Interference of Cocal Virus with Friend Leukemia Virus-Induced Splenomegaly in DBA/2J Mice[1] (34803)

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The successful use of viral interference as a method for suppression of murine leukemia (1, 2), and the preferential use of arboviruses as oncolytic agents in man and animals stimulated efforts to develop a comparable system with the arboviruses against a virus-induced murine leukemia.

In a survey (3) of all the major arbovirus serotypes for their cytolytic activity against L5178Y murine lymphoblasts *in vitro*, members of the vesicular stomatitis virus (VSV) serogroup were outstanding. Among the three virus serotypes of the serogroup, Cocal virus proved to be more consistently cytolytic for the murine lymphoblast than the other two viruses, *viz.*, VSV (Indiana) and VSV (New Jersey).

Cocal virus (prototype strain TRVL 40233), an agent serologically related to VSV (Indiana), was first isolated from a pool of 249 mites of the laeloptid genus Gigantolaelops (Acarina, Laeloptidae) combed off 11 terrestrial rice rats, (*Oryzomys laticeps velutinas,* Allen and Chapman) which were trapped in September 1961, on Bush Bush Island, Trinidad (4).

This report describes limited aspects of the antagonism between Cocal arbovirus and Friend virus in DBA/2J mice as monitored by (1) the inhibition of Friend virus-induced splenomegaly, (2) reduction or increase of survival time as reflected by Friend LD_{50} dose and (3) reduction or increase of Friend virus latent period as reflected by Friend virus ED_{50}, or percentage of mice exhibiting splenomegaly at a given time.

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Materials and Methods. General experimental design. The experimental mouse groups were composed of 8 to 16 weanling DBA/2J male mice. In all experiments inoculation was intraperitoneal (ip) and the volume of inoculated virus suspension was 0.2 ml. The ip route of inoculation was selected because of other investigators' preference for this route (1). The arbovirus or placebo control was injected from 5 days before to 5 days after the inoculation of Friend leukemia virus (FLV). The date of FLV inoculation was routinely counted as Day 0. The development of splenomegaly was followed by spleen palpation during the course of the experiment and/or by direct spleen weight at the termination of the experiment (10–14 days after FLV administration). The source of the Cocal virus (either 10% normal mouse brain in 7.5% bovine plasma albumin or cell culture medium) was used as control inoculum.

Mice. Following the recommendation of Dr. E. Frederick Wheelock, 6- to 8-week-old male DBA/2J mice bred by the Jackson laboratory were selected as the interference assay host system because of their uniformity in response to FLV.

Cell cultures. Continuous cell lines of green monkey (Vero) and rhesus monkey (LLC-MK_2) kidney were maintained in 32-oz bottles, using Eagle's basal medium with Hanks' salts supplemented with antibiotics (100 μg per ml of penicillin, streptomycin, and tylomycin), and 5% fresh unheated calf serum. Cells from 32-oz bottles were used to prepare 3-oz cell culture bottles, used for plaque assays of the Cocal virus.

Viruses. Cocal virus. Lyophilized Cocal virus stocks, prepared as 10% mouse brain in

7.5% bovine plasma albumin by the World Health Organization Arbovirus Reference Center at the Yale Arbovirus Research Unit, were used as the source of both lyophilized and wet frozen virus stocks. All virus stocks were stored at —70°.

Friend leukemia virus. An eighth serial passage of the original virus was obtained from Dr. Charlotte Friend (5) by Dr. Edwin Mirand and carried several passages in DBA/2J mice in our laboratory. A single lot (20% infected spleen homogenate) of FLV was the source of all the cell-free filtrates employed for all experiments. The spleens were homogenized in cold Hanks' lactalbumin hydrolyzate medium, the homogenate was clarified at 5000 g/10 min at 4°, and the supernatant fluid was passed through a 0.45 μ (average pore diameter) grid membrane.[2]

Virus titrations. Cocal virus–plaque assay. Virus dilutions (0.2 ml) were adsorbed onto the cell sheets for 1–2 hr and overlaid with medium previously described (6, 7), but with the following modification: oxalacetate and arginine were omitted, ionagar No. 2[3] was substituted for Noble agar and 100 μg/ml DEAE added to the nutrient overlay. Virus titers are expressed in plaque-forming units (PFU) per 0.2 ml inoculum.

Friend leukemia virus. Groups of eight 6- to 8-week-old DBA/2J mice were inoculated ip with 0.2 ml of serial log dilutions of FLV virus. The development of splenomegaly was followed by spleen palpation starting on Day 7 after inoculation and thereafter every 2 or 3 days. "Leukemic mice" are designated animals having enlarged spleens as measured by direct weight or palpation. The titer was calculated according to the Kaerber method (8) and expressed as ED_{50} (9)—the dose required for induction of any enlarged spleen in 50% of the mice at t days (day when splenomegaly was observed).

Parameters used to measure degree of interference. Direct spleen weight. Direct spleen weights were used when the protocol called for killing the mice at a prescribed time. When the experiment was allowed to proceed without a designated sacrifice time, leukemogenesis was monitored by spleen palpation.

Spleen palpation. Results using spleen palpation were recorded in two ways: (1) recording the percentage of the protected mice (absence of splenomegaly) at a time when 100% of the Friend-infected mice displayed enlarged spleens or (2) comparison of ED_{50} values at various intervals during the experiment. The spleen-palpation method was of great value in monitoring the duration of an interference effect or when various doses of FLV were used.

LD_{50}. When the mice were not sacrificed, the experiment was allowed to progress until LD_{50} determinations could be obtained, *i.e.*, using death as an endpoint instead of splenomegaly.

Results. Results of inoculation of Friend virus only. Different cell-free filtrates of the same lot of FLV were used in these experiments. The ED_{50} was time dependent and varied 0.9–2.0 logs in different experiments at the same observation period with different filtrates.

The rate of splenomegaly due to FLV was directly related to the dilution of FLV administered. Mice infected with high doses (2.0–2.5 ED_{50}) of the standard FLV lot developed palpable spleens as early as 7–10 days after infection, whereas mice infected with low doses (1.0–1.5 ED_{50}) of the FLV lot required 15–56 days for splenomegaly to occur.

In the analysis of some of our experiments, we waited until 100% of the Friend control mice infected with a particular Friend leukemia virus dose developed splenomegaly before conclusions were drawn about protection by the treating arboviruses.

Protection against splenomegaly by infectious Cocal virus. In preliminary experiments, not reported here, 7 logs (PFU/0.2 ml) of infectious Cocal virus killed DBA/2J weanling mice. Experiments were repeated wherein 2 and 4 logs (PFU/0.2 ml) of Cocal virus were employed as the interfering arbovirus in FLV-infected mice. Both virus doses gave comparable results when assessing the

[2] The Nalge Company, Rochester, New York.

[3] Colab Laboratories, Inc., Box 66, Chicago Heights, Illinois 60411.

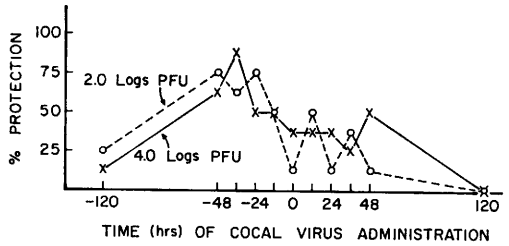

FIG. 1. Effect of two different infectious Cocal virus doses at various time intervals on the inhibition of Friend leukemia virus-induced splenomegaly 14 days after Friend leukemia virus inoculation. Percentage of protection is based on the percentage of mice with spleen weights below 50% of the control Friend leukemia virus-induced splenomegaly mean (16 mice per group.)

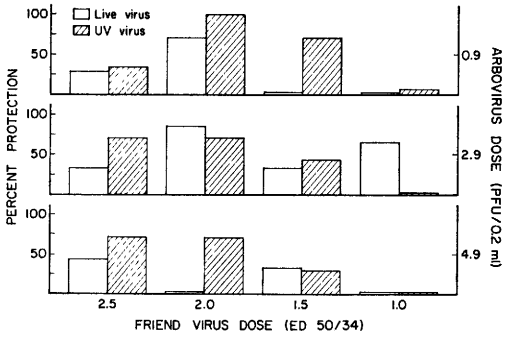

FIG. 2. Antileukemic action of infectious and uv-inactivated Cocal virus at three Cocal virus concentrations against four different Friend leukemia virus doses at a time when 100% of the Friend leukemia virus controls exhibit splenomegaly.

percentage (62–87%) of mice having spleen weights below ½ of the control FLV average (See Fig. 1). Those groups of mice demonstrating the greatest reduction in spleen enlargement were mice receiving Cocal virus 36–48 hr prior to FLV.

Use of UV-irradiated Vero cell-propagated Cocal virus for inhibiting FLV-induced splenomegaly. A suspension of Vero-propagated Cocal virus stock in unheated calf serum, containing $10^{5.9}$ PFU/0.2 ml (in LLC-MK_2 cells) was irradiated under a 15-W germicidal lamp for 4 min at a distance of 4 cm, resulting in a total loss of infectivity as measured in LLC-MK_2 cell cultures. In the experiments, mice were inoculated with 10^{-1},- 10^{-3},- and 10^{-5}-fold dilutions of the infectious Cocal virus suspension, calculated to contain 4.9 logs, 2.9 logs, and 0. 9 log PFU/0.2 ml in LLC-MK_2. The UV-treated undiluted virus was similarly diluted and used as virus inocula.

All Cocal-treated mouse groups were inoculated with the arbovirus 36 hr prior to inoculation with various FLV doses (2.5, 2.0, 1.5, 1.0, and 0.5 ED_{50} per 34 days).

The antileukemic action of infectious and UV-inactivated Cocal virus at four Cocal dosages may be seen in Fig. 2. This is the percentage of protection (absence of splenomegaly) seen at a time when the FLV controls are 100% positive.

At FLV doses of 2.5, 2.0, and 1.5 logs ED_{50} per 34 days, the greatest protection (100%) against splenic enlargement was seen with the smallest dose of UV-treated Cocal virus. This did not hold true for the infectious Cocal virus where best protection was seen with 2.9 logs Cocal virus. Protection ranging from 0 to 100% was seen with the UV-treated Cocal virus, whereas 0–87% protection was given with infectious Cocal virus. In most Cocal–Friend combination regimens, the UV-treated Cocal virus conferred more protection than did the infectious virus. Four out of twelve infectious Cocal regimens failed to protect, whereas protection was seen with 10 of 12 regimens with the UV-treated Cocal virus. Delay in the latent period to splenomegaly was short, from 0 to greater than 25 days. Protection was of greater duration with UV-treated Cocal virus (Table I).

The protection of Cocal virus against FLV-induced splenomegaly depicted in Fig. 2 was protection based on the incidence of splenomegaly in the FLV controls. What was actually done in this experiment was the titration of FLV in the presence of three different Cocal doses. The log ED_{50} was calculated against progressing observation time (Table II), and a picture of early protection by UV-irradiated Cocal resulted with a loss of this protection in time.

When the $ED_{50}/_t$ range of the Cocal (infectious and UV-irradiated)-treated mice was plotted with respect to the control FLV (Fig. 3) one visually sees this protection be-

TABLE I. Duration of Protection Offered by Cocal Virus Against Friend Leukemia Virus.

Cocal dose (PFU/0.2 ml)	Friend dose (ED_{50}/34 days)	Infectious Cocal		Uv-irradiated Cocal	
		Range[a]	Days protection	Range	Days protection
None		25–34	—	25–34	—
0.9	0.5	25–27	0	25–27	0
2.9		25–>46	>12	25–>46	>12
4.9		25–>46	>12	25–46	12
None		18–25	—	18–25	—
0.9	1.0	18–25	0	18–34	9
2.9		18–>46	>21	25–25	0
4.9		18–25	0	18–25	0
None		14–18	—	14–18	—
0.9	1.5	14–18	0	14–43	25
2.9		18–25	7	18–27	9
4.9		14–25	7	14–25	7
None		11–14	—	11–14	—
0.9	2.0	11–14	0	13–14	0
2.9		12–25	11	11–18	4
4.9		11–12	0	11–25	11
None		11–11	—	11–11	—
0.9	2.5	11–13	2	11–25	14
2.9		11–14	3	11–25	14
4.9		11–18	7	11–14	3

[a] Ratio of time (days) splenomegaly first develops to time (days) 100% mice develop splenomegaly.

TABLE II. Effect of Infectious and UV-Irradiated Cocal Virus on Friend Virus $ED_{50}/_t$.

Cocal treatment	ED_{50}/days				
	11	14	18	25	36
Untreated					
Friends control	1.5	1.6	2.5	2.9	3.5
Treated					
Infectious virus					
0.9 logs[a]	1.2	1.9	2.5	3.4	3.5
2.9 logs	1.2	1.5	2.2	3.3	3.4
4.9 logs	1.5	1.7	2.2	3.0	3.1
UV-inactivated virus					
0.9 logs	0.8	0.9	1.5	3.2	3.5
2.9 logs	0.8	0.8	1.8	2.9	3.2
4.9 logs	1.2	1.8	2.2	3.2	3.4

[a] Logs PFU/0.2 ml in Vero cells.

tween 11 and 18 days and the fading away of this protection between 18 and 36 days.

Sparing or potentiation of mortality in mice administered FLV and Cocal virus. The effect of Cocal dosage alone and in combination with FLV are tabulated (for three FLV doses only) in Table III. It is important to note that mice singly infected with 2.5 logs ED_{50}–FLV begin dying of leukemia 4 days earlier than mice infected with the same FLV dose plus a nonkilling dose of infectious Cocal virus. This sparing effect of low Cocal dosage, however, did not apply when lower FLV doses (1.5 and 0.5 ED_{50}) were used; with 1.5 logs ED_{50}–FLV, death from leukemia occurred 8 days earlier in the dually infected mice. Death did not occur in mice with single virus doses of either FLV (0.5 logs ED_{50}) or infectious Cocal (0.9 log PFU/0.2 ml). When these two nonkilling virus doses were given together, 43% of the mice died. Hence, depending on the FLV dosage, one may see either a sparing effect of an arbovirus or potentiation of the killing effect.

If the LD_{50} values of the FLV control mice and UV-irradiated Cocal-treated mice are

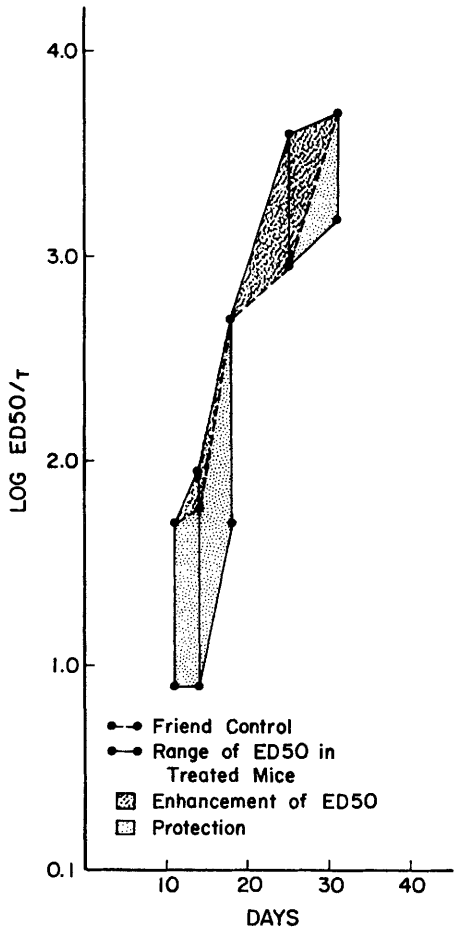

FIG. 3. Reduction and potentiation of Friend leukemia virus $ED_{50}/_t$ by Cocal virus (infectious and uv-irradiated.)

calculated (Table IV), one again sees that the small amount of protection as measured by the LD_{50} calculation occurred between 15 and 22 days. Protection was negated by Day 25, however, and potentiation of the LD_{50} endpoint occurred.

Neither the 0.9 log nor 4.9 log doses of UV-irradiated Cocal significantly influenced the FLV LD_{50} but in the presence of an infectious Cocal dose of 0.9 log PFU/0.2 ml or a 2.9 log dose of inactivated Cocal, the FLV LD_{50} is increased from 0.9 log to 2.4 logs at 36 days post-Friend inoculation.

Discussion. The experiments presented here have demonstrated and confirmed the use of viral interference as a method for the temporary suppression of murine leukemia. Although it has been reported that lymphocytic choriomeningitis virus (1, 2) and Sendai (1) virus interfere with murine leukemia viruses, this is the first report on the successful use of an arbovirus for inhibition of a virus-induced leukemia. This work is a direct extension of the experiments (3) wherein arboviruses exerted a cytolytic effect on L5178Y murine lymphoblasts *in vitro*. Arboviruses have also been reported to inhibit nonviral-associated transplantable AK4 and 9421 murine leukemias (10–12).

The delay in latent period to appearance of splenomegaly was dependent on the dose and state (infectious or inactivated) of the Cocal virus. Arbovirus infectivity was not a requirement for initiation of protection against leukemogenesis, since UV-irradiated Cocal virus exerted as great a degree of protection as did infectious Cocal virus.

The interfering activity of UV-irradiated Cocal virus now allows us to pursue the arbovirus–Friend leukemia virus interference problem with the knowledge that paralysis or death due to the interfering arbovirus during the experiment will not occur.

This lack of a requirement for infectivity of the interfering virus suggests that a chemically mediated inhibitor may be involved. Reports have been published (13, 14, 16) suggesting that suppression of FLV splenomegaly might be attributed to the inhibitory effects of interferon.

Preinoculation with Cocal virus reduced significantly the splenomegalic response of mice to subsequent infection with FLV. The greatest interference (reduction of splenomegaly) was seen when Cocal virus was administered 24–48 hr prior to FLV. Whereas administration of the Cocal virus 120 hr after Friend inoculation completely negated the splenomegaly inhibition effect, virus given 12–48 hr after FLV resulted in some degree of antileukemic effect. Wheelock found (13) that repeated administration of interferon or an injection of Statalon prolonged the life of mice with established FLV-induced leukemia. In interference studies with nonarboviruses (Sendai and LCM) and murine leukemia viruses (1, 2) inhibition of splenomegaly was dependent on inoculation of the virus prior

TABLE III. Effect of Infectious Cocal Virus on Mortality of Mice Dually Infected with Friend and Cocal Virus.

Virus	Dose	% Mortality at given day											
		14	18	22	25	32	34	35	36	39	41	43	46
Controls													
Cocal[a]	0.9	0	→										
	2.9	43	57	→									
	4.9	29	29	43	→								
Friend[b]	0.5	0	→										
	1.5	0	→							57	71	71	71
	2.5	0	14	29	29	43	43	43	43	57	57	57	71
Dual infection													
Friend dose[b]	Cocal dose[a]												
	0.9	0	0	0	0	43	43	43	43	43	43	43	43
0.5	2.9	43	43	43	43	57	57	57	57	57	57	57	57
	4.9	14	14	14	14	28	28	28	28	28	28	28	28
	0.9	0	0	0	43	43	57	57	57	57	57	57	71
1.5	2.9	14	14	14	14	14	43	43	43	57	57	57	88
	4.9	14	14	14	28	28	28	28	43	43	43	43	43
	0.9	0	0	14	28	28	43	43	43	57	57	71	71
2.5	2.9	14	14	28	43	43	43	43	43	43	57	57	57
	4.9	0	0	57	71	71	71	71	71	71	71	71	100

[a] Dose given as PFU/0.2 ml.
[b] Dose given as ED_{50}/34 days.

TABLE IV. Effect of Cocal Virus on Friend Virus LD_{50}.

Cocal treatment	LD_{50}/days											
	15	18	22	25	32	34	35	36	39	41	43	46
None												
Friend control	0.0	0.6	0.8	0.8	0.9	0.9	0.9	0.9	1.6	1.8	1.8	1.9
Treated												
Infectious virus												
0.9 logs[a]	0.0	0.0	0.0	1.1	1.7	1.8	1.9	1.9	2.1	2.2	2.2	2.5
UV-inactivated virus												
0.9 logs	0.0	0.0	0.8	0.8	1.1	1.1	1.1	1.1	1.4	1.5	1.8	2.2
2.9 logs	0.6	0.6	0.8	0.8	1.6	1.8	1.9	1.9	1.9	1.9	1.9	2.1
4.9 logs	0.0	0.0	0.6	0.8	1.0	1.3	1.3	1.3	1.4	1.7	1.8	1.8

[a] Logs PFU/0.2 ml in Vero cells before inactivation by uv.

to the murine leukemic virus, but in these same studies, no leukemia-interfering effect was seen when the antileukemic virus was inoculated after the leukemogenic agent.

The interfering effect of Cocal virus was dependent on the concentration of the leukemia virus. When infectious Cocal virus was used as the interfering agent, 2.9 logs of Cocal exerted a greater antileukemic effect than either the high arbovirus (4.9 logs) or low arbovirus dose (0.9 logs). From this we can interpret that a specific level of infection is required for a protective effect against leukemia.

When the treating arbovirus was inactivated by ultraviolet irradiation, Cocal vi-

rus dose was no longer of paramount importance for antileukemic effect. Within limits (leukemia doses of 1.0, 1.5, and 2.0 $ED_{50}/_{34}$ days), the protective effect of inactivated Cocal virus was inversely proportional to the concentration of the leukemic dose, *i.e.*, best protection against splenomegaly was seen at the highest leukemic doses. The antileukemic effect of the inactivated Cocal virus when used in conjunction with the high leukemia doses did not significantly diminish, despite a 4-log dilution of the arbovirus inoculum.

Inoculation of DBA/2J mice with Cocal virus induced significant temporary protection against an otherwise rapid development of leukemic disease due to inoculation with FLV. This was evident by a delayed latent period (25 days) to splenomegaly. By no means, however, was protection permanent or of long duration. In most cases the protected mice eventually developed splenomegaly or died of leukemia. The LD_{50} of the FLV was potentiated by either the 2.9-log inactivated or 0.9-log infectious Cocal virus, but not by the 0.9 or 4.9 inactivated Cocal doses. The LD_{50} enhancing effect of the 2.9 and 4.9 infectious Cocal virus doses could not be determined, since deaths due to the Cocal virus did occur with these doses.

The data presented here demonstrate both suppression and enhancement of FLV-induced splenomegaly, depending on the dose of both the Friend leukemia and Cocal viruses. Enhancement of FLV-induced splenomegaly with Guaroa virus (15) and Poly I: Poly C (16) has been reported.

This finding that an arbovirus interferes with virus-induced leukemia will allow investigators to pursue more than 250 additional arboviruses which have not yet been explored for their interfering effect against virus-induced leukemias. The interfering effect of Cocal virus in mice and the failure of this virus to naturally produce overt disease in man, in spite of apparent infection means that Cocal virus may be a potential therapeutic agent in human leukemia, used either alone or in conjunction with interferon, known antileukemic drugs, and other dietary (17) measures (*e.g.*, thiamin-free diet) used for controlling leukemia.

In some of these experiments there have been some arbovirus-treated FLV-infected mice that never came down with leukemia, but the small numbers defy significance. Most of the treated leukemic mice died of leukemia, but the interference established by a single arbovirus dose in these experiments is sufficiently encouraging to justify continuation of the experiments.

Summary. In studying the inhibitory effect of a single infectious or UV-inactivated arbovirus (Cocal) dose on Friend leukemia virus-induced leukemia in DBA/2J mice, the greatest degree of interference (reduction of splenomegaly) occurred when Cocal virus was administered 24–48 hr prior to Friend leukemia virus. The percentage and duration of splenomegaly inhibition was dependent on the dose and state (infectious or inactivated) of the Cocal virus.

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