Mucosal immunization of calves with recombinant bovine adenovirus-3: induction of protective immunity to bovine herpesvirus-1

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To determine the potential of replication-competent (E3-deleted) bovine adenovirus-3 (BAV-3) as a delivery system for vaccine antigens in calves, we evaluated the ability of recombinant BAV-3 expressing different forms of bovine herpesvirus-1 (BHV-1) glycoprotein gD to protect against BHV-1 infection in calves that had pre-existing BAV-3 specific antibodies. Three- to four-month-old calves, vaccinated intranasally with recombinant BAV-3 expressing full-length gD (BAV3.E3gD) or a truncated version of gD (gDt) (BAV3.E3gDt), or with E3-deleted BAV-3 (BAV3.E3d; control), were challenged with BHV-1 strain 108. Vaccination with BAV3.E3gD or BAV3.E3gDt induced gD-specific antibody responses in serum and nasal secretions, and primed calves for gD-specific lymphoproliferative responses. In addition, all calves developed complement-independent neutralizing antibodies against BHV-1. Protection against viral challenge was observed in calves vaccinated with recombinant BAV3.E3gD or BAV3.E3gDt as shown by a significant reduction in body temperature and clinical disease, and a partial reduction in the amount and duration of virus excretion in nasal secretions. These results indicate that replication-competent BAV-3-based vectors can induce protective immune responses in calves (the natural host) that have pre-existing BAV-3-specific antibodies.

Introduction

Vaccination has proved to be an effective means of controlling respiratory viral diseases. Although the control of viral diseases in veterinary medicine conventionally uses killed (KV) or modified live viral (MLV) vaccines, these are far from ideal. MLV vaccines may cause abortions, immunosuppression and establish latent infection, and KV vaccines do not provide complete protection.

Recent efforts have concentrated on developing genetically engineered vaccines, which are safe, economical and efficacious (Babiuk et al., 1996). One way to produce vaccines is by developing genetically engineered virus genomes as vectors to express and deliver genes of other pathogens in vivo (Ertl & Xiang, 1996).

Since recombinant human adenoviruses have been proven to be excellent mucosal vaccine vectors (Imler, 1995), we have been studying bovine adenovirus-3 (BAV-3) with the aim of using it as a live viral vector for bovine vaccines. BAV-3, a representative of subgroup I of BAVs (Bartha, 1969), has been shown to replicate in the respiratory tract of cattle producing mild or no clinical symptoms (Darbyshire, 1968). Analysis of the complete DNA sequence of BAV-3 suggested that the size and overall organization of the BAV-3 genome was similar to human adenovirus-5 (HAV-5) except for a few differences (Reddy et al., 1998). To facilitate the development of BAV-3 as a vector, we recently constructed a replication-competent BAV-3 in which 1-245 kb of the E3 region was deleted (Zakhartchouk et al., 1998).

To test the feasibility of using replication-competent BAV-3 as a live viral vector, we used bovine herpesvirus-1 (BHV-1) infection of calves as a disease model. BHV-1 causes a number of clinical manifestations in cattle (Yates, 1982). Any of the major glycoproteins (gB, gC and gD) can induce some degree of immune protection against disease caused by BHV-1 infection in cattle (Babiuk et al., 1987; Israel et al., 1992). However, as gD is a major target of neutralizing antibodies, efforts have centred primarily around developing and using recombinant forms of gD as a sub-unit vaccine (van Drunen
Previously, calves inoculated intranasally with wild-type BAV-3 did not show any clinical symptoms (S. K. Mittal and A. N. Zakhartchouk and others). The 108 strain of BHV-1 was propagated and quantified as described previously (Rouse & Babiuk, 1974).

We have previously constructed a replication-competent BAV-3 expressing full-length and truncated forms of BHV-1 gD, and showed that intranasal immunization of cotton rats with these recombinant viruses induces both gD-specific mucosal and systemic immune responses (Zakhartchouk et al., 1998). In this report, we describe the usefulness of recombinant BAV-3 in the induction of antigen-specific protective immune responses when administered intranasally in calves (the natural host).

**Methods**

**Cells.** Madin–Darby bovine kidney (MDBK) cells were grown in Eagle’s minimum essential medium supplemented with 5% foetal bovine serum (FBS). The wild-type (WBR-1 strain) and recombinant BAV-3 were propagated in MDBK cells as described previously (Mittal et al., 1995). The 108 strain of BHV-1 was propagated and quantified as described previously (Rouse & Babiuk, 1974).

**Recombinant BAV-3.** The construction of recombinant BAV3.E3d, BAV3.E3gD and BAV3.E3gDt has been described previously (Zakhartchouk et al., 1998). Briefly, recombinant BAV3.E3d contains a 1-245 kb deletion in the E3 region (Zakhartchouk et al., 1998). The recombinant BAV3.E3gD and BAV3.E3gDt contain genes encoding the full-length (Tikoo et al., 1990) and truncated form (Tikoo et al., 1993) of BHV-1 gD, respectively. These genes have been inserted in the E3 region of the BAV3.E3d genome in the same transcriptional orientation as E3 without any exogenous regulatory elements (Fig. 1).

**Immunization and challenge.** The calf experiments were carried out in accordance with the regulations of the Canadian Council for Animal Care and the calves involved were housed in high-security isolation rooms throughout these experiments. Nine BHV-1-negative calves (3–4 months old) were randomly allocated into three groups, with three calves in each group. At day 0, the calves were exposed intranasally for 5 min to an aerosol of 10⁶ p.f.u. BAV-3.E3d, BAV3.E3gD or BAV3.E3gDt using a Devilbiss Nebulizer, model 65 (Devilbiss Barry, Ontario, Canada). Twenty-eight days later the calves were boosted. Two weeks later the calves were challenged with an aerosol of 10⁷ p.f.u./ml of BHV-1 strain 108.

Calves were bled at the times of the first immunization, booster immunization and BHV-1 challenge as well as 10 days after challenge for analysis of antibody responses and lymphoproliferative responses.

**Virus isolation.** Nasal secretions were collected using nasal swabs every third day for 10 days post-primary immunization, and every alternate day for 10 days post-BHV-1 challenge. BAV-3 was recovered from nasal fluids and quantified by the 50% endpoint method (Graham & Prevec, 1991). BHV-1 was recovered from nasal fluids and quantified by plaque titration in 24-well plates with 0.5% agarose overlay.

**Clinical evaluation.** Calves were clinically evaluated by a veterinarian for 10 days following challenge. The clinician was blind to vaccine groups. Rectal temperatures, nasal scores and clinical scores were measured daily. Nasal and clinical score were given between 0 (normal) and 4 (severe). The clinical score was based on levels of conjunctivitis and rhinitis.

**ELISA.** BHV-1-gD- and BAV-3-specific antibodies were determined in sera and nasal secretions by ELISA as described previously (van Drunen Littel-van den Hurk et al., 1997). Briefly, 96-well Immulon-2 microlitre plates were coated with either purified truncated gD (0.01 µg per well) or BAV-3 (0.5 µg per well) and incubated with serial dilutions of each sample. Antigen-specific IgG was detected by alkaline phosphatase-conjugated goat anti-bovine IgG (Kirkegaard and Perry Laboratories) followed by p-nitrophenyl phosphate (PNPP). Antigen-specific IgA was measured by incubating with monoclonal anti-bovine IgA (provided by K. Nielson, Agriculture Canada, Animal Disease Research Institute, Nepean, Ontario, Canada) followed by goat antimouse IgG–HRP conjugate. The reaction was visualized with ABTS [2,2’-azino bis(3-ethylbenzthiazoline-6-sulfonic acid, dia monium salt) substrate.

**Virus-neutralization test.** Doubling dilutions of heat-inactivated serum samples were mixed with 100 p.f.u. BHV-1 and incubated for 1 h at 37 °C. The virus–serum mixture was plated on a confluent monolayer of MDBK cells in 24-well plates and a 0.5% agarose overlay. After 2 days incubation, titres were expressed as reciprocals of the highest serum dilution that caused a 50% reduction in the number of plaques relative to the control well.

**Proliferation assay.** Bovine blood was collected into citrate–dextan and peripheral mononuclear cells (PBMC) were isolated on 60% Percoll (Pharmacia Biotech). PBMCs were dispensed to 2 × 10⁶ cells/ml into a culture consisting of AIM-V medium (Gibco/BRL) supplemented with 2% FBS and 200 µM 2-mercaptoethanol (Sigma), and 5 × 10⁵ p.f.u./ml BAV-3 or purified gD at 2 µg/ml was added in a 100 µl volume to triplicate wells. After 3 days in culture the wells were incubated with 0.4 µCi/ml [H³]thymidine (Amersham) for 6 h. The cells were harvested and thymidine uptake was measured by scintillation counting. Proliferative responses were calculated as the mean value of triplicate cultures and was expressed as a stimulation index (SI; counts/min in the presence of antigen divided by counts/min in the absence of antigen). A stimulation index of > 2 was considered positive.

**Preparation of lymphocytes from tonsils and lymph nodes.** Aseptically removed nasopharyngeal tonsils, platinine tonsils and retropharyngeal lymph nodes were teased through nylon mesh screens. The isolated cells were centrifuged through a discontinuous Percoll gradient and washed with Hanks’ buffered salt solution. The number of gD-specific IgA antibody-secreting cells present in the above tissues and isolated PBMCs were determined by an ELISPOT as described by Czerkynski et al. (1983) and Papp et al. (1997).

**Statistical analyses.** All data were analysed with the aid of the GraphPad Prism program (GraphPad Software)

**Results**

Pathogenicity of recombinant BAV-3 in calves

Previously, calves inoculated intranasally with wild-type BAV-3 did not show any clinical symptoms (S. K. Mittal and A. N. Zakhartchouk and others). The 108 strain of BHV-1 was propagated and quantified as described previously (Rouse & Babiuk, 1974). The 108 strain of BHV-1 was propagated and quantified as described previously (Rouse & Babiuk, 1974).
others, unpublished). In order to determine if an E3 deletion modulates the pathogenesis of BAV-3, calves were inoculated intranasally with an aerosol of $10^8$ p.f.u. BAV3.E3d, BAV3.E3gD or BAV3.E3gDt. Calves were examined daily for clinical signs of disease. None of the calves showed any clinical symptoms including temperature increases (data not shown). One of the calves shed BAV-3 for 6 days post-primary immunization as determined by plaque assay and PCR analysis (data not shown). These results suggest that: (a) BAV-3 may establish inapparent infection with virus excretion, and (b) neither E3 deletion nor gD gene insertion affected the pathogenesis of BAV-3 in calves.

**Immune response in calves**

To determine the ability of BAV-3 recombinants to induce BAV-3- or gD-specific immune response, calves were twice inoculated intranasally, 4 weeks apart, with $10^8$ p.f.u. BAV3.E3d, BAV3.E3gD or BAV3.E3gDt. Serum samples were collected at days 0, 28 and 40 post-inoculation and tested for anti-BAV-3 IgG by ELISA. As seen in Fig. 2, all calves had pre-existing BAV-3 serum IgG antibodies. After the first immunization, there was an increase in the BAV-3-IgG antibody titre. However, there was no difference in the BAV-3-IgG antibody titre between the 28th and 40th day following primary immunization. In addition, serum samples collected at day 0 showed a range of BAV-3 neutralization titres from 320 to 1260.

Serum samples collected at days 0, 28, 40 and 52 were tested for gD-specific IgG antibodies by ELISA, and BHV-1-neutralizing antibodies by a neutralization assay. As seen in Fig. 3A, calves immunized with BAV3.E3gD or BAV3.E3gDt showed gD-specific IgG titres after the first immunization. After the second immunization, all vaccinated calves had significant levels of BHV-1 gD-specific IgG whereas the control calves (immunized with BAV3.E3d) did not. Similarly, calves immunized with BAV3.E3gD or BAV3.E3gDt de-
Fig. 4. Frequency of gD-specific IgA antibody-secreting cells (SC). Calves were immunized intranasally with BAV3.E3d (hatched bars), BAV3.E3gD (white bars) or BAV3.E3gDt (black bars) at days 0 and 28 and subsequently challenged with BHV-1 at day 42 post-primary immunization. Lymphocytes were isolated from retropharyngeal lymph nodes (LN), nasopharyngeal tonsils (NPhT) and PBMCs and used in an ELISPOT assay as described in the text. Results are expressed as the mean no. gD-specific IgA-secreting cells per $5 \times 10^5$ cells. Error bars represent the standard deviation of the means.

Developed significantly higher ($P < 0.05$) BHV-1-neutralizing antibody responses than control calves after two immunizations (Fig. 3B). No significant difference in antibody titre was observed between BAV3.E3gD- and BAV3.E3gDt-immunized calves.

To assess the mucosal immune responses, nasal secretions collected at days 0, 28, 40 and every alternate day after challenge were analysed for gD-specific IgA antibodies by ELISA (Fig. 3C). No detectable level of gD-specific IgA was found in calves after the first immunization. However, after the second immunization with BAV3.E3gD or BAV3.E3gDt, calves developed significantly higher ($P < 0.05$) gD-specific IgA response than control calves (immunized with BAV3.E3d). Both BAV3.E3gDt and BAV3.E3gD induced similar IgA responses. Analysis of the kinetics of gD-specific IgA responses after BHV-1 challenge showed that there was a dramatic increase in gD-specific IgA in the nasal secretions from the 4th to 10th day post-challenge (Fig. 3D). In addition, a significant number of gD-specific IgA-secreting cells were also detected in nasopharyngeal tonsils of calves immunized with BAV3.E3gD and BAV3.E3gDt at day 52 post-immunization (Fig. 4).

To determine the specificity of the immune response, ELISA titres to BHV-1 gB were determined. All calves immunized with BAV3.E3gD or BAV3.E3gDt had gD-specific antigen response in the nasal secretions from the 4th to 10th day post-challenge (Fig. 3D). In addition, a significant number of gD-specific IgA-secreting cells were also detected in nasopharyngeal tonsils of calves immunized with BAV3.E3gD and BAV3.E3gDt at day 52 post-immunization (Fig. 4).

**Antigen-specific proliferation**

To determine the presence of a gD-specific cellular immune response, PBMCs were isolated from BAV3.E3gD-, BAV3.E3gDt- and BAV3.E3d-immunized calves 28 days after the first immunization. Antigen-specific responses were measured by cellular proliferation after *in vitro* stimulation of PBMCs with gD. As shown in Table 1, the calves immunized with BAV3.E3gD or BAV3.E3gDt developed a significant proliferative response whereas the BAV3.E3d-immunized calves did not.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Stimulation index*</th>
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<tbody>
<tr>
<td>BAV3.E3d</td>
<td>$1.33 \pm 0.03$</td>
</tr>
<tr>
<td>BAV3.E3gD</td>
<td>$6.3 \pm 2.6$</td>
</tr>
<tr>
<td>BAV3.E3gDt</td>
<td>$11.7 \pm 5.1$</td>
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* Measured 28 days after the first immunization.

Table 1. gD-specific proliferation of PBMCs from calves immunized with BAV-3 recombinants

Fig. 5. Specificity of the immune response of calves immunized with BAV-3 recombinants. (A) gB-specific IgG ELISA titres in sera of calves immunized with BAV3.E3d (■), BAV3.E3gD (△) or BAV3.E3gDt (○). Error bars represent the standard deviation of the mean. (B) Reactivity of bovine sera tested at a 1:10 dilution in a Western blot after two immunizations with BAV3.E3d (lane 1), BAV3.E3gD (lane 2) or BAV3.E3gDt (lane 3). The control included BHV-1 specific hyperimmune sera tested at a 1:100 dilution (lane 4).
Protection from challenge with BHV-1

Fourteen days after the second immunization, calves were challenged by an intranasal aerosol of $10^7$ p.f.u. of BHV-1 per animal. Following challenge, calves were clinically evaluated for temperature, depression, appearance and severity of nasal lesions. For 10 days post-challenge, the mean rectal temperature of the BAV3.E3d-immunized calves exceeded that of the calves immunized with BAV3.E3gD or BAV3.E3gDt (Fig. 6B). The differences were significant ($P < 0.05$) between BAV3.E3gD- and BAV3.E3d-immunized calves on days 3–6, 8 and 9 post-challenge. There was also a significant difference between BAV3.E3gDt- and BAV3.E3d-immunized calves on days 4–6 post-challenge. There was no significant difference between BAV3.E3gD and BAV3.E3gDt.

Clinical examination of the calves demonstrated that the nasal scores of calves immunized with BAV3.E3d showed a sharp increase during the first 6 days post-challenge and did not return to pre-challenge values by day 10, the end of the trial (Fig. 6D). However, the nasal scores of calves immunized with BAV3.E3gD or BAV3.E3gDt did not show much of an increase, except on day 5 in BAV3.E3gD-immunized calves (Fig. 6D). In addition, calves immunized with BAV3.E3d were mildly depressed (6A).

In order to determine whether the calves were protected from viral infection, the extent of virus shedding from the nose was also assessed. As shown in the Fig. 6C, there was no significant difference in virus shedding between the groups for the first 4 days post-challenge. However, the BAV3.E3gD- and BAV3.E3gDt-immunized calves shed significantly less ($P < 0.05$) virus than the BAV3.E3d-immunized calves on days 6 and 8 post-challenge.

Discussion

Although recombinant human adenoviruses have been shown to be effective vectors for the delivery of vaccine antigens to animals (Callebaut et al., 1996; Prevec et al., 1989; Torres et al., 1996), their use as a vaccine-delivery system in domestic animals is limited in part due to safety concerns. As non-human adenoviruses are species specific, the development of animal-specific adenovirus would be a logical choice. Recently, we reported the construction and characterization of replication-competent recombinant BAV-3 expressing BHV-1 gD or gDt (Zakhartchouk et al., 1998). In this report, we investigated the potential of intranasal immunization with recombinant BAV-3 for inducing protective immune responses against BHV-1 challenge. While animal studies using recombinant human adenoviruses have been reported (Callebaut et al., 1996; Mittal et al., 1996; Prevec et al., 1989), the present study differs in two aspects. First, the experiment was done in calves, the natural host of BAV-3. Secondly, the calves...
contained significant levels of pre-existing BAV-3 specific antibodies, a situation similar to field conditions.

One of the salient findings of the present study is that calves immunized with recombinant BAV-3 showed a significant increase in both BAV-3- and gD-specific immune responses, despite containing preexisting neutralizing antibodies against BAV-3. In addition, recombinant BAV-3 excretion was observed only in one animal at day 6 post-immunization. These results strongly suggest that the presence of vector-specific antibodies should not hinder the use of recombinant BAV-3 vectors for immunization in calves.

Intranasal immunization of calves with BAV3.E3gD or BAV3.E3gDt induced gD-specific immune responses of a similar magnitude. Similarly, intramuscular immunization of calves with purified gD or gDt also induced gD-specific immune responses of a similar magnitude (van Drunen littel-van den Hurk et al., 1997). This is not surprising since all known antigenic epitopes of gD are retained in gDt (Tikoo et al., 1993). However, intradermal immunization of cotton rats with recombinant HAV-5 induced a lower immune response to gDt than to gD (Mittal et al., 1996). In contrast, intranasal immunization of cotton rats with recombinant BAV-3 induced a lower immune response to gD than to gDt (Zakhartchouk et al., 1998). These studies strongly suggest that the ability of gD or gDt to induce an effective immune response may depend on the route of immunization and the animal species used. Whether the immunogen is anchored in the membrane (gD) or secreted as a soluble protein (gDt) may not in itself be exclusively responsible for the immunogenicity of the protein.

Adenoviruses have been shown to be excellent vaccine vectors for the induction of mucosal immunity (Imler, 1995; Rosenthal et al., 1996), which is thought to be crucial in protecting the host from respiratory and enteric infections. Intranasal immunization of calves induced a gD-specific IgA response in the nasal secretions. However, the magnitude of the IgA response was not sufficient to eliminate BHV-1 shedding, but it reduced the duration of shedding. This may suggest that gD/gDt expressed by recombinant BAV-3 was sufficient to prime the antigen-specific IgA response in the nasal passage. Support for this prediction comes from the fact that the IgA titre increased sharply after the 4th to 10th day post-challenge. Immunization of calves with detergent-solubilized virion glycoproteins also produced very low levels of BHV-1-specific IgA (Israel et al., 1992). In addition, this high level of IgA in the nasal secretions and the presence of gD-specific IgA-secreting cells in the nasopharyngeal tonsil and not in the lymph node or PBMC at day 52 suggests that antibody was locally produced in the nasal passage.

To determine the vaccine potential of these BAV-3 vectors, immunized calves were challenged with BHV-1 and observed for development of clinical symptoms (fever, depression, rhinitis, conjunctivitis) and virus excretion. Our results indicate that protection against clinical disease was induced in calves immunized with either BAV3.E3gD or BAV3.E3gDt as shown by significant reduction in temperature, depression and clinical scores. There was no difference in the amount of virus shedding between the groups during the first 4 days post-challenge. Interestingly, no virus shedding was observed in one animal immunized with BAV3.E3gD. Virus shedding was significantly reduced in calves immunized with either BAV3.E3gD or BAV3.E3gDt than in calves immunized with BAV3.E3d after day 4 post-challenge. This reduction in virus shedding correlated with the gD-specific IgA levels in the nasal secretions. This is consistent with a previous report (Papp et al., 1997, 1998) where the highest levels of IgA in the nasal passage correlated with reduced BHV-1 replication.

In conclusion, we have shown that recombinant BAV-3 can induce an antigen-specific immune response in calves containing BAV-3-specific antibodies. In addition, we have shown that intranasal immunization of calves with recombinant BAV-3 expressing BHV-1 gD or gDt partially protected calves from clinical disease and reduced the time of viral shedding. Further work is in progress to improve the levels of mucosal immunity to further reduce virus replication in the nasal passages.

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