Synergistic Toxicity of Triethylene Thiophosphoramide and Folic Acid in the IRC 741 Leukemia*

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SUMMARY

The purpose of this study was to determine the influence of folic acid or vitamin B-12 on the course of the IRC 741 rat leukemia treated with thioTEPA. On the first day immediately following tumor implantation into Fischer rats individual series of these animals were given 6 mg/kg thioTEPA, in a single injection, or 1 mg/kg, every other day for six doses without and with simultaneous injections of folic acid or vitamin B-12. ThioTEPA alone, in multiple doses, increased the rat survival time, but these animals eventually died of leukemia. ThioTEPA in a single injection, although more toxic, nevertheless produced remissions consistent with experimental cure. The most important observation, however, was that the concomitant administration of folic acid and a single dose of thioTEPA was associated with premature death of all animals so treated, suggesting that under certain experimental conditions folic acid may interfere with the ameliorative or curative properties of this particular antileukemic agent. Vitamin B-12 appeared inactive in this experimental system.

The purpose of this study was to determine whether folic acid or vitamin B-12, when given in combination with triethylene thiophosphoramide (thioTEPA), would modify the course of the Dunning, IRC 741, acute histiocytic rat leukemia (5). This experimental tumor arose spontaneously in an inbred Fischer rat which had been carrying a chemically induced mammary carcinoma and since 1957 has been propagated without interruption. As observations were made in this laboratory of the serial morphologic bone marrow changes which occur during the evolution of this type of animal leukemia it was noted that progression of the leukemia was accompanied by a reversal of the myeloid:erythroid ratio. In addition, the erythroid precursors late in the disease consisted mainly of rubriblasts and prorubricytes, many of which had a megaloblastoid appearance.

In view of the fact that folic acid and vitamin B-12 have been used both clinically and experimentally to modify this type of erythropoiesis, these agents were used in a like manner in this investigation. Of associated interest was the possibility, predicated on clinical inference (6), that either or both of these substances might materially effect the growth of the tumor or alter its response to antileukemic therapy. ThioTEPA was used as the prototype chemotherapeutic agent because it significantly increases the leukemic animal survival time, there is less toxicity from it with the minimal effective dose, it is relatively more stable than nitrogen mustard, and it has been employed in the clinical management of human malignant disorders.

MATERIALS AND METHODS

Inbred male and female Fischer rats weighing 70–120 gm., obtained by brother-to-sister matings, housed in stainless steel cages in groups of six to ten animals, were employed in the experimental procedures. They were given supplemental vitamins1 from birth to insure adequate nutritional

1 As Deca-Vi-Sol, Mead Johnson and Company, Evansville, Indiana.
status in addition to the ordinary laboratory diet of purina chow pellets and tap water ad libitum. As the method for inter-animal transfer, a 2-cu. mm. solid tumor fragment from the donor rat was transplanted subcutaneously by trocar into the axillary region of the recipient animal. Serial in vivo bone marrow aspirations were performed at 2-day intervals on rats intraperitoneally anesthetized with 0.2 ml. of a 0.6 per cent solution of sodium pentobarbital. Either a 5-mm. incision was made over the lateral femur or the ilium and the skin, fascia, and muscles were separated to expose the periosteum. With the use of a dental burr attached to a Handee 600 electric drill the bone was rapidly penetrated and entrance gained to the medullary cavity. A 20-gauge, unbeveled needle attached to a 5-cc. glass syringe was introduced through the drill opening and marrow obtained by rapid plunger withdrawal. Marrow particles were smeared on coverslips and stained with Wright-Giemsa stain. A single suture approximated the skin edges, and the animal was returned to the cage to be used again 2 days later.

To determine the survival effect of thioTEPA on leukemic and normal rats, this agent was injected subcutaneously in two doses, as a single injection of 6 mg/kg (1.2 mg/ml sesame oil suspension) and six injections of 1 mg/kg, one every other day (0.2 mg/ml suspension). ThioTEPA was given to the tumor recipient on day 1 immediately following the transplantation of the tumor and simultaneously to the healthy animal. In all procedures outlined the course of the untreated leukemic rat was used as a comparative, survival control. Similarly, folic acid and vitamin B-12 were given intraperitoneally to determine the effect of these substances on both the nontreated leukemic and normal animals. In the normal animals 2.5 mg/kg of folic acid and 10 µg/kg of vitamin B-12 were given daily for 10 days. The doses for the leukemic animals were identical, but the drugs were given in separate animal series, on day 1 following the transplantation for 10 days and on day 7 after transplant for one injection only. This particular treatment regimen was selected because the 7th day is the earliest time when the leukemia could be detected visibly in the bone marrow and death of untreated leukemic rats may occur initially on the 10th day after tumor transfer. In addition, both vitamins were administered in various combinations, as indicated in Table 1, to leukemic and normal animals. Code letters were assigned to these experiments to facilitate recording of results.

RESULTS

Bone Marrow Alterations

The normal Fischer rat bone marrow is cellular, with few fatty areas. The myeloid:erythroid ratio is 5:1, and the red cell precursors are small and characterized by dark staining, clumped nuclear chromatin with a thin rim of homogeneous, basophilic cytoplasm. Mature histiocytes are usually scattered throughout the bone marrow (Fig. 1). On the 10th post-transplant day, when the leukemia is visibly well established, the myeloid:erythroid ratio is reversed, and many of the erythroid elements appear megaloblastoid (Fig. 2). The majority of the remaining cells are histiocytes which resemble those of the normal animal. Nucleolated blast forms are conspicuous by their absence. Within the next 24-48 hours the erythroid precursors disappear, and the marrow consists almost entirely of histiocytes (Fig. 3). Death of the animal occurs during or immediately following this period.

TREATMENT

ThioTEPA.—Table 1 represents the survival response of leukemic (and normal) rats to thioTEPA when given in two dosage regimens. At 1 mg/kg every other day for six injections the medication is innocuous to the normal animal and prolongs the life of the animal with leukemia, although a cure is not obtained. Conversely, when 6 mg/kg was given in a single dose, a number of toxic deaths (10 per cent) was present, but, more important, 90 per cent of the animals had a remission compatible with an experimental cure. The term “experimental cure” is defined as an animal surviving more than 60 days after treatment without a palpable tumor mass and with normal peripheral blood and bone marrow.

Folic acid and vitamin B-12.—The apparent inactivity of folic acid and vitamin B-12 in untreated leukemic (and normal) animals is shown in Table 1. By comparing the survival figures of the folic acid- and vitamin B-12-treated animals with a mean survival time of 12.9 days in the untreated leukemic rat it is evident that neither of these agents has a significant influence on the leukemia course when administered alone in this manner. This inactivity was also reflected in the unchang-
ing bone marrow characteristics of the normal and leukemic rats.

**Combined administration.**—When combinations

**TABLE 1**

<table>
<thead>
<tr>
<th>Condensed Experimental Protocols Used with Leukemic Rats</th>
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<tbody>
<tr>
<td>Drug*</td>
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</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>ThioTEPA</td>
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<tr>
<td>ThioTEPA</td>
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<tr>
<td>Folic acid</td>
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<td>Folic acid</td>
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<td>Vitamin B-12</td>
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<td>Vitamin B-12</td>
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<td>ThioTEPA + (A)</td>
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<td>ThioTEPA + (C)</td>
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<td>ThioTEPA + (E)</td>
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<td>Folic acid</td>
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<tr>
<td>ThioTEPA + (F)</td>
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<td>Folic acid</td>
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* 24 leukemic animals and 24 healthy, normal controls were used for each experiment (total, 624). Capitals in parentheses are code letters for specific drug combinations.
† From day after transplantation of tumor.
‡ The number indicates total survival time subsequent to which, unless the animal was cured, death resulted. 00 denotes unaltered life span.

**DISCUSSION**

The most important observation of this study was that folic acid, when given concurrently and continually with the specific curative dose of thioTEPA, led to premature death of all animals so treated. The basis for this unexpected outcome is not known at present, and only relevant suppositions may be made to explain these findings. This result might be due to an inherent toxicity of each agent. However, folic acid itself apparently is not toxic, since normal and leukemic animals tolerate it with impunity. In addition, thioTEPA is lethal to just 10 per cent of both normal and leukemic animals and only when given in optimum amounts. An alternative hypothesis may be that, in this particular experimental system, as the result of leukemic cell growth, the nonmalignant bone marrow elements may have developed a deficiency of folic acid (1, 3, 4, 7, 12). This change could conceivably protect the bone marrow in the following manner: Administration of folic acid to this animal might accelerate the growth of normal reserve bone marrow stem cells by correcting the suspected metabolic defect and, since antileukemic drugs are more potent against rapidly proliferating tissues (7), the hiatus between the toxic and beneficial results of therapy would be narrowed. The chemotherapeutic agent, therefore, presumably would inhibit not only leukemic cells but also might depress the normal marrow components necessary for recovery from the cytotoxic agent.

**FIG. 1.**—The normal Fischer rat bone marrow (X980). The erythroid elements appear as small, dark-staining cells with a narrow rim of cytoplasm. The larger, vesicular cells, exemplified by the two cells in the central, lower portion of the photomicrograph, are histiocytes normally found in the bone marrow.

**FIG. 2.**—The bone marrow on day 10 of the leukemia (X980). The megaloblastoid precursors are located mainly in the upper portion of the microscopic field and contain large, dark-staining nuclei. Some rubricytes are present and, combined with the earlier forms of erythropoiesis, have caused a reversal of the myeloid:erythroid ratio.

**FIG. 3.**—Just prior to death, the bone marrow is composed mainly of histiocytic elements (X980). The small, dark granules result from trauma to marrow mast cells during the making of the smear.
The failure to observe concurrent morphologic changes in the serial bone marrow examinations of these leukemic animals suggests that the folic acid effect may be mediated at a cellular, biochemical level and may not be delineated by microscopy. The absence of a deleterious consequence with vitamin B-12 suggests that it is inactive in this particular drug relationship, despite the fact that both folic acid and vitamin B-12 are involved in similar metabolic processes (8).

The associated observation that the fatal results in combined therapy occurred only under specific dosage regimens and time relationships indicates that these factors are of critical importance in the production of apparent synergistic toxicity. A similar outcome has been recently observed between methyl-bis-guanylhydrazone (CH₄-G) and citrovorum factor (9). In this instance the high mortality associated with the experimental use of this agent may be prevented or decreased if citrovorum factor is administered intraperitoneally 30 minutes before each dose of CH₄-G. On the other hand, earlier pre-administration of folic acid greatly increased the lethality from CH₄-G, as did N₁⁰-formyl folic acid.

Finally, this type of experiment may serve as a laboratory prototype so that the more detailed, specific modes of action of other chemotherapeutic agents may be defined. Although the various parenteral and oral forms of polyfunctional alkylating agents, including thioTEPA, appear to be interrelated, the mechanisms by which they exert their effectiveness are not completely known (10). The differential activity which they and other agents might demonstrate in relation to folic acid and vitamin B-12 would help to classify these drugs on the basis of their metabolic and biochemical roles in therapy and might, when used with other experimental tumors, reveal basic patterns of neoplastic growth.

REFERENCES
Synergistic Toxicity of Triethylene Thiophosphoramide and Folic Acid in the IRC 741 Leukemia

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