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Pathogenesis of Adenovirus Type 5 Pneumonia in Cotton Rats (*Sigmodon hispidus*)

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Cotton rats (*Sigmodon hispidus*) were inoculated intranasally with $10^{2.0}$ to $10^{10.0}$ PFU of human adenovirus type 5. The virus replicated to a high titer in pulmonary tissues, with the peak titer being proportional to the input dose. The 50% lethal dose was $10^{9.4}$ PFU. Histopathologic changes were proportional to the infecting inoculum and included the infiltration of interstitial and intra-alveolar areas, moderate damage to bronchiolar epithelium, and cellular infiltration of peribronchiolar and perivascular regions. These changes could be divided into two phases: an early phase (affecting alveoli, bronchiolar epithelium, and peribronchiolar regions) with an infiltrate consisting primarily of monocytes-macrophages and neutrophils, with occasional lymphocytes, and a later phase (affecting peribronchiolar and perivascular regions) with an infiltrate consisting almost exclusively of lymphocytes. In both phases, the predominant process was the response of the host to infection, rather than direct viral damage to infected cells. An infecting inoculum of $10^{8.0}$ PFU or larger caused severe damage to type II alveolar cells, which were swollen, showed a loss of lamellar bodies, and were surrounded by polymorphonuclear leukocytes and macrophages. No evidence of complete viral replication was found in type II alveolar cells.

Adenoviruses are important human pathogens producing a wide variety of diseases, pneumonia being among the most serious (10). Shortly after the initial report of adenovirus isolation in 1953, an attempt was made to develop an animal model for human adenovirus diseases by use of a variety of laboratory animals, including primates (chimpanzees and rhesus monkeys), carnivores (ferrets and domestic cats), rabbits, and rodents (guinea pigs, hamsters, cotton rats, white rats, and mice). None developed clinical disease, although seroconversion was seen in the chimpanzees, guinea pigs, and cotton rats (28). Subsequent reports described adenovirus inoculation of rabbits (23), dogs (30), pigs (3), newborn hamsters (22), mice (24), and guinea pigs (9), but none proved to be a useful model for pulmonary disease.

Recently, cotton rats (*Sigmodon hispidus*) were shown to be susceptible to infection by at least four serotypes of human adenovirus (types 1, 2, 5, and 6) and to develop pulmonary histopathology following adenovirus type 5 (Ad5) inoculation (20). The development of an extensive array of genetically defined adenovirus mutants (10), coupled with a convenient small-animal model of pulmonary disease, suggested that it might be possible to determine the molecular mechanism by which adenoviruses produce pneumonia by examining the role of selected viral genes in the pathogenesis of the disease. This paper describes the sequential development of pathologic changes during wild-type Ad5 pulmonary infection in cotton rats.

MATERIALS AND METHODS

Animals. Cotton rats (*S. hispidus*) were obtained from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health, housed in large polycarbonate rat cages, and fed a diet of standard rat chow and water.

Virus and cell culture. A previously identified wild-type strain of Ad5 (7) was used. The virus was propagated in monolayer cultures of KB cells grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and then in medium supplemented with 10% chicken serum at the time of infection (7). A549 cells (kindly supplied by E. J. Dubovi) were grown under the same conditions as KB cells.

Experimental design. Young adult cotton rats were anesthetized with methoxyflurane and inoculated intranasally with 0.1 ml of viral suspension. At intervals following inoculation, animals were sacrificed by carbon dioxide asphyxiation. Tissues for viral assays were homogenized in 10 volumes of Hanks' balanced salt solution and stored at −70°C. Lungs for histopathologic analysis were inflated transtracheally with 10% neutral buffered formalin and embedded in paraffin for sectioning. Histologic sections were stained with hematoxylin and eosin (H&E). Lungs for immunofluorescence microscopy were inflated with a 1:1 mixture of phosphate-buffered saline and O.C.T. compound (Tissue Tek II; Miles Laboratories), snap frozen, and stored at −70°C until sectioning was done at −20°C. Tissue for electron microscopy was fixed and stored in glutaraldehyde and embedded in Epon.

Viral assays. Infectivity was determined by indirect immunofluorescence focus assays on KB cells with polyclonal rabbit antiserum to intact virus (31) or by plaque assays (18) on A549 cells (20). The same reagents were used for the demonstration of virion antigens in tissues.

RESULTS

Kinetics of viral replication. Animals were inoculated intranasally with $10^{6.0}$, $10^{7.0}$, or $10^{8.0}$ PFU of Ad5 and

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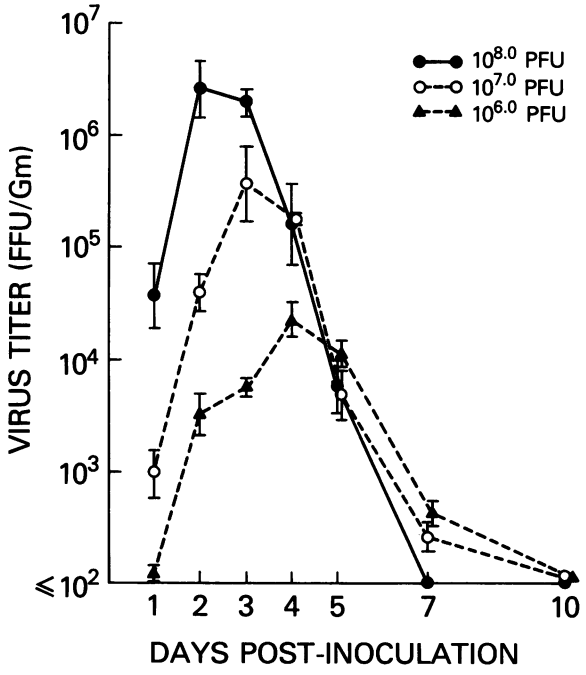


FIG. 1. Viral replication in lungs of cotton rats inoculated intranasally on day 0 with $10^{6.0}$, $10^{7.0}$, or $10^{8.0}$ PFU of human Ad5. Each point, indicated as fluorescent-focus units (FFU) per gram of tissue, represents the geometric mean titer for five animals ± the standard error.

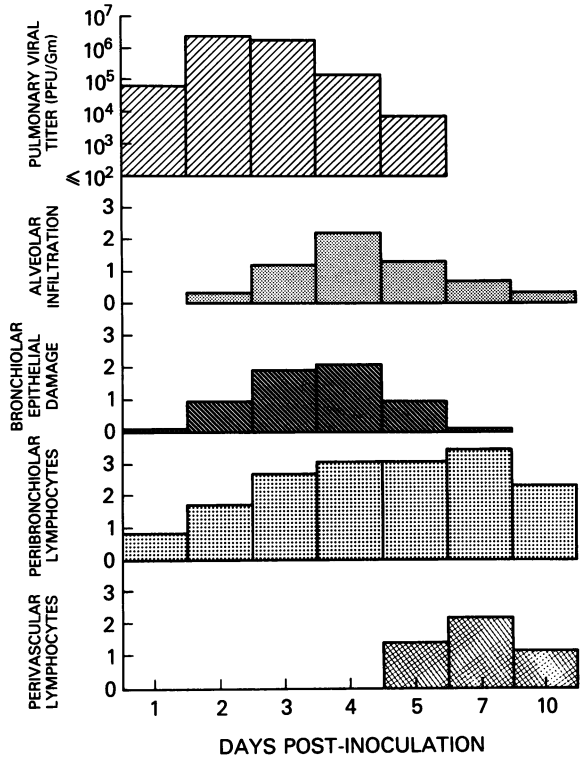


FIG. 2. Quantitation of histopathologic changes in cotton rat lungs following intranasal inoculation of $10^{8.0}$ PFU of Ad5. Each category was scored on a scale of 0 (normal tissue) to 4 (maximum pathology), and each bar represents the arithmetic mean score for five animals.

sacrificed at intervals over the next 10 days. Viral titers (Fig. 1) are represented by the geometric mean of titers in lungs from five animals for each datum point. The duration of infection did not differ among the three dosages, but the peak titer correlated directly and the time required to reach peak titer correlated inversely with the magnitude of the challenge dose.

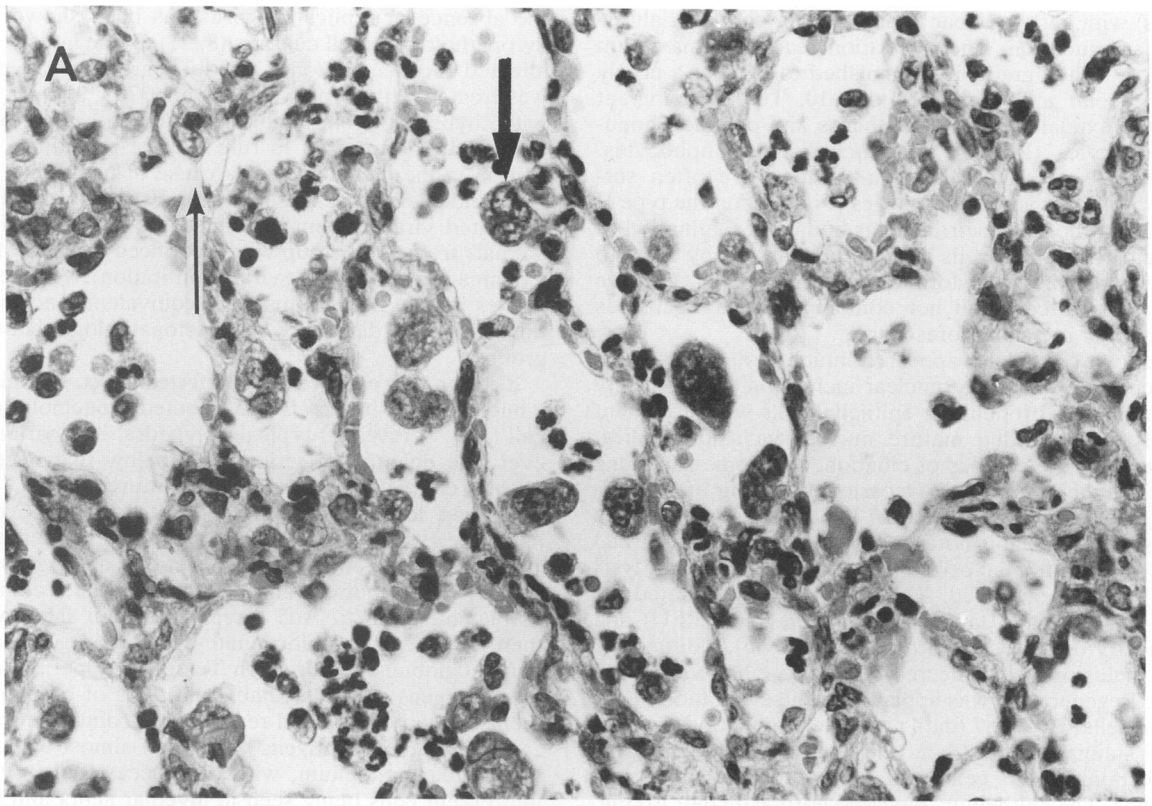
Infectious dose. Cotton rats were inoculated intranasally with 10^2 to 10^8 PFU of wild-type Ad5, and four animals from each group were sacrificed 3, 5, and 7 days postinfection. Each lung was homogenized in Hanks' balanced salt solution to prepare a 10% suspension and stored at −70°C. Viral infectivity titers were determined by an immunofluorescence focus assay (31) on KB cell monolayers. The fluorescence-negative lung homogenates were passaged once in triplicate KB cell monolayer cultures, which were observed for the development of cytopathic effects. Lung homogenates from animals infected with 10^5 to 10^8 PFU were found uniformly positive for viral replication when assayed by immunofluorescence. Homogenates of lungs from cotton rats infected with 10^4 or 10^5 PFU showed typical cytopathic effects when passaged in KB cells, but those from animals infected with smaller amounts of virus were found uniformly negative. These data indicated that the 50% infectious dose of Ad5 for *S. hispidus* was approximately $10^{3.5}$ PFU.

Lethal dose. Groups of 12 animals each were inoculated intranasally with serial twofold dilutions of Ad5, the largest dose being $10^{10.0}$ PFU per animal. All animals receiving $10^{10.0}$ and $10^{9.7}$ PFU died within 7 days, with deaths at the higher dose beginning 2 days postinoculation. The 50% lethal dose, calculated by the Reed-Muench method (25), was $10^{9.4}$ PFU. No deaths occurred with inocula smaller than $10^{8.8}$ PFU per animal.

Histopathology. Histopathologic changes are described and illustrated for animals receiving $10^{8.0}$ PFU. Changes in animals receiving $10^{7.0}$ or $10^{6.0}$ PFU were qualitatively similar to those seen at the higher dose but were significantly reduced in magnitude, while animals that received 10^9 to 10^{10} PFU showed enhanced epithelial cell injury and alveolar cell infiltration, with some degree of diffuse alveolar damage. Four basic types of tissue change were seen: alveolar infiltration, epithelial cell injury, peribronchiolar infiltration, and perivascular infiltration. Each was scored on a scale of 0 (normal tissue) to 4; the scores for five animals per time point were averaged, and the mean scores were plotted as a function of time after viral inoculation (Fig. 2, results obtained with an inoculum of $10^{8.0}$ PFU).

Alveoli appeared normal on day 1. By day 2, a pathologic change was apparent; it consisted of interstitial thickening, swelling of type II pneumocytes, some cells (probably mac-

FIG. 3. Cotton rat lung 3 days after inoculation of $10^{8.0}$ PFU of Ad5. (A) Alveolar walls are thickened, and the alveolar spaces are filled with inflammatory cells, predominantly PMNs and macrophages. Swollen type II pneumocytes are surrounded by inflammatory cells (arrows). H&E stain; magnification, ×760. (B) Electron micrograph of a swollen type II pneumocyte (II) surrounded by macrophages (M) and PMNs (P). One macrophage has ingested a dead cell (D). Magnification, ×7,200.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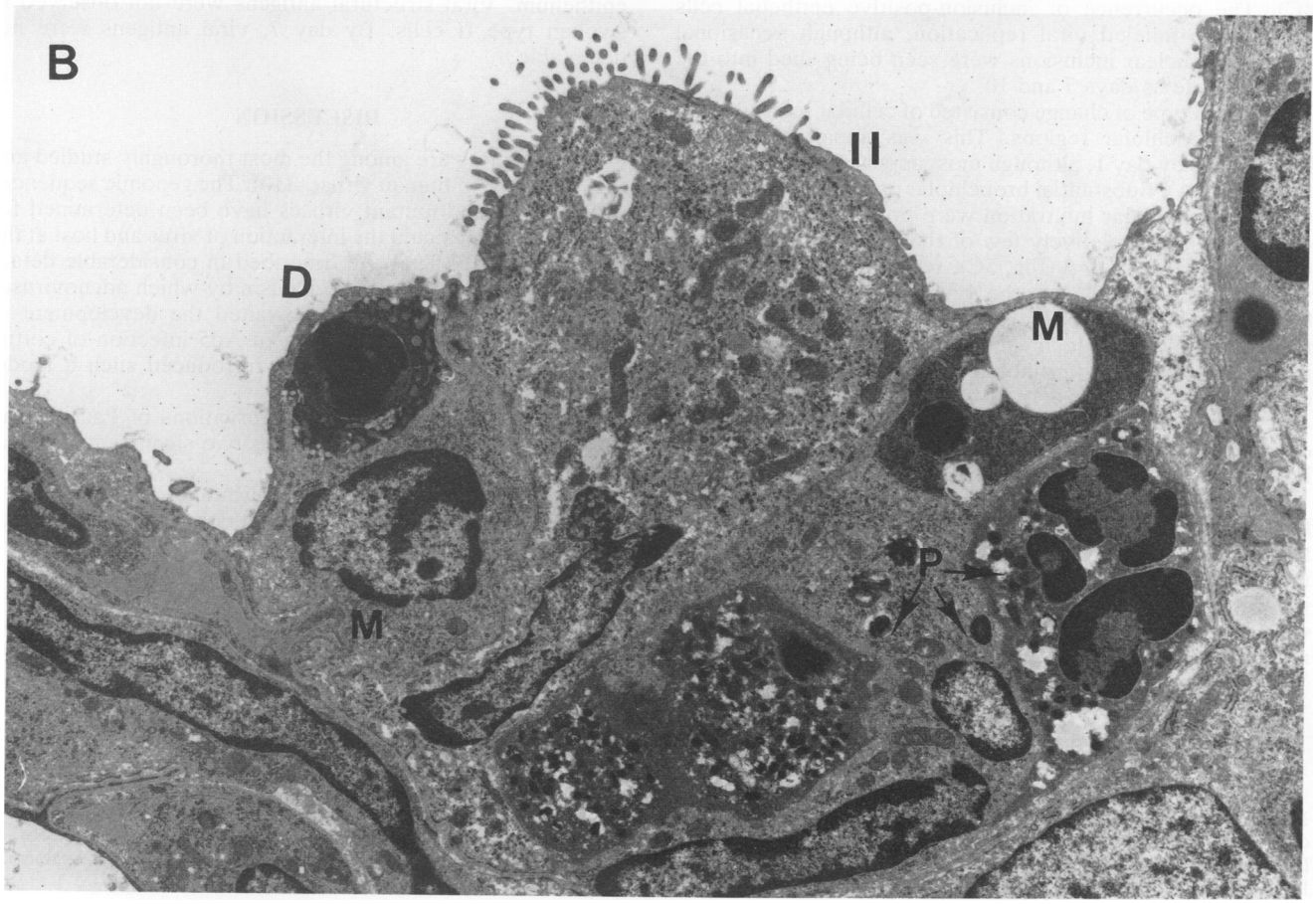
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rophages) with early nuclear condensation, and intra-alveolar cellular exudation. The infiltration reached a maximum on day 4 and then gradually diminished to present a nearly normal alveolar appearance by day 10. The predominant cells in the exudate were macrophages and polymorphonuclear leukocytes (PMNs), with occasional lymphocytes. Markedly enlarged type II pneumocytes were often surrounded by PMNs and macrophages (Fig. 3), and the type II cells had lost most of their lamellar bodies. Examination of over 40 affected type II cells by electron microscopy failed to show evidence of virion formation in these cells, and the affected type II cells did not contain virion antigens, as determined by immunofluorescence.

Bronchiolar epithelium appeared intact on day 1, but 5 to 10% of cells showed early nuclear inclusions (Fig. 4A). By day 3, damage to bronchiolar epithelial cells was prominent (Fig. 4B) and included mature nuclear inclusion bodies, cytoplasmic vacuoles, loss of ciliation, and some sloughing into the bronchiolar lumen. Occasional clefting of the epithelium and cellular infiltration of the basement membrane (arrow) were noted. It is noteworthy that despite the marked changes in the epithelial cells in which virus replicated, lysis of cells was not observed. This result was also noted for animals that received larger inocula (e.g., 10^{10} PFU) and subsequently died. By day 5 (data not shown), clefting and nuclear inclusions were decreased, while ciliation was increased. Regeneration was apparent in the elongation of the epithelial cells. By day 7 (data not shown), ciliation, nuclear size and staining density, and the layering of nuclei in the basal portion of the cells had all approached a normal appearance. The epithelium appeared normal on day 10 (Fig. 4C). The occurrence of inclusion-positive epithelial cells generally paralleled viral replication, although occasional cells with nuclear inclusions were seen being shed into the lumen as late as days 7 and 10.

The third type of change consisted of cellular infiltration of the peribronchiolar regions. This was apparent in some bronchioles by day 1, although most appeared normal at this time (Fig. 5A). Substantial bronchiolar epithelial cell damage and peribronchiolar infiltration were present on day 3 (Fig. 4B and 5B), but relatively few of the infiltrating cells were lymphocytes. By day 5 (Fig. 5C), when alveolar infiltration was greatly reduced and the bronchiolar epithelium had begun to heal, a cuff of lymphocytes had begun to encircle the bronchioles and to invade the bronchiolar walls. This infiltrate became most pronounced on day 7 (Fig. 5D), often extending to a thickness of 10 to 15 cells. By day 10 (Fig. 4C), when bronchiolar epithelial and alveolar tissues were essentially normal, there were still significant numbers of lymphocytes around the bronchioles.

Lymphocytic infiltration around the blood vessels was the final change observed. Perivascular infiltration began later than peribronchiolar lymphocytic infiltration, with minimal infiltration being seen prior to day 5 (Fig. 6A) and maximal infiltration being seen on day 7 (Fig. 6B). By day 10, there were few lymphocytes remaining around blood vessels (data not shown).

Role of penton in pathologic responses. Purified penton in the absence of replicating virus has been shown to cause cytopathology in cell cultures (8, 21). Since infectivity is not affected and free penton is rapidly degraded by trypsin, Ad5 was treated with trypsin (0.1 mg/ml, 37°C, 30 min) and then with soybean trypsin inhibitor (0.1 mg/ml) prior to inoculation of the cotton rats to rule out the possibility that free penton in the inoculum was a cause of pathology. Animals were inoculated intranasally with $10^{9.0}$ PFU of treated or untreated viral suspension. On days 1, 2, 3, and 5, eight animals from each group were sacrificed (four for histologic examination and four for viral quantitation). Viral replication curves for the two groups were equivalent, and there were no significant differences in histopathology between the groups.

Electron microscopy of Ad5-infected lungs. Examination of a nuclear inclusion in an Ad5-infected bronchiolar epithelial cell (Fig. 7) revealed typical polyhedral Ad5 virions. However, the quantity of virions was below that necessary to effect a crystalline array in a cell culture (19).

Antigen localization in Ad5-infected lungs. Cotton rats inoculated with $10^{8.0}$ PFU were sacrificed at intervals of 1 to 7 days after infection. Frozen sections of lung tissue were stained by an indirect immunofluorescence technique with rabbit antiserum to Ad5 (prepared in our laboratory) and then fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Organon Teknika-Cappel). Viral structural antigens were detectable in nuclei of bronchiolar epithelial cells by day 1 and reached a maximum level on days 2 and 3 (Fig. 8). Antigens were seen almost exclusively in bronchiolar epithelium, with only occasional (<1%) single fluorescent cells being seen in alveolar septa and bronchial epithelium. Viral structural antigens were not observed in swollen type II cells. By day 7, viral antigens were not detectable.

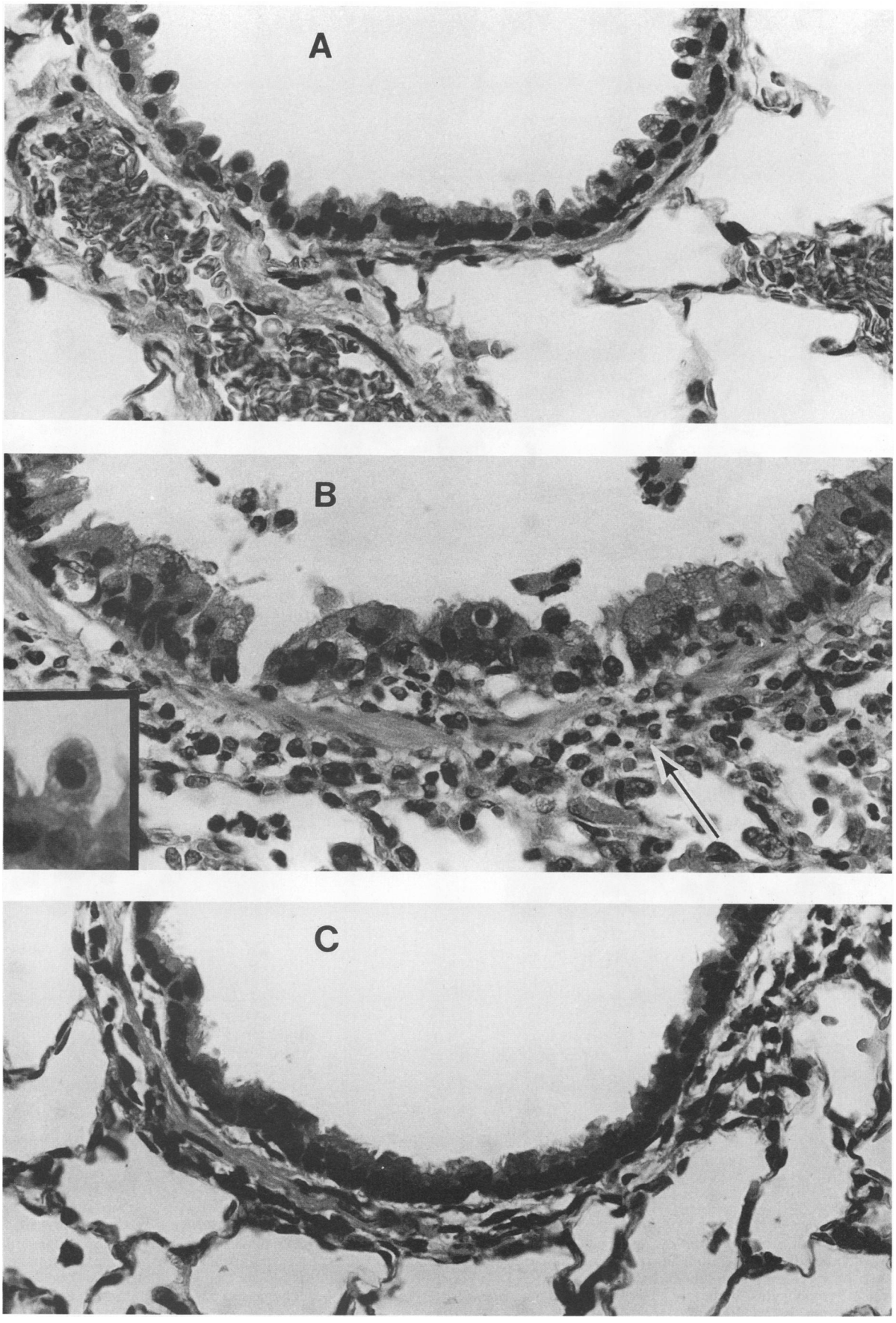
DISCUSSION

Adenoviruses are among the most thoroughly studied and characterized of human viruses (10). The genomic sequences of wild-type and mutant viruses have been determined for several serotypes, and the interaction of virus and host at the subcellular level has been described in considerable detail. An understanding of the mechanism by which adenoviruses cause disease, however, has awaited the development of animal models. The description of Ad5 infection of cotton rats, with attendant pneumonia, introduced such a model (20).

While confirming the basic observations of Pacini et al. (20), the current investigation adds two significant observations.

First, the magnitudes of viral replication and of pneumonia are directly proportional to the input dose of virus. Although such an observation seems logical, it contrasts with the results of earlier animal studies of influenza virus and pneumonia virus of mice, in which a lower input dose delayed the attainment of the peak viral titer, but did not diminish the magnitude of that titer (11, 17). The studies with influenza virus and pneumonia virus of mice were done with

FIG. 4. Cotton rat bronchiolar epithelium following inoculation of $10^{8.0}$ PFU of Ad5. (A) Day 1. The epithelium appears intact, but 5 to 10% of cells show early nuclear inclusions. (B) Day 3. Changes include mature nuclear inclusion bodies, cytoplasmic vacuoles, loss of ciliation, and sloughing into the bronchiolar lumen. Clefting of the epithelium is apparent, as is cellular infiltration of the basement membrane (arrow). (Inset) Bronchiolar epithelial cell with a mature basophilic Ad5 nuclear inclusion body. (C) Day 10. The epithelium appears normal. H&E stain; magnification, ×760; inset magnification, ×1,242.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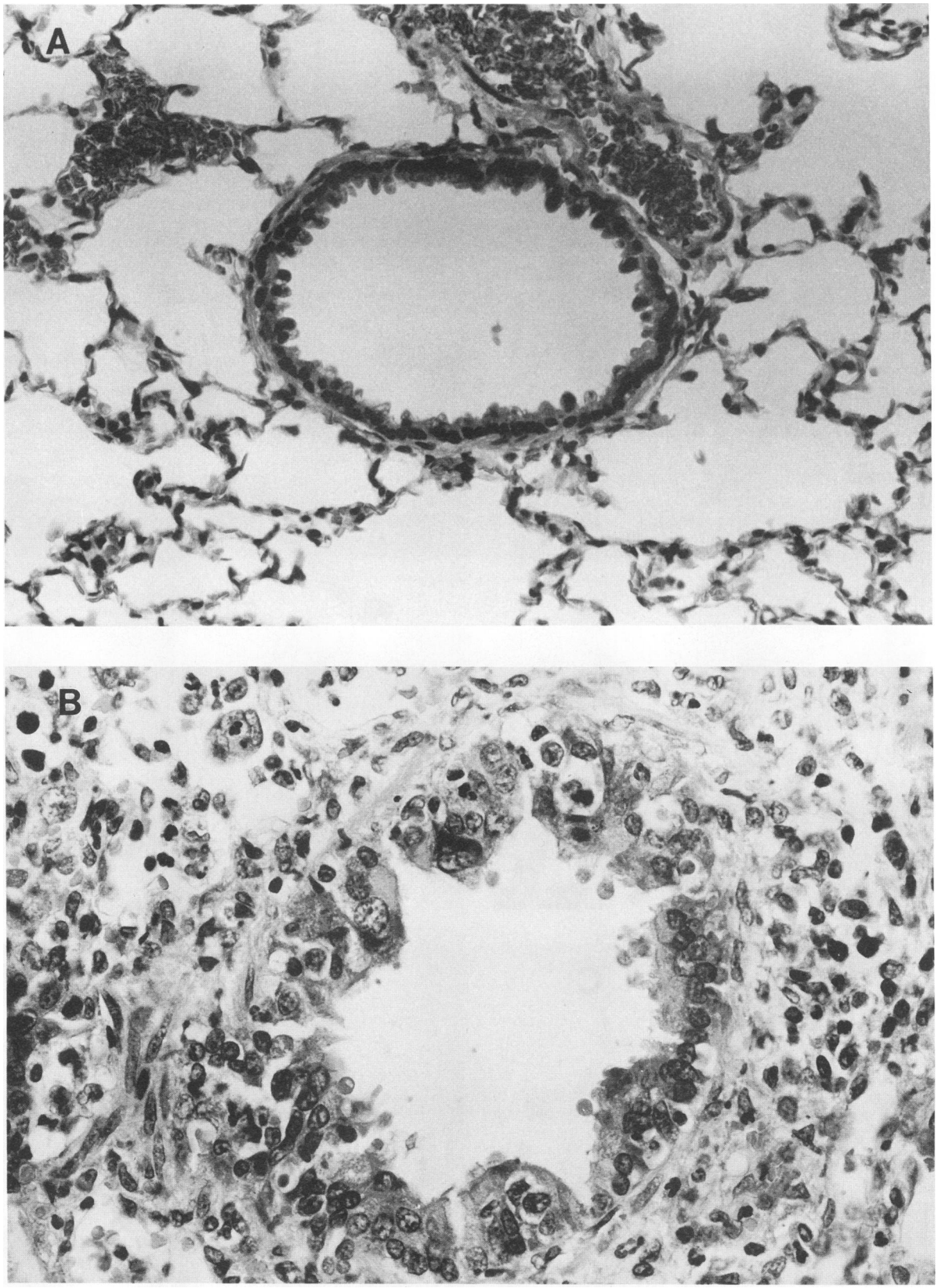
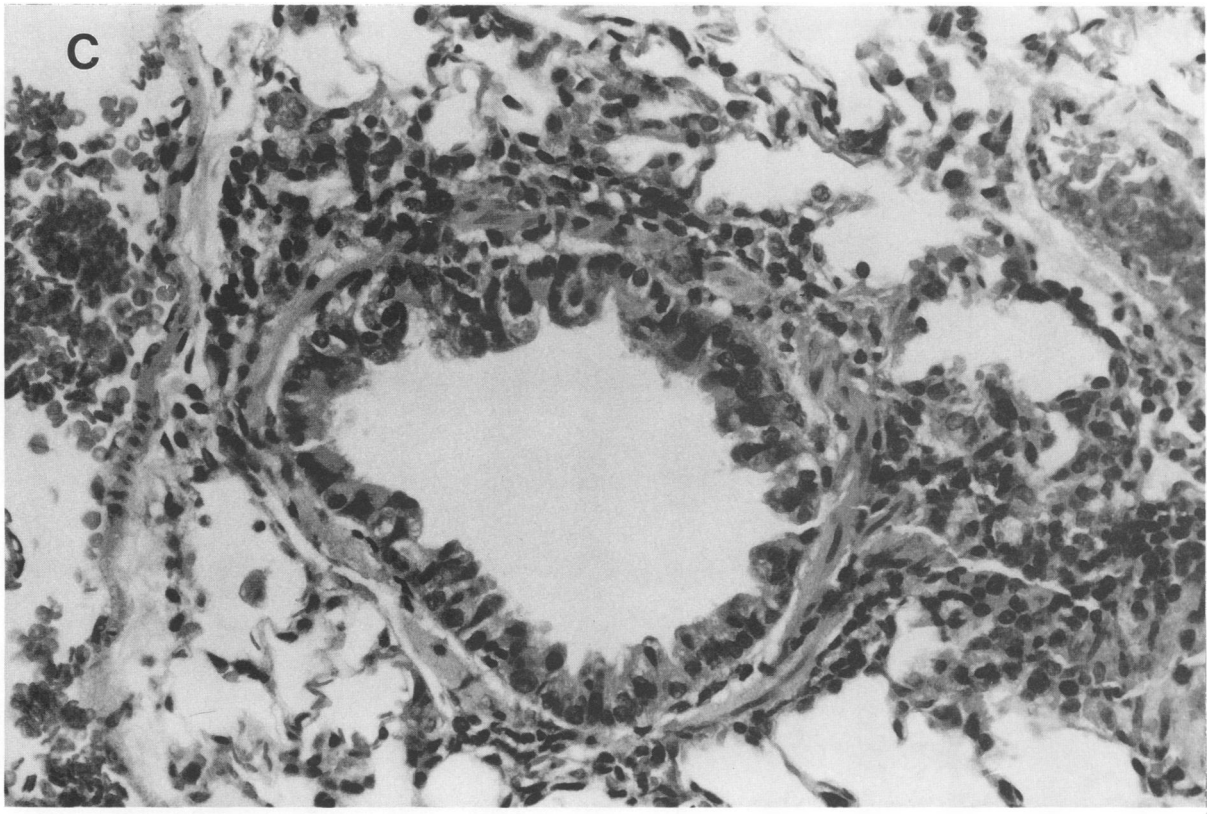


FIG. 5. Cotton rat bronchioles following inoculation of $10^{8.0}$ PFU of Ad5. (A) Day 1. Bronchioles have a nearly normal appearance. (B) Day 3. Substantial damage to bronchiolar epithelium and peribronchiolar infiltration, consisting primarily of macrophages and scattered PMNs, with only occasional lymphocytes, were noted. (C) Day 5. Peribronchiolar infiltration has changed in character, consisting almost exclusively of lymphocytes, with only a few PMNs and macrophages. (D) Day 7. Peribronchiolar lymphocytic infiltration is maximal. H&E stain; magnification, ×475.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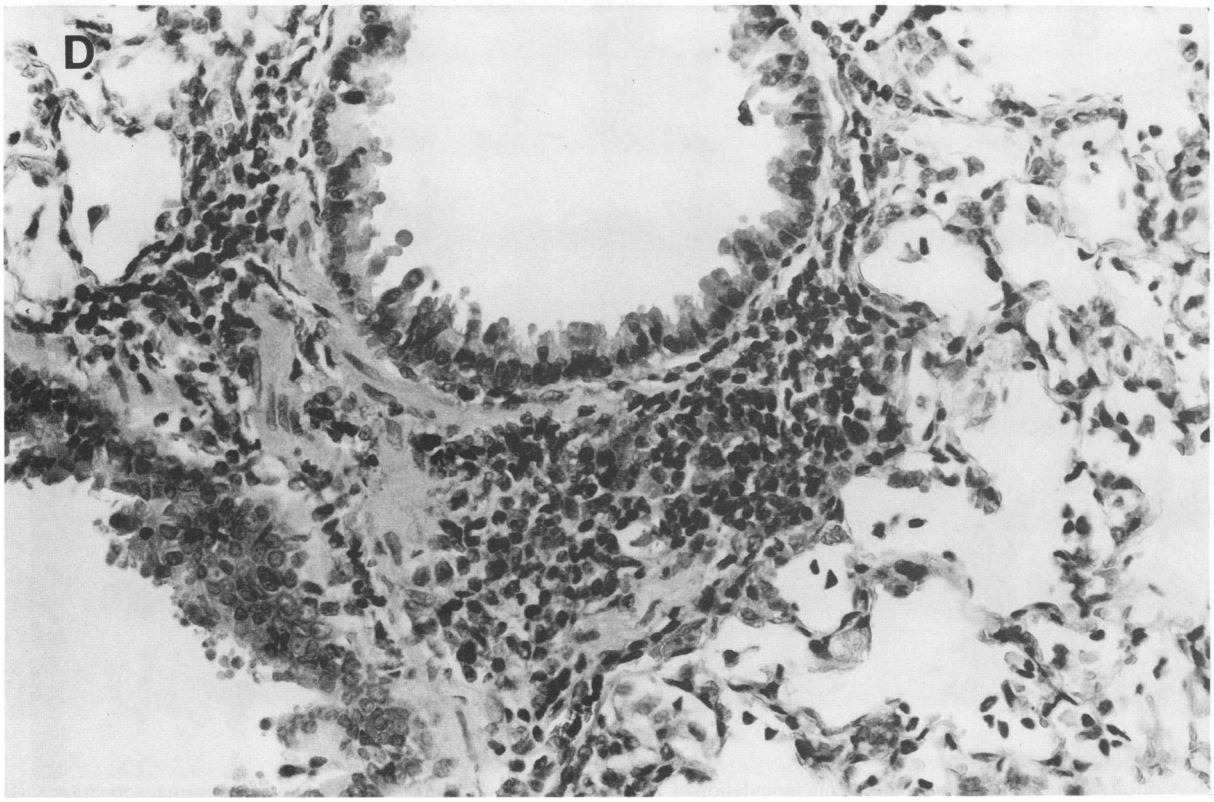
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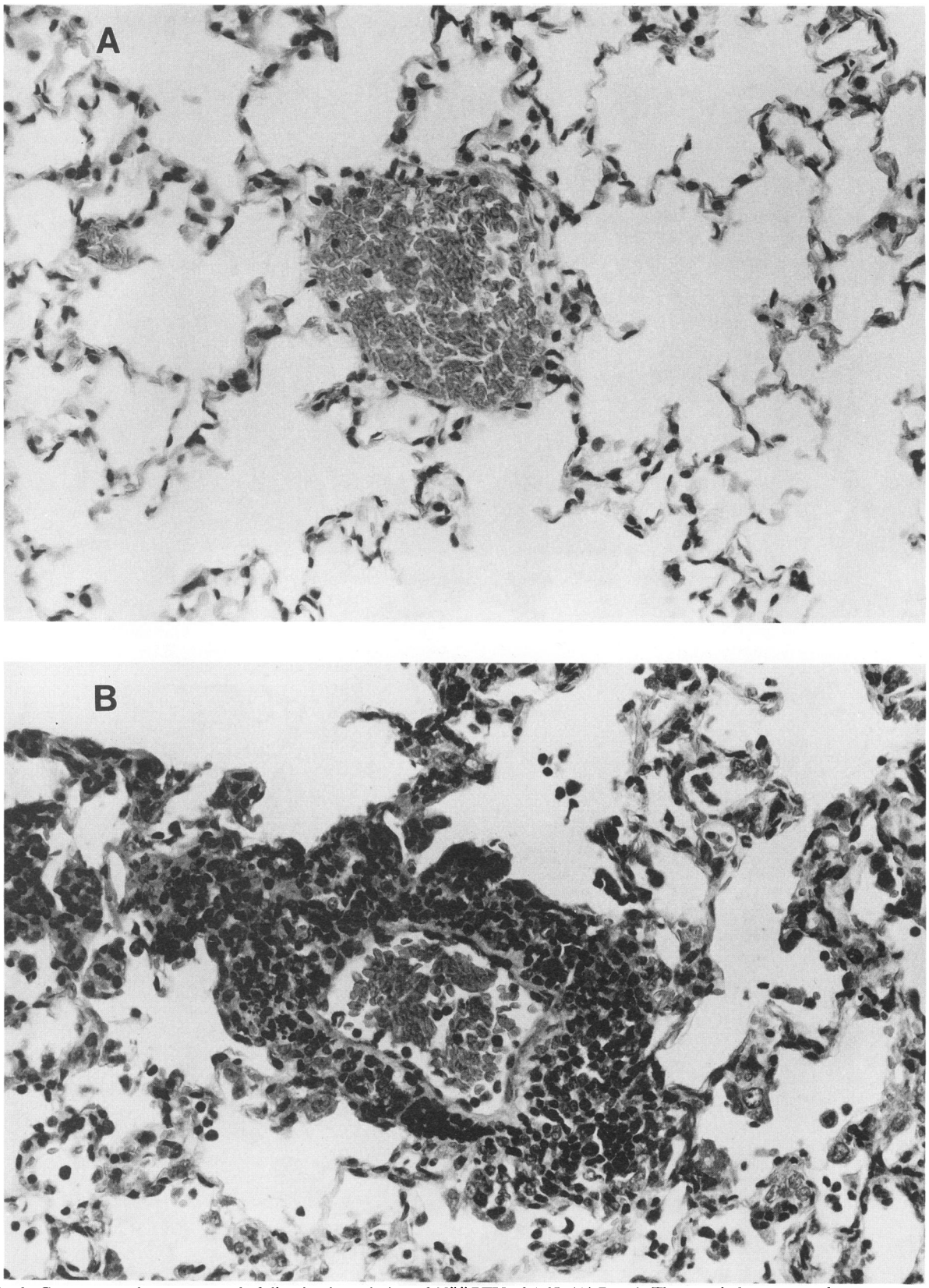

FIG. 6. Cotton rat pulmonary venule following inoculation of $10^{8.0}$ PFU of Ad5. (A) Day 1. The venule has a normal appearance. (B) Day 7. Intense perivascular lymphocytic infiltration is apparent. H&E stain; magnification, ×475.

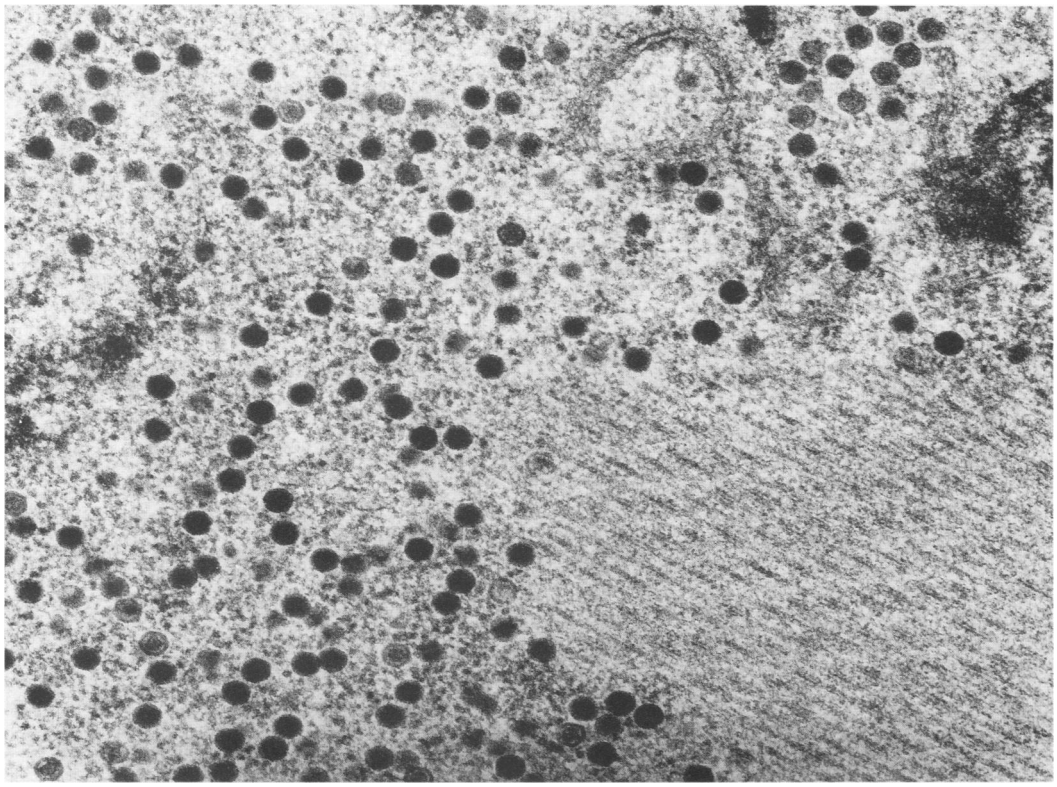

FIG. 7. Electron micrograph of a cotton rat bronchiolar epithelial cell showing polyhedral Ad5 virions 2 days after inoculation of $10^{8.0}$ PFU. Magnification, ×100,000.

viruses that were adapted to the host used, mice. The results with Ad5 suggest that this agent is not fully adapted to cotton rats, even though it multiplies and produces disease in this host. Efforts are now being made to adapt Ad5 to *S. hispidus*.

Second, the disease produced in cotton rats by adenovirus has two distinct histologic components. The earlier phase, beginning 2 days following inoculation of 10^8 PFU, consists of mild to moderate damage to bronchiolar epithelial cells and diffuse cellular infiltration of peribronchiolar and alveolar regions. Lysis of epithelial cells is not apparent, for although occasional clefting of the epithelium is observed, ulceration or denudation does not occur. It should be noted that adenoviruses do not lyse infected host cells in cultures, although as many as 10^4 infectious virions per cell may be produced (4). Cell types involved in the early infiltration include monocytes-macrophages, neutrophils, and occasional lymphocytes. Swelling of type II pneumocytes is prominent, and these cells are surrounded by macrophages and PMNs. Electron microscopy and immunofluorescence for Ad5 structural proteins failed to show evidence of viral replication in type II pneumocytes. In situ hybridization studies, however, demonstrated that Ad5 early genes are expressed in these cells (29).

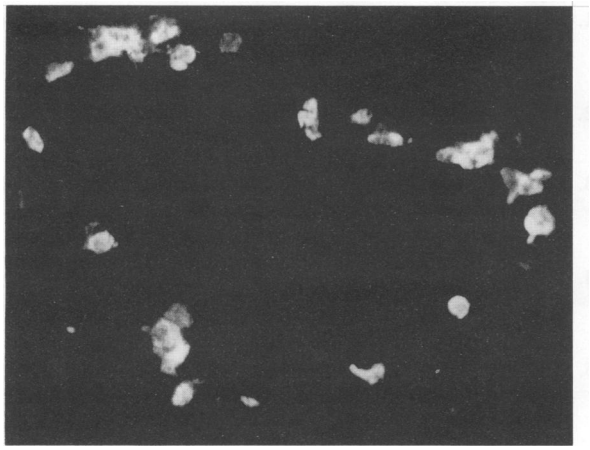

FIG. 8. Immunofluorescence photograph of a cotton rat lung 2 days after inoculation of $10^{8.0}$ PFU of Ad5. Only the nuclei of bronchiolar epithelial cells are stained for virion antigens. Magnification, ×760.

The later phase of the disease does not become significant until 2 or 3 days after the peak of the earlier phase, i.e., 5 to 7 days postinoculation. This phase consists almost exclusively of an infiltration of lymphocytes, which first cluster around bronchioles and invade their walls and then reach a peak level by day 5. Lymphocytes also produce an infiltrate around blood vessels and reach a peak level by day 7.

The timing of these two phases suggests that the earlier one is not a specific immunologic reaction, whereas the later one is, involving specifically sensitized T (and perhaps B) lymphocytes. Since the earlier phase reflects primarily the response of the host to infection, rather than direct viral damage of host tissues (such as is seen in widespread cytolysis and ulceration of respiratory epithelium by influenza virus), it may be prudent to avoid a label such as

nonimmunologic. It is clear that the two phases are different, in terms of timing (early versus late), cell types (multiple versus single), and cell distribution (diffuse versus focal). The early phase is probably a nonspecific immunologic reaction reflecting the action of cytokines (which might account for the presence of neutrophils and macrophages as well as lymphocytes), while the later phase is probably an immunologically specific T-lymphocyte response. Indeed, since it has been shown that deletion of the early region 3 19-kDa glycoprotein gene, which in wild-type Ad2- or Ad5-infected cells markedly reduces expression of the class I major histocompatibility antigen complex on the cell surface (1, 5, 13), greatly increases the lymphocytic invasion of the walls of infected bronchioles, it seems likely that the later phase is a cytotoxic T-cell response to Ad5 infection (15). A model Ad5 infection in mice, in which there was no viral replication, showed early and late lesions similar to those described in the cotton rat model (14). The early lesions in mice were associated with markedly increased levels of cytokines, including tumor necrosis factor alpha, interleukin 1, and interleukin 6. Nude mice showed a normal development of early lesions, but the late lesions were nearly abolished, providing further evidence that the late lesions represent a specific immune response to Ad5 infection.

There is increasing interest in using adenoviruses as vectors for delivering specific genes to the lung in vivo, and the functioning of the transferred genes has been demonstrated (26, 27). The present study and experiments with the mouse model of adenovirus pneumonia (14) suggest that caution be used with adenovirus vectors, since they might produce disease when partially permissive or nonpermissive for complete replication.

A comparison of the histopathology of adenovirus pneumonia in cotton rats (Ad5) and humans is made difficult by the scarcity of reported human cases, the preponderance of compromised patients (e.g., immunodeficient, immunosuppressed, or immature) among reported cases, incomplete knowledge of the interval between infection and death, and the diversity of adenovirus serotypes reported in human pneumonias. Furthermore, all reported human cases involved fatal adenovirus pneumonia, whereas the dose of Ad5 primarily used in the histologic studies with *S. hispidus* ($10^{8.0}$ PFU) was well below the 50% lethal dose for this species ($10^{9.4}$ PFU). Nonetheless, the cotton rat model bears the following similarities to human disease (2, 6, 16, 32, 33). First, the primary target tissue is bronchiolar epithelium, although the extensive epithelial necrosis and ulceration reported in many human cases are not seen in cotton rats. Second, mononuclear cell infiltration around affected bronchioles is prominent in both species. Third, both types of intranuclear inclusions seen in adenovirus infection in tissue cultures (4) and in fatal human adenovirus pneumonia are seen in *S. hispidus*. They consist of the early development of smaller, eosinophilic inclusions, often surrounded by an irregularly shaped halo, and (more commonly) larger, basophilic inclusions, which frequently force the chromatin to the periphery of the nucleus and create a ring-like appearance or completely obliterate the chromatin and enlarge the nucleus to as much as double the diameter of adjacent nuclei.

Future studies of adenovirus pathogenesis with cotton rats may benefit from three manipulations of the model. First, the observation that both viral yield and disease magnitude are directly proportional to viral input dose allows for a made-to-order severity of disease. This property may be of particular significance in studies of prophylactic or therapeutic intervention.

Second, the reproducibility of disease caused by wild-type virus allows comparison with mutant viruses to determine the role of specific genes in either ameliorating or exacerbating pneumonia (12, 15), and experiments have shown that the production of infectious virus is not required for pneumonia in cotton rats (13).

Third, the major role apparently played by the immune system in Ad5 pneumonia suggests that immunologic depletion studies would help define the mechanisms of immunopathology. Although cotton rats are not the ideal animals for immunologic depletion (particularly in contrast to mice), the development in our laboratory of inbred cotton rats significantly broadens the scope of potential studies.

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