Effect of Sequence of Administration of Methotrexate, Leucovorin, and 5-Fluorouracil on Mammary Tumor Growth and Survival in Syngeneic C3H Mice

G. H. Heppner and P. Calabresi

Department of Medicine, Roger Williams General Hospital, Providence 02908, and Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

SUMMARY

The administration of methotrexate (1 mg/kg), leucovorin (1 mg/kg), and (after a 1-hr interval) 5-fluorouracil (50 mg/kg) selectively suppresses antibody production in C3H mice without inhibiting, but even stimulating, cell-mediated immunity. The effect of this regimen, given at weekly intervals, was tested on the growth of recently arising syngeneic C3H/HeJ mammary tumors. Inhibition of growth was found in three types of experiments: (a) when treatment was begun 2 days after tumor implantation, (b) when it was begun 3 weeks after implantation, and (c) when it was begun after surgical enucleation of the tumor. The extent of the effect varied from tumor to tumor, but in all cases tumor incidence or growth was significantly inhibited. Comparison was made between the above regimen and two other sequences of administration of the same drugs, which are only weakly immunosuppressive of antibody production and which do not stimulate cell-mediated immunity. Survival of tumor-bearing mice was greater with the administration of methotrexate, then leucovorin, followed by 5-fluorouracil, than it was with the other two administration schedules.

INTRODUCTION

DiLorenzo et al. (5, 6) have described a drug regimen that in mice inhibits the production of 19 S and 7 S antibody to sheep RBC without inhibiting allograft rejection or contact sensitivity to oxazolone and that markedly stimulates delayed hypersensitivity to methylated bovine serum albumin. This regimen consists of MTX (1 mg/kg), LCV (1 mg/kg), and 5-FU (50 mg/kg); the 5-FU must be given at least 1 hr after the other 2 drugs. The purpose in developing this selectively immunosuppressive drug regimen was to provide a method to inhibit production of serum blocking factors, through the antibody part of the antigen-antibody complex (17), without interfering with CMI against tumor antigens (10). Thus, the development of the regimen was based on tests of immune function, not on pharmacological effects or theory (12). Since the initiation of this project, new information has appeared showing that humoral antibodies may also play a protective role against solid cancers (15), that blocking factors may also be antigens not complexed to antibody (4, 23), and that a suppression of humoral immunity may be associated with enhanced CMI, irrespective of the involvement of blocking factors (3). Further, it seems probable that humoral immunity may interfere with metastatic dissemination of tumor cells. We have, nevertheless, been testing the MTX-LCV-5-FU combination on early transplants of spontaneously arising C3H/HeJ mammary tumors in syngeneic C3H/HeJ mice, and we compared the effects with those obtained with the same drug combination administered in such a way as to have minimal immunological consequences.

MATERIALS AND METHODS

Mice. Male and retired breeder female C3H/HeJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The males were used when they were approximately 2 months old. The females were maintained as a source of mammary tumors.

Tumors. Mammary tumors were surgically excised and prepared for transplantation into either tumor-bank or experimental C3H/HeJ males by first being minced and grossly washed free of RBC in minimal essential medium (Difco Laboratories, Detroit, Mich.) and then being placed on a Bélico rocker at 37° for 1 hr in 0.25% Bactotrypsin (Difco), pH 7.2. Two % fetal calf serum was next added, followed by Worthington crude collagenase (3.3 mg/ml). The cells were again rocked at 37° for 1 hr and then were placed in suspension by being pulled up and down through a 3-ml syringe. The cells were filtered through sterile gauze, suspended in Eagle’s medium (Grand Island Biological Co., Grand Island, N. Y.), and centrifuged at 1000 rpm for 10 min. They were washed again in medium, centrifuged for 5 min at 800 rpm, and finally resuspended in 10 ml of medium. The cells were adjusted to an appropriate concentration of viable cells in medium after counting in trypan blue in a hemocytometer. Tumor cells (5 × 10⁶) were injected s.c. into recipient mice in volumes of 0.2 to 0.5 ml.

Treatment. All drugs were given i.p. MTX (Lederle Laboratories, Pearl River, N. Y.) was used at 1 mg/kg, LCV (Lederle Laboratories) was used at 1 mg/kg, and 5-FU (Roche Laboratories, Nutley, N. J.) was used at 50 mg/kg. Control mice received 0.9% NaCl solution. The surgical enucleation procedure used is described by Stolfi et al. (20).
RESULTS

The Effect of the Administration of MTX, LCV, and Then 5-FU on Growth of Syngeneic Mammary Tumors. The effect of the administration of MTX, LCV, and then 5-FU on the growth of C3H/HeJ mammary tumors was studied in 3 different protocols. In the first type of experiment, $5 \times 10^5$ syngeneic mammary tumor cells were implanted into male C3H/HeJ mice. Two days later and at weekly intervals throughout the experiment, the mice were treated with MTX, LCV, and 5-FU. Fifteen min separated the administration of MTX and LCV; 1 hr separated the administration of MTX and 5-FU. Control animals received 0.9% NaCl solution. The mice were checked twice a week for the appearance of palpable tumor outgrowths. There were 10 to 13 animals/group. In all, 8 different tumors were tested. One tumor was tested twice with essentially identical results. The tumors were all of recent origin, being in either their first or second transplant generation.

Results from all experiments were similar in showing a delay in the appearance of palpable tumors in the drug-treated mice, although the magnitude of the effect varied from tumor to tumor. In the experiment depicted in Chart 1, tumors were first detected in the mice receiving the combination 3 weeks after they were detected in the controls, and a significantly lower (at least $p < 0.05$, binomial distribution analysis) incidence was seen throughout the experiment. With 2 other tumors tested in separate experiments, although there was again a 3-week delay in tumor appearance, the final tumor incidence in the drug groups was not significantly different from that in controls. In 3 tumors tested separately, there was only a 5- to 10-day difference in tumor appearance.

Although drug treatment was usually continued throughout an experiment, this was not mandatory. In 1 experiment the treatment was stopped after 4 weeks. At 13 weeks, the tumor incidence in the treated mice was 23% (9 of 39 mice), whereas it was 52% (22 of 42) in the controls ($p < 0.001$, $\chi^2$).

The second type of protocol varied from the first in that drug treatment was withheld for 3 weeks, until just before palpable tumors were expected to appear. Drugs were then given at weekly intervals until the end of the experiment. The experiment shown in Chart 2 gave results similar to those in which treatment was begun after 2 days. In this experiment both the time of tumor appearance and the ultimate incidence were affected by the administration of MTX, LCV, and then 5-FU.

The third type of protocol attempted to simulate a surgery-drug adjuvant procedure. In this case the tumors were allowed to reach a size of 100 sq mm, surface area. They were then surgically enucleated, a procedure that leaves behind viable tumor cells (20). Two days after surgery and at weekly intervals thereafter, MTX, LCV, and 5-FU were administered. This protocol proved to be technically troublesome because of the difficulty of controlling the extent of enucleation. However, in 1 experiment there was definite evidence that the drug treatment retarded the rate of reappearance. In this experiment, 5 of 9 control mice had palpable tumors 20 days after surgery, as opposed to 1 of 10 test animals ($p < 0.05$). By 27 days, however, the difference was no longer significant.

In all experiments test mice were weighed weekly. No loss of weight due to drug treatment was ever observed.

The Effect of the Sequence of Administration of MTX, LCV, and Then 5-FU on Syngeneic Mammary Tumor Growth and Survival. The administration of MTX, LCV, and (after 1 hr) 5-FU results in strong, selective suppression of antibody production and either stimulation of or lack of effect on CMI to a number of antigens. Reversal of the drug order (5-FU, then MTX and LCV) or simultaneous administration (MTX + LCV + 5-FU) has a relatively weak suppressive effect on antibody production (1) and no effect on CMI (5).

To assess the effect of drug sequence on tumor growth inhibition by MTX, LCV, and 5-FU, we performed experiments in which the time and order of administration of the drugs were varied. Syngeneic mammary tumor cells ($5 \times 10^5$) were implanted into C3H/HeJ males. Two days later...
and at weekly intervals throughout the experiment, the mice were treated with either: (a) MTX, LCV, and (after 1 hr) 5-FU; (b) 5-FU and then (after 1 hr) MTX and LCV; or (c) MTX, LCV, and 5-FU given in rapid succession. Control animals received 0.9% NaCl solution. There were 10 animals/group. The mice were checked twice weekly for appearance of palpable tumor outgrowths. The tumors were then measured with calipers. This experiment was performed with 3 different tumors, each in its first transplant generation. The experiments were terminated at the end of 15 weeks. At this time no new tumors had appeared for at least 1 month, and the tumors in the surviving tumor bearers were at least 400 sq mm in surface area.

The results from these experiments show that tumor growth was inhibited in all of the 3 drug groups, with no difference in either direction between them with respect to either the length of time before tumors reached a palpable size or the growth rate of the tumors after that point. There was a difference, however, in the survival time and number of survivors. As Table 1 indicates only 33% of the control mice survived the 15-week experimental period, compared to 75% of the mice that received the regimens capable of weakly suppressing antibody production and compared to 93% of the mice given the treatment capable of strong humoral immunosuppression and enhanced CMI. Table 2 presents the survival time data for each of the 3 experiments. Survival was prolonged by administration of MTX and LCV prior to 5-FU in each experiment. The length of prolongation ranged from 6.5 days in Experiment 1 to 27.5 days in Experiment 3.

The data were further analyzed to determine whether the mice that died with tumors were bearing large (400-sq mm surface area) tumors at the time of death or whether the tumors were relatively small (100 sq mm). As can be seen in Table 1, the proportion of mice dying with large (400-sq mm) tumors at the time of death or whether the tumors were relatively small (100 sq mm). As can be seen in Table 1, the proportion of mice dying with large (400-sq mm) tumors was greater in the weakly immunosuppressed groups than did those mice given the drugs either simultaneously or in reverse order that died did so with small tumors than did even the control animals suggests that many of the deaths in these groups were not a result of primary tumor mass per se.

**DISCUSSION**

The idea that cancer chemotherapy need not be immunosuppressive and, indeed, may be more effective when it is not is becoming more widespread (13, 14, 21). A further extension of this idea is to use cancer chemotherapeutic agents in such a way as to modify immune responses to maximize the antitumor effect. The demonstration of blocking factors, some of which are antigen-antibody complexes, in the sera of animals and patients with progressively growing tumors suggested that, if one could selectively inhibit humoral immunity, one might be able to strengthen host resistance to cancer. Cyclophosphamide and 1-β-D-arabinofuranosylcytosine are both capable of such selective immunosuppression (8, 22), and both interfere with tumor growth under circumstances in which selective, but not general, immunosuppression is operative (9, 18). Both have also been shown to be associated with suppressed produc-
the administration sequence of MTX and 5-FU on antitumor activity have been presented by Martin et al. (11) and Bertino et al. (2). The reason for the superiority of the MTX-before-5-FU regimen has been postulated (2) to be an enhancement by preexisting, intracellular MTX of the binding of 5-fluorodeoxyuridine monophosphate, the active metabolite of 5-FU (16), to the site of action, thymidylate synthetase. Thus, the explanation for the superior effectiveness of the MTX-before-5-FU sequence may lie wholly in biochemistry, which has not been explored by us, and may be unrelated to immunological considerations. Alternatively, both factors may be involved. In any event the practical consequences of sequence of administration of these drugs are clearly evident.

ACKNOWLEDGMENTS

We wish to thank Harry Mourachian, Marilyn Wilson, Cheryl Miller, and Lily Chou for their expert technical assistance and Dr. Lowell Kopp for his help in statistical analysis. We also thank Dr. Ruth Fugmann for assistance in learning surgical aneculene.

REFERENCES


Sequence of MTX and 5-FU Administration
Effect of Sequence of Administration of Methotrexate, Leucovorin, and 5-Fluorouracil on Mammary Tumor Growth and Survival in Syngeneic C3H Mice

G. H. Heppner and P. Calabresi


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/37/12/4580

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.