MUCOSAL AND SYSTEMIC IMMUNE RESPONSES INDUCED BY IMMUNISATION OF COTTON RATS WITH RECOMBINANT ADENOVIRUSES

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ABSTRACT

Replication-defective and replication-competent recombinant human adenovirus type 5 vectors efficiently expressed the glycoprotein D (gD) or the transmembrane anchor truncated gD (tgD) of bovine herpesvirus type 1 (BHV-1) *in vitro*. To facilitate the evaluation of the efficacy of immunisation with these recombinant adenoviruses in conferring protection against BHV-1 infection, a cotton rat (*Sigmodon hispidus*) model for intranasal BHV-1 challenge was developed. I used this model to assess the ability of different routes of immunisation with the recombinant adenoviruses to elicit gD-specific systemic and mucosal immunity and confer protection against BHV-1 challenge. Immunisation with gD-expressing vectors induced better immunity and protection than immunisation with tgD-expressing viruses. Mucosal immunisation with the replication-competent virus was more efficient than that with the replication-defective vector in inducing gD-specific antibody in the serum and the respiratory tract. In contrast, systemic immunisation with the two vectors stimulated similar gD-specific antibody levels. These results indicate that the route of immunisation was crucial when assessing the efficacy of recombinant adenoviruses as vaccine vectors. The importance of the route of administration was further demonstrated by the finding that intranasal immunisation with the replication-competent vector stimulated higher antigen-specific IgA levels and antibody-secreting cell numbers in the respiratory tract than intradermal, intraperitoneal or enteric immunisation. Protection correlated with gD-specific antibody levels such that intranasal immunisation, even 3 months following vaccination, conferred complete, while intradermal or enteric immunisation conferred partial protection of the lungs of cotton rats against intranasal BHV-1 challenge. Pre-existing active adenovirus-specific immunity stimulated by intranasal administration of wild type adenovirus significantly inhibited the development of gD-specific antibody responses and protection against BHV-1 challenge

following immunisation with recombinant adenovirus. In contrast, passive transfer of adenovirus-specific antibody caused only a slight inhibition. Overall, mucosal and systemic immunisation with adenovirus vectors could induce antigen-specific immunity and protection against BHV-1 challenge. The level of gD-specific immune responses and protection from challenge were, however, dependent on the characteristics of the heterologous protein, the replication-capability of the viruses, the route of immunisation and the presence or absence of pre-existing adenovirus-specific immunity in the cotton rat.

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To cotton rat # 96-108-VIDO

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LIST OF ABBREVIATIONS

Ab	antibody
Ad	adenovirus
APC	antigen presenting cell
ARDS	acute respiratory distress syndrome
ASC	antibody-secreting cell
bAd	bovine adenovirus
BCV	bovine coronavirus
BHV-1	bovine herpesvirus type 1
BLV	bovine leukaemia virus
bPIV-3	bovine parainfluenza virus type 3
BSA	bovine serum albumin
CD	clusters of differentiation, designation for cell surface markers
CFTR	cystic fibrosis transmembrane conductance regulator
CMI	cell-mediated immunity
CMIS	common mucosal immune system
CRL cells	cotton rat lung cells
CTL	cytotoxic lymphocyte
dE1	E1-deleted
dE1E3	E1- and E3-deleted
dE3	E3-deleted
DNA	deoxyribonucleic acid
E1	early transcription region 1
E3	early transcription region 3
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
FBS	fetal bovine serum
GALT	gut-associated lymphoid tissue
gD	glycoprotein D
gB	glycoprotein B
gC	glycoprotein C
g.i.	gastrointestinal
GP	glycoprotein
h	hour(s)
HA	hemagglutinin
HAd	human adenovirus
HB	hepatitis B
HCMV	human cytomegalovirus

HE	hemagglutinin esterase
HIV	human immunodeficiency virus
HN	hemagglutinin neuraminidase
HPIV3	human parainfluenza virus type 3
HRSV	human respiratory syncytial virus
HRV	human rotavirus
HSV	herpes simplex virus
IBR	infectious bovine rhinotracheitis
i.cerebr	intracerebral
i.d.	intradermal, intradermally
i.duod	intraduodenal
IFN	interferon
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
i.m.	intramuscular, intramuscularly
i.n.	intranasal, intranasally
i.p.	intraperitoneal, intraperitoneally
i.t.	intratracheal
i.v.	intravenous, intravenously
i.vag	intravaginal
kbp	kilobase-pairs
kDa	kilo-Dalton
LCMV	lymphocytic choriomeningitis virus
LN	lymph node(s)
LP	lamina propria
mAb	monoclonal antibody(ies)
MALT	mucosa-associated lymphoid tissue
MDBK	Madin Darby bovine kidney
MEM	minimum essential medium
MHC	major histocompatibility complex
min	minutes
MLP	major late promoter
MOI	multiplicity of infection
MV	measles virus
NP	nucleoprotein
OD	optical density
PBS	phosphate buffered saline
PBS-T	PBS-tween
pfu	plaque forming unit(s)
p.i.	postinfection
PRV	pseudorabies virus
RNA	ribonucleic acid
RSV	respiratory syncytial virus

s.c.	subcutaneous, subcutaneously
SD	standard deviation
SI	stimulation index
SN	serum neutralising
SV40	simian virus 40
TBEV	tick-borne encephalitis virus
tgD	truncated gD
Th	T helper (lymphocyte)
UV	ultraviolet light
VP	viral protein
VSV	vesicular stomatitis virus
wt	wild-type

1.0 INTRODUCTION

Live recombinant human adenoviruses are an excellent delivery system for viral vaccine antigens (Imler, 1995, Rosenthal *et al.*, 1996). A great advantage of recombinant adenoviruses over other vaccination strategies is their capability to express large quantities of heterologous viral antigen *in vivo* in a similar manner as during a natural viral infection. Furthermore, adenoviruses naturally infect mucosal tissues, and therefore can induce mucosal immunity.

Induction of mucosal immunity is crucial in protecting against infections with mucosal pathogens such as bovine herpesvirus type 1 (BHV-1). Therefore, mucosal immunisation with recombinant adenoviruses that express BHV-1 proteins capable of inducing neutralising antibodies has the potential to serve as an effective vaccine strategy against infection and disease caused by BHV-1. Since immunisation of cattle with purified glycoprotein D (gD) of BHV-1 stimulates protective immunity against BHV-1 infection (Babiuk *et al.*, 1987), gD expressed by adenovirus vectors would serve as a good vaccine model to investigate mucosal immunity induced by recombinant adenoviruses.

Cotton rats (*Sigmodon hispidus*) have been widely used in immunisation and gene-therapy research involving recombinant human adenoviruses because cotton rats support human adenovirus replication (Pacini *et al.*, 1984). However, for evaluating gD-expressing adenovirus vectors in inducing protection against BHV-1 challenge, the animal model must also support BHV-1 replication. The development of a rodent model that supported the replication of both HAd5 and BHV-1 made it possible to investigate the efficacy of different routes of immunisation with replication-defective and replication-competent recombinant adenoviruses expressing gD in inducing gD-specific mucosal immunity and protection against mucosal BHV-1 infection.

2.0 LITERATURE REVIEW

2.1 Bovine herpesvirus type 1 and its control

Bovine herpesvirus type 1 (BHV-1), a member of the Alphaherpesviridae subfamily, can cause a variety of diseases in cattle including infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis, conjunctivitis, encephalitis, generalised systemic infections, and abortions (Gibbs & Rweyemamu, 1977, Yates, 1982). In addition, BHV-1 infection of the respiratory tract may render cattle susceptible to potentially fatal secondary bacterial pneumonia, a respiratory disease syndrome called "shipping fever" (Yates, 1982).

2.1.1 Conventional vaccines against BHV-1

Inactivated whole virus or attenuated live virus vaccines have been used commercially in the past decades to reduce economic losses due to BHV-1 infection (Tikoo *et al.*, 1995a). Killed virus preparations may be safer than attenuated live vaccines; one of their disadvantages has been, however, the need to combine them with strong adjuvants in order to induce protective immune responses. In addition, immunity to killed vaccines is usually of short duration.

Modified live virus vaccines have been developed by passage *in vitro* to produce attenuated isolates or by mutagenesis to produce temperature-sensitive mutants. They are used to control IBR in cattle and appear to induce some level of protective immunity to BHV-1 without the use of adjuvants (Gerber *et al.*, 1978, Jericho & Babiuk, 1983). They can be administered not only systemically, but also intranasally (i.n.) (Gerber *et al.*, 1978), providing the potential to induce strong local immunity in the respiratory tract. However,

these vaccines do not prevent the establishment of a latent infection or reinfection with wild type virus (Kahrs, 1976, Nettleton & Sharp, 1980, Pastoret *et al.*, 1980). Furthermore, they may induce abortions and adverse post-vaccinal reactions (Hyland *et al.*, 1974, Mitchell, 1974). An additional disadvantage of conventional killed or live attenuated vaccines is that it is impossible to differentiate, by serodiagnosis, between vaccinated and infected (e.g., latent carrier) animals, which may be crucial for eradication purposes.

2.1.2 New generation BHV-1 vaccines

Since conventional vaccines do not always prevent infection or disease, new strategies of vaccination have been investigated. Advanced molecular biological and biochemical techniques have enabled us to develop genetically engineered modified live virus vaccines, subunit protein vaccines, recombinant live viral vectors and polynucleotide vaccines. All these new generation vaccination strategies provide the means to differentiate between vaccinated and naturally infected, carrier animals.

Having identified viral encoded genes that influence the virulence of BHV-1, genetically engineered modified live viruses have been constructed by altering or deleting these genes (Tikoo *et al.*, 1995a). The advantages of this approach over conventional live viral vaccines are that the virus is attenuated without the possibility of backmutation.

Advances in protein chemistry and molecular biology have also resulted in the ability to identify, produce and purify large quantities of individual viral components necessary to elicit a protective immune response against the virus. It has been shown that a subunit vaccine consisting of one or several of the three major BHV-1 envelope glycoproteins (gB, gC and gD) induced a better level of immunity than a killed virus vaccine. This resulted in protection of all vaccinated cattle from mortality following challenge with BHV-1 and *Pasteurella haemolytica* (Babiuk *et al.*, 1987). To enhance the protective immune responses induced by subunit BHV-1 vaccines and to enable mucosal administration, lipid-based vehicles (e.g., liposomes) as delivery systems have also been investigated (Baca-Estrada *et al.*, 1997).

Delivering the purified subunit protein to the animal is not the only way to induce immunity to one particular viral component. A viral protein can also be delivered by using live recombinant viral vectors expressing the gene for the desired protein (Perkus & Paoletti, 1996). Depending on the choice of the live vector, this strategy often has the advantage of inducing strong mucosal immune responses following mucosal administration of the recombinant virus (discussed in more detail in 2.4).

Finally, the technique of DNA immunisation involves the *in vivo* delivery of the gene of a certain viral protein, usually in a circular plasmid form. DNA vaccines have induced cellular and humoral immune responses against different antigens, and protection against different diseases in animals (Hassett & Whitton, 1996, Shiver *et al.*, 1996). Genes of BHV-1 glycoproteins delivered intramuscularly (i.m.) or intradermally (i.d.) have also been shown to stimulate antigen-specific immune responses in cattle and mice (Cox *et al.*, 1993, Lewis *et al.*, 1997; Ralph Braun and Sylvia van Drunen Littel-van den Hurk, personal communication).

2.2 BHV-1 glycoproteins gD and tgD

Protection of cattle from disease following challenge with BHV-1 and *Pasteurella haemolytica* has been achieved by immunisation with a subunit vaccine consisting of BHV-1 gD (van Drunen Littel-van den Hurk *et al.*, 1993). Immunisation with gD was found to be more efficient in reducing viral replication and clinical signs, and inducing cellular immune responses than vaccination with other BHV-1 glycoproteins, such as gB and gC (Babiuk *et al.*, 1987, Hutchings *et al.*, 1990, van Drunen Littel-van den Hurk *et al.*, 1990, van Drunen Littel-van den Hurk *et al.*, 1993). Therefore, investigations have been concentrated on the further characterisation of gD *in vitro* and *in vivo* as a potential vaccine candidate against BHV-1.

2.2.1 The characteristics of gD and its roles in viral replication

Glycoprotein D is a homologue of herpes simplex virus 1 (HSV-1) gD (Leung-Tack *et al.*, 1994, Tikoo *et al.*, 1990) and is one of the major glycoproteins present in the envelope of BHV-1 virions and plasma membrane of virus-infected cells (Marshall *et al.*, 1986, van Drunen Littel-van den Hurk *et al.*, 1984). Its gene maps to the early transcription region of the BHV-1 genome and encodes a polypeptide of 417 amino acids (Tikoo *et al.*, 1990). Glycoprotein D is synthesised as a 63-kDa partially glycosylated precursor that leads to the formation of a 71 kDa mature protein, containing both N-linked and O-linked oligosaccharides (van Drunen Littel-van den Hurk & Babiuk, 1986). Analysis of purified gD by immunoprecipitation and Western blot assays indicated that gD produced by different mammalian expression systems can form dimers (Hughes *et al.*, 1988, van Drunen Littel-van den Hurk *et al.*, 1993, van Drunen Littel-van den Hurk *et al.*, 1997, van Drunen Littel-van den Hurk *et al.*, 1984). Glycoprotein D is essential for viral replication (Fehler *et al.*, 1992). It may be involved in attachment (Liang *et al.*, 1991), penetration (Chase & Letchworth III, 1994, Fehler *et al.*, 1992), and fusion with cells (Tikoo *et al.*, 1990). Some B lymphocyte (Tikoo *et al.*, 1993) and T lymphocyte epitopes (Leary & Splitter, 1990, Tikoo *et al.*, 1995b) have been identified on gD.

2.2.2 The rationale for the construction of truncated gD

Since gD remains mainly cell-associated following its production in BHV-1 infected cells, it must be extensively purified to be used as a vaccine. In addition, effective production of gD is problematic since gD is toxic to cells expressing it (Tikoo *et al.*, 1990). Recently, an economical method to produce gD has been developed by engineering the gD gene to function under the control of a bovine heat-shock protein 70 gene promoter, which regulates the expression of gD in transfected Madin Darby bovine kidney (MDBK) cells (Kowalski *et al.*, 1993). This system was designed to produce a transmembrane anchor truncated version of gD (truncated gD, tgD) in an expression system that is not cell-destructive. Truncated gD could be efficiently secreted into the medium of transfected

MDBK cells. The tgD produced by the heat-shock system is 61 kDa in molecular weight and is antigenically similar to the authentic gD glycoprotein when analysed *in vitro* by a panel of gD-specific monoclonal antibodies (mAb) (Kowalski *et al.*, 1993). Furthermore, immunisation of cattle with tgD conferred protection against BHV-1 challenge (van Drunen Littel-van den Hurk *et al.*, 1994).

The different cellular localisation of gD and tgD may result in different induction of immune responses. Questions about the influence of the antigen form on the induction of immune responses have been investigated in non-challenge mouse models: adjuvant formulation with the two subunit glycoproteins (Baca-Estrada *et al.*, 1996), DNA immunisation with the genes of gD and tgD (Lewis *et al.*, 1997; Braun, personal communication), and adenovirus-expressed tgD or gD (Papp et al., manuscript in preparation).

2.3 Recombinant adenovirus vectors

Live viral vectors provide an alternative for the delivery of individual viral antigens without the need for producing large quantities of purified protein. In addition, live recombinant viruses offer several other advantages compared to conventional or subunit vaccines. Most importantly, they provide the expression of the required antigen intracellularly – analogous to infection by the viral pathogen – without the danger of disease through loss of attenuation or improper inactivation of the vaccine.

Among the different viral vectors available (e.g., pox, polio, adeno), adenovirus (Ad) vectors are often preferred because of their relatively low pathogenicity, stability, efficient large-scale production and the ease of manipulation of their genomes. They may also be more efficacious than poxvirus vectors (vaccinia) in inducing mucosal immunity (Gonin *et al.*, 1996). Since adenoviruses are common in not only human but also in animal populations, research on their use may provide a potentially widely applied strategy for foreign gene delivery.

2.3.1 Infections caused by wild-type human adenoviruses

Adenoviruses constitute a large group of DNA viruses that infect mammals and birds. Human adenoviruses (HAd) are endemic in human populations around the world, with about a dozen out of the known 47 serotypes responsible for epidemic outbreaks of mainly respiratory and ocular infections. By the age of one year, more than 60 % of children have antibodies against at least one HAd serotype. By adulthood, 99% of the people are seropositive for HAd (for a review see Straus, 1984). The various Ad serotypes can infect and replicate at a number of locations in the body including the respiratory tract, the gastrointestinal tract, the eye and the urinary bladder (Horwitz, 1996, Straus, 1984). Adenovirus infection typically exhibits a short incubation period of 5-10 days, but in more than 30 % of the cases, Ad continue to be excreted in the feces for months or years following the initial infection (Fox *et al.*, 1969). It is not known whether abortive infection of certain cell types such as freshly isolated peripheral blood lymphocytes by Ad (Flomenberg *et al.*, 1997, Schranz *et al.*, 1979, Silver & Anderson, 1988) may be related to persistence or latency *in vivo*. Nevertheless, Ad infections often remain subclinical, rarely spreading beyond the draining lymph nodes (LN) of the site of primary infection. On rare occasions, however, patients have died from acute infection of the lungs or liver, or from widely disseminated infection.

Research interest began to focus on human adenoviruses after the finding that some serotypes were tumorigenic in rodent models; no association between Ad infection and human tumours, however, has been detected to date. Subsequent studies lead to the use of adenoviruses as the model of choice for molecular biologists to study DNA replication, RNA transcription and splicing, and the molecular basis of cell transformation. Hence the biology of these viruses is well known (Shenk, 1996).

2.3.2 Biology of human adenoviruses

Adenoviruses contain a single, double-stranded, linear DNA genome of approximately 30-40 kilobase pairs within a nonenveloped, icosahedral capsid. The

replication cycle of the virus can be divided into two phases. The early phase corresponds to events occurring before the onset of viral DNA replication, while the late phase corresponds to the period after initiation of DNA replication. During infection, the Ad virion enters the cell through receptor-mediated endocytosis and replication occurs in the nucleus, usually without integration of the viral DNA into the host genome. Late gene-expression occurs at approximately 8 hr post-infection (p.i.). Transcription of the genes of most of the viral structural proteins is mainly driven by the major late promoter (MLP). Although adenoviruses do not lyse infected cells, one cell can often produce 10,000 virions (Shenk, 1996). Cultured human cells generally support the complete HAd infectious cycle; most simian and rodent cells do not (Blair *et al.*, 1989). Interestingly, infection of monkey and rodent cells with HAd has shown that this restricted host range is not due to a lack of receptors. It is rather due to intracellular blocks arising from inappropriate interactions between cellular and viral components, for example, a block of an early step of viral DNA replication (Lucher, 1995). Therefore, early proteins are usually still expressed in cells of semipermissive species.

Human adenovirus type 5 (HAd5) possesses several advantages, which renders it a generally useful vector to express foreign genes in different mammalian systems. First, it replicates to high titres in human cells *in vitro*. The virions remain cell-associated well after the production of new virus is completed, which makes the concentration of large volumes of virus possible, simply by sedimenting infected cells. The handling and storage of adenoviruses are not difficult since the Ad virion is relatively stable. Furthermore, inserts of foreign genes are generally maintained without change through successive rounds of viral replication (Graham & Prevec, 1992).

2.3.3 Replication-defective and replication-competent HAd5 vectors

Recombinant human adenoviruses have become an attractive gene delivery system because of the ease of manipulation of their genome and the well-characterised methods for generating recombinant Ad vectors. To render the Ad virion capable of accommodating larger than 2 kbp of foreign DNA, either the early transcription region 1 or 3 (E1 and E3,

respectively) is most commonly deleted (Graham & Prevec, 1991, Graham & Prevec, 1992). Deletion of the E1 region results in a vector (dE1) that is replication-defective since E1 gene products are essential for the onset of DNA replication. Therefore, the vector can be propagated only in a cell line, which contains and expresses the left end of the Ad genome thus complementing the deficiency. Approximately 3 kbp can be deleted from E1, with a replacement insertion of up to 5 kilobase-pairs (kbp) of foreign DNA. Transcription of foreign genes inserted in the E1 deletion must be driven by a promoter introduced as part of the insert (Graham & Prevec, 1992). Since transcription of the foreign gene is completed before DNA replication, these vectors efficiently express their inserts in any normally HAd5 permissive or semi-permissive cell without the need for viral replication.

Deletion of the E3 region (dE3) does not dicrease the replication-competency of the vector *in vitro*, since E3 is not required for viral replication. E3-deleted vectors, therefore, can be propagated in any normally HAd5-permissive cells. The function of E3 region proteins is not known; they probably play a role in modulating the host immune response. For example, infection with dE3 vectors results in an increased inflammatory response in the lungs of cotton rats compared to inoculation with HAd5 that contains the E3 region (Berencsi *et al.*, 1994, Ginsberg *et al.*, 1990, Ginsberg *et al.*, 1989). It is possible that the absence of the 19 kDa glycoprotein encoded by the E3 region of HAd5, which is known to downregulate expression of MHC-I molecules on the surface of infected cells, results in an increase in the Ad-specific cytotoxic lymphocyte (CTL) response (Burgert & Kvist, 1987). Generally, dE3 vectors can accommodate 4 kbp of foreign DNA in their genome. In many replication-competent vectors used today, an SV40 promoter is introduced as part of the expression cassette. A heterologous promoter is not usually required, however, since expression of the foreign gene is efficiently driven by the MLP or E3 promoters (Graham & Prevec, 1992). Recombinant HAd5 systems are also used in which both E1 and E3 regions are deleted (dE1E3 vectors). They may accommodate up to 8 kbp of foreign DNA (Bett *et al.*, 1994, Bett *et al.*, 1993).

Recombinant HAd5 vectors have been used to express antigens from a wide variety of heterologous viruses (Perkus & Paoletti, 1996). High-level expression of foreign genes has been obtained from both dividing and quiescent cells of a variety of animal species,

including human and other primates, rodent, canine and bovine cells infected with HAd5 vectors. In recent years, Ad vectors have been studied as a recombinant vaccine delivery system against viral diseases (Imler, 1995) and tumours (Bischoff *et al.*, 1996, Chen *et al.*, 1996, Zhai *et al.*, 1996), and as gene transfer vectors for gene therapy (Bramson *et al.*, 1995, Brody & Crystal, 1994, Rosenfeld *et al.*, 1991).

2.3.4 Recombinant human adenoviruses as vaccine vectors

The popularity of human adenovirus as a vaccine delivery system is largely due to the successful and safe immunisation of millions of US military recruits with enteric coated wild-type (wt) HAd4 and HAd7 as a prevention against acute respiratory disease outbreaks (Top *et al.*, 1971a, Top *et al.*, 1971b). Following these first trials, a number of recombinant adenoviruses have been constructed and tested in animals. Since HAd5 has been studied extensively and is less pathogenic compared to HAd4 and HAd7, it is often the strain of choice for the development of recombinant vaccines.

Recombinant HAd5 vectors – even if unable to replicate – have proven to be effective in expressing foreign genes *in vitro* and *in vivo*. Systemic immunisation with both replication-competent and replication-defective Ad vectors has induced humoral and cell mediated immune responses to the expressed viral antigen (Table 2.1), (Imler, 1995). In many cases, immunisation resulted in protection from viral challenge (Table 2.1). Immunisation with recombinant HAd5 has even induced CTL to an intracellular parasite (malaria) antigen and protected mice against parasite challenge (Rodrigues *et al.*, 1997). Systemic immunisation alone, with recombinant HAd5 vectors, however, did not induce effective mucosal immune responses and protection from mucosal challenge (Table 2.1) (Gallichan *et al.*, 1993, Rosenthal *et al.*, 1996).

Since it is important to induce local immunity at the site of pathogen entry, it may be necessary to administer recombinant adenoviruses to mucosal surfaces. A great advantage of HAd5 vectors as a delivery system is that they naturally infect mucosal tissues (Prince *et al.*, 1993, Rosenfeld *et al.*, 1991); therefore, they have the potential to deliver the foreign gene effectively to mucosal induction sites. Indeed, imunisation with recombinant

Table 2.1 Replication-competent and replication-defective recombinant human adenoviruses as a vaccine delivery system

antigen	vector	animal	replic.	admin. route	immune response to antigen	challenge (route)	protect.	reference
HBsAg	dE3	hamster	+	i.n.	serum Ab	-		(Morin *et al.*, 1987)
		chimpanzee	+	oral	serum Ab		yes, d	(Chengalvala *et al.*, 1997, Lubeck *et al.*, 1989)
		dog	+/-	oral, i.t.	serum Ab	-		(Chengalvala *et al.*, 1994, Chengalvala *et al.*, 1991)
		man	+	oral	no Ab	-		(Tacket *et al.*, 1992)
		mouse	-	i.m.	serum Ab, splenic CTL	-		(He *et al.*, 1996)
VSV gp	dE3	cow	+	s.c., i.n.	serum Ab	-		(Prevec *et al.*, 1989)
		pig	+/-	s.c., i.n.	serum Ab	-		(Prevec *et al.*, 1989)
		dog	-	s.c., i.n.	serum Ab	-		(Prevec *et al.*, 1989)
		mouse	-	i.p.	serum Ab	i.v.	yes, d	(Prevec *et al.*, 1989)
RSV F,G	dE3	cotton rat	+	i.n.	serum Ab	i.n.	yes, i	(Collins *et al.*, 1990)
				i.duod.	serum Ab	i.n.	partial, i	(Collins *et al.*, 1990)
		chimpanzee	+	oral	serum Ab	-		(Hsu *et al.*, 1992)
		dogs	+/-	i.t.	serum Ab	i.n.	yes, i	(Hsu *et al.*, 1992)
		ferret	+	i.n.	serum Ab	i.n.	yes, i	(Hsu *et al.*, 1994)
HSV gB	dE3	mouse	-	i.p.	serum Ab	i.d.	yes, d	(McDermott *et al.*, 1989)
				i.p.	serum IgG, splenic CTL	i.n.	yes, d	(Gallichan *et al.*, 1993)
				i.p.	serum Ab, CTL	i.cerebr	yes, d	(Endresz *et al.*, 1995)
				i.n.	serum IgG, lung & nose IgA, splenic and med. LN CTL	i.n.	yes, d*	(Gallichan *et al.*, 1993)
				i.n.	high serum IgG, vaginal IgA	-		(Gallichan & Rosenthal, 1995)
				i.vag	low serum IgG, no vag. IgA	-		(Gallichan & Rosenthal, 1995)

Table 2.1 (cont)

antigen	vector	animal	repl.	admin. route	immune response to antigen	challenge (route)	protect.	reference
HSV gD epi.	dE3	mouse	-	i.p.	serum Ab	i.p.	partial, d	(Zheng *et al.*, 1993)
rabies gp	dE1E3	mouse	-	s.c.	serum Ab, CTL	s.c.	yes, d	(Xiang *et al.*, 1996)
				i.n.	serum Ab, CTL	s.c.	yes, d	(Xiang *et al.*, 1996)
				oral	no Ab	s.c.	no	(Xiang *et al.*, 1996)
	dE3	mouse	-	oral	serum Ab in some animals	i.cerebr	partial, d	(Prevec *et al.*, 1990)
				i.p.	serum Ab	i.cerebr	yes, d	(Prevec *et al.*, 1990, Yarosh *et al.*, 1996)
		skunk	-	oral	serum Ab	i.cerebr	yes, d	(Charlton *et al.*, 1992, Yarosh *et al.*, 1996)
				i.m.	serum Ab	i.cerebr	yes, d	(Charlton *et al.*, 1992)
		fox	-	oral	serum Ab	i.cerebr	yes, d	(Charlton *et al.*, 1992)
		dog	-	s.c.	serum Ab	-		(Prevec *et al.*, 1990)
				i.n.	serum Ab	-		(Prevec *et al.*, 1990)
HCMV gB	dE3	mouse	-	i.p.	CTL	-		(Berencsi *et al.*, 1993)
BCV HE	dE3	cotton rat	+	i.n.	serum and lung Ab, CTL, spleen cell proliferation	-		(Baca-Estrada *et al.*, 1995)
				i.duod	serum and intestinal Ab	-		(Baca-Estrada *et al.*, 1995)
HRV VP7	dE3	mouse	-	i.n.	serum Ab in dams and suckling neonates	oral	yes, d	(Both *et al.*, 1993)
				i.v., i.p., oral	serum Ab	-		(Both *et al.*, 1993)
bPIV F, HN	dE3	cotton rat	+	i.n.	serum Ab	i.n.	yes, i of lungs	(Breker-Klassen *et al.*, 1995)

Table 2.1 (cont)

antigen	vector	animal	repl.	admin. route	immune response to antigen	challenge (route)	protect.	reference
HIVEnv, Gag	dE3	chimpanzee	+	i.n.	serum, nasal, vaginal, salivary Ab, spleen cell proliferation, PBL CTL	-		(Lubeck *et al.*, 1994, Natuk *et al.*, 1993)
						i.v.	yes, i	(Lubeck *et al.*, 1997)
		dog	+/-	i.t.	serum Ab	-		(Natuk *et al.*, 1992)
PRV gD	dE3	cotton rat	+	i.m.	serum Ab	i.p.	yes, d**	(Eloit & Adam, 1995)
		mouse	-	i.m.	serum Ab	i.p.	yes, d	(Eloit & Adam, 1995)
	dE1E3	cotton rat	-	i.m.	serum Ab	i.p.	yes, d	(Eloit & Adam, 1995)
		mouse	-	i.m.	serum Ab	i.p.	yes, d	(Eloit & Adam, 1995, Gonin *et al.*, 1996)
				i.p.	serum Ab	i.p.	partial	(Ganne *et al.*, 1994)
				i.n.	serum Ab	i.n.	yes, d	(Gonin *et al.*, 1996)
	dE1E3	rabbit	-	i.m., i.n.	serum Ab	i.m.	partial	(Eloit *et al.*, 1990)
TBEV NS1	dE1E3	mouse	-	i.p.	no Ab	s.c.	yes, d	(Jacobs *et al.*, 1994)
MV N	dE1E3	mouse	-	i.p.	serum Ab, CTL	i.cerebr	partial, d	(Fooks *et al.*, 1995)
EBV env	dE1E3	cottontop tamarin	-	i.m.	serum Ab	i.m., i.p.	yes, d	(Ragot *et al.*, 1993)
HBsAg	dE1E3	chimpanzee	-	i.v.	no Ab, only priming	i.v.	partial	(Levrero *et al.*, 1991)
HC ag-s	dE1E3	mouse	-	i.p.	serum Ab	-		(Makimura *et al.*, 1996)
				i.p.	CTL	-		(Bruna-Romero *et al.*, 1997)
malaria CSP	dE1E3	mouse	-	s.c, i..m.	Ab, $CD8^+$ IFN-γ SC	i.v.		(Rodrigues *et al.*, 1997)
				ip, iv, i.n.	low Ab, no $CD8^+$ IFN-γ SC	-		(Rodrigues *et al.*, 1997)

repl. = replication-capability of vector in the animal; d = protection (protect.) from disease or death; i = protection from infection measured by viral recovery
* better and longer lasting protection than after i.p. immunisation; ** lower doses protect than dE1E3 immunisation or imm. in mice

adenoviruses in the respiratory as well as in the gastrointestinal tract has stimulated not only systemic but also mucosal immune responses to several foreign viral antigens (Table 2.1). Most of the studies using mucosal immunisation with recombinant viral vectors have focused on the generation of serum neutralising antibodies and short-term protection against challenge. Gallichan and co-workers (Gallichan *et al.*, 1993) also investigated the generation of herpesvirus-specific CTL and the duration of immune responses following immunisation with a dE3 recombinant HAd5 expressing gB of HSV-1. Long term HSV serum neutralising antibody responses were induced by both intraperitoneal (i.p.) and intranasal (i.n.) immunisation with the recombinant Ad, and splenic CTL responses were maintained for as long as 58 weeks following i.p. immunisation. They also found that i.n. administration was more efficient than i.p. immunisation in inducing mucosal IgA responses and protection against i.n. HSV challenge. In other studies, i.n. immunisation was more effective than systemic immunisation in inducing local Ad- and β–galactosidase-specific antibody production in the respiratory tract (van Ginkel *et al.*, 1995). The importance of local stimulation in the mucosa is further indicated by the finding that i.n. administration of recombinant HAd5 was more effective than gastrointestinal (g.i.) inoculation in inducing immunity in the respiratory tract (Collins *et al.*, 1990). However, immunisation with recombinant Ad at a distant mucosal site can be more effective than local stimulation. Intravaginal immunisation with recombinant Ad expressing HSV gB was less effective than i.n. immunisation in inducing vaginal gB-specific antibodies (Gallichan & Rosenthal, 1995).

2.4 The efficacy of recombinant viral vectors in the presence of antigen-specific immunity

Immunity to a pathogen can be passively derived (maternal antibodies) or actively acquired (natural infection). Both passive and active immunity can significantly modify infection, viral gene expression, and disease caused by viruses (Renegar & Small, 1994, Zinkernagel, 1993). Since the success of immunisation with recombinant live viral vectors

depends on infection with the virus followed by foreign gene expression, immunity to the vector is expected to influence the efficacy of vaccination with recombinant viral vectors.

2.4.1 Passive immunity to viral vectors

The level of both mucosal and systemic antibody responses to a viral infection that takes place in the presence of passively acquired IgG can be reduced significantly (Kimman *et al.*, 1987, Murphy *et al.*, 1986, Sabin *et al.*, 1963, Xiang & Ertl, 1992). The suppressive effect of virus-specific serum antibody on the induction of resistance to challenge is greatest when the virus vaccine is administered parenterally rather than mucosally (Murphy *et al.*, 1989). One possible explanation for less inhibition following mucosal immunisation is that serum IgG is less efficient in blocking mucosal than systemic viral infections. Therefore, the local induction of a mucosal IgA response, by mucosal infection, is less suppressed than is the induction of systemic IgG responses (Jayashree *et al.*, 1988, Kimman & Westenbrink, 1990).

Similar to the neutralisation of any virus by virus-specific antibodies, live recombinant viruses may be inactivated by antibodies before they could enter cells and express their foreign gene. Studies with recombinant adenoviruses in mice showed that passive transfer of Ad-specific antibody into the venous circulation blocked the expression of the heterologous gene in hepatocytes, but did not affect gene-transfer into the lung (Yang *et al.*, 1995a). In contrast, instillation of Ad-specific antiserum into the trachea blocked gene transfer to airway epithelia without affecting gene transfer to hepatocytes. Therefore, passively derived antibody is effective in neutralising virus depending on the site of the antibody transfer and virus infection.

Since neutralising antibody to the virus vector suppresses the expression of the foreign gene, it is likely that the induction of immune responses to the foreign gene product will be suppressed as well. Intraperitoneally administered vaccinia virus-specific antibodies inhibited, in a dose-dependent manner, HA-specific CTL responses following intravenous (i.v.) administration of a recombinant vaccinia virus expressing the HA protein of influenza (Johnson *et al.*, 1993). They had no effect on the humoral response to HA or vaccinia,

although inhibition was shown in a previous study (Johnson *et al.*, 1988). The effect of Ad-specific passive antibody immunity on immunisation with recombinant adenoviruses remains to be determined.

2.4.2 Active immunity to viral vectors

Active immunity to a virus develops following natural infection or experimental administration of the virus to the host. It involves the stimulation of different regulatory and effector immune responses including the expansion of antigen-specific lymphocytes and the development of immunological memory. Active immune responses specific for a virus vector may inhibit the foreign gene expression following administration of recombinant virus more than passive vector-specific antibody discussed earlier. First, active immunity is longer lasting than passively acquired antibody. Second, local production of antibodies following infection by the virus may be more effective in inhibiting viral infection than passively transferred antibody. Third, a virus infection induces not only humoral but also cell mediated immune responses. In accordance, adenoviruses have been shown to induce not only Ad-specific serum antibody, but also mucosal antibody responses (van Ginkel *et al.*, 1995, Yang *et al.*, 1995b) and cell mediated immune responses. Adenoviruses are able to stimulate T cell proliferative responses, MHC class II-restricted $CD4^+$ cells of the Th1 subset, MHC class-I restricted $CD4^+$ and $CD8^+$ CTL (Flomenberg *et al.*, 1995, Ginsberg & Prince, 1994, Rawle *et al.*, 1989, Sparer *et al.*, 1997, van Ginkel et al., 1997, Yang *et al.*, 1995a, Yang *et al.*, 1994, Yang *et al.*, 1995b, Yang & Wilson, 1995).

Since adenoviruses are very common pathogens, it is very likely that many individuals (animals or humans) have developed some level of Ad-specific immunity by natural infection. Furthermore, if repeated administration of Ad vectors is necessary for optimal vaccination or gene therapy results, the host may develop substantial levels of Ad-specific immunity by the time of second administration of the recombinant virus. Therefore, it is important to investigate the effect of active Ad-specific immunity on the efficacy of immunisation and gene-transfer by recombinant adenoviruses.

Immune responses induced by administration of adenovirus can substantially diminish the efficiency of gene transfer following a second administration of recombinant Ad (Dong *et al.*, 1996, Kozarsky *et al.*, 1994, Mittal *et al.*, 1993, Setoguchi *et al.*, 1994, Smith *et al.*, 1993, Yang *et al.*, 1995a, Yei *et al.*, 1994). In spite of Ad-specific immunity, however, repeated administrations have been successful in expressing foreign genes *in vivo* (Bout *et al.*, 1994a, Crystal *et al.*, 1995, Mastrangeli *et al.*, 1993, Setoguchi *et al.*, 1994, Zabner *et al.*, 1994). The level of Ad-specific immunity at the site of the second administration of the virus largely depends on the route and dose of, and the time passed since the first administration of the virus. Intraperitoneal immunisation with Ad did not affect foreign gene expression following administration of the vector to the lung (Setoguchi *et al.*, 1994). In addition, inhibition of second foreign gene expression was less evident 3 months after the first administration compared to 1 month later (Setoguchi *et al.*, 1994). The level of adenoviral gene expression may also influence the induction of Ad-specific immunity. Schulick *et al.* (1997) showed that i.v. pre-exposure to ultraviolet light (UV)-irradiated adenoviral vectors (with reduced viral gene expression but preserved capsid function) did not block foreign gene expression following subsequent i.v. administration of recombinant Ad as much as pre-exposure to intact virus.

Several immune effector mechanisms may be responsible for the elimination of Ad-infected cells. Transgene expression was shown to be completely or partly diminished as a result of destruction of virus-infected cells by MHC class I-restricted CTL (Kaplan *et al.*, 1997, Yang *et al.*, 1995a). In accordance, $CD8^+$ T cell-deficient mice had prolonged transgene expression in hepatocytes (DeMatteo *et al.*, 1997). Adenovirus-specific neutralising antibody in the airways, however, was sufficient to fully block gene transfer to the airways (Yang *et al.*, 1995a). Furthermore, it is not surprising that mice transiently depleted of $CD4^+$ T cells before first administration supported longer expression of the foreign gene following second administration of the vector than control mice (Yang *et al.*, 1995b, Yang & Wilson, 1995), since $CD4^+$ T cells are important in the development of both humoral and cellular effector mechanisms against adenoviruses (Yang *et al.*, 1995a, Yang *et al.*, 1995c).

The role of Ad-specific mucosal IgA in blocking gene-transfer to the lung of mice has been suggested. Yang *et al.* (1995b) showed that transient $CD4^+$ cell depletion and intratracheal (i.t.) interleukin (IL)-12 or interferon (IFN)-γ administration completely inhibited the formation of Ad-specific IgA following Ad administration. These treatments caused more efficient gene transfer to occur following a second administration of recombinant adenovirus. $CD4^+$ T cell depletion did not affect the development of systemic CTL responses and only partially inhibited Ad-specific IgG responses in the lung. Intratracheal IL-12 and IFN-γ administration did not inhibit the levels of lung IgG. These results may suggest that Ad-specific mucosal IgA may be primarily responsible for the blockade of foreign gene expression by recombinant Ad in the mouse lung.

Most research groups investigating the inhibitory effect of active vector-specific immunity on the efficacy of foreign gene-delivery have been mainly concerned about the level of expression of the foreign gene. Only a few have studied how immune responses induced by the foreign gene may be influenced by pre-existing vector-specific immunity. Kundig and co-workers (1993) immunised mice by the i.v. route with a recombinant vaccinia virus expressing lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP). At different timepoints following first immunisation, they i.v. inoculated the animals with another recombinant vaccinia virus expressing vesicular stomatitis virus (VSV) glycoprotein (GP). They found that GP-specific B cell responses were suppressed for more than 9 months. The level of suppression depended on the dose of the first vaccinia virus administration (Kundig *et al.*, 1993). Others infected mice i.v. with different doses of vaccinia virus, and later immunised them i.v. with recombinant vaccinia virus expressing haemagglutinin (HA) of influenza virus. Antibody titres specific for HA and survival rate after i.n. influenza challenge were reduced the greatest in mice infected with a high dose of non-recombinant vaccinia compared with titres in the control group (Andrew, 1989). Interestingly, there was no difference in vaccinia-specific CTL activity following infection with high or low dose of non-recombinant vaccinia virus, which supports the idea that the protective efficacy of the recombinant vector is dependent upon the level of pre-existing vaccinia-specific antibody, rather than CTL.

The effect of active vector-specific immunity on immunisation with recombinant Ad was also investigated. Systemic pre-infection of mice with 10^5 plaque forming units (pfu) of HAd5 slightly inhibited antibody and CTL responses specific for the expressed foreign gene induced by a subsequent systemic administration of recombinant Ad (Xiang *et al.*, 1996). The possible inhibitory effect of infection with higher doses of Ad has not been investigated. It appears important to perform these experiments, since the usual dose of recombinant Ad used for immunisations or gene therapy experiments is 10^7 to 10^9 pfu per animal (see references in Table 2.1).

2.4.3 Immunity to the expressed antigen

Both active and passive immunity to the expressed foreign gene may also inhibit the efficacy of immunisation with recombinant viral vectors. Indeed, immune responses to transgene-encoded proteins limited the stability of gene-expression after injection of replication-defective Ad vectors (Tripathy *et al.*, 1996). Passive transfer of respiratory syncytial virus (RSV) antiserum suppressed the humoral immune response to RSV proteins expressed by recombinant vaccinia viruses (Galetti *et al.*, 1995, Murphy *et al.*, 1988), but did not inhibit CTL responses to the proteins (Galetti *et al.*, 1995). In contrast, vaccination with recombinant vaccinia viruses has been successful despite existing active immunity to the expressed antigens (Flexner *et al.*, 1988). In addition, the efficacy of vaccination of lambs in surgically created gut-loops with HAd5 expressing gD of BHV-1 was not inhibited by the presence of maternal antibodies to gD and Ad (Philip Griebel, manuscript in preparation). Data in the literature, therefore, seem contradictory but indicate that not only immunity to a viral vector but also to the foreign protein can cause suppression of the efficacy of vaccination with recombinant virus.

2.5 Concerns about the safety of recombinant adenoviruses

There are two main issues identified up to date regarding the limitation of application of recombinant adenovirus vectors. One, their efficacy in the presence of

Ad-specific immunity, has been discussed. Another issue is their safety; it is important that recombinant adenoviruses do not shed extensively following *in vivo* administration. This would increase the possibility of infection of individuals and the recombination with wild type viruses. For gene therapy purposes, it is also desirable to maintain control over the extent of foreign gene expression.

Replication-defective recombinant adenoviruses are often considered safer than replication-competent vectors because foreign gene expression by replication-defective dE1 viruses always correlates with the infectious viral dose. Furthermore, their spread *in vivo* and shedding to the environment have been suggested to be more limited than that of dE3 adenoviruses (Oualikene *et al.*, 1994). However, replication of a dE1 virus may also take place during a simultaneous infection with wt HAd5, which theoretically raises similar safety concerns as administration of a replication-competent virus. Evidence for phenotypic complementation of recombinant dE1 adenovirus expressing the cystic fibrosis transmembrane conductance regulator (CFTR) or β-galactosidase with wt HAd5 was found in human cells *in vitro* (Imler *et al.*, 1995, Oualikene *et al.*, 1995) and *in vivo* following i.n. administration of the viruses to cotton rats (Imler *et al.*, 1995). No *in vivo* trans-complementation of the recombinant Ad with wt HAd5 was observed, however, when recombinant virus was delivered i.v. or i.m. and wt HAd5 was administered i.n. (Oualikene *et al.*, 1995).

In vivo spreading and shedding to the environment of both replication-competent and replication-defective adenoviruses can take place, even though replication-defective vectors have been suggested to be limited in their capability of *in vivo* dissemination. Replication-defective dE1E3 virus was not isolated from any organs of the cotton rat following i.m. delivery (Oualikene *et al.*, 1994, Oualikene *et al.*, 1995), although foreign gene expression was found at the site of administration in the muscle (Huard *et al.*, 1995). Following i.v. inoculation in the tail, no dE1E3 vector was isolated from any organ (Oualikene *et al.*, 1995), although others detected foreign gene expression in the liver, kidney, tail and some muscles (Bout *et al.*, 1994b, Brody *et al.*, 1994, Goldman *et al.*, 1995, Huard *et al.*, 1995). Following i.n. delivery, no virus was found in tissues other than those of the respiratory tract (Yei *et al.*, 1994). Others, however, found dE1E3 virus

following i.n. inoculation in both the respiratory tract and in the small bowel or faeces (Bout *et al.*, 1994a, Zabner *et al.*, 1994), in kidneys and liver (Oualikene *et al.*, 1994) and in the oesophagus and spleen (Imler *et al.*, 1993). Foreign gene expression following i.n. inoculation of recombinant dE1E3 was also detected in the lung, liver and heart (Huard *et al.*, 1995). Huard et al. (1995) also investigated many other routes of administration of a dE1E3 vector: they found foreign gene expression in the heart, liver, lung, thymus and muscles following intracardiac delivery; in the peritoneum, diaphragm and liver following i.p. inoculation; in the lung, heart and thymus following buccal administration; and in liver, spleen, kidney, peritoneum, stomach, rectum, thymus and muscles following gastric-rectal inoculation. Thus, spreading of replication-defective viruses is not always limited to the site of infection as generally believed, even following mucosal administration.

Dissemination of replication-competent and replication-defective adenoviruses were compared in only one detailed investigation, using i.m. and i.n. administration of adenoviruses in cotton rats (Oualikene *et al.*, 1994). Following i.m. delivery, they detected dE3 virus in the liver, spleen, lungs, nasal-washes, poplietal and inguinal LN, while dE1E3 virus was not isolated from any organs. Following i.n. administration of the dE3 vector, all tested samples (liver, spleen, kidneys, lungs, nasal-wash, brain, gut, poplietal and inguinal LN) were positive for adenovirus. In contrast, dE1E3 was found only in the lungs, nasal-wash, kidneys and LN after i.n. administration of the virus. Others have shown that following i.p. inoculation of the dE3 vector, adenovirus could be detected in the liver, kidneys, spleen and lungs (Mittal *et al.*, 1993).

Oualikene et al. (1994) found that wt HAd5 disseminated similarly to dE3 HAd5 virus in the cotton rat. While wt HAd5 was detected in every organ tested 3 days following i.n. delivery, at 7 days following inoculation it was found only in the lungs and nasal-washes (Oualikene *et al.*, 1995). Interestingly, in a previous experiment, they detected HAd5 in every organ tested 28 days following i.n. inoculation (Oualikene *et al.*, 1994).

In people, wt HAd5, HAd7 and recombinant dE3 viruses were shed in the stool and also in the pharynx following oral immunisation (Schwartz *et al.*, 1974, Tacket *et al.*, 1992, Top *et al.*, 1971b). In contrast, no evidence for Ad dissemination to the throat was found following oral administration of type 21 HAd in humans (Dudding *et al.*, 1972). Extensive

investigations and many years of experience with oral vaccination using HAd type 4 and 7 in military recruits showed that replication-competent Ad can be used safely to immunise humans (Top *et al.*, 1971b). Dissemination of recombinant dE3 adenoviruses to different tissues in either humans or animals, however, has not been studied following gastrointestinal routes of administration. Furthermore, since different routes of administration, dose, detection systems and animal models have provided contradictory data (as described above), further studies are necessary to determine the capability of adenoviruses to disseminate following different routes of administration.

2.6 Mucosal immunity and protection against viral infection

It is important to discuss what elements may constitute protective immune responses against viruses, for two reasons. First, the immunisation strategy has to be such that the foreign gene expressed by the live vector would induce protective immunity against the respective mucosal viral infection. Second, understanding what the protective immune responses are against the viral vector itself, one may be able to design strategies to optimise the efficiency of vaccination using the viral vector. Since both BHV-1 and HAd5 primarily infect mucosal surfaces, it is important to investigate the induction and effector mechanisms of immune responses that protect the mucosa against viruses.

2.6.1 Inductive and effector mechanisms of mucosal immunity

The first line of defence against mucosal pathogens involves mechanical and physicochemical cleansing mechanisms such as peristalsis or ciliary movement, mucous coat, proteolytic enzymes, innate humoral factors, and the physical characteristics of mucosal epithelial cells and junctions, which degrade and repel most pathogens. In addition, a large and highly specialised immune system protects the mucous membranes, and thereby also the body's interior, against potential insults from the environment (Sanderson & Walker, 1994). Since the aim of vaccination is to stimulate antigen-specific host-defenses, this section will address the inductive and effector mechanisms of antigen-

specific mucosal immune responses and their role in protection against mucosal viral infection.

A mucosal immune response involves priming at inductive sites, dissemination in the circulation and homing to effector sites of antigen-specific lymphocytes (for a review see Tomasi, 1994). These events are studied in the most detail for the gut-associated lymphoid tissue (GALT) but are thought to be similar for the organised mucosa-associated lymphoid tissues (MALT) of the respiratory tract. Briefly, live replicating and dead particulate antigens are thought to be taken up from the lumen of the gut or airways through specialised areas, microfold (M) cells, of the follicle associated epithelium. The antigen, usually unaltered in M cells, is then transported into the underlying lymphoid tissue containing professional antigen presenting cells (APC) and B and T cells, where antigen-priming of the lymphocytes takes place. After antigen-induced activation, proliferation, and partial differentiation, both B and T cells migrate to the regional LN, from which – probably after further differentiation – they go via the lymph into the peripheral blood. These stimulated memory cells migrate to intestinal lamina propria (LP) or to mucosal tissues and exocrine glands outside the gut, notably also in the respiratory tract and in lactating mammary glands (Phillips-Quagliata & Lamm, 1994). The integrated dissemination of immune cells from GALT to all exocrine sites, the so called common mucosal immune system (CMIS), is the functional basis for oral vaccines. Evidence for the existence of a CMIS comes from studies of cell trafficking and from the observation that immunisation of one mucosal site often leads to detectable immune responses at distant mucosal sites (McDermott & Bienenstock, 1979, Mestecky *et al.*, 1994). In addition to lymphocyte activation in MALT, antigen taken up at mucosal surfaces may be directly transferred to draining LN and other peripheral lymphoid tissue, where activation of antigen-specific lymphocytes may occur (Dharakul *et al.*, 1988, Phillips-Quagliata & Lamm, 1994). Immune induction by antigen at these sites may contribute to the development and regulation of systemic and mucosal immunity following mucosal viral infection.

Effector immune mechanisms against viruses that infect mucosal surfaces include both cell-mediated and humoral immune responses. Cellular elements such as natural killer

cells, $CD8^+$ and $CD4^+$ T cells residing in the LP or as intraepithelial lymphocytes (IEL) have been proposed to play a role in the clearance of virus infected cells (Kiyono & McGhee, 1994, London, 1994). Cytotoxic lymphocytes are induced following viral infection of the mucosa and are able to mediate protection against viral induced disease (Murphy, 1994). However, for viruses that replicate rapidly at mucosal surfaces, such as BHV-1 and adenoviruses, the proliferation of memory CTL may not be sufficiently rapid to alter significantly the peak titre of virus in the respiratory tract. Since disease is usually experienced when peak titres of viruses are attained, immunisation with antigens that induce predominantly CTL activity in the absence of antibody is expected to be less successful in restricting replication of challenge virus at mucosal surfaces than vaccination that induces sustained antibody response as well (London, 1994, Murphy, 1994). Furthermore, antibodies are thought to play an important role not only as a first line of defence in preventing viral infection of the mucosal epithelium and underlying tissues, but also in the induction of immune responses (uptake of viral antigens via immunoglobulin receptors on M cells) and in the clearance of viral infections (e.g., antibody dependent cellular cytotoxicity) (Kilian & Russell, 1994).

2.6.2 Effector mechanism of antigen-specific antibodies at mucosal surfaces

At mucosal surfaces, antibodies may neutralise the infectivity of viruses by aggregation of virus (immune exclusion) or by prevention of their attachment and penetration to target cells. In addition, both IgG and IgA antibodies may act to neutralise viruses after their penetration to the host cell (Outlaw & Dimmock, 1991). The major viral antigens that induce neutralising antibody responses are the surface proteins and glycoproteins of viruses. Although the same antigens and epitopes appear to be recognised by different classes of antibodies, IgA and IgG are more efficient than IgM in neutralising virus (Murphy, 1994).

In primates and rodents, antiviral IgA is likely to play the major role in clearance of primary viral infections and prevention of infection on re-exposure to virus. Compared to IgG, IgA is actively transported through the mucosal epithelium to the lumen, is less likely

to cause local inflammation and is more stable at mucosal surfaces. These characteristics are likely due to IgA's secretory component acquired in the epithelium, its poor capability to fix complement and to its polymeric nature (Underdown & Mestecky, 1994).

Although serum IgG of primates and rodents is not transported actively across epithelium, IgG antibodies may gain excess to mucosal surfaces by passive diffusion (transudation). This is suggested by the correlation between serum and mucosal IgG titres (Wagner *et al.*, 1987) and by the observation that parenterally transferred IgG antibody can protect mice from mucosal virus infection (Eis Hubinger *et al.*, 1993). In addition, virus-specific IgG produced by mucosal B cells may also contribute to total antiviral activity in mucosal secretions (Fazekas *et al.*, 1994).

Evidence for the association of mucosal antibodies with resistance to viral infections have been provided by studying the effect of passively transferred and host derived antibodies on virus infection (Murphy, 1994, Renegar & Small, 1994). Furthermore, IgG and IgA antibodies have been compared for their efficacy *in vivo*. In one study, using monoclonal antibodies that recognise the same viral glycoprotein, mice passively immunised i.n. with IgG or IgA were protected equally against pulmonary Sendai virus infection (Mazanec *et al.*, 1992). Monomeric and polymeric IgA were equally efficacious. These data are consistent with the similarities of IgG and IgA in their *in vitro* neutralising activities. In contrast, a neutralising polymeric IgA mAb specific for influenza HA, transferred systemically, was more effective in restricting the replication of influenza virus in the upper respiratory tract of the mouse than a comparable amount of IgG mAb of the same specificity (Renegar & Small Jr., 1991). An important advantage of IgA antibodies in protecting against mucosal infections of the respiratory tract appears to be their abundance at mucosal surfaces due to their selective induction and their ability to be transported selectively across mucosal surfaces, rather than an inherently greater antiviral activity than that of IgG.

2.6.3 Immunisation at mucosal surfaces

Evidence strongly supports the concept of relative compartmentalisation of systemic and mucosal immune responses in mammalian species (Tomasi, 1994). Many experimental results have shown that mucosal immunisation is superior to parenteral immunisation in inducing immunity and protection at mucosal surfaces (Moldoveanu *et al.*, 1993, Ogra, 1996, Tamura & Kurata, 1996). Mucosal immunisation with live viruses is able to stimulate strong mucosal IgA responses in rodents and humans, which usually correlate better than serum antibody induced by systemic immunisation with protection against mucosal viral infections.

In addition, compartmentalisation also exists within the common mucosal immune system. There is especially a dichotomy between the gut and the upper aerodigestive tract with regard to homing properties and terminal differentiation of B cells (Mestecky *et al.*, 1994, Phillips-Quagliata & Lamm, 1994). Such a disparity may be explained by microenvironmental differences in the antigenic repertoire, or by differences in the lymphoid and vascular adhesion molecules involved in local B-cell extravasation. For example, although trafficking of B cells between mucosal sites occurs, the concentration of virus-specific IgA-producing B cells at the site of antigenic stimulation is often much higher than that at more distant sites (Dharakul *et al.*, 1988, Kantele *et al.*, 1997). Such compartmentalisation within the CMIS may provide answers to why local immunisation of the respiratory tract is usually more efficacious than immunisation at a distant mucosal site such as the gut in inducing respiratory mucosal immunity and protection against challenge (Brownlie *et al.*, 1993, McLean et al., 1996, Collins *et al.*, 1990). There are exceptions, however: mucosal immunisation in the respiratory tract is sometimes more effective than local inoculation of liposome-vaccine or recombinant Ad in the vagina (de Haan *et al.*, 1995, Gallichan & Rosenthal, 1995).

Mucosal administration of live replicating viral vaccines has been found to induce stronger mucosal immune responses and protection against challenge than inactivated vaccines (Couch *et al.*, 1996, Ogra, 1996, Tamura & Kurata, 1996, Weeks-Levy & Ogra, 1996). Non-replicating viruses usually induce immunity only following systemic

immunisation (Morrison & Knipe, 1996, Xiang *et al.*, 1996; and see Table 2.1.), although there are a few reports for replication-defective viruses inducing immunity following mucosal administration (Bender *et al.*, 1996, Gonin *et al.*, 1996). Inactivated viral or subunit vaccines usually induce immune responses following mucosal vaccination only if combined with strong adjuvants such as cholera toxin or if delivered by liposomes (Michalek *et al.*, 1994). It is possible that the MALT possesses selective regulatory and effector mechanisms to recognise and respond with induction of immunity only to potentially dangerous agents to avoid bystander tissue damage and immunologic exhaustion.

The mucosal immune system is thought to be not only hyporesponsive compared to the systemic immune system but mucosal immune responses are also considered shorter lived than serum antibody responses (Slifka & Ahmed, 1996). However, mucosal immunisation of mice with high doses of live virus may result in long-term antibody production both in the serum (Irie *et al.*, 1992, McNeal & Ward, 1995) and the respiratory tract (Hyland *et al.*, 1994, Jones & Ada, 1987, Liang *et al.*, 1994). Furthermore, recombinant HAd5 expressing gB of HSV-1 was capable of inducing long term humoral and cellular immunity to gB and protection against lethal doses of i.n. HSV-1 challenge in mice (Gallichan *et al.*, 1993).

2.7 The cotton rat as a model for mucosal adenoviral infections

The cotton rat (*Sigmodon hispidus*) has been used as an animal model to study the pathogenesis of various human and animal respiratory viruses such as human parainfluenza virus type 3 (HPIV3), bovine parainfluenza virus type 3 (bPI3), human respiratory syncytial virus (HRSV), HAd5 and bovine adenovirus (bAd) (Breker-Klassen *et al.*, 1995, Mittal *et al.*, 1995, Murphy *et al.*, 1981, Pacini *et al.*, 1984, Porter *et al.*, 1991, Prince *et al.*, 1978, Prince *et al.*, 1993). In fact, the only rodents known to support HAd5 replication are cotton rats and hamsters (Hjorth *et al.*, 1988). Mice are only semi-permissive for HAd5 replication (Blair *et al.*, 1989, Ginsberg *et al.*, 1991). Using an i.n. challenge model in the cotton rat, HAd5 replication in the lungs was shown to peak between day 2 and 5 p.i.

depending on the dose of virus administered intranasally (Pacini *et al.*, 1984, Prince *et al.*, 1993). Immunofluorescent and electronmicroscopic studies demonstrated that the virus primarily replicated in the bronchiolar epithelial cells of cotton rats (Prince *et al.*, 1993). Histopathological changes were also observed. These changes could be divided into two phases: early, non-specific infiltration of neutrophils, macrophages and lymphocytes to the peribronchiolar and alveolar regions, and a later phase of the disease, which consisted almost exclusively of infiltration of lymphocytes (Prince *et al.*, 1993). The molecular basis of adenovirus pathogenesis has been studied in cotton rat and mouse models (Ginsberg & Prince, 1994).

With the establishment of the i.n. HAd5 infection model, the cotton rat has become available for the preliminary evaluation of recombinant HAd5 vectors as vaccine delivery systems. Although recombinant adenoviruses have successfully delivered vaccine antigens to several mammals (primates, monkeys, carnivores, ruminants and rodents) (Graham & Prevec, 1992, Imler, 1995, Rosenthal *et al.*, 1996), only the species which are permissive for HAd5 replication, such as cotton rats, hamsters, cattle and primate species, are suitable for studies comparing replication-defective and replication-competent HAd5 vectors.

3.0 HYPOTHESES AND OBJECTIVES

A laboratory rodent that supports the replication of both adenovirus and bovine herpesvirus type 1 (BHV-1) would provide a model to evaluate the efficacy of replication-defective and replication-competent adenovirus vectors expressing the glycoprotein D (gD) of BHV-1 in inducing gD-specific immunity and protection against BHV-1 challenge. Cotton rats are already known to support the replication of human adenovirus. Since the cotton rat (*Sigmodon hispidus*) has served as a rodent model for many viral pathogens, the first hypothesis was that cotton rats can be used as a model for experimental BHV-1 infection as well.

Immunisation of cattle with purified gD and transmembrane anchor truncated gD (tgD) of BHV-1 stimulates protective immunity against BHV-1 infection. These glycoproteins expressed by a recombinant adenovirus, therefore, have the potential to serve as a vaccine model to investigate mucosal immunity induced by recombinant adenoviruses. The second hypothesis was that recombinant human adenoviruses expressing gD or tgD of BHV-1 can induce mucosal immunity and protection against BHV-1 challenge in cotton rats.

For gD-specific immunity to develop it is crucial that cotton rat cells become infected with recombinant adenovirus and express the gD or tgD protein. Since adenovirus-specific immunity can prevent an infection or effective foreign gene expression by recombinant adenovirus, the third hypothesis was that pre-existing adenovirus-specific immunity inhibits the efficacy of immunisation with recombinant adenovirus.

To examine whether the above hypotheses were correct, the following objectives were set:

1. To investigate the capability of BHV-1 to replicate and cause pathological changes in cotton rats

2. To determine the *in vitro* expression of gD and tgD in cotton rat cells infected with recombinant adenoviruses

3. To assess the ability of recombinant adenoviruses expressing gD or tgD to elicit gD-specific systemic and mucosal immunity and confer protection against intranasal BHV-1 challenge

4. To determine the effect of adenovirus-specific active immunity and passive antibody-transfer on immunisation with recombinant adenovirus

4.0 MATERIALS AND METHODS

4.1 Virological assays

4.1.1 Cell lines

Cotton rat lung (CRL) cells were cultured and maintained as a stable cell line as previously described (Baca-Estrada *et al.*, 1995). Madin Darby bovine kidney (MDBK) cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The 293 cell line, which is a human embryonic kidney derived, human adenovirus type 5 (HAd5) transformed cell line expressing the E1 region proteins of HAd5, was a gift from Dr. Frank L. Graham (McMaster University, Hamilton, ON). All three cell lines were propagated in minimal essential medium (MEM; with Earle's salts; Gibco/BRL Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco/BRL), 2mM L-glutamine and penicillin streptomycin solution (Sigma Chemical Co., St. Louis, MO) (growth medium). Each cell line was used up to passage number 50.

4.1.2 Stock virus preparation and purification of BHV-1

The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA, and propagated in MDBK cells. The field isolate 108 strain of BHV-1 was obtained from Animal Diseases Research Institute (ADRI), Lethbridge, Alberta, Canada and passaged twice in MDBK cells.

For use *in vivo* and most *in vitro* experiments, the Cooper strain of BHV-1 was concentrated by ultracentrifuging the culture supernatant of infected cells through a sucrose cushion. The pelleted virus was resuspended in phosphate buffered saline (PBS). For

infection of CRL cells and cotton rats with BHV-1 108 strain, the culture supernatant of infected cells was used. For enzyme-linked immunosorbent assay (ELISA), BHV-1 was purified by ultracentrifugation on discontinuous Na/K tartrate gradients as described (Misra *et al.*, 1981). Total protein content of the purified virus preparation was estimated by a Bio-Rad (Cambridge, MA) protein assay. Stocks of both strains of BHV-1 were titrated on MDBK cells and were stored in small aliquots at -70 °C.

4.1.3 Infection of CRL cells and MDBK cells with BHV-1

To determine the kinetics of BHV-1 growth, different multiplicity of infection (MOI) of BHV-1 were added to CRL or MDBK cell monolayers. After 1 hour (h) of adsorption at 37 °C, excess virus was removed by washing cells twice with PBS and replaced with MEM supplemented with 2% FBS (Gibco/BRL). At various timepoints postinfection (p.i.), cells and culture supernatants were harvested and stored at -70 °C. All samples were further processed and analysed simultaneously.

4.1.4 BHV-1 titration

Quantification of BHV-1 was performed by a plaque assay. Samples obtained following *in vitro* (cell culture inoculum) or *in vivo* (lung and trachea extracts) infection with BHV-1 were serially diluted in PBS, absorbed onto MDBK cell monolayers for 1 h, then overlaid with growth medium containing 1% agarose. After 3 days of incubation at 37 °C, plaques were counted, and titres expressed as plaque forming units (pfu) per ml of culture inoculum or per gram of tissue.

4.1.5 Preparation and purification of adenoviruses

The construction of HAd5 vectors containing the gene of BHV-1 gD or tgD in the E1 (gD-dE1E3 and tgD-dE1E3, respectively) or the E3 regions (gD-dE3 and tgD-dE3, respectively) were described previously (Mittal *et al.*, 1996, van Drunen Littel-van den

Hurk *et al.*, 1993). The characteristics of these vectors are summarised in Table 4.1. Adenoviruses were propagated in 293 cells, and released from infected cells by 3 rounds of freezing and thawing. The cell debris was removed by centrifugation. The supernatant was used for administration to cotton rats in experiments described in sections 5.3.4, 5.3.5, 5.4, 5.5.1, 5.5.2, 5.5.3, 5.5.5. In other experiments, the recombinant vectors were partially purified by centrifugation in a discontinuous cesium chloride gradient. Adenoviruses were titrated by plaque assay and stored at -70 °C. The adenovirus preparations contained no more than 0.1 μg gD per 10^8 pfu virus, as measured by capture ELISA. For the *in vitro* assays the wild type (wt) HAd5 was purified by two rounds of continuous cesium chloride gradient centrifugation (Graham & Prevec, 1991). Total protein content of each purified virus preparation was estimated by a Bio-Rad protein assay. For experiment 5.3.1, the purified adenoviruses were provided by Suresh K. Mittal.

4.1.6 Titration of adenoviruses

To quantify the amount of adenovirus in culture-inoculum or in tissue samples, adenovirus was titrated on 293 cells under a 1% agarose overlay (Graham & Prevec, 1991). After 5-8 days of incubation at 37 °C, plaques were counted, and titres were expressed as pfu per ml of culture inoculum or per gram of tissue.

4.1.7 Immunoprecipitation and gel-electrophoresis

Glycoprotein D and tgD expression by CRL cells infected by recombinant adenoviruses was determined by immunoprecipitation and gel-electrophoresis. Adenoviruses were adsorbed onto CRL cells for 1 h at 37 °C. Then the inoculum was removed and replaced with methionine-free MEM (ICN Biomedicals Inc., Mississauga, ON). CRL cells infected with BHV-1 were used as a positive control. At 3 h p.i. 75 μCi of EXPRESS Protein Labelling Mix containing L-[^{35}S]-methionine (NEN Life Science

Table 4.1 Recombinant human adenoviruses used in the studies

HAd5 virus:	gD or tgD		deletion	regulatory sequences		references:
	gene:	insertion in:	in:	promoter:	poly A:	
dE1E3	-	-	E1, E3	-	-	Graham and Prevec, 1992
dE3	-	-	E3	SV40	SV40	Mittal et al., 1996
gD-dE1E3	gD	E1 region	E1, E3	MLP	multiple	van Drunen Littel van den Hurk et al., 1993
tgD-dE1E3	tgD	E1 region	E1, E3	MLP	multiple	Mittal et al., 1996
gD-dE3	gD	E3 region	E3	SV40	SV40	Mittal et al., 1996
tgD-dE3	tgD	E3 region	E3	SV40	SV40	Mittal et al., 1996

Products, Boston, MA) in 1.5 ml methionine-free medium was added to the plates. At 36 h or 20 h p.i. with adenoviruses and BHV-1, respectively, supernatants were collected and cells were harvested and pelleted by centrifugation. Samples were mixed with RIPA buffer (150 mM NaCl; 50 mM Tris-HCl, pH 7.5; 10 mM EDTA; 1 % v/w sodium deoxycholate; 1 % v/v Triton X-100; 0.5 % w/v sodium dodecyl sulphate (SDS)) containing pooled gD-specific mAb and incubated on a rocker for 2 h at room temperature. Protein A coupled Sepharose beads (Pharmacia, Uppsala, Sweden), 100 mg/ml in RIPA buffer, were added and the mixture incubated on a rocker at 4 °C overnight. The protein A Sepharose was pelleted by centrifugation and the pellet washed 5 times with RIPA buffer. Thirty µl sample buffer (70 mM Tris-HCl, pH 6.8; 10 % v/v glycerol; 2 % w/v SDS; 2 % β-mercaptoethanol; 0.05 % w/v bromophenol blue) was added to the pellet. Protein samples were boiled for 5 minutes before separation by gel-electrophoresis. Polyacrylamide gel-electrophoresis (PAGE) was performed by using denaturing, discontinuous Laemmli gels (Laemmli, 1970). A stacking gel of 4 % and a separating gel of 10 % were used. After the separation was complete, the gel was fixed (methanol:water:acetic acid = 25:65:10) and soaked in Amplify (Amersham Canada, Inc., Oakville, ON) prior to drying. The dried gel was then exposed to X-ray film (Eastman Kodak Company, Rochester, NY) at -70 °C.

4.1.8 Immunocytochemistry

Immunocytochemical staining of CRL cells infected with recombinant adenoviruses was used to demonstrate *in situ* expression of gD and tgD *in vitro*. Infection of CRL cells with BHV-1 at an MOI of 1 was used as a positive control. CRL cells were grown to confluence on LAB-TEK chamber slides (Nunc Inc., Naperville, IL, USA) and incubated with virus at an MOI of 5 for 1 hour. Non-adsorbed virus was removed by washing with MEM and the slides were further incubated with growth medium. Slides were fixed in cold acetone at different time points p.i., air dried, then incubated for 30 min with MEM with 1 % horse serum (Gibco). Slides were washed with PBS between each following step. Slides were incubated for 1 h at room temperature with a pool of monoclonal antibodies (mAb) specific for gD, described by Hughes *et al.* (1988) and van

Drunen Littel-van den Hurk & Babiuk (1986). Then, biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was added for 1 hour. Detection of bound antibody was performed by adding an avidin biotin complex (ABC)-horseradish peroxidase (HRP) (ABC-HRP kit, Vector). Slides were developed by a peroxidase substrate kit (DAB, Vector), counterstained with toluidine blue (Aldrich, Milwaukee, WI, USA), rinsed with distilled water and air dried. Brown staining indicating the presence of gD was examined by a light microscope (Olympus AH2).

4.1.9 Quantification of gD and tgD by ELISA

CRL cells were infected with recombinant adenoviruses to assess the kinetics of gD and tgD expression in vitro. Adenoviruses were adsorbed onto CRL cell monolayers at an MOI of 10 for 1 hour at 37 °C. Excess virus was removed by washing cells twice with PBS, followed by the addition of MEM supplemented with 2% FBS (Gibco/BRL). Cell monolayers were observed visually with the aid of a dissecting microscope. Cells and culture supernatants were harvested at various time-points p.i. and were frozen at -70 °C until all the samples could be processed simultaneously.

The amount of gD and tgD in the samples was measured by ELISA. Cell pellets were mixed with 2x volume of RIPA buffer, centrifuged and the supernatants containing the cell extracts were used in the assay. Immulon-2 microtiter plates (Dynatech Laboratories, Inc., Chantilly, Va.) were coated overnight at 4 °C with a pool of mAb specific for gD in coating buffer (50 mM $NaHCO_3/Na_2CO_3$, pH 9.6). Between each step, plates were washed three times with PBS containing 0.05 % Tween-20 (Sigma; PBS-T). Culture supernatants and cell extracts were serially diluted in PBS containing 0.5 % bovine serum albumin (BSA; Boehringer-Mannheim, Quebec, Canada) (PBS-BSA) and incubated for two hours at 37 °C. Purified gD was used as a positive control (a gift from Sylvia van Drunen Littel-van den Hurk). Plates were incubated with gD-specific polyclonal rabbit IgG (a gift from Sylvia van Drunen Littel-van den Hurk), then with horseradish peroxidase-conjugated goat anti-rabbit IgG (Gibco/BRL) for 2 h at room temperature for each step. Colour was developed using 0.15 mg/ml of 2,2'-azino-di-{3-ethyl-benzthiazoline

Colour was developed using 0.15 mg/ml of 2,2'-azino-di-{3-ethyl-benzthiazoline sulfonate} (ABTS; Boehringer-Mannheim) as a substrate. Absorbance was read at 405 nm with a reference wavelength at 490 nm using a microplate reader (Bio-Rad model 3550).

4.2 *In vivo* procedures

4.1.2 Cotton rats

Inbred male and female hispid cotton rats (*Sigmodon hispidus*), (Figure 4.1), 3-5 months of age and seronegative to adenovirus and BHV-1, were obtained from barrier-maintained pathogen-free colonies at the Veterinary Infectious Disease Organization (VIDO, University of Saskatchewan) or from Virion Systems, Inc. (Rockville, MD). Animals were handled according to the guidelines of the Canadian Council on Animal Care and the University of Saskatchewan Committee on Animal Care and Supply.

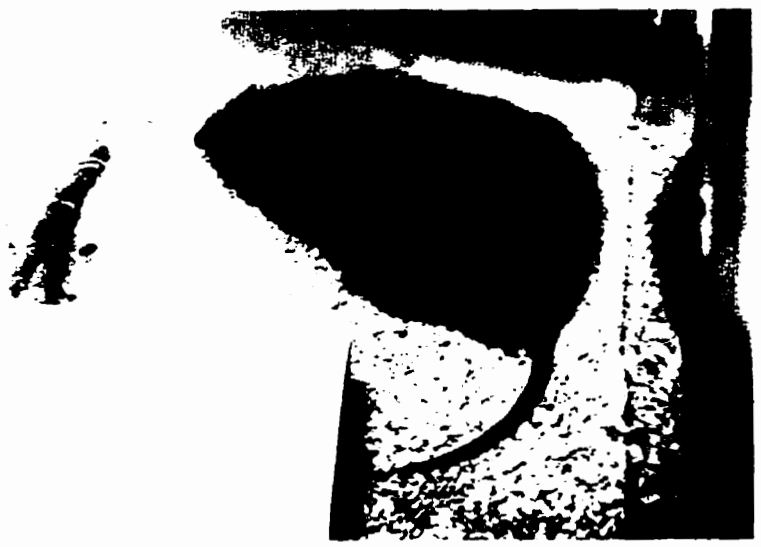


Figure 4.1 Cotton rat (*Sigmodon hispidus*)

4.2.2 Immunisation and challenge protocols

Cotton rats were anaesthetised with nitrous oxide-halothane (MTC Pharmaceuticals, Cambridge, Canada) (1:2) during all immunisations and challenges. Blood samples were obtained by cardiac puncture. Animals were euthanised by a halothane overdose.

For intranasal (i.n.) immunisation and challenge, viruses were administered in 50 µl volumes into the nares of cotton rats. Intradermal (i.d.) immunisation was performed by injecting 2 x 25 µl virus suspension into two spots of the back skin of cotton rats. Intraperitoneal (i.p.) administration was given by injecting 500 µl virus inoculum into the peritoneal cavity. For the primary enteric immunisation, the duodenum was surgically exposed and 10^8 pfu of adenovirus (500 µl), or PBS as a control, was injected into the duodenum. The incisions were closed upon completion of the procedure. The animals were allowed to recover and were housed under normal conditions. The secondary, oral immunisation was performed by delivering 10^8 pfu of virus suspended in 500 µl of 0.4 M $NaHCO_3$ into the oesophagus with a 20 gauge gavage needle. Evans blue dye was used to confirm that the inoculum was restricted to the site of administration and did not spread to the peritoneum or the respiratory tract following intraduodenal or oral delivery, respectively. Before intraduodenal or intragastric immunisation, cotton rats were starved overnight.

4.2.3 Production of adenovirus-specific hyperimmune serum

Adenovirus-specific serum was raised in cotton rats by subcutaneous (s.c.) inoculation with complete Freund's adjuvant containing 10^7 pfu of HAd5. Animals were reimmunised with the same amount of virus in incomplete Freund's adjuvant four weeks later. Four weeks after the second immunisation, serum from all animals was collected and pooled. The titre of Ad-specific antibodies was 2×10^5, as determined by ELISA. The serum was stored at -20 °C until it was used in passive transfer experiments.

4.3 Assessment of viral replication and spread *in vivo*

4.3.1 Histopathology and immunohistochemistry

The left lung of cotton rats was fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. For histological evaluation, 5 μm sections were cut and stained with hematoxylin and eosin (H&E).

Unstained sections of formalin-fixed lung from the same blocks were stained immuno-histochemically using an avidin-biotin complex method (Haines & Chelack, 1991). The slides (three per animal) were incubated with 1/5000 or 1/10000 dilutions of a pool, three parts to one, of two monoclonal antibodies directed against BHV-1 gB (3F11) and gC (1H6) glycoproteins respectively (gift from Dr. V. Misra) or with normal mouse serum at a 1/5000 dilution as a negative control. Formalin-fixed bovine fetal liver from a case of natural BHV-1 infection was used as a positive control. Slides were treated with 3,3'diaminobenzidine-H_2O_2 solution as chromogen and counterstained with hematoxylin. A positive reaction consisted of brown deposits, indicating the presence of BHV-1 antigens.

A scoring system of 0 (normal), 1 (mild), 2 (moderate), and 3 (severe) based on the severity of lesions in the lower respiratory tract was used for histological evaluation of the lungs. Lesions were graded separately for the bronchioles and alveoli. Bronchiolar lesion scores considered the development of intranuclear inclusion bodies, degree and extent of epithelial necrosis and inflammation. Alveolar lesion scores were based on intranuclear inclusion bodies, necrosis, neutrophils and macrophages in alveolar septa, and type II pneumocyte hyperplasia.

4.3.2 Preparation of trachea and lung extracts

One or three days after challenge with BHV-1, trachea and lungs were aseptically removed from euthanized cotton rats, placed in 1 ml MEM and frozen at -70 °C. When all samples were collected, they were thawed, weighed and homogenised (Polytron

homogeniser, Brinkman Industries, Rexdale, Canada). Lungs were homogenised for 2 x 10 seconds, while tracheae were homogenised for 3 x 10 s. Tissue-homogenates were centrifuged to remove debris. Supernatants were collected and analysed for the presence of BHV-1 by plaque assay or the presence of antigen-specific antibodies by ELISA.

4.3.3 Adenovirus isolation from cotton rat tissues

One or three days after inoculation of cotton rats with recombinant adenoviruses, various organs and tissues were removed under aseptic conditions and placed in MEM. All samples were frozen and kept at -70 °C until they could be processed simultaneously. All samples were then thawed, homogenised by a Polytron homogeniser (Brinkman Industries) and centrifuged to remove debris. To quantify adenovirus, the supernatants were analysed by plaque assay. Samples with no plaques were incubated on 293 cells in 6 well tissue culture plates and cells were observed daily for 7 days for plaque-formation. In case of negative results, cultures were frozen and thawed 3 times to release any adenovirus. The resulting crude cell suspensions were passaged in 293 cells one more time. If no plaque formation was noted 7 days p.i. following the second passage, the sample was considered to be negative for adenovirus.

4.4 Assessment of immune responses

4.4.1 Isolation of lymphocytes

Spleens, lymph nodes and bone marrow were removed aseptically, chopped, then gently pushed through a fine plastic mesh. Erythrocytes were lysed by Tris-buffered 0.83% NH_4Cl and the resulting lymphocyte suspensions were washed twice with MEM. Lung lymphocytes were isolated from a piece of tissue that contained the right lung, the bronchi and the lower part of the trachea, which remained following separation of most of the trachea and the left lung for other assays. The tissue was cut into small pieces and incubated for 1 h in complete medium [MEM supplemented with 10% FBS (Sigma), 2

mM L-glutamine (Gibco/BRL), 1 mM sodium pyruvate (Gibco/BRL), 100 μM non-essential amino acids (Gibco/BRL), 10 mM HEPES buffer, and 100 U/ml penicillin G, 100 μg/ml streptomycin solution (Sigma), final pH of 7.2] containing 150 U/ml collagenase A (Worthington Biochemical Co., Freehold, NJ) and 50 U/ml DNase I (Sigma). The digested lung tissue was then gently pushed through a plastic mesh. The lung cell suspension was centrifuged through a discontinuous Percoll (Pharmacia) gradient and washed twice with MEM. Cells were resuspended in complete medium and incubated for one hour in a tissue culture flask to remove the adherent cell population. The purified lymphocyte population was then collected, washed, resuspended in complete medium containing 50 μM 2-ME (Sigma) and used in the ELISPOT and proliferation assays.

4.4.2 Lymphocyte proliferation assay

Antigen-specific T cell function in spleens was measured by lymphocyte proliferation assay. Lymphocytes were dispensed in 100 μl volumes (2 to 4 x 10^5 cells/well) into 96 well tissue culture treated plates (Costar, Cambridge, MA). Irradiated BHV-1, HAd5 (20 mW/cm^2 UV irradiation for 8 min, 1000 mJ) or various concentrations of purified gD (0.1-1 μg/ml) were added in 100 μl to triplicate wells. After 2 days in culture, 0.4 μCi [methyl-^{3}H]-thymidine (Amersham Canada, Inc.) was added to all wells for the last 24 h of culture. Incorporation of thymidine into cellular DNA was assessed by harvesting cells onto glass fibre filter mats (Saktron, Sterling, VA) and the radioactivity determined using a β scintillation counter (Beckman, Richmond, BC). Proliferative responses were expressed as stimulation indices (SI).

$$SI = \frac{\text{counts per minute in the presence of antigen}}{\text{counts per minute in the absence of antigen}}$$

4.4.3 Antigen-specific ELISPOT assay

An enzyme-linked immunospot (ELISPOT) assay was used to determine the number of gD- and adenovirus-specific antibody-secreting cells (ASC) in the spleen, bone marrow and the right lung. Nitro-cellulose based, hydrophobic, 96-well microtiter plates (Polyfiltronics, Rockland, MA) were coated overnight with purified gD (0.3 μg/well) or purified hAd5 (1 μg/well) in coating buffer at 4 °C. After being washed three times with PBS under sterile conditions, plates were incubated with complete medium for one hour at room temperature. Lymphocytes were then added to the plates (5 to 100 x 10^4 cells/well) and incubated in complete medium for 8 hours at 37 °C. Following incubation, cells were removed by washing the plates with cold distilled water and PBS-T. The plates were extensively washed between each following step. To detect bound IgG, biotinylated anti-rat IgG (Zymed, Mississauga, Ontario, Canada) in PBS-BSA was added to the plates for a 2 h incubation at room temperature. To detect bound IgA, rabbit anti-rat IgA (a gift from Dr. B. Underdown, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada) was added for 2 h, followed by incubation with biotinylated goat anti-rabbit antibody (Zymed). All plates were then incubated with streptavidin-alkaline phosphatase, followed by spot development with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)-Nitro Blue Tetrazolium (NBT) substrate. The numbers of spots in triplicate cultures were counted with the aid of a dissecting microscope.

4.4.4 Collection of nasal- and lung-washes

Nasal- and lung-washes were collected by using 2 cm long pieces of Teflon TFE 16 TW Natural tubes (Cole-Parmer Instrument Company, Niles, IL) connected to 16 gauge needles (Baca-Estrada, personal communication). Cotton rats were euthanised before the procedure. For the nasal-wash, the tube was placed into an incision of the trachea and 0.3 ml PBS was flushed through and collected at the nares. To obtain lung-washes, the tube was inserted in the trachea and 1 ml PBS was flushed in and out of the lungs 2 times. Wash samples were stored at -20 °C until they were analysed simultaneously with serum samples.

4.4.5 Antigen-specific ELISA

Antibody levels specific for BHV-1 and adenovirus in sera, washes or extracts from the lung and trachea were determined by ELISA. Immulon-2 microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated overnight with purified tgD (0.05 μg/well), purified BHV-1 or purified HAd5 (0.5 μg/well) in coating buffer at 4 °C. The plates were washed three times with PBS-T between each step. The plates were incubated with PBS-BSA for 1 hour at room temperature, then all the samples were serially diluted in PBS-BSA and incubated overnight at 4 °C. Bound IgG was detected using horseradish peroxidase-conjugated goat anti-rat IgG (Zymed). Bound IgA was measured by rabbit anti-rat IgA (a gift from Dr. B. Underdown), followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Gibco/BRL). Colour was developed using 0.15 mg/ml of 2,2'-azino-di-[3-ethyl-benzthiazoline sulfonate] (ABTS; Boehringer-Mannheim) as a substrate. Absorbance was determined at 405 nm with a reference wavelength at 490 nm using a microplate reader (Bio-Rad model 3550). In experiments 5.3.1., antibody levels were expressed as the optical density (OD) at 405 nm. In other experiments, antibody titres represent the $\log_{10}$ of the inverse of the last dilution that resulted in an absorbance which was greater than the sum of the mean background absorbance plus 3 standard deviations (SD). Antibody titres specific for gD were identical regardless of whether purified BHV-1, gD or tgD was used to coat the ELISA plates.

4.4.6 BHV-1 serum neutralisation assay

BHV-1 neutralising assays were performed as described by van Drunen Littel-van den Hurk et al. (1984). Briefly, doubling dilutions of heat inactivated (56 °C, 30 minutes) serum samples were mixed with 100 pfu of BHV-1 and incubated for 1 hour at 37 °C. The virus-sample mixture was then plated on confluent MDBK cells in 96 well microtiter plates and incubated for 2 days. Serum neutralising (SN) titre was defined as the reciprocal of the highest serum dilution resulting in reduction of virus plaque formation by at least 50% relative to the virus control.

4.4.7 HAd5 serum neutralisation assay

Adenovirus-neutralising assays were performed as described previously by Mittal et al., 1993, with minor modifications. Briefly, ten-fold dilutions of heat inactivated (56 °C, 30 minutes) serum samples were mixed with 100 pfu of hAd5 and incubated for 1 hour at 37 °C. The virus-sample mixture was then plated on confluent 293 cells in 24 well plates, incubated for 1 hour, then incubated for 8 days under a 1% agarose overlay until plaques were formed. Serum neutralising titre was defined as the reciprocal of the highest serum dilution resulting in reduction of virus plaque formation by at least 50% relative to the virus control.

4.4.7 Statistical analysis

Statistical analysis of the data was performed using a Student t-test.

5.0 RESULTS

5.1 Establishment of an intranasal BHV-1 challenge model in cotton rats

The primary goal of this thesis was to assess mucosal and systemic immune responses induced by replication-defective and replication-competent recombinant HAd5 vectors expressing gD of BHV-1. Since cotton rats support HAd5 replication, they provide a suitable model for such a project. However, to evaluate the efficacy of immunisation with recombinant adenoviruses in conferring protection against BHV-1 challenge, a laboratory animal that supported BHV-1 replication was necessary. The first objective of this thesis was to find out whether cotton rats are a suitable laboratory animal model for BHV-1 infection.

5.1.1 BHV-1 glycoprotein expression in CRL cells following infection

Viral replication can take place only if the cell becomes infected with the virus and viral proteins are appropriately expressed. To find out whether CRL cells expressed a major glycoprotein of BHV-1 following adsorption of the virus, gD expression in CRL cells was monitored by immunoprecipitation, immunocytochemistry and ELISA. Results of immunoprecipitation provided evidence that gD was synthesised *de novo* in BHV-1 infected CRL cells (see next section, Fig. 5.2.1). Immunocytochemical staining of BHV-1 infected CRL cells indicated that gD was abundant in the perinuclear region as early as 2 h p.i. (Fig. 5.1.1.a). The cell membrane stained positive for gD from 4 h p.i. on (data not shown). The peak production of gD was at 24 h p.i., as measured by ELISA (Fig. 5.1.1.b).

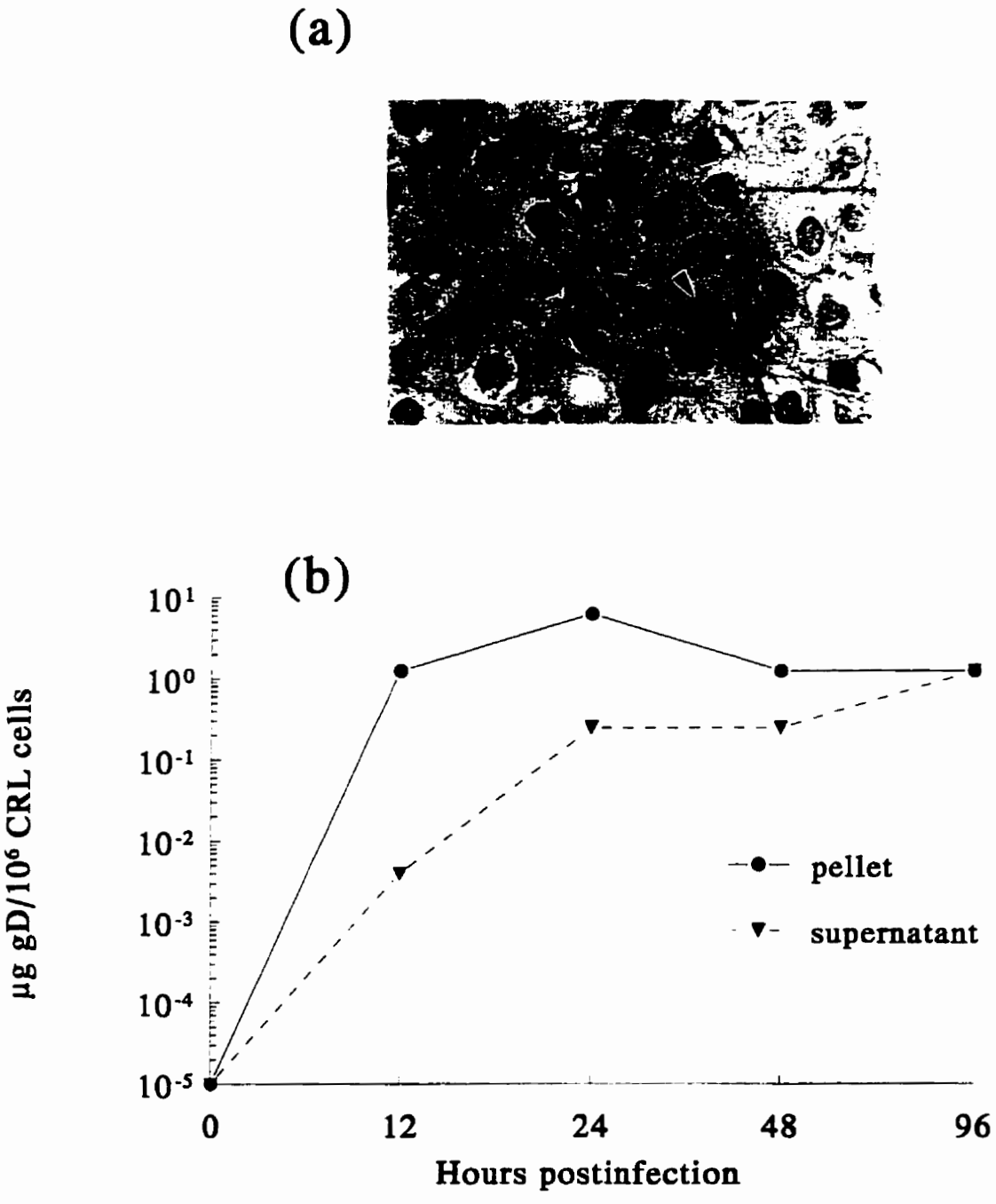


Figure 5.1.1 gD-expression by BHV-1 infected CRL cells. CRL cell monolayers were infected with the Cooper strain of BHV-1 at an MOI of 1. (a) At 2 h p.i., gD-expression was detected by imunocytochemistry. Dark precipitation in the perinuclear region indicates gD-specific staining (arrow). (b) gD-production in culture supernatants and cells was measured by ELISA at different timepoints p.i. Data are expressed as µg gD/10^6 CRL cells in single cultures, and represent results from 2 experiments.

5.1.2 BHV-1 replication in CRL cells *in vitro* and in cotton rats *in vivo*

To determine whether BHV-1 could replicate in CRL cells *in vitro*, the kinetics of virus production was monitored. The growth of BHV-1 in CRL cells was similar to that observed in MDBK cells (Fig. 5.1.2). In both cell lines, isolate 108 replicated to a slightly higher titre than the laboratory Cooper strain (Fig. 5.1.2).

Since BHV-1 could replicate efficiently in CRL cells *in vitro*, next I assessed the capability of BHV-1 to replicate *in vivo*. Cotton rats were inoculated i.n. with the two different strains of BHV-1 and virus growth was monitored in the respiratory tract. Viral recovery from the trachea and lung increased compared to that at the beginning of infection. BHV-1 titres peaked between day 1 and 2 postinfection (Fig. 5.1.3). Although both strains could replicate in the cotton rat, the 108 strain replicated to higher titres than the Cooper strain. From day 2 on, viral titres declined and virus was completely cleared from all animals by day 8.

Cotton rats developed cellular and humoral immune responses to BHV-1 glycoproteins. (Table 5.1.1). By day 8 p.i., both the Cooper and 108 strains induced gD- and gB-specific antibody in the serum (Table 5.1.1) and the lung- and trachea-extracts (data not shown). The animals did not show any apparent signs of disease or changes in behaviour at any stage of the BHV-1 infection period.

5.1.4 Histopathological and immunohistochemical evaluation of BHV-1 infected cotton rat lungs

To examine whether BHV-1 infection and replication caused any cell damage in the lung, sections of lungs from infected cotton rats were examined histologically. The results of histological evaluation of cotton rat lungs infected with the Cooper and 108 strains of BHV-1 are summarised in Fig. 5.1.4. No lesions were seen in the lungs of rats killed at 6 hours following infection. Randomly, rare intra-alveolar macrophages and indistinct intranuclear inclusion bodies in bronchiolar epithelial cells were seen on day 1 postinfection. By day 2 p.i., rats infected with the 108 strain exhibited multifocal moderate

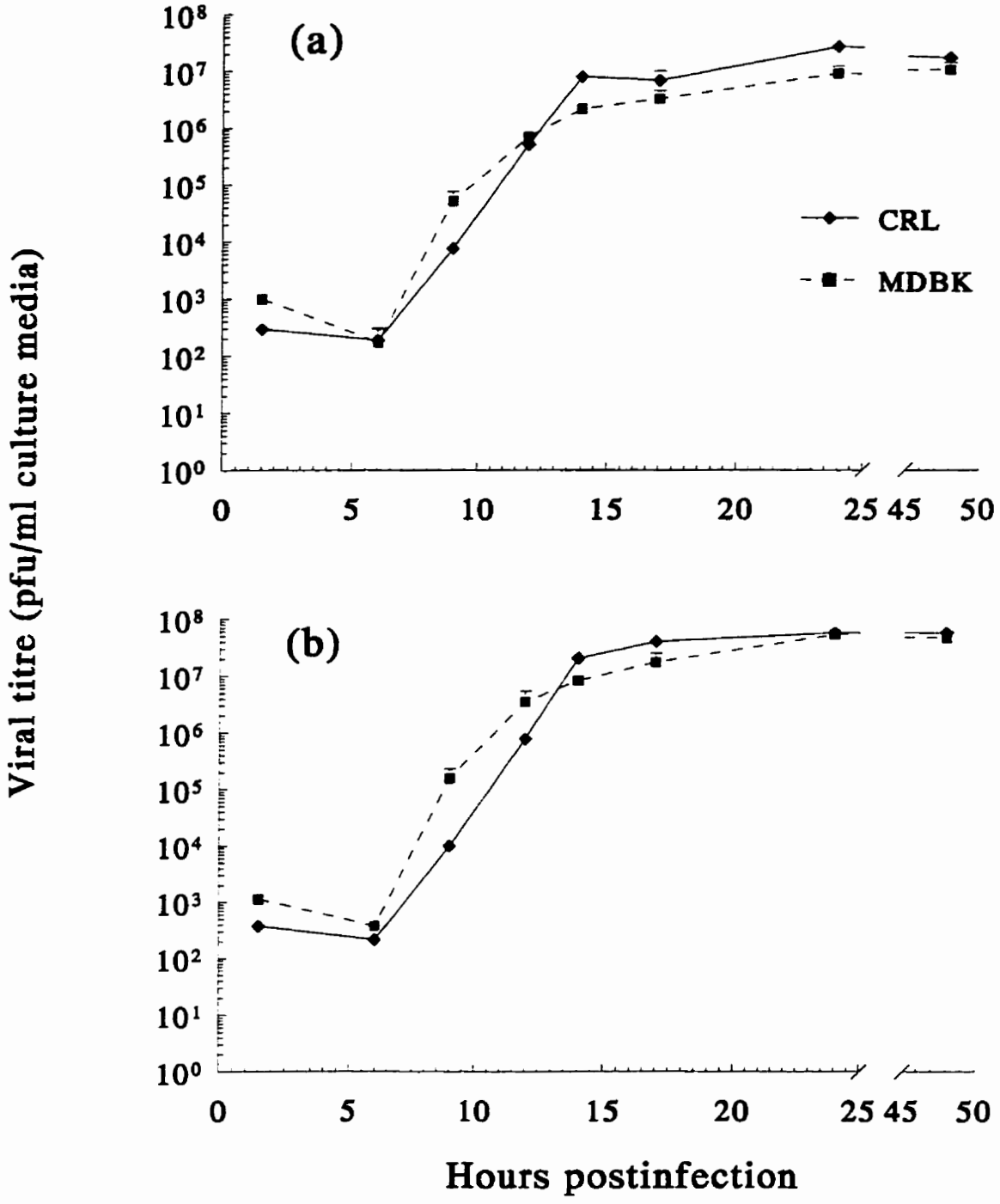


Figure 5.1.2 Kinetics of *in vitro* replication of BHV-1 in cotton rat and bovine cells. CRL and MDBK cells were infected with the Cooper (a) and 108 (b) strains of BHV-1 at an MOI of 1. Total virus production at different timepoints p.i. was measured by plaque assay on MDBK cells. Data are expressed as the mean ± SD pfu/ml of triplicate cultures.

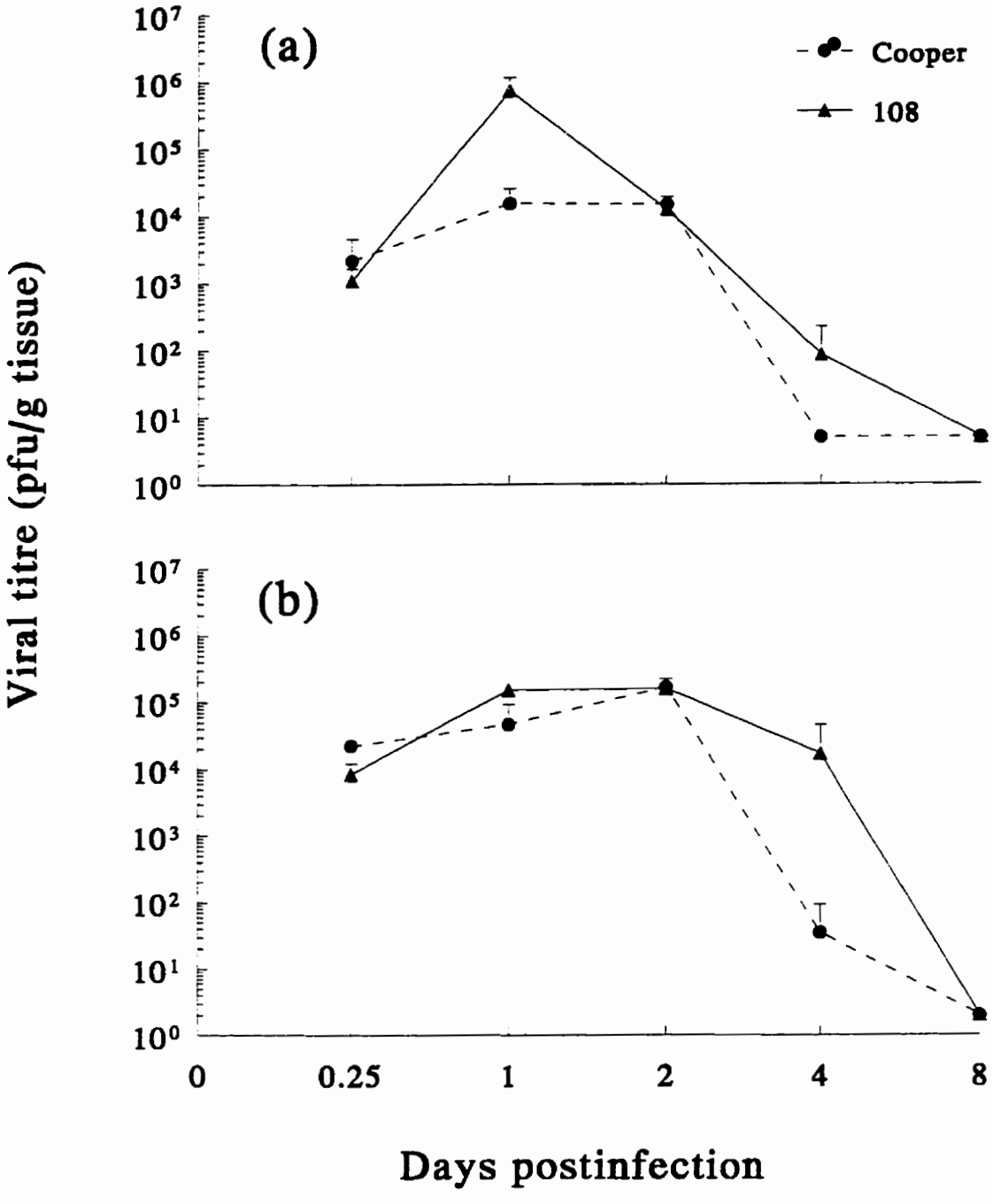


Figure 5.1.3 Replication of BHV-1 in the respiratory tract of cotton rats. Viral titres in the trachea (a) and the lung (b) were determined by plaque assay at different timepoints after i.n. inoculation of $5x10^7$ pfu of the Cooper and the 108 strains. Results are expressed as the mean pfu/g tissue from 2 or 3 animals per group.

Table 5.1.1 Immune responses induced by intranasal inoculation of BHV-1 in cotton rats

virus	gD-specific serum Ab IgG / IgA OD-s*	gB-specific serum Ab IgG / IgA OD-s*	gD-specific spleen lymphocyte proliferation (SI)**	BHV-1 specific spleen lymphocyte proliferation (SI)**
Cooper	0.57 / 0.09	0.365 / 0.09	2.9	6.01
108	0.61 [§] / 0.13	0.465 / 0.154	3.05	6.49
control	0.05 / 0.08 [†]	0.05 / 0.08 [†]	1.1 ± 0.3 [‡]	1.1 ± 0.4 [‡]

Cotton rats were inoculated with 5 x 10^7 pfu of the Cooper or 108 strains of BHV-1. Spleen lymphocyte proliferative responses and serum antibody responses specific for BHV-1 or BHV-1 glycoproteins were determined at 8 days post-challenge.

* Mean OD-s at 1:10 dilution of serum samples measured by ELISA.

** Mean stimulation index (SI) at 1 μg gD/ml and 0.5 μg BHV-1/ml concentrations from 2 animals/group. *In vitro* stimulation was performed at different antigen concentrations with similar results.

[§] OD of 0.61 value was equivalent with gD-specific serum titre of 500.

[†] Mean OD-s of samples on day 0 or day 2 p.i. (controls) at 1:10 dilution.

[‡] Mean stimulation index ± SD of PBS controls or non-immunised animals as determined by experimental data from numerous experiments.

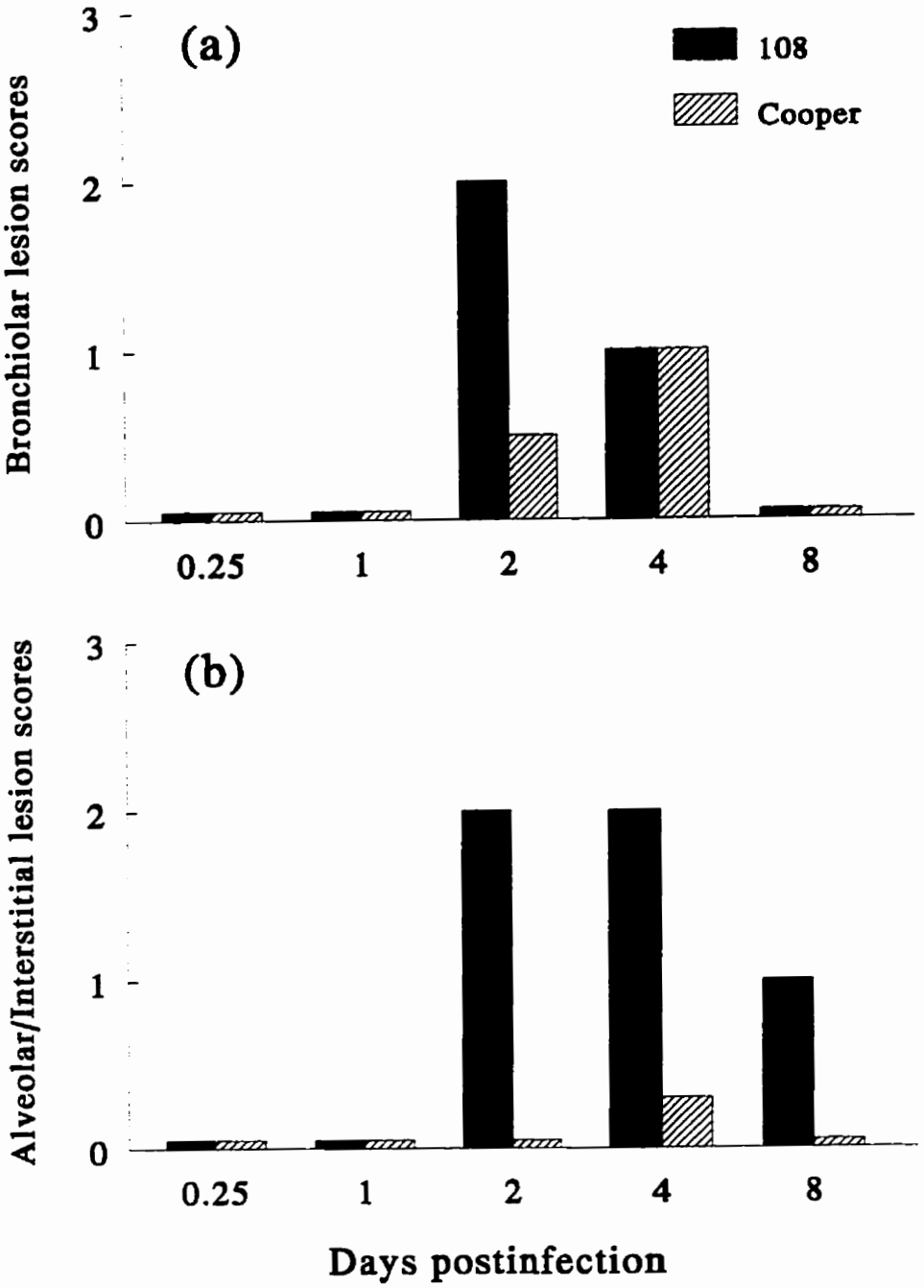


Figure 5.1.4 Quantitation of histological changes in the lungs of cotton rats inoculated with BHV-1. Lungs were collected at various time points after i.n. infection with $5x10^7$ pfu of the Cooper or the 108 strains and evaluated for histological lesions in the bronchioli (a) and alveoli (b). Lesions were scored according to the severity on a scale of 0 (normal) to 3 (severe). Each bar denotes a mean of scores for 3 animals at 6 h, day 1 and day 4 p.i. and for 2 animals at day 2 and day 8 p.i.

bronchiolitis with the presence of intranuclear inclusion bodies in bronchiolar epithelial cells, epithelial cell necrosis and infiltration of neutrophils into the mucosa and lumen (Fig. 5.1.5.a.). Multifocal, moderate interstitial pneumonia with macrophage and neutrophil infiltration of alveolar septa, along with a few inclusion bodies in alveolar epithelial cells and occasional intra-luminal cells were also seen (Fig. 5.1.5.b.). An inflammatory response, mild within the airways (1 of 3 rats) and moderate in the alveoli (3 of 3 rats), was present at day 4 p.i. Type II pneumocyte hyperplasia and macrophage infiltration of the septa were evident but inclusion bodies were absent. By day 8, a mild peribronchiolar lymphocytic infiltration was present along with alveolar septal infiltration of macrophages or macrophages and lymphocytes. The Cooper strain was less pathogenic for the cotton rats than the 108 strain, producing only mild focal lesions. Lesions persisted through day 4 and waned by day 8 p.i.

To correlate histological changes with BHV-1 infection and replication, lung sections were examined by immunohistochemical staining for the presence of BHV-1 antigens. In the lungs of cotton rats infected with the Cooper strain, only a few cells, randomly distributed throughout the alveoli, stained positively for BHV-1 antigens at 6 hours. By day 1, in all three infected rats, there were several small positively-staining foci of bronchiolar epithelial cells, and in one animal alveolar epithelial cells were also positive. Such foci were moderately numerous by day 2, involving both bronchiolar and alveolar epithelium. They were also present in similar numbers and distribution on day 4, but were absent by day 8.

Lungs of cotton rats infected with the 108 strain did not contain any detectable BHV-1 antigens at 6 h postinfection. Several moderately-sized foci of positive staining cells within the bronchiolar and alveolar epithelial cell population were found at day 1. Staining was widespread with moderate to large-sized bronchiolar and alveolar positive foci by day 2 (Fig. 5.1.5. a & b). Many moderately sized foci were still present on day 4 but were absent by day 8. Specific staining was generally cytoplasmic in distribution and corresponded for the most part with histological lesions.

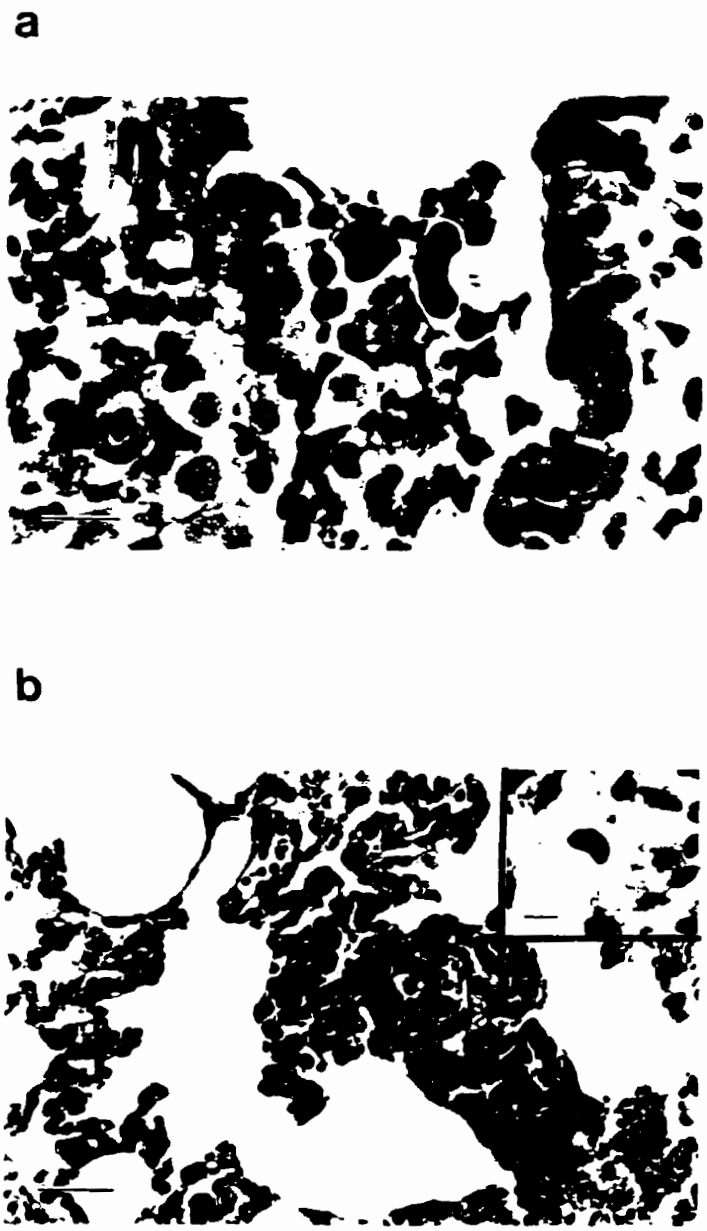


Figure 5.1.5 Histological lesions in a BHV-1 infected cotton rat lung. Lesions shown are at day 2 p.i. with the 108 strain. Note: (a) epithelial necrosis and luminal debris in a bronchiole (Bar, 10 μm) and (b) marked cellular infiltration of alveolar septa (Bar, 40 μm). Inset: intranuclear inclusion body in a desquamated alveolar epithelial cell (Bar, 5 μm).

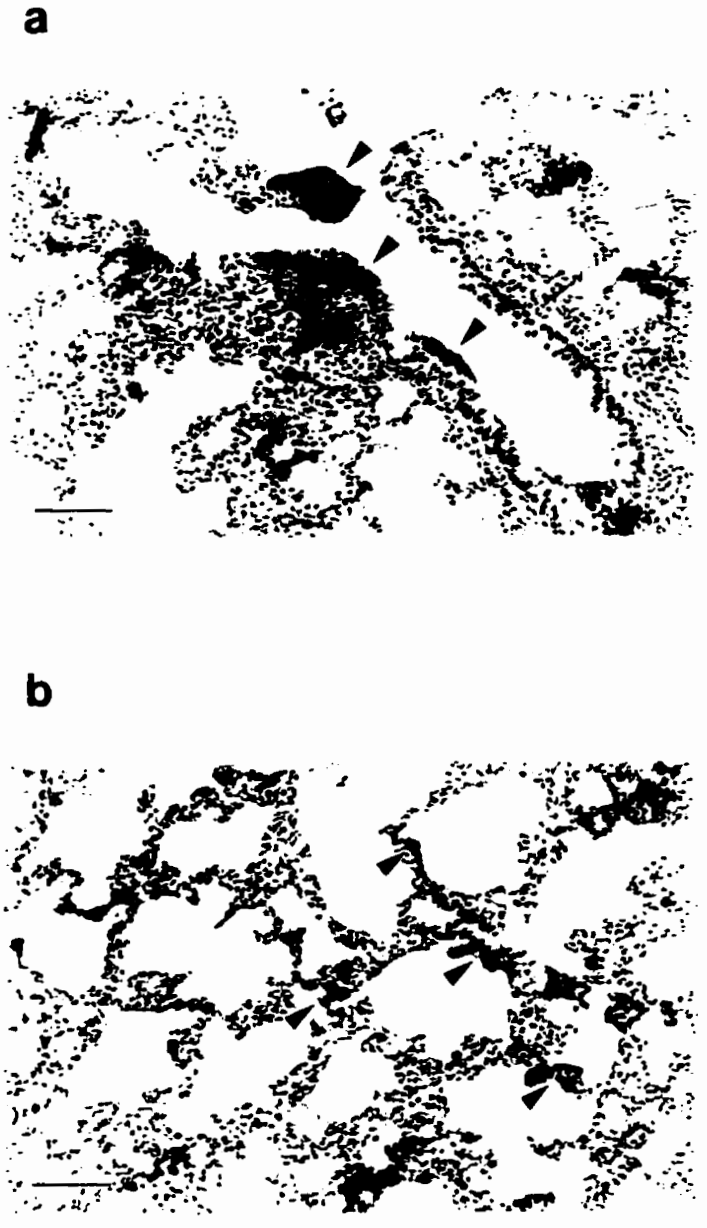


Figure 5.1.6 Immunohistochemical staining in a BHV-1 infected cotton rat lung. Immunohistochemical staining shown is at day 2 p.i. with the 108 strain. Positive immunohistochemical staining (arrows) was noted multifocally in (a) the bronchiolar epithelium and (b) the alveolar walls (Bars, 150 μm).

5.1.5 Conclusions

A BHV-1 i.n. challenge model in cotton rats was established by demonstrating that BHV-1 replicated in CRL cells *in vitro* and in the respiratory tract of cotton rats *in vivo* without requiring prior adaptation of the virus. The 108 strain of BHV-1 replicated better than the Cooper strain both *in vitro* and *in vivo*. Furthermore, lesion development was greater and viral antigen was more widespread in the lungs of animals challenged with the 108 strain than in those challenged with the Cooper strain. Based on the observed differences between the two strains, I used the 108 strain in the majority of the challenge experiments.

5.2 *In vitro* characterisation of recombinant adenoviruses

Replication-competent and replication-defective recombinant adenoviruses carrying the gD or the tgD gene of BHV-1 were planned to be used for studying mucosal immune responses induced by adenovirus vectors in cotton rats. Since the level of gD or tgD expression, and the replication capability of the vector may influence the induction of immune responses, it was important to characterise the vectors *in vitro*.

5.2.1 Replication capability of recombinant HAd5 vectors

Although the capability of adenovirus vectors to replicate in CRL cells had been previously determined (Mittal et al., 1996), it was necessary to confirm these results for the new stocks of adenoviruses that were to be used in the following experiments. Such studies were important because of the theoretical possibility of the replication-defective viruses (gD-dE1E3 and tgD-dE1E3) rescuing the E1 region during propagation in 293 cells. Table 5.2.1 summarises the replicative capability of recombinant adenoviruses in different cells. Both gD-dE3 and tgD-dE3 viruses formed plaques on CRL cells, although the number of pfu as determined on CRL cells was 2-4 times lower than on 293 cells. The gD-dE1E3 and tgD-dE1E3 constructs did not form any detectable plaques on CRL cells.

Table 5.2.1 Replicative capability of recombinant adenoviruses in cells of different species.

CRL : cotton rat lung
MDBK: Madin Darby bovine kidney
293 : human embrionic kidney cells expressing E1 proteins

HAd5 recombinant virus	replicates in 293 cells	CRL cells	MDBK cells
dE1E3	✓	-	-
dE3	✓	✓	✓
gD-dE1E3	✓	-	-
tgD-dE1E3	✓	-	-
gD-dE3	✓	✓	✓
tgD-dE3	✓	✓	✓

5.2.2 gD and tgD expression by CRL cells infected with adenovirus vectors

Expression of gD or tgD by 293 and CRL cells infected with recombinant adenoviruses has been previously shown (Mittal et al., 1996). It was important, however, to confirm expression of the foreign genes by the new virus stocks, after a few passages of the viruses in 293 cells. Expression of the foreign genes in CRL cells was also further characterised for the kinetics of expression and localisation of the foreign protein to provide background information for later *in vivo* experiments. All vectors expressed the appropriate foreign glycoproteins, detected by immunoprecipitation and SDS-PAGE (Fig. 5.2.1). Furthermore, gD was abundant in the pellet fraction of CRL cell cultures infected with gD-dE1E3 and gD-dE3 vectors, while tgD expressed by the tgD-dE1E3 and tgD-dE3 vectors was only detectable in the culture supernatants.

gD-specific immunocytochemical staining was used to determine the localisation of gD in CRL cells at different timepoints p.i. with the gD-dE1E3 or gD-dE3 viruses. Cells infected by gD-dE3 expressed gD in different membrane compartments of the cells, and in the cell membrane, by 8 h p.i. (Fig. 5.2.2.a). However, expression of gD in CRL cells infected with gD-dE1E3 was evident later and at lower levels than gD-expression following gD-dE3 infection (Figure 5.2.2). Furthermore, infection with gD-dE1E3 caused significantly less cytopathic effect on CRL cells than infection with gD-dE3 at 22 h (Fig. 5.2.2) and at 48 h p.i. (evaluated by visual observation of numerous cultures, data not shown). In contrast to gD-expression, tgD expressed by tgD-dE1E3 or tgD-dE3 was hardly detectable; the cell-cultures appeared similar to the ones with gD-dE1E3 at 8 h p.i. (results not shown). However, flow cytometric analysis of CRL cells infected with recombinant adenoviruses showed that not only gD, but also tgD (although at lower levels) was present on the cell surface (data not shown). Interestingly, infection with tgD-dE1E3 did not cause any cytopathic effect on CRL cells, while infection with tgD-dE3 caused a cytopathic effect by 48 h p.i., similar to that following gD-dE1E3 infection (data not shown).

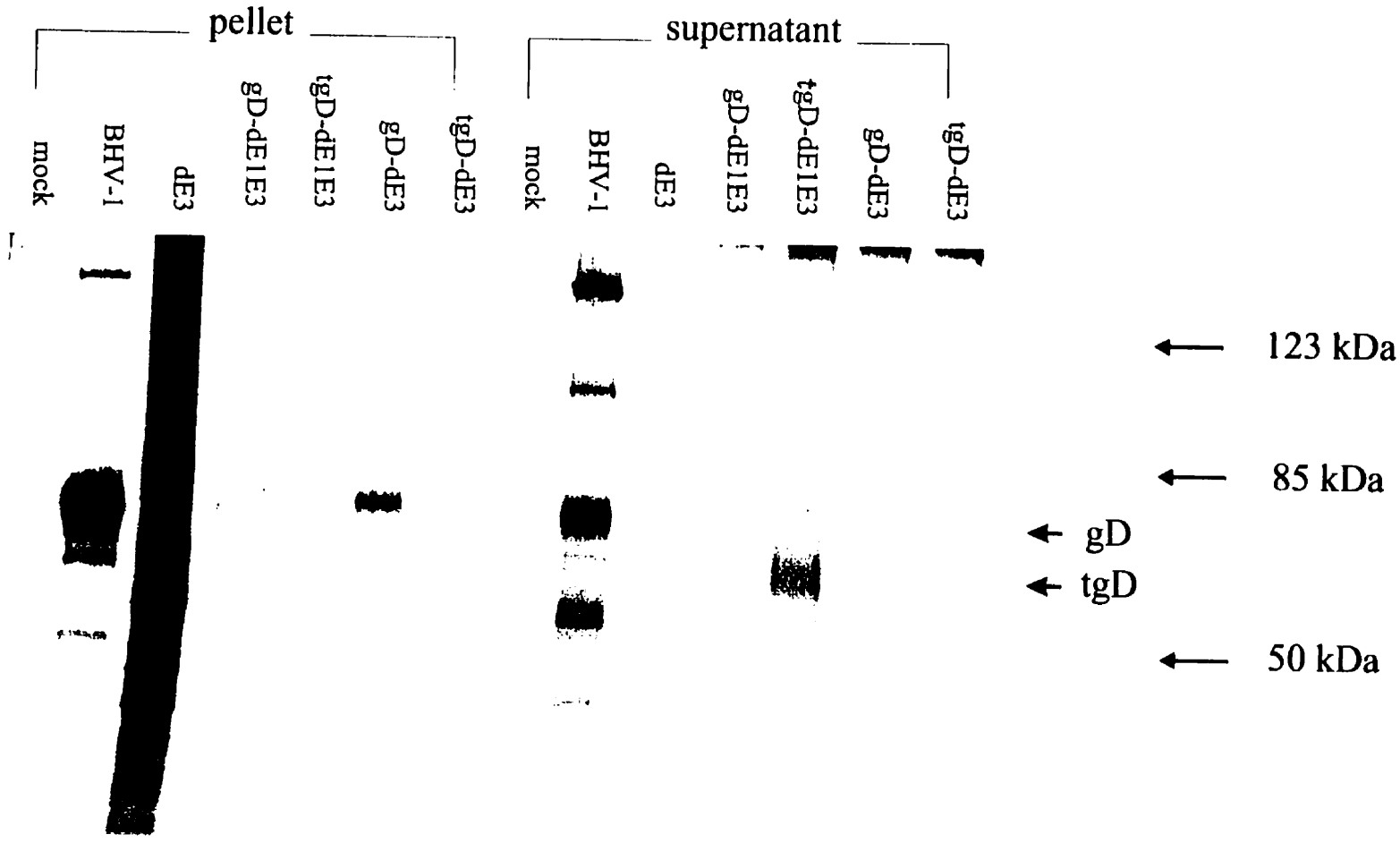


Figure 5.2.1 Expression of gD and tgD by CRL cells infected with recombinant adenovirus vectors. Cells were infected with mock, BHV-1, dE3, gD-dE1E3, gD-dE3, tgD-dE1E3 or tgD-dE3 viruses and labeled with ^{35}S-methionine. Supernatants and cell pellets were immunoprecipitated with a pool of gD-specific MAbs at 36 hours p.i. and analysed by SDS-PAGE. Molecular weight markers are shown on the right. Arrows indicate gD (71 kDa) and tgD (61 kDa).

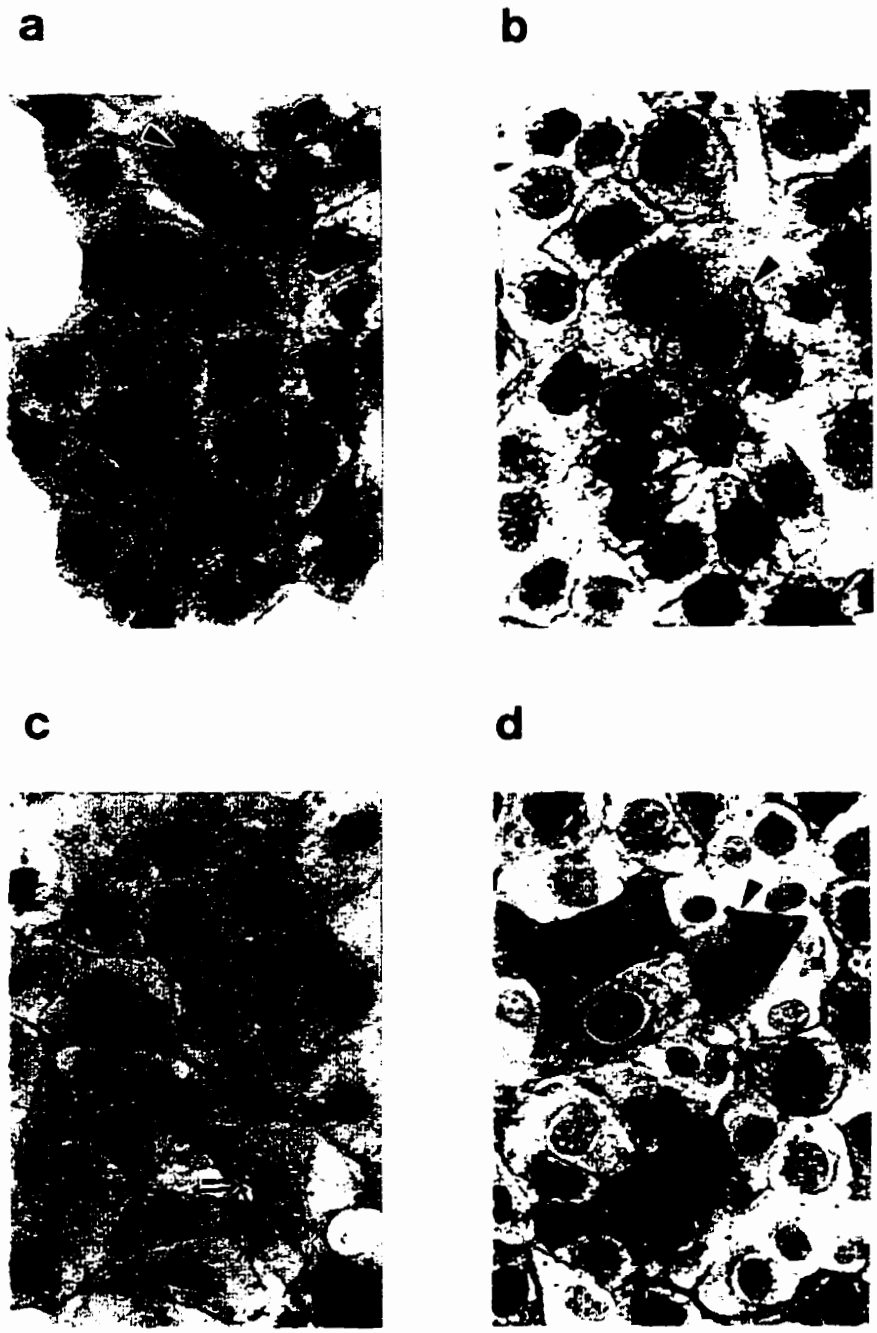


Figure 5.2.2 Expression of gD in CRL cells infected with recombinant adenoviruses. CRL cells were infected with gD-dE1E3 or gD-dE3 viruses at an MOI of 5. At different times p.i., expression of gD was demonstrated by immunocytochemistry using a pool of gD-specific mAbs. Infection with a) gD-dE3, 8 h p.i. b) gD-dE1E3, 8 h p.i. c) gD-dE3, 22 h p.i. d) gD-dE1E3, 22 h p.i. Dark precipitation (full arrows) indicate presence of gD in the cells. Non-infected controls did not show gD-specific staining, similarly to the cell indicated with open arrow on (c).

5.2.3 Kinetics of gD- and tgD-expression

Immunocytochemical staining had suggested that differences in the kinetics of gD expression may exist between gD-dE1E3- and gD-dE3-infected CRL cells. Furthermore, the total amount of tgD appeared less than that of gD following infection of CRL cells with the different adenoviruses. Such differences in the kinetics and quantity of foreign gene expression may influence the induction of immune responses *in vivo*; therefore, the production of gD and tgD at different timepoints p.i. with the recombinant adenoviruses was quantified by ELISA. While a hundred times more gD was produced by gD-dE3 than by gD-dE1E3 at 12 h p.i., similar amounts of gD were found in the cell cultures at 48 h p.i. (Fig. 5.2.3. a & b). Therefore, as the immunocytochemical staining indicated, the kinetics of gD-expression was slower in the gD-dE1E3- than in gD-dE3-infected CRL cells. Furthermore, the total production of gD by either gD-dE1E3- or gD-dE3-infected cells was higher than the production of tgD by the tgD-expressing vectors (Fig. 5.2.3). Interestingly, the supernatant and the cell fraction of cultures contained similar amounts of tgD.

5.2.4 Conclusions

The above results confirmed the replication-competence of dE3 and replication-deficiency of dE1E3 viruses in CRL cells. Furthermore, expression of gD and tgD was demonstrated in CRL cells following infection with the recombinant adenoviruses. Although higher total quantities of gD than tgD were produced, gD and tgD were found both cell-associated and in the culture medium. As expected, gD was more abundant in the cell fraction than in the supernatant of CRL cell-cultures. The kinetics of gD-expression by CRL cells infected with gD-dE1E3 was slower compared to that following infection with gD-dE3. In addition, the gD-expressing vectors and the replicating vectors caused more severe cytopathic effect on CRL cells following infection than tgD-expressing and non-replicating vectors. The characterisation of foreign gene expression and capability of replication is important because these features may influence the induction of immune responses by recombinant adenoviruses.

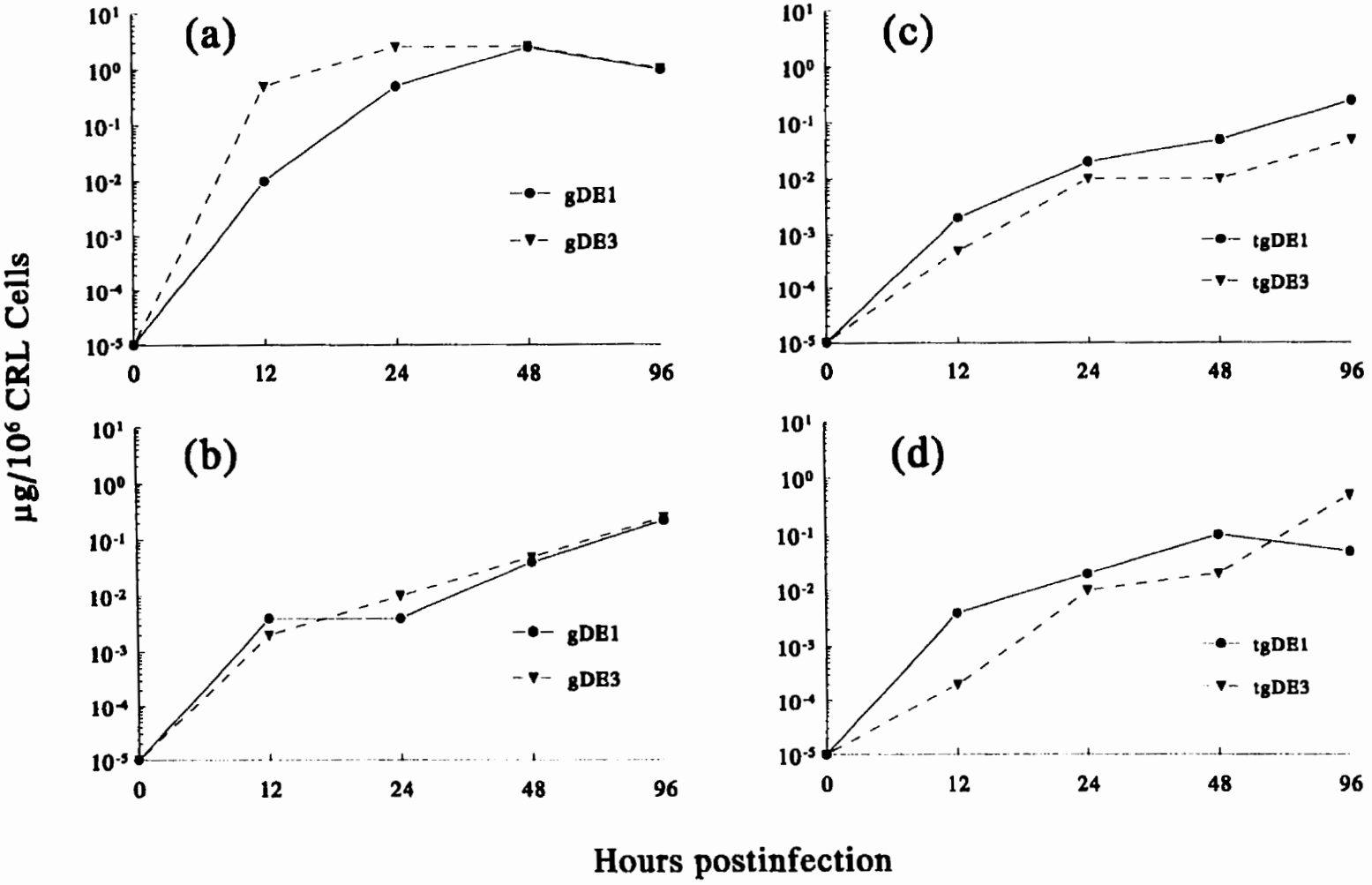


Figure 5.2.3 Kinetics of gD- and tgD-expression by CRL cells infected with recombinant adenoviruses. CRL cells were infected with gD-dE1E3 or gD-dE3 (a & b) and tgD-dE1E3 or tgD-dE3 (c & d) viruses at an MOI of 5. At different times p.i., gD or tgD in the cell pellet (a and c) and the culture supernatant (b and d) were detected by ELISA. Values represent µg gD or tgD/ 10^6 cells.

5.3 Immunity induced by recombinant adenoviruses in cotton rats

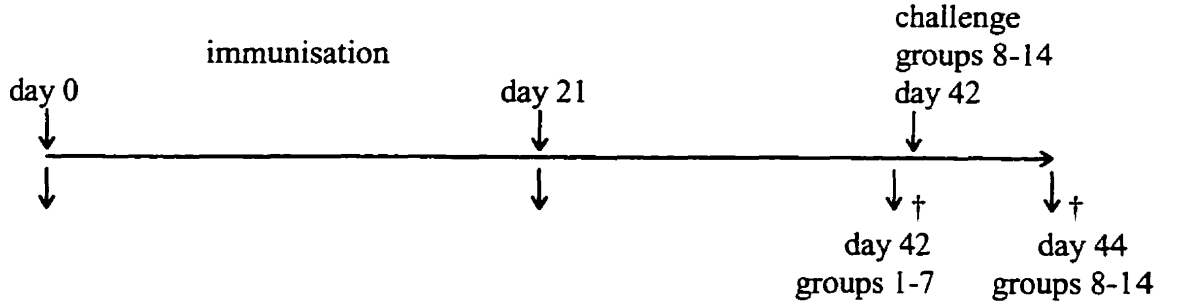
Recombinant adenoviruses gD-dE1E3, tgD-dE1E3, gD-dE3 and tgD-dE3 were shown to express their foreign genes in CRL cells *in vitro*. Furthermore, an i.n. BHV-1 infection model was established in cotton rats, which provided a means of assessing the ability of immunisation to confer protection against BHV-1 challenge in a laboratory animal model. Therefore, the next objective was to investigate the capability of recombinant HAd5 vectors expressing gD or tgD to induce immunity to BHV-1 and to confer protection against i.n. BHV-1 challenge in cotton rats. Since the induction of mucosal immunity is crucial in protection against infection with mucosal pathogens, the most important goal was to measure mucosal immune responses induced by recombinant adenoviruses.

5.3.1 Immunity induced by recombinant adenoviruses expressing gD and tgD

The first experiment was designed to compare the efficacy of gD- and tgD-expressing recombinant adenoviruses in inducing gD-specific immune responses and protection against BHV-1 challenge. Furthermore, it was important to determine whether both replication-defective and replication-competent vectors could induce antigen-specific immune responses. To induce both systemic and mucosal immunity, adenoviruses were administered i.d. followed by an i.n. boost 3 weeks later. Figure 5.3.1 describes the experimental design.

5.3.1.1 Systemic immune responses

Antigen-specific systemic humoral immune responses in cotton rats were assessed by measuring serum antibody responses by ELISA. Both serum IgG and IgA specific for gD were induced by immunisation with gD- and tgD-expressing recombinant adenoviruses, but not with dE3, dE1E3 and PBS controls (Figure 5.3.2). The levels of these responses were higher following the secondary (day 42) than after the primary (day 21)



group #	# of cotton rats per group	immunisation				i.n. BHV-1 challenge	
		with	dose (pfu)	route		strain	dose (pfu)
				primary	secondary		
1	3	dE1E3	10^7	i.d.	i.n.	Cooper	10^7
2	3	dE3	10^7	i.d.	i.n.	Cooper	10^7
3	3	gDE1	10^7	i.d.	i.n.	Cooper	10^7
4	3	tgDE1	10^7	i.d.	i.n.	Cooper	10^7
5	3	gDE3	10^7	i.d.	i.n.	Cooper	10^7
6	3	tgDE3	10^7	i.d.	i.n.	Cooper	10^7
7	3	PBS	-	i.d.	i.n.	Cooper	10^7
8	3	dE1E3	10^7	i.d.	i.n.	-	-
9	3	dE3	10^7	i.d.	i.n.	-	-
10	3	gDE1	10^7	i.d.	i.n.	-	-
11	3	tgDE1	10^7	i.d.	i.n.	-	-
12	3	gDE3	10^7	i.d.	i.n.	-	-
13	3	tgDE3	10^7	i.d.	i.n.	-	-
14	3	PBS	-	i.d.	i.n.	-	-

Figure 5.3.1 Experimental design: immunity induced by recombinant adenoviruses expressing gD and tgD. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples collected from euthanised animals. i.n. = intranasal; i.d. = intradermal.

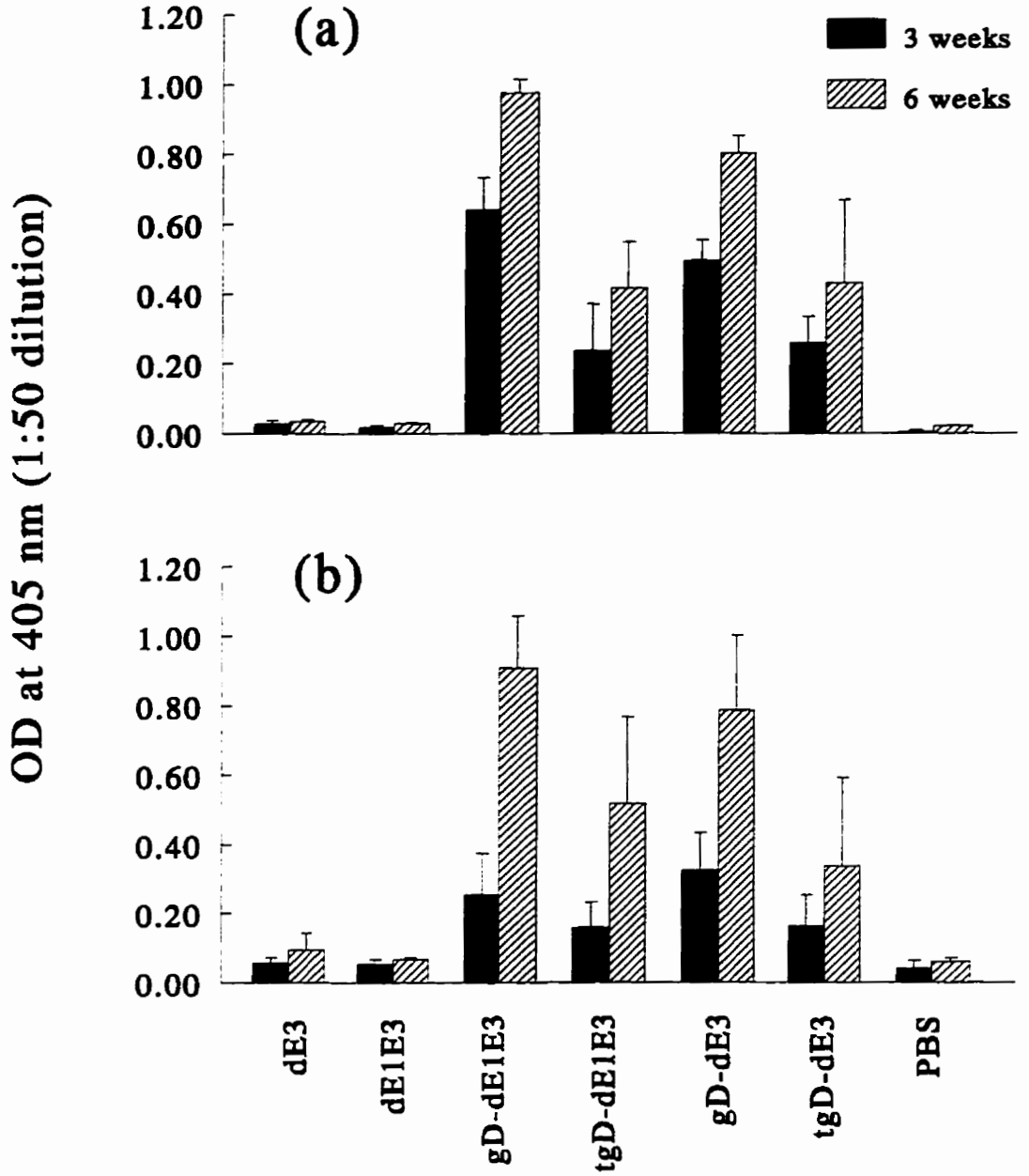


Figure 5.3.2 gD-specific serum antibody responses in cotton rats immunised with recombinant adenoviruses. Cotton rats were inoculated with recombinant adenoviruses expressing gD or tgD, or with PBS by the intradermal route and boosted intranasally 3 weeks later. gD-specific (a) IgG and (b) IgA levels in the serum at 3 and 6 weeks following the primary immunisation were measured by ELISA. The graphs show OD-s at 1:50 dilution of the samples. Bars represent the mean OD ± SD for 3-6 animals per group.

immunisation. Immunisation with the gD-expressing vectors induced significantly higher serum IgG at day 42 than immunisation with the tgD-expressing vectors ($P < 0.01$ for gD-dE1E3 vs. tgD-dE1E3; $P < 0.05$ for gD-dE3 vs. tgD-dE3). The levels of gD-specific serum IgA were not significantly different between groups immunised with any of the four gD- or tgD-expressing vectors, but they were all significantly higher than those of the PBS and control Ad groups. The recombinant adenoviruses induced similar Ad-specific serum IgG and IgA responses (Fig. 5.3.3).

To determine the biological activity of antigen-specific serum antibody, BHV-1- and Ad-neutralising antibody assays were performed. Immunisation with all Ad vectors resulted in Ad-neutralising antibody in the serum but only immunisation with gD-expressing vectors induced BHV-1 SN titres significantly different from controls (Figure 5.3.4). SN titres induced by immunisation with gD-dE1E3 and gD-dE3 were significantly higher ($P < 0.05$) than those by tgD-dE1E3 and tgD-dE3 (Fig. 5.3.4).

Another way of evaluating systemic antibody responses is quantification of antigen-specific antibody-secreting B cells in the spleen. In addition, such information would help more accurately describe immune responses induced by the different recombinant adenoviruses. Therefore, the frequency of gD- and Ad-specific IgA and IgG secreting cells in the spleen were measured by ELISPOT. As expected, only those animals inoculated with gD- or tgD-expressing vectors had gD-specific ASC in their spleens, while all contained Ad-specific ASC, except for the PBS control (Fig. 5.3.5). Interestingly, the replication-competent vectors induced higher frequency of gD-specific ASC in the spleen than did the replication-defective adenoviruses (Fig. 5.3.5). The difference between gD-dE1E3 and gD-dE3 groups was statistically significant ($P < 0.01$) for both IgG and IgA ASC frequencies (Fig. 5.3.5).

Serum antibody and ELISPOT assays were performed on samples taken at both day 42 (no challenge) and 44 (after challenge). The results were equivalent at these two timepoints.

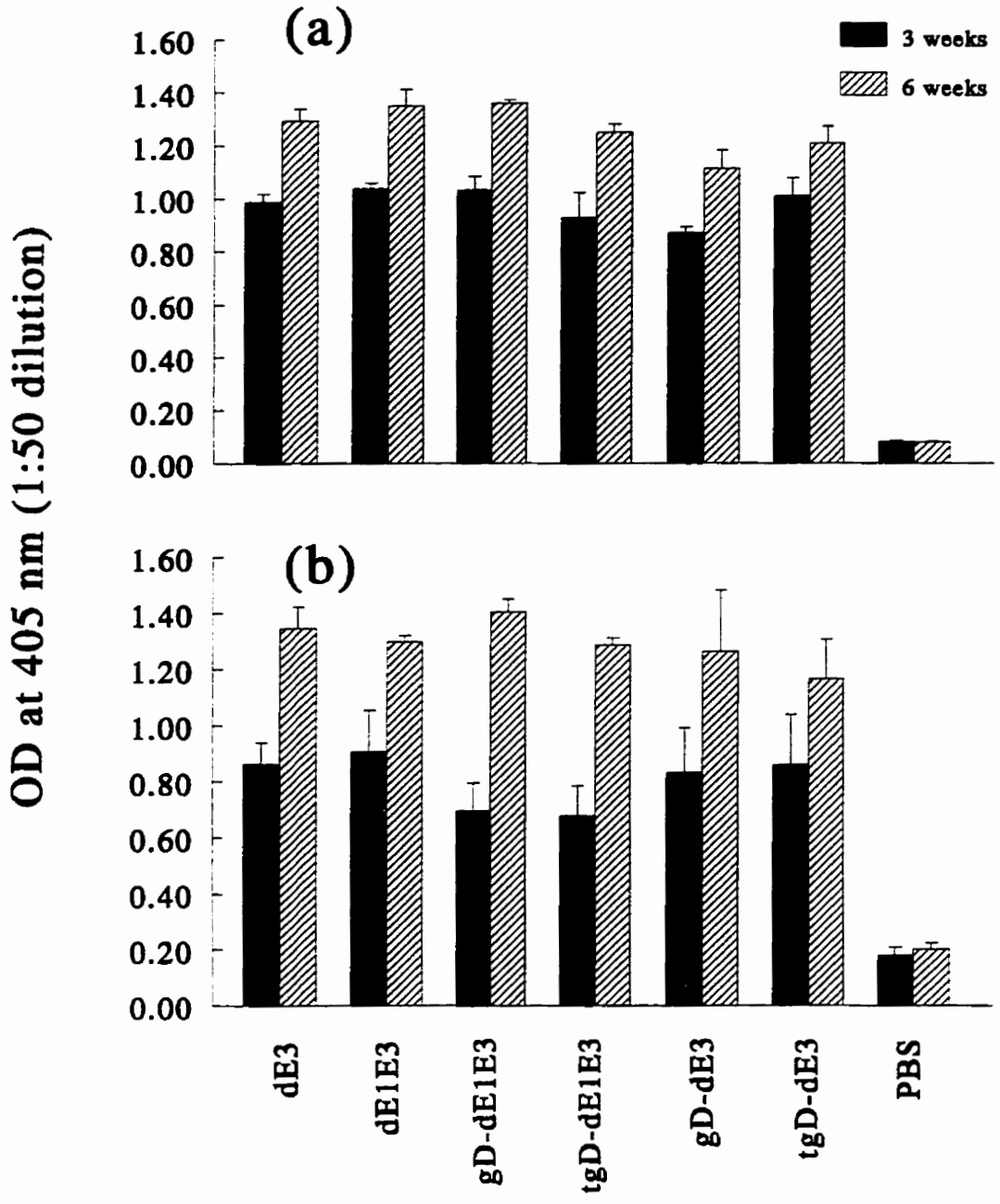


Figure 5.3.3 Adenovirus-specific serum antibody responses in cotton rats immunised with recombinant adenoviruses. Cotton rats were inoculated with recombinant adenoviruses expressing gD or tgD, or with PBS by the intradermal route and boosted intranasally 3 weeks later. Ad-specific (a) IgG and (b) IgA levels in the serum at 3 and 6 weeks following the primary immunisation were measured by ELISA. The graphs show OD-s at 1:50 dilution of the samples. Bars represent the mean OD ± SD for 3-6 animals per group.

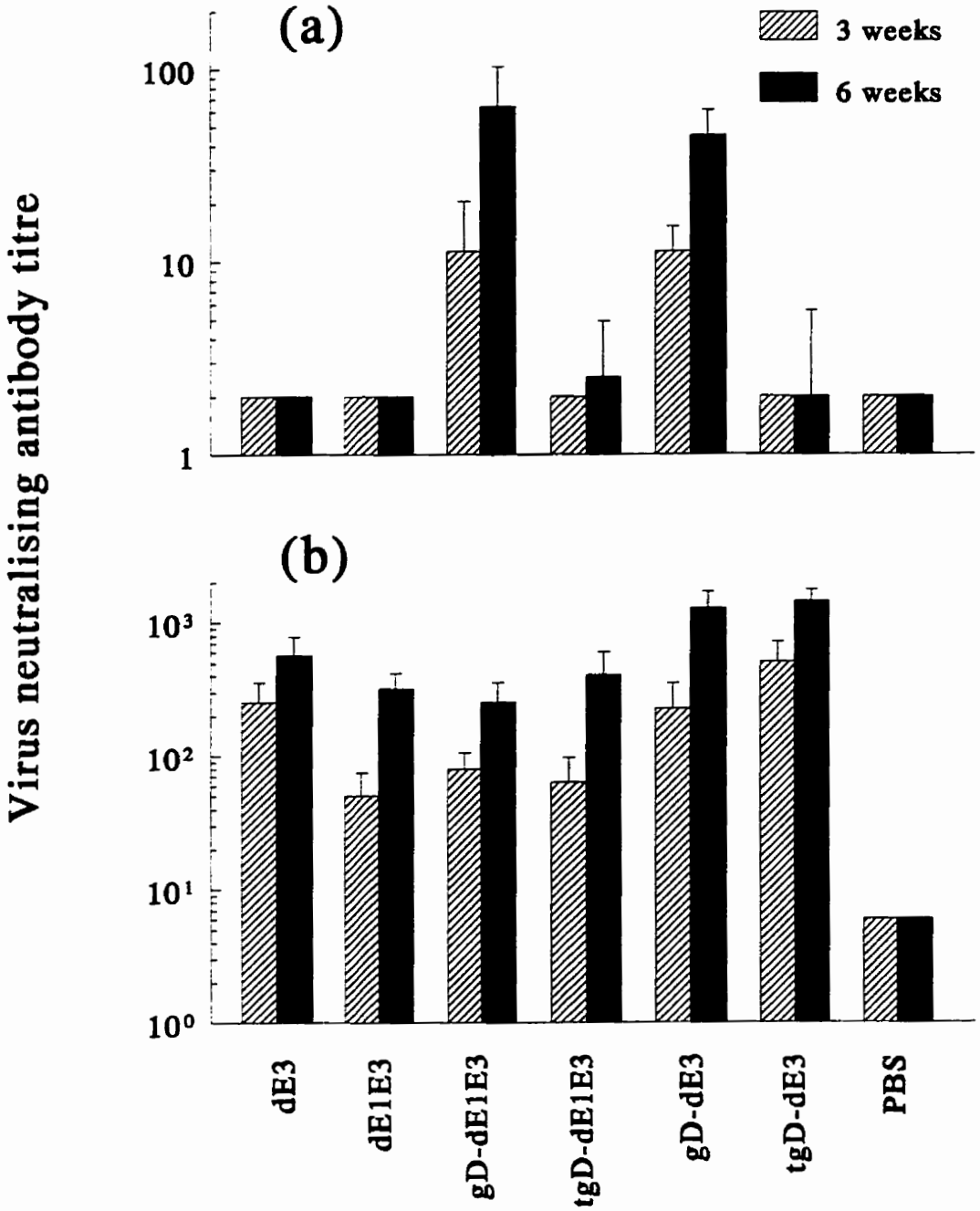


Figure 5.3.4 BHV-1- and Ad-neutralising antibody titres in sera of cotton rats immunised with recombinant adenoviruses. Cotton rats were inoculated with recombinant adenoviruses expressing gD or tgD, or with PBS by the intradermal route and boosted intranasally 3 weeks later. Serum samples collected at 3 and 6 weeks following primary immunisation were analysed for (a) BHV-1- and (b) HAd5-neutralising antibodies by virus neutralisation assays. Bars represent the mean value for 3 animals per group ± SD.

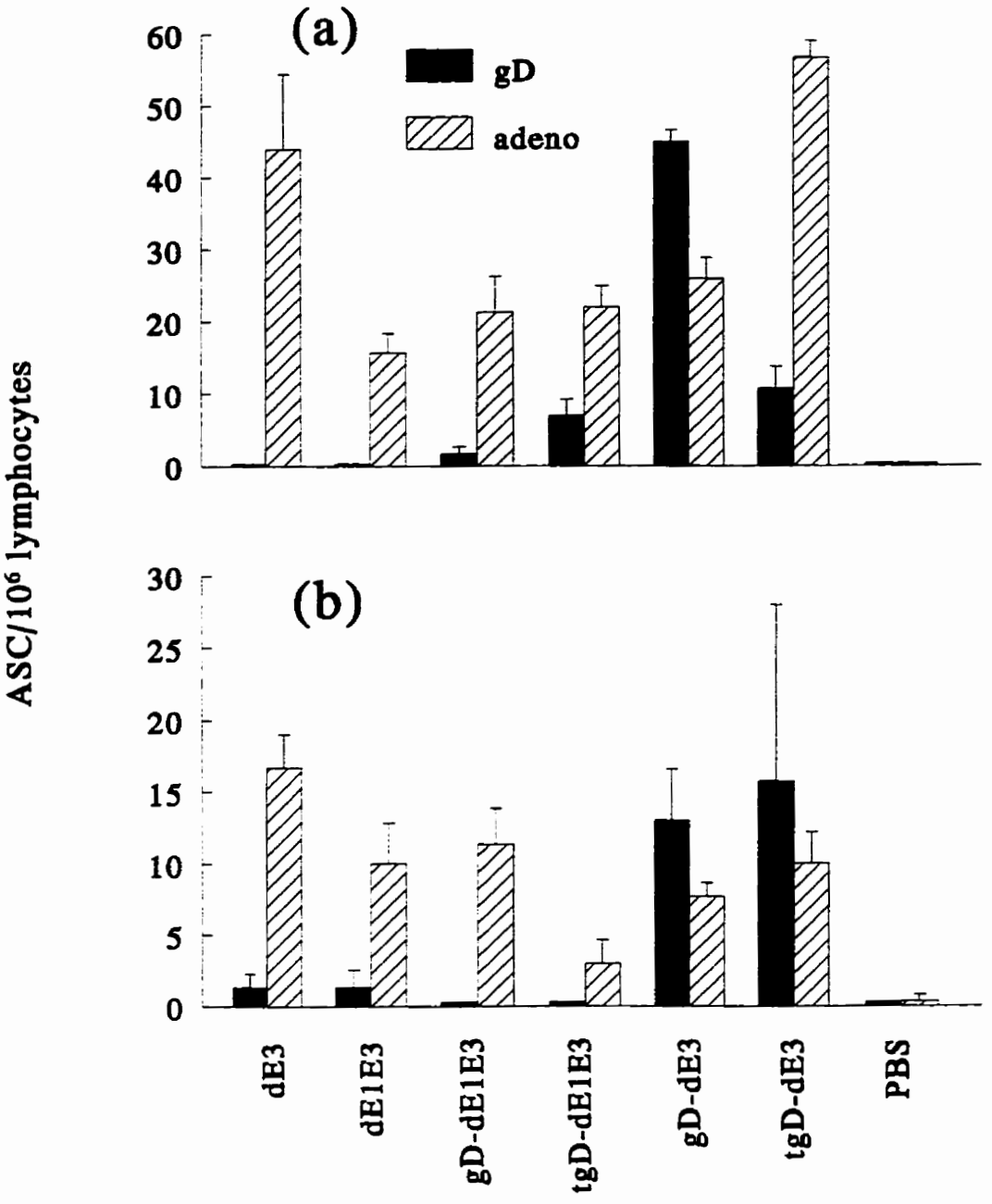


Figure 5.3.5 Frequency of gD- and Ad-specific antibody-secreting cells in the spleen of cotton rats immunised with recombinant adenoviruses. Cotton rats were inoculated with recombinant adenoviruses expressing gD or tgD, or with PBS by the intradermal route and boosted intranasally 3 weeks later. The frequency of (a) IgG and (b) IgA antibody-secreting cells (ASC) in spleen lymphocytes isolated 3 weeks after the secondary immunisation were determined by ELISPOT assay. Bars represent the mean number of ASC/million lymphocytes ± SD for 3 animals per group.

Antigen-specific T cell activation induced by immunisation with recombinant adenoviruses was measured by spleen cell proliferation assays. At day 42, spleen cells of only those animals immunised with gD-expressing vectors responded with proliferation to *in vitro* stimulation with gD (Fig. 5.3.6). The stimulation indices (SI) in the gD-dE1E3 and gD-dE3 groups were higher than those in other groups ($P < 0.01$). In addition, Ad-specific proliferation of spleen cells was observed in all Ad-immunised groups, with statistically higher stimulation indices than the PBS control (Fig. 5.3.6). At day 44, antigen-specific proliferative responses were similar to those detected at day 42, except for the mean gD-specific SI in the tgD-dE1E3 group, which was not statistically different from SI in the gD-dE3 and gD-dE1E3 groups at this later timepoint (data not shown).

5.3.1.2 Antibody levels in the respiratory tract

Local mucosal immune responses play a significant role in protection against respiratory viral infections; therefore, it was important to determine gD-specific antibody levels in the respiratory tract. Animals that received gD-expressing adenoviruses developed significantly higher levels of gD-specific IgG and IgA in the lung-washes ($P < 0.05$) and gD-specific IgA in the nasal-washes ($P < 0.001$) than the other groups (Figure 5.3.7). No animals contained gD-specific IgG in nasal-washes (Fig. 5.3.7.a.). In contrast to gD-specific antibody responses, Ad-specific IgG and IgA levels in the lung- and nasal-washes did not differ significantly between all groups immunised with recombinant adenoviruses (Fig. 5.3.8), but were significantly higher than those in the PBS group. BHV-1 neutralising antibody titres in the lung- and nasal-washes were below detection level.

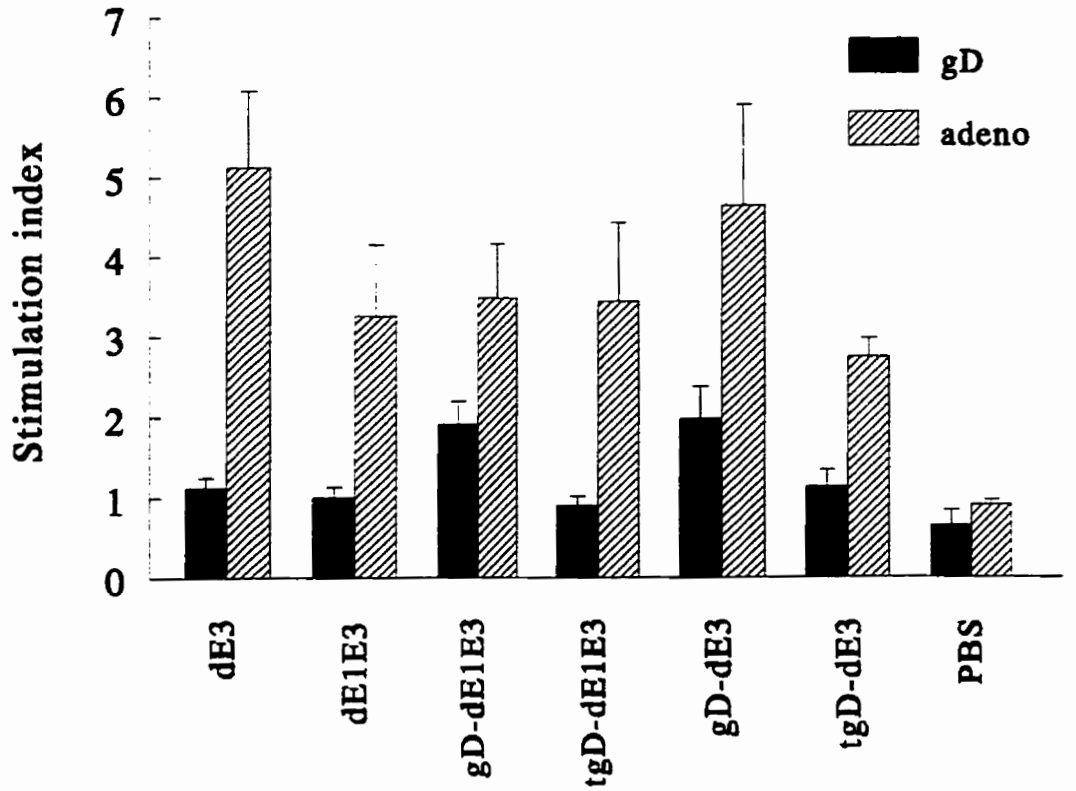


Figure 5.3.6 gD- and HAd5 specific proliferative responses of spleen-lymphocytes of cotton rats immunised with recombinant adenoviruses. Cotton rats were immunised with recombinant adenoviruses expressing gD or tgD or inoculated with PBS by the intradermal route and boosted intranasally 3 weeks later. Spleen lymphocytes isolated 3 weeks after the secondary immunisation were cultured in the presence of 0.05 μg/ml purified gD or 2.5 μg/ml UV-inactivated HAd5. Bars represent the mean stimulation index (SI) ± SD for 3 animals per group.

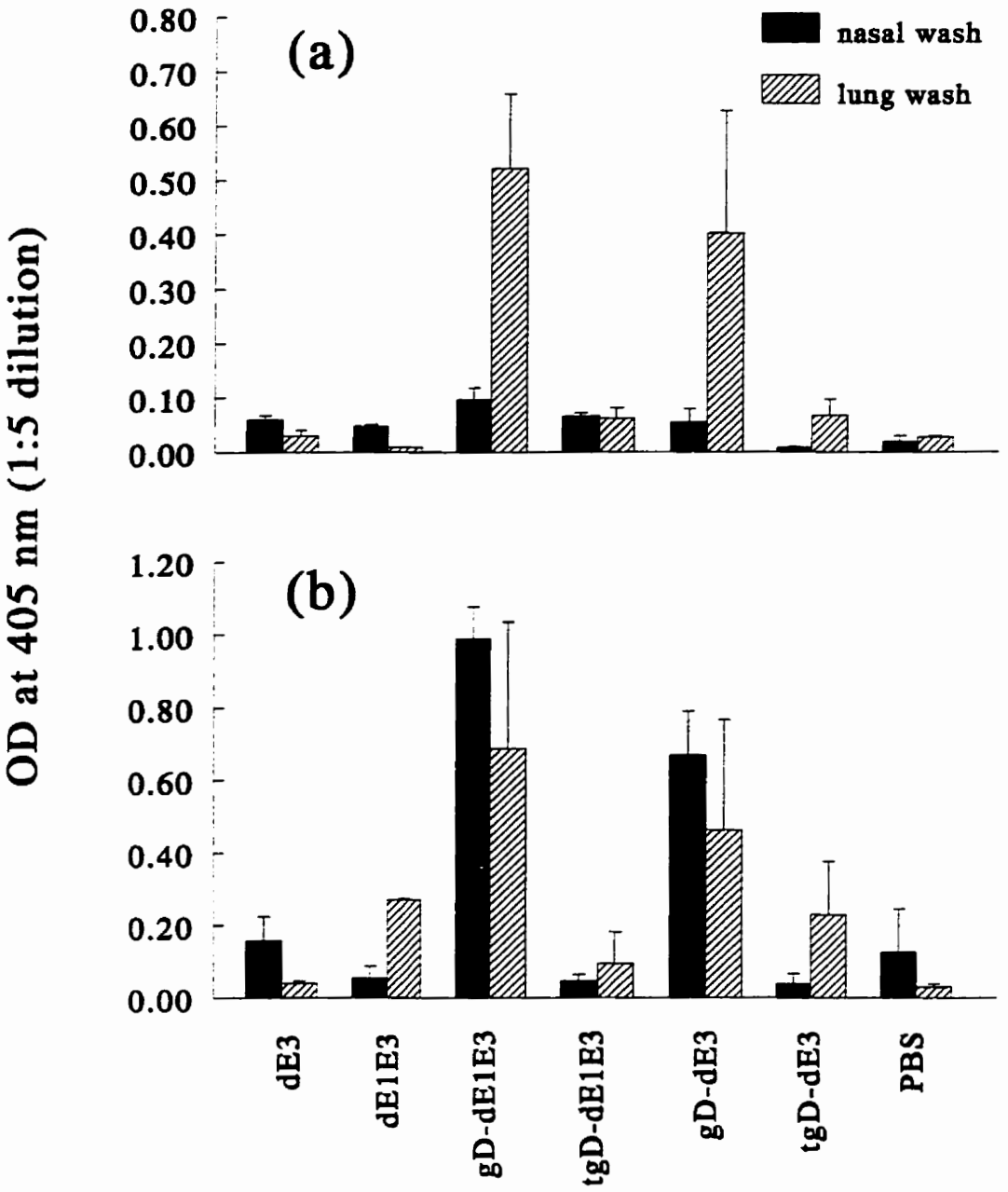


Figure 5.3.7 gD-specific antibody levels in nasal- and lung-washes in cotton rats immunised with recombinant adenoviruses. Cotton rats were immunised with recombinant adenoviruses expressing gD or tgD or inoculated with PBS by the intradermal route and boosted intranasally 3 weeks later. Washes were collected at 3 weeks after the secondary immunisation. gD-specific (a) IgG and (b) IgA levels at different dilutions were measured by ELISA. The graphs show OD-s at 1:5 dilution of the samples. Bars represent the mean OD ± SD for 3 animals per group.

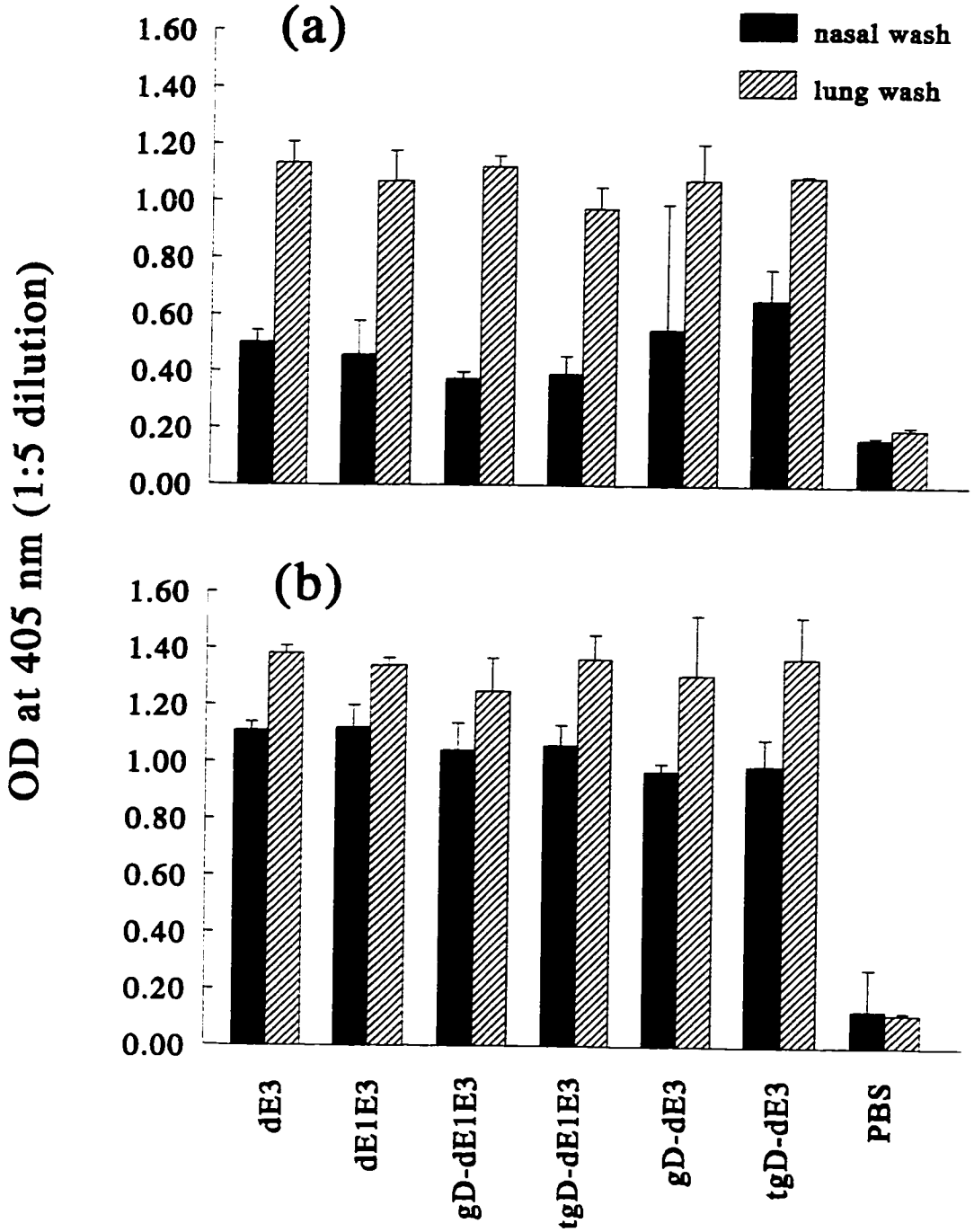


Figure 5.3.8 Adenovirus-specific antibody levels in nasal- and lung-washes in cotton rats immunised with recombinant adenoviruses. Cotton rats were inoculated with recombinant adenoviruses expressing gD or tgD, or with PBS by the intradermal route and boosted intranasally 3 weeks later. Washes were collected at 3 weeks after the secondary immunisation. Ad-specific (a) IgG and (b) IgA levels at different dilutions were measured by ELISA. The graphs show OD-s at 1:5 dilution of the samples. Bars represent the mean OD ± SD for 3 animals per group.

5.3.1.3 Protection against BHV-1 challenge

Since immunisation of cotton rats with various HAd5 vectors expressing gD or tgD resulted in gD-specific immune responses, next, protection of the lungs against BHV-1 challenge was evaluated. Titres of infectious BHV-1 recovered from the lungs were lower in all groups immunised with gD- or tgD-expressing vectors than in the lungs of the dE3, dE1E3 and PBS control groups ($P < 0.05$) (Table 5.3.1). There was more than a 2 $\log_{10}$ decrease in virus titres from the lungs of cotton rats immunised with the gD-expressing vectors, and approximately a 1 $\log_{10}$ reduction in animals inoculated with the tgD-expressing vectors. BHV-1 titres were significantly lower in the gD-dE1E3 and gD-dE3 groups compared to the tgD-E1E3 and tgD-dE3 groups ($P < 0.05$).

Table 5.3.1 BHV-1 recovery from lungs of cotton rats immunised with recombinant adenoviruses expressing gD and tgD and challenged with BHV-1.

Immunisation with:	Virus recovery (pfu/g lung tissue)
dE1E3	$4.50 \pm 2.02 \times 10^6$
dE3	$4.11 \pm 1.91 \times 10^6$
gD-dE1E3	$1.49 \pm 1.33 \times 10^4$
tgD-dE1E3	$3.36 \pm 1.68 \times 10^5$
gD-dE3	$1.62 \pm 1.07 \times 10^4$
tgD-dE3	$6.28 \pm 1.75 \times 10^5$
PBS	$4.83 \pm 1.80 \times 10^6$

Cotton rats were inoculated with 10^7 pfu of recombinant adenoviruses twice, 3 weeks apart by the intradermal and intranasal routes, respectively. One group was similarly inoculated with PBS. At 6 weeks after primary inoculation, animals were challenged with 10^7 pfu of BHV-1. Lungs were collected at 42 h post-challenge for virus isolation. Values are the means ± SD for 3 animals per group.

5.3.1.4 Immunity induced by UV-irradiated recombinant adenovirus

To investigate whether BHV-1-specific immune responses observed *in vivo* could be the result of the administration of residual gD present in Ad preparations (even following purification), groups of cotton rats were inoculated i.p. with gD-dE3 or UV irradiated gD-dE3, both containing the same amount of gD (0.1 μg/animal). Immunisations were repeated 20 days later. Serum antibody responses specific for gD and Ad were measured by ELISA 20 and 27 days following the first immunisation. Intraperitoneal immunisation with intact gD-dE3 induced both gD- and Ad-specific serum IgG while the irradiated gD-dE3 preparation induced only Ad-specific antibody (Figure 5.3.9). Since protein antigens alone are even poorer immunogens following mucosal immunisation than systemic immunisation, intranasal immunisation with 0.1 μg gD was not performed. Instead, cotton rats were inoculated by the i.n. route with irradiated non-purified gD-dE1E3 containing a higher amount of gD (4 μg gD per cotton rat). Neither gD- nor Ad-specific serum antibody responses developed following i.n. administration with such inoculum (data not shown). These results suggested that residual gD glycoprotein delivered together with adenovirus did not induce detectable gD-specific antibody responses.

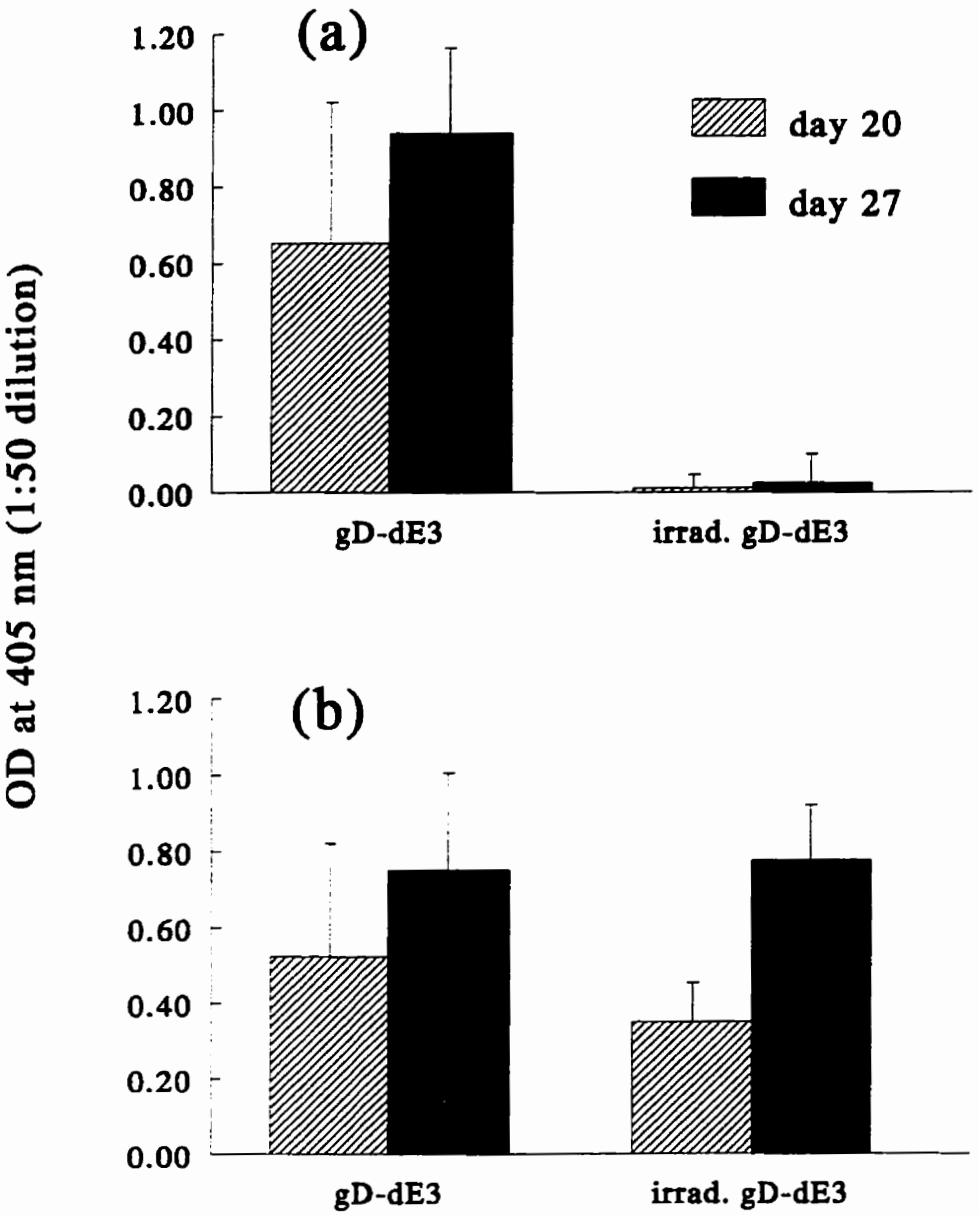


Figure 5.3.9 Antigen-specific serum antibody responses in cotton rats immunised with live and UV-irradiated recombinant adenovirus. Cotton rats were immunised intraperitoneally with gD-dE3 (10^7 pfu) and boosted i.p. 20 days later (10^8 pfu). For the inoculation of another group of cotton rats, equivalent amounts of gD-dE3 were exposed to 20 mW/cm^2 UV-radiation for 8 min before administration to animals. Serum levels of (a) gD-specific and (b) Ad-specific IgG at day 20 and 27 following primary immunisation were detected by ELISA. The graphs show OD-s at 1:50 dilution of the samples. Bars represent the mean OD ± SD for 3 animals per group.

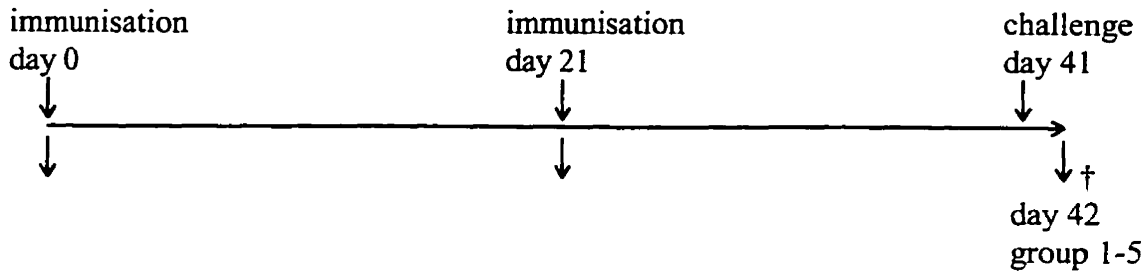
5.3.2 Immunity induced by mucosal administration of replication-defective and replication-competent recombinant adenoviruses

Recombinant adenoviruses expressing gD were shown to induce higher levels of immunity and better protection against BHV-1 challenge than tgD-expressing vectors. Therefore, gD-expressing adenoviruses were used to address further questions about the induction of mucosal immunity in this thesis. Following i.d.-i.n. immunisation the replication-defective vectors induced similar antibody levels as the replication-competent vectors. Next, I investigated the capability of replication-defective and replication-competent adenoviruses following only mucosal administration to induce gD-specific immunity and protection against BHV-1 challenge. Cotton rats were immunised with gD-dE1E3 or gD-dE3 either by the i.n. or gastrointestinal (g.i.) route followed by i.n. BHV-1 challenge (Figure 5.3.10), and systemic and mucosal immune responses were determined.

5.3.2.1 Serum antibody responses

To determine antigen-specific humoral responses, serum antibody titres specific for gD and Ad were measured 3 weeks after the primary intranasal or intraduodenal immunisation and 3 weeks after the secondary i.n. or oral boost. Both gD-dE3 and gD-dE1E3 vectors induced gD-specific IgG and IgA in the serum following primary immunisation (Fig. 5.3.11). Titres increased or decreased in some animals by 3 weeks after the boost, but the mean titres within a group did not change significantly (Fig. 5.3.11). The gD-dE3 vector induced significantly higher levels of gD specific IgG than the gD-dE1 vector ($P < 0.001$). All four immunisation regimens stimulated similar levels of Ad-specific IgG in the serum; i.n. immunisation, however, stimulated higher Ad-specific IgA than g.i. immunisation (data not shown).

To measure biological activity of the gD-specific serum antibody, BHV-1 SN titres were determined. Immunisation by the i.n. and the g.i. routes with the gD-dE3 vector induced significantly higher BHV-1 SN titres than either immunisation regimen with the gD-dE1E3 vector or the PBS control (Fig. 5.3.12).



group #	# of cotton rats per group	immunisation				i.n. BHV-1 challenge	
		inoculum	dose (pfu)	route		strain	dose (pfu)
				primary	secondary		
1	3	gD-dE1E3	10^8	i.n.	i.n.	108	5×10^7
2	4	gD-dE1E3	10^8	i.duod.	oral	108	5×10^7
3	3	gD-dE3	10^8	i.n.	i.n.	108	5×10^7
4	4	gD-dE3	10^8	i.duod.	oral	108	5×10^7
5	4	PBS	-	i.n.	i.n.	108	5×10^7

Figure 5.3.10 Experimental design: immunity induced by mucosal administration of replication-defective and replication-competent recombinant adenoviruses. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples from euthanised animals.
i.n. = intranasal; i.duod. = intraduodenal. In the text, tables and other figures intraduodenal-oral immunisation is referred to as gastrointestinal (g.i.) immunisation.

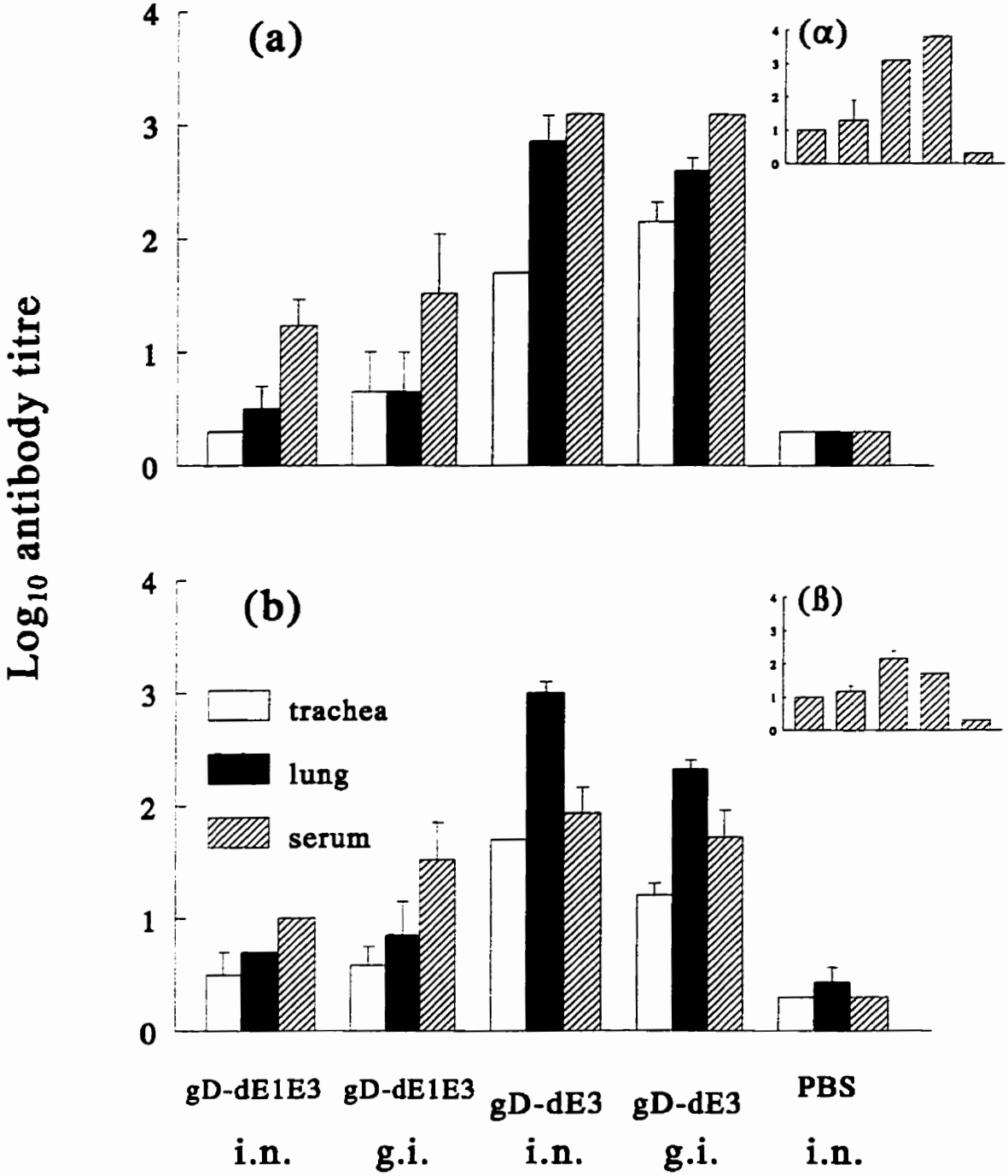


Figure 5.3.11 Antibody responses in cotton rats immunised with recombinant adenoviruses by mucosal routes. Cotton rats were inoculated with recombinant adenoviruses gD-dE1E3, gD-dE3 or with PBS twice, 3 weeks apart by intranasal (i.n.) or gastrointestinal (g.i.) routes. gD-specific (α) IgG and (β) IgA levels 3 weeks after the primary immunisation, and gD-specific (a) IgG and (b) IgA titres 3 weeks after the secondary immunisation in trachea-, lung-extracts and serum were measured by ELISA. Bars represent the mean $\log_{10}$ antibody titre for 3-4 animals/group ± standard error of the mean.

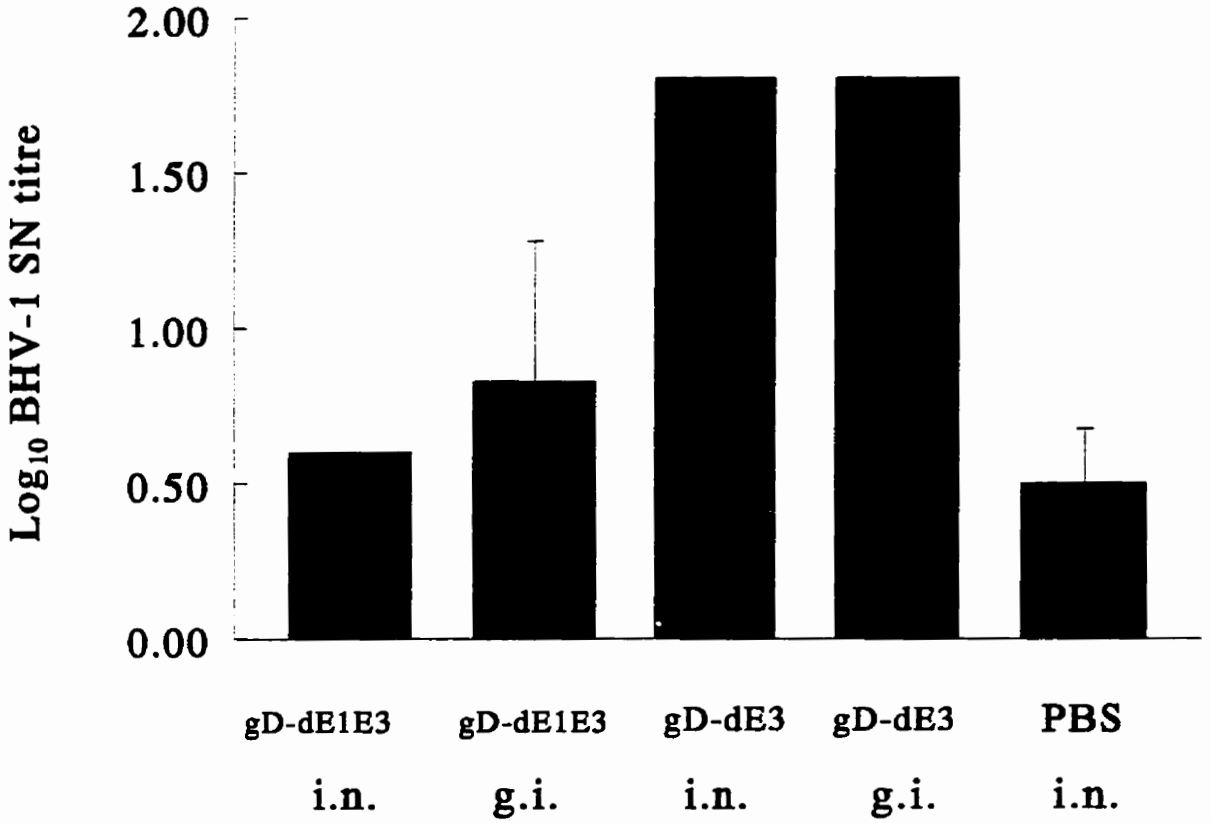


Figure 5.3.12 BHV-1-neutralising antibody titres in sera of cotton rats immunised with recombinant adenoviruses by different mucosal routes. Cotton rats were inoculated with recombinant adenoviruses gD-dE1E3, gD-dE3 or with PBS twice, 3 weeks apart by intranasal (i.n.) or gastrointestinal (g.i.) routes. Serum samples collected at 6 weeks following primary immunisation were analysed for BHV-1-neutralising antibodies by virus neutralisation assay. Bars represent the mean $\log_{10}$ SN titre ± SD for 3-4 animals per group.

5.3.2.2 Antibody levels in the respiratory tract

To determine the ability of the different immunisation regimens to induce antigen-specific humoral immune responses in the respiratory tract, lung- and trachea-extracts were analysed for the presence of gD- and Ad-specific antibody. Immunisation with the gD-dE3 vector induced significantly higher gD-specific IgA and IgG in the lung ($P < 0.001$) and trachea ($P < 0.01$) than immunisation with the gD-dE1E3 virus and the PBS control (Figure 5.3.11). Titres of gD-specific IgG in the respiratory tract correlated with those in the serum, while the relative ratio of lung/serum gD-specific IgA compared to lung/serum IgG suggested that IgA was locally produced in the lung (Figure 5.3.11). gD-specific ASC were found in the lungs and mediastinal LN of animals immunised i.n. with gD-dE3 but not with gD-dE1E3 (data not shown). BHV-1 neutralising antibody titres in the lung- and trachea-extracts were below detection level.

5.3.2.3 Protection against BHV-1 challenge

Since mucosal immunisation with the adenovirus vectors induced immune responses in the serum and the respiratory tract, we assessed the ability of these immune responses to confer protection against i.n. BHV-1 challenge. Table 5.3.2 shows the results of BHV-1 recovery from the trachea and lungs 24 hours after i.n. BHV-1 challenge. Intranasal immunisation with the gD-dE3 vector resulted in BHV-1 titres in the lung below detection limit in 2 of 3 animals and a minimal titre in one animal: the mean virus titre was significantly different from that in the gD-dE1E3 i.n. and the PBS control groups ($P < 0.001$). In addition, g.i. immunisation with the gD-dE3 vector partially reduced BHV-1 titres in the lung of 3 out of 4 animals. In the trachea, BHV-1 titres were only partially reduced in each immunisation group with the vectors, and means were not significantly different from the PBS control.

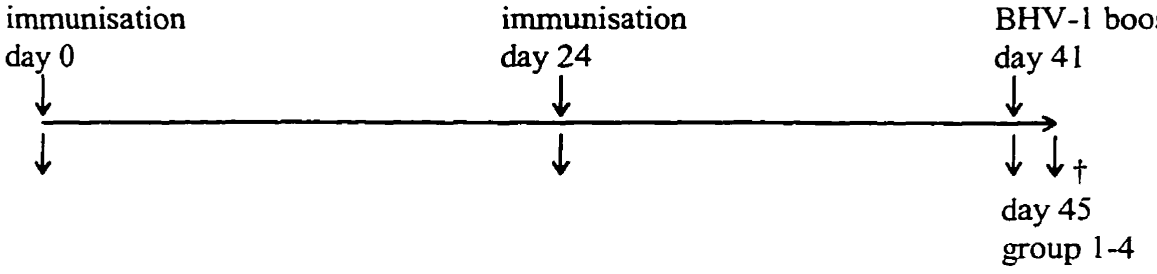
Table 5.3.2 BHV-1 recovery from the trachea and lung of cotton rats immunised with recombinant adenoviruses by the mucosal route and challenged with BHV-1

immunisation		mean ± S.E.* log_{10} BHV-1 titres	
vector	route	trachea	lung
PBS control	intranasal	4.68 ± 1.15	5.12 ± 0.15
gD-dE1E3	intranasal	2.79 ± 0.55	5.22 ± 0.18
gD-dE1E3	gastrointestinal	3.09 ± 0.45	5.10 ± 0.27
gD-dE3	intranasal	1.00 ± 1.22	0.70 ± 0.86
gD-dE3	gastrointestinal	2.89 ± 1.22	3.48 ± 0.76

Animals were immunised twice with recombinant adenovirus or PBS and challenged with BHV-1. Trachea and lung homogenates obtained one day after challenge were tested for the presence of BHV-1 by plaque assay. *S.E. = standard error of the mean

5.3.3 Immunity induced by different routes of immunisation with gD-dE3

The results of the previous experiment indicated that following mucosal administration, the replication-competent Ad was more efficient in inducing immunity to BHV-1 than the replication-defective vector. In the following studies, therefore, the gD-dE3 virus was used to investigate the potential of different routes of immunisation with recombinant Ad to induce gD-specific immune responses in the respiratory tract. Cotton rats were inoculated with gD-dE3 by the i.d., i.n. and g.i. routes (Figure 5.3.13) and both systemic and mucosal immunity were determined.



group #	# of cotton rats per group	immunisation				BHV-1 i.n. boost	
		inoculum	dose (pfu)	route		BHV-1 strain	dose* (pfu); µg
				primary	secondary		
1	5	gD-dE3	2×10^7	i.n.	i.n.	Cooper	10^4; 5
2	5	gD-dE3	2×10^8	i.duod.	oral	Cooper	10^4; 5
3	4	gD-dE3	2×10^7	i.d.	i.d.	Cooper	10^4; 5
4	5	PBS	-	i.n.	i.n.	Cooper	10^4; 5

Figure 5.3.13. Experimental design: immunity induced by different routes of immunisation with gD-dE3. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples from euthanised animals.
i.n. = intranasal; i.duod. = intraduodenal. In the text, tables and other figures intraduodenal-oral immunisation is referred to as gastrointestinal (g.i.) immunisation
* Purified BHV-1 was used for this inoculation.

5.3.3.1 Serum antibody responses

Serum antibody titres specific for gD and Ad 3 weeks following secondary immunisation were determined by ELISA. All routes of immunisation with gD-dE3 induced gD-specific IgG and IgA in the serum, which were significantly different from the PBS control ($P < 0.001$) (Figure 5.3.14). Serum gD-specific IgG levels were not different between groups immunised by different routes. However, the mean gD-specific IgA titre in the serum of i.n. immunised animals was higher than that in the i.d. group ($P < 0.02$), (Figure 5.3.14). In contrast, all three immunisation regimens stimulated similar levels of Ad-specific IgG and IgA in the serum (data not shown).

To measure biological activity of the gD-specific serum antibody, BHV-1 SN titres were determined. Immunisation by each regimen induced similar BHV-1 SN titres, which were significantly higher than that in the PBS control ($P < 0.01$), (Figure 5.3.15).

5.3.3.2 Antibody levels in the respiratory tract

To determine the ability of the different immunisation regimens to stimulate antigen-specific humoral immune responses in the respiratory tract, lung- and nasal-washes were analysed for the presence of gD- and Ad-specific antibodies. Each route of immunisation with the gD-dE3 vector induced significantly higher titres of gD-specific IgA and IgG in the lung than the PBS control ($P < 0.001$); (Figure 5.3.14). However, only mucosal immunisation, and not i.d. immunisation, resulted in significantly higher levels of antibody in the nasal-washes than the PBS control ($P < 0.01$), (Figure 5.3.14). Both regimens of mucosal immunisation induced significantly higher levels of gD-specific IgA in the lung- and nasal-washes than the i.d. route of immunisation ($P < 0.001$), (Figure 5.3.14). Similarly, both i.n. and g.i. immunisation induced higher titres of Ad-specific IgA in the lung- and nasal-washes than the i.d. immunisation ($P < 0.05$), (data not shown). BHV-1 neutralising antibody titres in the lung- and nasal-washes were below detection level.

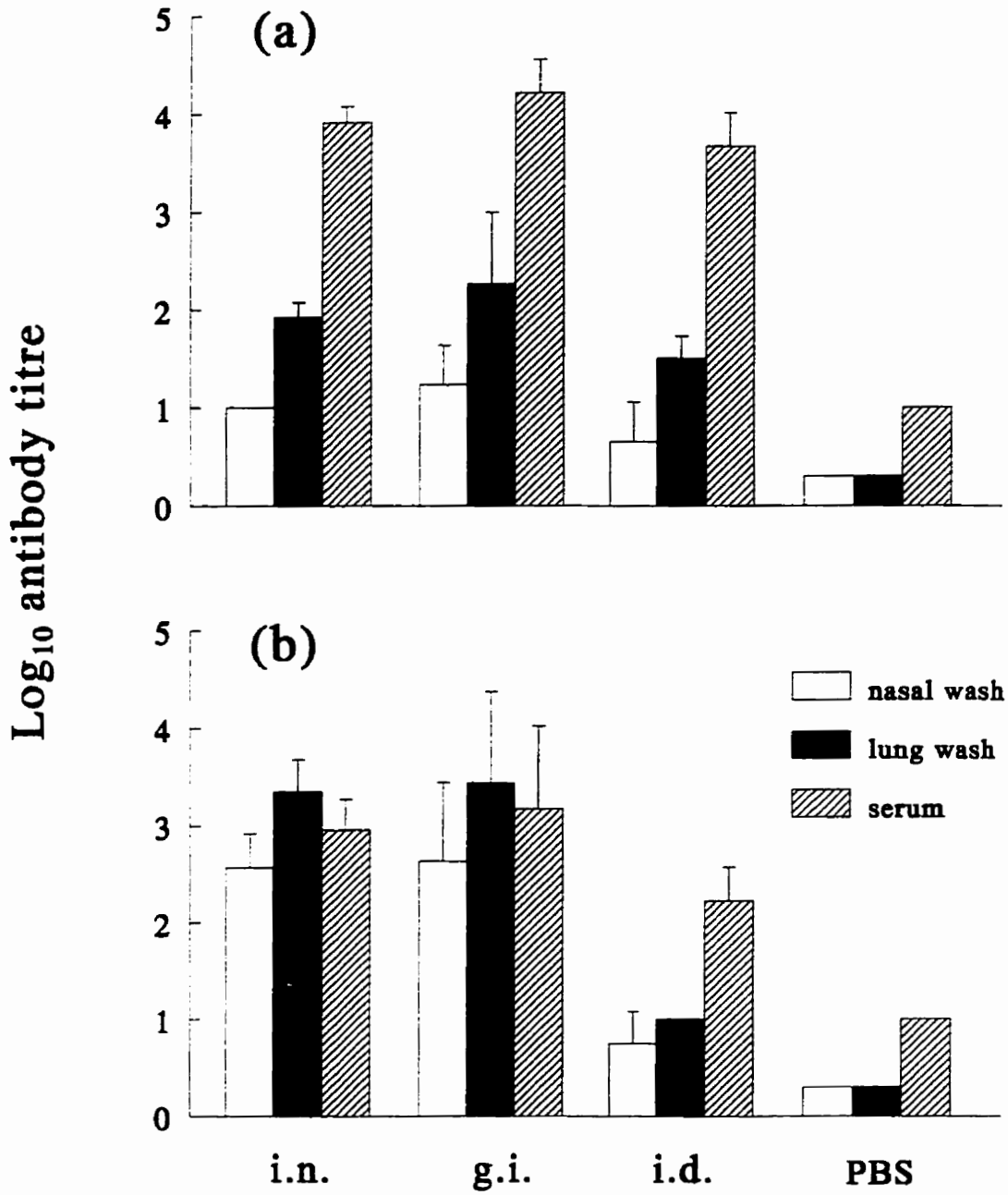


Figure 5.3.14 Antibody responses in cotton rats immunised with recombinant adenovirus by different routes. Cotton rats were inoculated with recombinant adenovirus gD-dE3 or with PBS twice, 3 weeks apart by intranasal (i.n.), gastrointestinal (g.i.) or intradermal (i.d.) routes. gD-specific (a) IgG and (b) IgA titres in nasal-, lung-washes and serum at 6 weeks after primary immunisation were measured by ELISA. Bars represent the mean $\log_{10}$ ± SD antibody titre for 4-5 animals/group.

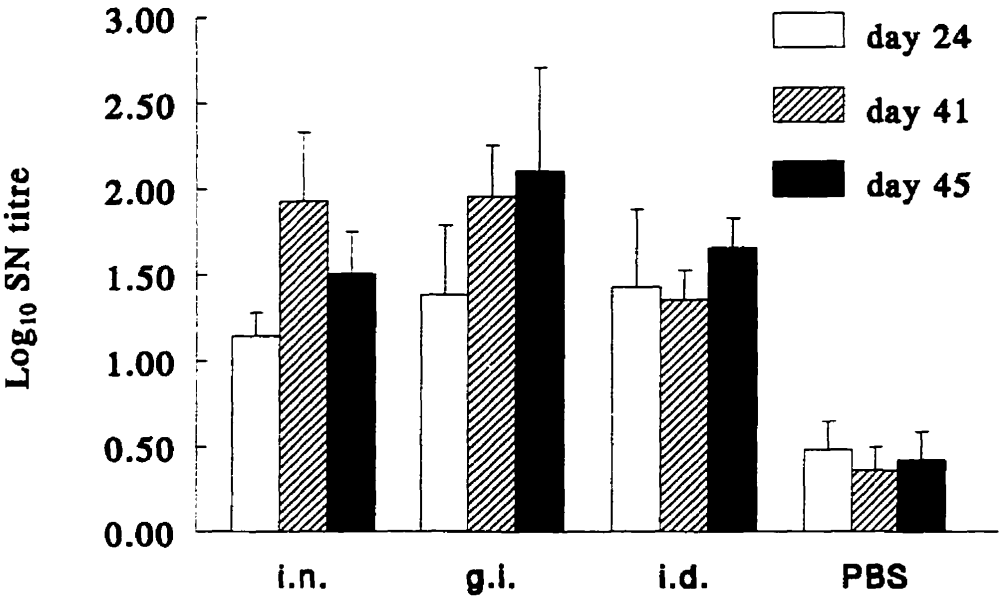


Figure 5.3.15 BHV-1-neutralising antibody titres in sera of cotton rats immunised with recombinant adenovirus by different routes. Cotton rats were inoculated with recombinant adenovirus gD-dE3 or with PBS twice, 3 weeks apart by intranasal (i.n.), gastrointestinal (g.i.) or intradermal (i.d.) routes. Serum samples collected at different timepoints were analysed for BHV-1-neutralising antibodies by virus neutralisation assay. Bars represent the mean $\log_{10}$ ± SD SN titre for 4-5 animals per group.

The higher ratio of lung/serum gD-specific IgA compared to lung/serum gD-specific IgG levels indicated that antibody may be locally produced in the lung of cotton rats immunised by the mucosal route but not of those inoculated intradermally. Therefore, the frequency of gD- and Ad-specific ASC in lung lymphocytes as well as in the mediastinal LN and spleen was determined by ELISPOT assay. The frequency of antigen-specific IgG and IgA secreting cells in the lung was higher following mucosal than i.d. immunisation (Table 5.3.3). There was no statistical difference in the numbers of ASC in the spleen between the gD-dE3-immunisation groups. Mediastinal lymph nodes contained only a low number of antigen-specific IgA secreting cells from cotton rats immunised with gD-dE3 by every route. Generally, the frequency of IgA committed ASC was higher than that of IgG producing ASC in each lymphocyte population examined (Table 5.3.3).

Table 5.3.3 Frequency of gD- and adenovirus-specific antibody-secreting cells in lymphocytes from tissues of cotton rats immunised with recombinant adenovirus by different routes

lympho-cyte source	immunisation	gD-specific ASC/million*		adenovirus-specific ASC/million*	
		IgG	IgA	IgG	IgA
lung	gD-dE3 / i.n.	95	460	44	900
	gD-dE3/ g.i.	140	400	32	900
	gD-dE3/ i.d.	17	25	2.5	59
	PBS / i.n.	4	22	2	22
MLN	gD-dE3/ i.n.	†nd	1	nd	7
	gD-dE3/ g.i.	nd	4.5	nd	10
	gD-dE3/ i.d.	nd	2.5	nd	3
	PBS / i.n.	nd	nd	nd	1
spleen ‡	gD-dE3/ i.n.	nd	5.1 ± 2	3.4 ± 3.2	15 ± 5.6
	gD-dE3/ g.i.	6 ± 8.8	26 ± 24.6	31.7 ± 28.1	59 ± 58
	gD-dE3/ i.d.	2.2 ± 3.86	7 ± 5.2	11 ± 4.5	21 ± 9.3
	PBS / i.n.	nd	nd	nd	nd

Cotton rats were inoculated with gD-dE3 or with PBS twice, 3 weeks apart by the intranasal (i.n.), gastrointestinal (g.i.) or intradermal (i.d.) route. Six weeks after the primary immunisation spleen, lung and mediastinal lymph node (MLN) lymphocytes were isolated and used in the ELISPOT assays.

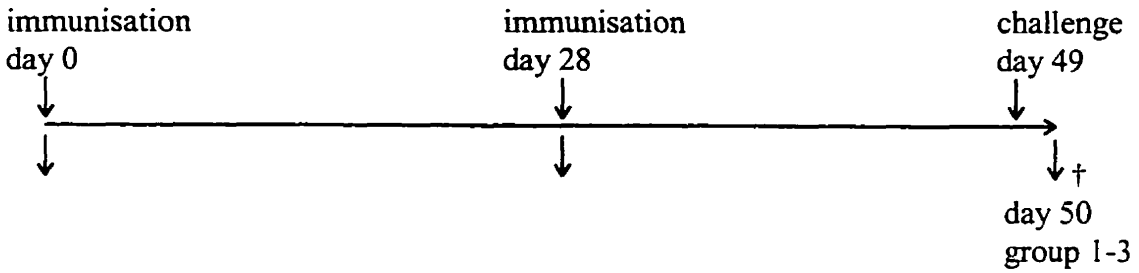
*Mean values of the number of antigen-specific antibody-secreting cells (ASC) per million lymphocytes in the lung and the MLN were determined in cultures of pooled cell populations.

‡ Mean ± SD of ASC/million lymphocytes from individual spleen cell populations.

† nd = not detected (less than 1 ASC/million)

5.3.4 Immunity and protection induced by different routes of immunisation

Results of the previous experiment suggested that i.n. immunisation with gD-dE3 induced better BHV-1-specific immunity in the respiratory tract of cotton rats than i.d. immunisation. The next experiment was designed to determine whether different routes of immunisation with gD-dE3 resulted in different protection of the cotton rat respiratory tract against BHV-1 challenge. Cotton rats were inoculated with gD-dE3 i.n. or i.d. followed by i.n. BHV-1 challenge (Figure 5.3.16).



group #	# of cotton rats per group	immunisation				i.n. BHV-1 challenge	
		inoculum	dose (pfu)	route		strain	dose (pfu)
				primary	secondary		
1	5	gD-dE3	2×10^7	i.n.	i.n.	108	5×10^7
2	4	gD-dE3	2×10^7	i.d.	i.d.	108	5×10^7
3	5	PBS	-	i.n.	i.n.	108	5×10^7

Figure 5.3.16. Experimental design: immunity and protection induced by different routes of immunisation. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples collected from euthanised animals.
i.n. = intranasal; i.d. = intradermal.

5.3.4.1 Antibody responses in the serum and the respiratory tract

To confirm the results of the previous experiment, gD- and Ad-specific antibody responses were measured in the serum and lung- and trachea-extracts of cotton rats immunised with gD-dE3 by the i.n. and i.d. routes. Similarly to earlier data, gD-and Ad-specific serum IgG levels in animals immunised i.n. did not differ from those immunised by the intradermal route; however, gD-specific IgA levels in the respiratory tract were significantly higher in animals immunised intranasally compared to those inoculated intradermally ($P < 0.001$); (Fig. 5.3.17).

5.3.4.2 Antigen-specific antibody-secreting cells in different tissues

To identify potential sites for antibody production in the cotton rat following different routes of immunisation with gD-dE3, the frequencies of gD- and Ad-specific ASC in lung, mediastinal LN, bone marrow and spleen lymphocytes were determined. In the lung, frequencies of antigen-specific IgG and IgA secreting cells were higher following i.n. immunisation than i.d. immunisation (Table 5.3.4), similarly to previous observations. Mediastinal lymph nodes also contained higher number of antigen-specific IgG secreting cells from cotton rats immunised i.n. compared to those inoculated intradermally. In contrast, spleens contained higher numbers of antigen-specific ASC following i.d. immunisation than i.n. inoculation. Both routes of immunisation with gD-dE3 resulted in gD- and Ad-specific IgA and IgG secreting cells in the bone marrow, while inoculation with PBS did not.

5.3.4.3 Protection of cotton rats against BHV-1 challenge

Since both routes of immunisation with gD-dE3 induced immune responses in the serum and the respiratory tract, we assessed the ability of these immunisation protocols to confer protection against BHV-1 challenge. Table 5.3.5 shows the results of BHV-1 recovery from the trachea and the lung one day after intranasal BHV-1 challenge.

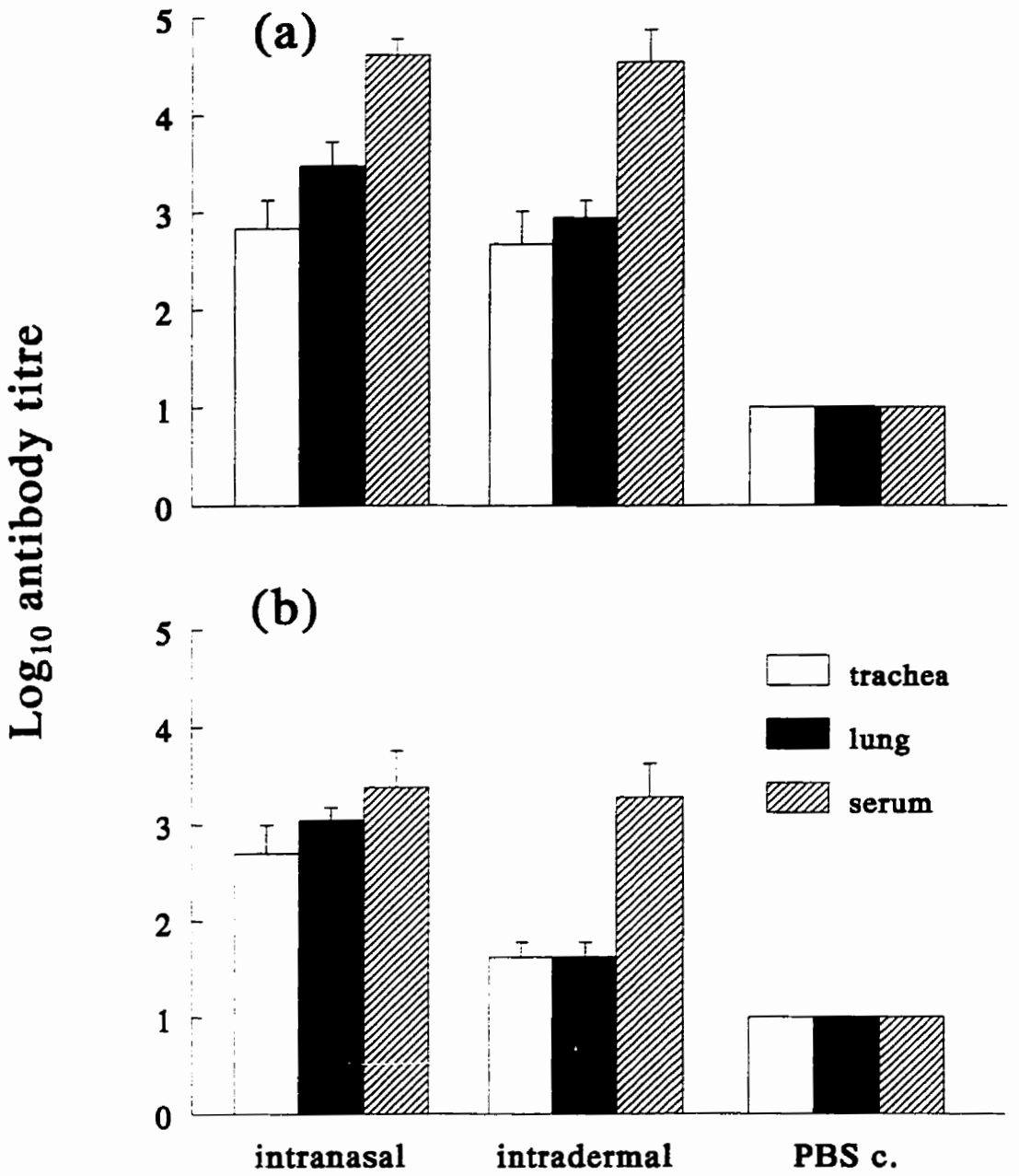


Figure 5.3.17 Antibody responses in cotton rats immunised with recombinant adenovirus by different routes. Cotton rats were inoculated with recombinant adenovirus gD-dE3 or with PBS twice, 3 weeks apart by intranasal (i.n.) or intradermal (i.d.) routes. gD-specific (a) IgG and (b) IgA titres in trachea-, lung-extracts and serum at 6 weeks after primary immunisation were measured by ELISA. Bars represent the mean $\log_{10}$ ± SD antibody titre for 4-5 animals/group.

Table 5.3.4 Frequency of gD- and adenovirus-specific antibody-secreting cells in lymphocytes from tissues of cotton rats immunised with recombinant adenovirus intranasally or intradermally

lymphocyte source	immunisation	gD-specific ASC/million*		adenovirus-specific ASC/million*	
		IgG	IgA	IgG	IgA
lung	gD-dE3 / i.n.	46	600	62	1600
	gD-dE3 / i.d.	†nd	35	15	200
	PBS / i.n.	nd	nd	nd	20
MLN	gD-dE3 / i.n.	25	40	10	30
	gD-dE3 / i.d.	nd	40	nd	20
	PBS / i.n.	nd	20	nd	5
bone marrow	gD-dE3 / i.n.	4.5	3	9	23
	gD-dE3 / i.d.	3	2	17	3
	PBS / i.n.	nd	nd	nd	nd
spleen	gD-dE3 / i.n.	nd	1	5.5	9
	gD-dE3 /i.d.	35	10	35	5
	PBS / i.n.	nd	nd	nd	nd

Cotton rats were inoculated intranasally (i.n.) or intradermally (i.d.) twice, 3 weeks apart, with recombinant adenovirus gD-dE3 or PBS as a control. Six weeks after the primary immunisation all animals were challenged i.n. with BHV-1. Spleen, bone marrow, lung and mediastinal lymph node (MLN) lymphocytes, isolated 1 day after challenge, were used in the ELISPOT assay.

*Mean values of the number of antigen-specific antibody-secreting cells (ASC) per million lymphocytes were determined in cultures of pooled cell populations of 5 animals/group.

†nd = not detected (less than 1 ASC/million)

Table 5.3.5 Effect of the route of immunisation with gDE3 recombinant adenovirus on the protection of cotton rats against intranasal BHV-1 challenge

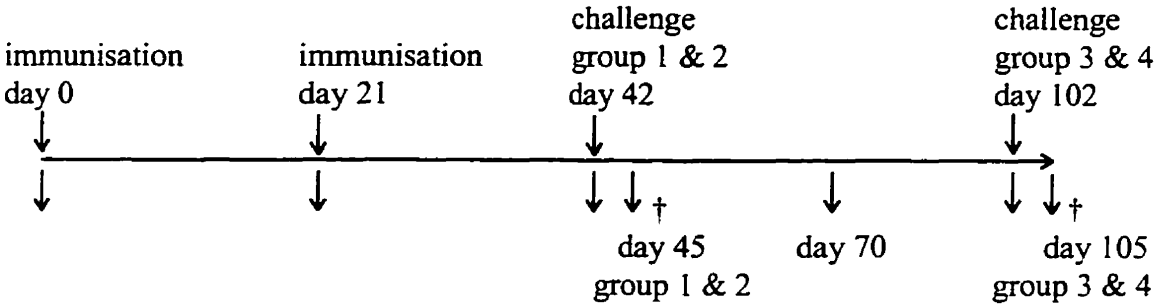
immunisation / route	virus isolation (log_{10} pfu/g tissue)	
	trachea	lung
gD-dE3 / i.n.	5.336 ± 0.227*	1.140 ± 0.313*
gD-dE3 / i.d.	5.917 ± 0.385	3.948 ± 0.641*
PBS / i.n.	6.151 ± 0.440	5.482 ± 0.504

Cotton rats were inoculated intranasally (i.n.) or intradermally (i.d.) twice, 3 weeks apart with gD-dE3 or PBS as a control. Six weeks after the primary immunisation animals were challenged with BHV-1 intranasally. One day post-challenge trachea and lung were removed, homogenised and tested for the presence of BHV-1 by plaque assay.
* Significant difference from other groups ($P < 0.05$)

Intranasal immunisation with the gD-dE3 vector resulted in BHV-1 titres below detection limit in the lung of 4 of 5 animals and a minimal titre in one animal; the mean virus titre was significantly different from the PBS control group ($P < 0.001$). The i.d. immunisation also resulted in reduced BHV-1 titres in the lung compared to the PBS control ($P < 0.01$) (Table 5.3.5). The level of protection in i.d. immunised groups was similar to that determined in a separate experiment (data not shown). BHV-1 titres in the lung were significantly lower in animals immunised i.n. than in those inoculated i.d. ($P < 0.001$). BHV-1 titres in the trachea were partially reduced in the i.n. immunisation group compared to the i.d. and PBS groups ($P < 0.05$).

5.3.5 Duration of immunity induced by intranasal administration of gD-dE3

In order to confirm protection of the lungs against BHV-1 challenge histologically and to further characterise the kinetics and duration of antigen-specific immunity induced by i.n. administration of gD-dE3, cotton rats were immunised i.n. with gD-dE3 followed by i.n. BHV-1 challenge 6 and 15 weeks following primary immunisation (Figure 5.3.18).



group #	# of cotton rats per group	immunisation				i.n. BHV-1 challenge	
		inoculum	dose (pfu)	route		strain	dose (pfu)
				primary	secondary		
1	7	gD-dE3	10^8	i.n.	i.n.	108	5×10^7
2	7	PBS	-	i.n.	i.n.	108	5×10^7
3	7	gD-dE3	10^8	i.n.	i.n.	108	5×10^7
4	5	PBS	-	i.n.	i.n.	108	5×10^7

Figure 5.3.18 Experimental design: duration of immunity induced by intranasal administration of gD-dE3. In the flowchart, arrows above the timescale describe time of treatment while arrows below describe time of serum sampling. The symbol † represents tissue samples collected from euthanised animals.
i.n. = intranasal

5.3.5.1 Kinetics of serum antibody responses

Figure 5.3.19 shows gD- and Ad-specific serum IgG and IgA levels at different time points following i.n. immunisation with gD-dE3. Serum antibody specific for gD (Figure 5.3.19.a) and BHV-1 SN antibodies (Fig. 5.3.20) were induced by the primary immunisation with gD-dE3 and their levels increased following secondary immunisation. These levels were maintained for at least 3 months. The kinetics of Ad-specific responses were similar to gD-specific antibody responses (Figure 5.3.19.b).

5.3.5.2 Duration of antibody responses in the respiratory tract

Interestingly, not only the levels of antigen-specific serum antibodies were maintained, but the titres of antibodies in lung-extracts were similar at 3 weeks and at 12 weeks following secondary immunisation (data not shown). To determine whether antigen-specific ASC were maintained locally in the lung and in peripheral lymphoid tissues, ELISPOT assays were performed 15 weeks following primary immunisation. Both gD- and Ad-specific ASC were found in the lung, bone marrow and spleen of gD-dE3-immunised animals (Table 5.3.6). The numbers of antigen-specific ASC in the lungs at 15 weeks following primary immunisation were similar to those at 6 weeks after immunisation determined in previous experiments (5.3.3; 5.3.4).

5.3.5.3 Protection against BHV-1 challenge determined histologically

Previous results of BHV-1 recovery from the respiratory tract have indicated that protection of the lungs against BHV-1 challenge was achieved by i.n. immunisation with gD-dE3. However, evidence for the lack of infection of cotton rat lungs *in situ* was necessary to confirm the protective ability of i.n. immunisation with gD-dE3 against BHV-1 infection. Therefore, lungs of cotton rats were examined by histological and immunohistochemical methods for evidence of virus-associated tissue damage and BHV-1 replication 3 days after challenge. Lungs of cotton rats in the control groups had lesions of

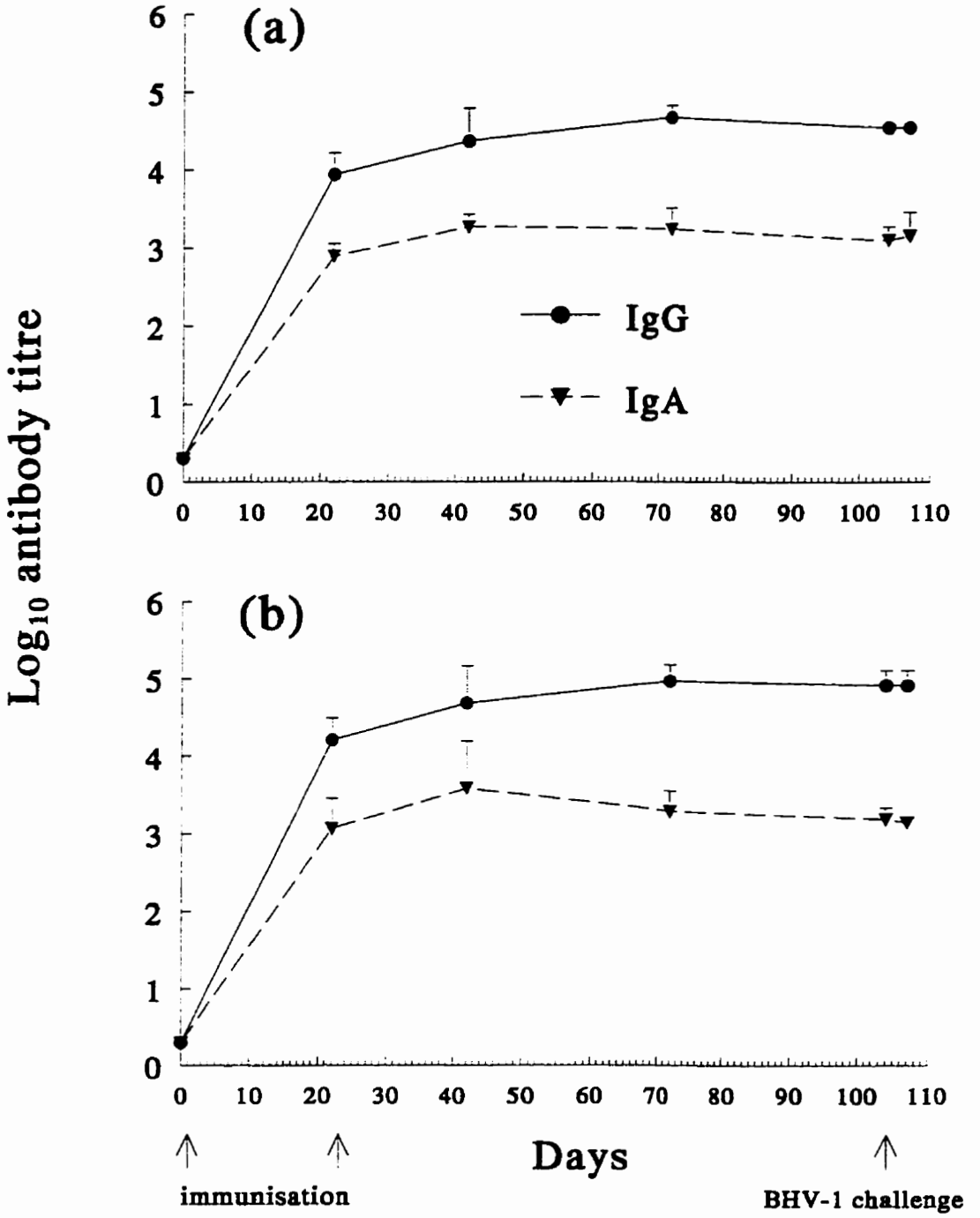


Figure 5.3.19 Kinetics of gD- and Ad-specific antibody responses in the serum of cotton rats immunised intranasally with recombinant adenovirus. Cotton rats were inoculated with gD-dE3 or with PBS twice, 3 weeks apart. Titres of IgG and IgA antibodies specific for (a) gD and (b) HAd5 at different timepoints p.i. were determined by ELISA. Error bars represent the SD of $\log_{10}$ antibody titres for 7 animals/group. Control animals maintained background levels of antibody during the study (not shown).

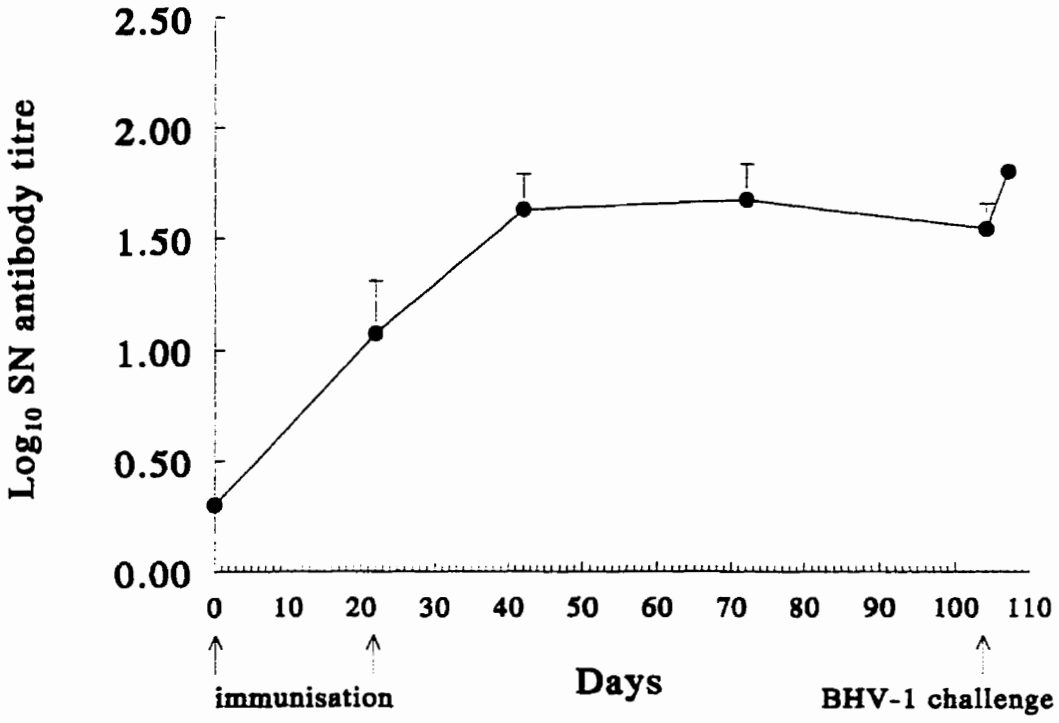


Figure 5.3.20 Kinetics of BHV-1-neutralising antibody titres in the serum of cotton rats immunised with recombinant adenovirus intranasally. Cotton rats were inoculated with gD-dE3 or PBS twice, 3 weeks apart. Serum samples collected at different timepoints following immunisation were analysed for BHV-1-neutralising antibodies by virus neutralisation assay. Points represent the mean $\log_{10}$ SN titre, while error bars show the SD for 7 animals per group ± SD. Control animals maintained background levels of SN antibody during the study (not shown).

Table 5.3.6 Frequency of gD- and adenovirus-specific antibody-secreting cells in lymphocytes from different tissues of cotton rats immunised intranasally with recombinant adenovirus

lymphocyte source	immunisation	gD-specific ASC/million*		adenovirus-specific ASC/million*	
		IgA	IgG	IgA	IgG
lung	gD-dE3	600	16	1100	36
	PBS	14	nd†	19	nd
bone marrow	gD-dE3	4	2	29	17
	PBS	nd	nd	2	nd
spleen	gD-dE3	2	nd	7	11
	PBS	nd	nd	nd	nd

Cotton rats were inoculated intranasally twice, 3 weeks apart, with recombinant adenovirus gD-dE3 (7 animals) or PBS as a control (5 animals). Twelve weeks after the secondary immunisation all animals were challenged intranasally with BHV-1. Spleen, bone marrow and lungs lymphocytes were isolated 3 days after challenge and used in the ELISPOT assays.

*Mean values of the number of antigen-specific antibody-secreting cells (ASC) per million lymphocytes were determined in cultures of pooled cell populations.

†nd = not detected (less than 1 ASC/million)

a multifocal mild interstitial pneumonia with type II pneumocyte proliferation and infiltration of few macrophages (Table 5.3.7). Small foci involving one or two cells positive for BHV-1 antigen were also demonstrated, usually associated with foci of interstitial pneumonia. In contrast, lungs from the two groups of immunised cotton rats did not have any lesions or BHV-1 replication foci, indicating significant protection following vaccination. In contrast to the lung, no lesions associated with BHV-1 infection were found in the lower part of the trachea of either immunised or control animals.

In accordance with the results of *in situ* lung examination, BHV-1 was isolated only from lungs of control animals but not from lungs of immunised animals, challenged at either 6 or 15 weeks following the primary immunisation (Table 5.3.7). In addition, mean virus titre in the trachea of immunised animals was significantly lower than that in PBS controls ($P < 0.01$) (data not shown).

Table 5.3.7. Effect of intranasal immunisation with recombinant adenovirus on the protection of the lungs of cotton rats against intranasal BHV-1 challenge

weeks following first immunisation	immunisation	virus isolation (pfu/g lung tissue ± SD)	BHV-1 specific foci	interstitial pneumonia
6	gD-dE3	nd*	0/7	0/7
6	PBS control	5.086 ± 0.286	7/7	7/7
15	gD-dE3	nd	0/7	0/7
15	PBS control	3.815 ± 0.941	4/5	5/5

Cotton rats were inoculated intranasally twice, 3 weeks apart with gD-dE3 or PBS as a control. Six or fifteen weeks after the primary immunisation animals were challenged with BHV-1 intranasally. Three days post-challenge lungs were removed and were tested for BHV-1 replication (virus isolation and BHV-1 specific foci) and pathological changes (interstitial pneumonia) in the lungs.

*nd = not detected (< 10 pfu/lung)

5.3.6 Conclusions

Results in sections 5.3.1 - 5.3.5 indicated that immunisation with recombinant adenovirus vectors expressing BHV-1 gD or tgD was capable of inducing gD-specific immune responses in the cotton rat. Furthermore, immunisation by the i.d.-i.n. route resulted in partial protection of the lungs of cotton rats against i.n. BHV-1 challenge. Immunisation with gD-expressing vectors induced better immunity and protection than tgD-expressing recombinant adenoviruses. Results from these experiments also showed that after mucosal administration, the replication-competent gD-dE3 vector induced higher levels of gD-specific immunity and reduced BHV-1 titres from the respiratory tract after challenge to a higher degree than the replication-defective gD-dE1E3 vector.

Different routes of immunisation with gD-dE3 (i.p., i.d., i.n., g.i.) were capable of inducing antigen-specific immune responses. The highest level of immunity in the respiratory tract and protection against i.n. BHV-1 challenge was achieved by the i.n. route of immunisation. Furthermore, the immunity induced by i.n. administration of gD-dE3 may have been long-lasting as indicated by the fact that antigen-specific immune responses were maintained at high levels for at least 12 weeks following secondary immunisation.

5.4 Adenovirus dissemination following different routes of delivery

In the previous experiments, several interesting findings appeared regarding the induction of immune responses following inoculation with recombinant adenoviruses. First, the replication-defective gD-dE1E3 virus was less efficient than the replication-competent gD-dE3 vector in inducing immunity following mucosal administration, while they were equally efficient following systemic inoculation. Second, intradermal immunisation with recombinant adenovirus induced gD-specific immunity in the respiratory tract, even though systemic immunisation with protein antigens rarely induces mucosal immunity (Michalek et al., 1994). Third, intraduodenal-oral immunisation induced almost equivalent levels of antibody in the respiratory tract as did the i.n. immunisation, although enteric immunisation is usually less efficacious in inducing immunity in the respiratory tract than i.n. immunisation (Mestecky et al., 1994).

Based on these observations one of my hypotheses was that replication-defective and replication-competent vectors had different capabilities of spreading systemically following mucosal immunisation. Furthermore, adenovirus may be able to spread to the respiratory tract following intradermal, intraduodenal and oral immunisation, thereby inducing local immunity directly in the respiratory tract. Therefore, the next objective was to determine the capability of replication-defective and replication-competent adenoviruses to disseminate in the cotton rat following different routes of inoculation.

5.4.1 Adenovirus isolation from different organs

Tissues of cotton rats were examined for the presence of adenovirus following administration of gD-dE1E3 or gD-dE3 by different routes. Cotton rats received 10^8 pfu of adenovirus by the i.d., i.n., oral or intraduodenal route. Tissues were collected at day 3 following inoculation with gD-dE3, because the peak of adenovirus replication in cotton rats has been determined to be around day 3 following i.n. inoculation (Pacini et al., 1984). Both day 1 and day 3 samples were collected following inoculation with gD-dE1E3,

because more virus was expected to be present in the tissues at an earlier timepoint p.i. in the case of a non-replicating adenovirus (Ginsberg et al., 1991). Following isolation from tissues, adenoviruses were detected by plaque assay on 293 cells. Results are shown in Tables 5.4.1, 5.4.2 and 5.4.3. The day 1 and day 3 data for gD-dE1E3 are described together, because the main interest of this study was to provide evidence for the presence of adenovirus in tissues, and not the kinetics of spreading of adenovirus.

Following intradermal inoculation with gD-dE1E3, adenovirus was isolated from most samples except for the alimentary tract and spleen (Tables 5.4.1 and 5.4.2). Intradermal administration of gD-dE3 resulted in virus spread to most tissues except for the alimentary tract and nasal-washes (Table 5.4.3).

Following intranasal inoculation with gD-dE1E3, adenovirus was isolated from all tested samples at either day 1 or day 3 (Tables 5.4.1 and 5.4.2). Intranasal administration of gD-dE3 provided similar results at day 3 (Table 5.4.3). Data obtained at day 1 following i.n. inoculation with gD-dE3 were similar to those at day 3 (results not shown). In a separate experiment, adenovirus was isolated from the lung in 2 of 7 animals even at 3 weeks following i.n. inoculation with gD-dE3. Intranasal inoculation of wild type HAd5 provided similar results at day 3 to those obtained by i.n. administration of gD-dE3 at day 3 p.i. (data not shown).

After intragastric inoculation with gD-dE1E3, adenovirus was isolated from most tested organs except for the mediastinal LN, ileum, liver and the skin (Tables 5.4.1 and 5.4.2). All tested samples proved to be positive for adenovirus following intragastric delivery of gD-dE3 (Table 5.4.3). However, the stomach of babies suckling a mother infected by the intragastric route by gD-dE3 did not contain detectable adenovirus (data not shown).

Intraduodenal inoculation of cotton rats with gD-dE1E3 resulted in adenovirus spread to each tested organ (Tables 5.4.1 and 5.4.2). Adenovirus was even detected in the vaginal wash from one animal (data not shown). Following intraduodenal administration with gD-dE3, adenovirus was isolated from all samples except for nasal-washes or only from the duodenum and the mesenteric LN in one animal (Table 5.4.3).

Table 5.4.1 Adenovirus isolation from tissues of cotton rats 1 day following inoculation with gD-dE1E3 by different routes

tissue	pfu/g tissue adenovirus following (route of inoculation)			
	intradermal	intranasal	intragastric	intraduodenal
nasal-wash	nd/nd	3000	500	nd/nd
trachea	20/nd	1×10^5	3000	nd/nd
lung	nd/nd	200	1600	nd/nd
mediastinal LN	nd/nd	+	nd	nd/nd
oesophagus	nd/nd	3000	20	nd/nd
stomach	nd/nd	nd	nd	nd/nd
duodenum	nd/nd	+	+	+/+
ileum	nd/nd	+	nd	+/+
feces	nd/nd	not tested	+	+/+
mesenteric LN	nd/nd	+	20	+/+
spleen	nd	nd	50	nd/nd
liver	nd/200	nd	nd	nd/nd
skin injection site	1000/2000	n.a.	n.a.	n.a.
skin	+/100	nd	nd	+/+

Values are from individual animals (animal #1 / #2 in the same group).
+ = First or second passage was positive for adenovirus
nd = not detected (second passage was negative for adenovirus)
n.a. = not applicable

Table 5.4.2 Adenovirus isolation from tissues of cotton rats 3 days following inoculation with gD-dE1E3 by different routes

tissue	pfu/g tissue adenovirus following (route of inoculation)			
	intradermal	intranasal	intragastric	intraduodenal
nasal-wash	+	4000	nd	nd/+/nd
trachea	+	2000	+	nd/100/+
lung	+	1×10^5	100	nd/+/+
mediastinal LN	+	+	nd	nd/70/nd
oesophagus	10	2000	20	nd/+/nd
stomach	nd	+	+	nd/100/500
duodenum	nd	+	nd	200/500/1000
ileum	nd	+	nd	nd/+/nd
feces	nd	20	nd	nd/+/nd
mesenteric LN	+	nd	nd	+/+/nd
spleen	nd	100	10	nd/+/+
liver	30	40	nd	nd/+/+
skin injection site	50	n.a.	n.a.	n.a.
skin	nd	+	nd	nd/+/+

Values are from individual animals (animal #1/ #2 / #3 in the same group).
+ = First or second passage was positive for adenovirus
nd = not detected (second passage was negative for adenovirus)
n.a. = not applicable

Table 5.4.3 Adenovirus isolation from tissues of cotton rats 3 days following inoculation with gD-dE3 by different routes

tissue	pfu/g tissue adenovirus following (route of inoculation)			
	intradermal	intranasal	intragastric	intraduodenal
nasal-wash	nd/nd	3000/3000	400/2000	nd/nd/nd/nd
trachea	nd/20	3000/1000	2000/2000	20/nd/20/20
lung	20/40	$10^5/10^5$	$10^5/1.2 \times 10^5$	nd/nd/+/20
mediastinal LN	+/nd	+/+	nd/+	10/nd/200/200
oesophagus	+/10	300/200	1500/40	nd/nd/10/20
stomach	nd/nd	10/+	nd/+	nd/nd/300/2000
duodenum	nd/nd	+/+	nd/+	+/+/100/2000
ileum	nd/nd	nd/+	+/+	+/nd/10/1000
feces	nd/nd	nd/+	nd/nd	nd/nd/100/1000
mesenteric LN	20/10	50/1000	20/40	+/+/200/+
spleen	100/80	nd/+	nd/+	nd/nd/20/1000
liver	50/30	10/+	nd/10	nd/nd/500/+
skin injection site	1000/40	n.a.	n.a.	n.a.
skin	+/nd	nd/+	nd/+	nd/nd/10/40

Values are from individual animals (animal #1 / #2 / #3 / #4 in the same group)
+ = first or second passage was positive for adenovirus
nd = not detected (second passage was negative for adenovirus)
n.a. = not applicable

5.4.2 Conclusions

Results of adenovirus isolation following inoculation of gD-dE1E3 or gD-dE3 indicated that both recombinant adenoviruses are capable of systemic dissemination following different routes of administration. As a result, adenovirus could be found in the respiratory tract even following inoculation at a distant site (intradermal or intraduodenal). Higher levels of adenovirus were found in the respiratory tract following intragastric administration than after intraduodenal administration. This observation indicated that adenovirus may have spread to the airways following administration of the virus to the oesophagus. Therefore, this method of inoculation may not exclusively involve the delivery of adenovirus to the alimentary tract.

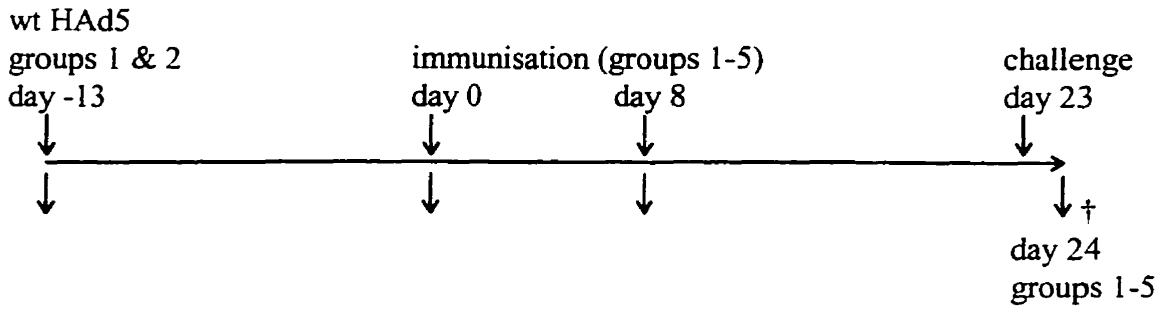
5.5 Effect of adenovirus-specific immunity on immunisation with recombinant adenovirus

As described in section 5.3, immunisation with recombinant adenovirus vectors expressing gD induced gD-specific immunity and partial protection of cotton rats against i.n. BHV-1 challenge. Outside laboratory conditions, however, one has to take into consideration the possibility that the vaccinee population may have acquired Ad-specific active or passive immunity prior to immunisation. Furthermore, the efficacy of repeated administration of adenovirus in inducing immunity to the heterologous protein may be limited because of Ad-specific immunity, which developed following a previous immunisation. Therefore, it is important to investigate the effect of immunity to HAd5 on the efficacy of immunisation with recombinant HAd5 vectors. The cotton rat model is especially suitable to conduct these studies because these laboratory animals support HAd5 replication.

5.5.1 Effect of HAd5-specific active immunity on intranasal and gastrointestinal immunisation with recombinant adenovirus

The most common way for an animal or human to acquire Ad-specific immunity is to be naturally infected by the virus through the respiratory tract. Following infection, memory type Ad-specific active immunity develops. Since HAd5 preferentially targets respiratory mucosal tissues in cotton rats, intranasal infection with wt HAd5 in cotton rats was chosen as a model to induce active Ad-specific immunity. The experiment described in Figure 5.5.1 was designed to assess whether i.n. infection of naive cotton rats with wt HAd5 had an effect on the immunity induced by i.n. immunisation with recombinant adenovirus. Considering the importance of local mucosal stimulation, I also wanted to find out whether immunisation at a different mucosal site (enteric) would be affected by i.n. infection with wt adenovirus. Animals were infected with wt HAd5 2 weeks before immunisation with gD-dE3 in order to induce strong cellular and humoral immunity

against Ad by the time of immunisation. The efficacy of immunisation with gD-dE3 by the i.n. and g.i. routes was then assessed by measuring gD- and Ad-specific immune responses and protection of lungs against BHV-1 challenge.



group #	# of cotton rats per group	i.n. HAd5 infection dose (pfu)	immunisation				i.n. BHV-1 challenge	
			inoculum	dose (pfu)	route of primary	route of second.	strain	dose (pfu)
1	4	2×10^7	gD-dE3	5×10^7	i.n.	i.n.	108	5×10^7
2	3	2×10^7	gD-dE3	5×10^7	i.duod.	oral	108	5×10^7
3	4	-	gD-dE3	5×10^7	i.n.	i.n.	108	5×10^7
4	3	-	gD-dE3	5×10^7	i.duod.	oral	108	5×10^7
5	4	-	PBS	-	i.n.	i.n.	108	5×10^7

Figure 5.5.1 Experimental design: effect of HAd5-specific active immunity on intranasal and gastrointestinal immunisation with recombinant adenovirus. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples collected from euthanised animals.
i.n. = intranasal; i.duod. = intraduodenal. In the text, tables and other figures intraduodenal-oral immunisation is referred to as gastrointestinal (g.i.) immunisation.

5.5.1.1 Serum antibody responses

At the time of immunisation with gD-dE3, 2 weeks following HAd5-infection, all HAd5-infected animals developed Ad-specific serum antibody responses similar to those in Figure 5.5.10. These results indicate that cotton rats were successfully infected with HAd5 and induced Ad-specific immune responses. Furthermore, Ad-specific IgG and IgA titres were significantly higher in the serum of HAd5-infected cotton rats compared to non-infected animals at all timepoints following immunisation with gD-dE3, as expected (Figure 5.5.2).

Three weeks after primary immunisation with gD-dE3, all immunised animals developed significantly higher levels of gD-specific IgG and IgA in the serum than those measured in the serum of PBS-control animals ($P < 0.001$) (Figure 5.5.2). Non-infected cotton rats, however, developed higher mean gD-specific antibody titres than HAd5-infected animals when the route of gD-dE3 immunisation was i.n. ($P < 0.01$). In contrast, mean gD-specific antibody titres were not significantly higher in non-infected compared to HAd5-infected groups, when immunised with gD-dE3 by the gastro-enteric route (Figure 5.5.2).

To measure the biological activity of the gD-specific antibody, BHV-1 SN titres were determined. BHV-1 SN titres were significantly lower in HAd5-infected than in non-infected animals between groups immunised with gD-dE3 by the same route ($P < 0.001$) (Figure 5.5.3).

5.5.1.2 Antibody responses in the respiratory tract

To determine the ability of the different immunisation regimens to induce antigen-specific humoral immune responses in the respiratory tract, lung and trachea extracts were analysed for the presence of gD- and Ad-specific antibody. Intranasal immunisation with gD-dE3 induced significantly lower gD-specific IgG and IgA levels in the trachea of HAd5-infected than non-infected animals ($P < 0.001$) (Figure 5.5.4). In contrast, gD-specific tracheal antibody levels induced by g.i. inoculation were not significantly affected

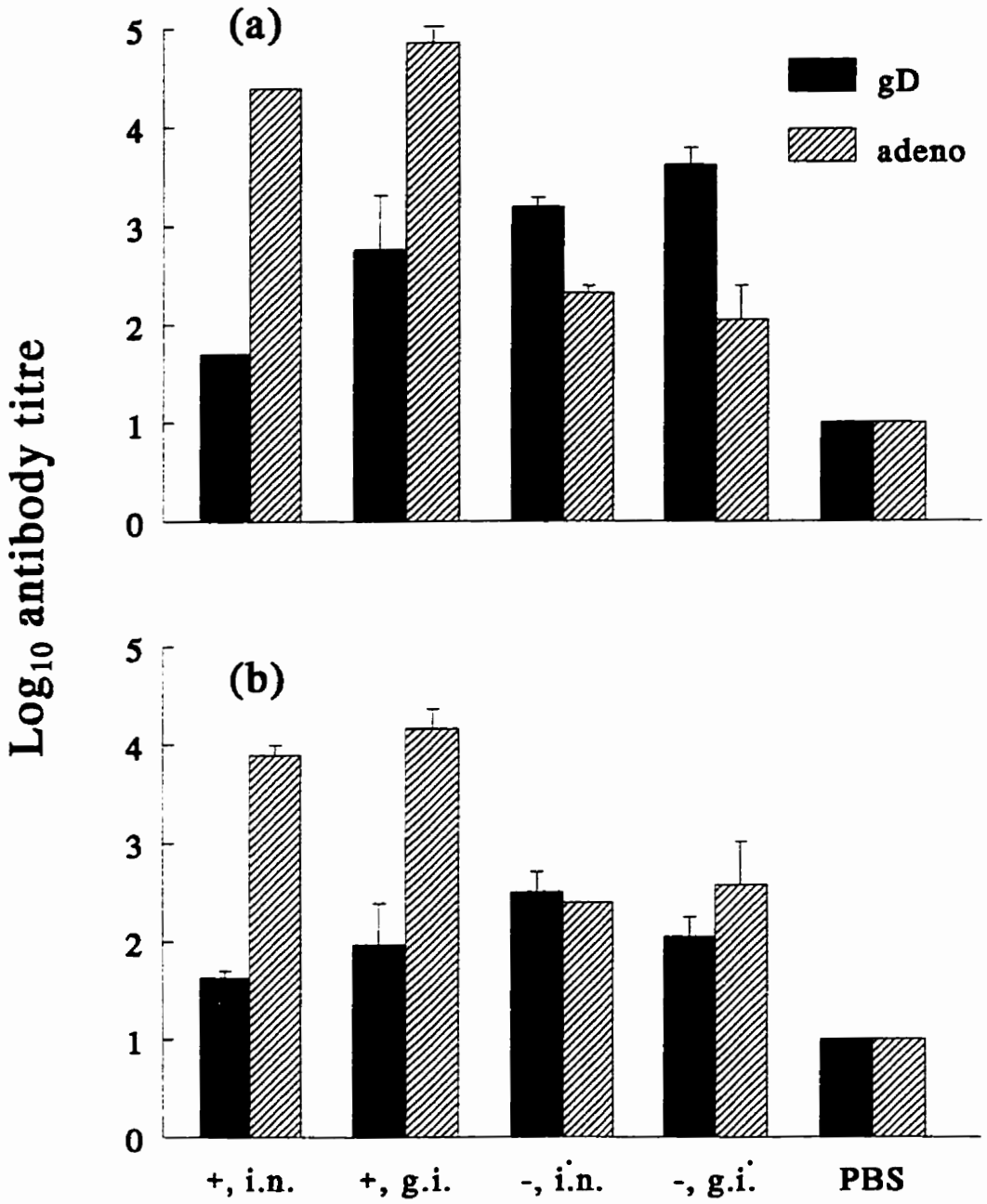


Figure 5.5.2 gD- and Ad-specific serum antibody responses in cotton rats immunised with gD-dE3 by different routes following HAd5 infection. Cotton rats were intranasally inoculated with wt HAd5 (+ symbol) or PBS (- symbol) as a control. Two and three weeks following infection, cotton rats were immunised with gD-dE3 by the intranasal (i.n.) or gastrointestinal (g.i.) routes or with PBS as a control. gD- and Ad-specific (a) IgG and (b) IgA levels in the serum at 3 weeks following the primary immunisation were measured by ELISA. Bars represent the mean $\log_{10} \pm$ SD antibody titre for 3-4 animals/group.

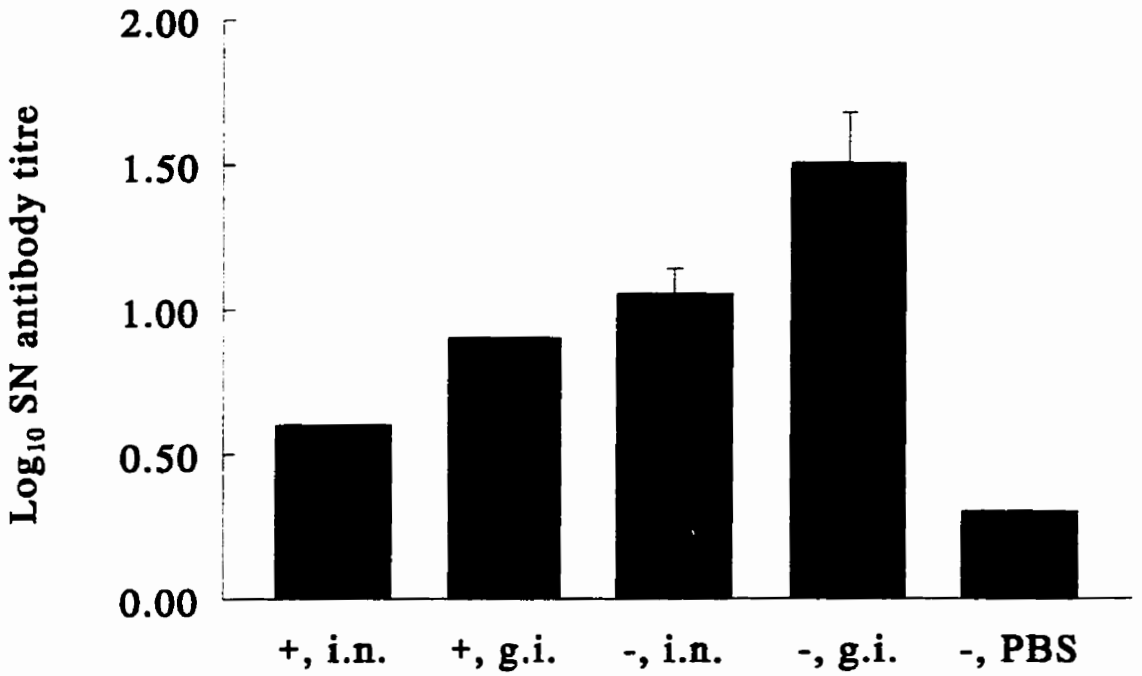


Figure 5.5.3 BHV-1-neutralising antibody titres in the sera of cotton rats immunised with gD-dE3 by different routes following HAd5 infection. Cotton rats were intranasally inoculated with wt HAd5 (+ symbol) or with PBS as a control (- symbol). Two and three weeks following infection the animals were immunised with gD-dE3 by the intranasal (i.n.) or gastrointestinal (g.i.) routes or with PBS as a control. Serum samples collected at 3 weeks following primary immunisation were analysed for BHV-1-neutralising antibodies by virus neutralisation assay. Bars represent the mean $\log_{10}$ SN titre ± SD for 3-4 animals per group.

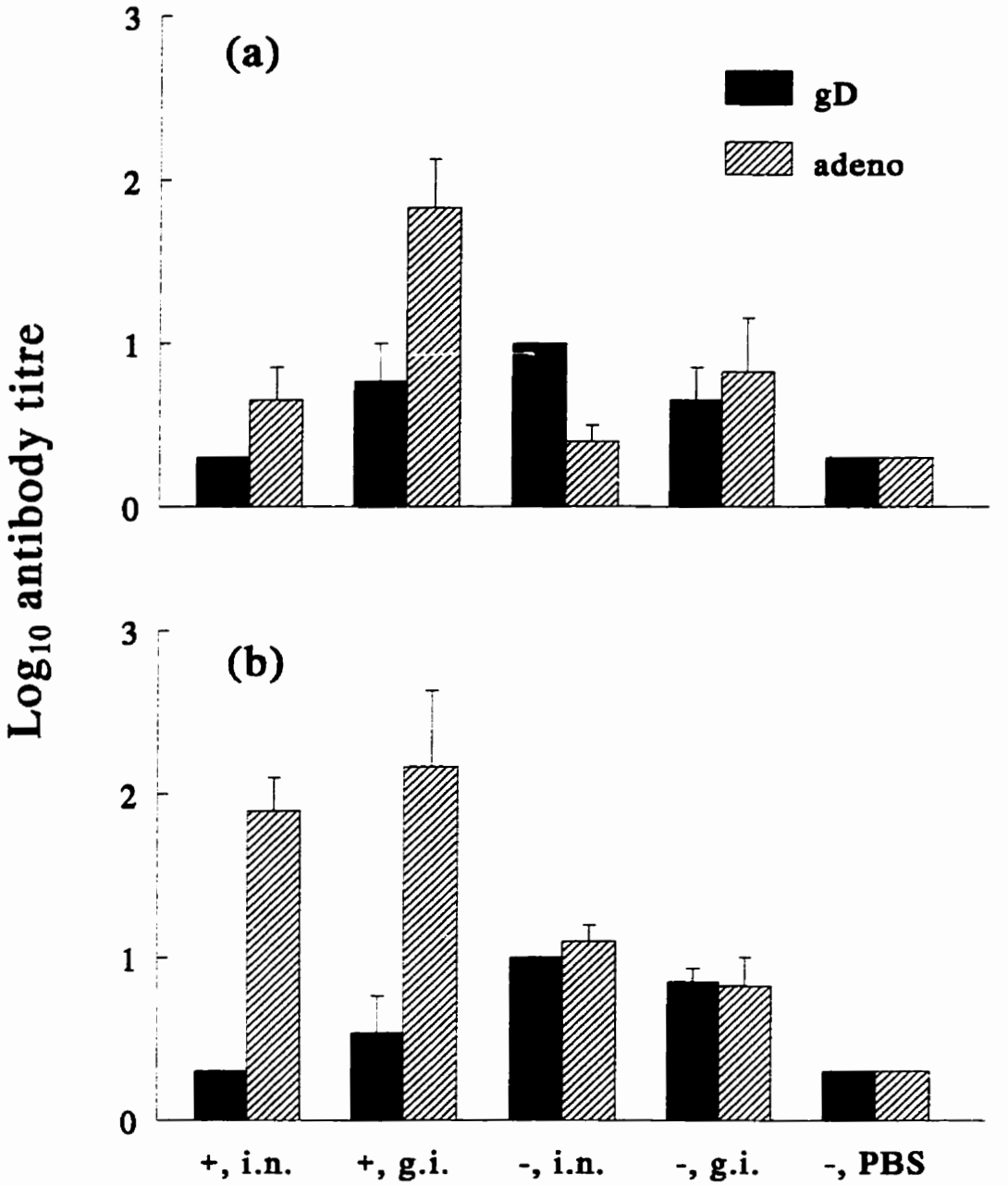


Figure 5.5.4 gD- and Ad-specific antibody responses in the trachea of cotton rats immunised with gD-dE3 by different routes following HAd5 infection. Cotton rats were intranasally inoculated with wt HAd5 (+ symbol) or with PBS as a control (- symbol). Two and three weeks following infection cotton rats were immunised with gD-dE3 by the intranasal (i.n.) or gastrointestinal (g.i.) routes or with PBS as a control. gD- and Ad-specific (a) IgG and (b) IgA levels in tracheal-extracts at 3 weeks following the primary immunisation and 1 day after BHV-1 challenge were measured by ELISA. Bars represent the mean $\log_{10} \pm$ SD antibody titre for 3-4 animals/group.

by pre-infection with HAd5. Interestingly, higher Ad-specific IgG levels were detected in the trachea of g.i. immunised, than i.n. immunised HAd5-infected animals ($P < 0.01$).

The mean of gD-specific lung-IgG titres in each immunisation group was higher than that in the PBS controls ($P < 0.01$); (Figure 5.5.5). However, HAd5-infected animals had significantly lower gD-specific IgG levels in their lungs than non-infected animals ($P < 0.01$). Furthermore, gD-specific lung IgA was induced only in non-HAd5-infected animals, with significantly higher titres than those in the HAd5-infected and PBS control groups ($P < 0.01$); (Figure 5.5.5). In accordance, only non-infected animals contained gD-specific IgA and IgG ASC in the lung tissue (similar numbers in the i.n. and g.i. immunisation groups) as determined by ELISPOT (data not shown). Adenovirus-specific IgG and IgA levels in the lung, measured by ELISA, resembled those observed in the serum (Figures 5.5.2 and 5.5.5).

5.5.1.3 BHV-1 recovery from lungs following challenge

Since HAd5 infection affected antigen-specific immune responses induced by a subsequent immunisation with gD-dE3, its effect on protection of lungs against BHV-1 challenge was investigated. Indeed, significantly more BHV-1 was isolated from the lungs of HAd5-infected than non-infected animals immunised with gD-dE3; (Figure 5.5.6). BHV-1 titres in the gastro-intestinal immunisation group were not significantly affected by pre-infection with HAd5. Each immunisation group contained less BHV-1 in the lung than the PBS control ($P < 0.05$).

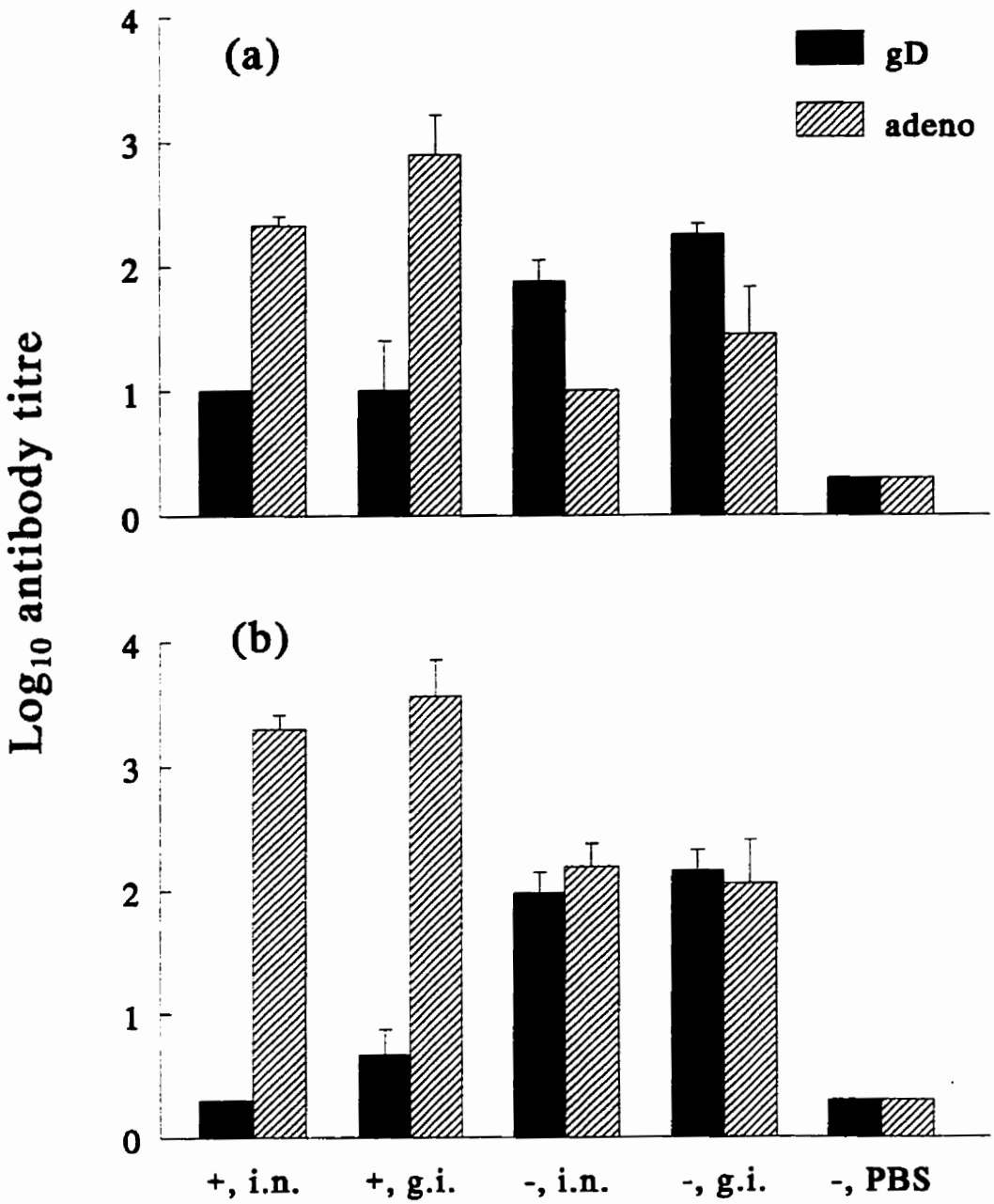


Figure 5.5.5 gD- and Ad-specific antibody responses in the lung of cotton rats immunised with gD-dE3 by different routes following HAd5 infection. Cotton rats were inoculated intranasally with wt HAd5 (+ symbol) or with PBS as a control (- symbol). Two and three weeks after infection cotton rats were immunised with gD-dE3 by the intranasal (i.n.) or gastrointestinal (g.i.) routes or with PBS as a control. gD- and Ad-specific (a) IgG and (b) IgA levels in lung-extracts at 3 weeks following the primary immunisation and 1 day after BHV-1 challenge were measured by ELISA. Bars represent the mean $\log_{10}$ antibody titre ± SD for 3-4 animals/group.

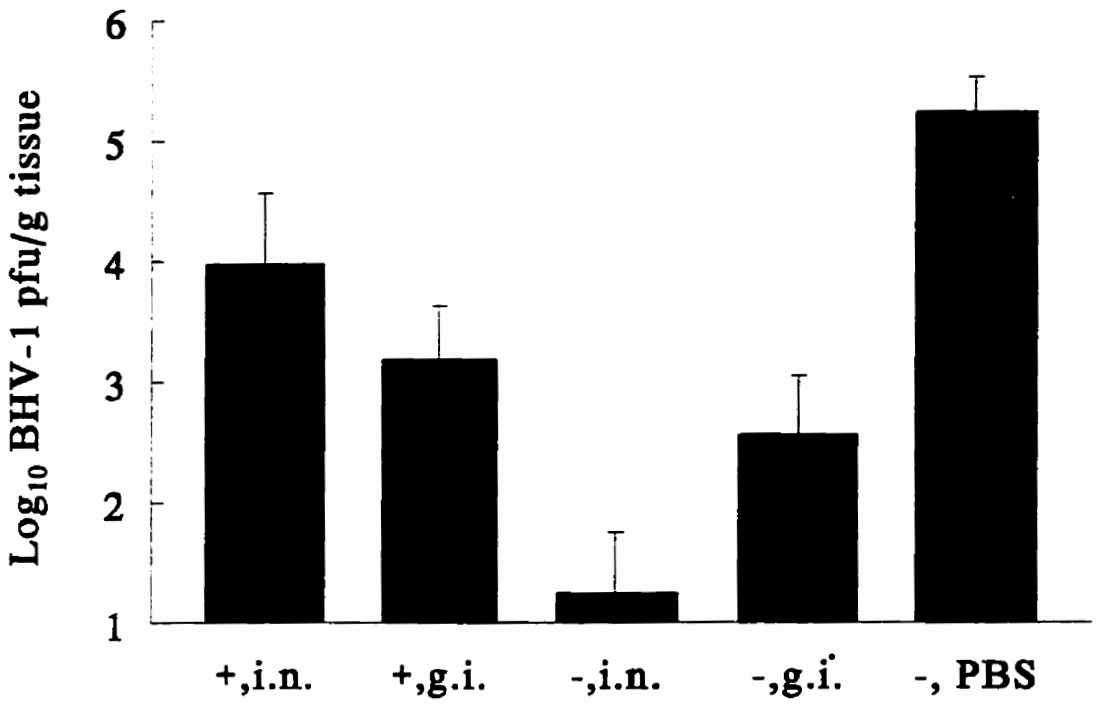
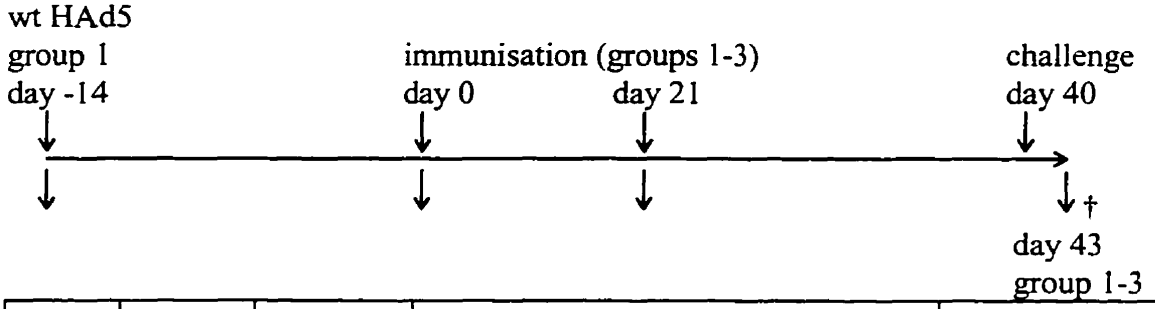


Figure 5.5.6 BHV-1 recovery from lungs of cotton rats immunised with gD-dE3 by different routes following HAd5 infection. Cotton rats were inoculated intranasally with wt HAd5 (symbol +) or with PBS as controls (symbol -). Two and three weeks following infection the animals were immunised with gD-dE3 by the intranasal (i.n.) or gastrointestinal (g.i.) routes or with PBS as a control. Three weeks after the primary immunisation with gD-dE3 animals were challenged with BHV-1 intranasally. One day post-challenge lungs were homogenised and tested for the presence of BHV-1 by plaque assay. Bars represent the mean $\log_{10}$ antibody titre ± SD for 3-4 animals per group.

5.5.2 Effect of active HAd5-specific immunity on intraduodenal immunisation with recombinant adenovirus

Results described in section 5.4 suggested that it is more appropriate to administer adenoviruses intraduodenally rather than orally if one wants to study the induction of immune responses induced by immunisation in the alimentary tract. By doing so, direct spread of adenovirus to the respiratory tract can be avoided. Therefore, the next experiment had two aims: first, to study gD-specific immune responses induced by intraduodenal immunisation alone; second, to determine the effect of Ad infection on the efficacy of a subsequent intraduodenal immunisation with gD-dE3 (Figure 5.5.7). HAd5-infected and uninfected cotton rats were immunised with gD-dE3 by the intraduodenal route and challenged with BHV-1 3 weeks following secondary immunisation. gD- and Ad-specific immune responses and protection against BHV-1 infection were determined.



group #	# of cotton rats per group	i.n. HAd5 infection dose (pfu)	immunisation				i.n. BHV-1 challenge	
			inocu-lum	dose (pfu)	route of primary	route of second.	strain	dose (pfu)
1	6	2×10^7	gD-dE3	10^8	i.duod.	i.duod.	108	5×10^7
2	6	-	gD-dE3	10^8	i.duod.	i.duod.	108	5×10^7
3	5	-	PBS	-	i.duod.	i.duod.	108	5×10^7

Figure 5.5.7 Experimental design: effect of active HAd5-specific immunity on intraduodenal immunisation with recombinant adenovirus. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples from euthanised animals.

5.5.2.1 Antibody responses

At the time of immunisation with gD-dE3, HAd5-infected animals developed Ad-specific serum antibody responses similar to those in Figure 5.5.11, 2 weeks following HAd5-infection. These animals did not develop gD-specific antibody responses (Fig. 5.5.8) or BHV-1 SN titres (background level of 0.3 $\log_{10}$ titre) following intraduodenal immunisation with gD-dE3. Intraduodenal immunisation with gD-dE3, however, induced gD-specific IgG and IgA titres in the serum, lung- and nasal-washes (Fig. 5.5.8) and BHV-1 SN titres ($\log_{10}$ titre 1.29 ± 0.415, significantly different from background) in those animals that were not pre-infected with HAd5. Interestingly, the two immunisation groups had similar levels of Ad-specific lung IgG and serum IgA following immunisation with gD-dE3 (Fig. 5.5.9). Serum antibody responses including SN titres were almost identical following primary and secondary immunisations (data not shown).

Both gD-specific (Figure 5.5.8) and Ad-specific (not shown) antibody levels in lung- and nasal-washes correlated with those measured in lung- and tracheal-extracts prepared from the same animals, respectively. These observations confirmed that the two methods for assessing antibody levels in the respiratory tract are equally appropriate.

To determine local immune responses in the gut, the frequency of gD- and Ad-specific ASC in the mesenteric LN was measured by ELISPOT. Interestingly, significant numbers of gD- and Ad-specific ASC in the mesenteric LN were detected in only those animals that were not infected with HAd5 before immunisation with gD-dE3 (Table 5.5.1). These results are in accordance with ELISPOT data obtained from animals of the previous experiment (data shown here, in Table 5.5.1). The frequency of antigen-specific ASC in the spleens was not different between infected and non-infected groups (Table 5.5.1).

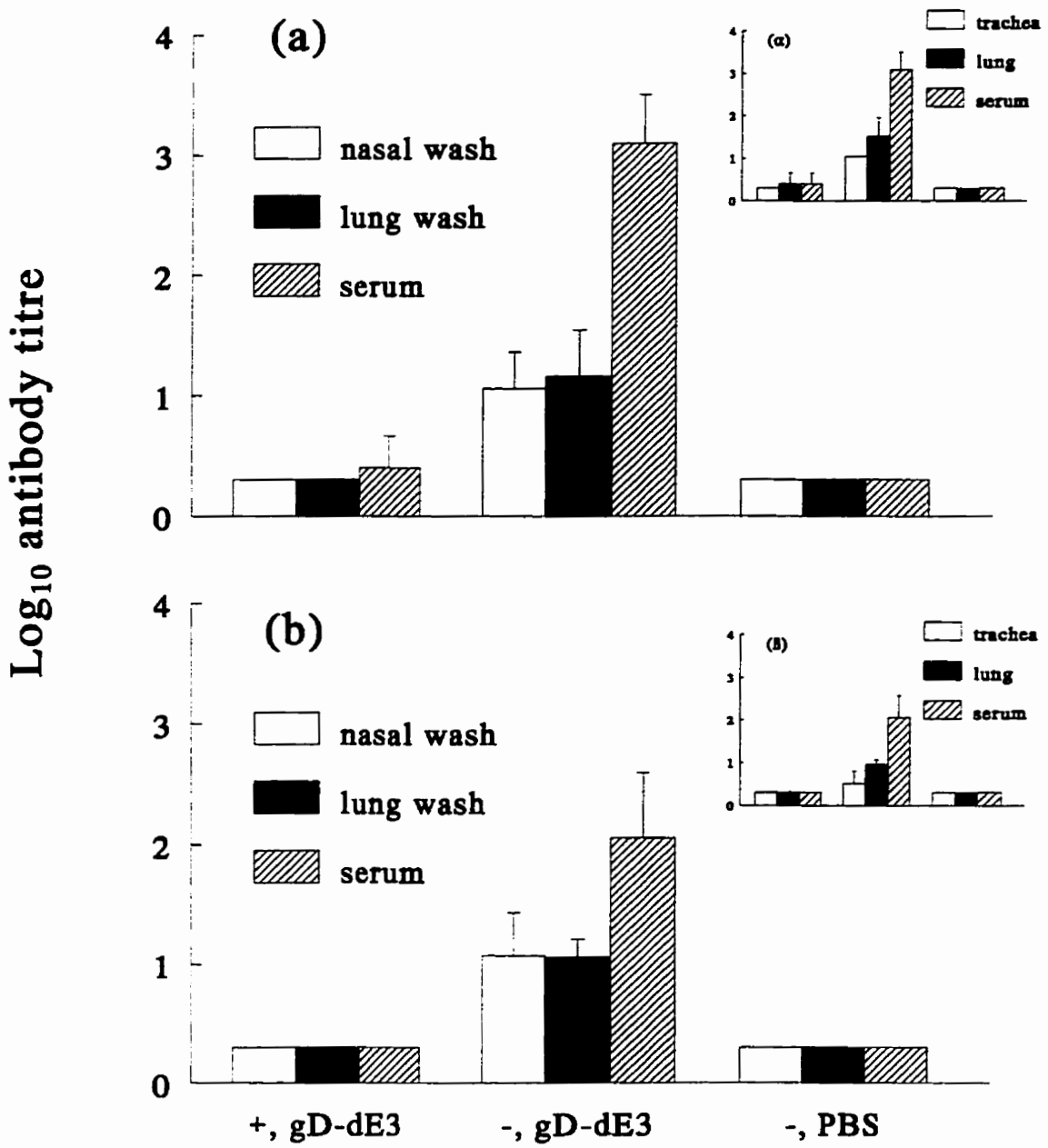


Figure 5.5.8 Effect of adenovirus-specific active immunity on the induction of gD-specific antibody responses following immunisation with gD-dE3 by the intraduodenal route. Cotton rats were inoculated i.n. with wt HAd5 (+ symbol) or with PBS as controls (- symbol). Two and five weeks following infection the animals were immunised with gD-dE3 or with PBS as a control by the intraduodenal route. gD-specific (a) IgG and (b) IgA levels in the nasal- and lung-washes and the serum at 6 weeks following the primary immunisation were measured by ELISA. Insets: (α) trachea-, lung-extract and serum IgG; (β) trachea-, lung-extract and serum IgA. Bars represent the mean $\log_{10}$ antibody titre ± SD for 6 animals/group.

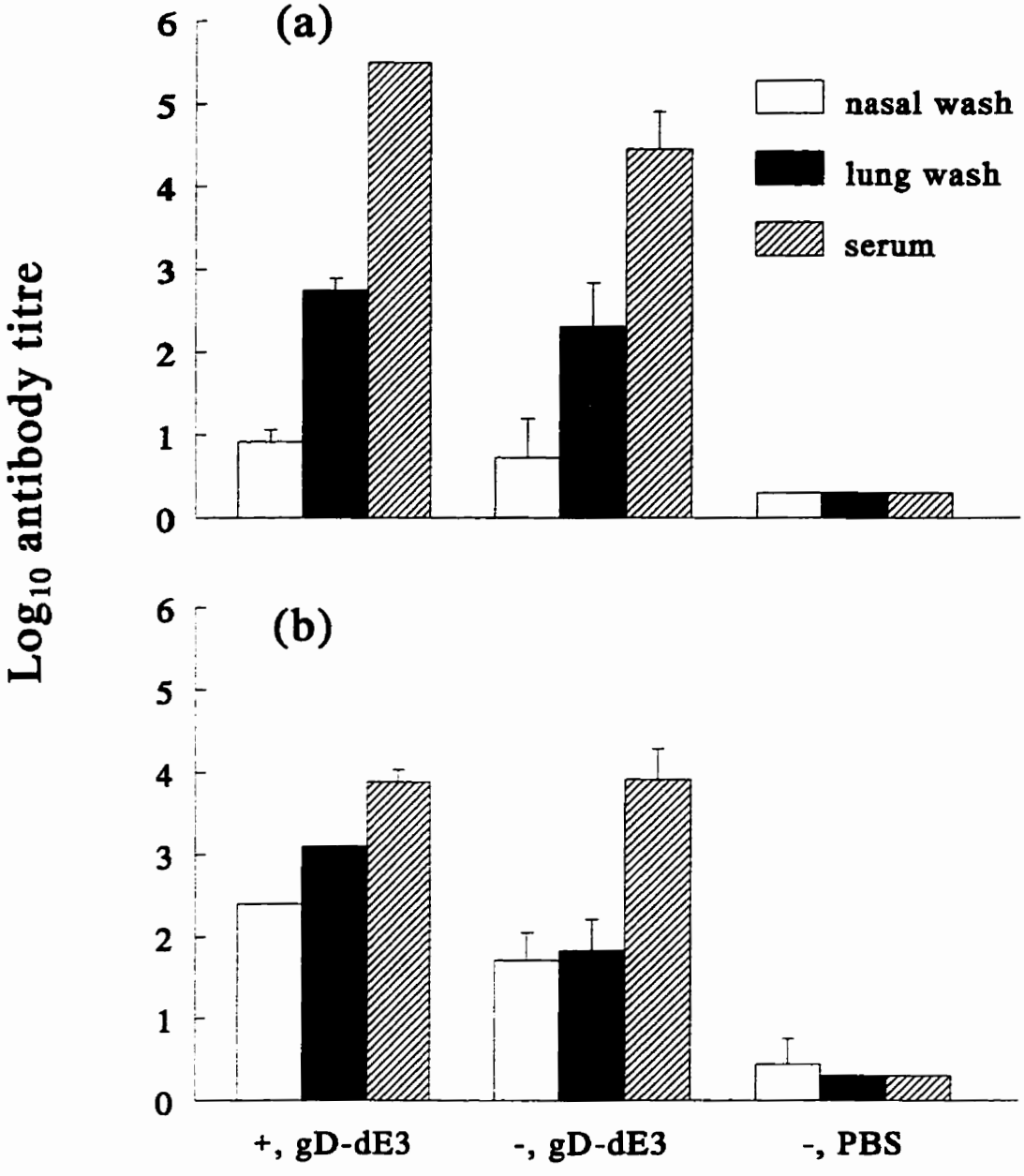


Figure 5.5.9 Adenovirus-specific antibody responses in cotton rats immunised with gD-dE3 by the intraduodenal route following i.n. HAd5 infection. Cotton rats were inoculated i.n. with wt HAd5 (+ symbol) or with PBS as controls (- symbol). Two and five weeks following infection the animals were immunised with gD-dE3 or with PBS as a control by the intraduodenal route. Ad-specific (a) IgG and (b) IgA levels in the nasal- and lung-washes and the serum at 6 weeks following the primary immunisation were measured by ELISA. Bars represent the mean $\log_{10}$ antibody titre ± SD for 6 animals/group.

Table 5.5.1 Effect of active adenovirus-specific immunity on the frequency of gD- and adenovirus-specific antibody-secreting cells in lymphocytes of cotton rats immunised with recombinant adenoviruses

experiment	immunisation	gD-specific IgG/IgA ASC/million*		HAd5-specific IgG/IgA ASC/million*	
		mes LN	spleen	mes LN	spleen
†5.5.1.	+, i.duod-oral	nd / nd	2.2 / 3	nd / 1.5	12 / 7
	-, i.duod-oral	7 / 9	6 / 8	8.5 / 17.5	10 / 7
‡5.5.2.	+, i.duod-i.duod	n / 1	nd / nd	n / 3	4 / 4.5
	-, i.duod-i.duod	n / 11	nd / 2.2	n /25	2 / 4.3
5.5.1. -5.5.2.	-, PBS	nd / nd	nd / nd	nd / nd	nd / nd

Cotton rats were inoculated with wild type HAd5 (+ symbol) or with PBS as controls (- symbol) intranasally.

† Two and three weeks after infection with HAd5 the animals were immunised with gD-dE3 by the gastrointestinal route (intraduodenal-oral). Three weeks after the primary immunisation animals were challenged with BHV-1 intranasally. One day post-challenge mesenteric LN and spleen lymphocytes were isolated and used in the ELISPOT assays.

‡ Two and five weeks after infection with HAd5 cotton rats were immunised by the intraduodenal route with gD-dE3 or PBS as a control. Three weeks after the secondary immunisation animals were challenged with BHV-1 intranasally. Three days post-challenge mesenteric LN and spleen lymphocytes were isolated and used in the ELISPOT assays.

• Mean values of the number of antigen-specific antibody-secreting cells (ASC) per million lymphocytes were determined in cultures of pooled cell populations from 4-8 animals.

nd = not detected (less than 1 ASC/million)

n = not tested

5.5.2.2 Protection against BHV-1 challenge

Since HAd5 infection affected antigen-specific immune responses, I investigated whether it affected protection of the respiratory tract against BHV-1 challenge. Significantly less BHV-1 was isolated from the lungs of animals that were not pre-immune to HAd5 before gD-dE3 immunisation compared to those that were (Table 5.5.2). Virus titres in the trachea, however, were not decreased by either immunisation regimen compared to the control.

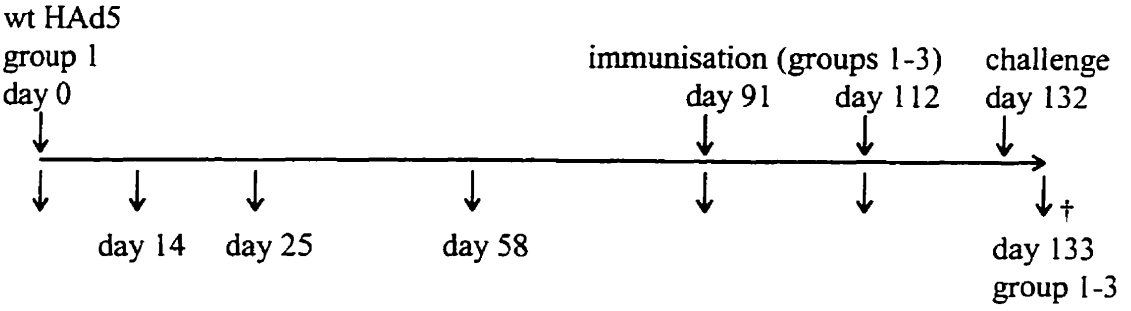
Table 5.5.2 Effect of adenovirus-specific immunity on immunisation with gD-dE3 as reflected in BHV-1 recovery following challenge

immunisation	virus isolation (log_{10} pfu/g tissue)	
	trachea	lung
+ , gD-dE3 intraduodenal	2.942 ± 0.533	2.997 ± 0.715
- , gD-dE3 intraduodenal	2.756 ± 0.381	< 1.0 ± 0.0 *
- , PBS	2.494 ± 0.275	4.061 ± 0.430

Cotton rats were inoculated intranasally with 2 x 10^7 pfu wt HAd5 (+ symbol) or with PBS as controls (- symbol). Two and five weeks later cotton rats were immunised with gD-dE3 or PBS as a control intraduodenally. Three weeks after the secondary immunisation animals were challenged with BHV-1 intranasally. Three day post-challenge trachea and lung were removed, homogenised and tested for the presence of BHV-1 by plaque assay.
* Significant difference from other groups ($P < 0.001$).

5.5.3 Long-term effect of infection with HAd5 on intranasal immunisation with recombinant adenovirus

The previous experiments have shown that active immunity to HAd5 inhibited the efficacy of different routes of immunisation with gD-dE3. It is possible, however, that by a later timepoint p.i. with HAd5, some components of Ad-specific cellular or humoral immunity decline resulting in less inhibition of the induction of gD-specific immunity by immunisation with gD-dE3. To test this hypothesis, cotton rats were infected with HAd5 13 weeks before immunisation with gD-dE3 (Fig. 5.5.10) and the effect of HAd5-infection on the level of immunity induced by recombinant adenovirus was assessed.



group #	# of cotton rats per group	i.n. HAd5 infection dose (pfu)	immunisation				i.n. BHV-1 challenge	
			inoculum	dose (pfu)	route of primary	route of second.	strain	dose (pfu)
1	8	2×10^7	gD-dE3	5×10^7	i.n.	i.n.	108	5×10^7
2	6	-	gD-dE3	5×10^7	i.n.	i.n.	108	5×10^7
3	3	-	dE3	5×10^7	i.n.	i.n.	108	5×10^7
4	5	-	PBS	-	i.n.	i.n.	108	5×10^7

Figure 5.5.10 Experimental design: long-term effect of infection with HAd5 on intranasal immunisation with recombinant adenovirus. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples from euthanised animals.
i.n. = intranasal

5.5.3.1 Kinetics of adenovirus-specific serum antibody responses

To determine the levels of Ad-specific serum antibody at 13 weeks compared to that at 2 weeks following i.n. infection with HAd5, Ad-specific IgG and IgA titres in the serum were measured by ELISA. Ad-specific IgG titres continually increased during the study (Figure 5.5.11). IgA titres did not rise further following the eighth week of the experiment (Figure 5.5.11). Mean Ad-specific antibody titres were significantly higher at week 13 compared to those at week 2 p.i. ($P < 0.001$).

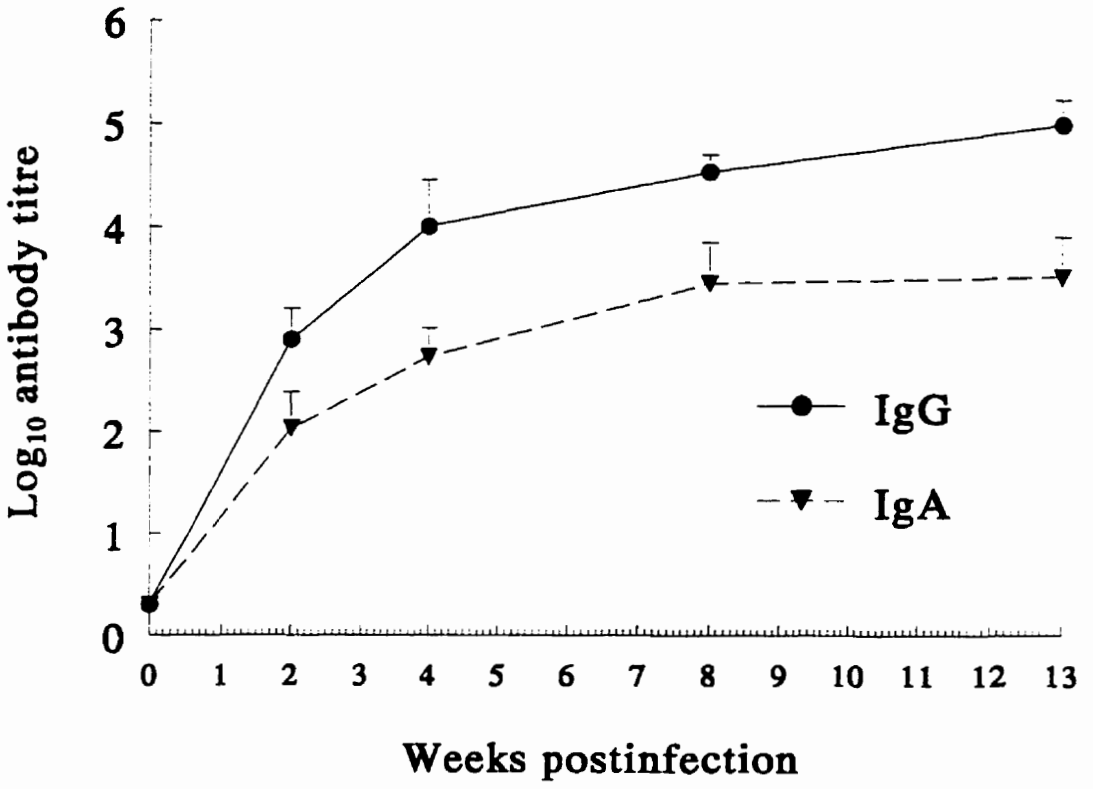


Figure 5.5.11 Kinetics of adenovirus-specific serum antibody responses in cotton rats infected with wild type HAd5. Sera collected at different timepoints p.i. i.n. with 10^7 pfu of HAd5 were analysed for Ad-specific (a) IgG and (b) IgA by ELISA. Values represent the mean $\log_{10}$ antibody titre ± SD for 10 animals.

5.5.3.2 Antibody responses following immunisation with gD-dE3

Although immunisation with gD-dE3 induced gD-specific IgG and IgA antibodies in the serum, lung- and tracheal-extracts in both HAd5-infected and non-infected animals (significantly higher titres than the PBS control, $P < 0.001$), HAd5-infected animals developed significantly lower gD-specific antibody than non-infected cotton rats ($P < 0.01$). IgA levels in the trachea were not significantly different between the two immunisation groups (Figure 5.5.12). BHV-1 neutralising titres in the serum were also lower in the HAd5-infected group than in the non-infected group ($P < 0.001$); (Figure 5.5.12). As expected, immunisation with dE3 control virus did not induce any gD-specific antibody (data not shown). In contrast to gD-specific humoral responses, all animals immunised with adenovirus developed similar levels of Ad-specific antibody by week 19 (data not shown).

5.5.3.3 Antibody-secreting cell frequencies

To determine whether the frequencies of gD- and Ad-specific ASC were affected by a previous HAd5 infection, ELISPOT assays were performed. Although immunisation with gD-dE3 induced antigen-specific ASC in the lung, bone marrow and spleen of both HAd5-infected and non-infected animals, gD-specific ASC frequencies in the lung were higher in the non-infected compared to the HAd5-infected group (Table 5.5.3). Adenovirus-specific ASC frequencies were similar or higher in the HAd5-preinfected group compared to other groups.

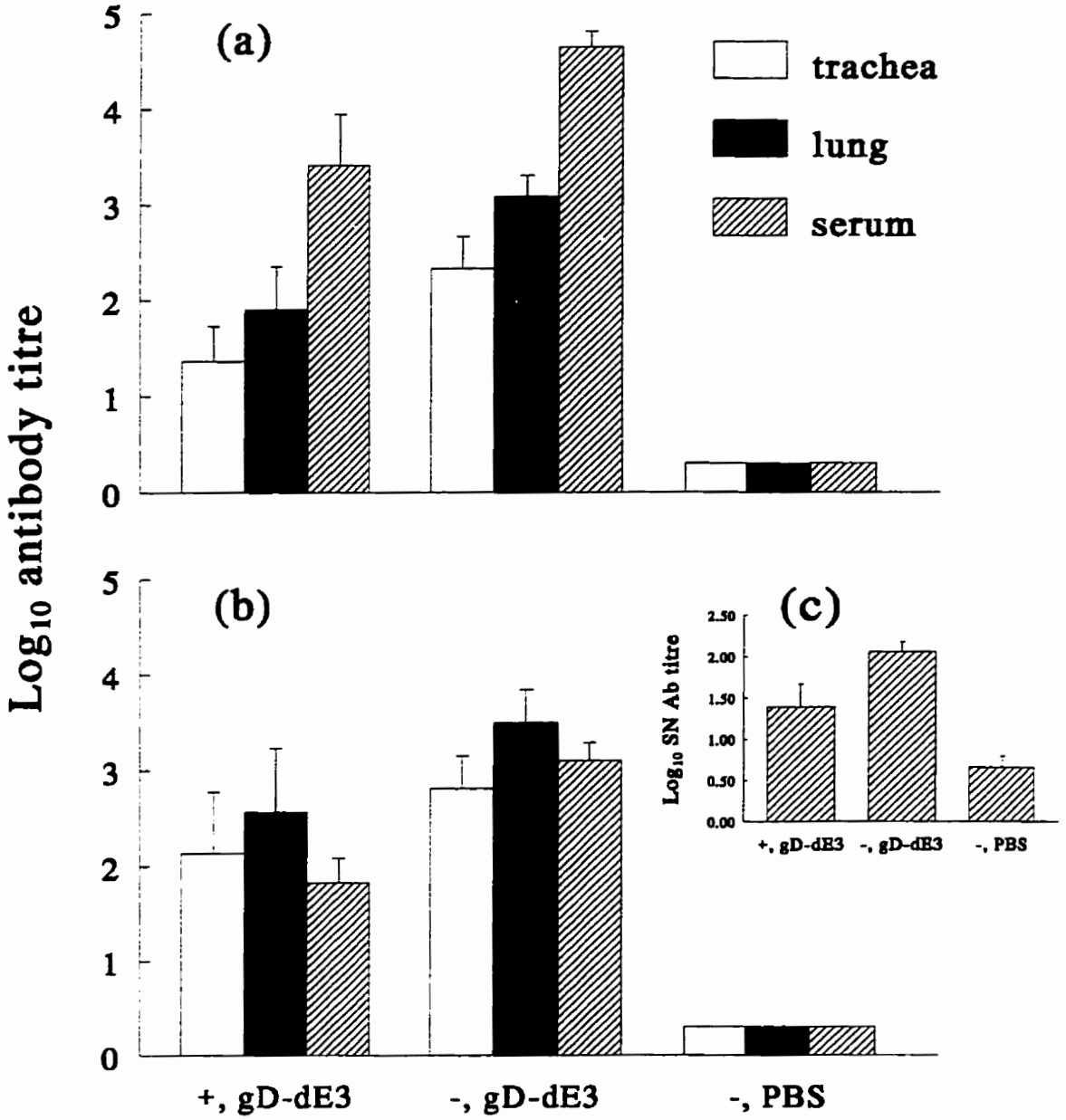


Figure 5.5.12 Effect of adenovirus-specific active immunity stimulated 13 weeks earlier on the induction of gD-specific antibody responses. Cotton rats were inoculated with wt HAd5 (+ symbol) or PBS as a control (- symbol) intranasally. Thirteen and sixteen weeks following infection the animals were immunised i.n. with gD-dE3 or with PBS as a control. gD-specific (a) IgG and (b) IgA levels in the trachea- and lung-extracts and the serum at 6 weeks following the primary immunisation with gD-dE3 were measured by ELISA. (c) Serum samples collected at 6 weeks following primary immunisation with gD-dE3 were analysed for BHV-1-neutralising antibodies by virus neutralisation assay. Bars represent the mean $\log_{10}$ antibody titre ± SD for 5-8 animals/group.

Table 5.5.3 Effect of active adenovirus-specific immunity on the frequency of gD- and adenovirus-specific antibody-secreting cells in lymphocytes of cotton rats immunised with recombinant adenoviruses

lymphocyte source	immunisation	gD-specific ASC/million*		adenovirus-specific ASC/million*	
		IgG	IgA	IgG	IgA
lung	+ , gD-dE3	4	26	280	4600
	- , gD-dE3	20.5	186	100	1940
	- , dE3	not tested			
	- , PBS	nd	nd	nd	nd
bone marrow	+ , gD-dE3	nd	1.3	30	41
	- , gD-dE3	nd	2	4.5	18
	- , dE3	nd	nd	3.5	24
	- , PBS	nd	nd	nd	1
spleen	+ , gD-dE3	1.7	nd	8	20
	- , gD-dE3	1	1.7	14	9
	- , dE3	nd	nd	14	11.5
	- , PBS	nd	nd	nd	nd

Cotton rats were inoculated with wild type HAd5 (+ symbol) or with PBS as a control (- symbol) intranasally. Thirteen and sixteen weeks later the animals were immunised i.n. with gD-dE3 or with PBS. Three weeks after the secondary immunisation animals were challenged with BHV-1 intranasally. One day post-challenge lung, bone marrow and spleen lymphocytes were isolated and used in the ELISPOT assays.

* Mean values of the number of antigen-specific antibody-secreting cells (ASC) per million lymphocytes were determined in cultures of pooled cell populations from 3-8 animals.

nd = not detected (less than 1 ASC/million)

5.5.3.4 BHV-1 recovery after challenge

Since antigen-specific immune responses induced by immunisation with gD-dE3 were affected by a previous i.n. HAd5 infection, we investigated whether protection of the respiratory tract against BHV-1 challenge was different between the immunisation groups. Table 5.5.4 shows that immunisation with gD-dE3 significantly reduced BHV-1 titres in the lung compared to the dE3 and PBS controls. Infection with HAd5 before immunisation with gD-dE3, however, resulted in significantly more BHV-1 in the lungs compared to those in animals immunised with gD-dE3 alone. In addition, virus titres in the trachea were lower compared to the controls only in those animals that had not received HAd5 infection before gD-dE3 immunisation ($P < 0.001$); (Table 5.5.4).

Table 5.5.4 Effect of adenovirus-specific immunity on immunisation with recombinant adenovirus as reflected in BHV-1 recovery following challenge

immunisation	virus isolation (log_{10} pfu/g tissue)	
	trachea	lung
+, gDE3 intranasal	5.271 ± 0.560	3.934 ± 0.555 *
-, gDE3 intranasal	4.578 ± 0.316 *	< 1.3 ± 0.0 *
-, dE3	5.935 ± 0.402	5.496 ± 0.617
-, PBS	6.042 ± 0.403	5.450 ± 0.556

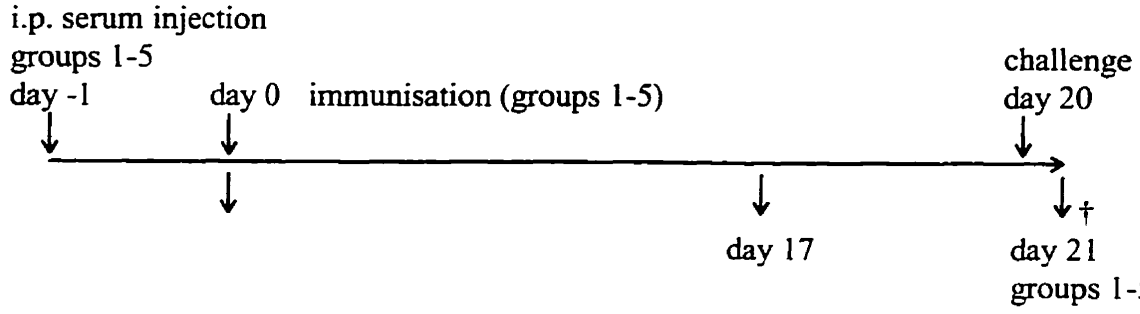
Cotton rats were inoculated with wt HAd5 (+ symbol) or PBS (- symbol). Thirteen and sixteen weeks later the animals were immunised intranasally with gD-dE3, dE3 or with PBS. Three weeks after the secondary immunisation animals were challenged with BHV-1 intranasally. One day post-challenge trachea and lung were removed, homogenised and tested for the presence of BHV-1 by plaque assay.
*Significant difference from other groups ($P < 0.001$)

5.5.4 Effect of adenovirus-specific passive immunity on systemic and mucosal immunisation with recombinant adenovirus

Previous results showed that i.n. infection with HAd5 had an inhibitory effect on gD-specific humoral immune responses and protection against BHV-1 infection induced by immunisation with gD-dE3. Infection with a pathogen, however, is not the only way to acquire immunity to the pathogen. Young animals, for example, may have obtained Ad-specific antibodies from their mother without having been infected with adenovirus. To investigate the effect of passive Ad-specific immunity on subsequent immunisation with recombinant adenovirus by different routes, serum-transfer experiments were designed. In the first experiment, cotton rats were inoculated with Ad-hyperimmune or normal cotton rat serum i.p., followed by i.n. and i.p. immunisation with gD-dE3 (Fig. 5.5.13). Induction of antigen-specific immune responses and protection against BHV-1 challenge were determined.

5.5.4.1 Halflife of adenovirus-specific antibodies following passive transfer

Before immunisation with gD-dE3, it was important to examine whether cotton rats contained Ad-specific antibody following i.p. passive transfer of Ad-specific hyperimmune serum. Furthermore, knowing the kinetics of the decay of this antibody would help interpret results and facilitate future studies. Figure 5.5.14 shows Ad-specific IgG and IgA titres in the serum at different timepoints after inoculation of Ad-specific hyperimmune serum. At day 1 after the passive transfer, both IgG and IgA, specific for HAd5, were detected in the serum. These titres were higher than those detected in the serum of cotton rats 2 weeks following i.n. infection with HAd5 (see 5.5.3.1). The halflife of both serum IgG and IgA of the cotton rat was estimated to be 6-7 days.



group #	# of cotton rats per group	injection of (cotton rat serum)	immunisation				i.n. BHV-1 challenge	
			inocu-lum	dose (pfu)	route		strain	dose (pfu)
					primary	second.		
1	3	ad-spec*	gD-dE3	2×10^7	i.p.	-	Cooper	10^7
2	3	ad-spec*	gD-dE3	2×10^7	i.n.	-	Cooper	10^7
3	3	normal	gD-dE3	2×10^7	i.p.	-	Cooper	10^7
4	3	normal	gD-dE3	2×10^7	i.n.	-	Cooper	10^7
5	3	normal	PBS	-	i.p.	-	Cooper	10^7

Figure 5.5.13 Experimental design: effect of adenovirus-specific passive immunity on the efficacy of immunisation with recombinant adenovirus. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples from euthanised animals.
i.n. = intranasal; i.p. = intraperitoneal.
* Transfer of adenovirus-specific hyperimmune cotton rat serum.

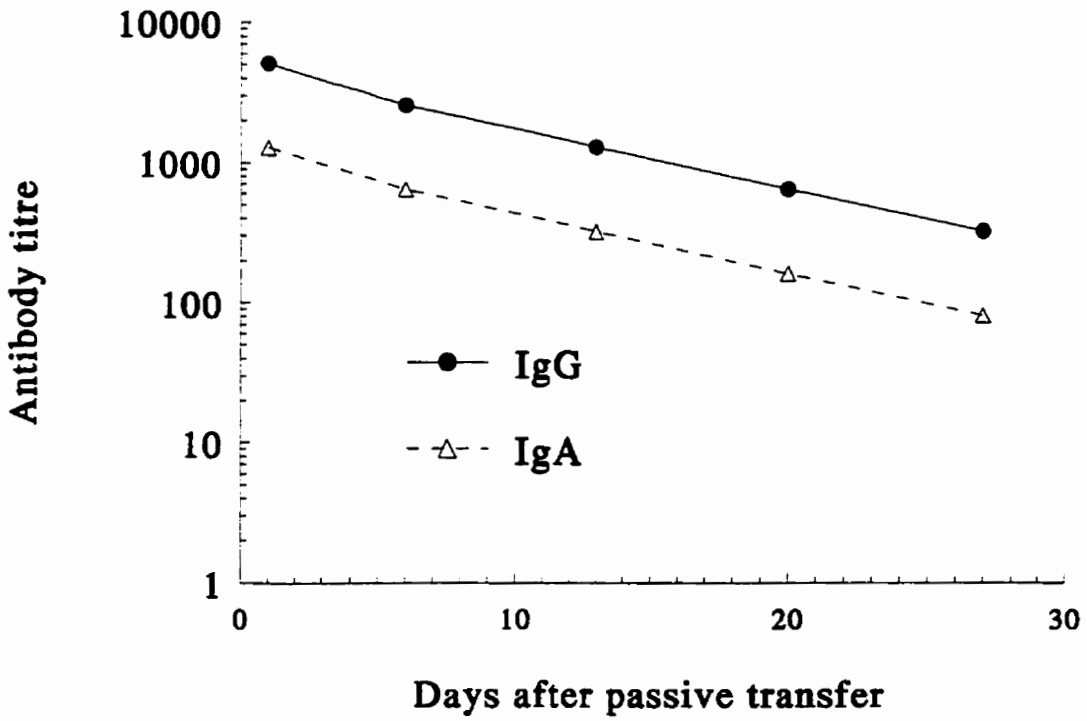


Figure 5.5.14 Kinetics of the decay of adenovirus-specific antibodies in the serum of cotton rats following passive transfer of adenovirus-specific serum. Two animals were i.p. injected with 1 ml of pooled Ad-specific hyperimmune serum. Adenovirus-specific serum antibody levels at different timepoints following passive transfer were measured by ELISA. Values represent the mean titre from 2 animals.

5.5.4.2 Antibody responses following immunisation with gD-dE3

To determine whether the presence of Ad-specific antibodies in the cotton rat affected the levels of gD-specific antibody responses induced by immunisation with gD-dE3, antigen-specific antibody titres in the serum, trachea- and lung-extracts were measured. Each immunisation regimen resulted in gD- and Ad-specific antibodies in the serum and the lung-extract, as well as BHV-1 SN antibodies, of significantly different mean titres from PBS controls (Table 5.5.5). Serum neutralising antibody titres and gD-specific IgG levels, however, were significantly higher in those animals that had received control serum compared to those that had been injected with Ad-immune serum before the i.n. immunisation with gD-dE3. In addition, there was a trend in all other samples that Ad-specific antibody inhibited the induction of gD-specific immunity, even though these differences were not significantly different (Table 5.5.5).

5.5.4.3 BHV-1 recovery following challenge

Since the presence of Ad-specific antibodies in the cotton rat slightly affected gD-specific immune responses, I investigated whether it affected protection of the respiratory tract against BHV-1 challenge. BHV-1 titres in both the lung and trachea were significantly lower in those i.p. immunised animals that were naive for adenovirus compared to those that had received Ad-specific serum ($P < 0.01$) (Figure 5.5.15). In addition, BHV-1 titres in the trachea were significantly lower in those i.n. immunised cotton rats that had been injected with control serum compared to those with Ad-specific serum ($P < 0.01$); (Figure 5.5.15). However, mean virus titres from the lungs were lower in each immunisation group than in the PBS control. In addition, the mean BHV-1 titre in the trachea was significantly lower in animals immunised intranasally with gD-dE3 than in the PBS control ($P < 0.05$).

Table 5.5.5 Effect of adenovirus-specific passive immunity on antigen-specific antibody titres from cotton rats immunised with gD-dE3

sample	immuni -sation	gD-specific log_{10} ELISA titre ± SD		adenovirus-specific log_{10} ELISA titre		BHV-1 log_{10} SN
		IgG	IgA	IgG	IgA	
serum	+ , i.p.	3.4 ± 0.17	0.76 ± 0.4	3.2 ± 0.0	1.8 ± 0.18	0.6 ± 0.0
	+ , i.n.	2.9 ± 0.3*	1.33 ± 0.9	3.5 ± 0.0	1.69 ± 0.0	0.6 ± 0.0*
	- , i.p.	3.8 ± 0.8	1.23± 0.8	3.4 ± 0.17	1.83 ± 1.15	1.2 ± 0.5
	- , i.n.	3.8 ± 0.52*	1.76 ± 0.7	3.6 ± 0.17	2.2 ± 0.17	1.2 ± 0.3*
	- , PBS	1.0 ± 0.0	0.3 ± 0.0	1.0 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
lung	+ , i.p.	1.5 ± 0.17	0.9 ± 0.17	1.83 ± 0.23	1.7 ± 0.0	0.3 ± 0.0
extract	+ , i.n.	1.39 ± 0.0	1.46 ± 0.4	2.2 ± 0.17	1.7 ± 0.0	0.3 ± 0.0
	- , i.p.	1.7 ± 0.35	1.13 ± 0.5	2.3 ± 0.55	1.23 ± 0.4	0.3 ± 0.0
	- , i.n.	2.0 ± 0.57	2.16 ± 0.4	2.86 ± 0.4	3.13 ± 0.63	0.3 ± 0.0
	- , PBS	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
trachea	+ , i.p.	0.3 ± 0.0	0.3 ± 0.0	0.8 ± 0.55	1.0 ± 0.7	0.3 ± 0.0
extract	+ , i.n.	0.3 ± 0.0	0.3 ± 0.0	1.0 ± 0.0	0.77 ± 0.8	0.3 ± 0.0
	- , i.p.	0.67 ± 0.3	0.3 ± 0.0	1.36 ± 0.35	0.77 ± 0.4	0.3 ± 0.0
	- , i.n.	0.77 ± 0.4	0.3 ± 0.0	1.5 ± 0.17	1.6 ± 0.17	0.3 ± 0.0
	- , PBS	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0

Cotton rats were inoculated with hyperimmune adenovirus-specific (+ symbol) or normal (- symbol) cotton rat serum. One day later the animals were immunised with gD-dE3 intranasally (i.n.) or intraperitoneally (i.p.) or with PBS as a control. Three weeks after immunisation animals were challenged with BHV-1 intranasally. One day post-challenge trachea and lung were removed, homogenised and tested for the presence of antigen-specific antibody simultaneously with serum samples by ELISA or serum neutralisation assay.

* Significant difference between the two groups ($P < 0.05$)

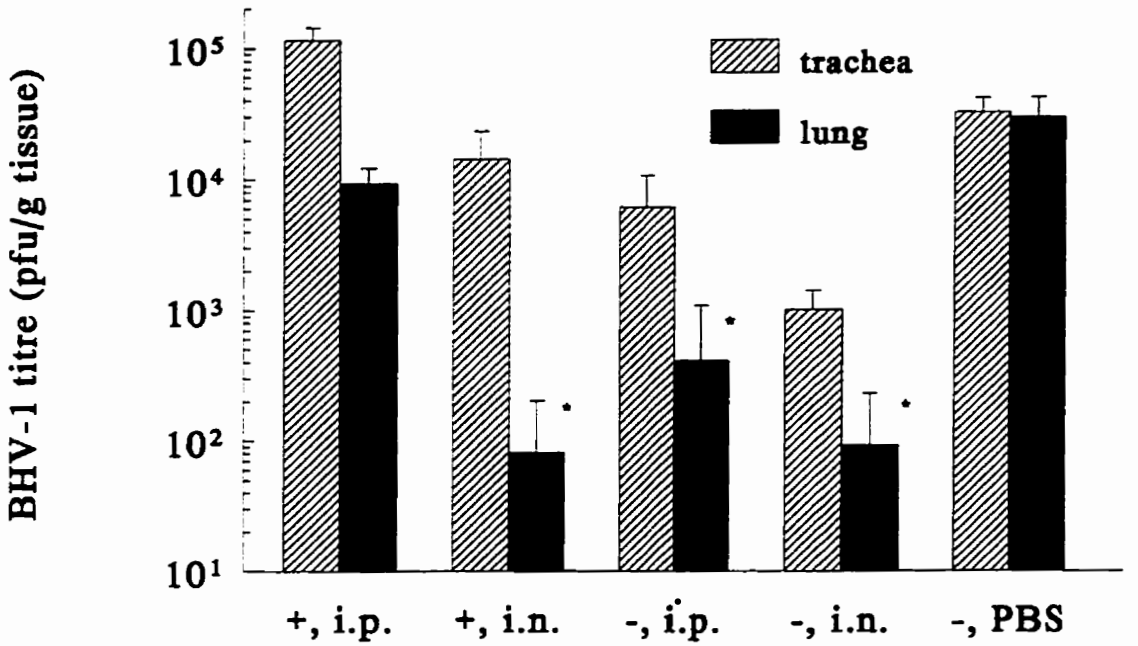


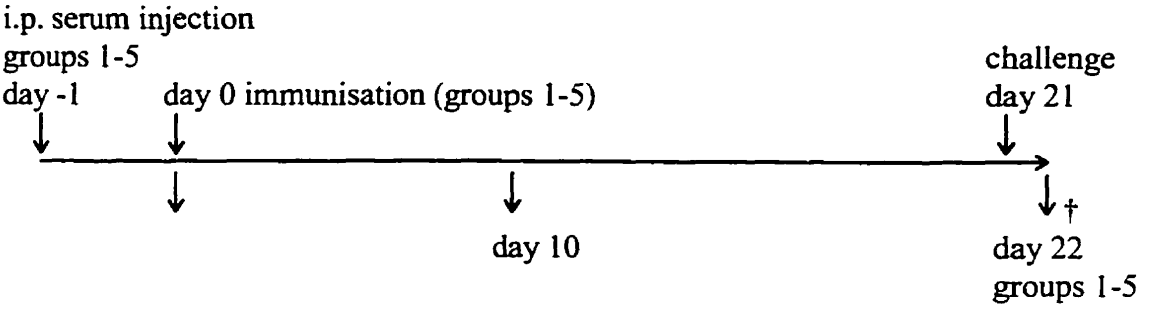
Figure 5.5.15 Effect of adenovirus-specific passive immunity on the protection of cotton rats immunised with gD-dE3 against BHV-1 infection. Cotton rats were inoculated intraperitoneally with Ad-specific (+ symbol) or normal (- symbol) cotton rat serum. Twenty-four hours later cotton rats were immunised by the i.n. or the i.p. routes with gD-dE3 or with PBS as a control. Three weeks after immunisation with gD-dE3 animals were challenged with BHV-1 intranasally. One day post-challenge lungs and tracheae were homogenised and analysed for the presence of BHV-1 by plaque assay. Bars represent the mean $\log_{10}$ antibody titre ± SD for 3 animals per group.
*Significantly different from PBS controls ($P < 0.001$)

5.5.5 Effect of adenovirus-specific passive immunity on different routes of mucosal immunisation with recombinant adenovirus

Results of the previous experiment indicated that pre-existing Ad-specific passive immunity inhibited gD-specific immune responses induced by immunisation with gD-dE3 in some animals. Furthermore, there was less protection against BHV-1 infection in Ad-immune compared to naive animals. To confirm these results and to determine whether the route of immunisation influenced the level of gD-specific immunity in animals with passive immunity to Ad, cotton rats were immunised with gD-dE3 by the i.n. and the intraduodenal routes following passive transfer of Ad-specific or normal cotton rat serum (Figure 5.5.16). Induction of antigen-specific immune responses and protection against BHV-1 challenge were determined.

5.5.5.1 Antibody responses

Interestingly, no statistical differences were observed 3 weeks following immunisation with gD-dE3 between the means of gD-specific antibody titres from animals that had received normal cotton rat serum before immunisation compared to those that received Ad-specific serum (Figure 5.5.17). Intranasal immunisation with gD-dE3 in both groups induced gD-specific IgG and IgA in the serum and lung-washes and IgA in the nasal-washes. However, there was a trend that animals that recieved Ad-specific hyperimmune sera had less gD-specific antibodies. Intraduodenal immunisation also induced gD-specific serum IgG but it did not stimulate IgA in any of the samples. Adenovirus-specific antibodies were detected in each gD-dE3-immunised group. However, the mean Ad-specific titres were higher in the samples from intranasally immunised animals (Figure 5.5.18).



group #	# of cotton rats per group	injection of (cotton rat serum)	immunisation				i.n. BHV-1 challenge	
			inocu-lum	dose (pfu)	route		strain	dose (pfu)
					primary	second		
1	4	ad-spec*	gD-dE3	5×10^7	i.n.	-	108	5×10^7
2	5	ad-spec*	gD-dE3	5×10^7	i.duod.	-	108	5×10^7
3	5	normal	gD-dE3	5×10^7	i.n.	-	108	5×10^7
4	5	normal	gD-dE3	5×10^7	i.duod.	-	108	5×10^7
5	4	normal	PBS	-	i.n.	-	108	5×10^7

Figure 5.5.16 Experimental design: effect of adenovirus-specific passive immunity on different routes of mucosal immunisation with recombinant adenovirus. In the flowchart, arrows above the timescale indicate the time of treatment while arrows below indicate the time of serum sampling. The symbol † represents tissue samples collected from euthanised animals.
i.n. = intranasal; i.duod. = intraduodenal; i.p. = intraperitoneal.
* Transfer of adenovirus-specific hyperimmune cotton rat serum.

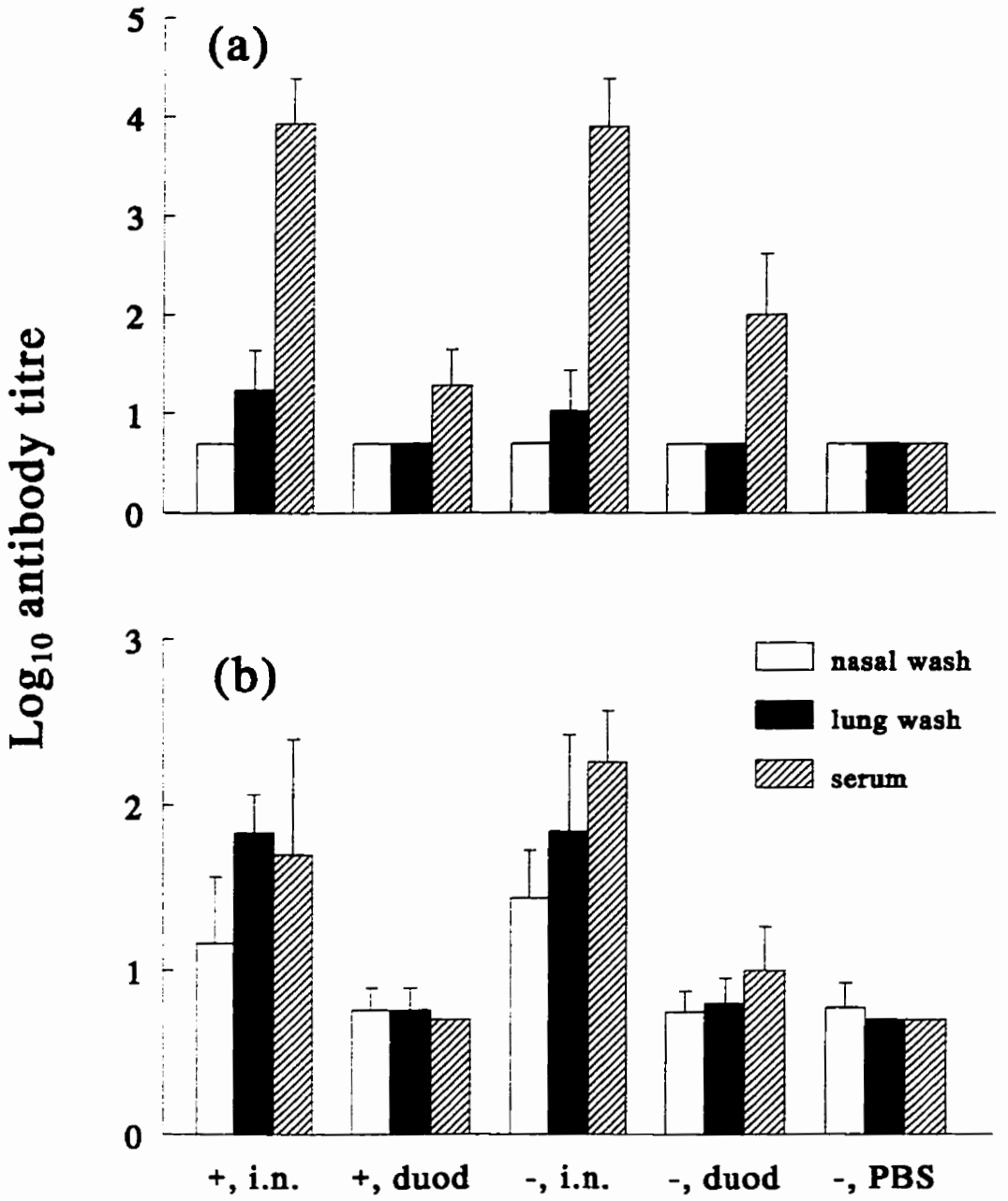


Figure 5.5.17 Effect of adenovirus-specific passive immunity on the induction of gD-specific antibody responses in cotton rats immunised with gD-dE3 by different mucosal routes. Cotton rats were inoculated intraperitoneally with Ad-specific (+ symbol) or normal (- symbol) cotton rat serum. Twenty four hours later cotton rats were immunised with gD-dE3 by the intranasal (i.n.) or the intraduodenal (i.duod.) routes or with PBS as a control. gD-specific (a) IgG and (b) IgA levels in the nasal- and lung-washes and the serum at 3 weeks following immunisation with gD-dE3 were measured by ELISA. Bars represent the mean $\log_{10}$ antibody titre ± SD for 4-5 animals/group.

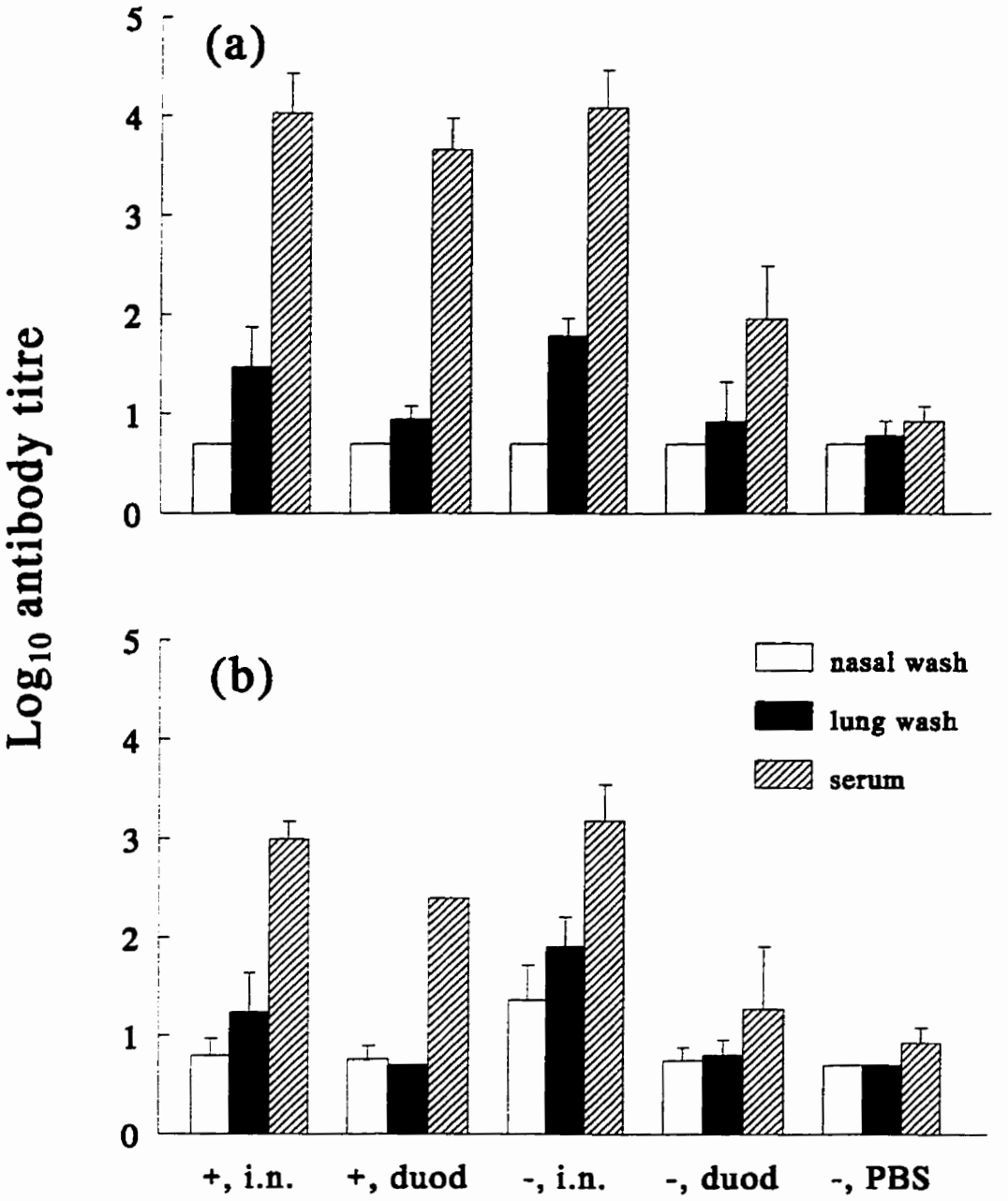


Figure 5.5.18 Adenovirus-specific antibody responses in cotton rats immunised with recombinant adenovirus by the intraduodenal route following passive transfer of adenovirus-specific serum. Cotton rats were inoculated intraperitoneally with Ad-specific (+ symbol) or normal (- symbol) cotton rat serum. Twenty four hours later the animals were immunised with gD-dE3 by the intranasal (i.n.) or the intraduodenal (i.duod.) routes or with PBS as a control. Adenovirus-specific (a) IgG and (b) IgA levels in the nasal- and lung-washes and the serum at 3 weeks following immunisation with gD-dE3 were measured by ELISA. Bars represent the mean $\log_{10}$ antibody titre ± SD for 4-5 animals/group.

5.5.5.2 Antibody-secreting cell frequencies

To determine whether the frequency of antigen-specific ASC in the lung and the spleen were affected by passive immunity against adenovirus, ELISPOT assays were performed. Each immunisation regimen induced gD- and Ad-specific lung-ASC while the PBS control did not (Table 5.5.6). Intraduodenal immunisation induced only IgA secreting, but not IgG secreting cells in the lung. In contrast, i.n. immunisation induced high numbers of both IgA and IgG gD- and Ad-specific ASC in the lung. These numbers were somewhat lower in the Ad-specific serum-transfer groups than in the normal immunised groups. Spleens contained only a low frequency of antigen-specific ASC (Table 5.5.6).

5.5.5.3 BHV-1 recovery after challenge

To determine whether Ad-specific passive immunity influenced the protection of the respiratory tract against BHV-1 infection, BHV-1 titres were measured in the lung and trachea 1 day after viral challenge. Intranasal immunisation with gD-dE3 resulted in reduced BHV-1 titres from the respiratory tract compared to the PBS control ($P < 0.01$), while intraduodenal immunisation did not (Fig. 5.5.19). Interestingly, animals inoculated with Ad-specific serum contained significantly higher BHV-1 titres in their lungs than animals that received normal cotton rat serum before i.n. immunisation with gD-dE3 ($P < 0.05$), despite no significant differences in gD-specific and BHV-1 SN antibody levels (Fig. 5.5.17).

Table 5.5.6. Effect of passive adenovirus-specific immunity on the frequency of gD- and adenovirus-specific antibody-secreting cells in lymphocytes of cotton rats immunised with recombinant adenoviruses

lymphocyte source	immunisation	gD-specific ASC/million*		adenovirus-specific ASC/million*	
		IgG	IgA	IgG	IgA
lung	+ , gD-dE3 i.n.	200	900	370	1050
	+ , gD-dE3 i.duod	nd	2.5	nd	6
	- , gD-dE3 i.n.	510	1250	360	460
	- , gD-dE3 i.duod.	nd	20	nd	25
	- , PBS c.	nd	nd	nd	nd
spleen	+ , gD-dE3 i.n.	1.5	nd	10	8.3
	+ , gD-dE3 i.duod	1	nd	1.2	nd
	- , gD-dE3 i.n.	1	nd	22	4.8
	- , gD-dE3 i.duod.	nd	nd	nd	nd
	- , PBS c.	nd	nd	nd	nd

Cotton rats were inoculated intraperitoneally with hyperimmune Ad-specific (+ symbol) or normal (- symbol) cotton rat serum. One day later the animals were immunised with gD-dE3 intranasally (i.n.) or intraduodenally (i.duod.) or with PBS as a control. Three weeks after immunisation the animals were challenged with BHV-1 intranasally. One day post-challenge lung and spleen lymphocytes were isolated and used in the ELISPOT assays.

* Mean values of the number of antigen-specific antibody-secreting cells (ASC) per million lymphocytes were determined in cultures of pooled cell populations from 4-5 animals/group.

nd = not detected (less than 1 ASC/million)

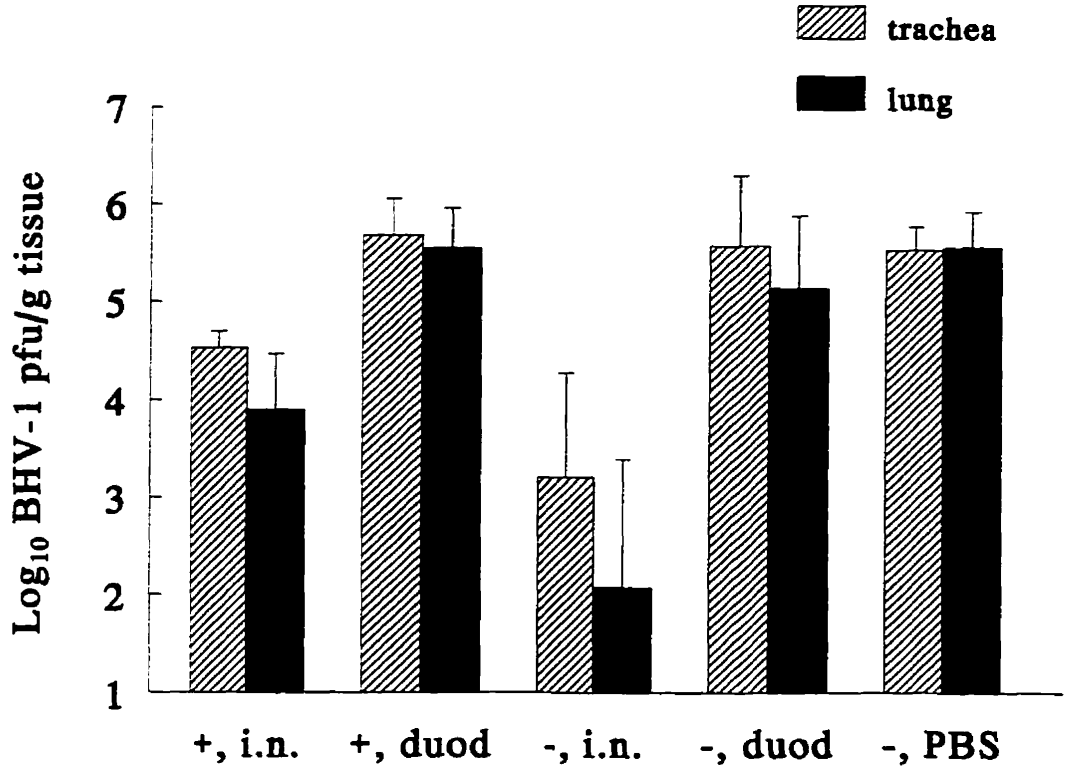


Figure 5.5.19 Effect of adenovirus-specific passive immunity on the protection against BHV-1 infection in cotton rats immunised with recombinant adenovirus. Cotton rats were inoculated intraperitoneally with adenovirus-specific (+ symbol) or normal (- symbol) cotton rat serum. Twenty four hours later the animals were immunised with gD-dE3 by the intranasal (i.n.) or the intraduodenal (i.duod.) routes or with PBS as a control. Three weeks after immunisation with gD-dE3 animals were challenged with BHV-1 intranasally. One day post-challenge lungs and tracheae were homogenised and tested for the presence of BHV-1 by plaque assay. Bars represent the mean $\log_{10}$ antibody titre ± SD for 4-5 animals per group.

5.5.6 Conclusions

Results indicate that immunity to adenovirus may inhibit gD-specific antibody responses induced by subsequent immunisation with gD-dE3. Pre-infection with HAd5 i.n. negatively affected the efficacy of i.n. immunisation not only at 2 weeks, but also at 13 weeks after HAd5 infection. At 13 weeks following infection, however, this inhibitory effect was smaller than at 2 weeks after HAd5-infection. Inhibition of gD-specific antibody responses and protection against BHV-1 challenge was also observed after intraduodenal-oral and intraduodenal immunisations following HAd5-infection.

In contrast to active immunity, passively acquired Ad-specific antibody had little inhibitory effect on the level of immune responses induced by immunisations with gD-dE3 by different routes. Interestingly, however, protection against BHV-1 challenge was significantly inhibited by passive transfer of Ad-specific antibody.

Results also indicate that even one i.n. immunisation with gD-dE3 induced gD-specific antibody responses in the respiratory tract and partial protection against i.n. BHV-1 challenge. Furthermore, intraduodenal immunisation alone was able to induce gD-specific immunity in the serum and the respiratory tract.

6.0 DISCUSSION

6.1 Establishment of an intranasal BHV-1 challenge model in cotton rats

Cotton rats provide an excellent model for investigating immune responses induced by recombinant HAd5 vectors because they support HAd5 replication. However, to evaluate the protective efficacy of immunisation with recombinant HAd5 expressing gD of BHV-1, an animal model that supports BHV-1 replication is necessary. Since cotton rats have served as a rodent model for many viral pathogens, there was a probability that they could also support BHV-1 replication.

BHV-1 replicated in the respiratory tract of cotton rats without requiring prior adaptation of the virus. The histological lesions seen in the lung of each rat, namely necrotising bronchiolitis with the presence of intranuclear inclusion bodies in epithelial cells, were consistent with lesions observed following BHV-1 infection in cattle (Jericho & Darcel, 1978). Pulmonary lesions in cattle are highly variable and inconsistently present, both in natural and experimental infections (Yates, 1982). In the cotton rats, immunohistochemical staining confirmed that BHV-1 became established in the lung and was the cause of the pathological changes as specific viral staining was demonstrated in the lesions in the terminal bronchioles and alveoli. The normal behaviour and absence of clinical signs in the cotton rats indicated that while BHV-1 became established and could replicate within the respiratory tract, the infection was mild and subclinical. Nevertheless, the fact that BHV-1 infection in cotton rats mimics the disease in cattle in terms of pathology suggests that this may be a valid laboratory animal model for use in development and evaluation of new vaccination strategies against BHV-1.

I have not investigated whether *in vivo* passage of BHV-1 in cotton rats would increase BHV-1 infectivity in these animals. I have shown, however, that different BHV-1

strains have slightly different capabilities to replicate in cotton rats. The 108 strain replicated better than the Cooper strain in both CRL cells and in cotton rats. Lesion development was greater, viral antigen was more widespread and higher titres of BHV-1 were evident in the lungs of animals challenged with the 108 strain than with the Cooper strain. The more virulent nature of the 108 strain as compared to the Cooper strain in cattle has been previously suggested (unpublished observation), further supporting the similarity of the cotton rat model to bovine disease. Considering the observed differences between the two strains, I used the 108 strain in most of the challenge experiments.

Since vaccination-challenge studies are very expensive in cattle, a useful model for i.n. BHV-1 infection was established by determining that BHV-1 can replicate in cotton rats. Furthermore, since no other laboratory rodent has been shown to support the replication of both HAd5 and BHV-1, the cotton rat became the most appropriate small animal model for comparing replication-defective and the replication-competent vectors regarding their effectiveness in inducing immunity to gD and conferring protection against BHV-1 challenge.

6.2 Immunity induced by recombinant adenoviruses in cotton rats

Immunisation of cotton rats with recombinant adenoviruses induced protective immune responses against i.n. BHV-1 challenge. Protection of the lung from infection measured by BHV-1 recovery correlated with the absence of BHV-1 replication *in situ* (Table 5.3.7), which provided evidence that measuring BHV-1 titres in the lung is a satisfactory method to assess protection from infection. The level of gD-specific immune responses and protection from BHV-1 challenge were dependent, however, on the type of foreign gene expressed by the vectors, the replication-capability of the viruses and the route of immunisation. These differences and the possible mechanisms involved are discussed below.

6.2.1 Immunity induced by tgD- and gD-expressing recombinant adenoviruses

Expression of the foreign gene is a prerequisite for the development of an antigen-specific immune response. Therefore, before immunising cotton rats, I tested the ability of recombinant adenoviruses to express gD or tgD in CRL cells *in vitro*. All vectors expressed the appropriate foreign gene. The total production of gD was higher, however, than that of tgD by 3 days p.i. (Fig. 5.2.3.). Furthermore, the majority of gD was cell associated, while tgD was found in similar amounts in the culture-supernatant and in the cell pellet (Fig. 5.2.1-3). Glycoprotein D has been known to be abundant in membrane compartments of cells it is expressed by (van Drunen Littel-van den Hurk *et al.*, 1990, van Drunen Littel-van den Hurk *et al.*, 1984) while tgD is efficiently secreted into the medium (Kowalski *et al.*, 1993). The more efficient accumulation of gD in cells was expected since authentic gD contains a transmembrane anchor region while tgD does not. Finally, infection with tgD-expressing adenoviruses caused less cytopathic changes than gD-expressing adenoviruses in CRL cells. This could be the reflection of the toxic nature of gD described before (Tikoo *et al.*, 1990).

To determine whether differences between tgD- and gD-expressing vectors observed *in vitro* influenced gD-specific immune responses and protection against BHV-1 challenge *in vivo*, cotton rats were immunised with recombinant adenoviruses and then challenged with BHV-1. gD-specific antibody levels, spleen cell proliferative responses and protection against BHV-1 challenge were lower in animals immunised with tgD- than gD-expressing vectors (Fig. 5.3.2-8). These results may simply be explained by lower levels of expression of tgD than gD following immunisation, suggested by *in vitro* data (Fig. 5.2.3). Such an explanation is plausible since a high dose of subunit gD vaccine induced higher levels of antigen-specific immune responses than a low dose of gD (Baca-Estrada *et al.*, 1996). However, the actual amount of gD expressed *in vivo* following immunisation with live vectors is not known. It is possible that the cumulative production of tgD (Fig. 5.2.3) resulted in similar total amounts of tgD as gD in the cotton rat. Furthermore, several observations and published data suggest that the antigen-dose is not

the only explanation for the lower immunogenecity of secreted molecules compared to cell-associated proteins. There are examples in the literature demonstrating that the cellular localisation of an antigen – whether it is retained intracellularly, expressed on the surface of cells or secreted into the medium – influenced its immunogenicity. Cell surface anchoring increased the level of immune responses induced by VP7 of rotavirus compared to those stimulated by a genetically engineered secreted form of the protein, or the authentic form, which is retained in the endoplasmic reticulum (Andrew *et al.*, 1990). Cell surface expression of a dengue virus and a bovine leukaemia virus (BLV) envelope protein resulted in enhanced immunogenicity of the protein compared to the authentic form which is retained intracellularly (Gatei *et al.*, 1993, Men *et al.*, 1991). In addition, a cell surface-anchored and a secreted protein may stimulate different levels of antigen-specific antibody isotypes and cytokines (Cardoso *et al.*, 1996, Inchaupse *et al.*, 1997). Immunisation of mice with recombinant adenoviruses expressing gD induced higher gD-specific IgG2a and IFN-γ production than immunisation with vectors expressing tgD. In contrast, vaccination with both recombinants induced similar levels of gD-specific IgG1 (Papp et al., manuscript in preparation). Lewis *et al.* (1997) obtained similar results following immunisation with polynucleotides. In this case, i.m. inoculation of plasmids encoding gD induced predominantly IgG2a isotype responses, while plasmids encoding tgD stimulated mainly IgG1 antibodies. This reference is especially noteworthy since the level of expression of tgD was equivalent to that of gD following *in vitro* transfection of mouse cells (Jeffrey Lewis, personal communication). However, the most clear evidence against the exclusive influence of the dose of the antigen on the level of immune responses was obtained by immunisation of mice with purified gD and tgD proteins. Hundred times as much tgD induced similar levels of gD-specific IgG and BHV-1 neutralising serum Ab responses as gD (Baca-Estrada *et al.*, 1996). *In vivo* results described in this thesis and the above references are also in accordance with *in vitro* experimental data showing that purified tgD is less efficient than gD in stimulating proliferative responses of spleen cells from gD- or tgD-immunised mice or cattle (Baca-Estrada *et al.*, 1996, Tikoo *et al.*, 1995b). These results suggest that not only the amount, but also the characteristics of the protein may

affect immune responses to an antigen. Authentic gD can, perhaps, induce higher antibody levels than tgD because extracellular gD-aggregates or gD accumulated in cell membranes might form repeated B-cell epitopes which could trigger T-cell independent B-cell responses. Particulate antigens and highly organised viral envelope structures bearing repeated B cell epitopes, indeed, are more likely to stimulate efficient B cell responses than soluble antigens (Bachmann & Zinkernagel, 1996). Alternatively, gD's capability to form dimers and to concentrate in cell membranes may make it more accessible for professional APC such as dendritic cells. The more efficient uptake of gD than that of tgD by these APC may lead to more efficient presentation and processing, and subsequently higher levels of immune responses induced by gD than tgD. Better presentation of professional APC might also lead to stronger Th1 type responses, often characterised by the predominance of IgG2a and IFN-γ, and better protection than presentation by mainly B cells (Rossi-Bergmann *et al.*, 1993). There is evidence that IgG2a provides better protection against viral challenge than the presence of other isotypes (Ishizaka *et al.*, 1995). Furthermore, live respiratory tract virus infections usually induce strong IgG2a and IFN-γ levels, which correlate with protection against a second viral challenge (Zinkernagel, 1993).

Inflammation is often necessary for the induction of immune responses. The need for adjuvants in most protein based vaccines can also be explained by the capability of the adjuvant to induce an inflammatory response. Since gD-expression resulted in a stronger cytopathic effect than tgD-expression (section 5.2.2), it is possible that gD-expression caused stronger inflammation than tgD-expression at the site of inoculation. The potential of gD to elicit strong inflammation and to be presented effectively by professional APC may explain why immunisation with recombinant adenoviruses expressing gD induced better antigen-specific immune responses than immunisation with adenoviruses expressing tgD. The above information and data in the literature suggest that gD may be a more suitable vaccine antigen than tgD following administration by a delivery system based on the *in vivo* expression of antigen, such as Ad vectors and DNA immunisation.

6.2.2 Immunity induced by replication-defective and replication-competent recombinant adenoviruses

Replication-defective and replication-competent adenoviruses were both efficacious, following i.d.-i.n. immunisation, in inducing immunity to gD and conferring protection against BHV-1 challenge (Fig. 5.3.4). The gD-dE3 vector was more efficient, however, than gD-dE1E3 in stimulating gD-specific ASC in the spleen than gD-dE1E3 (Fig. 5.3.5). The difference between the capacity of replication-defective and replication-competent adenoviruses to induce gD-specific immunity was further demonstrated following mucosal administration. The gD-dE3 virus was more effective than gD-dE1E3 in inducing systemic and mucosal antibody responses following mucosal immunisation alone (Figures 5.3.11-12).

The dose of adenoviruses used in my studies (10^7 to 10^8 pfu/animal) was based on information in the literature. It is possible that mucosal administration of a higher dose of gD-dE1E3 could induce comparable immune responses to those induced by a low dose of gD-dE3. Data in the literature indicate, however, that replication-defective and replication-competent adenoviruses usually induce different levels of immunity or protection, especially following mucosal administration. For example, although i.m. immunisation with both dE3 and dE1E3 vectors expressing gD of pseudorabies virus (PRV) could induce antibody responses against gD of PRV (Eloit & Adam, 1995), protection against viral challenge required immunisation with a hundred times as much dE1E3 as dE3 (Eloit & Adam, 1995). Moreover, oral administration of a dE3 vector expressing a rabies glycoprotein induced immunity to rabies glycoprotein while dE1E3 virus did not (Prevec *et al.*, 1990, Xiang *et al.*, 1996). Intranasal immunisation with a dE1E3 vector induced few antigen-specific $CD8^+$ T cells and low serum antibody responses, while i.m. or s.c. immunisation induced strong immune responses (Rodrigues *et al.*, 1997). In general, replication-defective dE1E3 vectors have rarely been reported to be effective following mucosal administration, while such references are plentiful using replication-competent dE3 vectors (Table 2.1). All these data in the literature are consistent with my findings, and

indicate that the route of immunisation is crucial when assessing the efficacy of recombinant adenoviruses as vaccine vectors.

There are several possibilities as to why systemic, but not mucosal immunisation with the replication-defective vectors induced high levels of gD-specific antibody responses. First, systemic spread of Ad vectors may have been necessary for the induction of efficient gD-expression and induction of gD-specific immunity. In this case, only low gD-specific immunity could have developed following immunisation with gD-dE1E3 because the mucosal barrier would limit the systemic spread of the non-replicating vector. In contrast, a replication-competent Ad may disseminate equally well following mucosal and systemic immunisation due to replication. Indeed, replication-competent dE3 Ad has been isolated from more organs than replication-defective dE1E3 Ad following i.n. administration to cotton rats (Oualikene *et al.*, 1994). According to other data, however, the dE1E3 vector expressed foreign genes very efficiently in different tissues following mucosal administration (Huard *et al.*, 1995). Information in the literature about the capability of replication-defective Ad to disseminate systemically is controversial and seems to be a function of the route and dose of Ad inoculation, detection assays and animal model. Moreover, information regarding the spread of adenoviruses following i.d. administration has not been reported. My data indicate that the dissemination of both gD-dE3 and gD-dE1E3 is similar following i.d., i.n. and intraduodenal inoculation (Table 5.4.1.-3.). In addition, the two vectors stimulated similar Ad-specific immune responses following both systemic and mucosal administration (data not shown) suggesting that both vectors were accessible to all compartments of the immune system. Nevertheless, it is possible that gD-dE1E3 induced better immunity following systemic than mucosal administration because it may have more easily accessed tissues in which it could express gD effectively after systemic immunisation.

Alternatively, the dE3 virus may have caused more severe inflammation in the mucosa than the dE1E3 vector (more severe cytopathic changes *in vitro*; section 5.2.2). This could result in stronger signals for an immune response to develop or a more effective uptake of gD by APC in the mucosa or the draining LN-s due to higher cellular infiltration.

Indeed, exposure of the mucosa to non-inflammatory doses or type of antigen, such as soluble antigens and inactivated viruses, often fails to induce immune responses (Lipscomb *et al.*, 1995), while live replicating pathogens usually stimulate both mucosal and systemic immunity following mucosal administration (Ogra, 1996). The systemic immune system may be less able to distinguish between harmless antigens and potentially dangerous sources of antigen and may respond to weaker immunostimulatory signals as well. For example, systemic immunisation with either killed or replicating particulate antigens often induces good immune responses (Morrison & Knipe, 1996). Therefore, systemic immunisation with gD-dE1E3 may have been able to induce better immunity than mucosal immunisation.

Another possibility is that the replication-defective gD-dE1E3 adenovirus might not had a chance to express enough gD *in vivo* in the mucosa to induce gD-specific immunity. This may be because the natural early clearance of Ad is very effective (Worgall *et al.*, 1997) and the expression of gD is driven by a late promoter. A more invasive infection by gD-dE3, due to replication, could have overcome the fast clearance mechanisms because it probably resulted in increasing number of cells expressing gD. This could result in a higher amount of gD available for uptake by APC in the MALT and the draining LN following mucosal infection. Furthermore, the ability of gD-dE3 to produce higher amounts of gD during the first day p.i. than the gD-dE1E3 virus (Fig. 5.2.1, 5.2.2, 5.2.3) may have resulted in very different kinetics of gD-expression *in vivo* by gD-dE3 and gD-dE1E3. Consequently, higher levels of gD-specific antibody responses would be induced by gD-dE3 compared to gD-dE1E3 following mucosal administration (Fig. 5.3.11-12). In contrast to mucosal administration, the replication-defective virus may have induced immunity following systemic administration because the systemic immune system may have a lower threshold for the dose of gD it responds to. The above mechanisms could explain the efficacy of the replication-defective gD-dE1E3 virus to induce good gD-specific immunity following systemic, but not following solely mucosal immunisation.

6.2.3 Mucosal antibody responses induced by mucosal and systemic immunisation with recombinant adenoviruses

I have shown that both mucosal (i.n. and g.i.) and systemic (i.p. and i.d.) routes of immunisation with gD-dE3 were capable of inducing gD-specific immune responses. However, the different routes stimulated different levels of antibody responses, especially with regard to respiratory tract IgA. For example, i.n. immunisation with gD-dE3 was more effective than i.d. or i.p. immunisation in inducing respiratory tract IgA responses. In contrast, i.n., i.d. and i.p. immunisation induced similar levels of gD-specific IgG antibody in the serum and lung-washes (Figures 5.3.14 & 17, Tables 5.3.3, 5.3.4 & 5.5.5). The high levels of IgA in the respiratory tract compared to those in the serum following i.n., but not after systemic immunisation, and the correlation between the level of IgA in lung-washes and the number of IgA ASC in the lung indicated that IgA was mainly locally produced in the respiratory tract (Fig. 5.3.14 & Table 5.3.3, Fig. 5.3.17 & Table 5.3.4). Local gD-specific IgA levels and the number of gD-specific ASC in the lung correlated with protection of the lungs against i.n. BHV-1 challenge. Cotton rats that contained the highest levels of respiratory tract IgA in the lung were best protected against i.n. BHV-1 challenge, while the lack of IgA in the respiratory tract corresponded with low levels of protection. Intranasal immunisation resulted in better protection against i.n. BHV-1 challenge than i.d. or i.p. inoculation (Fig. 5.5.15 & Table 5.3.5). These findings are consistent with other reports and suggest that mucosal IgA is important in protecting the host from a mucosal viral infection (reviewed by Murphy, 1994). However, partial protection was sometimes observed in the presence of very low levels of mucosal IgA (Figures 5.3.11, 5.3.14, 5.5.5, 5.5.8, etc.). In addition, the titre of gD-specific IgA in the lung, and especially in the trachea, did not always directly correspond with the number of BHV-1 pfu recovered following challenge. These observations suggest that effector mechanisms other than IgA may play a role in protection of cotton rats against i.n. BHV-1 challenge.

Antigen-specific IgG can bind and inactivate viruses before they infect the mucosal epithelium (Murphy, 1994). Intradermal immunisation induced gD-specific IgG in the

serum and the lung (Figures 5.3.14 and 5.3.17), which correlated with partial protection of the lung against i.n. BHV-1 challenge (Table 5.3.5). The presence of IgG at mucosal surfaces in my experiments, following any route of immunisation, is probably largely due to exudation of serum IgG to mucosal surfaces. This is likely since gD-specific IgG levels in the respiratory tract of cotton rats correlated with gD-specific IgG levels in the serum, but not with the number of gD-specific IgG ASC in the lung (Fig. 5.3.14 & Table 5.3.3, Fig. 5.3.17 & Table 5.3.4). Several findings in mice support this hypothesis (Papp et al., manuscript in preparation; Baca-Estrada, manuscript in preparation; Eis Hubinger *et al.*, 1993, Wagner *et al.*, 1987). The presence of a few IgG ASC in the lung of cotton rats indicated, however, that some of the IgG at mucosal surfaces may have been locally produced. In addition, lower levels of IgG in nasal than lung-washes indicated that IgG may have more easily transudated through the lung epithelium than the nasal and tracheal mucosa. This may be an explanation for why serum antibody is usually more protective against pulmonary than nasal infection (Graham *et al.*, 1993, Murphy, 1994). Therefore, the usually better protection of lungs than of tracheae following immunisation suggest that IgG may play a role in the protection of the cotton rat lung against i.n. BHV-1 challenge. In addition, other effector immune mechanisms such as cell-mediated immunity (CMI) might play a role in an accelerated clearance of BHV-1 from the respiratory tract.

Data in the literature are consistent with my results regarding the capability of i.n. immunisation to induce similar systemic, but better mucosal immunity and protection against respiratory mucosal viral challenge than systemic immunisation (Gallichan *et al.*, 1993, McGhee *et al.*, 1992, Michalek *et al.*, 1994, Tamura & Kurata, 1996). The reason for this observation probably is that following i.n. immunisation, specialised lymphoid tissues in the respiratory tract of rodents such as nasal- and bronchus-associated lymphoid tissues and lung-associated LN-s serve as inductive sites for antigen-specific immune responses by local stimulation with antigen. Antigen-specific lymphocytes stimulated locally are thought to leave these inductive sites, enter the systemic circulation and then return to the area of stimulation as effector cells. Inflammation may be important in the recruitment of activated lymphocytes, while the continued presence of specific antigens within the tissue would

help retaining recruited antigen-specific cells (Lipscomb *et al.*, 1995). It is not surprising therefore, that i.n. immunisation resulted in a higher number of gD-specific ASC in the lung than i.d. inoculation since persistence of gD in the lung and lung-associated LN is more likely following administration of antigen to the respiratory tract. In addition, memory lymphocytes stimulated in the respiratory tract may have unique homing specificities that favour their entry into the site of stimulation (Lipscomb *et al.*, 1995, McDermott & Bienenstock, 1979, Picker, 1994).

Although systemic immunisation usually does not induce local respiratory tract immune responses, i.d. immunisation with recombinant Ad induced a few gD-specific IgG and IgA secreting cells in the lung (Tables 5.3.3-4). It is possible, that local stimulation of gD-specific lymphocytes could take place in the lung even following i.d. inoculation of cotton rats with gD-dE3, since adenoviruses are able to spread systemically to the lung tissue following systemic immunisation (Table 5.4.3 and Mittal *et al.*, 1993). Alternatively, mild inflammation in the lung caused by a low level of Ad persistence or i.n. BHV-1 challenge may be enough to recruit gD- and Ad-specific lymphocytes to the lung induced in systemic lymphoid tissues. In fact, initiation of a mild inflammatory response in the lung can result in the accumulation of antigen-specific lymphocytes in the lungs of animals immunised via an extrapulmonary route (Hillam *et al.*, 1985, Lipscomb *et al.*, 1995).

Despite the presence of gD-specific antibodies in the respiratory tract following i.d. and i.p. inoculation, intranasal immunisation better protected cotton rats against i.n. BHV-1 challenge than systemic immunisation. This protection correlated with high levels of gD-specific IgA in the respiratory tract following i.n. administration of the recombinant adenovirus. Therefore, although I can not exclude the possibility that gD-specific CMI may have played a role in protection of the lungs against BHV-1 challenge, it is likely that the mechanism of protection against i.n. BHV-1 challenge involved gD-specific antibodies, especially mucosal IgA.

6.2.4 Mucosal antibody responses induced by the gastrointestinal route of immunisation

Based on the concept of the common mucosal immune system (CMIS) (Mestecky *et al.*, 1994) and the success of oral adenoviral immunisation of military recruits against acute respiratory distress syndrome (ARDS) (Top *et al.*, 1971a), g.i. immunisation with recombinant Ad expressing gD was expected to induce mucosal immunity and protection of the cotton rat respiratory tract against i.n. BHV-1 challenge. Indeed, administration of gD-dE3 by the oral and intraduodenal routes induced gD-specific antibody in the serum and the respiratory tract (Figures 5.3.11 & 5.5.4). In addition, intraduodenal-oral (Table 5.3.3) and intraduodenal (Table 5.5.6) administration of gD-dE3 could also induce gD-specific ASC in the lung. I found no other report in the literature on induction of immunity to a foreign protein in the respiratory tract following g.i. immunisation with recombinant adenovirus. However, wt HAd and other viruses have been shown to induce immunity in the respiratory tract following g.i. immunisation (Bender *et al.*, 1996, VanCott *et al.*, 1994; reviewed by Mestecky *et al.*, 1994).

According to the concept of the CMIS, antigen-specific lymphocytes stimulated in the gut following enteric immunisation would disseminate systemically and seed distant mucosal sites such as the respiratory tract. Before discussing the CMIS as a mechanism of induction of gD-specific immunity in the respiratory tract, it is important to consider that gD antigen may not have exclusively stimulated lymphocytes in the gut mucosa. First, I can not exclude the possibility that a low amount of Ad may have directly entered the peritoneum from the surface of the needle used in an intraduodenal injection, even though there was no evidence of virus inoculum leaking from the gut during or following injection to the duodenum (Evans blue dye). Even if minimal leakage took place, virus could access and express gD in different organs, inducing systemic gD-specific immune responses. Although the procedure of intraduodenal immunisation is widely used in rodents, I have not seen this issue addressed. This possibility could be examined by administering traces of recombinant Ad i.p. and monitoring whether any immune response develops. In addition,

there is a possibility that animals that had received intragastric inoculation regurgitated some virus, resulting in exposure of the respiratory tract to infection and foreign gene expression. Although there was no evidence of virus suspension in Evans blue dye spreading to the pharynx or further into the respiratory tract following administration of the virus in the oesophagus, adenovirus isolation results indicated that gD-dE3 may have spread to the respiratory tract. The amount of gD-dE3 virus in the lung 3 days following intragastric immunisation was similar to that following i.n. inoculation (Tables 3.4.1-3). Others have also suggested that live virus may have spread to the respiratory tract following oral or intragastric administration (Huard *et al.*, 1995, Kanesaki *et al.*, 1991, Schwartz *et al.*, 1974, Tacket *et al.*, 1992). Therefore, an oral capsule that delivers Ad directly into the stomach, and does not release virus till the capsule enters the gut may be necessary if one needs to use a completely enteric immunisation procedure. This way the possibility of regurgitation of virus from the stomach would be reduced. Even then, however, systemic adenoviral dissemination may take place following enteric administration, which would result in gD-expression not only in the gut mucosa, but also in systemic lymphoid tissue. My results clearly demonstrated that adenovirus spread systemically following either intranasal, intraduodenal or oral routes of administration with gD-dE1E3 and gD-dE3. This resulted in live infectious Ad (and potentially gD-expression) in the lungs as well as other organs (Table 3.4.1-3). These data suggest that both replication-defective and replication-competent adenoviruses have the capability to cross the mucosal (or lung) barrier and potentially infect other mucosal or systemic sites. It is possible, that many viruses have such capabilities for dissemination, such as rotavirus (Uhnoo *et al.*, 1990). This issue should be further investigated in vaccination and in tumor- and gene-therapy research to address safety concerns and to understand basic immunological mechanisms of induction of mucosal immunity.

Although gD-specific antibody induced in the respiratory tract, after g.i. immunisation with recombinant Ad, can not be exclusively explained by the migration of antigen-specific lymphocyte precursors to remote mucosal sites, it is very likely that the CMIS was, at least partly, responsible for the presence of mucosal antibody responses in

the respiratory tract following intraduodenal inoculation (Fig. 5.5.8 and Table 5.5.6). Adenovirus- and gD-specific ASC were induced in the mesenteric LN (Table 5.5.1) and Ad-specific antibodies were detected in gut washes (data not shown) following g.i. inoculation with recombinant adenovirus. It is possible that gD- and Ad-specific lymphocytes primed in the Peyer's patches of the gut or in the mesenteric LN migrated to the lung where they became plasma cells. It would be interesting to find out whether gD-specific ASC or memory T cells can be recruited into the lung without the presence of BHV-1, gD or local inflammation caused by virus infection in the lung. Such research would clarify whether antigen persistence, local inflammation or the homing specificities of lymphocytes are more important in the accumulation of antigen-specific lymphocytes in the lung. Whether immune responses in the respiratory tract induced by the g.i. route of immunisation with gD-dE3 are mainly due to the migration of lymphocytes induced in the gut or also to the spread of the virus itself to other mucosal sites also require further studies.

Local stimulation of gD-specific immunity in the respiratory tract seems very important in the protection of cotton rats against i.n. BHV-1 challenge. In contrast to complete protection following i.n. immunisation, only partial protection of the lung was observed against BHV-1 challenge in most animals immunised by the g.i. route (Table 5.3.2). Protection results varied following intraduodenal immunisation depending on the experimental design (Table 5.5.2 and Fig. 5.5.19). My findings are in accordance with other reports showing that i.n. delivery of live vaccines is often more effective than gastroenteric administration in protecting the respiratory tract against challenge (Brownlie *et al.*, 1993, Collins *et al.*, 1990, Couch *et al.*, 1996, McLean *et al.*, 1996, Meitin *et al.*, 1994, Tamura & Kurata, 1996). It is possible that following g.i. immunisation only a few viruses could access mucosal inductive sites due to dilution of the inoculum and effective non-immune exclusion of the virus in the gut. I used high (10^8) pfu of gD-dE3 for g.i. inoculation of cotton rats to overcome such effective clearance; however, even more virus or the development of new delivery techniques may be necessary to obtain similar levels of immunity and protection in the lung following g.i. as those following i.n. inoculation. It is

also possible that human adenovirus, especially the dE3 vector, does not replicate as well in the cotton rat gut (no data available) as in the respiratory tract (Pacini *et al.*, 1984). Nevertheless, the capability of g.i. immunisation to induce immunity in the respiratory tract is remarkable and has a great potential as a vaccine strategy against respiratory tract diseases. It would be important, however, to further explore what the mechanism of induction of immunity is at a distant mucosal site.

6.2.5 The duration of antibody responses induced by recombinant adenovirus

An important requirement from a good vaccine is that it should induce long-lasting protective immune responses. Antibody responses induced by immunisation with subunit protein vaccines are usually of short duration and repeated inoculations are necessary to maintain high antibody levels. One great advantage of recombinant adenoviruses over other vaccination strategies is their capability to deliver subunit viral proteins *in vivo* in such a manner that the antigens will be presented to the immune system similar to that following natural viral infection. This may change the regulation, and increase the levels and duration of immune responses to the heterologous antigen.

Although mucosal viral infections usually stimulate immunity of shorter duration than systemic infections (Slifka & Ahmed, 1996), long term humoral responses and protection from mucosal viral challenge have been reported following mucosal administration of live viruses or viral vectors (Gallichan *et al.*, 1993, Hyland *et al.*, 1994, McNeal & Ward, 1995). My results also suggest that immunity induced by i.n. administration of gD-dE3 in cotton rats may be long lasting: gD- and Ad-specific antibody responses were maintained at high levels for at least 12 weeks following immunisation (Figures 5.3.19 & 5.5.11). Antigen-specific ASC in the lung and the bone marrow were present at similar levels at 12 weeks as at 3 weeks following immunisation (Tables 5.3.6 & 5.3.4). Furthermore, complete protection of the lungs was achieved at 3 and 12 weeks following immunisation. It would be interesting to determine the capability of recombinant

human adenoviruses to induce long term immunity in an animal model with a longer life-span than that of rodents.

Long term antibody responses and protection against challenge may be due to the presence of ASC maintained for a long period of time in the bone marrow, draining mediastinal LN or the lung following respiratory viral infection (Hyland *et al.*, 1994, Jones & Ada, 1987, Liang *et al.*, 1994). My results indicate that significant numbers of gD-specific ASC persisted for at least 3 months in cotton rats following i.n. immunisation with gD-dE3 (approximately 10^3 in the lung, 10^3 in the bone marrow and 10^2 in the spleen). The numbers were calculated by assuming that there are approximately 2 million lymphocytes in the lung, 150 million in the bone marrow and 50 million in the spleen of cotton rats. These data suggest that at least as many gD-specific ASC seed systemic sites following i.n. immunisation with gD-dE3 as those retained in the lung.

Antigen-specific antibody continues to be produced in the bone marrow and in the lung for several years after the last exposure to antigen (Bice *et al.*, 1993, Bice *et al.*, 1991, Slifka & Ahmed, 1996). It is possible that the microenvironment of the bone marrow and the lung can provide the right signals and cytokines to sustain antibody-secreting plasma cells for long periods, even for years. In fact, antibody-secreting plasma cells from the bone marrow are as long lived as memory B cells (Manz et al., 1997). ASC are known to selectively express syndecan, which binds collagen and fibronectin (Lalor *et al.*, 1992). This may help retain ASC in the lung interstitium, alveoli, alveolar septa or pleura or to be scattered in the mucosa of the upper respiratory tract (Bice *et al.*, 1987). It is more likely, however, that memory B cells are involved in the mechanism of maintenance of long-term antibody responses. Memory B lymphocytes induced by immunisation with gD-dE3, recirculating throughout the lymphoid system, are perhaps exposed to persisting antigen in the lung, lung-associated LN, mediastinal LN or systemic lymphoid tissue. They would then proliferate, develop into ASC and plasma cells. In the meantime they would lose their capability to repeatedly recirculate in the lymphoid system and to enter lymph nodes via high endothelial venules: they would rather enter the bone marrow or the lung, which lack such venules. It has been suggested that ASC preferentially migrate to the bone marrow or

remain restricted to the site of persisting antigen, while memory B cells freely recirculate (Bachmann *et al.*, 1994). Although inflamed lungs non-specifically recruit both T and B cells, the presence of specific antigens within the tissue is an important factor in retaining recruited immune cells in the lung (Lipscomb *et al.*, 1995).

The mechanism of maintaining antibody levels and the number of antigen-specific ASC in the lung may be the result of the continuing presence of gD in the lung or draining LN. Indeed, 2 out of 7 cotton rats tested contained infectious adenovirus 3 weeks following i.n. immunisation with gD-dE3. In addition, the capability of certain viruses, such as adenoviruses, to induce long-term systemic immune responses following mucosal administration may be associated with their ability to spread systemically (Oualikene *et al.*, 1994, Slifka & Ahmed, 1996; and Tables 5.4.1-3). Therefore, adenovirus – and consequently gD – may persist at different sites in the systemic lymphoid tissue. Indeed, infectious dE3 Ad was isolated from different tissues 4 weeks following i.n. inoculation of cotton rats (Oualikene *et al.*, 1994). Adenoviral DNA was detected in the nasal mucosa for 70 days p.i., while foreign gene expression continued for 18 days following i.n. administration of a replication-defective recombinant Ad2 virus (Zabner *et al.*, 1994). In some cases, expression of the foreign gene was observed for 12 months in skeletal muscle of mice following i.m. administration of Ad (Stratford-Perricaudet *et al.*, 1990). Moreover, HAd shedding has been observed for several months following infection (Fox *et al.*, 1969, Fox *et al.*, 1977). The site of adenoviral persistence is not known. Adenoviruses may be able to persist *in vivo* in lymphocytes (Flomenberg *et al.*, 1997) such as they do *in vitro* (Flomenberg *et al.*, 1996, Silver & Anderson, 1988). In addition, antigens such as adenoviral proteins and gD may be associated with dendritic cells in the form of antigen-antibody complexes (Tew *et al.*, 1980, Unanue, 1993).

Direct evidence that maintenance of B cell memory depends on antigen persistence has come from the finding that memory responses decay rapidly when primed lymphocytes are adoptively transferred in the absence of antigen; cotransfer with antigen lead to maintenance of memory (Gray, 1994, Gray & Skarvall, 1988). However, antigen persistence is not the only possible mechanism to explain long term immunological

memory. There is evidence that qualitatively special long term memory B and T cells may exist, independently of persisting antigen (Sprent, 1994). Maybe, such memory B cells would be restimulated by local antigenic challenge, proliferate and become plasma cells in the lung. Understanding the mechanism of maintenance of long term immunological memory is important for the development of more efficacious vaccines.

Studies about immunity induced by recombinant adenoviruses has only recently been started in animals of long life-span or in humans (Table 3.1). It is not known whether mucosal or systemic immunisation with recombinant adenoviruses can induce long-term immune responses in such species. Studying the mechanism of immunological memory should help design appropriate vaccination protocols to utilise the potential of recombinant adenoviruses expressing gD in inducing long-term protection against BHV-1 infection in cattle.

6.2.6 gD-specific T cell responses induced by recombinant adenoviruses

Virus-specific humoral immune responses are known to play an important role in preventing secondary viral infections and extracellular spread of virus within the host, while cell mediated immunity usually contributes to the control and resolution of already established infections. Cytotoxic T cells have been shown to reduce the replication of certain viruses and protecting animals from disease caused by mucosal or systemic viral challenge (Zinkernagel, 1993). In some viral infections, $CD4^+$ T cells play a relatively minor role in the protection against disease (Zinkernagel, 1993). However, the induction of antigen-specific $CD4^+$ T cells is essential for inducing humoral immunity against most protein antigens since B cell responses to most viral antigens are T cell dependent (Bachmann & Zinkernagel, 1996, Zinkernagel, 1993). T cells also produce cytokines and are able to provide help for CTL (Fitch *et al.*, 1993).

Antigen-specific T lymphocytes are known to proliferate *in vitro* upon presentation of antigen. Although proliferation is not a specific effector function of T cells, proliferation assays have been widely used to assess the overall immunocompetence of an animal. Data obtained from such assays may reflect proliferation of CTL, cytokine-producing $CD4^+$ T

cells or bystander B cells. Nevertheless, the population that constitutes proliferating spleen cells is usually antigen-specific $CD4^+$ lymphocytes. They can also amplify bystander proliferation in an antigen-specific manner.

I was able to consistently demonstrate low and not always significant, levels of gD-specific proliferative responses of spleen cells in every experiment following immunisation with gD-expressing recombinant adenoviruses (Fig. 5.3.6 or not shown). To optimise the conditions for proliferative responses of cotton rat spleen cells, many culture-conditions and antigen doses were tested. Proliferative responses to Concanavalin A and antigen-specific proliferative responses to the Ad vector itself were good, showing that the cause of low gD-specific proliferative responses was not unsatisfactory *in vitro* culture conditions. Instead, the proliferation assay simply may not be an optimal assay for assessing the level of gD-specific T-cell activation. Cytokine production by T lymphocytes may better represent the potential of immunisation to induce gD-specific CMI. Indeed, immunisation of mice with gD-dE3 induced only low or no proliferative responses but high numbers of gD-specific IFN-γ cytokine producing cells in the spleen and the lungs (Papp et al., manuscript in preparation). Immunisation of mice with plasmids carrying the gene of gD was also able to induce only low gD-specific proliferative responses, while they induced high IFN-γ production of spleen and LN cells (Lewis *et al.*, 1997). In contrast, purified gD in an oil-based adjuvant formulation was capable of consistently inducing higher proliferative responses and IL-4 production by spleen cells of mice than control-inoculation (Baca-Estrada *et al.*, 1996). In cattle, immunisation with the gD or tgD protein usually, but not always, induces gD-specific proliferative responses in spleen cells (Tikoo *et al.*, 1995b, Baca-Estrada and Marlene Snider, personal communication). In sheep, i.d. or intrajejunal immunisation with gD-dE3 induced Peyer's patch and spleen cell proliferative responses of high SI in some animals and lower or none in others (Philip Griebel et al., manuscript in preparation). These references indicate that gD is capable of inducing T cell proliferative responses, but depending on the experimental set-up and the animal model used, this assay may not reflect the differences in gD-specific T cell induction between immunised and control groups.

It is also possible that, the genetic background of cotton rats is such that certain antigens such as gD, may induce low proliferative responses of their spleen cells, and maybe low T cell activation in general. Certain individual cattle and sheep, and certain strains of mice are low-responders to gD antigen (Philip Griebel, Sylvia van Drunen-Littel van den Hurk, Maria Baca-Estrada and Jeffrey Lewis, personal communication). Immunisation of cotton rats with recombinant Ad expressing haemagglutinin (HE) of coronavirus also induced very low spleen cell proliferative responses in cotton rats (Baca-Estrada *et al.*, 1995). To test whether gD expressed by adenoviruses *in vivo* can induce CMI, reagents for measuring cytokine production or development of other assays (e.g., delayed-type hypersensitivity assay) in cotton rats would be necessary.

Although gD of herpesviruses is known to induce proliferative responses and gD-specific cytokine production in different animal models (mentioned above), no gD-specific CTL responses has been demonstrated following either HSV or BHV infection, or immunisation with gD using different delivery systems (Johnson, 1991, and Baca-Estrada, Jeffrey Lewis and Sylvia van Drunen-Littel van Den Hurk, personal communication). I could not detect gD-specific CTL responses from spleens of cotton rats immunised with gD-dE3 either (data not shown). In contrast, gB of BHV-1 (Maria Baca-Estrada, personal communication) and gB and gC of HSV (Gallichan *et al.*, 1993, Witmer *et al.*, 1990) can stimulate CTL responses in mice. To understand why measuring gD-specific CTL responses has been unsuccessful needs further investigations. In addition, the role of gD-specific CMI in the protection against infection or disease caused by BHV-1 in cotton rats and cattle remains to be studied.

6.3 Effect of pre-existing adenovirus-specific immunity on immunisation with recombinant adenovirus

For gD-specific immunity to develop it is crucial that cotton rat cells become infected with recombinant adenovirus and express the gD protein. Since Ad-specific neutralising antibody can prevent an infection by adenovirus and Ad-specific CMI may

limit the duration of foreign gene expression, it is not surprising that pre-existing Ad-specific immunity reduced the efficacy of immunisation with gD-dE3 in cotton rats. Active Ad-specific immunity significantly inhibited the development of gD-specific antibody responses following immunisation with gD-dE3 and the protection of cotton rats against BHV-1 challenge, while only a slight inhibition of the efficacy of immunisation was observed by passive transfer of Ad-specific antibody.

These results are consistent with data in the literature. Although the effect of passive and active vector-specific immunity has rarely been compared, observations indicate that active immunity to a live vector usually more significantly reduces the efficiency of foreign gene-expression or the development of antigen-specific immune responses than passive transfer of Ad-specific serum antibodies (Andrew, 1989, Johnson *et al.*, 1993, Kundig *et al.*, 1993, Yang *et al.*, 1995a). It would be interesting to determine whether passive transfer of vector-specific antibody in a natural situation (maternal antibody) would inhibit the efficacy of immunisation with live recombinant vectors in new-born cotton rats. In this case, maternal antibody would be transferred both through the placenta (mainly IgG) and through milk (mainly IgA) and may affect immunisation with recombinant Ad differently than passive transfer of merely serum IgG into an adult rat. In addition, pre-immunisation with lower doses of HAd5, which probably resembles a natural infection better than that with high doses used in my experiments, may not interfere with the success of immunisation with recombinant adenovirus vaccines (such as in Xiang *et al.*, 1996).

Intranasal administration of 2 x 10^7 pfu wt HAd5 almost completely blocked the development of gD-specific immune responses following i.n. immunisation with gD-dE3. This suggests that in each experiment where I used the same route for a secondary immunisation as for a primary immunisation with gD-dE3, the second immunisation might have not provided much booster effect. Although gD-specific serum antibody levels were sometimes slightly higher following a second immunisation than those following primary immunisation (data not shown), this increase may only reflect the kinetics of the immune responses developing following the first immunisation. In fact, both gD- and Ad-specific

serum-IgG levels kept rising beyond the timepoint when primary antibody responses were assessed 3 weeks following immunisation (Figures 5.3.19 and 5.5.11). Whether the affinity of mucosal antibody was significantly increased by a second mucosal immunisation with recombinant Ad remains to be further investigated.

There are several possibilities to improve the efficacy of a second immunisation with gD. First, a primary immunisation with recombinant Ad later followed by immunisation with gD using a different delivery system (liposomes, DNA, etc.) may give better results than immunisation twice with recombinant Ad by the same route. Second, Ad-specific neutralising immunity may be circumvented by the administration of a second adenoviral vector of an alternate serotype (Mack *et al.*, 1997, Mastrangeli *et al.*, 1996). Third, a primary oral and a secondary i.n. immunisation might provide better immune responses than immunisation twice intranasally. Fourth, if the immunisations with gD-dE3 are far apart in time, expression of gD following the second immunisation may not be blocked and gD-specific immune responses may be enhanced. Immunisation with gD-dE3 twice, several months apart were not performed, but my results suggest that by 3 months following the first immunisation with adenovirus the inhibitory effect of Ad-specific active immunity decreased even if the two immunisations are performed by the same route (Fig. 5.5.12).

The mechanism by which Ad-specific immunity inhibits the efficacy of immunisation with recombinant adenovirus may involve neutralisation of the recombinant adenovirus or accelerated elimination of recombinant Ad-infected cells by HAd5-specific CTL. Since neither serum nor lung antibody levels were found to decrease during a 3 month period following immunisation (section 5.3.5.2), it is possible that other Ad-specific effector immune responses declined in the respiratory tract, allowing more extended gD-expression following immunisation with gD-dE3. For example, gB-specific CTL responses in the spleen declined following i.n. immunisation of mice with recombinant Ad expressing gB (Gallichan *et al.*, 1993) even though gB-specific memory CTL were present in the lymph nodes draining the vagina for more than a year. The duration of Ad-specific CTL responses in the respiratory tract is not known.

Data in the literature support the notion that immune responses specific for live viral vectors decline with time. Although the efficacy of immunisation with recombinant vaccinia virus was inhibited for at least 9 months following the first infection with vaccinia, the level of inhibition was lower at 5-9 months than at 7-42 days following infection (Kundig *et al.*, 1993). In addition, although the efficiency of heterologous gene-expression in the lung or liver was lower following the second administration of recombinant adenovirus, inhibition of foreign gene expression was less evident 12 weeks than 4 weeks after the first administration of recombinant adenovirus (Setoguchi *et al.*, 1994). Therefore, it may be more efficacious to apply a booster immunisation with recombinant Ad at a later timepoint following the first immunisation to achieve high levels of immune responses to the vaccine antigen.

Considering the importance of local mucosal immunity in protection against viruses, the possibility existed that i.n. exposure to wt HAd5 would not inhibit immunisation by the g.i. or intraduodenal route as much as it inhibits i.n. immunisation. Interestingly, the efficacy of intraduodenal-oral and intraduodenal immunisation was also decreased by pre-immunisation with wt HAd5 (Figures 5.5.4 and 5.5.8). The most likely explanation for such distant inhibition is that some wt Ad was swallowed during i.n. immunisation or was transported to the gut through the circulation, which then induced Ad-specific immunity in the GALT and mesenteric LN. Indeed, adenovirus was detected in the gut and mesenteric LN following i.n. administration of adenovirus (Table 5.4.3). Adenovirus-specific neutralising antibody in the gut could reduce infection of the gut with gD-dE3 following g.i. immunisation. Alternatively, Ad-specific CMI may have restricted both the quantity and duration of gD-expression. This may have caused the presence of less gD-specific ASC in the mesenteric LN than in animals without pre-existing HAd5-specific immunity (Table 5.5.1). It is also possible that Ad-specific immunity in the gut or antibodies in the serum have inhibited the systemic dissemination of gD-dE3 following g.i. administration, which, as I have suggested before, may have played a role in the induction of systemic and respiratory mucosal immunity. Another explanation for the inhibitory effect of i.n. administration of wt HAd5 on immunisation by the g.i. route is that i.n.

HAd5-infection induced Ad-specific immunity in the gut by the common mucosal immune system. Lymphocytes specific for Ad induced in the lung- or nasal-associated lymphoid tissue or draining LN-s of the respiratory tract may have migrated to the gut and mediated Ad-specific immunity against gD-dE3 infection in the gut.

In summary, passive transfer of Ad-specific antibodies had a lower inhibitory effect than active Ad-specific immunity on the development of gD-specific immunity induced by immunisation with gD-dE3. Therefore, a recombinant adenovirus vaccine may be more efficacious in young animals, which only have maternal antibodies, than in older animals, which may have encountered live Ad and have developed active Ad-specific immunity by the time of immunisation.

6.4 General conclusions and future applications

Recombinant adenoviruses have a great potential to induce mucosal immunity and protection against respiratory viral infections. Replication-defective and replication-competent recombinant adenoviruses expressing gD or tgD of BHV-1 could induce gD-specific systemic and mucosal immune responses and confer at least partial protection against i.n. BHV-1 challenge in the cotton rat. However, the characteristics of the antigen, the replication capability of the vector and the route of immunisation had a significant impact on the level of immune responses. The highest levels of immunity and protection were achieved by i.n. immunisation with the replication competent vector expressing the authentic form of gD. Pre-existing active Ad-specific immunity significantly inhibited the efficacy of immunisation while passive Ad-specific antibody interfered less with the success of immunisation.

The information in this thesis should be helpful in designing and testing recombinant adenovirus vaccines used in the cattle industry. Application of live human Ad-based vaccines may be somewhat limited, however, because of safety concerns for the human population. To partly overcome safety problems in cattle, a bovine adenovirus vector expressing gD of BHV-1 has been constructed (Suresh Tikoo, manuscript in

preparation) and will soon be assessed for its capability to induce immunity and protection against BHV-1 challenge in cotton rats, sheep and cattle. Similar safety concerns to human adenoviruses will have to be addressed, however, to use the bovine live vector as a commercial vaccine in the future.

The results of my studies may also be useful for vaccine research in other animal species and humans. Although the immune system of every species is somewhat different, I may have been able to identify and confirm basic features of adenovirus vector-induced immunity that could be applied to different biological systems. Furthermore, results in this thesis may also contribute to other applications of human adenoviruses since the influence of Ad-specific immunity on the efficacy of recombinant human adenoviruses and the systemic dissemination of Ad vectors raise a great concern in both gene- and tumor-therapy research.

7.0 REFERENCES

Andrew, M. E. (1989). Protective efficacy of a recombinant vaccinia virus in vaccinia-immune mice. *Immunol. Cell Biol.* **67**, 339-341.

Andrew, M. E., Boyle, D. B., Whitfeld, P. L., Lockett, L. J., Anthony, I. D., Bellamy, A. R. & Both, G. W. (1990). The immunogenicity of VP7, a rotavirus antigen resident in the endoplasmic reticulum, is enhanced by cell surface expression. *J. Virol.* **64**, 4776-4783.

Babiuk, L. A., L'Italien, J., van Drunen Littel-van den Hurk, S., Zamb, T., Lawman, M. J. P., Hughes, G. & Gifford, G. A. (1987). Protection of cattle from bovine herpesvirus type 1 (BHV-1) infection by immunization with individual viral glycoproteins. *Virology* **159**, 57-66.

Baca-Estrada, M. E., Foldvari, M., Snider, M., van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1997). Effect of IL-4 and IL-12 liposomal formulations on the induction of immune response to bovine herpesvirus type-1 glycoprotein D. *Vaccine* (in press).

Baca-Estrada, M. E., Liang, X., Babiuk, L. A. & Yoo, D. (1995). Induction of mucosal immunity in cotton rats to hemagglutinin-esterase glycoprotein of bovine coronavirus by recombinant adenovirus. *Immunology* **86**, 134-140.

Baca-Estrada, M. E., Snider, M., Tikoo, S. K., Harland, R., Babiuk, L. A. & van Drunen Littel-van den Hurk, S. (1996). Immunogenicity of bovine herpesvirus 1 glycoprotein D in mice: effect of antigen form on the induction of cellular and humoral immune responses. *Viral Immunol.* **9**, 11-22.

Bachmann, M. F., Kundig, T. M., Odermatt, B., Hengartner, H. & Zinkernagel, R. M. (1994). Free recirculation of memory B cells versus antigen-dependent differentiation to antibody-forming cells. *J. Immunol.* **153,** 3386-3397.

Bachmann, M. F. & Zinkernagel, R. M. (1996). The influence of virus structure on antibody responses and virus serotype formation. *Immunol. Today* **17**, 553-558.

Bender, B. S., Rowe, C. A., Taylor, S. F., Wyatt, L. S., Moss, B. & Small, P. A., Jr. (1996). Oral Immunization with a replication-deficient recombinant vaccinia virus protects mice against influenza. *J. Virol.* **70**, 6418-6424.

Berencsi, K., Rando, R. F., deTaisne, C., Paoletti, E., Plotkin, S. A. & Gonczol, E. (1993). Murine cytotoxic T cell response specific for human cytomegalovirus glycoprotein B (gB) induced by adenovirus and vaccinia virus recombinants expressing gB. *J. Gen. Virol.* **74**, 2507-2512.

Berencsi, K., Uri, A., Valyi-Nagy, T., Valyi-Nagy, I., Meignier, B., Vaux Peretz, F., Rando, R. F., Plotkin, S. A. & Gonczol, E. (1994). Early region 3-replacement adenovirus recombinants are less pathogenic in cotton rats and mice than early region 3-deleted viruses. *Lab. Invest.* **71**, 350-358.

Bett, A. J., Haddara, W., Prevec, L. & Graham, F. L. (1994). An efficient and flexible system for the construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl. Acad. Sci. USA* **91**, 8802-8806.

Bett, A. J., Prevec, L. & Graham, F. L. (1993). Packaging capacity and stability of human adenovirus type 5 vectors. *J. Virol.* **67**, 5911-5921.

Bice, D. E., Gray, R. H., Evans, M. J. & Muggenburg, B. A. (1987). Identification of plasma cells in lung alveoli and interstitial tissues after localized lung immunization. *J. Leukocyte Biol.* **41**, 1-7.

Bice, D. E., Jones, S. E. & Muggenburg, B. A. (1993). Long-term antibody production after lung immunization and challenge. *Am. J. Respir. Dis.* **138**, 565-571.

Bice, D. E., Weismann, D. N. & Muggenburg, B. A. (1991). Long-term maintenance of localized antibody responses in the lung. *Immunology* **74**, 215-222.

Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johannes, A., Fattaey, A. & McCormick, F. (1996). An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* **274**, 373-376.

Blair, E. G., Dixon, S. C., Griffiths, S. A. & Blair Zajdel, M. E. (1989). Restricted replication of human adenovirus type 5 in mouse cells. *Virus Research* **14**, 339-346.

Both, G. W., Lockett, L. J., Janardhana, V., Edwards, S. J., Bellamy, A. R., Graham, F. L. & Prevec, L. (1993). Protective immunity to rotavirus-induced diarrhoea is passively transferred to newborn mice from naive dams vaccinated with a single-dose of a recombinant adenovirus expressing rotaavirus VP7sc. *Virology* **193**, 940-950.

Bout, A., Imler, J.-L., Schultz, H., Perricaudet, M., Zurcher, C., Herbrink, P., Valerio, D. & Pavirani, A. (1994a). In vivo adenovirus-mediated transfer of human CFTR cDNA to Rhesus monkey airway epithelium: efficacy, toxicity and safety. *Gene Therapy* **1**, 385-394.

Bout, A., Perricaudet, M., Baskin, G., Imler, J.-L., Scholte, B. J., Pavirani, A. & Valerio, D. (1994b). Lung gene therapy: in vivo adenovirus-mediated gene transfer to Rhesus monkey airway epithelium. *Hum. Gene Ther.* **5**, 3-10.

Bramson, J. L., Graham, F. L. & Gauldie, J. (1995). The use of adenoviral vectors for gene therapy and gene transfer. *Curr. Opin. Biotechnol.* **6**, 590-595.

Breker-Klassen, M. M., Yoo, D., Mittal, S. K., Sorden, S. D., Haines, D. M. & Babiuk, L. A. (1995). Recombinant type 5 adenoviruses expressing bovine parainfluenza virus type 3 glycoproteins protect Sigmodon hispidus cotton rats from bovine parainfluenza virus type 3 infection. *J. Virol.* **69**, 4308-4315.

Brody, S. L. & Crystal, R. G. (1994). Adenovirus-mediated in vivo gene transfer. *Ann. NY Acad. Sciences* **716**, 90-101.

Brody, S. L., Metzger, M., Danel, C., Rosenfeld, M. A. & Crystal, R. G. (1994). Acute responses of non-human primates to airway delivery of an adenovirus vector containing the human cystic fibrosis transmembrane conductance regulator cDNA. *Hum. Gene Ther.* **5**, 821-836.

Brownlie, R. M., Brahmbhatt, H. N., White, D. C., Fountain, M. W., Rohde, M., Wehland, J. & Timmis, K. N. (1993). Stimulation of secretory antibodies against Bordetella pertussis antigens in the lungs of mice after oral or intranasal administration of liposome-incorporated cell-surface antigens. *Microb. Pathog.* **14**, 149-160.

Bruna-Romero, O., Lasarte, J. J., Wilkinson, G., Grace, K., Clarke, B., Borras-Cuesta, F. & Prieto, J. (1997). Induction of cytotoxic T-cell response against hepatitis C virus structural antigens using a defective recombinant adenovirus. *Hepatology* **25**, 470-477.

Burgert, H. G. & Kvist, S. (1987). The E3/19K protein of adenovirus type 2 binds to the domains of histocompatibility antigens required for CTL recognition. *EMBO J.* **6**, 2019-2026.

Cardoso, A. I., Blixenkrone-Moller, M., Fayolle, J., Liu, M., Buckland, R. & Wild, T. F. (1996). Immunization with plasmid DNA encoding for the measles virus hemagglutinin and nucleoprotein leads to humoral and cell-mediated immunity. *Virology* **225**, 293-299.

Charlton, K. M., Artois, M., Prevec, L., Campbell, J. B., Casey, G. A., Wandeler, A. I. & Armstrong, J. (1992). Oral rabies vaccination of skunks and foxes with a recombinant human adenovirus vaccine. *Arch. Virol.* **123**, 169-179.

Chase, C. C. & Letchworth III, G. J. (1994). Bovine herpesvirus 1 gIV-expressing cells resist virus penetration. *J.Gen. Virol.* **75**, 177-181.

Chen, P. W., Wang, M., Bronte, V., Zhai, Y., Rosenberg, S. A. & Restifo, N. P. (1996). Therapeutic antitumor response after immunization with a recombinant adenovirus encoding a model tumor-associated antigen. *J. Immunol.* **156**, 224-231.

Chengalvala, M. V., Bhat, B. M., Bhat, R. A., Dheer, S. K., Lubeck, M. D., Purcell, R. H. & Murthy, K. K. (1997). Replication and immunogenicity of Ad7-, Ad4-, and Ad5-hepatitis B virus surface antigen recombinants, with or without a portion of E3 region, in chimpanzees. *Vaccine* **15**, 335-339.

Chengalvala, M. V., Bhat, B. M., Bhat, R. A., Lubeck, M. D., Mizutani, S., Davis, A. R. & Hung, P. P. (1994). Immunogenicity of high expression adeno-hepatitis B virus recombinant vaccines in dogs. *J. Gen. Virol.* **75**, 125-131.

Chengalvala, M. V., Lubeck, M. D., Davis, A. R., Mizutani, S., Molnar-Kimber, K., Morin, J. & Hung, P. P. (1991). Evaluation of adenovirus type 4 and 7 recombinant hepatitis B vaccines in dogs. *Vaccine* **9**, 485-490.

Collins, P. L., Prince, G. A., Camargo, E., Purcell, R. H., Chanock, R. M. & Murphy, B. R. (1990). Evaluation of the protective efficacy of recombinant vaccinia viruses and adenoviruses that express respiratory cyncytial virus glycoproteins. In *Vaccines: Modern Approaches to New Vaccines including Prevention of AIDS*, pp. 79-84. Edited by F. Brown, R. M. Chanock, H. Ginsberg & R. A. Lerner. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory Press.

Couch, R. B., Cate, T. R. & Keitel, W. A. (1996). Oral immunization with influenza virus vaccines. In *Mucosal Vaccines*, pp. 303-310. Edited by H. Kiyono, P. L. Ogra & J. R. McGhee. San Diego, California: Academic Press, Inc.

Cox, G. J. M., Zamb, T. J. & Babiuk, L. A. (1993). Bovine herpesvirus 1: immune responses in mice and cattle injected with plasmid DNA. *J. Virol.* **67**, 5664-5667.

Crystal, R. G., Mastrangeli, A., Sanders, A., Cooke, J., King, T., Gilbert, F., Henschke, C., Pascal, W., Herena, J., Harvey, B. G. & al., e. (1995). Evaluation of repeat administration of a replication deficient, recombinant adenovirus containing the normal cystic fibrosis transmembrane conductance regulator cDNA to the airways of individuals with cystic fibrosis. *Hum. Gene Ther.* **6**, 667-703.

de Haan, A., Renegar, K. B., Small Jr., P. A. & Wilschut, J. (1995). Induction of secretory IgA response in the murine female urogenital tract by immunization of the lungs with liposome-supplemented viral subunit antigen. *Vaccine* **13**, 613-616.

DeMatteo, R. P., Chu, G., Ahn, M., Chang, E., Burke, C., Raper, S. E., Barker, C. F. & Markmann, J. F. (1997). Immunologic barriers to hepatic adenoviral gene therapy for transplantation. *Transplantation* **63**, 315-319.

Dharakul, T., Riepenhoff-Talty, M., Albini, B. & Ogra, P. L. (1988). Distribution of rotavirus antigen in intestinal lymphoid tissues: potential role in development of the mucosal immune response to rotavirus. *Clin. Exp. Immunol.* **74**, 14-19.

Dong, J. Y., Wang, D., van Ginkel, F. W., Pascual, D. W. & Frizzell, R. A. (1996). Systematic analysis of repeated gene delivery into animal lungs with a recombinant adenovirus vector. *Hum. Gene Ther.* **7**, 319-331.

Dudding, B. A., Bartelloni, P. J., Scott, R. M., Top Jr., F. H., Russell, P. K. & Buescher, E. L. (1972). Enteric immunization with live adenovirus type 21 vaccine. *Infect. Immun.* **5**, 295-299.

Eis Hubinger, A. M., Schmidt, D. S. & Schneweis, K. E. (1993). Anti-glycoprotein B monoclonal antibody protects T cell-depleted mice against herpes simplex virus infection by inhibition of virus replication at the inoculated mucous membranes. *J. Gen. Virol.* **74**, 379-385.

Eloit, M. & Adam, M. (1995). Isogenic adenoviruses type 5 expressing or not expressing the E1A gene: efficiency as virus vectors in the vaccination of permissive and non-permissive species. *J. Gen. Virol.* **76**, 1583-1589.

Eloit, M., Gilardi-Hebenstreit, P., Toma, B. & Perricaudet, M. (1990). Construction of a defective adenovirus vector expressing the pseudorabies virus glycoprotein gp50 and its use as a live vaccine. *J. Gen. Virol.* **71**, 2425-2431.

Endresz, V., Berencsi, K. & Gonczol, E. (1995). An adenovirus-herpes simplex virus glycoprotein B recombinant (Ad-HSV.gB) protects mice against a vaccinia HSV.gB and HSV challenge. *Acta Microbiol. Immunol. Hung.* **42**, 247-254.

Fazekas, G., Rosenwirth, B., Dukor, P., Gergely, J. & Rajnavolgyi, E. (1994). IgG isotype distribution of local and systemic immune responses induced by influenza virus infection. *Eur. J. Immunol.* **24**, 3063-3067.

Fehler, F., Herrmann, J., Saalmuller, A., Mettenleiter, T. C. & Keil, G. M. (1992). Glycoprotein IV of bovine herpesvirus 1 expressing cell line complements and rescues a conditionally lethal viral mutant. *J. Virol.* **66**, 831-839.

Fitch, F. W., Lancki, D. W. & Gajewski, T. F. (1993). T-cell-mediated regulation: help and suppression. In *Fundamental Immunology 3rd*, pp. 733-762. Edited by W. E. Paul. New York: Raven.

Flexner, C., Murphy, B. R., Rooney, J. F., Wohlenberg, C., Yuferov, V., Notkins, A. L. & Moss, B. (1988). Successful vaccination with polyvalent live vector despite existing immunity to an expressed antigen. *Nature* **335**, 259-262.

Flomenberg, P., Gutierrez, E., Piaskowski, V. & Casper, J. T. (1997). Detection of adenovirus DNA in peripheral blood mononuclear cells by polymerase chain reaction assay. *J. Med. Virol.* **51**, 182-188.

Flomenberg, P., Piakowski, V., Truitt, R. L. & Casper, J. T. (1995). Characterization of human proliferative T cell responses to adenovirus. *J. Infect. Dis.* **171**, 1090-1096.

Flomenberg, P., Piaskowski, V., Harb, J., Segura, A. & Casper, J. T. (1996). Spontaneous, persistent infection of a B-cell lymphoma with adenovirus. *J. Med. Virol.* **48**, 267-272.

Fooks, A. R., Schadeck, E., Liebert, U. G., Dowsett, A. B., Rima, B. K., Steward, M., Stephenson, J. R. & Wilkinson, G. W. G. (1995). High-level expression of the measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: induction of an MHC-1-restricted CTL response and protection in a murine model. *Virology* **210**, 456-465.

Fox, J. P., Brandt, C. D., Wassermann, F. E., Hall, C. E., Spigland, I., Kogon, A. & Elveback, L. R. (1969). The virus watch program: a continuing surveillance of viral infections in metropolitan New York families. VI. Observations of adenovirus infections: virus excretion patterns, antibody response, efficiency of surveillance, patterns of infections, and relation to illness. *Am. J. Epidemiol.* **89**, 25-50.

Fox, J. P., Hall, C. E. & Cooney, M. K. (1977). The Seattle virus watch. VII. Observations of adenovirus infections. *Am. J. Epidemiol.* **105**, 362-368.

Galetti, R., Beauverger, P. & Wild, T. F. (1995). Passively administered antibody suppresses the induction of measles virus antibodies by vaccinia-measles recombinant viruses. *Vaccine* **13**, 197-201.

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

Gallichan, W. S., Johnson, D. C., Graham, F. L. & Rosenthal, K. L. (1993). Mucosal immunity and protection after intranasal immunization with recombinant adenovirus expressing herpes simplex virus glycoprotein B. *J. Inf. Dis.* **168**, 622-629.

Ganne, V., Eloit, M., Laval, A., Adam, M. & Trouve, G. (1994). Enhancement of the efficacy of a replication-defective adenovirus-vectored vaccine by the addition of oil adjuvants. *Vaccine* **12**, 1190-1196.

Gatei, M. H., Naif, H. M., Kumar, S., Boyle, D. B., Daniel, R. C. W., Good, M. F. & Lavin, M. F. (1993). Protection of sheep against bovine leukemia virus (BLV) infection by vaccination with recombinant vaccinia viruses expressing BLV envelope glycoproteins: correlation of protection with CD4 T-cell response to gp51 peptide 51-70. *J. Virol.* **67**, 1803-1810.

Gerber, G., Marron, A. E. & Kucera, C. J. (1978). Local and systemic cellular and antibody immune responses of cattle to infectious bovine rhinotracheitis virus vaccines administered intranasally or intramuscularly. *Am. J. Vet. Res.* **39**, 753-760.

Gibbs, E. P. J. & Rweyemamu, M. M. (1977). Bovine herpesvirus. I: Bovine herpesvirus-1. *Vet. Bull. (London)* **47**, 317-343.

Ginsberg, H. S., Horswood, R. L., Chanock, R. M. & Prince, G. A. (1990). Role of early genes in pathogenesis of adenovirus pneumonia. *Proc. Natl. Acad. Sci. USA* **87**, 6191-6195.

Ginsberg, H. S., Lundholm-Beauchamp, U., Horswood, R. L., Pernis, B., Wold, W. S. M., Chanock, R. M. & Prince, G. A. (1989). Role of early region 3 in the pathogenesis of adenovirus disease. *Proc. Natl. Acad. Sci. USA* **86**, 3823-3827.

Ginsberg, H. S., Moldawer, L. L., Pravinkumar, S. B., Redington, M., Kilian, P. L., Chanock, R. M. & Prince, G. A. (1991). A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia. *Proc. Natl. Acad. Sci. USA* **88**, 1651-1655.

Ginsberg, H. S. & Prince, G. A. (1994). The molecular basis of adenovirus pathogenesis. *Infectious Agents and Disease* **3**, 1-8.

Goldman, M. J., Litzky, L. A., Engelhardt, J. F. & Wilson, J. M. (1995). Transfer of the CFTR gene to the lung of nonhuman primates with E1-deleted, E2-defective recombinant adenoviruses: a preclinical toxicology study. *Hum. Gene Ther.* **6**, 839-851.

Gonin, P., Oualikene, W., Fournier, A. & Eloit, M. (1996). Comparison of the efficacy of replication-defective adenovirus and Nyvac poxvirus as vaccine vectors in mice. *Vaccine* **14**, 1083-1087.

Graham, B. S., Davis, T. H., Tang, Y. W., Bunton, L. A. & Gruber, W. C. (1993). Immunoprophylaxis and immunotherapy of respiratory syncytial virus-infected mice with RSV-specific immune serum. *Pediatr. Res.* **21**, 270-274.

Graham, F. L. & Prevec, L. (1991). Manipulation of adenovirus vectors. In *Methods in Molecular Biology*. Edited by E. J. Murray. Clifton, New Jersey: The Humana Press, Inc.

Graham, F. L. & Prevec, L. (1992). Adenovirus-based expression vectors and recombinant vaccines. In *Vaccines: New Approaches to Immunological Problems*, pp. 363-390. Edited by R. W. Ellis. Butterworth-Heinemann, MA.

Gray, D. (1994). Regulation of immunological memory. *Curr. Opin. Immunol.* **6**, 425-430.

Gray, D. & Skarvall, H. (1988). B-cell memory is short lived in the absence of antigen. *Nature* **336**, 70-73.

Haines, D. M. & Chelack, B. J. (1991). Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology. *J. Vet. Diagn. Invest.* **3**, 101-112.

Hassett, D. E. & Whitton, J. L. (1996). DNA immunization. *Trends in Microbiology* **4**, 307-312.

He, X.-S., Chen, H.-S., Chu, K., Rivkina, M. & Robinson, W. S. (1996). Costimulatory protein B7-1 enhances the cytotoxic T cell response and antibody response to hepatitis B surface antigen. *Proc. Natl. Acad. Sci. USA* **93**, 7274-7278.

Hillam, R. P., Bice, D. E. & Muggenburg, B. A. (1985). Lung localization of antibody-forming cells stimulated in distant peripheral lymph nodes. *Immunology* **55**, 257-261.

Hjorth, R. N., Bonde, G. M., Pierzchala, W. A., Vernon, S. K., Wiener, F. P., Levner, M. H., Lubeck, M. D. & Hung, P. P. (1988). A new hamster model for adenoviral vaccination. *Arch. Virol.* **100**, 279-283.

Horwitz, M. S. (1996). Adenoviruses. In *Virology*, pp. 2149-2171. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. New York: Raven.

Hsu, K.-H. L., Lubeck, M. D., Bhat, B. M., Bhat, R. A., Kostek, B., Selling, B. H., Mizutani, S., Davis, A. R. & Hung, P. P. (1994). Efficacy of adenovirus-vectored respiratory cyncytial virus vaccines in a new ferret model. *Vaccine* **12**, 607-612.

Hsu, K.-H. L., Lubeck, M. D., Davis, A. R., Bhat, R. A., Selling, B. H., Bhat, B. M., Mizutani, S., Murphy, B. R., Collins, P. L., Chanock, R. M. & Hung, P. P. (1992). Immunogenecity of recombinant adenovirus-respiratory syncytial virus vaccines with adenovirus types 4, 5, and 7 vectors in dogs and a chimpanzee. *J. Infect. Dis.* **166**, 769-775.

Huard, J., Lochmuller, H., Acsadi, G., Jani, A., Massie, B. & Karpati, G. (1995). The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Therapy* **2**, 107-115.

Hughes, G., Babiuk, L. A. & van Drunen Littel-van den Hurk, S. (1988). Functional and topographical analyses of epitopes on bovine herpesvirus type 1 glycoprotein IV. *Arch. Virol.* **103**, 47-60.

Hutchings, D. L., van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1990). Lymphocyte proliferative responses to separated bovine herpesvirus 1 proteins in immune cattle. *J. Virol.* **64**, 5114-5122.

Hyland, L., Sangster, M., Sealy, R. & Coleclough, C. (1994). Respiratory virus infection of mice provokes a permanent humoral immune response. *J. Virol.* **68**, 6083-6086.

Hyland, S. J., Easterday, B. C. & Pawlisch, R. (1974). Antibody levels and immunity to infectious bovine rhinotracheitis virus (IBR) infection in Wisconsin dairy cattle. *Dev. Biol. Stand.* **28**, 510.

Imler, J.-L. (1995). Adenovirus vectors as recombinant viral vaccines. *Vaccine* **13**, 1143-1151.

Imler, J.-L., Bout, A., Dreyer, D., Dieterle, A., Schultz, H., Valerio, D., Mehtali, M. & Pavirani, A. (1995). Trans-complementation of E1-deleted adenovirus. A new vector to reduce the possibility of codissemination of wild-type and recombinant adenoviruses. *Hum. Gene Ther.* **6**, 711-721.

Imler, J.-L., Bout, B., Perricaudet, M. & al., e. (1993). . In *Clinical Ecology of Cystic Fibrosis*, pp. 21-25. Edited by H. Escobar, F. Baquero & L. Suarez. Amsterdam: Elsevier Science Publishers.

Inchaupse, G., Vitvitski, L., Major, M. E., Jung, G., Spengler, U., Maisonnas, M. & Trepo, C. (1997). Plasmid DNA expressing a secreted or a nonsecreted form of hepatitis C virus nucleocapsid: comparative studies of antibody and T-helper responses following genetic immunization. *DNA and Cell Biology* **16**, 185-195.

Irie, H., Harada, Y., Kataoka, M., Nagamuta, M., Moriya, Y., Handa, M., Saito, M., Matsubara, S., Kojima, K. & Sugawara, Y. (1992). Efficacy of oral administration of live herpes simplex virus type 1 as a vaccine. *J. Virol.* **66**, 2428-2434.

Ishizaka, S. T., Piacente, P., Silva, J. & Mishkin, E. M. (1995). IgG subtype is correlated with efficiency of passive protection and effector function of anti-herpes simplex virus glycoprotein D monoclonal antibodies. *J.Infect. Dis.* **172**, 1108-1111.

Jacobs, S. C., Stephenson, J. R. & Wilkinson, W. G. (1994). Protection elicited by a replication-defective adenovirus vector expressing the tick-borne encephalitis virus non-structural glycoprotein NS1. *J. Gen. Virol.* **75**, 2399-2402.

Jayashree, S., Bhan, M. K., Kumar, R., Raj, P., Glass, R. & Bhandari, N. (1988). Serum and salivary antibodies as indicators of rotavirus infection in neonates. *J. Infect. Dis.* **158**, 1117-1119.

Jericho, K. W. F. & Babiuk, L. A. (1983). The effect of dose, route and virulence of bovine herpesvirus 1 vaccine on experimental respiratory disease in cattle. *Can. J. Comp. Med.* **47**, 133-139.

Jericho, K. W. F. & Darcel, C. l. Q. (1978). Response of the respiratory tract of calves kept at controlled climatic conditions to bovine herpesvirus 1 in aerosol. *Can. J. Comp. Med.* **42**, 156-167.

Johnson, D. C. (1991). Adenovirus vectors as potential vaccines against herpes simplex virus. *Reviews of Inf. Dis.* **13**, S912-S916.

Johnson, M. P., Meitin, C. A., Bender, B. S. & Small Jr, P. A. (1993). Recombinant vaccinia immunization in the presence of passively administered antibody. *Vaccine* **11**, 665-669.

Johnson, M. P., Meitin, C. A., Bender, B. S. & Small, P. A. (1988). Passive immune serum inhibits antibody response to recombinant vaccinia virus. In *Vaccines 88: Modern Approaches to New Vaccines Including Prevention of AIDS*, pp. 189-192. Edited by R. M. Chanock, R. A. Lerner, F. Brown & H. Ginsberg. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Jones, P. C. & Ada, G. L. (1987). Persistence of influenza virus-specific antibody-forming cells and B-cell memory after primary murine influenza virus infection. *Cell. Immunol.* **109**, 53-64.

Kahrs, R. F. (1976). Vaccination programs for bovine respiratory diseases. *Modern Vet. Pract.* **57**, 29.

Kanesaki, T., Murphy, B. R., Collins, P. L. & Ogra, P. L. (1991). Effectiveness of enteric immunization in the development of secretory immunoglobulin A response and the outcome of infection with respiratory cyncytial virus. *J. Virol.* **65**, 657-663.

Kantele, A., Kantele, J. M., Savilathi, E., Westerholm, M., Arvilommi, H., Lazarovits, A., Butcher, E. C. & Makela, H. (1997). Homing potentials of circulating lymphocytes in humans depend on the site of activation. *J. Immunol.* **158**, 574-579.

Kaplan, J. M., Armentano, D., Sparer, T. E., Wynn, S. G., Peterson, P. A., Wadsworth, S. C., Couture, K. K., Pennington, S. E., St.George, J. A., Gooding, L. R. & Smith, A. E. (1997). Characterization of factors involved in modulating persistence of transgene expression from recombinant adenovirus in the mouse lung. *Hum. Gene Ther.* **8**, 45-56.

Kilian, M. & Russell, M. W. (1994). Function of mucosal immunoglobulins. In *Handbook of Mucosal Immunology*, pp. 127-140. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Kimman, T. G. & Westenbrink, F. (1990). Immunity to human and bovine respiratory syncytial virus. *Arch. Virol.* **112**, 1-25.

Kimman, T. G., Westenbrink, F., Schreuder, B. E. C. & Straver, P. J. (1987). Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. *J. Clin. Microbiol.* **25**, 1097-1106.

Kiyono, H. & McGhee, J. R. (1994). T helper cells for mucosal immune responses. In *Handbook of Mucosal Immunology*, pp. 263-273. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Kowalski, J., Gilbert, S. A., van Drunen Littel-van den Hurk, S., van den Hurk, J., Babiuk, L. A. & Zamb, T. J. (1993). Heat-schock promoter-driven synthesis of secreted bovine herpesvirus glycoproteins in transfected cells. *Vaccine* **11**, 1100-1107.

Kozarsky, K. F., McKinley, D. R., Austin, L. L., Raper, S. E., Stratford-Perricaudet, L. D. & Wilson, J. M. (1994). In vivo correction of low density lipoprotein receptor deficiency in the Watanabe heritable with recombinant adenoviruses. *J. Biol. Chem.* **269**, 1-8.

Kundig, T. M., Kalberer, C. P., Hengartner, H. & Zinkernagel, R. M. (1993). Vaccination with two different vaccinia recombinant viruses: long-term inhibition of secondary vaccination. *Vaccine* **11**, 1154-1158.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-683.

Lalor, P. A., Nossal, G. J. V., Sanderson, R. D. & McHeyzer-Williams, M. G. (1992). Functional and molecular characterization of single, (4-hydroxy-3-nitrophenyl) acetyl (NP)-specific, IgG1+ B cells from antibody-secreting and memory B cell pathways in the C57BL/6 immune response to NP. *Eur. J. Immunol.* **22**, 3001.

Leary, T. P. & Splitter, G. A. (1990). A method for the rapid identification of T lymphocyte epitopes. *Pept. Res.* **3**, 259-261.

Leung-Tack, P., Audonnet, J. C. & Riviere, M. (1994). The complete DNA sequence and the genetic organization of the short unique region (US) of the bovine herpesvirus type 1 (ST strain). *Virology* **199**, 409-421.

Levrero, M., Barban, V., Manteca, S., Ballay, A., Balsamo, C., Avantaggiati, M. L., Natoli, G., Skellekens, H., Tiollais, P. & Perricaudet, M. (1991). Defective and nondefective adenovirus vectors for expressing foreign genes in vitro and in vivo. *Gene* **101**, 195-202.

Lewis, J. p., Cox, G. j. M., van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1997). Polynucleotide vaccines in animals: enhancing and modulating responses. *Vaccine* (in press).

Liang, S., Mozdzanowska, K., Palladino, G. & Gerhard, W. (1994). Heterotypic immunity to influenza type A virus in mice. *J. Immunol.* **152**, 1653-1661.

Liang, X. P., Babiuk, L. A., van Drunen Littel-van den Hurk, S., Fitzpatrick, D. R. & Zamb, T. J. (1991). Bovine herpesvirus 1 attachment to permissive cells is mediated by its major glycoproteins gI, gIII, and gIV. *J. Virol.* **65**, 1124-1132.

Lipscomb, M. F., Bice, D. E., Lyons, C. R., Schuyler, M. R. & Wilkes, D. (1995). The regulation of pulmonary immunity. *Adv. Immunol.* **59**, 369-455.

London, S. D. (1994). Cytotoxic lymphocytes in mucosal effector sites. In *Handbook of Mucosal Immunology*, pp. 325-331. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Lubeck, M. D., Davis, A. R., Chengalvala, M., Natuk, R. J., Morin, J. E., Molnar-Kimber, K., Mason, B. B., Bhat, B. M., Mizutani, S., Hung, P. P. & Purcell, R. H. (1989). Immunogenicity and efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus. *Proc. Natl. Acad. Sci. USA* **86**, 6763-6767.

Lubeck, M. D., Natuk, R., Myagkikh, M., Kalyan, N., Aldrich, K., Sinangil, F., Alipanah, S., Murthy, S. C. S., Chanda, P. K., Nigida, S. M., Markham, P. D., Zolla-Pazner, S., Steimer, K., Wade, M., Reitz, M. S., Arthur, L. O., Mizutani, S., Davis, A., Hung, P. P., Gallo, R. C., Eichberg, J. & Robert-Guroff, M. (1997). Long-term protection of chimpanzees against high-dose HIV-1 challenge induced by immunisation. *Nat. Med.* **3**, 651-658.

Lubeck, M. D., Natuk, R. J., Chengalvala, M., Chanda, P. K., Murthy, K. K., Murthy, S., Mizutani, S., Lee, S. G., Wade, M. S., Bhat, B. M. & al., e. (1994). Immunogenicity of recombinant adenovirus-human immunodeficiency virus vaccines in chimpanzees following intranasal administration [published erratum appears in AIDS Res Hum Retroviruses 1995 Jan;11(1):189]. *AIDS Res. Hum. Retroviruses* **10**, 1443-1449.

Manz, R. A., Thiel, A. & Radbruch, A. (1997). Lifetime of plasma cells in the bone marrow. *Nature* **388**, 133-134

Lucher, L. A. (1995). Abortive adenovirus infection and host range determinants. In *The Molecular Repertoire of Adenoviruses*, pp. 119-154. Edited by W. Doerfler & P. Bohm. Berlin, Heidelberg: Springer.

Mack, C. A., Song, W.-R., Carpenter, H., Wickham, T. J., Kovesdi, I., Harvey, B.-G., Magovern, C. J., Isom, O. W., Rosengart, T., Falck-Pedersen, E., Hackett, N. R., Crystal, R. G. & Mastrangeli, A. (1997). Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. *Hum. Gene Ther.* **8**, 99-109.

Makimura, M., Miyake, S., Akino, N., Takamori, K., Matsuura, Y., Miyamura, T. & Saito, I. (1996). Induction of antibodies against structural proteins of hepatitis C virus in mice using recombinant adenovirus. *Vaccine* **14**, 28-36.

Marshall, R. L., Rodriguez, L. L. & Letchworth, G. J. (1986). Characterization of envelope proteins of infectious bovine rhinotracheitis virus (bovine herpesvirus 1) by biochemical and immunological methods. *J. Virol.* **57**, 745-753.

Mastrangeli, A., Daniel, C., Rosenfeld, M. A., Stratford-Perricaudet, L., Pericaudet, M., Pavirani, A., Lecocq, J. P. & Crystal, R. G. (1993). Diversity of airway epithelial cell targets for in vivo recombinant adenovirus-mediated gene transfer. *J. Clin. Invest.* **91**, 225-234.

Mastrangeli, A., Harvey, B. G., Yao, J., Wolff, G., Kovesdi, I., Crystal, R. G. & Falck-Pedersen, E. (1996). "Sero-switch" adenovirus-mediated in vivo gene transfer: circumvention of anti-adenovirus humoral immune defenses against repeat adenovirus vector administration by changing the adenovirus serotype. *Hum. Gene Ther.* **7**, 79-87.

Mazanec, M. B., Lamm, M. E., Lyn, D., Portner, A. & Nedrud, J. G. (1992). Comparison of IgA versus IgG monoclonal antibodies for passive immunization of the murine respiratory tract. *Virus Res.* **23**, 1-12.

McDermott, M. R. & Bienenstock, J. (1979). Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. *J. Immunol.* **122**, 1892-1898.

McDermott, M. R., Graham, F. L., Hanke, T. & Johnson, D. C. (1989). Protection of mice against lethal challenge with herpes simplex virus by vaccination with an adenovirus vector expressing HSV glycoprotein B. *Virology* **169**, 244-247.

McGhee, J. R., Mestecky, J., Detzbaugh, M. T., Eldridge, J. H., Hirasawa, M. & Kiyono, H. (1992). The mucosal immune system: from fundamental concepts to vaccine development. *Vaccine* **10**, 75-88.

McLean, C. S., Ni Challanain, D., Duncan, I., Boursnell, M. E. G., Jennings, R. & Inglis, S. C. (1996). Induction of a protective immune response by a mucosal vaccination with a DISC HSV-1 vaccine. *Vaccine* **14**, 987-992.

McNeal, M. M. & Ward, R. L. (1995). Long-term production of rotavirus antibody and protection against reinfection following a single infection of neonatal mice with murine rotavirus. *Virology* **211**, 474-480.

Meitin, C. A., Bender, B. S. & Small, P. A. (1994). Enteric immunization of mice against influenza with recombinant vaccinia. *Proc. Natl. Acad. Sci. USA* **91**, 11187-11191.

Men, R., Bray, M. & Lai, C.-J. (1991). Carboxy-terminally truncated dengue virus envelope glycoproteins expressed on the cell surface and secreted extracellularly exhibit increased immunogenicity in mice. *J. Virol.* **65**, 1400-1407.

Mestecky, J., Abraham, R. & Ogra, P. L. (1994). Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces. In *Handbook of Mucosal Immunology*, pp. 357-372. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Michalek, S. M., Eldridge, J. H., Curtiss III, R. & Rosenthal, K. L. (1994). Antigen delivery systems: new approaches to mucosal immunization. In *Handbook of Mucosal Immunology*, pp. 373-390. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Misra, V., Blumenthal, R. M. & Babiuk, L. A. (1981). Proteins specified by bovine herpesvirus 1 (infectious bovine rhinotracheitis virus). *J. Virol.* **40**, 1046-1054.

Mitchell, A. (1974). An outbreak of abortion in a diary herd following inoculation with an intramuscular infectious bovine rhinotracheitis vaccine. *Can. Vet. J.* **15**, 148.

Mittal, S. K., McDermott, M. R., Johnson, D. C., Prevec, L. & Graham, F. L. (1993). Monitoring foreign gene expression by a human adenovirus-based vector using the firefly luciferase gene as a reporter. *Virus Res.* **28**, 67-90.

Mittal, S. K., Middleton, D. M., Tikoo, S. K. & Babiuk, L. A. (1995). Pathogenesis and immunogenecity of bovine adenovirus type 3 in cotton rats (*Sigmodon hispidus*). *Virology* **213**, 131-139.

Mittal, S. K., Papp, Z., Tikoo, S. K., Baca-Estrada, M. E., Yoo, D., Benko, M. & Babiuk, L. A. (1996). Induction of systemic and mucosal immune responses in cotton rats immunized with human adenovirus type 5 recombinants expressing the full and truncated forms of bovine herpesvirus type 1 glycoprotein gD. *Virology* **222**, 299-309.

Moldoveanu, Z., Novak, M., Huang, W.-Q., Gilley, R. M., Staas, J. K., Schafer, D., Compans, R. W. & Mestecky, J. (1993). Oral immunization with influenza virus biodegradable microspheres. *J. Inf. Dis.* **167**, 84-90.

Morin, J. E., Lubeck, M. D., Barton, J. E., Conley, A. J., Davis, A. R. & Hung, P. P. (1987). Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. *Proc. Natl. Acad. Sci. USA* **84**, 4626-4630.

Morrison, L. A. & Knipe, D. M. (1996). Mechanisms of imunizations with a replication-defective mutant of herpes simplex virus 1. *Virology* **220**, 402-413.

Murphy, B. R. (1994). Mucosal Immunity to Viruses. In *Handbook of Mucosal Immunology*, pp. 333-339. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Murphy, B. R., Collins, P. L., Lawrence, L., Zubak, J., Chanock, R. M. & Prince, G. A. (1989). Immunosuppression of the antibody response to respiratory syncytial virus (RSV) by preexisting serum antibodies: partial prevention by topical infection of the respiratory tract with vaccinia virus-RSV recombinants. *J. Gen. Virol.* **70**, 2185-2190.

Murphy, B. R., Graham, B. S., Prince, G. A., Walsh, E. E., Chanoch, R. M., Karzon, D. T. & Wright, P. F. (1986). Serum and nasal-wash immunoglobulin G and A antibody response of infants and children to respiratory syncytial virus F and G glycoproteins following primary infection. *J. Clin. Microbiol.* **23**, 1009-1014.

Murphy, B. R., Olmstead, R. A., Collins, P. L., Chanock, R. M. & Prince, G. A. (1988). Passive transfer of respiratory syncytial virus (RSV) antiserum suppresses the immune response to the RSV fusion (F) and large (G) glycoproteins expressed by recombinant vaccinia viruses. *J. Virol.* **62**, 3907-3910.

Murphy, T. F., Dubovi, E. J. & Clyde Jr., W. A. (1981). The cotton rat as an experimental model of human parainfluenza virus type 3 disease. *Exp. Lung Res.* **2**, 97-109.

Natuk, R. J., Chanda, P. K., Lubeck, M. D., Davis, A. R., Wilhelm, J., Hjorth, R., Wade, M. S., Bhat, B. M., Mizutani, S., Lee, S., Eichberg, J., Gallo, R. C., Hung, P. P. & Robert-Guroff, M. (1992). Adenovirus-human immunodeficiency virus (HIV) envelope recombinant vaccines elicit high-titered HIV-neutralizing antibodies in the dog model. *Proc. Natl. Acad. Sci. USA* **89**, 7777-7781.

Natuk, R. J., Lubeck, M. D., Chanda, P. K., Chengalvala, M., Wade, M. S., Murthy, S. C., Wilhelm, J., Vernon, S. K., Dheer, S. K., Mizutani, S. & al., e. (1993). Immunogenicity of recombinant human adenovirus-human immunodeficiency virus vaccines in chimpanzees. *AIDS Res. Hum. Retroviruse* **9**, 395-404.

Nettleton, P. F. & Sharp, J. M. (1980). Infectious bovine rhinotracheitis virus excretion after vaccination. *Vet. Rec.* **107**, 379.

Ogra, P. L. (1996). Mucosal Immunoprophylaxis: An Introductory Overview. In *Mucosal Vaccines*, pp. 3-14. Edited by H. Kiyono, P. L. Ogra & J. R. McGhee. San Diego, California: Academic Press, Inc.

Oualikene, W., Gonin, P. & Eloit, M. (1994). Short and long term dissemination of deletion mutants of adenovirus in permissive (cotton rat) and non-permissive (mouse) species. *J. Gen. Virol.* **75**, 2765-2768.

Oualikene, W., Gonin, P. & Eloit, M. (1995). Lack of evidence of phenotypic complementation of E1A/E1B-deleted adenovirus type 5 upon superinfection by wild-type virus in the cotton rat. *J. Virol.* **69**, 6518-6524.

Outlaw, M. C. & Dimmock, N. J. (1991). Insights into neutralization of animal viruses gained from study of influenza virus. *Epidemiol. Infect.* **106**, 205-220.

Pacini, D. L., Dubovi, E. J. & Clyde, W. A. (1984). A new animal model for human respiratory tract disease due to adenovirus. *J. Inf. Dis.* **150**, 92-97.

Pastoret, P. P., Babiuk, L. A., Misra, V. & Griebel, P. (1980). Reactivation of temperature-sensitive and non-temperature-sensitive infectious bovine rhinotracheitis vaccine virus with dexamethasone. *Infect.Immun.* **29**, 483-488.

Perkus, M. E. & Paoletti, E. (1996). Recombinant virus as vaccination carrier of heterologous antigens. In *Concepts in Vaccine Development*, pp. 379-421. Edited by S. H. E. Kaufmann. Berlin, New York: Walter de Gruyter.

Phillips-Quagliata, J. M. & Lamm, M. E. (1994). Lymphocyte homing to mucosal effector sites. In *Handbook of Mucosal Immunology*, pp. 225-242. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Picker, L. J. (1994). Control of lymphocyte homing. *Curr. Opin. Immunol.* **6**, 394-406.

Porter, D. D., Prince, G. A., Hemming, V. G. & Porter, H. G. (1991). Pathogenesis of human parainfluenza virus 3 infection in two species of cotton rats: Sigmodon hispidus develops bronchiolitis, while Sigmodon fulviventer develops interstitial pneumonia. *J. Virol.* **65**, 103-111.

Prevec, L., Campbell, J. B., Christie, B. S., Belbeck, L. & Graham, F. L. (1990). A recombinant human adenovirus vaccine against rabies. *J. Infect. Dis.* **161**, 27-30.

Prevec, L., Schneider, M., Rosenthal, K. L., Belbeck, L. W., Derbyshire, J. B. & Graham, F. L. (1989). Use of human adenovirus-based vectors for antigen expression in animals. *J. Gen. Virol.* **70**, 429-434.

Prince, G. A., Jenson, A. B., Horswood, R. L., Camargo, E. & Chanock, R. M. (1978). The pathogenesis of respiratory syncytial virus infection in cotton rats. *Am. J. Pathol.* **93**, 771-792.

Prince, G. A., Porter, D. D., Jenson, A. B., Horswood, R. L., Chanock, R. M. & Ginsberg, H. S. (1993). Pathogenesis of adenovirus type 5 pneumonia in cotton rats (*Sigmodon hispidus*). *J. Virol.* **67**, 101-111.

Ragot, T., Finerty, S., Watkins, P. E., Perricaudet, M. & Morgan, A. J. (1993). Replication-defective recombinant adenovirus expressing the Epstein-Barr virus (EBV) envelope glycoprotein gp340/220 induces protective immunity against EBV-induced lymphomas in the cottontop tamarin. *J. Gen. Virol.* **74**, 501-507.

Rawle, F. C., Tollefson, A. E., Wold, W. S. & Gooding, L. R. (1989). Mouse anti-adenovirus cytotoxic T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. *J. Immunol.* **143**, 2031-2037.

Renegar, K. B. & Small Jr., P. A. (1991). Passive transfer of local immunity to influenza virus infection by IgA antibody. *J. Immunol.* **146**, 1972-1978.

Renegar, K. B. & Small, P. A. (1994). Passive immunization: systemic and mucosal. In *Handbook of Mucosal Immunology*. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Rodrigues, E. G., Zavala, F., Eichinger, D., Wilson, J. M. & Tsuji, M. (1997). Single immunizing dose of recombinant adenovirus efficiently induces CD8+ T cell-mediated protective immunity against malaria. *J. Immunol.* **158**, 1268-1274.

Rosenfeld, M. A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L. E., Paakko, P. K., Gilardi, P., Stratford-Perricaudet, L. D., Perricaudet, M., Jallet, S., Pavirani, A., Lecoq, J.-P. & Crystal, R. G. (1991). Adenovirus-mediated transfer of a recombinant alfa 1-antitrypsin gene to the lung epithelium *in vivo*. *Science* **252**, 431-434.

Rosenthal, K. L., Copeland, K. F. T. & Gallichan, W. S. (1996). Recombinant adenoviruses as vectors for mucosal immunity. In *Mucosal Vaccines*, pp. 147-158. Edited by H. Kiyono, P. L. Ogra & J. R. McGhee. San Diego, California: Academic Press, Inc.

Rossi-Bergmann, B., Muller, I. & Godinho, E. B. (1993). TH1 and TH2 T-cell subsets are differentially activated by macrophages and B cells in murine leishmaniasis. *Infect. Immun.* **61**, 2266-2269.

Sabin, A. B., Michaels, R. H., Ziring, P., Krugman, S. & Warren, J. (1963). Effect of oral poliovirus vaccine in newborn children. II. Intestinal resistance and antibody response at 6 months in children fed type I vaccine at birth. *Pediatrics* **31**, 641-654.

Sanderson, I. R. & Walker, W. A. (1994). Mucosal barrier. In *Handbook of Mucosal Immunology*, pp. 41-52. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego: Academic Press, Inc.

Schranz, V., Kulcsar, G., Dan, P., Horvath, J., Nasz, I., Barinsky, I. F. & Ugryumov, E. P. (1979). Interaction of human lymphocytes and viruses in vitro. *Acta. Microbiol. Acad. Sci. Hung.* **26**, 1-9.

Schulick, A. H., Vassalli, G., Dunn, P. F., Dong, G., Rade, J. J. & Zamarron, C. (1997). Established immunity precludes adenovirus-mediated gene transfer in rat carotid arteries. *J. Clin. Invest.* **99**, 209-219.

Schwartz, A. R., Togo, Y. & Hornick, R. B. (1974). Clinical evaluation of live, oral types 1, 2, and 5 adenovirus vaccines. *Am. Rev.Resp. Dis.* **109**, 233-238.

Setoguchi, Y., Jaffe, H. A., Chu, C.-S. & Crystal, R. G. (1994). Intraperitoneal in vivo gene therapy to deliver alfa1-antitrypsin to the systemic circulation. *Am. J. Respir. Cell. Mol. Biol.* **10**, 369-377.

Shenk, T. (1996). *Adenoviridae*: The Viruses and Their Replication. In *Virology*, pp. 2111-2148. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. New York: Raven.

Shiver, J. W., Ulmer, J. B., Donnelly, J. J. & Liu, M. A. (1996). Naked DNA vaccination. In *Concepts in Vaccine Development*, pp. 423-436. Edited by S. H. E. Kaufmann. Berlin, New York: Walter de Gruyter.

Silver, L. & Anderson, C. W. (1988). Interaction of human adenovirus serotype 2 with human lymphoid cells. *Virology* **165**, 377-387.

Slifka, M. K. & Ahmed, R. (1996). Long-term humoral immunity against viruses: revisiting the issue of plasma cell longevity. *Trends in Microbiology* **4**, 394-400.

Smith, T. A. G., Mehaffery, M. G., Kayda, D. B., Saunders, J. M., Yei, S., Trapnell, B. C., McLelland, A. & Kaleko, M. (1993). Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. *Nat. Gen.* **5**, 397-402.

Sparer, T. E., Wynn, S. G., Clark, D. J., Kaplan, J. M., Cardoza, L. M., Wadsworth, S. C., Smith, A. E. & Gooding, L. R. (1997). Generation of cytotoxic T lymphocytes against immunorecessive epitopes after multiple immunizations with adenovirus vectors is dependent on haplotype. *J. Virol.* **71**, 2277-2284.

Sprent, J. (1994). T and B memory cells. *Cell* **76**, 315-322.

Stratford-Perricaudet, L. D., Levrero, M., Chasse, J. F., Perricaudet, M. & Briand, P. (1990). Evaluation of the transfer and expression in mice of an enzyme-encoding gene using a human adenovirus vector. *Hum. Gene Ther.* **1**, 241-256.

Straus, S. E. (1984). Adenovirus infections in humans. In *The Adenoviruses*, pp. 451-496. Edited by H. S. e. a. Ginsberg. New York: Plenum Press.

Tacket, C. O., Losonsky, G., Lubeck, M. D., Davis, A. R., Mizutani, S., Horwith, G., Hung, P., Edelman, R. & Levine, M. M. (1992). Initial safety and immunogenicity studies of an oral recombinant adenohepatitis B vaccine. *Vaccine* **10**, 673-676.

Tamura, S.-I. & Kurata, T. (1996). Intranasal immunisation with influenza vaccine. In *Mucosal Vaccines*, pp. 425-436. Edited by H. Kiyono, P. L. Ogra & J. R. McGhee. San Diego, California: Academic Press.

Tew, J. G., Phipps, R. P. & Mandel, T. E. (1980). The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells. *Immunol. Rev.* **53**, 175.

Tikoo, S. K., Campos, M. & Babiuk, L. A. (1995a). Bovine herpesvirus 1 (BHV-1): biology, pathogenesis and control. *Adv.Virus Res.* **45**, 191-219.

Tikoo, S. K., Campos, M., Popowych, Y. I., van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1995b). Lymphocyte proliferative responses to recombinant bovine herpes virus type 1 (BHV-1) glycoprotein gD (gIV) in immune cattle: identification of a T cell epitope. *Viral Immunol.* **8**, 19-25.

Tikoo, S. K., Fitzpatrick, D. R., Babiuk, L. A. & Zamb, T. J. (1990). Molecular cloning, sequencing, and expression of functional bovine herpesvirus 1 glycoprotein gIV in transfected bovine cells. *J. Virol.* **64**, 5132-5142.

Tikoo, S. K., Zamb, T. J. & Babiuk, L. A. (1993). Analysis of bovine herpesvirus 1 glycoprotein gIV truncations and deletions expressed by recombinant vaccinia viruses. *J. Virol.* **67**, 2103-2109.

Tomasi, T. B. (1994). Introduction: an overview of the mucosal system. In *Handbook of Mucosal Immunology*, pp. 4-10. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

Top, F. H., Jr., Buescher, E. L., Bancroft, W. H. & Russell, P. K. (1971a). Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7. *J. Inf. Dis.* **124**, 155-160.

Top, F. H., Jr., Grossmann, R. A., Bartelloni, P. J., Segal, H. E., Dudding, B. A., Russell, P. K. & Buescher, E. L. (1971b). Immunization with live types 7 and 4 adenovirus vaccines. I. Infectivity, antigenicity, and potency of adenovirus type 7 vaccine in humans. *J. Inf. Dis.* **124**, 148-154.

Tripathy, S. K., Black, H. B., Goldwasser, E. & Leiden, J. M. (1996). Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat. Med.* **2**, 545-560.

Uhnoo, I., Riepenhoff-Talty, M., Dharakul, T., Chegas, P., Fisher, J. E., Greenberg, H. B. & Ogra, P. L. (1990). Extramucosal spread and development of hepatitis in immunodeficient and normal mice infected with rhesus rotavirus. *J. Virol.* **64**, 361-368.

Unanue, E. R. (1993). Macrophages, antigen-presenting cells, and the phenomena of antigen handling and presentation. In *Fundamental Immunology 3rd*, pp. 111-144. Edited by W. E. Paul. New York: Raven.

Underdown, B. J. & Mestecky, J. (1994). Mucosal immunoglobulins. In *Handbook of Mucosal Immunology*, pp. 79-98. Edited by P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. R. McGhee & J. Bienenstock. San Diego, California: Academic Press, Inc.

van Drunen Littel-van den Hurk, S. & Babiuk, L. A. (1986). Synthesis and processing of bovine herpesvirus 1 glycoproteins. *J. Virol.* **59**, 401-410.

van Drunen Littel-van den Hurk, S., Gifford, G. A. & Babiuk, L. A. (1990). Epitope specificity of the protective immune response induced by individual bovine herpesvirus-1 glycoproteins. *Vaccine* **8**, 358-368.

van Drunen Littel-van den Hurk, S., Parker, M. D., Massie, B., van den Hurk, J. V., Harland, R., Babiuk, L. A. & Zamb, T. J. (1993). Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. *Vaccine* **11**, 25-35.

van Drunen Littel-van den Hurk, S., Tikoo, S. K., van den Hurk, J. V., Babiuk, L. A. & van Donkersgoed, J. (1997). Protective immunity in cattle following vaccination with conventional and marker bovine herpesvirus-1 (BHV-1) vaccines. *Vaccine* **15**, 36-44.

van Drunen Littel-van den Hurk, S., van den Hurk, J. V., Gilchrist, J. E., Misra, V. & Babiuk, L. A. (1984). Interactions of monoclonal antibodies and bovine herpesvirus type 1 (BHV-1) glycoproteins: characterization of their biochemical and immunological properties. *Virology* **135**, 466-479.

van Drunen Littel-van den Hurk, S., van Donkersgoed, J., Kowalski, J., van den Hurk, J. V., Harland, R., Babiuk, L. A. & Zamb, T. J. (1994). A subunit gIV vaccine, produced by transfected mammalian cells in culture, induces mucosal immunity against bovine herpesvirus-1 in cattle. *Vaccine* **12**, 1295-1302.

van Ginkel, F. W., Liu, C., Simecka, J. W., Dong, J.-Y., Greenway, T., Frizzell, R. A., Kiyono, H., McGhee, J. R. & Pascual, D. W. (1995). Intratracheal gene delivery with adenoviral vector induces elevated systemic IgG and mucosal IgA antibodies to adenovirus and beta-galactosidase. *Hum. Gene Ther.* **6**, 895-903.

van Ginkel, F. W., McGhee, J. R., Liu, C., Simecka, J. W., Yamamoto, M., Frizzell, R. A., Sorscher, E. J., Kiyono, H. & Pascual, D. W. (1997). Adenoviral gene delivery elicits distinct pulmonary-associated T helper cell responses to the vector and to its transgene. *J. Immunol.* **159**, 685-693.

VanCott, J. L., Brim, T. A., Lunney, J. K. & Saif, L. J. (1994). Contribution of antibody-secreting cells induced in mucosal lymphoid tissues of pigs inoculated with respiratory or enteric strains of coronavirus to immunity against enteric coronavirus challenge. *J. Immunol.* **152**, 3980-3990.

Wagner, D. K., Clements, M. L., Reimer, C. B., Snyder, M. H., Nelson, D. L. & Murphy, B. R. (1987). Analysis of IgG antibody responses after live and inactivated influenza A vaccine indicate that nasal-wash IgG is a transudate from serum. *J. Clin Microbiol.* **25**, 559-562.

Weeks-Levy, C. & Ogra, P. L. (1996). Polioviruses and Mucosal Vaccines. In *Mucosal Vaccines*, pp. 283-294. Edited by H. Kiyono, P. L. Ogra & J. R. McGhee. San Diego, California: Academic Press, Inc.

Witmer, L. A., Rosenthal, K. L., Graham, F. L., Friedman, H. M., Yee, A. & Johnson, D. C. (1990). Cytotoxic T lymphocytes specific for herpes simplex virus (HSV) studied using adenovirus vectors expressing HSV glycoproteins. *J. Gen. Virol.* **71**, 387-396.

Wòrgall, S., Wolff, G., Falck-Pedersen, E. & Crystal, R. G. (1997). Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum. Gene Ther.* **8**, 37-44.

Xiang, Z. Q. & Ertl, H. C. J. (1992). Transfer of maternal antibodies results in inhibition of specific immune responses in the offspring. *Virus Res.* **24**, 297-314.

Xiang, Z. Q., Yang, Y., Wilson, J. M. & Ertl, H. C. J. (1996). A replication-defective human adenovirus recombinant serves as a highly efficacious vaccine carrier. *Virology* **219**, 220-227.

Yang, Y., Li, Q., Ertl, H. C. J. & Wilson, J. M. (1995a). Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* **69**, 2004-2015.

Yang, Y., Nunes, F. A., Berencsi, K., Furth, E. E., Gonczol, E. & Wilson, J. M. (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* **91**, 4407-4411.

Yang, Y., Trinchieri, G. & Wilson, J. M. (1995b). Recombinant IL-12 prevents formation of blocking IgA antibodies to recombinant adenovirus and allows repeated gene therapy to mouse lung. *Nat. Med.* **1**, 890-893.

Yang, Y. & Wilson, J. M. (1995). Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. *J. Immunol.* **155**, 2564-2570.

Yang, Y., Xiang, Z., Ertl, H. C. J. & Wilson, J. M. (1995c). Upregulation of class I major histocompatibility complex antigens by interferon gamma is necessary for T-cell-mediated elimination of recombinant adenovirus-infected hepatocytes in vivo. *Proc. Natl. Acad. Sci. USA* **92**, 7257-7261.

Yarosh, O. K., Wandeler, A. I., Graham, F. L., Campbell, J. B. & Prevec, L. (1996). Human adenovirus type 5 vectors expressing rabies glycoprotein. *Vaccine* **14**, 1257-1264.

Yates, W. D. G. (1982). A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Can. J. Comp. Med.* **46**, 225-263.

Yei, S., Mittereder, N., Tang, K., O'Sullivan, C. & Trapnell, B. C. (1994). Adenovirus-mediated gene transfer for cystic fibrosis: quantitative evaluation of repeated in vivo vector administration to the lung. *Gene Ther.* **1**, 192-200.

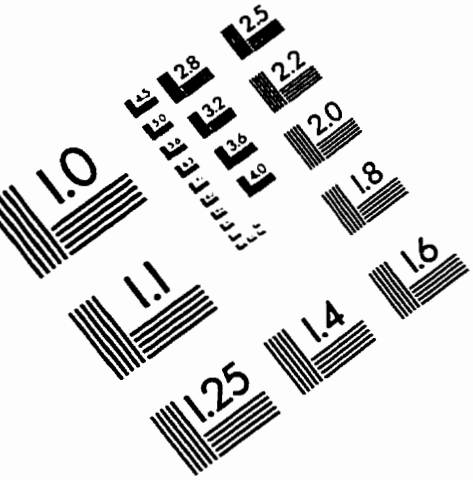
Zabner, J., Petersen, D. M., Puga, A. P., Graham, S. M., Couture, L. A., Keyes, L. D., Lukason, M. J., St. George, J. A., Gregory, R. J., Smith, A. E. & Welsh, M. J. (1994). Safety and efficacy of repetitive adenovirus-mediated transfer of CFTR cDNA to airway epithelia of primates and cotton rats. *Nat. Gen.* **6**, 75-83.

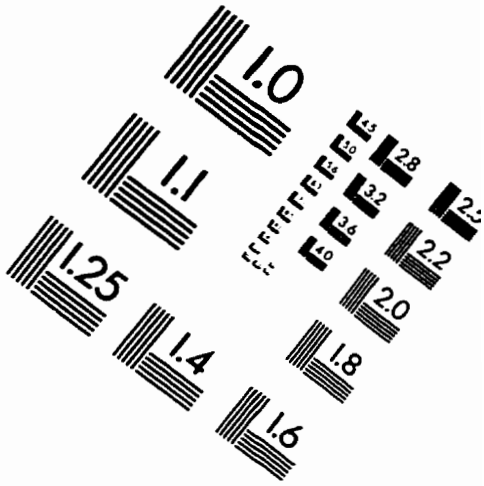
Zhai, Y., Yang, J. C., Kawakami, Y., Spiess, P., Wadsworth, S. C., Cardoza, L. M., Couture, L. A., Smith, A. E. & Rosenberg, S. A. (1996). Antigen-specific tumor vaccines. *J. Immunol.* **156**, 700-710.

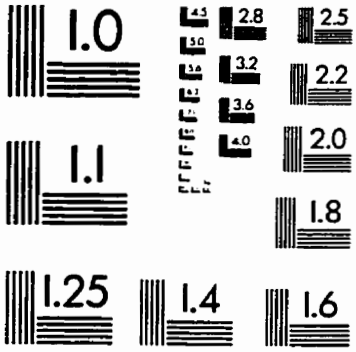
Zheng, B., Graham, F. L., Johnson, D. C., Hanke, T., McDermott, M. R. & Prevec, L. (1993). Immunogenicity in mice of tandem repeats of an epitope from herpes simplex gD protein when expressed by recombinant adenovirus. *Vaccine* **11**, 1191-1198.

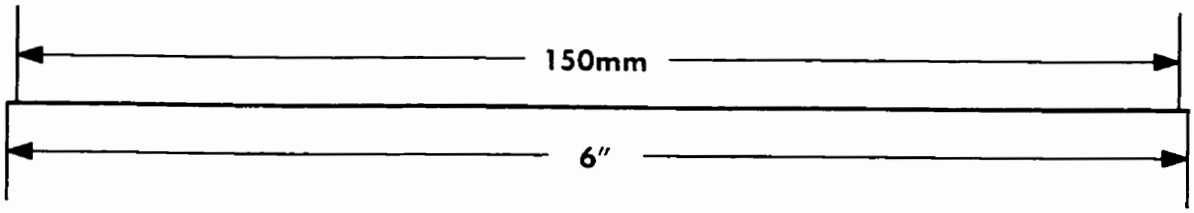
Zinkernagel, R. M. (1993). Immunity to viruses. In *Fundamental Immunology 3rd*, pp. 1211-1250. Edited by W. E. Paul. New York: Raven.

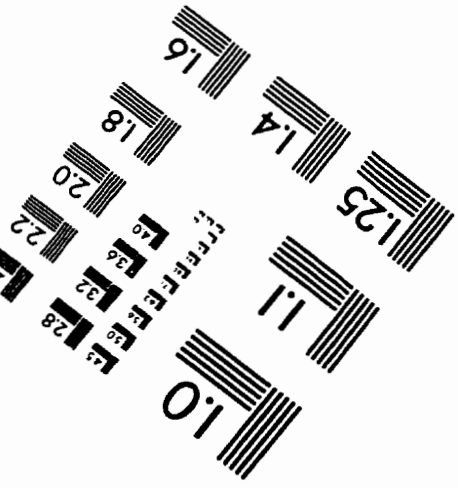
IMAGE EVALUATION TEST TARGET (QA-3)

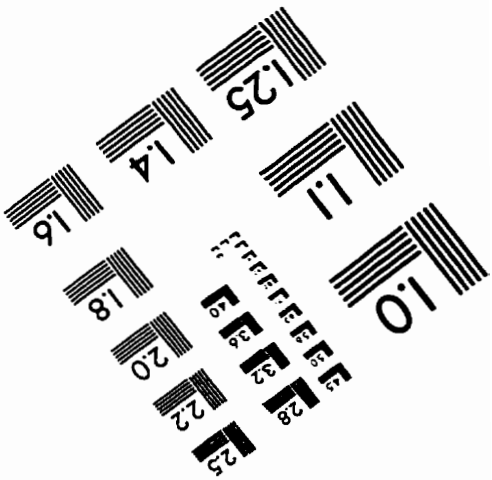












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