INFECTION AND IMMUNITY, Nov. 1972, p. 819-823

Vol. 6, No. 5
Printed in U.S.A.

Target-Organ Treatment of Neurotropic Virus Disease with Interferon Inducers

LOIS B. ALLEN[1] AND KENNETH W. COCHRAN

Department of Epidemiology and Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104

Received for publication 5 April 1972

Interferon inducers were used against vaccinial encephalitis to study the target-organ treatment of neurotropic disease and to correlate interferon levels and the antiviral state following such treatment. A 45-μg amount of statolon, 30 μg of polyribinosinic-polyribocytidylic acid complex (poly I·poly C), or 0.0154 HA unit of Sendai virus given intracerebrally protected 100% of mice challenged the next day with 1,000 median lethal doses (LD_{50}) of vaccinia virus. Significant protection against 1,000 LD_{50} of vaccinia virus persisted for 1, 4, or 3 weeks after poly I·poly C, statolon, or Sendai virus (154 HA units), respectively. These doses of poly I·poly C and statolon were also used to study postinfection treatment. Mice challenged with 1, 10, 100, or 1,000 LD_{50} were treated intracerebrally with poly I·poly C or statolon 24 or 48 hr later. Significant increases in survival time were seen in mice challenged with 1 to 100 LD_{50} of vaccinia virus and treated 24 hr later. At challenges of 10 or 100 LD_{50}, statolon was more effective than poly I·poly C in increasing survival times. When treatment was delayed until 48 hr after infection, significant increases in survival time occurred only when the challenges were in the range of 1 to 10 LD_{50}, with poly I·poly C and statolon being equally effective. Interferon was measured by Finter's dye-uptake method, with L-929 cells and Semliki Forest virus. Poly I·poly C, statolon, or Sendai virus, given intracerebrally to mice, produced serum interferon peaks of 5,120 units/ml at 2 hr, 2,560 units/ml at 12 hr, or 320 units/ml at 18 hr, respectively. Corresponding brain interferon peaks were 640 units/g at 2 hr, 640 units/g at 4 to 24 hr, and 960 units/g at 72 hr.

Recent studies on interferon (IF) and host antiviral resistance have involved direct administration of interferon inducers to the organ most immediately affected, such as the eyes in viral keratitis (18) or the lungs in viral pneumonitis (12, 16). Application of target-organ treatment to the central nervous system has been relatively unexploited. In an earlier study of IF and viral encephalitis (9), where both peripheral viral challenge and treatment routes were employed, significant protection occurred only when circulating IF preceded viremia. In other work (4), two peripheral injections of polyriboinosinic-polyribocytidylic acid complex (poly I·poly C), at 300 μg per 20-g mouse, prior to the onset of encephalitis symptoms, increased the survival of mice intranasally inoculated with one median lethal dose (LD_{50}) of vesicular stomatitis virus. In experiments where virus challenge was directly into the brain (1, 3), significant increases in survival of mice were seen only when peripheral treatment of inducer or IF preceded virus inoculation. From these reports it seemed that, once virus reached the brain, the likelihood of successful treatment by peripheral routes was slight. Useful information was gained from work with M5-8450, a statolon predecessor. M5-8450 was active (19) when it and poliovirus were inoculated peripherally. Other work (K. W. Cochran, 1954, Fed. Proc. **13**:343) revealed that it required intracerebral (ic) administration to affect the disease when virus was inoculated ic. This latter finding of the value of ic therapy was the stimulus for the present application of target-organ treatment to viral encephalitis. The material in this report is from a dissertation submitted in partial fulfillment of requirements for the degree of Doctor of Philosophy, Rocklam School of Graduate Studies, University of Michigan, Ann Arbor.

MATERIALS AND METHODS

Laboratory-bred male Swiss Webster or BALB/c mice, weighing 15 to 25 g, were used in these studies.

[1] Present address: ICN Nucleic Acid Research Institute, Irvine, Calif. 92664.

The IF inducers included poly I·poly C purchased from Microbiological Associates, Bethesda, Md.; statolon, lot 354-1014B-74, containing 6% nondialyzable solids, provided by W. J. Kleinschmidt, Lilly Research Laboratories, Indianapolis, Ind.; and Sendai virus, supplied by E. Minuse of this laboratory. The Sendai virus was used as infected allantoic fluid, stored at −70 C, with sorbitol added for stability. The virus preparation used was nonpathogenic given ic, but had a hemagglutinating titer of 512 units/ml.

Viruses. Semliki Forest virus, used in the IF assays, was obtained from D. C. DeLong, Lilly Research Laboratories. Prior to use in this study, it had been passed in BS-C-1 cells two times. Virus was prepared by infecting L-929 cells in minimal essential medium supplemented with 2% fetal calf serum (Flow Laboratories, Rockville, Md.). Virus-containing medium was separated from cell material and stored at −70 C. It had a titer of 10^8 median tissue culture infection doses ($TCID_{50}$)/ml. The Western Reserve strain of vaccinia virus, obtained from the American Type Culture Collection, Rockville, Md., was used as the challenge agent in mice. Prior to use in this study, it had undergone 24 mouse brain and three mouse lung passages. Mice were infected intranasally with a dose of virus known to produce lung consolidation. Lungs were aseptically removed, homogenized, and diluted to 20% in nutrient broth (Difco Laboratories, Detroit, Mich.). Virus-containing broth was separated from lung debris and stored at −20 C. This virus had an ic LD_{50} of $10^{6.5}$/0.03 ml.

Sample preparation. Blood was collected from ether-anesthetized mice by cardiac puncture by the method of Falabella (6). Blood samples were pooled from three mice; serum was separated from cells and stored at −20 C. After bleeding, brains of the same three mice were removed aseptically, ground with alundum, and diluted to 25% in a balanced salt solution. After standing at 4 C for 6 to 24 hr, the brain suspensions were centrifuged to remove cellular debris, and the supernatant fluid was stored at −20 C. When mice had been injected with Sendai virus, the brain samples were diluted in 0.1 M tartaric acid to give a *p*H of 3, and the samples were kept at 4 C for 48 hr before neutralization and storage at −20 C.

IF assay. IF was assayed in L-929 cells infected with Semliki Forest virus. The dye-uptake method of Finter (10) was modified to use 0.03 M NaH_2PO_4 in 50% ethanol to release and take up the dye. IF titers are expressed as DU_{50} units, representing the reciprocal of the dilution at which the dye uptake was midway between the levels of uninfected and virus-infected cell monolayers.

Treatment and challenge. IF inducers and vaccinia virus were injected ic in volumes of 0.03 ml. Control mice received placebo injections of buffered saline. Mice were observed for 21 days after virus challenge. Response to vaccinia virus was evaluated in terms of the harmonic mean survival time of each group of mice, calculated as described by Gard (11). Activity was expressed as a survival index, the ratio of the harmonic mean survival time of the treated group to that of the control. Increased values of the index represent increased effectiveness, and a survival index of 1.5 or greater was considered to indicate significant protection. When all treated animals survived for 21 days, a minimal harmonic mean survival time was calculated by assuming that one animal died on day 22.

RESULTS

In vivo protection studies. Various doses of poly I·poly C, statolon, or Sendai virus were injected ic at 24 hr before mice were challenged with 1,000 LD_{50} of vaccinia virus. The results are presented in Table 1. The minimal ic doses protecting 100% of the mice against this relatively severe challenge were 30 μg of poly I·poly C, 45 μg of statolon, or 0.0154 HA unit of Sendai virus. To determine the relative effect of peripheral versus local treatment, the minimal ic 100% protective doses of each inducer were then injected intraperitoneally into mice. One day later, the animals were challenged with 1,000 LD_{50} of vaccinia virus. All of the control mice died as did all of those treated with statolon or poly I·poly C; 1 of 15 Sendai virus-treated mice survived. There were no significant delays in time of death. Calculated survival indices were 1.2 for statolon, 1.3 for poly I·poly C, and 1.2 for Sendai virus, when each was given ip, compared to ≥90, ≥116, and ≥107, respectively, when approximately equal amounts were given ic.

To observe the duration of the antiviral status resulting from these inducers, mice were challenged ic with vaccinia virus at various intervals after the ic injection of 30 μg of poly I·poly C, 45 μg of statolon, or 1.54 HA units of Sendai virus. For poly I·poly C and statolon, these were the lowest doses that had protected all the mice when given as a single, 24-hr prophylactic dose. An effective antiviral state, measured against this significant challenge, existed 1 week after poly I·poly C, 4 weeks after statolon, and 3 weeks after Sendai virus (Table 2).

Therapeutic, i.e., postinfection, treatment was also examined. Groups of mice were inoculated ic with vaccinia virus representing challenges of 1 to 1,000 LD_{50}, and 24 or 48 hr later were treated with poly I·poly C or statolon (Table 3). When treatment occurred 24 hr after infection, both substances significantly improved survival in mice which received challenges of 1 to 100 LD_{50}. At this time, with challenges of 10 or 100 LD_{50}, statolon-treated animals had survival indices four to six times greater than poly I·poly C-treated animals. After 48 hr, increased survival was seen only in groups challenged with 1 or 10 LD_{50}.

IF stimulation. Serum and brain samples were collected at various intervals after the ic injection

TABLE 1. *Target-organ treatment of vaccinial encephalitis[a] with various doses of interferon inducers*

Treatment[b]	Dose	Mortality	Harmonic mean survival time[d] (days)	Survival index[d]
Poly I·poly C	0 (Saline)	38/38	3.8	
	30 μg	0/20	⩾440[c]	⩾116
	3	2/20	90.9	24
	0.3	5/38	52.6	14
	0.03	9/18	11.1	2.9
	0.003	9/18	10.3	2.7
Statolon	0 (Saline)	38/38	4.4	
	135 μg	0/18	⩾397	⩾90.2
	90	0/18	⩾397	⩾90.2
	45	0/18	⩾397	⩾90.2
	22.5	2/19	45.7	10.4
	4.5	5/20	21.4	4.9
	0.45	7/20	14.7	3.4
Sendai virus	0 (Saline)	20/20	4.1	
	1.54 HA units	0/20	⩾440	⩾107
	0.154	0/20	⩾440	⩾107
	0.0154	0/20	⩾440	⩾107
	0.00154	7/20	14.0	3.4

[a] WR vaccine virus, ca. 1,000 LD_{50} ic.
[b] Given ic 1 day before virus.
[c] Where all animals survived 21 days, a minimum possible survival time was calculated, assuming one mouse died on day 22.
[d] See Materials and Methods for explanation.

TABLE 2. *Duration of protection against vaccinial encephalitis in mice[a]*

Treatment[b]	Interval[c]	Mortality	Harm mean survival time[d]	Survival index[d]
Saline	W-3 and W-1	30/30	4.2	
Poly I·poly C, 30 μg	W-1	14/19	6.9	1.6
	W-2	18/18	4.1	1.0
	W-3	19/19	4.3	1.0
Saline	W-1	20/20	3.5	
Statolon, 45 μg	W-1	2/20	50.0	14.3
	W-2	3/20	24.6	7.0
	W-3	10/20	9.3	2.7
	W-4	11/20	8.7	2.5
Saline	W-1, 2, or 3	60/60	4.0	
Sendai virus, 1.54 HA units	W-1	6/20	14.5	3.6
	W-2	9/20	10.6	2.7
	W-3	16/20	5.9	1.5

[a] WR vaccinia virus, ca. 1,000 LD_{50} inoculated ic.
[b] Given ic at interval designated.
[c] W-1, -2, -3, -4 indicates treatment 1, 2, 3, or 4 weeks before virus.
[d] For explanation see Materials and Methods.

TABLE 3. *Postinfection treatment of vaccinial encephalitis in mice*

Challenge[a] dose	Survival index[b] (treated after 24 hr)		Survival index[b] (treated after 48 hr)	
	Poly I·poly C[c]	Statolon[d]	Poly I·poly C[c]	Statolon
1,000	1.3	1.2	1.1	1.0
100	4.1	25	1.1	1.0
10	12.6	55	7.9	7.9
1	⩾40	⩾42	⩾42	⩾40

[a] WR vaccinia virus inoculated ic.
[b] See Materials and Methods for explanation.
[c] Poly I·poly C (30 μg) given ic at interval indicated.
[d] Statolon (45 μg) given ic at interval indicated.

of poly I·poly C, statolon, or Sendai virus. Results of IF assays are presented in Fig. 1. Serum IF peaks occurred 2, 18, and 12 hr after the ic injection of poly I·poly C, Sendai virus, or statolon, respectively. The corresponding peaks for brain IF were 2 and 72 hr for poly I·poly C and Sendai virus. After statolon, however, the level remained at a plateau 4 to 24 hr.

DISCUSSION

The purpose of the present work was to explore the general feasibility of target-organ treatment of the central nervous system. Precise comparison of local and peripheral treatments would require a more extensive investigation. The data at hand, however, provide some direct evidence of relative efficiency. Concentrations of inducer approxi-

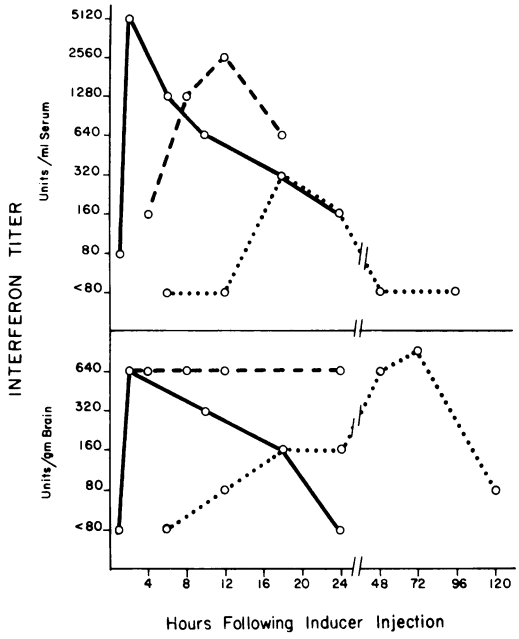

FIG. 1. *Effect of intracerebral administration of interferon inducers on brain and serum interferon levels. Solid line, Poly I·poly C; dashed line, statolon; dotted line, Sendai.*

mating the ic minimal 100% protective doses were ineffective given intraperitoneally.

The serum IF curves after intracerebral stimulation (Fig. 1) resemble those reported to follow intravenous injection of inducers (25, 26). This might be expected, since Field (7) and Mims (17) reported that material injected ic in mice is immediately taken up into the cerebrospinal fluid and then passes into the circulation. Consequently, ic injection of IF inducers could exert peripheral as well as local effects. The response of brain IF (Fig. 1) is also similar to that seen after intravenous stimulation. The later peak in brain due to Sendai virus may indicate stimulation by newly replicated, endogenous but nonpathogenic virus, whereas the prompt response in serum may be stimulation by the exogenous viral input. The time of occurrence of IF in brain after Sendai virus (Fig. 1) is similar to that following the ic inoculation of Semliki Forest or West Nile viruses (8). The similarity in peak levels after the various inducers may explain the similar effectiveness of poly I·poly C and statolon when their administration was delayed to 48 hr after infection (Table 3). At 24 hr postinfection, statolon was the more effective. At an advanced stage of disease, such as 48 hr postinfection, the magnitude of the response to treatment may be as important as duration. It is of interest that low concentrations of inducers were effective against vaccinia virus ic. Hilleman (13) suggested that low concentrations of inducers might leave some cells unstimulated and capable of response at a later time. Low doses, therefore, should not only minimize toxicity but might also be less likely to produce hyporeactivity.

When one considers approximately equipotent doses of statolon and poly I·poly C, the longer duration of protection from statolon or Sendai virus, compared to poly I·poly C (Table 2), may reflect the greater ability of a virion (5) to survive than a naked ribonucleic acid, such as poly I·poly C. These data do not delimit the total duration of the protective effect of statolon ic. Statolon and pyran copolymer (21) produced protection against MM virus for 30 or more days when the challenge dose of virus was approximately one LD_{50}. In the present study, the high level of protection at the longest interval tested suggests that the period of effectiveness of statolon, given ic, extends well beyond 4 weeks. Sendai virus-induced protection declined to a minimal level of significance, survival index of 1.5, at 3 weeks. It has been claimed (2, 20) that interferon is the probable mechanism through which inducers provide protection; others (4, 14, 15, 22, 24) have suggested alternative or supplementary activities. The marked contrast between the periods of detectable IF and demonstrable protection support the idea that induction of IF may not be the only means by which IF inducers exert antiviral activity.

This report shows that a viral encephalitis can be effectively countered by target-organ treatment. Since IF inducers used in this study were effective in low concentrations when given ic, this method of administration may provide a useful experimental tool for evaluating the relative antiviral activity of potential IF inducers. Although direct injection into the central nervous system may seem heroic or unrealistic when the results are extrapolated to clinical use, one should keep in mind that viral encephalopathies frequently threaten biological or intellectual life and, therefore, justify drastic action.

ACKNOWLEDGMENTS

This research was supported by Public Health Service training grant 5T01AI00060-13 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Baron, S., C. E. Buckler, R. M. Friedman, R. V. McClosky. 1966. Role of interferon during viremia. II. Protective action of circulating interferon. J. Immuncl. 96:17–24.
2. Buckler, C. E., K. T. Wong, and S. Baron. 1968. Induction of the interferon system by various inducers. Proc. Soc. Exp. Biol. Med. 127:1258–1262.
3. Catalano, L. W., Jr., and S. Baron. 1970. Protection against herpes virus and encephalomyocarditis virus encephalitis with a double stranded RNA inducer of interferon. Proc. Soc. Exp. Biol. Med. 133:684–687.

4. DeClercq, E., and T. C. Merigan. 1969. Local and systemic protection by synthetic polyanionic interferon inducers in mice against intranasal vesicular stomatitis virus. J. Gen. Virol. **5**:359–368.
5. Ellis, L. F., and W. J. Kleinschmidt. 1967. Virus-like particles of a fraction of statolon, a mold product. Nature (London) **215**:649–650.
6. Falabella, F. 1967. Bleeding mice: a successful technique of cardiac puncture. J. Lab. Clin. Med. **70**:981–982.
7. Field, E. J. 1967. Spread of scrapie agent by the blood stream. Vet. Rec. **81**:495.
8. Finter, N. B. 1964. A rich source of mouse interferon. Nature (London) **204**:1114–1115.
9. Finter, N. B. 1966. Interferon as an antiviral agent *in vivo*: quantitative and temporal aspects of the protection of mice against Semliki Forest virus. Brit. J. Exp. Pathol. **47**:361–371.
10. Finter, N. B. 1969. Dye-uptake methods for assessing viral cytopathogenicity and their application to interferon assays. J. Gen. Virol. **5**:419–427.
11. Gard, S. 1940. Encephalomyelitis of mice. II. A method for the measurement of virus activity. J. Exp. Med. **72**:69–77.
12. Gerone, P. J., D. A. Hill, L. H. Appell, and S. Baron. 1971. Inhibition of respiratory virus infections of mice with aerosols of synthetic double-stranded ribonucleic acid. Infect. Immunity **3**:323–327.
13. Hilleman, M. R. 1970. Double-stranded RNAs (poly I:C) in the prevention of viral infections. Arch. Intern. Med. **126**: 109–124.
14. Kjeldsberg, E., and M. Flikke. 1971. Antiviral activity of polyinosinic-polycytidylic acid in the absence of cell-controlled RNA synthesis. J. Gen. Virol. **10**:147–154.
15. Kleinschmidt, W. J. 1970. In vitro and in vivo studies with statolon. Ann. N.Y. Acad. Sci. **173**:547–556.
16. Kleinschmidt, W. J., and F. Streightoff. 1971. Inhibition of influenza virus and interferon response intranasally with statolon. Infect. Immunity **4**:738–741.
17. Mims, C. A. 1960. Intracerebral injections and the growth of viruses in the mouse brain. Brit. J. Exp. Pathol. **41**:52–59.
18. Park, J. H., and S. Baron. 1968. Herpetic keratoconjunctivitis: therapy with synthetic double-stranded RNA. Science **162**:811–813.
19. Powell, H. M., and C. G. Culbertson. 1953. Action of an antiviral mold filtrate against MEF1 poliomyelitis virus in mice. Proc. Soc. Exp. Biol. Med. **83**:161–163.
20. Schafer, T. W., and R. Z. Lockart, Jr. 1970. Interferon required for viral resistance induced by poly I:C. Nature (London) **226**:449–450.
21. Schmidt, J. P., F. F. Pindak, D. J. Giron, and R. R. Ibarra. 1971. Duration of resistance to viral infection following administration of interferon inducers. Tex. Rep. Biol. Med. **29**:133–140.
22. Turner, W., S. P. Chan, and M. A. Chirigos. 1969. Stimulation of humoral and cellular antibody formation in mice by poly I_r:C_r. Proc. Soc. Exp. Biol. Med. **133**:334–338.
23. Wheelock, E. F. 1967. Effect of statolon on Friend virus leukemia in mice. Proc. Soc. Exp. Biol. Med. **124**:855–858.
24. Woodhour, A. F., A. Friedman, A. A. Tytell, and M. R. Hilleman. 1969. Hyperpotentiation by synthetic double-stranded RNA of antibody responses to influenza virus vaccine in adjuvant 65. Proc. Soc. Exp. Biol. Med. **131**:809–817.
25. Youngner, J. S., and J. V. Hallum. 1968. Interferon production in mice by double-stranded synthetic polynucleotides: induction or release? Virology **35**:177–179.
26. Youngner, J. S., and W. R. Stinebring. 1966. Comparison of interferon production in mice by bacterial endotoxin and statolon. Virology **29**:310–316.