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# Inhibition of Murine Leukemia Virus with Poly-2′-*O*-(2,4-Dinitrophenyl)Poly[A]

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**Poly-2′-*O*-(2,4-dinitrophenyl)poly[A] (DNP-poly[A]) is a potent inhibitor of reverse transcriptases from a variety of sources (I. Kang and J. H. Wang, J. Biol. Chem. 269:12024–12031, 1994). In the present study, its inhibitory effect on the reverse transcriptase (RT) from Moloney murine leukemia virus (MuLV) was investigated. DNP-poly[A] was found to enter the virus spontaneously and to completely inhibit the RT within 30 min at 0°C. The inhibitor was also spontaneously transported into isolated human lymphocytes and leukocytes at 37°C. Animal studies have demonstrated the effectiveness of DNP-poly[A] as an antiviral drug when administered intraperitoneally at various doses from 1 to 100 mg/kg of body weight. MuLV-infected mice show the presence of RT in their blood as well as increased numbers of leukocytes. After the administration of DNP-poly[A] at a dosage of 100 mg/kg of body weight three times a week over a 3-week period, RT could no longer be detected by an ultrasensitive RT-PCR assay. Autopsy showed that the spleens of infected but untreated mice were enlarged 2- to 10-fold, with fused nodules and the proliferation of large abnormal lymphocytes, whereas the spleens of infected but treated mice resembled the normal spleens of uninfected control mice. These observations indicate that further study of DNP-poly[A] as a general antiretroviral agent is desirable.**

Since human immunodeficiency virus (HIV) is believed to be the causative agent of AIDS, arresting its replication has been the focus of much research. The currently approved anti-HIV drugs zidovudine, didanosine, and zalcitabine serve as inhibitors or chain terminators of the reverse transcriptase (RT) reaction (5, 6, 13). However, the virus is able to mutate in such a way as to elude these drugs. In a novel attempt to overcome the development of drug resistance because of the rapid mutation of HIV, the macromolecular inhibitor poly-2′-*O*-(2,4-dinitrophenyl)-poly[A] (DNP-poly[A]) was designed and synthesized (7) on the basis of crystallographic data on the active site of HIV type 1 (HIV-1) RT (1, 8). This inhibitor was designed to fit the gross structure of the entire, ~7.0-nm binding cleft of HIV-1 RT instead of fitting precisely the detailed structure of a small region in the enzyme. The specific binding of a precisely fitted small inhibitor molecule can be abolished by mutation of one or two amino acid residues at the binding site. However, since the entire binding cleft in RT cannot be abolished by mutation if the virus is to remain viable, DNP-poly[A] is expected to be not only a mutation-insensitive inhibitor of HIV but also an effective inhibitor of other retroviruses with similar RT structures. DNP-poly[A] was indeed found to be a highly potent inhibitor of RTs isolated from HIV-1, HIV-2, and zidovudine- and nevirapine-resistant strains of HIV, as well as from avian myeloblastosis virus (14) and Moloney murine leukemia virus (Mo-MuLV) for which the 50% effective concentration ($EC_{50}$) is in the subnanomolar range (7). Mo-MuLV originated from sarcoma 37, a transplantable connective tissue neoplasm of mice (9). It has been reported to produce a generalized lymphocytic neoplasm in mice within a short period (3, 4) and is specific with regard to age, strain, and species. Both Mo-MuLV and HIV belong to the group of negative-stranded RNA viruses which replicate through a DNA intermediate. In the present work the effect of DNP-poly[A] on Mo-MuLV was studied both in vitro and in Mo-MuLV-infected mice as a convenient animal model relevant to AIDS research.

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## MATERIALS AND METHODS

**Compounds and enzymes.** [$^3$H]poly[A] (22.8 Ci/mmol) was from Amersham (Arlington Heights, Ill.), and [$^3$H]dTTP (83.8 Ci/mmol) was from NEN DuPont (Boston, Mass.). GF/C filters were from Whatman (Hillsboro, Oreg.). Oxirane acrylic beads (250 μm), epoxy-activated Sepharose 6B, poly[A]-$[dT]_{12}$, ultrapure reagents for PCR, and human plasma were all from Sigma Chemical Co. (St. Louis, Mo.). RNasin was from Promega (Madison, Wis.), MS2 RNA was from Boehringer Mannheim (Indianapolis, Ind.), *Taq* DNA polymerase and gel electrophoresis reagents were purchased from Life Technologies (Grand Island, N.Y.), and HIV RT was purchased from Worthington Biochemical Corp. (Freehold, N.J.) and was stored in 10 mM potassium phosphate (pH 7.1) with 1 mM dithiothreitol (DTT) and 50% (vol/vol) glycerol at −20°C.

**Virus and cells.** Ecotropic Mo-MuLV particles, counted by electron microscopy and suspended in Dulbecco's modified Eagle medium (high concentration of glucose with 10% fetal bovine serum and 50 μg of gentamicin per ml) and elutriated cell preparations of human lymphocytes and leukocytes were purchased from Advanced Biotechnologies (Columbia, Md.).

**Assay of RT activity in solution.** The RNA-dependent DNA polymerase activity of RT was assayed by injecting 3 μl of enzyme solution into 23 μl of an assay mixture at 37°C containing 125 mM Tris-HCl (pH 8.2), 1 mM $MgCl_2$, 125 mM KCl, and 250 μM [$^3$H]dTTP as the substrate and 23 nM poly[A]-$[dT]_{12}$ as the template. After 10 min, the amount of [$^3$H]poly[dT] that had formed was determined by precipitation with 10% trichloroacetic acid, removal of the ligand with glass microfiber filters, and washing of the precipitate with cold 10% trichloroacetic acid and 50 mM sodium pyrophosphate and then ethanol. The radioactivity in the washed precipitate was then determined by liquid scintillation counting (7).

**Assay of RNA-dependent DNA polymerase activity of Mo-MuLV.** The RNA-dependent DNA polymerase activity of Mo-MuLV was assayed as described previously (12). Briefly, 5 μl of medium containing $6 \times 10^8$ virus particles was pelleted by centrifugation at 100,000 × *g* for 15 min in a Beckman Airfuge through a cushion of 20% glycerol containing 0.05 M Tris HCl (pH 7.8) and 0.1 M KCl. The pellet was resuspended in 100 μl of a solution containing 0.05 M Tris HCl (pH 7.8), 0.1 M KCl, 20 mM DTT, and 0.1% Triton X-100. A 10-μl aliquot was added to a 60-μl total reaction mixture containing 0.05 M Tris HCl (pH 7.8), 0.06 M KCl, 2 mM DTT, 0.6 mM manganese acetate, 0.02 $A_{260}$ units of poly[A]-$[dT]_{12}$, and 1.5 mM [$^3$H]dTTP. The reaction mixture was then incubated for 60 min at 37°C, and the reaction was terminated by the addition of 3 ml of 10% trichloroacetic acid (TCA). The acid-precipitated product was collected on GF/C

filters and was washed extensively with TCA, water, and ethanol and then dried, mixed with counting cocktail, and assayed in a Wallac scintillation counter.

**Testing the stability of DNP-poly[A] in 0.01 M HCl at 37°C.** DNP-poly[A] was incubated in 0.01 M HCl at 37°C for 4 and 24 h. At the end of the incubation periods, each tube was immersed in liquid nitrogen to stop any further inactivation. The 50% inhibitory concentrations ($IC_{50}$s) of HIV RT (at 25 nM) were determined by the procedure reported elsewhere (7). Control experiments were also run with diethyl pyrocarbonate-treated water.

**Preparation of Sepharose 6B with covalently attached DNP-poly[A].** About 200 mg of epoxy-activated Sepharose 6B beads was suspended in 4 ml of DNP-poly[A] solution (2.5 mg/ml). The suspension was mixed with 1 ml of 1.0 M $K_2CO_3$ solution, adjusted to pH 10 to 11 with $KHCO_3$, and gently shaken for 2 days at 25°C. The derivatized resin suspension was transferred to a 3-ml syringe fitted with a filter. From the decreased $A_{259}$ of the filtrate, the yield was estimated to be 2 mg of DNP-poly[A] per g of resin. The resin was sequentially washed with water–1.0 M KCl solution–buffered medium before use.

**Preparation of oxirane beads with covalently attached DNP-poly[A].** DNP-poly[A]-activated oxirane beads were prepared by derivatization as described previously (11).

**Binding of Mo-MuLV to DNP-poly[A] covalently attached to oxirane acrylic or Sepharose beads.** Epoxy-activated Sepharose or oxirane acrylic beads with covalently attached DNP-poly[A] were preequilibrated with 2 ml of buffer (20 mM Tris HCl [pH 7.5]) or 1 ml of reconstituted human plasma for 72 h at 25°C. Each mixture was kept in a capped sterilized container on a shaker. At each titration step, a mixture of $10^8$ virus particles in 100 μl of buffer or plasma was added to the resin suspension, and the mixture was shaken at 0°C for 60 min. An aliquot (50 μl) of the supernatant was then removed, centrifuged at 100,000 × *g* in an Airfuge through a glycerol layer (20% glycerol, 0.05 M Tris HCl [pH 7.8], 0.1 M KCl) to pellet the virus, and assayed for RT activity (12).

**Inhibition of RT in intact virus by DNP-poly[A].** A sample containing $6 \times 10^8$ virus particles in 5 μl of medium was incubated at 0°C with 10 μl of a 2 μM DNP-poly[A] stock solution. At each stated time, the virus particles were centrifuged at 100,000 × *g* for 10 min through a glycerol layer to separate the virus particles from DNP-poly[A] in the external liquid. The supernatant was carefully removed, and the pellet was washed with 200 μl of Tris HCl buffer (50 mM; pH 7.8) and again centrifuged. The pellet was then homogenized in 12 μl of 0.5% Triton X-100 at 0°C for 3 min and incubated with the Mo-MuLV RT assay mixture (12) at 37°C for 60 min. The assay reaction was terminated by the addition of 3 ml of 10% TCA. The incorporated radioactive product was collected on a GF/C filter, washed, and assayed by liquid scintillation counting.

**Transport of DNP-poly[A] into human lymphocytes and leukocytes at 37°C.** A 400-μl suspension containing $8 \times 10^6$ cells in 100 μl of fetal serum albumin, 3.2 ml of RPMI 1640, and 300 μl of [$^{14}$C]DNP-poly[A] (0.05 mg/ml; 3024 cpm/μg) was divided into several aliquots and incubated at 37°C for various lengths of time. At specified times each incubation mixture was centrifuged at 3,000 × *g* for 5 min in an Eppendorf centrifuge, and the supernatant was discarded. The pellet was carefully washed with 3,000 μl of phosphate-buffered saline (PBS) and centrifuged, and the supernatant was discarded. This washing procedure was performed three times. The homogenized pellet was then immersed in 5 ml of counting cocktail, and the radioactivity was counted in a Wallac liquid scintillation counter.

**Animal experiments.** Fifty-four male BALB/c mice (age ~3 weeks) were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.); they weighed 12 to 15 g prior to inoculation and/or treatment. Thirty mice were inoculated intravenously (via the tail vein) with $10^5$ to $10^8$ virus particles in saline. DNP-poly[A] was administered intraperitoneally in saline at various doses of 1 to 100 mg/kg of body weight. Blood was periodically drawn from the tail and was used in the product-enhanced RT (PERT) assay described below.

**Sample pretreatment for the PERT assay.** The PERT assay method of Pyra et al. (10) was used, with some modifications. Briefly, 5 μl of whole blood was collected into 5 μl of EDTA (13.8% [wt/vol]) plus 45 μl of saline and frozen at −20°C overnight. The hemolyzed blood was thawed and centrifuged at 4,000 × *g* for 30 min at 4°C. The supernatant was removed and again centrifuged at 100,000 × *g* for 30 min. The pellet was homogenized in 20 μl of buffer A containing 50 mM KCl, 50% glycerol, 25 mM Tris HCl (pH 7.5), 0.25 mM EDTA, 0.5% Triton X-100, and 5 mM DTT. This suspension was kept at 0°C for 15 min, and a 3-μl aliquot was removed and used in the PERT assay.

**PERT assay and product amplification by PCR.** cDNA was synthesized and amplified by the PERT assay and PCR, respectively, by using genomic MS2 RNA as described previously (10).

## RESULTS

**Synthesis of DNP-poly[A].** The inhibitor was synthesized from poly[A] supplied by Sigma (average molecular weight by light scattering, $10^5$). After derivatization and purification the product mixture was dialyzed against water via a membrane with a cutoff molecular weight of 12,000. From the spectrophotometrically determined DNP-to-adenine ratio, we estimate the average molecular weight of DNP-poly[A] to be $1.1 \times 10^5$, with a lower limit of $1.2 \times 10^4$.

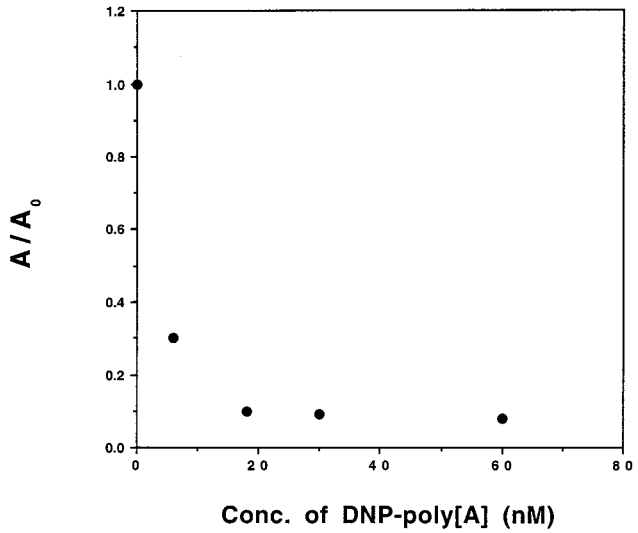


FIG. 1. Inhibition of HIV-1 RT in solution by DNP-poly[A] at 25°C. $A$ and $A_0$ represent the catalytic activities of the RT in the presence and absence of DNP-poly[A], respectively. The total concentration of RT is 25 nM. The concentration of DNP-poly[A] indicated by the abscissa corresponds to the total concentration of inhibitor (bound and free). The DNP-poly[A] used in this experiment has an average molecular weight of $1.1 \times 10^5$, and the DNP/adenine molar ratio is 1:1.2.

**Inhibition of RT in solution by DNP-poly[A].** Using poly[A]-oligo[dT]$_{12}$ as the template, we found that DNP-poly[A] is a potent inhibitor of RT in solution. The dependence of the observed ratio of the catalytic activity of inhibited enzyme ($A$) to that of the uninhibited enzyme ($A_0$) on the total inhibitor concentration is illustrated in Fig. 1; the apparent $IC_{50}$ was ~2 nM. It is noteworthy from the data that 5 nM DNP-poly[A] can cause 70% inhibition of the activity of 25 nM RT, which implies that each long DNP-poly[A] molecule can bind and inhibit several RT molecules.

The actual dissociation constants ($K_d$s) for the inhibitor bound to RT should be much lower than the apparent $IC_{50}$ given in Fig. 1, because the abscissa of Fig. 1 gives the total inhibitor concentration, which is sometimes much higher than the concentration of the free inhibitor. It is very difficult to treat the data in Fig. 1 quantitatively, because for [RT] > [inhibitor], the RTs that bound successively to the same inhibitor probably have different $K_d$ values. For an [RT] of <1 nM, catalytic activity is too low to be measured reliably.

In order to overcome this difficulty, we attached DNP-poly[A] covalently to oxirane acrylic or Sepharose beads and equilibrated the affinity beads that were obtained with different concentrations of RT or Mo-MuLV. The $K_d$ of the first binding sites on each anchored inhibitor molecule was calculated by measuring the observed removal of RT activity from the liquid phase.

**Inhibition of RT inside Mo-MuLV by DNP-poly[A].** Previous experimental studies showed that DNP-poly[A] can protect susceptible lymphocytes from HIV-1 in cell cultures with an $EC_{50}$ of 0.2 μg/ml or 2 nM (7). The observed protection could be due to the transport of DNP-poly[A] into HIV and inhibition of the RT inside, the transport of the inhibitor into lymphocytes and the prevention of reverse transcription after infection, or the prevention of attachment of HIV to lymphocytes by DNP-poly[A]. To explore these possibilities, the rates of transport of DNP-poly[A] into Mo-MuLV or lymphocytes were measured.

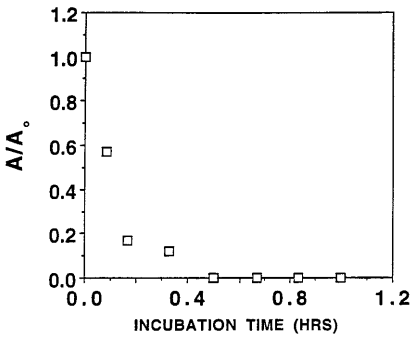


FIG. 2. Transport of DNP-poly[A] into intact Mo-MuLV and inhibition of the RT inside the cell at 0°C.

Intact Mo-MuLV particles in aqueous buffer exhibit no RT activity toward substrate and primer-template in external medium. However, the activity can be measured by first releasing the endogenous RT with low concentrations of Triton X-100 (0.1 to 0.5%). In the present work, the transport of DNP-poly[A] into Mo-MuLV was monitored by mixing the virus in the absence of detergent, taking aliquots of the mixture at different time intervals after mixing, centrifuging each aliquot, and rinsing each sediment to remove external DNP-poly[A]. The washed virus was subsequently treated with Triton X-100 to release the RT and was finally incubated in the assay solution for determination of RT activity. The rate of transport of DNP-poly[A] into Mo-MuLV was measured by monitoring the decrease in RT activity with the time of incubation with inhibitor. At 37°C we found that this decrease was too fast to follow. At 0°C, however, the decrease in RT activity with time was slow enough to be monitored in this way. The result of a typical experiment is illustrated in Fig. 2, in which the observed ratio of RT activity after incubation ($A$) and before incubation ($A_0$) is shown as a function of incubation time with the inhibitor.

The first point at zero incubation time in Fig. 2 was obtained by adding the Mo-MuLV suspension to DNP-poly[A] solution, within 5 s putting the mixture into an Airfuge centrifuge tube containing a layer of glycerol at the bottom, and immediately centrifuging the virus through the glycerol layer to remove the bulk of adhering liquid. It was found repeatedly that the RT activity of the washed pellet from the zero-time sample ($A$) was essentially the same as that from the virus incubated with buffer in the absence of the inhibitor ($A_0$). These control experiments indicate that the removal of externally adsorbed inhibitor by this procedure was sufficiently complete. The curve in Fig. 2 indicates that at 0°C the decrease in endogenous RT activity is slow enough to be monitored by this procedure.

The DNP-poly[A] used in the experiment whose results are presented in Fig. 2 has an average molecular weight of $1.1 \times 10^5$, and the adenine/DNP molar ratio was 1.5. Additional control experiments with [$^3$H]poly[A] with a similar molecular weight show that this underivatized polymer is not transported into Mo-MuLV under the same conditions, because the level of radioactivity of the washed virus was only three times the background counting rate. Since this low radioactivity level was reached within 5 min (2), we conclude that it was due to external adsorption of [$^3$H]poly[A] by Mo-MuLV.

It is also worth pointing out that the concentration of DNP-poly[A] in the incubation mixture (1.3 μM) is 1,000 times higher than the apparent $IC_{50}$ of the inhibitor for RT in solution or the $EC_{50}$ for HIV in microculture plates. This high inhibitor concentration was chosen deliberately to eliminate slow binding as a possible rate-limiting step in the inhibition process.

In an attempt to measure the backward diffusion of DNP-poly[A] out of Mo-MuLV, we found that after the virus has been incubated with the inhibitor at 1.3 μM for longer than 30 min at 0°C, no detectable RT activity can be recovered, even by reincubation of the inhibited Mo-MuLV in fresh medium for 24 h at 0°C. Under the same conditions the RT activity of uninhibited virus decreased less than 10%.

When considered together, the observations presented above indicate that the rate-limiting step in the observed time-dependent inhibition of endogenous RT in Mo-MuLV is membrane transport, not a slow rate of absorption or binding.

**Transport of DNP-poly[A] into mammalian cells.** The transport of DNP-[$^{14}$C]poly[A], [$^{14}$C]poly[A], and [$^3$H]poly[A] into human lymphocytes or leukocytes was measured at 37°C. The data are summarized in Fig. 3. These results indicate that the hydrophobic effect of a large number of DNP groups can compensate for the hydrophilic effect of the charged phosphate groups in each long DNP-poly[A] molecule sufficiently to allow it to permeate the membrane. By contrast, the hydrophilic poly[A] of equal chain length was not transported at all under the same conditions. Natural oligonucleotides often have a poor ability to permeate the membranes. The present observations suggest a convenient derivatization method for converting them to a membrane-permeable form.

**Binding of Mo-MuLV to DNP-poly[A] attached to solid support.** To exploit its ability to permeate the membrane, we linked each DNP-poly[A] molecule at one end covalently to oxirane acrylic resin and found that the resulting affinity resin can bind Mo-MuLV selectively from its suspension in human plasma. Presumably, the covalently anchored DNP-poly[A] (approximately 0.06 μm in length) can penetrate the retrovirus (~0.1 μm) and catch it by binding to the endogenous RT, as illustrated in the inset of Fig. 4. Equilibration studies indicate that this DNP-poly[A] affinity resin can selectively remove Mo-MuLV suspended in PBS or reconstituted human plasma, whereas the underivatized oxirane acrylic resin exhibited no binding at all under the same conditions. In order to determine the free energy of binding, a suspension of the affinity resin was equilibrated with different concentrations of the retrovirus. At each equilibrium state, the concentration of free virus ($C$), the number of moles of virus bound per milligram of resin ($n$), and the virus-binding capacity ($N$) are related by the Scatchard equation: $n/C = (N - n)\ (1/K_d)$, where $K_d$ is the dissociation constant of a virus bound at a specific binding site. The corresponding linear plot of the experimental data in Fig. 4 gives

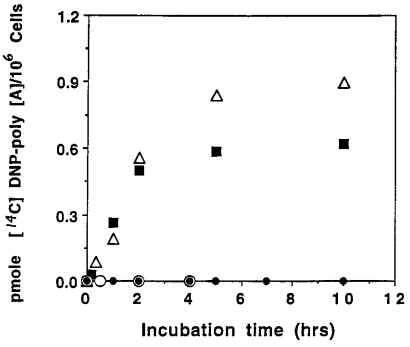


FIG. 3. Measurement of the transport of labeled poly[A] derivatives into human cells at 37°C. Symbols: ■, [$^{14}$C]DNP-poly[A] into lymphocytes; △, [$^{14}$C] DNP-poly[A] into leukocytes; ○, [$^{14}$C]poly[A] into lymphocytes; ●, [$^3$H]poly[A] into lymphocytes.

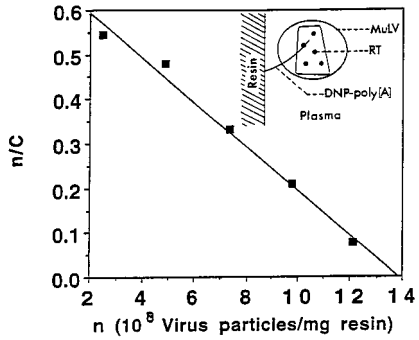


FIG. 4. Equilibrium binding of Mo-MuLV in human plasma to acrylamide beads with covalently attached DNP-poly[A]. (Inset) Assumed mode of binding.

a $K_d$ value of $3.4 \times 10^{-12}$ M and an $N$ value of $1.4 \times 10^9$ virus particles per mg. The linearity of the Scatchard plot indicates that in these very dilute mixtures, Mo-MuLV particles were bound to only one type of very tightly binding site, with a $K_d$ in the subnanomolar range. This $K_d$ is compatible with the $IC_{50}$ observed for the binding of DNP-poly[A] to very dilute Mo-MuLV RT in solution (7), but many orders of magnitude lower than the half-saturation concentration expected from any nonspecific binding or adsorption.

**Stability of DNP-poly[A].** Unlike the natural polynucleotides, DNP-poly[A] is completely stable in solutions containing RNases A, B, S, $T_1$, $T_2$, and H as well as phosphodiesterases 1 and 2 (11). DNP-poly[A] was also found to be quite stable in acidic medium, in that it was able to retain its inhibition potency when it was incubated for several hours in 0.01 M HCl or water at 37°C (15).

**Animal study.** The observed antiretroviral activity of DNP-poly[A] in cell cultures as well as its permeation through the membrane and stability in the presence of RNase or hydrochloric acid suggest that this RT inhibitor could be used as an effective antiretroviral agent. As a test case, we treated Mo-MuLV-infected mice with doses of DNP-poly[A] in Dulbecco's PBS solution by intraperitoneal injection and followed their blood chemistry and spleen histology in comparison with those for the control mice (infected and untreated, uninfected and untreated, and uninfected and treated).

The hematocrit measurements for 11 mice in the four groups were obtained (data not shown). The readings gave some indication of the progress of infection during the early stages of the disease, although the total number of mice ($n = 11$) was too small to be able to provide conclusive results. At about 3 months after infection, the infected and control mice were sacrificed. Mice that were treated immediately after viral infection with a dosage of 10 mg/kg of body weight three times at 3-day intervals did not manifest other leukemic symptoms such as large splenic cells or the large spleens which were observed in infected and untreated mice. Another group of infected mice that were treated 4 months after infection had lower spleen weights and fewer abnormal cells than the infected but untreated mice. The data are summarized in Table 1.

The spleens were preserved in formalin and were subsequently stained with hematoxylin and eosin for microscopic examination. Figure 5 indicates that under low magnification, the spleen from a healthy mouse (Fig. 5A1) and the spleen from an uninfected but treated mouse (Fig. 5D1) have similar spleen architectures, but the greatly enlarged spleen from an infected but untreated mouse (Fig. 5B1) has lost the normal spleen architecture completely. The spleen of an infected mouse which was treated at a later stage of infection (Fig. 5C1) has some regions of hypernodularity but still maintains some regions of red and white pulp. Under high magnification, the spleens from a healthy mouse (Fig. 5A2), an infected and treated mouse (Fig. 5C2), and an uninfected but treated mouse (Fig. 5D2) are quite similar. However, large abnormal cells can be seen to proliferate in the spleen from an infected but untreated mouse (Fig. 5B2) to such an extent that normal-size lymphocytes are very rare in the whole field.

The effect of continued treatment of Mo-MuLV-infected mice with DNP-poly[A] was also determined by monitoring the retroviral load in blood by the PERT assay as described in Materials and Methods. The results are summarized in Fig. 6. About 4 months after infection, blood samples from infected but untreated mice all showed strong ethidium bromide-stained bands in the electrophoresis gel (Fig. 6A). These bands correspond to about 100 bp according to the marker ladder which was run alongside it, and the intensities of the bands are proportional to the concentration of RT in the sample. The different intensities of the ~100-bp band in lanes 2 to 7 of Fig. 6A reflect different steady-state concentrations of RT in blood. Our positive controls with pure virus (lane 8 in all panels of Fig. 6) gave strong bands, and negative controls with normal, uninfected blood showed no band (not shown in Fig. 6) in the electrophoresis gel at the 100-bp position. Figure 6B, C, and D indicates that these bands progressively decreased in intensity after the treatment with DNP poly[A] was introduced and eventually disappeared. Indeed, viral RT was still not detectable 2 weeks after the treatment was terminated (data not shown), when all the mice were sacrificed for autopsy.

As toxicity controls, uninfected mice were also administered 1 to 100 mg of DNP-poly[A] per kg by intraperitoneal injection. No visible ill effect was observed, and the hematocrit, leukocyte, and erythrocyte levels for these mice remained within normal limits.

## DISCUSSION

The use of macromolecular inhibitors as therapeutic agents is often hampered by their failure to cross biological transport barriers. We have now found that derivatives of poly[A] with DNP groups attached to the 2′-*O* positions via an ether linkage can be transported freely and rapidly into viruses and cells, whereas poly[A] itself cannot. This poly[A] derivative was pre-

TABLE 1. Spleen weight and percentage of abnormal cells at autopsy

| Class of mice | No. of mice | Average wt (g) | % Abnormal cells[a] |
|---|---|---|---|
| Normal | 4 | 0.10 ± 0.00 | 0.0 ± 0.0 |
| Treated immediately after virus inoculation[b] | 4 | 0.12 ± 0.1 | 7.0 ± 2.0 |
| Treated 4 months after virus inoculation[c] | 6 | 0.20 ± 0.1 | 37.0 ± 7.0 |
| Inoculated with virus but not treated for 4 months[d] | 6 | 0.42 ± 0.3 | 59 ± 19 |

[a] The cells were counted from four different areas of each spleen.

[b] The mice were inoculated intravenously with $10^4$ to $10^5$ virus particles and were then treated intraperitoneally with one 10-mg/kg dose of DNP-poly[A] and sacrificed 3 months after infection for autopsy to see the effect of inhibitor on free retrovirus in blood.

[c] The mice were inoculated intravenously with $10^9$ virus particles. Four months after infection, they were treated intraperitoneally with 100 mg of DNP-poly[A] per kg three times per week for 3 weeks. These mice were sacrificed 5 months after infection for autopsy to see the effect of inhibitor on virus-infected cells.

[d] The mice were infected intravenously with $10^9$ virus particles, never treated with any drug, and sacrificed either 3 or 5 months after infection.

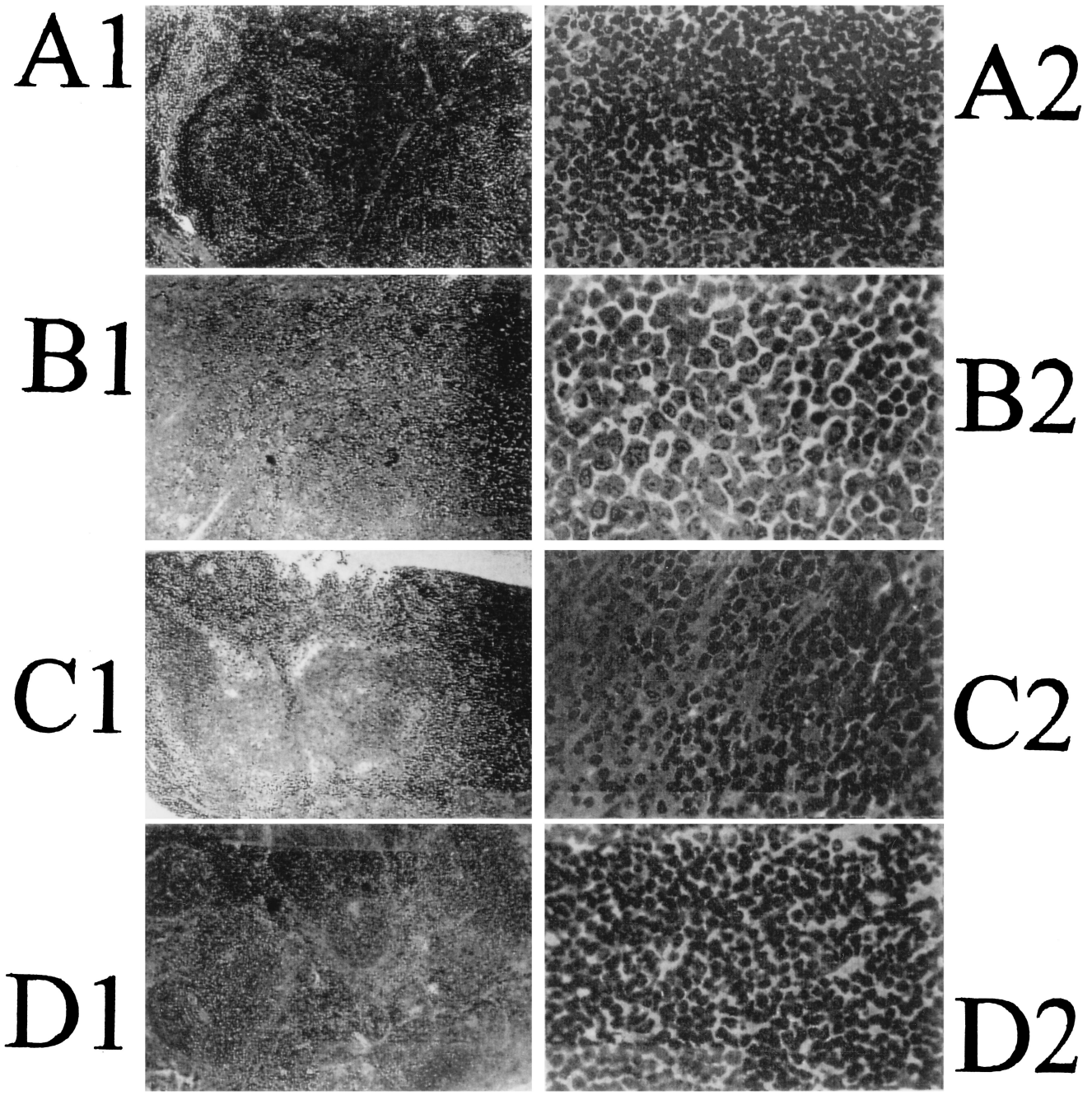


FIG. 5. Spleens from four sets of mice used for the RT-PCR studies whose results are presented in Fig. 6. All the spleens were preserved in formalin and stained with hematoxylin and eosin. Magnifications, ×160 for panels A1, B1, C1, and D1 and ×1,600 for panels A2, B2, C2, and D2. The four spleen samples were from normal mouse spleen (A); infected ($10^9$ virus particles), untreated mouse (B); a mouse infected with $10^9$ virus particles, after which we waited for leukemia to develop and treated the mouse intraperitoneally with a 10-mg/kg dose of DNP-poly[A] three times per week for 3 weeks (C); and a mouse infected with $10^8$ virus particles but treated intraperitoneally immediately with a 10-mg/kg dose of DNP-poly[A] three times per week for 1 week (D).

viously shown to protect susceptible lymphocytes in the presence of HIV (7). The new transport data suggest a protection mechanism involving the permeation of the inhibitor through viral and cellular membranes and then inhibition of the RT inside the cell.

Previous experiments have indicated that DNP-poly[A] can protect susceptible lymphocytes from HIV-1 in cell cultures, with an $EC_{50}$ of ~2 nM (7). This protection is most likely due to inhibition of RT, because the titration of 25 nM HIV-1 RT with nanomolar concentrations of DNP-poly[A] also gave an $IC_{50}$ of about 2 nM (Fig. 1). If the observed protection of lymphocytes was due to the antifusion activity of the compound, it would be a rare coincidence for it to exhibit nearly the same $EC_{50}$. Furthermore, inhibition of internal RT requires transport of the inhibitor across the viral envelope or cell membrane, or both, whereas antifusion activity by oligonucleotides does not. We found that the presumably membrane-soluble amphipathic DNP-poly[A] can be spontaneously

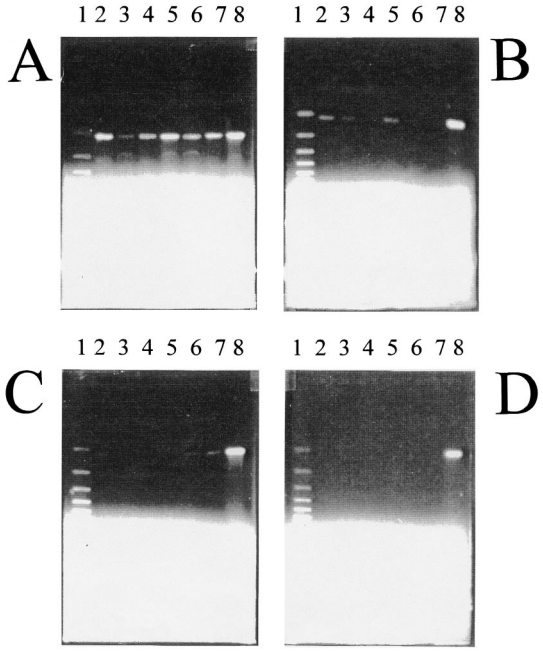


FIG. 6. Effect of DNP-poly[A] on viremia of Mo-MuLV-infected mice. Each of six mice was infected intravenously with $10^8$ virus particles. Four months later, when leukemia symptoms were fully developed, the mice were treated intraperitoneally with DNP-poly[A] (100 mg/kg at 3-day intervals). In all panels the ethidium bromide-stained bands in lanes 2 to 7 were from infected and treated mice. Lanes 1 and 8, PCR marker and pure virus (positive control), respectively. (A) Prior to treatment; (B) 1 week after treatment; (C) 2 weeks after treatment; (D) 3 weeks after treatment.

transported across the viral envelope (Fig. 2) and cell membrane (Fig. 3), and it can also protect lymphocytes against HIV in cell culture and inhibit RTs in solution. On the other hand, the presumably membrane-insoluble poly[A] cannot be spontaneously transported across cell membranes under the same conditions (Fig. 3). We found that it neither protects lymphocytes from HIV in cell cultures nor inhibits RTs in solution (7). For all these reasons it seems very unlikely that DNP-poly[A] functions as an antifusion agent.

In Mo-MuLV-infected but untreated mice, more abnormal giant cells were observed within the spleen, while the erythrocyte population decreased, eventually giving rise to anemia. The mice that were treated at 4 months after infection had presumably already developed the disease to such an extent that although the viral load in blood was markedly decreased, the spleen was still infiltrated by cells that looked abnormal. The statistical tests that were performed indicated that DNP-poly[A] is significantly more effective when it is administered immediately following infection, although the therapy was still somewhat beneficial when it was started 4 months after infection.

Since the mouse has a very low blood volume (5.5 to 8.0% of its body weight of ~15 g), multiple tests can be performed only on a microscale. In the present work, RT-PCR was used to monitor viral load because it requires only 5 μl of whole blood per assay. When Mo-MuLV replicates, resulting in more virus particles, the amount of RT increases. Consequently, the cDNA produced by reverse transcription also increases. Conversely, a decrease in intensity of the 100-bp band on an ethidium bromide-stained gel demonstrates a decrease in cDNA and therefore RT. Our results indicate that multiple treatments with DNP-poly[A] can decrease the viral load to an undetectable level.

The following additional properties suggest that DNP-poly[A] should be further studied as a potential antiretroviral agent.

**Specificity.** Previous studies indicate that DNP-poly[A] is a potent inhibitor of RTs (7) and a moderate inhibitor of RNases (11), but it does not inhibit DNP-dependent DNA polymerase, DNA-dependent RNA polymerase, glucose-6-phosphate dehydrogenase, pyruvate kinase, hexokinase, adenylate kinase, etc. This functional specificity is also demonstrated by the experiment whose results are illustrated in Fig. 4, in which the $K_d$ for equilibrium binding of MuLV in human plasma to acrylamide beads with covalently attached DNP-poly[A] was determined. When a similar experiment was conducted with Mo-MuLV in phosphate buffer, a $K_d$ of the same order of magnitude was obtained, which implies that none of the protein in human plasma can compete with Mo-MuLV RT for binding to the inhibitors.

On the other hand, DNP-poly[A] does not show species specificity. It was found to inhibit all the wild-type and mutant RTs that we tested, and hence may be a mutation-insensitive inhibitor of HIV.

**Bioavailability.** In addition to the transport data in Fig. 2 and 3, the bioavailability of DNP-poly[A] is also supported by the observed gradual disappearance of leukemic symptoms following the intraperitoneal administration of DNP-poly[A]. In order to stop viral propagation, the inhibitor must permeate many membrane barriers to reach all affected organs of the leukemic mouse.

**Stability.** DNP-poly[A] is resistant to all the nucleases that we tested and is quite stable at 37°C in 0.01 M HCl for several hours. Pharmacokinetic studies will be conducted to map its biodegradation path.

**Low level of toxicity.** In cell cultures no toxic effect was detected in T4 lymphocytes when the DNP-poly[A] concentration was varied from 0.05 to 200 mg/liter (7). In healthy mice treated with DNP-poly[A] as a toxicity control, no apparent toxic symptoms were observed when the intraperitoneal dosage was varied from 1 to 100 mg/kg three times per week over a 5-month period.

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