Tumor and Marrow Damage Occurring from Methotrexate, Monofluoromethotrexate, Difluoromethotrexate, and Dichloromethotrexate

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SUMMARY

The effects of methotrexate, 3',5'-dichloromethotrexate (DCM), and two new anti-folic acid compounds, monofluoromethotrexate (MFM) and difluoromethotrexate (DFM), on the C3H mouse marrow count and a transplanted tumor, the 6C3HED lymphosarcoma, were studied. The data were presented in the following forms: plots of the regression lines of marrow counts and tumor weight responses against dose of compound used; slopes of the regression lines; relative potencies of the bone marrow destruction; and antitumor activity as compared with methotrexate as a standard.

MFM caused the greatest damage to the marrow; DFM was almost equal to methotrexate in its destructive effect on the marrow. DCM caused no detectable destruction of the marrow in a dose range of 6.6 to 33 mg/kg; at a range of 75 to 168 mg/kg mild destruction was detected. All compounds caused suppression of tumor weight with increase in dose. The slopes of the plots of the regression lines of tumor weight against dose were all close to \(-1.0\). The relative potency of the antitumor activity of MFM and DFM and DCM compared with that of methotrexate showed that it took 3, 19, or 99 times as much compound, respectively, to cause the same amount of tumor suppression as resulted from methotrexate (dose range, .195-\(\approx\).25). The marrow destructive effect required 1.7, 31, or \(\approx00\) times as much MFM, DFM, or DCM as methotrexate, respectively.

DCM, 33 mg/kg/day for 54 days or a total dose of 1782 mg/kg, did not cause depression of the marrow, white, red, or peripheral blood differential counts.

Interest in the antifolic acid series of analogs in experimental cancer chemotherapy has been stimulated by the investigations of Goldin (5, 6). This paper is concerned with methotrexate and three of its analogs: 3',5'-dichloromethotrexate (DCM), 3'-monofluoromethotrexate (MFM), and 3',5'-difluoromethotrexate (DFM). The latter two compounds were recently synthesized by these laboratories (15). The use of mouse femur marrow as a measure of the damage to a host tissue which occurs concomitantly with treatment of the tumor in the same animal is the main basis of this study and has been presented in another paper (16).

MATERIALS AND METHODS

Dose response.—All animals used were C3H (18-20 gm.) male mice. Intraperitoneal injection.

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(\(\frac{1}{2}\) cc.) of a compound made up in buffered starch solution was started 3 days following transplantation of the 6C3HED lymphosarcoma and continued once a day for 6 days. Groups of eight animals were used at each dose. The tumors were removed and weighed on the 7th day. Body weights were taken of each group of animals on each dose at the time of the first injection and on the last day (7th) of the experiment. Bone marrow nucleated cell counts were done on one femur from each of five animals of each group. The method of removing and counting these elements was the same as that developed by Gerarde (4). Each dose response was replicated 3 times. The doses used are indicated in the section concerning results and as the abscissa entries in the chart for each compound.

Prolonged dosage of dichloromethotrexate.—DCM
was given intraperitoneally in doses of 17 or 33 mg/kg/day for 54 days to two groups of normal 18- to 20-gm. male mice. On the day that the experiment was started five mice were killed, and the following studies performed: marrow count, total white blood cell count (blood from neck stump following decapitation), total red cell and differential counts (granulocyte and lymphocyte counts—total of 100 cells/smear). These studies were done once a week, on five animals from each group, starting on day 14 of the experiment. Dosage was terminated on day 54, and the last studies were done on day 59.

Carcinostatic index.—This index was calculated from the body weight changes and tumor weights at each dose according to the formula (13):

\[
\text{Carcinostatic index} = \frac{\text{Dose of compound causing a toxic weight loss}}{\text{Dose of compound causing suppression of the treated tumor weight to 50 per cent of that of the controls}}
\]

RESULTS

DOSE RESPONSES

Methotrexate.—Chart 1 shows the plots of the marrow counts and tumor weights against doses used. Both the tumor tissue and marrow count were dose-responsive. The relative loss of marrow tissue was less than that of the tumor tissue, as can be seen by visual comparison of the two slopes, which were −.22 and −.72, respectively. The marrow count at the highest dose was \(8.0 \times 10^6\), whereas that at the lowest dose was \(12 \times 10^6\) as compared with the control count of \(14 \times 10^6\).

The tumor weight at 1.0 mg/kg was 79 mg, as compared with the control mean value of 1270 mg. The appearance of the marrow as it was expressed from the femur was not remarkably different from that of the control animals, except that occasionally it appeared to be slightly bloody. The ratio of the mean control/mean treated value at the highest dose was 1.8 and 16 for the marrow and tumor, respectively.

Monofluoromethotrexate.—Chart 2 shows the plots of the regression lines of tumor weight and nucleated cell count on the doses of MFM used in this experiment. With increase in dose there was progressive loss of each tissue. The two slopes were −.66 and −1.0 for the marrow and tumor, respectively. The marrow counts at the highest dose were markedly depressed as compared with the control value of 7.5 (6.4–9.0) \(\times 10^6\). The marrow count at the lowest dose was essentially the same as that of the untreated animals. The mean control value/mean treated value for the tumor and marrow tissues was 25 and 3.2, respectively, at the highest doses used. The gross appearance of the marrow as it was expressed for counting and imprints indicated that damage was occurring—i.e., it was slightly pinkish to red at the highest dose and somewhat watery in contrast to the orange-
red and solid normal marrow. It was similar to that described in acute folic acid deficiency of the rat (11).

**Difluoromethotrexate.**—The slope for the marrow (−.25) was the same as that from methotrexate and considerably less than that of the tumor tissue (−1.1). The marrow count at the lowest dose was essentially the same as the control value (Chart 3). That at the highest dose was slightly lower. The gross appearance of the marrow on expression of the marrow for counts or imprints was not strikingly different from that of the normal animals. The tumor weights were close to those found with MFM. The ratio of the mean control value/mean treated value at the highest dose was 23 and 1.5 for the tumor and marrow tissues, respectively.

**Dichloromethotrexate.**—a) 6.6 to 33 mg/kg.—This compound was not used at its highest tolerated dose because the ensuing marked inhibition of tumor tissue found in a preliminary test (33–112 mg/kg) resulted in minimal amounts of dissectible tissue at all levels above 30 mg/kg. Accordingly, DCM was given in the dose range of 6.6–33 mg/kg. Chart 4 shows the marrow and 6C3HED lymphosarcoma tissue responses from animals treated with this compound. It can be seen that there was no slope to the marrow count plot. In contrast, there was a marked regression of the tumor tissue weight on dose (−1.2). It was possible that had marrow counts been done at higher doses decrease in the quantity of cells of the femur marrow would have resulted. Grossly the marrow was slightly thicker and somewhat paler than the usual orange-red color seen in the normal mouse.

b) 75 to 168 mg/kg.—An attempt was made to determine the amount of DCM required to cause depression of the C57 mouse marrow nucleated cells. The change in the slope of the marrow count plot was not significant (−.40) when compared to controls. The mean control value/mean treated value at the highest dose was 23 and 1.5 for the tumor and marrow tissues, respectively. Actual bone marrow counts can be obtained by dividing any ordinate value by .9.

Chart 3.—Plot of the tumor and marrow regression lines and their 95 per cent confidence limits, obtained from animals treated with 4.9, 11.1, and 25 mg/kg of difluoromethotrexate. Each point on the tumor plot is for 24 animals, and for fifteen animals on the marrow plot. Control mean tumor weight and marrow count were 1015 mg and 10 × 10^6 nucleated cells, respectively. Actual marrow counts can be obtained by dividing any ordinate value by 3.6.

Chart 4.—Plot of the tumor and marrow regression lines and their 95 per cent confidence limits, obtained from animals treated with 1.48, 2.22, 3.34, and 5 mg/kg of monofluoromethotrexate for 6 days. Each point on the tumor plot is the mean value for 24 animals, and for fifteen animals on the marrow plot. Control mean tumor weight and marrow count were 1065 mg and 7.3 × 10^6 nucleated cells, respectively. The marrow line was shifted for easier visual comparison. Actual marrow counts can be obtained by dividing any ordinate value by 2.9.

Chart 5.—Plot of the tumor and marrow regression lines and their 95 per cent confidence limits, obtained from animals treated with 4.9, 11.1, and 25 mg/kg of difluoromethotrexate. Each point on the tumor plot is for 24 animals, and for fifteen animals on the marrow plot. Control mean tumor weight and marrow count were 1015 mg and 10 × 10^6 nucleated cells, respectively. Actual bone marrow counts can be obtained by dividing any ordinate value by 3.6.

elements within the 6-day treatment period of these response studies. The doses used were 75, 112, and 168 mg/kg. Chart 5 shows the results of this experiment. It can be seen that the marrow line was not horizontal as it was in the dose range from 6.6 to 33 mg/kg. In the preliminary dose response studies cited above in which no regression of tumor weight on dose was obtained, the dose range was from 33 to 112 mg/kg. The fact that in this instance a significant regression of tumor weight on dose did occur is probably due to two (or more) factors: test-to-test variability of the 6C3HED tumor system dose response studies and differences in technic of harvesting minute amounts of tumor tissue remnants. The appearance of the marrow was similar to that found in the first dose response with DCM. No deaths occurred at any of the levels during the time period.
of this experiment. Detectable destruction of the mouse marrow within a 6-day dosage period did occur from a total dose ranging from 450 mg/kg to 672 mg/kg/week. The slopes of the tumor and marrow plots were \(-.81\) and \(-.40\), respectively.

**Effect of Prolonged Treatment with Dichloromethotrexate**

Table 1 shows the marrow, peripheral white, red, and differential cell counts of animals treated intraperitoneally with 33 mg/kg daily for 54 days or a total dosage of 1782 mg/kg. The values found for animals treated with 17 mg/kg/day, which were also run, are not shown, because they were essentially the same as those found for the higher dose. The control values shown for each of the measured parameters are the mean and 95 per cent confidence limits of the studies done on days 0, 28, 49, and 59. It can be seen that there was no confidence limits were calculable for the marrow plot, since a nonsignificant regression occurred. Control mean tumor weight and marrow count was 1344 mg and 10 \(\times 10^6\) nucleated cells, respectively.

**TABLE 1**

**Marrow, Peripheral White, Red, and Differential Counts from Animals Treated with Dichloromethotrexate**

<table>
<thead>
<tr>
<th>Day</th>
<th>Marrow count (\times 10^6)</th>
<th>White blood* cell count (\times 10^6)</th>
<th>Red blood cell count (\times 10^6)</th>
<th>Differential count (gran./l.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.1±1.2</td>
<td>4.0±2.2</td>
<td>9.6±.4</td>
<td>34/66±3.5</td>
</tr>
<tr>
<td>14</td>
<td>6.0±1.5</td>
<td>2.3±.6</td>
<td>9.4±.5</td>
<td>31/69±8.9</td>
</tr>
<tr>
<td>21</td>
<td>8.5±.5</td>
<td>4.5±2.5</td>
<td>7.0±1.0</td>
<td>45/55±8.0</td>
</tr>
<tr>
<td>28</td>
<td>8.0±2.0</td>
<td>2.5±.9</td>
<td>7.6±.6</td>
<td>55/65±17</td>
</tr>
<tr>
<td>35</td>
<td>9.0±1.6</td>
<td>1.9±.6</td>
<td>11.5±1.2</td>
<td>57/43±20</td>
</tr>
<tr>
<td>49</td>
<td>6.0±1.7</td>
<td>4.0±2.0</td>
<td>6.1±1.8</td>
<td>36/42±15</td>
</tr>
<tr>
<td>59†</td>
<td>10.6±1.2</td>
<td>1.8±.8</td>
<td>8.9±.3</td>
<td>44/50±41</td>
</tr>
<tr>
<td>Control‡</td>
<td>9.0±.7</td>
<td>3.4±.7</td>
<td>9.6±.3</td>
<td>36/64±5.0</td>
</tr>
</tbody>
</table>

* Neck stump blood; following decapitation.
† 5 days following last injection.
‡ Days 0, 28, 49, and 59.

**Chart 4.**—Plot of the tumor and marrow response to doses of dichloromethotrexate ranging from 6.6 to 33.3 mg/kg for 6 days. Each point on the tumor plot is the mean value for 24 animals, and fifteen animals on the marrow plot. No confidence limits were calculable for the marrow plot, since a nonsignificant regression occurred. Control mean tumor weight and marrow count was 825 mg and 7.3 \(\times 10^6\) nucleated cells, respectively.

**Chart 5.**—Plot of the tumor and marrow response to doses of dichloromethotrexate ranging from 75 to 168 mg/kg for 6 days. Each point on the tumor plot is the mean value for 24 animals, and fifteen animals on the marrow plot. Control mean tumor weight and marrow count was 1844 mg and 10 \(\times 10^6\) nucleated cells, respectively.
little difference in the successive marrow counts taken on the indicated days. The lowest counts occurred on days 14 and 49 and were separated by normal counts. A general and consistent tendency toward the depression of the marrow count was not apparent in this study. The normal orange-red color of the marrow gradually changed during the course of the experiment to a buff-pale pink hue which was evident by the middle and continued throughout the rest of the experiment. The white cell counts (taken from neck stump blood following decapitation) were considerably lower than those taken from the tail vein and were consistent with the values found by others in the normal mouse heart (ventricular) blood (14). There was no difference on any of the test days from the control values.

In general, there were indications of possible lowering of the red cell and marrow counts and questionable elevation of the granulocyte/lymphocyte ratio, but these findings were not definite as measured in these studies.

**Carcinostatic Index**

The individual carcinostatic indexes (13) obtained from each replicate of the dose responses performed with these compounds, the mean carcinostatic index, and the 95 per cent confidence limits are shown in Table 2. DCM had the highest mean carcinostatic index (dose range, 6.6–33 mg.). The remaining two replicates were very close to the value found by Sloboda (13) or 10.4 (7.1–15.0). The means of the indexes for the fluoro derivatives were very close, and their confidence limits overlapped, so that it cannot be said that there was any real difference between the two, by this measurement of effectiveness. Their indexes were very close to those of 3-bromo-5-chloromethotrexate (6.0) and bromomethotrexate (5.7) (13). Methotrexate had the lowest carcinostatic index, indicating that its toxicity in the time period and dose range of these experiments is more readily shown by body weight loss than marrow damage.

**Relative Potencies**

Table 3 shows the amounts of MFM, DFM, or DCM needed to cause the same antitumor or marrow-destructive effect as compared with methotrexate (.198–2.25 mg/kg) for 6 days. For example, it took 200 times as much DCM to cause the same marrow destruction as methotrexate, while it took only 1.7 times as much MFM to cause the same effect. There were also marked differences between the antitumor potencies of these compounds as compared with the standard methotrexate; i.e., it took 99 times as much DCM to cause the same suppression of the tumor.

**DISCUSSION**

Marked species resistance to antifolic acid congeners has been observed by many investigators. For example, those derivatives of the parent compound, folic acid, which occur in nature have been shown to cause different responses in different species of test organisms (8). Marrow changes induced by a series of folic acid derivatives were found to vary in a single species—the guinea pig. Aminopterin caused pronounced effects when administered over a prolonged period of time, while the N15-labeled methyl pteric acid, pteroyl aspartic acid (active form), and pteroyl aspartic acid (racemic form) caused little or no effect (7). The responses of several species to the same drug (antifolic)—aminopterin—were also found to vary. Of three species treated, the rabbit showed the least marrow depression; the dog the most; the guinea pig was slightly more sensitive than the rabbit (9). In another experiment ami-
nopterin (0.5 mg/kg for 12 days for a total dose of 6 mg/kg) was given to 30- to 35-kg. swine (1), with only minor toxic effects. This is in contrast to the mouse and rat, in which 1 mg/kg/day caused death in 8 days (3, 7, 10), and in the monkey, where 0.5 mg/kg/day caused death after 7 days (8). It should not be completely unexpected that, of the numerous antifolic acid compounds which have been synthesized, a few will prove to be less toxic to normal mouse tissues and yet retain marked efficacy against a transplanted tumor. The reasons for the ability of the mouse to tolerate massive doses of DCM, and the inability of the 6C3HED tumor and L1210 leukemia (5, 6) to do likewise, await clarification.

The lack of definite quantitative changes in marrow, white, and red cell differential counts during treatment with dichloromethotrexate for 53 days with 17 and 33 mg/kg/day is confirmation of the findings of Goldin (5, 6) of the tolerance of the mouse to tremendous doses and prolonged therapy without markedly deleterious effects. The antitumor and bone marrow-destructive potency of DCM relative to that of methotrexate is similar to the findings of Schrecker (12). It should be emphasized that these findings are the quantitative aspect of the effects of these compounds on the marrow nucleated cell count and do not indicate qualitative changes.

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