The Effect of Drug Therapy against a Histologically Defined Rat Leukemia

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SUMMARY

A spontaneous rat leukemia, free of oncornaviruses, was defined pathologically and shown to be useful as a model system for testing three clinically active drugs. Histologically, the disease is a lymphoblastic leukemia, with virtually all organs densely infiltrated by leukemic cells. Treatment with specific doses of Cytoxan and melphalan resulted in a complete disappearance of the established tumor for a period of time before subsequent recurrence of the leukemia and eventual death of all animals. The use of nonspecific immune stimulators as adjuncts to effective chemotherapy failed to be any more effective, on groups of animals, than treatment with drug alone. The implications of these results to cancer therapy are discussed.

INTRODUCTION

Very few cases of spontaneous leukemia in rats have been reported following their exposure to irradiation, 3-methylcholanthrene, and other leukemogenic agents. Results from controlled studies indicate that, in Wistar/Furth and Fischer rats, an incidence of spontaneous leukemia as high as 25% has been observed (13, 14). Moloney et al. (12) suggested that the 20% incidence of spontaneous leukemia they observed in aged Wistar/Furth rats may have a viral etiology.

The histological changes that occur in Fischer rats inoculated with NRL strongly resemble those which occur in human acute lymphoblastic leukemia. This similarity prompted us to investigate the use of this rat leukemia as a possible model system for evaluating the effectiveness of drug and immunotherapy. Three drugs proven clinically active were selected to test in this system.

MATERIALS AND METHODS

Tumor. The NRL, which is transplantable in the Fischer 344 rat, was kindly supplied by Dr. Joel Warren, the Leo Goodwin Institute for Cancer Research, Fort Lauderdale, Fla. The tumor arose spontaneously in a Fischer 344 rat that was maintained in an isolator for over 1.5 years. This rat was part of a line of germ-free Fischer rats originally established at the National Cancer Institute, maintained by brother-sister matings, and reared in germ-free isolators. This colony has been periodically monitored for indigenous viruses. Tumors arising in this colony have been examined by electron microscopy and found to be free of the oncornavirus. The NRL has been maintained in Fischer 344 rats for over 80 passages (24). The transplantable leukemia has been maintained in our laboratory by s.c. inoculation of a homogenous tumor cell suspension passed every 12 to 15 days, or as an ascitic tumor that is passed i.p. at 10-day intervals in conventionally reared male Fischer 344 rats.

Rats. Young male Fischer rats, approximately 4 weeks old, were obtained through the Mammalian Genetics and Animal Production Section, Drug Research and Development, National Cancer Institute, NIH, Bethesda, Md. The animals were housed in stainless steel cages and fed Purina laboratory chow with water ad libitum. All animals were aged until they weighed 150 to 200 g before use in experimentation.

Drugs. Cytoxan (cyclophosphamide), melphalan (L-phenylalanine), and BCNU were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. The agents were dissolved in phosphate-buffered saline (pH 7.0) and administered i.p. in a constant volume of 0.01 ml/g of body weight.

Nonspecific Immunostimulators. The Phipps strain of BCG was obtained from the Trudeau Institute Mycobacterial Culture Collection, Saranac Lake, N. Y. The preparation and storage of BCG has been previously described (19). The quantitation of the viable BCG preparation was determined by the method of Rosenthal et al. (23). For use in experiments, frozen vials of BCG were thawed rapidly in a 37° water bath and diluted in phosphate-buffered saline (pH 7.0). Rats were inoculated i.d. contralateral to the primary site of tumor inoculation with approximately 8 x 10^7/0.2 ml viable BCG organisms. CG was kindly supplied by Dr. J. E. Chermann, Institute Pasteur, Garches, France. The killed bacteria were prepared by heating the organisms at 60° for 1 hr in the presence of 2% formalin. Animals received CG, 200 or 400 μg i.d., contralateral to the primary site of tumor cell inoculation.

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Pathology. Animals bearing tumors (30 to 40 mm) were sacrificed 17 days postinoculation, a time near death. The following tissues were removed: tumor, lymph nodes, liver, spleen, kidneys, adrenal, pancreas, bladder, testes, lung, thymus, and heart. The excised tissues were fixed in neutral formalin and routinely stained with hematoxylin and eosin. In addition, blood smears were prepared at different times during the course of the disease and were stained with either Giemsa or Wright’s stain. Hematocrits were determined in micropipettes. Osmotic fragility curves of the erythrocytes were determined according to the method of Danon (3).

Electron Microscopy. Samples of tumors, lymph nodes, and spleens were fixed in 3% glutaraldehyde followed by 1% chrome osmium (2). The tissues were dehydrated in ethyl alcohol and embedded in a mixture of Epon 816 and Araldite (15). Sections were cut on an LKB ultratome and stained with uranyl acetate and then with lead citrate (5, 22). Electron micrographs were taken with an Elmiskop 101 microscope.

RESULTS

Pathology. Grossly, the 1st manifestation of the tumor was at the site of inoculation. Growth was very rapid, with invasion and replacement of soft tissues and striated muscles. The tumor appeared poorly defined and relatively soft and creamy pink. Histologically, it was composed of densely packed, fairly uniform lymphoblasts (Fig. 1). The tumor cells showed well-defined borders and relatively narrow basophilic rims of cytoplasm. The nuclei possessed distinct nucleoli and were frequently indented. Mitotic figures were numerous. On microscopic examination, there was neoplastic involvement of virtually all organs, particularly the spleen, liver, and lymph nodes. These organs were either diffusely infiltrated by the leukemic cells or in most cases, large areas of the normal tissues were entirely replaced by lymphoblasts (Figs. 2 and 3). Other organs such as the kidney (Fig. 4), heart, lung, and adrenals showed a lesser degree of involvement. Tumor cells were not observed in the thymus, although they were present around the gland and invaded its interstitium (Fig. 5).

No striking changes in the peripheral blood appeared until 10 days postinoculation, although a gradual increase in lymphocytes were noted. From Day 10 to death (Days 17 to 25), the peripheral blood showed increasingly large numbers of undifferentiated lymphoblast-stem cells (Fig. 6). These primitive blast cells, which dominated in the terminal stages of the disease, exhibited large nuclei, prominent nucleoli, and a scanty light blue cytoplasm. Smudge cells were usually present. Until the terminal stages of the disease no anemia (normal hematocrit) was noted; however, at a time near death, anisocytosis and some nucleated red blood cells were noted. This observation initiated the examination of the osmotic resistance of the erythrocyte population (3, 21). However, fragility measurements exhibited a normal osmotic distribution of the red blood cell population, reflecting no increase in the number of very young erythrocytes in the blood of tumor-bearing rats.

Electron Microscopy. Electron microscopic examination of tumor, lymph nodes, and spleens removed from leukemic animals revealed no virus particles. Further evidence for the lack of a RNA tumor virus was the absence of the reverse transcriptase enzyme and high-molecular-weight RNA in the tumor samples. The ultrastructure appearance of the predominant cell type of the tumor was consistent with the structure of lymphoblasts. The cells showed large nuclei sometimes with extensions, nucleocytoplasmic bridges, and nuclear bodies. The cytoplasm of these cells contained many free ribosomes and polyribosomes but showed a poorly developed endoplasmic reticulum and relatively few but large mitochondria.

Drug Therapy against the NRL. Since there have been few reported chemotherapy studies against a spontaneous rat leukemia apparently free of oncorna viruses, it was of interest to test this rat model system to ascertain the therapeutic effect of 3 clinical agents.

Adult male rats were inoculated s.c. with approximately $5.2 \times 10^4$ tumor cells on Day 0. Thirteen days later, a time when tumor size ranged between 10 and 15 mm, individual groups of animals were administered 1 dose of either Cytoxan, melphalan, or BCNU. Data in Chart 1 show an antitumor response to these 3 drugs.

As may be seen, tumors grew progressively in the untreated control group, with subsequent development of generalized leukemia. All animals succumbed to the leukemia within 18 to 23 days. One administration of 15 mg of BCNU per kg, the maximum tolerated dose, resulted only in slight tumor regression followed by progressive tumor growth and death of all animals by Day 30. Interestingly, treatment with melphalan or Cytoxan at 2 drug levels resulted in complete regression of the established tumor within a 4-day period following therapy. A dose response to the drugs was observed with regards to recurrence of the tumor at the initial site of inoculation as well as to host toxicity. Melphalan at 4 mg/kg produced a short remission period (tumor disappearance) of approximately 4 to 6 days before relapse (recurrence of tumor), as well as a MST of 41 days. At the higher dose of melphalan (6 mg/kg), the remission period was extended to approximately 8 to 10 days before relapse and subsequent death of the leukemic animals, with a MST of 39 days. However, 3 of 7 animals died within 4 days following treatment, an indication of drug toxicity to the host. The remaining animals in the group exhibited severe body weight loss, anorexia, and loss of hair during the course of the study. Treatment with Cytoxan, 70 mg/kg, resulted in a similar toxic response, as described for the higher dose of melphalan, as 4 of 5 animals eventually died from the side effects of the drug, characterized again by severe body weight loss and anorexia. It was apparent from these studies that 60 mg of Cytoxan per kg was the maximum effective tolerated dose. A remission period of approximately 8 to 10 days was obtained with no apparent signs of host toxicity. However, all animals eventually succumbed with the leukemia.

Since the overall response elicited by Cytoxan at 60 mg/kg appeared to be the most beneficial to the host with regard to control of the tumor as well as to the absence of host toxicity, it was decided to see what antitumor response
Drug Therapy against a Rat Leukemia

Table 1. Drug therapy against NRL tumor. Adult male rats inoculated s.c. on Day 0 with approximately 5.2 x 10^4 tumor cells on Day 0. On Day 13, a time when tumor size averaged 10 to 15 mm, specific groups of animals were treated with either Cytoxan, melphalan, or BCNU. Animals were weighed and monitored for tumor response to the therapy throughout the experiment. Numbers in last column represent only leukemic deaths.

<table>
<thead>
<tr>
<th>Drug dose</th>
<th>No. of toxic deaths (days)</th>
<th>MST (days)</th>
<th>No. of survivors inoculated</th>
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<td>BCNU (15 mg/kg)</td>
<td>0 23-30 26</td>
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<td>0</td>
</tr>
<tr>
<td>Melphalan (4 mg/kg)</td>
<td>0 40-53 41</td>
<td>0</td>
<td>0</td>
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<td>Melphalan (6 mg/kg)</td>
<td>3 38-49 39</td>
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<td>0</td>
</tr>
<tr>
<td>Cytoxan (60 mg/kg)</td>
<td>0 38-49 43</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cytoxan (10 mg/kg)</td>
<td>4 33</td>
<td>0</td>
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</tbody>
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Chart 1. Drug therapy against NRL tumor. Adult male rats inoculated s.c. on Day 0 with approximately 5.2 x 10^4 tumor cells on Day 0. On Day 13, a time when tumor size averaged 10 to 15 mm, specific groups of animals were treated with either Cytoxan, melphalan, or BCNU. Animals were weighed and monitored for tumor response to the therapy throughout the experiment. Numbers in last column represent only leukemic deaths.

would be elicited when lower doses of the drug were administered. Adult male rats were inoculated s.c. with approximately 1.0 x 10^6 tumor cells on Day 0, followed by treatment with varying doses of Cytoxan on Day 13, a time when tumor size was approximately 15 mm. Individual groups of animals were then followed for tumor disappearance; relapse was evidenced by recurrence of tumor at the site of inoculation and MST. The results are presented in Chart 2.

Again, Cytoxan was effective against the NRL tumor, regardless of the dose administered, in producing a marked regression of the established tumor within a 3-day period following therapy. Tumors grew progressively in the untreated control group, with all animals succumbing to the leukemia within 19 to 22 days. The effectiveness of Cytoxan, 60 mg/kg, is reflected by the 100% increase in MST (40 days) attained compared to the untreated control group (20 days). Likewise, a complete tumor remission period for approximately 11 days occurred before recurrence of the tumor, without any apparent signs of host toxicity. Although 40- and 50-mg doses of Cytoxan per kg were effective against the tumor growth, neither dose produced a remission period as long as that observed with the higher level of the drug. Regardless of dose, there was a 100% tumor relapse, with all animals eventually dying with the disease.

Combined Chemoinmunostimulation Therapy. It was apparent that melphalan and Cytoxan at specific doses were effective against the NRL. Since all animals eventually relapsed and higher drug levels could not be used, we considered whether nonspecific immune stimulators, when used as adjuncts to effective chemotherapy, would increase the period of remission and/or obtain long-term survivors free of leukemia. Recently, Pearson et al. (19, 20), utilizing a murine leukemia, reported a substantial number of long-term survivors when 2 nonspecific immune stimulators were used in combination with drug therapy.

In the present study, a large number of rats were inoculated s.c. with approximately 3.6 x 10^4 NRL cells on Day 0. On Day 11, when the tumor size ranged between

![Graph](image)

Chart 2. Drug therapy against NRL tumor. Adult male rats inoculated s.c. on Day 0 with 1.0 x 10^6 tumor cells. On Day 13, a time when tumor size was approximately 15 mm, specific groups of animals were treated with either Cytoxan ranging from 40 to 60 mg/kg. Animals were observed daily for tumor regression and recurrence.
10 to 15 mm, specific groups of rats were treated with Cytoxan, 60 mg/kg, alone or in combination with BCG. BCG was administered in a shaved area at different time intervals following drug therapy. The results of this experiment are shown in Chart 3. The range of individual deaths in the untreated control group was 22 to 28 days with a MST of 25 days. Treatment with Cytoxan, 60 mg/kg, on Day 11 resulted in an increase of MST to 44 days, with the range of death protracted over a longer period of time (42 to 54 days). Administration of $8 \times 10^7$ viable BCG organisms on either Day 14, 17, 20, or 23, a period of tumor remission, following drug administration on Day 11, was no more effective than the drug used alone.

Similar results were obtained when 2 different doses of CG were administered at specific time intervals following chemotherapy (Chart 4). In this study, animals were inoculated s.c. with $1.5 \times 10^4$ NRL cells on Day 0, followed by Cytoxan treatment on Day 11. CG at doses of 200 or 400 µg was given i.d. on either Day 14, 17, 20, 23, or 26, a period of tumor remission, at a site contralateral to the primary site of tumor cell inoculation. As may be seen in Chart 4, the control group exhibited a MST of 20 days, with all animals dead by Day 21. No significant difference was observed in the MST's or individual days of death in groups of rats that received drug alone or drug plus CG.

Since BCG or CG therapy given following drug therapy appeared to be no more effective than drug treatment alone, it was decided to determine whether presensitization of rats with BCG before tumor challenge would augment the subsequent therapy with drug alone or with drug followed by a 2nd administration of BCG during the remission period. A specific group of animals were inoculated i.d. with $8 \times 10^7$ viable BCG organisms. Twelve days later, the BCG-sensitized rats, as well as a group of unsensitized animals, were inoculated s.c. with $2.2 \times 10^4$ Nova ascites cells. Ten days later, when tumor size ranged between 12 and 15 mm, all animals were treated with Cytoxan, 60 mg/kg. On either Day 15, 18, or 21, when the tumor was no longer palpable or macroscopically observable, rats were again inoculated i.d. with $8 \times 10^7$ viable BCG organisms contralateral to the primary site of tumor inoculation. As is shown in Chart 5, all untreated control animals were dead by Day 19, with a MST of 14 days. Presensitization with BCG failed to augment the subsequent therapy with drug, or drug followed by a 2nd treatment with BCG. All groups, regardless of treatment, exhibited similar MST's, with no long-term survivors.

**DISCUSSION**

The purpose of this study was 2-fold: (a) to describe the pathology of a spontaneous rat leukemia and (b) to attempt to control this neoplastic disease by drug therapy. It is well known that drug therapy has been successful in achieving prolonged periods of remission in the treatment of neoplastic disease. However, complete cures are uncommon and difficult to achieve because of the failure to totally eliminate residual viable tumor cells. In this investigation, a similar chemotherapeutic response was observed. Cytoxan and melphalan at specific dose levels induced a period of...
remission. However, all animals eventually relapsed and died from the disease. When higher drug levels of Cytoxan (70 mg/kg) and melphalan (6 mg/kg) were administered in hopes of extending the period of remission and/or cures, drug-induced host toxicity occurred. It was apparent from these studies that 60 mg of Cytoxan per kg was the most effective tolerated dose although all animals eventually succumbed to the leukemia.

The demonstration of tumor-specific antigens derived from human and animal cancers (6, 7, 18) suggests that immunological control measures might be valuable adjuncts to other forms of therapy. BCG has previously been reported to protect against viral-induced transplantable tumors (11, 17) and tumor viruses (8, 9, 25, 26). Certain species of Corynebacterium used as nonspecific stimulators have also been shown to be effective in the treatment of experimental animal tumor systems (4, 16, 27, 28). Recently, the successful use of BCG and different Corynebacterium species in combination with drug therapy against animal and human neoplasias has created much interest (1, 10, 20).

Since drug therapy alone failed to yield any long-term survivors free of leukemia in this study, attempts were made to see what effect the use of nonspecific immune stimulators as adjuncts to effective chemotherapy would have in either increasing the period of remission and/or obtaining long-term survivors free of the disease. An earlier study showed that BCG or CG, when administered alone, had no protective effect when given to NRI tumor-bearing rats (unpublished observations). In the present studies, it was felt that treatment with Cytoxan, 60 mg/kg, effectively lowered the tumor load to a point at which immunostimulation therapy would be advantageous to the host. However, the administration of BCG or CG at various times following drug therapy in this rat leukemia system failed to achieve any additional protection.

The effectiveness of chemoimmunostimulation therapy depends on several factors. Two very important prerequisites are: (a) the retention of a satisfactory immune status of the host, particularly after drug therapy, and (b) the ability of the host to develop an appropriate immune response to the tumor. Drug immunosuppression was an unlikely explanation for the poor response observed in these studies since rats presensitized to BCG, given injections of NRI tumor cells, and subsequently treated with Cytoxan, 60 mg/kg, reacted strongly to tuberculin when given i.d. injections 3 days after drug therapy (unpublished observations). The questions of whether the tumors studied possess tumor-specific transplantation antigens and whether a possible lack of such antigens was the basis for the failure of immunotherapy are now being studied.

REFERENCES

26. Sjogren, H. O., and Ankerst, J. Effect of BCG and Allogeneic Tumor


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Fig. 1. Section through center of the tumor, 16 days postinoculation. H & E, × 250.
Fig. 2. Section of an axillary lymph node, 17 days postinoculation. Large areas are replaced by leukemic tumor cells. H & E, × 250.
Fig. 3. Section of a liver, 16 days postinoculation. Note the high rate of mitotic figures of the tumor cells. H & E, × 250.
Fig. 4. Section of a kidney, 16 days postinoculation (same animal as in Fig. 3). H & E, × 250.
Fig. 5. Section shows tumor cells surrounding the thymus with no parenchymal infiltration. H & E, × 250.
Fig. 6. Blood smear from a leukemic rat in advanced phase of the disease (Day 15). Undifferentiated stem cells are present. Wright's stain, × 250.
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[Image of histological slides]
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