Effects of Poly(2'-O-Methyladenylic Acid) on Susceptibility and Autogenous Immunity to RNA Tumor Virus Oncogenesis In Vivo

(immune enhancement/Moloney sarcoma virus/Rauscher leukemia virus)

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ABSTRACT Poly(2'-O-methyladenylic acid) [poly(Am)] inhibited tumor development and death induced by the Moloney sarcoma virus-leukemia virus complex in newborn mice. The compound was effective at 10 μg per mouse when given at least 1 hr before inoculation of virus, but the greatest inhibition was seen in mice treated at least 4 hr before infection. Poly(2'-O-methyluridylic acid) and poly-(vinyladenine) also inhibited sarcoma development and death but were less effective than poly(Am). Poly(Am) also enhanced the antibody response of newborn mice to endogenous leukemia virus envelope antigens, which we refer to as autogenous immunity. The results of these preliminary studies suggest that poly(Am) altered the oncogenic potential of the Moloney sarcoma-leukemia virus complex in vivo, and the effect appears to be mediated through an enhancement of the immune response of the treated animals.

Synthetic polyribonucleotides, including poly(U), poly(C), poly(A), poly(G), poly(2'-O-methyladenylic acid) [poly(Am)] (1–3), and poly(vinyladenine) (4), have been shown to competitively inhibit mouse leukemia virus reverse transcriptase in vitro. Of the four polyribonucleotides tested in cell culture, poly(A) most effectively inhibited leukemia and sarcoma virus replication and this effect was even greater with poly(Am) (2, 5). When added to cells after infection, poly(Am) inhibited only within the first 4 hr, indicating an effect on some early, specific virus function such as the viral reverse transcriptase (5).

To test the effects of poly(Am) on RNA tumor-virus oncogenesis in vivo, experiments were performed with the Moloney pseudotype of mouse sarcoma virus, which induces solid tumors at the site of inoculation and also includes an excess of helper Moloney leukemia virus (6–8). Rauscher leukemia virus was also used in these studies to evaluate the effects of poly(Am) on virus-induced splenomegaly (9). The results of these preliminary studies indicate that the action of poly(Am) in vivo in altering the oncogenic potential of the viruses tested is the result of an effect on specific immunological functions of the treated animals.

MATERIALS AND METHODS

Animals. BALB/c strain mice were obtained from Cumberland View Farms, Clinton, Tenn. Newborn mice were used within 48 hr of birth and young adult mice were generally 6–8 weeks of age.

Virus. Rauscher leukemia virus (RLV) was obtained from Dr. Richard Tyndall, Oak Ridge Associated Universities, and was prepared from spleens of BALB/c mice infected for at least 21 days. The spleens were removed aseptically, homogenized in phosphate buffered saline, clarified by centrifugation at 1000 × g and stored at −70°. The titers of the stocks were at least 10^6 ID₅₀/ml in a 14-day mouse splenomegaly assay. RLV was inoculated into 6- to 8-week old BALB/c mice and the effects of the virus were assayed by spleen weight increases (9).

Moloney sarcoma virus (MSV) was obtained from Dr. Wallace P. Rowe, NIH, and was grown in NIH Swiss mouse embryo cells. The titer of the sarcoma virus pool was approximately 10^6 focus-forming units (FFU) per ml on BALB/c 3T3 cells by focus assay (7) and contained Moloney leukemia virus (MLV) the titer of which was 2 × 10^9 plaque-forming units (PFU) per ml by the XC cell-plaque assay technique (10) on Swiss mouse embryo cells. We refer to this stock as MSV-MLV. MSV-MLV was incubated subcutaneously into 48-hr-old BALB/c mice and was assayed by tumor induction and mortality.

Compounds. Poly(vinyladenine) was a gift of Dr. Paula Pitha, Johns Hopkins University. Poly(Am) and poly(2'-O-methyluridylic acid) [poly(Um)] were prepared by methods described previously (5, 11).

Radioimmune Precipitation Assay. Induction of antibody to envelop antigens of mouse leukemia virus was assayed by measuring the amount of [³H]leucine-labeled Moloney leukemia virus precipitated by dilutions of mouse serum. [³H]Leucine-labeled virus was prepared by methods described previously (5, 12). Dilutions of test serum were first incubated with 3 × 10^4 cpm of labeled virus at 37° for 60 min and then with anti-mouse gamma globulin (rabbit serum obtained from Cappel Laboratory) for 60 min at 37° and 2 hr at 4°. The precipitates were collected by centrifugation at 1200 × g for 15 min, washed three times in TNE buffer (0.5 M Tris-HCl at pH 7.5, 0.1 M NaCl, 1 mM ethylenediaminetetraacetate, resuspended in 0.5 ml of TNE, and dissolved in 10 ml of “Aquasol” (New England Nuclear Corp.) for counting in a scintillation spectrometer.

RESULTS

Effects of Poly(Am) on MSV(MLV) Tumorigenesis. Inoculation of newborn mice with MSV results in the progressive
development of sarcomas at the site of inoculation and subsequent death. In adult mice the virus induces sarcomas which usually regress (8). To test the effects of poly(Am) on MSV tumorigenesis, mice born within a 48-hr period were pooled and redistributed randomly according to original litter number. In control experiments up to 50 μg of poly(Am) had no toxic effect and did not affect the growth rate of newborn mice. The mice were given a single, arbitrarily chosen, dose of 10 μg of poly(Am) 1 hr before inoculation with 10^6 or 10^4 FFU of MSV. The MSV stock was prepared in cell culture and also includes helper Moloney leukemia virus (MLV) in 100-fold excess and thus is referred to as MSV(MLV). With the highest virus dose, poly(Am) had little effect on the rate of tumor development or death (Fig. 1A and B). At the lower virus titer, poly(Am) significantly delayed the development of sarcomas and death (Fig. 1C and D).

The results of this experiment indicate that poly(Am) at 10 μg can effectively alter the oncogenicity of the MSV-(MLV) complex, depending on the dose of the virus challenge. Postmortem examination of some poly(Am)-treated mice, that died during the experiment, showed large, infiltrating, undifferentiated sarcomas at the site of inoculation and extensive spleen involvement indicative of leukemia virus. Thus, the cause of death may be related to the leukemic process, rather than to the localized sarcomas.

In another experiment in which poly(Am) was given at a concentration of 33 μg 1 hr before MSV(MLV) at approximately 10^4 FFU, sarcomas developed to a palpable size (approximately 1 mm) in 60-70% of the mice in both the treated and untreated groups within 20 days. However, in the group treated with poly(Am), the tumors regressed to an undetectable size within 40 days. The tumors redeveloped, however, and all mice with tumors in both groups died within 100 days. These results have not yet been reconfirmed.

The effects of poly(Am) at 10 μg were also observed when given 4 hr before MSV(MLV) or at the time of virus inoculation. Two virus doses were used and, at the highest concentration (approximately 10^4.7 FFU), tumors developed in all of the untreated mice within 20 days; tumors developed within 50 days in mice given poly(Am) on the opposite side at the same time virus was injected. In the group given poly(Am) 4 hr before virus infection, only 9% developed sarcomas for the duration of the experiment (Fig. 2A). In the group pretreated with poly(Am) (Fig. 2B), mortality reached 90% by the time the experiment was terminated (248 days) but occurred at a much slower rate than in the other two groups. It is important to emphasize that many of the animals that died,
were devoid of palpable sarcomas, again indicating a primary role of the leukemia-virus component in mortality.

At the lower dose (approximately $10^4$ FFU), the rate of sarcoma development and mortality was similar in the untreated group and in the group where poly(Am) was injected at the time of infection (Fig. 2C and D). However, in the group pretreated with poly(Am), no sarcomas developed and only 10% of the mice had died by the end of the experiment. These results indicate that pretreatment with poly(Am) at 4 hr is much more effective in inhibiting sarcoma development and death than the pretreatment given at 1-hr as in the first experiments.

One other polynucleotide analogue and a polyadenine analogue were also tested against MSV(MLV). Poly(Um) at 10 μg decreased the rate of tumor development and death, but was much less effective than poly(Am) at the same dose. Poly(vinyladenine) at 250 μg was effective in inhibiting tumor development and mortality, but lower doses were not tested.

**Effect of Poly(Am) on the Immune Response to MSV(MLV).**

The importance of pre-exposure to poly(Am) and the regression of MSV(MLV) sarcomas described above suggested that poly(Am) might in some way influence the host immune response and we, therefore, tested the antibody response of treated mice. Newborn BALB/c mice (approximately 24-48 hours old) were pooled and redistributed by original litter size (9 mice per group). The mice were treated with poly(Am) at a dose of 10 μg, or with Eagle’s Basal Medium (EBM) subcutaneously, followed 4 hr later by infection with MSV(MLV) administered subcutaneously on the side opposite the poly(Am) treatment. At 2, 3, and 4 weeks after infection, three mice per group were selected randomly, bled, and the sera were tested for reactivity to MLV antigens by radioimmune-precipitation assay. The results (Table 1) showed that control mice gradually developed an antibody response to endogenous leukemia virus envelope antigens which cross react with MSV (MLV). These results are in agreement with previous studies on the spontaneous antibody response of mice to endogenous RNA tumor viruses, which we have referred to as autogenous immunity (12, 13).

A significant increase in anti-mouse leukemia virus antibody was detected in animals assayed 2 weeks after injection of poly(Am) alone, but not in MSV(MLV)-infected mice (Table 1). At 3 weeks after treatment with poly(Am), the MSV(MLV) infected group showed a similar response. Thus, it appears that newborn mice can be stimulated by poly(Am) to produce antibodies to mouse RNA tumor virus envelope antigens before maturation of immune competence, which normally occurs between 3 and 5 weeks of age (13). It is possible that the lower peak precipitation titers that occurred two weeks after infection in the poly(Am) + MSV(MLV)-treated group as compared to poly(Am) alone, may be due to antigen excess. By 4 weeks after treatment, the antibody titers were approximately equal in all four groups, presumably due to the normal development of humoral immunity to endogenous leukemia virus antigens. Thus, these results suggest that, 2 weeks after injection of poly(Am) or poly(Am) + MSV(MLV), the humoral immune capacity of BALB/c mice is enhanced by poly(Am) treatment in such a way that the animals developed a premature precipitating antibody response to viral antigens.

**Effect of Poly(Am) on RLV-Induced Splenomegaly.**

The effect of poly(Am) was also tested against two concentrations of RLV. Ten weanling BALB/c mice per group were treated with 50 μg of poly(Am) or Eagle’s basal medium (EBM) by intraperitoneal inoculation, followed 1 hr later by virus infection; a second injection of 10 μg of poly(Am) was given 24 hr later. The spleen weights were determined 21 days after infection and the results are given in Table 2. Splenomegaly was enhanced in both groups treated with poly(Am), but the greatest increase (47%) was seen with the lower virus concentration. In a second experiment, a 10-μg dose of poly(Am) was given 4 hr before virus inoculation. In this experiment (Fig. 3) treatment with poly(Am) resulted in a small, but not significant, decrease in spleen weight. Thus, RLV-induced

### Table 1. Radioimmune precipitation assay of mouse sera

<table>
<thead>
<tr>
<th>Time after injection (weeks)</th>
<th>Serum dilution</th>
<th>Mean percent counts/min precipitated*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>MSV(MLV)</td>
</tr>
<tr>
<td>2</td>
<td>1:4</td>
<td>15.2 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>16.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>15.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>1:4</td>
<td>25.5 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>18.5 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>16.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>16.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>1:64</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>1:4</td>
<td>54.2 ± 10.3</td>
</tr>
<tr>
<td></td>
<td>1:8</td>
<td>37.0 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>1:16</td>
<td>28.0 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>1:32</td>
<td>23.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>1:64</td>
<td>18.4 ± 0.9</td>
</tr>
</tbody>
</table>

Newborn BALB/c mice were given EBM (for controls) or 10 μg of poly(Am), followed in 4 hr by approximately $10^6$ FFU of MSV(MLV). At the indicated times, mice were selected randomly and the serum was collected and assayed by radioimmune precipitation with approximately $3 \times 10^6$ cpmin of H-labeled Moloney leukemia virus.

* Each point represents the mean ± standard error of three to five mice. In this test, 20% precipitation is background.
spleenomegaly was not inhibited by poly(Am) treatment and a larger dose produced enhancement of the disease. The paradoxical effects of poly(Am) in MSV(MLV)- and RLV-infected mice could be due to competing effects of the compound on the target cells of the viruses.

**DISCUSSION**

An initial purpose of these studies was to determine if the capacity of poly(Am) to competitively inhibit viral reverse transcriptase in vitro and to inhibit viral infection in cell culture could be used to determine the requirement for reverse transcriptase for viral tumorigenesis in vivo. It is clear from the experiments reported that poly(Am) also has an effect on host immune functions which are virus specific. It is also possible that, at lower MSV(MLV) doses, inhibition of viral functions may also account for the effect on tumorigenesis.

The in vitro stimulation of immune functions by double-stranded synthetic polynucleotides has been explored extensively by Braun (14-17) and others (18, 20). However, single-stranded polynucleotides have not been found to be very effective in enhancing immune competence, due to their high sensitivity to nuclease (15). Associated homopolymers of poly(A)·poly(U), poly(C)·poly(G), and poly(I)·poly(C) in particular were found to enhance both primary and secondary humoral immune competence. Poly(A)·poly(U) and poly(I)·poly(C) also induced premature antibody formation in hyporesponsive newborn mice (21).

In the present study the effects of poly(Am) in vivo demonstrate that the single-stranded polynucleotide is capable of enhancing immune competence, and may be related to the nuclease resistance (11) or some other effect conferred by methylation. The immune enhancement by poly(Am) appears to involve the effector components of humoral immunity since poly(Am) induces a premature response to endogenous viral antigens in newborn mice. We have also found that poly(Am) enhances the rate of response of mice to sheep erythrocytes (unpublished data). The mechanism by which the single-stranded polynucleotides enhance immune responsiveness is not entirely clear from these studies, but it may involve either an effect on cells which process antigens or by direct action on antibody-synthesizing cells. The effect on RLV infection which resulted in enhancement of virus-induced splenomegaly is consistent with this interpretation. A primary target-cell population by RLV has been shown to be nonthymus-dependent, B-cells (bone marrow derived cells), which are responsible for the humoral immune response (22-24). Thus, if poly(Am) acts in some manner to stimulate B-cell proliferation, target-cell pool for this virus would be increased and would enhance the proliferative effects of the virus. Hanna et al. (24) reported enhancement of RLV-mediated splenomegaly in both germfree and conventional mice by immunization with sheep erythrocytes prior to RLV infection. It was interpreted from these studies that, while the significant splenomegaly seen 2-3 weeks after virus infection was a consequence of the virus-replicating hematopoietic component of the spleen, it was a secondary aspect of the disease process and was directly related to RLV infection of the B-cell compartment of the spleen white pulp (23). Also, a previous lactic dehydrogenase virus infection which specifically enhances the B-cell compartment (as shown by enlarged germinal centers) rendered mice more susceptible to RLV-mediated splenomegaly (24, 25). However, the tumorigenicity of other leukemia viruses, including MLV is dependent on thymic function but not B-cell proliferation (24). Thus, if poly(Am) stimulates B-cell proliferation, one of its effects may be to enhance the response to viral antigens and inhibit MSV(MLV) replication, whereas in the case of RLV, the stimulation provides a pool of replicating cells susceptible to virus transformation.

The beneficial effect of treatment of mice with poly(Am) before infection with MSV(MLV) appears to be due to enhancement of the immune response to the virus and/or inhibition of virus replication. However, the lack of any significant inhibition of RLV oncogenesis suggests that the effect of poly(Am) in vivo is related to specific cellular effects rather than direct inhibition of virus replication. We have reported previously that poly(Am) does not react directly with virus (5).

In one experiment, tumor regression occurred in all treated mice, which suggests a reaction to virus-induced tumor cells. Since the MSV stocks contain helper leukemia virus (MLV) which also replicates in these cells (26), it is possible that this effect is due to antibody-mediated destruction or inhibition of tumor cells expressing viral antigens (27). However, these experiments do not exclude a concomitant enhancement of cell-mediated immunity, and this possibility is now being tested.

**Table 2. Effect of poly(Am) on RLV-induced splenomegaly**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean spleen weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>157</td>
</tr>
<tr>
<td>Poly(Am)</td>
<td>145</td>
</tr>
<tr>
<td>RLV(10⁴)</td>
<td>1582</td>
</tr>
<tr>
<td>RLV(10⁴) + poly(Am)</td>
<td>1932</td>
</tr>
<tr>
<td>RLV(10⁵)</td>
<td>658</td>
</tr>
<tr>
<td>RLV(10⁵) + poly(Am)</td>
<td>1409</td>
</tr>
</tbody>
</table>

Ten weanling BALB/c mice per group were given 50 μg of poly(Am) intravenously. One hour later the mice were inoculated (intravenously) with RLV and, 24 hr later, were treated again with 10 μg of poly(Am). Spleen weights were determined 21 days after inoculation.

* Spleen-enlarging dose.
A significant observation in these experiments was the premature induction of an antibody response to the endogenous leukemia virus. It has been shown in several strains of mice that there is a chronic immune response to a variety of virion and virus-induced cell surface antigens (13, 28, 29). One important question is whether this response functions in the control and regulation of the endogenous virus burden and in pathogenesis. We have suggested that autogenous immunity to endogenous leukemia virus antigens may be beneficial in RF strain mice (13). In those studies, a marked decrease in mouse leukemia virus antigens in the thymus and spleen was correlated with the age-dependent (3-5 weeks) development of humoral immune competence, which in turn was related to the occurrence of immune-complex glomerulonephritis. Furthermore, data correlating the incidence of the natural lymphoid neoplasia with the severity of these glomerular lesions indicated an inverse relationship in aged animals (13, 30). The specificity of this natural antibody to leukemia virus envelope antigens was demonstrated by a radioimmune precipitation assay (12) and by immune electron microscopy (31) in several strains of mice, including BALB/c. Treatment with poly(Am) appears to enhance the rate of this response in newborn mice, which results in a level of resistance to MSV oncogenesis seen in adult animals.

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