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Polymeric Drugs: Direct Compared with Indirect Inhibition of Leukemia Virus Replication in Mice

VITOLIS E. VENGRIS, PAULA M. PITHA, AND LYLE L. SENSENBRENNER

The Johns Hopkins Oncology Center, Baltimore, Maryland 21205

JOSEF PITHA

National Institute on Aging, Gerontology Research Center, Baltimore, Maryland 21224

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SUMMARY

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Inhibition of viral replication by macromolecular drugs may occur by direct interference or by aid of the body's defenses. Poly-9-vinyladenine, an electroneutral and nontoxic analogue of polyadenylic acid, seems to interfere directly with replication of Friend leukemia virus in mice. This polymer did not change the host immune response (cellular or humoral), induce interferon or affect the induction of interferon by other agents, inhibit the replication of a lytic (Semliki Forest) virus, or inhibit the growth of transplanted L1210 tumor (presumably of nonviral origin). Antiviral activity was observed only when the polymer was administered daily, but the drug did not have to be applied at the time or via the route of infection. Statolon, a polynucleotide-containing polyanionic macromolecule, inhibited the same virus by multifaceted action, as this macromolecule both enhanced the immune response and induced interferon. The antiviral activity of statolon could be observed even after a single dose of this macromolecule.

INTRODUCTION

The antiviral action of macromolecules in an organism can be the result of at least three mechanisms: (a) interference with an essential step in the virus replication cycle, (b) induction of interferon, and (c) activation of the immunosystem (1-3). The last two mechanisms have been well documented (2, 3); the first mechanism has been suggested by comparing the effectiveness of macromolecular inhibitors on viral infection *in vitro* and *in vivo* (4, 5). In this work the effects of two macromolecules on viral infection in mice were studied and compared. The first was poly(vA),[1] an electroneutral, high molecular weight analogue of polyadenylic acid that is stable to chemical and enzymatic hydrolysis. It was shown previously that this polymer selectively inhibited replication of exogenous murine leukemia virus

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[1] The abbreviations used are: poly(vA), poly-9-vinyladenine; FLV, Friend leukemia virus; SFFV, spleen focus-forming virus; LLV, lymphatic leukemia virus; SED_{50}, spleen enlarging dose (50%); poly(I·C), complex of polyinosinate with polycytidylate; SRBC, sheep red blood cells.

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in primary mouse embryo cells (6) but was without any effect on the induction of endogenous murine leukemia virus by 5-iodo-2'-deoxyuridine (7) and did not affect the replication of lytic RNA viruses (6). The second compound studied, statolon, which was used for comparison, is a fermentation product containing a polynucleotide (8). It was shown that this polymer protects animals against oncogenesis induced by RNA (9) and DNA (10) oncogenic viruses.

MATERIALS AND METHODS

Compounds. Poly(vA) was prepared as described previously (11), and statolon was a gift from Dr. W. J. Kleinschmidt, lot 354-1080B-220.

Mice and cells. Inbred strains of DBA/2, C57BL/6, $CD2F_1$ (Balb/c × DBA/2), and $B6D2F_1$ (DBA/2 × C57BL/6) females, 9–11 weeks of age, were obtained from Cumberland View Farms (Cumberland, Tenn.), and a partly inbred strain of Swiss female mice, 10–12 weeks old, was obtained from Buckberg Laboratory Animals (Tomkins Cover, N. Y.). The SC-1 mouse cell line, mouse L cells, and XC cells were grown in Eagle's minimal essential medium with 10% heated fetal bovine serum.

Viruses and viral assays. FLV was obtained from Dr. W. P. Rowe and propagated by intravenous inoculation into DBA/2 mice. The SED_{50} was determined 7 days after inoculation. SFFV was assayed in DBA/2 or Swiss mice by the spleen focus assay as described previously (12) 7 days after inoculation. The amount of LLV in mouse plasma was assayed by titration on an SC-1 mouse cell by the XC test (13). Semliki Forest virus was obtained from Dr. S. Baron. Vesicular stomatitis virus, New Jersey serotype, was propagated and titrated on mouse L cells (14).

Effects of poly(vA) and statolon on FLV infection. Five DBA/2 or Swiss mice were inoculated with either 10 or 400 LD_{50} of FLV and at the times indicated were injected with poly(vA) or statolon. On day 7 after inoculation, the mice were killed and the LLV in the plasma was assayed by the XC test. The spleens of the animals were weighed and the number of foci counted.

Effects of statolon and poly(vA) on growth of L1210 tumor. L1210 transplantable tumor cells were maintained by intraperitoneal passage of ascitic fluid in $B6D2F_1$ mice. To assay the effect of polymers, mice treated with 1×10^6 L1210 cells were injected with poly(vA) and statolon, and mean survival time was used as the criterion of effectiveness.

Effects of statolon and poly(vA) on Semliki Forest virus infection in mice. Virus at a dose of 10 LD_{50} was given intraperitoneally to Swiss mice (10 mice/group); the polymer treatment was started immediately after virus inoculation, and repeated daily. Survival time was used as the criterion of antiviral effect.

Interferon induction and assay. The ability of poly(vA) to induce interferon in mice was tested in groups of 15 mice (DBA/2), which were given a single dose of poly(vA) (1 or 4 mg/mouse, intravenously or intraperitoneally). Three mice from each group were then bled 2, 6, 12, 24, and 48 hr after induction. The sera obtained at each time interval were pooled and assayed for interferon activity in mouse L cells (15). To test the effect of poly(vA) on interferon induction by poly(I·C), DBA/2 mice were given either a single dose of poly(vA) intraperitoneally (1 mg/mouse) or the same dose intravenously daily for 6 days before induction by poly(I·C) (50 μg/mouse, intravenously), and interferon in serum was assayed.

Toxicity studies. The toxicity of poly(vA) was determined in DBA/2 mice. Mice (15 mice/group) were given 5.0, 250, or 1000 mg/kg doses intraperitoneally and observed for clinical symptoms and body weight changes. The criteria used for toxicity were the observed clinical symptoms, changes in body weight, survival time, total leukocyte counts, and blood differential counts. Furthermore, the tissues were examined macroscopically and microscopically for pathological changes, and splenomegaly and hepatomegaly were measured.

Hematopoietic spleen colony assay. $CD2F_1$ mice (10 mice/group) were irradi-

ated with 950 rads of γ-rays, using a dual ^{137}Cs source irradiator at a dose rate of 136 rads/min. After irradiation, mice were given 3×10^6 viable nucleated syngeneic spleen cells intravenously, and hematopoietic spleen colonies were counted on day 7 (16).

Antibody, graft-vs.-host disease, delayed hypersensitivity, and skin graft rejection assays. Irradiated mice (10 mice/group, 950 rads) were given 16×10^6 viable nucleated syngeneic spleen cells intravenously and immunized with 1 ml of a 1% suspension of SRBC injected intraperitoneally. The anti-SRBC hemagglutinin titers were determined 7 days after immunization.

Graft-vs.-host disease was initiated by the intravenous injection of 10×10^6 viable spleen or bone marrow cells of C57BL/6 mice into lethally irradiated (950 rads) $B6D2F_1$ mice (10 mice/group). Body weight changes and survival time were used as criteria for the disease. Histological sections of tissues were examined to ascertain the cause of death.

The footpad-swelling method was used to detect humorally mediated immunity to SRBC in the mouse (17). DBA/2 mice (five mice per group) were immunized by intravenous injection of 1×10^5 SRBC, and hypersensitivity was measured (18) by the increase in footpad thickness after injection of an eliciting dose of 1×10^8 SRBC (in 50 μl) on day 4 by a dual-caliper gauge A02T (Schnelltaster, Kropkin GmGH, Schluchtern, Hessen, Germany). Averages of five independent measurements are given. To check the effect on cell-mediated immunity, the skin allograft rejection test was used. A circular full-thickness skin graft (1 cm in diameter) of $B6D2F_1$ mice was placed on a $CD2F_1$ recipient mouse. Survival time was defined as the day of complete graft rejection.

RESULTS

Toxicity. Poly(vA) did not show any toxic effect in mice when the compound was given in a single dose in amounts up to 1000 mg/kg; higher doses could not be tested because of the limited solubility of poly(vA). Mice were found to tolerate these levels without exhibiting clinical signs, hematological changes, or statistically significant changes in spleen or liver size. Macroscopic and microscopic pathological examination of spleen, liver, thymus, lung, kidney, brain, and gastrointestinal tract did not reveal any lesions that could be attributed to toxicity of poly(vA). The toxicity of statolon was studied previously (19); the LD_{50} for mice is about 1500 mg/kg.

Effect on FLV infection. A single injection of poly(vA) (0.1, 1, 4, or 10 mg/mouse), given intraperitoneally either simultaneously or 24 hr after infection with FLV (10 SED_{50}), did not significantly affect FLV infection as measured by the number of spleen foci on day 7 after infection. Multiple injections of poly(vA) (1 mg/mouse, intraperitoneally) given 0, 72, and 120 hr after infection reduced both the number of spleen foci and the amount of helper virus present in the serum. Maximum inhibition was achieved when poly(vA) was given daily (Table 1). Statolon, on the other hand, in a single intravenous injection administered before or after FLV, significantly reduced the number of spleen foci (Table 2).

FLV-induced reticulum cell leukemia is associated with great enlargement of the spleen. A single dose of poly(vA) did not reduce splenomegaly. Daily doses of poly(vA) caused only a slight reduction in spleen size under conditions in which the polymer significantly reduced both the number of spleen foci and the amount of helper virus in mouse plasma. FLV-induced splenomegaly, however, was significantly reduced by a single dose of statolon (Table 3).

Effect on growth of transplantable tumor and infection with Semliki Forest virus. A single dose of statolon (4 mg/mouse) given intravenously, or daily intravenous inoculations of poly(vA) (1 mg/mouse), did not protect mice against death following the injection of L1210 cells (1×10^6) or prolong survival. The mean survival time of the control group (10 mice/group) was 8.2 ± 0.1 days, whereas the mean survival times in the poly(vA)-treated and statolon-treated groups were

TABLE 1

Effect of poly(vA) on number of FLV-induced foci in spleen and its titer in serum

Mice (five per group) were inoculated intravenously with 10 SED_{50} units of FLV, and 1 hr later they were inoculated either intravenously or intraperitoneally with poly(vA) (1 mg/mouse). The poly(vA) treatment was repeated daily, and the mice were killed 7 days after infection. The titers of LLV and SFFV were estimated as described in MATERIALS AND METHODS.

Treatment	SFFV		LLV	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
FLV	48.1 ± 10.1	25.6 ± 2.1	1.3×10^6	1.7×10^6
FLV, poly(vA) i.v.	16.6 ± 4.9	8.2 ± 4.3	8.6×10^5	4.0×10^5
FLV, poly (vA) i.p.		13.8 ± 1.24		1.9×10^5

7.9 ± 0.2 and 8.0 ± 0.2 days, respectively.

Daily administration of poly(vA) also did not protect mice against death following Semliki Forest virus (10 LD_{50}) infection or prolong survival time. The mean survival time of the control group was 8.0 ± 0.3 days, whereas the mean survival time in the poly(vA)-treated group was 8.9 ± 0.4 days. Statolon was reported previously to be effective against a similar viral infection (9).

Effect on hematopoietic spleen colony formation. Poly(vA) or statolon did not inhibit normal hematopoietic spleen colony formation in mice regardless of the route of infection and concentration. The injection of 3×10^6 spleen cells into untreated, irradiated, syngeneic mice gave 22.7 ± 1.4 hematopoietic spleen colonies on day 7 after injection. In mice given either a single dose (1 mg/mouse injected on the same day or 1 day after the spleen cells) or repeated doses of poly(vA) (1 mg/mouse for 6 days), the number of colonies on the seventh day was 23.3 ± 1.4. Injection of recipient mice with a single dose of statolon (4 mg/mouse on day 0 or day 1) was also without any effect (24.2 ± 1.6 colonies).

Effect on host defense mechanisms. Administration of statolon, either simultaneously or 1 or 3 days after the immunizing dose of SRBC, increased the primary antibody response in mice by 30%, 23%, or 23%, respectively (Table 4). Poly(vA), on the other hand, when given at different times in relation to antigen administration, had insignificant effects; all changes were done to the combined standard errors of experiments and were under 8% (Table 4).

TABLE 2

Effect of statolon on spleen foci induced by FLV

Mice (five per group) were injected intravenously with 10 SED_{50} units of FLV, and then with statolon (4 mg/mouse, intravenously) at the times indicated. The titer of SFFV was estimated on the seventh day after infection as described in MATERIALS AND METHODS.

Statolon	Day of administration	No. of SFFV spleen foci
−		50.0 ± 4.7
+	−1	1.3 ± 0.8
+	0	3.5 ± 0.9
+	1	5.7 ± 4.7
+	2	9.6 ± 2.5

Lethally irradiated mice given allogeneic bone marrow or spleen cells develop signs and symptoms of graft-vs.-host disease (20). Poly(vA) did not protect mice significantly against graft-vs.-host disease, and only slight prolongation of survival was usually observed.

The footpad swelling reaction to SRBC was used to monitor specific humoral (3 hr, immediate hypersensitivity response) and cellular (24 and 48 hr, delayed hypersensitivity response) immunity (17). Statolon did not alter the delayed hypersensitivity response but significantly increased footpad swelling at 3 hr, indicating a specific humoral response (Table 4). Poly(vA) neither altered swelling at 3 hr nor prevented delayed hypersensitivity when given prior to or simultaneously with the immunizing dose or eliciting dose, or when administered daily. Control footpads showed no swelling and no cellular infiltration response, as determined histologically.

Single doses of statolon (4 mg/mouse,

TABLE 3

Effects of poly(vA) and statolon on FLV infection in mice

The results below were obtained 10 days after administration of virus (FLV, 400 SED_{50}) or plasma. Similar results were found on day 7. A single dose of statolon (4 mg/mouse, intravenously) was injected 1 hr after virus, or daily doses of poly(vA) (1 mg/mouse, intravenously) were administered. Five mice each from the control and drug-treated groups were killed on day 7 (data not shown), and the other five mice from each group, on day 10. Their spleens were weighed, and diluted plasma [1:100 for the statolon groups and 1:1000 for the poly(vA) groups] was injected intravenously into recipient mice for SFFV, LLV, and splenomegaly determinations.

Treatment	Spleen weight after FLV	Plasma recipients		
		SFFV	LLV	Spleen weight
	mg			*mg*
Statolon	133 ± 10	0	0	138 ± 12
FLV, statolon	318 ± 41	0	3.0×10^3	248 ± 16
FLV	1260 ± 65	–[a]	3.9×10^6	803 ± 68
Poly(vA)	133 ± 4	0	0	208 ± 8
FLV, poly(vA)	1161 ± 105	4.0 ± 2	9.0×10^4	204 ± 3
FLV	1551 ± 208	19.8 ± 8	6.7×10^5	216 ± 16

[a] Too numerous to count.

TABLE 4

Effects of poly(vA) and statolon on antibody production and delayed hypersensitivity in response to SRBC

Statolon was given in a single dose of 4 mg/mouse, intravenously. Poly(vA) was given in a single dose of 1 mg/mouse, intraperitoneally; similar results were obtained when it was given daily (1 mg/mouse, intravenously).

Treatment	Day of treatment	Antibody titer		Footpad swelling after injection with eliciting dose of SRBC		
		Expt. 1	Expt. 2	3 hr	24 hr	48 hr
				mm	*mm*	*mm*
Control		5.6 ± 0.3	6.5 ± 0.4	0.22 ± 0.14	0.80 ± 0.11	0.37 ± 0.06
Statolon	0	7.3 ± 0.2		0.49 ± 0.05	0.78 ± 0.15	0.37 ± 0.06
	1	6.9 ± 0.3				
	3	6.9 ± 0.4				
Poly(vA)	–1			0.22 ± 0.08	0.76 ± 0.16	0.35 ± 0.06
	0		6.5 ± 0.3	0.30 ± 0.06	0.82 ± 0.12	0.45 ± 0.06
	1		6.7 ± 0.2			
	3		7.0 ± 0.2	0.30 ± 0.08	0.82 ± 0.05	0.39 ± 0.06

intravenously) and poly(vA) (1 mg/mouse, intraperitoneally) given at the time of skin grafting did not prolong the survival of mouse skin allografts made across H-2 differences. Allograft survivals of 12.4 ± 0.9 days in the control group and 12.0 ± 0.4 days in the poly(vA)- and 12.8 ± 0.9 days in the statolon-treated groups were observed.

Poly(vA) did not induce detectable circulating interferon in mice (more than 4 units/ml) and did not alter the interferon induction by poly(I·C) [100 units/ml with or without poly(vA) treatment]. Statolon was shown to be an effective interferon inducer in a similar experiment (19).

DISCUSSION

Polynucleotides were shown to inhibit the replication of leukemic viruses both in tissue cultures and in mice (21, 22). In cell cultures the viral RNA-directed DNA polymerase may be the drug target. In the whole animal the effect of polynucleotides on leukemia virus infection is more complex. The main antiviral mechanism seems to be enhancement of the host immune response (22); however, the induc-

tion of interferon (23) and a direct effect of the polymer on the virion-associated RNA-dependent DNA polymerase may also contribute to the antiviral effect.

Statolon contains a polynucleotide component and apparently has polynucleotide-like effects. Statolon inhibited both FLV replication and FLV-induced splenomegaly in mice (Tables 2 and 3) at a dosage that did not affect normal hematopoietic cell proliferation, and thus the observed inhibition was not due to the toxic effect. The level of inhibition by statolon of FLV replication reported here is comparable to that of polynucleotide inhibitors (22); one dose of drug was satisfactory in both cases. Statolon enhanced humoral antibody response as measured by the Arthus reaction to SRBC (Table 4), but did not significantly affect cell-mediated immunity, as measured by delayed hypersensitivity to SRBC, by graft-vs.-host disease, and by skin allograft rejection. Statolon was previously found to be active against oncogenic (9, 10) and numerous lytic viruses, probably through its ability to induce interferon (19). It seems, therefore, that the mechanism of antiviral activity of statolon is pleiotropic. Through the interferon system it interferes effectively with viral replication at the early stages of infection, and the stimulation of immune responses permits the mobilization of an effective immune response in the later stages of viral infection.

The effects of poly(vA) are distinctly different from those of polynucleotides or statolon. This polymer has very low toxicity and effectively inhibits both the transforming and the helper virus of the FLV complex (Tables 1, 3, and 4). The antiviral effect was specific for FLV. Poly(vA) did not affect the growth of a transplantable tumor of nonviral origin or the replication of a lytic RNA virus, such as Semliki Forest virus. Poly(vA) also did not affect normal hematopoietic cell proliferation, as measured by its effect on hematopoietic spleen colonies. This lack of activity against hematopoietic spleen colonies, a transplantable tumor, and a lytic virus indicates that poly(vA)-induced inhibition of FLV infection is not caused by the cytotoxicity of the polymer.

The inhibition of FLV did not also appear to be mediated through the interferon system. Poly(vA) did not induce circulating interferon in mice or alter interferon induction in response to poly(I·C). Furthermore, the results indicate that neither the humoral nor the cellular host immune response was altered by poly(vA). The lack of poly(vA) effect on virus adsorption was reported previously (6). These results suggest that the observed inhibition of murine leukemia virus infection may be due to the direct inhibition of some essential step in the early stages of the viral replication cycle, such as inhibition of the activity of the RNA-dependent DNA polymerase.

The inhibition of FLV by poly(vA) was less effective than that produced by statolon or polynucleotides; nevertheless it has an important implication for the design of polymeric drugs. Since this compound is stable to hydrolysis and is not metabolized, it is probable that the polymer itself is the antiviral agent. Another important observation is the dependence of the antiviral effects of poly(vA) on the schedule of administration. A large single dose of poly(vA) was not effective, whereas a small dose, when administered daily, significantly reduced virus replication. These results contrast with previous findings on the fate of poly(vA) in the animal (24). It was shown that shortly after intraperitoneal injection poly(vA) appeared in the bloodstream. The majority of the polymer was cleared within a day into the reticuloendothelial organs, where it could be found in undegraded form even a month after injection. The fast clearance of the polymer from the bloodstream is probably the reason why a single dose of poly(vA) was not effective.

Several potentially applicable drug systems have been developed in which a drug of small molecular size is released slowly from a macromolecular carrier. Using both insoluble and soluble carriers can prolong the half-life of the drugs in the blood (25, 26). The polymer examined in this study has an activity span similar to the soluble drug-carrier system. It is possible that further delay in clearance from the bloodstream may be achieved by chem-

ical modification of the polymer, as was recently shown for polymers containing sulfoxide groups (27).

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