Prophylactic and Therapeutic Use of Poly(I)·Poly(C) [Poly-D-lysine] Against Herpesvirus Encephalitis in Mice (36396)

LOUIS W. CATALANO, JR.,[1] WILLIAM T. LONDON, JERRY M. RICE, AND JOHN L. SEVER

Section on Infectious Diseases, Perinatal Research Branch, National Institute of Neurological Diseases and Stroke, Bethesda, Maryland 20014; and the Experimental Pathology Branch, National Cancer Institute, Bethesda, Maryland 20014

A variety of substances have been demonstrated experimentally to induce interferon. Polyriboinosinic·polyribocytidylic acid [poly(I)·poly(C)] has been shown to be among the most active of the synthetic substances and induces interferon both *in vivo* and *in vitro* (1, 2). Recently, poly(I)·poly (C) has been shown to eradicate established herpes simplex virus (HSV) keratoconjunctivitis in the rabbit (3) and to protect against herpesvirus encephalitis in the mouse (4). This material has also been used in the treatment of herpes simplex encephalitis in a human infant with possible therapeutic benefit (5).

A recent study has indicated that a complex of poly(I)·poly(C) and poly-D-lysine can enhance the *in vivo* induction of interferon in mice compared to poly(I)·poly(C) alone (6). This effect is most pronounced when the poly(I)·poly(C) polymer is of low molecular weight (7).

Because of the potential therapeutic usefulness of such a substance in treating HSV infections or other viral diseases of man, a study was undertaken to investigate the ability of poly(I)·poly(C) [poly-D-lysine] complexes to afford protection against, and to treat mice with, experimental herpesvirus encephalitis.

Materials and Methods. Specific pathogen free male C3H/HeN mice (20 g) were used for all experiments. Stocks of a type 1 HSV (strain VR_3) were grown in primary rabbit cell monolayers in 32-oz glass bottles and titered by observation of cytopathic effects in tube cultures of the same cells as previously described (8).

[1] Present address: Box 155, Neurological Institute, 710 W. 168th Street, New York 10032.

Polyriboinosinic acid and polyribocytidylic acid were purchased as the potassium salts (Schwarz Bio-Research, Inc., Orangeburg, NY, lot 6701). Both homopolymers were of relatively low molecular weight ($\sim 10^5$, manufacturer's data). Each was dissolved individually in phosphate buffered saline (PBS = 0.15 *M* NaCl, 0.006 *M* Na_2HPO_4, pH 7.0) at a concentration of 2 mg/ml (5 m*M* polynucleotide phosphorus). Equal volumes of the two solutions were combined, heated to 70° in a water bath, cooled slowly to room temperature, and stored in portions at —20°. The identities and concentrations of the homopolymers, and the formation of the hypochromic double-stranded complex [poly(I)·poly(C)] after mixing, were confirmed by scanning the ultraviolet absorption spectra of each preparation in a Cary Model 15 spectrophotometer. Poly-D-lysine of molecular weight $\sim 1.6 \times 10^5$ was purchased as the hydrobromide salt (Nutritional Biochemicals Corp., Cleveland, OH) and dissolved in PBS at a concentration of 1.1 mg/ml (5 m*M* ε-amino nitrogen), dialyzed overnight at 4° against 40 vol of PBS to remove bromide ion, and stored in portions at 20°. To prepare poly(I)·poly(C) [1:1 poly-D-lysine] complexes, equal volumes of stock poly(I)·poly(C) and poly-D-lysine solutions were thawed, heated to 70°, allowed to cool to ~50°, and slowly combined by adding the poly-D-lysine solution dropwise to the poly(I)·poly(C) solution while stirring vigorously. Some precipitate invariably formed when the final third of the poly-D-lysine solution was added; most of this slowly redissolved when the mixture was heated to ~75° and stirred for a period of time. Any in-

TABLE I. Design of Four Studies.

Study	Pre-treatment:[a] polymer solutions (ip)	Virus challenge (ml):[a] (day 0)	Posttreatment: polymer solutions (ip)
1. Protection-therapy	0.2 ml day before challenge (day −1)	0.5, sc	0.1 ml, days 1, 3, 5, 7 and 9
2. Protection-therapy	0.1 ml (day −1)	0.02, ic	0.1 ml, days 1, 3, 5, 7 and 9
3. Therapy	None	0.5, sc	0.1 ml, days 2, 4, 6, 8 and 10 or days 4, 6, 8, 10 and 12
4. Rechallenge			
A. Survivors of group 3	None	Rechallenge: 0.5, sc, day 25	None
B. Controls (no prior virus inoculation)	None	0.5, sc	None

[a] Abbreviations: ip = intraperitoneally; ic = intracranially; sc = subcutaneously.

soluble remnants were removed by centrifugation; these never amounted to more than 20% of the poly(I)·poly(C) originally present, on the basis of ultraviolet spectroscopic measurements. The complex solutions were stored at 4° until used, at which time they were warmed to 56° to redissolve a gelatinous precipitate which tended to form during refrigeration. The final concentration of each solution used for antiviral therapy was therefore: poly(I)·poly(C), 1.0 mg/ml; poly(I)·poly(C) [poly-D-lysine], 1.0 mg of poly(I)·poly(C) plus 0.55 mg of poly-D-lysine HBr/ml; poly-D-lysine HBr, 0.55 mg/ml. A previous publication has reported the levels of interferon achieved in mice with comparable dosages of each solution used in the following experiments (6).

Four studies were conducted using the above polymer solutions and herpes virus challenge (Table I). These included two protection-therapy experiments in which the drug was given before and following virus challenge; one therapy experiment; and one rechallenge experiment using the survivors of the therapy experiment plus a control group. Animals were observed for at least 21 days after the final virus inoculation.

Statistical analysis was performed using the chi-square method. Protection was defined as control mortality minus experimental mortality, divided by control mortality.

Results. Protection-therapy experiments. In order to ascertain clearly whether poly(I)·poly(C) [poly-D-lysine] could afford protection against HSV encephalitis, two initial experiments were conducted by pretreating the mice 24 hr *prior to* either subcutaneous (sc) (Fig. 1) or intracerebral (ic) (Fig. 2) inoculation of virus.

Animals were given an initial intraperitoneal dose of 0.2 ml of the polymer solutions (Fig. 1). This dose of poly(I)·poly(C), alone (group A) or complexed with poly-D-lysine (group B), was acutely toxic, and within 24 hr resulted in 11 deaths among the 60 mice in group A and 7 deaths among the 60 mice in group B. No acute toxicity was observed with poly-D-lysine alone (group C) or with PBS (group D). Subsequent doses of 0.1 ml, begun the day after viral inoculation, caused no apparent mortality.

The mortality observed for each day following sc challenge on day 0 with approximately $10^{4.5}$ $TCID_{50}$ of HSV is shown in Fig. 1. Groups of animals given either poly(I)·poly(C) [poly-D-lysine] (Group B) or poly(I)·poly(C) alone (Group A) on days −1, 1, 3, 5, 7 and 9 showed significantly lower mortality than those animals treated with PBS ($X^2 = 57.82$, $p < .0001$ and $X^2 =$

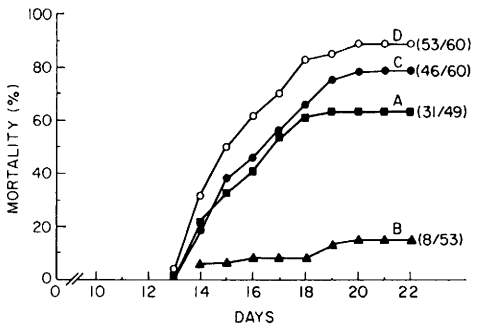

FIG. 1. Protection and therapy experiment; mortality of groups receiving approximately $10^{4.5}$ $TCID_{50}$ of HSV subcutaneously on day 0. Treatment groups as follows: (A) poly(I)·poly(C) alone, (B) poly(I)·poly(C) [poly-D-lysine] complexes, (C) poly-D-lysine alone (D) PBS only, on days −1, 1, 3, 5, 7 and 9. Numbers in parentheses show number of dead animals over total effective number of animals in each group, which originally consisted of 60 mice.

8.211, $p = 0.004$, respectively). The calculated percentage protection for Group B was 82.9% and for Group A was 25.0%. There was no significant difference between those mice treated with poly-D-lysine (Group C) and PBS (Group D) ($X^2 = 2.074$, $p > .10$). Animals treated with poly(I)·poly(C) [poly-D-lysine] complexes had significantly less mortality than mice treated with poly(I)·poly(C) alone ($X^2 = 23.02$, $p < .0001$).

To avoid the acute toxicity encountered in the previous experiment, all initial injections in a second protection experiment were reduced to 0.1 ml intraperitoneally on the day before intracerebral viral challenge. No apparent deaths resulted from this treatment; early mortality followed ic inoculation of virus in a few animals, and was attributed to trauma. Figure 2 illustrates the mortality observed on each day following ic challenge with approximately 10 $TCID_{50}$ (8 LD_{50}) of HSV. This dose was chosen because previous experiments had indicated that only relatively low levels of protection were achieved in those mice treated solely with poly(I)·poly(C) of higher molecular weight (approx 10^7) (4); such virus challenge levels would then permit testing the efficacy of the poly (I)·poly(C) [poly-D-lysine] complex solution (Group B) under the most stringent circumstances.

The difference in mortality between Group B and PBS-treated animals (Group D) was found to be only marginally significant with the number of animals used ($X^2 = 3.478$, $p = 0.6$). The calculated percent protection of Group B compared to Group D was 28.4%. The mortality of Groups A and C did not differ from that of the PBS-treated mice. Since there was no statistical difference between the mortality of the two control treatment groups (Group C, poly-D-lysine; and Group D, PBS), the combined mortality of these two groups was compared to the mortality of Group B. Under these circumstances, a significant difference was present for the poly(I)·poly(C) [poly-D-lysine] treatment group ($X^2 = 4.1067$, $p = .04$).

Therapy experiments. In order to investigate whether poly(I)·poly(C) [poly-D-lysine] might be therapeutically beneficial against HSV encephalitis in mice, an experiment was performed in which the animals received treatment beginning 2 and 4 days *after* subcutaneous inoculation of virus. Previous experiments had shown that an occasional animal displayed signs of central nervous system infection (lethargy and irritability) as early as the fourth day following sc inoculation of HSV. Virtually all animals which subsequently die following sc infection with HSV have histopathologic and virologic

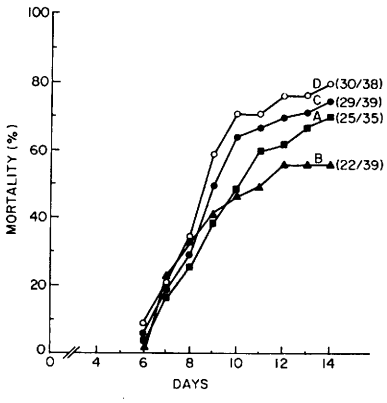

FIG. 2. Protection and therapy experiment; mortality of groups receiving approximately 10 $TCID_{50}$ (8 LD_{50}) of HSV intracerebrally on day 0. Treatment times and groups (A, B, C, D) as given as Fig. 1. There were initially 39 animals in each group.

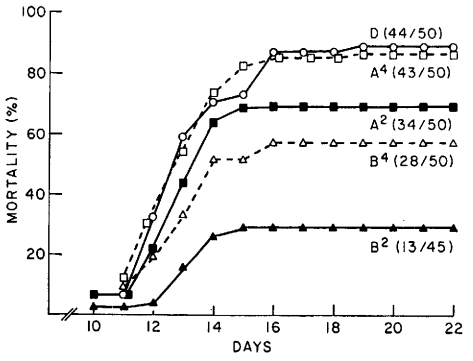

FIG. 3. Therapy experiment; mortality of groups receiving approximately $10^{4.5}$ $TCID_{50}$ of HSV subcutaneously on day 0 and treatment (5 injections on alternate days) instituted either 2 or 4 days following viral inoculation (as indicated by superscript). Treatment groups A, B, D as given in Fig. 1. The original number of animals in each group was either 45 or 50, as indicated.

evidence of herpetic infection in their brains (L. S. Catalano, unpublished data). The results of this experiment are given in Fig. 3. Since the above experiments had demonstrated the lack of apparent prophylactic benefit of poly-D-lysine treatment alone (Group C), this additional control was eliminated. A therapeutic effect of poly(I)·poly(C) [poly-D-lysine] was demonstrated when therapy was instituted 2 days (Group B^2) or 4 days (Group B^4) after sc inoculation of virus ($X^2 = 32.038$, $p < .0001$ and $X^2 = 11.161$, $p < .001$, respectively). The calculated percentage protection for group B^2 was 67.2% and for Group B^4 was 36.4%. A therapeutic effect of poly(I)·poly(C) alone was also seen if treatment was instituted 2 days (Group A^2) following sc HSV inoculation ($X^2 = 4.720$, $p < .05$); this effect was not present when therapy was begun 4 days (Group A^4) following virus inoculation ($p > .10$). The calculated percentage protection for Group A^2 was 22.7%, and for Group A^4 was 2.3%.

Effect of treatment on the immune system. In order to determine whether treatment with either poly(I)·poly(C) [poly-D-lysine] complexes or poly(I)·poly(C) alone might alter the immune response in a detrimental way following sc inoculation with HSV, a further investigation was carried out with the survivors of the above experiment. Animals were rechallenged with an additional sc inoculation of HSV ($10^{4.5}$ $TCID_{50}$) on day 25 following the initial sc inoculation of virus. Previous studies (8) have shown that significant levels of anti-HSV neutralizing antibodies appear as early as 9 days following sc inoculation with HSV, and that such levels of antibody are sufficiently high by 21 days after infection to protect against ic challenge with HSV. An additional PBS-treated control group of mice (Group D^{25}, not previously infected with HSV) was added on day 25 to determine the base line mortality of the dose of virus used for rechallenge. These mice were comparable in age and weight to the animals which were reinfected. The results are shown in Fig. 4. Animals previously infected with HSV, whether treated with poly (I)·poly(C) [poly-D-lysine], poly(I)·poly (C) alone, or PBS only, displayed clear evidence of resistance to rechallenge, whereas the mice not previously infected displayed an 86% mortality ($p < .0001$ for all three previously infected groups).

Discussion. The results of the present study indicate an enhanced anti-HSV effect

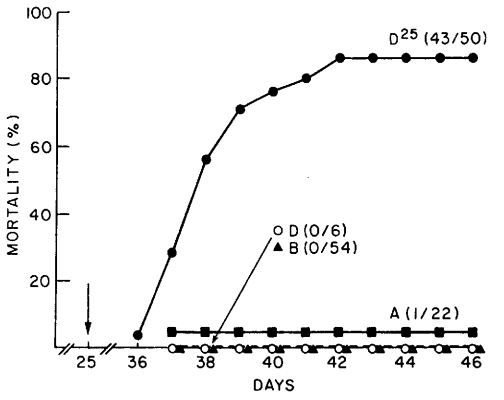

FIG. 4. Virus rechallenge experiment; surviving animals from therapy experiment (Fig. 3) reinoculated subcutaneously with approximately $10^{4.5}$ $TCID_{50}$ of HSV on day 25 following original infection; a PBS control treated group (D^{25}) was not previously infected with HSV. Treatment groups A, B, D as given in Fig. 1. Total numbers of animals in each group are shown, and in the cases of groups A, B and D represent all survivors from Fig. 3.

in mice treated either prophylactically or therapeutically with poly(I)·poly(C) [poly-D-lysine] complexes, compared to poly(I)·poly(C) administration alone. This effect was most marked for animals pretreated with this material one day prior to sc virus inoculations; nevertheless, a therapeutic effect was clearly evident if treatment was begun as late as 4 days following sc infection when an occasional mouse was found to have early signs of central nervous system (CNS) infection. A prophylactic effect of poly(I)·poly (C) [poly-D-lysine] was also seen against ic inoculation of mice with HSV when compared to the pooled control treatment group. It should be noted that it is difficult to investigate the antiviral activity of any compound when the route of HSV challenge is intracerebral since approximately 1–3 $TCID_{50}$ of virus equals 1 LD_{50} (4).

Slight *in vitro* enhancement of the ability of poly(I)·poly(C) to induce interferon has been reported with certain low molecular weight polycations such as streptomycin and neomycin (9). This enhancement is not seen, however, when such polycations are combined with poly(I)·poly(C) and administered *in vivo*. When the high molecular weight polycation diethylaminoethyl dextran is used instead of the polyamine antibiotics, interferon induction is markedly enhanced both *in vitro* (10 and *in vivo* (11). However, other workers have noted lethal toxic effects in mice with this compound, including liver damage, abdominal adhesions and ascites (6). No such toxicity has been observed, as yet, with poly-D-lysine.

A previous report has demonstrated a 10–100-fold increase in the *in vivo* inducing capabilities of poly(I)·poly(C) when combined with poly-D-lysine (6) using dosages comparable to those used in the present study. As was previously pointed out in the introduction, however, this enhancement appears to be a function of the molecular weight of the poly(I)·poly(C) being studied. In the present study, a poly(I)·poly(C) prepared from homopolymers of molecular weight of approximately 10^5 was used; other reports studying the anti-HSV of poly(I)·poly(C) have used a compound with a molecular weight of about 10^7 (4). At the present time, it is not established whether there is a possible increased toxicity of the higher molecular weight poly(I)·poly(C) duplexes. However, because of the small degree of toxicity observed with solutions composed of lower molecular weight poly(I)·poly(C) complexed with poly-D-lysine, such materials should be considered as a potentially useful therapeutic anti-HSV compound.

Herpes simplex virus has been previously regarded as relatively resistant to interferon (12–14). More recent studies indicate that either induced interferon (4) or homologous viral interference (8) can protect mice from developing fatal HSV encephalitis following ic challenge with this virus. The present study demonstrates that poly(I)·poly(C) [poly-D-lysine] protected mice from developing fatal CNS infection following sc infection with HSV. In light of these observations and those of others (3, 15) HSV should be considered sufficiently sensitive to the effects of this polymer to warrant additional investigation with various synthetic polynucleotides in life-threatening infections of humans. One patient with HSV encephalitis has been treated with poly(I)·poly(C) with possible therapeutic benefit (5). Further studies may provide additional evidence of the role and usefulness of interferon and interferon inducers in this common, and frequently fatal, disease.

Summary. Polyriboinosinic·polyribocytidylic acid [poly(I)·poly(C)] complexed with poly-D-lysine was studied as a prophylactic and therapeutic material in mice inoculated with herpes simplex virus (HSV). With the treatment as described, a higher proportion of mice treated with poly(I)·Poly(C) [poly-D-lysine] survived than when treatment consisted of poly(I)·poly(C) alone, or phosphate buffered saline (PBS) alone, following subcutaneous (sc) inoculation with HSV. This enhanced protection was still detectable if treatment was begun as late as 4 days following sc inoculation of HSV, at which time an occasional mouse was showing signs of early central nervous system illness. The treatment

regimen did not appear to adversely alter the immunological responsiveness of sc infected animals as judged by subsequent ic rechallenge with HSV. No mortality was observed as a result of repeated injections of 100 μg of poly(I)·poly(C) complexed with poly-D-lysine.

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