Effect of West Nile and Ilheus Viruses on Mouse Leukemias*

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An inhibitory effect of certain viruses on the growth of transplantable tumors in mice has been demonstrated by Moore and co-workers (6—8) and by Kaprowski and Norton (4). Turner and Mulliken (11) have reported a slightly increased survival rate in one strain of mouse leukemia (9417) after inoculation of vaccinia virus, but two other strains (1394 and C1498) were unaffected. Sharpless (9) has demonstrated complete eradication of leukemia in fowl following inoculation of Russian spring-summer encephalitis virus. The present communication reports the suppressive effect of West Nile and Ilheus encephalitis viruses on two strains of mouse leukemia, as determined by serial blood counts.

MATERIALS AND METHODS

Mice were of the Akm strain, obtained from Carworth Farms. They were about 45 days old and weighed 18—22 gm. at the start of each experiment. Both sexes were used, but each experimental group of 30 mice was of a single sex. Purina Laboratory Chow and water were available ad libitum.

Leukemia Ak4 has been previously described (1). Leukemia Ak 9421 arose spontaneously in a 6-month-old male mouse of Akm stock at this institute in April, 1946. Both strains are maintained in this laboratory by intraperitoneal transplantation of a suspension of cells from minced leukemic spleen into Akm mice. In the studies reported here all inocula were standardized to deliver 100,000 cells per mouse. This inoculum produced fatal leukemia of remarkably uniform course in over 95 per cent of the mice.

Most experimental groups in this study consisted of 30 mice: 10 controls, 10 to be infected with West Nile virus, and 10 with Ilheus virus.

Blood cells were counted by standard clinical technics. Blood was obtained by nicking one of the tail veins with a razor blade. For ½ hour prior to taking blood counts, the mice were warmed under an electric lamp for several minutes to insure vasodilation and free flow of blood. Blood counts were usually done 3 times per week in the early stages of leukemia and daily after the leukocyte counts started to rise. Leukocyte counts were done on all mice. Erythrocyte counts and differentials were done on three randomly selected mice from each group of ten. All data in the charts and tables are arithmetic means. Since differential counts were not done on all mice, the sum of the averaged differential counts is not identical with the averaged total leukocyte counts. Obviously, the blood counts representing the last stages of leukemia represent only the few animals surviving to that time. The control curves in Charts 3—6 represent the combined data from controls of all experiments with the indicated strain of leukemia. Control data are tabulated separately for each experiment in the table.

West Nile virus (10) and Ilheus virus (5) were obtained from Dr. Hilary Kaprowski of Lederle Laboratories and maintained by intracerebral inoculation of CFW (Carworth Farms) Swiss mice. In all experiments here reported a uniform stock of either virus was utilized, prepared by emulsifying about 50 infected mouse brains in 0.85 per cent sterile saline to give a 20 per cent suspension. Suspensions were centrifuged for 10 minutes at 2,000 r.p.m. to remove large particles and were then sealed in ampoules in 5-cc. lots and maintained at about —76° C. until immediately before use. Both virus preparations after freezing and thawing had an intracerebral titer of approximately 10⁴—¹⁰⁴.

In the experimental studies the viruses were inoculated intraperitoneally in a dose of 0.5 cc. of the 20 per cent mouse brain preparations. This dosage is approximately an LD₉₅ for West Nile virus and an LD₉₀ for Ilheus virus, as titered intraperitoneally in normal Akm or CFW mice.

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The interval between inoculation with leukemia and inoculation with virus was varied in the several experimental groups in order to observe differences in effect of virus infection initiated at various stages of the leukemic process.

Dead mice were autopsied. Wet weights of liver and spleen were recorded but are not tabulated in this presentation. If leukemia was not grossly apparent, histologic sections of liver and spleen were examined. Four mice in which leukemia was not found were discarded from the study.

Leukemic tissues were examined for the presence of virus, when indicated, by preparation of 10 per cent tissue suspensions in sterile saline and inoculation intracerebrally into CFW (leukemia resistant) mice. Virus titers are expressed as the logarithm of the greatest dilution causing death by encephalitis.

RESULTS

Effect of viruses on normal Ak4 mice.—Nonleukemic Ak4 mice infected with West Nile virus appeared well until the third day, when some animals exhibited paralysis of the hind quarters and one died. Three days later eight of the ten mice were dead, and all were dead by the ninth day after inoculation.

Of ten mice inoculated with Ilheus virus, only six succumbed to infection. In these six, hind quarter paralysis appeared about the sixth or seventh day. One mouse died on the fourth day, and the others died between 9 and 13 days after inoculation. Only the six fatally infected mice were considered in the study of hematologic effects.

Both viruses caused slight leukopenia and lymphopenia by the seventh day, as compared to the uninjected control group. Granulocytes and erythrocytes showed no variation attributable to virus. Liver and spleen weights were within normal limits at time of death.

Course of untreated Ak4 leukemia.—Ak4 leukemia, as used in this study, caused death in 6–14 days in nearly 100 per cent of inoculated Ak4 mice. Mean survival time was about 8 days. For about 2 days before death there was ruffling of the fur and marked inanition and paresis. Terminally, respiration was usually slow and gasping. On autopsy the striking gross findings were enlargement of axillary and inguinal nodes to about 1 mm. in diameter and marked enlargement of liver and spleen. The leukemic livers were paler than normal or were spotted with pale areas if the leukemic infiltration was not complete. Signs of hemorrhage were not usual. Histologic sections revealed widespread infiltration with leukemic cells, most consistent and most obvious in the liver.

The erythrocyte count showed considerable fluctuation but no consistent change. The course of this leukemia is so short that depression of erythropoiesis would not be expected to be reflected in the peripheral blood. The wide fluctuations observed probably represent the summation of blood loss from the tail incision and hemococoncentration due to poor water intake during the period of inanition, as well as the inaccuracies inherent in the blood counting technic. There appeared to be increased bleeding from the tail incisions in the later stage of leukemia, but thrombocytes did not appear reduced, as judged by the stained blood smears. The leukocyte count showed no abnormality for about 5 days after inoculation, but thereafter rose rapidly to levels of 100,000–400,000 cells per cubic millimeter on the day of death. The increased leukocytes were predominantly very immature cells (probably prolymphocytes and lymphoblasts), which we have arbitrarily called "blasts," but at the onset of leukocytosis there was also an increase in mature granulocytes and lymphocytes to as much as 40,000 cells per cubic millimeter. Terminally, granulocytes and mature lymphocytes disappeared.

Effect of normal brain injections on Ak4 leukemia.—One milliliter of a 20 per cent normal (CFW) mouse brain suspension was injected intraperitoneally into each of ten mice on the second day after inoculation with Ak4 leukemia, and into another group on the sixth day. There were no significant differences between the blood counts of these injected groups and of their uninjected controls. Leukemic infiltration of liver and spleen, as judged by organ weight, was just as great in the injected as in the uninjected mice, and survival times were slightly shorter. Thus, it appears that any hematologic effects observed in virus-treated mice must be attributed to the virus and not to the brain tissue.

Effect of West Nile virus on Ak4 leukemia.—West Nile virus was inoculated, in several experiments, 4 hours, 2 days, 5 days, 6 days, and 7 days after inoculation of leukemia. In each experiment leukocyte counts remained at normal or near-normal levels for 2–3 days after the blood had become leukemic in the untreated controls. There was no depression of granulocytes and only moderate depression of lymphocytes below normal levels during this period. The effect was a selective inhibition of leukemic blast cell proliferation. Since the virus usually kills in 5–9 days, studies of leukemia beyond the tenth day were rarely possible. In those few mice that did survive to the twelfth day, leukocytes and blast counts
leukemic effect of the virus is merely suppressive, rather than "curative" as in fowl leukosis (9). Since it is impractical to publish complete data of these studies, those data which best illustrate the anti-leukemic effect have been chosen for presentation (Table 1 and Chart 1).

Inhibition of leukemic cell proliferation in the virus-infected mice was also evidenced by the absence of hepatosplenomegaly and adenopathy in the treated mice. Weights of liver and spleen were normal at time of death in the virus-treated mice and were 50—100 per cent above normal in uninfected mice dying of leukemia. This effect has also been observed by Moore1 in Ak4 leukemic mice infected with Russian spring-summer encephalitis virus.

**Effect of Ilheus virus on Ak4 leukemia.**—The results seen with Ilheus virus in Ak4 leukemia paralleled those seen with West Nile virus. Hematologic and survival data from those studies which best demonstrated the anti-leukemic effect are presented in Table 1 and Chart 2. The absence of hepatosplenomegaly in virus-infected mice was again observed. Suppression of the leukemia was usually not quite so complete as with West Nile virus, probably because the Ilheus virus inoculum did not cause infection in all animals. Uninfected animals could not be excluded from these groups, because death from virus and death from leukemia could not be distinguished, and routine testing for virus was impractical.

**Localization of viruses in leukemic mice.**—The site of virus proliferation was studied in Ak4 leukemic mice in two experiments.

First, since the spleen of the leukemic mouse presents accessible and numerous leukemic cells, virus titers were determined for this organ and compared with parallel titers of brain and whole blood. Virus was inoculated intraperitoneally on the fourth day of Ak4 leukemia, and one mouse infected with each virus was sacrificed 2, 4, and 7 days later for virus titration. The data demonstrate that West Nile virus was present in blood and spleen throughout the study, with spleen showing slightly higher titers (10^{-2} to 10^{-4}) than blood (10^{0} to 10^{-2}). Brain (10^{-1} suspension) contained no detectable virus at 48 hours, but on the fourth and seventh days the brain showed higher titers (10^{-4} and 10^{-6}) than blood or spleen. Ilheus virus never attained a titer over 10^{-2} in this study but was present in spleen and 4 days after inoculation in higher titers (10^{-2}) than in blood or brain. Ilheus virus was not detected in the mouse sacrificed on the seventh day, probably because of failure to establish infection in the animal tested.

Second, five mice were inoculated with Ilheus virus on the fourth day of Ak4 leukemia, and after another 2 days were bled by decapitation. Heparin was added to prevent coagulation, and all blood was pooled. Erythrocytes were agglutinated by adding a few drops of a rabbit anti-mouse erythrocyte serum and were sedimented by slow centrifugation. This procedure left a large proportion of leukocytes and only a few erythrocytes in the supernatant. This supernate was decanted and then centrifuged at high speed to sediment the leukocytes. The leukocyte-rich sediment and the erythrocyte sediment were washed twice with saline, and then these preparations and the plasma were serially diluted in saline and titered for virus. No virus was detected in the erythrocytes, even in 10 per cent dilution. Virus was present in the leukocytes in a titer of 10^{-4}. Virus was present in the plasma at a titer of approximately 10^{-2}.

These two experiments indicate that the tested viruses invade leukocytes, presumably the leukemic leukocytes.

**Course of untreated Ak 9421 leukemia.**—Ak 9421 leukemia, as used in this study, is somewhat less acute than Ak4 leukemia. Death usually occurs between the eleventh and twentieth days after inoculation. Mean survival time after inoculation is about 14 days. Gross and microscopic pathology are as described for Ak4 leukemia. Hematologic findings differ from those of Ak4 leukemia. Hematologic virus-infected mice in that the total leukocyte count rarely exceeds 50,000, and this leukocytosis is due to approximately equal increase in mature lymphocytes and lymphoblasts.

**Effect of West Nile virus on Ak 9421 leukemia.**—When West Nile virus was inoculated in the early stages of development of Ak 9421 leukemia, mice died of virus encephalitis before untreated leukemic controls showed any signs of leukemia.

When West Nile virus was inoculated on the seventh day after inoculation of leukemia, the blood counts remained essentially normal for 2 days after the controls showed leukemic counts, but there was no significant difference in survival time. When virus was given 11 days after leukemia, the same suppression of leukemic leukocytosis was observed, and in this experiment the mean survival time of the virus-infected mice was slightly longer (doubtful significance) than that of the controls. In this strain of leukemia the inhibitory effect was against both mature and immature lymphoid cells (Chart 3). (Data from

1 A. E. Moore, personal communication.


<table>
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<th>Days after Leukemia inoculation</th>
<th>Virus</th>
<th>Leukocytes*</th>
<th>Blasts*</th>
<th>Lymphocytes*</th>
<th>Granulocytes*</th>
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**Table 1: Effect of West Nile and Ilheus Viruses on Ak4 Leukemia**

*All blood counts are expressed as thousands of cells per cubic millimeter of blood. The figures in parentheses following the mean blood cell counts indicate the number of mice averaged, and in the leukocyte column this figure also indicates the numbers of mice surviving on that day. Since differential counts were not done on all mice, the sum of the average differential counts is not identical with the average total leukocyte counts.

† Survival times are computed from inoculation of leukemia to death, regardless of time of virus inoculation.
Chart 1.—The effects of West Nile virus infection on survival and blood picture of mice bearing Ak 4 leukemia.

Chart 2.—The effects of Ilheus virus infection on survival and blood picture of mice bearing Ak 4 leukemia.

Chart 3.—The effects of West Nile virus infection on survival and blood picture of mice bearing Ak 9421 leukemia.

Chart 4.—The effects of Ilheus virus infection on survival and blood picture of mice bearing Ak 9421 leukemia.
studies with 9421 leukemia are not tabulated because of space limitations.) Inhibition of leukemic infiltration of liver and spleen was again apparent, since weights of these organs were within normal limits in the virus-infected mice at times when the control mice showed marked hepatosplenomegaly.

**Effect of Ilheus virus on Ak 9421 leukemia.**—When Ilheus virus was inoculated on the eleventh day of Ak 9421 leukemia, the leukemic leukocytosis was partially suppressed (Chart 4). When the virus was given on the seventh day, no effect on the course of the leukemia was observed.

**Effect of viruses on Ak4R leukemia.**—Ak4R leukemia (3) differs from its parent strain Ak4 in that it is not affected by treatment with folic acid antagonists, whereas this treatment will cause a 100 per cent increase in survival time in the parent strain. This resistance to anti-folic compounds was produced by serial passage of Ak4 leukemia in A-methopterin–treated mice (2). In untreated mice the course of Ak4R leukemia is slightly longer than that of Ak4 leukemia, but in other respects it is almost identical.

West Nile virus or Ilheus virus inoculated on the seventh day after inoculation of Ak4R leukemia caused inhibition of leukemic leukocytosis for 3 days, as compared to untreated controls. Mice surviving beyond this period showed leukocytosis equal to that in the untreated controls. Survival time was not prolonged by either virus (Chart 5).

**DISCUSSION**

This work extends the spectrum of neoplasms which are inhibited by neurotropic viruses to include the mouse leukemias. This anti-neoplastic effect is evidenced by a delay of 2–3 days in the development of leukemic leukocytosis and visceral infiltration. The interval of 2–3 days represents 20–40 per cent of the total duration of this neoplastic process. Such an effect is analogous to the slowing of growth of localized tumors, such as the Sarcoma 180, which has previously been reported for these viruses (4). There was a very slight but quite consistent increase in survival time of the virus-treated mice in most of these studies. These differences are too small to have statistical significance in groups of only ten mice, but the occurrence of even a slight increase in survival is suggestive of inhibition of the leukemic process, when one remembers that the “treatment” is of itself rapidly lethal. It seems quite possible that other viruses might exhibit a greater anti-leukemic effect, and it may be that nonlethal viruses will have this effect, as has been reported for leukosis of fowl (9). It is clear from these data that these viruses exerted their anti-leukemic effect even when inoculated late in the course of the disease.

The mechanism of any anti-neoplastic effect is of utmost interest. The current excitement over the wide therapeutic effects of adrenal steroids makes every investigator alert to the possibility that his manipulations may cause adrenal hyperactivity. In this connection it is of interest that in studies now in progress with Semliki virus no anti-leukemic effect has been demonstrated. This virus is rapidly lethal in the mouse and hence might be expected to produce as great an adrenal stress as West Nile or Ilheus virus. Hence, it seems unlikely that the anti-leukemic effects observed with the latter viruses can be attributed to a nonspecific stress.

The observations indicating that the virus was in the leukemic cells (in spleen and blood) suggest that the viruses act by entering and destroying, or preventing multiplication of, these cells. Even if we accept this as proved, however, the mechanism by which the virus inhibits the cells remains to be explained. The possibilities that the virus blocks an essential step in cellular metabolism or competes with the cells for essential metabolites deserve consideration. It is thought that the anti-neoplastic effects of various vitamin and purine
analogs is caused by such interference with metabolic processes. The fact that West Nile and Ilheus viruses were effective against both Ak4 and Ak4R leukemia indicates that the viruses do not act through the same mechanism as the folic acid antagonists.

SUMMARY
Infections with West Nile or Ilheus viruses were established in mice bearing Ak4, Ak4R, or Ak 9421 leukemia of varying duration. It was demonstrated that both of these viruses temporarily inhibit leukemic leukocytosis and infiltration, even when injected in the late stage of those leukemias. There was no significant increase in survival time in the virus-treated mice. Evidence was obtained which indicates that virus was present in, or on, the leukemic cells.

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