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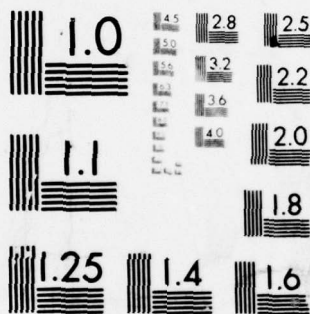
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Activity of Amantadine, Rimantadine and Ribavirin
Against Swine Influenza in Mice and Squirrel Monkeys

G. H. SCOTT,* E. L. STEPHEN and R. F. BERENDT

From The U. S. Army Medical Research Institute of Infectious Diseases
Frederick, Maryland 21701

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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9 June 1977

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ABSTRACT

Amantadine, rimantadine, and ribavirin given orally, either prophylactically or therapeutically, reduced mortality and increased the survival time of mice infected with the type A/New Jersey (swine) strain of influenza virus. In addition, amantadine and rimantadine therapy increased the rate of virus clearance from lungs of infected mice. Amantadine treatment initiated either before or after virus challenge ameliorated the illness in squirrel monkeys; when therapeutically-administered it stopped virus shedding from infected monkeys within hours.

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The outbreak of influenza in early 1976, caused by a virus serologically related to the "swine" virus implicated in the 1918-19 pandemic, stimulated an accelerated national vaccination program (10). As part of this program our laboratory evaluated several candidate vaccines on the basis of protective efficacy in laboratory animal models and evaluated chemoprophylactic and chemotherapeutic drugs in animal models. The results of studies to evaluate the vaccines will be reported separately.

Amantadine, rimantadine and ribavirin have demonstrated activity against disease caused by several strains of type A influenza virus (6, 8, 9, 12). This paper reports results of studies on the efficacy of these 3 drugs given by the oral route for the prevention and/or treatment of type A/New Jersey (swine) influenza virus infections in mice. Amantadine, which is the only one of these drugs currently approved for use in humans, was also evaluated in a squirrel monkey model for influenza virus infection (4).

MATERIALS AND METHODS

Mice. Outbred, female Swiss mice (Cr1:COBS CD1[ICR]BR) were obtained from the "Sendai-free" colony of Charles River Laboratories. Two ages of mice were used in separate experiments: weanlings (21 days old) and adults (6 to 8 weeks old). Mice were randomly selected and housed 20 to a cage in biological containment cabinets operated under negative pressure. Lighting in the cabinets was controlled to give 12 h of light and 12 h of darkness each day. Commercial mouse pellets and water were provided ad libitum.

Monkeys. Male squirrel monkeys (*Saimiri sciurius*), weighing 0.5 to 0.9 kg were used. Housing and feeding arrangements have been described (4).

Virus. Influenza virus, strain A/NJ/8/76 (Hsw1 N1), with a history of 6 passages in embryonated eggs was adapted to mice in 9 serial passages. Mice were infected by intranasal (i.n.) instillation of supernatant fluid obtained by centrifugation of a suspension of homogenized lungs removed from mice infected 3 to 4 days previously. After the ninth passage, allantoic cavities of 10-day-old embryonated chicken eggs were inoculated with supernatant fluid from infected lungs. After incubation for 48 h at 35 C, infected allantoic fluid was harvested and clarified by centrifugation at $1200 \times g$ for 15 min at 4 C. Antibiotics were added to the clarified fluid to achieve a final concentration of 250 units penicillin/ml and 100 μg streptomycin/ml; aliquots of the suspension were stored at -60 C. Titrations in embryonated chicken eggs indicated that the infected allantoic fluid

contained $10^{7.7}$ egg median infectious doses (EID_{50}) of virus per milliliter.

Drugs used and treatment schedule. Amantadine hydrochloride and its structural analogue, rimantadine hydrochloride, were obtained from E. I. duPont de Nemours and Co., Inc., Newark, N.J. Ribavirin was obtained from the Nucleic Acid Research Institute of ICN Pharmaceuticals, Inc., Irvine, Cal. For tests in mice the drugs were dissolved in sterile, triple-distilled water at a concentration of 0.25 mg/ml and provided to groups of mice in drinking water at selected times beginning as early as 48 h before or as late as 96 h after challenge with infectious virus. Treatment was discontinued 14 days postinfection in all studies. Based on preliminary experiments, daily consumption of water was estimated at 6 ml per mouse. On this basis, each mouse ingested 60 mg of drug/kg body wt/day.

Amantadine was administered to monkeys by means of a nasogastric tube connected to a syringe and passed through a steel speculum inserted between the monkey's teeth and into the esophagus. Two doses of amantadine were tested; 7.5 mg/kg/day and 15.0 mg/kg/day. One-half milliliter containing one-half of the prescribed dose was given in the morning and the balance in the afternoon. Infected control monkeys were given the same volume of sterile water. Treatment, which was continued for a total of 7 days in each study, was initiated either 24 h prior to virus challenge or 48 h after challenge, a time when clinical illness was apparent.

Virus challenge. Lightly anesthetized mice were given $10^{6.0}$ EID_{50} of virus in 0.05 ml by the i.n. route. Monkeys were challenged with 10^7 EID_{50}

of virus by the intratracheal (i.t.) route as previously described (4).

Sampling and assay procedures. At selected intervals after infection, lungs removed from mice were scored for gross lesions, weighed, and assayed for virus by established procedures (11). Virus was isolated from monkeys by swabbing the oropharynx. The swabs were washed in 1.0 ml of heart infusion broth (HIB) containing 50 µg/ml of gentamicin, 100 units/ml of penicillin and 100 µg/ml of streptomycin; these samples were assayed for virus by established procedures (11).

Clinical determinations and illness scoring. Beginning at least 2 days prior to infection the rectal temperature, hematocrit, total and differential leukocyte counts, respiratory rate, pharyngeal virus isolation, food consumption, body weight, nasal discharge, coughing and sneezing, labored breathing, and activity for monkeys were recorded daily. To facilitate analysis of treatment effects, the system devised by Berendt and Hall (2) was employed to score the response of monkeys over the first 7 days of infection. With this system, a critically ill monkey would score approximately 77 (assuming a 20% weight loss and maximum values for the other parameters); sham-inoculated control monkeys scored <5.0.

RESULTS

Experiments in mice. Preliminary experiments revealed marked differences in survival between untreated weanling and adult mice following infection with the New Jersey strain of influenza virus. Intranasal doses of $10^{3.8}$ EID₅₀ routinely killed one-half of the 21-day-old mice with a mean time to death of <6 days. In contrast, the LD₅₀ for 6- to 8-week-old mice was $>10^{5.8}$ EID₅₀ of virus. Virus titers in the lungs of both weanling and adult mice exceeded 10^7 EID₅₀ at 3 days; thereafter, lung virus concentrations gradually declined to undetectable levels by 9 to 11 days postinoculation. Extensive pulmonary consolidation and a significant increase in lung weight was observed by 6 days postchallenge.

Lung virus titers, lung lesion scores and lung weights of adult mice infected with type A/NJ/influenza virus and given each of the drugs are summarized in Table 1. Virus replicated rapidly in the lungs of untreated mice, and the lungs of these mice weighed almost 3 times as much as those from noninfected mice. Approximately 40% of each infected lung had plum-colored lesions typical of influenza by the seventh day. Despite extensive pathologic changes, however, 90 to 100% of the infected adult mice survived.

None of the drugs altered tissue virus levels measured 3 days postinfection. By 7 days, however, virus titers were significantly lower in mice treated therapeutically with ribavirin. The development of lung pathology, as reflected by lung weight, was less extensive when rimantadine

was given prophylactically and when ribavirin was given therapeutically. Prophylactic administration of amantadine and rimantadine resulted in fewer lung lesions than were observed in untreated mice.

In contrast to adult mice, type A/NJ influenza infections in untreated weanling mice were uniformly lethal with a mean time to death of 5.8 days (Table 2). Amantadine, rimantadine, and ribavirin used prophylactically delayed the time to death and significantly increased survival to 80 to 90%. None of the drugs studied significantly affected virus titers at 3 days in the lungs of infected mice. However, compared to untreated mice, treatment with either amantadine or rimantadine significantly reduced lung virus titers at 7 days after virus challenge, suggesting that both drugs increased the rate of virus clearance from the respiratory tract.

Fig. 1 depicts the survival of infected mice as a function of time when drug treatment was initiated relative to virus challenge. These survival data clearly indicate that while early treatment was desirable, and survival rates declined as treatment was delayed, each of the drugs effectively reduced mortality rates even when treatment was delayed for as long as 4 days.

Experiments in monkeys. Preliminary experiments suggested that the activity of rimantadine on swine influenza differed little from that of amantadine. For this reason, and because amantadine is approved for use in humans by the Food and Drug Administration (FDA), we concentrated on the latter drug for primate studies.

Following i.t. instillation of 10^7 EID₅₀ of virus, monkeys became febrile within 24 h; fever then slowly subsided. Most other changes in clinical parameters reached a maximum in 2 to 5 days, and then slowly returned to prechallenge values. Although there was considerable variation in the duration of convalescence, all clinical values approached normal by day 10. Illness scores for these infected, untreated monkeys averaged 45.9 in contrast to scores of <5 for uninfected monkeys.

Illness scores for infected monkeys treated with either 7.5 or 15.0 mg/kg/day of amantadine beginning either 24 h before or 48 h after virus challenge are shown in Table 3. The scores of treated monkeys were significantly lower than those of untreated monkeys, indicating that amantadine was effective both prophylactically and therapeutically. No clear cut effect of dose was observed.

In an effort to determine the predominating drug effect, we subtracted the contribution made by virus shedding from the illness scores (Table 4). Virus shedding was considered to be indicative of infection; the other parameters were signs of illness. After this adjustment the average scores for all groups of treated monkeys were still lower than those calculated for controls, indicating that a major effect of the drug was a reduction in the severity of illness. Data on the effect of drug treatment on the duration of virus shedding are also summarized in Table 4. Prophylactically administered drug did not significantly alter the duration of virus shedding. Surprisingly, however, when treatment was initiated 48 h after virus challenge, the period of virus shedding was shortened significantly. Analysis

of the other parameters that constitute the illness score revealed a lessening in all after prophylaxis or therapy rather than an effect on any particular one.

DISCUSSION

Amantadine, rimantadine and ribavirin given orally either prophylactically or therapeutically reduced mortality and increased the mean time to death of mice infected with A/NJ (swine) influenza virus. None of these drugs prevented infection, but amantadine and rimantadine therapy increased the rate of virus clearance from the lungs of young infected mice. In the present study, virus clearance in young mice was not significantly affected by oral ribavirin treatment. In contrast, previous reports (14) have attributed considerable antiviral activity to ribavirin administered as small-aerosol particles directly to the respiratory tract of infected animals. The reason for this discrepancy is not known, but may be due to the difference in drug level in the lungs after treatment by 2 different routes.

As previously reported (2), the New Jersey strain of influenza virus caused a milder illness in mice and squirrel monkeys than that observed in our laboratory after infection with an H3N2 serotype virus (11); the observation of mild illness in experimental animals is consistent with the report of Beare and Craig following the i.n. inoculation of humans (1). Amantadine treatment initiated either before or after virus challenge ameliorated the illness, and therapeutically administered amantadine apparently stopped virus shedding from infected monkeys within hours after treatment was started. Successful amantadine therapy for A/NJ virus infections in both mice and monkeys contrasts with the reports of other

workers that therapeutically administered amantadine has only a minimal effect on the shedding of other strains of influenza virus (5). This observation suggests that the NJ strain may be more sensitive to amantadine than other type A viruses, especially during stages of the infection when the virus is replicating rapidly. The increased survival seen in groups of mice in which treatment was not initiated until 96 h, and the dramatic cessation of virus shedding from infected monkeys when treatment was initiated at 48 h after virus challenge, suggest that amantadine need not be limited to a prophylactic role in influenza. The fact that amantadine-treated monkeys did not shed virus deserves special attention. Any reduction in virus dissemination from infected individuals could, of course, curtail epidemic spread of the virus.

Clearly, the therapeutic efficacy of amantadine cannot be explained wholly on the basis of antiviral activity. In our animal models peak virus titers in the lung were often achieved before treatment was started. It is possible that the host's response to the drug played an important role in ameliorating the illness. This is consistent with findings by Little et al. (7) who observed that amantadine treatment increased the rate of recovery from disease in small airways and improved lung function in individuals suffering from naturally acquired influenza infections. Our animals apparently benefited not only from this response, but from a degree of antiviral activity of the drug as well. Although none of the drugs prevented infection, amantadine reduced the severity of illness in monkeys, and all

3 drugs significantly increased survival of mice even when treatment was initiated after the onset of bronchopneumonia. The beneficial effect of treatment, obtained in 2 widely differing animal models, gives strong support to the hypothesis that these drugs might also be effective in treating influenza infections in humans.

Although vaccination continues to be the most widely used prophylaxis against influenza, immunological prevention and control of the disease is not wholly adequate. Because of the capacity of influenza virus to undergo mutations which circumvent specific immunity established through vaccination with previously prevalent strains, vaccines are usually only partially protective. Despite the partial efficacy of existing vaccines, a need for effective therapeutic measures remains. This study supports the mounting evidence that amantadine, rimantadine and ribavirin used alone or in conjunction with vaccine prophylaxis might offer better management of influenza than can be expected through vaccination procedures alone.

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TABLE 1. Effect of drugs given orally to 8-week-old mice infected with type A/NJ influenza virus.

Group	Lung virus titer ^a (log ₁₀ EID ₅₀ /infected lung)		Mean lung lesion _b scores ^b 7 days	Mean lung weights, mg 7 days
	3 days	7 days		
Noninfected controls	-	-	0	142 ^c
Prophylactic				
Amantadine	7.7	5.8	0.9	310
Rimantadine	7.3	5.2	0.6	272 ^c
Ribavirin	7.0	4.8	2.0	332
Therapeutic				
Amantadine	6.8	4.2	1.2	324
Rimantadine	6.8	5.0	1.7	352
Ribavirin	7.1	3.6 ^c	1.0	266 ^c
Infected controls	7.1	5.5	1.7	390

^a Geometric mean.

^b Scale of 0-4 from negative to total consolidation.

^c P < 0.05 compared to infected controls.

TABLE 2. Effect of drugs given orally to 3-week-old mice (n=5) infected with type A/NJ influenza virus.

Group	Lung virus titer ^a (log ₁₀ EID ₅₀ /lung)		Mean lung lesion score	% Survival (n=30)	Mean day of death
	3 days	7 days	7 days		
Prophylactic					
Amantadine	7.7	6.0	1.4	83	10.6
Rimantadine	7.5	5.8	1.0	93	10.5
Ribavirin	7.4	6.0	2.6	90	9.0
Therapeutic					
Amantadine	7.2	4.1 ^b	1.0	63	7.4
Rimantadine	7.3	4.2 ^b	1.5	83	6.0
Ribavirin	6.6	5.2	2.8	43	5.3
Infected					
controls	7.4	5.8	3.0	0	5.8

^a Geometric mean.

^b P < 0.05 compared to controls.

TABLE 3. Effect of orally administered amantadine upon illness scores of squirrel monkeys infected with type A/NJ influenza virus.

Treatment	(n)	Dose (mg/kg/day)	Illness score ^a	p ^b
Water control	8	0	45.9 $\pm$ 2.9	-
Prophylactic	4	7.5	21.2 $\pm$ 4.9	<0.005
Prophylactic	4	15.0	23.4 $\pm$ 3.8	<0.005
Therapeutic beginning at 48 h	4	7.5	24.9 $\pm$ 2.5	<0.005
Therapeutic beginning at 48 h	4	15.0	18.2 $\pm$ 1.4	<0.005

^a Mean $\pm$ standard error of the mean.

^b Compared to water control.

TABLE 4. Effect of amantadine treatment on duration of virus shedding and adjusted illness scores^a of squirrel monkeys infected with type A/NJ influenza virus.

Treatment	(n)	Dose (mg/kg/day)	Days virus shedding ^b	Adjusted illness score ^b
Water control	8	0	5.75 $\pm$ 0.67	38.9 $\pm$ 3.2
Prophylactic	4	7.5	6.25 $\pm$ 0.75 ^d	15.4 $\pm$ 3.7 ^d
Prophylactic	4	15.0	4.50 $\pm$ 0.65 ^d	16.4 $\pm$ 2.8 ^d
Therapeutic beginning at 48 h	4	7.5	2.75 $\pm$ 0.85 ^c	20.9 $\pm$ 4.6 ^d
Therapeutic beginning at 48 h	4	15.0	2.0 $\pm$ 0 ^d	14.7 $\pm$ 1.3 ^d

^a Total illness score less virus shedding contribution.

^b Mean $\pm$ standard error of the mean.

^c P < 0.025 compared to water control.

^d P < 0.005 compared to water control.

FIG. 1

Effects of initiation time on drug efficacy for the
treatment of type A/NJ influenza infection in mice.

