Replication of Clinical Measles Virus Strains in Hispid Cotton Rats (44384)

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Abstract. **An alternative model to nonhuman primates to study measles virus (MV) pathogenesis, to evaluate potential MV vaccines, or to screen for potential antivirals effective against this virus is highly desirable. The laboratory-adapted Edmonston strain of MV has been reported to replicate in the lungs of hispid cotton rats following intranasal inoculation, immunosuppress infected animals, and disseminate widely from the lungs, making these animals a candidate model. However, clinical MV strains have generally not been found to grow in these animals, limiting the utility and acceptance of this model. In the present studies we demonstrate reproducible replication of several clinical MV strains in hispid cotton rats. As with the Edmonston strain, leukocytes appear to be the primary target cells of these viruses following intranasal inoculation, and extrapulmonary dissemination is common. It is also demonstrated that prior MV infection or immunization of test animals with MV vaccine prevents pulmonary tract infection. These findings should make the MV-cotton rat model more acceptable.** [P.S.E.B.M. 1999, Vol 221]

Over one million children die annually of measles despite the availability of measles virus (MV) vaccines (1, 2). Most of these deaths are due to pneumonia (3). However, MV frequently translocates from the lungs and can cause an array of debilitating disorders, including those involving the liver, pancreas, immune system, and central nervous system (3–10). Dissemination of MV from the lungs is probably mediated by infected leukocytes, especially macrophages (5, 10, 11). MV-leukocyte interactions probably also play a critical role in two other prominent aspects of MV pathogenesis, marked immunosuppression of the host and the frequent occurrence of secondary infections (5, 10).

The continued medical impact of MV has led to renewed interest in the pathogenesis of this virus (11–13), as well as attempts to elucidate better measles vaccines (14–17) and antiviral compounds (18–20). There is also an interest in the comparative pathogenesis of MV and other leukocyte-associated viruses (e.g., human immunodeficiency, cytomegalovirus, and Epstein-Barr virus) (21, 22). Monkeys would appear to be most suitable for these studies since many of these primates develop disease manifestations similar to those seen in humans after experimental inoculation with this virus (23–25). However, their cost, limited availability, and restrictions on the use of many nonhuman primates for medical research makes them impractical for many studies. This has led to a search for alternative, particularly rodent, models to carry out MV studies. In fact, rodent models have been used to study aspects of MV pathogenesis, particularly MV infection of the central nervous system (CNS) (26–28). However, these models are generally restricted to the use of immunologically immature (newborn or very young) animals, the use of CNS-adapted MV, and/or inoculation by an unnatural inoculation route (i.e., intracerebral).

Cotton rats have been used to study the pathogenesis of two viruses related to MV, respiratory syncytial (RSV) (29) and parainfluenza type 3 (PIV3) (30, 31). More recent reports have indicated that these animals can also support growth of the highly laboratory-adapted Edmonston strain of MV, but not most clinical MV strains (32, 33). In one of these studies (33), it was shown that like natural infection,

This work was supported primarily by Public Health Service contract no. NO1-AI-65292.

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Received June 22, 1998. [P.S.E.B.M. 1999, Vol 221]
Accepted December 22, 1998.

0037-9727/99/2211-0053$14.00/0

the inoculated virus was immunosuppressive and frequently disseminated from the lungs. Support for the latter finding was obtained using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

In this report, in contrast to what has been reported previously, we describe reproducible replication in the lungs of cotton rats of several different clinical MV strains following intranasal inoculation of these animals. Also presented is evidence that cotton rat leukocytes are the primary target cells of the inoculated viruses and that dissemination of virus from the lungs is frequent. The potential utility of the model for vaccine evaluation is shown by the demonstration that cotton rats previously infected with MV or immunized with a standard MV vaccine are protected from pulmonary infection by the clinical MV strains.

Materials and Methods

Tissue Culture. Vero (African green monkey kidney, ATCC CCL 81) and B95–8 (Epstein Barr virus-transformed marmoset leukocytes; ATCC CRL 1612) tissue culture cells were obtained from the American Type Culture Collection (ATCC). Earle's minimum essential medium (Sigma Chemical Co., St. Louis, MO) supplemented with 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 g/ml), sodium bicarbonate (0.2%), and L-glutamine (2 m*M*/ml; 5% FCS-MEM) was used to grow the Vero cells. Identically supplemented Dulbecco's modified Eagle medium (5% FCS-DMEM; Gibco BRL) was used to propagate the B95–8 cells.

Viruses. Enders-Moraten (E-M) vaccine virus was obtained from the Acute Respiratory Disease Center, Baylor College of Medicine. This live virus vaccine was produced by Merck, Sharp, and Dohme in chick cells and sold under the tradename Attenuvax. MV isolates AC705, 11498, and 11233 were obtained from E. C. Herrmann, Ph.D. (Mobilab, Peoria, IL). The Howard MV isolate was obtained from Gail Demmler, M.D. (Department of Pediatrics, BCM). These four viruses were isolated in primary monkey kidney cells and then passaged several times in Vero cells before being sent to us. All of the other measles viruses used in these studies were generously given to us jointly by Drs. Hiroshi Suzuki (Department of Public Health, Niigata University, Niigata, Japan), Katsumi Mizuta (Virus Research Center, Sendai National Hospital, Sendai, Japan), and Mwila E. Mpabalwani (Virology Laboratory, The University Teaching Hospital, Lusaka, Zambia). These viruses were all isolated in Zambia, Africa, between 1991 and 1994 during studies performed under the auspices of the Japan International Cooperation Agency. All were initially isolated in B95–8 cells and then passed several times in this cell line prior to being sent to us.

A working stock of each MV strain mentioned above was prepared in B95–8 cells as described previously (32). The M02 and A99 viruses also were serially passaged in Vero cells to "adapt" them to grow well in these cells. Ultraviolet light (UV)-inactivated virus was prepared by exposing thin films of virus for seven minutes to a UV light source (intensity 320 (W cm-2) kept at a distance of 15 cm. All of the virus preparations were stored at –700C°. The median tissue culture infectious dose (TCID50) of each preparation was determined using B95–8 cells and a 96-well microplate assay described briefly below, and in detail elsewhere (32).

Animals. Six- to eight-week-old cotton rats (50–100 g) of either sex were used in these studies. All of the animals were descendants of two 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. Comparably aged inbred BALB/c or outbred CD-1 mice were used in two studies. These animals were purchased from Charles River Laboratories (Wilmington, MA). All of the animals were housed in the Baylor College of Medicine (BCM) vivarium in cages covered with barrier filters. All were given food and water *ad libitum*.

Inoculation of Animals. Animals were inoculated with MV either intranasally (i.n.) or intraperitoneally (i.p.). Intranasal inoculations were performed by gradually introducing 50 μl of the desired virus suspension into the nares of cotton rats lightly anesthetized with Metofane (methoxyfurane, Pitman-Moore, Mundelein, IL) using a P-200 pipettor (Rainin Instruments Co., Inc., Woburn, MA). For intraperitoneal inoculations, the animals were anesthetized using CO_2 and then injected with 100 μl i.p. of the appropriate virus.

Virus Quantitation. Quantitation of virus in inocula and test suspensions was determined in 96-well tissue culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) as described previously (32). Care was taken to ensure that the cells in each sample were kept suspended during the course of each titration. This was necessary since preliminary studies indicated that most of the MV present in lung cell (LC) and peritoneal exudate (PE) cell suspensions was cell associated. The amount of virus in inocula was expressed as median cotton rat infectious doses ($\log_{10}$)/ml of suspension, whereas levels of virus in LC and PE cell suspensions were expressed as $TCID_{50}(\log_{10})$/g lung or/ml of suspension, respectively. Because 10 ml of medium were used during each peritoneal lavage, the total amount of virus obtained from this site was always 10X the stated titer.

Collection and Preparation of Samples for Virus Isolation. Only peritoneal exudate cells (PEC) were collected from animals inoculated i.p. with virus. These were obtained by lavaging the peritoneal cavity as described by Stuart *et al.* (34). The cells in each suspension were then pelleted by centrifugation (450*g*), washed using phosphate buffered saline (PBS), and resuspended in 1 ml of 2% FCS-DMEM.

Lungs were removed from all animals inoculated i.n. with virus. These were weighed, rinsed in PBS and transpleurally lavaged as described previously (35). If looking for extrapulmonary dissemination of virus, one lobe of liver, the mediastinal lymph nodes (MDLN), thymus, spleen, and

blood were also collected from each animal. Each organ was individually teased into a single cell suspension that was then centrifuged at 450*g*. The resulting cell pellet was washed and resuspended in 1 ml of 2% FCS-DMEM medium. The blood was added to test tubes containing sufficient heparin sulfate (Sigma Chemical Co.) to give approximately 10 units of anticoagulant/ml of blood. Each blood suspension was then layered onto gradients containing Lymphocyte M density separation medium (Accurate Chemical Co., Hicksville, NY) and centrifuged for 20 min at 450*g*. The leukocytes remaining at the interfaces of these gradients were removed, washed, and resuspended in 1 ml of 5% FCS-DMEM. Each cell suspension was tested for virus using the 96-well microplate assay.

Fractionation of Lung and Peritoneal Cells. In some experiments, the lung and PE cell suspensions obtained by lavage were separated into fluid and cellular fractions by centrifugation (450*g* for 10 min). The fluid portions were decanted and placed on ice until assessed for virus. The pelleted cells were resuspended in medium and separated into nonadherent and adherent cell fractions using adherence to plastic (36). Each of the resulting nonadherent and adherent cell fractions were collected, washed, and resuspended in 1 ml of 2% FCS-DMEM. These suspensions were tested for virus using the 96-well microplate assay.

Identification of Leukocyte Subpopulations. Acridine orange stain (0.01%) and the classification system of Golstein and Blomgren (37) were generally used to identify the cell types present in suspensions or different cell fractions. However, in some experiments the cells in the different suspensions or fractions were pelleted onto glass slides using a cytocentrifuge (Shandon-Elliot Cytospin, Pittsburgh, PA) and stained with Leukostat stain (i.e., a modified Wright stain; Fisher Diagnostics, Orangeburg, NY) for morphologic identification. The latter preparations were observed with a Bausch and Lomb Balplan light microscope (Rochester, NY). Preparations stained with acridine orange were observed using a Leitz Orthoplan fluorescent microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with Ploem illumination, an HBO-200 W mercury burner, a BG12 excitation filter and a 470 nm barrier filter.

Preparation of Primary LC and PE Cell Cultures. In a number of experiments, lung and PEC not used for other purposes were suspended in 10 ml of 20% FCS-MEM and placed in 25-cm^2 flasks (Corning, Cambridge, MA). These flasks were incubated at 36°C (5% $C0_2$) until they formed confluent monolayers (usually 2–3 weeks). At that time, the cells in the monolayers were removed from the surface of the plastic flasks using trypsin and standard tissue culture procedures (38), washed 3X, and resuspended in 1 ml of medium. A portion of each cell suspension was tested for virus. The remaining cells were added to either new flasks or slide chambers (Nunc, Inc., Naperville, IL). When these subcultured cells formed confluent monolayers, the entire process was repeated. In this manner up to seven passage levels of different lung or PE cell suspensions were obtained and tested for virus.

Confirmation of Virus Identity. Although the viruses isolated from cotton rats experimentally inoculated with MV nearly always produced "typical" CPE (namely prominent syncytia or giant cell formation) in B95–8 cells, these isolates were intermittently checked for identity using MV-specific rabbit antisera (Accurate Chemical Co.). In all instances, the isolated virus was completely neutralized by >1:160 dilutions of this antisera, but not by heterologous antisera (i.e., rabbit antiserum to Herpes simplex virus [HSV] 1 and 2; Chemicon International, Inc., Temecula, CA, used at dilutions >1:4).

Histologic Processing and Staining. Lungs collected for histopathological evaluations were fixed in Omnifix II (An-Con Genetics, Inc., Melville, NY) for a minimum of 24 hr, embedded in low-melting-point paraffin, sectioned at 5 μM thickness, and stained with hematoxylin and eosin (H&E). These preparations were observed in a blinded fashion for hispathology using a standard light microscope. The presence of MV antigens in lung and PE cell suspensions was determined using standard indirect fluorescence antibody procedures, MV-specific rabbit antisera (i.e., Accurate Chemical Co.) and fluorescein-conjugated goat antirabbit antisera (Cappel, Durham, NC) (32). The fluorescent staining was performed on cytocentrifuged cells. The nonspecific esterase staining procedure of Yam *et al.* (39) was used to identify macrophages in lung and PE cell cultures.

Phagocytosis Assays. Glutaraldehyde-stabilized, lyophilized sheep red blood cells (Sigma Chemical Co.) were reconstituted and coated with 19S (IgM) antibodies specific for sheep red blood cells (Cordis Laboratories, Hialeah, FL). The coated erythrocytes were washed three times and added to slide chambers containing monolayers of lung, PE, or B95–8 tissue culture cells. After incubation of these slide chambers at 360°C for either 120 min or overnight, the adherent cells present in them were removed using trypsin, washed and resuspended in 1 ml of 2% FCS DMEM medium. Samples from each suspension were added to standard hemocytometers and observed for internalized (phagocytized) antibody-coated erythrocytes. A cell was considered to be phagocytic if it contained at least three internalized erythrocytes. The percentage of the total number of cells observed that were phagocytic was then recorded.

Statistics. Descriptive statistics, the Kruskal–Wallis nonparametric analysis of variance and Student's *t* tests were all performed using True Epistat, a statistical program for IBM-compatible computers designed by T.L. Gustavson of Epistat Services, (Richardson, TX).

Results

Clearance of African-Derived MV Strains from Cotton Rats. Groups of four cotton rats were inoculated either i.n. or i.p. with approximately 10^4 $TCID_{50}$ of different

clinical MV strains obtained from Zambia. Four days later the inoculated animals were sacrificed, and the lungs of the cotton rats inoculated i.n. and the peritoneal cavities of the animals inoculated i.p., were lavaged. The mean titers of virus detected in the resulting lung (hatched bars) and PE (open bars) cell suspensions are shown in Figure 1.

As the hatched bars in Figure 1 indicate, significant levels of MV were detected in all of the LC suspensions except those obtained from animals inoculated with the 94Y1099 virus strain. Highest titers (i.e., $10^{4.1}$ and $10^{3.9}$ $TCID_{50}$/g lung, respectively) were measured in the samples collected from animals inoculated with MV strains M02 and A99. Less virus was detected in the suspensions derived from animals inoculated with MV strains 90Y288 and 91Y761 (i.e., $10^{3.1}$ and $10^{3.3}$ $TCID_{50}$/g lung, respectively), and minimal virus was detected in suspensions collected from cotton rats inoculated with MV strains M407 and G1171 (i.e., $10^{1.4}$ $TCID_{50}$/g lung).

As the open bars in Figure 1 suggest, virus was detected in every PE cell suspension collected from the animals inoculated i.p. Suspensions from animals inoculated with MV02 had the highest mean virus titer (i.e., $10^{4.1}$ $TCID_{50}$/ml of PEC suspension; $10^{5.1}$ $TCID_{50}$/suspension). However, equivalent amounts of virus were also detected in suspensions collected from cotton rats inoculated i.p. with MV strains A99 and 90Y288 (i.e., $10^{3.9}$ and $10^{4.0}$ $TCID_{50}$/ml of suspension, respectively). Samples from the groups of cotton rats inoculated with MV strains M407 and G1171 had the lowest mean virus titers (i.e., $10^{3.1}$ and $10^{2.3}$ $TCID_{50}$/ml of PEC suspension, respectively).

Clearance of Strains M02 and A99 from Mice. Markedly contrasting results were obtained when comparable doses of MV strains M02 and A99 were injected similarly into outbred CD-1 or inbred BALB/c mice. As indicated by the values displayed in Table I, no virus was detected on Day 4 in any of the lung or PE cell suspensions collected from mice. This was true although significant levels of MV were detected in both experiments on Day 4 in the lungs and peritoneal cavities of control cotton rats inoculated i.n. or i.p. with these viruses.

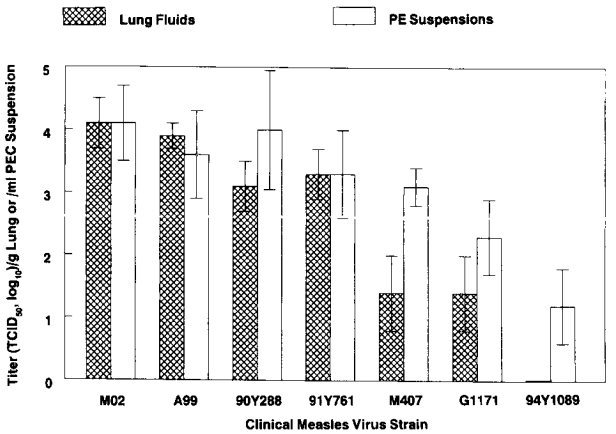

Figure 1. Geometric mean virus titers (GMT + one standard deviation) measured in lungs of cotton rats inoculated intranasally with different clinical measles virus strains obtained from Zambia (hatched bars), or in peritoneal lavage fluids of animals inoculated intraperitoneally with these viruses (open bars). GMT for lungs are expressed as $TCID_{50}(log_{10})$/g of lung. For peritoneal lavage suspensions, the GMT are expressed as $TCID_{50}(log_{10})$/ml of peritoneal suspension (10 ml of medium was used to lavage each peritoneal cavity); number of animals/group = 4.

Clearance of U.S.-Derived MV Strains from Cotton Rats. The geometric mean pulmonary virus titers seen on Day 4 in cotton rats inoculated i.n. with either different U.S.-derived MV strains or the African-derived M02 strain are shown in Table II. As the bolded values in this table indicate, the mean pulmonary virus titers seen in the groups of cotton rats inoculated with MV strains Howard, 11498, and 11233 were significantly less than those detected on this day in the animals inoculated with MV M02. The mean pulmonary virus titer of the group of cotton rats inoculated with AC705, another MV strain originally isolated in the United States, had a *P*-value less than 0.1, and thus just missed statistical significance using the Kruskal–Wallis analysis of variance test.

Determination of the $CRID_{50}$ of MV Strains M02 and A99. The $CRID_{50}$ for MV strains M02 and A99 were determined to appraise the ability of these viruses to infect cotton rats. Groups of four cotton rats were inoculated i.n. or i.p. with serial 10-fold dilutions of each virus. Four days later these animals were sacrificed, and the number of cotton rats in each group infected with MV was determined. As indicated by the data in Table III, the $CRID_{50}$ for the M02 MV pool used in these studies was estimated to be $10^{2.1}$ (or ≈126) $TCID_{50}$ following intranasal inoculation and $10^{1.1}$ (or ≈13) $TCID_{50}$ when administered to cotton rats i.p. The $CRID_{50}$ values for the A99 strain varied only slightly (i.e., $10^{2.2}$ $TCID_{50}$ following i.n. inoculation and $10^{1.3}$ TCID50 following i.p. inoculation; raw data not shown).

Kinetics of Replication of MV Strains M02 and A99 in Cotton Rats. The kinetics of replication of MV strains M02 and A99 in the lungs of cotton rats inoculated i.n. with 10^4 $TCID_{50}$($10^{2.1}$–$10^{2.2}$ $CRID_{50}$) of these viruses are shown in Figure 2. As indicated, significant levels of both viruses were detected in the lungs (mean titer = $10^{2.5}$ $TCID_{50}$/g lung for MV M02 and $10^{2.1}$ for MV A99) 1 day after virus inoculation. Maximal virus titers occurred on Day 4 (i.e., mean = $10^{4.3}$ $TCID_{50}$/g of lung for both viruses). The virus then declined and appeared to be cleared from the lungs between Days 7 and 10.

Association of Recovered Virus with Leukocytes. In selected experiments the lung and PE cell suspensions obtained on Day 4 from cotton rats experimentally inoculated with MV M02 were assayed for virus before and after being separated into fluid (supernatant), nonadherent cell and adherent cell fractions. As the data in Table IV indicate, minimal (<3% of the virus measured in the unfractionated suspensions), or no virus was detected in the fluid portion of these suspensions. Instead, with only one exception (Animal 6), much (>32%), and sometimes all, of the

Table I. Comparison of Virus Titers Measured in Lung or Peritoneal Suspensions Obtained from Cotton Rats or Mice 4 Days After these Animals were Experimentally Inoculated with Measles Virus Strains M02 or A99[a]

Expt. no.[b]	Species/strain inoculated	Virus/Inoc route	Suspension tested	Mean virus titer[c] (/g lung)	Mean virus titer[c] (/ml suspension)
1	Cotton rat	M02I.N.	Lung	4.3 ± 0.4	—
	Cotton rat	M02I.P.	Peritoneal	—	4.6 ± 0.3
	Mouse (ICR)	M02I.N.	Lung	0	—
	Mouse (ICR)	M02I.P.	Peritoneal	—	0
	Mouse (Balb/c)	M02I.N.	Lung	0	—
	Mouse (Balb/c)	M02I.P.	Peritoneal	—	0
2	Cotton rat	A99 I.N.	Lung	4.1 ± 0.5	—
	Cotton rat	A99 I.P.	Peritoneal	—	4.4 ± 0.5
	Mouse (ICR)	A99 I.N.	Lung	0	—
	Mouse (ICR)	A99 I.P.	Peritoneal	—	0
	Mouse (Balb/c)	A99 I.N.	Lung	0	—
	Mouse (Balb/c)	A99 I.P.	Peritoneal	—	0

[a] Each animal was inoculated intranasally (i.n.) or intraperitoneally (i.p.) with approximately 10^4 median tissue culture infectious doses of the appropriate measles virus (MV) strain. Four days later the lungs of the animals inoculated i.n., and the peritoneal cavities of those inoculated i.p., were lavaged. Each of the resulting suspensions was tested for virus.
[b] Abbreviations: no. = number; expt. = experiment; MV = measles virus; inoc = inoculation; g = gram; NA = not applicable; I.N. = intranasal; I.P. = intraperitoneal.
[c] Mean titer ($TCID_{50}$, log_{10}) ± standard deviation; 0 = no virus detected (minimum detection being $10^{1.4}$ $TCID_{50}$/g lung or $10^{1.3}$ $TCID_{50}$/ml of peritoneal fluid; number of animals/group = 4.

Table II. Comparison of Virus Titers Measured in Lungs of Cotton Rats 4 Days After these Animals were Experimentally Inoculated with Different Measles Virus Strains Isolated in the United States or MV Strain M02[a]

Measles virus inoculated	Source	No. animals positive/ no. animals inoculated[c]	Mean virus titer[b] all animals	Mean virus titer[b] positive animals
M02	Africa	5/5	4.0 ± 0.4	4.0 ± 0.4
Howard	U.S.	1/5	**1.0 ± 0.5**[d]	1.9 ± 0
AC705	U.S.	2/5	1.3 ± 0.8	2.2 ± 0.4
11498	U.S.	1/5	**1.0 ± 0.5**	1.9 ± 0
11233	U.S.	1/5	**0 ± 0**	0 ± 0

[a] Each animal was inoculated intranasally with approximately 10^4 median tissue culture infectious doses of the appropriate measles virus (MV) strain. Four days later, the animals were sacrificed, and their lungs were removed, lavaged, and tested for virus.
[b] Mean virus titers ($TCID_{50}$, log_{10}) ± standard deviation; 0 = no virus detected (minimum detection being $10^{1.4}$ $TCID_{50}$/g lung); number of animals/group = 5.
[c] Abbreviations: No. = number; U.S. = United States.
[d] The mean virus titers of each group were compared using the Kruskal-Wallis nonparametric analysis of variance test; a value of 0.8 was assigned to lungs in which no virus was detected; bolded values indicate means with P values ≤ 0.05 compared to the mean value obtained for the group of mice inoculated with M02 virus.

virus was found associated with the cellular portions of these suspensions, particularly the adherent cell fraction.

Persistence of MV in Cultures of Cotton Rat Lung and PE Cells. Levels of virus seen in six successive subcultures of lung cells derived from a lung lavage suspension obtained from a cotton rat 4 days after this animal was inoculated are shown in Figure 3. As the bars in this figure indicate, virus titers in excess of 10^4 or 10^5 $TCID_{50}$/ml of suspension were detected in five of the six subcultures. (The virus titer in the original LC suspension was $10^{4.2}$ $TCID_{50}$/g lung). This was true although testing occurred over a 3.5-month period, involved six subcultures and numerous washes of the cells being passaged. These results were not unusual. Similar levels of virus were detected in virtually every subculture of lung and PEC derived from an infected animal and tested similarly.

Characteristics of the Cells Present in Lung and PE Cell Cultures. Although initial cultures of lung and PE cells usually appeared to contain a variety of cell types, a single cell form commonly predominated after one subculture. As Figure 4(A) shows, this cell was generally nondescript except for its large size and relatively abundant cytoplasm. However, as shown by cytochemical staining and their ability to internalize opsonized sheep erythrocytes, virtually all of these cells were highly phagocytic (see Fig. 4b) and positive (to varying degrees; see Fig. 4C) for nonspecific esterase.

MV Dissemination from the Lungs. Several experiments were performed in which extrapulmonary dissemination of MV was looked for in animals inoculated i.n. with MV M02. In these experiments, blood, brains, MDLN, thymuses, lungs, spleens, and portions of liver were col-

Table III. Determination of the Median Cotton Rat Infectious Dose of Measles Virus Strain M02 for Cotton Rats[a]

$TCID_{50}$ of MV (log_{10}) injected[b]	No. lungs positive/ no. lungs tested	Mean GMT on Day 4	No. peritoneal cavities positive/no. tested	Mean GMT on Day 4[c]
4.5	4/4	5.3 ± 0.3	4/4	5.6 ± 0.3
3.5	4/4	4.1 ± 0.3	4/4	4.6 ± 0.3
2.5	4/4	2.9 ± 0.4	4/4	3.6 ± 0.3
1.5	0/4	0	4/4	3.4 ± 0.2
0.5	0/4	0	0/4	0
0.05	0/4	0	0/4	0

Note. Calculated $CRID_{50}$ for lungs = $10^{2.1}$ $TCID_{50}$; for peritoneal fluids = $10^{1.1}$ $TCID_{50}$.

[a] Six groups of four cotton rats were inoculated intranasally or intraperitoneally on Day 0 with serial 10-fold dilutions of the M02 strain of measles virus (MV). Four days later these animals were sacrificed, and their peritoneal cavities or lungs were lavaged and tested for MV in B95-8 tissue culture cells.

[b] Abbreviations: $TCID_{50}$ = median tissue culture infectious dose (B95-8 cells); $CRID_{50}$ = median cotton rat infectious dose; no. = number; GMT = geometric mean virus titer.

[c] $TCID_{50}$ (log_{10})/g lung or ml of peritoneal lavage fluid.

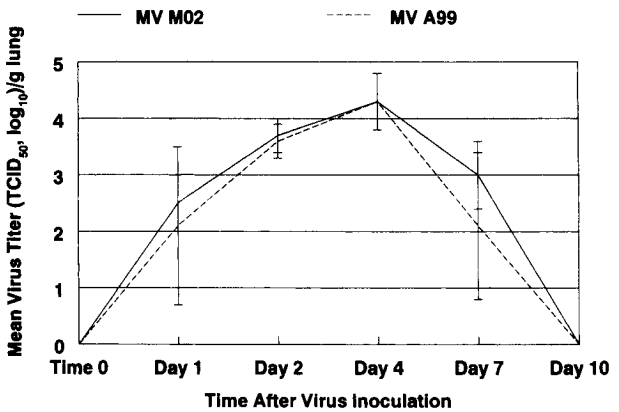

Figure 2. Kinetics of replication of measles virus strains M02 (_____) and A99 (– – – – – – –) in lungs of cotton rats inoculated intranasally with approximately 10^4 $TCID_{50}$ of these viruses. Lung titers are expressed as geometric mean pulmonary lung virus titer ($TCID_{50}$ log_{10})/g of lung + one standard deviation. Virus titers in peritoneal lavage fluids are expressed as geometric mean virus titer ($TCID_{50}$ log_{10})/ml of suspension + one standard deviation. The number of experiments for each time point shown was ≥ 2.

lected from test cotton rats 4 and 7 days after virus inoculation. As indicated by the data presented in Table V, viable MV was recovered on Day 4 from 16 of 16 lungs, 9 of 16 MDLN (56%) and 2 of 16 (13%) spleens. No viable virus was detected on Day 4 in brains, peripheral blood leukocytes (PBL), thymuses, or liver lobes. On Day 7, viable virus was only detected in the lungs.

Histologic Findings. Significantly greater numbers of inflammatory cells (i.e., polymorphonuclear neutrophiles [PMN], lymphocytes, and macrophages) were consistently observed on Days 4 and 7 postinfection in H&E-stained lung sections from virus-inoculated animals than sham-inoculated, or noninoculated animals. These cells generally occurred in foci and were most evident around bronchi and bronchioles. However, individual and small numbers of inflammatory cells were also seen scattered randomly throughout the aveoli. Photomicrographs of the stained lung sections are not presented, as the changes seen were virtually identical to those seen in cotton rats infected with the Edmonston strain of MV and published previously (1).

Fluorescent Antibody Findings. Brightly (apple green) fluorescing cells were commonly seen on glass (cytocentrifuge) slides containing lung and PEC collected on Day 4 from virus-inoculated animals and stained for MV antigens using an indirect fluorescent antibody procedure. These fluorescing cells were only evident in samples obtained from virus-inoculated animals and were not present in similar preparations stained using control (anti-HSV) antisera in the primary staining step (data not shown).

Immunization or Infection with Live, but not Inactivated, Virus Protect Cotton Rats from Pulmonary MV Infection. As indicated in Table VI, cotton rats inoculated i.m. with 100 $TCID_{50}$ of E-M vaccine virus, or i.n. with a similar dose of M02 *MV*, produced significant levels of MV-specific neutralizing antibodies (i.e., mean $titer_s$ = $2^{7.9}$ or $2^{9.3}$/0.05 ml of sera, respectively), and were completely protected from pulmonary virus infection when challenged i.n. with 10^4 $TCID_{50}$ of MV M02 3 weeks later. In contrast, uninoculated cotton rats, or cotton rats inoculated i.n. with UV-inactivated MV M02, did not produce significant levels of circulating virus-specific neutralizing antibodies and were not protected from pulmonary infection when comparably challenged.

Discussion

Previous efforts by us (32) and others (33) to infect cotton rats with clinical MV strains have been largely unsuccessful. In our hands (see Table II for an example), virus was usually rapidly cleared from inoculated cotton rats so that by Day 4 after virus administration, viable virus was only occasionally detectable in any test animal. It was thus surprising to find in the present studies that significant quantities of virus could be detected in the lungs and peritoneal cavities of cotton rats 4 days after these animals were inoculated with most (six of seven) of the Zambian-derived virus strains tested (see Fig. 1). Indeed, the $CRID_{50}$ values obtained for two of the strains (i.e., M02 and A99) were reasonably low (i.e., between 100 and 200 $TCID_{50}$) follow-

Table IV. Comparison of Measles Virus Titers in Cellular and Fluid Portions of Lung and Peritoneal Suspensions Obtained 4 Days After Virus Inoculation[a]

Animal number	Source of suspension	Titer (log_{10})[b]	% MV detected in supernatant[c]	% MV detected in nonadher cells[c]	% MV detected in adher cells[c]
1	Lung	4.1	0	10	90
2	Lung	4.1	0	3	32
3	Lung	4.1	0	10	90
4	Lung	3.6	0	0	32
5	Lung	4.1	0	10	32
6	Lung	4.4	0	3	10
7	Lung	3.9	3	10	90
8	Lung	4.4	0	10	90
9	Peritoneum	4.8	0.03	3	90
10	Peritoneum	4.8	0.01	1	32
11	Peritoneum	4.3	0.03	0.3	99

[a] The lungs of animals inoculated intranasally were collected 4 days after virus inoculation and lavaged. Peritoneal fluids were collected from animals inoculated intraperitoneally on Day 4. These suspensions were titered and then separated into fluid, nonadherent (nonadher) and adherent (adher) cell fractions as described in Materials and Methods. Each fraction was then tested for virus.
[b] The values displayed are titers measured in the original (unfractionated) suspension; lung titers are expressed as $TCID_{50}$ (log_{10})/g lung; titers in peritoneal fluids are expressed as $TCID_{50}$/ml peritoneal lavage fluid.
[c] These percentages were based on the mean virus titers detected in the original (unfractionated) suspensions.

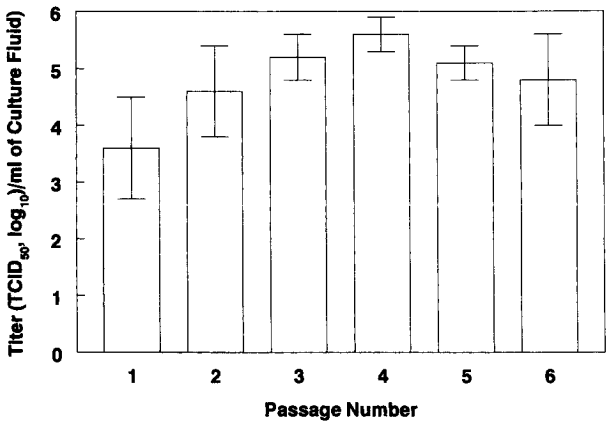

Figure 3. Geometric mean measles virus titers ($TCID_{50}$ log_{10}/ml of culture fluid) in six successive subcultures of lung cells originally obtained by lavage from the lungs of a cotton rat inoculated intranasally 4 days previously with measles virus strain M02. Each subculture was made 2–4 weeks apart.

ing intranasal inoculation, and 10-fold lower following intraperitoneal inoculation (see Table III). The kinetics of replication of these two MV strains in lungs of inoculated cotton rats (see Fig. 2) proved to be similar to that observed in cotton rats infected with the Edmonston strain of MV (32), RSV (29) and PIV3 (31).

As with the Edmonston MV strain, there was considerable evidence that leukocytes were the primary target cells for the inoculated virus. First, infectious virus was readily isolated from lung and peritoneal lavage suspensions, both of which could be shown by acridine orange or Leukostat stain to consist primarily of leukocytes. Second, all of the sites from which viable MV was isolated (i.e., the lungs, peritoneal cavity, mediastinal lymph nodes, and spleen) contain large numbers of leukocytes. Third, more virus was consistently recovered from the peritoneal cavities of inoculated animals than from the lungs (Fig. 1). The simplest explanation for this is that approximately10-fold more leukocytes (target cells) were routinely obtained from the peritoneal cavities of test animals than from their lungs (data not shown). Fourth, in fractionation studies, most of the virus detected in lung and PE suspensions was closely associated with cellular fractions, particularly those that adhered to plastic (Table IV). These appeared to be primarily macrophages. (It should be noted that in these studies, 100% of the virus detected in the original [unfractionated] suspensions was seldom recovered. This was most likely due to loss of cells during the fractionation and washing procedures.) However, the most convincing evidence of MV multiplication in cotton rat leukocytes was the demonstration of persistent infection of cultured lung and PE cells obtained from virus-infected cotton rats (Fig. 3). Based on their morphology (relatively large cells with abundant cytoplasm), phagocytic capability and positive staining for nonspecific esterase (see Fig. 4, panels A – C), the cells in these cultures appeared to be overwhelmingly macrophages or cells of the same lineage (35, 39).

Because MV Edmonston has been reported to translocate from the lungs of cotton rats inoculated i.n. (33), extrapulmonary dissemination of virus was looked for in the present studies. As indicated in Table V, viable MV was isolated from all lungs, 13% of the spleens and 56% of the MDLN collected on Day 4 from cotton rats inoculated i.n. No viable virus was detected in the other organs collected on this day (i.e., brain, blood, liver, or thymus), or in any organ other than the lungs on Day 7. These findings are not very disparate from those reported by others (33) who, using an RT-PCR assay, found evidence of MV RNA in most of the organs of cotton rats experimentally infected with MV Edmonston 4 and 7 days after virus inoculation, but viable virus only in lungs. However, it does not appear that the group looked for virus in the MDLN.

The clinical MV strains used in these studies did not

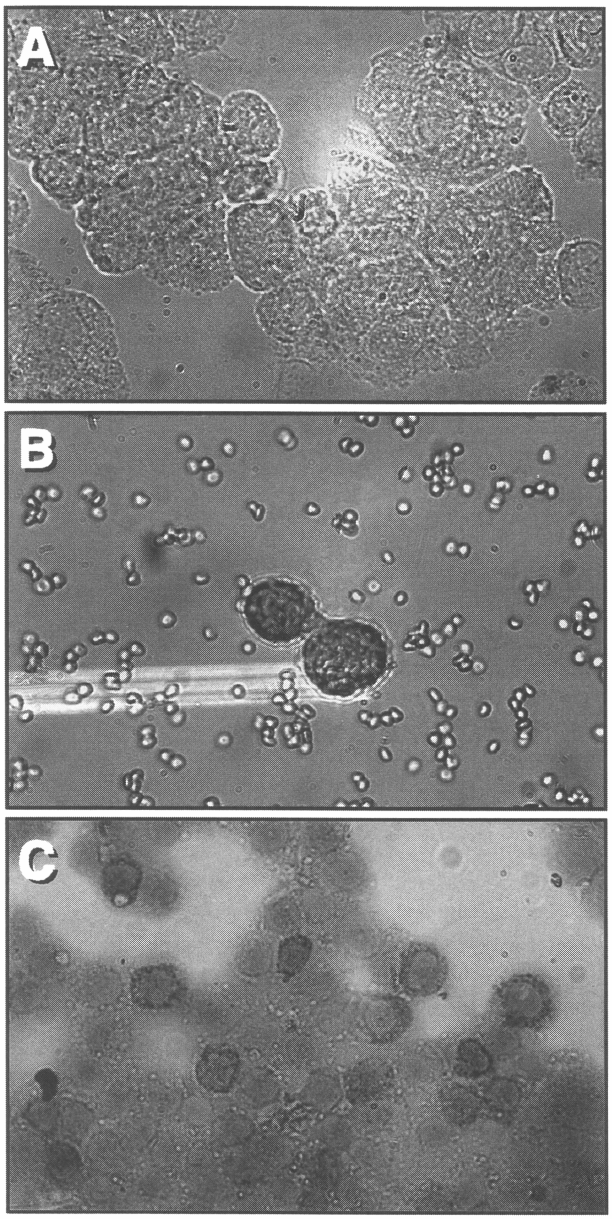

Figure 4. (Panel A) Photomicrograph (100X) of unstained cultured cotton rat lung cells. These cells were present in the third subculture of cells obtained by lavage from the lungs of a cotton rat 4 days after this animal was intranasally inoculated with measles virus M02. (Panel B) Photomicrograph (100X) of cells taken from the same culture as those shown in Panel A, but after these cells were exposed to antibody coated (opsonized) sheep red blood cells (rbc) for 4 hr. The cotton rat cells were then removed from their culture flasks, added to standard hemacytometers, and observed for phagocytized rbc. Every cell observed (100) had the appearance of the two shown (i.e., they were filled with rbc). (Panel C) Photomicrograph (20X) of lung cells from the same culture shown in Panel A, but pelleted by cytocentrifugation onto glass slides and stained for nonspecific esterase using the procedure of Yam *et al.* (1970). All of the cells observed were positive, to varying degrees, for this enzyme. Unactivated U937 monocytoid cells stained similarly (negative control) did not exhibit any of the red color indicative of nonspecific esterase (not shown).

cause overt disease. Evidence of infection could only be obtained by sacrificing test animals and showing increased inflammation in lung tissues compared to that seen in sections of lung from uninfected or sham-inoculated animals, or by measuring viable virus in lung or PE suspensions. Despite this limitation, the results obtained suggest that the cotton rat may be useful to evaluate candidate MV vaccines and potential antivirals, particularly since virus levels were consistent, and similar results were obtained with several clinical MV strains. As an example of the model's potential utility, we were able to show that either prior infection or immunization of the animals with standard E-M vaccine several weeks prior to virus challenge was able to induce virus-specific neutralizing antibodies and protect animals from pulmonary MV infection (Table VI). In contrast, UV-inactivated M02 virus administered i.n. was neither immunogenic nor protective, although the amount of inactivated M02 virus administered to the test animals was equivalent to that of the live M02 virus used in this experiment.

The cotton rat model may also be useful for MV pathogenesis studies. The data obtained in the earlier (32, 33) and present studies indicate that MV infection of cotton rats has at least three characteristics in common with natural MV infections: There is 1) primary infection of leukocytes; 2) virus translocation from the lungs; and 3) immunosuppression of the infected host. (The last was not investigated in these studies, but has been reported to occur in cotton rats infected with MV Edmonston (2)). Thus despite the failure to see overt disease in this model, many of the hallmarks of measles are present.

The reason(s) for the discordant results obtained in our earlier and present studies are not entirely clear. Both were performed similarly, with only two major exceptions: 1) Vero cells were used to prepare all of the working stocks of virus in the initial studies, whereas B95–8 cells were used in the present ones; and 2) all of the MV strains used in the 1992 studies were isolated in the United States. In contrast, all of the strains used in the present studies were originally isolated in Zambia.

The use of different tissue culture cells in the two studies could have affected their outcomes. It has been reported that measles viruses that are grown in different tissue culture cells lines may use different host cell receptor site(s) (40). Thus CD46 has been reported to be the primary host cell receptor for measles viruses grown in Vero cells and other common tissue culture lines (41), but apparently not for wild-type measles viruses isolated and then propagated exclusively in B95–8, other monkey B lymphocyte lines, or human B lymphocytes (42). However, it is unlikely that the use of different tissue culture cell lines in the two studies was a significant factor affecting the different outcomes. First, the Edmonston MV strain shown to grow in cotton rats (32, 33), replicates well in Vero cells. Second, both the M02 and A99 MV strains that we adapted to grow well in Vero cells by serially passaging them in this cell line, grew as well in cotton rats as the original isolates that could only grow well in B95–8 cells (data not shown).

Use of virus strains from different geographic areas could have affected the outcomes of these studies. Genetic

Table V. Frequency of Detection of Virus in Different Organs Collected 4 Days After Intranasal Inoculation of Cotton Rats with Measles Virus M02[a]

Number of animals tested for virus	Organ tested	Number of organs positive/ no. organs tested	Percentage MV positive
16	Brain	0 of 16	0
16	Blood	0 of 16	0
16	Thymus	0 of 16	0
16	Lung	16 of 16	100
16	MDLN[b]	9 of 16	56
16	Spleen	2 of 16	13

[a] Lightly anesthetized cotton rats were intranasally inoculated with approximately 100 $CRID_{50}$ of measles virus strain M02. Four days later, each animal was sacrificed, and the appropriate organs were collected and tested for the presence of viable measles virus (MV) as described in Materials and Methods.
[b] Abbreviations: MDLN = mediastinal lymph node; MV = measles virus.

Table VI. Comparison of Serum-Neutralizing and Pulmonary Virus Titers in Uninoculated Cotton Rats, or Cotton Rats Inoculated with Live or Inactivated Measles Virus Preparations[a]

Group no.	Virus administered	Mean MV-specific neutralizing antibody in sera (log_2 $TCID_{50}$/0.05 ml)[b]	Mean pulmonary MV titer (log_{10} $TCID_{50}$/g lung)[b]
1	None	1.3 ± 0.6	4.0 ± 0.5
2	UV-Inac. MV M02	1.3 ± 0.6	4.1 ± 0.3
3	Live MV E-M vaccine	**7.9 ± 0.5**	**0**
4	Live MV M02	**9.3 ± 0.9**	**0**

[a] Cotton rats were inoculated intramuscularly (i.m.) with approximately 10^4 $TCID_{50}$ of measles virus (MV) Enders-Moraten (E-M) or intranasally (i.n.) with a similar dose of live MV M02. Control animals were given placebo (media) i.m. and i.n. or UV-inactivated MV M02 (10^4 $TCID_{50}$ prior to inactivation) i.n. Three weeks later all of the animals were bled and challenged i.n. with 10^4 $TCID_{50}$ of MV M02. Four days later the animals were sacrificed, and the lungs were assayed for virus.
[b] The mean serum neutralizing and pulmonary virus titers obtained for Groups 2–4 were compared to those of Group 1 using the Kruskal-Wallis nonparametric analysis of variance. Bolded values indicate statistical significance $P \leq 0.05$; number (no.) of animals/group = 3 or 4; 0 = no virus detected (minimum detection = $10^{1.4}$ $TCID_{50}$/g lung).

differences among wild-type MV strains that can affect their transmission and epidemiology have been reported (43–46). Moreover, these variations tend to cluster according to geographic regions (44, 45). Perhaps more importantly, different MV strains may alternatively use CD46 or another (unknown) molecule as cell receptors, and the degree of usage of these may be MV strain–specific (47). Thus it is possible that the variability seen in the different studies, or even within the present study (see Fig. 1), is due to differences in the ability of the different MV strains to use whatever receptor site is present on cotton rat cells. If true, it may be possible to investigate these patterns more fully and gain some insight into the identity of the alternative MV receptor site.

As the discussion above indicates, there is a need to find an alternative to nonhuman primate models for studying MV pathogenesis, and especially for at least initial evaluation of potential MV vaccines and antivirals. The data presented in this report suggest that the cotton rat may provide such an alternative.

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