

Pathogenesis of pneumovirus infections in mice: detection of pneumonia virus of mice and human respiratory syncytial virus mRNA in lungs of infected mice by *in situ* hybridization

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The pathogenesis of pneumonia virus of mice (PVM) and human respiratory syncytial virus (HRSV) in BALB/c mice were investigated by using *in situ* hybridization to detect virus mRNA in fixed lung sections. Following intranasal inoculation with 120 p.f.u. PVM the pattern of hybridization showed that virus mRNA was initially detected within 2 days in alveolar cells. As the infection progressed the number of hybridizing alveolar cells increased and signal was also detected in cells lining the terminal bronchioles. By days 4 to 5 post-infection areas of morphological abnormality could be seen, particularly in the strongly hybridizing regions of the lung, and this correlated with the appearance of clinical

signs of infection. In animals which survived the infection virus-specific mRNA could not be detected 10 days post-infection. Mice infected with 1500 p.f.u. HRSV showed significant differences in the distribution of virus-specific mRNA when compared to the pattern seen with PVM. HRSV mRNA was detected over large areas, but predominantly in peribronchiolar and perivascular regions of the lungs 5 days post-infection. The yield of PVM from infected mouse lungs was considerably higher than that of HRSV. The possible implications of these results for the use of the mouse model for pneumovirus infections are discussed.

Introduction

Pneumonia virus of mice (PVM) is a common pathogen in laboratory animal colonies, particularly those containing athymic mice (Homberger & Thomann, 1994; Kraft & Meyer, 1990). The virus was first isolated from apparently healthy animals and while the naturally acquired infection is thought to be asymptomatic, passage of virus in mice lungs resulted in overt signs of disease ranging from an upper respiratory tract infection (Smith *et al.*, 1984) to a fatal pneumonia (Carthew & Sparrow, 1980a; Richter *et al.*, 1988; Weir *et al.*, 1988). Despite its name the natural host of PVM is not yet established and serological evidence indicates that many rodent species can be infected (Eaton & van Herick, 1944; Horsfall & Curnen, 1946). In addition, the presence of neutralizing antibodies indicates that some primates, including man, are infected with an antigenically indistinguishable virus (Horsfall & Curnen, 1946; Pringle & Eglin, 1986). Up to 80% of serum samples taken from human adults elicit a PVM-specific neutralizing and haemag-

glutination-inhibiting response, though the clinical consequences of human infection are unclear (Pringle & Eglin, 1986).

PVM was the first described example of the viruses now classified in the subfamily *Pneumovirinae* of the family *Paramyxoviridae*, which include human, bovine and ovine respiratory syncytial viruses (HRSV, BRSV, ORSV) and avian pneumovirus (Pringle, 1996). Molecular cloning has shown that the organization of the PVM genome is similar to that of HRSV (Chambers *et al.*, 1990). Restricted serological cross-reactivity between the nucleocapsid protein (N) and the phosphoprotein (P) of PVM and HRSV has been described but none of the external proteins show cross-reactivity, and the viruses can be distinguished from each other in neutralization assays (Gimenez *et al.*, 1984; Ling & Pringle, 1989). While the amino acid sequence identity of some of the PVM and HRSV proteins can be high, the nucleotide sequence identity between genes of PVM and RSVs is much lower and ranges from 60% for the most highly conserved N gene (Barr *et al.*, 1991) to the nonstructural protein genes which show little or no identity (Chambers *et al.*, 1991). However, despite the high degree of sequence conservation there is no cross-hybridization between N genes of PVM and HRSV.

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HRSV is the major cause of viral lower respiratory tract disease in infants and young children, causing bronchiolitis and bronchopneumonia (Aherne *et al.*, 1970). In attempts to understand the mechanisms of pathogenesis of HRSV infection, several animal models have been used, but none is able to reproduce the disease seen in human infants (McIntosh & Chanock, 1990). HRSV is able to multiply in the lungs of several species of rodent under laboratory conditions and, despite not being a natural host for HRSV, the mouse has been used to evaluate several features of HRSV infection. A model using BALB/c mice is favoured due to its reproducibility, among other factors, but the mice show few, if any, signs of respiratory illness (Taylor *et al.*, 1984). BRSV in cattle has been used as a model for HRSV but this is limited for practical reasons. PVM in mice may offer an amenable model system for the analysis of a mammalian pneumovirus infection in a natural host, and this model can be compared to the frequently used model of HRSV infection in mice. A comparison of the two may indicate differences which direct the different outcomes of infection.

We describe here an analysis of the progress of PVM infection in BALB/c mice by using *in situ* hybridization to follow the location of virus-specific RNA in the lung, and compare this with mice infected with HRSV. The two viruses exhibited differences in the distribution of virus RNA in the lung.

Methods

■ **Virus and cells.** A pathogenic strain of PVM, J3666, was supplied by D. Harter (Rockefeller Institute, New York). This strain has been passaged entirely in mice, and was grown once in BS-C-1 cells to increase the volume of the stock immediately prior to use in the studies reported here. The A2 strain of HRSV was used to infect mice.

■ **Infection of animals.** Six-week-old specific-pathogen-free BALB/c mice were purchased from Harlan Olac (Bicester, UK). The mice were certified free of PVM and were transported in filtered cages. Animals were housed in an isolator maintained at a negative pressure of 2.5 mm H₂O. Mice were lightly anaesthetized with diethyl ether and intranasally inoculated with 50 µl virus inoculum on the external nares. After animals were sacrificed one lung from each animal was removed and fixed immediately by immersion in formal saline. A 10% (w/v) suspension in PBS was generated by Dounce homogenization of the remaining lung, and the suspension was stored at -70 °C. The lower limit of sensitivity of the assay was 200 p.f.u./g. For the determination of the clinical outcome of infection five mice were infected with 60 p.f.u., 70 with 120 p.f.u. and 22 with 600 p.f.u. PVM strain J3666.

■ ***In situ* hybridization.** The fixed lungs were processed overnight in open cassettes on a Shandon Processor model 2LE with standard processing times. The tissues were embedded in paraffin wax on a Tissue Tek III Thermal Console and stored at 4 °C. Sections (4 to 6 µm) were mounted on glass slides coated with 3-aminopropyltriethoxysilane (Easton & Eglin, 1991).

The pre-treatment of sections was based on methods provided by H. Goram (Institute of Molecular Medicine, Oxford) and F. Lewis (Institute of Pathology, University of Leeds). After de-waxing in xylene and rehydration, the sections were post-fixed in 4% (w/v) paraformaldehyde in PBS for 20 min. The sections were digested in 0.001% (w/v)

proteinase K in 0.1 M Tris-HCl pH 8, 0.05 M EDTA pH 8 (preheated to 37 °C) for 8 min at 37 °C and re-fixed in paraformaldehyde. The slides were rinsed in 0.1 M triethanolamine (TEA) pH 8, and acetylated in 0.25% (v/v) acetic anhydride in 0.1 M TEA pH 8 for 10 min at room temperature. After rinsing in two changes of 2 × SSC the sections were dehydrated by immersion in a series of increasing concentrations of fresh ethanol.

Virus-specific probes were prepared from a plasmid containing a full-length copy of the PVM N gene inserted into plasmid pGEM-1 (Promega) under the control of the SP6 RNA polymerase promoter and from a 1.08 kbp fragment of the N gene of HRSV strain RS-S2 inserted into pBluescribe (Stratagene) at the T7 polymerase promoter site. Plasmid DNA was used as template and negative-sense radioactive RNA probes were prepared by *in vitro* transcription in the presence of [³⁵S]UTP. The purified probes, in buffer containing 1% SDS, 10 mM Tris-HCl pH 7.4, 1 mM EDTA and 10 mM DTT, were added to hybridization buffer [50% (v/v) formamide, 10% (w/v) dextran sulphate, 1 × Denhardt's solution (Sambrook *et al.*, 1989), 150 mM NaCl, 0.5 mM Tris-HCl pH 8, 0.1 M DTT and 100 µg/ml tRNA]. After heating at 65 °C for 10 min, and centrifuging at 2000 g for 10 min, 75 µl of the supernatant was placed on each slide. A coverslip was placed on the section and the edges were sealed. The slides were incubated at 65 °C overnight.

Slides were next washed in 4 × SSC, digested with RNase A for 30 min at 37 °C to remove excess unbound probe and washed successively twice in 2 × SSC, once in 1 × SSC and once in 0.5 × SSC for 10 min each. All washes contained 1 mM DTT. The slides were washed in 0.1 × SSC, 1 mM DTT (preheated) at 60 °C for 30 min and cooled in 0.1 × SSC, 1 mM DTT at room temperature. The sections were dehydrated by immersion in an ethanol series containing 0.08 × SSC and 1 mM DTT. After drying the slides, radioactivity was visualized with liquid emulsion (LM-1 Hypercoat; Amersham) following the manufacturer's instructions. After 2 weeks the photographic emulsion was developed and the sections were stained with haematoxylin and eosin.

Results

Characteristics of experimental infections of mice with PVM and growth of PVM in the lungs of infected animals

In preliminary experiments the clinical signs of infected mice were noted and used to devise a system for assigning a score (Table 1). Mice, in groups of 5 to 12, were infected with

Table 1. Scores associated with clinical signs caused by infection of mice with PVM strain J3666

Clinical score	Clinical signs
1	Healthy with no signs of illness
2	Consistently ruffled fur, especially on neck
3	Piloerection, breathing may be deeper and mice less alert
4	Laboured breathing. Frequently showing tremors and lethargy
5	Abnormal gait and reduced mobility. Laboured breathing. Frequently emaciated. May show cyanosis of tail and ears
6	Death

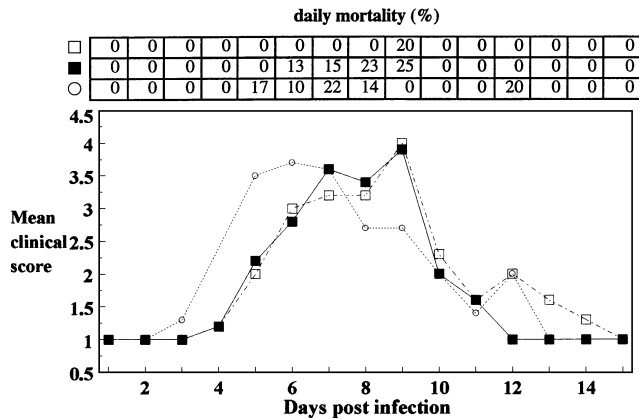


Fig. 1. Effect of quantity of infectious virus on the average clinical scores of mice following infection with PVM strain J3666. Mice were examined at daily intervals and clinical scores were assigned, according to the scheme described in Table 1, following infection with 60 (□), 120 (■) or 600 (○) p.f.u. in an inoculum volume of 50 μ l. Results are expressed as means from five mice. The daily mortality is given as a percentage of the number of animals infected with a given dose of virus.

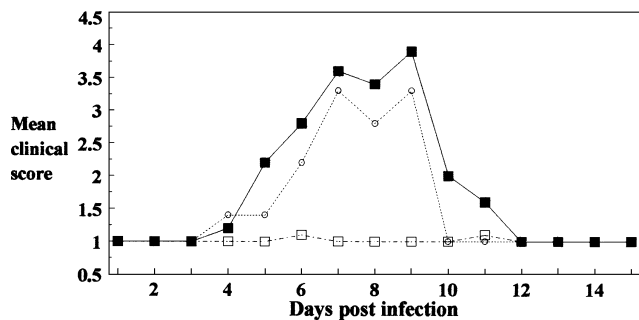


Fig. 2. Effect of inoculum volume on the average clinical scores of mice following infection with PVM strain J3666. Mice were infected with 120 p.f.u. in an inoculum volume of 10 (□), 25 (○) or 50 μ l (■), examined at daily intervals and clinical scores assigned, according to the scheme described in Table 1. Results are expressed as means from five mice.

60, 120 or 600 p.f.u. PVM strain J3666 by intranasal inoculation, and observed for 15 days. The external observable clinical signs of the mice were noted and a mean score for the animals was calculated on each day (Fig. 1). Depending on the quantity of virus in the inoculum, mice showed the first signs of illness between days 3 and 5, with laboured breathing, wasting and cyanosis at their greatest between days 6 and 9. Fatalities began to occur on day 6 rising to a maximum on day 9, although some deaths occurred as early as day 5 in mice inoculated with 600 p.f.u. Signs of recovery were seen in the surviving mice from days 5 to 11, with all mice appearing healthy after day 12. The onset and severity of disease were related to the amount of virus in the inoculum, with illness occurring earlier in mice infected with the highest dose of 600 p.f.u. than in those infected with lower doses. Overall, similar disease profiles were seen for all doses in a 50 μ l inoculum.

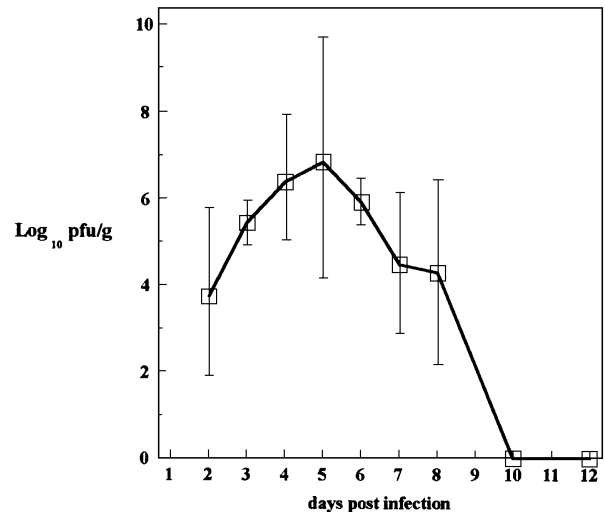


Fig. 3. Replication of PVM strain J3666 in the lungs of infected mice. Titre of virus (p.f.u./g) in lung homogenates prepared at daily intervals is shown following infection of mice with 120 p.f.u. PVM. The line is drawn through the geometric mean and standard deviations are indicated. The lower limit of detection of the assay was 200 p.f.u./g.

The volume of inoculum used had a significant effect on the outcome of infection. Fig. 2 shows the results of infection with three different volumes of inoculum containing the same (120 p.f.u.) dose of virus. As can be seen, reducing the inoculum volume from 50 to 25 μ l diminished the severity and duration of the clinical signs. Reducing the inoculum volume even further to 10 μ l almost completely abolished signs of infection, with just one mouse showing mild signs of infection. For subsequent experiments mice were inoculated with 120 p.f.u. in 50 μ l.

The rate of multiplication of PVM in mouse lungs has not been reported before and it was of interest to determine this fundamental characteristic. Following infection, mice were sacrificed on days 2 to 8, day 10 and day 12 and lung homogenates were prepared. The mean titre of each homogenate, plotted against time after infection, are given in Fig. 3. Virus titres were below detectable levels (less than 200 p.f.u./g) after day 10. The results indicate that the virus replicates efficiently in the lung, reaching a peak mean titre of 6.7×10^6 p.f.u./g on day 5 post-infection. This peak occurred prior to the highest clinical score achieved with the same dose of virus (Fig. 2).

Detection of PVM RNA in infected mouse lungs by *in situ* hybridization

Single lungs from mice infected with 120 p.f.u. PVM strain J3666 and sacrificed on days 2 to 12 post-infection were fixed in formal saline and hybridized with the PVM N gene riboprobe designed to detect virus mRNA and antigenome RNA. In addition, further sections were taken from each

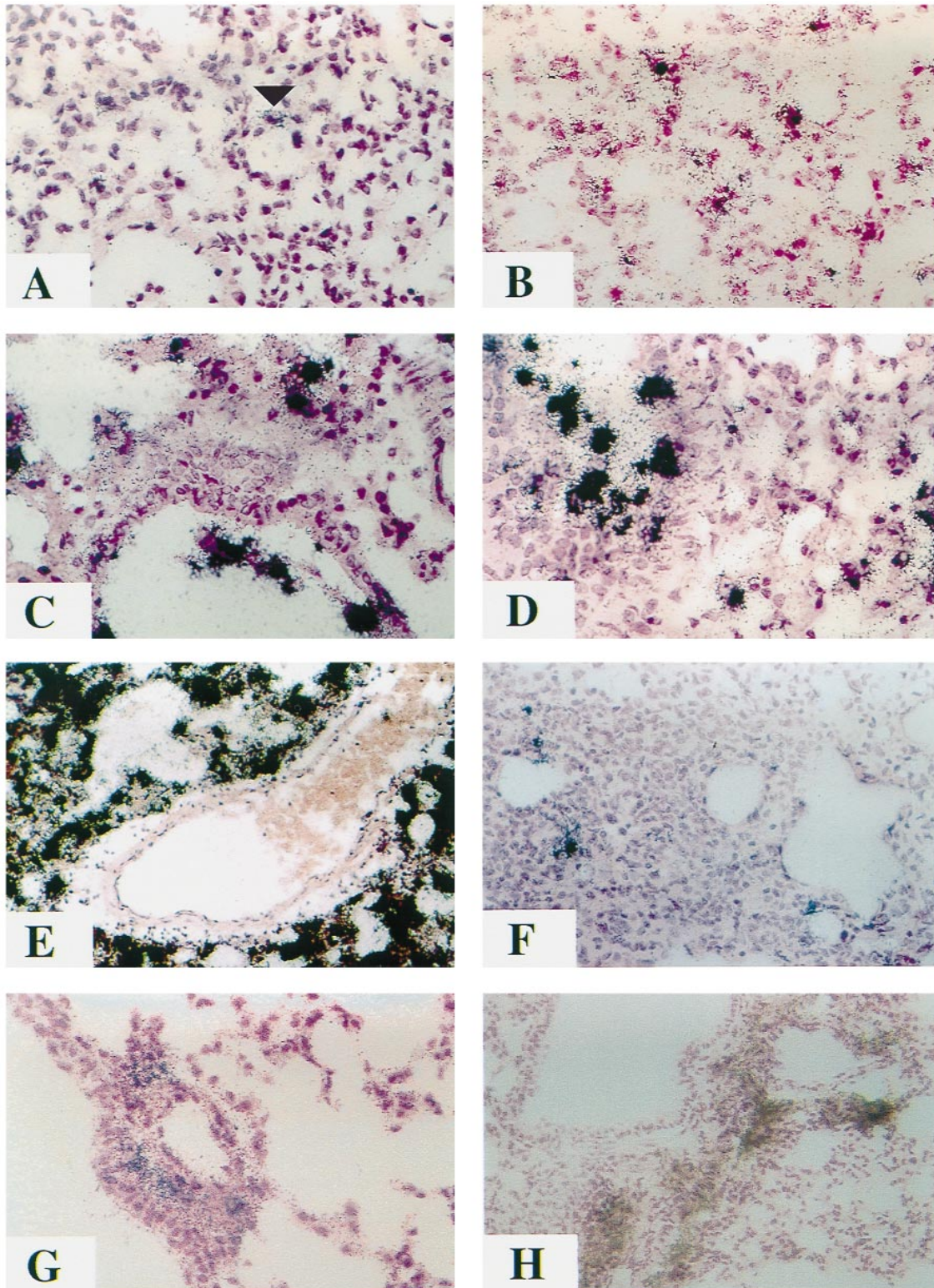


Fig. 4. Detection of pneumovirus RNA by *in situ* hybridization. Sections from lungs of mice infected with PVM or HRSV were hybridized with radioactively labelled virus-specific probes. Signal was detected by autoradiography. Lung sections were prepared from PVM-infected mice 2 (A; a hybridizing cell is arrowed), 3 (B), 4 (C), 5 (D) and 8 days (F) post-infection and hybridized with a PVM-specific probe. A sample was also prepared from a mouse which had died as a result of PVM infection 6 days post-infection (E). Lung sections were prepared from HRSV-infected mice 5 days post-infection and hybridized with an HRSV-specific probe (G-H). Magnification $\times 400$ (A-D, G-H) or $\times 200$ (E-F).

sample and hybridized with a probe from the HRSV N gene to detect non-specific hybridization. There was no cross-hybridization between the PVM and HRSV N genes under the conditions used, and no non-specific hybridization was seen (not shown).

Viral RNA was detected in tissue sections as early as day 2 post-infection. The dark silver grains, indicating a positive signal, appeared localized to individual cells, with little background. Most of the positively hybridizing cells were in the alveoli (Fig. 4A), with a considerably smaller number of cells in terminal bronchioles also showing a positive signal. By day 3 post-infection the signal was localized in strongly hybridizing regions while other areas of the lung showed no hybridization. The number of cells showing a positive signal had increased when compared with sections from day 2, but the infection was still localized, with individual cells appearing positive while adjacent cells remained negative (Fig. 4B).

By days 4 and 5 post-infection, when signs of respiratory distress were seen in the animals, the tissue sections showed morphological changes in the epithelium of some terminal bronchioles, and in places the epithelium had pulled away from the basement membrane. In these sections eosinophilic material was frequently seen in the lumen of the bronchioles. Hybridization was seen in cells of the terminal bronchiolar epithelium with large areas of some bronchioles, including those showing morphological abnormalities, giving a very strong signal (Fig. 4C–D). The hybridization signal in alveolar regions was less strictly localized, with a faint signal often seen in cells adjacent to the alveolar septa. This suggests that the fainter signal may be the result of secondary infection. In many areas of the sections the deposition of silver grains was concentrated on the apical surface of the terminal bronchiolar epithelium. This could be seen more clearly when sections were examined by dark-field microscopy (not shown).

On day 6 post-infection one mouse died as routine checks were taking place. Its lungs were excised and fixed immediately. An unusually strong positive signal was observed in up to three-quarters of the area of sections prepared from the lungs of this animal. All types of alveolar and bronchial cells were infected, while the vascular tissue remained uninfected (Fig. 4E). The signal was much stronger and more widely spread than that seen in mice sacrificed on day 5 post-infection, or in other mice sacrificed on day 6 or later.

From day 8 post-infection onwards the number of positively hybridizing cells was considerably less than that seen earlier, with virus RNA detected only in scattered cells in very few parts of the lung (Fig. 4F). From day 10 onwards no positive signal could be detected by *in situ* hybridization. At this late time-point alveolar tissue in the lungs of some animals appeared normal, whereas in others there were signs of haemorrhage and mononuclear lymphocyte infiltration. Similarly, most of the terminal bronchioles appeared normal while a small number of others contained eosinophilic exudate. By day 12 post-infection the lungs from all mice appeared normal.

Detection of respiratory syncytial virus RNA in infected mice lungs by *in situ* hybridization

Six mice were infected intranasally with 1500 p.f.u. HRSV in a 50 μ l inoculum volume. Four mice were sacrificed on day 5. At this time the mice had no detectable external clinical signs of disease and appeared well. A plaque assay of lung homogenates prepared from each mouse gave a mean titre of 4.8×10^3 p.f.u./g, indicating that the virus had replicated within the lungs.

In situ hybridization was carried out using the HRSV N gene probe, with the PVM N gene probe acting as the non-specific control probe, and as before no non-specific hybridization was seen (not shown). The positive signal appeared predominantly in peribronchiolar and perivascular regions of the lungs and was distributed over fairly large areas. Typical examples are shown in Fig. 4(G–H). While a few terminal bronchiolar epithelia and alveolar cells were individually positive, the overall number was considerably lower than that seen in mice infected with PVM on day 5 post-infection. Overall, the pattern of hybridization seen with HRSV-infected mice was considerably different to that seen with PVM-infected mice, suggesting that HRSV was restricted in its ability to replicate in certain areas of the lung.

Discussion

Infection of BALB/c mice with as little as 60 p.f.u. PVM strain J3666 led to a productive infection in which the virus grew to high levels with significant morbidity and mortality. The external signs of infection ranged from the mildest, where piloerection of fur could be seen, to severe pneumonia with emaciation which was sometimes fatal. The time of onset of overt disease and of recovery were dependent on the infectious dose used, with higher doses leading to earlier onset of clinical signs but also to slightly earlier indications of recovery (Fig. 1). This is consistent with earlier analysis of PVM infections in mice (Horsfall & Ginsberg, 1951). It is likely that the early onset results from a higher virus load reaching the lungs, and this is consistent with the observation that the inoculum volume was also important in determining the outcome of infection, with smaller volumes resulting in milder clinical signs (Fig. 2). The tissues showed clear signs of pneumonia and this was particularly marked in the terminal bronchioles. This contrasts with HRSV infection of human infants in which a fatal outcome has been proposed to be due to blockage of the bronchiolar lumen with squamous plugs, leading to constriction of the airways and collapse of the alveolar sacs (Aherne *et al.*, 1970). It is possible that the choice of mouse strain for these studies may influence the pathological changes seen. For example, it is known that the T-cell response to virus antigens of different strains of mice may differ.

Following infection with PVM, *in situ* hybridization detected virus positive-sense RNA, mRNA and antigenome in

cells after 2 days. The number of hybridizing cells in the lungs suggests that by this very early time the virus had already undergone one or more rounds of replication, and this is supported by the infectivity assay of lung homogenates (Fig. 3). At this time virus was detected most frequently in single cells, primarily in the alveoli. Unfortunately, the thickness of the sections necessary to ensure a positive hybridization signal prevented identification of the type of alveolar cell that was infected. As the infection progressed the number of infected sites increased, indicating further spread of the infection. The route and mechanism of spread of pneumoviruses from the initial site of infection to these presumably secondary sites is not known. It is possible that the virus was already present at these sites on day 2, but below the level of detection of *in situ* hybridization. The infection was localized in that while some areas of the lung showed a number of infected cells there were many areas which showed no signs of infection, and this was consistent throughout the course of infection in the sections analysed. As the infection continued, the hybridization signal suggested that the virus was able to spread from the initially infected cell into adjacent cells, leading to some regions of the lung having very intense signal. The type of cells infected also altered with time, with some of the columnar epithelia of the terminal bronchioles becoming infected by days 4 and 5 post-infection (Fig. 4C–D). The tissues also showed marked histopathological abnormalities, for example nuclei became acentrally located in some bronchiolar epithelia and in other regions cells had detached from the basement membrane. The damage to the bronchiolar epithelia was apparent immediately before the peak in the clinical scores, which assessed the severity of the resultant disease (Figs 1 and 2), and these significant histopathological changes may be directly associated with the more severe external signs of disease. In many sections eosinophilic exudate could be seen in the lumen of several terminal bronchioles, and haemorrhaging in the region of both alveoli and bronchioles was evident in some mice, consistent with bronchiolitis. The number of sites of infection continued to rise to a peak on day 6, coinciding with the peak in lung virus titre. The most extreme example of this was seen in the lungs of the mouse that died naturally of the infection. In the lung of this animal (Fig. 4E) there was no significant hybridization to cells of the vascular system, despite very intense hybridization to the adjacent lung tissue. This suggests that haematogenous spread of PVM is unlikely and may explain, in part, the strict pneumotropism of this and related viruses.

It is interesting to note that in many bronchiolar epithelial cells the distribution of silver grains was not random but appeared to be preferentially localized at the apical surface. The reason for this is not clear, and while it is tempting to suggest that this reflects budding of virus, as has been reported for HRSV in polarized cells (Roberts *et al.*, 1995), the hybridization probe detected mRNA and not genomic RNA. The localization was specific since it was not seen with the control probes.

In tissue samples taken from day 8 onwards the animals had passed the point at which they were likely to succumb to a fatal infection, and these animals should be regarded as recovering from the infection. At these later times the number of sites at which virus RNA was detected was significantly reduced when compared to those seen on days 5 and 6 (Fig. 4F). The cells showing hybridization to the probe, which were frequently single, were scattered throughout the sections, with large areas of apparently normal uninfected tissue. On day 10 and later no hybridizing cells were detected, indicating that virus clearance had occurred to a level below that detectable by *in situ* hybridization. Some small regions of the tissue sections showed indications of minor pathological abnormalities but most of the tissue was normal, suggesting that tissue damage seen at the early stages of infection was in the process of being repaired.

Mortality occurred at the same time as lung virus titre and the number of infected sites began to decline, and this might be taken to suggest a role for immune-mediated pathology in the most severely affected animals. Such immune-mediation of disease was seen in HRSV-infected mice receiving adoptive transfer of primed CD8⁺ MHC-I-restricted cytotoxic T cells (Cannon *et al.*, 1988, 1989). Similarly, the potentiation of HRSV disease by formalin-inactivated virus in cotton rats is thought to have been immune-mediated (Murphy *et al.*, 1990). PVM-infected athymic mice also die exhibiting clinical signs similar to those described here, and the absence of inflammatory cells in the lungs of PVM-infected mice in this study leaves the involvement of the immune system open to question (Carthew & Sparrow, 1980*b*; Richter *et al.*, 1988; Weir *et al.*, 1988). Once again, different strains of mice may give a different result.

Infection of mice with HRSV has been used as a model for the human infection. It was therefore of interest to compare the pattern of multiplication of HRSV with that of PVM. Previous studies have indicated that RSV antigen is found only in the alveoli, but not the bronchiolar epithelium, of BALB/c mice (Taylor *et al.*, 1984). A mild cellular infiltration of mononuclear cells and polymorphs was also observed (Anderson *et al.*, 1990). The time-scale of infection of mice with PVM strain J3666 was similar to that reported for HRSV infection; however, mice infected with HRSV showed no clinical signs of disease (Anderson *et al.*, 1990; Taylor *et al.*, 1984). In our study only a few HRSV-infected alveolar and bronchial epithelium cells were seen by *in situ* hybridization. The strongest signal was in the peribronchiolar and perivascular regions (Fig. 4G–H). This was different to the situation seen with PVM. *In situ* hybridization using an HRSV N gene in infected cotton rats detected virus RNA in both bronchiolar and alveolar regions (Murphy *et al.*, 1990). The presence of HRSV in perivascular and peribronchiolar regions has previously been seen in samples from human infants (Aherne *et al.*, 1970) and from calves (Taylor *et al.*, 1984), but these were accompanied by a severe inflammatory response, interstitial pneumonia and bronchial hyperplasia, which was not seen in BALB/c mice.

This suggests that the use of HRSV in mice as a model for infection of human babies and infants may be of limited use, possibly because the infection is more restricted.

It is clear that the pattern of HRSV infection in animals is host-dependent and is different to that of PVM in mice. Although PVM induces severe disease and a productive infection in BALB/c mice, it is uncertain whether PVM infection in mice is analogous to HRSV infection in babies and infants. Further work on human infections may help to resolve this. Investigation of the process of PVM infection in its natural host offers an amenable system for further study which may identify aspects which are of relevance to HRSV infection in humans. Of particular interest will be the nature of the immune response in infected animals, and the differences which occur during infection with non-pathogenic PVM strains.

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