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Use of Cotton Rats to Evaluate the Efficacy of Antivirals in Treatment of Measles Virus Infections

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No practical animal models for the testing of chemotherapeutic or biologic agents identified in cell culture assays as being active against measles virus (MV) are currently available. Cotton rats may serve this purpose. To evaluate this possibility, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR) and poly(acrylamidomethyl propanesulfonate) (PAMPS), two compounds that have been reported to inhibit MV in vitro, and ribavirin, an established antiviral drug with MV-inhibitory activity, were evaluated for their antiviral activities against MV and respiratory syncytial virus (RSV) in tissue culture and in hispid cotton rats. A single administration of PAMPS markedly inhibited pulmonary RSV or MV replication (>3 $\log_{10}$ reduction in pulmonary titer compared to that for controls), but only if this compound was administered intranasally at about the time of virus inoculation. Both EICAR and ribavirin exhibited therapeutic activity against RSV and MV in cotton rats when they were administered parenterally. However, both of these compounds were less effective against MV. On the basis of the pulmonary virus titers on day 4 after virus inoculation, the minimal efficacious dose of EICAR against MV (120 mg/kg of body weight/day when delivered intraperitoneally twice daily) appeared to be three times lower against this virus than that of ribavirin delivered at a similar dose (i.e., 360 mg/kg/day). These findings correlated with those obtained in vitro. The data obtained suggest that cotton rats may indeed be useful for the initial evaluation of the activities of antiviral agents against MV.

Despite the availability of efficacious vaccines and eradication efforts, between 500,000 and 1 million children still die annually due to measles virus (MV)-related infections (6, 7, 9). No chemotherapeutic agents are currently approved for use for the prophylaxis or treatment of measles, although there are case reports that ribavirin used alone and administered intravenously (i.v.) or orally (4, 11, 13) or ribavirin combined with immune serum globulin (27, 30) administered i.v. can provide clinical benefit in patients with this disease. No clinical trials have been performed to substantiate these findings, and there have been case studies in which no clear clinical benefit was seen when ribavirin was administered to patients with measles either by intravenous administration (19) or by continuous small-particle aerosol (spa) (3). Regardless of their clinical efficacies, both ribavirin and immune serum globulin are costly (10, 21) and require hospitalization for administration (26), limiting the use of either of these agents for the treatment of measles, particularly in developing countries where this disease is most preponderant. The continued prevalence of measles and the paucity of available agents for the prevention or treatment of MV infections make the elucidation and development of new chemotherapeutic and/or biologic agents effective against this virus highly desirable.

Potential agents with activity against MV can readily be identified by cell culture-based antiviral assays. However, no practical animal model is currently available for the testing of materials identified in these assays for safety and efficacy or evaluation of the effects of altering routes of administration, drug doses, schedules, and regimens. This inability may be a factor in the failure to develop compounds that might be effective against MV. Monkeys could be used to test these materials since a number of monkey species are susceptible to MV infection and develop a disease very similar to measles in humans (2, 5, 15). However, it is difficult and costly to use these animals to work out the most basic of questions (e.g., optimal drug doses, routes of inoculation, schedules, or the safety and efficacy of different drug combinations).

Recently, MV strains that can replicate in the lungs of hispid cotton rats, albeit without causing overt illness, have been elucidated (25, 37, 38), thus opening the possibility that these animals can be used for initial in vivo testing of potential candidates with activity against MV. To help evaluate this potential, three compounds—ribavirin, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (EICAR), and poly(acrylamidomethyl propanesulfonate) (PAMPS)—reported to have good activity against MV in tissue culture-based assays and possibly in vivo (i.e., ribavirin), were tested for efficacy against MV in tissue culture and in hispid cotton rats experimentally infected with this virus. The results of these tests and the potential of using cotton rats as an animal model for initial testing of materials found to be active against MV in tissue culture assays are discussed below.

MATERIALS AND METHODS

Cell culture. HEp-2 (human epithelial carcinoma; ATCC CCL23) and B95-8 (Epstein-Barr virus-transformed marmoset leukocytes; ATCC CRL1612) culture cells were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). The former cells were used to prepare stocks of respiratory syncytial virus (RSV) and to perform assays that involved that virus. The latter cells were used to prepare stocks of MV and to carry out assays that involved that virus. Minimum essential medium (MEM; catalog no. M4655; Sigma Chemical Co., St. Louis, Mo.) supplemented with 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 g/ml), sodium bicarbonate (0.2%), and L-glutamine (2 mM/ml) (5% FBS–MEM) was used to grow the HEp-2 cells and was used for all

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assays and procedures involving these cells. RPMI 1640 medium (catalog no. 51501-78P; JRH Biosciences, Lexana, Kans.) supplemented exactly as described for MEM was used to grow the B95-8 cells and was used for all procedures with this cell line. All of the media supplements were purchased from Sigma Chemical Co.

Viruses. Seed RSV A2 virus was obtained from ATCC (catalog no. VR1302). A working stock of this virus was prepared by infecting monolayers of HEp-2 cells with this virus and placing the infected monolayers in a 36°C (5% CO_2) incubator for 4 to 6 days until they exhibited >90% syncytium formation. At that time, the flasks were removed, placed in a Branson model 220 sonicating water bath, and subjected to six 15-s bursts of sonication (each at 50 to 60 Hz). The medium was then collected from each flask, pooled, and centrifuged at 450 × *g* for 20 min. The virus-rich supernatant was passed through a 0.45-μm-pore-size filter (Acrodisc; catalog no. 4184; Gelman Scientific, Inc., Ann Arbor, Mich.), portioned, labeled, and frozen at −70°C.

The M02 strain of MV used in these studies was originally isolated in B95-8 cells in 1993 from a Zambian child with measles. The virus was passaged three times in B95-8 cells before being sent to us by Hiroshi Suzuki (Department of Public Health, Niigata University, Niigata, Japan), with permission of his collaborators, Katsumi Mizuta (Virus Research Center, Sendai National Hospital, Sendai, Japan) and Mwila E. Mpabalwani (Virology Laboratory, The University Teaching Hospital, Lusaka, Zambia). The characterization of this virus and its growth in cotton rats have been described in detail elsewhere (38).

A working pool of the M02 strain of MV was prepared in the same manner that was described above for the preparation of stocks of RSV A2, except that B95-8 cells were used to support MV growth. The median cell culture infectious dose ($CCID_{50}$) of each virus pool was determined with 96-well plates (Falcon 3072) as described previously (36).

Animals. All of the cotton rats (*Sigmoden hispidus*) used in these studies were obtained from the Baylor College of Medicine cotton rat colony. This colony was started in 1984 with six pairs of animals obtained from the Small Animal Section, Veterinary Research Branch, Division of Research Services, National Institutes of Health. All of the animals were housed in cages covered with barrier filters and were given food and water ad libitum. Six- to 10-week-old (60- to 110-g) cotton rats of either sex were used in all experiments.

Compounds. PAMPS was obtained from Monomer-Polymer and Dajac Laboratories Inc. (Trevose, Pa.) as a sterile solution in distilled water. Ribavirin was acquired from ICN Pharmaceuticals (Costa Mesa, Calif.). EICAR was supplied by A. Matsuda, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan. The synthesis and characterization of this material have been described in detail elsewhere (23, 24). Sterile solutions of ribavirin and EICAR were prepared by weighing out the appropriate amounts of each and suspending them in tissue culture-grade distilled water (catalog no. W-3500; Sigma Chemical Co.). The resulting solutions were then filter sterilized with 0.2-μm-pore-size hollow-fiber syringe filters (DynaGard; Microgon, Inc., Laguna Hills, Calif.).

Inoculation of animals. Intranasal (i.n.) inoculations of virus and PAMPS were performed by gradually introducing 50 μl of virus or drug solution into the nares of cotton rats anesthetized with methoxyflurane (Metofane; Pitman-Moore, Mundelein, Ill.) with a P-2000 pipetting aid (Rainin Instruments Co., Inc., Woburn, Mass.). For intraperitoneal (i.p.) inoculations, the cotton rats were rendered unconscious with CO_2, and then the appropriate material was injected i.p. with 1-ml tuberculin syringes (catalog no. 9602; Becton-Dickinson and Co., Rutherford, N.J.).

Aerosol treatments. spa-generating units equipped with Collison nebulizers were used to deliver ribavirin by continuous spa. The use of these units to deliver antiviral agents, including ribavirin, has previously been described in detail (35). In these studies, the animals were inoculated i.n. on day 0 with RSV or MV M02 and were then exposed to ribavirin aerosols for 16 h a day on days 1 through 3 after virus inoculation. At the start of each day of treatment, a suspension of ribavirin containing 20 mg of ribavirin/ml of water was prepared and was placed into the aerosol delivery reservoir. An air compressor connected to the spa-generating unit was then started and was regulated to deliver 12.5 liters of aerosol per min to the test animals, which were housed in plastic cages covered with plastic tops. The placebo control animals were comparably inoculated with MV M02 but received aerosols containing distilled water only. All of the animals were killed on day +4, at which time their lungs were tested for virus levels.

The estimated maximum dosage of ribavirin delivered per cotton rat on each treatment day was calculated by using the formula maximum dosage of drug = respiratory volume of cotton rats per minute per kilogram of body weight (14) × 60 min/h × 16 h × the estimated retention value (31) × concentration of ribavirin in the aerosol (22, 34). For example, by using 20 mg of ribavirin/ml in the aerosol reservoir, 0.7 liter-min/kg × 60 (min/h) × 16 h × 0.5 × 200 μg/liter is equal to 67.2 mg of drug/kg/day.

In vitro cytotoxicity assays. The test compounds were tested for cytotoxicity in quadruplicate in 96-well flat-bottom plates (Falcon 3072). In these assays, ribavirin, EICAR, or PAMPS was added to the initial wells of the plates so that the final concentration of each was 1,000 μg/ml. Vehicle (H_2O) was added to adjacent wells. The compounds and vehicle were then diluted longitudinally up the plate by using serial twofold dilutions. Approximately 3×10^3 tissue cells were added to each well, including those that contained just medium (cell control). With this concentration, the monolayers that formed following settlement and attachment of the cells were 20 to 30% confluent.

Wells containing the cell controls were observed daily. When the monolayers in these wells became confluent, all of the wells were observed for cytotoxicity or inhibition of monolayer formation. The median cytotoxic concentration (CC_{50}) was calculated (in micrograms per milliliter) for each cell type by determining the mean concentration of test compound in the last wells of replicate rows that exhibited >20% cytotoxicity or <50% confluence. The latter was confirmed by using the fluorophore Alamar Blue (AB; catalog no. DAL1100; Biosource International, Camarillo, Calif.). Briefly, after the wells were observed microscopically for cytotoxicity, 20 μl of AB was added to each. The plates were then placed in the CO_2 incubator for 6 h. At the end of that period, they were removed and put in a Fluorolite 1000 microplate fluorometer (Dynatech Laboratories, Chantilly, Va.) equipped with a 546DF10 excitation filter and a 590DF35 emission filter. The amount of fluorescence present in each well was then determined. This fluorescence was directly proportional to the level of reduction of AB that occurred during the 6-h incubation, which in turn was dependent on the number and metabolic activity of the viable cells present in each well at the time that the AB was added (1, 32).

In vitro antiviral assays. Assays for assessment of the antiviral activities of the test compounds were performed in 96-well flat-bottom tissue culture plates (Falcon 3072). In these assays, the test compounds or vehicle was added to the initial wells in quadruplicate (test compounds) or duplicate (vehicle) and serially diluted (2-fold) in 2% FBS–MEM (assays with RSV and HEp-2 cells) or 2% FBS–RPMI 1640 medium (assays with MV and B95-8 tissue cells) longitudinally up the test plates. Equal volumes (50 μl) of RSV or MV containing approximately 100 $CCID_{50}$s of virus were then added to all wells except those set aside as antiviral agent and cell control wells. Approximately 3×10^3 HEp-2 or B95-8 cells (100 μl) were then added to each well. Control wells that contained antiviral agent and no virus (antiviral agent control), virus but no antiviral agent (virus control), or medium but no virus or antiviral agents (cell control) were included in each test. The plates were placed in a 36°C (5% CO_2) incubator for 5 to 7 days, during which time the virus control wells were observed daily. When virus-induced cytopathic effects (CPEs) in these wells were 70 to 100% evident, every well in the assay was observed for CPEs. The median efficacious concentration (EC_{50}) was calculated after determining the final concentration of antiviral agent in the last wells of each set of replicate rows that exhibited <50% CPE compared to the CPEs in virus control wells. The selectivity index (SI) for each compound and virus was calculated by dividing the appropriate CC_{50} by the appropriate EC_{50} (e.g., the SI of ribavirin for RSV is equal to the CC_{50} of ribavirin in HEp-2 cells/EC_{50} of ribavirin against RSV).

In vivo antiviral assays. On day 0 of the in vivo antiviral assays, the animals were weighed, anesthetized with methoxyflurane, and inoculated i.n. with 50 μl of medium containing approximately 10^4 $CCID_{50}$s of MV M02 or RSV A2. In experiments with ribavirin or EICAR, the animals were generally treated i.p. twice daily (b.i.d.) with test compound or vehicle on days 1 through 3. The exception to this was when ribavirin was administered on days 1 through 3 by spa. In experiments with PAMPS, drug was always administered i.n. Moreover, different administration schedules were used. Thus, some groups of animals were inoculated with PAMPS only on day 0, others were inoculated on days 0 through 3, and still other groups were inoculated on days 1 through 3. On day 0, the time of administration of this compound relative to the time of administration of virus varied. For example, sometimes the animals were inoculated with PAMPS 5 min after virus inoculation, sometimes the animals were inoculated with PAMPS 15 min after virus inoculation, and sometimes the animals were inoculated with PAMPS 60 min after virus inoculation. Regardless of the compound, route of drug administration, or inoculation schedule, the cotton rats were always killed on day 4 after virus inoculation, when peak virus levels normally occur in placebo-treated animals (37, 38). The lungs of these animals were removed, weighed, rinsed in phosphate-buffered saline, and then transpleurally lavaged with 3 ml of 5% FBS–MEM as described previously (33). The resulting lung fluid suspensions were placed on ice and assessed as soon as possible (usually between 1 and 2 h after collection) for RSV or MV levels as described above. Special care was taken with the suspensions obtained from animals inoculated with MV to keep the cells in the test fluids suspended during the titrations. This was done because it has been our experience that this virus remains cell associated until very late in infection and either is not detectable or is detectable only at a low titer in lung lavage fluids free of lung cells (37). Lung virus titers ($\log_{10}$ $CCID_{50}$ per gram of lung) were calculated for each lung. These were used to calculate the geometric mean virus titer for each group. The minimal amount of virus detectable in these assays was 100 $CCID_{50}$s/g of lung.

Statistics. The geometric mean virus titers in the experimental groups were compared to the mean virus titer in the placebo control group by the Kruskal-Wallis nonparametric analysis of variance if there were more than two treatment groups in the experiment. If there were only two groups in an experiment, the geometric mean virus titers were compared by the Mann-Whitney nonparametric statistic test. General statistics (e.g., means and standard deviations) were calculated by using Instat (GraphPad Software, San Diego, Calif.), a software program for International Business Machines Corp.-compatible computers.

TABLE 1. Comparison of the cytotoxicities and antiviral efficacies of ribavirin, EICAR, and PAMPS against RSV and MV in HEp-2 and B95-8 cell cultures[a]

Compound	Virus	Cell culture	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI
Ribavirin	RSV	HEp-2	500 ± 0	10 ± 3	50
Ribavirin	MV	B95-8	750 ± 0	47 ± 21	16
EICAR	RSV	HEp-2	750 ± 0	1.4 ± 0.9	536
EICAR	MV	B95-8	750 ± 0	8 ± 0	94
PAMPS	RSV	HEp-2	>1,000 ± 0	0.3 ± 0.9	>3,300
PAMPS	MV	B95-8	>1,000 ± 0	0.5 ± 0	>2,000

[a] The CC_{50}s and EC_{50}s of each test compound were determined as described in Materials and Methods. Shown are the mean values obtained in two replicate experiments in which each compound was tested in quadruplicate by using serial twofold dilutions; SIs were calculated by dividing the mean CC_{50} by its respective mean EC_{50}.

RESULTS

Cytotoxicities and antiviral activities of test compounds in vitro. The cytotoxicities of ribavirin, EICAR, and PAMPS in proliferating HEp-2 and B95-8 cell cultures are shown in Table 1. As indicated by the CC_{50}s obtained for ribavirin (i.e., 500 μg/ml) and EICAR (i.e., 750 μg/ml), both compounds either were lethal to these cells or inhibited their growth at the higher test concentrations. In contrast, PAMPS did not inhibit either cell line, even at the highest concentration of this compound tested (i.e., 1,000 μg/ml).

Despite their cytotoxicities, both ribavirin and EICAR manifested significant selective antiviral activity. As indicated in Table 1, the EC_{50} of ribavirin for RSV, 10 μg/ml, was 50-fold lower than its CC_{50} for HEp-2 cells, and its EC_{50} for MV, 47 μg/ml, was 16-fold less than the CC_{50} of this compound for B95-8 cells (i.e., 750 μg/ml). The SI obtained for EICAR against RSV in HEp-2 cells was 536, and the SI was 94 for MV in B95-8 cells, suggesting that this compound was 5- to 10-fold more active against these viruses than ribavirin. Because PAMPS had a CC_{50} of >2,000 μg/ml for both cell lines and was inhibitory to both RSV and MV at concentrations of <1 μg/ml, this compound had the highest SIs obtained in these tests (>3,300 versus RSV in HEp-2 cells and >2,000 versus MV in B95-8 cells).

Antiviral activity of ribavirin in vivo. In initial experiments with ribavirin against MV in cotton rats, the drug was delivered by continuous spa by using reservoir concentrations and delivery schedules that have proven to be effective in significantly reducing pulmonary virus titers in cotton rats experimentally infected with RSV (12, 39) (i.e., daily exposure of virus-infected animals on days +1 through +3 following virus inoculation for ≥12 h/day to ribavirin aerosols generated from delivery reservoirs containing 20 mg of ribavirin/ml). However, as the data in Table 2 indicate (experiment 1), under these conditions ribavirin did not significantly reduce pulmonary MV titers compared to the mean virus titers obtained for the animals treated with placebo (i.e., distilled water). (The estimated maximum dose of ribavirin that each cotton rat received was 67.2 mg/kg/day.) In contrast, this same regimen and dose of ribavirin significantly reduced the pulmonary RSV titers in the positive control group (group 4).

Because it was not practical to administer higher concentrations of ribavirin for such sustained periods by spa, in subsequent experiments, ribavirin was administered i.p. to the cotton rats. In the representative experiment shown in Table 2 (i.e., experiment 2), the animals were injected twice daily on days +1 through +3, with groups 2 to 4 receiving doubling doses of ribavirin starting with 90 mg/kg/day (the standard dose of ribavirin used in this laboratory to inhibit RSV replication in cotton rats when ribavirin is delivered parenterally). As indicated in Table 2, no significant reduction in mean pulmonary MV titer was seen in the groups of infected cotton rats inoc-

TABLE 2. Pulmonary virus titers 4 days after virus challenge of cotton rats administered ribavirin i.p. or by continuous spa daily[a]

Expt no. and group	Challenge virus	Treatment	Daily dose[b] (mg/kg)	Route of inoculation	Virus titer (log_{10}/g lung) in cotton rat no.:				Mean ± SD titer
					1	2	3	4	
Expt 1									
1	MV	Placebo	0	spa	4.6	4.6	4.1	4.6	4.5 ± 0.3
2	MV	Ribavirin	67	spa	4.6	4.6	3.6	4.1	4.2 ± 0.5
3	RSV	Placebo	0	spa	4.0	4.5	3.5	4.0	4.0 ± 0.4
4	RSV	Ribavirin	67	spa	2.0	0[c]	3.0	2.5	**2.3 ± 0.6**[d]
Expt 2									
1	MV	Placebo	0	i.p.	4.5	4.0	4.0	4.5	4.3 ± 0.3
2	MV	Ribavirin	90	i.p.	3.5	3.5	4.0	4.0	3.8 ± 0.3
3	MV	Ribavirin	180	i.p.	3.0	4.0	3.5	3.5	3.5 ± 0.4
4	MV	Ribavirin	360	i.p.	3.5	3.5	3.0	3.5	**3.1 ± 0.5**[d]
Expt 3									
1	RSV	Placebo	0	i.p.	4.1	4.6	4.6	4.1	4.0 ± 0.4
2	RSV	Ribavirin	90	i.p.	3.5	3.5	2.5	2.6	**3.0 ± 0.6**[d]
3	RSV	Ribavirin	180	i.p.	3.1	2.5	2.0	2.5	**2.5 ± 0.5**[d]

[a] Animals were inoculated i.n. on day 0 with 10^4 $CCID_{50}$s of MV or RSV. On each of the next 3 days, the animals in experiment 1 were exposed for 16 h to continuous spas of placebo (distilled water) or ribavirin (reservoir concentration of 20 mg/ml), while those in experiments 2 and 3 were inoculated i.p. b.i.d. with either placebo (distilled water) or the indicated dose of ribavirin. All of the animals were killed on day 4 after virus inoculation, at which times their lungs were removed and tested for virus.

[b] The daily dose for animals exposed to spa represents the estimated maximum dosage of ribavirin delivered per cotton rat. The calculation used to obtain this value is provided in Materials and Methods.

[c] A titer of zero indicates that no virus was detected in the lung lavage sample. For statistical analyses, lungs without detectable virus were assigned a value of 1.5 (the minimal detectable level of virus in these assays was 2.0 log_{10}/g of lung).

[d] Boldfaced means and standard deviations indicate statistical significance between means ($P \leq 0.5$) for these groups and the placebo controls as determined by the Mann-Whitney (experiments involving two groups) or Kruskal-Wallis (experiment 2) nonparametric analysis of variance test; the number of animals per group was 4.

TABLE 3. Pulmonary virus titers 4 days after virus challenge in cotton rats administered EICAR i.p. daily[a]

Expt no. and group	Challenge virus	Treatment	Daily dose (mg/kg)	Virus titer (log_{10}/g lung) in cotton rat no.:				Mean ± SD titer
				1	2	3	4	
Expt 1								
1	MV	Placebo	0	4.0	4.5	4.1	3.5	4.0 ± 0.4
2	MV	EICAR	50	4.0	3.5	4.0	4.0	3.9 ± 0.6
3	MV	EICAR	100	3.6	3.0	3.0	3.5	3.3 ± 0.3
4	RSV	Placebo	0	4.0	4.5	4.1	3.6	4.1 ± 0.4
5	RSV	EICAR	100	3.1	2.0	2.5	3.0	**2.7 ± 0.5**[b]
Expt 2								
1	MV	Placebo	0	4.5	3.6	4.0	4.0	4.0 ± 0.4
2	MV	EICAR	120	3.5	2.0	3.0	3.0	**3.0 ± 0.6**[b]

[a] All of the cotton rats used in these experiments were inoculated intranasally on day 0 with 10^4 median tissue culture infectious doses of MV or RSV. On each of the next 3 days, the animals were inoculated i.p. b.i.d. with placebo (distilled water) or the indicated dose of EICAR. All of the animals were killed on day 4 after virus inoculation, at which times their lungs were removed and tested for virus.

[b] Boldfaced means and standard deviations indicate statistical significance between means for these groups and the placebo control group ($P \leq 0.05$) as determined by the Kruskal-Wallis nonparametric analysis of variance test (experiment 1) or the Mann-Whitney test (experiment 2); the number of animals per group was 4.

ulated b.i.d. with 90 or 180 mg of ribavirin/kg. However, a significant ($P = 0.03$) reduction in mean pulmonary virus titer of 1.2 log_{10}/g of lung was observed in the group of animals inoculated in this manner with 360 mg of ribavirin/kg/day. This reduction was comparable to that seen in cotton rats experimentally infected with RSV and treated i.p. b.i.d. with 90 or 180 mg of ribavirin/kg/day (experiment 3, Table 2).

Antiviral activity of EICAR in vivo. Because of the limited supply of EICAR, it was not tested for its activity against MV by spa in cotton rats, nor was it tested parenterally at doses of >120 mg/kg/day. Regardless, ostensible antiviral efficacy was seen in our studies. In two of two experiments in which animals were administered 100 mg of EICAR/kg b.i.d. by i.p. injection, we observed 0.5 to 0.7 log_{10} reductions in mean pulmonary virus titers compared to the mean virus titer determined for control animals infected with MV and treated with placebo (e.g., experiment 1, Table 3). When cotton rats experimentally inoculated with MV were treated i.p. with 120 mg of EICAR/kg, in two of three experiments (e.g., experiment 2 in Table 3), statistically significant reductions in mean pulmonary MV titers were observed ($P = 0.03$ in both of these experiments). The minimal efficacious dose (MED) of EICAR against RSV was not determined. However, 100 mg of this compound/kg administered i.p. b.i.d. reduced pulmonary RSV titers 1.3 log_{10}/g of lung compared to the mean pulmonary virus titer measured in the placebo control animals (see experiment 1, Table 3).

Antiviral activity of PAMPS in vivo. In the initial experiment with PAMPS against MV in cotton rats, PAMPS was administered only once, 5 or 60 min after virus infection. As the data in Table 4 indicate, the cotton rats experimentally inoculated with MV and administered PAMPS (100 mg/kg) i.n. only 5 min later (group 2) had 3.3 log_{10} less virus on day +4 than control animals comparably infected and inoculated with distilled water 5 min after virus inoculation (group 1; $P < 0.01$). In contrast, no significant reduction in mean pulmonary MV titer was observed in cotton rats given 100 mg of PAMPS/kg i.n. 60 min after virus inoculation compared to the titer in those given placebo at this time (groups 4 and 5; $P > 0.05$). Similar findings were seen with RSV. A nearly 4 log_{10} reduction in pulmonary virus titer compared to the mean RSV titer in control animals occurred in cotton rats inoculated with RSV and PAMPS 5

TABLE 4. Pulmonary virus titer on day 4 in cotton rats administered placebo or PAMPS at different intervals after experimental inoculation of the animals with MV[a]

Expt no. and group	Challenge virus	Treatment	Dose (mg/kg)	Time of dosing	Virus titer (log_{10}/g lung) in cotton rat no.:				Mean ± SD titer
					CR1	CR2	CR3	CR4	
Expt 1									
1	MV	Placebo	0	+5 min	4.0	4.5	4.1	3.5	4.1 ± 0.3
2	MV	PAMPS	100	+5 min	0	0	3.0	0	**0.8 ± 1.5**[b]
3	RSV	PAMPS	100	+5 min	0	0	0	0	**0 ± 0**[b]
4	MV	Placebo	0	+60 min	4.0	5.0	4.0	4.0	4.3 ± 0.5
5	MV	PAMPS	100	+60 min	4.0	4.1	4.0	4.5	4.1 ± 0.3
Expt 2									
1	MV	Placebo	0	Days 0–3	5.0	4.5	4.5	5.1	4.0 ± 0.4
2	MV	PAMPS	100	Day 0	3.0	3.5	3.5	3.6	**3.3 ± 0.3**[b]
3	MV	PAMPS	100	Days 1–3	4.0	4.5	4.6	4.0	4.3 ± 0.3
4	MV	PAMPS	100	Days 0–3	3.1	3.0	3.5	3.0	**3.1 ± 0.3**[b]

[a] All of the cotton rats used in these experiments were inoculated i.n. on day 0 with 10^4 median $CCID_{50}$s of MV or RSV. In experiment 1, the animals in groups 1 to 3 were inoculated i.n. with either placebo (distilled water) or PAMPS 5 min after virus inoculation, while those in groups 4 and 5 were inoculated with PAMPS 55 min later. In experiment 2, the animals were inoculated i.n. with PAMPS on the indicated days, with all of the inoculations on day 0 being given 30 min after virus inoculation. All of the cotton rats were sacrificed on day 4 after virus inoculation, at which times their lungs were removed and tested for virus.

[b] Boldfaced means and standard deviations indicate statistical significance between means of these groups and the placebo control ($P \leq 0.05$) as determined by the Kruskal-Wallis nonparametric analysis of variance test; number of animals per group was 4.

min apart, and no significant reduction was seen in animals given RSV and PAMPS an hour apart (data not shown).

In subsequent experiments with PAMPS, more varied delivery schedules were used. As the representative results of an experiment shown in Table 4 indicate (i.e., experiment 2), both of the groups of animals to which PAMPS was first administered on day 0 exhibited >1.5 $\log_{10}$ reductions in MV titers on day +4 compared to the mean pulmonary virus titers determined for the control group (group 1; $P < 0.05$ for both groups). Interestingly, the mean virus titer in the group of cotton rats given PAMPS on days 0 through 3 (i.e., group 4) was not significantly different from mean virus titer calculated for the animals given PAMPS only on day 0 (group 2). In contrast to these reductions, no significant decrease in mean pulmonary virus titer was observed in the group of animals administered PAMPS daily, starting 1 day after virus inoculation and continued through day +3 (i.e., group 3; $P > 0.05$). It should be noted that in this experiment, PAMPS was not administered to the animals in groups 2 and 4 until 15 min after virus inoculation on day 0.

DISCUSSION

The primary goal of the studies described here was to initiate evaluation of hispid cotton rats for use in preliminary in vivo testing of materials that have been identified as having potential activity against MV. Ribavirin was initially considered for inclusion in these studies as a positive drug control since it has been reported to inhibit MV replication both in vitro (17, 29) and in vivo in a clinical setting (4, 11, 13). However, because of conflicting reports about its clinical efficacy (3, 19) and the lack of controls in those studies, ribavirin was included in our experiments, not as a positive control but as a test compound. Although it could not be used as a positive control in in vivo testing, it still acted as a standard to whose antiviral activity the other compounds could be compared. This was because it was easily the best studied of the three test compounds. As an additional point of reference, testing against RSV was also incorporated into most experiments. In addition to acting as a measure of comparison, RSV served in these experiments as a positive paramyxovirus control.

EICAR and PAMPS were picked for study because they, too, have been reported to have significant antiviral activity against MV in in vitro assays. Indeed, the former compound is structurally and functionally related to ribavirin and has been reported to have 10 to 50 times more antiviral activity against MV than ribavirin (8, 28). However, there are no reports on the activity of either of these compounds against MV or RSV in vivo. PAMPS has been tested against influenza virus in a mouse model. In those studies, this compound strongly inhibited influenza virus replication, if it was administered close to the time that the animals were inoculated with virus (18).

All three of the test compounds exhibited selective antiviral activity against MV and RSV in our cell culture assays (Table 1). Ribavirin had the lowest SIs of the three compounds studied in these tests. EICAR appeared to be 5.7-fold more active than ribavirin against RSV and approximately 10-fold more active than this compound against MV. Extraordinarily high selective antiviral activity (i.e., SI of >2,000) was exhibited by PAMPS against both these viruses. All three compounds appeared to be more effective in inhibiting RSV than in inhibiting MV.

Testing of ribavirin against MV was begun with doses that were known to inhibit RSV (12, 38). Thus, in the spa experiments, ribavirin was delivered 16 h/day on days 1 through 3 after virus inoculation by using 20 mg of ribavirin/ml in the aerosol delivery reservoir. By using this regimen and drug concentration, significant inhibition of pulmonary RSV replication was seen (e.g., in experiment 1, Table 2, a reduction in mean pulmonary virus titer of 1.7 $\log_{10}$/g of lung was seen for group 4 compared to that seen for group 3, the placebo control; $P \leq 0.05$). In contrast, no significant inhibition of pulmonary MV replication was observed in the spa experiments (e.g., experiment 1, Table 2). Despite the failure to see reduced MV titers, the results obtained in these experiments were concordant with those obtained in the one published clinical study in which aerosolized ribavirin was evaluated for efficacy against MV infection (3). In that study, no clear clinical benefit was observed in any of the 13 patients exposed to the aerosolized drug.

Daily i.p. administration of 90 mg of ribavirin/kg also failed to inhibit MV replication in lungs of cotton rats (e.g., group 2, experiment 2, Table 2), although this is the dose, regimen, and schedule that, as routinely found in our laboratory, inhibits RSV replication in these animals (see experiment 3 for an example). This result was not unexpected since the in vitro data suggested that it could take up to five times higher concentrations of ribavirin to significantly inhibit MV compared to those required to inhibit RSV. In fact, the apparent MED of ribavirin administered i.p. b.i.d. appeared to be four times that seen with RSV (i.e., 360 mg of ribavirin/kg/day for MV versus 90 mg/kg/day for RSV; experiments 2 and 3, Table 2).

Although EICAR could not be evaluated at doses of >120 mg/kg/day, an apparent MED was determined for this compound. As the data depicted in Table 3 indicate, daily administration of 50 mg of EICAR/kg did not result in a significant reduction of mean pulmonary virus titer compared to the mean titer in control animals (groups 2 and 1, respectively). However, marginal reductions in virus titer were consistently seen in animals administered 100 mg of this compound/kg/day in divided doses (e.g., group 3, experiment 1), and significant reductions occurred in animals treated b.i.d. with 120 mg of drug/kg (group 2 versus group 1, experiment 2). In fact, significant reductions in mean virus titer were observed in only two of three experiments in which the animals were given this dose. It thus appeared that the MED for this compound was about 120 mg/kg/day, or about threefold lower than the MED of ribavirin against MV in cotton rats (i.e., 360 mg/kg/day). These data were in relative accord with the results obtained in our in vitro tests, in which EICAR was found to be approximately sixfold more active against MV than ribavirin. Up to 10- to 50-fold differences in in vitro antiviral activities have been reported between these two nucleoside analogs elsewhere (8, 28).

As in the in vitro experiments, PAMPS markedly inhibited MV replication in cotton rats (Table 4). However, this antiviral activity was highly dependent on the interval of time between virus inoculation and administration of the drug. Thus, when animals were inoculated with PAMPS i.n. 5 min after virus challenge (group 2, experiment 1), a >3 $\log_{10}$ reduction in mean pulmonary MV titer per g of lung was observed compared to the mean virus titer for the placebo control group (group 1, experiment 1). In contrast, increasing the interval just 55 min, to 1 h (group 5), resulted in a complete loss of antiviral activity. Even a quarter of an hour delay caused a significant diminution in the antiviral effect. For example, only a 1.5 $\log_{10}$ reduction in mean virus titer occurred in animals administered PAMPS 15 min after virus inoculation (i.e., group 2, experiment 2).

Interestingly, PAMPS appeared to be effective only in preventing primary virus infection. Two findings support this view: (i) there was no significant reduction in mean pulmonary virus

titer in the group of animals that was inoculated with PAMPS starting on day 1 and continuing through day 3 (i.e., group 3, experiment 2, Table 4), but there was a significant reduction in both groups that received PAMPS on day 0 (i.e., groups 2 and 4), and (ii) there was no significant difference in the mean pulmonary virus titers found in cotton rats treated with PAMPS on days 0 through 3 (e.g., group 4, experiment 2, Table 4) and those treated with this compound only once on day 0 (e.g., group 2, experiment 2). The reasons for this are not clear. However, PAMPS is thought to prevent virus replication by inhibiting virus attachment (18). Thus, the ability of MV to spread from cell to cell following fusion of host cell cytoplasmic membranes without exiting the host cell (thus bypassing the need for the virus to bind to host cell receptors to cause subsequent infection) (20) may have been a contributing factor. It is also possible that following intranasal inoculation PAMPS was not distributed evenly or sufficiently enough to effectively protect the respiratory tract from the logarithmically increasing and more dispersed infectious particles that occur in the respiratory tract during virus infections. Regardless, the findings obtained with PAMPS are similar to those reported by Ikeda et al. (18), who tested the activity of this compound against influenza virus in mice.

Unless one finds a way to maintain PAMPS in the respiratory tract, use of this compound as an antiviral agent for the prevention of MV infections is very improbable. Ribavirin is also an unlikely candidate for further development as an anti-MV agent. The data that support its efficacy against MV are too limited and mixed to warrant implementation of large-scale, controlled clinical trials. In addition, this compound is expensive (10, 26) and a potential teratogen (16). Use of increased doses is not practical since this would also increase the chances for adverse effects.

Of the three compounds used in these studies, EICAR has the most potential for development as an agent for use against MV. In its favor, it appeared to be severalfold more active than ribavirin against MV in these studies and has been reported to be many times more active than that drug by others (8, 28). However, this compound is structurally and functionally related to ribavirin and may share some of ribavirin's teratogenic and toxic properties. It should be noted that although no overt cytotoxicity was apparent in any of the cotton rats used in these studies, no histologic, serum enzyme, or other cytotoxicity testing was performed. Moreover, in none of the experiments did the drug treatment period exceed 4 days.

In toto, our findings suggest that hispid cotton rats may indeed be useful for initial evaluation of compounds that have been identified in tissue culture assays as potential MV inhibitors. In a previous study (38), it was demonstrated that these animals could be reproducibly infected with clinical isolates of MV (e.g., the M02 strain used in these studies). In this study we showed that pulmonary MV infection could be suppressed by treatment with compounds with known in vitro anti-MV activity. Moreover, this antiviral activity paralleled that seen in vitro (i.e., PAMPS had greater activity than EICAR, which had greater inhibitory activity than ribavirin). In addition, the results obtained with PAMPS were similar to those published previously on the activity of this compound against influenza in mice. Both studies demonstrate that PAMPS must be given nearly concomitantly with the initiation of virus infection to have an effect. Of the three test compounds, only ribavirin has been used in clinical settings. Our finding that ribavirin was efficacious against MV in cotton rats when given parenterally, if given at four times the MED for this compound against RSV, is also not inconsistent with the mixed results obtained in the studies with humans, in which this nucleoside analog was administered i.v. In the one instance in which a direct comparison can be made since the same dose, administration regimen, and scheduling were used in both these studies and the clinical one (3), similar results were obtained; there was no apparent benefit to either cotton rats or patients. Additional studies will be done as new compounds become available.

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