Synergism of Methotrexate with Imuran and with 5-Fluorouracil and Their Effects on Hemolysin Plaque-forming Cell Production in the Mouse

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SUMMARY

The present study has provided evidence that the administration of methotrexate (MTX) and 5-fluorouracil (5-FU) together in the proper regimen results in a synergistic suppression of 19 S hemolysin plaque-forming cell production. A similar effect was demonstrated when a combination of Imuran (IM) and MTX was used although the degree of potentiation was not as great. The administration of MTX (1 mg/kg) 15 min prior to the administration of 5-FU (50 mg/kg) resulted in very strong (90%) inhibition of hemolysin plaque-forming cell production in C3HeB/FeJ female mice. If the direction of administration was reversed, that is 5-FU before MTX, particularly when the interval between the two was less than 6 hr, little inhibition was observed (12 to 30%). The interaction of IM and MTX was synergistic if IM was given prior to MTX but additive if IM was given after MTX. These types of drug-drug interactions may prove useful in the control of immune reactions, in particular, the production of serum-blocking factor. Such a potent inhibitor of humoral antibody production may provide a beneficial shift of tumor immunity in favor of the host.

INTRODUCTION

Increased efficacy gained by using antiproliferative agents in combination has been appreciated for many years. In 1957, a substantial review concerned with the definition and characterization of synergism and antagonism of antiproliferative agents in in vitro neoplastic cell systems was written by Goldin and Mantel (10). In vitro and in vivo work was initiated in the 1960's and culminated in the therapeutic trial of many combinations in human neoplastic disease (3, 4, 7, 9, 11, 12, 17, 18, 23). However, much of this work was of a theoretical and empirical nature and only in the late 1960's did the actual interaction of these drugs begin to be characterized in such a way as to shed light on the mechanism of the drug-drug interaction. Time and direction of administration of drugs have become important parameters in drug combinations, vastly altering their ability to kill tumor cells both in vitro (5, 6) and in vivo (20).

The proliferative event in hemolysin plaque production has been characterized by its rapid cell division (22). It would therefore seem possible to use this system as a model proliferative system for manipulation by drug combinations. Indeed, almost as soon as many antineoplastic agents were discovered they were tested on the immune response (1). There is also substantial literature on the use of paired drug combinations in manipulating the immune response (2, 14, 16, 19, 21). The immune response is a valuable tool in studying the antiproliferative effects of drug combinations and may provide important information about the effects of these combinations on neoplastic disease.

Regimens that alter immunological systems may also play an important role in altering tumor immunity. The recent work on serum-blocking factor and its relationship to neoplastic disease suggests that the control of the production of serum-blocking factor might be of benefit to the tumor host (13).

Thus, information concerning the control of immune reactivity (particularly humoral immunity) is important because the immune responses may be a model for proliferative disease and because control of serum-blocking factor can be of benefit to the cancer host.

The work presented here is concerned with the interaction of the antifolate, MTX, and the antipyrimidine, 5-FU, and with the interaction of MTX with the antipurine, IM. We have studied the ability of these combinations to act synergistically to inhibit, in vivo, the production of 19 S HPFC as assayed by the Jerne plaque technique.

MATERIALS AND METHODS

Animals. C3HeB/FeJ female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and were used in a weight range of from 18 to 23 g.

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2The abbreviations used are: MTX, methotrexate; 5-FU, 5-fluorouracil; IM, imuran; HPFC, hemolysin plaque-forming cells; SRBC, sheep red blood cells.
Sensitization to SRBC: Animals were given 0.2 ml of a 10% suspension of SRBC on Day 0 and the spleen HPFC were assayed on Day 4.

Drug Treatment. All drugs were given on Day 2 following SRBC. MTX was given in a dosage of 1 mg/kg, 5-FU at 50 mg/kg, and IM at 80 mg/kg. Time and direction of administration of the 2 agents given together were varied. In the case of the IM experiments, all animals received 0.02 N NaOH as a vehicle control.

Assay of 19 S HPFC. Assay was done according to the Jerne plaque technique as modified by Uyeki and Long (22). The data are presented as percentage inhibition of HPFC production, calculated from the HPFC/10^6 spleen cells plated by dividing the difference between the drug-treated group and the control by the control value. The data were analyzed with the use of the Student’s t test.

RESULTS

Effect of MTX and 5-FU on HPFC Production. Seen in Chart 1 are the results of the treatment of mice sensitized to SRBC with either MTX alone, 5-FU alone, 5-FU followed in 1 hr by MTX, MTX followed in 1 hr by 5-FU, or MTX and 5-FU given concomitantly. MTX given alone in a dosage of 1 mg/kg resulted in a 35.9% inhibition of HPFC production. A dosage of 5-FU at 50 mg/kg proved to be approximately equipotent and caused a 35.4% inhibition of HPFC production. When the 2 agents were given concomitantly, 61.8% inhibition was observed. This value is very near the theoretical additive effect of the 2 (71.3% inhibition). When 5-FU was given 1 hr prior to MTX, 29.8% inhibition resulted. In contrast, the opposite direction (MTX followed in 1 hr by 5-FU) caused an 87.8% inhibition of HPFC production. The group given MTX then 5-FU and the group given MTX plus 5-FU are significantly different from all other groups (including each other), and as indicated by the line through the bars, the groups given MTX alone, 5-FU alone, and 5-FU then MTX are not significantly different.

Thus it appeared that we could generate the additive effect by giving the 2 agents concomitantly, demonstrate potentiation by giving MTX followed in 1 hr by 5-FU, and show “antagonism” by giving the agents 1 hr apart in the direction of 5-FU then MTX. It was then of interest to investigate some of the variables of this system.

Effect of the Time Interval between MTX and 5-FU on Synergism. This series of experiments was designed to investigate the effect of time upon the synergism demonstrated by the MTX followed by 5-FU regimen. Chart 2 illustrates the results. The group which received MTX plus 5-FU concomitantly functions as a 0 time control and showed 61.8% inhibition. When 15 min were allowed to elapse between the administration of MTX and 5-FU, 65.9% inhibition, a value not significantly different from MTX plus 5-FU was observed. However, when the interval was increased to 30 min, 1 hr, and 6 hr, 92.3, 87.8, and 89.5% inhibition resulted respectively. These values were not significantly different from one another but were significantly different from the concomitant group. From these data, it appeared that 15 min were not long enough to result in potentiation but 30 min were sufficient. The potentiation was still demonstrable at a 6-hr interval. The next series of experiments was directed toward a similar investigation of the effect of time upon the antagonistic direction, 5-FU followed by MTX.

Effect of the Time Interval between 5-FU and MTX on “Antagonism.” In Chart 1, there was a striking difference between the group which had MTX followed by 5-FU (synergism) and the group which had 5-FU followed by MTX. The 2nd group has been referred to as the antagonistic group since the result was much less than the expected theoretical additive effect and the actual concomi-
tant effect. The consequence of varying the time interval between the 2 agents in this direction is shown in Chart 3. Once again the concomitant value is 61.8% inhibition. When an interval of 15 min was allowed, 64.9% inhibition was observed. This value was not significantly different from the concomitant administration group. In contrast, in the case where MTX was given 30 min after 5-FU, only 12.4% inhibition of HPFC production was seen. At 1 hr between the 2 drugs, 29.8% inhibition was observed. These 2 values (30 min and 1 hr) were significantly different from either concomitant administration or the 15 min interval group. It seems apparent that 15 min were not long enough to observe antagonism and that the antagonism was decreasing with time and was absent at a 6-hr interval.

Having determined at least in part the effect of time upon the MTX-5-FU system, it was of interest to see whether another drug pair could generate a similar set of data. Our choice for this series of experiments was MTX and IM.

**Effect of IM Followed at Various Times by MTX on HPFC Production.** In this series of experiments, as shown in Chart 4, MTX (1 mg/kg) given alone caused a 40.9% inhibition of HPFC production; whereas, IM (80 mg/kg) gave a 26.9% inhibition. The theoretical additive effect would then be 67.8% inhibition. As in the previous experiments, the concomitant administration of MTX plus IM generated very nearly the additive value (66.6% inhibition). When MTX was followed by IM with a 15-min interval, 78.1% inhibition of HPFC production resulted. At an interval of 30 min, 80.1% inhibition was seen, and at an interval of 1 hr, 91.2% inhibition was observed. When the interval was lengthened to 6 hr, 59.3% inhibition was seen. The 15-min interval group, the 30-min interval group, and the 1-hr interval group are all significantly different from the concomitant administration group, but the 15-

and 30-min groups are not significantly different from one another. The 6-hr interval group is not significantly different from the concomitant administration group. These data seem to indicate that there is significant synergism in this drug pair if intervals between the 2 agents are less than 6 hr. The synergism is in the opposite direction from the MTX-5-FU study, in that IM must precede MTX in order to see potentiation. The effect of the opposite direction (MTX then IM) is seen in Chart 5.

**Effect of MTX Followed at Various Times by IM on HPFC Production.** As shown in the previous figure, the concomitant administration of IM and MTX results in 66.6% inhibition of HPFC production. When MTX preceded the administration of IM by 15 min, 30 min, 1 hr, or 6 hr, there was no marked difference from the concomitant administration group. The 15-min interval group, however, did result in a slightly greater percentage inhibition (76%) which was significantly different from the concomitant group. Thus in this direction there was no antagonism nor was there marked synergism.

**DISCUSSION**

For the purposes of this investigation, potentiation and antagonism have been defined with respect to the results of concomitant drug administration. When a significant difference between any group and the group given the drugs concomitantly occurred, it was called potentiation if the inhibition seen was greater and antagonism if the inhibition seen was less. If there was no significant differ-

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**Chart 3.** Effect of time upon the demonstration of "antagonism" of MTX and 5-FU. Again, 19 S HPFC as assayed at 4 days after antigen with drug administration on Day 2 are shown. The same groups as shown in Chart 2 are shown in this chart except that the direction of administration of MTX and 5-FU is reversed. The administration of 5-FU precedes the administration of MTX. Mean ± S.E. are shown. Any bar that is connected by a line to another bar is not significantly different from that bar. The number of animals used is included in the bar. EAV, expected additive value.

**Chart 4.** Effect of IM (80 mg/kg) followed at various times by MTX (1 mg/kg) on HPFC production as assayed at 4 days after antigen (SRBC). The drugs were given on Day 2 after antigen. The following regimens were done: MTX alone, IM alone, IM plus MTX given concomitantly, IM followed in 15 min by MTX, IM followed in 30 min by MTX, IM followed in 1 hr by MTX, and IM followed in 6 hr by MTX. The expected additive value was 67.7% and represents the sum of MTX alone plus IM alone. The dashed line separates the individual administration groups from the combination groups. Among the combination groups, any group which is connected by a line to another group is not statistically significantly different from that group. In addition, the 2 groups with asterisks are not significantly different. The mean ± S.E. are shown. The animal numbers are as in Charts 1 to 3, in that the concomitant administration group has 8 and all others 4. EAV, expected additive value.
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Chart 5. Effect of MTX followed at various times by IM on HPFC production as measured on Day 4 after antigen (SRBC). The experimental design was as in Chart 4 (right section) except that MTX preceded IM at the times studied. All comparisons revealed nonsignificant differences except for the 1st 2 bars (concomitant administration versus MTX then IM, 15 min). Mean ± S.E. are shown. The number of animals in the concomitant group is 8 and all other groups had 4. EAV, expected additive value.

It appears, from the preceding, that MTX is not just preparing the cells for an attack by any antiproliferative agent, since IM does not work in that manner. If, as suggested by others (15), the potentiation of 5-FU's action by MTX is due to a type of sequential blockade (N<sup>5</sup>,N<sup>10</sup>-methylene tetrahydrofolate depletion and thymidylate synthetase inhibition), then it appears that cofactor depletion must precede, in time, the inhibition of thymidylate synthetase in order to see a positive interaction. From our data, if thymidylate synthetase inhibition (5-FU action) is followed by MTX-induced cofactor depletion, then the cofactor depletion does not have an effect of further limiting thymidylate production. This speculation includes the assumption that the limitation of thymidylate and resultant inhibition of DNA synthesis are the mechanism of the inhibition of HPFC production.

The problem that one would anticipate from such a regimen (MTX-5-FU) would be an increased toxicity along with a potentiated effect. This has been observed by others (15). The application of this regimen to other immunological systems has not revealed any toxicity at the dose levels used, but this has not been rigorously pursued. It has, however, been determined that this regimen while almost abolishing a response to SRBC has little or no effect upon cell-mediated immunity (8). Although tumor cells and SRBC may have little in common antigenically, an inhibition of SRBC antibody production by a drug regimen may, in our experience, be expected to correlate with an inhibition of the humoral response to mouse mammary tumors. This may be because the drug regimen is affecting B-cell function in general. Thus the potential benefit of this regimen may lie not in its ability to kill tumor cells with greater efficiency but in its ability to selectively reduce the production of serum-blocking factor and, by doing so, allow full expression of host-protective, cell-mediated immunity.

REFERENCES

Synergism in Immunosuppression


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