Late Therapy of an Arbovirus Encephalitis in Mice with Interferon and Interferon Stimulators (37382)

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Numerous studies have demonstrated that interferon and interferon stimulators are highly effective when used prophylactically (1, 2), and in certain situations therapeutically, in a variety of experimental viral infections (3, 4). It has been suggested that passively administered interferon may exert a greater protective effect *in vivo* than does the interferon stimulator polyinosinic·polycytidylic (In·Cn) acid (5, 6). This interpretation was based on the greater protective effect of mouse interferon as compared with In·Cn observed in two studies (5, 6). In a previous study, we reported that the use of multiple doses of In·Cn resulted in a protective effect which clearly exceeded the protection reported for passive interferon administration in experimental Semliki Forest virus (SFV) infection of mice (7). In the present study, we report the results of experiments in which these multiple doses of In·Cn were directly compared with multiple doses of large amounts of mouse interferon in protecting SFV infected mice. We also report attempts to increase the amount of interferon stimulated by In·Cn and thus improve the therapeutic effectiveness of an optimal schedule of In·Cn in treating SFV infected mice.

Materials and Methods. NIH general purpose Swiss female mice of 20–25 g were used throughout. They were inoculated with SFV subcutaneously (sc). All inoculations of mouse interferon or interferon stimulators were intraperitoneal (ip). Semliki Forest virus (SFV) was obtained from Dr. Robert Friedman and grown on chick embryo monolayers. The titer was $10^{6.7}$ plaque-forming units (pfu)/ml on chick embryo cell cultures and $10^{5.0}$ 50% lethal doses (LD_{50})/ml when titered sc in groups of six 20–25 g mice.

Interferon assays. At various time intervals after injection of In·Cn or In·Cn combined with poly-D-lysine groups of 5 mice were bled by decapitation and their blood pooled. The blood was centrifuged at 1500 rpm for 20 min and serum was then stored at −20°. The serum interferon titers were expressed as the reciprocal of the highest dilution of serum which inhibited the hemagglutinin yield of GD-7 virus during a single growth cycle in mouse L-cells by 0.5 $\log_{10}$ (8). Titers were adjusted in accordance with the titer of a laboratory reference interferon which was titered in each assay. The international reference mouse serum interferon titered $10^{4.5}$ units/ml.

Mouse serum interferon. Mouse serum interferon was obtained from Abbott Laboratories. This interferon pool was obtained by injecting Swiss mice iv with $10^{8.3}$ pfu of Newcastle disease virus; the mice were bled 8 hr later by decapitation and their serum pooled. The serum was acidified to pH 2.0 for 5 days, and then brought back to pH 7.4. The titer of this pool was 25,000 units/ml.

In·Cn and poly-D-lysine. Double-stranded polyinosinic·polycytidylic ribonucleic acid of high molecular weight (mol wt of individual copolymers 10^6) was prepared as described previously (9). The concentration of this material before it was combined with poly-D-lysine was 200 μg/ml. Poly-D-lysine HBR (Nutritional Biochemicals Corp., Cleveland, Ohio; mw approximately 160,000) was dissolved in phosphate-buffered saline (PBS) at 0.11 mg/ml, or 500 μ*M* E-amino nitrogen, and stored at 4°. Solutions for injection were prepared by adding In·Cn stock solution to twice its volume of 0.5% (w/v) PBS (pH 7.0) solution of carboxymethylcellulose 7HSP (CMC;

Hercules Powder Co., Wilmington, Del.). A volume of poly-D-lysine stock solution equal to that of the stock solution was then added slowly with stirring at approximately 40°. This procedure yields solutions in which precipitates either do not form or redissolve within 24 hr at room temperature, and in which all poly-D-lysine is associated with the synthetic double-stranded RNA (Rice *et al.*, in preparation). This procedure permits the use of the poly-D-lysine complex technique with preparations of much higher molecular weight than those previously described (10, 11).

COAM. Chlorite Oxidized Amylose is obtained by oxidizing amylose with Na chlorite as described by Claes (12). Multiple carboxyl groups are inserted into the amylose molecule by the oxidation. COAM is a moderately good antiviral agent (13). It leads to the production *in vivo* of small amounts of interferon and is a good enhancer of antibody production (14). When given ip to mice before ip administration of In • Cn it leads to a 6–100-fold increase in the titer of circulating interferon as compared to that obtained by In • Cn alone. (H. B. Levy, J. Duenwald, and C. E. Buckler, in press). The COAM used in these experiments was kindly supplied by Dr. A. Billiau of Leuven.

Statistics. In the protection experiments the percentage survivals were compared by the chi square test for proportions (1 degree of freedom).

Experimental design. In the protection experiments a large number of mice were inoculated sc with 2 LD_{50} of SFV in 0.2 ml of Eagle's medium with 2% fetal calf serum (FCS). A group of 40 mice was kept as infected controls, while groups of 20 mice were started on treatment with mouse interferon or an interferon stimulator, as described. Therapy was begun from 1 to 5 days after virus inoculation. Mice were observed for a minimum of three weeks. No deaths occurred after the 16th day after virus challenge; most deaths occurred within the first 10 days.

Results. The effect of daily doses of In • Cn or mouse serum interferon on mortality from SFV infection in mice was studied. The first experiments were designed to compare directly the effect of continuous therapy with In • Cn with continuous therapy with interferon. On different days after injection of SFV groups of 20 mice were injected intraperitoneally with either 100 μg of In • Cn daily for 4 days or 0.25 cc of mouse serum interferon (25,000 units/ml) twice a day for 5 days. Table I summarizes three such experiments. Therapy with interferon was effective only if begun one day after injection of SFV; treatment begun on Day 2 after SFV infection did not significantly reduce mortality. Therapy with daily doses of In • Cn beginning on Day 1 after infection was significantly more effective than continuous interferon therapy. In addition, when all three experiments are combined, therapy with In • Cn

TABLE I. Comparison of In • Cn and Interferon in Treatment of SFV Infection in Mice.

	% Mortality relative to day therapy started[a]						
	Saline	In • Cn[b]				Interferon[c]	
Expt.	D1[f]	D1	D2	D3	D4	D1	D2
1	70	15[d]	25[d]	25[d]	70	55	60
2	75	0[d]	10[d]	25[d]	40[e]	20[d]	87
3	70	5[d]	20[d]	30[d]	50	40	67
Average	72	7[d]	18[d]	27[d]	53[e]	38[d]	71

[a] Groups of 20 mice; all mice were injected sc with 2 LD_{50} of SFV on Day 0.
[b] 100 μg, once daily for 4 days, ip.
[c] 0.25 ml containing 25,000 units/ml, twice daily for 5 days, ip.
[d] $p < 0.01$.
[e] $p < 0.05$.
[f] D1 = Day 1.

was effective even when begun as late as Day 4 after infection ($p < 0.05$). These results with In • Cn are similar to those previously reported by us (7).

These results indicate that continuous therapy with In • Cn is considerably more effective at protecting SFV infected mice than is continuous therapy with high titered mouse serum interferon. This is consistent with the observation that considerably greater quantities of interferon would be stimulated by this dose schedule of In • Cn than were passively administered (15).

Effect of poly-D-lysine on stimulation of interferon by In • Cn. It has previously been reported that combining In • Cn with poly-D-lysine significantly enhanced the ability of In • Cn to induce serum interferon (10). The serum interferon responses of mice injected ip with a single dose of 100 μg of In • Cn or a single dose of 100 μg of In • Cn combined with 55 μg of poly-D-lysine are shown in Fig. 1. Mice receiving In • Cn plus poly-D-lysine had about 10-fold higher serum interferon levels at 6 and 12 hr after In • Cn injection, while serum titers 24 hr after In • Cn injection were similar in the two groups. Mice receiving just poly-D-lysine did not have detectable serum interferon levels.

In a second experiment, groups of mice were injected with either 100 μg of In • Cn ip or 100 μg of In • Cn plus 55 μg of poly-D-lysine ip daily for 4 days. Serum interferon levels were determined six hours after each injection of In • Cn. Six hours after the first injection serum interferon titers in the group receiving the In • Cn plus poly-D-lysine were

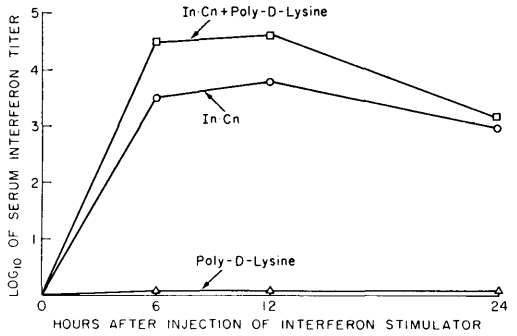

FIG. 1. Effect of poly-D-lysine on the stimulation of interferon by a single dose of In•Cn in mice.

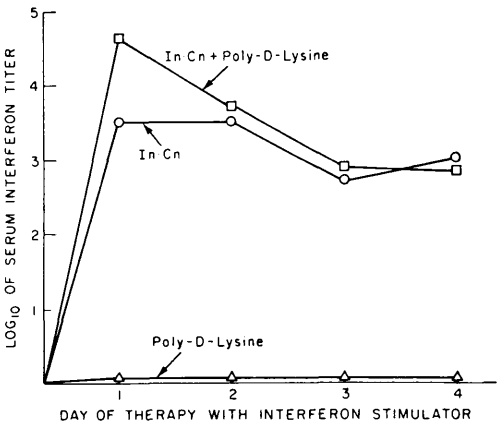

FIG. 2. Effect of poly-D-lysine on the stimulation of interferon by multiple doses of In•Cn in mice.

again about 10-fold higher than in the serum of mice receiving just the In • Cn (Fig. 2). However, on each of the subsequent three days there was no difference in the serum levels in the two groups (Fig. 2).

Effect of poly-D-lysine on the therapeutic effectiveness of In • Cn. In order to determine if any correlation existed between the ability of the In • Cn combined with poly-D-lysine to induce interferon and its ability to protect SFV-infected mice, several protection studies were performed. In the first two experiments mice were injected sc with 2 LD_{50} of SFV as previously described. One or 2 days after virus infection groups of 20 mice were injected with a single dose ip of either 100 μg of In • Cn or 100 μg of In • Cn combined with 55 μg of poly-D-lysine. Mice receiving the In • Cn plus poly-D-lysine on Day 1 had a significantly lower mortality than mice receiving just the In • Cn (Table II). There was no protection in either group when therapy was delayed until Day 2 after infection (Table II). This result is consistent with the greater ability of In • Cn combined with poly-D-lysine to induce the formation of interferon.

In the next two experiments, mice were injected with SFV as before and then given 4 daily doses of either In • Cn or In • Cn plus poly-D-lysine beginning 4 or 5 days after virus challenge. Mice receiving In • Cn plus poly-D-lysine did not have a significantly lower mortality on either day than did mice receiving just In • Cn (Table III). These re-

TABLE II. Effect of Poly-D-lysine on the Therapeutic Effectiveness of a Single ip Dose of In • Cn Against SFV Infection in Mice.

	% Mortality relative to day therapy administered[a]				
	Saline	In • Cn[b]		In • Cn (poly-D-lysine)[c]	
Expt.	D1[f]	D1	D2	D1	D2
1	80	75	90	45[e]	85
2	75	50	70	20[d]	65
Average	78	63	80	33[d]	75

[a] Groups of 20 mice; all mice were injected sc with 2 LD_{50} of SFV on Day 0.
[b] 100 μg.
[c] 100 μg In • Cn combined with 55 μg poly-D-lysine.
[d] $p < 0.01$.
[e] $p < 0.05$.
[f] D1 = Day 1.

sults indicate that increased interferon stimulation by In • Cn plus poly-D-lysine only on Day 1 was not sufficient to provide increased protection. This finding confirms the observation that repeated doses of In • Cn are necessary for an optimal protective effect (7).

Effect of combined therapy with In • Cn and COAM on protection of SFV infected mice. Previous studies have demonstrated that COAM may induce small amounts of interferon *in vivo* and is an effective antiviral agent in a variety of experimental situations (13, 14). Studies from this laboratory have shown that pretreatment of mice with 3 mg of COAM ip significantly increased the interferon response to In • Cn administered ip three hours after COAM treatment (H. B. Levy, J. Duenwald, and C. E. Buckler, in press). The ability of COAM treatment to improve the therapeutic effectiveness of In • Cn therapy in SFV infected mice was therefore evaluated. Mice were injected with SFV as before. One or two days after virus challenge groups of 20 mice were injected ip with either COAM, COAM followed 3 hr later by In • Cn, or just In • Cn. Significantly lower mortality ($p < 0.01$) occurred among mice receiving COAM plus In • Cn on either Day 1 or Day 2 after virus infection, than among mice treated with either COAM or In • Cn alone (Table IV), although some protection was observed in each of the treatment groups. These results are consistent with the previously observed enhancement of interferon stimulation by In•Cn in mice pretreated with COAM. However, COAM is also an effective antiviral agent, and so it is possible that these improved results are just the addition of the antiviral effect of the two drugs, rather than the result of an enhanced interferon response.

Discussion. The purpose of these experiments was to determine directly the comparative efficacy of an optimal dose schedule of a potent interferon stimulator, In•Cn, as compared with an intensive dose schedule of mouse interferon itself. Daily treatment with 100 μg of In•Cn intraperitoneally was significantly more effective than twice daily doses of 0.25 ml containing 25,000 units/ml of mouse interferon. Similarly, modifications of In•Cn which increased the stimulation of interferon were correlated with increased protection. A single dose of In•Cn plus poly-D-lysine induced formation of 10-fold more serum interferon than did a single dose of In•Cn alone; similarly a single dose of In•Cn plus poly-D-lysine was significantly more

TABLE III. Effect of Poly-D-lysine on the Therapeutic Effectiveness of Multiple ip Doses of In • Cn Against SFV Infection in Mice.

	% Mortality relative to day therapy started[a]				
	Saline	In • Cn[b]		In • Cn (poly-D-lysine)[c]	
Expt.	D4[f]	D4	D5	D4	D5
1	70	45	60	30[d]	55
2	80	30[d]	40[d]	25[d]	50[e]
Average	75	38[d]	50[e]	28[d]	53[e]

[a] Groups of 20 mice; all mice were injected sc with 2 LD_{50} of SFV on Day 0.
[b] 100 μg, daily.
[c] 100 μg In•Cn combined with 55 μg poly-D-lysine, daily.
[d] $p < 0.01$.
[e] $p < 0.05$.
[f] D4 = Day 4.

TABLE IV. Effect of a Single Dose of COAM on the Therapeutic Effectiveness of a Single ip Dose of In·Cn in SFV Infection in Mice.

	% Mortality when therapy administered on Day 2 after infection[a]			
Expt.	Saline	In·Cn[b]	COAM[c]	In·Cn + COAM[d]
1	90	50[e]	50[e]	25[e]
2	95	90	90	65[e]
3	100	75[e]	75	50[e]
Average	95	72[e]	72[e]	47[e]

[a] Groups of 20 mice; all mice were injected sc with 2 LD_{50} of SFV on Day 0.
[b] 100 μg, ip.
[c] 3 mg, ip.
[d] 100 μg In·Cn ip, 3 hr after 3 mg COAM ip.
[e] $p < 0.01$.

effective at protecting SFV infected mice than was a single dose of In·Cn. Multiple doses of In·Cn plus poly-D-lysine did not stimulate the formation of sustained higher levels of serum interferon, and likewise were no more effective therapeutically than were multiple doses of In·Cn by itself. Pretreatment with 3 mg of COAM, which enhances the interferon response to In·Cn, significantly increased the therapeutic effect of a single dose of In·Cn.

It has been suggested that passively administered interferon may exert a greater protective effect *in vivo* than does the interferon stimulator In·Cn (5, 6). This interpretation was based on the greater protective effect of mouse serum interferon as compared with In·Cn observed in two studies (5, 6). In these studies the dose of In·Cn used was relatively small. In a previous study we demonstrated that large, multiple doses of In·Cn resulted in a much greater protective effect which exceeded the protection reported for passive interferon administration in SFV infection in mice (7). In the present study, multiple doses of In·Cn were considerably more effective therapeutically than twice daily doses of 0.25 ml containing 25,000 units/ml of mouse interferon. Based on previous observations of serum interferon levels after multiple doses of In·Cn and the half-life of interferon in the serum (15, 16), we estimate that the release into serum of between 2×10^6 and 6×10^6 units of interferon was stimulated by the dose schedule of In·Cn used; mice receiving passive interferon, on the other hand, received a total of about 6×10^4 units of interferon. The greater therapeutic effect of this dose schedule of In·Cn is thus consistent with the observation that considerably larger amounts of interferon were stimulated by this dose schedule of In·Cn than were passively administered. Therefore, in a situation where an interferon stimulator gave rise to more interferon than was applied passively, the inducer exerted a much greater protective effect. The addition of either poly-D-lysine or COAM increased the therapeutic effect of a single dose of In·Cn in SFV infection in mice. This correlates with the higher serum interferon levels obtained, and suggests that an even greater therapeutic effect would be observed if some way were found to obtain sustained higher serum interferon levels or to increase the initial serum interferon levels. Methods which either enhance the interferon-inducing ability of inducers or decrease the toxicity of interferon stimulators may make possible even greater protective effects late in the course of experimental viral infections.

Summary. The therapeutic effectiveness of daily doses of 100 μg of polyinosinic acid·polycytidylic acid (In·Cn) intraperitoneally was compared with two daily doses each of 0.25 ml containing 25,000 units/ml of mouse serum interferon intraperitoneally in protecting Semliki Forest virus infected mice. Mice receiving daily doses of In·Cn had a significantly lower mortality than did mice receiving the multiple doses of interferon. Combining In·Cn with poly-D-lysine resulted in a significantly enhanced interferon response to a single dose of In·Cn, and a similar greater therapeutic effect of a single dose of In·Cn plus poly-D-lysine was noted. Neither sustained higher levels of serum interferon nor increased protection were observed following multiple doses of In·Cn plus poly-D-lysine, as compared with multiple doses of In·Cn by itself. Pretreatment with 3 mg of COAM, which enhances

the interferon response to In·Cn, significantly increased the therapeutic effect of a single dose of In·Cn.

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