Mucosal immunization with recombinant adenoviruses: induction of immunity and protection of cotton rats against respiratory bovine herpesvirus type 1 infection

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To facilitate the evaluation of vaccines against bovine herpesvirus type 1 (BHV-1), a cotton rat model of intranasal (i.n.) BHV-1 infection was established. Cotton rat lung cells were similar to bovine cells in their ability to support BHV-1 replication in vitro. Furthermore, i.n. inoculation of cotton rats with BHV-1 resulted in pulmonary lesions comparable to BHV-1 infection in cattle. Using this model, the potential of i.n. and gastrointestinal (g.i.) immunization was examined with recombinant human adenoviruses expressing glycoprotein D (gD) of BHV-1 to induce protective immunity against BHV-1. The replication-competent virus (gD-dE3) was more efficient than the replication-defective virus (gD-dE1E3) in inducing gD-specific antibody in the serum and in the respiratory tract. Furthermore, i.n. immunization with gD-dE3 stimulated antigen-specific antibody-secreting cells in the lung 12 weeks following immunization. Protection against BHV-1 challenge correlated with gD-specific antibody levels such that i.n. immunization with gD-dE3 conferred complete protection, while g.i. immunization conferred only partial protection of the lungs of most animals against BHV-1 challenge. In comparison, immunization with gD-dE1E3 by either route resulted in only a partial reduction of BHV-1 titre in the respiratory tract. The results obtained demonstrate that mucosal immunization with replication-competent recombinant adenovirus expressing gD of BHV-1 can induce immunity and protection against BHV-1 challenge.

Introduction

Bovine herpesvirus type 1 (BHV-1), a member of the subfamily Alphaherpesviridae, can cause a variety of diseases in cattle, including acute respiratory infections which render cattle susceptible to potentially fatal secondary bacterial pneumonia (Yates, 1982). In order to develop new vaccination strategies, individual viral components, such as glycoprotein D (gD), that evoke effective protective immune responses to BHV-1 have been identified (Babiuk et al., 1987; van Drunen Littel van den Hurk et al., 1990) and reviewed (Tikoo et al., 1995). Since evaluation of vaccination strategies against BHV-1 in cattle is both costly and difficult, there is a need to develop a laboratory animal model for BHV-1 infection; a rodent model would also be more feasible than cattle for investigating the mechanism of induction of mucosal immune responses. A candidate for developing such a model, the cotton rat (Sigmodon hispidus), has been used to study the pathogenesis of various human and animal respiratory viruses, including human adenovirus type 5 (hAd5) (Breker-Klassen et al., 1995; Mittal et al., 1995; Pacini et al., 1984; Porter et al., 1991; Prince et al., 1978, 1993).

Live hAd5 has been shown to be an excellent delivery system for vaccine antigens (Imler, 1995; Rosenthal et al., 1996). Both replication-defective and replication-competent hAd5 have been used for insertion of foreign genes (Graham & Prevec, 1992); however, replication-defective viruses are often preferred to replication-competent vectors because of their greater safety. Adenoviruses deleted of the non-essential E3 early transcription region (dE3) remain replication-competent in permissive host cells, while deletion of the E1 region as well...
(dE1E3) results in a replication-defective virus. The replication-defective virus can be propagated only in cells which express the E1 region proteins thus complementing the deficiency of the virus. Although unable to replicate in vivo, such dE1E3 viruses have been able to induce immunity to inserted gene products after systemic immunization (Eloit & Adam, 1995; Eloit et al., 1990; Fooks et al., 1995; Jacobs et al., 1994; Mittal et al., 1996; Ragot et al., 1993; Xiang et al., 1996) and sometimes following administration to the respiratory tract (Eloit et al., 1990; Gonin et al., 1996; Xiang et al., 1996). Replication-competent dE3 adenoviruses, in contrast, have often proven effective not only following systemic immunization, but also after mucosal administration in the respiratory or alimentary tract (Baca-Estrada et al., 1995). The construction of recombinant hAd5 containing the BHV-1 gD gene in the E3 region utilizing an E3-deleted vector (gD-dE3) or in the E1 region utilizing an E1–E3-deleted vector (gD-dE1E3) has been described previously (Mittal et al., 1996; van Drunen Littel van den Hurk et al., 1993). The recombinant viruses were propagated in 293 cells. For immunization, the viruses were purified by centrifugation in a discontinuous caesium chloride gradient. Virus titres were determined by plaque assay on 293 cells. For the in vitro assays, the wild-type hAd5 was purified by two rounds of continuous caesium chloride gradient centrifugation. Total protein content of each virus preparation was estimated by a Bio-Rad protein assay. The gD-dE1E3 and gD-dE3 virus preparations contained no more than 0.02 μg gD protein per 10^9 p.f.u. virus, estimated by a capture ELISA. UV-irradiated gD-dE1E3 and gD-dE3 virus preparations do not induce gD-specific immunity in cotton rats (unpublished observations), indicating that gD present in the virus inoculum is insufficient to induce an immune response. 

**Methods**

**Animals.** Inbred male and female cotton rats (S. hispidus), 3–5 months of age, seronegative to adenovirus and BHV-1, were obtained from barrier-maintained pathogen-free colonies at the Veterinary Infectious Disease Organization and Virion Systems, Inc. (Rockville, Maryland, USA). 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.

**Cells and viruses.** Cotton rat lung (CRL) cells were cultured and maintained as a stable cell line as previously described (Baca-Estrada et al., 1995). The Cooper strain of BHV-1 was obtained from the National Veterinary Services Laboratories (Ames, Iowa, USA). The virus was propagated in Madin–Darby bovine kidney (MDBK) cells using minimal essential medium (MEM) with Earle’s salts (Gibco/BRL) supplemented with 10% foetal bovine serum (FBS; Gibco/BRL) and 2 mM l-glutamine. The field isolate 108 strain of BHV-1 was obtained from the Animal Diseases Research Institute (Lethbridge, Alberta, Canada) and passaged twice in MDBK cells. For inoculation of cotton rats, the supernatants of cultures of the 108 and Cooper strains were used. For ELISA, BHV-1 was purified by ultracentrifugation on discontinuous Na/K tartrate gradients as described (Misra et al., 1981). Stocks of both strains of BHV-1 were titrated on MDBK and CRL cells and were stored in small aliquots at −70 °C.

**Virus infection and titration.** BHV-1 was adsorbed onto CRL or MDBK cell monolayers for 1 h 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). At various time-points, cells and culture supernatants were harvested and frozen at −70 °C until all the samples could be processed simultaneously. Samples were titrated for BHV-1 by plaque assay on MDBK cells.

**In vivo challenge and immunizations.** Cotton rats were anesthetized with nitrous oxide–halothane (MTC Pharmaceuticals) (1:2) during all immunizations and challenges. Blood samples were obtained by cardiac puncture. Animals were euthanized by halothane overdose. For i.n. immunization (10^4 p.f.u. of purified adenovirus) and challenge (5 × 10^7 p.f.u. of BHV-1), viruses were administered in 50 μl volumes into the nares of cotton rats. For the intraduodenal immunization, 10^4 p.f.u. of adenovirus in 500 μl PBS, or PBS alone, was injected into the duodenum following its surgical exposure under aseptic conditions. The abdomen was then closed and the animals were housed under normal conditions. For oral immunization, 10^3 p.f.u. of adenovirus suspended in 500 μl of 0.4 M NaHCO_3 was delivered into the stomach with a 20 gauge gavage needle. Before intraduodenal and oral immunization, cotton rats were starved overnight.

**Preparation of trachea and lung extracts.** After euthanasia, the trachea and right lung from each rat were aseptically removed and frozen in 1 ml MEM at −70 °C. When all samples were collected, they were thawed, weighed, homogenized (Polytron homogenizer; Brinkman Industries, Rexdale, Canada) and centrifuged to remove debris. The resulting supernatants were assayed for the presence of BHV-1 and antibodies. In our experiments, antigen-specific antibody levels in lung and trachea extracts always correlated with those in lung and nasal washes, respectively (unpublished observation).

**Histopathology and immunohistochemistry.** The left lung was fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin for histological evaluation.

Unstained sections of formalin-fixed lung from the same blocks were stained immunohistochemically using an avidin–biotin complex method.
supplemented with 10% FBS (Hyclone Laboratories), 2 mM.

The tissue was cut into small pieces and incubated in complete medium: MEM washed with MEM. To obtain lymphocytes from the right lung, the

Preparation of lymphocytes from the spleen, bone marrow

Preparation of lymphocytes from the spleen, bone marrow and lungs. Spleens and bone marrow were removed aseptically, chopped, then gently pushed through a plastic mesh with a 5 ml syringe plunger and washed with MEM. Erythrocytes were lysed by Tris-buffered 0.83% NH₄Cl, and the resulting lymphocyte suspensions were washed with MEM. To obtain lymphocytes from the right lung, the tissue was cut into small pieces and incubated in complete medium: MEM supplemented with 10% FBS (HyClone Laboratories), 2 mM t-glutamine, 1 mM sodium pyruvate, 100 µM non-essential amino acids, 10 mM HEPES buffer (Gibco/BRL), 50 µM 2-mercaptoethanol and 100 U/ml penicillin G, 100 µg/ml streptomycin solution (Sigma), with 150 U/ml collagenase A ( Worthington) and 50 U/ml DNase I (Sigma) for 1 h, then pushed through a plastic mesh. The lung cell suspension was centrifuged through a discontinuous Percoll (Pharmacia) gradient, then washed with MEM. Cells were resuspended in complete medium and incubated for 1 h in a tissue culture flask to attach adherent cells. The non-adherent cell population was then resuspended and used in the ELISPOT assay.

Antigen-specific ELISPOT assay. An enzyme-linked immuno-

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Results

Kinetics of BHV-1 replication in cotton rats

Before inoculation in vivo, we determined whether BHV-1 could replicate in CRL cells in vitro. The growth of BHV-1 in CRL cells was comparable to that observed in MDBK cells (Fig. 1). In both cell lines, isolate 108 replicated to a slightly higher titre than the laboratory Cooper strain (Fig. 1).

Since BHV-1 could replicate efficiently in CRL cells in vitro, we inoculated cotton rats i.n. with the two different strains of BHV-1 and monitored virus growth in the respiratory tract. Virus recovery from the trachea and lung showed increasing titres of the 108 strain of BHV-1, resulting in a peak between days 1 and 2 post-infection (p.i.) (Fig. 2). From day 2 onwards, virus titres of both the Cooper and 108 strains declined and the virus was completely cleared by day 8 in all animals. The cotton rats did not show any apparent signs of disease or change in behaviour at any stage of the BHV-1 infection period. However, they developed cellular and humoral immune responses to BHV-1 glycoproteins by day 8 p.i. (data not shown).

Histopathological and immunohistochemical

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

To demonstrate virus-induced cell damage arising from BHV-1 infection and replication in the cotton rat lung, sections of lung 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 summarized in Fig. 3 (a, b). No lesions were seen in the lungs of rats killed at 6 h following infection. Randomly, rare intra-
alveolar macrophages and indistinct intranuclear inclusion bodies in bronchiolar epithelial cells were seen on day 1 p.i. By day 2, rats infected with the BHV-1 108 strain exhibited multifocal moderate bronchiolitis with presence of intranuclear inclusion bodies in bronchiolar epithelial cells, epithelial cell necrosis and infiltration of neutrophils into the mucosa and lumen (Fig. 3c). 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, was also seen (Fig. 3d). An inflammatory response, mild within the airways (one of three rats) and moderate in the alveoli (three of three 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.

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 the BHV-1 antigens at 6 h. By day 1, in all three rats infected there were several small, positively staining foci of bronchiolar epithelial cells, and in one animal alveolar epithelial cells were also positive. Such foci indicating BHV-1 replication 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 p.i. Several to many moderately sized foci of positively 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. 3e, f). 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.

**Antibody response after immunization**

Groups of cotton rats (three animals per group) were inoculated twice i.n., 3 weeks apart, with $10^7$ p.f.u. of the gD-dE1E3 or the gD-dE3 recombinant adenoviruses. Separate groups of cotton rats (four animals per group) were immunized with $10^7$ p.f.u. of gD-dE1E3 or gD-dE3 by the intraduodenal route, and then boosted with a second dose of the same preparation orally 3 weeks later (g.i. immunization groups). Control animals were inoculated with PBS. Evans blue dye was
Fig. 3. Histological changes and immunohistochemical staining in the lungs of cotton rats inoculated with BHV-1. Lungs were collected at various time-points after i.n. infection with \(5 \times 10^7\) p.f.u. of Cooper or 108 strains and evaluated for histological lesions in the (a) bronchioli and (b) alveoli. Lesions were scored according to severity on a scale of 0 (normal) to 3 (severe). Each bar denotes a mean of scores for three animals at 6 h, days 1 and 4 p.i., and for two animals at days 2 and 8 p.i. Lesions (c, d) and immunohistochemical (IHC) staining (e, f) shown are at day 2 p.i. with the 108 strain. Note (c) epithelial necrosis and luminal debris in a bronchiole (bar, 10 \(\mu m\)) and (d) marked cellular infiltration of alveolar septa (bar, 40 \(\mu m\)). Inset: intranuclear inclusion body in a desquamated alveolar epithelial cell (bar, 5 \(\mu m\)). Positive IHC staining (arrows) was noted multifocally in (e) the bronchiolar epithelium (bar, 150 \(\mu m\)) and (f) the alveolar walls (bar, 150 \(\mu m\)).

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.

To determine antigen-specific systemic humoral responses, serum antibody titres specific for gD and adenovirus were measured 3 weeks after the secondary immunization (Fig. 4). Both gD-dE3 and gD-dE1E3 induced gD-specific IgG and IgA in the serum regardless of the route of administration. The gD-dE3 virus, however, induced significantly higher levels of gD-specific IgG in the serum than did gD-dE1E3 (\(P < 0.05\)). All
four immunization regimens stimulated similar levels of adenovirus-specific IgG in the serum (data not shown).

To measure biological activity of the gD-specific serum antibody, BHV-1 SN titres were determined. Immunization by the i.n. or the g.i. routes with gD-dE3 induced a BHV-1 log10 SN titre of 1.5 ± 0. In contrast, the gD-dE1E3 virus induced a log10 SN titre of 0.7 ± 0.3 following i.n., and no detectable titre following g.i. immunization.

**Antibody production in the respiratory tract**

To determine the ability of the different immunization regimens to induce antigen-specific humoral immune responses in the respiratory tract, lung and trachea extracts were analysed for the presence of gD- and adenovirus-specific antibody. Immunization 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 immunization with the gD-dE1E3 virus and the PBS control (Fig. 4). 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 (Fig. 4).

**Protection of cotton rats against BHV-1 challenge**

Since mucosal immunization with the recombinant adenoviruses induced immune responses in the serum and the respiratory tract, we assessed their ability to confer protection against BHV-1 challenge. Cotton rats were challenged with 5 × 105 p.f.u. of the 108 strain of BHV-1 i.n., 3 weeks after secondary immunization. Table 1 shows the results of BHV-1 recovery from the trachea and the lung 24 h after BHV-1 challenge. Immunization with the gD-dE3 vector i.n. resulted in complete reduction of BHV-1 titres in the lungs of two out of three animals (different from PBS controls, P < 0.05) and partial reduction of BHV-1 titres in one animal. In addition, g.i. immunization with the gD-dE3 virus partially reduced BHV-1 titres in the respiratory tract of three out of four animals. In contrast, gD-dE1E3 induced a partial reduction in mean BHV-1 titres only in the trachea compared to the PBS control, regardless of the route of immunization.

**Immunity and protection induced by i.n. administration of gD-dE3**

In order to confirm protection against BHV-1 challenge and to further characterize the antigen-specific immunity induced by i.n. administration of gD-dE3, groups of cotton rats were inoculated twice i.n. with 10⁸ p.f.u. of gD-dE3 (14 animals per group) or with PBS (12 animals per group), 3 weeks apart. Seven animals from each group were challenged with BHV-1 3 weeks after the second immunization, and the other animals were challenged 12 weeks after the second immunization.

G-specific serum IgG and IgA levels at different time-points following immunization are shown in Fig. 5. Serum antibody specific for gD was induced by the primary immunization with gD-dE3 and its level increased slightly following the secondary immunization. Serum levels of gD-specific IgG and IgA (Fig. 5) and BHV-1 log10 SN titres of 1.6 were maintained during the 12-week period, as well as the levels of antigen-specific antibodies in lung extracts (data not shown). The kinetics of adenovirus-specific responses were similar to gD-specific antibody responses (data not shown).

**Table 1. Effect of mucosal immunization with adenovirus vectors on the protection of cotton rats against intranasal BHV-1 challenge**

Animals were immunized twice with the adenovirus vectors and challenged with BHV-1. Trachea and lung homogenates obtained 1 day after challenge were tested for the presence of BHV-1.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Route</th>
<th>Trachea log 10 BHV-1 titres</th>
<th>Lung log 10 BHV-1 titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS control</td>
<td>Intranasal</td>
<td>4.68 ± 1.15</td>
<td>5.12 ± 0.15</td>
</tr>
<tr>
<td>gD-dE1E3</td>
<td>Intranasal</td>
<td>2.79 ± 0.55</td>
<td>5.22 ± 0.18</td>
</tr>
<tr>
<td>gD-dE1E3</td>
<td>Gastrointestinal</td>
<td>3.09 ± 0.45</td>
<td>5.10 ± 0.27</td>
</tr>
<tr>
<td>gD-dE3</td>
<td>Intranasal</td>
<td>1.00 ± 1.22</td>
<td>0.70 ± 0.86</td>
</tr>
<tr>
<td>gD-dE3</td>
<td>Gastrointestinal</td>
<td>2.89 ± 1.22</td>
<td>3.48 ± 0.76</td>
</tr>
</tbody>
</table>

Fig. 4. Antibody responses in cotton rats. (a) IgG and (b) IgA titres specific for gD in trachea, lung extracts and serum 3 weeks after secondary immunization with adenovirus recombinants were measured by ELISA. Error bars represent the standard error of the mean of three to four animals per group.
Recombinant adenoviruses and BHV-1

Fig. 5. Kinetics of gD-specific antibody responses in the serum. Serum samples were collected at different time-points following i.n. administration of gD-dE3. IgG (●) and IgA (■) titres were measured by ELISA. Error bars represent the standard deviation of log_{10} antibody titres for seven animals per group. Control animals maintained background levels of antibody during the study (not shown) equal to that of gD-dE3-immunized animals at day 0.

To determine whether antigen-specific ASC were maintained locally in the lung or in peripheral lymphoid tissues in the cotton rat, ELISPOT assays were performed 12 weeks following immunization. Both gD- and adenovirus-specific ASC were found in the lung, bone marrow and spleen of gD-dE3-immunized animals (Table 2). The number of ASC in the lung 12 weeks after immunization was similar to that in the lung of animals 3 weeks following immunization (data not shown).

To assess the protective ability of i.n. immunization with gD-dE3 against BHV-1 infection, lungs of cotton rats were examined for evidence of BHV-1 replication 3 days after challenge. Virus was isolated from lungs of control animals but not from immunized animals challenged at either 3 weeks or 12 weeks following the second immunization (Table 3). Lungs of cotton rats in the control groups had lesions of a multifocal mild interstitial pneumonia with type II pneumocyte proliferation and infiltration of macrophages. Small foci positive for BHV-1 antigen were also demonstrated randomly, usually (but not invariably) associated with foci of interstitial pneumonia. In contrast, lungs from the two groups of immunized cotton rats did not have any lesions or BHV-1 replication foci, indicating significant protection following vaccination.

Discussion

Since vaccination-challenge studies are expensive in cattle, it is important to evaluate potential vaccine candidates in an experimental animal model. To establish the BHV-1 i.n. challenge model in cotton rats, we demonstrated that 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 necrotizing bronchiolitis with the presence of intranuclear inclusion bodies in epithelial cells, were consistent with lesions observed following BHV-1 infection in cattle (Jéricho & Darcel, 1978). Reviewing the evidence on pulmonary histopathological changes in cattle, Yates (1982) found the lesions to be highly variable and inconsistently present, both in natural and experimental infections. In the cotton rats, immunohistochemical staining confirmed that BHV-1 became established in the lung and was the cause of the changes seen, as specific virus staining was

Table 2. Frequency of gD- and adenovirus-specific antibody-secreting cells in lymphocytes from different tissues of cotton rats immunized intranasally with recombinant adenoviruses

<table>
<thead>
<tr>
<th>Lymphocyte source</th>
<th>Immunization</th>
<th>gD-specific ASC/10^6*</th>
<th>Adenovirus-specific ASC/10^6*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgA</td>
<td>IgG</td>
</tr>
<tr>
<td>Lung</td>
<td>PBS control</td>
<td>600</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>gD-dE3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone marrow</td>
<td>PBS control</td>
<td>14</td>
<td>ND†</td>
</tr>
<tr>
<td></td>
<td>gD-dE3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Spleen</td>
<td>PBS control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>gD-dE3</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PBS control</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

* Mean values of the number of antigen-specific IgG and IgA committed ASC per 10^6 lymphocytes were calculated from cultures of pooled cell populations.
† ND, Not detected (less than 1 ASC/10^6).
suggested that the two types of vector may be equally effective replication-competent recombinant adenoviruses have sug-
immunization studies comparing replication-defective and replication-competent viruses with regard to their effectiveness in inducing immunity to gD and conferring protection against BHV-1 challenge. Systemic immunization studies comparing replication-defective and replication-competent recombinant adenoviruses have suggested that the two types of vector may be equally effective for the induction of antigen-specific immune responses (Eloit & Adam, 1995; Mittal et al., 1996). Eloit & Adam (1995) observed, however, that immunization with a replication-defective adenovirus expressing gD of pseudorabies virus (PRV) induced protection of cotton rats and mice against PRV challenge only at much higher doses of immunization than with a replication-competent vector. Furthermore, oral immunization with replication-competent adenovirus expressing rabies glycoprotein induced antibody responses and partial protection against experimental rabies infection in mice, while replication-defective virus did not (Prevec et al., 1990; Xiang et al., 1996). However, no studies have directly compared the efficacy of replication-defective and replication-competent adenoviruses expressing the same foreign gene in inducing mucosal and systemic immunity following mucosal administration.

In the present study, we found that i.n. administration of the replication-competent gD-dE3 virus induced gD-specific antibody responses and resulted in complete protection of the lungs of most cotton rats against i.n. challenge with BHV-1. The replication-defective gD-dE1E3 virus, however, induced significantly lower levels of antibody than gD-dE3 and resulted in only a partial reduction of BHV-1 titres compared to controls. Previous experiments have shown gD-dE1E3 and gD-dE3 to be similar in their ability to induce immune response and partial protection to BHV-1 challenge following intradermal administration (Mittal et al., 1996). These observations, together with the results of the present study, suggest that the route of immunization is crucial when assessing the efficacy of recombinant adenoviruses as vaccine vectors. Intradermal inoculation with the replication-defective virus may have allowed for a more efficient expression of gD in the skin, and possibly other tissues, than mucosal administration. Alternatively, the higher level of gD-specific antibody responses induced by gD-dE3 compared to gD-dE1E3 following mucosal

<table>
<thead>
<tr>
<th>Weeks following immunization</th>
<th>Immunization</th>
<th>Virus isolation (log&lt;sub&gt;10&lt;/sub&gt; p.f.u./g lung tissue)</th>
<th>BHV-1-specific foci</th>
<th>Interstitial pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>gD-dE3</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>12</td>
<td>PBS control</td>
<td>5.086 ± 0.286</td>
<td>7/7</td>
<td>7/7</td>
</tr>
<tr>
<td>12</td>
<td>gD-dE3</td>
<td>ND</td>
<td>0/7</td>
<td>0/7</td>
</tr>
<tr>
<td>12</td>
<td>PBS control</td>
<td>3.815 ± 0.941</td>
<td>4/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, Not detected (< 10 p.f.u./lung).
administration may be the result of a more invasive infection by gD-dE3 than gD-dE1E3, allowing for higher gD-expression in the mucosa-associated lymphoid tissue and in draining lymph nodes following mucosal infection with gD-dE3. Finally, the ability of the gD-dE3 virus to produce larger amounts of gD than the gD-dE1E3 virus during the first day of infection of CRL cells in vitro (unpublished observation) may influence the immune responses induced.

Induction of mucosal immunity may be crucial in protecting a host from a respiratory virus infection. Mucosal antibody, such as secretory IgA, has usually been found to correlate with resistance to such infections (Kraehenbuhl & Neutra, 1994; Murphy, 1994). Our results are consistent with reports in the literature: the highest levels of IgA in trachea and lung extracts correlated with the best protection against BHV-1 challenge in animals immunized i.n. with gD-dE3 (Fig. 4). Furthermore, higher levels of IgA in the respiratory tract compared to those in the serum, and the presence of ASC in the lung, suggest that antibody was locally produced in the lung.

Interestingly, antigen-specific ASC in the lung were present at similar levels at 12 weeks following immunization as at 3 weeks following immunization. Serum antibody titres were also maintained (Fig. 5) and ASC in the bone marrow (Table 3) were detected. Others have found that long term humoral response and even protection from mucosal virus challenge could be achieved following live virus administration to the mucosa (Gallichan et al., 1993; McNeal & Ward, 1995). This may be due to ASC maintained for the lifetime of rodents in the bone marrow and in the draining mediastinal lymph nodes following respiratory virus infection (Hyland et al., 1994). These observations are interesting because mucosal virus infections usually stimulate immunity of shorter duration than systemic infections (Slifka & Ahmed, 1996). Certain viruses, such as adenoviruses, may be unique in that they can induce long-term mucosal and systemic immune responses following mucosal administration. This may be associated with their ability to spread systematically (Huard et al., 1995; Oualikene et al., 1994) even though they primarily infect mucosal tissues.

Similarly to i.n. immunization, i.g. administration of gD-dE3 could also induce gD-specific antibody in the serum and in lung and tracheal extracts. In addition, intraduodenal–oral administration of gD-dE3 induced ASC in the lung (unpublished observation). Only partial protection was observed, however, against BHV-1 challenge in these animals. This finding is in accordance with other reports showing that i.n. delivery of live virus is often more effective than gastroenteric administration (Collins et al., 1990; Kanesaki et al., 1991; Meitin et al., 1994). Perhaps local induction of antigen-specific immune responses in the lung is necessary to achieve complete protection.

Observations that gastroenteric immunization with live viruses can induce protective immunity in the respiratory tract are thought to support the concept of a common mucosal immune system (Mestecky et al., 1994). Antibody induced in the respiratory tract after g.i. immunization with adenoviruses, however, cannot be exclusively explained by the migration of antigen-specific lymphocytes to remote mucosal sites, since systemic adenovirus dissemination can take place following either the intraduodenal or oral route of administration of gD-dE1E3 and gD-dE3. This results in adenovirus particles in the lungs as well as other organs (unpublished data). Whether immune responses induced in the respiratory tract by the g.i. route of immunization with gD-dE3 are mainly due to the migration of lymphocytes primed in the gut, or whether the spread of the virus to other mucosal sites is also involved, requires further study.

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