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Respiratory Syncytial Virus Infection in Mice

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The A2 strain of human respiratory syncytial virus replicated in the nose and lung of BALB/c mice, with virus growing to higher titers in older animals than in younger animals. Virus was recovered from the nose between days 2 and 7 with peak titers on days 3 and 4, and from the lungs between days 2 and 9, with peak titers on days 4 through 6. Serum antibody developed 2 weeks after infection. Viral antigen was demonstrated in the alveolar cells of the lung by immunofluorescence. Histopathological changes included infiltration by mononuclear cells of the peribronchiolar and perivascular tissue, some interstitial thickening, and formation of multinucleated giant cells. Virus could not be recovered from the respiratory tract of mice inoculated with bovine strains of respiratory syncytial virus. Growth of the A2 strain of human respiratory syncytial virus in different cell lines affected its infectivity for mice. Infection of BALB/c mice with respiratory syncytial virus provides a highly reproducible model for the study of the pathogenesis of and mechanisms of immunity to this virus.

Respiratory syncytial (RS) virus is a major cause of bronchiolitis and pneumonia in children less than 6 months old (3), and there is increasing evidence that this virus is also important in respiratory disease of young calves (12). Thus, there is a need for an effective vaccine for use in both humans and cattle. A number of animal models of RS virus infection have been developed to gain information on immunity to and the pathogenesis of this virus. Experimental infection with RS virus has been described in ferrets (8), cotton rats (7), chimpanzees (2), cebus monkeys (11), owl monkeys (10), and cattle (L. H. Thomas, E. J. Stott, A. P. Collins, S. Crouch, and J. Jebbett, Arch. Virol., in press; L. H. Thomas, E. J. Stott, A. P. Collins, and J. Jebbett, Br. J. Exp. Pathol., in press). However, there are disadvantages with most of these models. For example, primates are scarce. The histopathological changes after infection in some animal species do not have all the features of the disease in humans. None of the animal species is inbred, and there are few specific immunological reagents available for them. It was because of these disadvantages that Prince et al. (6) examined a large number of inbred mice for their susceptibility to infection with the Long strain of human RS virus. These workers used 3-day-old mice that were examined 4 days after inoculation for virus in the lung and nasal passages. They showed that there were considerable differences between mouse strains in susceptibility to RS virus infection. We have extended these observations and report here the effects of a number of other variables, such as age and sex of the mouse, the strain of RS virus, and the type of cell used to grow the inoculum, on the susceptibility of BALB/c mice to RS virus infection.

MATERIALS AND METHODS

Mice. Specific-pathogen-free BALB/c mice of the Medical Research Council Laboratory Animal Center's category 4 standard were obtained from the specific pathogen free unit at the Institute for Research on Animal Diseases, Compton, (BALB/c Com) and from the Clinical Research Centre, Harrow (BALB/c Crc). Mice 3 to 7 days of age were anesthetized with ether and inoculated intranasally (i.n.) with approximately 10^4 PFU of RS virus in a volume of 10 μl. Mice greater than 3 weeks of age were anesthetized by intraperitoneal inoculation of 0.06 mg of Pentobarbitone Sodium (May and Baker Ltd., England) per g of body weight and inoculated i.n. with RS virus in a volume of 50 μl. Control mice were inoculated i.n. with equivalent amounts of tissue culture supernatant from uninfected cells or with UV-inactivated RS virus. Mice were used in groups of five.

Virus strains. The human A2 isolate (5) of RS virus was obtained from C. Pringle of the Institute of Virology, Glasgow. A single pool of virus, prepared in HeLa cell cultures, that contained approximately $10^{6.3}$ PFU/ml was stored in liquid nitrogen and used in most experiments.

The Compton 127 and Snook isolates of bovine RS virus have been described in detail elsewhere (Thomas et al., in press). For inoculation into mice, virus was prepared in calf kidney cells. The 9007 strain of RS virus was isolated at Compton in February 1982 from a calf with severe respiratory disease and had been passed three times in bovine tissue culture before inoculation into mice. All isolates of bovine RS virus were stored at −70°C before use.

Microbiology. At intervals after inoculation mice were killed with 0.6 mg of pentobarbitone inoculated intraperitoneally per g of body weight and exsanguinated from the axilla. The lungs and the snout from each animal were removed and homogenized separately in Hanks balanced salt solution containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.8), 200 U of penicillin, streptomycin, and mycostatin per ml, 0.218 M sucrose, 4.8 mM glutamate, and 30 mM magnesium chloride to give 10% (wt/vol) suspensions of lung and 20% (wt/vol) suspensions of nose. Homogenates were centrifuged at 9,000 × *g* for 1 min, and the supernatant was removed and assayed immediately. Plaque assays were performed on secondary calf kidney cells, plaques being counted after 7 days at 33°C for human RS virus and after 10 days for bovine RS virus.

In some experiments, lungs from infected and control mice were homogenized in medium not supplemented with antibiotics. The lung homogenate was inoculated onto blood agar plates, which were incubated at 37°C aerobically, and into mycoplasma medium (4) to detect glucose-fermenting

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TABLE 1. Effect of age on susceptibility of mice to i.n. inoculation with $10^{4.2}$ PFU human RS virus

Expt	Age of mice	Mean titer of RSV isolated from[a]: Nose	Mean titer of RSV isolated from[a]: Lung	Lung lesions
1	3 to 4 days	3.9 ± 0.02	3.4 ± 1.14	ND
	7 days	3.7 ± 0.13	4.0 ± 0.13	ND
	3 to 4 weeks	3.5 ± 0.27	4.2 ± 0.15	ND
	8 to 10 weeks	2.5 ± 0.49	4.5 ± 0.11	ND
2	3 to 4 days	ND	2.9 ± 0.29	−
	7 days	ND	3.2 ± 0.12	±
	3 to 4 weeks	ND	4.3 ± 0.22	+
	8 to 10 weeks	ND	4.2 ± 0.28	+

[a] Mean $\log_{10}$ PFU per gram of tissue ± standard deviation for 5 mice 4 days after i.n. inoculation. ND, Not determined.

mycoplasmas. Samples in mycoplasma medium were diluted to 10^{-4} and observed for up to 4 weeks for the development of mycoplasma growth. Bacteria and mycoplasmas were not isolated from any mice.

Histology. Lungs were inflated by intratracheal injections of Formol sublimate to preserve the pulmonary architecture in an expanded state. After 24 h of immersion in Formol sublimate, lungs were transferred to 70% ethanol. Histological sections were stained with hematoxylin and eosin.

Immunofluorescence. Lungs were inflated by intratracheal injection of OCT compound (Tissue-Tek II; Lab-Tek Products, Westmont, Ill.) diluted 1:2 in phosphate-buffered saline, quick-frozen in liquid nitrogen, and stored at −20°C until sectioned. Cryostat sections were cut at 10 μm. To localize viral antigen in tissues, acetone-fixed sections were incubated sequentially with gnotobiotic calf antiserum to human RS virus and fluorescein isothiocyanate-labeled rabbit anti-bovine immunoglobulin G (IgG; Nordic Immunological Laboratory, Ltd.). As controls, sections were incubated with gnotobiotic calf antiserum to Pi-3 virus followed by fluorescein isothiocyanate-labeled rabbit anti-bovine IgG.

Serology. Antibody to RS virus was measured by a modification of the single radial hemolysis test (9). Trypsin-treated bovine erythrocytes were coupled to the purified IgG fraction of a gnotobiotic calf antiserum to RS virus by using chromic chloride and then incubated with a suspension of bovine RS virus. Control sera were tested against IgG-coupled, trypsin-treated erythrocytes not incubated with RS virus.

RESULTS

Effect of age and sex of mice on susceptibility to human RS virus. Mice were inoculated i.n. with $10^{4.2}$ PFU of human RS virus given as 10 μl of undiluted virus for animals up to 7 days old or as 50 μl of 1/5 dilution of virus for mice greater than 3 weeks of age. Animals were examined 4 days after inoculation. There was little difference in the titer of virus recovered from the nasal passages of mice of different ages, although the oldest animals yielded less virus than did those under 3 weeks of age (Table 1). In contrast, significantly higher titers of virus were found in the lung of mice greater than 3 weeks of age (Table 1; $P < 0.05$ for experiment 1 and $P < 0.001$ for experiment 2 by Students' t test). Lung lesions that developed in RS virus-infected mice also appeared to be most severe in mice 3 to 10 weeks of age.

The mean titer of virus in the lungs was $10^{4.5}$ ± 0.11 PFU/g in male mice and $10^{4.3}$ ± 0.10 PFU/g in female mice. These values were not significantly different. Thus, in future experiments mice of either sex and greater than 3 weeks of age were used.

Pattern of viral replication. The course of respiratory tract infection in mice inoculated i.n. with $10^{4.6}$ PFU of human RS virus is shown in Fig. 1. The amount of virus recovered from the lungs increased rapidly to a peak on days 4 to 6. The titers then declined until infectious virus was undetectable by day 11. Peak titers of virus in the nasal passages occurred slightly earlier and were lower than those in the lung. Infectious virus in the nose was undetectable by day 9.

Immunofluorescence. Lung tissue was examined by immunofluorescence for viral antigen on days 4 to 5 and on day 7 of infection. At days 4 to 5, viral antigen was detected more frequently in the lungs of mice greater than 3 weeks of age than in the younger animals and appeared as small foci of fluorescent cells in the alveoli (Fig. 2). Little viral antigen

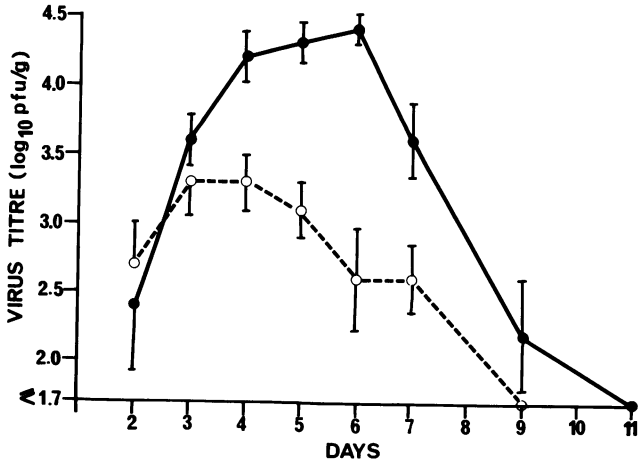

FIG. 1. Virus replication in nose (○) and lungs (●) of mice inoculated i.n. with $10^{4.6}$ PFU of human RS virus. Each point represents the geometric mean titer per gram of tissue ± standard deviation for 5 to 15 mice.

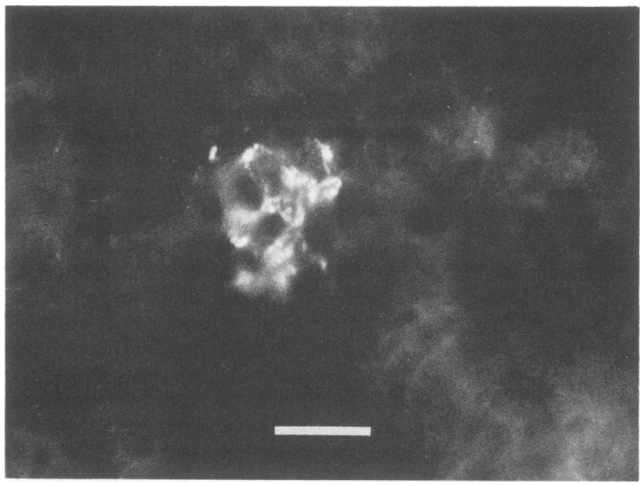

FIG. 2. RS virus antigen-positive cells as detected by indirect immunofluorescence in the alveolar walls of the lung from a mouse inoculated i.n. 4 days previously with $10^{4.2}$ PFU of human RS virus. Bar, 20 μm.

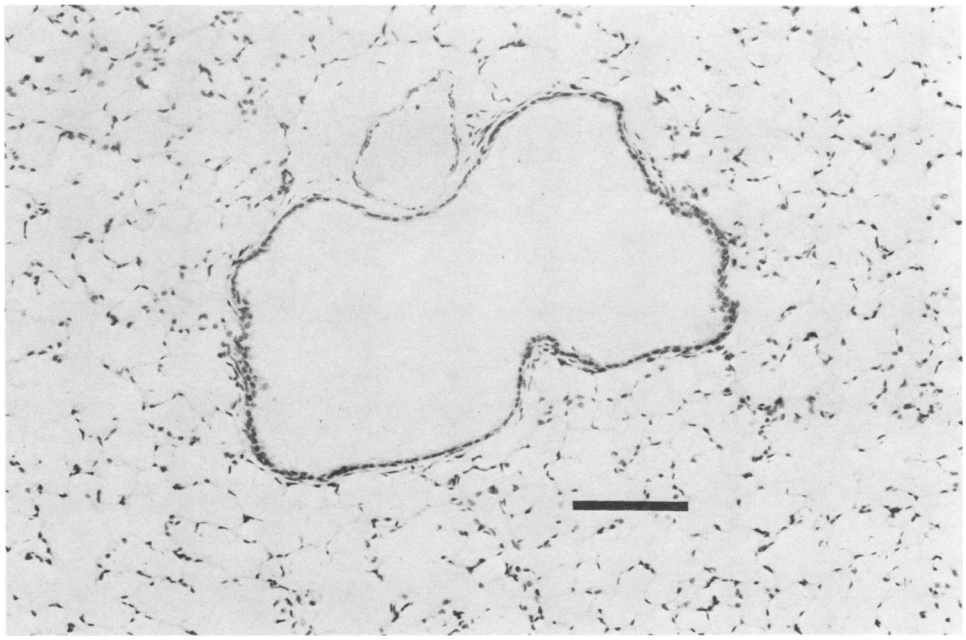

FIG. 3. Section of control lung from a mouse inoculated i.n. 7 days previously with tissue culture fluid from uninfected HeLa cells. The bronchial epithelial cells have little cytoplasm, there is no peribronchiolar or perivascular infiltration, and the alveolar walls are thin. Bar, 100 μm.

was detected in cells of the bronchiolar epithelium. At day 7, little or no viral antigen was detected in the lungs.

Histology. The lungs from control mice inoculated i.n. with tissue culture fluid from uninfected HeLa cells (Fig. 3) were the same as those from uninoculated animals. Similarly, lungs from mice inoculated 7 days previously with UV-inactivated RS virus were no different from those of uninoculated controls. Gross pathological changes in the lungs were not detected after i.n. inoculation of mice with approximately 10^4 PFU of human RS virus. However, mice developed histological lung lesions which were maximum at days 6 to 7 post-inoculation. These changes in lung histology were not extensive and were resolving 9 to 11 days after infection. The predominant lesion consisted of an infiltration of the perivascular and of the peribronchiolar tissue by mononuclear cells (Fig. 4). The infiltrating cells were predominantly

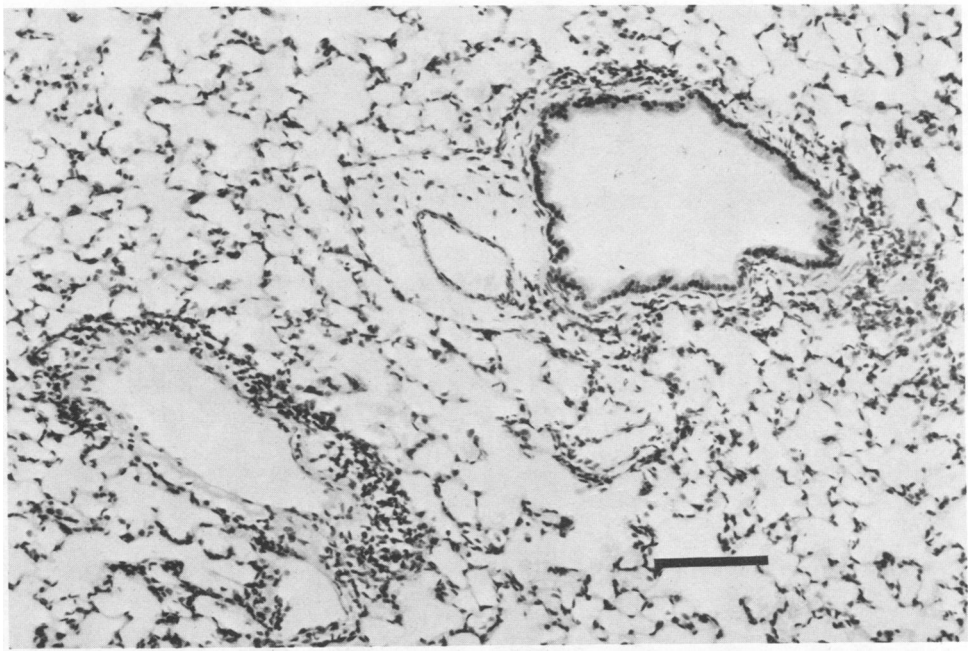

FIG. 4. Section of lung from a mouse inoculated i.n. 7 days previously with $10^{4.6}$ PFU of the A2 strain of human RS virus. The bronchial epithelial cells have increased cytoplasm, and there are peribronchiolar and perivascular accumulations of mononuclear cells. Bar, 100 μm.

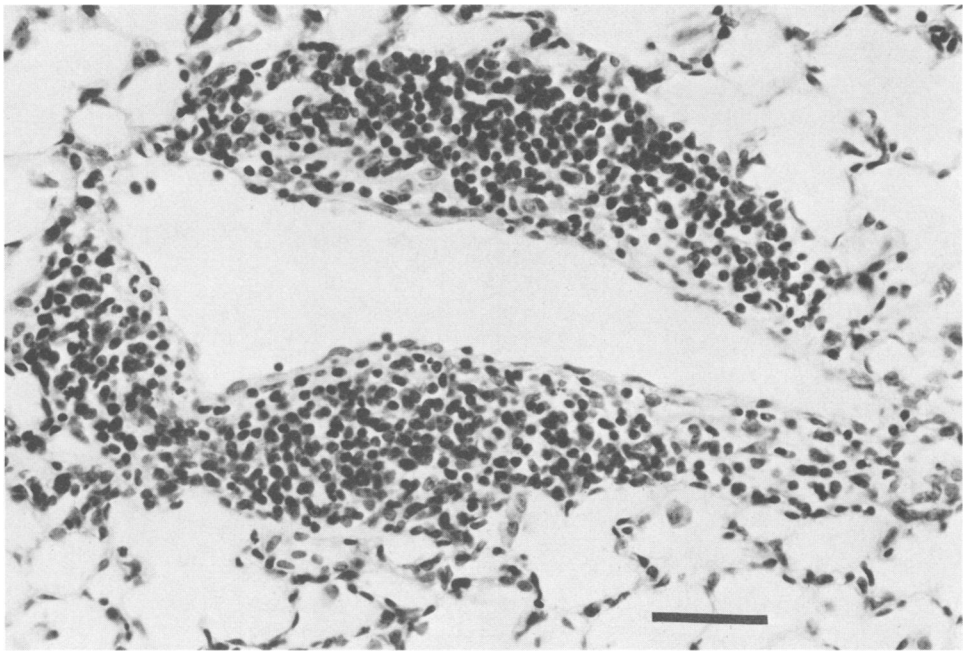

FIG. 5. Small lymphocytes infiltrating the perivascular tissue in the lung of a mouse inoculated i.n. 7 days previously with human RS virus. Bar, 40 μm.

small lymphocytes with a few polymorphonuclear leukocytes and macrophages, but with few or no plasma cells (Fig. 5). The epithelial cells of affected bronchioles were markedly swollen, and occasionally an exudate composed of desquamated epithelial cells, macrophages, and polymorphonuclear leukocytes could be seen in the lumen of some of the smaller bronchioles (Fig. 6). In some areas the alveolar walls were infiltrated with mononuclear cells, and there was an alveolar exudate and enlarged septal cells (Fig. 7). It was in such areas that giant cells were occasionally found (Fig. 8).

Antibody response. With the single radial hemolysis test, antibody to RS virus was first detected in the sera of 6 week-old-mice 2 weeks after i.n. inoculation with RS virus. The level of antibody continued to increase up to 4 weeks postinfection when the experiment was terminated (Fig. 9). Antibody was also detected 3 weeks after inoculation of 2- to

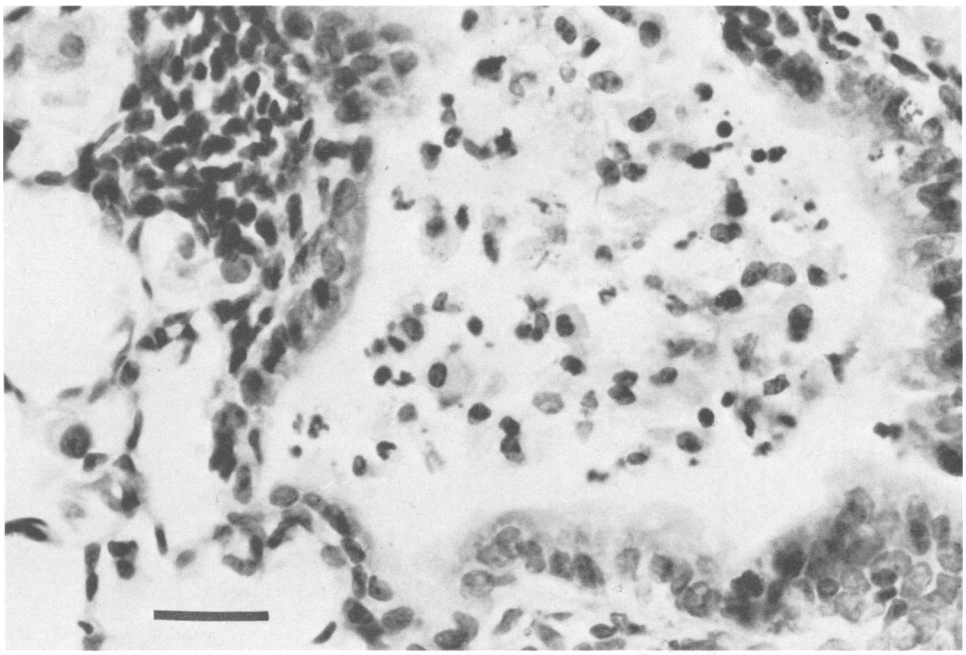

FIG. 6. Bronchial exudate composed of desquamated epithelial cells, macrophages, and a few polymorphonuclear leukocytes in the lung of a mouse inoculated i.n. 7 days previously with human RS virus. Bar, 25 μm.

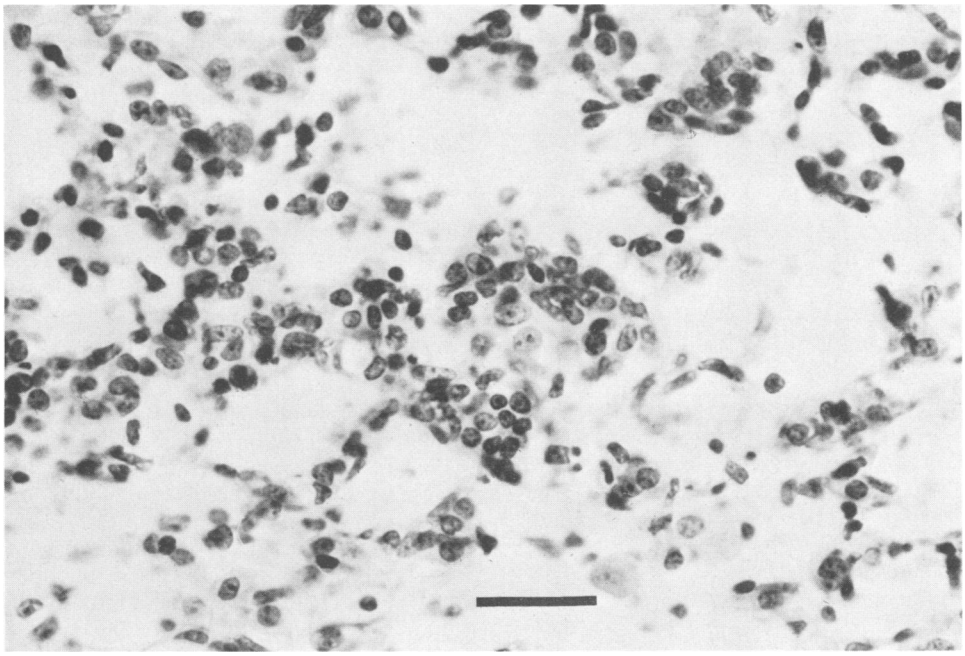

FIG. 7. Some alveolar exudate, enlarged septal cells, and thickening of alveolar walls in the lung of a mouse inoculated i.n. 7 days previously with human RS virus. Bar, 25 μm.

4-day-old mice; the mean single radial hemolysis zone of 11 mice was 24 ± 8.2 mm^2. Sera from infected mice did not lyse control erythrocytes (i.e., IgG-coupled erythrocytes incubated without RS virus).

Infectivity for mice of human RS virus grown in different cell lines. Preliminary experiments suggested that human RS virus grown in different cell lines varied in its ability to replicate in mice. To examine this more fully, human RS virus was grown in Vero, MA104, HeLa, and CK cells. Undiluted virus and a 1/10 dilution of virus were inoculated i.n. into groups of mice, and animals were examined 5 days later for virus in the lungs. RS virus was recovered in higher titers from the lungs of mice given virus grown in HeLa and CK cells than from the lungs of animals inoculated with similar titers of virus grown in Vero or MA104 cells (Table 2). Lungs were not examined for the development of lesions.

Growth of bovine RS virus in mice. Groups of mice were inoculated i.n. with three different isolates of bovine RS virus. Although the titers of two of the inocula (Compton 127 and Snook) were comparable to that of the human RS virus

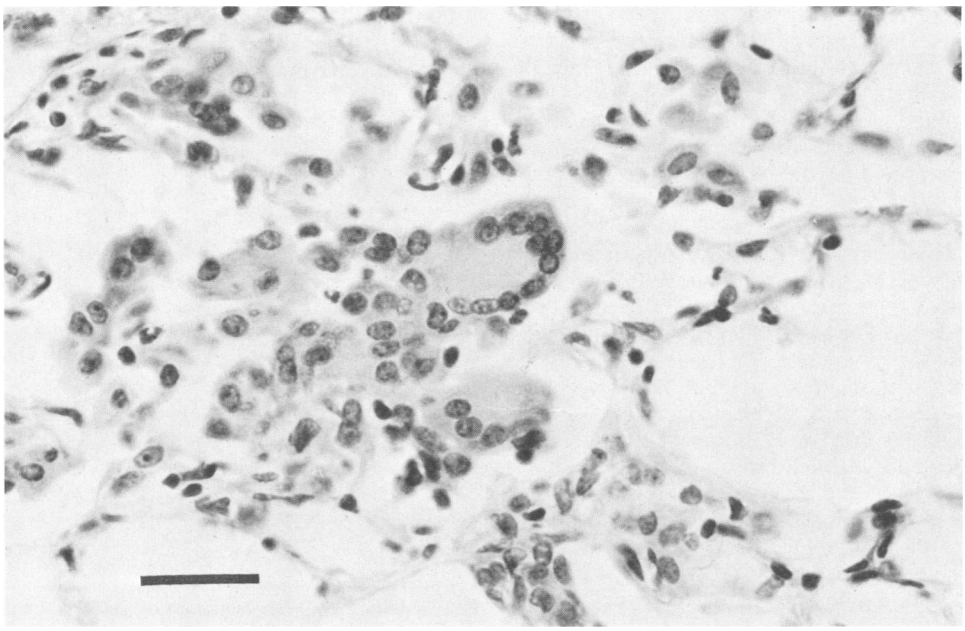

FIG. 8. Giant cell formation in the lung of a mouse inoculated i.n. 7 days previously with human RS virus. Bar, 25 μm.

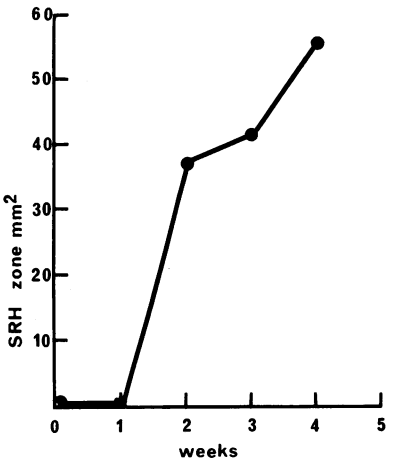

FIG. 9. Development of serum antibody to RS virus as measured by the single radial hemolysis (SRH) test in mice inoculated i.n. with $10^{4.6}$ PFU of human RS virus. Each point represents the mean value for 5 mice.

grown in CK (Table 2), virus was not recovered by plaque assay from the lungs of any of the mice 5 days after inoculation (Table 3). However, virus was recovered from mouse lungs after two to three blind passages of lung homogenate in calf testis cells.

DISCUSSION

Infection of 3-week-old BALB/c mice with the A2 strain of human RS virus was highly reproducible. The standard deviation of the mean titer of virus recovered from the lungs was usually less than 6%. The titer of virus in the lungs of 4-day-old BALB/c mice inoculated with the A2 strain of human RS virus was similar to that reported by Prince et al. (6) for the Long strain.

Mice <7 days of age are immunologically immature and therefore might be more susceptible to infection than older animals. In fact, the yield of RS virus was greater from the lungs of older mice. This age-related difference in susceptibility may be related to the inoculation procedure. Thus, the ether used to anesthetize mice <7 days old may have inactivated some of the inoculum. In addition, younger mice were inoculated with 1/5 of the volume of that given to older animals, and this may have resulted in less virus reaching the lungs. The presence of viral antigen in the lungs and the development of lung lesions in mice greater than 3 weeks of age reflected the higher level of virus replication in the lungs of these animals when compared with younger mice.

The pattern of virus replication in the respiratory tract was examined in more detail. The higher titer of virus in the lungs than in the nasal passages may reflect a tissue tropism by the virus. Alternatively it may be an artifact associated with isolation procedures, since nasal turbinates were not dissected from surrounding tissues before homogenization. Virus titers peaked earlier and declined more rapidly in the nasal passages than in the lungs.

The distribution of viral antigen in the lungs of mice 4 to 5 days after inoculation resembled that reported for infant ferrets inoculated i.n. with the Long strain of RS virus (8). Viral antigen occurred in small scattered foci in cells of the alveolar walls. This distribution is in contrast to that seen in children (1), cebus monkeys (11), and calves (Thomas et al., in press), where viral antigen is present in bronchial epithelial cells as well as in alveolar cells. Furthermore, antigen is only seen in bronchial epithelial cells in cotton rats (7). The failure to detect viral antigen in the bronchial epithelium of mice was surprising since the nature of the lung lesions suggested that the bronchial epithelial cells were infected. However, Richardson et al. (11) observed that in cebus monkeys viral antigen was detected only in those lungs from which more than $10^{4.5}$ PFU were isolated per g of tissue. Virus recovered from the lungs of mice rarely exceeded this titer. Further work is in progress to determine the distribution of RS virus in lungs from mice.

The histological changes seen in the lungs of human infants with severe RS virus infection are composed of necrosis of the bronchiolar epithelium with a peribronchiolar inflammatory response and an interstitial pneumonia (1). With the exception of the bronchial epithelial necrosis, similar lesions were seen in mice infected with RS virus in these experiments and also in gnotobiotic calves inoculated with bovine RS virus (Thomas et al., in press). However, the lung lesions in mice were mild in comparison with that reported for children and calves. In contrast, lesions appear to be limited to the alveoli in infant ferrets (8) and to the bronchiolar epithelium in the lungs of cotton rats (7). It may be possible to increase the severity of the lung lesions in mice by increasing the dose inoculated as for cebus monkeys (11).

The development of lung lesions appeared to be due to RS virus alone since bacteria and mycoplasmas were not recovered from the lungs of RS virus-infected mice. Further, we failed to detect antibody to *Mycoplasma pulmonis* in sera from RS virus-infected mice with a radioimmunoassay test.

The poor replication in mice of RS virus grown in Vero and MA104 cells may be due to defective interfering particles or to the selection of virus with a low infectivity for mice. It is unlikely that defective interfering particles were produced in Vero or MA104 cells, as all cells were inoculated at low multiplicity of infection (13). Further, dilution of the inoculum failed to increase the infectivity of the virus for mice. Failure by monkey cells to cleave the fusion protein of RS virus could render the virus noninfectious. However, this is an unlikely explanation as virus grown in Vero or MA104 cells is still infectious for these and CK cells. It may be that carbohydrate moieties of the virus derived from host cells

TABLE 2. Comparison of the infectivity for mice of human RS virus grown in different cells

Cells	Titer ($\log_{10}$ PFU/ml)	Inoculum dilution	Mean titer[a] of RS virus isolated from lungs
Vero	6.3	None	3.4 ± 0.56
		1/10	2.7 ± 0.70
MA104	5.4	None	2.2 ± 0.55
		1/10	1.7 ± 1.14
HeLa	6.1	None	4.5 ± 0.06
		1/10	3.9 ± 0.33
CK[b]	6.4	None	4.5 ± 0.14
		1/10	4.3 ± 0.15

[a] Mean $\log_{10}$ PFU per gram of tissue ± standard deviation for 5 mice 5 days after i.n. inoculation.

[b] CK, Calf kidney cells.

TABLE 3. Comparison of the infectivity for mice of three strains of bovine RS virus

Virus strain	Virus grown in:	Inoculum[a]	Mean titer[b] of RS virus isolated from lungs
Compton 127	CK[c]	5.7	<1.7
Snook	CK	3.5	<1.7
9007	CTe[d]	1.2	<1.7

[a] Log_{10} PFU per mouse.
[b] Mean log_{10} PFU per gram of tissue for 5 mice 5 days after i.n. inoculation.
[c] CK, Calf kidney cells.
[d] CTe, Calf testis cells.

influence the infectivity of RS virus for the mouse respiratory tract.

The bovine RS virus strains, Compton 127 and Snook, could only be recovered from lungs after several blind passages. The difficulty in recovering bovine RS virus from mice is likely to be a property of the virus strain rather than the host cells used to produce it since human A2 virus grown in CK cells was readily recovered from mice.

The development of a highly reproducible model of RS virus infection in inbred mice should be of great value in examining the pathogenicity of and immunity to this virus. For example, passive immunization of BALB/c mice with monoclonal antibodies has helped to define the protective epitopes of RS virus (G. Taylor, E. J. Stott, M. Bew, B. F. Fernie, A. P. Collins, M. Hughes, and J. Jebbett, J. Immunol., in press), and this will help the development of an effective vaccine against RS virus infection.

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