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Induction of mucosal immunity in cotton rats to haemagglutinin–esterase glycoprotein of bovine coronavirus by recombinant adenovirus

M. E. BACA-ESTRADA,* X. LIANG,* L. A. BABIUK*† & D. YOO* *Veterinary Infectious Disease Organization and †Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

SUMMARY

An effective vaccine against enteric bovine coronavirus (BCV) must be able to induce mucosal immunity. We recently described the construction of recombinant human adenovirus type 5 (hAd5) carrying the BCV haemagglutinin–esterase (HE) gene in the early transcription region 3 of the adenovirus genome. In this study, we examined the induction of systemic and mucosal immune responses to the hAd5 vector carrying the BCV HE gene (AdBcHE) following intranasal or enteric immunization of cotton rats. Regardless of the route of administration, mucosal immunization with AdBcHE induced significant levels of anti-HE IgG antibodies in serum. In addition, following intranasal immunization with AdBcHE, significant levels of anti-HE IgA antibodies were found in lung washes of immunized cotton rats. Furthermore, the specific anti-HE antibodies in sera and mucosal secretions efficiently neutralized BCV infectivity *in vitro*. T-cell proliferation and cell-mediated cytotoxic responses against the BCV HE were elicited in the spleen of intranasally immunized animals. The results demonstrate that mucosal immunization with AdBcHE is capable of inducing both systemic and mucosal immunity to the BCV HE. These immune responses may be important in protecting animals from BCV infection.

INTRODUCTION

Enteric coronavirus is an important cause of neonatal diarrhoea in calves, resulting in significant economic losses due to the mortality and decreased productivity of the survivors. The membrane glycoprotein haemagglutinin–esterase (HE; 65 000 MW) is found in certain species of coronaviruses, such as bovine coronavirus (BCV),[1–3] haemagglutinating encephalomyelitis virus[4] and human coronavirus OC43.[5] The BCV HE protein has been studied extensively and it appears to be a major membrane-associated glycoprotein thought to be essential for virus multiplication.[6] The BCV HE glycoprotein shows a significant sequence homology with the haemagglutinin protein of human influenza type C virus.[7–9] Monoclonal antibodies directed against the BCV HE glycoprotein efficiently neutralized BCV infectivity *in vitro*[10] and protected the intestinal epithelium of cattle from virus infection *in vivo*, indicating that the HE protein of BCV may play a significant role in the initiation of BCV infection in animals.[11]

The immune mechanisms involved in protection against enteric coronavirus in cattle are not yet fully understood. It has been suggested that passive transfer of BCV neutralizing antibodies through colostrum protects newborn calves against the disease.[12] However, susceptibility to enteric coronavirus infection occurs when maternal antibody titres in the newborn calf decline,[12] indicating that stimulation of active mucosal immunity may be needed to confer long-term protection in these animals.

Adenovirus has been proven effective in inducing mucosal immunity and in preventing acute respiratory disease in US Army recruits when administrated orally.[13,14] Based on these observations adenoviruses appear to be an ideal mucosal delivery system for vaccine antigens. Indeed, the effectiveness of recombinant adenovirus vectors in inducing mucosal immunity has been demonstrated in numerous systems.[15–18] Recently, Gallichan *et al.*[19] demonstrated that intranasal immunization of mice with recombinant adenovirus expressing herpes simplex virus glycoprotein B induced mucosal and systemic immune responses and provided protection from intranasal challenge.

We have recently described the construction of recombinant human adenovirus type 5 (hAd5) carrying the BCV HE gene in the early transcription region 3 of the adenovirus genome (AdBcHE).[20] Evaluation of recombinant adenovirus in experimental animals has been difficult due to the restricted host range of human adenovirus. However, cotton rats have been shown to support the replication of human adenovirus[21] and have been used as a model to study the pathogenesis of adenovirus pneumonia.[22] Indeed, efficient replication of human recombinant adenovirus in the airway epithelium of cotton

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Correspondence: Dr M. E. Baca-Estrada, Veterinary Infectious Disease Organization, 124 Veterinary Road, Saskatoon, Saskatchewan, Canada S7N 0W0.

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rats mediated the expression of the human cystic fibrosis transmembrane conductance regulator gene product and the α-1-antitrypsin protein.[23,24] These observations support the use of the cotton rat model to assess the effectiveness of adenovirus vectors as mucosal vaccine delivery systems. In the present study we examined the induction of mucosal and systemic immune responses to the BCV HE glycoprotein following intranasal and enteric immunization of cotton rats with AdBcHE.

MATERIALS AND METHODS

Cells and viruses

HAd5 (dl309, deleted for the E3 gene) and recombinant AdBcHE were propagated and titrated in human lung epithelial carcinoma A549 cells or 293 cells in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco), amino acids and vitamins (Flow Laboratories, McLean, VA). BCV was grown in Mardin–Darby bovine kidney cells (MDBK) in minimal essential medium (MEM) containing 10% FBS. BCV plaque assay was performed in Georgia bovine kidney (GBK) cells. For animal immunization, adenovirus was pelleted through a 30% sucrose cushion and purified by subsequent centrifugation in a discontinuous caesium chloride gradient with a density of 1·2–1·4 g/ml. Virus bands were collected and the caesium chloride was exchanged with 1 mM EDTA, 100 mM Tris–HCl, pH 8·0, by Sephadex G-25M PD-10 column chromatography (Pharmacia, Uppsala, Sweden). Purified virus was titrated on 293 cells and stored at −70° in the presence of 10% glycerol.

Immunoprecipitation and SDS–PAGE

Antibodies were preincubated for 2 hr at room temperature with cell lysates prepared from mock-infected cells. The preincubated antibody was then added to the radioactively labelled samples prepared from virus-infected cells, and the mixture was further incubated for 2 hr at room temperature. Protein A–Sephadex beads (10 mg; Pharmacia) were added to the mixture and incubated overnight at 4° with continuous shaking in RIPA buffer [1% Triton-X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl, pH 7·5, 10 mM–EDTA, 0·5% sodium dodecyl sulphate (SDS)]. Immune complexes bound to Sepharose beads were washed three times with RIPA buffer and dissociated by boiling for 5 min in 2% SDS, 10% glycerol, 0·02% bromophenol blue, 62·5 mM Tris–HCl, pH 6·8, in the presence or absence of 5% β-mercaptoethanol (β-ME). Polypeptides were resolved on 10% SDS–polyacrylamide gel electroph (PAGE) at 15 V/cm^2. The gels were fixed in 40% methanol containing 10% acetic acid, treated with Amplify (Amersham Int., Oakville, Ont., Canada) and autoradiographed at −80°.

Primary culture of cotton rat lung cells

Lungs from a cotton rat were removed surgically and mechanically disaggregated. Lung cells were loosened with a rubber policeman and digested with 1 mg/ml collagenase for 20 min at 37° in RPMI-1640 media supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 2·5 μg/ml of amphotericin B (complete media). Cells were washed twice with complete media and plated in tissue culture plates. When a monolayer of fibroblast like-cells was formed, cells were trypsinized and cultured in complete MEM. After 25 passages, a single cell was cloned by limited dilution and amplified. The cloned cells were maintained as a stable cell line designated CTRL.

Animals

A pair of inbred cotton rats (*Sigmodon hispidus*) was obtained originally from the Veterinary Resources Branch (Division of Research Services, NAtional Institute of Health, Bethesda, MD) and was used to establish a breeding colony behind a germ-free barrier in the animal facility of the Veterinary Infectious Disease Organization (University of Saskatchewan, Canada). Male and female animals aged between 4 and 10 weeks were used in this study. Animals were handled in accordance with the guidelines of the Canadian Council on Animal Care and the University Committee on Animal Care and Supply.

Animal immunization

For intranasal immunizations, cotton rats were anaesthetized by inhalation of halothane (MTC Pharmaceuticals, Cambridge, ON). Fifty microlitres of virus suspension containing 10^7 plaque-forming units (PFU) of hAd5 or AdBcHE was placed in their nostrils for inhalation. Animals were immunized twice at 4-week intervals and killed by a halothane overdose at 7 days after the second immunization. For enteric immunization, cotton rats were starved overnight and anaesthesized with halothane by inhalation; the abdomen was then opened under aseptic conditions, and 300 μl containins 3×10^6 PFU of Ad5 or AdBcHE inoculated into the duodenum. The abdomen was then closed, and the rats were allowed to recover and housed under normal conditions.

Collection of lavages and serum samples

Blood samples were periodically obtained by cardiac puncture. For collection of bronchoalveolar lavages, animals were anaesthetized by inhalation with halothane (MTC) Pharmaceuticals), and the trachea and thoracic cavity were exposed surgically. The trachea just below the larynx was tied off with a fine surgical suture, and 0·5 ml of phosphate-buffered saline (PBS) was gently injected into the trachea below the suture using a 25-gauge needle, thus expanding the lungs. The lavage was pulled back into the syringe and reinjected into the lungs for a total of three cycles before final withdrawal. Recovery of bronchoalveolar lavages averaged 0·3–0·4 ml. The fluids were immediately clarified by centrifugation in a microfuge for 5 min at 15 000 *g* and stored at −20° for later evaluation for antibody titres by enzyme-linked immunosorbent assay (ELISA) and virus neutralization assays.

Intestinal secretions were collected using the method described by Nedrud *et al.*[25] Briefly, animals were killed and the small intestines were removed and clamped on each end. After being rinsed twice in PBS, the intestines were injected using a 27-gauge needle, with 3 ml of a solution containing 25 mM NaCl, 40 mM Na_2SO_4, 10 mM KCl, 20 mM $NaHCO_3$, 50 mM EDTA, 0·1 mg/ml soybean trypsin inhibitor (Sigma, St Louis, MO) and 162 mg/ml polyethylene glycol (approximate MW 3350; Sigma). The intestines were emptied after 10 min by pushing the contents out into a tube. After vigorous vortexing, the suspensions were clarified by centrifugation at 700 *g* for 10 min. The intestinal washes were adjusted to a final

concentration of 1 mM phenylmethyl sulphonyl fluoride (PMSF; Sigma) and further centrifuged for 15 min at 15 000 *g* in an Eppendorf microfuge. Ten microlitres of 0·1 M PMSF was added to each millilitre of supernatant and the samples were incubated on ice for 15 min before adding 50 μl of 3·5% bovine serum albumin (BSA) per millilitre. The samples were stored at −20°.

ELISA

Antibody titres specific for the BCV HE in sera and lung lavages were determined by ELISA. Approximately 100 ng of BCV virions in coating buffer (50 mM $NaHCO_3/Na_2CO_3$, pH 9·6) was adhered onto each well of Immulon 2 microtitre plates (Dynatech Laboratories, Chantilly, VA) at 4° overnight. The plates were washed four times with PBS containing 0·05% Tween-20 (PBS-T; Sigma), and incubated with 3% heat-inactivated horse serum for 1 hr at room temperature. The plates were washed with PBS-T and the samples, serially diluted in dilution buffer (PBS-T plus 1% BSA), were added and incubated for 2 hr at room temperature. After removing unbound antibodies by washing the plates three times with PBS-T, horseradish peroxidase-conjugated goat anti-rat IgG (Zymed, Mississauga, Ontario, Canada) was added for 1 hr at room temperature. For IgA detection, rabbit anti-rat IgA (a gift from Dr B. Underdown, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada) was added, followed by 1 hr incubation at room temperature. Plates were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody for 2 hr and, after an extensive wash, 0·15 mg/ml of ABTS 2,2′-azino-di-[3-ethyl-benzthiazoline sulphate(6); Boehringer-Mannheim, Mannheim, Germany] in 50 mM citric acid, pH 4·0, containing 0·01% hydrogen peroxide, was added for colour development, and the enzyme reaction was terminated by the addition of an equal volume of 10% SDS. The absorbance at 405 nm with a reference wavelength at 490 nm was determined using a microplate reader (Bio-Rad model 3550; Cambridge, MA).

Virus neutralization assay

Samples were heat-inactivated at 56° for 30 min, and duplicates of the twofold serial dilutions were mixed with 1000 PFU/ml of BCV in MEM containing 10% FBS. The virus-sample mixtures were incubated for 1 hr at 37° and 100 μl of the mixture was used to infect GBK cell monolayers grown in six-well plates. Virus inoculum was removed after 1 hr, and the cell monolayer was washed with MEM and overlaid with 1% agarose in MEM containing 10 μg/ml trypsin. Plates were incubated for 2 days and stained with 0·01% neutral red overnight. Virus neutralization titre was determined as the reciprocal serum dilution demonstrating a mean plaque number less than 50% of the plaques observed in the virus control well.

T-cell proliferative response

Spleen cells from immunized animals were cultivated at 2×10^5 cells/well in flat-bottomed 96-well plates (Nunclon, Roskilde, Denmark) with various dilutions of UV-irradiated BCV in MEM supplemented with 10% FBS (HyClone, Logan, UT), 50 μM β-ME and 2 mM L-glutamine. Cultures were incubated for 3–4 days at 37° and pulsed with 1 μCi/ml of [^{3}H]thymidine (Amersham, Oakville, Canada) for 18 hr. Cells were washed and harvested, and the radioactive incorporation was determined by liquid scintillation counting. Stimulation index was determined by dividing the experimental c.p.m. by the media control c.p.m.

Cytotoxicity assay

Cotton rats were intraperitoneally or intranasally inoculated with 10^7 PFU of AdBcHE and boosted by the same route 4 weeks later. Spleen cells were isolated 7 days after the boost and restimulated for 4 days *in vitro* in the presence of 10 μg/ml BCV, or stimulated with irradiated Ad-infected cotton rat fibroblasts. One to five million target cells were labelled with 100 μCi of radioactive sodium chromate (Amersham) for 90 min at 37°. Cells were then washed three times, and 5×10^3 cells were incubated with effector cells at different ratios for 6 hr at 37° in a volume of 0·2 ml of MEM containing 10% FBS. In each experiment, appropriate controls were run simultaneously. The percentage of specific ^{51}Cr-release from target cells was calculated from the mean of triplicate wells using the formula: (mean c.p.m. effector cells − mean c.p.m. spontaneous release)/(mean c.p.m. total release − mean c.p.m. spontaneous release) × 100. Spontaneous release was determined from target cells in the absence of effector cells, and was less than 15% of the maximum release.

RESULTS

Synthesis of HE in cotton rat cells

Properties of the recombinant HE protein produced by the recombinant AdBcHE have described previously.[20] The recombinant HE produced in 293 cells and MDBK cells retained enzymatic activities indistinguishable from the authentic HE and was immunoreactive with the HE-specific BCV neutralizing monoclonal antibodies (BD9, KD9, HC10, KC4).[20] In order to evaluate whether the AdBcHE vector was capable of expressing BCV HE glycoprotein in cotton rat cells, we first established a stable cell line derived from the lungs of a cotton rat (CTRL cells), and used these cells to assess synthesis of the HE protein by the AdBcHE vector. BCV-specific rabbit antisera immunoprecipitated a 65 000 MW protein from cells infected with recombinant virus (Fig. 1, lane 3) similar to the HE protein of BCV virions (Fig. 1, lane 1). As the HE protein is homodimeric in nature, we also examined the potential of the HE protein to form homodimers in CTRL cells. In the absence of the reducing agent β-ME, migration of the 65 000 MW protein was shifted to 130 000 MW (Fig. 1, lane 6), indicating that the HE formed dimers in CTRL. A monomeric 65 000 MW protein was also identified from BCV-infected cells even in the absence of the reducing agent (lane 4). This monomeric form was frequently observed in BCV preparations and may be the result of partial denaturation of the dimeric HE glycoprotein during the preparation steps.

Antibody responses following intranasal immunization

Cotton rats, six animals per group, were inoculated intranasally with PBS or 10^7 PFU of hAd5 or AdBcHE vector. Four weeks following primary immunization, animals were boosted with the same dose of virus intranasally. One week after the boost, the antibody responses specific for BCV HE and hAd5 were determined by ELISA in sera and bronchoalveolar lavages.

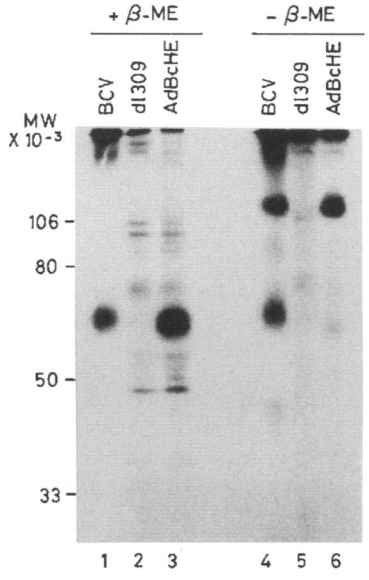

Figure 1. Expression of the BCV HE protein by recombinant AdBcHE vector in cotton rat lung cells. BCV HE glycoprotein was immunoprecipitated with a rabbit anti-BCV serum from lysates of radiolabelled CTRL infected with BCV (lanes 1 and 4), dl309 (lanes 2 and 5) and AdBcHE (lanes 3 and 6.

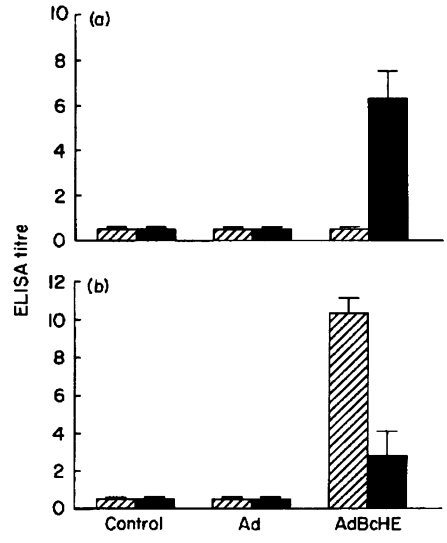

Figure 2. Anti-BCV HE antibody responses in sera (▨) and bronchoalveolar lavages (■) after intranasal immunization. Cotton rats were immunized intranasally with PBS, 10^7 PFU of hAd5 or AdBcHE, and boosted in the same way 4 weeks later. Samples were collected 1 week after the boost, and the anti-BCV HE IgA (a) and IgG (b) antibodies were determined by ELISA and are represented as mean titre ($\log_2$) ± SD.

Only those animals immunized with AdBcHE generated significant levels of anti-BCV HE IgA and IgG antibodies, in both sera and lung lavages (Fig. 2). In addition, specific anti-hAd5 IgA and IgG responses were induced following immunization with either hAd5 or AdBcHE (data not presented). These results demonstrated that intranasal administration of AdBcHE induced both local and systemic antibody responses to the BCV HE protein. No antibody responses to hAd5 or BCV HE were found in the control animals (Fig. 2).

Sera, lung washes and nasal washes were analysed for virus-neutralizing antibodies in a plaque-reduction assay. Cotton rats immunized with AdBcHE had BCV neutralizing antibodies in sera and broncheoalveolar lavages (Table 1).

Cellular immune response

To determine whether mucosal immunization with the AdBcHE vector was capable of inducing systemic cell-mediated immunity to BCV, we assessed the proliferative and the cell-mediated cytotoxic responses following intranasal immunization of cotton rats. Cotton rats, six animals per group, were inoculated intranasally with PBS or 10^7 PFU of hAd5 or AdBcHE. Four weeks following primary immunization, animals were boosted with the same dose of virus intranasally and 1 week later the animals were killed and spleen cells were isolated to determine the proliferative response to BCV. *In vitro* stimulation of spleen cells demonstrated that immunization with AdBcHE elicited significant proliferative responses to BCV compared to control and hAd5 immunized groups (Table 2).

In this study we demonstrated that immunization of cotton rats with AdBcHE induced effector cells in the spleen capable of lysing BCV-infected targets (Fig. 3). Evidence that BCV could infect CTRL cells was shown by the immunoprecipitation of BCV proteins (Fig. 1). Furthermore, spleen cells from hAd5- and AdBcHE-intranasally immunized groups exhibited lytic activity against hAd5-infected target cells (Fig. 3). Lytic activity in the control non-immunized group was low for infected (hAd5 or BCV) and mock-infected targets (data not presented).

Antibody response following enteric immunization

To evaluate whether enteric immunization of cotton rats with AdBcHE could elicit an immune response to BCV HE glycoprotein, cotton rats were intraduodenally inoculated with 3×10^7 PFU and the antibody responses against BCV and hAd5 were evaluated by ELISA. Enteric immunization with AdBcHE elicited anti-BCV HE IgG and IgA responses, in serum and intestinal secretions, respectively (Table 3). In

Table 1. Neutralization of BCV by sera and bronchoalveolar lavages*

Animal group	Serum	Lung wash
Control	< 3·3	< 1
hAd5	< 3·3	< 1
AdBcHE	10·3	4

* Cotton rats were immunized intranasally as described in the footnote of Fig. 2. BCV neutralizing antibody titres were calculated by a plaque-reduction assay. The neutralization titres were expressed as the $\log_2$ of the highest dilutions at which the virus plaque formation was reduced more than 50%.

Table 2. Proliferative responses of splenic lymphocytes to BCV after intranasal immunization*

	CPM × 10^2		
	Immunization		
In vitro stimulation BCV (μg/ml)	Control	Ad	AdBcHE
0	4·2 ± 1·5	4·8 ± 1·5	7·3 ± 3·8
5	9·6 ± 1·4	4·9 ± 2	10·4 ± 4·6
10	7·9 ± 1·6	5·6 ± 1·6	12·8 ± 4·7
20	6·5 ± 1·9	4·7 ± 1·7	27·5 ± 8·7

* Cotton rats were immunized as described in the footnote of Fig. 3. Proliferative responses of spleen cells were determined by cultiring spleen cells in the presence of different concentrations of purified UV-irradiated BCV. Results are expressed as the mean ± SD (c.p.m. × 10^2) of three individual animals/group.

addition, immunization with hAd5 or AdBcHE induced anti-hAd5 IgG and IgA antibody responses in sera (Table 3).

Immunization of cotton rats with AdBcHE by the enteric route stimulated the development of serum BCV neutralizing

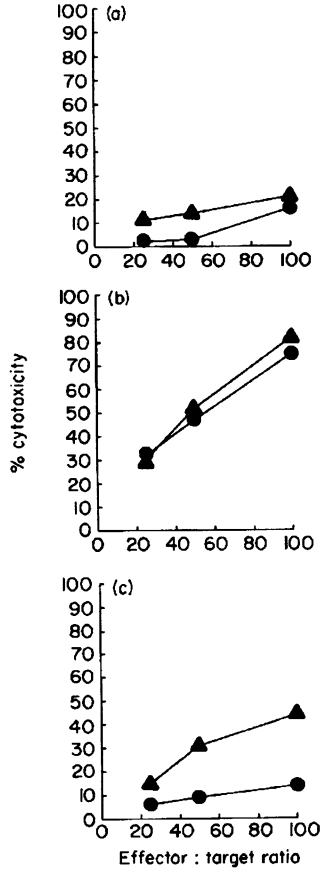

Figure 3. Generation of cytotoxic activity in spleen cells from intranasally immunized cotton rats with hAd5 (▲) and AdBcHE (●). Lytic activity against: (a) mock-infected CTRL; (b) hAd5-infected CTRL; and (c) BCV-infected CTRL. Results represent the mean percentage cytotoxicity of three animals/group.

Table 3. Anti-BCV HE antibody responses elicted by intraduodenal immunization of cotton rats with AdBcHE

Virus used for immunization	Serum (IgG)	Intestinal secretions (IgA)
hAd5	1·9 ± 1·2	1·2 ± 0·8
AdBcHE	11·32 ± 2·31	4·8 ± 1·3

Specific anti-BCV HE antibody responses following intraduodenal immunization were measured by ELISA. Results are expressed as $\log_2$ mean titre ± SD of five animals/group.

antibodies (mean titre of 10 in five rats/group), compared to that of control hAd5-immunized animals (mean titre of < 7·9 in five rats/group). However, due to the toxicity of the intestinal washes we were unable to measure neutralization titres in these samples.

DISCUSSION

The present study demonstrates that mucosal immunization of cotton rats with recombinant adenovirus carrying the HE gene induces both mucosal and systemic immune responses to BCV HE.

Cotton rats have been shown to support the replication of hAd5 in the respiratory tract.[21] For this reason, we chose the cotton rat as a model to assess the immunogenicity of our recombinant AdBcHE vector following mucosal immunization. Our results demonstrate that both intranasal and enteric immunization induce anti-BCV IgG and IgA in sera and mucosal secretions. In addition, serum BCV-neutralizing antibodies were generated by both routes of immunization. These results are consistent and extend previous findings emphasizing the usefulness of the cotton rat model in the study of mucosal immunization with recombinant adenovirus vectors.

Cell-mediated defence mechanisms, such as cytotoxic T lymphocyte (CTL) activity or T-cell-derived cytokines, are important in the control of viral infections (reviewed in ref. 26). Delivery of vaccine antigens by recombinant viral vectors has been shown to induce cell-mediated immune responses to the foreign gene product efficiently;[19,27,28] therefore, we evaluated the capacity of the AdBcHE vector to generate proliferative and cytotoxic responses to BCV. Immunization with AdBcHE induced proliferative responses to BCV in the spleen, suggesting that intranasal immunization generates systemic cell-mediated immune responses to the vaccine antigen. In addition, compared to hAd5-immunized rats, only the spleens of AdBcHE-immunized animals contained cells capable of lysing hAd5- and BCV-infected cells. Whether this lytic activity was mediated by antigen-specific major histocompatibility complex (MHC)-restricted CTL, or by other lytic mechanisms such as T-cell-derived cytokines, has not been determined due to the lack of cotton rat-specific immunological reagents. However, the fact that the cotton rats used in this study have been derived from an inbred population[29] and the lack of response in mixed lymphocyte reactions between rats of our colony (data not shown), provide indirect evidence of MHC compatibility among the cotton rats in our colony. Nevertheless, the possibility that other specific or non-specific mechanisms rather than CTL accounted for this lytic activity remains.

The induction of systemic immune responses following intranasal immunization with AdBcHE may be explained by the ability of both wild-type hAd5 and replication-proficient mutants Ad-dl327 (deleted for the E3 gene) to disseminate systemically, including to peripheral lymphoid organs, after intranasal inoculation.[30]

The current vaccines used against BCV appear to have limited effectiveness.[31] One possible reason, that active immunization of calves is of limited value, is directly related to the presence of anti-BCV antibodies in the milk. These passively transferred antibodies quickly neutralize the vaccine virus and thereby prevent induction of immunity. Studies in cotton rats have demonstrated that mucosal immunization with a recombinant vaccinia vector could overcome the suppressive effects of passively transferred antibodies when administrated intranasally.[32] Whether mucosal immunization of cattle with recombinant adenovirus vectors can overcome the effects of virus-specific maternal antibodies remains to be explored.

The demonstration that mucosal delivery of BCV HE antigen by recombinant adenovirus can stimulate mucosal and systemic immune responses in cotton rats offers an ideal model to evaluate the mechanisms involved in the induction of mucosal immune responses by recombinant adenovirus vectors; studies based on this cotton rat model will provide information necessary to develop more effective vaccines to protect the susceptible animal population.

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