A Comparison of the Efficacy of Endogenous, Exogenous, and Combined Endogenous–Exogenous Interferon in the Treatment of Mice Infected with Encephalomyocarditis Virus (33529)

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It was recently stated "since interferon per se (i.e., *exogenous interferon*) shows little promise as a prophylactic or therapeutic agent, interest has shifted to a search for acceptable inducers by which the body might be stimulated to make its own interferon" (i.e., *endogenous interferon*) (1). This view appears to be shared by many investigators interested in the therapeutic applications of interferon (2–5). We have been impressed, however, by the efficacy of *exogenous interferon* in delaying the evolution of murine leukemia (6–8) and in protecting mice infected with encephalomyocarditis (EMC) virus (9). The present paper reports the results of investigations designed to compare the efficacy of endogenous, exogenous, and combined endogenous–exogenous interferon in the treatment of mice preinfected with EMC virus.

Methods and Materials. Viruses. Encephalomyocarditis (EMC) virus was obtained from Dr. J. Huppert and propagated in monolayer cultures of L cells. One month old male mice (Institut de Cancer I.C. strain) were inoculated intraperitoneally (i.p.) with 0.2 ml of a given viral dilution. Inoculated mice were examined twice daily and kept for 3 weeks. The lethal dose (LD_{50}) of EMC virus inoculated in each experiment was determined by simultaneous titration.

Newcastle Disease virus (NDV) (Karzon-Kan. Leavenworth 48, laboratory strain) was propagated in the allantois of 10-day-old embryonated eggs and titered by standard plaque assay methods utilizing secondary cultures of chick embryo fibroblasts. Titers were expressed in plaque forming units (pfu).

Cell culture. Mouse and chick embryo fibroblasts and mouse L cells were prepared by standard techniques and maintained in modified Eagle's basal medium supplemented with 5–10% heat inactivated calf serum.

Induction of endogenous interferon (a) Newcastle disease virus (0.2 ml containing $5 \times 10^7 - 4 \times 10^8$ pfu) was inoculated intravenously (i.v.) according to the technique of Baron and Buckler (10). To test for the presence of interferon in the serum, mice were bled 5 hr after inoculation of NDV. Sera were diluted 1:5 in phosphate buffered saline (PBS) and maintained at pH 2 at 4° for 4 days prior to interferon assay. On no occasion was residual infectious NDV detected in the serum after this treatment.

(b) Statolon (an extract of the mold *Penicillium stoloniferum*) was obtained through the courtesy of Dr. W. J. Kleinschmidt and prepared as previously described (11). Twenty five mg of statolon (9.5% active statolon) in 0.5 ml was inoculated intraperitoneally. Mice were bled 11 hr after inoculation of statolon to test for the presence of serum interferon.

(c) Complexed polyinosinic and polycytidylic acids [poly(I)·poly(C)] were obtained through the courtesy of Dr. A. M. Michelson and inoculated i.v. (5.6 or 8 μg in 0.2 ml) (the concentration of each preparation was determined by spectrophotometry). To determine the presence of interferon in the serum, mice were bled 2 hr after inoculation (12).

Preparation of exogenous interferon. Mouse brain interferon was prepared by techniques described by Finter (13) with modifications as previously reported (14). After centrifugation and treatment at pH 2 interferon was concentrated 10-fold by pressure dialysis and centrifuged at 80,000*g* for 1 hr. It was shown that these interferon preparations fulfilled the

TABLE I. Presence of Interferon in Serum of Mice Inoculated Daily with NDV, Statolon, or Poly(I) • poly(C).

Expt.	Day:	Interferon titer[a] 1	2	3	4	5	6
I	NDV (2×10^8 pfu/day)	1200	230	120	120	15	<20
	Statolon (25 mg/day)	120	NT[b]	NT	NT	NT	NT
	Poly(I) • poly(C) (5.6 μg/day)	110	110	15	25	30	20
II	NDV (5×10^7 pfu/day)	1280	640	100	65	NT	NT
	Statolon (25 mg/day)	160	80	20	<10	NT	NT
	Poly(I) • poly(C) (8 μg/day)	640	160	40	40	NT	NT

[a] Titers are expressed as the reciprocal of the dilution (see "Methods and Materials"). Each value represents the average of the serum interferon titers of 3 mice sacrificed each day. Mice were bled 5 hr after i.v. inoculation of NDV, 11 hr after i.p. inoculation of statolon, and 2 hr after i.v. inoculation of poly(I) • poly(C).

[b] NT = not tested.

basic criteria for interferon (15, 16).

In the experiments described below mice were inoculated twice daily (09:30 and 17:00 hr) i.p. with 0.2 ml of the interferon preparation.

Assay of interferon. Interferon was assayed by inhibition of cytopathic effect (CPE) of vesicular stomatitis virus (VSV) in monolayer cultures of L cells in plastic Disposo-trays.[1] A standard reference mouse brain interferon was included in each test and its titer (1:1600/0.2 ml) rarely varied by more than one dilution in the different assays. *Tenfold* concentrated mouse brain interferon preparations usually titered 1:8000–1:16,000/0.2 ml. (The same interferon preparations titered 1:48,000–1:96,000/2 ml when tested in simultaneous 50% plaque reduction assays utilizing L cells and VSV).

Statistical analysis. The experimental results were analyzed by Mr. Philippe Lazar and Mrs. Suzanne Guéguén 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 infected mice.

Results. Induction of endogenous interferon by NDV, statolon, and poly(I)•poly(C). Prior to undertaking experiments on the therapeutic effects of endogenous and exogenous interferon, the interferon response to a single or repeated daily inoculation of NDV, (5×10^7 or 2×10^8 pfu), statolon (25 mg), and poly(I)•poly(C) (5.6 or 8 μg) was determined. Considerable amounts of interferon were observed after a single inoculation of a given inducer (Table I), but as has been previously reported by others (17–19), less interferon was detected after daily reinoculation of inducer. Appropriate controls demonstrated that this interferon resulted from reinoculation of inducer and was not due to the presence of residual interferon from inoculation of inducer on the preceding day. The kinetics of the appearance of serum interferon in mice receiving 1 or 3 injections of NDV are illustrated in Table II. It is apparent that even on the fourth day, in the period of relative resistance to reinduction of interferon, considerable amounts of interferon may be produced since a relatively constant serum interferon level was maintained at least between hours 3 and 15 after inoculation. Other preliminary experiments demonstrated that the interferon response of mice to a single or repeated i.v. inoculation of NDV was not significantly modified either by preinfection of mice with EMC virus or by pretreatment of mice with exogenous interferon.

Comparison of the efficacy of endogenous, exogenous, and combined endogenous–ex-

[1] Disposo-trays FB-48 Gateway International, Los Angeles, California.

TABLE II. Comparison of the Kinetics of Induction of Serum Interferon after 1 or 3 Injections of NDV.

Day of injection	No. of injections of NDV[b]	Titer of interferon[a]; hours after inoculation of NDV							
		0	1	3	6	9	12	15	24
First	1	<10[d]	160	2560	5120	10,240	2560	640	160
		<10	40	1280	5120	5120	1280	640	80
Fourth	3[c]	<10	<10	80	160	80	160	40	<10
		<10	<10	40	80	40	80	20	<10

[a] Two mice from each group were sacrificed at the hours indicated and their serum was assayed for interferon.

[b] A dose of 4×10^8 pfu of NDV inoculated i.v. each day.

[c] NDV inoculated on days 1, 2, and 4.

[d] Titer expressed as the reciprocal of the dilution.

ogenous interferon (Expts. I–III). To compare the therapeutic efficacy of endogenous, exogenous, and combined endogenous–exogenous interferon, mice were inoculated with 40–80 LD_{50} of EMC virus, and treatment was initiated 3 hrs thereafter. Endogenous interferon was induced either by a single or by daily repeated inoculations of NDV ($5 \times 10^7 - 4 \times 10^8$ pfu), statolon (25 mg) or poly(I)·poly(C) (5.6 or 8 μg). Exogenous interferon was administered twice daily.

The results of 3 experiments (Table III) may be summarized as follows: (i) All of the various treatments increased the survival of viral infected mice, expressed either as an overall increase in the number of mice surviving viral infection at 21 days or as a delay in the day of death (harmonic mean survival) compared to control viral infected mice. (ii) No significant difference was observed between the different groups of mice treated with NDV, statolon, or poly(I)·poly(C). (iii) The repeated daily injection of an inducer did not add significantly to the protective effect observed after a single injection. (iv) Treatment with exogenous interferon proved more effective than treatment with inducers of endogenous interferon, regardless of the inducer utilized. Thus, the number of mice surviving infection after endogenous interferon treatment ranged from 0/25 (0%) to 4/20 (20%) (harmonic mean survival 4.9–9.4 days) whereas the number of mice surviving infection after exogenous interferon treatment ranges from 7/19 (37%) to 15/18 (83%) (harmonic survival 12.4–36.0 days). (v) No significant difference could be demonstrated between exogenous interferon and combined endogenous–exogenous interferon. The number of surviving mice in this latter group was 14/25 (56%) and 13/19 (68%) (harmonic mean survival 19.8–28.9 days).

Further comparison of the efficacy of exogenous interferon and endogenous–exogenous interferon (Expt. IV). Since the use of potent interferon preparations in Expts. I and II may have masked the added benefit resulting from daily injections of NDV (combined endogenous–exogenous treatment) viral infected mice were treated either with varying amounts of interferon alone or together with daily injections of NDV.

As shown in Table IV a significant protective effect was observed in mice inoculated with 2000 or more units of interferon per day. Treatment of mice with exogenous interferon and daily inoculation of NDV (combined endogenous–exogenous interferon) resulted in a greater degree of protection at each dilution of interferon. Furthermore in this experiment, repeated daily inoculation of NDV alone proved as effective (4/20 mice surviving harmonic mean survival 9.3 days) as inoculation of 8000 or 32,000 units of interferon (6/20 and 5/19 mice surviving; harmonic mean survival 7 and 10.1 days, respectively).

Efficacy of a combined endogenous–exogenous interferon treatment initiated 18 or

TABLE III. A Comparison of the Efficacy of Endogenous, Exogenous, and Combined Endogenous–Exogenous Interferon (IF) in the Treatment of Mice Infected with EMC Virus.[a]

	Expt.:[d]	I		II		III	
	EMC (LD_{50}, i.p.):	40		80		50	
		No. of mice surviving[b]/no. inoculated	Harmonic mean survival (days)	No. of mice surviving[b]/no. inoculated	Harmonic mean survival (days)	No. of mice surviving[b]/no. inoculated	Harmonic mean survival (days)
Virus control		0/20	4.1	0/25	4.2	0/24	4.4
Endogenous interferon	NDV, once	3/19	8.3	2/25	7.6	0/20	7.3
	NDV, repeated	2/20	9.1	3/25	8.0	3/21	9.4
	Statolon, once	NT		2/20	6.8	4/20	8.7
	Statolon, repeated	NT		NT		2/20	7.0
	Poly(I) • poly(C), once	NT		0/25	4.9	2/20	6.5
	Poly(I) • poly(C), repeated	NT		1/25	6.8	3/20	8.3
Exogenous interferon	IF, repeated	15/18	36.0	8/25	12.4	7/19	13.2
Combined endogenous and exogenous interferon	NDV, repeated + IF, repeated	13/19	28.9	14/25	19.8	NT	

[a] All treatment initiated 3 hr *after* inoculation of virus (+ 3 hr).
[b] No. of mice surviving 21 days.
[c] NT = not tested.
[d] Expt. I: NDV (5×10^7 pfu) inoculated either once (+3 hr) or repeated (+24 hr); interferon repeated: 8000 units inoculated +3 hr and twice daily for 11 days.
II: NDV (2×10^8 pfu) and poly(I) • poly(C) (5.6 μg) inoculated either once (+3 hr) or repeated daily thereafter for 6 days; statolon (25 mg) inoculated once (+3 hr); interferon (16,000 units) inoculated +3 hr and twice daily for 6 days.
III: NDV (5×10^7 pfu), statolon (25 mg), and poly(I) • poly(C) (8 μg) inoculated either once (+3 hr) or repeated daily thereafter for 4 days; interferon (13,000 units) inoculated +3 hr and twice daily for 12 days.

TABLE IV. Treatment of EMC Viral Infected Mice with Varying Amounts of Exogenous Interferon Alone or Together with Repeated Inoculation of NDV (Expt. 4).[a]

	Interferon inoculated (units/day)									
	0		500		2000		8000		32,000	
Interferon alone	0/20[b]	4.0[c]	0/19	4.4	1/19	5.7	6/20	7.0	5/19	10.1
Interferon + NDV	4/20	9.3	4/20	9.5	12/20	23.3	6/19	13.9	8/18	19.1

[a] All mice were inoculated i.p. with 50 LD_{50} EMC virus. Dilutions of interferon (as indicated) or dilutions of interferon and NDV (4×10^8 pfu) were inoculated 3 hr thereafter and daily for 5 days.

[b] Number of mice surviving at 21 days/number of mice inoculated.

[c] Harmonic mean survival (days).

24 hr after inoculation of EMC virus (Expts. V, VI). In the preceding experiments, relatively large doses of EMC virus (40–80 LD_{50}) were inoculated in order to emphasize the differences between the various therapeutic regimens. It is likely however, that under natural conditions the animal or human host is infected with much smaller viral inocula. It was considered of interest therefore to inoculate mice with small doses of EMC virus and to delay initiation of interferon therapy for 18 or 24 hr. For these experiments only the combined endogenous–exogenous interferon treatment was tested since this therapy had proven as effective (Expts. I and II) or more effective (Expt. IV) than exogenous interferon alone.

Table V shows that this combined interferon treatment increased the number of mice surviving viral infection with 8 and 2.5 LD_{50} from 7/25 (28%) and 9/25 (36%) to 13/25 (52%) and 14/25 (56%) (in both experiments $p = 0.05$).

Discussion. The purpose of these experiments was to compare the therapeutic efficacy of endogenous, exogenous, and combined endogenous–exogenous interferon in the treatment of mice infected with EMC virus. To accentuate the differences between these therapeutic regimens, mice were inoculated i.p. with relatively large doses of EMC virus (40–80 LD_{50}) and treatment was begun 3 hr thereafter. Newcastle Disease virus, statolon, and complexed polyinosinic and polycytidylic acids were chosen as inducers of endogenous interferon, since all have been shown by others to afford some degree of protection in *in vivo* experiments (12, 20, 21, 22). In our experiments, these inducers were all equally effective in increasing the survival of viral infected mice. Daily reinoculation of an inducer did not add significantly to the protective effect observed after a single injection (Table III).

Although many investigators have assumed that the induction of endogenous interferon would prove more effective than exogenous interferon (1–5), repeated administration of potent interferon preparations was clearly more effective than endogenous interferon in 3 experiments (Table III) and equally effective in a fourth experiment (Table IV). Considering the results of these 4 experiments together, 0–20% of mice survived viral infection after treatment with any one of the interferon inducers, whereas 26–83% of mice survived after treatment with exogenous interferon. Endogenous interferon treatment proved more effective than exogenous interferon only when relatively weak preparations of the latter were employed (Expt. IV, Table IV). Thus the efficacy of exogenous interferon in our experiments was probably due to the high titer of the concentrated preparations utilized and to the continuation of treatment after viral infection (9). It is important however to emphasize that under different experimental conditions [i.e., different strains of mice, different viruses, or addition of DEAE to poly(I)·poly(C) (23)] endogenous interferon therapy might have proven more effective.

The kinetics of the appareance of serum interferon after repeated inoculation of NDV

TABLE V. The Efficacy of a Combined Endogenous–Exogenous Interferon Treatment Initiated 18 or 24 Hr after Inoculation of EMC Virus.

Expt.:	V		VI	
EMC (LD_{50}, i.p.):	8		2.5	
Treatment initiated (+ hours) after inoculation of virus	+18		+24	
	No. of mice surviving[a]/no. inoculated	Harmonic mean survival (days)	No. of mice surviving[a]/no. inoculated	Harmonic mean survival (days)
Virus control	7/25	7.4	9/25	8.0
NDV and exogenous[b] interferon	13/25	13.0	14/25	13.9

[a] No. of mice surviving 21 days.

[b] NDV (4×10^8 pfu) inoculated 18 or 24 hr after viral inoculation and also on the following day. Interferon: 80,000 units inoculated 18 or 24 hr after viral inoculation and daily for 11 days.

(Table II) demonstrated that a significant interferon level[2] was maintained for at least 12 hr (and probably longer). Since exogenous mouse interferon disappears rapidly from the circulation (24–26) the total amount of interferon produced, even during this period of resistance to interferon induction, must have been considerable. We are unable at present to explain why repeated inoculation of an inducer such as NDV did not therefore prove more effective or at least as effective as exogenous interferon. [Previous experiments (9, 14) demonstrated that there was no significant difference *in vivo* between exogenous NDV induced interferon (either L cell or serum/spleen) and *exogenous* mouse brain interferon of comparable titer]. Two possibilities may be considered: (a) repeated inoculation of NDV may induce the formation of a factor(s) which inhibits interferon activity (27). This factor(s) may have been eliminated from exogenous interferon in the course of preparation. (b) the total amount of interferon produced after repeated inoculation of an inducer may have been considerable but the concentration at any given time may have been below a necessary threshold. This threshold may have been attained immediately after inoculation of potent interferon preparations. These possibilities are currently being investigated.

From the limited number of experiments undertaken, it was not possible to determine whether treatment with combined endogenous–exogenous interferon was more effective than exogenous interferon alone. No significant difference between these 2 regimens was detected in 2 experiments (Expts. I and II, Table III), whereas in a third experiment (Expt. IV, Table IV) combined treatment proved superior. The therapeutic value of endogenous–exogenous interferon was illustrated by Expts. V and VI (Table V) in which a significant increase in the number of mice surviving infection (and a delay in death) was observed even when treatment was initiated 18 or 24 hr *after* viral inoculation (2.5 or 8 LD_{50})[3]. These results seem of some practical interest to us, since there are a number of instances in clinical medicine when the time of exposure to a given virus is known and antiviral therapy could be instituted shortly thereafter.*

[2] The serum interferon level was "low" compared to the level observed after the initial inoculation of NDV, but comparable to the serum levels observed after inoculation of concentrated exogenous interferon (24).

[3] The cycle of EMC viral multiplication in monolayer cell cultures is approximately 8 hr (28, 29) and it is probably of comparable duration *in vivo* (30).

* *Note Added in Proof:* See also experiments of N. B. Finter in "Interferon" (Ciba Foundation) p. 204. Churchill, London (1967).

Summary. The efficacy of endogenous, exogenous, and combined endogenous–exogenous interferon was compared in the treatment of mice preinfected with 40–80 LD_{50} of EMC virus. The different interferon inducers, Newcastle Disease virus, statolon, and poly(I) • poly(C) proved equally effective in increasing the survival of viral infected mice. Daily reinoculation of an inducer afforded no significant added protection. Administration of potent interferon preparations proved more effective than any of the interferon inducers in 3 experiments and equally effective in a fourth experiment. In 2 experiments no significant difference was observed between the therapeutic efficacy of combined endogenous–exogenous interferon and exogenous interferon alone, whereas in a third experiment the combined treatment proved more effective. A combined interferon treatment initiated 18 or 24 hr *after* inoculation of 2.5 or 8 LD_{50} of EMC virus still afforded a significant degree of protection.

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1. Lampson, G. P., Tytell, A. A., Field, A. .K, Nemes, M. M., and Hilleman, M. R., Proc. Natl. Acad. Sci. U.S. **58**, 782 (1967).

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

3. Ho, M. and Postic, B., "First International Conference on Vaccines against Viral and Rickettsial Diseases of Man," Vol. 147, p. 632. PAHO/WHO Scientific Publ., Washington, D.C. (1967).

4. Merigan, T.C., New Engl. J. Med. **276**, 913 (1967).

5. Hilleman, M. R., *in* "Perspectives in Leukemia," p. 272. Grune and Stratton, New York (1968).

6. Gresser, I., Falcoff, R., Fontaine-Brouty-Boyé, D., Zajdela, F., Coppey, J., and Falcoff, E., Proc. Soc. Exptl. Biol. Med. **126**, 791 (1967).

7. Gresser, I., Coppey, J., Fontaine-Brouty-Boyé, D., Falcoff, E., Falcoff, R., Zajdela, F., Bourali, C., and Thomas, M. T., *in* "Interferon," (Ciba Foundation), p. 240. Churchill, London (1967).

8. Gresser, I., Berman, L., De-Thé, G., Fontaine-Brouty-Boyé, D., Coppey, J., and Falcoff, E., J. Natl. Cancer Inst. **41**, 505 (1968).

9. Gresser, I., Bourali, C., Thomas, M. T., and Falcoff, E., Proc. Soc. Exptl. Biol. Med. **127**, 491 (1968).

10. Baron, S. and Buckler, C. E., Science **141**, 1061 (1963).

11. Kleinschmidt, W. J. and Murphy, E. B., Virology **27**, 484 (1967).

12. Field, A. K., Tytell, A. A., Lampson, G. P., and Hilleman, M. R., Proc. Natl. Acad. Sci. U.S. **58**, 1004 (1967).

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

14. Gresser, I., Coppey, J., Falcoff, E., and Fontaine-Brouty-Boyé, D., Proc. Soc. Exptl. Biol. Med. **124**, 84 (1967).

15. Lockart, R. J., Jr., *in* "Interferons" (N. B. Finter, ed.), p. 1. North-Holland Publ. Amsterdam (1966).

16. Chany, C., Fournier, F., and Falcoff, E., *in* "Interferon" (Ciba Foundation), p. 64. Churchill, London (1967)

17. Ho, M. and Kono, Y., J. Clin. Invest. **44**, 1059 (1965).

18. Ho, M., Kono, Y., and Breinig, M. K., Proc. Soc. Exptl. Biol. Med. **119**, 1227 (1965).

19. Youngner, J. S. and Stinebring, W. R., Nature **208**, 456 (1965).

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

21. Kleinschmidt, W. J. and Murphy, E. B., Bacteriol. Rev. **31**, 132 (1967).

22. Schmidt, J. P. and Pindak, F. F., Appl. Microbiol. **15**, 654 (1967).

23. Dianzani, F., Rita, G., Cantagalli, P., and Gagnoni, S., personal communication.

24. Gresser, I., Fontaine-Brouty-Boyé, D., Coppey, J., Falcoff, R., and Falcoff, E., Proc. Soc. Exptl. Biol. Med. **124**, 91 (1967).

25. Subrahmanyan, T. P. and Mims, C. A., Brit. J. Exptl. Pathol. **47**, 168 (1966).

26. Baron, S., Buckler, C. E., McCloskey, R. V., and Kirschtein, R. L., J. Immunol. **96**, 12 (1966).

27. Chany, C. and Brailovsky, C., C. R. Acad. Sci., Paris **261**, 4282 (1965).

28. Henry, C. and Franklin, R. M., Virology **9**, 84 (1959).

29. Amako, K. and Dales, S., Virology **32**, 184 (1967).

30. Dickinson, L. and Griffiths, A. J., Brit. J. Exptl. Pathol. **47**, 35 (1966).

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