Post-Exposure Prophylaxis of Murine Rabies with Polyinosinic-Polycytidylic Acid and Chlorite-Oxidized Amylose¹

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Chlorite-oxidized amylose (COAM), polyinosinic-polycytidylic acid [poly(I:C)], and combinations of the two drugs were evaluated for their interferon-inducing properties and their ability to protect mice against rabies infection. Post-exposure administration of one or two doses (100 μ g each) of poly(I:C) significantly protected mice against rabies infection. Pretreatment of mice with COAM 3 h before poly(I:C) stimulation resulted in an enhancement of the interferon response. However, the increased interferon titers were not reflected by increased protection against rabies infection over that achieved with poly(I:C) therapy alone. Therapy with COAM alone did not protect mice against rabies but, rather, was associated with enhanced mortality.

The oxidation of amylose by sodium chlorite produces a polyanionic compound, chlorite-oxidized amylose (COAM). This product has been shown to have antiviral activity and to induce small amounts of interferon *in vivo* (3). In addition, COAM enhances antibody production (2) and was recently shown to potentiate the interferon response to polyinosinic-polycytidylic acid [poly(I:C)] (10). Poly(I:C), itself an excellent inducer of interferon, is also an adjuvant for both humoral and cellular immunity (15).

Our laboratory has been investigating the use of poly(I:C) in post-exposure prophylaxis of street rabies virus infection in mice, an infection that is inhibited by this drug (8). The protective effect of poly(I:C) appears to be related primarily to its capacity to induce interferon; however, the possibility has not been eliminated that the adjuvant effect may also be playing a role in protecting animals from infection.

This report evaluates the interferon response and protection against rabies infection engendered by COAM alone and in combination with poly(I:C). In addition, the serum-neutralizing antibody responses in treated animals were determined in an effort to document an adjuvant effect on antibody production by poly(I:C), COAM, or both.

MATERIALS AND METHODS

Animals. Three-week-old outbred Swiss-Webster mice purchased from Simonson Laboratories (Gilroy, Calif.) were used.

Rabies virus. The street rabies virus used in the challenge experiment was obtained from the salivary gland of a naturally infected skunk. The challenge inoculum was prepared by passing this virus intracerebrally once in suckling mice. Animals were challenged intramuscularly in the lower right leg with 0.1 ml of a 1:50 dilution of this material.

Poly(I:C) and COAM. Poly(I:C) and COAM were supplied by the Antiviral Substances Program of the National Institutes of Health. The stock concentration of poly(I:C) was 1 mg/ml. Each poly(I:C) dose consisted of 0.1 ml (0.1 mg) of this solution and was given intramuscularly. In the challenge experiment, poly(I:C) was injected into the same site as viral inoculation. COAM was supplied as a 30-mg/ml solution that was diluted 10-fold in phosphate-buffered saline, and 0.1 ml (0.3 mg) was injected by the intraperitoneal route.

Experimental design. The temporal sequence of the prophylactic regimens used in the interferon response and challenge experiments is presented in Table 1. To determine the kinetics of the interferon response, uninfected mice were given the indicated treatment regimens, and three mice per group were bled at 3, 6, 12, 24, and 48 h after the last indicated dose. Two mice from each group in the challenge experiment were bled 3 h after the last indicated dose to document the interferon response. In the challenge experiment, virus was injected 3 h before time zero.

The experiment was observed for 143 days, and dead animals were verified as having died from rabies by examination of their brains with the fluorescent

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antibody technique (7). Brain samples negative for rabies by this technique were injected intracerebrally into 3-week-old mice, which were observed for 3 weeks for evidence of rabies infection. Animals that were negative for rabies by both techniques were not included in the results.

Interferon assay. Pooled serum samples from two or three mice from each bleeding time were assayed for interferon by the plaque reduction technique. Monolayers of mouse L-929 cells in petri dishes (10 by 35 mm; Falcon Plastics, Oxnard, Calif.) were overlaid with 1-ml portions of appropriate dilutions of serum samples in minimal essential medium (MEM) and incubated for 24 h at 37 C. Cells were then washed with phosphate-buffered saline and challenged with 70 to 90 plaque-forming units of vesicular stomatitis virus. After a 1-h virus adsorption period, monolayers were overlaid with 3 ml of MEM containing 0.5% agarose and reincubated at 37 C for another 24 h. Uninfected cells were then stained with a neutral red solution (0.2 mg/ml), and plaques were counted 12 to 24 h later. The serum dilution inhibiting 50% of the plaques as compared with control plates was used as the end point, and the reciprocal of that dilution was used to express interferon titers as units per milliliter.

Neutralizing antibody assay. During the course of the challenge experiment, two mice from each group were bled 2, 7, 18, 30, 60, and 90 days after challenge. Neutralizing antibody levels of sera from individual mice were determined by the rapid fluorescent focus inhibition technique described by Smith et al. (13), who demonstrated that it correlated with the mouse neutralization test.

Serial dilutions (fivefold) of heat-inactivated serum were prepared in eight-chambered Lab-Tek slides (Miles Laboratories), using modified MEM as diluent (11). Each chamber was inoculated with an equal volume (0.1 ml) of medium containing 6×10^4 plaque-forming units of rabies virus strain CVS-11 (supplied by George Baer) and incubated for 90 min at 37 C. Control slides consisted of normal mouse serum and a laboratory antibody standard, each challenged with the same virus preparation. After incubation, 10^5 BHK-21/13S cells (11) in 0.2 ml of medium containing $10 \mu\text{g}$ of diethylaminoethyl-dex-

tran per ml were added to each chamber and incubated for 24 h at 35 C. The chambers were then removed from the slides, and the cells were rinsed in phosphate-buffered saline and fixed in cold acetone for 10 min. Infected cells were stained with fluorescein isothiocyanate-conjugated antirabies serum and examined through a Zeiss microscope using BG-38 and UG-1 exciter filters and barrier filters no. 65 and 41. An OSRAM HBO 200 high-pressure mercury vapor lamp was used for illumination. Twenty ($\times 250$) microscopic fields for each dilution were examined, and the number of fields containing fluorescing cells was tabulated. Control slides contained fluorescing cells in 18 to 20 of the 20 fields examined. A reduction of 50% of the number of fields containing fluorescing cells was indicative of neutralizing antibody. The

FIG. 1. Kinetics of the interferon responses after administration of the prophylactic regimens of poly(I:C) and COAM indicated in Table 1. Three mice per group were bled 3, 6, 12, and 24 h after the last indicated drug injection. The first plotted point for each drug regimen represents a sample obtained 3 h after the last drug injection. Group A, 1 dose of COAM; group B, 1 dose of poly(I:C); group C, 2 doses of poly(I:C); group D, 1 dose of COAM and 1 dose of poly(I:C); group E, 1 dose of COAM and 2 doses of poly(I:C); group F, 2 doses of COAM and 3 doses of poly(I:C).

TABLE 1. Temporal sequence of administration of prophylactic regimens used in the interferon response^a and virus challenge^b experiments

Group	Prophylactic regimen	No. of doses	Time (h) of drug administration						
			0	3	6	9	12	96	99
A	COAM	1	COAM						
B	Poly(I:C)	1	Poly(I:C)						
C	Poly(I:C)	2	Poly(I:C)				Poly(I:C)		
D	COAM	1	COAM	Poly(I:C)					
E	Poly(I:C)	1							
	COAM	1	COAM	Poly(I:C)			Poly(I:C)		
	Poly(I:C)	2							
F	COAM	2	COAM	Poly(I:C)			Poly(I:C)	COAM	Poly(I:C)
	Poly(I:C)	3							

^a Three mice per group were bled at 3, 6, 12, 24, and 48 h after the last indicated drug injection.

^b Rabies virus was injected 3 h before time zero in the challenge experiment.

reciprocal of the highest serum dilution exhibiting this end point was taken as the neutralizing antibody titer of that sample.

RESULTS

The kinetics of the interferon response to the six prophylactic regimens are plotted in Fig. 1 and illustrate the interferon titer observed at varying intervals after initiation of each given drug regimen. The first sampling point for each individual regimen was obtained 3 h after completion of the final injection. The injection of COAM alone did not induce measurable levels of interferon. One dose of poly(I:C) produced peak interferon titers 3 and 6 h later. Two doses of poly(I:C) produced peak titers of interferon 15 h after the first injection (3 h after the second injection). When an injection of COAM preceded a single injection of poly(I:C), we observed a peak interferon titer 6 h later that was twice the maximal titer seen with a single injection of only poly(I:C). When an injection of COAM preceded two injections of poly(I:C), we observed a peak titer of 5,000 U 3 h after the second poly(I:C) injection. This titer was fivefold greater than the highest titer observed after two injections of poly(I:C). When a dose of COAM was followed by two doses of poly(I:C) and then 4 days later a second dose of COAM was given followed by a third dose of poly(I:C), we observed a peak interferon titer of 500 U 6 h after the final poly(I:C) injection or 105 h after the initiation of that drug regimen.

The protective effect of the six prophylactic regimens was evaluated in animals challenged with rabies virus (Table 2). Each drug regimen was instituted 3 h after virus inoculation. Animals were observed for 143 days, and in each group the majority of rabies deaths occurred

from 15 to 40 days postinoculation. Both single- and two-dose therapy with poly(I:C) provided significant protection against rabies virus infection. Combination therapy with COAM and poly(I:C) did not result in any significant increase in protection over that achieved with poly(I:C) therapy alone. COAM alone did not protect mice against rabies infection and, in fact, seemed to enhance rabies mortality. No mortality occurred in the COAM drug control. Although COAM pretreatment of our poly(I:C)-injected animals produced enhanced interferon responses, there was no significant difference in the observed protection.

The neutralizing antibody responses to rabies virus during the course of the challenge experiment are shown in Table 3. In this model of rabies infection, significant antibody titers are usually not observed until clinical symptoms develop. As the results indicate, antibody was not detectable until some time after day 7. By day 18, nearly all groups showed the presence of antibody at similarly low levels. Group F, however, which received two doses of COAM and three doses of poly(I:C), showed a fivefold greater antibody titer at this time than any other group. Although suggestive of an early enhancement of antibody production, the titer was only one dilution greater than the other groups, and the number of animals bled was too small to state conclusively that antibody enhancement occurred. The same situation also pertains to day 30, where the only animal with an antibody titer of 125 was in group F.

DISCUSSION

In recent years, a number of antiviral agents have been developed that are effective against a

TABLE 2. *Protective effect of poly(I:C) and COAM against street rabies virus challenge^a*

Group	Prophylactic regimen	No. of doses	Rabies deaths/total	Percent mortality	P value ^b	Interferon ^c (U/ml)
A	COAM	1	18/33	54.5	NS	< 10
B	Poly(I:C)	1	2/32	6.2	< 0.001	400
C	Poly(I:C)	2	5/34	14.7	< 0.01	1,600
D	COAM	1	5/34	14.7	< 0.01	1,600
E	Poly(I:C)	1	5/33	15.1	< 0.02	3,200
	COAM	2				
F	Poly(I:C)	1	1/32	3.1	< 0.001	50
	COAM	2				
	Poly(I:C)	3				
	Controls		12/32	37.5		< 10

^a Intramuscular injection of 0.1 ml of a 1:50 dilution of the first suckling mouse brain pool of a virus obtained from the salivary gland of a naturally infected skunk.

^b Chi-square test with Yates correction factor. NS, Not significant.

^c Reciprocal of interferon titer measured 3 h after the last indicated dose. Control titers were obtained 6 h after virus injection.

TABLE 3. Rabies-neutralizing antibody response^a of mice after virus challenge and treatment with COAM, poly(I:C), or both

Group	Prophylactic regimen	No. of doses	Days postinoculation					
			2	7	18	30	60	90
A	COAM	1	— ^b	—	—	5 ^c	25	25
B	Poly(I:C)	1	—	—	5	5	5	5
C	Poly(I:C)	2	—	—	5	25	25	25
D	COAM	1	—	—	5	5	25	—
E	COAM	1	—	—	5	—	5	5
F	Poly(I:C)	2	—	—	5	25	25	25
	COAM	2	—	—	25	25	25	25
	Poly(I:C)	3	—	—	25	125	125	25
	Controls		—	—	5	5	25	25

^a Antibody determinations were made individually on sera from two mice at each time point.

^b —, No neutralizing activity at a 1:5 serum dilution.

^c Numbers represent reciprocal of antibody titer.

variety of viral infections (14). The occurrence of rabies in humans after optimal therapy (1, 4) establishes the fact that currently recommended treatment is not always adequate and has prompted a number of investigators to look for new methods to treat this disease.

The use of poly(I:C) has been shown to be highly effective in preventing rabies in mice and rabbits (5, 8). The results reported in this paper further emphasize the efficacy of therapy with poly(I:C) alone in preventing rabies infection when given after exposure to virus, which is analogous to the condition under which most humans are treated.

The data presented indicate that the magnitude of the interferon response is not proportional to the degree of protection observed. It is evident that both one- and two-dose therapy with poly(I:C) protected animals despite differences in the interferon response. Furthermore, enhancement of the interferon response to poly(I:C) with COAM did not significantly increase the level of protection. This observation is in agreement with those of Pindak et al. (12), who, after studying four interferon inducers, concluded that the amount of interferon induced and the degree of protection against virus infection were both dose dependent, but that protection was not directly correlated with the interferon titers.

It has been suggested (10) that the mecha-

nism through which COAM enhances the interferon response to poly(I:C) may be analogous to the "priming" phenomenon described *in vitro* in which pretreatment of cells with small amounts of interferon results in the production of enhanced amounts of interferon after induction (6, 9). Since COAM has been reported to induce small amounts of interferon *in vivo*, the suggestion is quite logical that this interferon could serve as a primer. However, we attempted to prime both rabbits and mice with small amounts of homologous interferon and failed to see enhanced interferon titers after poly(I:C) stimulation (unpublished observations).

Since both COAM and poly(I:C) have been reported to have an immunoenhancing effect (2, 15), we expected to see more evidence than we did of an adjuvant effect on antibody production in all treatment groups. Interpretation of our antibody results is made difficult because of the different effects these drugs might have on the immune system. For example, it is likely that the antiviral interferon-inducing effects of poly(I:C) or COAM caused a reduction in replication of the challenge virus and hence antigenic load. This effect might have masked an immunoenhancing effect. Therefore, the near equivalence of the antibody titers in poly(I:C)-treated and control animals may represent, on the part of poly(I:C)-treated animals, an enhancement of antibody production since they may have actually been exposed to less antigen.

The lack of evidence of an adjuvant effect of COAM may be related to the route by which the drug was administered. It has been demonstrated that COAM does not elicit adjuvant activity unless injected simultaneously with and into the same site as antigen (2). In our studies, neither of these criteria were met, since COAM was injected 3 h after the antigen (rabies virus) and was administered intraperitoneally, whereas virus was injected intramuscularly. Furthermore, the dosage of COAM used in this study was lower (0.3 mg/mouse) than the doses that have been reported to elicit an adjuvant effect (4 to 40 mg/mouse) (2).

In summary, the experiments reported here demonstrate no advantage in the use of COAM, either alone or in combination with poly(I:C), over the use of poly(I:C) alone in post-exposure therapy of street rabies virus-infected mice.

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