JOURNAL OF VIROLOGY, Jan. 1996, p. 541–548 Vol. 70, No. 1
0022-538X/96/$04.00+0

Reovirus Infection in Rat Lungs as a Model To Study the Pathogenesis of Viral Pneumonia

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Received 11 July 1995/Accepted 11 October 1995

We undertook the present study to elucidate the pathogenesis of the pathologic response to reovirus infection in the lungs and further understand the interactions of reoviruses with pulmonary cells. We found that reoviruses were capable of causing acute pneumonia in 25- to 28-day-old Sprague-Dawley rats following intratracheal inoculation with the reoviruses type 1 Lang (T1L) and type 3 Dearing (T3D). The onset of the pneumonia was rapid, marked by type I alveolar epithelial cell degeneration, type II alveolar epithelial cell hyperplasia, and the infiltration of leukocytes into the alveolar spaces. More neutrophils were recruited into the lungs during T3D infection than during T1L infection, and the serotype difference in the neutrophil response was mapped to the S1 gene of reovirus. Viral replication in the lungs was required for the development of pneumonia due to T1L and T3D infections, and replication occurred in type I alveolar epithelial cells. T1L grew to higher titers in the lungs than did either T3D or type 3 clone 9, and the S1 gene was found to play a role in determining the level of viral replication. We propose that experimental reovirus infection in the lungs can serve as a model for the pathogenesis of viral pneumonia in which pulmonary inflammation results following direct infection of lung epithelial cells.

Mammalian reoviruses are associated with pulmonary infections, having been isolated from humans and various animals experiencing respiratory illnesses. Albert Sabin originally coined the name for reoviruses from respiratory enteric orphan viruses and described the isolation of reoviruses from chimpanzees, monkeys, and a child experiencing respiratory illnesses (27). In a report by Jackson and Muldoon (17), reoviruses were listed as a causative agent for the common cold. Reoviruses have also been associated with respiratory illnesses in dogs, cats, cattle, and pigs (for a review, see reference 30). In spite of the association of reoviruses with respiratory infections, little work characterizing the pulmonary pathologic response to reovirus infection has been done.

We have shown previously that reoviruses enter bronchus-associated lymphoid tissue through pulmonary M cells and spread from the airways to regional lymph nodes (21). In the present study, we explored reovirus interactions with other pulmonary host cells and the host response to reovirus infection in the lungs, and we established a model for the study of viral pneumonia. Viral pneumonia, in which inflammation of the lungs results from virus infection, is a serious human disease, and representative laboratory models are needed to better understand the pathogenesis of the disease.

Reoviruses have been used extensively for studies of viral pathogenesis in a number of organs, including the intestines, liver, heart, and brain, and much has been learned about viral entry and spread through the host, tissue tropism, and disease pathogenesis (30). Reoviruses are nonenveloped, icosahedral viruses with a segmented double-stranded RNA genome (for a review, see reference 28), and by using reovirus reassortants, the molecular bases of many of the steps in the pathogenesis of virus infections have been determined (10, 30). The ability to readily study the genetics of reovirus infections, and reovirus association with pulmonary infections, makes reovirus ideal for the study of viral interactions with pulmonary cells and for development of a laboratory model for viral pneumonia.

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MATERIALS AND METHODS

Viruses and cell culture. We used laboratory stocks of the type 1 Lang (T1L), type 3 Dearing (T3D), and type 3 clone 9 (T3C9) (15) reoviruses. We also used the reassortant viruses 1HA3 and 3HA1 (33), E3 (6), EB144 (4), and KC19 (5), which were generated previously in our laboratory from crosses between T1L and T3D. Purified stocks of virus were made by infecting mouse L cells, and after sonication of infected cells and Freon extraction, virus was purified over cesium chloride gradients, as described previously (32). Top component particles, reovirus particles that lack the double-stranded RNA genome of the virus, are empty capsids found in a band above that of virus particles on cesium chloride gradients (18). Top component particles from T1L and T3D purifications were also collected and used to inoculate rats. The particle-to-PFU ratio for purified virus was 200:1, whereas for top component, the particle-to-PFU ratios ranged from 10^5:1 to 10^6:1.

Rats and inoculations. Virus antigen-free juvenile rats were purchased from Taconic Farms (Germantown, N.Y.) and were 25 to 28 days old (weight, 75 to 100 g). The rats were anesthetized with aerosolized halothane (Halocarlson Laboratories, North Augusta, S.C.) and inoculated intratracheally (i.t.), intranasally (i.n.), or intravenously (i.v.) with reovirus diluted in sterile, endotoxin-free saline (Abbott, Chicago, Ill.). For i.t. inoculations, the virus inoculum was instilled into the pulmonary airways in a volume of 100 μl by using a blunt-ended, 5-cm catheter. i.n. inoculations of virus were administered to anesthetized rats in a volume of 100 μl, and for i.v. delivery, reovirus was injected via the tail vein. The rats were euthanized at experimental time points by intraperitoneal administration of an overdose of pentobarbital (Anthony Product Co., Arcadia, Calif.).

Titration of virus in lung tissue. Lungs were removed from infected rats and placed in gelatin saline (136 mM NaCl, 2.7 mM $CaCl_2$, 0.08 mM $MgCl_2 \cdot 6H_2O$, 19 mM H_3BO_3, 0.13 mM $Na_2B_4O_7 \cdot 10H_2O$, 0.3% gelatin). After three freeze-thaw cycles, the tissue was homogenized manually in a Dounce homogenizer. The debris was pelleted, and the supernatant was titered for reovirus by plaque assay on mouse L cells (32).

Histopathology and ultrastructure studies. Lungs from the rats were fixed by inflating the lungs via the trachea with 2.5% glutaraldehyde in 0.85 M sodium cacodylate buffer, pH 7.4, at a pressure of 25 cm of H_2O. Blocks of lung tissue were cut for embedding in glycol methacrylate for light microscopy or Epon for electron microscopy. Glycol methacrylate-embedded tissue was sectioned (1- to 2-μm sections) and stained with methylene blue and basic fuchsin. Ultrathin sections (80 nm) were made through Epon-embedded tissue, stained with uranyl acetate, and analyzed with a Philips 300 electron microscope.

BAL and differential analysis of recovered cells. Cells present in and recruited into the airways of the lungs were recovered by bronchial alveolar lavage (BAL). After barbiturate overdose, the lungs were collapsed by piercing the diaphragm, and the trachea was cannulated with a 2-cm-long 18-gauge catheter to which a piece of polyethylene tubing (PE-190) had been fitted. Two 3-ml volumes of phosphate-buffered saline (PBS) were slowly instilled into and withdrawn from the lungs to lavage the airways. The two lavage fluid samples were pooled for analysis, and aliquots of the lavage fluid were centrifuged onto microscope slides for 5 min at 80 rpm in a Shandon cytocentrifuge. After the slides were air dried, the cells were fixed with Leukostat (Fisher Scientific, Pittsburgh, Pa.) and differentially stained with Diff-Quik (Baxter, Miami, Fla.).

Immunoperoxidase staining of frozen lung sections. To prepare frozen sections of rat lungs, the lungs were inflated with prewarmed embedding medium, Polyfreeze (Polysciences, Warrington, Pa.), and then removed and washed with PBS. Blocks of lung tissue were mounted onto pieces of corkboard with Polyfreeze and immersed in 2-methylbutane in a dry-ice bath. Six-micrometer sections were made through the blocks in a precooled (−20°C) cryostat and placed onto gelatin-coated slides. The tissue sections were air dried, fixed for 10 min with cold 2% paraformaldehyde in PBS, pH 7.3, and subsequently stained by using a Vectastain Elite kit (Vector, Burlingame, Calif.). The primary antibody for the stain was antireovirus rabbit sera (laboratory stock), and the biotinylated anti-rabbit antibody and peroxidase reagents were kit supplied. Diaminobenzidine (5 mg/ml in 0.1 M Tris-HCl, pH 7.6) was the substrate used for antigen localization, and Giemsa was used as a counterstain. All washes were performed with ELISA III (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.05% Tween 20, 0.1% bovine serum albumin, pH 7.4).

RESULTS

Inoculation of reovirus T1L by various routes to determine viral effects in the lungs. To determine whether we could generate a reproducible animal model of pulmonary reovirus infection, we inoculated juvenile rats with reovirus T1L, 10^{10} PFU per rat, using three different routes: i.t., i.n., and i.v. Rats inoculated i.t. exhibited noticeable respiratory distress following reovirus administration, indicating pneumonia. The pneumonia was most severe, as assessed by histopathology, at day 3 following i.t. inoculation. Of six animals inoculated i.t., however, two animals died before day 3 (33 1/3% mortality).

Rats inoculated with T1L either i.n. or i.v. did not appear to have any signs of respiratory distress during the course of the experiment, and no histological changes were seen in their lung sections at any time point (days 3, 4, 6, and 7 for i.n. inoculation and days 3, 5, 7, and 11 for i.v. inoculation). Apparently, i.n. inoculation did not deliver enough virus to the lungs to produce pneumonia, i.e., much of the inoculum may have been swallowed or remained in the upper respiratory tract, where a subclinical infection may have ensued. Additionally, the lack of detectable disease after i.v. inoculation indicated that T1L was not capable of infecting the lungs following hematogenous spread. The i.t. route of virus delivery was therefore used for subsequent experiments to develop a model for viral pneumonia that results from direct infection of the lungs.

Description of the pneumonia resulting from i.t. inoculation with T1L. For our subsequent studies to characterize the pulmonary response to reovirus infection, a reduced inoculum of 10^8 PFU of reovirus per rat was administered to generate marked pulmonary pathologic changes without the clinical disease or lethality seen at the higher dose described above. Lung sections for analysis were taken from rats 3 days following inoculation.

The pneumonia in response to i.t. inoculation of reovirus T1L was characterized by an influx of leukocytes into the air spaces and was multifocal, with primarily alveolar rather than airway involvement (Fig. 1). There was extensive epithelial damage in the alveoli in response to the infection, as indicated by type II alveolar epithelial cell hyperplasia. Such type II cell proliferation represents replacement of destroyed type I alveolar epithelial cells, which are terminally differentiated cells responsible for gas exchange (12, 29). Edema around blood vessels and hyaline membrane were further characteristics of the pulmonary inflammation and tissue injury. Hyperplasia of the bronchial epithelium was also noted, but inflammation in the airways was minimal. No hemorrhage was observed. At lower doses of virus, the morphologic characteristics of the pneumonia were similar to those with the higher dose of 10^8 PFU per rat, except that the foci of inflammation were smaller and less frequent (data not shown).

i.t. inoculation of rats with T3D. For a direct comparison of T1L infection and infection with a different serotype, T3D, we inoculated 25- to 28-day-old rats with T3D, 10^8 PFU per rat, and examined their lungs histologically. As with T1L, a severe pneumonia, with predominantly alveolar involvement, resulted by 3 days following inoculation with T3D. Infiltration of leukocytes into the alveoli, with no or little hemorrhage, and multifocal epithelial damage marked by type II cell hyperplasia were characteristic (Fig. 1). Additionally, we saw perivascular edema, hyaline membrane, and bronchial epithelial cell hyperplasia.

Despite the similarities in the pneumonias observed with T1L and T3D, there were important differences. The alveolar infiltrate following T3D inoculation had a more prominent neutrophil component than that following T1L inoculation (Fig. 1). In addition, the type II cell hyperplasia was more marked and the interstitium was somewhat more cellular in the lungs following infection with T3D. With the noted morphologic differences after T1L and T3D respiratory infections, a foundation was laid for conducting reassortant analyses to determine the molecular basis of the pathogenesis of reovirus infection in the lungs.

Differential analysis of cells recovered by BAL. Leukocytes recruited into the lung air spaces following inoculation with reovirus were recovered by BAL and were differentially stained. While BAL fluid from normal rats contains close to 100% alveolar macrophages, 1 day following inoculation with either T1L or T3D, an influx of neutrophils was detected in the BAL fluid (Fig. 2). The BAL fluid from T1L-infected rats at 1 day following inoculation contained 24% neutrophils and 76% mononuclear cells, including mature macrophages and smaller monocytes. In contrast, 66% of the cells recovered following T3D infection were neutrophils, while the remainder were mononuclear cells. The profile of cells recovered by BAL therefore corroborated the histologic observations.

Reassortant analysis of the different patterns of neutrophil recruitment. We performed a reassortant analysis to determine which gene(s) might have been responsible for the neutrophil response in the lungs. A panel of reassortants, 1HA3, 3HA1, E3, EB144, and KC19, were used at a dose of 10^8 PFU per rat, and the lungs of infected rats were lavaged 1 day following i.t. inoculation. We determined the percentages of neutrophils in the BAL fluid and arranged the results in decreasing order (Table 1). By comparing the reassortant data with results from T1L and T3D infections, we found that infection with reassortants that resulted in more than 55% neutrophils in the BAL fluids had the S1 gene of T3D, whereas the reassortants with less than 47% neutrophils had the S1 gene of T1L (Table 1). Although there was a gradation in the results from the highest to the lowest percentage of neutrophils, suggesting that more than one gene was involved in the phenotype, the segregation pattern of the other reovirus gene segments showed a random distribution. The S1 gene of reoviruses, therefore, played a prominent role in determining the degree of neutrophil recruitment into the airways following i.t. inoculation with reovirus.

Replication of T1L, T3D, and T3C9 in the lungs. We determined whether reoviruses were capable of replicating in rat lungs by assaying for virus in lung homogenates from days 0 to 10 following i.t. inoculation of rats with 10^4 PFU of either T1L

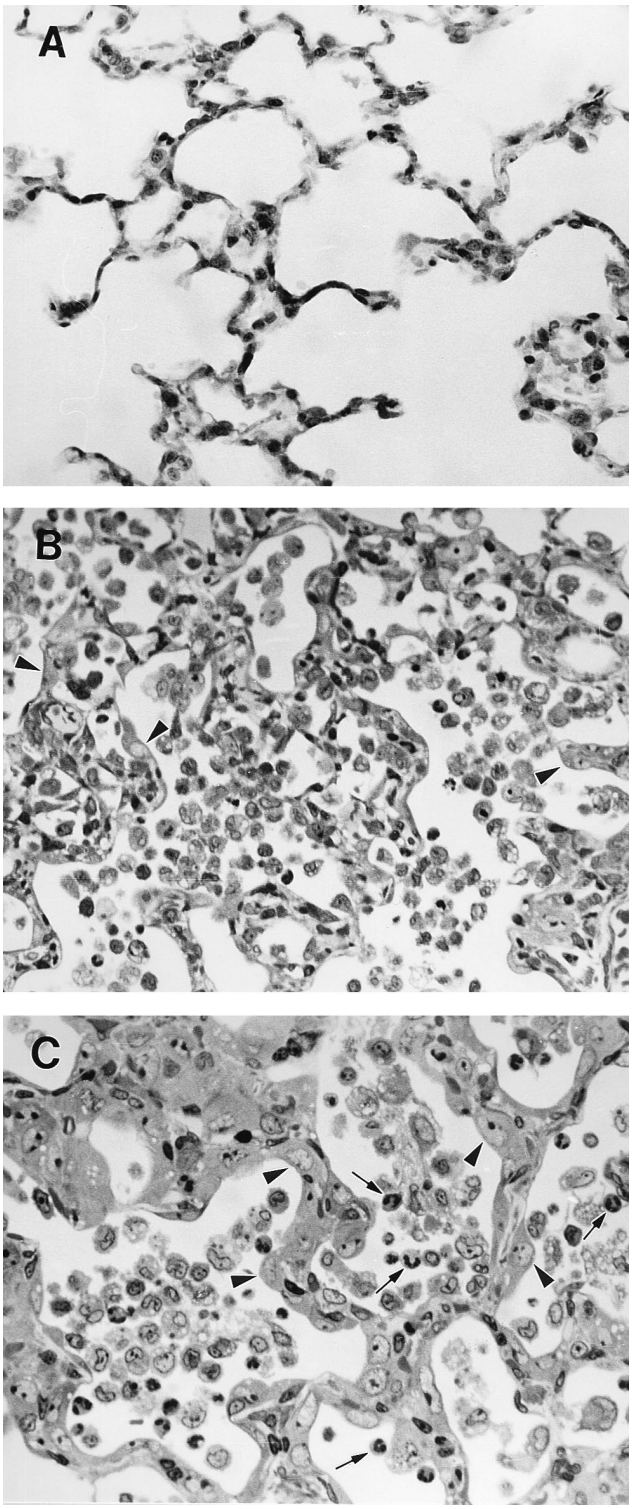

FIG. 1. Lung sections from normal and reovirus-infected rats. Photomicrographs are of glutaraldehyde-fixed, glycol methacrylate-embedded lung tissue sections, 3 days after inoculation with 10^8 PFU of reovirus T1L or T3D. (A) Lung section from an uninfected rat showing the normal lung morphology, i.e., open air spaces in the alveoli and thin alveolar walls. (B) Lung section from a rat 3 days following i.t. inoculation with T1L, showing air spaces of the alveoli filled with leukocytes, the majority of which are mononuclear cells, and an increased number of type II alveolar cells (arrowheads). (C) Lung section from rat 3 days following i.t. inoculation with T3D, showing air spaces filled with leukocytes, of which a larger proportion of cells were neutrophils (arrows). There was marked type II cell hyperplasia (arrowheads) and a more cellular interstitium. Magnification, ×340.

or T3D (day 0 was 90 min following inoculation). The amount of virus used for inoculation was lower than the dose for the pathology studies described above in order to facilitate detection of an increase in the amount of virus in the lungs due to replication. We also inoculated rats with T3C9 for comparison to T3D and T1L.

A rapid rise in viral titer in the lungs was seen from day 0 to day 1 for all three viruses (Fig. 3). The rise in titer, however, was more marked with T1L, which grew to about 1 $\log_{10}$ greater than both T3D and T3C9 beginning at day 1. Throughout the course of the experiment, the titer of T1L in the lungs remained higher than the titer of either T3D or T3C9, which replicated to similar levels throughout the experiment. The titer for all three viruses peaked at day 3 and then declined until day 10, when some virus was still detected. Reoviruses were therefore capable of replicating in rat lungs, and there was a difference in the level of replication depending on the serotype.

Replication of the reassortants 1HA3 and 3HA1 in the lungs of rats: role of the S1 gene. To determine whether the serotype difference for replication could be mapped to the S1 gene of reovirus, which has been shown to play a role in the level of replication in other organs such as the brain (33) and the intestine (2), we inoculated rats with 1HA3 or 3HA1, as well as T1L or T3D (10^4 PFU per rat), and measured the amount of viral replication in the lungs. We chose 1HA3 and 3HA1 for comparison to T1L and T3D because they are single gene reassortants for S1. The reassortant 3HA1, which has a T3D background with the S1 gene of T1L, reached titers in the lungs similar to those of T1L (Fig. 4). The reassortant 1HA3, which has a T1L background with the S1 gene of T3D, reached levels similar to those of T3D, especially over the first 3 days (Fig. 4). The S1 gene of reovirus, therefore, played a role in determining the level of viral replication in the lungs.

Requirement of reovirus replication for the development of pneumonia. To determine if replication of reovirus was required for the development of pneumonia, we inoculated rats with top component particles (virus particles lacking the double-stranded RNA genome), generated from both T1L and T3D virus purifications, adjusted to the same particle number as that in the virus inocula used for determining the morphological characteristics of the pneumonia. Because the outer capsid is the same for top component and infectious virus, using top component allowed us to determine if the replication of the virus in the lungs was required for the development of pneumonia or whether the proteins of the reovirus outer capsid alone induced the pulmonary inflammation. We examined histological lung sections from rats 3 days following inoculation with top component from either T1L or T3D and did not detect any inflammation, indicating that reovirus replication was necessary for the development of pneumonia (data not shown).

Localization of viral antigen in lungs of rats infected with T1L and T3D. To determine the site of viral replication in the lungs, frozen lung sections were made from rats inoculated with 10^8 PFU of either T1L or T3D. The lung sections were stained with an immunoperoxidase stain to localize reovirus antigen in the lungs. At 1 day following inoculation with either T1L or T3D, viral antigen was detected in some of the pulmonary alveolar septa, as indicated by the presence of diaminobenzidine precipitate on alveolar cells (Fig. 5). There also appeared to be some very light staining of alveolar cells at 1 and 4 h following inoculation; however, the staining was very close to background levels (data not shown). The heavier staining of the alveoli at 1 day than at 1 and 4 h following inoculation suggested an increase in the amount of viral antigen,

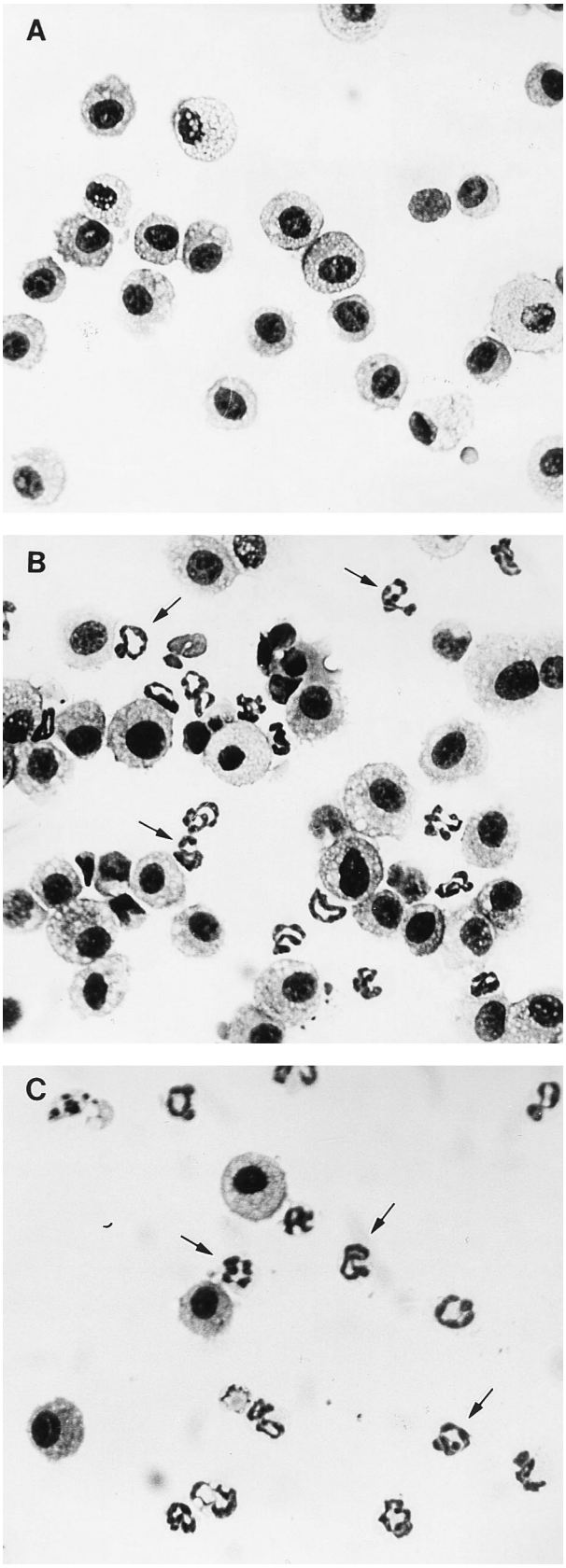

FIG. 2. BAL cytology from normal and virus-infected rats. (A) Cells in the BAL fluid from an uninfected control rat showing approximately 100% alveolar macrophages. (B) Cells in the BAL fluid from a rat inoculated with 10^8 PFU of T1L, indicating an influx of neutrophils (arrows) into the airways 1 day following inoculation. (C) Cells in the BAL fluid from a rat inoculated with 10^8 PFU of T3D, also indicating an influx of neutrophils (arrows) into the airways 1 day following inoculation. The percentages of neutrophils in the BAL fluid were 24% after T1L inoculation (mean for eight rats) and 66% after T3D inoculation (mean for six rats). Magnification, ×376.

TABLE 1. Reassortant analysis of neutrophil recruitment into lung airways 1 day following i.t. inoculation with 10^8 PFU of virus

Virus	% Neutrophils[a]	Parental origin of gene segment[b]									
		L1	L2	L3	M1	M2	M3	S1	S2	S3	S4
T3D	66	D	D	D	D	D	D	D	D	D	D
1HA3	58	L	L	L	L	L	L	D	L	L	L
KC19	57	L	L	L	L	D	L	D	L	D	L
E3	56	D	D	D	D	L	D	D	D	D	D
3HA1	46	D	D	D	D	D	D	L	D	D	D
EB144	34	L	L	L	L	D	D	L	L	D	L
T1L	24	L	L	L	L	L	L	L	L	L	L

[a] Percentage of neutrophils in the population of cells recovered by lung lavage 1 day following i.t. inoculation with reovirus.
[b] D, T3D; L, T1L.

which we interpreted as an indication of viral replication in alveolar cells.

Ultrastructure studies to demonstrate reovirus replication within alveolar cells. We examined infected lung tissue at the ultrastructural level at 90 min and 1 day following inoculation with reovirus T1L and T3D to determine which cells of the alveoli supported viral replication. The doses of virus per rat were 2×10^9 PFU in the study of the results at 90 min and 1×10^8 PFU in the study of the results at 1 day. A larger dose was used for the 90-min time point to facilitate the detection of virus particles by electron microscopy. By 90 min following inoculation with either T1L or T3D, we found virus particles in the alveoli entering type I epithelial cells (Fig. 6). Viral particles within the cytoplasm of the type I cells were membrane bound, indicating endocytosis of the particles. Both T1L and T3D entered type I cells, but neither was seen within type II cells.

At 1 day following inoculation with T1L, we found progeny virus (as distinguished from input virus by the lack of a membrane around the particles and by the paracrystalline array of virus particles) in the cytoplasm of type I alveolar epithelial cells (Fig. 6). At 1 day following i.t. inoculation with T3D, marked type II cell hyperplasia and type I cell loss caused difficulty in detecting progeny virus; however, at 12 h following T3D inoculation, we found progeny virus in type I epithelial cells (Fig. 6). Type I alveolar epithelial cells, therefore, supported replication of both T1L and T3D.

Within 90 min following inoculation of rats with either T1L or T3D, viral particles were also seen within alveolar macrophages in membrane-bound vesicles (Fig. 7). Although we found input virus particles within 90 min following inoculation, we did not find progeny virus within alveolar macrophages at later times, suggesting either that alveolar macrophages do not support replication of reovirus or that virus-laden macrophages may have migrated out of the alveoli.

DISCUSSION

We describe in the present report the pathogenesis of reovirus infection in rat lungs and thus introduce a laboratory

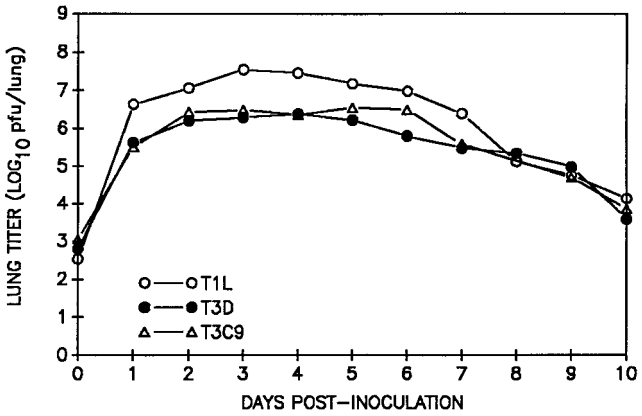

FIG. 3. Virus titers in rat lungs following inoculation with reovirus. Juvenile rats were inoculated i.t. with 10^4 PFU of either T1L, T3D, or T3C9, and lung homogenates from the infected rats were assayed for virus by plaque assay at the indicated times. Day 0 was 90 min following inoculation. The datum at each time point is the mean of the titers from two rats.

model for viral pneumonia in which reoviruses are used. Prior to the present report, the pathogenesis of reovirus infection in the lungs had not been well described. We reported previously the entry of reoviruses into bronchus-associated lymphoid tissue through pulmonary M cells and spread to regional lymph nodes (21). We have shown here that in addition to using the respiratory tract as a portal of entry into the host, reoviruses directly infect the alveolar epithelium following i.t. inoculation, causing severe pneumonia in juvenile Sprague-Dawley rats. The host response was characterized by an infiltration of leukocytes into the pulmonary alveoli, viral replication in type I alveolar epithelial cells, and type II alveolar cell hyperplasia. At sufficiently high doses (10^{10} PFU per rat), pulmonary infection due to reovirus T1L can be lethal.

We propose that reoviruses can serve as a model of viral pneumonia in which there is direct infection of the pulmonary epithelium without prior viremia. The development of reovirus pneumonia was rapid, with extensive lung injury by 3 days following inoculation. The kinetics of the pneumonia resembled those of other models in which pneumonia follows direct

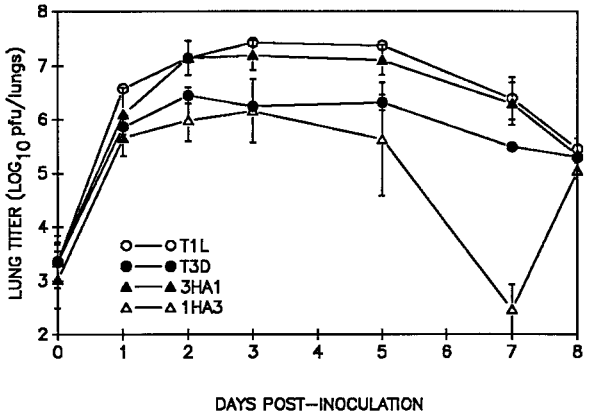

FIG. 4. Replication of reassortant reoviruses in the lungs of rats. Juvenile rats were inoculated with 10^4 PFU of either T1L, T3D, 1HA3, or 3HA1, and lung homogenates were assayed for virus by plaque assay. Day 0 was 90 min following inoculation. Four rats were used for each time point up to day 5, and two rats were used per time point on days 7 and 8. Data are the means and standard errors for the titers from each group of rats.

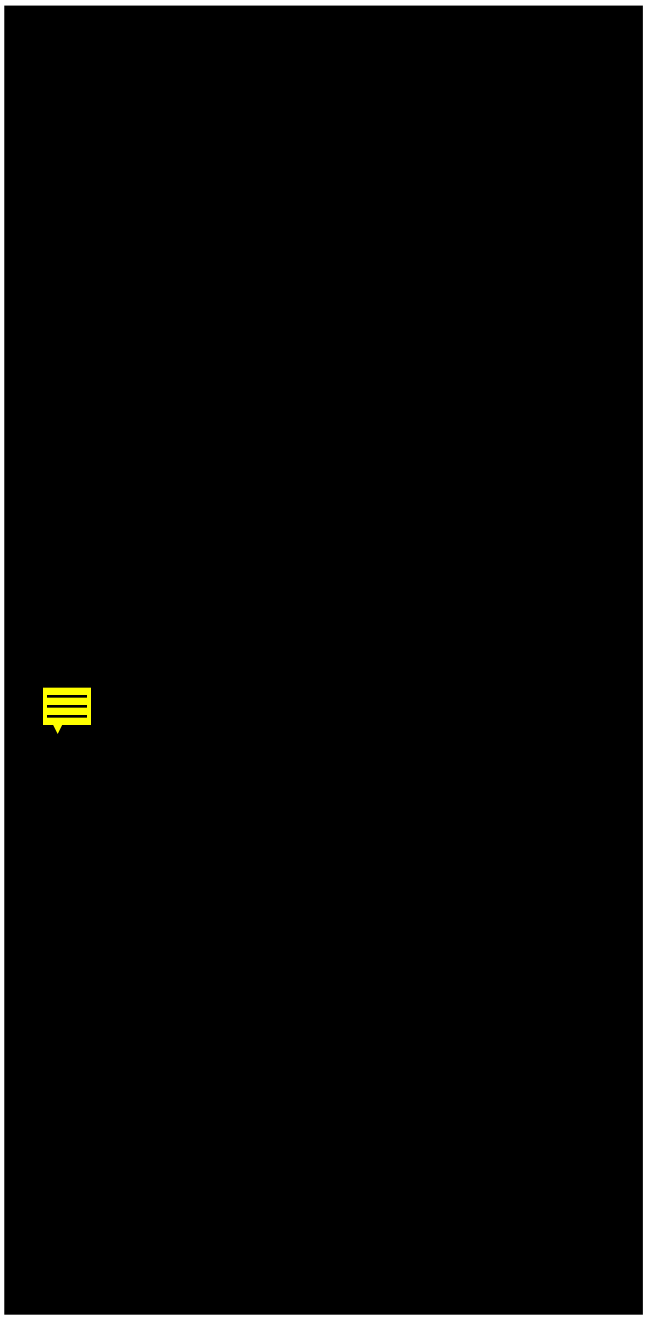

FIG. 5. Localization of viral antigen in the pulmonary alveoli 1 day following i.t. inoculation with T1L or T3D (4×10^8 PFU per rat). Cryostat sections from frozen lung tissue were stained for viral antigen by an immunoperoxidase assay. (A) Lung section from an uninoculated control rat. (B) Lung section from a rat 1 day following inoculation with T1L. (C) Lung section from a rat 1 day following inoculation with T3D. In panels B and C, viral antigen in the alveoli is indicated by the presence of a brown precipitate on alveolar cells. Magnification, ×288.

infection of lung epithelial cells. For example, in both the mouse model of influenza virus infection (22) and the cotton rat model for adenovirus infection (23), the pulmonary changes are also rapid, with severe pathology by day 3.

Reovirus infection in the lungs can be contrasted with infections with other viruses, such as measles, smallpox, and rubella viruses (20), as well as the polyomavirus K virus (14), in which pulmonary infection occurs after systemic spread of virus. For

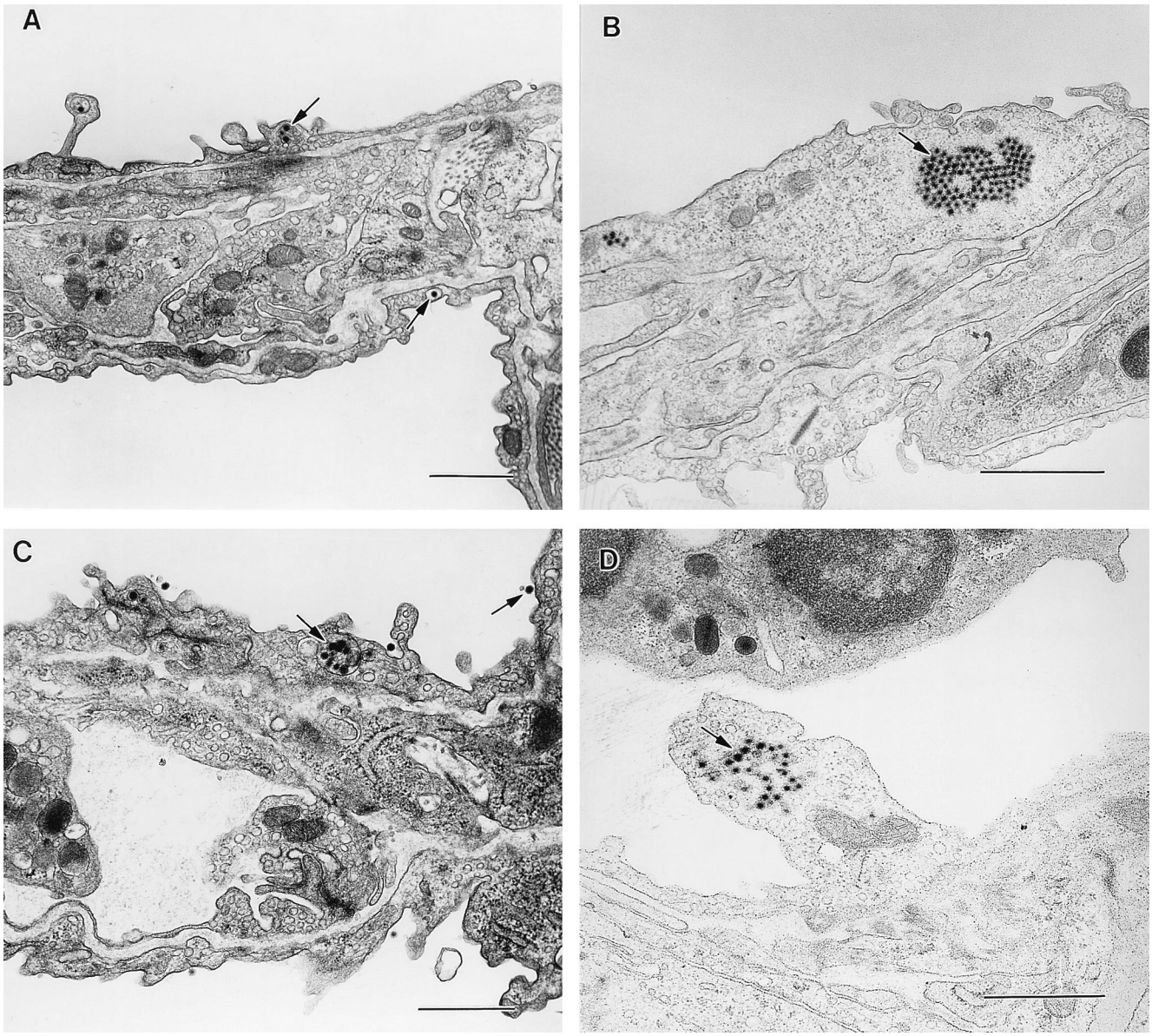

FIG. 6. Reovirus infection of type I alveolar epithelial cells: entry and replication as demonstrated in ultrathin sections from juvenile rats infected with reovirus. (A) Lung section from a rat 90 min following inoculation with 2×10^9 PFU of T1L, showing entry of viral particles (arrows) into type I alveolar epithelial cells; viral particles were present in membrane-bound vesicles within type I cells. (B) Progeny virus (arrow) found in the cytoplasms of type I cells from a rat 1 day following inoculation with 10^8 PFU of T1L, indicating replication of T1L in type I cells. Progeny virus was readily distinguished from input virus by the lack of a membrane surrounding the particles. (C) T3D virus particles (arrows) adhering to and entering type I cells from a rat inoculated with 2×10^9 PFU of T3D. (D) T3D progeny virus (arrow) in type I cells, as in panel B, except 12 h following inoculation with 10^8 PFU of T3D. Bars, 1 μm.

example, mice inoculated i.n. with K virus do not develop respiratory symptoms and pulmonary pathologic changes until 9 days after infection (14). Reoviruses have been shown to localize in the lungs after inoculation into the blood; by 5 min following i.v. inoculation of reovirus, 90% of the virus is cleared from the blood, and of that, 20% is found in the lungs (31). In the present study, however, in spite of potential localization of reovirus at the lungs, T1L was not capable of causing detectable pulmonary disease following i.v. inoculation.

Pulmonary reovirus infection was also similar to influenza virus, Sendai virus, and adenovirus infections with regard to viral replication in the lungs, where peak virus titers were achieved by day 3 postinoculation. In the mouse model of influenza virus infection (1), the mouse model of Sendai virus infection (3, 24), and the cotton rat model of adenovirus infection (23), viral replication in the lungs has been demonstrated, with viral titers peaking between days 3 and 5. Reovirus replication in pulmonary epithelial cells, in turn, contributed to the epithelial destruction. We demonstrated that reovirus replication was a prerequisite for pulmonary pathologic changes, whereas in murine adenovirus pneumonia, pulmonary inflammation develops without viral replication (13).

After i.t. inoculation with T1L and T3D, viral particles were found within type I alveolar epithelial cells as well as in alveolar macrophages. The latter observation was not surprising because alveolar macrophages are responsible for clearing the

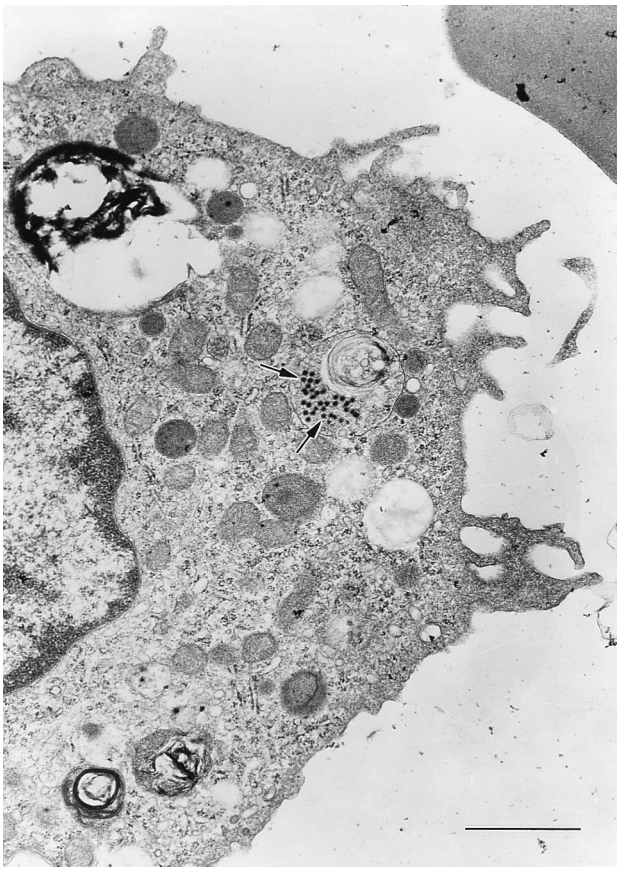

FIG. 7. Uptake of viral particles by alveolar macrophages. Ultrathin sections from rat lungs infected with T1L or T3D, and examined within 90 min following inoculation, revealed the presence of alveolar macrophages containing virus particles (arrows) within membrane-bound vesicles. Shown is an alveolar macrophage from a rat infected with T1L. Bar, 1 μm.

alveolar surface of foreign material, essentially keeping the alveoli sterile, and they are efficient in this regard (12). We were unable to ultrastructurally demonstrate replication of reoviruses in alveolar macrophages in vivo, although we have ultrastructural evidence for what appeared to be viral factories in alveolar macrophages cultured in vitro (unpublished observations). However, during reovirus infection in cultured alveolar macrophages, there was only a 1-$\log_{10}$ rise in titer in the T1L-infected cells and supernatants and a barely detectable rise, if any, with T3D and T3C9. These observations bear remarkable similarity to observations of influenza virus. With influenza virus, alveolar macrophages are infected by the virus and virus antigens are produced; however, replication is abortive, producing no infectious virus (25, 26).

The capacity of viruses to infect alveolar macrophages is an important issue for understanding the pathogenesis of respiratory infections. For example, the neutrophil chemotactic factors KC and MIP-2 are produced by alveolar macrophages (11, 16, 34). KC and MIP-2 are elicited in cultured alveolar macrophages treated with reovirus and in rats inoculated i.t. with reovirus (8), thus defining a potential role for alveolar macrophages in the host response to reovirus infection. Furthermore, levels of KC and MIP-2 elicited during reovirus infection are higher in T3D infection than in T1L infection (8), indicating a serotype-dependent response consistent with our observations of BAL cells recovered from reovirus-infected rats.

Although T1L and T3D both caused pulmonary inflammation, T3D infection elicited more neutrophils and more marked type II epithelial cell hyperplasia, while T1L grew to higher titers in the lungs. We determined that the S1 gene, which codes for the outer capsid protein σ1 and the nonstructural protein σ1s, played a prominent role in initiating neutrophil recruitment, although another gene(s) may be involved as indicated by the gradation in the results (Table 1). How S1 mediates neutrophil chemotaxis into the airways is as yet unclear, although after intraperitoneal inoculation of purified σ1 from T3D, neutrophils are recruited into the peritoneum (9). σ1 itself may therefore possess neutrophil-chemotactic properties or directly stimulate cells to produce chemokines. Type II cell hyperplasia (indicating type I cell destruction) was much more marked in the histological lung sections from T3D- than from T1L-infected rats. The more extensive type I cell destruction may have been a function of the greater number of neutrophils recruited into the lungs of T3D-infected rats. Neutrophils release proteolytic enzymes in response to infection that can, in turn, have a detrimental effect on the lung tissue (19). Although type I epithelial cell destruction was primarily a direct result of viral infection, recruited neutrophils may have contributed to epithelial damage in T3D-infected rats.

The S1 gene was also found to influence viral replication in the lungs, which is similar to its demonstrated role in replication in the brain and intestine. In the brain, differences between the levels of replication of T1L and T3D were mapped to the S1 gene, and the differences were related to the tropism of the viruses (33). T1L virus infected ependymal cells, whereas T3D virus infected neuronal cells. In the intestine, the difference in replication between T1L and T3D was proposed to be a function of the sensitivity of σ1 (the S1 gene product) to chymotrypsin digestion (2). The σ1 protein of T1L is more resistant to enzymatic digestion than that of T3D, conferring the increased infectivity of T1L (7). Although we found that the S1 gene influenced the level of replication in the lungs, the mechanism is yet unclear. In the lungs, the tropism of reovirus to the type I alveolar epithelial cells was not serotype specific; therefore, cellular tropism differences cannot explain how S1 mediated differences in virus replication in the lungs. If the stability of the σ1 protein in the pulmonary environment is different depending on the serotype, the difference in replication between T1L and T3D might be explained. We examined only the contribution of S1 on the level of virus replication and therefore cannot rule out a contribution from other genes to this phenotype. Further studies are needed to elucidate the role of S1 in pulmonary viral replication.

We used juvenile rats, aged 25 to 28 days, for the above-described experiments. When adult rats (weighing 250 to 300 g) were inoculated i.t. with T1L or T3D, the pulmonary inflammation results indicated pathological changes similar to those in juvenile rats, and the serotype differences were consistent (unpublished observations). The mechanism by which reoviruses caused pulmonary pathologic changes in the adult rats appeared to be the same as that in the younger rats, i.e., viral infection of type I alveolar epithelial cells, with replication and epithelial destruction followed by type II cell hyperplasia. In our model and under our experimental conditions, we did not detect a difference in the pathogenesis of the infection in rats within the age range used.

In the present report, we have provided some understanding of reovirus pathogenesis in the lungs and provided a laboratory model for viral pneumonia in which direct infection of the pulmonary epithelium results in inflammation and tissue injury. The different host responses to T1L and T3D provided a basis for a genetic analysis of the pulmonary response to viral

infection, and further genetic studies will build upon our model. The reovirus model can be used to further our understanding of the host response to viral cytopathic effect in the pulmonary epithelium and the host inflammatory and repair responses in the lungs. The similarities to other respiratory viruses, coupled with the capability to determine the molecular basis of viral pathogenesis by using virus reassortants, make reovirus an attractive virus for a model for the study of viral pneumonia.

ACKNOWLEDGMENTS

The research was supported by U.S. PHS grant 5R37AI13178 from the NIAID and the Shipley Institute of Medicine.

We thank Elaine Freimont, Bonnie Meek, Caroline Snowman, Carol Mathieu, and Laurie Graham for their excellent technical support. We also thank David Knipe, Anthony Farone, and Dale Spriggs for review of the manuscript.

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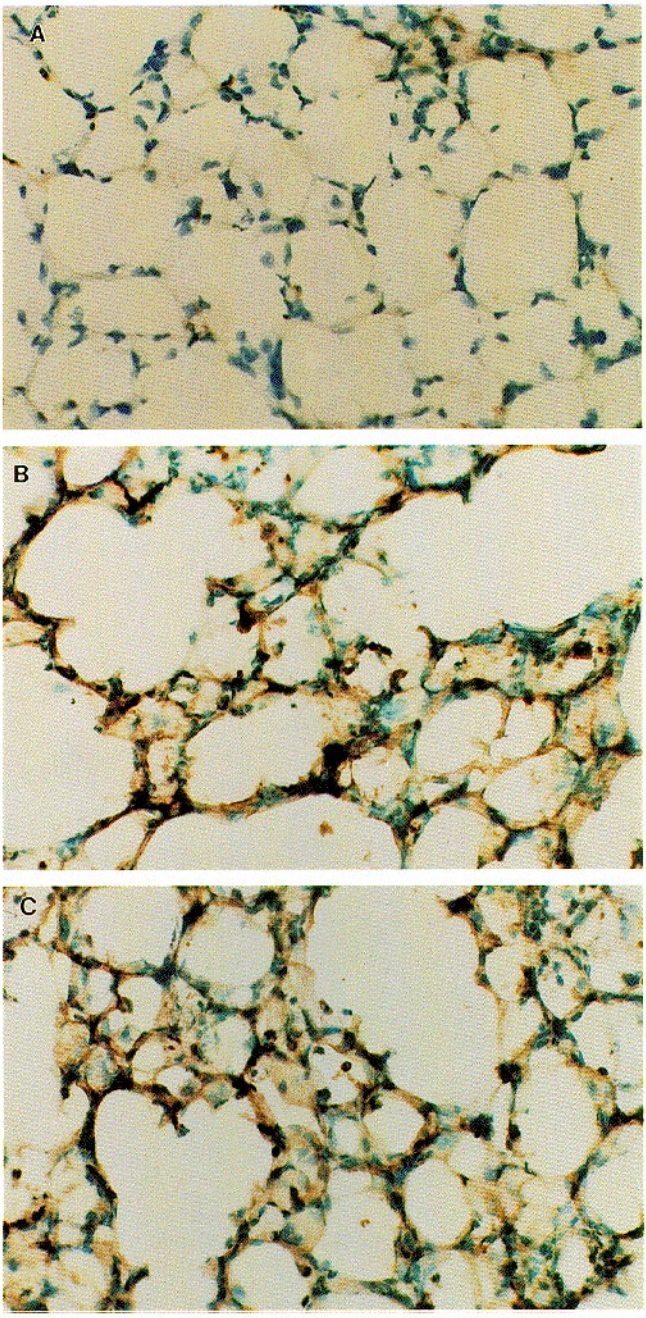
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