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Inhibition of Respiratory Virus Infections of Mice with Aerosols of Synthetic Double-Stranded Ribonucleic Acid

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Aerosols of double-stranded complexes of polyinosinic and polycytidylic acids (poly I:C) were useful in protecting mice infected with aerosols of influenza (A_2/Taiwan/64) and parainfluenza type 1 (Sendai) viruses. Administration of poly I:C as an aerosol offers an advantage, particularly in therapy, by eliminating the risk of pulmonary dissemination of viral infections due to intranasally instilled fluids. Treatment of mice with aerosols of poly I:C reduced the infection rate with influenza virus but did not inhibit virus multiplication in the lungs of most of those animals where infection became established. Sendai virus infection rates were undiminished in mice treated with poly I:C, but lung-virus titers were significantly suppressed as compared with those of untreated animals. The maximum poly I:C doses (40 μg) administered by aerosol produced no evidence of toxicity in the mice.

The discovery of interferon induction by double-stranded complexes of polyinosinic and polycytidylic acids (poly I:C; reference 2) has led to several reports demonstrating the effectiveness of this inducer in protecting laboratory hosts from viral infections (2, 7–10, D. A. Hill and S. Baron, Bacteriol. Proc., p. 149, 1969). Although poly I:C has been administered to animals by intranasal, intraperitoneal, and intravenous inoculations or by ocular instillation, there have been no reports of aerosol administration. Intranasal administration of poly I:C prior to infection protects mice against intranasal challenge with influenza virus. However, treatment after infection has not been effective (*unpublished observations*). This may be due to the known dissemination and enhancement of influenza virus infection by fluids administered intranasally after infection (13). Since aerosol administration may not disseminate virus, this method may ultimately be useful in the therapy of influenza infections. A study was therefore made of the efficacy of airborne administration of poly I:C during myxovirus infection of mice as a guide to its possible usefulness in therapeutic studies.

The following communication describes the effectiveness of poly I:C administered in aerosol form to mice in protecting against infections with influenza (A_2/Taiwan/64) and parainfluenza type 1 (Sendai) viruses. Continuation of poly I:C treatment up to the third day postinfection did not appear to enhance the viral infection nor overcome the protective effect of previous treatment.

MATERIALS AND METHODS

Poly I:C. The synthetic double-stranded poly I:C used in these studies was prepared by mixing equimolar concentrations of poly I and poly C (P. L. Biochemicals, Inc.). The single-stranded homopolymers had a molecular weight of approximately 10^5 daltons. The final concentration of poly I:C was 1.0 mg/ml in physiological saline.

Mice. All mice used in these experiments were males of the Swiss-Webster strain produced at the Fort Detrick Animal Farm. The mice were in the 16- to 20-g range at the beginning of each experiment.

Viruses. The influenza virus used in these experiments was the A_2/Taiwan/64 strain. The virus was propagated in 10-day-old embryonated hens' eggs that were inoculated by the chorioallantoic route and incubated for 48 hr at 35 C before the allantoic fluid was harvested. The working seeds contained $10^{8.0}$ median egg infectious doses (EID_{50}) per milliliter.

The Sendai strain of parainfluenza virus type 1 was obtained from the Research Reference Reagents Branch of the National Institutes of Health. For these experiments, virus was produced in embryonated eggs (third passage) as described above for influenza,

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except that the infected eggs were incubated for 72 hr instead of 48 hr. The Sendai virus pool contained $10^{9.5}$ EID_{50} per ml.

Virus assay. Both the Sendai and influenza viruses were assayed by injecting 0.1 ml of virus dilution into 10-day-old embryonated eggs by the chorioallantoic route. After 72 hr of incubation, the allantoic fluids were harvested and individually tested for hemagglutinin by using chicken erythrocytes as an indicator of infection. The EID_{50}'s were calculated by the Karber method (6).

Exposure of mice to viral aerosols. Mice were exposed to viral aerosols generated with a Collison atomizer in a modified Henderson apparatus (5). Sendai virus was diluted 10^{-3} in beef heart infusion broth (BHIB) and the influenza virus was atomized either as undiluted allantoic fluid or as a 10^{-1} dilution in BHIB. The mice were exposed to the dynamic aerosols for a 3-min period. An all-glass impinger containing 10 ml of BHIB was used to sample the virus aerosols for 1 min at the midpoint of the animal exposure. The virus concentration in the impinger fluid was used to determine the aerosol concentration. The inhaled dose of the mice was calculated by multiplying the aerosol concentration per unit volume by the length of the exposure period and by the breathing rate (per minute) of the mice (4).

Exposure of mice to aerosols of poly I:C. Mice were treated with static aerosols of poly I:C in a 90-liter rotating drum (3). Ten to 20 mice were placed in each of four rectangular wire mesh baskets suspended in the interior of the aerosol drum. The total time exposure to aerosol for each treatment was 1 hr. At the beginning of the exposure period and at 15-min intervals thereafter 4 ml of poly I:C (1 mg/ml, total of 16 ml) was aerosolized with a University of Chicago Toxicological Laboratory glass atomizer (11). Using sodium fluorescein in the poly I:C solution as a tracer for aerosol recovery data, it was determined that the maximum inhaled dose per mouse per treatment did not exceed 8 μg of poly I:C.

Serology. Infection of mice with either virus was determined by the appearance of hemagglutinating-inhibiting (HI) antibodies in plasma 3 to 4 weeks after virus inoculation. The plasma was collected from retro-orbital blood vessels in heparinized micropipettes. The HI tests were performed by the microtiter technique (12) using 2 to 4 units of antigen. A proportion of mice were bled before each experiment and were found to be invariably negative for HI antibody.

RESULTS

Susceptibility of poly I:C-treated mice to influenza virus. In two experiments, mice were treated with aerosols of poly I:C 24 hr before exposure to aerosols of A_2/Taiwan/64 virus. The doses of virus used in these experiments infected 73% of the control, untreated mice (Table 1). The single treatment with poly I:C reduced the overall infection rate to 32%. The geometric mean titer of HI antibody in plasma was found to be somewhat lower in the treated animals in one experiment (PIC-1), but the reverse was found in the second experiment (PIC-3). The protective effect of poly I:C could not be measured in terms of reduced mortality because it was impractical to achieve high enough doses of virus by aerosol administration.

In a subsequent experiment mice were given three treatments with poly I:C. The first was given 3 hr before administration of virus, and the second and third treatments were given 24 and 48 hr after the virus. Ten mice from the control and 10 mice from the treated groups that received the larger virus dose were sacrificed at 72 hr for assay of virus in lungs. The remaining mice were held for 4 weeks and then tested for the presence of HI antibody in plasma. The infection rates (Table 2) of the mice were similar to those of the previous experiments (Table 1). The geometric mean titers of the treated animals were only slightly lower than those of the controls. The virus titers of the lungs (Table 3) are similar in the control and treated groups except for three mice in the treated group that showed little or no virus (mice numbers 3, 5, and 9). The mean log virus titer of the remaining mice in the treated group was 4.10 EID_{50} compared with 4.35 EID_{50} for the controls. The mean log virus titer of all the treated mice was 3.09 EID_{50}.

Effect of poly I:C treatment on Sendai virus infections of mice. Two groups of mice (A and B) were treated with an aerosol of poly I:C on 2 consecutive days. After the second treatment, these mice and two untreated control groups

TABLE 1. *Susceptibility of mice treated with aerosols of poly I:C to challenge with airborne influenza virus*

Expt no.	A_2/Taiwan/64 aerosol dose[a]	Controls[b]		Poly I:C treated[c]	
		Infected/total[d]	GMT[e]	Infected/total	GMT
PIC-1	293	9/10	9.10	7/10	6.75
PIC-1	46	6/9	6.82	1/10	5.32
PIC-3	184	19/20	6.63	8/19	7.82
PIC-3	7	9/20	7.54	2/18	8.32

[a] Inhaled dose, median egg infectious dose.
[b] Total infected/total no. of mice = 43/59 = 73%.
[c] Exposure to poly I:C preceded virus inoculation by 24 hr. Total infected/total no. of mice = 18/57 = 32%.
[d] Based on seroconversion by hemagglutinating-inhibiting (HI) test.
[e] Geometric (log_2) mean HI titer of positive plasmas.

TABLE 2. *Effect of multiple treatments of mice with poly I:C on susceptibility to influenza virus*

Expt no.	A_2/Taiwan/64 aerosol dose[a]	Controls[b]		Poly I:C treated[c]	
		Infected/total[d]	GMT[e]	Infected/total	GMT
PIC-4	184	15/15	7.72	11/15	7.23
PIC-4	12	9/15	7.21	4/15	6.57

[a] Inhaled dose, median egg infectious dose (EID_{50}).

[b] Total infected/total no. of mice = 24/30 = 80%.

[c] Treatments with poly I:C were given at −3, 24, and 48 hr relative to virus inoculation. Total infected/total no. of mice = 15/30 = 50%.

[d] Infected based on seroconversion by hemagglutinating-inhibiting (HI) test.

[e] Geometric (log_2) mean HI titer of positive plasmas.

TABLE 3. *Effect of multiple treatments of mice with poly I:C on influenza virus titers in lungs*

Mouse no.	Log EID_{50}/0.1 ml of 10% lung[a]	
	Controls	Poly I:C treated[b]
1	4.5	4.5
2	5.5	4.5
3	5.5	<0.5
4	5.3	3.0
5	4.5	<0.5
6	4.3	5.2
7	2.5	2.8
8	4.5	5.5
9	3.4	0.8
10	3.5	3.2
Mean log[c]	4.35	3.05 or <

[a] Virus titers of lungs 72 hr after inoculation. EID_{50}, median egg infectious dose.

[b] Treatments with poly I:C were given at −3, 24, and 48 hr relative to virus inoculation.

[c] F-value is 3.65 for 1,18 degrees of freedom, suggesting that the means are significantly different at the 5% level.

(C and D) were exposed to Sendai virus aerosols (77 to 194 EID_{50}). Groups A and B received additional treatments with poly I:C on days 1, 2, and 3 after virus inoculation. Five mice were sacrificed from each of the groups on day 1 and again on day 3. Lungs were removed for virus assay. The remaining mice from each group were bled at 14 days and again at 23 days for HI determinations on the plasmas.

At 24 hr none of the lungs from the groups treated with poly I:C had virus (Table 4). The mean log titers of the untreated control groups were 2.64 and 1.90 EID_{50}. At 72 hr most of the lungs in the treated groups had virus, but the mean log titers were at least 1,000-fold lower than those of the untreated controls. The HI response of treated mice was lower than that of controls, as seen by the geometric mean titers of plasmas collected at 14 days (Table 5). By the 23rd day, however, there appeared to be no differences between the control and the treated groups.

Effect of various poly I:C treatment regimens on Sendai infections in mice. Six groups of ten mice each were treated with poly I:C on various schedules. Group A was treated on days −1, 0, and 1. Group B was treated on days −1 and 0. Group C was treated on days 0 and 1. Groups D, E, and F were given single treatments on days −1, 0, and 1, respectively. Group G was not treated. On day 0, all mice were exposed to an aerosol dose of 95 EID_{50} of Sendai virus. All mice were sacrificed 72 hr after virus inoculation, and lungs were examined for virus content.

The results in Table 6 showed that all treatment groups except group F differed significantly from the control. Treatment groups A, B, and C were significantly different from D, E, and F. The

TABLE 4. *Effect of multiple treatments of mice with poly I:C on Sendai virus[a] titers in lungs*

Time[b] of lung harvest (hr)	Mouse no.	Log$_{10}$ EID_{50}/0.1 ml of 10% lung			
		Poly I:C treated groups[c]		Control groups	
		A	B	C	D
24	1	Neg	Neg	2.1	2.5
24	2	Neg	Neg	2.5	1.9
24	3	Neg	Neg	2.7	1.7
24	4	Neg	Neg	2.9	1.1
24	5	Neg	Neg	3.0	2.3
Mean log				2.64	1.90
72	6	1.1	0.5	>5.3	4.5
72	7	1.2	Neg	5.6	5.1
72	8	1.9	2.9	5.5	4.5
72	9	1.3	1.6	5.1	4.5
72	10	2.7	3.1	4.1	4.7
Mean log		1.64	1.62	>5.12	4.66

[a] Mice in groups A and C received an inhaled dose of 77 median egg infectious doses (EID_{50}); mice in groups B and D received 194 EID_{50}.

[b] Relative to virus inoculation.

[c] Mice treated on days −1, 0, 1, 2, and 3 relative to virus inoculation.

statistical analysis was done by the Duncan multiple F test method (1).

DISCUSSION

The present study was undertaken to determine whether aerosols of poly I:C could be used to protect mice infected with respiratory viruses. Although instillation of poly I:C has been reported to protect mice challenged with influenza viruses (7), administration of fluids could tend to disseminate and enhance pulmonary infections (13). The finding that airborne administration of an interferon inducer could be protective against influenza and Sendai virus infections of mice, even though small doses of poly I:C were given, indicates that this method of administration holds promise. It might be predicted that larger doses of poly I:C given by the airborne route would increase the protective effect. Doses of poly I:C that provide a stronger interferon response would be required to achieve treatment of an already established infection (M. Worthington and S. Baron, *personal communication*).

The administration of poly I:C to mice by the aerosol route effectively altered their responses to influenza and Sendai virus infections. The data suggest that the type of protection elicited against influenza virus was somewhat different from the protection observed when the animals were challenged with Sendai virus. In the case of influenza the poly I:C treatments appeared to prevent infection in some of the inoculated animals. Among treated mice that became infected, the virus titers of the lungs were no lower than those in the untreated groups. On the other hand, the poly I:C treatment did not alter the infection rates of mice exposed to Sendai virus but did markedly reduce virus titers in their lungs at 24 and 72 hr postinfection. Additional experimentation will be necessary to determine whether or not these differences are related to the biological characteristics of the viruses or to the relative doses employed in the viral challenges.

The best protection against Sendai virus infection was observed when the poly I:C treatments were given before or on the day of virus inoculation. Mice treated with poly I:C 24 hr after exposure to the virus showed no significant decreases in virus titers of the lungs compared to controls.

The doses of poly I:C administered in these experiments were only a fraction of those em-

TABLE 5. *Effect of poly I:C treatment on hemagglutinating-inhibiting (HI) response of mice exposed to Sendai virus*[a]

Days after virus inoculation	Group	Poly I:C treatment[b]	No. infected per total[c]	GMT[d]
14	A	Yes	9/9	4.54
14	B	Yes	9/10	3.99
14	C	No	11/11	6.78
14	D	No	12/12	6.99
23	A	Yes	9/9	5.99
23	B	Yes	10/10	4.92
23	C	No	10/10	5.22
23	D	No	11/12	4.55

[a] Mice in groups A and C received an inhaled dose of 77 median egg infectious doses (EID_{50}); mice in groups B and D received 194 EID_{50}.
[b] Mice were treated on days −1, 0, 1, 2, and 3 relative to virus inoculation.
[c] Based on seroconversion by HI test.
[d] Geometric (log_2) mean HI titer.

TABLE 6. *Effect of various poly I:C treatment regimens on lung virus titers of mice infected with Sendai*[a]

Mouse no.	A (−1, 0, 1)[b]	B (−1, 0)	C (0, 1)	D (−1)	E (0)	F (1)	G (none)
1	Neg	1.9[c]	3.4	2.3	3.5	4.9	5.5
2	1.5	1.0	3.5	3.5	3.8	3.5	3.5
3	2.7	Neg	1.1	3.5	3.7	4.0	5.0
4	1.5	2.5	Neg	3.5	3.0	4.3	4.9
5	.9	2.9	3.0	2.1	2.8	3.9	5.3
6	.7	1.3	2.7	1.5	2.8	4.5	4.9
7	1.8	.7	3.3	2.7	2.7	4.9	4.3
8	.2	1.8	Neg	4.1	4.1	4.4	5.5
9	1.8	2.1	2.2	2.1	3.9	2.5	4.6
10	1.9	2.2	1.2	3.2	3.5	4.0	4.9
Mean log[d]	1.30	1.64	2.04	2.85	3.38	4.09	4.84

[a] Average inhaled dose was 95 median egg infectious doses (EID_{50}).
[b] Days of poly I:C treatment relative to virus inoculation.
[c] $Log_{10}EID_{50}$ per 0.1 ml of 10% lung suspension.
[d] Means A, B, and C, C and D, E and F, D and E, and F and G are not significantly different at the 5% level. Other combinations of means are significantly different at the 1% level.

ployed by other investigators. A single aerosol treatment consisted of a maximum of 8 μg, and the longest treatments were continued for 5 consecutive days (40 μg, total). Since these doses were calculated from the concentration of poly I:C in the aerosols and respiratory volumes of the mouse, the actual dose retained by the mice may be considerably less than 8 μg. No evidence of toxicity was seen in mice given a 5-day aerosol treatment with poly I:C. Mice from the treated and untreated groups were sacrificed daily, and lung tissues were prepared for histopathological examination. Histologic alterations were not seen in poly I:C treated or untreated animals.

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