Interferon and Murine Leukemia. I. Inhibitory Effect of Interferon Preparations on Development of Friend Leukemia in Mice.* (31672)

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Interferon(1) has been shown to inhibit both the multiplication of oncogenic viruses and the cellular transformation induced by these viruses *in vivo* and *in vitro* (2,3,4,5,6,7, 8,9). To our knowledge the effect of interferon on the murine leukemia viruses has not been investigated. We report here the inhibitory effect of potent preparations of interferon on the evolution of Friend disease in mice.

Methods and materials. Viruses. Friend virus was obtained as 53rd mouse passage thru the courtesy of Dr. Raymond Latarjet. Viral suspensions were prepared by grinding leukemic spleens with phosphate buffered saline (PBS) (10 or 20% suspension) in a chilled mortar. After centrifuging twice at 2,000 rpm, the supernatant was utilized either immediately or sealed in ampoules and stored at −60°C. It was passaged and titered by intraperitoneal (i.p.) injection (0.2 ml) of 4-6-week-old Swiss mice. The average spleen weight of normal uninoculated mice was 170 mg, and the range was 100 to 250 mg. Spleen weight served as a criterion of infection(10) and weights above 350 mg were considered indicative of disease (titer was expressed as spleen dose SD_{50}). In all instances microscopic examination of the spleen confirmed this assumption. At one month the titer of different viral preparations varied between log 2.5 and 3.5 SD_{50}/ml (i.p.). *West Nile Virus* was obtained thru the courtesy of Dr. N. B. Finter as a 10% suspension of infected mouse brain. It was passaged by intracerebral inoculation of young adult Swiss on Institut du Cancer (I.C.) mice (titer log 6.5 LD_{50}/0.03 ml. *Vesicular Stomatitis virus* (V.S.V.), *Encephalomyocarditis virus* (E.M.C.) and *Newcastle Disease virus* (N.D.V.) were stock laboratory strains, passaged numerous times in mouse embryo fibroblasts (V.S.V.) (E.M.C.) or in the allantoic cavity of the embryonated egg (N.D.V.). Titrations of V.S.V. (log 7.0 PFU/ml) and E.M.C. (log 8.6/ml) were performed by standard plaque assay procedures. Titration of N.D.V. was performed either by plaque assay on monolayer cultures of chick embryo fibroblasts (log 9.3 PFU/ml) or by inoculation of the 9-10-day-old embryonated egg.

Cell culture. Primary and secondary culture of mouse embryo fibroblasts were prepared by standard techniques. A continuous line of mouse L cells was obtained thru the courtesy of Dr. André Lwoff. Cultured cells were maintained in a medium consisting of 10% heat inactivated calf serum and a modified Eagle's balanced salt solution.

Preparation of interferon. Mouse brain interferon was prepared by the technique of Finter(11,12,13). Weanling Swiss or I.C. mice were inoculated intracerebrally (i.c.) with a 10^{-1} dilution of stock West Nile Virus. When 10-20% mortality was observed, the remaining sick mice were killed by cervical spine extension, the brains removed immediately and placed in an ice chilled beaker. Brains were ground in a mortar or in an electric mixer and extracted 4 times with 2 ml, 1 ml, 0.5 and 0.5 ml of cold PBS/brain. After centrifugation at 2,500 rpm for 20 minutes the supernatant was treated at pH 2 for 18 hours and then centrifuged at 27,000 rpm for 17 hours in a Spinco L_1 centrifuge rotor 30 (80,000 *g*). The pH of the supernatant was adjusted to 7. To increase the interferon titer, preparations were concentrated by pressure dialysis. As a control, normal mouse brain was obtained from mice inoculated i.c. with PBS and the techniques of extraction were identical to those employed in the preparation of mouse brain interferon. In one experiment interferon was prepared from sera and spleens of mice inoculated with

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N.D.V.(14). The spleens were ground with 3 ml PBS/spleen and the pool of serum and spleen was treated as described above for mouse brain interferon. The pooled sera and spleens of uninoculated mice constituted the control preparation. All control and interferon preparations were tested for fungal and bacterial contamination. Susceptible mice were inoculated intracerebrally with an aliquot of each interferon preparation to determine the presence of residual West Nile Virus. In no instance was infectious virus recovered. The protein concentration of each preparation was determined by the method of Lowry(15). Preparations of interferon or normal mouse brain were distributed in 60 ml pyrex glass bottles in quantities sufficient only for the daily dose of interferon and stored at —60°C until utilized in the ensuing few days. A stock of interferon and control mouse brain was prepared for each experiment. In those experiments in which 2 different preparations of interferon were employed, the interferon titers were comparable.

It was shown that preparations of interferon resisted pH 2, were not dialyzable, and were not sedimented by centrifugation at 80,000 *g* for 17 hours. They inhibited the multiplication of several challenge viruses (V.S.V. *in vitro* and E.M.C. *in vivo*) provided the assay was performed in cells of mouse origin. They did not inhibit V.S.V. as assayed in monolayer cultures of chick embryo cells.

Assay of interferon. Two-fold dilutions of the test preparation were incubated with monolayer cultures of mouse embryo fibroblasts (MEF) or L cells, in plastic petri dishes (2 ml/dish). After incubation for 18 hours at 37°C, test material was removed and the cell sheet washed once with PBS. Test cultures were incubated with 50-100 PFU (0.2 ml) of V.S.V. for 1 hour at 37°C before addition of agarose-medium overlay. Interferon titers were expressed as the dilution/2 ml of the original preparation responsible for a 50% reduction in the number of plaques as compared to virus control cultures(16). Preparations of interferon utilized in each experiment were assayed several times. It was found that a given preparation of interferon varied at least 2-fold in titer in assays at different times under apparently similar conditions. Control normal mouse brain preparations did not inhibit V.S.V. at the initial dilution of 1:25.

Animals. A colony of Swiss mice was established at the Institut du Cancer with the assistance of Dr. Raymond Latarjet. DBA_2 and IC mice were supplied from the mouse colony of the Institut du Cancer.

A table of random permutations(17) was utilized in the distribution of homogenous (age and weight) lots of male and female mice (4-6 weeks) into standard mouse cages (6-8 mice/cage). At the termination of each experiment the weight and location of the cage of the animals was analyzed. There did not appear to be any relationship between these factors and the development of splenomegaly in infected mice. There was no significant difference in the weight of mice in different groups at the termination of the experiment.

Statistical analysis. The experimental results were analyzed by Mr. Philippe Lazar and Mrs. Suzanne Guéguen of the Unité de Recherche Statistique de l'Institut National de la Santé et de la Recherche Médicale. Standard tests of analysis of variance and comparison of means were employed. Calculations were based on logarithmic values of spleen weights. A distribution free method (Wilcoxon test) was employed when only a few animals were included in the experiments.

Results. The pathogenesis of Friend virus in mice has been previously described(18,19, 20). In the Swiss mice employed in the experiments to be described, Friend virus induced a characteristic and marked splenomegaly, usually more than 1000 mg, one month after viral inoculation. All mice however did not respond in a uniform manner. Ten-fifteen percent of the mice inoculated with Friend virus presented only limited splenomegaly (260-400 mg).

Microscopic examination of the spleen of mice with advanced disease revealed a loss of normal splenic architecture with replacement of much of the parenchyma by large irregularly shaped cells containing vesicular

TABLE I. Effect of Mouse Brain-Interferon on Development of Splenomegaly in Friend Disease of Swiss and DBA_2 Mice.

		NO VIRUS	VIRUS (1)	VIRUS + PBS	VIRUS + NORMAL MOUSE BRAIN	VIRUS + MOUSE BRAIN INTERFERON
EXP. I, 3 weeks	N° mice	7	7			7 (2)
	g.m. spleen w.	**117mg**	**759mg**			**363mg**
	m ± s. e.	2.07 ± 0.01	2.88 ± 0.03			2.56 ± 0.14
EXP. I, 5 weeks	N° mice	6	8			6 (2)
	g.m. spleen w.	**143mg**	**1100mg**			**471mg**
	m ± s. e.	2.16 ± 0.02	3.04 ± 0.04			2.67 ± 0.10
EXP. II	N° mice	14	15	15	14	18 (3)
	g.m. spleen w.	**190mg**	**598mg**	**607mg**	**638mg**	**410mg**
	m ± s.e.	2.28 ± 0.02	2.78 ± 0.08	2.78 ± 0.08	2.81 ± 0.09	2.61 ± 0.09
EXP. III	N° mice	5	13		13 (4)	13 (5)
	g.m. spleen w.	**150mg**	**704mg**		**520mg**	**267mg**
	m ± s. e.	2.18 ± 0.04	2.85 ± 0.09		2.72 ± 0.11	2.43 ± 0.06

EXP. I: Swiss mice, sacrificed at 3 and 5 wk after viral inoculation.
EXP. II: Swiss mice sacrificed at 4 wk after viral inoculation.
EXP. III: DBA_2 mice sacrificed at 3½ wk after viral inoculation.

(1) Virus 0.2 ml I.P. undiluted Friend virus (log 3.4 SD_{50}/ml) in Exp. I and II and log 3.0 SD_{50}/ml in Exp. III.
(2) Titer of interferon employed: 1:12.800/2 ml (MEF-plaque).
(3) Titer of interferon employed: 1:6.400/2 ml (MEF-plaque).
(4) Protein concentration 5.7 mg/ml.
(5) Titer of concentrated interferon employed: 1:64.000/2 ml (MEF-plaque). Protein concentration: 5.8 mg/ml.

g.m. = Geometric mean. m ± s.e. = Geometric mean in logarithms ± standard error.

nuclei with clumped chromatin at the periphery of the nucleus. A close correlation was observed between the size of the spleen and the degree of Friend cell involvement.

During the usual period of observation (4-6 weeks) only a negligible mortality attributable to the disease was encountered. A slight elevation in the number of circulating leucocytes (10,000-15,000/mm^3) and a slight anemia (hematocrit 38-40) were characteristic. "Friend cells" were observed in Giemsa stained preparations of peripheral blood. As a criterion of infection the increased weight of the liver and leucocytosis were less satisfactory than spleen weight.

Experiments I, II. Inoculation of mouse brain interferon intraperitoneally in Swiss mice once a day. Mice were inoculated intraperitoneally (0.25 ml) with mouse brain interferon, normal mouse brain, or PBS 24 and 3 hours prior to inoculation of Friend virus, and daily thereafter. In Experiment I, (Table I) the difference at 3 weeks between the geometric mean of the spleen weights of untreated leukemic mice (759 mg) and interferon treated mice (363 mg) was not considered significant. At 5 weeks however the difference was considered significant ($p = .02$ by Wilcoxon test).

In Experiment II (Table I) there was no significant difference between the geometric mean spleen weight of mice receiving Friend

virus alone; virus and daily inoculations of PBS; or virus and daily inoculation of a preparation of normal mouse brain. Thus neither the trauma nor possible metabolic derangements attendant upon daily inoculation of 0.25 ml influenced the development of splenomegaly. Although the difference between the interferon treated group and the virus control groups appeared encouraging, it was not considered statistically significant ($p = .10$).

Experiment III. Inoculation of concentrated mouse brain interferon intraperitoneally in DBA_2 mice twice daily. These results suggested that a more marked inhibition of splenomegaly might be achieved by a concentration of the interferon preparation and by a more frequent administration of interferon.

Accordingly, one-month-old DBA_2 mice were inoculated with a concentrated preparation of interferon or concentrated normal mouse brain 24, 17 and 3 hour before viral inoculation and twice daily thereafter (at periods separated by at least 7 hours). No significant difference at 3½ weeks (Table I) was noted between the 2 virus control groups. Administration of the concentrated preparation of interferon twice a day significantly ($p = .01$) inhibited the splenomegaly induced by Friend virus.

Experiment IV. Inoculation of concentrated mouse brain interferon intraperitoneally in Swiss mice twice daily. It was of interest therefore to compare within the same experiment the efficacy of a concentrated preparation of interferon to the standard unconcentrated interferon. As in Exp. III, preparations of interferon and normal mouse brain were inoculated intraperitoneally (0.2 ml) 24, 17 and 3 hours prior to viral inoculation and twice daily thereafter. There was no significant difference between the various control groups 1 month after viral inoculation (Fig. 1). Although unconcentrated interferon (titer 1:6400) did not inhibit splenomegaly to a significant degree, a concentrated preparation of interferon (titer 1:67,500) was shown to exert a marked inhibitory effect on the development of splenomegaly ($p = .0001$).

At autopsy ascites was observed in several mice inoculated with the concentrated preparation of interferon. Fluid was present in greatest amounts in those mice whose spleens were most involved by Friend disease.

Experiment V. Inoculation of serum-spleen interferon intraperitoneally in Swiss mice twice a day. The design of Exp. V differed from the preceding experiments as follows: a) the source of interferon was a mixture of sera and spleens of mice inoculated with N.D.V. Thus both the inducing virus and the tissue origin of the interferon were different from those previously employed. b) 2 groups of mice received interferon or normal serum-spleen extract (0.2 ml) twice daily for *only 3 days,* on the day prior to viral inoculation, on the day of viral inoculation and on the day following viral inoculation. Two other groups of mice also received serum-spleen interferon or normal serum-spleen extract at these times but in contrast to the 3-day group, treatment was *continued* twice daily thereafter.

As can be seen from Fig. 2, there was no significant difference one month after inoculation of Friend virus between the various viral control groups. Treatment with interferon for *only* 3 days, even though preceding viral inoculation, did not significantly alter the development of splenomegaly. However, when this treatment was continued throughout the test period a significant ($p = < .0001$ inhibition of splenomegaly was observed.

Ascites was present in all mice receiving interferon daily throughout the experiment. This was not unexpected in view of the greater concentration of protein in the serum-spleen interferon preparation than in concentrated preparations of mouse brain interferon. As observed in the preceding experiment, those mice with the greatest amount of peritoneal fluid presented the most marked splenic involvement. Ascites was not observed in mice inoculated with normal serum-spleen extract.

Experiment VI. Inoculation of concentrated mouse brain interferon intravenously in Swiss mice. Mice were inoculated (0.2 ml) with concentrated mouse brain interferon (titer 1:25,600) or concentrated normal mouse brain, *intravenously* and *intraperitoneally* 24

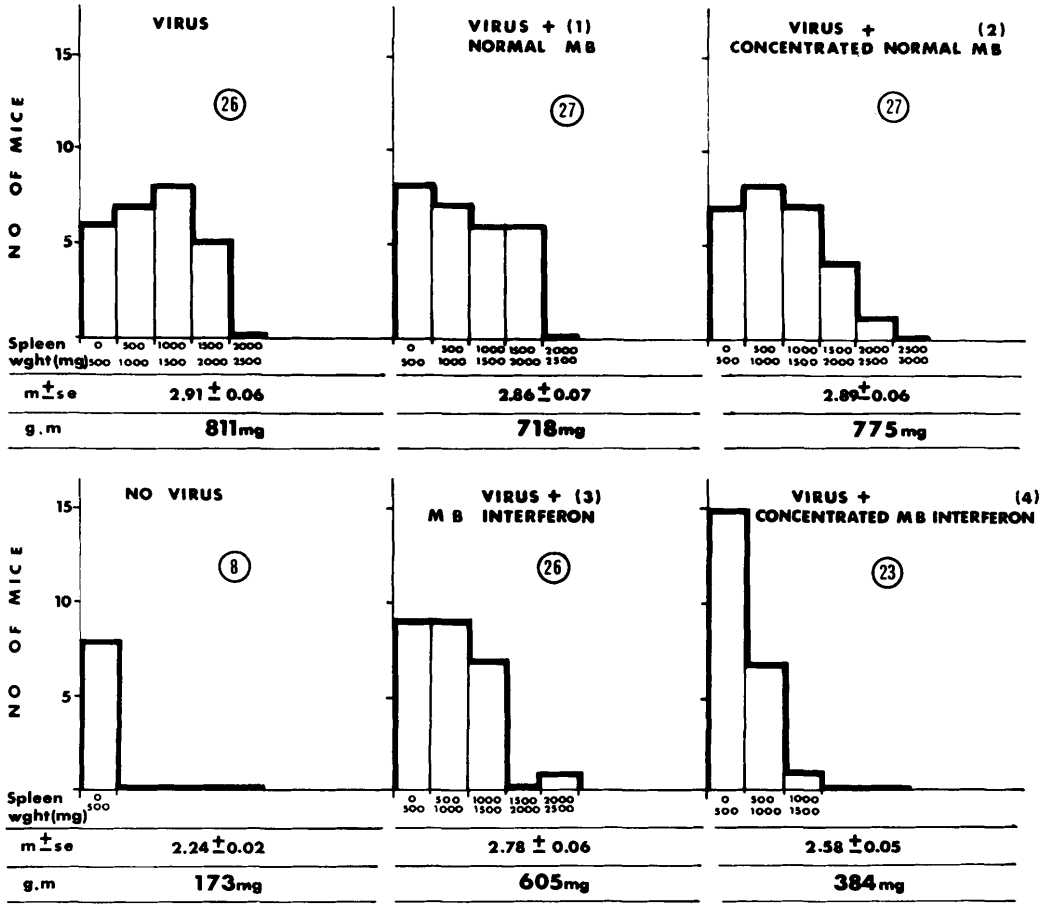

FIG. 1. Effect of concentrated and unconcentrated preparations of interferon on development of splenomegaly in Friend disease. Exp. 4. Virus = Friend virus undiluted 0.2 ml inoculated i.p. 0 : No of animals/group; M. B. = Mouse brain; (1) protein concentration 1.6 mg/ml; (2) protein concentration 5.3 mg/ml; (3) interferon titer 1:6,400/2 ml; (plaque-cell L) protein concentration 2.4 mg/ml; (4) interferon titer 1:67,500/2 ml; (plaque-cell L) protein concentration 8.2 mg/ml; G. M. = geometric mean of spleen weight. M ± S.e. = logarithmic mean ± standard error.

and 3 hours prior to inoculation of Friend virus. Thereafter these mice were inoculated daily only *intravenously* with 0.2 ml of interferon (titer 1:12,800) or normal mouse brain extract. At termination of the experiment (1 month) no significant difference in the geometric mean spleen weight of any of the groups was observed (virus control 589 mg; virus and concentrated normal mouse brain 794 mg, virus and concentrated mouse brain interferon 759 mg).

Discussion. The results of these experiments demonstrate that daily administration of mouse brain interferon of high titer (>1:12,000/2 ml) retarded the development of splenomegaly induced by Friend virus in Swiss and DBA_2 mice. There was a direct correlation between the interferon titer, as assayed *in vitro* (V.S.V) or *in vivo* (E.M.C.) and the inhibitory effect of this preparation on the development of Friend disease. This inhibitory effect was observed not only after administration of mouse brain interferon induced by West Nile Virus, but also after inoculation of an interferon preparation derived from the sera and spleens of mice infected with N.D.V. Extracts of normal mouse brain and normal mouse serum-spleen neither inhibited nor enhanced the development of splenomegaly in viral infected mice.

It is generally accepted that interferon is most effective when administered prior to, or

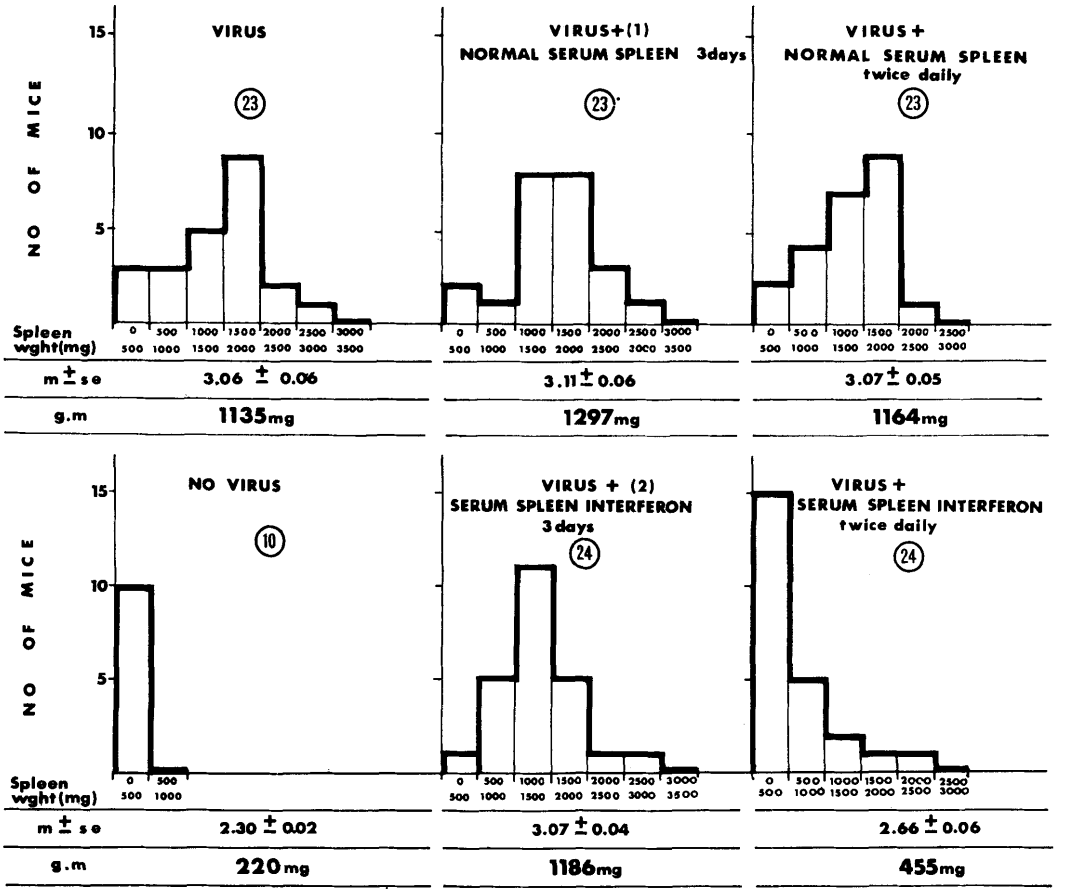

FIG. 2. Effect of serum-spleen interferon on development of splenomegaly in Friend disease. Exp. 5. Virus = Friend virus undiluted 0.2 ml inoculated i.p.; 0 = No. of animals/group; (1) = protein concentration 15 mg/ml; 2 = interferon titer = 1:14.000/2 ml (MEF - plaque assay) protein concentration = 15 mg/ml; G.M. = geometric mean; M ± S.e. = logarithmic mean ± standard error.

at the time of viral inoculation(21,22). To our knowledge no significant anti-viral activity has been observed *in vivo* when interferon has been administered more than several hours after viral infection has been established. Thus in the experiments of Lampson and his coworkers(6) interferon treatment initiated 6 hours after inoculation of Rous sarcoma virus "was without measurable effect." In our experiments preparations of interferon were injected prior to viral infection and daily thereafter. Two findings indicated that it was necessary to continue treatment of mice *after* viral inoculation in order to achieve a significant inhibitory effect. In one experiment interferon was administered to one group of mice twice daily for *only 3 days,* whereas another group of mice received interferon twice daily throughout the experiment. Marked inhibition of splenomegaly was observed only in the latter group of mice.

Secondly, in 2 experiments it was observed that ascites was present in several mice receiving concentrated interferon preparations and the spleens of these mice were much larger than those of the interferon treated mice which did not have ascites. Since interferon was recovered in large amounts from this ascitic fluid, one may speculate that the daily interferon dose inoculated i.p. had not diffused out from the peritoneum and had therefore been ineffective in inhibiting progressive splenomegaly.

In Friend disease infectious virus can be

consistently recovered in large amounts from the neoplastic spleen. If new foci of cellular transformation in this organ are engendered by successive cycles of viral multiplication it seems reasonable to continue interferon treatment beyond the period of viral inoculation. Thus if interferon were administered *only* for a few days, synthesis of virus would be inhibited initially but thereafter viral multiplication might ensue unchecked. In this respect Friend viral infection appears to differ from several other oncogenic virus-host systems in which cellular transformation is either not accompanied by synthesis of infectious virus *in vivo* (adenovirus 12(23)) or in which the presence of small amounts of infectious virus after an initial period of viral multiplication appears irrelevant to a progressive neoplastic process (polyoma virus(24)). Thus one might predict that administration of interferon might be ineffective *after* inoculation of polyoma or adenovirus 12 but might be effective if administered several days *after* infection with Friend virus. Experiments are in progress to determine the validity of this hypothesis.

When a concentrated preparation of interferon was inoculated intravenously, rather than intraperitoneally, no difference was discerned between interferon treated and untreated mice. Since the interferon titer of the preparation utilized was comparable to the interferon titer of preparations employed in other experiments, one may speculate that the inefficacy of interferon treatment was related to the intravenous route of administration. This problem will be discussed at more length in the succeeding article.

We have employed the term *preparations of interferon* rather than interferon, since there was no proof that the inhibition of splenomegaly observed in interferon treated mice was mediated by the same anti-viral factor that was assayed *in vitro* and *in vivo* employing V.S.V. and E.M.C. as challenge viruses. It is possible that inhibition of splenomegaly was achieved by mechanisms as yet undetermined. Experiments utilizing semi-purified preparations of interferon may clarify the role of interferon in this phenomenon.

Summary. Administration of concentrated preparations of interferon of high titer (extracted from either mouse brain or serum and spleen) inhibited the development of splenomegaly induced by Friend virus in Swiss and DBA_2 mice. Interferon was effective *only* when treatment was continued daily throughout the duration of the experiment, and when inoculated intraperitoneally (rather than intravenously).

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Interferon and Murine Leukemia. II. Factors Related to the Inhibitory Effect of Interferon Preparations on Development of Friend Leukemia In Mice. (31673)

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In the preceding communication we presented the results of experiments demonstrating an inhibitory effect by interferon preparations on the development of Friend leukemia in mice. We present here an analysis of various factors pertaining to the demonstration of this phenomenon.

Materials and methods. The viruses employed and their methods of assay, the techniques of cell culture, the preparation of interferon and its assay, the different strains of mice employed, have all been described in the preceding communication.

In some interferon assays a tube dilution technique was also employed. Two-fold dilutions of interferon in nutritive medium were incubated with L cells for 18-24 hours at 37°C. The medium was then removed, the cell cultures washed once with PBS, and medium containing 100 $TCID_{50}$ of vesicular stomatitis virus (V.S.V.) was added. The interferon titer was expressed as the highest dilution which protected more than 75% of the cell sheet at a time when complete cell destruction was observed in control cultures.

Diffusion of interferon after intraperitoneal (i.p.) and intravenous (i.v.) inoculation of interferon. Mice were inoculated i.p. with 0.25 ml of an interferon preparation. At intervals thereafter 2.25 ml of PBS were inoculated i.p. and light pressure applied to the abdomen to ensure dispersion of the fluid. After brief exposure to ether, the mice were exsanguinated by section of the carotid vessels and the blood collected for assay of serum interferon. The abdominal fluid was aspirated and centrifuged at 1500 rpm for 10 minutes to sediment the peritoneal macrophages. The supernatant was stored at −20°C prior to interferon assay. Spleen, kidney and liver to be tested for interferon were washed twice in 20 ml of PBS and frozen immediately in dry ice(1). A 10% (wet weight) tissue suspension was prepared in PBS, treated at pH 2, centrifuged at 110,000 *g* for 1 hour, and the pH of the supernatant adjusted to 7.

Results. Capacity of Friend virus to induce interferon. To determine whether Friend virus induced the formation of interferon, Swiss and IC mice were inoculated i.v. with 0.2 ml of undiluted virus (log 3.5 SD_{50}/ml). Equivalent numbers of mice were inoculated with NDV (log 9.0 PFU/ml) (positive control). Small quantities of an interferon-like substance (titer 1:10-1:40) were detected in both the serum and spleen of Swiss and IC mice, 24 but not 5 hours after inoculation of Friend virus.*

Large quantities of interferon were detected in mice inoculated with NDV (5 hours, 1:5120; and 24 hours 1:1280 in serum and spleen).

* The imprecise *in vivo* assay of Friend virus renders quantitative studies difficult. Thus the limited interferon response to the i.v. inoculation of Friend virus may reflect an inadequate(2) number of virions inoculated rather than an intrinsic property of the virus.