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Pathogenesis and Immunogenicity of Bovine Adenovirus Type 3 in Cotton Rats (*Sigmodon hispidus*)[1]

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Intranasal inoculation of cotton rats (*Sigmodon hispidus*) with 10^8 PFU of bovine adenovirus type 3 (BAd3) resulted in limited virus replication in the lung and trachea. Histopathological changes in the lungs were characterized by necrosis and hyperplasia of bronchiolar epithelium, eosinophilic intranuclear inclusions, pneumocyte type II hyperplasia in the alveoli, and mild peribronchiolar and perivascular lymphocytic infiltration. Immunohistochemically, viral antigens were observed more frequently in bronchiolar epithelial cells than in alveolar cells in cotton rat lung sections stained using a rabbit anti-BAd3 serum. Bronchiolar epithelial changes, intranuclear inclusion bodies, type II pneumocyte proliferation, peribronchiolar infiltration, and immunohistological staining were maximum at Day 3 or Day 4 postinoculation, whereas perivascular infiltration was first observed at Day 8 postinoculation. In addition to the histological study of the pathogenesis of BAd3 infection, we monitored the BAd3-specific immune response in cotton rats. Anti-BAd3 IgG and virus neutralizing antibodies were detected in sera, whereas anti-BAd3 IgA antibodies were found in the sera, lung, and nasal washes. Our results suggest that the cotton rat can serve as a useful small-animal model for investigating the pathogenesis of BAd3 infection, as well as immune responses to BAd3 recombinant virus vaccines.

INTRODUCTION

Bovine adenoviruses (BAd) are involved in subclinical to mild respiratory and enteric infections in calves (Darbyshire, 1968; Mattson, 1973a; Stott *et al.*, 1980; Mattson *et al.*, 1988). There are at least nine currently accepted serotypes of BAd which are divided into two subgroups on the basis of a number of characteristics that include antigenic properties, DNA homology with other adenoviruses, and replication in different primary and established cell lines (Bartha, 1969; Benko *et al.*, 1989a, b, 1990; Hu *et al.*, 1984a, b). BAd serotypes 1, 2, 3, and 9 are grouped into subgroup I, whereas BAd serotypes 4, 5, 6, 7, and 8 are in subgroup II.

BAd serotype 3 (BAd3) was first isolated in 1965 from the conjunctiva of a normal healthy cow (Darbyshire *et al.*, 1965a) and subsequently it has been isolated from normal cattle as well as from cattle showing signs of either respiratory or respiratory–enteric disease (Mattson, 1973a, b). Intranasal experimental inoculation of calves with BAd3 results in virus replication in the respiratory tract with unapparent to mild clinical symptoms accompanied with a varying degree of histopathological changes (Darbyshire *et al.*, 1965b, 1966; Ide *et al.*, 1969). Natural infection with BAd3 usually resolves without apparent clinical signs; however, following infection, secondary invaders may act synergistically with BAd3 to produce severe respiratory tract infection (Mattson, 1973a; Yates, 1982). The majority of older cattle have antibody against BAd3, suggesting that natural infection with BAd3 is usually subclinical (Lehmkuhl *et al.*, 1975, 1979).

Adenoviruses have excellent potential as vectors for recombinant viral vaccines and also for somatic gene transfer into human cells (Berkner, 1988; Graham, 1990; Graham and Prevec, 1992; Stratford-Perricaudet *et al.*, 1990; Rosenfeld *et al.*, 1991, 1992; Ragot *et al.*, 1993). Most of these currently available expression vectors are based on human adenoviruses (HAd). Recently we have developed a BAd3-based vector having a foreign gene insert into the early region 3 (E3) (Mittal *et al.*, 1995). Such vectors certainly hold considerable potential as live recombinant viral vaccines for veterinary use. Since we are currently in the process of constructing a number of BAd3 recombinant viruses containing foreign genes representing protective antigens from pathogens of veterinary interest, a small-animal model with which to evaluate the immune responses to and pathogenic potential of these recombinant viruses compared to those the wild-type virus would be of considerable importance. The development of such a model to study BAd3 pathogenesis has not been reported. Since the cotton rat (*Sigmodon*

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hispidus) serves as a good model for studying pathogenesis of a number of HAd serotypes (Pacini *et al.*, 1984; Prince *et al.*, 1993), this species was a natural choice for a potential model of BAd3 infection. In this report we describe the usefulness of the cotton rat as a small-animal model for studying the pathogenesis and immunogenicity of BAd3.

MATERIALS AND METHODS

Virus and cell culture

The wild-type (wt) isolate of BAd3 (Mittal *et al.*, 1992) was propagated and titrated in MDBK cell monolayers grown in GIBCO BRL minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 25 m*M* HEPES, and 50 μg/ml gentamicin. A purified preparation of BAd3 was obtained following a cesium chloride density-gradient centrifugation (Graham and Prevec, 1991).

Animals

An inbred colony of cotton rats (*S. hispidus*), established from three breeding pairs of cotton rats obtained as a gift from the Veterinary Resources Branch, Division of Research Services, National Institute of Health, Maryland, and maintained at the Veterinary Infectious Disease Organization, Saskatoon, was the source of the animals used. Animals were housed individually in polycarbonate rat cages and fed a standard ration of rat feed pellets and water.

Experimental design

A total of 27 6- to 8-week-old cotton rats of either sex were randomly grouped into nine groups (three animals/group) and anesthetized with halothane, and seven groups were inoculated intranasally with 50 μl of a purified BAd3 preparation (10^8 PFU/animal). At 0.25, 1, 2, 3, 4, 8, and 10 days postinoculation (p.i.), animals were killed by an overdose of halothane and the right lung was inflated with and fixed in 10% neutral-buffered formalin for histopathological and immunohistochemical analyses. The trachea and the left lung from inoculated animals were collected in MEM for virus isolation. The two remaining groups were inoculated intranasally either with 0.1 *M* phosphate-buffered saline (PBS), pH 7.2, or with a heat-killed preparation originating from 10^8 PFU of BAd3, and the animals were killed at 3 days p.i. The right lung from each animal was processed similarly for histopathological and immunohistochemical studies, whereas the trachea and left lung were collected in MEM for virus isolation.

In a second experiment, five cotton rats were inoculated intranasally with 50 μl of a purified BAd3 preparation (10^8 PFU/animal) at 0 and 4 weeks p.i. Similarly a first control group (two animals/group) was inoculated with PBS and a second control group with a heat-killed preparation originating from 10^8 PFU of BAd3. At 4 and 8 weeks p.i., blood samples were collected to monitor development of BAd3-specific antibodies by ELISA and virus neutralization assays. Nasal and lung washes were also collected at 8 weeks p.i. to determine mucosal antibody responses against BAd3.

Raising anti-BAd3 antibodies in rabbits

Four milligrams of cesium chloride density-gradient-purified BAd3 suspension in 1 ml PBS containing Mg^{2+} and Ca^{2+} (0.137 *M* NaCl, 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 2.7 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$) was mixed thoroughly with an equal volume of Freund's complete adjuvant and the mixture (1 ml/animal) was injected subcutaneously (sc) into two New Zealand white rabbits. At 4 weeks following the first injection, 1 mg of BAd3 preparation was emulsified with an equal volume of Freund's incomplete adjuvant and injected sc into each rabbit. The animals were boosted 2 weeks later and were bled under anesthesia 10 days after the last injection. The development of anti-BAd3 bodies was monitored by ELISA. Immunoprecipitation of extracts of [^{35}S]-methionine-labeled mock- or BAd3-infected MDBK cells with anti-BAd3 sera raised in rabbits showed that both antisera obtained were BAd3-specific (data not shown).

ELISA

Ninety-six-well microtiter plates (Dynatech Laboratories) were coated with 100 μl of a purified BAd3 preparation (5 μg/ml in 0.05 *M* sodium carbonate, pH 9.6), incubated overnight at 4°, and washed with washing buffer (WB) [PBS, 0.05% Tween 20, 0.1% bovine serum albumin (BSA)]. The plates were incubated with 200 μl of dilution buffer (DB) (PBS, 0.05% Tween 20, 1% BSA)/well at 37° for 1 hr. The serum samples collected from mock- or virus-inoculated cotton rats were serially diluted with DB, 100 μl was added to each well, and the plates were incubated at 37° for 1 hr. Following washing with WB, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-rat IgG serum (Zymed) 1:2500 diluted in DB was added to each well, and the plates were incubated at 37° for 2 hr and washed with WB. One hundred microliters of ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulfonate(6)]) substrate (1 mg/ml ABTS in ABTS buffer, Boehringer-Mannheim) was added to each well and the plates were incubated at room temperature for 20 min. The optical density (OD) at 405 nm of each well was measured with an ELISA reader (Bio-Rad). The serum dilution having an OD reading of at least the mean + 2 SD above negative control sera was taken as the ELISA antibody titer.

The titers of BAd3-specific IgA antibodies in the serum, nasal, and lung washes collected from virus-inoculated cotton rats were determined using a polyclonal rabbit anti-rat IgA (obtained as a gift from Dr. B. Underdown,

Department of Pathology, McMaster University, Hamilton, Canada) and HRP-conjugated goat anti-rabbit IgG (Boehringer-Mannheim) sera.

Virus neutralization assays

Blood samples were collected from virus-inoculated cotton rats by cardiac puncture. The virus neutralization assays were similar to those described previously (Mittal *et al.*, 1993). Briefly, 100 μl of serially diluted serum samples in PBS was mixed with an equal volume of BAd3 suspension containing 100 PFU. Following incubation at 37° for 1 hr, the mixture was added to MDBK cell monolayers and the residual virus was allowed to adsorb for 1 hr. The cell monolayers were maintained under an agarose overlay and virus plaques were enumerated 10 days following infection. The highest serum dilution that resulted in reduction of virus plaque formation by at least 50% was considered the BAd3 neutralizing antibody titer. Assays were done in duplicate and serum samples collected from mock-inoculated cotton rats and an anti-BAd3 hyperimmune rabbit serum were used as negative and positive controls, respectively.

Virus isolation

The trachea and left lung were collected from cotton rats inoculated with BAd3 or PBS and stored at −70° until use. Tissues were homogenized with MEM by a tissumizer, sonicated, and supernatants were used for virus titration in MDBK cells by plaque assays (Mittal *et al.*, 1995).

Histopathology

The formalin-fixed cotton rat lung tissues were processed routinely, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H & E).

Immunohistochemical analysis

The avidin–biotin complex method for immunohistochemical staining of formalin-fixed paraffin-embedded tissues (Haines and Chelack, 1991), adapted to a robotic system, was used. Briefly, sections were deparaffinized and then hydrated by immersing the slides sequentially in xylene, ethanol, and water. The slides were treated with 0.5% H_2O_2 for 30 min at room temperature to inactivate endogenous peroxidases, immersed in a solution of protease XIV for 15 min at 37°, and washed with PBS. To avoid nonspecific reaction, the sections were incubated with normal goat serum. The slides (three/animal) were incubated for 1 hr at room temperature with 1:500 or 1:1000 dilutions of rabbit anti-BAd3 serum or with normal rabbit serum (negative control) and washed with PBS. Bovine lung sections with natural adenoviral pneumonia were used as a positive control. The slides were then incubated with HRP-conjugated goat anti-rabbit IgG serum for 30 min at room temperature. Following washing with PBS, the slides were treated with 3,3′ diaminobenzidine–H_2O_2 solution for 5 min at room temperature, washed with PBS, and counterstained with hematoxylin. A positive reaction consisted of brown deposits, indicating the presence of BAd3 antigens.

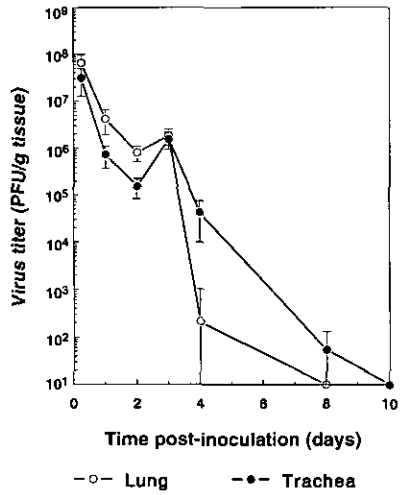

FIG. 1. Replication and clearance of virus from lungs and trachea of BAd3-inoculated cotton rats. Animals were inoculated intranasally with 10^8 PFU of BAd3. Lungs and tracheas were collected for virus titration. Each time point represents the mean virus titer for three animals ±SD.

RESULTS

Replication and clearance of BAd3 from cotton rat respiratory tract

Following intranasal inoculation of animals with 10^8 PFU of cesium chloride density-gradient-purified BAd3, animals were killed at various intervals over a 10-day period. The titers of infectious virus recovered from the lung and the trachea of virus-inoculated animals showed a declining trend until Day 2 p.i., followed by an approximately 5- to 10-fold rise in virus titers at Day 3 p.i. (Fig. 1). An increase in the amounts of virus recovered from the lung and the trachea at Day 3 p.i. compared to virus yields obtained at Day 2 p.i. appears due to active virus replication. At this time point, the amounts of infectious virus generated through active virus replication may be more than the number of infectious virus particles removed or rendered noninfectious as a result of host defense mechanisms. After Day 3 p.i., the infectious virus titers declined to reach undetectable levels by Day 8. The amounts of infectious virus isolated from the lung and the trachea showed similar patterns.

Histopathologic findings in BAd3-infected cotton rat lungs

Clinically, the virus-inoculated cotton rats showed no signs of respiratory distress and nasal discharge, nor

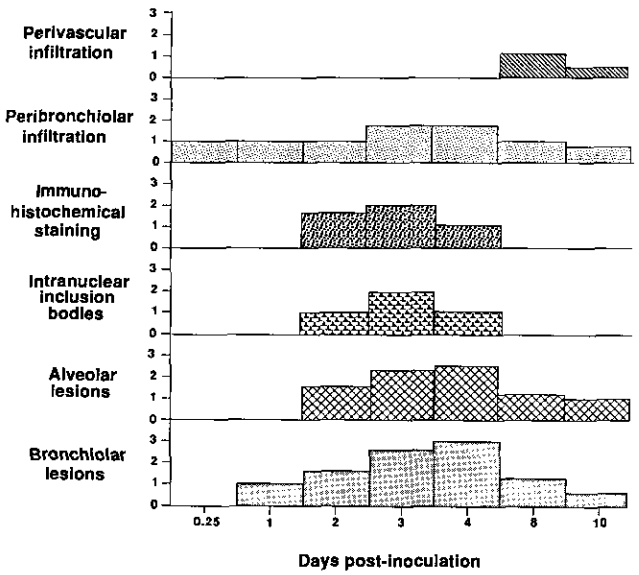

FIG. 2. Quantitation of histopathological changes and immunohistochemical staining in lungs of BAd3-inoculated cotton rats. Animals were inoculated intranasally with 10^8 PFU of BAd3 and at various times p.i., lungs were collected and processed for histopathology and immunohistochemical staining. The 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.

was there any change in feed and water intake and weight gain (data not shown). Bronchiolar and alveolar changes and immunohistochemical staining were absent in the lungs of animals inoculated with 10^8 PFU equivalents of heat-killed BAd3 (data not shown). In the lungs of cotton rats inoculated with infectious virus, the main histologic lesions were seen in the airways and alveoli, with only minor peribronchiolar and perivascular changes. Immunohistochemical staining was seen predominantly in the bronchiolar epithelial cells. A summary of histopathological changes and immunohistochemical staining observed in the lungs of cotton rats is illustrated in Fig. 2. These findings were scored on a scale of 0 (normal) to 3 (severe). In all of the animals, lesions were present in at least one of the three sections of lung examined.

Bronchiolar lesions. Changes were not seen in the bronchioles at 6 hr p.i. but by Day 1 the epithelium, especially in the larger airways, had a ragged appearance with the presence of a few pyknotic nuclei. Desquamation of a few epithelial cells and infiltration of a few neutrophils into the lumen were evident at Day 1 p.i. Focal loss of cilia was noted. No positive immunohistochemical staining was observed. At Day 2, clumps of desquamated epithelial cells and occasional neutrophils were present in bronchioles. The epithelium showed early changes of hyperplasia with stratification and presence of mitotic figures. Loss of cilia was more extensive. Eosinophilic intranuclear inclusion bodies were rare and were generally located superficially within the epithelium. Necrotic cells with cytoplasmic condensation, karyorhexis, or pyknosis were present and epithelial cells were frequently swollen and pale. Positive immunohistochemical staining was noted intracytoplasmically in epithelial cells. By Day 3, cellular debris was common in airways, along with the presence of a few neutrophils. Distinct intranuclear inclusion bodies were observed in cells both within the epithelium and in the lumen. The epithelium was hyperplastic and thickened, especially in the larger airways. Multifocal cysts containing shrunken necrotic cells were present within the epithelial layer. Immunohistochemical staining was more pronounced in luminal debris and in the superficial epithelium. In positive cells, intranuclear inclusion bodies were often stained less intensely than the cytoplasm. By Day 4, luminal debris was still present. Intranuclear inclusions were fewer and less distinct. Necrotic epithelial cells and cystic spaces within the epithelium were still present. The epithelium was hyperplastic with rare mitotic figures. Immunohistochemical staining was most intense at this stage. Typical bronchiolar histological changes and immunohistochemical staining are shown in Figs. 3c and 3d, respectively. At Day 8 postinfection, luminal cell debris was minimal, intranuclear inclusion bodies were absent, and epithelial hyperplasia was more focal. No specific immunohistochemical staining was noted. At Day 10, a minimal amount of luminal debris was noted. The epithelium was mildly hyperplastic, with rare necrotic cells present. Specific immunohistochemical staining was again absent. The lung sections from PBS-inoculated cotton rats used as a negative control for histopathology and immunohistochemical staining (Figs. 3a and 3b) displayed normal characteristics. Low numbers of eosinophils were present within the bronchiolar epithelium of the BAd3-, heat-killed BAd3-, and PBS-inoculated control animals.

Alveolar changes. The changes in alveoli were first noted on Day 2 and consisted of type II pneumocyte hyperplasia, characterized by a low number of large plump cells, with large basophilic nuclei, protruding into alveolar lumina. Neutrophils were occasionally present in alveolar walls. These changes became more extensive and more pronounced by Day 3. There was a concomitant light mononuclear cell infiltration, consisting mainly of macrophages, of alveolar walls. Rarely, single-cell necrosis and mitotic figures were seen. By Day 4 these alveolar changes were most marked (Fig. 3e) and were in decline by Day 8. Specific immunohistochemical staining, however, was minimal and noted only in a few alveolar cells, in both the cytoplasm and the nucleus, at Day 4 (Fig. 3f).

Peribronchiolar lymphocytic infiltration was noted at a low level in control animals; by Days 3 and 4, this became moderate in BAd3-inoculated cotton rats and waned at Day 8. Perivascular lymphocytic infiltration was a late and inconsistent development, first seen at Day 8 in two

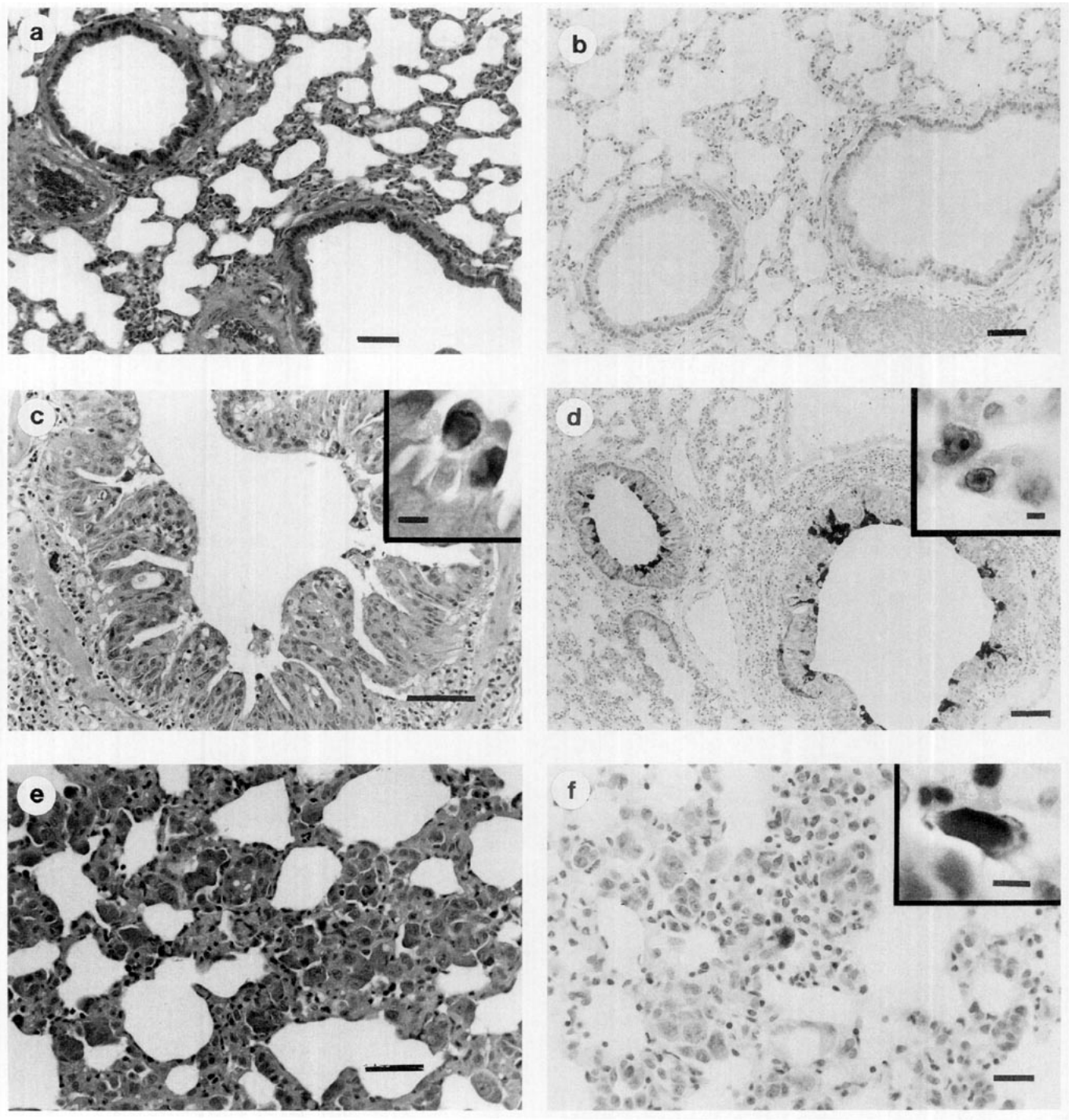

FIG. 3. Histopathological changes and immunohistochemical staining in lungs of BAd3-inoculated cotton rats. Animals were inoculated intranasally with 10^8 PFU of BAd3 or PBS and at various times p.i., lungs were collected and processed for histopathology (a, c, and e) and immunohistochemical staining (b, d, and f). (a and b) Lung 3 days after PBS inoculation. (a) Bronchioles and alveoli are normal and (b) there is absence of specific positive staining. Bar, 50 μm. (c) Lung 3 days after BAd3 inoculation. Note changes in the bronchiolar wall, consisting of hyperplasia of bronchiolar epithelium, epithelial cell necrosis, cystic spaces, and small amount of luminal debris. Bar, 50 μm. Inset: Eosinophilic intranuclear inclusions in bronchiolar epithelial cells. Bar, 5 μm. (d) Lung at 3 days after BAd3 inoculation: Extensive positive staining of the bronchiolar epithelial cells and surface debris. Bar, 100 μm. Inset: Positively stained intranuclear inclusions in bronchiolar epithelial cells. Bar, 5 μm. (e) Lung 4 days after BAd3 inoculation: Alveolar type II pneumocyte hyperplasia is marked and is accompanied by a light mononuclear cell infiltration. Bar, 50 μm. (f) Lung 4 days after BAd3 inoculation. Positive staining of an intranuclear inclusion in a alveolar epithelial cell. Bar, 50 μm. Inset: Positively stained intranuclear inclusions in an alveolar epithelial cell. Bar, 5 μm.

TABLE 1

BAd3 Antibody Response in Cotton Rats Inoculated Intranasally with BAd3[a]

Animal	Animal inoculated with	Time of inoculation (week)	Anti-BAd3 ELISA titers		BAd3 neutralizing antibody titers	
			4 weeks	8 weeks	4 weeks	8 weeks
1	BAd3	0, 4	800	3200	800	1600
2	BAd3	0, 4	3200	12800	1600	6400
3	BAd3	0, 4	800	3200	800	1600
4	BAd3	0, 4	800	12800	1600	3200
5	BAd3	0, 4	1600	12800	1600	6400
6	Heat-killed BAd3	0, 4	<25	<25	<50	<50
7	Heat-killed BAd3	0, 4	<25	<25	<50	<50
8	PBS	0, 4	<25	<25	<50	<50
9	PBS	0, 4	<25	<25	<50	<50

[a] Cotton rats were inoculated intranasally either with PBS or with 10^8 PFU of infectious virus or heat-killed preparation of cesium chloride density-gradient-purified BAd3 at 0 and 4 weeks p.i. The serum samples were collected at 4 and 8 weeks p.i. and tested for the presence of antibodies to BAd3 by ELISA and virus neutralization assays.

animals and by Day 10 in only one, and was never marked (Fig. 2).

Immune response against BAd3 in cotton rats

The results described above showed that the limited virus replication produced significant histopathologic changes in the lungs of cotton rats inoculated with BAd3 as described above. To determine whether limited replication of BAd3 in cotton rats could elicit a BAd3-specific immune response, animals were inoculated intranasally twice with BAd3 at 0 and 4 weeks p.i. The serum samples were collected at 4 and 8 weeks, and nasal and lung washes collected at 8 weeks after the primary inoculation. The sera were tested for anti-BAd3 IgG antibodies by ELISA and for BAd3 neutralizing antibodies by virus neutralization assays (Table 1). At 4 weeks p.i., both the ELISA and the virus neutralizing antibody titers were equal to or higher than 1:800. The booster inoculation at 4 weeks p.i. produced a further two- to fivefold rise in titers. The serum samples collected from the mock- or heat-killed BAd3-inoculated animals did not show antibody titers above background.

The serum samples and nasal and lung washes collected from BAd3-infected cotton rats were tested similarly for BAd3-specific IgA antibodies by ELISA as a measure of mucosal immunity (Table 2). The BAd3-specific IgA antibodies as measured by ELISA in the sera of BAd3-inoculated cotton rats were equal to or higher than 1:160 at 4 weeks p.i. The booster inoculation at 4 weeks p.i. resulted in a further two- to threefold rise in the BAd3-specific IgA antibodies. In the lung and nasal washes, the BAd3-specific IgA antibodies as measured by ELISA were equal to or higher than 1:320 and 1:40, respectively, at 8 weeks after the first inoculation. The sera and the lung and nasal washes collected from the mock- or heat-killed BAd3-inoculated animals did not show any BAd3-specific IgA antibodies above background.

DISCUSSION

The main objective of this study was to develop a small-animal model to study the pathogenesis of BAd3 infection. Our results clearly demonstrate that cotton rats have limited susceptibility to infection with BAd3 and develop pulmonary lesions microscopically similar to those of natural or experimental adenoviral disease in the bovine respiratory tract (Darbyshire *et al.*, 1965b, 1966; Ide *et al.*, 1969). Bronchiolar epithelium is a primary target tissue of bovine adenoviruses and the infection is characterized by bronchiolar hyperplasia, epithelial necrosis, and intranuclear inclusion bodies, along with peribronchiolar lymphocytic infiltration, in both cattle and cotton rats. Type II pneumocyte hyperplasia, however, is not a consistent feature of the bovine disease (Darbyshire *et al.*, 1966).

BAd3-inoculated cotton rats did not show any clinical signs of respiratory disease. A higher infective dose of virus may be required to produce clinical disease; however, experimental inoculation of calves with BAd3 usually results in clinically unapparent infection or only mild respiratory disease (Ide *et al.*, 1969; Mittal *et al.*, unpublished data). Even in colostrum-deprived young calves experimentally exposed to BAd, most showed only mild respiratory disease (Darbyshire *et al.*, 1965b, 1966), and natural exposure of seronegative older calves generally results in mild clinical signs or unapparent infection and seroconversion (Mattson, 1973b; Yates, 1982). Thus our studies indicate that the pathogenesis of BAd3 is not dramatically different in cotton rats and calves.

The cotton rat has been developed successfully as a small-animal model of HAd5 infection, with the pattern

TABLE 2

BAd3-Specific IgA Antibody Response in Cotton Rats Inoculated Intranasally with BAd3[a]

Animal	Animal inoculated with	Time of inoculation (week)	Serum BAd3-specific IgA ELISA titers		Mucosal BAd3-specific IgA ELISA titers	
			4 weeks	8 weeks	LW	NW
1	BAd3	0, 4	160	320	320	40
2	BAd3	0, 4	320	1280	640	80
3	BAd3	0, 4	160	640	640	80
4	BAd3	0, 4	320	1280	640	80
5	BAd3	0, 4	320	1280	640	80
6	Heat-killed BAd3	0, 4	<10	<10	<10	<10
7	Heat-killed BAd3	0, 4	<10	<10	<10	<10
8	PBS	0, 4	<10	<10	<10	<10
9	PBS	0, 4	<10	<10	<10	<10

[a] Cotton rats were inoculated intranasally either with PBS or with 10^8 PFU of infectious virus or heat-killed preparation of cesium chloride density-gradient-purified BAd3 at 0 and 4 weeks p.i. The serum samples were collected at 4 and 8 weeks p.i., whereas the lung and nasal washes were collected only at 8 weeks p.i. The sera and lung and nasal washes were tested for the presence of BAd3-specific IgA antibodies by ELISA. LW, lung wash; NW, nasal wash.

of viral replication and pulmonary histopathology being essentially similar to that of the natural human disease (Pacini *et al.*, 1984; Prince *et al.*, 1993). The microscopic lesions have been divided into an early and a later phase on the basis of the type of cells involved in the inflammatory response (Prince *et al.*, 1993). The bovine adenoviral disease in cotton rats is very similar with the bronchiolar and alveolar changes and necrosis, type II pneumocyte proliferation, and neutrophil infiltration, starting 2 days p.i., constituting the early phase while the later phase is characterized by mild lymphocytic infiltration, initially around bronchioles and by Day 8, around the blood vessels. Macrophage and neutrophil infiltration of the interalveolar septa and alveolar lumina, although present, was not a prominent feature in our studies with BAd3, and peribronchiolar and perivascular infiltration of lymphocytes were also less pronounced than that in HAd5-inoculated cotton rats (Prince *et al.*, 1993). In contrast, bronchiolar epithelial necrosis was more marked in the BAd3-inoculated cotton rats, perhaps reflecting a difference in pathogenicity of these two viruses for this species.

Intranuclear inclusions were numerous in bronchiolar epithelial cells in H & E sections, and both cytoplasmic staining and nuclear staining were noted immunohistochemically, using rabbit BAd3-specific antiserum. Such inclusions were rare in alveolar epithelial cells in histological sections, and viral antigens were only rarely identified immunohistochemically. Intranuclear inclusions are a criterion of productive adenovirus replication (Pacini *et al.*, 1984). Inoculation dose of infectious virus, type of cellular changes, and time p.i. are some of the factors which may affect development of intranuclear inclusion bodies in the animal models of adenovirus infection. The infective dose of 10^8 PFU which was used in the present study may not be optimum for the development of intranuclear inclusions in the alveolar cells. Necrosis of virus-infected cells, a feature for this animal model of BAd3 infection, may negatively affect the appearance of intranuclear inclusions. Necrosis of alveolar epithelial cells is indicated by the marked type II pneumocyte proliferation. No inclusions were seen in these cells. In a subsequent experiment, when we used a lower infective dose of BAd3 (3×10^7 PFU) to inoculate cotton rats, intranuclear inclusions were commonly observed in the alveolar cells (unpublished data). The kinetics of BAd3 replication in primary cultures of cotton rat lung fibroblast cells did not provide evidence of active virus replication (data not shown). However, the results of immunohistochemical staining of BAd3-infected cotton rat lung sections suggest that epithelial cells rather than fibroblast cells may be the preferable site of virus replication.

We do not know whether the limited BAd3 replication, mainly in the airways of cotton rats, is related to the severity of histopathological lesions in the lungs. A comparative study involving the wt BAd3 and a BAd3 E1-deleted mutant in producing histopathological lesions in lungs of cotton rats should help to clarify this point. Studies with replication-incompetent HAd5 mutants or E1-deleted HAd5 recombinant viruses have indicated that the synthesis of viral structural proteins is not essential to produce lesions of adenoviral pneumonia in mice and cotton rats (Ginsberg *et al.*, 1990, 1991); however, a higher virus infectious dose is usually needed to obtain histopathological changes comparable to those obtained with wt HAd5. The expression of early gene(s) of adenovirus plays an important role in induction of cytokines, such as tumor necrosis factor-α, interleukin (IL)-1, and IL-6 in the lung, which in turn may largely be responsible for the inflammatory response observed during the early phase of adenoviral pneumonia due to HAd5 (Ginsberg

et al., 1991). For HAd5 infections in cotton rats (Ginsberg *et al.*, 1989, 1990; Prince *et al.*, 1993) and mice (Ginsberg *et al.*, 1991) it has been argued that the earlier phase is the result of a nonspecific inflammatory reaction probably due to cytokines, and the later phase perhaps represents an immunologically specific T-lymphocyte response. This may also be true for BAd3 pneumonia in cotton rats.

Intranasal inoculation of cotton rats with BAd3 resulted in BAd3-specific IgG and IgA antibodies in the serum and lung and nasal washes, suggesting that BAd3 vectors do have the potential to be used as vehicles for the delivery of foreign antigens to mucosal surfaces. Mucosal delivery may be a better way to elicit a mucosal immune response, in addition to humoral and cellular immune responses, against respiratory and enteric pathogens, compared to the standard intramuscular route of vaccine administration. The cotton rat model of BAd respiratory tract infection should provide a useful system for monitoring pathogenesis of bovine adenoviral disease and also for investigating immune responses against the foreign antigens delivered through a BAd-vectored vaccine. Currently we are investigating the pathogenesis and the immune responses against firefly luciferase protein expressed by a BAd3–luciferase recombinant virus, having an E3 deletion replaced with the reporter gene (Mittal *et al.*, 1995), when delivered to the respiratory tract of cotton rats by the intranasal route. These studies should form the foundation for future studies of immune responses to other recombinant adenoviruses carrying genes of protective viral antigens.

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