*THE EFFECTS OF NONTUMOR VIRUSES ON VIRUS-INDUCED LEUKEMIA IN MICE: RECIPROCAL INTERFERENCE BETWEEN SENDAI VIRUS AND FRIEND LEUKEMIA VIRUS IN DBA/2 MICE**

By E. Frederick Wheelock

SCHOOL OF MEDICINE, WESTERN RESERVE UNIVERSITY, AND THE UNIVERSITY HOSPITALS, CLEVELAND, OHIO

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The virus etiology of certain murine leukemias and possible virus etiology of human leukemia make it reasonable to think of treatment and perhaps prevention of these diseases in terms of virus inhibition. One means of inhibiting virus replication in cells is by the introduction of a second virus which can either compete with the first virus for replicating sites and biosynthetic mechanisms or induce synthesis of interferon, a virus inhibitor. In a recent communication,[1] clinical and hematologic remissions in the course of acute myelogenous leukemia in a patient were reported to follow repeated inoculations of certain selected attenuated nontumor viruses, but the mechanism involved in the production of these remissions could not be determined. The possibility that viral interference was involved, however, stimulated the study, herein reported, of the effects of nontumor viruses on virus-induced leukemia in mice.

In recent years viral interference has been demonstrated in a variety of nontumor virus-cell culture and animal systems. In contrast to nontumor viruses, there has been great difficulty in growing murine leukemic viruses in cell culture systems, and *in vitro* studies of viral interference with this class of viruses have not been conducted. Such studies, however, are possible in *in vivo* systems.

During the past 15 years a number of reports have appeared in the literature describing inhibition of neoplastic disease in animals following inoculation of a variety of different viruses. One of the first in this series was an article by Sharpless, Davies, and Cox[2] who reported complete regression of a transplantable lymphoid tumor in chickens following inoculation of any of a number of arboviruses. The mechanism of tumor regression was thought to be direct destruction of tumor cells by virus multiplication.

In mice bearing transplantable leukemias, vaccinia virus[3] and a virus immunologically related to lymphocytic choriomeningitis[4] have been reported to prolong survival, whereas sublethal neurotropic viral infections had no consistent beneficial effects on the course of the disease.[5, 6] Guinea pigs with a transplantable leukemia were reported to have an increased survival rate and a decreased spleen size following inoculation with lymphocytic choriomeningitis virus.[7] It was not until recently, however, that viral interference experiments were conducted with a known virus-induced neoplasm. Rowe[8] reported that mice inoculated with the Moloney strain of mouse lymphoid leukemia virus showed marked diminution of spleen weight response to infection with Friend leukemia virus given 3–4 weeks later. However, conclusions about the mechanism of interference could not be made from the data obtained, since antigenic relationships between the two viruses have been demonstrated.[9]

The purpose of the present investigation was to study the effects of inoculation of

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nontumor viruses on virus-induced leukemia in mice and to determine, if possible, the mechanism of any changes produced in the leukemic disease by these viruses. In the experiments in DBA/2 mice reported here, a reciprocal interference was found between Sendai virus (one of the parainfluenza group of viruses which produces benign infections in mice) and Friend leukemia virus.

Materials and Methods.—Viruses: Friend leukemia virus (FV) was obtained from Dr. Charlotte Friend[10] in the form of infected DBA/2 mice. The enlarged spleens were removed from the mice 3 weeks after infection and a 20% suspension of them made in phosphate buffered saline (PBS). The suspension was then centrifuged at 6000 rpm for 1 hr, and the supernates were passed through a Millipore filter with a pore size of 450 μm, quick-frozen, and stored at −70°C.

Vesicular stomatitis virus (VSV) and *Sindbis virus* (Egypt AR 339 strain) were grown in mouse "L" cell monolayer cultures. The infected cultures were sonicated and clarified by centrifugation at 1000 rpm for 30 min; the supernates were then passed through a Millipore filter with a pore size of 450 μm, quick-frozen, and stored at −70°C.

Sendai virus (SV obtained from Dr. Fred Davenport) and Newcastle disease virus (NDV Hickman strain) were grown in 10-day-old embryonated chicken eggs. After 2 days' incubation at 37°C, infected allantoic fluids were pooled, clarified by centrifugation, and spun at 105,000 *g* for 7 hr. The pellets were then washed with saline, dispersed in PBS by sonication, filtered as described above, quick-frozen, and stored at −70°C.

Cell cultures: The "L" strain of mouse fibroblasts derived from normal mouse skin[11] was employed in the assay for interferon. Cells were grown in growth medium consisting of Eagle's minimum essential medium supplemented with tryptose phosphate broth (4%) and fetal calf serum (10%). The concentration of sodium bicarbonate was 1.75 gm/liter, and all cell-culture vessels were gassed with 5% CO_2 in air before incubation at 37°C.

Interferon assay: The specimens to be tested for interferon were diluted in growth medium, and 1 ml of each dilution was added to 1-day-old cultures of mouse "L" cells grown in incomplete monolayers in screw-cap tubes. After 20 hr of incubation at 37°C the cultures were washed once with 4 ml of PBS. To each tube, 1 ml of warm protein-free Eagle's medium was added, and then 1 ml of cold growth medium containing 2000 tissue-culture infective doses ($TCID_{50}$) of vesicular stomatitis virus was inoculated. This amount of virus produced gross cytopathic effects in cultures in 24–30 hr. Cultures were considered to be protected when less than 10% of the cells showed cytopathic effects at a time when more than 75% of control cells exhibited cytopathic effects.

Interferon titers are expressed in culture protecting units (CPU) as reciprocals of the highest dilution of the specimen, 1 ml of which protected cultures against challenge with VSV. No specimen was tested at less than 1:10 dilution.

Mice: Six- to 8-week-old DBA/2 female mice were obtained from the Jackson Memorial Laboratories.

Peritoneal washings: Mice were anesthetized with ether and exsanguinated, and the abdominal wall was shaved. The peritoneal cavity was opened and washed with 5 ml of PBS. The recovered washing was then clarified by centrifugation at 2000 rpm for 5 min and the supernate was acidified at pH 2 for 24 hr, neutralized, and processed as described above for the interferon assay.

Results.—Effect of Sendai virus on Friend virus leukemia: DBA/2 mice were inoculated intraperitoneally (IP) with Friend virus, 2×10^2 mouse infecting $doses_{50}$ (MID_{50}) per mouse. At intervals before and after Friend virus, groups of 20 mice were given intraperitoneal inoculations of Sendai virus, $10^{7.5}$ egg infective $doses_{50}$ (EID_{50}) per mouse. Three weeks after Friend virus inoculation all mice were sacrificed and their spleens weighed.

As seen in Figure 1, marked reduction of the splenomegalic response to Friend virus resulted when Sendai virus was administered as early as 3 weeks prior to Friend virus. There was, however, no reduction of splenic enlargement when Sendai virus was given together with or after Friend virus. A slight but statistically significant enhancement of splenomegaly was observed in mice inoculated with

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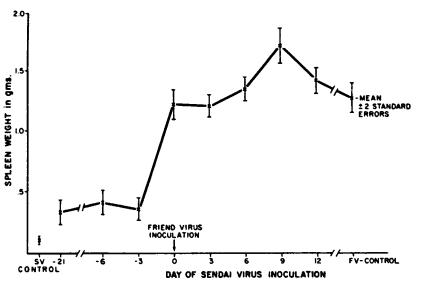

FIG. 1.—Effect of time of inoculation of Sendai virus on the splenomegalic response to Friend virus. Each point represents a group of 20 mice inoculated with Sendai virus at the designated interval in relation to Friend virus inoculation, and is calculated on the basis of spleen weights 21 days after Friend virus inoculation.

Sendai virus 9 days after Friend virus, when compared with the results in mice which received only Friend virus (*FV* in Fig. 1). In addition, preliminary experiments with Friend virus-infected mice indicate that prior inoculation with Sendai virus inhibits the lymphocytotic and erythroblastotic responses as well as the splenomegalic response.

In experiments with other nontumor viruses, intraperitoneal inoculations of Newcastle disease, Sindbis, and vesicular stomatitis at titers higher than those used with Sendai virus failed to reduce the splenomegalic response to subsequent Friend virus inoculations.

Effect of route of virus inoculations on Sendai versus Friend interference: In the experiments described above, Sendai virus reduced the splenomegalic response to Friend virus when both were inoculated by the intraperitoneal route. In order to determine whether interference between the two viruses was a local intraperitoneal phenomenon or a systemic one, varied routes of inoculation for each virus were tested.

As seen in Table 1, intraperitoneal inoculation was the only route by which Sendai virus reduced the splenomegalic response to Friend virus given intraperitoneally 7 days later. Intraperitoneal inoculations of Sendai virus, however, could inhibit only peritoneally administered and not intravenously administered Friend virus. Sendai virus, inoculated either intraperitoneally or intravenously, failed to inhibit intravenously administered Friend virus. Thus, interference between the two viruses was dependent on identical peritoneal routes of inoculation, suggesting that competition between the two viruses for the same peritoneal host cells might have been the basis of the interference mechanism. Alternatively, viral interference might have been mediated through interferon which would have been at highest concentrations near primary host cells available to both viruses.

The following experiments were therefore designed to elucidate the mechanisms

TABLE 1

EFFECT OF ROUTE OF INOCULATION OF SENDAI VIRUS ON THE SPLENOMEGALIC RESPONSE TO FRIEND LEUKEMIA VIRUS*

Route of Sendai virus†	Interval (days)	Route of Friend virus	Spleen weight (mg)‡	±2 Standard errors
IV	7	IP	1420	±90
IP	7	IP	340	±240
SC	7	IP	1250	±220
PN	7	IP	1290	±130
—	—	IP	1280	±320
IP	7	IV	1490	±320
IV	7	IV	1420	±180
—	—	IV	1310	±230

* Each experiment was performed with 7–10 mice
† IV, intravenous; IP, intraperitoneal; SC, subcutaneous; PN, intranasal.
‡ 21 days after Friend virus inoculation IP; 16 days after Friend virus inoculation IV.

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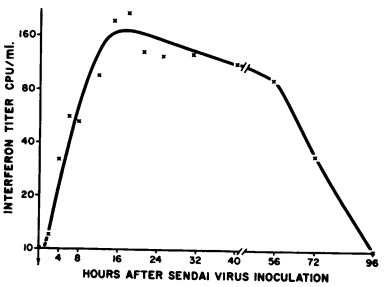

FIG. 2.—Production of interferon in the peritoneal cavities of mice following intraperitoneal inoculation of Sendai virus. CPU (Culture Protecting Units of interferon) = reciprocal of the highest dilution of the peritoneal wash which protects "L" cell cultures against cytopathic effects of vesicular stomatitis virus.

of suppression of Friend virus leukemia by prior inoculation of Sendai virus. The possible role of Sendai virus-induced interferon was first studied.

Interferon production by Sendai virus: Interferon production in the peritoneal cavities of mice in response to Sendai virus inoculation was measured. At intervals after Sendai virus inoculation IP, the peritoneal cavities of groups of five mice were opened and washed with saline. The washes were acidified overnight to destroy Sendai virus, then neutralized and assayed for interferon in "L" cell monolayers against a VSV challenge as described in *Materials and Methods.* As seen in Figure 2, interferon production began in the peritoneal cavity 2 hr after inoculation and increased logarithmically until the eighteenth hr. Thereafter a gradual decline in titer ensued over a 3-day period. The interferon produced in response to Sendai virus was identified as such by its acid stabilty, sensitivity to trypsin, nonsedimentability at high-speed centrifugation, and ability to protect "L" cell cultures on incubation before VSV inoculation.

It is of interest to note that the three viruses listed above as failing to reduce the splenomegalic response to Friend virus, viz., Newcastle disease, vesicular stomatitis, and Sindbis virus, also failed to produce detectable amounts of interferon in the peritoneal cavity.

Interferon production by Friend virus-infected mice: If Sendai virus-induced interferon were responsible for the suppression of Friend virus leukemia, then the failure of Sendai virus to reduce splenomegaly when given *after* Friend virus might be due to the inability of Friend virus-infected mice to produce interferon. The capabilities of Friend virus-infected mice to produce interferon in response to Sendai virus were therefore investigated. In the course of these experiments, it was found that Friend virus alone did not elicit interferon production in the peritoneal cavity.

Mice were inoculated intraperitoneally with Friend virus, 2×10^2 MID_{50}/mouse. At daily intervals thereafter, groups of five mice were inoculated IP with Sendai virus; 24 hr later, the peritoneal cavities were opened and washed with 5 ml of PBS; the washes were acidified overnight, then neutralized and assayed for interferon. Spleens of the sacrificed mice were weighed in order to relate interferon production to the stage of Friend virus disease.

As shown in Figure 3, mice inoculated with Sendai virus on the first day after Friend virus inoculation almost completely lost their ability to synthesize interferon in the peritoneal cavity. Between the second and twelfth day inteferon production increased in response to Sendai virus challenge, and normal levels of production were reached on the twelfth day. It was during the period of return of the in-

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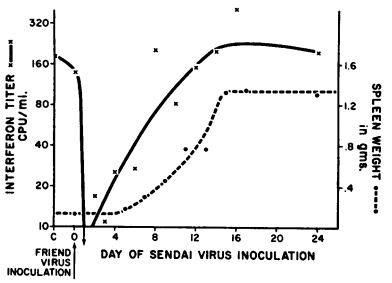

FIG. 3.—Production of interferon in response to Sendai virus administered intraperitoneally at daily intervals following Friend virus inoculation. Groups of 4 mice were sacrificed 24 hr after each Sendai inoculation, the peritoneal washings assayed for interferon, and spleens weighed.

terferon-producing function that the spleens rapidly enlarged, and maximum weights were reached on the fifteenth day.

Experiments were performed to determine whether or not the transient loss of peritoneal interferon production was related to the route of Friend virus inoculation. Mice were inoculated intravenously with Friend virus and 1 day later challenged with an intraperitoneal inoculation of Sendai virus. As in the previous experiment, a marked reduction in interferon production was observed. Thus, the transient loss of peritoneal interferon production in response to Sendai virus was apparently the result of Friend virus infection *per se*, since it occurred irrespective of the route of Friend virus inoculation.

Discussion.—The experiments described above represent studies of *in vivo* interference between a nontumor virus and a murine leukemia virus. The term *interference* used here refers to the inhibition by one virus of the cellular response to the second virus, e.g., (1) the splenomegalic response to Friend virus infection, and (2) the interferon response to Sendai virus infection. The reciprocal interference between Sendai and Friend viruses demonstrates that leukemia viruses have the capacity both to inhibit and be inhibited by nontumor viruses. Thus, these studies provide an experimental basis for attempts to use nontumor viruses in the treatment and prevention of virus-induced leukemias. Furthermore, determination of the mechanism of reciprocal virus interference in mice may assist in an understanding of the clinical and hematologic remissions which followed the repeated administration of viruses to a young adult patient with acute myelogenous leukemia.[1]

The present report describes only certain aspects of the reciprocal interference between Sendai and Friend viruses in mice. On the basis of the results obtained thus far, no definitive conclusions can be drawn as to the mechanisms involved. However, the demonstration that Sendai virus inoculation can protect mice against Friend virus leukemia when the interval between administration of the two viruses is as long as 3 weeks may provide important clues as to the mechanism of viral interference. Preliminary experiments have failed to detect interferon in any cells, organs, or body fluids of mice 3 weeks after Sendai virus inoculation, but undetectable amounts of interferon may be present and active against Friend virus. These preliminary experiments may therefore suggest, as an alternative possibility, that the basis of Sendai virus inhibition of Friend virus leukemia is a direct virus-virus interference within cells. In this type of interference, host cells utilized by both viruses for their replication may exist in the peritoneum and elsewhere, resulting in direct intracellular competition between the two viruses for replicating sites and

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biosynthetic mechanisms. This possibility, as well as the persistence of Sendai virus in cells for long periods of time following inoculation, is experimentally approachable and is under current investigation. In this respect it is of great interest to determine the maximum period of time following Sendai virus inoculation that mice are protected against a subsequent inoculation of Friend virus. Also of interest is a long term follow-up of mice in which the manifestations of Friend virus leukemia have been completely suppressed by the prior inoculation of Sendai virus. Finally, investigation of mechanisms of interference by Sendai virus against Friend virus should include a study of possible antigenic relationships between the two viruses. It should be pointed out that a direct oncolytic effect of Sendai virus on Friend virus-infected cells is unlikely, since Sendai virus does not reduce the splenomegalic response when administered after Friend virus.

The temporary loss of the ability of the Friend virus-infected mouse to produce interferon in response to Sendai virus may be an important factor in the pathogenesis of the leukemic disease in mice. It may be that the disruption of mechanisms for interferon production is necessary for the establishment of Friend infection in cells. However, the transient nature of the alteration may mean that the infection, once established, can persist even in the presence of interferon. It is interesting to note that Sendai virus did not reduce splenomegaly even when given after a return of the interferon-producing function, that is, by the twelfth day after Friend virus inoculation. The spleen by this time is rapidly enlarging (see Fig. 3), however, and may perhaps contain Friend virus that is refractory to inhibition.

The demonstration that Sendai virus inoculated after Friend virus cannot induce interferon production does not necessarily implicate interferon in the inhibition of Friend virus leukemia by the prior inoculation of Sendai virus. This conclusion is based on the observation that when Sendai virus is inoculated together with Friend virus, interferon is produced (Fig. 3), and yet no inhibition of the splenomegalic response to Friend virus occurs (Fig. 1).

Finally, the transient cutoff in Sendai virus-induced interferon production in the peritoneal cavity of Friend virus-infected mice suggests that Friend virus undergoes an initial transient infection of cells in the peritoneal cavity and then moves on to other sites in the body, thereby freeing-up peritoneal cells for infection and interferon production by Sendai virus.

If the interference between Sendai and Friend viruses demonstrated in the laboratory system employed in this work has a counterpart in nature, it may be in the form of a balance between leukemia viruses and certain nontumor viruses. Thus, in nature there may be constant inhibition of leukemia viruses by other viruses. Emergence of a leukemia virus into a disease-producing agent may be the result of either the removal of the inhibiting viruses or a breakdown in the interfering mechanism between viruses. The study in mice of a leukemia virus which is inhibited by the prior inoculation of a nontumor virus may assist in the discovery and understanding of such a balance between viruses in nature.

Summary.—Sendai virus inoculated into DBA/2 mice as long as 3 weeks prior to Friend virus markedly reduces the splenomegalic response to Friend virus. This effect is dependent upon the route of inoculation: both viruses must be administered intraperitoneally. Newcastle disease, vesicular stomatitis, and Sindbis viruses have *no* such Friend virus-inhibiting properties.

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Sendai virus inoculated into mice IP induces the production in the peritoneal cavity of $\approx 10^3$ culture protecting units of interferon within 24 hr. Within 1 day after Friend virus inoculation IP or IV, mice undergo a marked reduction of peritoneal interferon response to Sendai virus. By 12 days after Friend virus inoculation, mice can once again synthesize normal amounts of interferon in response to Sendai virus.

The interactions between these two viruses in the animal model may offer clues as to the development and suppression of human leukemia.

Note added in proof: In a paper in press in the *Journal of the National Cancer Institute*, J. K. Youn and G. Barski report inhibition of the splenomegalic, lymphocytotic, and erythroblastotic responses to Rauscher virus in mice inoculated with lymphocytic choriomeningitis virus 48 hrprior to Rauscher virus. No protection of mice against leukemic disease resulted when LCM virus inoculation followed Rauscher virus.

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PRODUCTION OF ALTERED CELL FOCI IN TISSUE CULTURE BY DEFECTIVE MOLONEY SARCOMA VIRUS PARTICLES

BY JANET W. HARTLEY AND WALLACE P. ROWE

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

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Harvey and Moloney have recently described the ability of passage lines of the Moloney strain of mouse leukemia virus to induce sarcomata in infant mice.[1] In attempting to propagate the Moloney sarcoma virus (MSV) in mouse embryo tissue culture, we found that it produced foci of altered cells. This report describes an assay procedure based on focus formation, and presents evidence that the focus-forming particles are defective.

Materials and Methods.—Viruses: MSV virus was given to Dr. A. C. Allison by Dr. Moloney, and passed in infant CDF_1 mice; we received the virus from Dr. Allison as extracts of tumor, liver, spleen, or salivary glands of tumorous mice. The virus used for most experiments was a

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