Pathology and immunogenicity in the cotton rat (Sigmodon hispidus) model after infection with a bovine adenovirus type 3 recombinant virus expressing the firefly luciferase gene

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The histopathology of adenovirus pneumonia in cotton rats (Sigmodon hispidus) due to bovine adenovirus type 3-luciferase recombinant virus (BAd3-Luc), which has a 0.7 kb deletion from the early region 3 (E3) replaced with the firefly luciferase gene, was compared with that produced by the parental wild-type (wt) bovine adenovirus type 3 (BAd3). After intranasal inoculation of cotton rats with 3 x 10^7 p.f.u. of BAd3-Luc, the infectious virus titres in the lungs at various times post-infection were similar to those of animals infected with the parental virus. Quantitative analysis of histopathological changes and immunohistochemical staining showed that the character and severity of the lesions were indistinguishable in the two infections. Luciferase activity was detected in the lungs of BAd3-Luc-inoculated animals until 4 days post-infection (p.i.). Antibodies to both BAd3 and luciferase were detected in sera collected from BAd3-Luc-infected animals until at least 6 weeks p.i. These results show that BAd3-Luc produces pulmonary lesions in cotton rats similar to those of wt BAd3 and suggest that BAd3-based vectors may be suitable for the development of live recombinant virus vaccines.

Introduction

The role of bovine adenovirus type 3 (BAd3) in the aetiology of naturally occurring respiratory disease in cattle appears to be minor, although the virus has been isolated from cattle showing signs of mild respiratory and respiratory-enteric disease and from apparently healthy cattle (Darbyshire, 1968; Mattson, 1973a, b; Stott et al., 1980; Mattson et al., 1988). Intranasal or intratracheal inoculation of colostrum-deprived calves with BAd3 resulted in only a mild clinical response characterized by pyrexia, respiratory distress, nasal and conjunctival discharges (Darbyshire et al., 1965, 1966; Ide et al., 1969) associated histologically with proliferative and necrotizing bronchiolitis, and pulmonary atelectasis. Experimental intranasal inoculation of calves at 1 week of age or older with BAd3 failed to produce signs of respiratory or enteric disease (Ide et al., 1969; S. K. Mittal and others, unpublished data). Since this virus does not cause significant disease, it has been considered a potential candidate for a live virus vector.

The potential of various adenoviruses as vectors for recombinant virus vaccines and somatic gene therapy is currently being extensively investigated (Chanda et al., 1990; Graham, 1990; Prevec et al., 1990; Graham & Prevec, 1992; Rosenfeld et al., 1992; Mittal et al., 1994, 1995b; Ragot et al., 1993; Engelhardt et al., 1994). Most of these studies are based on the use of human adenovirus type 5 (HAd5) as a foreign gene expression vector in mammalian cells. To generate recombinant viruses, foreign genes can be inserted into the adenovirus genome at either of two locations: the early region 1 (E1) or E3, (Berkner & Sharp, 1984; Haj-Ahmad & Graham, 1986; Ghosh-Choudhury et al., 1987; Bett et al., 1993). Since E3 gene products are not essential for virus growth in tissue culture or in experimental animals (Ginsberg et al., 1989), this region offers an attractive locus for foreign gene insertion to generate replication-competent recombinant viruses specifically for use as live recombinant virus vaccines.
Recently, we have demonstrated that, like HAd E3, BAd E3 is not required for virus replication in cultured cells and this site can be utilized for insertion of foreign DNA sequences (Mittal et al., 1995a). BAd3-based expression vectors have the potential to provide an excellent alternative to conventional vaccines for the protection of cattle against infectious respiratory diseases. However, since HAd E3 gene products are involved in modulating host immune responses to virus infection (Burgert & Khvost, 1987; Gooding et al., 1988; Carlin et al., 1989; Tollefson et al., 1991; Wold et al., 1994) and HAd E3-deletion mutants have been shown to have an increased pathogenic potential in cotton rats (Sigmodon hispidus) compared to their wild-type (wt) counterpart (Ginsberg et al., 1989), there is a need to evaluate the pathogenic potential of and immune responses to a BAd3-based recombinant virus. Previously, we established that the cotton rat is a useful small-animal model to study BAd3 pneumonia (Mittal et al., 1995a). In the studies presented here, we compare the pathology and immunogenicity of a BAd3-luciferase recombinant virus (BAd3-Luc) with those of the parental wt BAd3.

Methods

Viruses and cell culture. The WBR-1 isolate of BAd3 (Mittal et al., 1992) was used as the source of wt BAd3. The construction and characterization of BAd3-Luc, which contains the firefly luciferase gene in the E3 region of BAd3, have been described in detail elsewhere (Mittal et al., 1995a). The wt BAd3 and BAd3-Luc were propagated and titrated in Madin–Darby bovine kidney (MDBK) cells grown as monolayers in Gibco BRL minimum essential medium (MEM) supplemented with 25 mm-HEPES and 50 μg/ml gentamicin and 10% fetal bovine serum. Viruses were purified by caesium chloride density-gradient centrifugation (Graham & Prevec, 1991).

Animals. The source of animals was an inbred colony of cotton rats maintained at the Veterinary Infectious Disease Organization, Saskatoon, Canada (Mittal et al., 1995c).

Experimental design. A total of thirty three 6-8 week old cotton rats of either sex were randomly grouped into 11 groups (3 animals/group). Under halothane anaesthesia, five groups were inoculated intranasally with 100 μl of a purified BAd3-Luc preparation (3 × 10⁷ p.f.u./animal) at day 0. At 4 and 6 weeks p.i., blood samples were collected to monitor the development of BAd3-specific antibodies by ELISA and virus neutralization assays. Lung washes were also collected at 6 weeks p.i. to detect BAd3-specific IgA antibodies by ELISA. The development of antibody to luciferase was also monitored by ELISA.

Virus isolation. Tissues for virus isolation were homogenized in 1 ml of MEM and the supernatants were used for virus titration in MDBK cells by plaque assays (Mittal et al., 1995a).

Histopathological and immunohistochemical analyses. The formalin-fixed cotton rat lungs were processed for paraffin wax embedding, sectioned and stained with haematoxylin and eosin (H&E). The immunohistochemical staining of formalin-fixed paraffin-embedded tissue sections was carried out by the avidin–biotin complex protocol (Haines & Chelack, 1991) using an anti-BAd3 rabbit serum (Mittal et al., 1995c), adapted to a robotic system, described previously (Mittal et al., 1995c).

ELISA and virus neutralization assays. Blood samples were collected from mock- and virus-inoculated cotton rats by cardiac puncture under anaesthesia. Microtitre plates were coated either with a purified BAd3 or firefly luciferase preparation for the detection of BAd3 or luciferase antibody respectively, in the sera and lung washes obtained from mock- and virus-inoculated animals, after an ELISA protocol (Mittal et al., 1995c). The virus neutralization assays were carried out as described previously (Mittal et al., 1993b).

Luciferase assays. The assays to measure luciferase activity were essentially similar to those described elsewhere for virus-infected mouse tissues and infected cells in culture (Mittal et al., 1993b).

Results

Replication and clearance of BAd3-Luc from cotton rat lungs compared to wt BAd3

To determine whether a 0-7 kb E3 deletion present in BAd3-Luc affects the kinetics of virus replication and clearance from the lungs of inoculated cotton rats, titres of infectious virus recovered from the lungs of animals inoculated intranasally either with BAd3-Luc or wt BAd3 were compared. Viruses titres in inoculated animals declined until day 2 p.i., then increased by approximately 4-fold on day 3 p.i. probably due to active virus replication (Fig. 1). From day 3 p.i. onwards, virus titres declined and reached undetectable levels by day 7. Over the 7 day period, infectious virus titres recovered from the lungs of BAd3-Luc-infected animals were similar to those from the lungs of wt BAd3-inoculated cotton rats (Fig. 1).

Histopathological and immunohistochemical findings in BAd3-Luc- or wt BAd3-infected cotton rat lungs

In a previous histopathological and immunohistochemical study of the lungs of cotton rats inoculated with wt BAd3, we observed that bronchiolar epithelial lesions, intranuclear inclusion bodies, type II pneumocyte pro-
Pathogenesis of BAd3-luciferase recombinant 3

Fig. 1. Replication and clearance of BAd3-Luc compared to wt BAd3 from lungs of cotton rats. After intranasal inoculation of cotton rats either with 3 x 10^7 p.f.u. of purified BAd3-Luc (○) or wt BAd3 (□), lungs were collected at different time points for virus titration by plaque assays. Each time point is represented as the mean virus titre from three animals ± SD.

Liberation and immunohistochemical staining were maximally developed by day 3 or 4 p.i. (Mittal et al., 1995c). Therefore, in the present study, lungs of cotton rats inoculated with BAd3-Luc and wt BAd3 were collected only on days 3 and 4 p.i. for histopathological and immunohistochemical studies. As in our previous study of wt BAd3 infection in cotton rats, none of the cotton rats inoculated with BAd3-Luc or wt BAd3 showed any sign of respiratory distress or nasal discharge, nor were there any changes in the feed and water intake or in weight gain (data not shown). Histologically, however, by days 3 and 4 p.i., there were well-developed pulmonary lesions in both the BAd3-Luc and the wt BAd3-inoculated groups of cotton rats.

Mock-inoculated animals examined at day 3 p.i. had no lesions either in the airways or alveoli (Fig. 2a, f). At day 3 p.i., the BAd3-Luc-inoculated animals showed moderate to marked changes in the bronchiolar mucosa consisting of widespread hyperplasia of the epithelium with a mild infiltration of neutrophils, multifocal necrosis of individual epithelial cells, formation of cysts within the epithelium and occasional mitotic figures (Fig. 2b). Eosinophilic intranuclear inclusion bodies were seen in low number. In alveoli, multifocal mild to moderate locally extensive type II pneumocytes were present, which are large, plump and slightly cuboidal cells with large nuclei and abundant basophilic cytoplasm (Fig. 2d). Rare mitotic figures were present in alveolar cells. In two of the three animals, occasional alveolar lining cells contained indistinct intranuclear inclusions. A few neutrophils were present multifocally within the alveolar walls. By day 4, changes were essentially the same as those observed on day 3 p.i. Luminal debris was present in two of three animals. A few mitotic figures were present in epithelial cells. Eosinophilic intranuclear inclusions were present in all three animals in low to moderate number. Alveolar type II pneumocyte hyperplasia was extensive, mitoses were occasionally observed and indistinct intranuclear inclusions were present randomly within alveolar lining cells. At both days 3 and 4 p.i. there was a low to moderate degree of immunohistochemical staining, both intra- and extracellular, of the bronchiolar epithelial cells (Fig. 2g), using a BAd3-specific antibody raised in a rabbit against a caesium chloride density-gradient purified preparation of wt BAd3 (Mittal et al., 1995c). Intranuclear and intracytoplasmic staining was also observed in a number of alveolar lining cells (Fig. 2i), except in one animal which was negative on day 3. No specific immunohistochemical staining was observed with an anti-luciferase rabbit serum (data not shown). This may be due to alteration of epitopes by formalin fixation (Haines & Chelack, 1991) or alternatively, levels of luciferase expression in individual bronchiolar epithelial and alveolar cells may be below the sensitivity of immunohistochemical staining. However, luciferase expression in lungs of BAd3-Luc-inoculated cotton rats was easily detected by highly sensitive enzymatic assays (see below).

On day 3 p.i. cotton rats inoculated with wt BAd3 showed moderate to marked changes in the bronchioles (Fig. 2c) identical in nature to those described above for the BAd3-Luc-inoculated animals. Eosinophilic intranuclear inclusion bodies were more numerous in two of the three animals inoculated with wt BAd3 than in BAd3-Luc inoculated animals. The bronchiolar lumen contained necrotic cell debris in two of the three animals. By day 4 there were minor changes in the characteristics of the lesions in the airways. On day 3 alveolar changes due to wt BAd3 (Fig. 2e) were similar to those described above for BAd3-Luc and on day 4 were slightly increased. Immunohistochemical staining using an anti-BAd3 antibody (Fig. 2h, j) was similar on both days to that of BAd3-Luc.

Quantitative analysis of histological changes and immunohistochemical staining on a scale of 0 (normal) to 3 (severe) showed that bronchiolar epithelial and alveolar lesions, number of intranuclear inclusion bodies in bronchiolar epithelial and alveolar cells, immunohistochemical staining of bronchiolar epithelial and
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**Fig. 3.** Quantification of histopathological changes and immunohistochemical staining in lungs of BAd3-Luc-inoculated cotton rats compared to wt virus. Cotton rats were inoculated intranasally either with $3 \times 10^7$ p.f.u. of purified BAd3-Luc or wt BAd3 and at days 3 and 4 p.i. lungs were collected and processed for histopathological and immunohistochemical examinations. The changes were scored on a scale of 0 (normal) to 3 (severe) depending on the severity of lesions. Each bar denotes the mean score for three animals.

**Fig. 2.** Histopathological changes and immunohistochemical staining in cotton rat lungs caused by BAd3-Luc and wt BAd3 infections. After intranasal inoculation of cotton rats with either $3 \times 10^7$ p.f.u. of purified BAd3-Luc or wt BAd3, lungs were collected at days 3 and 4 p.i. and processed for histopathological and immunohistochemical studies. (a–e, H&E stain; f–j, immunohistochemical staining).

(a, f) Airway and alveoli at 3 days after mock-inoculation. Bronchioles and alveoli are normal (a) and there is no specific positive staining (f). Bar, 50 μm. (b, c) Airways at 3 days after BAd3-Luc (b) or wt BAd3 (c) infection. Similar changes of bronchiolar epithelial hyperplasia and epithelial cell necrosis are present. Bar, 50 μm. (d, e) Alveoli at 3 days after BAd3-Luc (d) infection or 4 days after wt BAd3 (e) infection. There is prominent Type II pneumocyte hyperplasia and mild mononuclear cell infiltration. Bar, 50 μm. (g, h) Bronchiolar epithelium at 3 days after BAd3-Luc (g) or wt BAd3 (h) infection, showing positive staining of the bronchiolar epithelial cells. Bars, 10 μm (g) and 20 μm (h). (i, j) Alveoli at 3 days after BAd3-Luc (i) or wt BAd3 (j) infection, showing positive staining in alveolar cells (open arrow). Bar, 10 μm.
alveolar cells and peribronchiolar lymphocyte infiltration were of similar magnitude in both the BAd3-Luc and the wt BAd3-inoculated animals (Fig. 3).

**Kinetics of luciferase expression in lungs of cotton rats inoculated with BAd3-Luc**

We have shown previously that in BAd3-Luc-infected MDBK cells, luciferase expression was detectable by luciferase assays and Western blot (Mittal et al., 1995a). After intranasal inoculation of cotton rats, lungs collected at various times p.i. were processed to monitor luciferase expression by enzymatic assays. In the BAd3-Luc-inoculated animals at day 1 p.i. luciferase activity was detected at a low level and activity increased at least until day 4 and was below detection level at day 7 p.i. (Fig. 4). We did not attempt to monitor luciferase expression in other organs of infected animals as our previous study after intratracheal inoculation of rats with a HAd5 vector expressing luciferase showed that approximately 98% of the total luciferase activity was confined to the lungs (Braciak et al., 1993). Luciferase activity was not detected in the lungs of cotton rats inoculated with wt BAd3 (data not shown).

### Table 1. BAd3-specific antibody response in cotton rats inoculated intranasally either with BAd3 or BAd3-Luc

<table>
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<th>Animal no.</th>
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<th>BAd3 neutralizing antibody titres</th>
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**Immune responses against BAd3 and luciferase in cotton rats inoculated with BAd3-Luc**

In the sera collected at 4 and 6 weeks p.i. from cotton rats inoculated intranasally either with BAd3-Luc or wt BAd3 at day 0 were tested for BAd3 antibody by ELISA and virus neutralization assays to determine whether the limited replication of BAd3-Luc observed in the lungs could elicit a BAd3-specific immune response. At 4 weeks p.i., BAd3-specific ELISA and virus neutralizing antibody titres were equal to or higher than 1:320 and 1:160, respectively, and showed a slight decrease at 6 weeks p.i. (Table 1). The serum samples and lung washes collected from BAd3-Luc and wt BAd3-infected cotton rats were similarly analysed to detect a BAd3-specific IgA response. BAd3-specific IgA antibody titres in the sera and lung washes were equal to or higher than 1:80 and showed a slight decrease at 6 weeks p.i. (Table 2). BAd3-specific immune responses observed in cotton rats due to BAd3-Luc were similar to or slightly reduced compared to wt BAd3 infection. The serum samples collected from the mock-inoculated animals failed to show BAd3 antibody titres above the background level.

To determine the usefulness of BAd3-based vectors in eliciting an immune response against foreign proteins in a mammalian host, the sera obtained from BAd3-Luc-inoculated cotton rats were also tested for luciferase antibody by ELISA. The luciferase ELISA antibody titres were equal to or higher than 1:40 at 4 weeks p.i. and were waning at 6 weeks p.i. (data not shown). The serum samples collected from the mock- or wt BAd3-
Discussion

As the E3 proteins of adenoviruses are not required for virus replication, this region is seen as an excellent potential target for foreign gene insertion particularly for the development of live recombinant virus vaccines. We have constructed a BAd3-based E3 insertion vector system (Mittal et al., 1995a) with the objective of exploring its potential as recombinant vaccines for cattle. An important advantage of BAd3 is that this virus can be grown to very high titres, thereby reducing the cost of vaccine production.

Since the adenovirus E3 region, approximately 5–10% of the genome (Cladaras & Wold, 1985), has been preserved in all representative adenovirus serotypes that have been analysed, it would be surprising if E3 gene products do not play an important role during natural infections that is beneficial for the virus. Indeed, E3 functions have been shown to play a role in modulating the host immune response to virus infection (Ginsberg et al., 1989; Wold et al., 1994). The E3 transcription unit of HAd2 and HAd5, which contains at least nine open reading frames (ORF), seven of which have been found to be expressed in infected cells, has been studied extensively (Cladaras & Wold, 1985; Wold et al., 1994). The biological functions of a number of the E3 proteins of HAd2 and HAd5 have been determined. The HAd E3 19 kDa glycoprotein, an integral membrane glyco-

protein, protects the virus-infected cell from lysis by cytotoxic T lymphocyte (CTL) attack by forming a complex with newly synthesized major histocompatibility complex class I antigens in the endoplasmic reticulum and thus inhibits their transport to the surface of infected cells (Burgert & Kvist, 1987). The E3 14.7 kDa protein prevents lysis of virus-infected mouse cells by tumour necrosis factor α (TNF-α) (Gooding et al., 1988). The E3 14.5 kDa and E3 10.4 kDa proteins together prevent cytolysis by TNF-α and also down-regulate the expression of the epidermal growth factor receptor in virus-infected cells (Carlin et al., 1989; Tollefson et al., 1991). In general, E3 proteins lower the immune response to virus and would presumably enhance virus survival in the infected host. The mutants with a deletion in the E3 19 kDa, E3 14.7 kDa and E3 14.5 kDa-10.4 kDa ORFs produce pronounced pathology in cotton rat lungs perhaps due to an increase in CTL and inflammatory response (Ginsberg et al., 1989).

The E3 transcription unit of BAd3 is approximately half the size of the corresponding unit of HAd5 and one of the BAd3 E3 ORFs is homologous to the E3 14.7 kDa protein of HAd (Mittal et al., 1992, 1993a). The 0.7 kb deletion in the E3 region of BAd3-Luc interrupts two ORFs which may encode functional proteins of 284 and 141 amino acids (Mittal et al., 1992, 1993a). The BAd3 E3 predicted protein of 284 amino acids contains eight potential N-glycosylation sites and a hydrophobic sequence of approximately 25–30 amino acids near the C-terminal end – a potential transmembrane domain. This putative glycoprotein may be functionally similar to E3 19 kDa of HAd2 or HAd5, although this has yet to be investigated. Therefore, it is important to determine the pathogenic potential of recombinant viruses that have deletions in the E3 region, compared to wt virus before E3 insertion vectors can be considered safe for live recombinant virus vaccines. The proposed use of the cotton rat, as an alternative to the natural host, to study the role of a BAd3 E3 deletion in the pathogenesis of the disease, arose from our previous observations that histopathological lesions in the lungs after intranasal inoculation of cotton rats with BAd3 were similar to those produced in calves (Mittal et al., 1995c). With this in mind, the present study was conducted to investigate the pathogenic potential of a recombinant BAd3 and immune responses to the foreign protein expressed by it in the cotton rat model.

Histologically and immunohistochemically, BAd3-Luc-infected cotton rat lungs showed pulmonary changes similar to those induced by the parental wt virus. The presence of intranuclear inclusion bodies was consistent with replication of virus in both instances. It appeared that the deletion affecting the predicted proteins of 284 and 141 amino acids did not have a significant effect on

Table 2. BAd3-specific IgA antibody response in cotton rats inoculated intranasally either with BAd3 or BAd3-Luc

Cotton rats were inoculated intranasally either with PBS or with 3 x 10⁷ p.f.u. of a caesium chloride density-gradient purified preparation of the wt BAd3 or BAd3-Luc recombinant at day 0. The serum samples were collected at 4 and 6 weeks p.i., whereas the lung washes were collected only at 6 weeks p.i. The sera and lung washes were tested for the presence of BAd3-specific IgA antibodies by ELISA. The serum dilution showing an absorbance reading of at least the mean + 2 s.d above the negative control serum was taken as the ELISA antibody titre.

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<th>Animal inoculated with:</th>
<th>Animal no.</th>
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<th>Mucosal BAd3-specific IgA ELISA titres</th>
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the nature and severity of the lesions of BAd3 pneumonia in cotton rats, unlike those observed with deletions in the gene encoding E3 19 kDa of HAd5, which showed distinctly more severe pneumatic lesions than the wt virus (Ginsberg et al., 1989). The ORF that encodes the putative BAd3 E3 14-7 kDa is intact in our present vector and therefore its role in virus pathogenesis could not be ascertained from the present studies. Vaccinia virus expressing TNF-α is less pathogenic to mice than vaccinia-based vectors lacking TNF-α and the virulence of the TNF-α expressing vector can be increased by the HAd E3 14-7 kDa protein, probably by counteracting the action of TNF-α (Tufariello et al., 1994). A study of BAd3 mutants, with deletions affecting one or more E3 ORFs, in cattle would certainly be helpful to determine the role of BAd3 E3 gene products in virus pathogenesis and modulation of the host immune responses to virus infection in the natural host.

Luciferase expression observed in the lungs of cotton rats inoculated intranasally with BAd3-Luc together with the development of serum and mucosal antibodies specific to BAd3 and luciferase suggest that BAd3-based vectors are likely to be useful as delivery vehicles for vaccine antigens. Since BAd3 naturally infects the respiratory and gastrointestinal tracts of cattle, effective mucosal protection against a number of respiratory and enteric virus pathogens of cattle could be induced by appropriately designed BAd3 vectors. The safety of such BAd3-based vaccines would not be a major concern as natural infection of calves with BAd3 is inapparent or very mild and experimental reproduction of clinical disease in calves is difficult to achieve (Ide et al., 1969). Furthermore, we have shown that in the cotton rat model BAd3-Luc does not appear to have any enhanced pathogenic potential compared to the parental wt virus.

As vaccination-challenge trials in cattle are very expensive, the cotton rat model provides a suitable alternative to evaluate the potential of a number of different BAd3-based vaccine candidates against a single pathogen before a more elaborate study is conducted in cattle. For bovine respiratory viruses, such as bovine herpesvirus type 1 and bovine parainfluenza virus type 3 that replicate in the respiratory tract of cotton rats (Breker-Klassen et al., 1995; Mittal and others, unpublished results), initial vaccination-challenge trials could also be conducted in this model. We are currently constructing a number of BAd3 recombinant viruses containing genes from bovine respiratory and enteric virus pathogens with the aim of developing novel BAd3-based recombinant vaccines for veterinary use.

We thank B. Carroll, L. Boyer and C. Toy for the care and handling of cotton rats, K. Caspell for preparation of photographs, and Dr D. Haines and the staff of Diagnostic Immunology Laboratory, Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, for immunohistochemical staining of the cotton rat lung sections. This work was supported by grants from the Saskatchewan Agriculture Development Fund, Natural Sciences and Engineering Research Council of Canada, National Agriculture Biotechnology Initiative, Western Economic Development, Medical Research Council of Canada and National Institutes of Health, USA. F. L. G. is a Terry Fox Research Scientist of the National Cancer Institute of Canada and S. K. M. was partially supported by a research fellowship from the Health Services Utilization and Research Commission of Saskatchewan, Canada.

This article is published with the permission of the director of the Veterinary Infectious Disease Organization as journal series no. 193.

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(Received 31 May 1995; Accepted 13 September 1995)