Suppression of Established Friend Virus Leukemia by Statolon VII. Relative Roles of Interferon and the Immune Response in Development of FV-Dormant Infections[1] (37401)

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Statolon, a potent inducer of interferon, derived from the double-stranded RNA mycophage-containing mold, *Penicillium stoloniferum,* (1, 2) has been employed experimentally to protect animals (3–6) and cell cultures (1, 3, 6) against a number of non-oncogenic viruses. Statolon has also been shown to inhibit virus-induced murine sarcomas *in vivo* (7–9) and *in vitro* (10).

Previous communications have reported that statolon administered to Friend leukemia virus (FV)-infected mice can completely suppress the development of overt leukemia (11). Following the administration of statolon, interferon reaches peak titers in the serum of infected mice within 6–12 hr and viremia abates within 24 hr (12). FV-transformed cells, however, can be found in the spleen for approximately 3 wk thereafter, declining in number only after the appearance of FV-cytotoxic antibodies (13, 14). The FV genome persists in a dormant state in these mice emerging late in life, when levels of FV-cytotoxic antibodies decline, to produce leukemia (15).

FV-infected mice are immunodepressed in their response to sheep red blood cells (16). Immunologic reactivity of FV-infected mice to sheep erythrocytes is restored 1 day after statolon treatment (17). The appearance of FV-cytotoxic antibodies in FV-infected mice after the administration of statolon suggests that abrogation of FV-induced immunodepression is crucial to the suppression of FV leukemia.

This sequence of events, *i.e.,* interferon production, clearance of FV from the bloodstream, abrogation of FV-induced immunodepression, and production of FV-cytotoxic antibodies, may be necessary for clinical remissions to be produced in leukemic mice treated with statolon. In contrast, Newcastle disease virus (NDV) or polyinosinic–polycytidylic double-stranded RNA (poly I: poly C), induced interferon in leukemic mice but failed to produce similar remissions (18). Therefore, it was of interest to examine these interferon inducers for their ability to clear virus from the blood of FV-infected mice, reestablish immunologic responsiveness and suppress FV to a dormant state. This report will demonstrate that (a) interferon induction and transient clearance of FV from the blood of leukemic mice are not sufficient by themselves to induce remissions, (b) of the interferon inducers tested, only statolon abrogates immunodepression and stimulates FV-cytotoxic antibody and (c) only statolon converts a rapidly lethal FV infection to a dormant state and produces prolonged clinical remissions.

Materials and Methods. Viruses. Friend virus (FV). FV was originally obtained from infected DBA/2J mice provided by Dr. C. Friend. Three weeks after infection, the enlarged spleens were removed and a 10% (w/v) disrupted cell suspension was made in Dulbecco's phosphate-buffered saline (PBS). The suspension was then centrifuged at 5000*g* for 1 hr and the supernatant fluids were passed through a membrane filter with a pore size of 0.45 μm (Millipore Filter Corp., Bedford, MA), frozen and stored at −70°. The FV inoculum used in all experiments was 2×10^3 leukemia-producing units/mouse and was given intraperitoneally (ip).

Vesicular stomatitis virus (VSV). VSV

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was grown in mouse L-cell monolayer cultures. The infected cultures were disrupted and then centrifuged at 5000*g* for 30 min. The supernatant fluids were filtered, frozen and stored as described for FV.

Newcastle disease virus (NDV). The Hickman strain of NDV was employed. The virus was grown in the allantoic sac of 11-day-old embryonated chicken eggs. The infected allantoic fluids were harvested 2–3 days following NDV inoculation and were clarified by low speed centrifugation. The virus was then pelleted at 90,000*g* for 6 hr. The virus pellets were washed with PBS, dispersed using a Branson Instruments "Sonifier" and passed through a 0.45 μm pore size membrane filter. The virus was stored at −70°. The titer of the virus seed employed in these studies was 2×10^9 EID_{50}/ml.

Mice. Female DBA/2J mice, 6 to 8 wk old, obtained from the Jackson Memorial Laboratories, Bar Harbor, ME, were used in all experiments.

Interferon assay. Serum specimens were assayed for interferon according to methods described previously (19). Interferon activity was determined on the basis of protection of mouse L-cell monolayer cultures against the cytopathic effects produced by VSV.

Assay for FV cytotoxic antibody. FV-cytotoxic antibodies were measured by the *in vitro* ^{51}Cr release method originally described by Wigzell (20) and modified by Holm and Perlmann (21). Spleens, removed from DBA/2J mice infected approximately 1 mo previously with FV, were finely minced with scissors, pipetted vigorously to free the cells from the spleen stroma and then the freed cells were washed 3× with 20 ml PBS. The cells were suspended in RPMI-1630 medium (Gibco) containing 10% fetal calf serum and the viable cell concentration was determined using erythrocin B strain and a hemocytometer. To each 3 ml containing 1×10^7 viable cells was added 100 μCi of ^{51}Cr in the form of sodium chromate (Amersham-Searle). The cells were incubated for 45 min at 37°. After this incubation, the cells were washed 2× with 20 ml PBS and then incubated for 30 min at 4° in the original volume of RPMI-1630 to release the loosely bound isotope from the cells. The cells were washed 2× with PBS and then resuspended in RPMI-1630 plus 10% fetal calf serum and 5% guinea pig complement at a cell concentration of 2×10^6 cells/ml. Incubation mixtures contained 1×10^6 labeled spleen cells in 0.5 ml of medium plus complement and 0.05 ml of mouse serum suspected of containing FV-cytotoxic antibodies. The reaction mixtures were incubated for 3 hr at 37° with occasional agitation. At the end of the incubation period, 1 ml PBS was added to each reaction mixture and mixed and then centrifuged for 10 min at 300*g*. The top 1 ml was removed from each reaction tube and counted in a Baird Atomic gamma scintillation counter. Labeled spleen cells incubated in the presence of 0.05 ml fetal calf serum or normal mouse serum served as controls. Tests were done in duplicate.

Assay for antibody plaque-forming cells. All animals were immunized ip with 2×10^8 sheep red blood cells (SRBC) suspended in normal saline 4 days prior to assay. Spleens were examined for plaque-forming cells (PFC) against SRBC employing Jerne and Nordin's (22) assay of localized hemolysis in agar. Briefly, spleen cells of individual animals were dispersed and suspended in Hanks' balanced salt solution. After dilution, 0.1 ml of the spleen cell suspension along with 0.1 ml of a 10% suspension of SRBC was added to 2.0 ml of molten 0.75% Bacto Agar (Difco) containing DEAE-dextran. This mixture was poured onto a base-layer of solid agar in petri dishes, allowed to solidify and incubated for 1 hr at 37°. Guinea pig complement (1:15) was then added to each plate and after an additional hour of incubation plaque numbers were determined with magnification.

Chemicals. Statolon. Statolon lot 354-869B-139 was donated by Dr. W. J. Kleinschmidt, Eli Lilly & Co., Indianapolis, IN. Statolon was freshly prepared 2 hr prior to use by dissolving 20 mg statolon/ml in 1% sodium bicarbonate at 4°.

Polyinosinic-polycytidylic double-stranded RNA (poly I:poly C). Poly I:poly C was

either prepared in this laboratory by mixing equimolar concentrations of polycytidylic and polyinosinic acids (Mann Research Laboratories, Inc., New York, NY) dissolved in 0.006 *M* $NaH_2PO_4 \cdot H_2O$–0.15 *M* NaCl buffer (pH 7.0) for 15 min (23) or purchased from P-L Biochemicals, Inc. (Milwaukee, WI).

Results. Relationships among interferon production, viremia and establishment of FV-dormant infection. Eight week old female DBA/2 mice were infected with 2×10^3 leukemia-producing units of FV. Three days later they were inoculated intravenously (iv) with either 4 mg of statolon, 25 μg poly I: poly C or 2×10^8 EID_{50} NDV, per mouse, At various time intervals thereafter, 3 mice from each group were exsanguinated and their sera were pooled and titered for levels of interferon.

At 24 hr after administration of the interferon inducer 3 mice from each group were exsanguinated and their plasmas were individually tested for the presence of FV. One-tenth milliliter of plasma from each FV-infected mouse was inoculated ip into each of 2 normal mice. The recipient mice were observed for development of leukemia 21–28 days following inoculation. Between 10 and 15 mice in each group inoculated with the various interferon inducers were kept for a minimum of 2 mo and observed for the development of FV leukemia.

As shown in Table I, all groups of mice receiving the inducers had interferon present

TABLE I. Relationships Among Interferon Production, Viremia and Establishment of a FV-Dormant Infection.

Treatment (hr)	Interferon (units/ml)[a]	Presence of FV in blood 24 hr after injection of interferon inducer[b]	Mice with FV-dormant infections[c] (%)
Control—no interferon inducer			
6	0	+++	0, 0
12	0		
24	0		
48	0		
Statolon, 4 mg iv			
6	6400	000	71, 33
12	1600		
24	800		
48	0		
Poly I·poly C, 25 μg iv			
6	1600	+++	0, 0
12	1600		
24	0		
48	0		
NDV, 2×10^8 EID_{50} iv			
6	6400	0+0	0, 0
12	12,800		
24	400		
48	0		

[a] Interferon titrations were carried out on pooled sera obtained from three mice at the times indicated.

[b] Three mice from each group were tested individually for viremia.

[c] The first percentage value represents number of mice protected after 2 wk; the second value represents number of mice protected 2 mo after infection (long-term survival). Mice which had normal sized spleens, as observed through the abdominal wall, were considered to be protected against FV leukemia.

in their sera by 6 hr following inoculation. NDV induced maximum titers of interferon 12 hr following inoculation, whereas statolon and poly I:poly C induced maximum titers of interferon earlier. The highest interferon titers were consistently found in sera taken from NDV-inoculated mice.

The data show that induction of interferon by statolon and NDV was related to clearance of viremia. Poly I:poly C, 25 μg iv, induced moderate levels of interferon and did not clear FV from the blood. Clearance of FV from the blood within 24 hr after inoculation of the interferon inducers was not by itself sufficient to suppress the FV infection completely; all mice receiving NDV or poly I:poly C developed leukemia within 2 wk of FV inoculation.

In order to relate prolonged absence of FV from the blood to establishment of a FV-dormant infection we tested plasma for FV on selected days after inoculation of the interferon inducers. Separate mice were used for each time point since bleeding of FV-infected mice affects leukemogenicity [(24) and unpublished results]. The results, given in Table II, indicate that disappearance of FV from the blood of NDV-inoculated mice was transient. None of the mice tested had a FV viremia 1 or 2 days following inoculation of NDV. FV was present, however, in the blood of most of the mice tested between 4 and 10 days following NDV and in all mice by Day 14. In contrast, only an occasional statolon-treated mouse had FV in its plasma when tested up to 14 days following inoculation and all mice were viremia negative by Day 21. Again, only statolon converted the rapidly lethal FV infection into a dormant one.

FV was present in the blood of all mice receiving 25 μg poly I:poly C. In other experiments, increasing the dose of poly I:poly C resulted in a transient clearance of FV but no long lasting remissions.

Relationship between abrogation of FV-induced immunosuppression and the development of FV-dormant infection in mice inoculated with various interferon inducers. FV-induced immunodepression, as measured by a diminished antibody response to sheep erythrocytes, can be abrogated by treatment with statolon (17). To relate the importance of statolon's restoration of immunocompetence to the suppression of FV leukemia, statolon and the other interferon inducers, NDV, and poly I:poly C, were compared for their ability to restore the immune response to SRBC in FV-infected mice. In these experiments poly I:poly C was inoculated at 150 μg/mouse ip, a dose which transiently cleared the blood of FV at 24 hr but did not lead to the establishment of a dormant FV infection.

Immunocompetence was determined by the Jerne–Nordin (22) anti-sheep PFC antibody plaque technique. Mice were inoculated with FV on Day 0, an interferon inducer on Day 3, SRBC on Day 6 and the spleen was removed for assay of anti-SRBC-PFC on Day 10.

The effects of statolon, NDV, and poly I:

TABLE II. Relationship Between Clearance of FV Viremia and Development of FV-Dormant Infection.

	Viremia on various days postinjection of interferon inducer[a]							Mice with FV-dormant infections/total no. infected (%)[b]
	1	2	4	7	10	14	21	
Control	+++	+++	+++	+++	nd	nd	nd	0/10 (0)
Statolon	000	+00	++0	000	+00	+00	000	7/10 (70)
Poly I·poly C	+++	+++	+++	nd	+++	nd	+++	0/9 (0)
NDV	000	000	+++	++0	++0	+++	+++	0/10 (0)

[a] Three mice from each group at each time were tested individually for viremia. nd = not done; + = Friend viremia; 0 = no Friend viremia.

[b] Leukemia determined on the basis of splenomegaly 1 mo after infection with Friend virus.

poly C on the immunocompetence of FV-infected mice are shown in Table III. The immunologic response of FV-infected mice to sheep RBC was approximately 10% of that in normal animals. Statolon, but not NDV or poly I:poly C, restored immunological competence to FV-infected mice. In this experiment all 3 agents induced interferon, cleared the blood of FV by 24 hr but, as previously shown, only the administration of statolon resulted in the establishment of a FV-dormant infection.

Reversal of FV-induced immunosuppression by statolon suggested that establishment of a FV-dormant infection might be dependent upon production of antibody against FV. In order to test this hypothesis, DBA/2 mice were inoculated with either normal rabbit serum or rabbit anti-mouse lymphocyte serum (ALS) 7 and 2 days before FV. These mice were then injected with statolon 3 days after FV and studied for interferon, viral clearance, FV-cytotoxic antibody production and the establishment of FV-dormant infections. The results, given in Table IV, show that ALS had no effect on the ability of statolon to induce interferon or effect viral clearance from the blood. However, all mice receiving ALS failed to produce FV-cytotoxic antibody, and all developed overt leukemia within 3 wk of treatment.

Discussion. The inhibitory effects of interferon and interferon inducers on viral induced murine leukemias has been well documented. Daily injections of high titered partially purified interferon begun after FV inoculation inhibited virus replication and subsequent disease (25, 26). Interferon administered when mice were beginning to die of FV leukemia also prolonged survival (27). Sendai virus, a parainfluenza virus of low virulence, induced interferon and inhibited development of leukemia when injected prior to FV (28). Prolonged survival of mice with advanced FV leukemia followed treatment with either Sendai virus or statolon (27). Other nonviral inducers of interferon, notably polyanions and synthetic polynucleotides have been reported to inhibit murine leukemia viruses both *in vivo* and *in vitro* (10, 29–33).

The inhibitory action of interferon on murine leukemia viruses remains unquestioned, but considerable evidence exists to indicate that suppression of tumorigenesis by interferon preparations may be mediated through other mechanisms. Weinstein, Gazdar and Sims (34) were unable to correlate interferon production with inhibition of tumor-

TABLE III. Correlation of Interferon Production, Clearance of FV Viremia and Reversal of Friend Virus-Induced Immunosuppression with Development of FV-dormant Infections[a] in Mice Inoculated with Interferon Inducers.[b]

Treatment	Interferon production	Clearance of FV viremia	Anti-sheep RBC PFC $\times 10^3$/spleen[c]	Development of FV-dormant infection[d]
No FV	0	0	126 ± 30	0
FV	0	0	13 ± 6	0
FV + statolon	+	+	103 ± 58	+
FV + NDV	+	+	7 ± 3	0
FV + poly I·poly C	+	+	10 ± 14	0

[a] 0 indicates no interferon production, no clearance of FV viremia or no development of dormant infection. + indicates interferon production, clearance of FV viremia or development of dormant infection.

[b] Statolon, 4 mg NDV, 2×10^8 EID_{50}, or poly I·poly C, 150 μg, was given intravenously 3 days after FV.

[c] All mice were immunized with 2×10^8 SRBC suspended in normal saline 3 days after interferon inducer and the plaque assay was performed 4 days after immunization.

[d] Suppression of FV into a dormant infection was measured by the presence of normal sized spleens 4 wk after FV inoculation.

TABLE IV. Effect of Anti-lymphocyte Serum on Suppression of Established FV Leukemia by Statolon.

Treatment[a] of FV-inoculated mice	Interferon[b] 6 hr after statolon	Viremia[c] 24 hr after statolon	FV-carrier[d] production (leukemic mice/total)	FV-Cytotoxic antibody, % ^{51}CR release[e]	
				FV-carrier mice	Leukemic mice
NRS-statolon	1600	$<10^{0.5}$	5/12	62	24
ALS-statolon	1600	$<10^{0.5}$	12/12	—	0
0-0	<4	$10^{3.5}$	5/5	—	0

[a] Mice were inoculated with either normal rabbit serum or anti-lymphocyte serum 2 and 7 days prior to FV inoculation. Statolon 4 mg/mouse was inoculated intravenously 3 days after FV.

[b] Serum interferon levels expressed as the reciprocal of the highest dilution per milliliter which protected L-cell monolayers against the cytopathic effect of vesicular stomatitis virus.

[c] Viremia expressed as the number of leukemia-producing doses/ml of plasma, as assayed in DBA/2 mice.

[d] Expressed as the number of mice with spleens greater than 0.25 g over total number of mice 3 wk after FV inoculation.

[e] Expressed as the percentage increase over controls of release of ^{51}Cr from labeled leukemic spleen cells treated with 1:10 dilution of pooled experimental serum.

igenesis in mice treated with poly I:poly C. In addition, as shown here, interferon inducers other than statolon could not completely suppress FV leukemia. Finally, statolon induced as much interferon in ALS-treated mice as in normal mice and yet could not inhibit the development of FV leukemia in the immunosuppressed hosts. These results indicate that a noninterferon mechanism of probable immune origin is operative in effecting complete suppression of virus-induced leukemia. In support of this hypothesis is the finding that statolon abrogates the immunodepressive effects of FV and permits the infected mouse to mobilize an effective humoral cytotoxic antibody response to leukemic cells (17).

A third mechanism by which interferon inducers can inhibit tumorigenesis is by a direct or lymphokinin-mediated indirect attack on tumor cells. Poly I:poly C has a potent inhibitory effect on both viral and nonviral murine tumors (35). Poly I:poly C has also been shown to enhance both the humoral and cellular responses to foreign antigens (36) and accelerates the graft vs host reaction in mice (37). However, experiments with immunosuppressed hosts reveal that some mechanism other than stimulation of the immune response is responsible for the tumor-inhibiting effects of poly I:poly C (38, 39). Toxic effects on tumor cells could result from a direct effect by poly I:poly C (40) or indirectly from lymphotoxin released from mouse lymphocytes which can be stimulated with poly I:poly C (41).

We show here that statolon suppresses FV leukemia by inducing interferon and reversing FV-induced immunodepression with associated production of FV-cytotoxic antibody. These experiments identify interferon as being operative for only a short period of time and accounting for clearance of FV from the blood of leukemic mice. These events, however, must be combined with an associated immunostimulation of the host and possibly other host defense mechanisms in order for the virus genome to be suppressed to a dormant state and for long lasting clinical remissions to ensue.

Summary. Interferon induction and transient clearance of FV from the blood of leukemic mice are not sufficient by themselves to induce remissions. Of the interferon inducers tested, only statolon abrogates immunodepression, stimulates FV-cytotoxic antibody, and converts a rapidly lethal FV infection to a dormant state resulting in pro-

longed clinical remissions.

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