Effect of Repeated Inoculation of Interferon Preparations on Infection of Mice with Encephalomyocarditis Virus (32723)

I. GRESSER, C. BOURALI, M. T. THOMAS, AND E. FALCOFF

Laboratory of Viral Oncology, Institut de Recherches Scientifiques sur le Cancer, 94-Villejuif, France; Groupe de Recherches sur les virus, Institut National de la Santé et de la Recherche Médicale, Hôpital Saint-Vincent de Paul, Paris

It was previously noted that continued administration of interferon to mice after inoculation of Friend virus was more effective than treatment limited only to the short period immediately before and after viral inoculation (1,2). It was considered of interest to extend these observations and determine whether continued interferon administration would also prove of value in an acute systemic viral disease. We present here the results of experiments on the effect of repeated inoculation of interferon preparations on infection of mice with encephalomyocarditis (EMC) virus and the immune response of interferon protected mice to this virus.

Methods and Material.. Viruses. Encephalomyocarditis virus (EMC) was obtained thru the courtesy of Dr. J. Huppert and passaged in monolayer cultures of L cells. One-month-old male and female weanling mice (IC strain) were inoculated intraperitoneally (ip) with 0.2 ml of a given viral dilution. Inoculated mice were examined twice daily and the number of paralyzed or dead mice was recorded. After viral inoculation, mice were kept up to 6 months.

Cell culture. Primary and secondary cultures of mouse embryo fibroblasts and L cells were prepared by standard techniques, and maintained in modified Eagle's balanced salt solution supplemented with 5–10% heat inactivated calf serum.

Serologic tests. Hemagglutination inhibition (HI). As previously described (3) EMC virus agglutinated washed guinea pig erythrocytes (1%) at 4°C employing a borate–KCl buffer at pH 8. HI tests were performed by incubating 2 fold dilutions of non absorbed heat inactivated (56°C for 30 min.) sera for 1 hour at 26°C with 8 units of EMC hemagglutinin (HA). A simultaneous antigen titration was included in all tests. A 1:20 dilution of sera from uninoculated IC mice did not inhibit 8 HA units of EMC virus and HI titers of 1:20 or greater were consequently considered significant.

Neutralization tests. Twofold dilutions of heat inactivated sera were incubated for 1 hour at 26°C and 1 hour at 4°C with 20 pfu of EMC virus (L Cells). The neutralization titer was expressed as the highest dilution responsible for a 50% reduction in the number of plaques as compared to viral control cultures.

A negative (normal mouse serum) and positive (hyperimmune anti-EMC rat serum) serum control were included in each HI and neutralization test. As previously described (3), it was found that HI and neutralization tests yielded equivalent results. Hyperimmune serum was obtained 21 days after the intracerebral inoculation of 1-month-old rats with .05 ml of a 1:5 dilution of stock EMC virus.

Preparation and assay of interferon. Mouse brain interferon was prepared by the technique of Finter (4) with modifications as previously described (2). After centrifugation and treatment at pH 2, interferon was concentrated 10-fold by pressure dialysis and centrifuged at 80,000*g* for 1 hour (2). It was shown that these preparations fulfilled various criteria for interferon (5), including inhibition of its activity by actinomycin D. Titers were expressed as the dilution/2 ml of the original preparation responsible for a 50% reduction in the number of plaques (vesicular stomatitis virus).

In a few experiments not presented in detail here, a highly purified mouse brain interferon was prepared by techniques to be described elsewhere[1] and an interferon from monolayer cultures of L cells inoculated with Newcastle Disease virus was prepared by techniques described by Youngner *et al.* (6).

[1] Falcoff, R. Fontaine-Brouty-Boye, D., and Falcoff, E., in preparation.

Statistical analysis. The experimental results were analyzed by Mr. Philippe Lazar and Mrs. Suzanne Guéguen of the Unité de Recherche Statistique de l'Institut National de la Santé et de la Recherche Médicale. The different groups of mice were compared by standard tests (analysis of variance and *t* test) based on the inverse of the survival of viral infected mice.

Results. Effect of interferon treatment on EMC injection. Treatment initiated prior to viral inoculation and continued thereafter. Preliminary experiments demonstrated that preparations of crude or highly purified mouse brain interferon, or L cell interferon (titers 1:6000) administered ip to mice 24 and 1 hour prior to inoculation of 100 LD_{50} of EMC virus conferred a slight but significant degree of protection compared to untreated viral infected mice or mice treated with normal mouse brain extract. The number of mice surviving infection was considerably increased by continuation of interferon treatment twice daily for 8 days following viral inoculation.

To increase these protective effects, experiments were undertaken utilizing *concentrated* preparations of mouse brain interferon (titers 1:26,000–1:56,000). It can be seen from Expts. I and II (Table I) that interferon administered ip 24 and 1 hour prior to inoculation of 250 LD_{50} of EMC virus protected 7 of 20 mice (35%) (Expt. I) and 5 of 14 mice (36%) (Expt. II). Continuation of interferon treatment twice daily (bid) after viral inoculation increased the number of surviving mice to 15/20 (75%) and 9/14 (64%), respectively.

Interferon administered subcutaneously (sc) prior to viral inoculation also proved effective[2] (Expt. V, Table I) and continuation of interferon treatment after viral inoculation increased the number of surviving mice from 1/25 (4%) to 8/25 (32%). (The results of other experiments demonstrated that the ip route of inoculation conferred greater protection than the subcutaneous route.)

[2] Although the number of interferon treated mice (1/25) surviving viral infection did not differ from the number of untreated mice surviving infection the mean day of death in these two groups was significantly ($p = 0.05$) different.

Treatment initiated after viral inoculation and continued thereafter. In the experiments outlined above (Table I) it was found that interferon treatment initiated even 3 hours *after* viral inoculation (100–250 LD_{50}) and twice daily thereafter protected 4/20 mice (Expt. I), 1/15 mice (Expt. II) and 3/25 mice (Expt. V). It was considered of interest to compare the protection conferred by only one or two doses of interferon after viral inoculation to the degree of protection conferred by continuation of interferon administration. For these experiments, mice were inoculated with only 8 or 13 LD_{50} of EMC virus instead of 100–250 LD_{50} of virus as employed in previous experiments.

The results of Expts. III and IV (Table I) demonstrated that a significant degree of protection was observed in mice receiving interferon 3 and/or 8 hours after inoculation of 8 or 13 LD_{50} of EMC virus. A far greater degree of protection was observed when this treatment was continued twice daily for the ensuing 4 days in Expt. III or for the ensuing 9 days in Expt. IV.

In Expt. IV, 3 groups of mice received interferon 3 hours after viral inoculation: in the first group, one dose of 0.25 ml of interferon was inoculated ip; in the second group 6 $\times$ this amount was inoculated simultaneously (0.75 ml ip and 0.75 ml sc); finally in the third group 0.25 ml of interferon was inoculated 3 hours after viral infection and continued bid for 9 days.

Interferon treatment conferred some protection in all 3 groups of mice compared to untreated viral infected mice. No significant difference ($p = .10$) was observed between the first 2 groups of interferon treated mice (1/25 and 5/25) despite the 6-fold difference in the amount of interferon administered. Repeated administration of interferon however in the third group significantly ($p = < 0.001$) increased the number of surviving mice to 17/25 (68%).

Residual paralysis in interferon treated mice surviving EMC infection. It has been reported that occasionally mice survive EMC infection with residual limb weakness or paralysis (7). In our experiments this syndrome has also been observed on rare occasions in mice inoculated with very small

TABLE I. Effect of Interferon Treatment on Infection of Mice with Encephalomyocarditis Virus.

	Interferon administered[a]									
	Intraperitoneally								Subcutaneously	
Experiment	I		II		III		IV		V	
EMC LD_{50} ip	250		250		8		13		100	
Titer of interferon employed	1:50,000		1:35,000		1:56,000		1:56,000		1:26,000	
Treatment	Survivors[b] at 14 days	Mean day death[c]	Survivors at 21 days	Mean day death	Survivors at 13 days	Mean day death	Survivors at 19 days	Mean day death	Survivors at 25 days	Mean day death
None	0/20	4.8	0/15	3.9	5/30	6.2	0/25	4.9	1/25	4.7
Normal Brain Extract[d] —24, —1 hour and continued bid	0/20	4.2	0/15	4.2	NT[k]		NT		0/25	5.8
Interferon only —24, —1 hour	7/20	7.5	5/14	7.2	NT		NT		1/25	6.9
Interferon —24, —1 hour and continued bid	15/20[e]	10.2	9/14[e]	7.2	NT		NT		8/25[j]	8.2
Interferon only + 3 hour (i. e., after viral inoculation)	NT		NT		18/30	8.0	1/25[g] 5/25[h]	7.0 7.2	NT	
Interferon only + 8 hour	NT		NT		14/30	7.6	NT		NT	
Interferon + 3 hour and + 8 hour	NT		NT		13/30	9.4	NT		NT	
Interferon + 3 hour and continued bid	4/20[e]	7.1	1/15[e]	6.4	26/30[f]	10.0	17/25[i]	11.6	3/25[j]	6.7

[a] 0.2 ml inoculated ip or sc unless otherwise indicated.
[b] No. of mice surviving infection/no. of mice inoculated.
[c] Average day of death calculated only for deceased mice.
[d] Inoculated —24, —1 hour (i. e., before viral inoculation) and twice daily (bid) thereafter.
[e] Interferon treatment for 13 days.
[f] Interferon treatment for 5 days.
[g] 0.25 ml interferon inoculated ip.
[h] 0.75 ml interferon inoculated ip and 0.75 ml sc simultaneously in each mouse in this group.
[i] 0.25 ml interferon inoculated ip (bid) for 10 days.
[j] Interferon treatment for 16 days.
[k] NT = Not tested.

TABLE II. Susceptibility of Surviving Interferon Treated Mice to Rechallenge with EMC Virus.

Day of rechallenge	21	33	48	
EMC LD_{50}	± 50	± 100	± 100	Total
Controls	0/15[a]	1/15	1/16	2/46
Interferon-treated mice surviving 1st EMC infection	3/15	4/15	2/23	9/53

[a] No. of mice surviving/no. of mice inoculated.

doses of virus (i.e., < 1–10 LD_{50}). It has not been observed in untreated mice inoculated with 100 or more LD_{50} since at this viral inoculum most mice succumb. Residual paralysis of one or more limbs has however occasionally been observed in *interferon treated* mice inoculated with 100 or more LD_{50}. In these experiments 40–70% of the interferon treated mice survived this large viral dose (thus in terms of "effective" lethal doses one may consider that 100 LD_{50} had been reduced by interferon treatment to 1 LD_{50}). Infectious virus was not recovered from either washed cells or tissue extracts of heart, liver, brain, and spleen from 8 interferon treated or untreated mice with residual paralysis 1–6 months after viral inoculation.

Effect of interferon treatment. 1) *On the development of HI antibodies to EMC virus.* In a series of 4 experiments sera were obtained from 44 interferon treated mice 26–36 days after inoculation of 100 LD_{50} of EMC virus. Fourteen of 44 mice (32%) surviving EMC virus infection developed HI antibodies. It was found that the interferon treatment schedule did not alter significantly the percentage of mice with HI antibody. Thus 21 mice survived EMC infection after 2 injections of interferon prior to viral inoculation and 6 of these mice developed HI antibody (29%). Twenty three mice received repeated inoculations of interferon after viral inoculation and 8 of these mice had antibody (35%).

2) *On susceptibility of surviving mice to rechallenge with EMC virus.* Although most interferon protected mice did not develop detectible HI antibody to EMC virus at the time serum was obtained, it was considered possible that these mice might nevertheless demonstrate an enhanced resistance to a second inoculation of EMC virus. Accordingly, 100 weanling mice were inoculated ip with 0.2 ml of an interferon preparation (titer 1:50,000) 24 and 1 hour prior to inoculation of 100 LD_{50} of EMC virus. Seventy mice survived viral infection. Twenty one to 48 days after the initial inoculation of EMC virus some of these mice and appropriate control mice were inoculated with 50–100 LD_{50} of EMC virus.

As can be seen from Table II only a few of the interferon treated mice surviving an initial inoculation of EMC virus were resistant to rechallenge with this virus 9/53 (17%)[3] (compared to 2/46 (4%) of control mice).

Absence of tolerance to exogenous interferon. It has been reported that cell cultures and animals become refractory to repeated attempts to induce the synthesis of interferon (8). It was considered of interest therefore to determine whether mice acquire a tolerance to exogenous interferon.

One-month-old male and female mice were inoculated subcutaneously twice daily for 9 days with 0.2 ml of a concentrated interferon preparation (titer 1:56,000). The last interferon dose was administered 1 hour prior to viral inoculation. Another group of mice received only 2 doses of interferon, 24 and 1 hour prior to viral inoculation. A third group served as untreated controls.

The 2 groups of interferon treated mice proved to be equally protected regardless of the amount of interferon that had been administered prior to viral inoculation (Table III). There was no evidence therefore that

[3] It is likely that the few mice surviving the rechallenge inoculation were those that had in fact developed antibody to EMC virus as a result of their initial infection.

TABLE III. Absence of Tolerance to Exogenous Interferon.

Group	No. of mice surviving/total no. of mice inoculated
Treated with interferon[a] twice daily for 9 days and 1 hour prior to viral inoculation[b]	15/24
Treated with interferon 24 and 1 hour prior to viral inoculation	14/24
Untreated	5/24

[a] Interferon (1:56,000/2 ml) inoculated twice daily 0.2 ml subcutaneously.

[b] EMC 64 LD_{50} inoculated intraperitoneally.

9-day pretreatment of mice with interferon (19 inoculations) decreased or enhanced resistance to EMC virus as compared to mice receiving only 2 inoculations of interferon prior to viral infection.

Discussion. There has been considerable doubt concerning the value of exogenous interferon in the therapy of systemic viral diseases (9–11) since in animal experiments interferon has usually been effective only when administered prophylactically (12–16). There are however several instances in which interferon has been effective after inoculation of virulent viruses. Denys (14) observed that concentrated interferon administered before and for the 3 days following inoculation of Sindbis virus "exhibited a significant protective effect" whereas an injection prior to and once after viral inoculation proved ineffective. In the experiments of Finter a single large dose of interferon was effective 4 hours after inoculation of 110 LD_{50} of Semliki Forest virus (17) and 18 hours after inoculation of approximately 1 LD_{50} of this virus (18).

The results of the experiments presented herein demonstrated that the administration of potent interferon preparations before or even 3 hours after viral infection conferred a significant degree of protection on mice inoculated with several hundred LD_{50} of EMC virus. Continuation of interferon treatment (ip or sc) for several days after viral inoculation considerably increased the number of surviving mice. These findings seem to us of some importance in the evaluation of the therapeutic usefulness of interferon. They suggest that intensive interferon treatment for a few days after viral inoculation may prove sufficient to enable the animal to survive an otherwise lethal infection. Thus in Expt. III (Table I), it was found that continuation of interferon treatment for 5 days after viral inoculation conferred a significant increase in protection. For practical reasons in the experiments described we have limited continued interferon treatment to two injections daily. An increase in the potency of the interferon administered (or the use of a slow release interferon preparation) might well have proven even more efficacious.

The onset of symptoms and death in mice infected with EMC virus is roughly proportional to the dose of virus inoculated. Interferon appeared to act by diminishing the "effective" dose of virus, so that interferon treated mice inoculated with 100 LD_{50} of EMC virus resembled untreated mice inoculated with 1 LD_{50} of virus, in the delay of symptoms, the number of mice surviving infection, and on occasion the presence of neurologic sequelae of viral infection. Furthermore most interferon treated mice (68%) failed to develop HI antibody to EMC virus or to resist rechallenge with EMC virus (83%), suggesting that viral multiplication had been repressed below a level necessary to induce a detectable immune response.

Lastly it should be emphasized that these concentrated interferon preparations were effective in mice inoculated with several hundred LD_{50} of a virulent virus. As pointed out by Finter (18) these experimental viral inocula probably far exceed those encountered in the course of natural viral infection. There are however several other therapeutic advantages of exogenous interferon therapy that seem worthy of mention: a) the absence of toxicity (19,20), b) the absence of detectable neutralizing antibody to interferon in "homologous" recipients (20,21), and c) the apparent absence of tolerance to exogenous interferon as reported in this communication. Considered together these results would seem to justify some optimism as to the eventual therapeutic value of interferon in some viral diseases.

Summary. Administration of mouse brain interferon prior to or after inoculation of 100–250 LD_{50} of encephalomyocarditis (EMC) virus conferred a significant degree of protection on weanling IC mice. Continuation of interferon treatment for several days after virial inoculation considerably increased the number of mice surviving infection. Most interferon protected mice did not develop HI antibody to EMC virus nor did they demonstrate resistance to viral rechallenge. By acting early in the course of viral infection interferon may have repressed viral multiplication below a level necessary to induce a specific immune response. Mice did not develop a "tolerance" to repeated inoculation of exogenous interferon. Considered together the experimental results suggested that *continued* interferon administration may prove of value in the treatment of some viral diseases.

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1. Gresser, I., Coppey, J., Falcoff, E., and Fontaine, D., Compt. Rend. Acad. Sci. Paris **263**, 586 (1966).

2. Gresser, I., Coppey, J., Falcoff, E., Fontaine, D., Proc. Soc. Exptl. Biol. Med. **124**, 84 (1967).

3. Craighead, J. E., Shekolov, A., Proc. Soc. Exptl. Biol. Med. **108**, 823 (1961).

4. Finter, N. B., Nature **204**, 1114 (1964).

5. Lockart, R. Z., Jr., in "Interferons," Finter, N. B., ed., p. 1. North-Holland Pub., Amsterdam, 1966.

6. Youngner, J. S., Scott, A. W., Hallum, J. V., Stinebring, W. R., J. Bacteriol. **92**, 862 (1966).

7. Perol-Vauchez, Y., Tournier, P., Lwoff, M., Compt. Rend. Acad. Sci. Paris **253**, 2164 (1961).

8. Ho, M., in "Interferons," Finter, N. B., ed., p. 21. North-Holland Pub. Amsterdam, 1966.

9. Hilleman, M. R., Am. J. Med. **38**, 751 (1965).

10. Wagner, R. R., Am. J. Med. **38**, 726 (1965).

11. Ho, M., Postic, B., Intern. Conf. Vaccines against Viral and Rickettsial Diseases of Man, 1st PAHO/WHO Scientific Publication, Washington, D. C. **147**, 632 (1967).

12. Isaacs, A., Advan. Virus Res. **10**, 1 (1963).

13. Finter, N. B., in "Interferon," Finter, N. B., ed., p. 232. North-Holland Pub. Amsterdam, 1966.

14. Denys, P., Lancet **2**, 174 (1963).

15. Baron, S., Buckler, C. E., Friedman, R. M., McCloskey, R. V., J. Immunol. **96**, 17 (1966).

16. Arakawa, J., Sawa, I., Fujiwara, K., Compt. Rend. Soc. Biol. **159**, 786 (1965).

17. Finter, N. B., Brit. J. Exptl. Pathol. **47**, 361 (1966).

18. Finter, N. B., J. Gen. Virol. **1**, 395 (1967).

19. Gresser, I., Coppey, J., Fontaine-Brouty-Boye, D., Falcoff, E., Falcoff, R., Zajdela, F., Bourali, C., Thomas, M. T., Ciba Found. Symp., Interferon, 1967, in Wolstenholme G. E. W. and O'Connor M., eds. p. 240, J. A. Churchill Ltd. (1967).

20. Falcoff, E., Falcoff, R., Fournier, F., Chany, C., Ann. Inst. Pasteur **111**, 562 (1966).

21. Gresser, I., Fontaine, D., Coppey, J., Falcoff, R., Falcoff, E., Proc. Soc. Exptl. Biol. Med. **124**, 91 (1967).

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