Influence of Synthetic Double-Stranded Ribonucleic Acid, Poly I:C, on Friend Leukemia in Mice[1] (34269)

V. M. LARSON, W. R. CLARK, G. E. DAGLE, AND M. R. HILLEMAN

Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486

Recent studies in these laboratories revealed that double-stranded ribonucleic acids of diverse origin were efficient inducers of interferon and resistance to lytic viral infections *in vivo* and *in vitro* (1–6). The complex of synthetic polyriboinosinic and polyribocytidylic acids (poly I:C, rI:rC, or $rI_n \cdot rC_n$) showed special promise and was chosen for further studies in virus-induced neoplasia in animals. The effect of Poly I:C and of certain other interferon inducers on the oncogenesis of adenovirus 12 in newborn hamsters has been reported elsewhere (7). The present report summarizes the findings in studies of the effect of Poly I:C on Friend leukemia in mice.

Materials and Methods. Mice. Four to 6-week old male BALB/c mice were obtained from several suppliers through the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. Healthy mice weighing 16–24 g were distributed at random into the experimental groups after 2 weeks of conditioning.

Virus. Friend leukemia virus was obtained from the Flow Laboratory Repository (Contract No. PH43-65-1012), Viral Oncology, Etiology Area, National Cancer Institute, National Institutes of Health, Bethesda, Maryland. The 10% suspension of infected spleen titered $10^{5.1}$ ID_{50} (50% infectious dose) per 0.2 ml when tested for spleen-enlarging activity by intraperitoneal (ip) inoculation into BALB/c mice.

Poly I:C. The individual homopolymers were purchased from Miles Laboratories, Elkhart, Indiana. Solutions of the homopolymers and the Poly I:C complex were prepared in phosphate buffered saline solution (PBS) (0.006 *M* sodium phosphate, 0.15 *M* NaCl) as described earlier (2) and were furnished by Drs. A. K. Field and A. A. Tytell of these laboratories.

Experimental design. In all experiments, the virus dose per mouse was $10^{4.1}$ ID_{50} given intraperitoneally in 0.2-ml volume at time "0." The Poly I:C or PBS (placebo control) was given intraperitoneally in 0.5 ml in single or multiple doses as indicated in the text. The various parameters used in the experiments to measure the effect of Poly I:C treatment included splenomegaly based on palpation and weight, histopathology of spleen and liver, survival time and percent, and titer of virus in the plasma. The mice were weighed and palpated for splenic enlargement at weekly intervals. Observations for death were made twice daily and the spleens from those animals which died spontaneously or were sacrificed were removed and weighed at autopsy. The spleens and livers were fixed in 10% formalin and stained with hematoxylin–eosin for histopathologic observation.

Virus infectivity titration. The ether-anesthetized mice were bled from the brachial artery employing a capillary pipette rinsed in heparin and the blood samples from 10 mice in each test group were pooled. The plasma was collected following centrifugation and was stored at −60° until assayed for virus. Serial 10-fold dilutions of plasma were inoculated intraperitoneally in 0.2-ml amount into groups of 5 mice. The mice were sacrificed by cervical fracture after 3 weeks, the spleens were removed and weighed, and the

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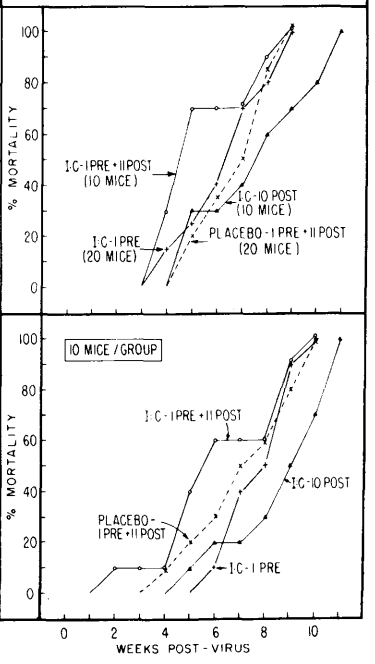
EXP	GROUP	TREATMENT: REGIMEN — POLY I:C DOSE = 263 μg; VIRUS INOCULATED AT "0" TIME; POLY I:C & VIRUS GIVEN INTRAPERITONEALLY	RESULTS: VIRUS TITER OF POOLED PLASMA = $\log_{10}$ ID_{50}/0.2 ml (10 MICE/GROUP) — DAY 10	DAY 14	SPLENOMEGALY (10 MICE/GROUP) 10 DAYS — MEAN SPLEEN WEIGHT (mg)	% * INHIBITION	14 DAYS — MEAN SPLEEN WEIGHT (mg)	% * INHIBITION
1	1	1 DOSE POLY I:C, -6 HR	ND**	5.5	ND	ND	1568	-53
	2	12 DOSES POLY I:C, -6 HR DA 2, 8 & 4 DAY INTERVALS THROUGH DAY 42 ***	ND	5.7	ND	ND	1346	-31
	3	10 DOSES POLY I:C, DA6, AND 4 DAY INTERVALS THROUGH DAY 42 ***	ND	4.5	ND	ND	751	+27
	4	12 DOSES PLACEBO, -6HR, DA2, & 4 DAY INTERVALS THROUGH DAY 42 ***	ND	4.9	ND	ND	1024	-
2	1	1 DOSE POLY I:C, -6 HR	4.3	5.3	790	-30	1458	-56
	2	12 DOSES POLY I:C, -6 HR DA 2, 8 & 4 DAY INTERVALS THROUGH DAY 42 ***	4.5	5.1	373	+39	1258	-35
	3	10 DOSES POLY I:C, DA6, AND 4 DAY INTERVALS THROUGH DAY 42 ***	4.1	4.7	197	+68	515	+45
	4	12 DOSES PLACEBO, -6 HR, DA 2, & 4 DAY INTERVALS THROUGH DAY 42 ***	5.3	4.7	610	-	933	-



* BASED ON COMPARISON WITH PLACEBO GROUP #4, MINUS SIGN INDICATES GREATER SPLENOMEGALY IN TREATED THAN IN PLACEBO CONTROL ANIMALS.
** NOT DONE
*** NO FURTHER TREATMENT AFTER DA 6 FOR MICE SACRIFICED ON DA 10 AND AFTER DA 10 FOR MICE SACRIFICED ON DA 14

FIG. 1. Effect of treatment with Poly I:C on viremia, splenomegaly, and mortality of BALB/c mice infected with Friend leukemia virus.

ID_{50} end point was calculated according to the Spearman-Karber procedure (8) in which a spleen weighing 180 mg or more was considered to be affected by the Friend virus.

Results. Effect of prophylactic and/or therapeutic treatment of Friend leukemia in mice with Poly I:C. Groups of BALB/c mice in 2 separate experiments were treated with large doses of Poly I:C (263 μg/dose) before and/or after inoculation with Friend virus, according to the regimens shown in Fig. 1. The schedules which were used included administration of a single dose of Poly I:C prior to virus (group 1), one dose before and multiple doses after virus (group 2), and multiple doses started after virus (group 3). The control animals (group 4) received PBS placebo in the same regimen as used for group 2. Additional control animals were included which received Poly I:C alone in the 3 regimens and no virus was given. In these animals, the mean spleen weights of the animals sacrificed on day 14 were 106 mg (group 1), 154 mg (group 2), 182 mg (group 3), and 119 mg (group 4, no drug controls). The drug alone was therefore shown to have only minor effect on the spleen weight.

The effect of Poly I:C treatment on plasma virus titer, splenomegaly, and mortality in the 2 experiments is shown in Fig. 1. Administration of a single dose of Poly I:C 6 hr before virus (group 1) increased splenomegaly at the time periods shown. This increased spleen weight in the Poly I:C treated animals compared with the controls at day 14 was significant in both studies, *viz.*, $p < .01$ in Expt. 1 and $p < .05$ in Expt. 2. There was no striking effect of Poly I:C on the death rate.

Treatment with Poly I:C both before and after Friend virus (group 2) resulted in significant ($p < .05$) inhibition of splenomegaly

on day 10 based on spleen weight in Expt. 2 (+39%) and on palpation findings in Expt. 1 (3.4% and 86.6% positive in the treated and control groups, respectively). This effect was only transitory since at day 14, there was actually increased splenomegaly in the treated animals (— 31 and —35% in Expts. 1 and 2, respectively). Additionally, there were earlier deaths in both experiments in a portion of the animals given Poly I:C in this pre- and post-treatment regimen.

The most evident protective effect was obtained when Poly I:C treatment was initiated after the virus was given (group 3). Inhibition of splenomegaly was observed both at 10 and 14 days in both experiments (27–68%) and this appeared to be associated with a slight extension of the survival time. The decrease in spleen weight in Expt. 2 was significant both at day 10 ($p < .001$) and day 14 ($p < .05$).

All 3 treatment regimens appeared to reduce the virus titer in the plasma 10 days following virus in Expt. 2 (1.0, 0.8, and 1.2 neg. $\log_{10}$) compared with the control group. This effect was either lost or reversed by day 14.

Histopathology. Spleens and livers of the mice in Expt. 1 (Fig. 1) as well as those of the control mice which received drug alone and were killed on day 14 were examined histologically. The degree of histologic change was noted and graded arbitrarily from none to extreme involvement. Averages were used in rendering judgment.

The differences in spleen weight between the various treatment groups of infected animals were associated principally with the amount of necrosis and hemorrhage. Necrosis was decreased in all treatment groups compared with the control group, especially in group 3. The hemorrhagic response following treatment with Poly I:C was increased in group 1, unchanged in group 2, and decreased markedly in group 3 as compared with the placebo control group. There was no marked alteration in reticulum-cell type (Friend leukemia cell) proliferation in the spleen by treatment. The reticulum cell changes in the liver were substantially reduced in treatment group 3 with no appreciable change in the others.

Poly I:C given alone had no apparent effect on the liver, but there were small changes in spleen weight (see above) and in the amount of extramedullary hematopoiesis in the spleen. The mean spleen weights for groups 2 and 3 were significantly greater ($p < .01$ and $p < .001$, respectively) than for placebo control group 4. Hematopoiesis was substantially increased for group 3 and to a lesser extent for groups 1 and 2 compared with placebo control group 4. The greater spleen weights for groups 2 and 3 compared with group 1 were probably related to the length of time which had elapsed since the last dose of Poly I:C was given and with the total amount of the drug administered. The observations with regard to spleen weight of animals which received Poly I:C alone in Expt. 1 were consistent with similar observations in Expt. 2, not included here. Even though increased hematopoiesis was noted in the animals which received Poly I:C only, there was no gross evidence for toxicity in any of the treatment groups based on weekly measurement of body weight.

Effect of Poly I:C and cortisone on splenomegaly in Friend leukemia. It was shown in Expts. 1 and 2 above that a single dose of 263 μg of Poly I:C given 6 hr before Friend virus increased the splenomegaly compared with the controls. It was suggested that such increase might have resulted from some adverse effect related to the known stimulatory effects of Poly I:C on the ordinary immune mechanisms (9). Studies were carried out, therefore, in which Poly I:C was given in Friend virus leukemia and the immunologic system was suppressed by administration of cortisone.

In two separate experiments, groups of 10 BALB/c mice were given a single intraperitoneal dose of 263 μg of Poly I:C, a single subcutaneous dose of 2.5 mg of cortisone acetate, or combined Poly I:C and cortisone 6 hr prior to inoculation with Friend leukemia virus. Control animals were given PBS as placebo. The mice were sacrificed on the fourteenth day following virus, the spleens were weighed, and the mean weights were calculated.

TABLE I. Effect of Poly I:C and Cortisone Treatment on Spleen Weight in Friend Leukemia in BALB/c Mice.[a]

Treatment (6 hr before virus)	Mean spleen wt (mg) 14 days after virus (10 mice/group)							
	Expt. 1				Expt. 2			
	Virus infected		Uninfected		Virus infected		Uninfected	
	Weight	Reduction[b]	Weight	Reduction	Weight	Reduction[b]	Weight	Reduction
Poly I:C, 263 μg	1311	−7%	144	−12%	1318	−34%	95	−6%
+ Cortisone, 2.5 mg	1891	−54%	128	+1%	1610[c]	−63%	61	+31%
Cortisone, 2.5 mg	2036	−67%	113	+12%	1220[d]	−24%	69	+22%
PBS placebo (control)	1229	—	129	—	987	—	89	—

[a] The virus and Poly I:C were given intraperitoneally. Cortisone was given subcutaneously in the scapular region. Placebo was administered at both sites.

[b] Compared with placebo control group: + = protection; and − = failure of protection.

[c] Mean of 9 surviving mice.

[d] Mean of 7 surviving mice.

Table I shows that Poly I:C and cortisone given alone and together exerted an adverse effect, *i.e.*, an increase in splenomegaly in mice infected with Friend leukemia virus. Greatest increase was found in Expt. 2 when the 2 substances were given together and in Expt. 1 when cortisone was administered alone. In uninfected mice, Poly I:C given alone caused a small increase in spleen weight (6–12%) whereas cortisone given alone or in combination with Poly I:C effected a slight to moderate reduction in spleen size (1–31%).

Effect of amount of Poly I:C given in a single dose on splenomegaly in Friend leukemia. The increase in splenomegaly following pretreatment of Friend leukemia in mice with Poly I:C was found to be dose-related. In two experiments, BALB/c mice were given a single dose of Poly I:C in graded doses 6 hr prior to Friend leukemia virus and the mean spleen weights were measured 14 days later. Appropriate controls were included. Figure 2 shows that there was substantial

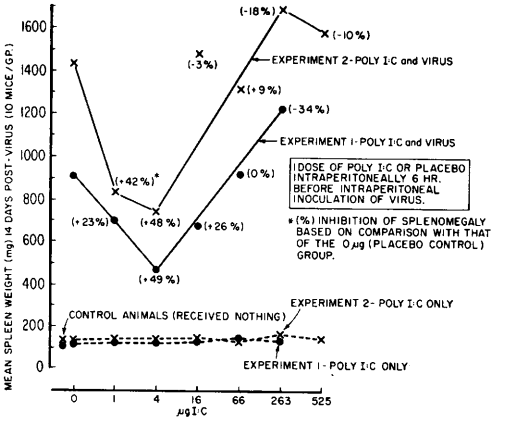


FIG. 2. Effect of graded doses of Poly I:C on spleen weight of mice infected with Friend Leukemia.

decrease in splenomegaly when 4 μg of Poly I:C were given (48–49%). Treatment with 1 μg of Poly I:C reduced the spleen weight by 23 and 42% in the 2 experiments compared with the placebo controls and a dose of 66 μg showed little or no effect. The larger doses (263 and 525 μg) tested led to enhanced splenomegaly (10–34%) though to a lesser extent than was shown in the experiments described in Fig. 1 and Table I.

Discussion. Friend leukemia of mice is an example of an RNA virus-induced cancer in which there is a short incubation period and apparent need for continuing replication of the virus in order to maintain the neoplasia (10). Such infection is an apt model for studies to attempt prevention and therapy of neoplasia by interruption of virus replication using the interferon mechanism and possibly also of the expression of neoplasia in cells infected with the virus.

The previous studies from this laboratory showed that Poly I:C is a highly effective inducer of interferon and of resistance to ordinary lytic viral infections *in vitro* and *in vivo* (1–6). Further, the substance was shown (7) to be protective against internal but not subcutaneous tumors in hamsters given adenovirus 12 by the subcutaneous route. The activity of Poly I:C against adenovirus-induced tumor might have been due either to induction of interferon or to stimulation of ordinary immune mechanisms (9). The greater activity in females compared with male animals and the fact that single-stranded Poly I and Poly C homopolymers were also active were interpreted to favor the latter possibility. Studies by Gresser *et al.* (11–14) and by Wheelock and Larke (10, 15) have shown rather conclusively that continued administration of concentrated preparations of mouse interferon may significantly delay the progress of leukemia caused by the Friend or Rauscher leukemia virus. It has been shown, further, that agents known to induce interferon including Sendai virus (10, 15–17), statolon (10, 15, 18, 19), phytohemagglutinin (19), pyran copolymer (19) and Poly I:C (20) may also be active in inhibiting leukemogenesis by these viruses. The role of interferon, however, has not been established for all these substances in leukemia.

It is clear from the present data that Poly I:C may exert small but significant influence on the course of Friend leukemia given in a high infectious dose and that the outcome is influenced by the time of initiating drug, the dose of drug given, and the number and frequency of injections. Clearly the most favorable results were obtained when drug treatment was started after virus. BALB/c mice are known to develop interferon following inoculation with Poly I:C as shown by Dr. A. K. Field in these laboratories (unpublished). Interferon effect is generally regarded as prophylactic (6) and the suppression of splenomegaly shown when treatment was delayed until 6 days after virus was given might reflect a suppression of the infection of new cells which is necessary for progress of the neoplastic process or a beneficial effect on the host immune response.

The effect of Poly I:C given prior to virus was clearly dose-related. Whether the enhanced splenomegaly following use of a large dose of Poly I:C was related to possible toxic effect is not known since no clear evidence for toxemia was noted at doses up to 263 μg. If the enhanced splenomegaly were due to an adverse effect resulting from the activation of the ordinary immune mechanisms by Poly I:C, this was not reversed by cortisone since cortisone combined with Poly I:C or given alone resulted in far greater splenomegaly than when Poly I:C was administered alone. The splenomegaly which was caused by the Friend leukemia virus was associated with necrosis and hemorrhage, and these effects were greatly reduced when multiple doses of Poly I:C were started 6 days after virus. Additionally, there were fewer Friend-type reticulum cells in the livers of the post-treatment than in the other treatment groups. Poly I:C given alone in the absence of virus caused little apparent effect other than slight increase, in certain instances, in spleen weight and extramedullary hematopoiesis in that organ.

It is evident from the studies that the relationships between the host, and the virus, and the Poly I:C are highly complex and are closely related to dosage of drug and to the time and amount given. Beneficial as well as adverse effects may be obtained and these may differ depending upon the kind of response being observed, and upon the time period in the course of the disease which is being observed. It was most evident that the dose of drug may be extremely important and that it is essential to reduce the dosage to optimal levels if beneficial rather than adverse effects are to be obtained.

Summary. Studies were carried out to measure the effect on Friend leukemia in BALB/c mice of synthetic double-stranded RNA, Poly I:C (rI:rC), given in different prophylactic and therapeutic regimens. The effect of Poly I:C was strongly influenced by the time of initiating treatment, the amount of drug given, and the number and frequency of doses administered. Necrosis and hemorrhage of the spleen were among the principal pathologic effects of Friend leukemia in mice and these were markedly reduced in those instances in which drug treatment decreased splenomegaly. When administered prior to virus, cortisone greatly increased splenomegaly in Friend leukemia both alone and in combination with Poly I:C. The effect of Poly I:C administration before virus was strongly dose-related and a significant beneficial effect was obtained when the amount of drug was reduced to 4 μg. *In toto,* Poly I:C was not strikingly beneficial in altering the events in experimental Friend leukemia. The complex and multifaceted relationships between virus and drug and their administration will require detailed examination before an optimal prophylactic or therapeutic regimen can be derived.

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