Treatment of Spontaneous Leukemia in AKR Mice with Chemotherapy, Immunotherapy, or Interferon¹

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Summary

AKR mice are genetically destined to develop Gross (RNA) virus-induced lymphatic leukemia. Leukemic AKR mice treated with combination vincristine, cyclophosphamide (Cytoxan), and 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-l-nitrosourea sustained a 180% increase of life-span. Combination chemotherapy plus immunization with neuraminidase-treated allogeneic (Gross virus-induced) G, G leukemic cells intradermally resulted in 35% of animals surviving beyond 150 days without evidence of the disease. It is significant that allogeneic E, G leukemic cells as immunogens were as effective in prolonging the life-span of the immunized leukemic AKR mice as were syngeneic leukemic thymocytes.

Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), an antiviral compound, alone showed no apparent antitumor effect. However, in experiments in which the clinically diagnosed leukemic AKR mice received a combination of cytoreductive therapy [vincristine plus prednisone or, more effectively, vincristine, Cytoxan plus 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-l-nitrosourea, followed by Virazole], there was a noticeable reduction of the viral titer, a delay in the reappearance of viable clonogenic cells, and an increase in the survival time for the leukemic AKR mice as compared to those receiving cytoreductive therapy alone.

The effectiveness of purified mouse interferon in AKR mice was also examined. The decrease in the viral titer of animals that received interferon treatment was markedly greater than that of those receiving a combination of cytoreductive therapy with Virazole or immunotherapy. The administration of mouse interferon had a direct effect on the appearance of the spontaneous leukemia in AKR mice. The median life-span of the control animals was 36 weeks, whereas 45% of the AKR mice treated with five doses of $5 \times 10^6$ units of interferon are still alive at 54 weeks of age. Thus, interferon not only reduces the Gross murine leukemia virus titer in the chronically infected AKR mice but also significantly delays the appearance of the primary lymphoma.

Introduction

Increased immunogenicity of transplantable (8, 33, 34) and autochthonous MuLV (Gross) and methylcholan-

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Materials and Methods

Animals. Male DBA/2/Ha mice, 8 to 10 weeks old, were obtained from the West Seneca Laboratory of Roswell Park Memorial Institute, Buffalo, N. Y. Male C57BL and female AKR mice, 7 to 8 weeks old, and female retired AKR breeders, 5 to 7 months old, were supplied by The Jackson Laboratories, Bar Harbor, Maine. The mice were kept in a room free of disease (2-4). The immunoprotection evoked by neuraminidase-treated cells was found to be specific for the tumor used for immunization (2-6, 29, 30, 36, 37). These experiments led to good understanding of the factors that determine the optimal conditions for therapy in a rapidly growing neoplasm in highly inbred animals. Recently, considerable attention has been directed to the study of the response to combined modality therapy of spontaneous leukemia of viral origin (26-28, 35, 41-43, 45). In many respects spontaneous leukemic AKR mice have characteristics similar to those of humans with leukemias. It is well documented that the vertically transmitted MuLV is the etiological agent for lymphoma in AKR mice (17, 19, 21-23, 25).

This work shows the effectiveness of cytoreductive drugs in combination with specific immunotherapy with neuraminidase-treated syngeneic and allogeneic leukemia cells in AKR mice. The effectiveness of preventive immunotherapy in MuLV-infected AKR mice as well as the therapeutic value of Virazole in combination with chemotherapy was tested. The suppressive effects of purified mouse interferon on the expression of Gross (RNA) leukemia virus and the appearance of primary lymphoma in AKR mice are currently under investigation.
thermostatically controlled at 20–22° and allowed Purina chow (breeders) and tap water ad libitum.

**Experimental Tumors.** Leukemia L1210 tumor cells have been maintained as highly virulent leukemia by weekly i.p. passage in DBA/2 mice. Gross virus-induced E$_2$G leukemia was maintained by weekly i.v. passage of 0.5 to 1 x 10$^8$ viable leukemic cells in syngeneic C57BL mice. Spontaneous leukemic AKR mice were selected from a colony of 3500 to 4000 female retired breeders. Clinical diagnosis of spontaneous leukemia in the AKR mice was made with 95% accuracy by splenic and lymph node palpation, followed by leukocyte counts. Animals were considered to be leukemic when their leukocyte counts were greater than 17,000/cu mm and they had splenic and lymph node enlargement. At the time of diagnosis, the average splenic weight was 470 mg, the thymus weight was 650 mg, and the average leukocyte count was 28,000/cu mm.

**Purification of V. cholerae Neuraminidase.** The enzyme is purified as described by Ada et al. (1) using V. cholerae filtrate (obtained from Sigma Chemical Co., St. Louis, Mo.) as a source of crude enzyme preparation. The enzyme showed maximum activity at pH 5.6 with different substrates and was free of other proteolytic activity including neuraminoalalidase. One unit of neuraminidase activity is defined as the amount of enzyme which hydrolyzes 1 ng of N-acetyleneuraminic acid from N-acetyleneuraminosylactose or orosomucoid for 15 min at 37°.

**Preparation of Tumor Cells for Neuraminidase Treatment.** Leukemia L1210 cells were harvested from DBA/2 mice 6 to 7 days after the animals were inoculated with 10$^6$ or 10$^8$ leukemic cells i.p. The tumor cells were freed from contaminating RBC by a 5-sec osmotic shock treatment. Tumor cell preparations were consistently free of erythrocytes, and 98% of the leukemic cell population existed trypan blue. E$_2$G leukemic cells were obtained from the spleens of C57BL mice 7 days after transplantation of 1,000,000 leukemic cells i.v. The spleens were removed and placed immediately in a plastic Petri dish containing Roswell Park Memorial Institute Medium 1640 supplemented with 20% heat-deactivated fetal bovine sera. The edges of the spleens were split at regular spacings and gently squeezed between blunt forceps to force the contents into the surrounding media. Cell suspensions were drawn through a 20-gauge needle in the plastic syringe to produce a single-cell suspension. This was filtered through a double layer of gauze to remove any aggregates of cells. Cells were washed twice with Roswell Park Memorial Institute Medium 1640. The separation of viable from nonviable tumor cells was achieved by flotation on bovine serum albumin as described by Wepsic (46). Viable single-cell suspensions from normal spleens or thymus and AKR lymphoma were achieved by the method described for the preparation of E$_2$G leukemic cells. The final viability of leukemic cells was 92 to 98% as determined by the trypan blue exclusion method.

**Incubation of Normal and Leukemic Cells with Neuraminidase.** Normal and leukemic cells were incubated at 37° in sodium acetate buffer (0.05 M sodium acetate, 0.154 M sodium chloride, and 0.005 M calcium chloride) at pH 5.6 to 6 in the presence of 25 to 35 units of neuraminidase per 2.5 x 10$^7$ cells per ml each. In certain experiments, tumor cells were incubated with neuraminidase in the presence of mitomycin C (25 μg/10$^8$ cells). At the termination of incubation the siliconized reaction flask was removed, cooled rapidly, and spun at 800 to 1000 x g for 55 sec in a clinical centrifuge. Cells thus incubated were washed twice with 50 volumes of 0.9% NaCl solution containing 0.005 M EDTA and used for immunization experiments. Supernatant and washings were combined and used for the quantitation of neuraminidase-susceptible N-acetylneuraminic acid.

**Prophylactic Therapy of MuLV-infected AKR Mice with Neuraminidase-treated Syngeneic and Allogeneic Leukemia Cells.** Eight-week-old female AKR mice were randomized into 6 groups of 40 mice each. Group 1 received 0.9% NaCl solution injections every 15 days for a total of 9 times. Group 2 received 2 x 10$^8$ neuraminidase-treated leukemia L1210 cells per injection i.d. Group 3 received 2 x 10$^7$ neuraminidase-treated allogeneic E$_2$G cells per injection i.d. Group 4 received 2 x 10$^8$ neuraminidase-treated normal AKR thymocytes per injection. Group 5 received 2 x 10$^7$ neuraminidase-treated leukemic AKR thymocytes i.p. while animals in Group 6 were immunized i.d. with the same number of neuraminidase-treated normal thymocytes per injection. The mice were checked daily for mortality until the termination of the experiment (450 days after the date of their birth). The thymus and spleens were removed from mice that died, and the weights were recorded.

**Production of Mouse Interferon.** Interferon was produced at 20,000 units/ml in C-243 mouse tissue culture line infected with Newcastle disease virus. Subsequently, the interferon was concentrated to approximately 1 x 10$^6$ units/ml. The concentrated mouse interferon preparation then was subjected to column chromatographic purification on a Sephadex G-200 column. The fractions representing the interferon were pooled and rechromatographed on a Sephadex G-100 column. With this simple gel filtration method, a relatively pure interferon preparation (1 x 10$^6$ interferon units/mg of protein) was obtained.

**Randomization of AKR Mice into Interferon Experiment.** Three-month-old female AKR mice were randomized into 5 equal groups of 20 animals each. Group 1 served as the control and received at the time of randomization and 2, 4, 6, and 8 days later 1 ml of 0.9% NaCl solution i.p. Group 2 received at the time of randomization and 2, 4, 6, and 8 days later 1 ml of 10% fetal bovine sera i.p. Group 3 received 5 x 10$^7$ units of interferon per injection i.p. at the time of randomization and 2, 4, 6, and 8 days later Group 4 received 5 x 10$^7$ units of interferon per injection i.p. at randomization and 2, 4, 6, and 8 days later. Group 5 received 5 x 10$^7$ units of interferon per ml per injection i.p. at randomization and 2, 4, 6, and 8 days later.

The MuLV titer in the experimental animals was determined by obtaining a 2% extract of a short section (4 to 6 mm) of mouse tails from 10 individual animals in each group. The samples were obtained prior to the treatment, at the time of randomization, during the treatment (on Days 4, 6, and 8), and at posttreatment periods (10, 30, and 85 days). Each tail specimen was individually assayed by the XC focus-forming assay as described by Rowe et al. (32). The MuLV titer is expressed per ml of 2% extract of a 4- to 6-mm section of AKR mouse tail.

Mice were checked for mortality daily. All animals were
Various Treatments of Spontaneous Leukemia in AKR Mice

Table 1
Change of MuLV titer in AKR mice after chemotherapy or chemotherapy + immunotherapy

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Pre-treatment (Day 0)</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 13</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Post-treatment (Day 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated leukemic AKR mice</td>
<td>1260</td>
<td>1317</td>
<td>1060</td>
<td>1520</td>
<td>1290</td>
<td>1620</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy + 0.9% NaCl solution</td>
<td>1310</td>
<td>1020</td>
<td>890</td>
<td>756</td>
<td>1100</td>
<td>960</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy + immunotherapy</td>
<td>950</td>
<td>680</td>
<td>420</td>
<td>385</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MuLV titer is expressed per 0.2 ml of 2% extract of a short section (4 to 6 mm) of mouse tails obtained from animals at days indicated, using XC focus assay.

Table 2
Change of MuLV in AKR mice after chemotherapy or chemotherapy + Virazole

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Pre-treatment (Day 0)</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 13</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Post-treatment (Day 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated leukemic AKR mice</td>
<td>1260</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy + 0.9% NaCl solution</td>
<td>1120</td>
<td>1020</td>
<td>890</td>
<td>975</td>
<td>1100</td>
<td>960</td>
<td>1310</td>
</tr>
<tr>
<td>Chemotherapy + Virazole</td>
<td>780</td>
<td>640</td>
<td>550</td>
<td>410</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MuLV titer is expressed per 0.2 ml of 2% extract of a short section (4 to 6 mm) of mouse tails at days indicated using XC focus assay.

Results

Prophylactic Therapy of MuLV-infected AKR Mice with Neuraminidase-treated Syngeneic and Allogeneic Leukemic Cells. The total covalently bound N-acetylneuraminic acid per 10^6 cells is: leukemia L1210, 0.11 µmole; E2G leukemia, 0.135 µmole; normal or leukemic AKR thymocytes, 0.020 to 0.025 µmole. Upon incubation of normal or leukemic cells under optimal conditions, about 65 to 70% of N-acetylneuraminic acid was removed by neuraminidase.

Optimal neuraminidase concentration for E2G, L1210, and AKR leukemic thymocytes was established at 25 to 35 units/2.5 x 10^5 tumor cells/ml of media. This enzyme concentration represents about 15- to 30-fold excess of the number of neuraminidase-susceptible N-acetylneuraminic acid molecules available on the L1210 or E2G or leukemic thymocytes. After incubation with neuraminidase 75 to 85% autopsied. Spleens, lymph nodes, and thymus of dead animals were removed, and the weights were recorded.

Combined Antiviral Chemoimmunotherapy in AKR Mice with Spontaneous Leukemia. The studies of the effectiveness of antiviral therapy alone or in combination with chemotherapy with or without immunotherapy using neuraminidase-treated allogeneic E2G leukemic cells in AKR mice with spontaneous lymphoma were carried out after positive confirmation of the disease (palpation and WBC count).

In the 1st experiment, diagnosed mice were treated with vincristine, 0.75 mg/kg on Day 1; palmO-ara-C, 125 mg/kg on Day 3; methyl-CCNU, 25 mg/kg on Day 7. On Day 9 they were randomized into the following groups: chemotherapy alone; chemotherapy followed by immunization at multiple sites with 2 x 10^7 neuraminidase-treated allogeneic E2G leukemic cells i.d.; and chemotherapy plus immunization with 2 x 10^7 X-irradiated (5000 R) allogeneic E2G leukemic cells i.d. at the times indicated.

In the 2nd experiment, AKR mice were treated with vincristine, 0.75 mg/kg on Day 1; or combination therapy with vincristine, 0.75 mg/kg on Day 1, and prednisone on Days 1, 2, 3, and 4; or with vincristine, 0.75 mg/kg on Day 1, Cytoxan, 125 mg/kg on Day 3, and methyl-CCNU, 25 mg/kg on Day 7. Two days after the termination of chemotherapy, the animals were randomized into the following experimental groups: chemotherapy alone, chemotherapy followed by treatment with Virazole, either 150 or 200 mg/kg i.p. at times indicated. In each experimental group the MuLV titer was determined at the time of clinical diagnosis, during the treatment and posttreatment periods as indicated in Tables 1 and 2 using the XC focus-forming assay (32).
of the treated cells excluded trypan blue but could not initiate tumor growth in a syngeneic host.

The efficacy of using allogeneic leukemia L1210 and MuLV-induced E2G leukemia cells treated with neuraminidase as immunoegen as prophylactic treatment of AKR mice was compared to the data obtained with neuraminidase-treated syngeneic normal or leukemic thymocytes from AKR mice. It is significant that both neuraminidase-treated syngeneic leukemic AKR thymocytes (Chart 1) and the allogeneic E2G leukemic cells (Chart 2) were equally effective in delaying the appearance of primary lymphoma in AKR mice (p < 0.05). Immunization carried out with neuraminidase-treated L1210 leukemic cells or neuraminidase-treated normal AKR thymocytes i.d. was ineffectual, and the treated mice died of leukemia at about the same rate as did the control group. This clearly establishes the immunoprophylactic and therapeutic value of neuraminidase-treated MuLV-induced spontaneous and allogeneic leukemic cells.

Immunotherapy in Spontaneous Leukemic AKR Mice after Cytoreductive Therapy. Without cytoreductive therapy 50% of AKR mice died by 14 days after diagnosis of spontaneous leukemia, 90% died by 33 days, and 96% died by 56 days. Immunotherapy alone using neuraminidase-treated spontaneous leukemic thymocytes or neuraminidase-treated allogeneic E2G leukemic cells i.p. without prior treatment with cytoreductive therapy did not change the survival pattern of leukemic AKR mice (Chart 3).

Good remission induction was achieved with the following drug combination: vincristine on Day 1, plus palmO-ara-C or Cytoxan on Day 3, plus methyl-CCNU on Day 7. Significant reduction in the thymus and spleen weights was observed after chemotherapy. Splenectomy of thymus and spleen of animals in the chemotherapy group to young nonleukemic AKR mice failed to show evidence of viable neoplastic cells before Day 11 for spleen and before Day 21 for thymus.

Leukemic AKR mice that received combination therapy using vincristine, palmO-ara-C, and methyl-CCNU sustained an increased life-span of about 180%, but less than 7% of the mice survived beyond 100 days (Chart 4). These animals then died of lymphoma as a result of reinduction of a secondary lymphoma where the spleen rather than thymus was evident.
the primary organ involved in the repopulation of clonal lymphocytes after chemotherapy.

Combination chemotherapy followed by immunization with $2 \times 10^6$ neuraminidase-treated allogeneic E$_2$G leukemia cells injected at multiple sites at the days indicated in Chart 4 resulted in the survival of 30% of treated mice free of disease beyond 120 days. Immunization alone carried out with $2 \times 10^6$ untreated allogeneic E$_2$G leukemia cells used as an immunogen was ineffectual, and the treated mice died at about the same rate as did the drug control group (Chart 4).

Using the above experimental protocols we established the relationship between the AKR virus titer and the type and clinical efficacy of the treatment. The results presented in Table 1 indicate that cytoreductive therapy alone did not significantly alter the viral titer in the treated AKR mice. Chemotherapy and immunotherapy with neuraminidase-treated leukemic thymocytes i.d. produced a noticeable decrease in the viral titer after the 13th day of the treatment, and this remained at a lower level at 45 days after the initiation of the treatment.

**Effect of Virazole in Combination with Cytoreductive Therapy in Leukemic AKR Mice.** When Virazole was tested for antiviral activity against MuLV in vitro by the XC focus-forming assay, 80% inhibition of the plaque formation was achieved with as low as 20 µg Virazole per ml of culture medium. For this reason we began to study the effect of Virazole with or without cytoreductive therapy in leukemic AKR mice. Virazole in doses as high as 800 mg/kg showed no quantitative or qualitative effect on the lymphocyte function of the treated animals (WBC, phytohemagglutinin, pokeweed blastogenesis, or thymus-derived lymphocytes). Virazole alone showed no apparent antitumor effect in clinically diagnosed AKR mice, and only a slight increase in the survival time was observed when used in combination with vincristine. When clinically diagnosed AKR mice received combination cytoreductive therapy (vincristine (0.75 mg/kg on Day 1), prednisone (30 mg/kg on Days 1, 2, 3, and 4), followed by Virazole (150 mg/kg on Days 9, 10, 11, and 12)), there was a delay in the reappearance of viable clonalogic lymphoma cells as measured by the explantation-splenomegaly assay and a moderate increase in the survival time of these animals as compared to the ones receiving cytoreductive therapy alone (Chart 5). The most effective combination was vincristine plus Cytoxan plus methyl-CCNU followed by 3 doses of Virazole (200 mg/kg), on Days 9, 10, and 11. The increase of life-span with chemotherapy alone was 183%, while with chemotherapy plus Virazole it was 280% (Chart 6). There was a short delay in the reappearance of the viable clonalogenic lymphoma cells as measured by the explantation-splenomegaly assay as compared to animals that received cytoreductive therapy alone (Chart 7).

In order to test whether weekly injections of Virazole could further increase the survival of leukemia mice, 30 AKR mice were first treated with a combination of vincristine plus Cytoxan plus methyl-CCNU followed by 3 doses of Virazole on Days 9, 10, and 11 followed by treatment with an additional 200-mg/kg dose of Virazole once a week. Results presented in Chart 6 clearly indicate that after the 7th injection of Virazole the mortality rate changed and most of the remaining treated AKR mice died, presumably because of Virazole-induced drug toxicity.

The results in Table 2 show that chemotherapy plus Virazole produced a decrease in the viral titer evident after the 13th day of treatment.

**Effectiveness of Purified Mouse Interferon on MuLV Titer and Subsequent Change on the Appearance of Primary Lymphoma in AKR Mice.** In the light of our partial success in spontaneous AKR leukemia using chemoinmunotherapy or antiviral agents in combination with cytoreductive therapy, we investigated the effectiveness of purified exogenous mouse interferon on the expression of Gross leukemia virus and the appearance of primary lymphoma in AKR mice. The viral titer was determined prior to and during the treatment as well as 10, 30, and 85 days after initiation of treatment with interferon. Table 3 summarizes the results obtained from each experimental group. The viral titer in experimental groups that received 0.9% NaCl
solution or fetal bovine sera did not change during the course of experimentation. On the other hand, exogenous mouse interferon showed a profound effect on the viral titer. This was particularly apparent in the group of animals that received 5 x 10^5 or 5 x 10^6 units interferon and, to a lesser degree, in animals receiving 5 x 10^5 units of interferon. It is significant that the reduction of viral titer from 85 days after the initiation of treatment showed no significant increase as compared to the viral titer obtained 4 days after starting the interferon therapy.

Similarly, administration of exogenous mouse interferon had a direct effect on the appearance of primary spontaneous leukemia in AKR mice. The median life-span of AKR mice receiving 0.9% NaCl solution or fetal bovine sera after randomization was 34 and 38 weeks, respectively (Chart B). All the animals in both control groups died of primary lymphoma which was confirmed by autopsy. While in Group 3, which received the highest concentration of interferon, the viral titer (Table 3) was drastically reduced or eliminated, the animals died at the same rate as did the animals receiving 0.9% NaCl solution or fetal calf sera injection, but only 6 of 20 animals died of leukemia. The remaining animals showed marked signs of atrophy of the spleen (5 to 10 mg) or thymus. On the other hand, 45% of animals in Group 4, which received 5 injections of 7 x 10^4 units of interferon, were still alive at 52 weeks of age. In this experimental group, only 2 animals showed any sign of organ atrophy. A lower concentration of interferon, that is, 7 x 10^3 units of interferon, also showed beneficial effects, and 25% of animals were still alive at 52 weeks of age. Thus, exogenous mouse interferon used in optimal doses not only reduces the viral titer in the chronically infected AKR mice but also significantly delays the appearance of primary lymphoma.

Discussion

Immunotherapy alone using neuraminidase-treated spontaneous leukemic thymocytes or allogeneic E2G leukemic cells i.d. without prior treatment with cytoreductive therapy did not change the survival pattern of the leukemic AKR mice.

The efficacy of using allogeneic MuLV-induced E2G leukemic cells as immunogen with or without treatment with neuraminidase was compared to the data obtained using AKR leukemic thymocytes in leukemic AKR mice that were brought to remission with vincristine plus Cytoxan plus methyl-CCNU. As a result of immunization of AKR mice i.d. with neuraminidase-treated E2G leukemic cells, approximately 30% of the treated leukemic mice survived beyond 160 days without evidence of the disease.

Comparable observations were made on immunoprotection in young AKR mice. The use of both neuraminidase-treated spontaneous leukemic thymocytes and allogeneic E2G leukemic cells resulted in a significant delay of the appearance of primary lymphoma in AKR mice. Immunization performed with untreated E2G leukemic cells or neuraminidase-treated L1210 tumor cells or normal (AKR) thymocytes was ineffectual, and the treated mice died at about the same rate as did the 0.9% NaCl solution control.

It is particularly significant that the allogeneic (MuLV-induced) E2G leukemic cells used as immunogen in animals brought into remission with combination chemotherapy was as effective in prolonging the life-span of the leukemic AKR mice as were the syngeneic leukemic thymocytes (2, 3). This system may therefore represent the best model for human leukemia for working out optimal conditions for chemoimmunotherapy using allogeneic leukemic blast cells as immunogen.

Combination drug therapy plus immunotherapy resulted in considerable prolongation of the life-span of AKR mice diagnosed with spontaneous lymphoma. Longer observation of the treated mice, however, showed that despite an
span and a delay in the reappearance of the viable clonogenic lymphoma cells as compared to those AKR mice that received cytoreductive therapy alone. Virazole inhibited the growth of some type of tumors (9-16, 24, 31). apparent “cell cure” relapse nonetheless follows, probably due to viral reinduction. For this reason, we studied the effectiveness of Virazole, a broad-spectrum antiviral agent (18, 20, 38, 39, 44, 47). The effectiveness of purified mouse interferon was also tested in vivo and in vitro against the MuLV.

Virazole showed antiviral activity against MuLV in vitro as determined by the XC focus-forming assay. A concentration of <10 μg Virazole resulted in 80% inhibition of MuLV replication without any apparent cytotoxicity or cell death of the treated mouse embryo cells. Experiments performed in clinically diagnosed spontaneous leukemic AKR mice with interferon was also tested in vivo and in vitro against the MuLV.

Virazole inhibited MuLV replication in vitro as well as in combination with chemotherapy in vivo and produced a decrease of viral titer in the treated AKR mice. Animals that received chemotherapy alone showed no change in their viral titer. We tested the effects of purified mouse interferon on the expression of MuLV, the etiological agent for spontaneous leukemia in AKR mice. The viral titer was determined prior, during and 10, 30, and 85 days after the treatment with various dosages of purified mouse interferon. The MuLV titer, as measured by the XC focus-forming assay utilizing mouse tail extracts, showed no change in experimental groups where the animals received 0.9% NaCl solution or 10% fetal bovine sera. There was a marked decreased in the viral titer of animals that received interferon treatment. This was particularly true in mice that received multiple injections of 5 x 10^4 or 5 x 10^5 units interferon. It is significant that the reduction of viral titer (2000 to <100) was not a transient one, since the animals tested on day 85 showed no significant difference from their MuLV titer observed only 4 days after the initiation of the interferon therapy. The magnitude of MuLV titer reduction in AKR mice achieved by exogenous interferon is far greater than that by the combination of cytoreductive therapy and Virazole with or without immunotherapy using syngeneic or allogeneic leukemic cells. Administration of purified mouse interferon in chronically infected AKR mice had a direct effect on the appearance of the primary spontaneous leukemia. The median lifespan of the control groups (0.9% NaCl solution or fetal bovine sera) was 36 weeks. All animals in these groups died of primary lymphoma as confirmed by autopsy. AKR mice treated with the highest dose of interferon (5 courses of 7 x 10^5 units of interferon) died at a faster rate than did animals given injections of 0.9% NaCl solution or fetal calf sera. However, only 7 of 20 mice died of leukemia; and the remaining ones showed marked signs of organ atrophy, became athymic, and had abnormally small spleens (5 to 10 mg) at the time of death. There were definite signs of wasting in these animals. On the other hand, 45% of the AKR mice treated with 5 doses of 5 x 10^5 units of interferon are still alive at 54 weeks of age. In this experimental group only 4 animals of 11 that died showed any sign of organ atrophy. Thus, interferon used under optimal conditions not only reduces the MuLV titer in the chronically infected AKR mice but also significantly delays the appearance of primary lymphoma. Our studies substantiate and further extend observations reported by several laboratories that interferon treatment appears to offer a certain degree of protection in animals infected with oncogenic viruses, and it appears to inhibit the growth of some type of tumors (9-16, 24, 31).

### Table 3
Change of MuLV titer in AKR mice after treatment with exogenous mouse interferon

<table>
<thead>
<tr>
<th>Experimental groups^a</th>
<th>Pre-treatment (Day 0)</th>
<th>During treatment</th>
<th>Posttreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.9% NaCl solution)</td>
<td>2020</td>
<td>2330</td>
<td>1725</td>
</tr>
<tr>
<td>Control (fetal bovine serum)</td>
<td>2245</td>
<td>2030</td>
<td>2000</td>
</tr>
<tr>
<td>Interferon (7 x 10^4 units/injection)</td>
<td>2010</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>Interferon (7 x 10^4 units/injection)</td>
<td>3010</td>
<td>35</td>
<td>190</td>
</tr>
<tr>
<td>Interferon (7 x 10^4 units/injection)</td>
<td>2655</td>
<td>85</td>
<td>135</td>
</tr>
</tbody>
</table>

^a Treatment of AKR mice was performed after randomization by injection of 0.9% NaCl solution or 10% fetal bovine sera, or exogenous mouse interferon i.p. on Days 0, 2, 4, 6, and 8.

^b MuLV titer is expressed per ml of 2% extract of a short section (4 to 6 mm) of AKR mouse tails obtained from animals at days indicated, using the XC focus assay.
Interferon has been shown to inhibit the multiplication and transformation in vitro and in vivo of many RNA and DNA viruses (7). Our observations based on the AKR system are now being translated in our laboratory into various therapeutic models in various animal systems.

The combined modality of therapy clearly offers a major improvement in the control of spontaneous MuLV-induced leukemia. The combined uses of chemotherapy, antiviral agents and interferon, and immunotherapy directed against tumor cells and viral etiological agents have direct implications for human neoplastic diseases.

References


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