Amelioration of Friend Virus Leukemia by Sequential Administration of Viral and Nonviral Interferon Inducers[1] (35710)

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Recent reports have focused on the feasibility of treating Friend virus leukemia with inducers of interferon (1–4). Certain of these have proved effective in either prolonging mean survival and/or ameliorating some manifestation of the disease, such as splenomegaly or the tissue titer of Friend virus. Although the role of these inducers in effecting remissions of the disease is not clear, events support the reasonable assumption that interferon is involved.

The present report summarizes the effects observed when DBA/2 mice with established Friend leukemia were treated sequentially with a series of viral and nonviral inducers of interferon. Included were polyriboinosinic:polyribocytidylic acid complex (Poly I:C) (5), statolon (6), sindbis virus (7), and tilorone hydrochloride (8, 9). Of these, statolon and Poly I:C have been reported to exert limited control on the progression of this murine leukemia (1, 3, 4).

Materials and Methods. Mice. Six–eight-week-old male DBA/2 mice weighing 14–18 g were obtained from the Jackson Laboratory, Bar Harbor, Maine. The mice were conditioned for 1 week and radomized into appropriate groups (178 mice/group).

Viruses. (1) Friend leukemia virus (FLV) was obtained from the American Type Culture Collection, Bethesda, Md., and passed several times in Swiss mice. Nine days following intravenous injection of 0.5 ml of a 20% homogenate of infected spleen tissue, the enlarged spleens were removed, pooled, and a 20% homogenate prepared in cold Locke–Ringer's solution. The homogenate was centrifuged at 3000 rpm for 1 hr, the supernate passed through a 450 mμ Millipore filter, quick frozen, and stored at −70°. The virus was titrated in DBA/2 mice using the spleen focus assay method of Axelrad and Steeves (10). The titer was expressed in focus-forming units (FFU), with 1 FFU defined as the quantity of virus required to induce an average of one focus per spleen. Friend virus, contained in 0.2 ml of Locke–Ringer's solution, was injected intraperitoneally at a dosage of 2800 FFU/mouse. (2) Vesicular stomatitis virus (VSV), provided by Dr. G. D. Mayer of The Wm. S. Merrell Co., was propagated in monolayer cultures of mouse L cells (Grand Island Biologicals, Grand Island, N. Y.). Infected monolayers were subjected to three cycles of freeze-thawing, centrifuged at 3000 rpm for 30 min, filtered, frozen, and stored as before. (3) Sindbis virus (SV), supplied by Dr. D. Yohn, The Ohio State University, was grown in the allantoic sacs of 10-day-old virus-free embryonated chicken eggs. Allantoic fluids were aspirated and clarified by centrifugation at 105,000*g* for 8 hr, washed, and subsequently dispersed in phosphate-buffered saline (PBS), filtered, quick frozen, and stored at −70°. SV was injected intraperitoneally at a concentration of 1×10^7 EID_{50}/mouse (50% egg infective dose).

Nonviral inducers of interferon. (1) Poly I:C, obtained from Miles Laboratories, Elkhart, Ind. was administered intraperitoneally at a dosage of 100 μg/mouse contained in 0.2 ml of 0.85% saline. (2) Statolon, supplied by Dr. W. J. Kleinschmidt of the Eli Lilly Co., was introduced intraperitoneally in 0.2 ml of PBS at a dosage of 15 mg/15 g of body wt.

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(3) Tilorone hydrochloride, supplied by Drs. R. F. Krueger and G. D. Mayer of The Wm. S. Merrell Co., was administered by gavage in 0.2 ml of aqueous solution at a dosage of 250 mg/kg of body wt.

Interferon assay. Interferon assays were performed in 24-hr monolayers of mouse L cells grown in microtissue culture plates (Falcon Plastics, Div. of Bioquest). Microtiter pipettes which deliver 0.05 ml were used to quantitate fluids in this microsystem. Growth medium consisted of Eagle's minimum essential medium supplemented with 10% inactivated calf serum. Samples to be assayed were routinely acidified to pH 2 for 24 hr at 4°, neutralized, and serially diluted in growth medium. Following 24 hr of incubation at 37°, the dilutions of the test material were removed, the monolayers washed once with Hanks' balanced salt solution, and challenged with 300 $TCID_{50}$ of VSV in 0.2 ml of growth medium. This concentration of VSV produced 100% cytopathogenic effect (CPE) in control cultures in 18–24 hr. All cultures were incubated at 37° for 24 hr in an atmosphere of 5% carbon dioxide. The interferon titer is expressed as the reciprocal of the dilution of test material, 0.2 ml of which protected 50% of the cells from CPE, at a time when control cultures exhibited 100% CPE. Several samples were assayed repeatedly, and the system was found to be both reproducible and efficient.

Friend virus assay of infected spleen extracts. The titer of FLV in splenic extracts from treated and placebo control groups was determined using the procedure of Chirigos *et al.* (11). Ten per cent homogenates of pooled infected spleen tissue were prepared in PBS and clarified by centrifugation at 3000 rpm for 15 min (2°). Serial 10-fold dilutions of the splenic extracts were prepared in PBS, and 0.2-ml aliquots of each test dilution were injected into each of five mice. After 14 days, the mice were sacrificed by cervical disarticulation and the spleens removed and weighed. Using the Spearman-Karber (12) procedure, spleens weighing 180 mg or more were scored positive in determining the 50% infectious dose (ID_{50}).

Results. Effect of a series of viral and nonviral interferon inducers on the survival of DBA/2 mice with established Friend's disease. Mice in the treated and placebo control groups were inoculated with 2800 FFU of FLV. Administration of the inducers was begun 7 days postinoculation of FLV when the mean spleen weight reached 180 mg (8 mice/sample). The regimen followed is outlined in Table I. The placebo animals received the appropriate diluent by the same route. Two additional groups of control mice were inoculated with (a) Friend virus only to establish mean survival, and (b) with the inducers alone to determine the response of normal DBA/2 mice. Survival time of the control group of mice which had received FLV only did not differ significantly from that observed for the infected mice which had received placebo.

At the periods indicated in Table II, mice were randomly selected (8 mice/sample group), anesthetized, decapitated, and their sera collected and pooled. In addition, spleens were weighed and averaged and a pooled 10% splenic homogenate was prepared in cold Locke–Ringer's solution. Both sera and splenic extracts were quick frozen and stored at −70° until assayed for FLV and interferon. Remaining animals were observed

TABLE I. Regimen of Interferon Inducers Used in the Treatment of Mice with Established Friend Virus Leukemia.

Agent	Dosage	Day administered[a]	Route
Poly I:C	100 μg/mouse	7 Days	Intraperitoneal
Sindbis virus	1×10^7 EID_{50}/mouse	13 Days	Intraperitoneal
Statolon	5 mg/15 g body wt	19 Days	Subcutaneous
Tilorone hydrochloride	250 mg/kg body wt	25 Days	Oral

[a] Times are postinoculation of Friend virus.

TABLE II. Effects of a Series of Interferon Inducers on Spleen Weights, Interferon Levels, and Virus Titers in DBA/2 Mice with Friend Virus Leukemia.

Sample time[a] (days)	Mean spleen wt (mg)			Interferon titer spleen[b]		Virus titer spleen[c]		Interferon titer serum	
	Treated	Placebo control	% Variation from control[d]	Treated	Placebo control	Treated	Placebo control	Treated	Placebo control
0	82	95	—	20	10	NT	NT	10	10
2	128	106	+21	10	20	NT	NT	10	20
4	162	125	+30	NT[e]	NT	NT	NT	NT	NT
6	182	178	+2	10	10	3.1	3.7	<10	10
7 (Poly I:C)									
8	206	343	—40	320	10	2.4	4.3	1280	<10
11	445	650	—31	40	10	NT	NT	80	<10
13 (Sindbis virus)									
14	1370	820	+67	40	10	4.1	4.3	80	<10
17	1670	1560	+7	20	<10	NT	NT	20	<10
19 (Statolon)									
20	1140	1780	—36	80	<10	3.0	3.7	640	<10
23	1420	2360	—40	20	<10	NT	NT	20	10
25 (Tilorone hydrochloride)									
26	1600	2200	—27	320	<10	2.1	3.6	320	10
29	1360	2440	—44	20	<10	NT	NT	40	<10
37	2160	2590	—17	10	<10	2.8	3.0	20	<10
90	2570	3250	—20	10	<10	3.3	3.1	10	<10

[a] Represents sample taken pre-FLV inoculation. All others are postinoculation of FLV (sample size = eight mice for all samples).
[b] Interferon titer is expressed as the reciprocal of the dilution of test material (0.2 ml) of which protected 50% of the cells from CPE.
[c] Titer expressed as — $\log_{10} ID_{50}$/0.2 ml.
[d] + and — represent percentage increased or decreased splenomegaly as compared with placebo controls.
[e] Not tested.

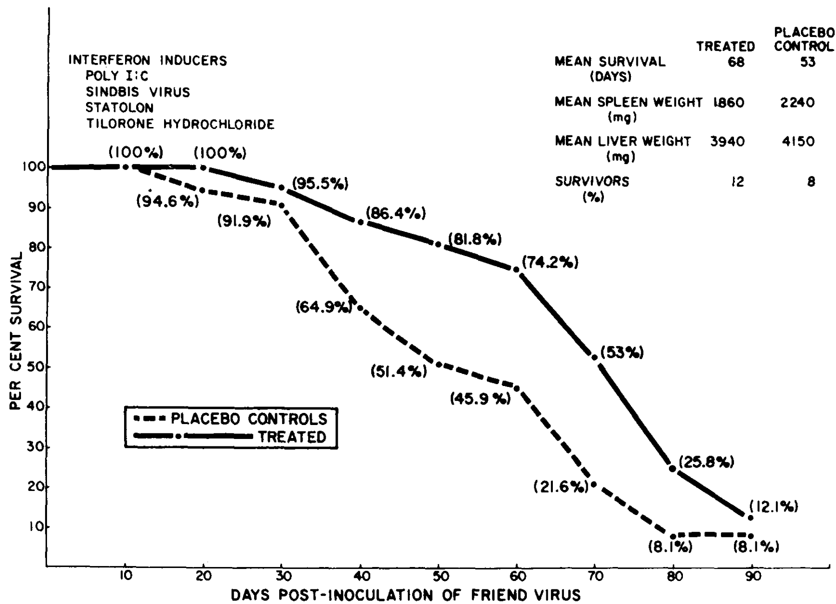

FIG. 1. Effect of a series of interferon inducers on survival rate, mean survival, and the degree of hepatomegaly and splenomegaly (mean) in DBA/2 mice with established Friend virus leukemia.

twice daily for 90 days, and animals dying from the disease were autopsied and spleen and liver weights determined. Animals surviving 90 days were treated similarly.

The effect of the regimen of inducers on mean survival and the survival rate is presented in Fig. 1. The treated group exhibited an increased mean survival of 15 days when compared with the placebo control group. From the Fig. 1 it is apparent that the treatment regimen affected the survival rate markedly between the 30th and 60th day post-FLV inoculation. Treated animals began dying 31 days after inoculation and died at a slower rate than did the control animals until day 60, with 74.2 and 45.9% survival in the treated and placebo control groups, respectively, to this point. The survival rate decreased markedly between 60 and 80 days, with 12.1% survival in the treated group at 90 days, as compared to 8.1% in the placebo group. Since the inducers were administered only through day 25, it appears that the therapeutic effects persisted and were effective in prolonging the survival of many animals which otherwise would have died between the 30th and 60th day.

Effect of the inducers on splenomegaly and hepatomegaly in mice with Friend leukemia. The effect of the series of viral and nonviral inducers of interferon on splenomegaly, splenic, and serum interferon titers, and splenic virus titers during the course of the study is also shown in Table II. Following treatment with Poly I:C, statolon, and tilorone hydrochloride, splenomegaly in the test animals was 40, 36, and 27% less than recorded for the placebo control group. This contrasts sharply with the significant increase in the extent of splenomegaly (67%) noted in the treated group following inoculation of SV. Mean spleen and liver weights of animals dying from Friend leukemia and those surviving the test period (Fig. 1) indicate that the administration of viral and nonviral inducers resulted in a somewhat lessened splenomegaly and hepatomegaly. Of further significance is the fact that the marked organomegaly observed in the placebo controls was due in part to diffuse hemorrhage and that the lesser degrees of organomegaly in the treated animals was associated with much less hemorrhagic diathesis. The histopathology of these phenomena will be described in detail elsewhere.

Interferon levels in Friend virus-infected

mice treated with a series of interferon inducers. Although not recorded here, normal DBA/2 mice were found to respond (with significant levels of interferon) to all of the inducers used. Friend virus-infected mice also were found to respond to the inducers, but titers were slightly less than were recorded for the normal animals. The notable exception was to the injection of SV, to which normal animals responded with an average serum titer of 1280 and an average spleen titer of 320. As can be seen in Table II, leukemic animals did not respond to this extent. Animals in the placebo group produced low titers of interferon (10–20) early in the course of the disease, but generally did not produce detectable quantities after the first week. In treated animals, interferon titers generally were higher in the spleen than in the serum. Increase in the titers of measurable interferon occurred in both serum and spleen following administration of Poly I:C, SV, statolon, and tilorone hydrochloride (Table II). Animals produced interferon upon restimulation at the times indicated. Following cessation of treatment, interferon levels decreased.

Virus titer in the spleens of DBA/2 mice treated with a series of viral and nonviral inducers of interferon. As shown in Table II, spleens were assayed for recoverable Friend virus before initiation and after termination of treatment, and at 24 hr following the administration of Poly I:C, SV, statolon, and tilorone hydrochloride. Virus was recovered from both treated and placebo control spleens prior to treatment (6 days postinfection). Thereafter, the titer of infectious virus in the spleens of control animals increased to a maximum of 4.3 ID_{50} and then decreased to a level of 3.1 ID_{50} at 90 days.

After treatment with Poly I:C, virus titer decreased from 3.1 to 2.4 ID_{50}, increased to 4.1 ID_{50} postinoculation of SV, and again decreased after administration of both statolon and tilorone hydrochloride. Following termination of treatment, the titer of infectious FLV in the spleen rose to a level comparable to that observed in the control mice on the 90th day.

SV enhanced the replication of FLV as evidenced by the increased virus titer in the spleen (4.1) and the associated increase in the degree of splenomegaly (67% when compared with the appropriate controls).

Discussion. One of the most studied of the experimental leukemia models is Friend leukemia, an erythroblastic leukemia, the etiology of which has been shown to be a viral complex (13). Control of this murine leukemia by viral interference, as mediated by interferon, has been attempted, using both endogenous inducers and exogenous preparations. Gresser *et al.* have reported that administration of exogenous mouse interferon can markedly inhibit the leukemic process (14, 15). Further, certain of the known interferon inducers have proved effective in suppressing the development of Friend leukemia for limited periods. Statolon has been reported to inhibit splenomegaly and exert a transitory suppressive effect on the development of Friends disease, when administered 2–5 days postinoculation (4). It is possible that the suppression of leukemogenesis noted at the earlier treatment periods (2nd and 3rd day) by these authors may have reflected the influence of interferon on the localization and replication of the viral complex in the target cells. For the most part, the remissions observed following treatment with endogenous or exogenous interferon have proved transitory and have been followed by exacerbations of disease with the usual or slightly decreased severity.

That the neoplastic transformation in Friend leukemia could be better controlled by administration of a series of interferon inducers seems appropriate, if the progressive increase in the number of tumor cells in Friend leukemia is dependent on successive cycles of viral replication, as has been suggested by Gresser *et al.* (16). In addition to providing an opportunity for restimulation of animals, a series of various inducers might result in stimulation of different target organs. The data presented indicate that the nonviral inducers of interferon indeed were effective in suppressing the development of Friend leukemia. The administration of these inducers resulted in a reduced splenomegaly and virus titer, which correlated with the

production of measurable quantities of interferon. The fact that ameliorative effects persisted for a significant period following termination of treatment, at a time when measurable interferon levels had decreased markedly, suggests that additional facets of the immune mechanism may be involved.

It is difficult to compare the relative effectiveness of the nonviral inducers, since all seemed to influence the course of the disease, but were introduced at different stages in the leukemic process. However, it is interesting that statolon and subsequently, tilorone hydrochloride were at least as effective as the other inducer (Poly I:C), particularly since they were administered following the introduction of SV, which had produced an enhancement of Friend virus production. This enhancement was evidenced by increased splenomegaly and an accompanying sharp rise in splenic titer of the virus.

Although Sendai virus has been reported to be effective in controlling leukemogenesis in mice (2), in this study the introduction of SV, also a confirmed interferon inducer (7), resulted in enhancement of Friend virus replication. This phenomenon has been reported to occur with FLV, Rauscher leukemia virus, and murine sarcoma virus following the administration of Guaroa virus (17–19). A similar enhancement was reported in a situation in which Newcastle disease virus and lactic dehydrogenase-elevating virus were administered prior to Friend virus (20). In our laboratory, an enhancement effect was observed when Newcastle disease virus was administered up to 20 days after inoculation of Friend virus (unpublished).

The mechanism of enhancement of Friend virus replication by sindbis virus is not understood at this time. However, it seems reasonable that the interferon produced is quantitatively or qualitatively insufficient for complete protection of the target cells against the total infectious dose of both viruses.

It cannot be concluded that failure to detect interferon in the serum and tissues indicates that it was not produced. To the contrary, this may suggest that the interferon was produced and cell-associated (unmeasurable) at the periods tested. It is possible also that enhancement may be attributable to an increase in target cells resulting from superimposed virus infection, as suggested by Steeves *et al.* (20). Still to be considered is the point that SV may influence antibody production to Friend virus. Suggestive of this is the study of De Somer *et al.* (21) in which interferon-inducing quantities of SV inhibited antibody to polio virus. More recently, Turner *et al.* (19) have suggested that enhancement observed when mice are dually infected with murine sarcoma and Guaroa viruses may be due to the production of "progressor" antibody, which results in protection of tumor cells from sensitized lymphocytes.

From the data, the sequential administration of a series of known interferon inducers beneficially modified the course of viral leukemia as measured by increased mean survival, decreased hepatomegaly and splenomegaly, and reduced splenic titers of Friend virus. Although the role of interferon in ameliorating virus-induced leukemia is not conclusive, these data indicate that the interferon response correlates with the altered progress of the disease. However, the role of other resistance mechanisms merits serious consideration.

Summary. This study was designed to test the effect of the sequential administration of a series of viral and nonviral inducers of interferon on the progression of Friend virus leukemia in DBA/2 mice. The inducers, Poly I:C, SV, statolon, and tilorone hydrochloride, were given at 6-day intervals following injection of Friend virus, beginning 7 days postinfection until the 25th day. Mean splenomegaly, hepatomegaly, virus titer, and interferon titer were monitored periodically during the treatment regimen. Mice dying from the leukemia were autopsied and their liver and spleen weights recorded. The treatment regimen used was successful in increasing mean survival time and in decreasing hepatomegaly and splenomegaly. Decreases in virus titer and splenomegaly were associated with increased interferon levels in the spleens of experimental animals following administration of Poly I:C, statolon, and tilorone hydrochloride, while an enhancement phenomenon was observed after inoculation of SV. The amelioration of established Friend's disease observed using this program

of therapy seems to offer promise, but other combinations of inducers (viral and nonviral) and protracted periods of treatment must be explored.

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