Selective Alteration of Immunocompetence with Methotrexate and 5-Fluorouracil

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SUMMARY

A drug combination comprised of methotrexate (1 mg/kg given on Day 2 of the sensitization period) followed in 1 hr by 5-fluorouracil (50 mg/kg) is capable of potentiative suppression of humoral antibody production without concomitant inhibition of cell-mediated immunity. Thus, this combination suppressed 19 S hemolytic antibody production in C3HeB/FeJ mice by 88% but had no inhibitory effect upon allograft rejection in A/J mice or upon contact sensitivity to oxazolone in C3HeB/FeJ mice. Treatment during the induction of hypersensitivity to methylated bovine serum albumin, a response apparently having a cell-mediated component opposed by a humoral blocking component, resulted in strong stimulation of the edematous end point in male C3HeB/FeJ mice. Stimulation of methylated bovine serum albumin-induced hypersensitivity was also found in female mice when leucovorin was added to the regimen. This selective immunosuppressive regimen might prove beneficial in attempts to control the production of serum blocking factors.

INTRODUCTION

Although the total immune response to solid tumors is complex, with many components interacting with one another, CMI\(^2\) and SBF seem to play primary roles (8-10). The CMI component of this response is largely responsible for the destruction of the cells of a solid tumor and therefore is viewed as a host protective response; whereas SBF, because of its ability to mitigate the effect of CMI, has been assigned a counterprotective role. Because of this relationship, there has been interest in finding means of controlling the production of SBF without compromising the effectiveness of CMI. One approach has been through the use of immunosuppressive agents.

With ara-C, as well as with 5-FU, it has been possible to demonstrate a depression of humoral antibody production without concomitant inhibition of CMI (2, 7, 11). Inhibition of the production of SBF in the mouse mammary tumor system has been achieved, along with a retardation of tumor growth in vivo.

The application of these regimens is, however, limited by technical difficulties. The dose of ara-C needed to achieve a selective inhibition of humoral antibody production varies from antigen to antigen. On the other hand, 5-FU causes a somewhat variable inhibition of humoral antibody production, particularly of 7 S hemolysin plaque-forming cells, and also is toxic at higher doses. In order to find a regimen that might give greater selectivity without these problems, we chose to investigate the immunosuppressive effect of MTX and 5-FU in combination.

The effect of MTX and 5-FU was studied in several model systems. First, hemolysin plaque-forming cell production as assayed by the Jerne plaque method was used as a measure of humoral antibody production. Next, the homograft reaction was used as an example of CMI. In addition, oxazolone-induced contact sensitivity, a response with an early cell-mediated immune component (5, 6, 13, 15), was also used. Finally, the effect of drug treatment on hypersensitivity to MBSA was studied. This reaction appears to have a humoral blocking component which is balanced in opposition to a cell-mediated immune component (2, 4). In our experience, MBSA-induced hypersensitivity has proven to be a valuable assay in that it has predicted the effect of drugs on the mouse mammary tumor system.

MATERIALS AND METHODS

Animals. A/J males and C3HeB/FeJ males and females used for this study were obtained from The Jackson Laboratory, Bar Harbor, Maine. All mice were 2 to 3 months old when used.

Hemolysin Plaque-forming Cell Assay. Animals were sensitized to sheep RBC (0.2 ml of a 10% suspension). The spleen hemolysin plaque-forming cells were determined on Day 4 after antigen according to the technique of Jerne as modified by Uyeki and Klassen (14). Details of this technique have been described previously (7).

Skin Grafting. Grafting and scoring of rejection conformed to the principles of Billingham and Silvers (1). Tail skin was used as the donor tissue. Strain C3HeB/FeJ mice were killed by cervical dislocation, and their tails were removed. The skin was peeled from the tails and laid, inner layer down, on sterile gauze soaked in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) The skin was cut from the middle of the back of each recipient (previously anesthetized with pentobarbital, 50 mg/kg). A C3HeB/FeJ...

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\(^2\)The abbreviations used are: CMI, cell-mediated immunity; SBF, serum blocking factor; ara-C, cytosine arabinoside; 5-FU, 5-fluorouracil; MTX, methotrexate; MBSA, methylated bovine serum albumin.
The Effect of MTX and 5-FU on Hemolysin Plaque-forming Cell Production. As seen in Chart 1, the administration of MTX (1 mg/kg) given on Day 2 of a 4-day sensitization period, resulted in a 35.9% inhibition of hemolysin plaque-forming cell production to sheep RBC in C3HeB/FeJ mice; 5-FU alone (50 mg/kg) caused a 35.4% inhibition. When MTX was followed in 1 hr by 5-FU, 87.8% inhibition resulted. This potentiated result was observed only when MTX was followed by 5-FU and, in fact, if 5-FU were given prior to MTX, only 29% inhibition resulted. When the 2 agents were given concomitantly, a value close to the theoretical additive value was obtained (61.8%). The inhibition seen with MTX followed in 1 hr by 5-FU was significantly different from that seen when the 2 agents were given concomitantly (p < 0.01). Thus it appeared that the combination of MTX followed in 1 hr by 5-FU could cause a profound inhibition of humoral antibody production even though the doses of drug involved were small.

The Effect of MTX and 5-FU on the Rejection of Allografts. Tail skin from C3HeB/FeJ mice was grafted onto the backs of A/J mice. Animals were treated with MTX followed in 1 hr by 5-FU on Day 4 after grafting and the bandages were removed on Day 7. The grafts were then observed for signs of rejection. The results of this experiment were as follows. The average day of rejection for untreated animals was 11.5 ± 0.2 (S.E.). Neither MTX (11.5 ± 0.5), 5-FU alone (11.9 ± 0.3), nor MTX followed by 5-FU (11.6 ± 0.4) significantly altered this response. Thus this regimen, which had been shown to cause a potentiated inhibition of humoral antibody production, appeared not to inhibit CMI as evidenced by allograft rejection.

The Effect of MTX and 5-FU on Oxazolone-induced Contact Sensitivity. Additional evidence for a lack of inhibition of CMI was obtained from studies of the effect of the combination on oxazolone-induced contact sensitivity. Animals were treated on Day 2 after sensitization with oxazolone, challenged on Day 6, and assayed 24 hr later. As seen in Table 1, the combination (MTX followed in 1 hr by 5-FU) did not inhibit this response. This supports the idea that the combination is a selective regimen which is capable of profoundly inhibiting humoral immunity while leaving CMI unaffected. Although the combination did not cause an inhibition of oxazolone-induced contact sensitivity, MTX alone had a slight, although not significant, inhibitory effect on CMI. On the other hand, 5-FU alone caused a small increase in the oxazolone response. The reverse combination (5-FU then MTX) and MTX followed by 5-FU also gave slight increases.

Effect of MTX and 5-FU on MBSA-induced Hypersensitivity. The effect of the combination was next studied in the MBSA system which appears to be a model of a "balanced" response, consisting of both humoral and cell-mediated components balanced in opposition to each other. The effects of MTX and 5-FU on MBSA-induced hypersensitivity in C3HeB/FeJ mice are shown in Tables 2 and 3 and on Chart 2. The male C3HeB/FeJ mice (Table 3) yielded striking results in that the individual agents caused only a mild stimulation of...
Table 1
The effect of MTX and 5-FU given 48 hr after sensitization on oxazolone-induced contact sensitivity in C3HeB/FeJ mice

Animals were challenged on Day 6 and assayed 24 hr later by paw edema plethysmography. The time interval between the 2 agents was 1 hr.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Edema (µl)</th>
<th>% change</th>
<th>Significancea</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>87.6 ± 4.8b</td>
<td>21.9 inhibition</td>
<td>N.S.c</td>
</tr>
<tr>
<td>MTX (1 mg/kg)</td>
<td>4</td>
<td>68.4 ± 13.1</td>
<td>21.9 inhibition</td>
<td>N.S.c</td>
</tr>
<tr>
<td>5-FU (50 mg/kg)</td>
<td>4</td>
<td>122.0 ± 9.3</td>
<td>39.4 stimulation</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>5-FU followed by MTX</td>
<td>5</td>
<td>96.3 ± 12.3</td>
<td>9.5 stimulation</td>
<td>N.S.</td>
</tr>
<tr>
<td>MTX followed by 5-FU</td>
<td>4</td>
<td>112.0 ± 10.1</td>
<td>27.9 stimulation</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

a As judged by Student's t test.
b Mean ± S.E.
c Nonsignificant.

Table 2
Effect of MTX and 5-FU given 48 hr after sensitization on MBSA-induced hypersensitivity in male C3HeB/FeJ mice

Animals were given 1 mg MBSA on Days 0 and 7 of a 14-day sensitization period. Challenge MBSA (200 µg) was given on Day 14 and edema was read plethysmographically 24 hr later. The time interval between MTX and 5-FU was 1 hr.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Edema (µl)</th>
<th>% change</th>
<th>Significancea</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>31.0 ± 6.3b</td>
<td>38.0 stimulation</td>
<td>N.S.c</td>
</tr>
<tr>
<td>MTX (1 mg/kg)</td>
<td>5</td>
<td>42.8 ± 7.2</td>
<td>38.0 stimulation</td>
<td>N.S.c</td>
</tr>
<tr>
<td>5-FU (50 mg/kg)</td>
<td>5</td>
<td>42.1 ± 8.4</td>
<td>35.6 stimulation</td>
<td>N.S.</td>
</tr>
<tr>
<td>MTX followed by 5-FU</td>
<td>5</td>
<td>68.2 ± 8.0</td>
<td>119.9 stimulation</td>
<td>p &lt; 0.01</td>
</tr>
</tbody>
</table>

a As judged by Student's t test.
b Mean ± S.E.
c Nonsignificant.

Table 3
Effect of MTX and 5-FU on MBSA-induced hypersensitivity in C3HeB/FeJ female mice

The procedure is as described in Table 2.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Edema (µl)</th>
<th>% change</th>
<th>Significancea</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>35.9 ± 6.5b</td>
<td>56.3 inhibition</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>MTX (1 mg/kg)</td>
<td>5</td>
<td>15.7 ± 5.5</td>
<td>1.6 inhibition</td>
<td>N.S.c</td>
</tr>
<tr>
<td>5-FU (50 mg/kg)</td>
<td>5</td>
<td>35.3 ± 4.8</td>
<td>65.4 stimulation</td>
<td>N.S.</td>
</tr>
<tr>
<td>MTX followed by 5-FU</td>
<td>5</td>
<td>59.4 ± 10.7</td>
<td>31.5 inhibition</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

a As judged by Student's t test.
b Mean ± S.E.
c Nonsignificant.

MBSA reactivity (MTX, 38%; 5-FU 35%), whereas the combination caused a marked degree of stimulation (119%) which was greater than the expected additive effect of these 2 drugs. The administration of these agents in the female mice (Table 4) revealed that MTX significantly inhibited the response (56%), whereas 5-FU had no effect. The combination of MTX followed in 1 hr by 5-FU actually reversed the inhibitory effect of MTX and caused a small stimulation. The reverse direction (5-FU followed by MTX) maintained an inhibition of the response. MTX followed by 5-FU thus produced results that were significantly different from the reverse direction (p < 0.02) as well as overcoming the inhibitory effect of MTX alone. These results confirm the importance of the direction of drug administration observed in the hemolysin plaque-forming cell experiments and also support the idea of the selectivity of this regimen.
Effect of the Addition of Leucovorin to the Combination. Because of the significant inhibition of MBSA by MTX alone in female C3HeB/FeJ mice and because of the slight inhibition of oxazolone-induced contact sensitivity by MTX, leucovorin, an antagonist to MTX (3), was added to the regimen 30 min after MTX in order to try to minimize this effect. The results of these experiments are seen in Table 4. The MTX-induced inhibition of the MBSA response was reduced by leucovorin to about one-half its previous level (data not shown). In addition, the combination including leucovorin caused a significant stimulation (109%) in the female animals, yielding results comparable to those achieved in the male animals without leucovorin. The addition of leucovorin to the combination neither helped nor hurt the response in the males, as these animals were apparently already maximally stimulated by the combination.

**DISCUSSION**

This study provides evidence that MTX (1 mg/kg) given 1 hr prior to 5-FU (50 mg/kg) can markedly inhibit humoral antibody production as measured by hemolysin plaque-forming cell production while having little effect upon CMI. The effect of the combination on humoral immunity indicates that the inhibition is potentiative; that is, the inhibition of humoral immunity observed is greater than both the theoretical additive effect of the individual drugs and the actual additive effect when the drugs are given concomitantly. This potentiative effect allows one to use low doses of drugs, minimizing the problems of toxicity found with high doses of 5-FU alone.

Although the combination had a strong inhibitory effect upon humoral immunity, studies in CMI systems (allograft rejection and oxazolone) show that this regimen does not inhibit CMI. Thus, not only is the combination potentiative with respect to inhibition of humoral immunity, but it is also selective in that the combination does not inhibit CMI.

The MBSA hypersensitivity experiments show that this selectivity extends even to responses induced to the same antigen. The effect of this combination on this balanced immune system resulted in a very strong stimulation of the response. Since the response to MBSA is thought to have a humoral blocking component which is balanced in opposition to a CMI component, stimulation can be interpreted as resulting from an inhibition of the humoral blocking component without inhibition of the cell-mediated component.

While speculation must await the investigation of this regimen in a tumor system, this potentiative and selective effect of the combination raises the possibility of its use in immunochemotherapy of solid tumors, where the CMI component seems to be largely responsible for the destruction of tumor cells and SBF seems to mitigate the effect of CMI.

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**Table 4**

Effect of leucovorin on the stimulation of MBSA-induced hypersensitivity seen after MTX and 5-FU treatment in C3HeB/FeJ mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Sex</th>
<th>Edema (µl)</th>
<th>% change</th>
<th>Significance* (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>M</td>
<td>31.0 ± 6.3</td>
<td>119.9 stimulation</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>MTX followed by 5-FU</td>
<td>5</td>
<td>M</td>
<td>68.2 ± 8.0</td>
<td>107.5 stimulation</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>MTX, then leucovorin followed by 5-FU</td>
<td>5</td>
<td>M</td>
<td>64.4 ± 8.0</td>
<td>107.5 stimulation</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>None</td>
<td>5</td>
<td>F</td>
<td>35.9 ± 6.5</td>
<td>65.4 stimulation</td>
<td>N.S.</td>
</tr>
<tr>
<td>MTX followed by 5-FU</td>
<td>5</td>
<td>F</td>
<td>59.4 ± 10.7</td>
<td>65.4 stimulation</td>
<td>N.S.</td>
</tr>
<tr>
<td>MTX, then leucovorin followed by 5-FU</td>
<td>5</td>
<td>F</td>
<td>75.1 ± 10.7</td>
<td>109.0 stimulation</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

* As judged by Student’s t test.

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[Image: Chart 2. The effect of MTX (1 mg/kg on Day 2) and 5-FU (50 mg/kg on Day 2) on MBSA-induced hypersensitivity as measured by paw edema at 24 hr after Day 14 challenge. Shown above the zero is the average % increase and below the zero the average % inhibition. Left, results in female C3HeB/FeJ mice; right, results in male C3HeB/FeJ mice. Differences that are statistically significant from the control are indicated by the p value as calculated by Student’s t test.]
The characterization of SBF is incomplete, but antibody and antigen-antibody complexes have both been shown capable of blocking CMI (10), suggesting that inhibition of humoral immunity would have therapeutic benefit. However, other humoral factors might also play beneficial roles in host resistance to tumors; i.e., cytotoxic, "arming," and unblocking antibodies. Therefore, such a regimen would be expected to sacrifice these responses. Mott (12) has suggested that chemotherapeutic suppression of "immune enhancement" (that is, selective inhibition of SBF) may be a primary determinant of successful cancer chemotherapy. We are currently testing the efficacy of this combination on spontaneously arising mouse mammary tumors.

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REFERENCES

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