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Development of a cotton rat–human metapneumovirus (hMPV) model for identifying and evaluating potential hMPV antivirals and vaccines

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Abstract

Hispid cotton rats were inoculated with two different human metapneumovirus (hMPV) subtype A strains and one subtype B hMPV. Although no overt disease was seen in any virus-inoculated animal, following an eclipse phase, significant pulmonary virus titers were observed in every hMPV-inoculated animal through day 7 post virus inoculation (p.i.) and in most through day 10. Peak virus titers occurred four days p.i., while virus-induced histopathology was most evident in lung sections obtained from animals 7 to 10 days p.i. The latter consisted primarily of desquamating and hypertrophic columnar epithelial cells lining the bronchi and bronchioles and the presence of large numbers of leukocytes in and around the bronchi and bronchioles. In fluorescent antibody studies, virus antigen-specific fluorescence was most evident in the desquamating tall columnar epithelial cells lining bronchi and bronchioles, in pneumocytes lining alveoli and in single or small groups of free cells, most probably leukocytes, present in the lumen of alveoli, bronchi and bronchioles. Virus was generally not detected in inoculated animals >10 days p.i. Although the pattern of virus replication in cotton rats was similar for all the three virus stains, the B subtype consistently grew to lower levels than the two A strains. Regardless, these findings indicate that hMPV replicates in cotton rats and that these animals may be used as a small animal model of hMPV infection and to facilitate the identification and development of vaccines and antivirals for preventing and/or ameliorating infections caused by this virus.

Keywords: hMPV; Human metapneumovirus; Cotton rats; Animal model; Antiviral testing; Vaccine evaluation

1. Introduction

Since the first report describing the existence of human metapneumovirus (hMPV) and its association with human respiratory disease in 2001 (van den Hoogen et al., 2001), numerous publications have followed indicating that this virus is both ubiquitous (Madhi et al., 2003; van den Hoogen et al., 2004) and a significant human respiratory pathogen (van den Hoogen et al., 2004) capable of causing infection and disease in infants (Xepapakaki et al., 2004), young children (Boivin et al., 2003; Falsey et al., 2003; van den Hoogen et al., 2003; Viazov et al., 2003; Esper et al., 2004), adults (including the elderly) (Falsey et al., 2003; Osterhaus and Fouchier, 2003) and immunocompromised individuals (Pelletier et al., 2002; van den Hoogen et al., 2003). No chemotherapeutic agents, antibody preparations or vaccines are currently approved for the treatment or prevention of infections caused by this virus. However, at least one potential hMPV vaccine candidate, a recombinant bovine/human parainfluenza type 3 virus expressing the hMPV fusion (F) protein, already has been described (Tang et al., 2003; MacPhail et al., 2004). The availability of a small animal model of hMPV infection could significantly help in the evaluation of such interventions. For

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example, the model could be used to indicate doses, administration schedules, route(s) of administration and other conditions to be utilized in clinical trials. Recently, hMPV has been found to grow up to 6 $\log_{10}$ in the respiratory tracts of several non-human primates (i.e., chimpanzees, African green monkeys and Cynomolgus macaques), and relatively well (i.e., 4–5 $\log_{10}$) there in at least two small animal species, Syrian golden hamsters and ferrets (Skiadopoulos et al., 2004; Kuiken et al., 2004; MacPhail et al., 2004). This virus has also been reported to replicate in the lungs of Balb/c mice, but to a lesser extent (MacPhail et al., 2004). Thus, there are already a number of potential animal models for carrying out hMPV studies. The results contained in this report indicate that hispid cotton rats may also be suitable for such studies. The potential advantages of a cotton rat–hMPV model are discussed.

2. Materials and methods

2.1. Animals

Male and female cotton rats (*Sigmoden hispidus*) weighing between 75 and 150 g were utilized randomly in all experiments. These animals were descendants of six pairs of cotton rats obtained in 1984 from the Small Animal Section of the Veterinary Research Branch, Division of Research Services, National Institutes of Health (NIH) and maintained as a discrete colony in the Baylor College of Medicine (BCM) central vivarium since that time. All were kept in cages covered with barrier filters and given food and water ad libitum. Blood samples obtained from representative animals housed in this colony at intervals before or during the course of these experiments were seronegative for adventitious viruses and other rodent pathogens. The BCM Investigational Animal Care and Use Committee (IACUC) approved all of the experimental protocols utilized in these studies.

2.2. Tissue culture

LLC-MK2 (Rhesus monkey kidney; ATCC CCL7) cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Eagle's Minimal Essential Medium (MEM; Sigma Chemical Co.; cat. no. M4465) supplemented with 10% fetal calf serum (FCS; Summit Biotechnology, Fort Collins, CO.; cat. no. FP-200-05), 100 U/ml penicillin (Sigma Chemical Co., cat. no. P-4458), 100 μg/ml gentamicin sulfate (Sigma Chemical Co., cat. no. G-1264), 2 mM L-glutamine (Sigma Chemical Co., cat. no. G7513) and 0.2% sodium bicarbonate (Sigma Chemical Co.; cat. no. S8761) was used to grow these cells. To prepare hMPV pools and to perform assays involving hMPV, this same media lacking FCS (−FCS) but supplemented with 2 μg TPCK-treated trypsin/ml (+WT; Worthington Biochemical Corp., Lakewood, NJ; cat. no. 32C5468) was used.

2.3. Viruses

hMPV 26575 and hMPV 26583 were originally isolated in Canada. They were obtained from the Centers for Disease Control (CDC), Atlanta, GA with permission from Dr. Guy Boivin located at the Research Center in Infectious Diseases, Laval University, Que., Canada. These viruses have been referred to in other publications (Bastien et al., 2003; Biacchesi et al., 2003; Skiadopoulos et al., 2004) as CAN98-75 and CAN97-83, respectively. Subtyping performed at the CDC indicated that the 26575 isolate was a subtype B (or 2) hMPV and 26583 a subtype A (or 1). The third hMPV strain utilized in the studies, RL Bx, was acquired from Gail Demmler, M.D., Department of Pediatrics-Infectious Diseases, Texas Children's Center, BCM. Based on viral sequences identified using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay, this virus was identified by Dr. Robert Atmar of the Respiratory Virus Pathogens Unit, BCM, as an A (or 1) hMPV subtype. Sucrose-purified (sp) pools of the two Canadian isolates were prepared as described in detail previously (Wyde et al., 2004). Using the virus quantification assay described below, it was determined that each of these preparations contained approximately 9.0×10^6 median tissue culture infectious doses ($TCID_{50}$)/ml. The working pool of hMPV RL Bx was similarly grown in LLC-MK2 cells utilizing MEM-FCS + WT. However, it was not sucrose-purified and only had a titer of 2.0×10^5 $TCID_{50}$/ml. The sp26575, sp26583 and RL Bx strains were utilized at passage level 5, 10 and 6, respectively. An A2 strain of respiratory syncytial virus (RSV) was used to check the specificity of the antibodies produced in cotton rats experimentally infected with the hMPV strains. This virus was obtained from the ATCC (cat. no. VR-1302) and working pools of it were prepared as described in detail previously (Wyde et al., 1993).

2.4. Collection of nasal washes and lungs

Cotton rats were sacrificed using CO_2. The lungs of these animals were removed, rinsed in sterile water and weighed. They were then transpleurally lavaged (Wilson et al., 1980), and the resulting lung lavage fluids (LF) were placed on ice until assayed for virus levels. Next, the cotton rats were decapitated, and the lower jaw from each head was disarticulated. Nose washes (NW) were individually collected from each head by pushing 1 ml of MEM through each naris and capturing the effluent from the posterior opening of the palate.

2.5. Virus quantification

Levels of infectious virus in working stocks of virus, LF and NW were determined as described previously (Wyde et al., 2003), with two exceptions: (1) each sample was tested in duplicate in sterile 48-well (Corning 25830) tissue culture plates using serial 0.5 $\log_{10}$ dilutions and (2) when using the latter plates, each tissue culture monolayer was inoculated with 0.125 ml volumes instead of the 0.05 ml added to the

wells of the 96-well plates. The cell monolayers in each well were observed and scored daily for 14 days for virus-induced cytopathic effects (CPE). After determining the last well in each row that had CPE, the amount of virus present in each test suspension was estimated using the interpolation method of Karber (Rhodes and Van Rooyen, 1953) and expressed as $TCID_{50}$ (log_{10})/ml (virus pools), $TCID_{50}$ (log_{10})/nose wash or $TCID_{50}$ (log_{10})/g lung. Using this assay, the minimum amount of virus detectable in virus pools was 0.9 log_{10}/ml, 1.1 log_{10} in NW and 1.9 log_{10}/g in LF.

2.6. hMPV-Specific neutralizing antibody assay

Blood was collected from the retro-orbital sinus plexus of animals anesthetized with IsoFlo (isoflurane; Abbott Laboratories, North Chicago). Serum was prepared from each sample, heat inactivated at 56 °C for 30 min and then stored at 4 °C until assayed for virus-specific neutralizing antibodies. Each serum was tested in duplicate in sterile 96-well tissue culture plates (Falcon 3072) as described in detail elsewhere (Wyde et al., 1995), with three modifications. One, confluent monolayers of LLC-MK2 cells were utilized in these assays. Second, after serially diluting the sera, approximately 100 $TCID_{50}$ of the appropriate hMPV strain was added to the test and virus control wells. Finally, the morning after setting up an assay, the medium in each well of each test plate was removed and the cell monolayers in them rinsed with phosphate buffered saline (PBS; pH, 7.2). Two hundred microliters of MEM-FCS+ WT was then added back to each well, and the plates were returned to the 36 °C incubator. The cell monolayers in the virus control wells were observed daily. When these monolayers exhibited 80–100% virus-induced CPE, all of the wells in the assay were observed for an additional 24 h. At the end of this period, the monolayers were fixed and stained with 10% formalin containing 0.1% crystal violet. The next morning each well was scored for the presence or absence of virus. Titers were expressed as log_2 (/0.05 ml of serum) of the reciprocal of the last dilution of antiserum that completely inhibited virus-induced CPE. The minimum detectable virus neutralization antibody titer possible in these assays was 2.5 log_2/0.05 ml sera.

2.7. Experimental designs

Experiments were initiated by anesthetizing the animals with isoflurane and inoculating them intranasally (i.n.) with 0.1 ml of virus. The day that the animals were inoculated with virus was always considered to be day 0. To determine the median cotton rat infectious dose ($CRID_{50}$) of the different virus pools, groups of four animals were inoculated i.n. with serial 10-fold dilutions ranging from 10^{-1} to 10^{-5} of the appropriate virus. Four days p.i., these animals were sacrificed, and their lungs processed and tested for virus as described above. The resulting data were used to estimate the minimal amount of each virus that infected the lungs of 1/2 of the replicate animals inoculated (i.e., the $CRID_{50}$). For studies designed to follow the kinetics of virus replication, LF and NW were collected from animals sacrificed using CO_2 at 6 h and 2, 4, 7, 10 and 14 days p.i. (four animals per time point). In some experiments, control animals were mock-infected i.n. with medium from LLC-MK2 cell monolayers that were processed in the same manner that each sp-virus pool was prepared. In other experiments, control animals were not inoculated with anything. To allow parallel histologic studies with virologic determinations, in some tests, two of the five lung lobes collected from selected cotton rats were placed in Streck tissue fixative (Streck Laboratories, La Vista, NE) and then processed for histopathologic assessment. The remaining lung lobes were weighed, transpleurally lavaged as described elsewhere (Wilson et al., 1980) and then assessed for virus levels. In experiments, where no histologic studies were planned, all the five lung lobes were weighed and lavaged. To permit comparisons between different sized animals and between lungs in which different numbers of lobes were utilized, all lung titers were calculated on per gram of lavaged lung basis.

2.8. Histologic studies

For assessment of pulmonary histopathology, lungs were removed and placed in Streck tissue fixative for 24 h. These were then dehydrated, embedded in low-melting point paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin (H&E). The stained sections were observed in a blinded fashion for histopathology using a light (Olympus Model CK2) microscope equipped with 10, 20 and 40× objectives. Pictures of stained H&E sections were made using an Olympus Magnafire digital camera mounted to the microscope and controlled with Olympus Magnafire 2.1C software.

2.9. Fluorescent antibody studies

Lungs designated for fluorescent antibody studies were similarly processed except that after the sections were rehydrated, they were treated with Antigen Unmasking Solution (cat. no. H3300, Vector Laboratories Inc., Burlingame, CA) using the manufacturer's directions. They were then overlayed for 10 min with Evans blue stain (60 mg/100 ml of PBS). This stain acts both as a counter stain and as a means to limit non-specific binding to the tissue due to charges on the fluorescein isothiocyantate (FITC)-conjugated antibody used in the assay. The sections were then tested for the presence of hMPV-specific antigens using an indirect antibody staining method (Coons, 1958). Accordingly, the sections were sequentially overlayed first with rabbit antisera-specific for hMPV antigens (Wyde et al., 2004) and then with FITC-conjugated goat anti-rabbit immunoglobulin G (Chemicon Corp., Temecula, CA, cat. no. AP132F). Two controls were run in these studies: (1) sections of lung obtained from uninfected cotton rats were stained in the exact same manner as was used to stain lung sections prepared from hMPV-inoculated animals and (2) sections of lung from infected

animals were stained similarly, but using normal rabbit antiserum in the primary staining step. Viewing of the stained sections was done using a Leitz Wetzlar Ortholux II UV microscope equipped with an HBO-200W mercury burner, a BG12 excitation filter and a 470 μM barrier filter. Pictures were taken using a Leitz camera connected to the microscope.

2.10. Statistics

Instat, a statistical program designed for IBM compatible computers (Version 3, Graphpad Software Inc., San Diego, CA), was used to calculate all means and standard deviations. This program was also utilized to perform Student's *t*-test to compare mean virus and antibody titers obtained on the same day, and to run non-parametric analysis of variance tests to compare mean titers acquired at different times after virus inoculation. For the analyses of virus titers, a negative sample was assigned a value 0.5 $\log_{10}$ $TCID_{50}$ below the minimal detectable titer for that assay.

3. Results

3.1. Kinetics of hMPV replication in cotton rats

Table 1 displays the results of a representative experiment performed to determine the kinetics of replication of hMPV in the upper (nasal) and lower (lungs) respiratory tracts of cotton rats following intranasal inoculation. In the experiment shown, the animals were inoculated i.n. with 100 μl of medium containing approximately 9×10^5 $TCID_{50}$ (~1000 median cotton rat infectious doses) of hMPV 26583. Four animals were sacrificed at each of the time intervals shown.

In the experiment shown, virus was detected in two of the four NW tested at 6 h p.i., and in three of the four LF. Assigning titers of 0.6 $\log_{10}$/nasal wash and 1.4 $\log_{10}$/g lung to samples, in which no virus was detected, the mean virus titers calculated for the NW and LF obtained at this first test interval were 1.3 ± 0.3 $\log_{10}$ $TCID_{50}$/nasal wash and 2.5 ± 0.8 $\log_{10}$ $TCID_{50}$/g lung. On days 2 and 4, virus was present in every nasal wash and lung lavage fluid tested. Moreover, mean virus titers in these samples increased to 3.2 ± 0.6 $\log_{10}$/nasal wash and 3.5 ± 0.8 $\log_{10}$ $TCID_{50}$/g lung on day 2 and peaked in both the upper and lower respiratory tracts on day 4, reaching 3.7 ± 0.4 $\log_{10}$/nasal wash and 4.4 ± 0.9 $\log_{10}$/g lung on this day. The mean titers in the NW and LF fell on day 7 to 1.5 ± 0.8 $\log_{10}$/nasal wash (two of four NW being positive for virus) and 3.3 ± 0.9 $\log_{10}$ $TCID_{50}$/g lung (four of four LF positive for virus), and declined further on day 10 to 1.2 ± 0.2 $\log_{10}$/nasal wash (one of four NW positive for virus) and 1.7 ± 0.2 $\log_{10}$ $TCID_{50}$/g lung (two of four cotton rats having virus-positive LF). On day 14, no virus was detected in any nose wash or lung fluid tested.

The kinetics of replication of hMPV 26575 and RL Bx are not shown since, with one exception, their replication kinetics were quite similar to that of hMPV 26583. The exception was that the 26575 strain consistently failed to replicate to as high a level in the lungs of cotton rats as either of the two A viruses (Fig. 1 provides an example). The possible reasons for this are addressed below in the Section 4.

3.2. Comparison of the ability of the different hMPV strains to infect and replicate in cotton rat lungs

Fig. 1 displays the results of two replicate experiments performed to compare the ability of the different hMPV strains to replicate in the lungs of cotton rats. Shown are the mean virus titers in LF samples collected on day 4 p.i. from groups of four cotton rats inoculated with approximately 9×10^5 $TCID_{50}$ of hMPV 26583, 9×10^5 $TCID_{50}$ of hMPV 26575 or 2×10^4 $TCID_{50}$ of hMPV RL Bx. As the heights of the bars displayed in Fig. 1 indicate, significant levels of virus were present in all test groups 4 days p.i. in both experiments. However, the mean virus titer obtained for hMPV 26575 in each experiment was statistically significantly lower than the mean titers determined in these experiments for the other two hMPV strains when these means were compared using Student's *t*-test. For example, when the mean virus titer of hMPV 26575 in Experiment 1 (i.e., 3.0 ± 0.6 $TCID_{50}$/g lung) was compared using Student's *t*-test to either the titer of the RL Bx virus (i.e., 4.4 ± 0.4) or the 26583 strain (i.e., 5.0 ± 0.3 $\log_{10}$/g lung) determined in this experiment, a p-value < 0.05

Table 1
Kinetics of replication of human metapneumovirus strain 26583 in the upper (nose) and lower (lungs) respiratory tracts of hispid cotton rats[a]

Time p.i. NW and LF (collected)	Mean virus titer of NW ± S.D.[b] ($\log_{10}$/nose wash)	Number of hMPV+ (samples)	Mean virus titer of LF ± S.D.[b] ($\log_{10}$/g lung)	Number of hMPV+ (samples)
6 h	1.3 ± 0.3	2 of 4	2.5 ± 0.8	3 of 4
Day 2	3.2 ± 0.6	4 of 4	3.5 ± 0.8	4 of 4
Day 4	3.6 ± 0.5	4 of 4	4.4 ± 0.9	4 of 4
Day 7	1.5 ± 0.8	2 of 4	3.3 ± 0.9	4 of 4
Day 10	1.2 ± 0.2	1 of 4	1.7 ± 0.2	1 of 4
Day 14	<1.1 ± 0	0 of 4	<1.9 ± 0	0 of 4

[a] The cotton rats were inoculated intranasally with approximately 9×10^5 $TCID_{50}$ of human metapneumovirus (hMPV) 26583. Six hours later, and again on days 2, 4, 7, 10 and 14 post virus inoculation (p.i.), four animals were sacrificed, and nose washes (NW) and lung lung fluids (LF) were obtained from each. These samples were tested for hMPV levels.

[b] Shown are the mean virus titers ± one standard deviation (S.D.).

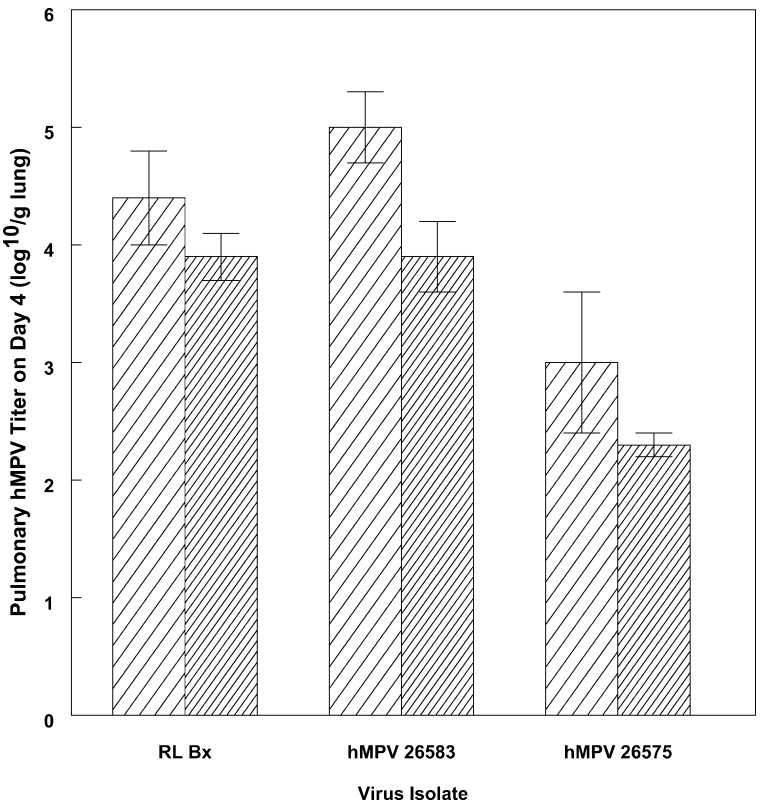

Fig. 1. Comparison of mean virus titers in lung fluids obtained from cotton rats 4 days after these animals were inoculated intranasally with 9×10^5 $TCID_{50}$ of hMPV 26583, 9×10^5 $TCID_{50}$ hMPV 26575 or 2×10^4 $TCID_{50}$ of hMPV RL Bx. Bars with the wide hatch pattern depict the results obtained in Experiment 1. Bars with the narrow hatch pattern indicate results determined in Experiment 2.

was obtained. Similarly, the mean virus titer on day +4 p.i. for the group of cotton rats inoculated with the 26575 strain was 2.3 ± 0.1 $\log_{10}$/g lung in Experiment 2, significantly lower ($p < 0.05$) than the mean pulmonary virus titers of either the RL Bx (3.9 ± 0.2 $\log_{10}$/g lung) or 26583 (3.9 ± 0.2 $\log_{10}$/g lung) viruses. In both experiments, the mean lung virus titers obtained for groups of cotton rats given the 26583 and RL Bx viruses were equivalent to each other (i.e., $p \geq 0.05$ when the means obtained in each experiment for these groups were compared using Student's *t*-test). Based on the lung infection data obtained after titrating the three different hMPV stains in cotton rats, $CRID_{50}$ values of 4900, 900 and 110 $TCID_{50}$ were calculated for hMPV 26575, 26583 and RL Bx, respectively.

3.3. Histologic findings

With the exception of an occasional mast cell, basophile or macrophage, H&E-stained sections of lungs prepared from uninfected animals were unremarkable (data not shown). Similarly processed and stained tissue sections prepared from lungs obtained 4, 7 or 10 days post inoculation from mock-infected cotton rats were also generally unexceptional except for a small increase in the number of randomly scattered individual or small clusters of leukocytes (Fig. 2, Panel A). Some of these leukocytes appeared to be polymorphonuclear neutrophiles (PMN). The bronchi and bronchioles in sections prepared from both un-inoculated and mock-inoculated animals were lined uniformly with densely packed tall columnar epithelial cells (Fig. 2, Panel A). In contrast, marked histopathologic changes were evident in the stained sections obtained 7 or 10 days p.i. from virus-infected animals. In these, there were numerous areas of obvious peribronchiolar infiltration of inflammatory cells, desquamating and hypertrophic bronchiole columnar epithelium (Fig. 3, Panels B and C), bronchiolitis (Fig. 2, Panel C) and even occasional sites of perivascular inflammation (Fig. 2, Panel D). Thickened septal walls and numerous scattered patches of leukocytes, including PMN, were also present (Fig. 2, Panels B–D). The number of inflammatory cells and changes in parenchymal cells seen in the sections of lung prepared from animals 4 days p.i. were intermediate, greater than the number seen in the lungs collected from control animals but less than the changes seen in days 7 and 10 virus-infected lungs (data not shown).

3.4. Fluorescent antibody results

With the exception of the diffuse red fluorescence of the Evans blue counter stain, virtually no fluorescence was seen in sections of lung obtained from cotton rats 4, 7 or 10 days after they were inoculated with hMPV 25683 and stained

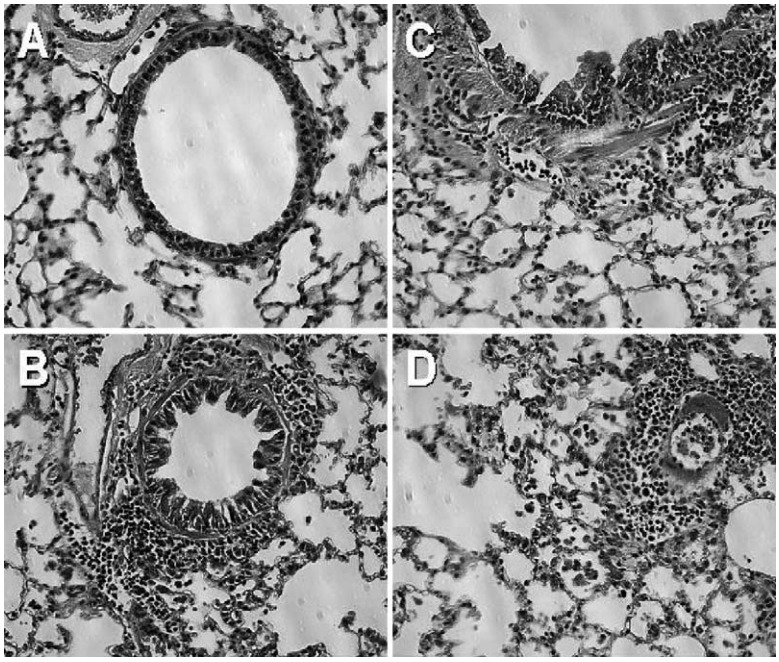

Fig. 2. *Panel A:* photomicrograph of a section of lung from a mock-infected cotton rat and stained with hemotoxylin and eosin. *Panels B–D:* photomicrographs of a similarly stained section of lung taken from a cotton rat 7 days after this animal was infected i.n. with 9×10^5 $TCID_{50}$ of hMPV 25683. Peribronchiolar infiltration of inflammatory cells is evident in Panel B, while in Panel C, the presence of these cells in alveoli and focal erosion of a bronchiole are shown. Panel D shows an area of prominent perivascular cuffing (magnification in all panels ∼55×).

using normal rabbit serum as the primary staining reagent (Fig. 3, Panel A). Similarly, only red fluorescing cells and tissues were observed on sections of lung prepared from uninfected or mock-infected animals and stained utilizing the rabbit hMPV antigen-specific sera in the first staining step (not shown). However, areas of bright green fluorescence were frequently present in sections of lung prepared from animals sacrificed 4, 7 or 10 days after being infected with hMPV and stained using the rabbit hMPV antigen-specific sera in the primary staining step. This fluorescence predominantly occurred in free (round) cells located within or attached to the lumina of alveoli (leukocytes?), in similar cells located around different bronchi and bronchioles amongst the tall columnar epithelial cells lining these structures and in some of the pneumocytes comprising the alveoli (see Fig. 3, Panels B and C). Fluorescence patterns differed in that the fluorescence tended to be diffuse throughout the entire cytoplasm of the free cells and primarily in the apical portions of the tall columnar cells (see Fig. 3, Panel C).

3.5. Neutralizing antibody

Table 2 displays the levels of hMPV-specific neutralizing antibody titers measured in the sera of cotton rats 19 and 35 days following intranasal inoculation of these animals with 9×10^5 $TCID_{50}$ of either hMPV 26575 or hMPV 26583. It can be seen that no cotton rat used in these studies had any detectable hMPV- or RSV-specific neutralizing antibodies in their serum prior to being inoculated with hMPV (row 1). However, significant levels of hMPV-specific neutralizing antibodies were present in every animal inoculated with either strain of this virus by day 19 p.i. These titers did not rise significantly over the next 16 days, suggesting that their levels were nearly at their peak by day 19. (In different experiments, animals were similarly inoculated with the RL Bx strain. The production pattern and levels of virus-specific serum neutralizing antibody titers in these animals were equivalent to those seen in the cotton rats inoculated with hMPV 26583.) As shown in Table 2, the cotton rats inoculated with the hMPV 26583 strain produced significantly higher levels of virus-specific neutralizing antibodies than the animals inoculated with the hMPV 26575 strain (range of means for animals inoculated with hMPV 26583 = 6.1–6.6 $\log_2$/0.05 ml versus a range of 3.0 to 3.7 $\log_2$/0.05 ml for cotton rats inoculated with the 26575 strain; $p \leq 0.05$ for each pair using Student's *t*-test). This was most likely, because the latter virus did not grow as well in cotton rats as the hMPV 26583 strain (see Fig. 1). Despite the differences in the levels of virus-neutralizing antibody induced by the two hMPV strains, the antibodies induced were virus-specific (i.e., no neutralization of RSV A2 was observed) and had equivalent neutralizing activity against virus belonging to either subtype.

4. Discussion

Although no overt disease was seen in any animal inoculated with hMPV in these studies, evidence was obtained that indicated that this virus could replicate relatively well in cotton rats: (1) significant pulmonary virus titers, particularly

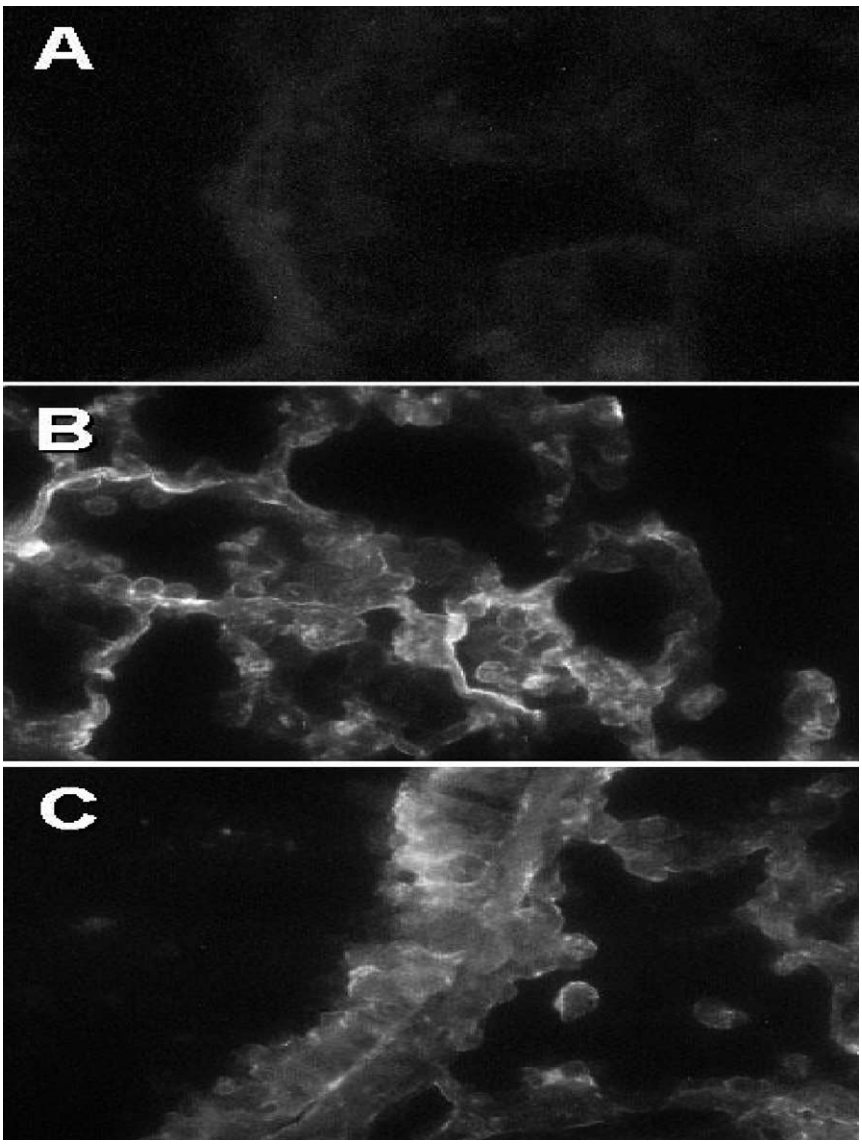

Fig. 3. Photomicrographs showing fluorescence in lung sections prepared from a mock-infected cotton rat (Panel A) or a cotton rat 7 days after it was inoculated intranasally with approximately 9×10^5 $TCID_{50}$ of hMPV 26583 (Panels B and C). These sections were stained using an indirect fluorescent staining procedure utilizing rabbit anti-hMPV in the primary staining step and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit serum in the second step. Evans blue stain was added prior to this staining both as a counter stain and to reduce non-specific staining of the tissue by FITC or FITC-conjugated protein. In Panel A, only the muted fluorescence of the Evans blue is evident. In Panel B, pneumocytes and mononuclear cells exhibit virus-specific (apple green) fluorescence. In Panel C, similar virus-specific fluorescence is seen predominantly in the apical portion of the tall columnar cells lining the bronchi and in the cytoplasm of some of the mononuclear cells located in the lumen of an alveolus (magnification in all panels ∼128×).

on day 4 p.i. but through day 7, were measured in every animal inoculated i.n. with any of the three hMPV strains tested (see Table 1 for the kinetic data obtained for the 26583 strain; these data are not shown for strains 26575 or RL Bx, but they had similar replication patterns). (2) These titers occurred following a true eclipse phase, indicating that the virus levels measured were not a result of virus clearance, but rather were due to virus replication. The data presented in Table 1 provides a good example. In this experiment, each animal was inoculated i.n. with approximately 9×10^5 $TCID_{50}$ of hMPV 26583. Six hours later, two of the four animals sampled had no detectable levels of virus in their lungs and the other two had only low levels (i.e., <3 $\log_{10}$ $TCID_{50}$/g lung). However, by day 4, the mean virus titer had risen to >4 $\log_{10}$/g lung. A similar pattern was seen in the upper respiratory tract (Table 1), albeit, to a lesser extent. (3) In histologic studies, marked histopathology was evident in H&E-stained lung sections prepared from virus-infected animals compared to the histopathology seen in similarly stained sections from un-inoculated or mock-inoculated animals. These changes were especially evident in lung sections obtained from animals infected with hMPV 7 or 10 days previously (see Fig. 3), but were also evident in sections of lungs collected at earlier time points from infected animals.

Table 2
hMPV-Specific serum neutralizing antibody responses induced in cotton rats following experimental infection[a]

Sera tested	Immunizing virus	Subtype of immunizing virus	Mean neut. antibody titer ± S.D.[b,c] (log_2/0.05 ml) vs.		
			RSV	hMPV26575	hMPV 26583
Pre-inoculation	N.A.	N.A.	<2 ± 0	<2 ± 0	<2 ± 0
Day 19 p.i.	hMPV 26575	B	<2 ± 0	**3.5 ± 0.7**	**3.0 ± 0**
	hMPV 26583	A	<2 ± 0	**6.3 ± 0.6**	**6.1 ± 0.8**
Day 35 p.i.	hMPV 26575	B	<2 ± 0	**3.7 ± 0.6**	**3.5 ± 0.7**
	hMPV 26583	A	<2 ± 0	**6.6 ± 0.5**	**6.3 ± 0.4**

[a] Eight cotton rats were inoculated intranasally with 100 μl containing approximately 9×10^5 log_{10} $TCID_{50}$ of hMPV 26583 and eight with the same dose of hMPV 26575; 19 and 35 days later, these animals were bled, and the sera obtained from them tested for virus-specific neutralizing antibody against both hMPV subtypes and against respiratory syncytial virus A2 (RSV A2).
[b] Bolded means were found to be statistically significantly different from the unbolded means ($p < 0.05$) using Student's *t*-test.
[c] *Abbreviations:* S.D., standard deviation; p.i., post virus inoculation; neut., neutralizing.

Interestingly, the changes seen in cotton rats appeared to be quite similar to those reported to occur in monkeys following experimental infection of these animals with hMPV (Kuiken et al., 2004). Thus, in both species, multi-focal erosive and inflammatory changes were seen in and around the bronchi and bronchioles. The latter were characterized with markedly increased numbers of leukocytes (macrophages, lymphocytes and PMN) in alveoli and around the tall columnar cells lining the bronchi and bronchioles. Virus-specific fluorescence indicated that virus antigen was present in the tall columnar epithelial cells lining bronchi and bronchioles, especially those exhibiting desquamation. Moreover, this fluorescence was predominant on the apical surfaces of the columnar cells. It was also evident in some of the pneumocytes lining alveoli and in single or small groups of free cells, most probably leukocytes, in the lumen of alveoli, bronchi and bronchioles (see Fig. 3). It was not determined, if the fluorescence in these cells was due to phagocytosis or replication of virus. (4) All of the cotton rats experimentally inoculated with virus produced significant levels of hMPV-specific neutralizing antibodies by day 19 p.i. (i.e., titers >3 log_2/0.05 ml; see Table 2). Importantly, more antibody was induced by the strain of virus that grew better in cotton rats (i.e., hMPV 26583) than the one that did not (hMPV 26575), although the animals were inoculated with the same amount of virus (i.e., 9×10^5 $TCID_{50}$). Moreover, the antibodies induced only neutralized hMPV virus strains and not RSV.

The fact that hMPV 26575, the only subgroup B strain tested, did not grow as well as the two subgroup A (or 1) viruses merits some thought. It is possible that subgroup B human metapneumovviruses do not grow as well as subgroup A viruses in cotton rats. However, it is not uncommon in our experience with influenza and respiratory syncytial virus to have different strains of human viruses grow more or less well in animals. In fact, there was a difference in virus replication between the two subtype A hMPV strains used in our study. The $CRID_{50}$ for the 26583 strain was determined to be 900 $TCID_{50}$, ~8.2-fold higher than the $CRID_{50}$ of the RL Bx virus (110 $TCID_{50}$) (for comparison, the $CRID_{50}$ for RSV A2, a well-adapted virus is in our hands ~100 $TCID_{50}$). Thus, it is likely that the results obtained were more likely due to strain, rather than subtype differences. We are currently isolating clinical strains of hMPV and subtyping them. When this has been accomplished, we will test multiple strains of each subtype for their ability to replicate in hispid cotton rats. Regardless, despite the diminished replication seen in these studies for the one B subtype strain assessed, it is clear that all three virus strains replicated in cotton rats and caused histopathology.

The fact that the antibodies induced in cotton rats following experimental infection with either the 26575 or 26583 strains equivalently neutralized both viruses would appear to run counter to the reports that there is significant variability in the antigenicity of subtype A and B hMPV strains (Peret et al., 2002; Viazov et al., 2003; van den Hoogen et al., 2004). However, the neutralizing epitopes on the F protein of human metapneumoviruses, as opposed to those on the attachment protein, are highly conserved even in viruses belonging to the different genetic lineages (Skiadopoulos et al., 2004). Thus, it is possible that the equivalent virus-neutralizing antibody responses seen in these studies to viruses belonging to the different subgroups may have been a result of responses to these (F protein) epitopes. Such a response could have masked disparate responses to other epitopes, including those on the G protein. Two other possibilities that may have lead to the results seen include that during experimental infection the G protein is not well recognized by the cotton rat, or that many of the antibodies induced by the other epitopes are not virus-neutralziing and thus are not measurable in a neutralizing antibody assay.

As discussed above, it has already been shown that hMPV can grow well in the respiratory tracts of several species of monkeys (Skiadopoulos et al., 2004; MacPhail et al., 2004; Kuiken et al., 2004), relatively well (i.e., between 4 and 5 log_{10}/g lung) in golden Syrian hamsters and ferrets and to a lesser extent in Balb/c mice (MacPhail et al., 2004; Skiadopoulos et al., 2004). Thus, it is fair to ask, if there is a need for another hMPV–animal model (e.g., an hMPV–cotton rat model). In fact, the finding that hMPV can replicate relatively well in cotton rats is important for several reasons:

(1) at least three other paramyxoviruses have been shown to grow in hispid cotton rats, including RSV (Dreizin et al., 1971; Prince et al., 1978), parainfluenza virus type 3 (Murphy et al., 1981) and measles virus (Wyde et al., 1999; Niewiesk, 1999). This fact means that it may be possible to more readily evaluate potential vaccines (e.g., the recombinant bovine/human parainfluenza type 3 virus expressing the hMPV fusion (F) protein mentioned above), antibody preparations (e.g., IVIG) or chemotherapeutic agents (e.g., ribavirin) for virus specificity and comparable antiviral activity against different paramyxoviruses in this species. (2) All of the agents that are presently approved for use against RSV in humans (i.e., ribavirin (Hruska et al., 1982), Respigam (Ottolini et al., 1999) and Synagis (palivizumab; (Ottolini et al., 1999), were tested extensively in cotton rats and shown to be efficacious in this model. Ribavirin and different polyclonal antibody preparations need to be assessed for their antiviral activity against hMPV, and the results derived from these studies compared with those obtained in the earlier RSV–cotton rat studies. This should facilitate interpretation of the findings. Similarly, if humanized monoclonal antibodies become available for potential use against hMPV, it will be desirable to compare the results obtained with the new preparations with what was seen during the testing of Synagis. (3) Cotton rats have been reported to develop vaccine-enhanced disease similar to what was observed in the 1960s clinical trials of a formalin-inactivated RSV vaccine (Prince et al., 1986; Murphy et al., 1990; Piedra et al., 1992, 1993). Thus, it is likely that this species can also be utilized to assess potential hMPV vaccine candidates for their ability to exacerbate disease. (4) The pulmonary histopathology induced during experimental infection of cotton rats by hMPV appears to be similar to what is seen in monkeys (see Fig. 3 and Ref. (Kuiken et al., 2004). Assuming that what occurs in monkeys accurately reflects what is seen in humans, this may indicate that the pulmonary histopathology induced in cotton rats following experimental hMPV infection also accurately mirror what occurs in humans infected with hMPV. (5) Finally, but not the least important, the finding of another potential animal model to study hMPV and potential interventions against this virus is both worthwhile and an advantage in itself. As stated by others (Byrd and Prince, 1997), having multiple animal models of virus infection is highly desirable as the diseases caused by these agents can be multi-faceted and no single model may readily allow study the different facets. The different models may each provide useful information.

It should be noted that in the studies of MacPhail (MacPhail et al., 2004), the ability of hMPV virus to replicate in cotton rats was tested and found not to occur. The reasons for the different results are not clear, but may be a result of the fact that different hMPV strains were utilized (i.e., Netherlands isolates versus North American), different cell lines (Vero versus LLC-MK2), different medium (non-trypsin-supplemented versus trypsin-supplemented), different preparation of samples (homogenization of turbinates and lungs compared to nose washes and lung lavaging), different technicians with different experience with this virus, or a combination of these factors. Regardless, the consistent positive results obtained in these studies are a fact and clearly indicate that the cotton rat–hMPV model is suitable for identifying, evaluating and aiding in the preclinical development of chemotherapeutics, vaccines and other types of antivirals (e.g., immune globulins) that can be used to prevent or ameliorate hMPV infections.

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References

Bastien, N., Normand, S., Taylor, T., Ward, D., Peret, T.C.T., Boivin, G., Anderson, L.J., Li, Y., 2003. Sequence analysis of the N, P, M and F genes of Canadian human metapneumovirus strains. Virus Res. 93, 51–62.

Biacchesi, S., Skiadopoulos, M.H., Boivin, G., Hanson, C.T., Murphy, B.R., Collins, P.L., Buchholz, U.J., 2003. Genetic diversity between human metapneumovirus subgroups. Virology 315, 1–9.

Boivin, G., De Serres, G., Cote, S., Gilca, Y., Abed, Y., Rochette, L., Bergeron, M.G., Dery, P., 2003. Human metapneumovirus infections in hospitalized children. Emerg. Infect. dis. 9, 634–640.

Byrd, L.G., Prince, G.A., 1997. Animal models of respiratory syncytial virus infection. Clin. Infect. Dis. 25, 1363–1368.

Coons, A.H., 1958. Flourescent antibody methods. In: Danielli, J.F. (Ed.), General Cytochemical Methods, first ed. Academic Press, New York, pp. 399–400.

Dreizin, R.S., Vyshnevetskaia, I.O., Bagdamian, E.E., Yankevich, O.D., Tarasova, L.B., Klenova, A.V., 1971. Experimental RS virus infection of cotton rats: a viral and immunofluorescent study. Vopr. Virusol. 16, 670–676.

Esper, F., Martinello, R.A., boucher, D., Weibel, C., Ferguson, D., Landry, M.L., Klenova, A.V., 2004. A 1-year experience with human metapneumovirus in children aged <5 years. J. Infect. Dis. 189, 1388–1396.

Falsey, A.R., Erdman, D., Anderson, L.J., Walsh, E.E., 2003. Human metapneumovirus infections in young and elderly adults. J. Infect. Dis. 187, 785–790.

Hruska, J.F., Morrow, P.E., Suffin, S.C., Douglas, R.G., 1982. In vivo inhibition of respiratory syncytial virus by ribavirin. Antimicrob. Agents Chemother. 21, 125–130.

Kuiken, T., van den Hoogen, B.G., van Riel, D.A.J., Laman, J.D., Van Amerongen, G., Fouchier, R.A.M., Osterhaus, A.D.M.E., 2004. Experimental human metapneumovirus infection of Cynomolgus macaques (*Macaca fascicularis*) results in virus replication in ciliated epithelial cells and pneumocytes with associated lesions throughout the respiratory tract. Am. J. Pathol. 164, 1893–1900.

MacPhail, M., Schickli, J.H., Tang, R.S., Kaur, J., Robinson, C., Fouchier, R.A.M., Osterhaus, A.D.M.E., Spaete, R.R., Haller, A.A., 2004. Identification of small-animal and primate models for evaluation of vaccine candidates for human metapneumovirus (hMPV) and implications for hMPV vaccine design. J. Gen. Virol. 85, 1655–1663.

Madhi, S.A.L.H., Abed, Y., Klugman, K.P., Boivin, G., 2003. Human metapneumovirus-associated lower respiratory tract infections among hospitalized human immunodeficiency virus type 1 (HIV-1)-infected and HIV-1-uninfected African infants. Clin. Infect. Dis. 37, 1705–1710.

Murphy, B.R., Sotnikov, A.V., Lawrence, L.A., Banks, S.M., Prince, G.A., 1990. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivate respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV months after immunization. Vaccine 8, 497–502.

Murphy, T.F., Dubovi, E.J., Clyde Jr., W.A., 1981. The cotton rat as an experimental model of human parainfluenza virus type 3 disease. Exp. Lung Res. 2, 97–109.

Niewiesk, S., 1999. Cotton rats (*Sigmodon hispidus*): an animal model to study the pathogenesis of measles virus infection. Immunol. Lett. 65, 47–50.

Osterhaus, A., Fouchier, R., 2003. Human metapneumovirus in the community. Lancet 361, 890–891.

Ottolini, M.G., Porter, D.D., Hemming, V.G., Zimmerman, M.N., Schwab, N.M., Prince, G.A., 1999. Effectiveness of RSVIG prophylaxis and therapy of respiratory syncytial virus in an immunosuppressed animal model. Bone Marrow Transplant. 24, 41–45.

Pelletier, G., Dery, P., Abed, Y., Boivin, G., 2002. Respiratory tract reinfections by the new human metapneumovirus in an immunocompromised child. Emerg. Infect. dis. 8, 976–978.

Peret, T.C., Boivin, G., Li, Y., Couillard, M., Humphrey, C., Osterhaas, A.D., Erdman, D.D., 2002. Characterization of human metapneumoviruses isolated from patients in North America. J. Infect. Dis. 185, 1660–1663.

Piedra, P.A., Wyde, P.R., Castleman, W.L., Ambrose, M.W., Jewell, A.M., Speilman, D.J., Hildreth, S.W., 1993. Enhanced pulmonary pathology associated with the use of formalin-inactivated respiratory syncytial virus vaccine in cotton rats is not a unique viral phenomenon. Vaccine 11, 1415–1423.

Piedra, P.A., Wyde, P.R., Jewell, A.M., Ambrose, M.W., Castleman, W.L., Hildreth, S.W., 1992. Enhanced lung pathology induced with cellular antigens in formalin-inactivated RSV vaccines in cotton rats. Antimicrob. Agents Chemother. In: 32nd International Conference, Anaheim, CA.

Prince, G.A., Jenson, A.B., Hemming, V.G., Murphy, B.R., Walsh, E.E., Horswood, R.L., Chanock, R.M., 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by intramuscular inoculation of formalin-inactivated virus. J. Virol. 57, 721–728.

Prince, G.A., Jenson, A.B., Horswood, R.L., Camargo, E., Chanock, R.M., 1978. The pathogenesis of respiratory syncytial virus infection in cotton rats. Am. J. Pathol. 93, 771–792.

Rhodes, A.J., Van Rooyen, C.E., 1953. Textbook of Virology, second ed. Williams and Wilkins, Baltimore, MD.

Skiadopoulos, M.H., Biacchesi, S., Buchholz, U.J., Riggs, J.M., Surman, S.R., Amaro-carambot, E., McAuliffe, J.M., Elkins, W.R., St. Claire, M., Collins, P.L., Murphy, B.R., 2004. The two major human metapneumovirus genetic lineages are highly related antigenically, and the fusion (F) protein is the major contributor to this antigenic relatedness. J. Virol. 78, 6927–6937.

Tang, R.S., Schickli, J.H., MacPhail, M., Fernandes, F., Bicha, L., Spaete, J., Fouchier, R.A., Osterhaus, A.D., Spaete, R., Haller, A.A., 2003. Effects of human metapneumovirus and respiratory syncytial virus antigen insertion in two 3′ proximal genome positions of bovine/human parainfluenza virus type 3 on virus replication and immunogenicity. J. Virol. 77, 10819–10828.

van den Hoogen, B.G., de Jong, J.C., Groen, J., Kuiken, T., De Groot, R., Fouchier, R.A.M., Osterhaus, A.D.M.E., 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med. 7, 719–724.

van den Hoogen, B.G., Osterhaus, A.D.M.E., Fouchier, R.A.M., 2004. Clinical impact and diagnosis of human metapneumovirus infection. Pediatr. Infect. Dis. 23, S25–S32.

van den Hoogen, B.G., Van Doornum, G.J.J., Fockens, J.C., Cornelissen, J.J., Beyer, W.E.P., De Groot, R., Osterhaus, A.D.M.E., Fouchier, R.A.M., 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J. Infect. Dis. 188, 1571–1577.

Viazov, S., Ratjen, F., Scheidhauer, M., Fiedler, M., Roggendorf, M., 2003. High prevalence of human metapneumovirus infection in young children and genetic heterogeneity of the viral isolates. J. Clin. Microbiol. 41, 3043–3045.

Wilson, S.Z., Knight, V., Wyde, P.R., Drake, S., Couch, R.B., 1980. Amantadine and ribavirin aerosol treatment of influenza A and B infection in mice. Antimicrob. Agents Chemother. 17, 642–648.

Wyde, P.R., Chetty, S.N., Jewel, A.M., Boivin, G., Piedra, P.A., 2003. Comparison of the inhibition of human metapneumovirus and respiratory syncytial virus by ribavirin and immune serum globulin in vitro. Antivir. Res. 60, 51–59.

Wyde, P.R., Meyerson, L.R., Gilbert, B.E., 1993. An in vitro evaluation of the antiviral activity of SP-303, an euphorbiaceae shrub extract, against a panel of respiratory virus. Drug Dev. Res. 28, 467–472.

Wyde, P.R., Moore, D.K., Hepburn, T., Silverman, C.L., Porter, T.G., Gross, M., Taylor, G., Demuth, S.G., Dillon, S.B., 1995. Evaluation of the protective efficacy of reshaped human monoclonal antibody RSHZ19 against respiratory syncytial virus in cotton rats. Pediatr. Res. 38, 543–550.

Wyde, P.R., MoorePoveda, D.K., Daley, N.J., Oshitani, H., 1999. Replication of clinical measles virus strains in hispid cotton rats. Proc. Soc. Exp. Biol. Med. 221, 53–62.

Wyde, P.R., Moylett, E.H., Chetty, S.N., Jewell, A., Bowlin, T.L., Piedra, P.A., 2004. Comparison of the inhibition of human metapneumovirus and respiratory syncytial virus by NMSO3 in tissue culture assays. Antivir. Res. 63, 51–59.

Xepapakaki, P., Psarras, S., Bossios, A., Tsolia, M., Gourgiotis, D., Liapi-Adamidou, G., Constantopoulos, G., Kafetzis, D., Papadopoulos, N.G., 2004. Human metapneumovirus as a causative agent of acute bronchiolitis in infants. J. Clin. Virol. 39, 267–270.