Local and Systemic Protection by Synthetic Polyanionic Interferon Inducers in mice against Intranasal Vesicular Stomatitis Virus

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SUMMARY
The effects of the synthetic polyanionic interferon inducers maleic acid/divinyl ether copolymer (MA/DVE) and polyinosinic/polycytidylic acid (polyrI/polyrC) were studied in mice challenged intranasally with vesicular stomatitis virus. Young (14-day or 24-day) animals were used. Either local (intranasal) or systemic (intraperitoneal) treatment protected significantly against virus infection. Optimal local protection was given by 40 μg. of polyrI/polyrC instilled 4 hr before virus challenge. Intraperitoneal MA/DVE exerted a more durable effect lasting for at least 10 days, and polyrI/polyrC proved to be active when applied several days after inoculation of virus. PolyrI/polyrC treatment started after virus in the brain had reached maximum concentration retarded progression of the disease, but when delayed until clinical signs of illness occurred, polyrI/polyrC treatment was not effective. The protective activity of MA/DVE and polyrI/polyrC injected intraperitoneally against virus infection at a distant site suggested the mediation by a systemic antivirus state, presumably due to interferon production.

INTRODUCTION
Intranasal inoculation of vesicular stomatitis virus may be a useful model for assessing the prophylactic and therapeutic efficacy of interferon inducers since it seems to follow a route of entry and spread to the central nervous system which might also be employed by certain natural virus infections causing meningo-encephalitis in animals and man (e.g. herpes simplex, rabies and poliomyelitis) (Johnson & Mims, 1968). Upon nasal instillation, vesicular stomatitis virus attacks primarily the olfactory mucosa and invades the central nervous system along the olfactory nerves; young mice die 4 to 7 days after infection with evidence of extensive necrosis of the olfactory brain areas (olfactory bulbs, tuberculum olfactorium and hypothalamus) (Sabin & Olitsky, 1938). Intranasal inoculation of virus was used by Jensen & Rasmussen (1963) to study the influence of stress on susceptibility of mice to vesicular stomatitis virus and in our experiments to examine the protective effect of the synthetic anionic polymers maleic acid/divinyl ether copolymer (NSC-46015-C) and polyinosinic/polycytidylic acid. Both compounds stimulate interferon production in vitro (Merigan & Finkelstein, 1968; Field et al. 1968) and in vivo (Regelson, 1967; Merigan, 1967a; Field et al. 1967) and induce either prolonged protection against subsequent virus challenge (Merigan, 1967b; Merigan & Finkelstein, 1968) or recovery from already established virus...

METHODS

Animals. Two- to four-week-old Swiss albino mice, from Berkeley-Pacific animal breeders, were used throughout this study. They were kept in an air-conditioned room at 20°C.

Compound tested. Maleic acid/divinyl ether copolymer NSC-46015-C (MA/DVE) mol. wt = 17,000 was synthesized and characterized at Hercules, Inc., Wilmington, Delaware and given by Dr William Regelson, Division of Oncology, Medical College of Virginia, Richmond, Virginia. The polymer was made up in minimal Eagle’s medium (MEM) immediately before use. Polyriboinosinic and polyriboctydilic acid homopolymers were purchased from Miles and Company, Elkart, Indiana. The synthetic polynucleotides were mixed in equimolar concentration in saline and allowed to react for 1 hr at 25°C. Formation of double-stranded polynucleotides/polyoctydilic acid complexes (polyrI/polyrC) was evidenced by a marked hypochromic effect at 260 nm. and a sharp transition point (Tm) at 62°C. The polyrI/polyrC preparation was further diluted in MEM to the appropriate concentration just before use.

Virus. The INDIANA strain of bovine vesicular stomatitis virus was propagated and titrated in primary chick embryo fibroblasts. The titre of stock virus was 10^8 p.f.u./ml. Small volumes (0.5 ml.) of stock virus were sealed in 2 ml. glass vials and stored at -70°C. Before inoculation a sample was thawed and diluted in MEM. Unanaesthetized mice were inoculated with the virus by applying a 0.01 ml. vol. in 4 to 6 drops to the external nares from a number 27 x ½ in. needle mounted on a 0.25 ml. syringe. Deaths were scored twice daily. Most animals died between the 4th and 7th days after inoculation (2-week-old mice), or between the 6th and 10th days (24-day-old mice). Animals surviving 15 days after infection without visible signs of illness were counted as survivors.

Statistical significance was assessed by the $\chi^2$ test with Yates’s correction employing daily death and survival counts as determined from the day the first animal died to the day the last animal died in either group.

RESULTS

Relation between age of mice and susceptibility to intranasal challenge with vesicular stomatitis virus

Young and old mice are equally susceptible to intracerebrally injected vesicular stomatitis virus; however, older mice are generally resistant to peripheral (intranasal, intraocular and intramuscular) inoculation of the virus (Sabin & Olitsky, 1938). Therefore, special attention was paid to the age of the animals used in our protection studies (Table 1).

Effect of intranasal instillation of MA/DVE or polyrI/polyrC on intranasal challenge with vesicular stomatitis virus

The local effect of MA/DVE and polyrI/polyrC on intranasal challenge with vesicular stomatitis virus was tested in several ways. When MA/DVE and polyrI/polyrC...
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were given intranasally to 14-day-old mice at concentrations of 50 μg. per mouse and 4 μg. per mouse respectively 3 hr before a 100 % lethal infection, the mean survival time was extended by 24 to 36 hr. The effect of these relatively low polymer concentrations was further explored in mice challenged with a 70 % lethal virus dose (Fig. 1). Survival rose from 33 % (control) to 55–60 % (treated groups). This protection was not statistically significant. The protective effect of intranasal treatment of polyrI/polyrC was then correlated with the concentration of the polymer. Two-week-old mice received either 40 μg., 4 μg., or 0.4 μg. of polyrI/polyrC intranasally and were challenged 4 hr later with a 100 % lethal dose of virus. Survival rose to 50 % for mice treated with the highest dose (Table 2). Repeated intranasal administrations of MA/DVE and polyrI/polyrC 40, 24, and 16 hr before virus challenge did not confer any protection, although both large and small doses of polymer were used (250 and 50 μg. per mouse for MA/DVE; 40 and 4 μg. per mouse for polyrI/polyrC). The protective activity of locally administered MA/DVE was not increased when MA/DVE was complexed to arginine (1:3 weight ratio), although arginine enhances the interferon production by MA/DVE.

Table 1. Relationship of age of mice and susceptibility to intranasal challenge with vesicular stomatitis virus

<table>
<thead>
<tr>
<th>Age (days) at time of infection</th>
<th>14</th>
<th>18</th>
<th>24</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD100 (p.f.u./mouse)</td>
<td>5×10^4</td>
<td>2×10^6</td>
<td>10^7</td>
<td>&gt;3×10^7</td>
</tr>
<tr>
<td>LD70 (p.f.u./mouse)</td>
<td>10^4</td>
<td>N.D.</td>
<td>2×10^6</td>
<td>&gt;3×10^7</td>
</tr>
<tr>
<td>Average weight (g.)</td>
<td>6.5±0.8</td>
<td>N.D.</td>
<td>1.31±0.9</td>
<td>N.D.</td>
</tr>
<tr>
<td>Onset of disease and death</td>
<td>4-7</td>
<td>N.D.</td>
<td>6-10</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

(continued on next page)
in peritoneal macrophages *in vitro* (Merigan & Finkelstein, 1968) and in the serum of the mouse *in vivo* (De Clercq & Merigan, unpublished data). Finally, repeated intranasal doses of 10 μg. of polyri/polyrc, started 1 day after virus challenge and continued twice daily for 4 days, failed to protect but instead potentiated the lethal effect of the vesicular stomatitis virus challenge: the mean survival time was reduced by 24 hr and survival from 30% to 10%.

*Effect of intraperitoneal injection of MA/DVE or polyri/polyrc on intranasal vesicular stomatitis virus challenge. MA/DVE and polyri/polyrc administered before virus challenge*

To establish the prophylactic activity of systemically administered MA/DVE and polyri/polyrc, both polymers were injected intraperitoneally several times before virus challenge. Twenty-four-day-old animals were used in this experiment. They had to be challenged with $2 \times 10^6$ p.f.u. vesicular stomatitis virus to give 65% death in the

Table 2. *Effect of intranasal instillation of polyri/polyrc on mortality induced by $5 \times 10^4$ p.f.u. of vesicular stomatitis virus in 14-day-old mice*

<table>
<thead>
<tr>
<th>Dose of polyri/polyrc per mouse</th>
<th>Control</th>
<th>40 μg.</th>
<th>4 μg.</th>
<th>0.4 μg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivors/number challenged</td>
<td>0/11</td>
<td>5/10</td>
<td>4/11</td>
<td>2/10</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>0%</td>
<td>50%</td>
<td>36%</td>
<td>20%</td>
</tr>
</tbody>
</table>

Table 3. *Effect of intraperitoneal injection of MA/DVE or polyri/polyrc on mortality induced by $2 \times 10^6$ p.f.u. of vesicular stomatitis virus in 24-day-old mice*

<table>
<thead>
<tr>
<th>Time (days) between injection of polymer and virus challenge</th>
<th>10</th>
<th>6</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA/DVE (125 mg./kg.)</td>
<td>17/31</td>
<td>14/31</td>
<td>21/30</td>
</tr>
<tr>
<td>Survivors/number challenged</td>
<td>55</td>
<td>45</td>
<td>70</td>
</tr>
<tr>
<td>Survival (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyri/polyrc (25 mg./kg.)</td>
<td>16/32</td>
<td>Not done</td>
<td>16/20</td>
</tr>
<tr>
<td>Survivors/number challenged</td>
<td>50</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>Survival (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated controls</td>
<td>18/52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors/number challenged</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

control group. Both MA/DVE and polyri/polyrc conferred maximal protection when given 1 day before inoculation ($P < 0.005$ and $P \sim 0.001$ respectively by comparison of final numbers of animals which died or lived following challenge in treated and control groups). For both MA/DVE and polyri/polyrc, a slight protective effect persisted 10 days after the injection of the polymer (Table 3). MA/DVE was consistently less effective when given 6 days before virus challenge. Three separate observations indicated that effectiveness of treatment decreased in the order $-1$ day, $-10$ days, and $-6$ days. An identical dip in the prolonged antivirus protection of MA/DVE was
observed in mice challenged intraperitoneally with Mengo virus (Merigan & Finkelstein, 1968) and might be related to the tolerance state which follows the injection of MA/DVE (Merigan & Finkelstein, 1968) and other plastic polymers (polyacrylic acid) (De Somer et al. 1968) and which seems to be most pronounced within 4 to 8 days after injection.

Effect of intraperitoneal injection(s) of polyrI/polyrC on intranasal challenge with vesicular stomatitis virus. PolyrI/polyrC administered after virus challenge

Additional experiments were made to correlate the protective effect of intraperitoneal polyrI/polyrC and the time that treatment began. Fourteen-day-old mice were inoculated with a 70% lethal dose of vesicular stomatitis virus. Five mg./kg. of polyrI/polyrC were administered every second day, from 1 day before infection in one group, from 1 day after infection in the second group, and from 3 days after infection in the third group. In all groups, treatment was continued until the 5th day after virus challenge (Fig. 2). The protection afforded was closely related to the time treatment was initiated. When started 1 day before infection, polyrI/polyrC increased the number of survivors by more than 40% (P < 0.001). An increase of 20% was observed if polyrI/polyrC treatment began 1 day after infection (P < 0.02). Treatment postponed until 3 days after infection increased the mean survival time by only 12 hr. A similar experiment explored further the minimal protection conferred by polyrI/polyrC treatment at the 3rd and 5th days. Ten mg./kg. of polyrI/polyrC were injected 3 and 5 days after a 100% lethal virus challenge. Mortality was delayed significantly (P < 0.005) in the treated group, although all treated and untreated mice eventually succumbed to the infection (Fig. 3).

Additional experiments were designed to correlate the effect of post-challenge treatment with polyrI/polyrC, the virus growth in the brain, and the onset of clinical symptoms of illness. Virus titres in the brains of 14-day-old mice were determined at

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**Fig. 2.** Effect of repeated intraperitoneal injection of polyrI/polyrC on mortality induced by 10^6 p.f.u. of vesicular stomatitis virus in 14-day-old mice. PolyrI/polyrC injected at 5 mg./kg. on alternate days starting: ○, 1 day before infection; ●, 1 day after infection; ○, 3 days after infection (10 mice per group). Treatment continued until 5th day. ●, control mice.
various intervals after a 100% lethal virus challenge. Groups of five mice were killed, their brains removed and homogenized in 10 volumes MEM. Homogenates were cleared by centrifugation and titrated in both L929 cells and primary chick embryo fibroblasts. No virus could be detected 1 day after infection (Fig. 4). Vesicular stomatitis virus titre reached its greatest value on day 2 and decreased slowly until onset of illness and death (5th to 6th day). The effect of late treatment with polyrI/polyrC (Fig. 3) compared with the course of virus growth in the brain (Fig. 4) showed that polyrI/polyrC may have conferred protection and retarded progression of the disease upon administration after maximal vesicular stomatitis virus multiplication in the brain had occurred. Clinical signs of disease were followed in 24-day-old mice ino-
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culated with a 50% lethal virus dose. After 5 to 7 days, mice developed fever (up to 39.8°C compared to 37.5°C to 38.0°C in uninfected mice), followed by either circling (predominantly left circling) or flaccid paralysis of the back legs. Some of the mice had convulsions, others fell dying without previous signs of paralysis or equilibrium disorders. Most of them died within 24 to 48 hr after onset of disease. Hypothermia (35.5°C to 36.0°C) preceded death. Polyrl/polyrC treatment, initiated as late as 4 days after inoculation, reduced mortality significantly (Table 4). However, if treatment was delayed until first signs of disease (fever, circling) occurred (6th day), there was no therapeutic effect. Polyrl/polyrC had, clearly, to be administered before illness became clinically evident to be fully active in this acute systemic virus infection.

Table 4. Effect of intraperitoneal injection of polyrl/polyrC on mortality induced by 10⁶ p.f.u. of vesicular stomatitis virus in 24-day-old mice

<table>
<thead>
<tr>
<th>Control</th>
<th>At 2 and 6 days</th>
<th>At 3 and 5 days</th>
<th>At 4 and 6 days</th>
<th>At 6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivors/number challenged</td>
<td>5/10</td>
<td>9/9</td>
<td>9/9</td>
<td>8/10</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

DISCUSSION

Prolonged protection against viruses has been reported to persist for 1 or 2 months after single injections of the synthetic carboxylate polymers MA/DVE (Merigan, 1967b; Merigan & Finkelstein, 1968) polyacrylic acid (De Clercq & De Somer, 1968a) and statolon (Kleinschmidt & Murphy, 1967). Although these observations raised interest in the use of interferon inducers in virus infections, they suffered from certain limitations in interpretation. The prolonged protective effect of MA/DVE was described in a system where both polymer and virus were given intraperitoneally (Merigan, 1967b; Merigan & Finkelstein, 1968). This raised the possibility of a direct interaction of polymer and virus as a cause of the prolonged protection. Stronger evidence for the development of a persistent systemic antivirus state was put forward by De Clercq & De Somer, who found that intraperitoneally injected polyacrylic acid was active for at least 2 months against intravenous challenge with vaccinia virus (1968a). Since virus-like particles have been demonstrated in statolon preparations (Ellis & Kleinschmidt, 1967; Banks et al. 1968; Kleinschmidt et al. 1968), the durable protection afforded by statolon (Kleinschmidt & Murphy, 1967) may be due to the mycophage. Kleinschmidt & Murphy administered both statolon and virus by the intraperitoneal route and a single intraperitoneal dose of one virus may interfere for a period of several weeks with another virus, challenged by the same route (Wheelock, 1966).

Vesicular stomatitis virus given by the intranasal route is more like natural virus infection than intraperitoneal or intravenous challenge. Both MA/DVE and polyrl/polyrC conferred significant protection against otherwise lethal intranasal challenge with vesicular stomatitis virus. The greatest effect was obtained when the polymer was given 1 day before infection. MA/DVE was somewhat less effective when injected 10 days before infection, as was polyrl/polyrC. On the other hand, polyrl/polyrC
offered protection when treatment began 1 day or even as late as 4 days after virus inoculation. When polyrI/polyrC treatment was started when the mice developed fever (5 to 7 days after infection), or clinical evidence of paralysis, convulsions, equilibrium disorders and prostration (6 to 10 days after infection), there was no therapeutic effect. These findings further confirmed the prolonged prophylactic activity of MA/DVE copolymer against intranasal virus challenge, as described for intraperitoneal virus challenge (Merigan & Finkelstein, 1968). However, the protection afforded by MA/DVE and polyrI/polyrC against intranasal challenge with vesicular stomatitis virus seemed to be less significant than the protection obtained against intraperitoneal Mengo virus. When injected 10 days instead of 1 day before infection, MA/DVE remained invariably active against intraperitoneal Mengo virus but lost some of its protective capacity against intranasal vesicular stomatitis virus. Our findings brought some support for the therapeutic efficacy of polyrI/polyrC in acute virus infections (meningo-encephalitis), as claimed for herpetic keratoconjunctivitis (Park & Baron, 1968). However, polyrI/polyrC failed to protect once gross symptoms of meningo-encephalitis appeared.

It is still unclear whether these synthetic polyanions act through the induction of interferon alone. Direct interaction of polymer and virus must be considered when both are administered by the same route (Merigan, 1967b; Merigan & Finkelstein, 1968; De Clercq & De Somer, 1968b), but could be excluded in our system in which intraperitoneally injected polymer protected against a virus challenge given by the nasal route which progresses through the olfactory neurons to the brain. The protection of MA/DVE and polyrI/polyrC in our system might have been due to interferon produced in the peritoneal cavity or, alternatively, to other as yet unestablished effects on host functions as can be seen after the peak of virus multiplication has been reached.

Intranasal application of MA/DVE or polyrI/polyrC conferred minimal and transient protection. Only a single dose, given 3 or 4 hr before infection, was effective. No protection was observed for either higher doses or repeated administration 40, 24 and 16 hr before virus challenge or twice daily 1, 2, 3 and 4 days after virus challenge. The mortality from infection by vesicular stomatitis virus was not influenced by intranasal administration of polyrI/polyrC after infection, unlike the favourable response of herpetic keratoconjunctivitis to local treatment with polyrI/polyrC (Park & Baron, 1968), and is probably due to the fact that in our animals the virus was already multiplying vigorously in the brain and hence therapy at the portal of entry was futile.

Others have found it difficult to establish protection by interferon inducers in virus infections in which the virus reached the central nervous system by the neural and not by the circulatory route. For example, Soave (1968) failed to demonstrate any protective effect of intraperitoneally injected statolon in mice challenged with rabies either intracerebrally or intramuscularly. Polycrylic and polymethacrylic acids only slightly influenced the mortality induced by intracerebral challenge with vesicular stomatitis virus. Injected intraperitoneally either 8 days or 1 day before infection, polycrylic and polymethacrylic acid increased the mean survival time by 2 to 3 days and the percentage survival from 10% to 30% (De Clercq & De Somer, unpublished data). Akers & Stirling (1968) found intraperitoneally administered statolon highly active against intraperitoneal Columbia-SK virus, moderately active against intranasal, and inactive against intracerebral virus challenge: the resistance induced by intraperitoneal
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Statolon in mice infected intranasally with Columbia-SK virus appeared to be blood-borne, since all organs contained much virus in the untreated mice, but none in the treated mice (lung tissue excepted). Hence, spread of Columbia-SK virus to the different tissues must have been controlled in the blood stream. The significant sparing effect of intraperitoneally injected statolon on intraperitoneally injected Mengo virus (Schmidt & Pindak, 1967, 1968) could also be explained by prevention of viraemia.

In our intranasal model the virus spreads from the olfactory mucosa to the central nervous system along a closed system of neurons, causing lesions in areas which are connected by the specific neurons, without lesions in unrelated areas contiguous to the affected zones (Sabin & Olitsky, 1938). It appears from the significant protection we obtained against intranasal virus challenge that interferon inducers can interact successfully at the neural level with viruses causing meningo-encephalitis.

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