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Treatment of Influenza Infection of Mice by Using Rimantadine Hydrochlorides by the Aerosol and Intraperitoneal Routes

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Rimantadine hydrochloride was administered for 4 days in a small-particle (95% < 6.5 μm) aerosol (8.8 mg/kg per day) or intraperitoneally (40 mg/kg per day) to mice previously infected with influenza A/Aichi/2/68 (H_3N_2), mouse adapted. Mean time to death and incidence of survival were significantly increased in all treated groups of mice. The rate of eventual disappearance of virus from lung tissue was also accelerated by therapy. However, maximal mean virus titer per lung, and lung histopathology, did not reveal any difference between control and either group of treated mice. Aerosol therapy initiated at 72 h postinfection was as effective as that initiated at 6 h, even though lung virus titers of these mice had already peaked by 72 h. In contrast, intraperitoneal therapy initiated at 72 h was not effective in all studies.

Rimantadine hydrochloride (α-methyl-1-adamantanemethylamine hydrochloride; E. I. duPont de Nemours and Co., Inc., Newark, N.J.) has been reported to be active against influenza A infections in tissue culture, animals, and man (11, 18, 19). This drug has the same antiviral spectrum and mode of action as its structural analogue amantadine hydrochloride (11). Both amantadine hydrochloride and rimantadine hydrochloride have therapeutic activity when given orally 48 to 72 h after infection (2, 4, 10, 11). Grunert et al. (4) reported that amantadine hydrochloride was effective by the aerosol route, but only within 1 h after infection.

The administration of vaccines or drugs as small-particle aerosols for prophylactic or therapeutic purposes is not a completely new concept. Live tularemia and live Venezuelan equine encephalomyelitis vaccines have been shown to be effective when given by the aerosol route, although this route of administration is not generally used (6, 9, 15). In addition, certain refractory pneumonias of bacterial origin have been favorably treated by using aerosolized antibiotics (13). This report describes the comparative effects of both intraperitoneally (i.p.) and aerosol-administered rimantadine hydrochloride on survival, lung virus titer, and histopathology of mice during experimental influenza infection induced by small-particle aerosol exposure to the virus.

MATERIALS AND METHODS

Mice. Five-week-old outbred female mice, Tac:(SW)fBR, were used for studies 1, 3, 4, and 5, and Crl:COBSCO^R-(ICR)BR mice were used for study 2. Upon arrival, mice were randomized and housed 15 to a cage. Serial sacrifice studies were accomplished by the addition of mice to each group.

Virus. The A/Aichi/2/68 (H_3N_2) strain (verified by the Communicable Disease Center) of influenza virus presented as a small-particle aerosol (mass median diameter, 2.2 μm) was used to infect the mice. The original isolate was passed ten times in eggs and eight times in mice, followed by two final egg passages to obtain a mouse-virulent strain. The final inoculum obtained from the allantoic fluid of 10- to 12-day-old embryonated eggs harvested 48 h after inoculation contained $10^{8.4}$ egg median infective doses (EID_{50})/ml. Aliquots were stored at −70 C.

Virus lung titers. At each sampling period, lungs from three mice in each group were collected. Both lungs from each mouse were harvested, pooled, and individually homogenized by using a Ten Broeck grinder in 4.5 ml of heart infusion broth containing 250 U of penicillin and 250 μg of streptomycin per ml. One-tenth milliliter of serial 10-fold dilutions of these homogenates was inoculated into the allantoic sac of 10- to 12-day-old embryonated chicken eggs, using six eggs per dilution. Lung titers are the geometric mean of the three mouse lung samples, expressed as EID_{50}/mouse lung.

Drug. Rimantadine hydrochloride (kindly supplied by E. I. duPont de Nemours and Co., Inc., Newark, N.J.) was solubilized in sterile, triple-distilled water before aerosol or i.p. administration in either of two treatment schedules, 6 or 72 h postinfection. Four

treatment schedules were evaluated in each of the first three studies: (1) i.p. injection 6 h postinfection at 40 mg/kg per day for 4 days; (2) i.p. injection 72 h postinfection, 40 mg/kg per day for 4 days; (3) aerosol started 6 h postinfection for 4 consecutive days; and (4) aerosol started 72 h postinfection for 4 consecutive days. Exposure periods for the small-particle aerosol phases of the experiment were 80 min in duration.

The dosage estimate by the aerosol route was based on the following calculation:

$$\text{spray factor} = \frac{\text{Concentration of agent/liter of aerosol}}{\text{Concentration of agent / liter of spray suspension}}$$

The concentration of rimatadine in the spray suspension was 50 mg/ml. The spray factor was unknown, since the concentration per liter of aerosol had not been determined. A spray factor of 10^{-5} determined from fluorescein dye studies for the aerosol dissemination system was used in lieu of definitive information specifically related to rimantadine hydrochloride in solution. The concentration of the drug in the exposure chamber was estimated to be 0.5 mg/liter of air. Since the mouse breathes approximately 2 liters in 80 min (7), the presented dose was estimated to be 1 mg/mouse, or 40 mg/kg.

The actual dosage was obtained by quantitative drug assay of aerosol samples performed by duPont, using gas-liquid chromatography. The mean aerosol concentration of rimantadine hydrochloride in four samples was 0.68 ± 0.20 mg/ml by this assay technique. Presented actual dose (PD) was calculated by the following formula:

$$\text{PD} = \frac{\text{Concentration/ml in sample} \times \text{volume of sample} \times \text{volume of inspired air during the treatment period}}{\text{sampling time} \times \text{airflow in the sampler}}$$

$$\text{PD} = \frac{0.68 \text{ mg/ml} \times 20 \text{ ml} \times 2.0 \text{ liters of air}}{10 \text{ min} \times 12.5 \text{ liters/min}}$$

$$\text{PD} = 0.22 \pm 0.06 \text{ mg/mouse, or } 8.8 \text{ mg/kg}$$

In study 4, rimantadine hydrochloride was administered as a continuous aerosol to mice from 72 to 168 h postinfection.

Dissemination system. Aerosols of virus suspension and rimantadine hydrochloride solution were generated by a Collison spray device and disseminated into a Henderson apparatus (12, 14). This system yields particles having a mass median diameter of 2.2 μm, with 95% less than 6.5 μm.

Aerosol sampling. Glass impingers were used to collect samples for virus assay and for quantitative analysis of rimantadine hydrochloride (20). Samples for rimantadine assay were collected for 10 min at the midpoint of each 80-min exposure period. Samples were collected for virus assay at the midpoint of the exposure period to determine the challenge dose of virus.

Histopathology. Mice were sacrificed at 7 and 14 days postinfection from each group. The lungs were fixed with 10% neutral buffered formalin, processed routinely for paraffin embedding, sectioned, stained with hematoxylin and eosin, and examined microscopically.

Data. Percentage of survival was based on deaths from 5 to 21 days postinfection. Mean time to death was calculated only for dead mice from day 5 through day 21 of the observation period and was thus unaffected by survivors.

RESULTS

Study 1: SW mice challenged with $10^{4.2}$ EID_{50}/mouse. In Table 1, the survival and time-to-death data are shown for the four groups of mice of study 1 treated with rimantadine hydrochloride and the untreated control group. Significant increases in percentage of survival ($P < 0.005$) occurred in all four treatment groups compared with untreated mice. There was no significant difference in survival between the 6- and 72-h-postexposure treatment groups and no significant differences between routes of administration. In Table 2, lung virus titers in the mice of study 1 treated with small-particle rimantadine hydrochloride aerosols were significantly lower ($P < 0.001$) than titers of untreated mice at 24 h and returned to levels of untreated mice by 72 h. The peak titer for all groups was approximately $10^{5.0}$ EID_{50}/lung. Ten days after virus challenge, untreated mice had $10^{4.0}$ EID_{50} of influenza virus per lung compared with no detectable virus levels in lungs of mice from the treatment groups. By 72 h after exposure, histopathological evidence of pneumonia included necrotic bronchitis with lymphocytic infiltration of the alveolar walls. Typical histopathological lesions in lungs of mice from control and treatment groups at 7 days postexposure were subacute bronchopneumonia and peribronchial lymphoid nodules. There was an overall increase in cellularity throughout the lung fields, owing to the presence of inflammatory cells. However, there was no difference in severity or type of pneumonia between treated and untreated mice. At 14 days, bronchopneumonia was still evident and many alveoli were lined by swollen epithelial cells, indicating a progressive process in both the treated and untreated mice.

Study 2: ICR mice challenged with $10^{3.6}$ EID_{50}/mouse. ICR mice used in this study were challenged with less virus than the SW mice in study 1. All treatment schedules showed an increase in percentage of survival and mean time to death in mice treated with rimantadine. Although aerosol therapy initiated at 6 h postinfection did not yield a significant increase in survival, the mean time to death for the mice in this group was significantly longer ($P < 0.025$). Lung virus titers (Table 2) of treated mice 24 h

TABLE 1. *Effect of rimantadine treatment by two routes on survival and mean time to death of mice infected with influenza virus*[a]

Study	Therapy		Survival		Mean time to death (days)
	Route	Time postexposure (h)	(Survivors/total)	%	
1	None		12/30	40	8.3
	Aerosol	6[b]	23/27	85***[c]	11.0
		72	24/26	92***	10.0***
	i.p.	6	24/29	83***	11.8*
		72	28/29	97***	7.0
2 (ICR mice)	None		7/30	23	8.5
	Aerosol	6	13/30	43	11.1**
		72	18/29	62**	10.7
	i.p.	6	17/30	56**	10.0
		72	14/26	54*	12.3**
3	None		1/30	3	7.4
	Aerosol	6	10/30	33**	8.1
		72	7/30	23	8.8*
	i.p.	6	5/30	16	8.3
		6[d]	13/30	43***	10.5**
		72	14/30	46***	9.5*
4	None		5/45	11	7.5
	Aerosol	72	8/41	19	7.8
		72[e]	11/30	36**	7.9
	i.p.	72	12/45	26	8.1
		72[f]	10/45	22	7.3

[a] Challenge doses (EID_{50}/mouse) were: study 1, $10^{4.2}$; study 2, $10^{3.6}$; study 3, $10^{5.4}$; and study 4, $10^{5.0}$.
[b] Therapy was continued for 4 days except as noted below.
[c] Symbols: *, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.005$.
[d] Therapy continued through 10 days postinfection.
[e] Rimantadine administered by continuous aerosol to achieve equivalent total dose.
[f] Rimantadine administered every 8 h to achieve three times the total dose previously used.

after exposure were slightly lower than titers of control mice. The decline in lung virus titer of mice in the control group was not different from that observed from the treated groups of mice. Histological findings were similar to those of study 1. In this study, drug control mice for both i.p. and aerosol routes of rimantadine administration were included, using the same dosages and time intervals as for the virus-infected mice. There was no observable evidence of clinical illness or toxicity during the 21-day observation period. The lungs, livers, and kidneys of five mice from each drug control group were normal on gross and histological examination at 7 and 14 days after treatment.

Study 3: SW mice challenged with $10^{5.4}$ EID_{50}/mouse. In this study, the challenge dose of virus was increased. Both time to death and survival decreased ($P < 0.005$) for all the mice compared with study 1 (Table 1). Rimantadine therapy by the aerosol and i.p. routes increased percentage of survival and time to death. There was no significant difference between the treatment schedules. In addition, treatment i.p. daily from 6 h to 10 days did not increase percentage of survival when compared with treatment i.p. for 4 days starting 72 h postinfection.

Study 4: SW mice challenged with $10^{5.0}$ EID_{50}/mouse. Percentage of survival was increased by all methods of treatment initiated at 72 h postinfection (Table 1). Mice treated by continuously disseminated aerosol showed a significant increase ($P < 0.025$) in percentage of survival. By decreasing the concentration of rimantadine in the disseminator, it was possible to achieve a daily dose equivalent to other aerosol treatments. Administration of rimantadine every 8 h from 72 h by the intraperitoneal route was not effective.

DISCUSSION

Rimantadine hydrochloride was effective in reducing mortality and increasing the time to

TABLE 2. *Effect of rimantadine treatment on lung titers in mice infected with influenza by small-particle aerosol*

Time postinfection (h)	Mean $\log_{10}$ lung titer ± SEM[a]				
	Virus control	Aerosol (6 h)	i.p. (6 h)	Aerosol (72 h)	i.p. (72 h)
SW mice (study 1)					
6	2.91 ± 0.47	ND[b]	ND	ND	ND
24	7.12 ± 0.09	5.75 ± 0.09[c]	6.10 ± 0.45	ND	ND
72	8.08 ± 0.12	7.83 ± 0.03	7.08 ± 0.34	ND	ND
96	7.68 ± 0.12	7.63 ± 0.17	7.84 ± 0.13	7.59 ± 0.47	7.80 ± 0.17
144	7.62 ± 0.43	6.73 ± 0.57	7.06 ± 0.12	6.83 ± 0.05	7.39 ± 0.26
168	6.75 ± 0.22	5.82 ± 0.48	6.29 ± 0.25	5.31 ± 0.31*	5.25 ± 0.64
240	4.80 ± 0	0	0	0	0
ICR mice (study 2)					
6	3.20 ± 0.35	ND[a]	ND	ND	ND
24	6.83 ± 0.40	4.84 ± 1.02	5.91 ± 0.05	ND	ND
72	7.94 ± 0.18	7.36 ± 0.28	7.48 ± 0.22	ND	ND
96	7.37 ± 0.06	7.33 ± 0.16	7.78 ± 0.18	6.89 ± 0.12	6.46 ± 0.23*
144	6.98 ± 0.20	5.10 ± 0.23**	5.39 ± 0.26*	6.83 ± 0.20	6.73 ± 0.60
168	5.05 ± 0.15	5.86 ± 0.13*	5.64 ± 0.57	4.63 ± 1.27	5.31 ± 0.38
192	2.92 ± 1.68	5.03 ± 0.98	3.84 ± 0.88	3.53 ± 0.62	4.80 ± 0.38
216	2.97 ± 0.11	4.78 ± 0.53	2.55 ± 0.72	0.53 ± 0.53*	1.18 ± 1.18
240	0	2.37 ± 0.58	0	0	0
336	0	0	0	0	0
432	0	0	0	0	0

[a] SEM, Standard error of the mean.
[b] ND, Not done.
[c] Symbols: *, $P < 0.025$; **, $P < 0.001$.

death in mice infected with influenza virus. Of particular interest was the increased survival in some groups treated 72 h after exposure, since histopathologic lesions characterized by bronchopneumonia resulting from the influenza infection were present at that time. Aerosol therapy to treat influenza in mice with amantadine hydrochloride was reported previously to be effective in mice only if initiated before 1 h postinfection (4). Since mice generally show dyspnea, hyperpnea, and histologically determined pneumonia earlier than 72 h postinfection, therapy that can be delayed to 72 h may have practical clinical value.

The traditional mechanism of antiviral action of amantadine hydrochloride (and rimantadine hydrochloride) is assumed to involve impaired viral penetration and/or uncoating. Indeed, in those mice treated at 6 h postinfection, lower-lung virus titers were found at 24 h; this effect was not apparent 48 h later. Peak titers were not affected by treatment with rimantadine hydrochloride. It is possible that small delays in virus replication can affect increased survival or that rimantadine hydrochloride has ameliorating effects such as improving functional capacity of treated lungs or increasing recovery from the pathological sequelae.

Rimantadine hydrochloride administered by either the aerosol or i.p. route was not effective in reducing histopathological lesions associated with influenza in either the SW or ICR mice. This contradicts previous reports indicating decreased gross lung pathology and lung lesion score in mice (3, 16). These latter observations were made in studies in which mice were treated earlier than 6 h postinfection or before one complete virus cycle and were apparently from a prophylactic rather than a therapeutic effect.

Since it is possible to show decreasing survival with increasing challenge dose of virus, it was not surprising that more mice died in studies 3 and 4 compared with study 1 when the challenge dose was increased. The ICR mice were less resistant to this strain of influenza virus than SW mice even though they were challenged with a lower dose of virus. Although differences in percentage of survival, mean time to death, and source of mice can be related to challenge dose of virus, the limits of the therapeutic effects of rimantadine hydrochloride were not exceeded since protective effects were obtained in each study.

The gas chromatographic results of rimantadine hydrochloride aerosol samples clearly demonstrate the fallacy of using an extrapolated

spray factor to estimate an unknown aerosol concentration. Therefore, the spray factor for each compound, each concentration of drug, and each dissemination system must be determined before accurate dosage estimates can be derived. In addition, the particle size of the aerosol must be determined since this is the primary determinant for site of deposition of the drug in the respiratory tract (5). Even though the mice treated by the aerosol route received approximately one-fifth the total dosage of rimantadine hydrochloride as mice treated by the i.p. route, survival was the same. Recent studies now in progress demonstrate improved efficacy when rimantadine is given in a continuous aerosol for 5 days at the same dosages used in the studies described above. Continuous administration of rimantadine results in significantly greater percentage of survival ($P < 0.005$) than when aerosols are given intermittently for 80 min or i.p. for 5 days beginning at 3 days postinfection (unpublished data). Hence, the aerosol route of administration of rimantadine hydrochloride appears to be an efficacious means of treating influenza.

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