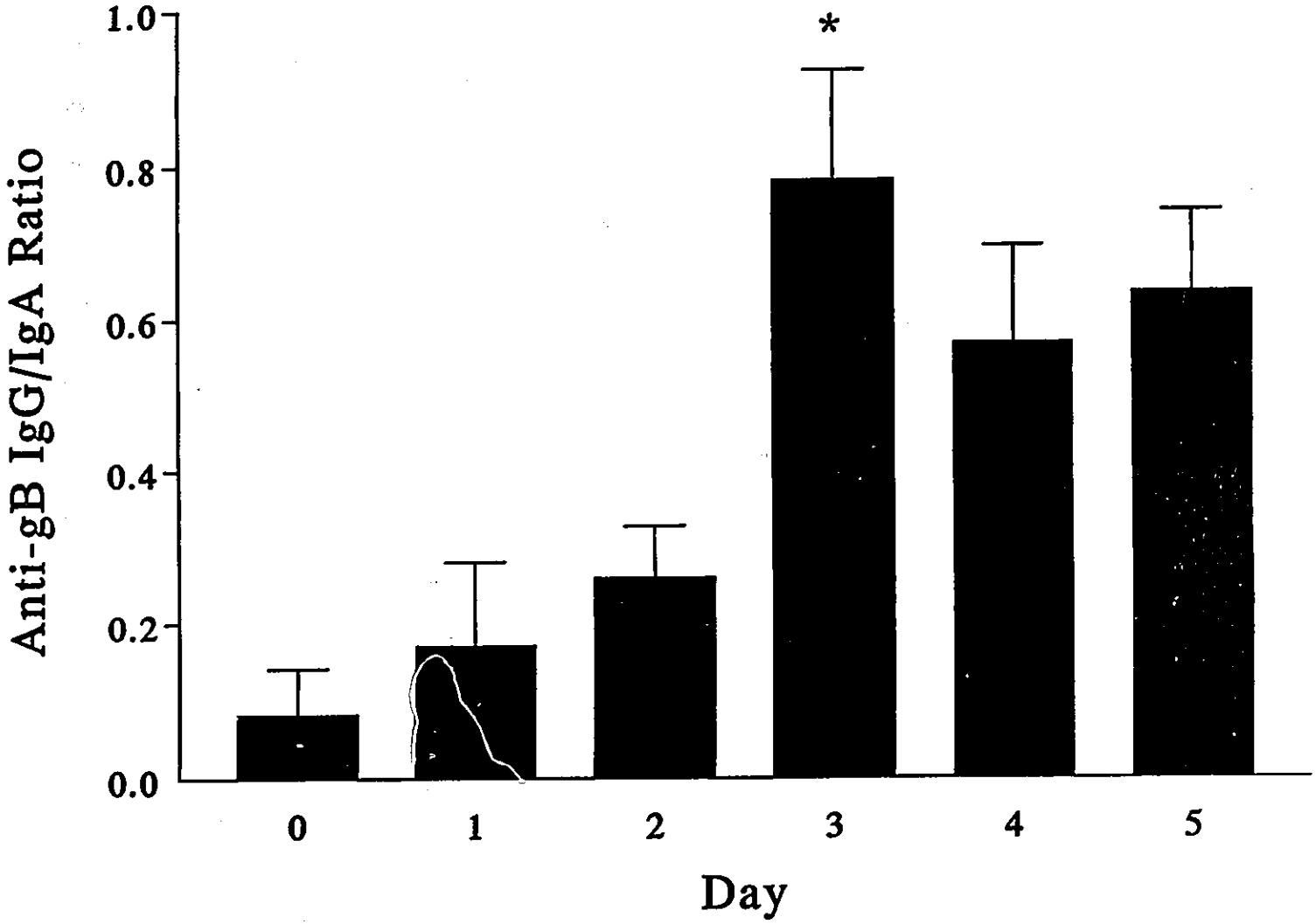
Anti-gB IgG/IgA Ratio
1.0
0.8
0.6
0.4
0.2
0.0
*
0
1
2
3
4
5
Day

TABLE 1

Effects Of The Estrous Cycle On HSV-2 Replication In The Gental Tract Following Intravaginal Infection.

Estrous Stage and Vaginal Wash HSV-2 Titre (10^2 pfu/ml)[b]							HSV-2 Titre (10^2 pfu/tissue)[c]		
Estrous Stage[a]	Day 1		Day 3		Day 5		Vagina	Uterus	Nerves
	Stage	Titre	Stage	Titre	Stage	Titre			
E	M	0	M	0	P	0	<0.05	<0.05	<0.05
M	D	1	D	2	D	110	310	0.3	<0.05
D	D	11	D	740	P	410	2300	32	5.0
P	E	1	E	0	M	0	<0.05	<0.05	<0.05

[a] Estrous staging was determined histologically; Estrus (E); Metestrus (M); Diestrus (D); Proestrus (P).

[b] Mice were inoculated intravaginally with 2×10^7 p.f.u. HSV-2 and vaginal washes were taken daily for determination of virus titres and the estrus stage.

[c] On the sixth day following infection tissues were isolated, homogenized, and viral titres determined.

TABLE 2

Protection and Virus Replication in the Genital Tracts of Mice Immunized Intranasally With AdgB8 and Challenged Intravaginally With HSV-2

Group[a]	Virus Isolation[b]		Vaginal Viral Replication[c]		Severity[d]	Protection[e]	Survival[f]
	days 1-4	day 5	day 3	day 5			
Unimmunized	14/14	14/14	3.6 (.50)	3.6 (.53)	8.9 (2.2)	0/14	0/14
Immunized	14/14	8/14***	3.5 (.57)	2.4 (.35)*	3.4 (3.1)*	5/14****	9/14**

[a] Mice were intravaginally challenged with 2×10^4 pfu of HSV-2; immunized mice were innoculated with AdgB8 3 weeks previously.
[b] Number of mice in which virus was detected/total.
[c] Mean ±SD $\log_{10}$ pfu; Calculated using only animals that experienced viral replication.
[d] Mean ±SD ; measured as area under the lesion score-day curve for first 6 days.
[e] Number of mice with that demonstrated no overt genital pathology/total (ie. score = 0).
[f] Number of mice surviving infection/total.
Significance between AdgB8 immunized and unimmuned mice: * $p<0.0001$; ** $p<0.005$; *** $p<0.01$; **** $p<0.025$.

TABLE 3

HSVgB-Specific Antibody Secreting Cells in Genital Tissues of Intranasally Immunized Mice 6 Days Following Intravaginal HSV-2 Challenge

	Mean HSVgB-specific ASC / million mononuclear cells (SD)[b]			
Immunization[a]	IgA	% of Total	IgG	% of Total
ILN				
i.n. AdgB	66.8 ± 6.3	65	1810 ± 151	34.5
Unimmunized	<1	<1	95.0 ± 56.5	1.6
Genital Tract				
i.n. AdgB	68 ± 4.0	8.1	1275 ± 170	91.6
Unimmunized	<1	<1	<1	<1

[a] Mice were unimmunized or immunized intranasally with AdgB8 3 weeks prior to ivag HSV-2 challenge. Six days following challenge the ILN and genital tracts were isolated from groups of 5 mice and the mononuclear cells were isolated and analyzed by ELISPOT for HSVgB-specific antibody secreting cells.

[b] Results are expressed as the mean ± SD of triplicat wells and as a percentage of total antibody secreting mononuclear cells observed.

2. Contributions to Gallichan, W.S., and Rosenthal, K.L. (1996a)

(A) Growth and purification of AdgB8 and HSV-2.

(B) Immunization of mice and collection and processing of all samples and tissues.

(C) Evaluation of gB-specific antibodies and the stages of the estrous cycle. Developed the ELISPOT assay to test for gB-specific ASC in the genital tissues and lymph nodes of mice.

(D) Determination of vaginal pathology and viral titres in HSV-2 challenged mice.

3. Summary

In Gallichan *et al.* (1996a) the levels of humoral immunity and protection from an HSV-2 challenge in the female genital tract were assessed in relation to the stage of the estrous cycle. It is clear from this report that the levels of specific antibodies in the vaginal washes of mice immunized intranasally with AdgB8 are dependent on the stage of the estrous cycle. During estrus, or the time of mating, IgA antibodies are at there highest levels, and conversely, IgG antibodies are at their lowest levels. During diestrus, the opposite is true, with IgA titres being relatively low and IgG titres relatively high. The dependence of antibody levels on the reproductive cycle was further demonstrated during progesterone treatment of intranasally immunized mice. Following the administration of progesterone, mice entered a diestrus-like stage and coincidentally the gB-specific IgG antibody levels increased while IgA titres decreased. These results have important implications with respect to the development of immunity in the female genital tract against sexually transmitted pathogens since it is obvious that in order to maintain a blanket of humoral immunity over the course of the reproductive

cycle the induction of both IgG and IgA will be required.

The evaluation of immunity to HSV-2 infection demonstrated a dependence on the stage of the estrous cycle. In agreement with other reports we found that mice were only susceptible to an intravaginal HSV-2 infection during diestrus or following progesterone treatment. In addition, mice immunized intranasally with AdgB8 were significantly protected from an intravaginal HSV-2 challenge. Interestingly, all mice became infected as indicated by the persistence of virus in vaginal washes for the four days following challenge. These results suggest that immunity to a sexually transmitted pathogen can be achieved following intranasal immunization despite the absence of sterile immunity. It is interesting to note that mice were challenged during a progesterone-induced diestrus like state and evaluation of antibodies revealed a predominance of gB-specific IgG. Subsequent assessment of antibody secreting cells demonstrated that intranasally immunized, but not unimmunized, mice contained gB-specific ASC in genital tissues during the period of viral clearance and resolution of any overt genital pathology. These observations indicate intranasal AdgB8 immunization successfully protects the female genital tract from primary infection with HSV-2. Furthermore, intranasally immunized mice are capable of providing IgG as well as IgA antibody secreting cells in genital tissues during HSV-2 infection despite being in a diestrus-like state. Therefore, the capacity of intranasal immunization to provide protection as well impart these immune responses to the female genital tract prior to and during infection strongly suggests that this route of immunization would be suitable for vaccination against sexually transmitted pathogens.

CHAPTER FIVE

COMPARISON OF INTRANASAL AND SYSTEMIC AdgB8 IMMUNIZATION FOR THE INDUCTION OF ANTIBODY SECRETING CELLS AND IMMUNITY TO INTRAVAGINAL HSV-2 INFECTION

Comparison of Intranasal versus Intraperitoneal Immunization with a Recombinant Adenovirus for Protection Against Intravaginal Herpes Simplex Virus Type-2 Infection (Gallichan, W.S., and Rosenthal, K.L., 1996b, Submitted to J. Virol.)

1. Background

As described in the introduction, the purpose of these studies was to determine not only whether mucosal immunization could induce immunity to, and provide protection from sexually transmitted viruses, but more specifically, whether mucosal immunization was superior to systemic immunization. To address this issue we examined the level of protection from an intravaginal HSV-2 infection in mice immunized intranasally or intraperitoneally. In addition, we evaluated the clearance of virus and the development of ASC specific for gB of HSV in the genital tissues following infection.

ABSTRACT

Resistance to sexually transmitted viral infections of the female genital tract can be examined following induction of immunity within the two main immune compartments of the immune system: systemic and mucosal. The focus of this study was to compare the degree and duration of resistance against herpes simplex virus (HSV) infection of the female genital tract following mucosal and systemic immunization. Female mice were immunized intranasally (i.n.) or intraperitoneally (i.p.) with a recombinant adenovirus vector expressing glycoprotein B (AdgB8) of herpes simplex virus (HSV) and examined for survival and signs of genital pathology. Following intravaginal HSV-2 challenge, virus replication was observed for 4 days in the vaginal washes of immunized as well as unimmunized mice, however, by day 5, virus titres had decreased significantly in immunized mice, and by day 7 virus was undetectable. Examination of antibody secreting cells (ASC) during the decline in virus titres revealed that gB-specific IgA ASCs were only observed in the genital tissues of i.n. immunized mice. Assessment of genital pathology and survival of mice indicated that i.n. immunization provided a significantly greater degree of protection when compared to naive or i.p. immunized mice. Moreover, when we examined long-term protection, only mice immunized i.n. were significantly protected when compared to naive controls. We conclude from these results that mucosal immunization provides optimal immunity, when compared to systemic immunization, from sexually transmitted viral infections of the female genital tract.

INTRODUCTION

The ability to provide protection in the female genital tract against diseases caused by sexually transmitted viruses such as HIV and HSV is likely to depend on the presence of both humoral and cellular immune functions (19, 20). More specifically, the unique immune functions associated with the mucosal immune system can provide optimal protection from sexually transmitted diseases (STD) (19,20). Secretory immunoglobulin A (sIgA) is considered the hallmark of mucosal immunity and can mediate protection of mucosal tissues through inactivation or neutralization of virus at the exterior surface of the mucosal epithelium, as well as intracellularly during transcytosis (2, 14, 15, 17, 24). Furthermore, the level of antibodies in external secretions has been shown to correlate with protection against diseases of the respiratory and gastrointestinal tracts (4, 11). As confirmation of the importance of mucosal IgA , anti-IgA but not anti-IgG or IgM antiserum instilled intranasally (i.n.) can abrogate immunity against influenza virus infection (23). In the genital tract, IgG antibodies are also an important component of protection, since IgG antibodies specific for HSV have been shown capable of protecting mice against intravaginal (ivag) HSV-2 infection (3, 25). Our own studies indicate that the levels of antigen specific IgA and IgG antibodies in the vaginal washes of mice are inversely related and dependent on the stage of the estrous cycle (8). These results suggest that the induction of both IgA and IgG will be required in order to maintain a blanket of humoral immunity in the female genital tract over the course of the estrous cycle. In addition, T cells are involved in protecting mucosal tissues, as has been shown for mucosally derived T cells in protecting the female genital tract against HSV-2

infection (16).

Within the immune system there exists two main immune compartments in which the development of an immune response can occur. The systemic immune compartment consists of the bone marrow, spleen, and lymph nodes, and the mucosal compartment, consists of lymphoid aggregates in mucosae and external secretory glands in addition to the lymph nodes that drain these tissues (1, 18, 19). The consequence of this compartmentalization is that antigen exposure within one immune compartment results in the predominant expression of subsequent immune functions within the tissues associated with that particular compartment (1, 18, 19).

As a result, there is an increasing emphasis on the development of novel vaccines designed to induce mucosal-specific immune functions and provide protection from STDs (19). Several groups pursuing this goal have demonstrated that intranasal (i.n.) immunization with live viral or bacterial vectors (10, 12, 16) or conjugates of antigen with cholera toxin (26) successfully induced mucosal specific humoral immunity in the female genital tract. In addition, SIV incorporated into microspheres and administered intratracheally, following systemic priming, induced protective immune responses against vaginal challenge with SIV (13). In our previous studies we demonstrated that i.n. administration of an adenovirus vector expressing glycoprotein B (AdgB8) of HSV-1 resulted in gB-specific IgA and IgG in the genital tract (6). In contrast, systemic administration of this same vector resulted in only IgG antibodies (6). In addition, we observed that although both routes of AdgB8 immunization induced neutralizing antibodies and protection from an i.n. HSV-2 challenge,

i.n. immunized mice were better protected 1 year later (7). Further, we recently reported that mice immunized i.n. with AdgB8 were able to resist an intravaginal HSV-2 infection (8), and long-term gB-specific CTL memory responses were observed in the mucosal immune compartments of the genital and respiratory tracts of i.n but not i.p AdgB8 immunized mice (9). Considering that intranasal immunization with AdgB8 appears to mediate mucosal-specific immune responses in the female genital tract, and resistance to HSV-2 infection, we addressed the issue of relative resistance to intravaginal HSV-2 infection in animals immunized systemically versus intranasally. Although evidence suggests that either systemic or mucosal immunization is capable of mediating resistance to STDs, a comparison of these routes is required to delineate the optimal route of vaccination against sexually transmitted viruses.

Viral titres in the vaginal washes of mice infected intravaginally with HSV-2. Following an intravaginal challenge with HSV-2 (strain 333), the vaginal washes of unimmunized mice or mice immunized i.n. or intraperitoneally (i.p.) with AdgB8, 6 weeks previously, were analyzed daily for virus by plaque formation on Vero cell monolayers, as previously described (8). We detected significant virus titres from all three groups of mice over the first 4 days, however, i.n. immunized mice contained marginally lower levels ($p \leq 0.05$) of detectable virus, relative to unimmunized mice, over the first 2 days (Fig. 1). By day 5, the virus titres in the vaginal washes of both i.n. and i.p. immunized mice had decreased dramatically (more than 2 logs, $p \leq 0.001$). On day 6 this trend continued and in addition, the number of immunized mice with detectable virus also decreased substantially such that by day

7 virus was undetectable in vaginal washes of any of the immunized mice. In contrast, virus was present in the vaginal washes of all the unimmunized control mice and remained at constant levels during the 6 days following challenge.

Survival from intravaginal HSV-2 challenge in mice immunized with AdgB8. Six weeks after AdgB8 immunization, mice were monitored for survival following a lethal intravaginal HSV-2 infection (8). None of the unimmunized control mice survived past day 6 due to the rapid onset of pathology which occurred in all mice and consisted of severe genital ulceration extending to the surrounding tissue (Fig. 2). Similarly, 8/10 mice immunized i.p. with AdgB8 developed genital pathology, and 7 of these mice succumbed to disease by day 9 while the eighth survived as did 2 others that did not display any overt symptoms (Fig. 2) ($p \geq 0.05$, compared to unimmunized mice). In contrast, a significant number of mice immunized i.n. with AdgB8 survived (8/10) the lethal HSV-2 challenge when compared to controls (0/10; $p \leq 0.005$) or i.p. (3/10; $p \leq 0.05$) immunized mice. Sixty percent of these mice were completely protected from any overt genital pathology ($p \leq 0.01$, compared to unimmunized mice). Interestingly, 1 of the i.p., and 2 of the i.n. immunized mice did develop genital swelling and redness (genital pathology score ≤ 2), however, none of these mice went on to develop severe symptoms. Further, all mice that did survive primary infection were found to be free of overt genital pathology and virus in vaginal washes for 3 months following challenge. Similar results were observed in 2 separate experiments (data not shown).

Long and short-term resistance to intravaginal HSV-2 infection in mice immunized

with AdgB8. Resistance to low and high dose intravaginal HSV-2 challenge was examined at short and long-time periods following AdgB8 immunization and evaluated based on the presence and severity of genital pathology (Table 1). Naive mice serving as controls were unimmunized and age-matched. All mice were examined over a 6 day period following HSV-2 challenge and genital pathology was scored in a blinded fashion using a 5 point scale as previously described (8). The presence of any overt genital pathology (score ≥ 1) as well as the severity of pathology over the 6 days following infection (area under the lesion score-day curve) was determined in each mouse as previously described (8). It is clear from the severity scores that in low dose challenge experiments both i.n. and i.p. immunized mice (at 4-6 weeks) displayed significantly less genital pathology than naive mice ($p \leq 0.0001$). However, when compared to mice immunized i.p. with AdgB8, more of the i.n. immunized mice remained disease free and the overall pathology severity scores were significantly lower (0.6 vs 3.7; $p \leq 0.05$). Furthermore, when mice immunized 9 to 10 months previously were challenged with a low dose of HSV-2, only i.n. immunized mice demonstrated a significant level of resistance based on severity of genital pathology ($p \leq 0.05$) (Table 1). Indeed, only one of the i.n. immunized mice displayed any genital pathology, whereas most of the i.p. immunized mice developed overt symptoms of infection (4/5) (Table 1).

Next, we assessed the development of genital pathology following a higher challenge dose of HSV-2. At this higher dose, both i.n. and i.p. immunized mice still displayed significantly less genital pathology than naive mice ($p \leq 0.0001$) at 4 to 6 weeks following AdgB8 immunization. In addition, mice immunized i.n. but not i.p. were significantly

protected at 9 to 10 months following AdgB8 immunization ($p \leq 0.0001$). However, the number of i.n. immunized mice displaying overt pathology increased, as did the overall severity of infection, over that observed at low challenge dose. The severity of infection in i.p. immunized did not change from the low challenge dose. As a result, there was no significant difference in severity of pathology between i.n. and i.p. immunized mice at this higher dose, at 4 to 6 weeks or 9 to 10 months post AdgB8 immunization. These results suggest that the mechanisms present i.n. immunized mice that mediated the superior low dose resistance became overwhelmed by the increase in challenge dose, whereas, the immune functions in i.p. immunized mice maintained a similar level of resistance.

Antibody secreting cells specific for HSV-gB in the genital tissues of AdgB8 immunized mice 6 days following intravaginal HSV-2 infection. Presumably, specific antibodies in the genital tracts of mice following AdgB8 immunization should neutralize and assist in clearance of virus during an infection. Therefore, the presence of gB-specific antibody secreting cells (ASC) was assessed in the iliac lymph nodes (ILN) that drain the genital tissues as well as the genital tissue itself (uterus, vagina, and fallopian tubes) of AdgB8 immunized and unimmunized mice 6 days following a lethal intravaginal HSV-2 infection by ELISPOT as previously described (8). At short (3 weeks) and long (9 months) times post AdgB8 immunization, mice immunized i.n. and i.p. contained IgG and IgA gB-specific ASCs in the ILNs (Table 2). The gB-specific ASCs in the ILNs represented a large percentage of the total ASCs, for both IgG and IgA memory responses, especially in mice immunized 3 weeks prior to HSV-2 challenge. Additionally, the ILNs of mice immunized i.n.,

as compared to i.p. contained far more IgA ASCs specific for gB, both at 3 weeks and 9 months post immunization. In contrast, the ILNs of naive mice undergoing a primary response to HSV-2 infection lacked any IgA ASCs and contained only a few IgG ASCs (1% of total) specific for gB of HSV. Examination of the genital tissues of mice on day 6 post HSV-2 infection revealed that only after intranasal immunization were gB-specific IgA ASCs found. Assessment of IgG ASCs demonstrated that both i.n. and i.p. immunized mice were capable of recruiting gB-specific IgG ASCs to the genital tissues following HSV-2 infection (Table 2). However, at long time periods following i.p. immunization (9 months) we were not always able to detect specific IgG ASCs in genital tissues. Although the numbers of specific ASCs found in the genital tissues were low, these results were consistent with 3 other similar experiments.

The resistance against genital pathology and the survival of mice from a lethal intravaginal HSV-2 infection that we observed following AdgB8 immunization was impressive. Although none of the unimmunized mice survived, we observed a large degree of resistance after AdgB8 immunization, especially in mice immunized intranasally. Interestingly, many different formulations and routes of antigen exposure have been explored for the induction of immunity in the female genital tract against HSV and other sexually transmitted pathogens (1, 17-20, 24). Several of these studies have explored the intranasal route of immunization for the induction of immune responses at distant mucosal surfaces, including the genital tract (6, 10, 12, 13, 21, 26). The results from these studies clearly demonstrate that intranasal immunization successfully induces humoral immunity in the female

genital tract of both IgA and IgG specificities. Our previous results using intranasal immunization with AdgB8 also demonstrated the presence of specific IgG and IgA antibodies in the serum and vaginal washes of mice (6). In contrast, systemic immunization only resulted in IgG antibodies. We also observed that i.n. but not i.p. AdgB8 immunization induced long-lived CTL memory responses in the genital associated lymphoid tissues of mice (9). This is likely a relevant finding since McDermott et al. (16) demonstrated that immune T cells from the genital lymph nodes of mice infected intravaginally with an attenuated strain of HSV-2 were capable of mediating protection in adoptive transfer studies against a subsequent intravaginal HSV-2 challenge. With our previous observations and those of other groups in mind, it is clear that intranasal immunization does provide optimal mucosal immune responses when compared to systemic immunization. Furthermore, the results presented here indicated that i.n. AdgB8 immunization also mediated the greatest survival of mice against lethal intravaginal infection with HSV-2 when compared to i.p. immunization. This was true shortly following AdgB8 immunization or for as long as 9 to 10 months after immunization, indicating that immunity was long-lived following intranasal immunization. In contrast, immunity in i.p. immunized mice was short-lived since mice were not significantly resistant 9 to 10 months after AdgB8 immunization.

Interestingly, the amount of virus used during challenge(s) was likely far in excess of that which would occur during a natural infection, and perhaps as a consequence of this, we observed a dose dependency on severity of infection in i.n. immunized mice. At a high HSV-2 challenge dose there was an increase in both the severity of infection and the number of mice

with overt genital pathology. Further, these symptoms were similar to those observed in i.p. immunized mice, suggesting that some immune functions may become overwhelmed while others can still maintain some level of protection at the higher challenge dose. Nevertheless, while these results are not the first demonstration that intranasal immunization provides protection in the female genital tract (5, 8, 22), they are the first demonstration that intranasal immunization provides superior resistance to intravaginal infection with a sexually transmitted virus when compared to systemic immunization. Consistent with these observations, Marx and collaborators demonstrated that intramuscular plus intratracheal or oral, but not intramuscular plus intramuscular, vaccination protected macaques against intravaginal SIV infection (13).

Secretory IgA is considered to be of primary importance in mucosal immunity and has been shown to be responsible for preventing infection of mucosal tissues (1, 17-20, 24). The main activity of IgA has generally been regarded as the ability to neutralize or prevent attachment of pathogens at the apical surface of mucosal tissues (1, 17-20, 24). However, more recently, in vitro (14, 15) and in vivo (2) models have demonstrated that secretory IgA is capable of forming complexes with viruses intracellularly, inhibiting virus replication, and subsequently preventing primary or resolving chronic infections. Interestingly, our studies demonstrate that immunized mice infected intravaginally with HSV-2 maintained virus replication in their genital tracts at levels similar to unimmunized mice for the first 4 days. Thus, AdgB8 immunization did not result in sterile immunity. By day 5 the levels of virus in vaginal washes had significantly decreased, and by day 7 none of the immunized mice

displayed any free virus. Of particular importance was the observation that virus became undetectable even in the vaginal washes of mice that developed severe genital pathology. We therefore assessed the ASCs present in the genital-associated lymph tissues and directly in the genital tract during this period of virus resolution in order to address whether there was local antibody production which may be responsible for neutralization of virus in vaginal washes late in infection. The genital tissues of mice immunized i.n. or i.p. with AdgB8, but not unimmunized mice, were found to contain gB-specific IgG ASCs. Perhaps more importantly, only i.n. immunized mice were capable of providing IgA ASCs in the genital tissues at this time. Furthermore, while both i.n. and i.p. immunized mice developed high numbers of gB-specific IgG ASC in the ILNs draining the genital tissues, i.n. immunized mice generated a much stronger IgA ASC response compared to i.p. immunized mice. These results would suggest that gB-specific antibodies, and in the case of i.n. immunized mice, IgA antibodies, are present locally during the period of virus clearance. Taken together, our observations may therefore reflect the development of local ASCs, the presence of specific antibodies, and the neutralization of intracellular and extracellular virus giving the perception of viral clearance even though tissues may still be infected.

The observed virus clearance following intravaginal infection occurred in both i.n. and i.p. immunized mice, despite the fact that i.p. immunization only induced secretory IgG (6) and IgG ASCs in genital tissues (Table 2). These results likely reflect the fact that in order to successfully infect mice intravaginally with HSV-2, it is necessary to induce them into a diestrus like state. This is achieved following progesterone administration, and as we have

demonstrated (8), this treatment results in increased levels of IgG antibodies and conversely, decreased levels of IgA antibodies in genital fluids. Therefore, the observed clearance of virus from vaginal washes that occurred at similar levels in i.n. and i.p. immunized mice is most likely related to the presence of IgG specific for gB of HSV. Indeed, IgG antibody levels were assessed in the vaginal washes of i.n. and i.p. immunized mice on the day of infection, however, there was no apparent difference in titres between the groups or correlation with subsequent resistance (data not shown). Interestingly, although there were specific antibodies present during infection, the reduction in initial virus load was not dramatic or sustained, suggesting that a reduced virus load did not contribute to eventual protection. Nevertheless, i.n. immunization resulted in long-term survival and resistance to intravaginal HSV-2 infection and this observation is consistent with the presence of IgA ASCs, but may also be attributable to increased CTL responses (9), or a combination of these and possibly other immune functions occurring in i.n. but not i.p. immunized mice.

In terms of the development of successful vaccine strategies for the induction of specific mucosal immune responses in the female genital tract our results indicate that mucosal immunization is the optimum route for antigen exposure. Indeed, we have demonstrated that i.n. immunization with an adenovirus vector expressing a single HSV glycoprotein is capable of inducing long-lived mucosal specific IgA and IgG antibodies as well as CTL (6-9). Protective immunity in the female genital tract against STDs has been investigated following numerous routes and antigen formulations (1, 17-20, 24), however, we have demonstrated here that when compared to systemic immunization, mice immunized i.n. with AdgB8 were

optimally resistant for a long time period from a lethal intravaginal HSV-2 challenge. Resistance occurred despite the fact that mice were initially infected and the optimal survival occurring in i.n. immunized mice correlated with the presence of herpes specific IgA producing B cells in the genital tissues, suggesting that mucosal-specific immune responses were responsible. These results may be important in the design of vaccines as well as immunotherapeutics against sexually transmitted viruses.

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FIG. 1. Replication of virus in the vaginal washes of AdgB8 immunized mice following an intravaginal HSV-2 infection. Mice immunized 6 weeks previously with AdgB8 were infected with a greater than 100% lethal dose of HSV-2 (2×10^4 pfu) and daily vaginal washes were analyzed for virus by plaque formation on Vero cell monolayers. * Naive mice were sacrificed on day 7 due to overt genital pathology. Comparisons among the means of groups was carried out using analysis of variance (ANOVA).

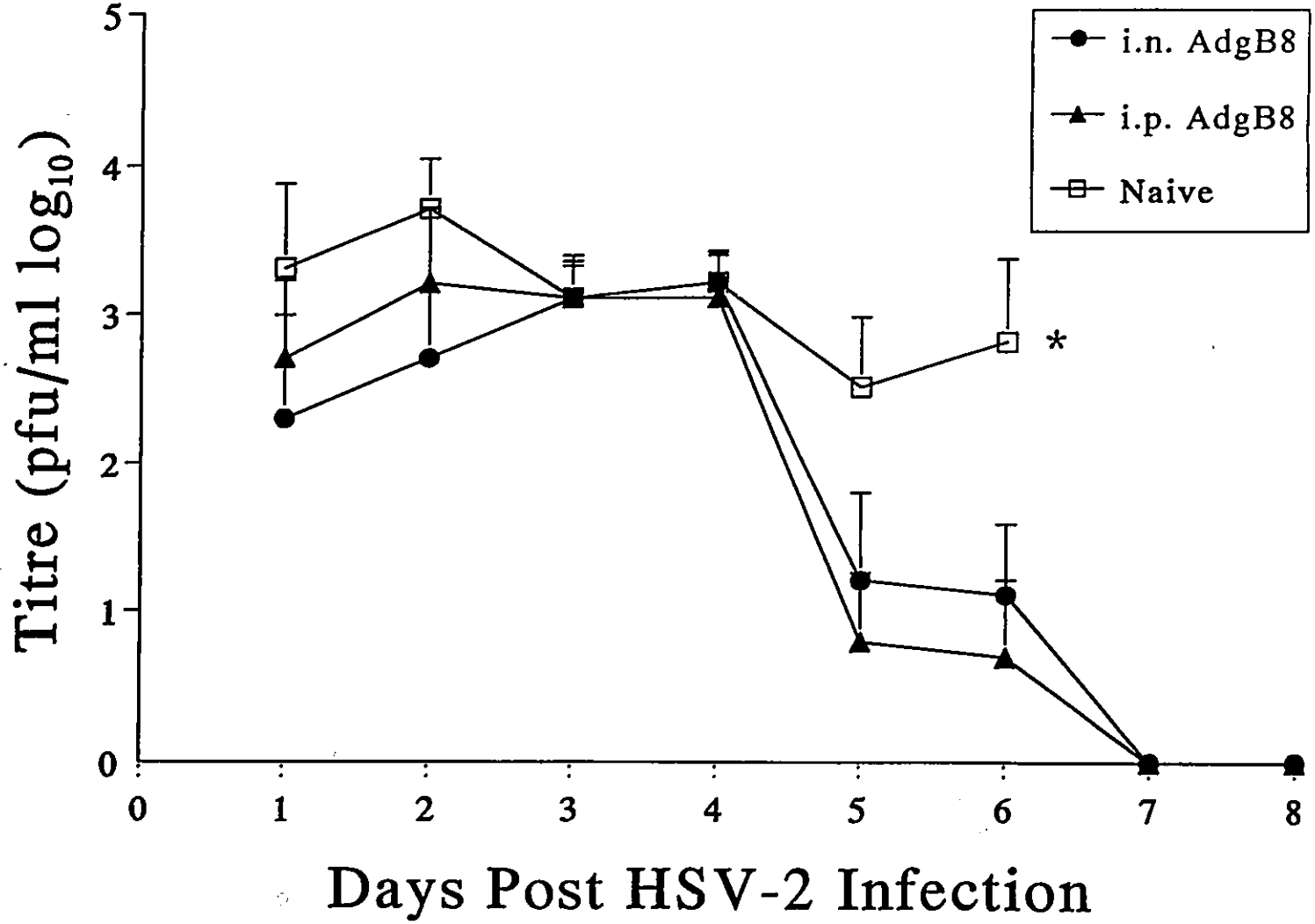

i.n. AdgB8
i.p. AdgB8
Naive
*
Titre (pfu/ml log10)
0
1
2
3
4
5
0
1
2
3
4
5
6
7
8
Days Post HSV-2 Infection

FIG 2. Survival of AdgB8 immunized mice following an intravaginal HSV-2 infection. Mice immunized 6 weeks previously with AdgB8 were challenged intravaginally with 2×10^4 pfu of HSV-2 and monitored daily for survival and signs of disease and if necessary sacrificed upon reaching a genital pathology score of 5. For comparisons between 2 groups, data were analyzed by Fisher's exact test.

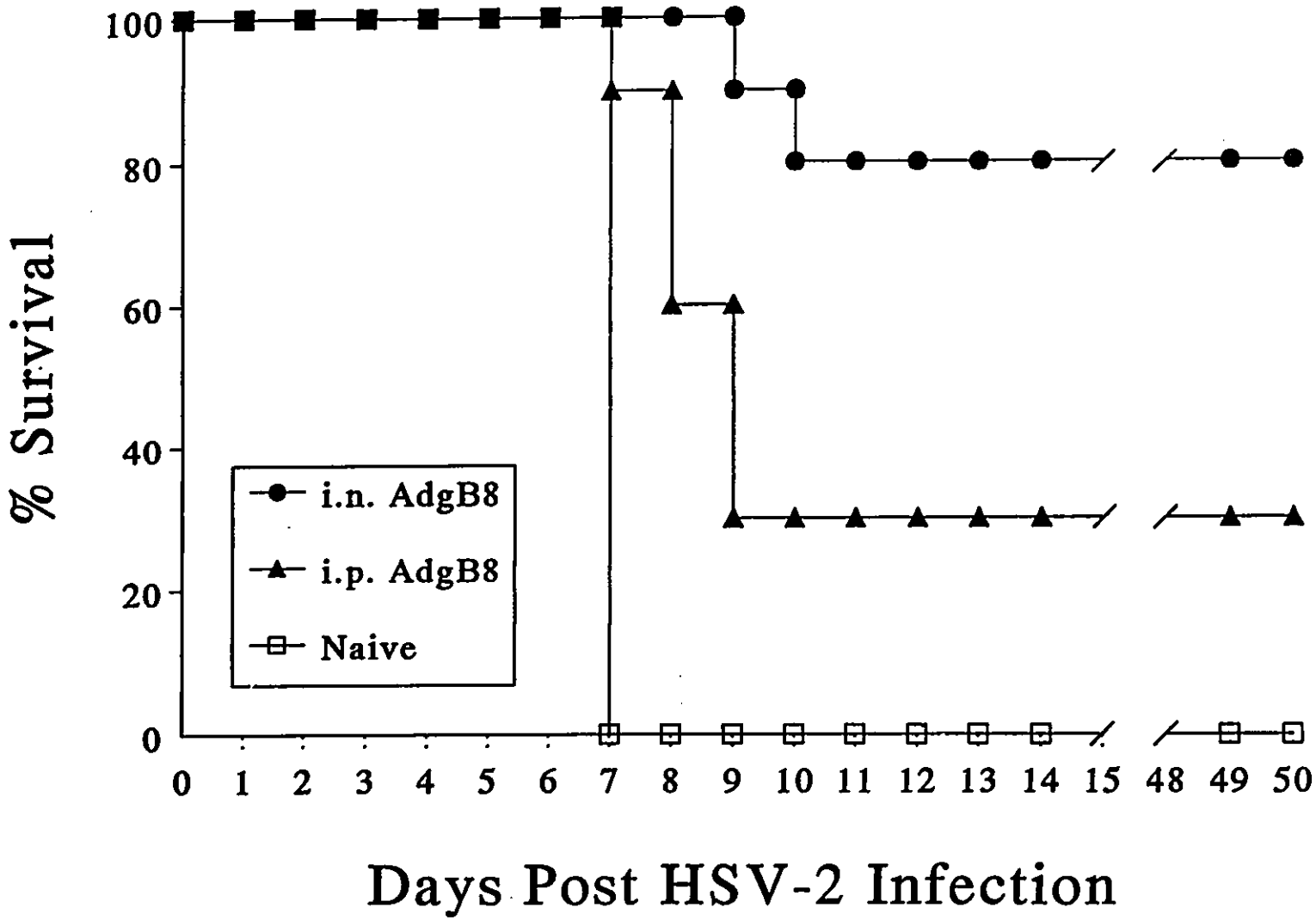

% Survival
100
80
60
40
20
0
i.n. AdgB8
i.p. AdgB8
Naive
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 48 49 50
Days Post HSV-2 Infection

Table 1: Genital Pathology Following Intravaginal HSV-2 Infection of AdgB8 Immunized Mice.

Group[a]	Time Post Immunization	Low Challenge Dose		High Challenge Dose	
		Pathology[b]	Severity[c]	Pathology	Severity
i.n.	4-6 wks	4/10	0.6 (0.8)[d, f]	7/10	2.3 (2.2)[d]
	9-10 mths	1/5	0.1 (0.2)[e]	4/9	1.5 (2.2)[d]
i.p.	4-6 wks	8/10	3.7 (3.0)[d]	9/10	3.3 (2.7)[d]
	9-10 mths	4/5	4.0 (3.0)	9/9	3.2 (2.6)
Naive	4-6 wks	9/9	8.5 (2.1)	10/10	8.3 (2.5)
	9-10 mths	5/5	6.2 (3.0)	5/5	6.2 (2.7)

[a] Mice were immunized i.n. or i.p. with AdgB8 and challenged intravaginally 4 to 6 weeks or 9 to 10 months later with 2×10^4 pfu (low dose) or 1×10^5 (high dose) of HSV-2.
[b] Number of mice that demonstrated overt genital pathology/total (ie. score ≥ 1).
[c] Mean ±SD ; measured as area under the lesion score-day curve for first 6 days.
Significance between AdgB8 immunized and unimmunized mice for same time period:
[d] $p<0.0001$, [e] $p<0.05$.
Significance between i.n. and i.p. immunized mice at 4-6 weeks:[f] $p<0.05$.
[b] For comparisons between 2 groups, data were analyzed by Fisher's exact test.
[c] Comparisons among the means of multiple groups was carried out using analysis of variance (ANOVA).

Table 2: HSVgB-Specific Antibody Secreting Cells in Genital Tissues of AdgB8 Mice Immunized 6 Days Following Intravaginal HSV-2 Infection

Tissue / Immunization[a]	Time Post Immunization	Mean HSVgB-Specific ASC / Million Mononuclear Cells[b]			
		IgA	% of Total	IgG	% of Total
ILN					
i.n.	3 wks	94 ± 11	70	1700 ± 131	48
	9 mths	29 ± 7	30	595 ± 76	17
i.p.	3 wks	18 ± 4	26	1540 ± 177	47
	9 mths	9 ± 2	14	2025 ± 237	38
Naive	-	< 2	< 2	65 ± 41	1
Genital Tract					
i.n.	3 wks	17 ± 3	11	30 ± 26	15
	9 mths	7 ± 3	4	10 ± 20	4
i.p.	3 wks	< 2	< 1	15 ± 10	5
	9 mths	< 2	< 1	< 2	< 2
Naive	-	< 2	< 1	< 2	< 1

[a] Naive or AdgB8 immunized mice were challenged intravaginally with 1 x 10^5 pfu HSV-2 and 6 days later the ILN and genital tracts were isolated from groups of 5 mice and the mononuclear cells were analyzed by ELISPOT for HSVgB-specific antibody secreting cells.
[b] Results are expressed as the mean ± SD of triplicate wells and as a percentage of total antibody secreting mononuclear cells observed.

2. Contributions to Gallichan, W.S., and Rosenthal, K.L. (1996b)

(A) Growth and purification of AdgB8 and HSV-2.

(B) Immunization of mice and collection and processing of all samples and tissues.

(C) Evaluation of gB-specific ASC in genital tissues and lymph nodes.

(D) Determination of vaginal pathology and viral titres in HSV-2 challenged mice.

3. Summary

In this paper, mice immunized intranasally or intraperitoneally with AdgB8 were assessed for protection and immune responsiveness following an intravaginal HSV-2 infection. It is evident from the results that neither intranasal nor intraperitoneal immunization provided sterile immunity at the doses of HSV-2 used for intravaginal challenge. However, whereas unimmunized mice were unable to clear the virus infection, both groups of immunized mice did clear any signs of virus replication. Evaluation of genital pathology demonstrated that both groups of immunized mice displayed protection from virus challenge, however, intranasally immunized mice were better protected and for a longer time, even at high challenge doses. Survival of mice also indicated that intranasal immunization provided a significantly greater level of protection when compared to intraperitoneal immunization.

Evaluation of gB-specific ASC in genital tissues during the period of virus resolution demonstrated that both groups of immunized mice contained gB-specific IgG ASC in genital tissues. However, only mice immunized intranasally contained IgA ASC and moreover, mice immunized by this routes displayed this ability for long time periods following AdgB8

immunization. These results indicate that mucosal or intranasal immunization with recombinant adenovirus vectors provides superior protection from sexually transmitted viruses. In addition, intranasal but not systemic immunization induced long-lived ASCs of both isotypes in the genital tissues of female mice during a primary infection.

CHAPTER SIX

CHARACTERIZATION OF CYTOTOXIC T LYMPHOCYTE MEMORY IN MUCOSAL AND SYSTEMIC IMMUNE COMPARTMENTS FOLLOWING AdgB8 IMMUNIZATION

Long-lived Cytotoxic T Lymphocyte Memory in Mucosal Tissues Following Mucosal But Not Systemic Immunization (Gallichan, W.S., Rosenthal, K.L., 1996c, J. Exp. Med. In Press)

1. Background

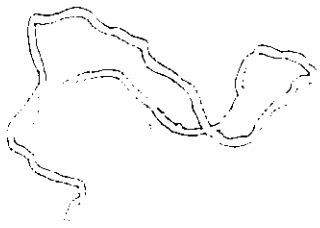
Immunological memory is the hallmark of the immune system and is recognized by the more rapid onset of specific immune responses following subsequent exposure to the same or closely related antigen. In the case of T cells, immune memory is typically based on the increased numbers of specific precursors present in the circulation at various time points following antigen exposure or immunization. As discussed in the introduction, stimulation of the mucosal immune system should lead to the maintenance of memory T cells within mucosal or related immune compartments. This section examines the maintenance of CTL memory within systemic and mucosal immune compartments following immunization with AdgB8 in each compartment.

In our previous work , outlined in chapter 2, we observed that mice immunized

intranasally with AdgB8 initially developed anti-HSV memory cytotoxic T lymphocytes that were present in the spleen. However, within several months of immunization we were unable to detect any anti-HSV splenic CTL following bulk stimulation. In contrast, mice immunized intraperitoneally maintained strong anti-HSV splenic CTL responses for longer than one year. To investigate whether intranasally immunized mice contained specific CTL in mucosal immune compartments, despite a lack of detectable splenic CTL, we assessed the development of recall responses in the draining lymph nodes of HSV infected tissues. We compared these memory responses to those occurring in unimmunized animals and reasoned that a more rapid generation of specific CTL in immunized animals would be indicative of the presence of CTL memory within that tissue, or having access to that tissue.

ABSTRACT

The induction and maintenance of long-term cytotoxic T lymphocyte (CTL) memory at mucosal surfaces may be a critical component of protection against mucosal pathogens and is one goal towards development of effective mucosal vaccines. In these studies we have functionally evaluated short and long-term T cell memory in the systemic and respiratory or genital-associated lymphoid tissues following mucosal or systemic routes of immunization. Our results indicate that shortly after immunizing mice with a recombinant adenovirus vector expressing glycoprotein B of herpes simplex virus (AdgB8), anti-gB cytotoxic T lymphocyte (CTL) memory responses were observed systemically and mucosally, regardless of the route of inoculation. In contrast, several months after immunization, memory CTL specific for gB of HSV compartmentalized exclusively to mucosal or systemic lymphoid tissues following mucosal or systemic immunization, respectively. Furthermore, the compartmentalized CTL memory responses in mucosal tissues were functionally observed for longer than 1.5 years following intranasal immunization, and CTL precursor frequencies one year after immunization were comparable to those seen shortly after immunization. Therefore, to our knowledge, this is the first functional demonstration that the maintenance of anti-viral memory T cells in mucosal tissues is dependent on the route of immunization and the time of assessment. These results have important implications for our understanding of the development, maintenance, and compartmentalization of functional T cell memory and the development and evaluation of vaccines for mucosal pathogens, such as HSV and HIV.



INTRODUCTION

Memory is a hallmark of both humoral and T cell-mediated immune responses and is typified by a more rapid and intense immune response on re-exposure to the same or closely related antigen. Indeed, a desirable property of vaccines is the ability to generate long-term immunological memory capable of preventing infection or limiting disease. Our current understanding of T cell memory stems from studies of systemic immune responses, however little is known concerning the induction and maintenance of T cell memory responses in mucosal tissues. Numerous pathogens, including respiratory, gastrointestinal and sexually-transmitted agents, such as herpes simplex virus (HSV) and human immunodeficiency virus (HIV), initiate infection at mucosal surfaces. Since the mucosal immune system is somewhat separate and distinct from systemic immunity (1-3), a better understanding of specific immunologic T cell memory in mucosal tissues should contribute to the development of effective mucosal vaccines and improved control of infections at these sites.

Mucosal surfaces are largely protected by secretory IgA as well as transudated IgG antibodies (4). The induction of B cells in mucosal tissues following antigen exposure or infection results in the migration of B lymphocytes within the common mucosal immune system, thus ensuring the maintenance of secretory immunity at mucosal surfaces (1-3). T cell-mediated immunity is also a critical component of protection against mucosal pathogens. In addition to the detection of cell-mediated cytotoxicity in mucosa-associated tissues (5, 6), the passive transfer of cytotoxic T lymphocytes (CTL) is associated with the clearance of

virus at mucosal surfaces and may reduce virus-related pathology (7-12). Furthermore, as with B cells, mucosally derived T cells migrate to mucosal tissues and this homing is even more pronounced during re-exposure to the same pathogen (2, 12).

The maintenance of T cell memory to viruses may be the result of long-lived antigen-specific lymphocytes (13-15). Alternatively, T cell memory may be maintained by constant stimulation as a result of antigen persistence (16, 17), idiotypic networks (18) or cross-reactions with other antigens (19). Of interest to us, and an often ignored issue is that the development of immune responses within the unique environments of secondary lymphoid tissues (20, 21), and the homing of effector and memory lymphocytes to the tissues in which antigen exposure originally occurred (12, 22, 23), may restrict the maintenance and observation of T cell memory to distinct immune compartments. Indeed, in our previous studies involving mice immunized intranasally (i.n.) against HSV, we observed a decline in the levels of splenic CTL such that one year following immunization we were unable to detect any anti-HSV CTL in the spleen. Interestingly, these mice were protected from an intranasal challenge with HSV-2 (24). We suspected that the lack of detectable splenic CTL at this time was a reflection of the recruitment, retention, and recirculation of specific CTL within the common mucosal immune system following intranasal immunization. In support of this theory was the concurrent observation that although systemically immunized animals displayed short-term protection from mucosal HSV-2 challenge, protection was not long-lived despite the fact that CTL specific for HSV-2 were readily detectable in the spleen (24).

Therefore, in this study we have examined the development and the long-term maintenance

of CTL memory within the distinct immune compartments of the mucosal and systemic immune systems following antigen exposure in either compartment. To induce immunity to HSV, mice were immunized with a recombinant adenovirus vector expressing the immunodominant HSV antigen glycoprotein B (AdgB8). The CTL immune responses that developed were evaluated in the spleen and mucosal tissues of the respiratory and genital tracts.

Cytotoxic T lymphocyte memory has been functionally observed as an enhanced secondary CTL response in vitro (25-27) and in vivo (13, 28-33) following re-exposure to antigen. Thus, by using the recall response as a functional indicator of specific memory in mucosal tissues we observed that i.n. immunization provided short and long-term mucosal anti-HSV CTL memory in respiratory and genital-associated lymphoid tissue. In contrast, following i.n. immunization, systemic (splenic) anti-HSV CTL memory dissipated over several months and was absent or at low levels in mice that maintained long-term mucosal CTL memory. Conversely, systemic immunization resulted in the long-term maintenance of splenic memory but only short-term mucosal memory. These results indicate that the functional presentation of short-term T cell memory is independent of the route of antigen exposure. Furthermore, the generation and maintenance of long-term CTL memory is dependent on the route of immunization, indicating that T cell memory at a functional level compartmentalizes over time and that the examination of T cell memory and development of T cell dependent vaccines should take into consideration the route of antigen exposure.

Materials and Methods

Animals and cell cultures. Inbred female C57BL/6 (H-2^b) mice (purchased from Charles River Canada, St. Constant, Quebec, Canada) were used for these studies. 293 and 293-N2S cells were grown in α-MEM (GIBCO Laboratories, Burlington, Canada), supplemented with 10% fetal calf serum (FCS; GIBCO), and 1% penicillin-streptomycin and L-glutamine (GIBCO). MC57 (H-2^b) and SVBalb (H-2^d) fibroblasts served as targets in the CTL assays.

Virus strains and inoculations. The construction of replication-competent recombinant adenovirus vectors, AdgB8 and AdE3$^-$, is reported elsewhere (33, 34). Briefly, AdgB8 contains the glycoprotein B (gB) gene from HSV-1 coupled to the SV40 promoter and inserted into the E3 region of human adenovirus type 5. AdE3$^-$ contains a deletion in the E3 region and served as a control. The recombinant adenoviruses were grown in 293-N2S cells, purified twice on CsCl gradients, and titered on 293 cells. The vaccinia vector expressing gB of HSV-1 (VacgB11) was kindly provided by B. Moss (NIH, Bethesda, MD) and its construction is reported elsewhere (36). HSV type 2 (HSV-2) strain 333 was propagated and virus titres were determined on Vero cells. Each mouse was immunized with a total of 10^8 pfu of AdgB8 or AdE3$^-$ in the given volumes of phosphate buffered saline pH 7.4 (PBS). Mice immunized intranasally (i.n.) were anaesthetized with Halothane, inverted, and 10-20μl of virus in PBS was introduced into the nares by means of a micropipet (24). Intraperitoneal (i.p.) and hind foot pad (h.f.p.) immunizations were performed by inoculation of virus in 0.2 ml and 50 μl of PBS, respectively. Mice were challenged intravaginally (ivag) or i.n. with 10^7 pfu of HSV-2 in 10μl or 20μl of PBS, respectively. Intravaginally challenged mice were first

inoculated subcutaneously with 2 mg of progestin/mouse (Depo-Provera, Upjohn, Don Mills, Ontario) five days prior to challenge, and then anesthetized using halothane, swabbed with a cotton applicator, placed on their backs and infected for 1 hour while under anesthetic.

Cytotoxic T-lymphocyte (CTL) assays. Spleen and lymph node 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 1:166 in RPMI 1640 medium (supplemented as above). Following stimulation splenic effector cells were incubated with uninfected and HSV-2 infected (MOI = 10, 6 h infection period) syngeneic (MC57) and allogeneic (SVBalb) targets at effector to target ratios of 80:1, 40:1, 20:1, and 10:1 in a 6 h ^{51}Cr release assay. Inhibition of cytotoxicity was determined by adding 100 µl of monoclonal antibody 145-2C11 (anti-CD3) to wells containing effector cells 1 h prior to addition of target cells at the 40:1 ratio. Data are expressed as percent specific lysis = 100 x [(experimental cpm - spontaneous cpm) / (maximum release cpm - spontaneous release cpm)]. Cytotoxic T-lymphocytes from lymph nodes draining the site of infection were examined in a primary CTL assay by the protocol of Pfizenmaier et. al. (37) with modifications (38). Briefly, in the primary CTL assay lymphocytes from lymph nodes draining the site of infection were harvested from mice 2 or 3 days post HSV-2 challenge and incubated for 3 days (without antigen stimulation) in RPMI 1640 medium with 10% fetal calf serum, 50 µM 2-mercaptoethanol, 1% L-glutamine, penicillin, and streptomycin. In vitro incubation (without antigen) has been shown to be

necessary in the herpes system for CTL to become fully cytolytic (37). Following incubation, the lymph node effectors were incubated with targets in a 6 h ^{51}Cr release assay as described above. VacgB11 (MOI = 10, 16 h infection period) as well as HSV-2 infected (as above) and uninfected fibroblasts served as targets.

Determination of CTL Precursor Frequencies and Lytic Units. For determining the CTL precursor frequencies of HSV-2 specific splenocytes limiting dilution analysis was performed. Briefly, splenocytes were isolated by Ficol gradient and titrated into round bottom, 96-well plates (NUNC, Roskilde, Denmark) with 12 replicates at each dilution. Feeders (irradiated splenocytes: 2000 rads) were isolated by Ficol gradient and added at 2×10^5 mononuclear cells per well. Stimulators were irradiated (2000 rads), HSV-2 infected splenocytes set at 2×10^5/well. Five days following incubation, the contents of each well was transferred to a corresponding well in V-bottomed NUNC plates and 5000 ^{51}Cr labelled and HSV-2 or VacgB infected syngeneic fibroblasts were added to each well. Plates were pulse spun up to 1500 rpm and incubated for 6 hours in a ^{51}Cr release assay. Positive wells were defined as those wells whose ^{51}Cr release exceeded the mean spontaneous release from control cultures, containing feeder cells but no responder cells, by at least 3 standard deviations.

The assay used for determining the CTL precursor frequencies of HSV-2 specific lymphocytes from the draining lymph nodes of HSV-2 infected mice was similar to that reported by Nugent et.al (31, 33). Lymphocytes from lymph nodes draining HSV-2 infected mucosal tissues (genital tract, ILN) were titrated into round bottom, 96-well plates in 100 *ul* of supplemented RPMI with 16 replicates for each lymphocyte concentration. Feeders

(irradiated splenocytes: 2000 rads) were isolated by ficol gradient and added at 2 x 10^5 mononuclear cells per well in 100 *ul* of 5 U of rIL-2 (Genzyme, Cambridge, MA), 10% (v/v) Rat T-Stim (Collaborative Biomedical Research Products, Cambridge, MA), and 50 mM α-methyl mannoside (Sigma). No exogenous antigenic stimulation was provided to the cultures, in order to restrict analysis to effector CTL expanded from CTLp exclusively activated in vivo. Five days following incubation cultures were assessed as above for precursor frequencies.

The lytic activity of CTL in the draining lymph nodes of HSV-2 infected tissues was estimated by determining the lytic units (LU). One lytic unit is defined as the number of mononuclear cells required to obtain 10% specific lysis of 5000 infected targets. The 10% level of lysis was chosen to accommodate the low levels of killing experienced by certain experimental groups. We have expressed the lytic activity of the mononuclear cells recovered from the draining lymph nodes as the number of lytic units per 10^7 cells. In short, mononuclear cells were harvested from the lymph nodes draining the sites of HSV-2 infection and incubated for 3 days (37) in RPMI 1640 medium with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 1% L-glutamine, penicillin, and streptomycin. The lymph node effectors were then plated in two-fold serial dilutions in 96-well V-bottomed plates (NUNC, Roskilde, Denmark) with 12 replicates at each dilution. Five thousand HSV-2 infected targets were added to each well and plates were briefly spun at 1500 rpm and incubated for 6 h at 37° C in a ^{51}Cr release assay. One lytic unit was determined by estimating the number of lymph node effectors required to cause 10% ^{51}Cr release by fitting a curve to the data using the equation

described by Clark et. al. (39) and a program generously supplied by D. A. Clark.

Statistics. Frequency estimates of CTLp were calculated using χ^2 analysis as described by Taswell (40), by using a computer program kindly provided by Richard Miller (University of Michigan, Ann Arbor). Estimates of CTLp frequencies were considered valid only if the plot of the logarithm of the fraction of negative cultures against the number of responder cells on a linear scale obeyed single-order kinetics with a probability (P) > 0.05 (41).

The lytic activity for each mouse is expressed as the mean lytic units (LU) /10^7 cells ± SEM. Statistical analysis of LU was carried out using analysis of variance (ANOVA).

Results

Dissipation of Splenic CTL Memory Following Intranasal AdgB8 Immunization. The maintenance of systemic anti-HSV-2 CTL was examined in C57BL/6 mice following intranasal (i.n.) or intraperitoneal (i.p.) immunization with AdgB8 at various time points over a 19 week period (Fig. 1). To determine whether memory CTL were present within the systemic immune system, HSVgB-specific splenocytes were first expanded in vitro following bulk stimulation with antigen. The expansion of the memory population permits qualitative evaluation of the extent of CTL memory in the spleen. Both i.p. and i.n. AdgB8 immunization resulted in the presence of splenic effectors at early time points post immunization as evidenced by the high levels of CTL-mediated killing of HSV-2 infected syngeneic targets (Fig. 1A and B). The lysis was T cell-mediated since anti-CD3 treatment markedly inhibited killing (Fig. 1A and B). Further, CTL-mediated lysis was MHC-restricted and virus-specific since uninfected syngeneic and allogeneic HSV-infected targets were not killed (data not shown). In mice immunized i.p. with AdgB8 the level of lysis of HSV-2 infected targets was maintained over the 19 week period (Fig. 1A). In contrast, in i.n. immunized mice the lysis of HSV-2 infected targets decreased to levels similar to that of control mice over the 19 week period (Fig. 1B). Moreover, in separate experiments, splenic CTL precursor frequencies were estimated at 1 in 21,280 (95% CI: 12,658 to 35,714) and 1 in 500,000 (95% CI: 250,000 to 10^6) mononuclear cells 14 months post i.p. or i.n. immunization, respectively. In addition, limiting dilution analysis using fibroblasts infected with AdgB8 as stimulators gave similar results (data not shown). These results indicate that while both routes of AdgB8

immunization initially resulted in the presence of anti-HSV CTL in the spleen, only i.p. immunized mice maintained long-term splenic anti-HSV memory CTL.

CTL Memory Responses in the Draining Lymph Nodes of the Respiratory Tract Following AdgB8 Immunization and Intranasal HSV-2 Challenge. T cell memory is typically a reflection of previous lymphocyte proliferation that is maintained through increased numbers of antigen specific lymphocytes. Therefore, to investigate antigen-specific CTL memory within the respiratory tract we took advantage of the antigen-induced alteration in the reactive state of the immune system following antigen exposure (i.n. AdgB8 immunization) by examining the levels of CTL in the draining lymph nodes following an HSV-2 challenge. We first determined that primary anti-HSV CTL responses in the draining lymph nodes of HSV-2 infected mucosal tissues appeared at low levels on days 2-3 and peaked by day 5 post infection (data not shown). Therefore, in assessing memory CTL responses in the respiratory tract of AdgB8 immunized mice, mice immunized 7 months previously were challenged i.n. with HSV-2 and 3 days later the mediastinal lymph nodes (MLN) which drain the respiratory tract were examined for CTL activity. Figure 2A shows that only lymphocytes from the MLNs of mice immunized i.n. 7 months previously with AdgB8 contained a strong anti-HSV-2 CTL recall response. In contrast, MLN cells from i.p. AdgB8 immunized mice failed to appreciably lyse HSV-2 infected targets (Fig. 2A). This indicates that long-term CTL memory, as observed through a functional recall response, existed within the local mucosal immune compartment of the respiratory tract following i.n. but not i.p. AdgB8 immunization. Interestingly, when the spleens of these same mice were examined for anti-

HSV CTL, only mice immunized i.p. demonstrated splenocytes that recognized and killed HSV-2 infected targets (Fig. 2B).

To further investigate the maintenance of long-term T cell memory in the respiratory tract we examined individual mice immunized with AdgB8 and compared them to unimmunized (naive) animals 2 days following i.n. HSV-2 challenge. Figure 3 demonstrates that at 2 days post i.n. HSV-2 challenge, only MLN lymphocytes from the mouse immunized i.n. with AdgB8 19 months previously demonstrated the ability to lyse HSV-2 infected syngeneic targets. In contrast, the lymphocytes from the mediastinal lymph nodes of the naive or h.f.p. immunized mouse failed to appreciably lyse HSV-2 infected targets (Fig. 3). Furthermore, when the lytic activity in the MLNs of the three mice were examined, the lymphocytes in the i.n. AdgB8 immunized mouse contained significantly ($p \leq 0.0001$) and more than 8 times the lytic activity than that found in either the naive or i.p. immunized mouse (Fig. 3). These results are representative of several experiments in which individual mice were examined at late time points post immunization and at no time did we observe killing from the MLNs of i.p. or h.f.p. immunized mice. HSV-2 specific lysis was T cell-mediated since it was completely inhibited with anti-CD3 antibody (Fig. 3). Killing was also virus-specific and MHC-restricted in the i.n. immunized mouse since uninfected and allo-infected targets were not lysed (data not shown). Figures 2 and 3 demonstrate that long-term T cell memory responses, as observed in the draining lymph nodes, are maintained in the respiratory tract following local (i.n.) but not systemic immunization.

In Figure 4 the presence of short-term CTL memory in the respiratory tract following

i.n. or h.f.p. AdgB8 immunization was evaluated. Three weeks post immunization both these groups demonstrated a CTL recall response that was greater than that of the naive mouse and was completely inhibitable with anti-CD3 (Fig. 4A). As well, the lytic activity in the MLNs of i.n. or h.f.p. immunized mice was significantly greater than in the naive mouse ($p \leq 0.0001$). In fact, there was more than 8 and 4 times the number of lytic units in i.n. or h.f.p. immunized mice, respectively, demonstrating that shortly after either mucosal or systemic AdgB8 immunization mice were able to mount anti-HSV-2 CTL memory responses in the mucosal-associated lymphoid tissue of the respiratory tract (Fig. 4A).

To determine whether the short-term CTL memory response to HSV-2 challenge was specific for gB of HSV, targets were infected with a vaccinia virus vector expressing gB of HSV (VacgB11). The lysis of VacgB11 infected targets was similar to that of HSV-2 infected targets (Fig. 4B), confirming the presence of short-term CTL memory responses in both i.n. and h.f.p. immunized mice. Moreover, since the lysis and lytic activity against VacgB infected targets was as high as against HSV-2 infected targets, the CTL memory response to HSV-2 challenge was for the most part directed against gB of HSV (Fig. 4B).

CTL Memory Responses in the Draining Lymph Nodes of the Genital Tract Following AdgB8 Immunization and Intravaginal Challenge with HSV-2. We next addressed the question of whether the maintenance of CTL memory in mucosal tissues was purely a local phenomena (ie. due to local immunization) or was also present at distant mucosal sites. Moreover, since HSV-2 is a sexually transmitted virus and since one of our main interests has been the development and characterization of anti-HSV-2 immune responses in the genital

tract, we investigated CTL memory responses at this site following AdgB8 immunization. Figure 5 consists of two representative experiments showing CTL recall responses in individual mice at early (5-9 weeks) and late (15-18 months) time points post immunization (Fig. 5A and B). The magnitude of the primary response is indicated by the CTL activity in the naive (unimmunized) mice. In these experiments, lymphocytes were isolated from the iliac lymph nodes (ILN), which drain the genital tract, three days following ivag HSV-2 challenge and examined for their ability to lyse HSV-2 infected targets. Figure 5A demonstrates that at early time points post immunization (ie. 5 weeks) both i.n. and i.p. AdgB8 immunized mice demonstrated lysis of HSV-2 infected targets that exceeded primary responses observed in naive mice. The killing was TCR-mediated as indicated by the inhibition of lysis with anti-CD3 antibody. The lytic activity in both groups was not only similar but was significantly ($p \leq 0.0001$) and more than 11 times greater than that observed in the naive mouse. Thus, as observed in the respiratory tract, shortly after immunization there exists a phase of CTL memory in the genital tract which is not dependent on the route of immunization. In the second experiment (Fig. 5B) mice were examined 9 weeks post immunization and again ILN lymphocytes from the mouse immunized i.n. maintained short-term CTL memory. However, at this time point the i.p. immunized mouse lacked a significant memory response when the lysis of targets or the lytic activity was compared to that observed in the naive mouse (Fig. 5B). Therefore, systemic immunization induced a phase of short-term memory in the genital tract, which we observed in the draining lymph nodes and which was absent by 9 weeks post immunization.

To examine the long-term maintenance of mucosal CTL memory, mice were challenged with HSV-2 intravaginally 15 or 18 months after AdgB8 immunization (Fig. 5A and B). At 18 and 15 months, i.n. immunized mice demonstrated much higher as well as anti-CD3 inhibitable levels of killing when compared to i.p. immunized mice. Indeed, the level of lysis and the lytic activity in i.n. immunized mice at 18 or 15 months post immunization was at least as high as that occurring at 5 or 9 weeks post i.n. immunization, respectively (Fig. 5A and B). Furthermore, in both experiments the lytic activity in ILN cells of i.n. immunized mice was significantly ($p \leq 0.0001$) and more than 4 times greater than that observed in i.p. immunized mice. In contrast, mice immunized i.p. 18 or 15 months prior to ivag HSV-2 challenge lacked a memory response and their lytic activity was not significantly different from that of the naive mice (Fig. 5A and B). These results indicate that only i.n. immunized mice maintained long-term anti-HSV CTL memory responses in the genital tract and the strength of these responses did not appear to decrease with time.

Splenic CTL Responses in AdgB8 Immunized Mice Following Intravaginal HSV-2 Challenge. The maintenance of splenic CTL memory in AdgB8 immunized and ivag challenged mice in Figure 5B was examined (Fig. 5C). Three days after ivag HSV-2 challenge, the splenocytes from mice immunized i.p. with AdgB8 (9 weeks and 15 months previously) demonstrated anti-HSV-2 CTL activity (following in vitro stimulation) (Fig 5C). This is in agreement with our previous results (Fig. 1A) which demonstrated that i.p. AdgB8 immunization induced long-term splenic CTL memory. Interestingly, neither of these mice demonstrated a CTL memory response in their ILNs following ivag HSV-2 challenge (Fig.

5B). In contrast, both mice immunized i.n. (ie. 9 weeks or 15 months previously) mounted CTL memory responses in their ILNs (Fig. 5B) yet failed to display high levels of anti-HSV-2 CTL in their spleens (Fig. 5C). In fact, the mouse immunized i.n. 15 months previously displayed a level of killing from the spleen similar to the naive mouse. These results suggest that immunological T cell memory can be observed functionally in mucosal tissues without being observed in the systemic system and vice-versa.

Specificity of the CTL Memory Responses in the Draining Lymph Nodes of the Genital Tract Following AdgB8 Immunization and Intravaginal Challenge with HSV-2. To examine the specificity and further characterize the long-term memory response in the genital tracts of i.n. immunized mice, the anti-HSV CTL activity in ILNs from mice immunized 5 months previously were examined 2 days post HSV-2 challenge and targets infected with VacgB11 were utilized (Fig. 6). In addition, two systemic routes of immunization (i.p., and h.f.p.) were assessed and a naive mouse was included to represent the primary response to gB of HSV. Figure 6 demonstrates that 2 days after ivag HSV-2 challenge the ILN cells in the i.n. immunized mouse lysed the VacgB11 infected targets and this lysis was completely inhibitable with anti-CD3 antibody. Furthermore, the lytic activity in the i.n. immunized mouse was significantly ($p \leq 0.0005$) and more than 4 times greater than in the naive or systemically immunized mice. In contrast, the level of lysis of VacgB11 targets in naive and systemically immunized mice were very low and not always inhibitable with anti-CD3. The lytic activity was also very low and not significantly different between the naive and systemically immunized mice (Fig. 6). These results confirm that long-term immunological T cell memory

is maintained in the genital tracts of mice immunized i.n., but not systemically, with AdgB8 and demonstrates that these memory CTL are specific for gB of HSV.

Limiting Dilution Analysis of CTLp Frequencies in Mice One Year Following AdgB8 Immunization. As a final step in examining the compartmentalization of memory CTL in AdgB8 immunized mice we determined the precursor frequencies of gB-specific CTL in the ILNs of mice immunized 1 year previously and 60 hours following an intravaginal HSV-2 challenge (Table 1). Analysis of the frequencies of gB-specific CTLp expanded under limiting dilution conditions showed that the ILNs of i.n. immunized mice (ranging from 1 in 3411 to 1 in 5946) contained significantly and several fold more gB-specific CTLp than the ILNs of control (unimmunized) (ranging from 1 in 23899 to 1 in 35350) or systemically (i.p. or h.f.p.) (ranging from 1 in 15643 to 1 in 31006) immunized mice (Table 1). Moreover, the CTLp frequencies demonstrated that the recall responses in systemically immunized mice, were no different than the primary responses, occurring in control animals, indicating that long-term mucosal T cell memory is maintained following mucosal but not systemic immunization.

Analysis of the splenocytes of these same animals expanded under limiting dilution conditions demonstrated that the frequencies of gB-specific CTLp in i.n. immunized mice were extremely low (ranging from 1 in 325543 to 1 in 667609). In fact, we were unable to detect any gB-specific CTL mediated lysis following bulk stimulation of splenocytes (Table 1). In contrast, mice immunized systemically maintained relatively high frequencies of CTLp (ranging from 1 in 55068 to 1 in 95959) and CTL mediated lysis was readily detectable following bulk stimulation.

Limiting Dilution Analysis of CTLp Frequencies in Mice Shortly Following AdgB8 Immunization. To evaluate the development of mucosal T cell memory in AdgB8 immunized mice at the CTLp frequency level, we examined the ILNs of mice 2 or 12 weeks following AdgB8 immunization. The results in Table 2 demonstrate that 60 hours following an intravaginal HSV-2 challenge, the gB-specific CTLp frequencies of ILN cells in i.n. immunized mice (ranging from 1 in 2523 to 1 in 6845) were significantly higher than in i.p. immunized mice (at either time point) (ranging from 1 in 13135 to 1 in 29292). Interestingly, the CTLp frequencies were similar in mice immunized i.n. at 2, 12 or 52 weeks previously (ranging from 1 in 2523 to 1 in 6845) (Tables 1 and 2). Although the gB-specific CTLp frequencies in the ILNs of i.p. immunized mice at 2 weeks (ranging from 1 in 13135 to 1 in 15311) were not as high as in i.n. immunized mice, they were higher than at 12 weeks (ranging from 1 in 17833 to 1 in 29292). Moreover, by 12 weeks the CTLp frequencies in the ILNs of i.p. immunized mice were similar to those observed in unimmunized mice in Table 1, suggesting that systemic immunization can provide mucosal T cell recall responses, however, the maintenance of this mucosal T cell memory is short lived.

The gB-specific splenic CTLp frequencies were also determined in these mice and demonstrate that both i.n. and i.p. immunization successfully induced short-term levels of T cell memory within the systemic immune system. However, the frequencies were generally 2 to 3 fold higher in i.p. immunized mice. In addition, the splenic CTLp frequencies in i.p. immunized mice only slightly decreased from 2 weeks (ranging from 1 in 40233 to 1 in 47904) to 1 year (ranging from 1 in 55068 to 72671), whereas, in i.n. immunized mice there

was a substantial decrease in CTLp frequencies over this period (2 to 7 fold) (Tables 1 and 2)

Discussion

T cell memory has been functionally viewed as the more rapid generation of recall responses following a second exposure to antigen (13, 28, 29, 31). Increased numbers of antigen-specific T cell precursors as well as qualitative differences in memory T cell activation requirements likely contribute to the magnitude of recall responses (42-46). The ability of memory T cells to persist for long periods of time may depend on several factors, including the persistence or complexity of antigen (16, 17), regulatory networks (18), cross-stimulation (19) or long-lived T cells (13-15). In addition, the maintenance of T cell memory may depend on the tissues in which exposure to antigen first occurred. Indeed, the analysis of surface markers on T cells has demonstrated the recirculation of activated and memory lymphocytes is selective and depends on the tissues and lymph nodes from which the lymphocytes originated (47). By using recall responses we have investigated CTL memory in mucosal and systemic immune compartments following various routes of immunization with an adenovirus vector expressing gB of HSV. Our results indicate that CTL memory, when examined functionally, has both an early and a late phase which manifests in the biphasic expression of memory CTL within a given tissue depending on the site of initial antigen exposure. More specifically, the presence of systemic CTL was observed to be short-lived following i.n. immunization, however, mucosally, CTL memory was long-lived. Similarly, systemic immunization resulted in short-lived mucosal memory CTL, but long-lived systemic CTL. In addition, intranasal immunization resulted not only in long-term CTL memory in the local mucosal tissues of the respiratory tract, but also distantly in the mucosal tissues of the genital

tract. This would imply that the genital tract is an implicit effector site within the common mucosal immune system. Therefore, our results demonstrate functionally the phenomenon of selective recirculation of memory lymphocytes to the tissue compartments in which sensitization to antigen first occurred. In addition, there is a short-lived period in which T cell memory can be functionally observed within the opposing immune compartment (ie. mucosal versus systemic).

Although we have established that AdgB8 immunization results in long-lived CTL, we have not explored the underlying nature of this memory. However, recent evidence (48, 49) suggests that infection with similar replication-competent recombinant adenoviruses (E3 inserts) results in only short-term expression of inserted antigen, suggesting that persistent antigen production is not the underlying mechanism responsible for the long-term maintenance of CTL memory within our system. Indeed, these studies also demonstrated that initially, vector derived antigen was present in both systemic and mucosal immune compartments regardless of the route of inoculation (48, 49). This would suggest that the persistence of antigen is unlikely driving the compartmentalized maintenance of CTL memory.

The primary observations that led to this study included the early detection of anti-HSV CTL in the spleens of i.n. or i.p. AdgB8 immunized mice, but the absence of long-term memory from the spleens of i.n. immunized mice (24). As well, mice immunized i.n. with AdgB8 were protected against heterologous intranasal challenge with HSV-2, and this protection lasted longer than in i.p. immunized mice (24). Moreover, we have recently observed that i.n. AdgB8 immunization protected mice from an intravaginal HSV-2 challenge,

and this protection was long-lived and significantly better than following i.p. immunization (submitted). By investigating these phenomena further, we show here that i.n. and i.p. routes of AdgB8 immunization initially induced high anti-gB CTLp frequencies and following bulk stimulation, similar levels of splenic anti-HSV CTL. However, several months later, bulk stimulation of splenocytes demonstrated that mice immunized i.n.had barely detectable CTL specific for HSV. Limiting dilution analysis of mice 12 to 14 months following i.n. immunization confirmed that the CTLp frequencies had decreased and had remained permanently low or undetectable. In contrast, i.p. immunization resulted in relatively high CTLp frequencies for longer than 14 months. Others have also shown the persistence of splenic CTL following systemic exposure to gB of HSV (50). However, the transient nature of splenic CTL following i.n. immunization may be due to the selective migration of memory T cells to mucosal sites within the common mucosal immune system (1-3, 11, 12, 20, 23, 47).

Thus, to address these seemingly conflicting observations of long-lived mucosal protection in the absence of systemic CTL, we examined short and long-term CTL memory in mucosal tissues by assessing recall responses within mucosal associated lymph nodes following local HSV-2 challenge. At less than nine weeks following either systemic (i.p. or h.f.p.) or mucosal (i.n.) routes of AdgB8 immunization, we observed rapid recall responses in the draining lymph nodes of the lungs and genital tract. In addition, CTLp frequencies indicated that mucosal memory responses were present in animals 2 weeks following either systemic or mucosal immunization. Thus, short-term CTL memory in mucosal tissues is not dependent on the route of immunization. In a similar manner, we examined short-term recall

responses in the systemic compartment and observed that mice immunized either i.n. or systemically developed memory responses to h.f.p. HSV-2 challenge (data not shown). These results suggest that functionally there exists within a population of recently activated lymphocytes, T cells that are capable of recirculating indiscriminately throughout the body. This is interesting since recently activated lymphocytes or blasts derived from skin or mucosal tissues have been shown to preferentially but not exclusively migrate back to the tissues in which antigen exposure originally occurred (1, 2, 3, 11, 12, 20, 23, 47). Our findings may then reflect the persistence of a population of recently activated lymphocytes that are capable of responding to sites of inflammation. This is quite possible in light of work demonstrating the increased penetration of memory or activated lymphocytes into sites of antigen challenge (2, 12). Alternatively, the routes of immunization utilized here may not reflect tissue restricted infection, resulting in some overlap of immune induction within opposing immune compartments. However, our observation of distinctively compartmentalized long-term memory responses suggests that this mechanism is unlikely to account for the observed short-term phase of T-cell memory. Moreover, it is interesting that as for short-term CTL memory, short-term protection from mucosal HSV-2 challenge (24) is not dependent on the route of immunization.

In our initial evaluation of long-term mucosal CTL memory, we examined recall responses in the respiratory-associated lymphoid tissue and observed a dissociation of memory responses in i.n. and i.p. immunized animals. Seven to 19 months following AdgB8 administration only mice immunized i.n. demonstrated CTL memory responses in the draining

mediastinal lymph nodes of the respiratory tract. Interestingly, in the mice immunized 7 months previously, only i.p. immunization resulted in the presence of long-term anti-HSV-2 CTL in the spleen. These results suggest that mucosally-induced memory lymphocytes are not maintained within the spleen but perhaps within the lymphoid or extralymphoid tissues of the respiratory mucosa.

Our results, as well as our previous observations of IgA induction in the respiratory tract (24), suggest that i.n. immunization with AdgB8 stimulates the induction of immune responses in the bronchus-associated lymphoid tissue (BALT) (51). The BALT serves as part of the mucosa-associated lymphoid tissues (MALT) and as such shares organizational as well as functional similarities with other mucosal surfaces as part of the common mucosal immune system. Lymphocytes derived from one mucosal tissue can recirculate through and localize selectively within other mucosal surfaces, including the respiratory tract and uterus (1, 2, 3, 52). In agreement with this, we recently demonstrated that i.n. AdgB8 immunization results in the presence of secretory anti-HSVgB IgA in the genital tract (53) and protection from intravaginal HSV-2 challenge (submitted).

To investigate the extent of mucosal CTL memory in distant mucosal tissues following i.n. AdgB8 immunization we evaluated long-term recall responses in the genital tract. Our results demonstrate that mice immunized i.n. with AdgB8 and challenged intravaginally up to 18 months later with heterologous HSV-2 developed strong anti-HSV-2 CTL memory responses. Furthermore, limiting dilution analysis of the recall responses confirmed that i.n. but not systemically immunized animals maintained memory CTL for as long as one year

either within the genital tract or its associated lymphoid tissues or capable of entering these sites following infection. To our knowledge, this is the first functional demonstration of long-lived CTL in local and distant mucosal tissues despite low or undetectable levels of systemic CTL.

Interestingly, the population of HSV-specific CTL that are maintained within the systemic immune system following i.p. immunization with AdgB8 are unable to mount memory responses to i.n. or ivag infection with HSV-2. This suggests that the population of lymphocytes present shortly following systemic immunization that contained T cells capable of mounting short-term mucosal memory responses were no longer present. Therefore, the indiscriminatory recirculating or inflammation responsive T cells present in the short-term phase of memory are no longer present in the systemically immunized animals. This leaves us to suggest that during the early stages of an immune response there are populations of functionally active T cells that have no tissue specific homing pattern or are able to respond or enter sites of inflammation and that either do not represent the long-term population of memory T cells or phenotypically change into that population.

Our results also demonstrate that the memory responses were predominantly specific for gB of HSV since the lytic activities and the lysis of targets infected with recombinant vaccinia virus expressing gB of HSV (VacgB11) were at least as high as for HSV-2-infected targets. Moreover, analysis of CTLp frequencies specific for gB of HSV occurring in the recall response demonstrated that the memory CTL were in fact predominantly gB-specific. Cytotoxic T lymphocyte precursor frequencies were also assessed against HSV infected

targets and the results confirmed that i.n. but not i.p. immunization induced long-term mucosal CTL memory but the absence of long-term splenic CTL memory. These results demonstrate that part of the primary CTL response to AdgB8 immunization and the recall response to HSV-2 challenge are directed towards gB of HSV. This is in contrast to recent reports by Nugent et.al (31, 33) where it was shown that although a large portion of the primary response to HSV-1 inoculation involves gB-specific CTL, early secondary responses did not. Unlike in our system, the lack of an early gB-specific recall response in their model is most likely due to the fact that HSV-1 was used for both sensitization and challenge and likely reflects the interactions and complexities of numerous neutralizing anti-HSV and CTL epitopes during both periods.

Currently, many vaccines or immunotherapeutics are given systemically with the objective of providing mucosal immune functions. Our studies demonstrate that mucosal (i.n.) administration of recombinant adenovirus vectors induced long-lived antigen-specific CTL in mucosal-associated lymphoid tissues, whereas systemic administration of this vaccine induced long-lived CTL systemically but not mucosally. Development of successful vaccines against mucosal pathogens, such as HSV and HIV, will require the induction of long-lived mucosal immune responses. Recently, we showed that intranasal immunization with AdgB8 induced secretory IgA specific for gB of HSV in both the respiratory (24) and genital tracts (53). The ability of recombinant adenoviruses to induce specific mucosal humoral responses and the long-term maintenance of anti-HSV-2 CTL in respiratory and genital tissues suggest that these vectors may serve as excellent mucosal vaccines.

Our results also show that short-term mucosal CTL memory was present following both i.n. and systemic immunization and only after several months did the memory CTL responses compartmentalize to mucosal or systemic tissues. These results have important implications with regard to the evaluation of vaccines since the time of assessment following vaccination may affect detection of CTL activity. Furthermore, the functional evaluation of memory CTL specific for mucosal pathogens should be based on assessment of CTL in mucosal-associated lymphoid tissues and not in the spleen.

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Figure 1. Maintenance of systemic CTL specific for HSV-2 in mice immunized intraperitoneally (i.p.) (A) but not intranasally (i.n.) (B) with AdgB8. Three mice were immunized at each time point over the course of 19 weeks at approximately 5 week intervals. As a control group 3 mice were i.n. immunized at 9 weeks with AdE3$^-$ (B). Following 6 days of in vitro culture with irradiated, AdgB8 infected stimulator cells the splenocytes were examined for CTL activity against HSV-2 infected MC57 targets. Effector to target ratios reported are 40:1■, 20:1▩, 10:1▨,and 40:1 + anti-CD3□.

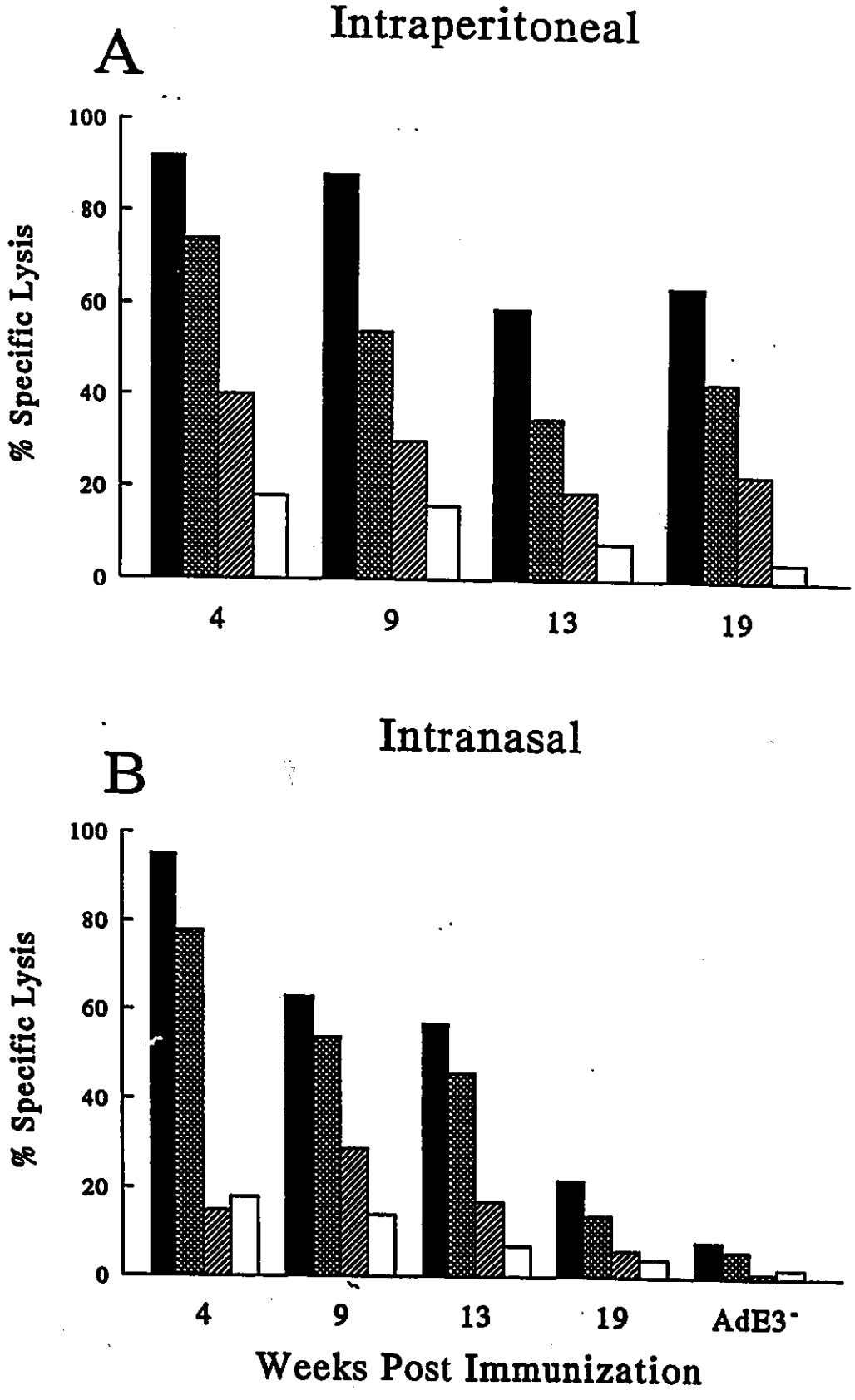

A
Intraperitoneal
% Specific Lysis
100
80
60
40
20
0
4
9
13
19
B
Intranasal
% Specific Lysis
100
80
60
40
20
0
4
9
13
19
AdE3-
Weeks Post Immunization

Figure 2. Systemic and mucosal CTL memory in the respiratory-associated lymphoid tissue (A) and spleens (B) of AdgB8 immunized mice. Mice were immunized intranasally (i.n.) or intraperitoneally (i.p.) and 7 months later challenged intranasally with HSV-2. The spleens and mediastinal lymph nodes (MLN) were isolated 3 days later. The spleens (B) were stimulated for 6 days with irradiated AdgB8 infected stimulators before being analyzed in a CTL assay. The MLNs (A) were cultured without antigen stimulation for 3 days before being assayed. CTL activity is reported at E/T ratios of 40:1 ■, 20:1 ▩, 10:1 ▨, and 40:1 + anti-CD3 □.

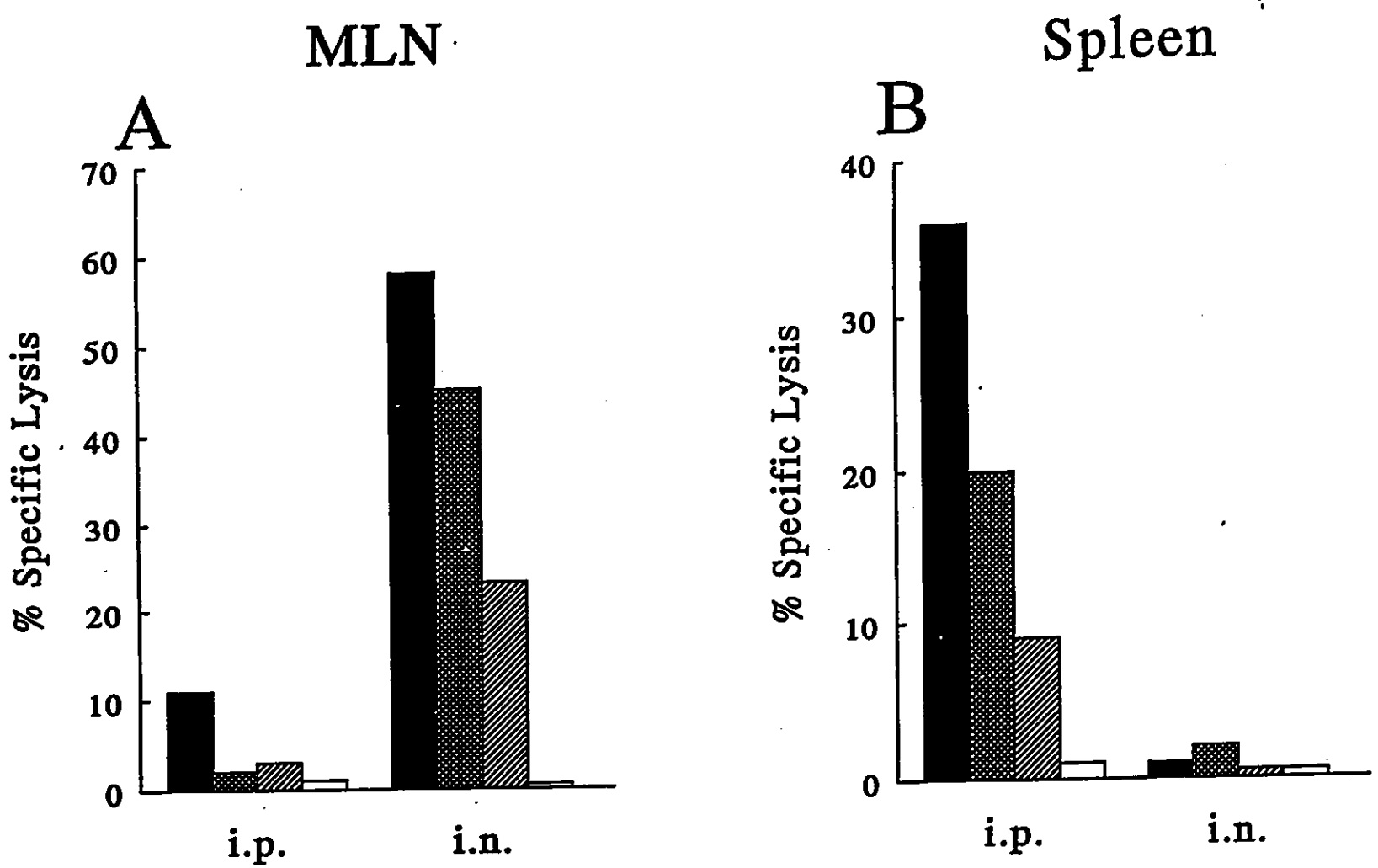

MLN
A
% Specific Lysis
70
60
50
40
30
20
10
0
i.p.
i.n.
Spleen
B
% Specific Lysis
40
30
20
10
0
i.p.
i.n.

Figure 3. Long-term CTL memory in the respiratory-associated lymphoid tissue of mice immunized intranasally (i.n.) with AdgB8. Two mice immunized either i.n. or in the hind foot pad (h.f.p.) with AdgB8 were i.n. challenged 19 months later with HSV-2. A naive (unimmunized) mouse was also challenged and served as a control representing the magnitude of a primary response. Two days following i.n. challenge the mediastinal lymph nodes (MLN) were isolated and cultured without antigen stimulation for 3 days before being examined for CTL activity. MC57 targets were infected with HSV-2 and the 40:1 ▨ and 40:1 + anti-CD3 ☐ E/T ratios are reported as well as the lytic activity ■ in mean lytic units per 10^7 mononuclear cells ± SEM. *Significantly different from naive and h.f.p. immunized mice ($P < 0.0001$)

Route	Time
Naive	-
i.n.	19 mths
h.f.p.	19 mths

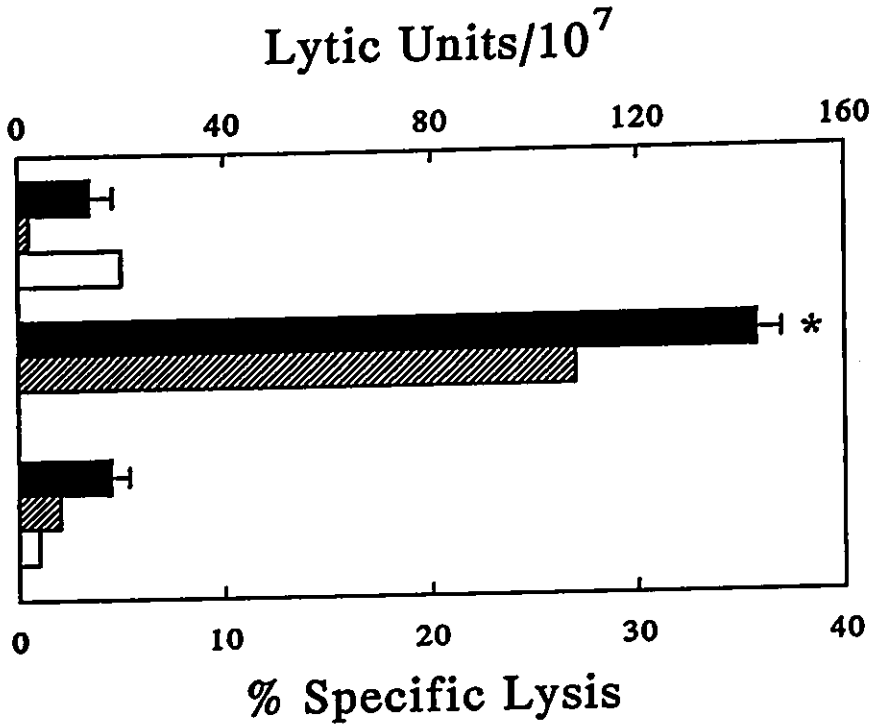


Figure 4. Short-term CTL memory in the respiratory-associated lymphoid tissue of mice immunized systemically or mucosally with AdgB8. Two mice immunized either intranasally (i.n.) or in the hind foot pad (h.f.p.) with AdgB8 were i.n. challenged 3 weeks later with HSV-2. A naive (unimmunized) mouse was also challenged and served as a control representing the magnitude of a primary response. Two days following i.n. challenge the mediastinal lymph nodes (MLN) were isolated and incubated for 3 days without antigen stimulation before being examined for CTL activity. MC57 targets were infected with HSV-2 (A) or VacgB11 (B) and the 40:1 ▨ and 40:1 + anti-CD3 □ E/T ratios are reported as well as the lytic activity ■ in mean lytic units per 10^7 mononuclear cells ± SEM. * Significantly different from the naive mouse ($P < 0.0001$).

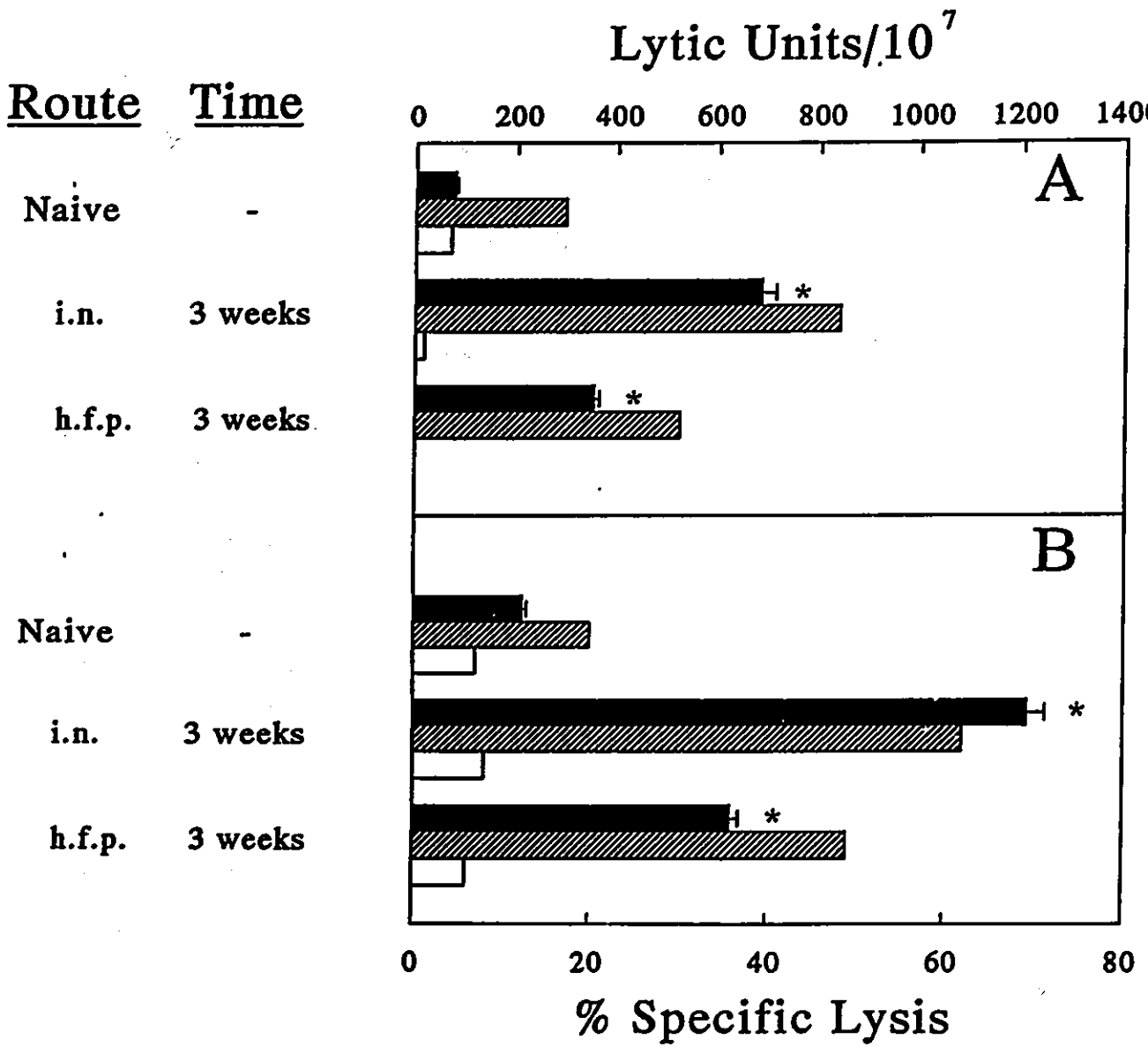
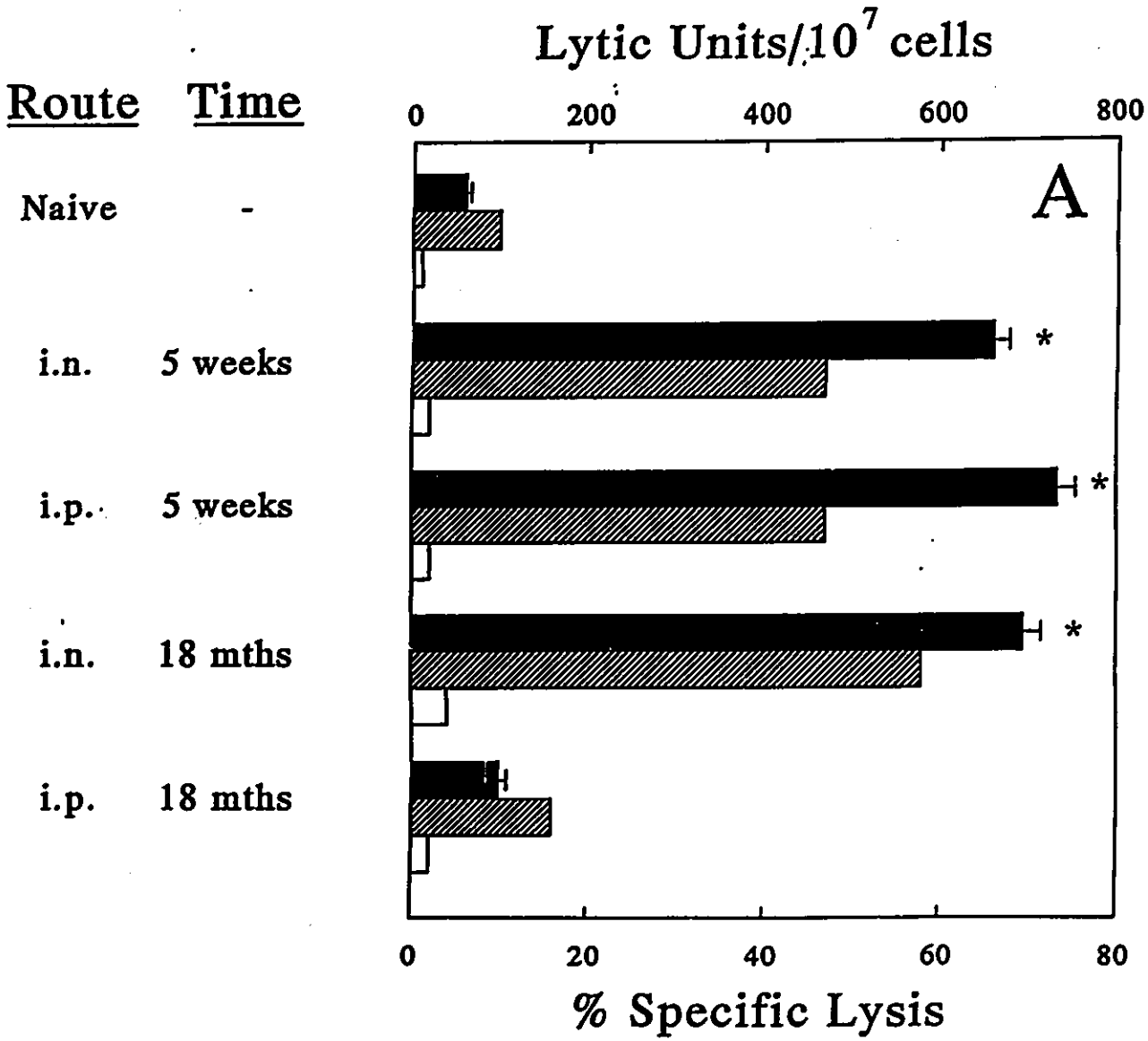

Route
Time
Naive
-
i.n.
3 weeks
h.f.p.
3 weeks
Naive
-
i.n.
3 weeks
h.f.p.
3 weeks
Lytic Units/10 7
0
200
400
600
800
1000
1200
1400
A
B
*
*
*
*
0
20
40
60
80
% Specific Lysis

Figure 5. Short and long-term CTL memory in the genital-associated lymphoid tissue (A and B) and spleens (C) of AdgB8 immunized mice. Individual mice in two separate experiments were intranasally (i.n.) or intraperitoneally (i.p.) immunized with AdgB8. At 5 weeks and 18 months (A) or 9 weeks and 15 months (B) post immunization mice were challenged intravaginally with HSV-2. Three days later the iliac lymph nodes (ILN) (A and B) draining the genital tract were isolated, cultured for 3 days without antigen stimulation, and examined for CTL activity against HSV-2 infected MC57 targets at E/T ratios of 40:1 and 40:1 + anti-CD3 (A and B). In addition, the lytic activity was determined and expressed in mean lytic units per 10^7 mononuclear cells ± SEM. *Significantly different from the naive and 18 month i.p. immunized mouse ($P < 0.0001$) (A) and from the naive and i.p. immunized mice at 9 weeks and 15 months ($P<0.0001$) (B). Figure C shows the CTL activity against HSV-2 infected targets from the spleens of mice in figure B. Splenocytes were stimulated as described in the Materials and Methods and incubated with targets at ET ratios of 40:1, 20:1, 10:1, 40:1 + anti-CD3 .


Route
Time
Naive
-
i.n.
5 weeks
i.p.
5 weeks
i.n.
18 mths
i.p.
18 mths
Lytic Units/10^7 cells
0
200
400
600
800
A
*
*
*
0
20
40
60
80
% Specific Lysis

Route	Time
Naive	-
i.n.	9 weeks
i.p.	9 weeks
i.n.	15 mths
i.p.	15 mths

Route	Time
Naive	-
i.n.	9 weeks
i.p..	9 weeks
i.n.	15 mths
i.p.	15 mths

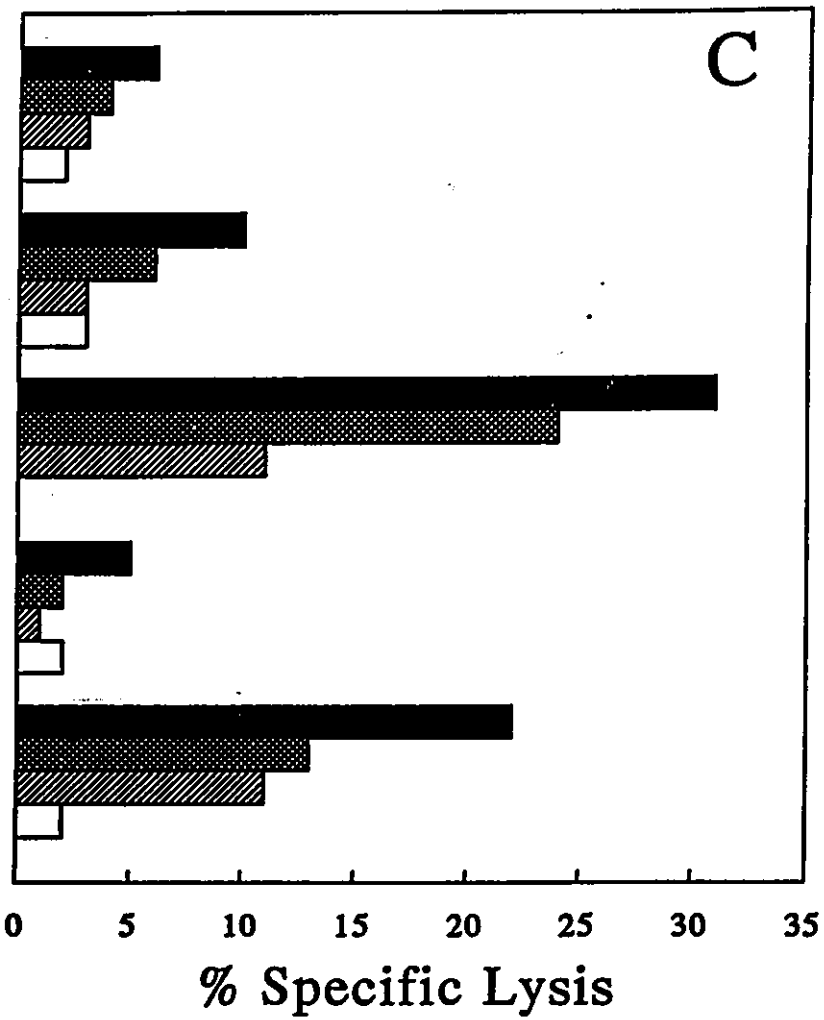


Figure 6. Specificity of long-term memory CTL in the genital-associated lymphoid tissue of intranasally immunized mice. Mice immunized 5 months previously with AdgB8 by the intranasal (i.n.), intraperitoneal (i.p.), or hind foot pad (h.f.p.) routes were challenged intravaginally with HSV-2. A naive (unimmunized) mouse was also challenged and served as a control representing the magnitude of a primary response. Two days later the MLNs were isolated, incubated for 3 days without antigen stimulation, and examined for CTL activity against VacgB11 infected targets. The 40:1 ▨, and 40:1 + anti-CD3 E/T ratios are shown as well as the lytic activity ■ expressed in mean lytic units per 10^7 mononuclear cells ± SEM. * Significantly different from the naive, i.p. and h.f.p. immunized mice ($P < 0.0005$).

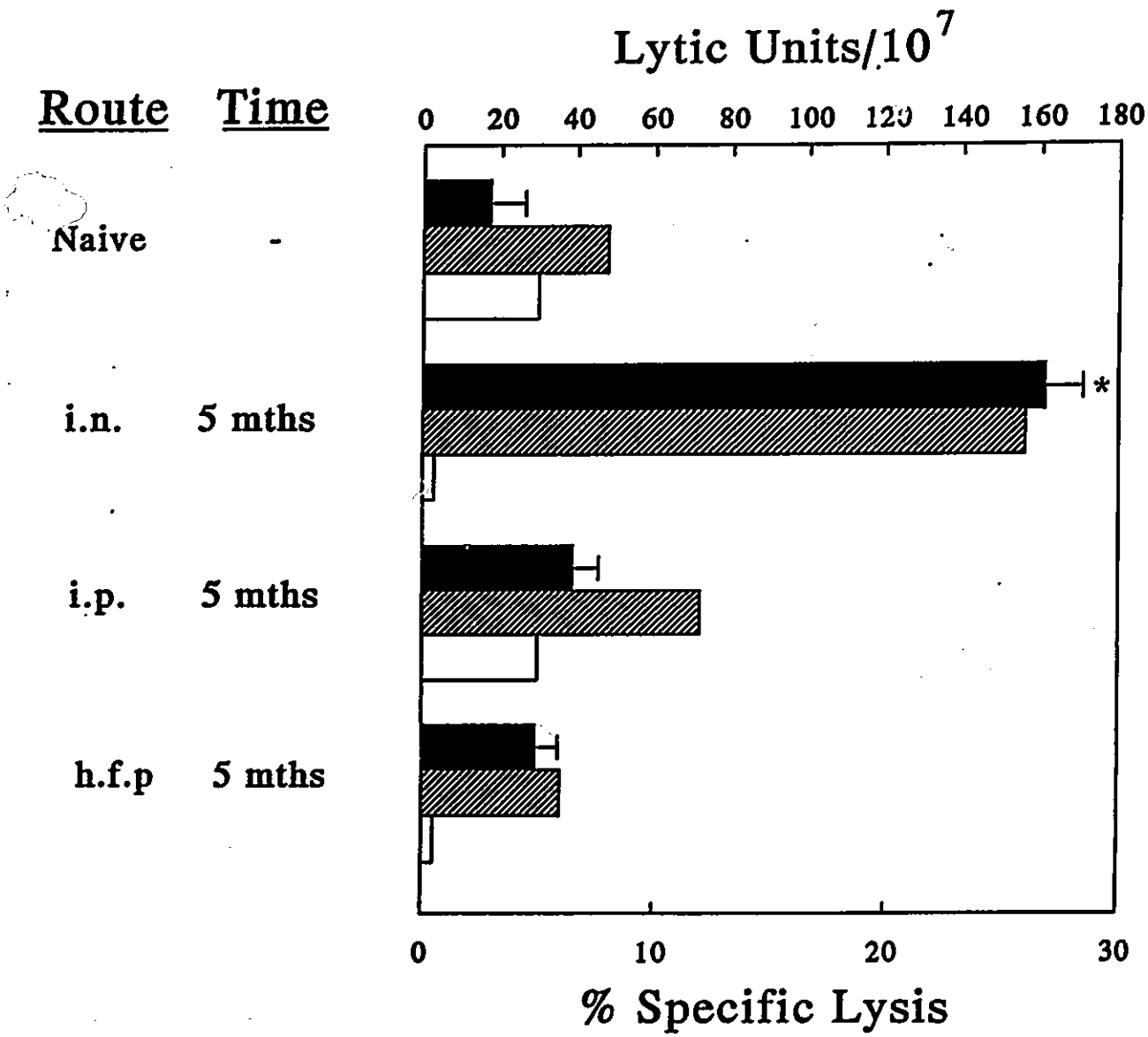

Route
Time
Naive
-
i.n.
5 mths
i.p.
5 mths
h.f.p
5 mths
Lytic Units/10^7
0
20
40
60
80
100
120
140
160
180
*
0
10
20
30
% Specific Lysis

Table 1. Cytotoxic T Lymphocyte Memory Responses in the Spleen and Iliac Lymph Nodes of Mice Immunized One Year Previously With AdgB8

Route of AdgB8 Immununization[a]	ILN		Spleen		
	Reciprocal CTLp Frequency (95% CI)[b]	Probability[c] (P)	CTL Lysis at 80:1 (SD)[d]	Reciporcal CTLp Frequency (95% CI)	Probability (P)
Intranasal	3411 (2645-4924)	0.99	3 (0.6)	667609 (421812-1599898)	0.82
	5946 (4497-8774)	0.41	2 (2.6)	325543 (325543-1006668)	0.52
Intraperitoneal	23607 (18271-33346)	0.84	52 (1.0)	66265 (51694-92273)	0.99
	30391 (23431-43232)	0.87	50 (3.4)	55068 (43388-75351)	0.54
	31006 (23790-44505)	0.76	37 (2.6)	72671 (55262-106092)	0.55
Hind Foot Pad	23555 (17586-35655)	0.70	75 (6.3)	95959 (67974-163100)	0.19
	15643 (12002-22453)	0.55	72 (6.8)	94844 (70597-144460)	0.72
Unimmunized	26106 (20118-37169)	0.71	-	-	
	23899 (18365-34208)	0.51	-	-	
	35350 (26590-52717)	0.99	-	-	

[a] One year following AdgB8 immunization mice were challenged intravaginally with 2×10^7 pfu of HSV-2 and 60 hours later the lymphocytes from the spleens and iliac lymph nodes (ILN) were isolated and the CTLp frequencies were determined in a limiting dilution assay. ILNs were cultured under expansion conditions without exogenous antigen.

[b] Reciprocal frequency of culture wells exhibiting positive cytolytic activity (3 standard deviations above the mean values obtained from cultures without responders) were determined by minimal χ^2 analysis. Each frequency and 95% confidence interval (CI) represents an individual mouse (2-3 per group) and all animals were analyzed together in one experiment against VacgB infected targets.

[c] Probability of obeying single-order kinetics, based upon χ^2 analysis for n-1 degrees of freedom, where n was always greater than 5 for ILNs and 4 for splenocytes and represents the number of responder cell dilutions tested.

[d] CTL mediated lysis was determined following bulk stimulation of splenocytes as described in the materials and methods.

Table 2. Cytotoxic T Lymphocyte Memory Responses in the Spleen and Iliac Lymph Nodes of Mice Recently Immunized with AdgB8

Route of AdgB8 Immunization[a]	Time Following Immunization	ILN		Spleen	
		Reciprocal CTLp Frequency (95% CI)[b]	Probability (P)[c]	Reciprocal CTLp Frequency (95% CI)	Probability (P)
Intranasal	2 weeks	4680 (3510-7000)	0.72	145600 (98510-278900)	0.10
		4030 (3030-5990)	0.44	95020 (64570-179900)	0.21
		6850 (5300-9650)	0.94	86470 (61670-144600)	0.61
	12 weeks	2520 (1980-3490)	0.33	127560 (91100-212800)	0.16
		5150 (3860-7760)	0.40	101890 (72000-174100)	0.26
		3590 (2850-4860)	0.88	142150 (100100-245100)	0.52
Intraperitoneal	2 weeks	13820 (10590-19900)	0.60	46130 (32180-81410)	0.32
		15310 (11530-22790)	0.97	40230 (27600-74170)	0.20
		13140 (10140-18630)	0.74	47900 (32530-90800)	0.64
	12 weeks	29290 (22090-43450)	0.64	29050 (19970-53260)	0.17
		17830 (13390-26680)	0.80	49880 (35360-84620)	0.08
		27170 (20920-38720)	0.84	45590 (31300-83890)	0.05

[a] Two or twelve weeks following AdgB8 immunization mice were challenged intravaginally with 2×10^7 pfu of HSV-2 and 60 hours later the lymphocytes from the spleens and iliac lymph nodes (ILN) were isolated and the CTLp frequencies were determined in a limiting dilution assay. ILNs were cultured under expansion conditions without exogenous antigen.
[b][c] See Table 1 legend.

2. Contributions to Gallichan, W.S., and Rosenthal, K.L. (1996c)

(A) Growth and purification of AdgB8 and HSV-2.

(B) Immunization of mice and collection and processing of spleens and lymph nodes.

(C) Developed limiting dilution culture conditions for the optimal expansion of herpes specific CTL

(D) Analysis of CTL activity and precursor frequencies in the spleens and lymph nodes of AdgB8 immunized mice.

3. Summary

In Gallichan *et al.*, 1996c, we compared the maintenance of CTL memory in mucosal and systemic immune compartments following intranasal or systemic AdgB8 immunization. It was found that only mice immunized intranasally with AdgB8 maintained long-lived CTL recall or memory responses following an HSV-2 challenge in the respiratory or genital tract. These same mice however, contained very low frequencies of splenic CTL precursors and undetectable levels of cytolysis following bulk stimulation of splenocytes. In contrast, mice immunized intraperitoneally or in the hind foot pad maintained high frequencies of splenic CTL precursors that were readily detectable following bulk stimulation. However, these same mice lacked a CTL memory response in mucosal associated tissues following a mucosal HSV-2 challenge. Together, these results demonstrate that CTL memory can compartmentalize exclusively within the mucosal or systemic immune systems depending on the route of initial antigen exposure. This suggests that the development of vaccines that are intended to induce

long-lived protective CTL responses will require initiation within mucosal tissues.

Short-lived CTL responses in were also examined following AdgB8 immunization in systemic and mucosal immune compartments. In confirmation of our previous studies we demonstrated here that mice immunized intranasally or systemically with AdgB8 initially developed strong splenic CTL responses. It wasn't until several months following intranasal immunization did levels of splenic CTL decrease. Similarly, mice immunized intraperitoneally with AdgB8 developed short term mucosal CTL memory responses, however, these too dissipated within several weeks following immunization. Therefore, shortly following AdgB8 immunization, memory CTL are not exclusively restricted to the immune compartment in which they were induced, however, within weeks to months there is a compartementalization that occurs. These results suggest that immune induction within the systemic immune system may lead to long-term CTL immunity in systemic tissues, but a lack of long-term immunity in mucosal tissues. The converse may also be true.

CHAPTER 7

SUMMARY

The aim of the studies carried out throughout this thesis work was to gain a better understanding of the immunological requirements for induction and maintenance of specific and protective immune responses in mucosal tissues. As a mucosal immunogen, these investigations utilized a recombinant adenovirus vector (AdgB8) capable of expressing the glycoprotein B gene of HSV-1. Adenovirus infections typically involve mucosal surfaces and result in subclinical or mild disease. Extensive studies of the molecular biology of adenoviruses has resulted in the development of recombinant adenovirus vectors capable of expressing foreign genes (Graham and Prevec, 1990). As discussed in the introduction, several animal models have employed adenovirus vectors as systemic vaccines, resulting in the induction of protective antibody and cellular immune responses within the systemic immune system. However, despite the fact that adenoviruses are mucosal pathogens with proven mucosal immunogenicity (Ogra *et al.*, 1980), and wild type adenoviruses have been used successfully as human oral vaccines against adenovirus-associated acute respiratory diseases (Chaloner-Larsson *et al.*, 1986; Top, 1975; Top *et al.*, 1971b), the induction of mucosal immune responses against foreign gene products contained in adenovirus vectors following intranasal administration remained to be investigated. The series of studies, described in the preceding chapters, examined intranasal and systemic routes of AdgB8

immunization for the induction of local and distal mucosal immune responses and protection against mucosal HSV-2 infection. The underlying premise of this thesis was based on the connectivity that exists between the mucosal tissues that make up the common mucosal immune system. The observation that the recirculation patterns of memory/activated lymphocytes are selective for the tissue compartments in which they were induced, likely accounts for this connectivity, and suggests that mucosal immunization is likely to result in the induction and maintenance of mucosal specific immune responses and optimal protection against mucosal pathogens. A discussion of the findings presented in the preceding chapters that are pertinent to this thesis is provided below.

As mentioned in the introduction, secretory IgA is the primary isotype found in mucosal secretions and is largely responsible for mediating protection against mucosal pathogens. Glycoprotein B of HSV is known to elicit protective antibody responses and McDermott *et al.*, (1989) demonstrated that intraperitoneal immunization with an adenovirus construct, AdgB2, resulted in serum neutralizing antibodies specific for gB of HSV-1 and 2. Therefore, following the first series of immunizations with AdgB8, mucosal tissues and surfaces were examined for the presence of gB-specific IgA. Nasal and lung washes of mice immunized intranasally but not intraperitoneally were shown to contain high levels of gB-specific IgA antibodies (Chapter 2; Fig. 2 and 3).

Some time previous to these studies, Waldman *et al.*, (1968, 1970) and Kasel *et al.*, (1969) demonstrated that intranasal application of inactivated influenza virus to volunteers resulted in local IgA antibodies. Several human studies since then have confirmed these

findings as well as noting the ability of natural or live attenuated influenza infections to induce local IgA production (Clements *et al.*, 1983; Clements and Murphy, 1986; Johnson *et al.*, 1986). Prior to reporting our observations, intranasal immunization of animals with live influenza virus (Novak *et al.*, 1992) or vaccinia recombinants (Meitin *et al.*, 1991) resulted in the production of local IgA, and a human trial reported that adding intranasal live attenuated cold-adapted influenza A vaccine to inactivated influenza vaccine provided additional protection (Treanor *et al.*, 1992). Therefore, these studies provided the biological bases for intranasal immunization, and it is not surprising that adenovirus vectors induced local IgA antibodies specific for the inserted antigens, especially considering the proven mucosal immunogenicity of wild type adenoviruses (Ogra, 1980). Nevertheless, the report by Gallichan *et al.*, (1993) (Chapter 2) represents the original study demonstrating the induction of antigen-specific IgA in the respiratory tract following intranasal administration of a recombinant adenovirus vector. Lubeck and collaborators immunized chimpanzees intranasally with an adenovirus vector expressing HIV antigens and, in confirmation of our findings, reported the presence of IgA in the respiratory tract (Lubeck *et al.*, 1994). Van Ginkel *et al.*, (1995), also demonstrated the mucosal immunogenicity of recombinant adenovirus vectors by observing local β-gal-specific IgA antibodies following intranasal administration of an adenovirus vector expressing β-galactosidase. Baca-Estrada *et al.*, (1995) used recombinant adenovirus vectors encoding haemagglutonin-esterase (HE) glycoprotein to induce anti-HE IgA antibodies in lung washes of cotton rats. Other live vaccine models using the intranasal route of immunization with vaccinia, influenza (Muster

et al., 1995), and Salmonella (Hopkins *et al.*, 1995) have since reported on the successful induction of IgA in the respiratory tract. Intranasal immunization with non-replicating vaccines utilizing cholera toxin (Walsh, 1994; Tamura *et al.*, 1992a, 1992b; Wu and Russell, 1993; Israel *et al.*, 1992), LTB/IL-2 conjugates (Hazama *et al.*, 1993) or liposomes (Gizurarson *et al.*, 1995) as adjuvants, have also demonstrated the successful induction of IgA in the respiratory tract. Many of the studies utilizing the intranasal route of immunization have reported the presence of specific serum antibodies. Following intranasal immunization with AdgB8, serum anti-gB IgG antibodies were observed and although titres were initially lower than in mice immunized intraperitoneally, several months later the titres had decreased in both groups to similar levels (Chapter 2; Fig. 1) (Chapter 3; Fig. 4). Neutralizing capacity was also similar to mice immunized intraperitoneally and to titres reported by McDermott *et al.*, (1989) (Chapter 2; Table 3). These results demonstrate that the intranasal administration of adenovirus vectors results in humoral immunity, both locally and systemically, providing the grounds for the development of vaccines against respiratory infections utilizing recombinant adenovirus vectors.

Interestingly, one major concern about using adenovirus vectors for repetitive gene delivery to lung epithelial cells is the induction of an immune response to the vector. Although targeted delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to the lung by an adenovirus vector has proven successful (Rich *et al.*, 1990; Rosenfeld *et al.*,, 1992; Zabner *et al.*, 1993), the results presented here, as well as those of other groups (Lubeck *et al.*, 1994; Van Ginkel *et al.*, 1995), suggest that the induction of local immune

responses may interfere with persistence of foreign gene expression and repetitive vector delivery. Since the gB gene in AdgB8 is inserted into the E3 region, AdgB8 is a replication competent vector. Arguably, replication defective vectors containing E1 deletions or inserts (Bett *et al.*, 1994), with perhaps other mutations, may not induce the same degree of immunity and be somewhat more successful as repetitive gene delivery vehicles.

The intranasal route of immunization has generated a lot of recent interest, due largely to the ability of this route to generate specific mucosal humoral immune responses locally, and more recently, in the female genital tract. Bacterial vectors (Pal *et al.*, 1994; Hopkins *et al.*, 1995), viral vectors (Gallichan and Rosenthal, 1995; Muster *et al.*, 1995), and cholera toxin or liposome formulations (Wu and Russell, 1993; Staats *et al.*, 1996; Haan *et al.*, 1995), have all successfully induced IgG and IgA antibody responses against target antigens in the female genital tract following intranasal immunization. Oral immunization with a number of live and protein antigen formulations has also been shown to induce IgG and IgA antibodies in genital secretions.

Since the main focus of our studies has been on immunity to HSV, a sexually transmitted pathogen, we therefore examined the genital tract for the presence of humoral immune responses following AdgB8 immunization. Vaginal washes of mice immunized intranasally, but not intraperitoneally or intravaginally were shown to contain anti-gB IgA antibodies (Chapter 3; Fig. 1 and 2). In contrast, anti-gB IgG antibodies were found in mice immunized by systemic or mucosal routes (Chapter 3; Fig. 3). Although antibody responses were detectable in vaginal washes for over a year, levels tended to decrease over the four months

following AdgB8 immunization. In a similar report, intranasal immunization with an adenovirus vector was shown to induce IgG and IgA antibodies in vaginal secretions of chimpanzees (Lubeck *et al.*, 1994). Several administrations of Ad-HIV, using different adenovirus serotypes, plus boostings with an HIV-subunit vaccine were required for the generation of HIV-specific IgA immune responses in the genital tract. Interestingly, as in our studies, the levels of specific IgA antibodies decreased over a 4 month period. The decrease in specific mucosal IgA antibody levels that apparently occurs with time in the female genital tract, likely reflects a decrease in the ability to maintain a high level of sterile immunity. Therefore, perhaps it is more relevant, when measuring immunity in the female genital tract following immunization, to examine the development of IgA antibodies and ASCs in response to an intravaginal challenge. Nevertheless, these results demonstrate the possibilities of using the intranasal route of immunization for providing immunity in the female genital tract.

In examining secretory immunity in the female genital tract it has been argued that there is a regionalized preference for immune induction that requires immunization in anatomically local mucosal compartments such as the intestinal tract or colon (Pierce and Cray, 1982). However, the results presented above as well as the protection studies and recall responses discussed later suggest that immunity initiated in the respiratory tract is capable of being expressed in the female genital tract. Furthermore, examination of the ratios of specific antibodies (Chapter 3; Table 1) in the serum and vaginal washes of intranasally immunized mice revealed that gB-specific IgG to IgA ratios were lowest in vaginal washes. The ratios

of gB-specific IgA to total IgA antibodies were highest in vaginal washes. These observations are consistent with the view that anti-gB IgA antibodies found in vaginal washes were secretory in nature and produced, in part, by local IgA plasma cells present within the genital lamina propria. These results do not disagree with the observations that a greater connectivity may exist between the intestinal and genital tract when compared to the respiratory and genital tract. They do however provide additional immunological evidence for the presence of a respiratory to genital tract exchange or immunological connection.

It is not surprising that specific immunity originating in the respiratory tract is expressed in the female genital tract. First, recent reports have provided evidence that the female reproductive tract has characteristics of mucosal effector tissues including IgA-producing cells, secretory component, and J chain (Kutteh and Mestecky, 1994; Parr and Parr, 1994). Second, as in the gut, the respiratory tract contains MALT (Kuper *et al.*, 1992), including the BALT and NALT. Intranasal administration of adenovirus vectors have been shown to infect respiratory epithelium and express inserted transcripts (Rich *et al.*, 1990; Rosenfeld *et al.*, 1992; Zabner *et al.*, 1993), and the isolation of humoral responses in lung and nasal tissues and lymph nodes shortly after immunization (Van Ginkel *et al.*, 1995) suggests that these immune responses were locally initiated within the BALT and NALT. Induction of effectors in the MALT is known to result in the dissemination of responses to other mucosal effector organs within the common mucosal immune system (McDermott and Bienenstock, 1979).

During investigations of antibody levels in vaginal washes of mice immunized intranasally with AdgB8, there appeared to be large fluctuations in gB-specific IgG and IgA levels from

day to day. As mentioned in the introduction, total antibody levels have been shown to be affected by the menstrual or estrous cycle. By examining the rat, Wira and collaborators found that the sex hormones estrogen and progesterone were responsible for these changes by directly affecting SC expression in the epithelium (Wira *et al.*, 1994). SC is responsible for transporting IgA across the epithelium and both components have been observed in the fluids of the uterus. Our studies concerning antibody levels with correlation to the stage of the estrous cycle, revealed that anti-gB IgG and IgA antibody titres in vaginal washes appeared highest during diestrus and estrous, respectively (Chapter 4; Fig. 1 and 2). Examination of the ratios of anti-gB IgG to IgA antibodies confirmed that during estrous, IgA levels were relatively higher than IgG levels and conversely, during diestrus, IgG levels were relatively higher than IgA levels. The same appeared true for total antibody levels (Chapter 4). These results are consistent with the findings of Wira and Sandoe, (1980, 1977), and Steele and Wira, (1989), who found that during the course of the estrous cycle, total IgA and IgG levels in the uterus were highest during proestrus, but unlike the IgG levels which sharply decreased during estrus, total IgA levels remained high and in fact have been reported to increase from levels during diestrus (Steele and Wira, 1989). Wira and Sandoe (1989) also examined specific anti-SRBC antibody levels and found an increase in specific IgA antibodies in the uterus following estradiol treatment. Estradiol levels increase during proestrus and estrus and therefore these results are consistent with our observations. These results are also consistent with the early observations of McDermott and Bienenstock (1980) demonstrating that B immunoblasts, and especially cells containing IgA, predominantly enter the genital tract during

proestrus and estrus. *In vivo* examination of IgA and IgG antibody levels following progesterone treatment in (Chapter 4) revealed a dramatic increase in anti-gB IgG levels with an even more dramatic decrease in anti-gB IgA levels in vaginal fluids. The changes in antibody levels are coincident with the animals entering into diestrus and consistent with the IgG and IgA antibody levels observed during natural diestrus (Chapter 4; Fig. 1 and 2). Therefore, specific anti-gB IgA and IgG levels appear to predominate during different stages of the estrous cycle and this is likely a reflection of the effects of hormones on SC expression and lymphocyte migration.

These findings have profound immunological implications. Currently, immunity in the female genital tract is believed possible following systemic immunization and, as reviewed in the introduction, generally results in IgG antibodies in sera and mucosal secretions. In addition, current immunotherapeutic approaches to controlling recurrent HSV-2 infections employ systemic administration of antigen (see section C). However, although IgG antibodies induced following systemic immunization may participate in protection against infections of the female genital epithelium (Whaley *et al.*, 1994; Eis-Hubinger *et al.*, 1993), our results imply that IgG and IgA mediated immunity at epithelial surfaces of the genital tract may predominate or be restricted to distinct stages of the estrous cycle. It is interesting to note that protection studies in the female genital tract generally require progesterone treatment to render animals susceptible and which, as we have demonstrated, increases IgG levels and takes IgA-mediated immunity out of the protection equation. Therefore, in order to maintain a blanket of humoral immunity at the epithelial surface of the genital tract, throughout the

estrous cycle, the expression of both IgA and IgG antibodies may be necessary. The observations presented here indicate that intranasal, but not systemic immunization, can fulfil this requirement.

In an effort to investigate the participation of memory/activated B cells in providing humoral immunity in genital tissues during an intravaginal challenge with HSV-2, in which sterile immunity is overcome, genital tissues and iliac lymph nodes were examined for ASCs producing anti-gB antibodies. Milligan and Bernstein (1995) have previously reported the presence of ASC in the genital tissues of mice immunized intravaginally with TK$^-$ HSV-2. Their work demonstrates that immunized mice challenged intravaginally with HSV-2 contained HSV-2 specific IgG and IgA ASC in the genital mucosa and lymph nodes. Data in Chapters 4 and 5 demonstrate that both IgG and IgA anti-gB ASC were also present in genital tissues of mice immunized intranasally with AdgB8 following an intravaginal HSV-2 challenge. In contrast, only anti-gB IgG ASC were present in mice recently immunized intraperitoneally with AdgB8. These observations represent one of the first demonstrations that intranasal immunization can provide specific IgA ASCs in the genital tissues during an infection. The discrepancy between intranasal and intraperitoneal immunization for the generation of IgA ASC in genital tissues indicates an obvious advantage for the former route of immunization with regards to protection against intravaginal HSV-2 infection. These results also indicate that despite being in diestrus (progesterone induced), which is characterized by a lack of secreted IgA (Chapter 4) and plasma cells (McDermott and Bienenstock, 1980; Parr and Parr, 1994; Wira *et al.*, 1994), gB-specific IgA ASC are able to

enter the genital tissues in response to HSV-2 infection. The mechanism responsible for this may be due to the observation that IFN-γ and IL-6 have been shown to up-regulate SC and IgA levels in the genital tract (Wira *et al.*, 1994). IFN-γ is involved in the control of many acute viral infections, including herpesviruses (Bouley *et al.*, 1995; Karupiah *et al.*, 1990; Karupiah *et al.*, 1993; Klavinskis *et al.*, 1989; Smith *et al.*, 1994). Further, HSV-1 infection of a murine epithelial cell line has been shown to selectively enhance IL-6 expression at the mRNA and protein level (Kanangat *et al.*, 1996), implying that HSV-2 infection of epithelial cells and the resulting inflammation is likely responsible for enhanced IgA-mediated humoral immunity, even during diestrus.

It is important to note that despite the fact that intraperitoneal immunization did not result in detectable anti-gB IgA ASCs in genital tissues following HSV-2 infection, the ILNs did contain significantly more anti-gB IgA ASCs than did naive challenged mice. These results suggest that while mice immunized intraperitoneally with AdgB8 may not contain anti-gB IgA memory/activated B cells, their immune systems are apparently primed for the ability to rapidly generate specific IgA ASCs. This is likely due to the presence of memory T cells which are not present in naive animals and which are therefore capable of supplying immediate help in the development of IgA producing B cells.

Investigations of immunity to HSV have indicated that protection is best mediated by a coordinated immune response involving humoral and cellular immune functions. Whereas HSV specific IgG and IgA antibodies have been implicated as an integral part of the first line of defence against infection (Balachandran *et al.*, 1982; Eisenberg *et al.*, 1985; Dix *et al.*,

1981; Rector *et al.*, 1982), cellular immune responses are essential in eliminating virus and controlling secondary infections or recrudescent episodes (Doherty *et al.*, 1992; Schmid and Rouse, 1992). In particular, virus-specific CTL can play a large role in mediating protection against HSV (Schmid and Rouse, 1992). In humans, cytotoxic T cells of the $CD4^+$ lineage tend to predominate in herpesvirus infections (Schmid, 1988; Yasukawa and Zarling, 1984), although $CD8^+$ have also been observed (Tigges *et al.*, 1992; Yasukawa *et al.*, 1989). In mice, $CD4^+$ cytotoxic T cells have also been observed and found to protect against HSV challenge (Doymaz *et al.*, 1991; Manickan *et al.*, 1995). $CD8^+$ T cells are however generally considered responsible for mediating cytotoxicity in mice and for providing protection against herpesvirus infections. The passive transfer of $CD8^+$ HSV-specific CTL enhances viral clearance in vivo if the donor lymphocytes are present prior to or immediately following infection (Bonneau and Jennings, 1989, 1990; Larsen *et al.*, 1983; Sethi *et al.*, 1983). In studying the development of cellular immunity following AdgB8 immunization we focused on $CD8^+$ T cell-mediated cytotoxicity with the understanding that protection against herpesviruses is multifactorial and involves other immune parameters such as cytokines, notably IFN-γ, in herpesvirus infections.

Previous studies have demonstrated that gB contains a major CTL epitope recognized by HSV-specific H-$2K^b$ CTL (Hanke *et al.*, 1991; Nugent *et al.*, 1994; Bonneau *et al.*, 1993), and immunization with AdgB2 results in the development of splenic CTL responses directed against gB (Witmer *et al.*, 1990; Hanke *et al.*, 1991). gB-specific CTL are also responsible for reducing infectious virus recovered during acute infection and from the dorsal root ganglia

(Bonneau and Jennings, 1990, 1989). Examination of splenocytes from mice immunized intranasally with AdgB8 found strong anti-HSV CTL responses specific for type 1 (Chapter 2; Table 1) and type 2 (Chapter 6) infected targets. Initial levels of splenic CTL-mediated lysis of targets was comparable to that found following intraperitoneal immunization. CTL precursor frequency analysis also suggested that CTL were present in the spleens of intranasally immunized mice, but were perhaps two-fold lower than in systemically immunized mice. Inspection of lymph nodes draining the respiratory tract shortly after immunization identified HSV-specific CTL in the mediastinal lymph nodes (MLN) of mice immunized intranasally, but not intraperitoneally (Chapter 6). HSV-specific CTL are not found in the spleen at this early time point following immunization, suggesting that following intranasal AdgB8 immunization, gB-specific CTL are generated in MALT and then migrate systemically to the spleen.

The obvious question at this point was whether gB-specific CTL induced following intranasal immunization migrate to local and distant mucosal tissues as a result of the common mucosal immune system. This is also important considering our emphasis on the generation of HSV-2 specific immunity in the female genital tract. To address this issue, we took advantage of the recall response that occurs following a second exposure to antigen and which can be observed in the draining lymph nodes of infected tissues (discussed in section D). A positive recall response is indicative of the presence of specific lymphocytes within that particular immune compartment. Alternatively, lymphocytes involved in a recall response may have access to the tissues involved in the infection. This has been clearly demonstrated by the

fact that predominantly memory-type T cells localize to inflammatory lesions, especially during the early phases (reviewed in Shimizu *et al.*, 1992). Although several groups have utilized the recall response in examining the spleen (Hill *et al.*, 1992; Mullbacher, 1994) or systemic tissues (Walker *et al.*, 1984; Nugent *et al.*,1994, 1995; Nash *et al.*, 1980) for the presence of memory/activated T lymphocytes, this concept has been rarely exploited to examine lymphocyte memory within mucosal tissues. In these experiments, AdgB8 immunized mice were challenged intranasally or intravaginally with HSV-2 and the development of HSV-specific CTL was monitored in the MLN and iliac lymph nodes (ILN), respectively. Recall or memory responses were observed in ILN and MLN shortly after immunization and were found to be specific for gB (Chapter 6). Although these results do not demonstrate that gB-specific CTL are continuously present within local and distant mucosal immune compartments, they do indicate that memory/activated CTL are able to participate in a recall response initiated within these tissues and are suggestive of their presence or recirculation in mucosal tissues throughout the mucosal immune system. Although these results may not seem unique since recirculation patterns of lymphocytes have established the connectivity of mucosal compartments (Butcher and Picker, 1996), this is perhaps the first functional demonstration that memory or recently activated lymphocytes can participate in a local or distal recall response in mucosal tissues.

Interestingly, mice immunized intraperitoneally with AdgB8 were also capable of mounting recall responses in mediastinal and iliac lymph nodes shortly after immunization (Chapter 6). These results are testimony of the ability of systemic immunization to provide

short-term immunity in mucosal tissues, and are reminiscent of the observations made by Offit *et al.*, (1991), and the model of immune maturation discussed by Ahmed and Gray (1996). Shortly after oral rotavirus infection, during the first and second phases of the immune response when effectors predominate, specific rotavirus-specific CTL are found in several systemic and mucosal immune compartments regardless of the route of immunization (Offit *et al.*, 1991). Close examination of surface receptors on $CD8^+$ T cells containing CTL activity following LCMV or Sendai virus infections shows that these lymphocytes are L-selectin$^-$ (memory phenotype) (Razvi *et al.*, 1995; Hou and Doherty, 1993), and the expression of various adhesion molecules is up-regulated on T cells activated *in vivo* by LCMV infection (Andersson *et al.*, 1994), suggesting a capacity for a broad tissue distribution (Butcher and Picker, 1996). Therefore, it appears that shortly following immunization, or during the first and second phases of the immune response, specific CTL are present throughout, or have access to, the immune compartments of the body regardless of the route of exposure and are capable of participating in recall responses.

Following immunization, the induction of both cellular and humoral immune functions specific for HSV is clearly desirable within mucosal tissues. Most importantly, the maintenance of these immune parameters within the tissues in which a second infection is likely to occur is a property that a vaccine intent on providing long-term immunity should elicit. In the introduction, the concept that the maintenance of immunological memory within distinct immunological immune compartments was dependent on the recirculation patterns of lymphocytes was developed. It was also pointed out that the development of an immune

response progresses through three phases; activation, death, and memory. The observations discussed above indicate that immune lymphocytes are present shortly after immunization in systemic and mucosal immune compartments regardless of the route of immunization. The period shortly after immunization, lasting several weeks, corresponds to the first and second phase of the immune response during which activated lymphocytes are still present (Ahmed and Gray, 1996). Interestingly, early observations on the maintenance of anti-HSV CTL in the spleens of mice immunized intranasally with AdgB8 indicated that several weeks to months after immunization, splenic CTL levels decreased dramatically (Chapter 2; Table 1). In contrast, strong CTL responses were observed for longer than one year in the spleens of mice immunized intraperitoneally. Taken together, these observations suggested that short-term CTL memory was independent of the route of antigen exposure, whereas the long-term maintenance of CTL memory in systemic immune compartments was dependent on systemic exposure. The counter argument implies that CTL induced in the mucosal immune system will be maintained in mucosal tissues.

A large part of this thesis originated from this hypothesis which states that the maintenance of CTL memory in distinct immune compartments is dependent on the recirculation patterns of memory lymphocytes and therefore on the tissues in which immune induction occurred. Again, the recall response was utilized to assess whether CTL memory existed within, or has access to, mucosal tissues. In Chapter 6, long-term CTL recall responses were observed in mucosal-associated immune compartments of mice immunized intranasally but not systemically with AdgB8. The CTL memory responses were maintained

for longer than one year in respiratory and genital associated lymphoid tissues (ie. MLN and ILN, respectively), and were confirmed by CTL precursor frequency analysis. The absence of significant CTL levels in the spleens of these same mice was also verified by CTL precursor analysis. Therefore, based on the ability to mount mucosal recall responses, and the presence or absence of systemic CTL, compartmentalization of CTL appears to occur in tissues as early as several weeks, and systemically up to three months following AdgB8 immunization. This time frame corresponds to the period involving the transition from the second to the third phase of the immune response (Ahmed and Gray, 1996). This period is characterized by the death of activated lymphocytes, which appear to recirculate nondiscreetly, leaving memory CTL to migrate to particular tissues depending on their original site of induction (Sprent, 1993).

Interestingly, recent studies on the surface phenotype of systemically induced LCMV-specific immune $CD8^+$ CTL, revealed that memory T cells were distributed into distinct subpopulations (Razvi *et al.*, 1995). The majority of memory CTL were L-$selectin^+$ and were either blast-size and cycling, or small-size and noncycling. In addition, a small-size, noncycling population was present that was L-$selectin^-$. The blast-size $CD8^+$ L-$selectin^+$ CTL are likely representative of a cycling population that has been shown to contain memory T cells (Sprent, 1993; Tough and Sprent, 1994; Mackay *et al.*, 1990; Mackay, 1993). Cycling is likely a result of being recently activated as a consequence of antigen stimulation (Gray and Matzinger, 1991; Oehen *et al.*, 1992), or in the absence of antigen (Jamieson and Ahmed, 1989; Lau *et al.*, 1994; Mullbacher, 1994), nonspecific stimuli such as cytokines or cross-reactive antigens

(Selin *et al.*, 1994), and/or perhaps as a consequence of recent migrational encounters in tissues (Butler and Picker, 1996). Blast-size $CD8^+$ L-$selectin^+$ CTL likely represent one of the states of memory T cells since they have been observed for as long as one year following LCMV infection (Razvi *et al.*, 1995), and long-term cycling T cells are considered a requirement for the maintenance of long-lived T cell memory (Sprent, 1993; Tough and Sprent, 1994; Mackay *et al.*, 1990; Mackay, 1993; Jamieson and Ahmed, 1989). These CTL may also represent a population of cells that have recently exited tissues were activation occurred. If this is true, then the small-size $CD8^+$ L-$selectin^+$ and $CD8^+$ L-$selectin^-$ lymphocytes may represent intermediate states and may contain CTL capable of entering tissues. The $CD8^+$ L-$selectin^-$ CTL may, in fact, contain lymphocytes with tissue specific homing patterns (Butler and Picker, 1996). Memory CTL that enter tissues would be visibly absent from the circulation. In the case of the results presented in Chapter 6, anti-gB CTL levels in the spleens of intranasally immunized mice decreased with time. This may be a reflection of activated lymphocytes dieing off during the second stage of the immune response (Gray and Ahmed, 1996; Sprent, 1993), and the migration of memory CTL to mucosal tissues. Certainly, this would explain the apparent absence of systemic CTL in mice immunized intranasally but not systemically, as well as the ability of intranasally immunized animals to mount rapid recall responses in mucosal-associated tissues during a mucosal infection. The small-size $CD8^+$ L-$selectin^-$ lymphocytes also express IL-2R and were found to be independent of the need for added IL-2 to develop into effectors (Razvi *et al.*, 1996), suggesting that this population is all the more ready to deal with antigen in tissues. The unique

results presented in Chapter 6 are therefore likely a functional demonstration of the maintenance of CTL memory in distinct immune compartments as a result of the homing and recirculation patterns of memory lymphocytes described in the literature (Butler and Picker, 1996; Mackay, 1993). These results provide a model for determining the requirements of tissue recirculation in the maintenance and presentation of T cell memory. Further examination of this phenomena by utilizing transgenic mice bearing specific antigen receptors may help delineate these issues. Owing to the recent and conflicting debate over the requirement for $CD4^+$ T cells in the maintenance of systemic CTL, the participation of $CD4^+$ T cells in maintaining the various subsets of memory CTL, systemically and in tissues should also be examined.

Long-term B cell memory responses were also examined in AdgB8 immunized mice. The results in Chapter 5 indicate that mice immunized intranasally but not systemically with AdgB8 were capable of rapidly mounting anti-gB IgA ASC in genital tissues. Anti-gB IgA ASC responses in ILN were also more numerous in intranasally immunized mice. Long-term anti-gB IgG ASC responses were present in the ILN of intranasally and intraperitoneally immunized mice, however intraperitoneally immunized mice lacked a long-term response in the genital tissues. These observations would suggest that intranasal AdgB8 immunization results in the long-term ability to mount IgA and IgG B cell memory responses in the tissues and lymph nodes draining the female genital tract. Intraperitoneal immunization does provide some level of B cell memory in the genital tract since, as mentioned previously, ASC responses in the ILN were far in excess of those observed in naive mice, however, responses

in the genital tissues themselves were clearly not as strong as in intranasally immunized mice.

The implications of the compartmentalization of CTL and B cell memory responses are several fold. First, the successful induction of long-term immunity in mucosal tissues, including the female genital tract, will likely rest on mucosal vaccination. Second, the visualization of T cell memory within the systemic immune system may be dependent on the route of antigen exposure. Third, examination of different routes of immunization for protection against mucosal pathogens may depend on how long following immunization protection is assessed. The second and third points may depend on whether or not the exposure to antigen occurs exclusively within one immune compartment. Apparently, the induction of immunity to adenovirus vectors in mice occurs within restricted immune compartments, perhaps as a consequence of the semipermissive nature of adenovirus infections in this species.

The final phase of this thesis examined protection against mucosal infections with HSV-2. These studies were carried out in an attempt to answer the above implications of the compartmentalization of immune responses as well as to compare the efficacy of intranasal to systemic AdgB8 immunization. Previous reports have clearly demonstrated that systemic, oral and local mucosal immunization can provide immunity in the respiratory and genital tract (see section C). In Chapter 2, mice immunized intranasally or intraperitoneally with AdgB8 were protected when challenged intranasally with HSV-2 eight weeks later. However, only mice immunized intranasally were significantly protected forty eight weeks post AdgB8 immunization. Superior local protection has also been observed in an influenza murine model

following intranasal immunization, however, protection was evaluated shortly after immunization (Tamura *et al.*, 1992a, 1992b).

To examine immunity in the female genital tract, protection models were developed that evaluated survival as well as the level and severity of genital pathology. Initial experiments revealed that mice immunized intranasally were significantly protected from an intravaginal HSV-2 challenge. These results are one of the few examples demonstrating that immunity to sexually transmitted pathogens in the female genital tract is possible following intranasal (Pal *et al.*, 1994; Furr and Taylor-Robinson, 1993), or intranasal plus systemic (Marx *et al.*, 1993) immunization.

Experiments examining the length of immunity following AdgB8 immunization clearly showed that survival and protection from genital pathology was long-lived following intranasal immunization. In contrast, although mice immunized intraperitoneally were initially protected following AdgB8 immunization, long-term survival and genital pathology was similar to that of controls. Interestingly, every immunized mouse infected intravaginally displayed signs of virus replication, regardless of whether or not they were ultimately protected or displayed any genital pathology. This indicates that sterile immunity was not observed for these challenge doses and that specific memory responses were likely responsible for mediating viral clearance. As discussed in the introduction, antigen-specific IgA antibodies and CTL are capable of clearing an established viral infection.

Taken together, these observations indicate that intranasal AdgB8 immunization provides long-lived secretory immunity (and IgG and IgA ASC) and CTL memory responses in the

female genital tract and long-term resistance against intravaginal HSV-2 infection. In contrast, intraperitoneal immunization induces serum derived IgG antibodies and short-term memory CTL and IgG ASC responses that were only present during the first and second phases of the immune response. Resistance against intravaginal HSV-2 infection in mice immunized intraperitoneally was also short-lived and this is likely attributable to the low levels of CTL and IgA ASC immune responses in mucosal tissues. These results therefore support the contention that intranasal immunization induces mucosal specific immune responses that are long-lived, maintained in mucosal immune compartments, and provide optimal protection, compared to systemic immunization, against local and distal mucosal infection.

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