Late Therapy with an Interferon Stimulator in an Arbovirus Encephalitis in Mice (35257)[1]

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Numerous studies have demonstrated that interferon and interferon stimulators are highly effective when used prophylactically in a variety of experimental viral infections (1, 2). It has, however, been much more difficult to demonstrate an effect of these agents when they are administered after virus inoculation. This may in part be due to the limited amounts of interferon which could be administered or stimulated and to the lack of systematic studies to determine the most effective dose schedules. In this study a comparison was made of a number of different dose schedules of a potent interferon stimulator, polyinosinic·polycytidylic acid (In·Cn), in experimental Semliki Forest virus infection of mice. The optimal dose schedule of In·Cn was then studied extensively in an attempt to determine how late in the course of this infection treatment could be begun and still be effective.

Materials and Methods. NIH general purpose Swiss female mice of 20–25 g were used throughout. They were inoculated with Semliki Forest virus (SFV) either intraperitoneally (ip) or subcutaneously (sc), as indicated. All inoculations of In·Cn were intraperitoneal. Semliki Forest virus was obtained from Dr. Robert Friedman at the NIH and grown on chick embryo monolayers. The titer was $10^{6.7}$ plaque-forming units/ml on chick embryo cell cultures and $10^{5.0}$ 50% lethal doses/ml when titrated intraperitoneally or subcutaneously in groups of six 20–25 g mice. For titrations of virus in blood and brain, a 10% suspension was made of blood or brain from individual mice. This suspension was centrifuged at 1500 rpm for 20 min; the supernatant was then stored at —70° in a mechanical freezer. Mice were exsanginated before removal of brains. The individual blood and brain suspensions were assayed for 50% tissue culture infectious doses ($TCID_{50}$) in chick embryo tissue culture; 0.2 ml of 10-fold serial dilutions were assayed in replicate culture tubes.

Double stranded In·Cn ribonucleic acid duplexes were prepared as described previously (3); the final concentration of this material was 0.5 mg/1.0 ml.

The same experimental design was used in all protection studies reported. A large number of mice were inoculated with about 2 LD_{50} of Semliki Forest virus in 0.2 ml of Eagle's medium with 2% fetal bovine serum, either ip or sc. A group of 40 mice were kept as infected controls, while groups of 20 mice each were started on In·Cn treatment every day beginning 1 to 5 days after virus inoculation. In each experiment two or three different dose schedules of In·Cn were compared. Mice were observed for a minimum of 3 weeks and in some experiments for 8 weeks. No deaths were noted to occur after the 16th day after virus challenge, and most deaths occurred during the first 10 days after virus challenge. In the protection experiments the percentage survivals were compared by the chi-squared test for proportions (1 degree of freedom).

Results. Protective effect of varying amounts of In·Cn given 1 to 3 days after infection. The first experiments were designed to determine whether large doses of In·Cn, which result in high levels of serum interferon (3, 4), would be therapeutically effective at times when small doses of In·Cn have been reported to be ineffective (5). Table I shows the effect of a single dose of 20–200 μg of In·Cn on mortality from SFV infection. No significant decrease in mortality

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TABLE I Effect of a Single ip Dose of In • Cn on SFV Infection in Mice.[a]

Dose In • Cn (μg)	(day):	% Final mortality; day In • Cn administered 1	2	3
0 (saline control)		75	—	—
200		25[b]	70	65
50		50	65	75
20		60	70	80

[a] The mice were injected with 2 LD_{50} of SFV sc. A group of 40 mice were kept as infected controls, while groups of 20 mice each were treated with a single ip dose of In • Cn on days 1, 2, or 3 after virus infection.

[b] $p < 0.01$ compared to saline-treated controls; if no [b], $p > 0.05$.

was noted when 20 or 50 μg of In·Cn were administered ip 1 day after sc virus challenge; significant protection was noted with a single dose of 200 μg of In·Cn at 1 day, but not at 2 days after virus challenge. This finding indicates that a single dose of 200 μg of In·Cn is more protective than are smaller doses. It also shows that protection can occur when treatment was given 1 day after infection.

Comparison of multiple dose schedules of In·Cn. In an attempt to increase the protective effect of In·Cn given after virus infection several multiple dose schedules were compared. Mice were injected with 2 LD_{50} SFV, as before. Groups of 20 mice each were started on In·Cn therapy every day beginning 1 to 4 days after virus inoculation and continuing until the 10th day after infection. In each experiment two different dose schedules of In·Cn were compared. Table II shows that a dose schedule of 100 μg of In·Cn daily gave significantly greater protection than did the same dose given twice a day, every other day, or as 1 dose. No greater protection was achieved by increasing the daily dose to 200 μg.

Treatment of mice beginning 1–5 days after sc infection with SFV. Table III shows the results of 5 experiments in which the apparently optimal regimen of 100 μg of In·Cn ip a day was used to treat mice challenged sc with Semlike Forest virus. In each experiment, a significant decrease in mortality was noted when In·Cn was begun 2 days after infection and in 4 of the 5 experiments a significant decrease in mortality was noted

TABLE II. Comparison of Multiple-Dose Schedules of In • Cn on SFV Infection of Mice.[a]

Expt. no.	Route of virus challenge	Dose (μg)	Schedule	(day):	% Final mortality relative to day therapy started 1	2	3	4
1	ip	0	(saline control)		65	—	—	—
		100	per day		0[b]	20[b]	10[b]	15[b]
		100	twice a day		15[b]	35	60	60
2	ip	0	(saline control)		92	—	—	—
		100	per day		40[b]	45[b]	95	95
		200	per day		30[b]	85	85	80
3	sc	0	(saline control)		72	—	—	—
		100	per day		0[b]	20[b]	25[b]	15[b]
		100	once		40[b]	45	75	65
4	sc	0	(saline control)		90	—	—	—
		100	per day		10[b]	30[b]	40[b]	60[b]
		100	every 2 days		10[b]	65[b]	75	75

[a] Mice were injected with 2 LD_{50} of SFV sc or ip. In each experiment 40 mice were kept as infected controls, while groups of 20 mice each were started on In • Cn treatment every day beginning 1 to 4 days after virus inoculation. Two different dose schedules were compared in each experiment.

[b] $p < 0.05$,

TABLE III. Postinfection Treatment of SFV Infection in Mice with In • Cn.[a]

Expt. no.	Saline-treated controls	% Final mortality relative to day therapy started (day): 1	2	3	4	5
1	72	0[c]	15[c]	30[c]	15[c]	35[b]
2	75	10[c]	40[b]	65	65	65
3	80	0[c]	20[c]	35[c]	45[c]	ND
4	72	0[c]	20[c]	25[c]	15[c]	75
5	90	10[c]	30[c]	40[c]	60[b]	65[b]
Av	78	2[c]	25[c]	35[c]	38[c]	60[c]

[a] Mice were injected with 2 LD_{50} of SFV sc. A group of 40 mice were kept as infected controls, while groups of 20 mice each received 100 μg of In • Cn ip a day beginning 1 to 5 days after virus inoculation and continuing until the 10th day after infection.

[b] $p < 0.05$.

[c] $p < 0.01$.

when treatment with In•Cn was begun on the 4th day after infection. In two of the four experiments in which In•Cn was begun on the 5th day, a significant decrease in mortality was also noted.

Figure 1 shows the combined cumulative mortality in these 5 experiments. The control mortality curve represents 200 mice, while the day 1 to day 4 curves represent 100 mice each. The day 5 curve represents 80 mice. Figure 1 shows only a slight delay in mean day of death in groups in which treatment was begun on day 3 or earlier. However, the difference in overall mortality between the control group and each of the other 5 groups is highly significant ($p = <0.01$).

These results indicate that significant protection can be obtained when treatment with an optimal dose schedule of In•Cn is begun as late as 4 or 5 days after sc challenge of mice with SFV.

Viremia and virus titers in the brains of untreated mice. To help determine the stages of pathogenesis of SFV infection in which

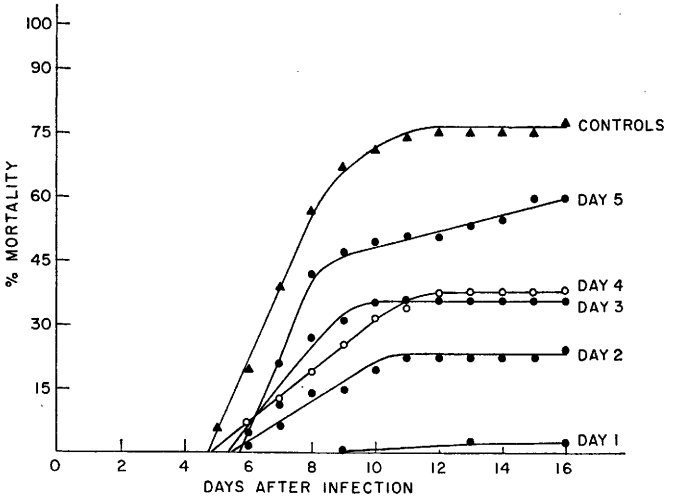

FIG. 1. Cumulative mortality of mice treated with In•Cn beginning 1 to 5 days after sc injection of SFV. The 5 experiments listed in Table III are combined and summarized. All mice were injected with 2 LD_{50} of SFV sc. In each experiment 40 mice were kept as infected controls, while groups of 20 mice each received 100 μg of In•Cn ip a day beginning 1 to 5 days after virus inoculation and continuing until the 10th day after infection.

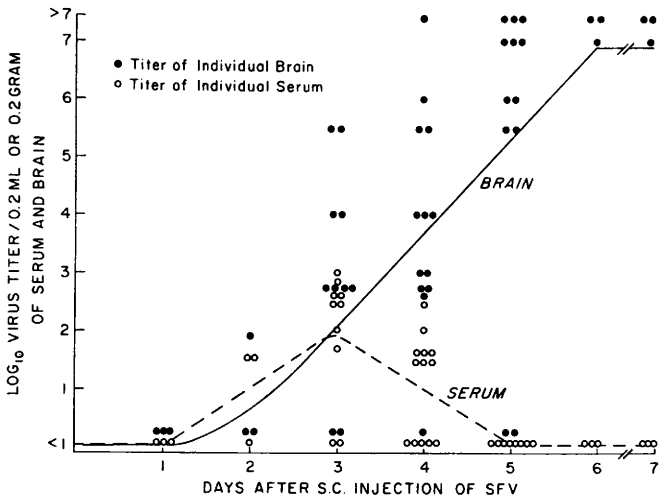

FIG. 2. Virus titers in the serum and brain of SFV infected mice. Mice were injected with 2 LD_{50} of SFV sc. Individual serum and brain specimens were collected each day after virus infection; virus titers of these specimens were assayed on chick embryo tisue.

therapy with In·Cn was effective, experiments were undertaken to establish the times of viremia and of virus growth in brain.

Figure 2 combines two separate experiments and shows the results of virus titrations of individual serum and brain specimens collected at varying times after inoculation of mice with 2 LD_{50} of virus sc. Viremia was first detected on day 2, reached a peak on day 3, and had terminated by day 5. Virus was first demonstrated in 1 of 3 brains on day 2 and was detected in 8 of 10 brains on day 3. Virus titers in the brains rose progressively until days 6 and 7. It is of particular interest that 12 of 13 brains on day 4 had at least fairly high titers of virus and that 10 of 12 brains on day five had somewhat higher titers of virus.

These results indicate that virus growth in the brain was well underway at a time when successful treatment with In·Cn could be begun.

Discussion. The original purpose of these experiments was to determine the therapeutic efficacy of an optimal dose schedule of a potent interferon stimulator, polyinosinic·polycytidilic acid (In·Cn). Daily treatment with 100 μg of In·Cn intraperitoneally was more effective than any of the other dose schedules tested. Using this regimen it was possible to significantly decrease the mortality of Semliki Forest virus-infected mice when treatment was begun 4 or 5 days after virus inoculation—a time at which high titers of virus were present in brains.

Although the low dose of SFV used did not infect all the mice, the evidence indicates that the late protective effects occurred in mice with infected brains. Specifically in inoculated mice which survived, virus did not multiply sufficiently to stimulate protective antibody in our unpublished experiments and in a previous report (6). Also the percentage without virus in the brain on days 4 and 5 and the percentage surviving agree. It seems reasonable to conclude that mice which did not develop virus in their brains by days 4 or 5 were those which were not infected and which therefore survived. Therefore mice which were protected when In·Cn was started on day 3 or later probably represent a protective effect in mice with already infected brains. Further support for this interpretation comes from the study of viremia. It has been reported that interferon can protect against viremic spread (7, 8). Therefore protection by In·Cn on days 1 to 3, prior to and up to peak viremia, probably represents inhibition of viremic spread. Protection on days 4 and 5, when viremia was diminishing or ended, probably represents a protective effect against virus already in the brain. It is noteworthy that protection obtained when treatment was started on days 4 or 5 represents an effect after high, but not maximum, virus titers in the brain had already developed.

Interferon and interferon stimulators have been much more effective when used prophy-

lactically in experimental viral infections than when administered after virus inoculation (1, 2). As more potent interferon and interferon stimulators became available, it became possible to protect infected animals when treatment was begun after virus infection (8–14; B. Postic, personal communication). It has been suggested that passively administered interferon may exert a greater protective effect *in vivo* than does the interferon stimulator In·Cn (5, 15). This interpretation was based on the greater protective effect of mouse interferon as compared with In·Cn. The present findings do not support this interpretation and they provide a possible explanation for the difference. In the earlier studies the ineffective dose of In·Cn was relatively small and a similar small dose was also not protective therapeutically in the present study. However larger and multiple doses of In·Cn resulted in a much greater protective effect which clearly exceeded the protection reported for passive interferon administration in SFV infection in mice (10). In unpublished preliminary studies we have also failed to observe protection of mice when twice a day treatment with 6000 units of interferon was begun later than 1 day after SFV challenge. The increased protective effect of the higher dose schedule of In·Cn correlates with markedly increased production of serum interferon (4). Thus in a situation where an interferon stimulator gave rise to more interferon than has been applied passively, the inducer exerted a much greater protective effect.

It is noteworthy that an increase of the In·Cn dose from 100 μg/day to 2 doses of 100 μg/day (Table II) resulted in a significantly decreased protection despite the fact that 2 doses of 100 μg a day gives rise to more serum interferon (4). This finding could indicate a disassociation between amount of interferon stimulated and the protective effect. However, we favor the interpretation that since the higher dose approaches the toxic level of In·Cn its decreased protective effect is more likely due to secondary actions of the stimulator. Methods which avoid the toxicity of inducers or the development of other less toxic interferon stimulators may make possible even greater protective effects late in the course of experimental viral infections.

Summary. A number of different dose schedules of a potent interferon stimulator, polyinosinic·polycytidylic acid, were compared in Semliki Forest virus infected mice. Daily treatment with 100 μg of In·Cn intraperitoneally was more effective than any of the other dose schedules tested. Using this regimen it was possible to significantly decrease the mortality of SFV infected mice when treatment was begun as late as 5 days after virus inoculation, at a time when high titers of virus were present in most brains. In experiments where the interferon stimulator gave rise to more interferon than had been applied passively in previous studies, the inducer exerted a much greater protective effect.

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