Effect of Feeder Cell-released Substances on the Survival of Clonogenic 9L Cells after Treatment with Antimetabolites

Michael Weizsaecker and Dennis F. Deen

Brain Tumor Research Center, The Department of Neurological Surgery, (M. W., D. F. D.) and Department of Radiation Oncology (D. F. D.), School of Medicine University of California, San Francisco, California 94143

ABSTRACT

Exponentially growing monolayer cultures of 9L rat brain tumor cells were treated with either 5-fluorouracil or methotrexate. The surviving fraction of cells was determined by a colony formation assay. After 5-fluorouracil treatment, 2 to 5 \( \times 10^5 \) feeder cells were required to maximize surviving fractions for each drug concentration and to generate a biphasic dose-response curve. If only 1 \( \times 10^4 \) feeder cells were used, the dose-response curve was steep. Uridine added to the dishes containing 1 \( \times 10^4 \) feeder cells restored the biphasic shape, while uridine and thymidine added to the dishes yielded the same curve obtained with 2 \( \times 10^3 \) feeder cells.

After methotrexate treatment, the surviving fraction of cells was dependent on feeder cell number when the medium in the dishes was supplemented with dialyzed fetal bovine serum, but it was not dependent on feeder cell number when nondialyzed fetal bovine serum was used. Biphasic dose-response curves were generated from methotrexate-treated cells plated in medium supplemented with either dialyzed or nondialyzed serum, but the drug was more toxic to cells plated in medium containing dialyzed serum. This additional toxicity could be reduced if either thymidine or 5-formyltetrahydrofolate were added to the dishes and eliminated if 1 \( \times 10^4 \) feeders were added. These results suggest that any cell culture system used to evaluate antimetabolites should be optimized for possible feeder cell and serum effects.

INTRODUCTION

Feeder cells were first used by Puck et al. (9, 10) to stimulate the growth of colonies from single mammalian tumor cells. Heavily irradiated feeder cells do not form colonies but increase PE's of untreated and treated test cells. They affect clonogenic cell survival after treatment with radiation (4) and alkylating agents (12). It has been suggested that feeder cells provide short-lived, diffusible factors that are necessary for cell growth (9), but these factors have not been identified. We report here the effect of feeder cells on the survival of clonogenic 9L rat brain tumor cells after treatment with 5-FU or MTX.

MATERIALS AND METHODS

Cell Culture Conditions. 9L rat brain tumor cells (2 \( \times 10^6 \)) were seeded into 75-cm\(^2\) culture flasks (Falcon Plastics, Oxnard, Calif.) and grown in complete medium consisting of MEM supplemented with nonessential amino acids, 10\% FBS, and gentamicin (50 \( \mu \)g/ml). After 24 hr, drugs were added to exponentially growing cells, which were routinely screened to assure the absence of Mycoplasma using the method of Chen (3).

Treatment. Dilutions of 5-FU (Roche Insectable; Roche Laboratories, Nutley, N. J.) or MTX (parenteral; Lederle Laboratories, Pearl River, N. Y.) were made in 1.5 ml of MEM, which were added to 13.5 ml of medium in the culture flask to achieve the final dilution. Cells were maintained at 35–37°C and pH 7.2 to 7.4. After a 24-hr exposure, the drug-containing medium was decanted, and the monolayer cells were washed twice with MEM and trypsinized. Single cells were suspended in MEM supplemented with nondialyzed or dialyzed FBS as indicated for each experiment.

Colony Formation Assay. The assay has been described (11, 12). In brief, single-cell suspensions from treated and untreated cultures were plated in 60-mm dishes (Falcon Plastics). The medium in the dishes was supplemented with either nondialyzed or dialyzed FBS as indicated, and varying numbers (1 \( \times 10^2 \) to 1 \( \times 10^6 \)) of irradiated (40 grays) 9L feeder cells were added 24 hr before test cell plating. In selected experiments, thymidine (20 \( \mu \)g/ml; Sigma Chemical Co., St. Louis, Mo.) and/or uridine (20 \( \mu \)g/ml; Sigma) were added to plated cells treated with 5-FU. Thymidine (20 \( \mu \)g/ml), hypoxanthine (20 \( \mu \)g/ml; Calbiochem Behring Corp, La Jolla, Calif.), or 5-N-formyltetrahydrofolate (leucovorin, 100 \( \mu \)g/ml; Lederle Laboratories, Pearl River, N. Y.) were added to plated cells treated with MTX. None of these substances in the administered doses affected the PE of cells from untreated cultures. Plated cells were incubated for 10 to 26 days in a humidified 95% air-5% CO\(_2\) environment at 37°C and pH 7.2 to 7.4.

PE's for untreated and treated cells were calculated as the ratio of the number of colonies observed to the number of cells plated. SF's were calculated as the ratio of the PE of treated cells to the PE of untreated cells.

RESULTS

Clonogenic Cell Survival after Treatment with 5-FU. When cells were plated with increasing numbers of feeder cells in medium containing nondialyzed FBS, the PE from untreated cells did not vary significantly, except that no colony growth
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was detected if $1 \times 10^8$ feeder cells were used (Chart 1). However, after treatment with 5-FU (1 $\mu$g/ml), the SF of treated cells increased 3-fold and plateaued as the number of feeder cells reached $5 \times 10^4$. After treatment with 5-FU (25 $\mu$g/ml), the SF of treated cells increased about 1000-fold and plateaued as the number of feeder cells increased to 2 to $5 \times 10^5$. Over the dose range of 5-FU, the SF was maximum for 2 to $5 \times 10^5$ feeder cells.

The SF’s for cells plated either with low ($1 \times 10^4$) or high ($2 \times 10^5$) numbers of feeder cells were constant with incubation times of up to 26 days (Chart 2).

When 9L cells treated with 5-FU were plated on Petri dishes containing medium supplemented with dialyzed FBS and $1 \times 10^5$ feeder cells, a steep dose-response curve resulted (Chart 3). When 5-FU-treated cells from the same experiment were plated into dishes containing medium supplemented with dialyzed FBS and $2 \times 10^5$ feeder cells, the dose-response curve was biphasic. This biphasic curve also could be obtained by adding both thymidine and uridine to the Petri dishes, but had a lower saturation value if uridine alone was added.

Clonogenic Cell Survival after Treatment with MTX. The SF after treatment with MTX (0.05 or 100 $\mu$g/ml) was unaffected by the number of feeder cells if the culture medium in the Petri dishes was supplemented with nondialyzed FBS (Chart 4). If the medium in the Petri dishes was supplemented with dialyzed FBS, the absence of feeder cells decreased the SF by about 10-fold with MTX (0.05 $\mu$g/ml), and by about 100-fold with MTX (100 $\mu$g/ml). SF’s obtained with nondialyzed FBS were achieved only if $1 \times 10^4$ to $1 \times 10^5$ feeder cells were added to the Petri dishes.

To assure that the observed decrease in the SF did not depend on the incubation time, cells treated with low or high drug doses were plated in feeder cell-free culture medium supplemented with dialyzed FBS and incubated for 10 to 26 days. The decrease in the SF under these conditions was consistent throughout the incubation period (Chart 5).

Dose-response curves were plotted for 9L cells treated with MTX (Chart 6). If cells were plated in Petri dishes supplemented with nondiazyed FBS, the curve plateaued at a SF of $3 \times 10^{-2}$; if cells were plated in Petri dishes supplemented with dialyzed FBS, the curve plateaued at a SF of $5 \times 10^{-4}$. Addition of hypoxanthine had no effect on the curve obtained using dialyzed FBS, but when thymidine was added, the curve plateaued at a SF of $2 \times 10^{-3}$. N-5-Formyltetrahydrofolate produced a plateau at a SF of $4.5 \times 10^{-3}$. Addition of $1 \times 10^4$ feeder cells to dishes containing dialyzed FBS produced the same dose-response curve as that obtained using nondialyzed FBS and no feeder cells, as predicted in Chart 4.

**DISCUSSION**

Using an in vivo colony formation assay, Bruce et al. (2) observed different-shaped dose-response curves for 5-FU and MTX. The survival curve of 5-FU-treated cells was characterized by an "exponential" shape, while the curve for MTX-treated cells decreased to a plateau at high doses. Madoc-Jones and Bruce (7) demonstrated with an in vitro clonogenic
cell assay for mouse L-cells that the cytotoxic activity of 5-FU was present several days after the drug-containing medium had been removed and cells had been plated for colony formation. They showed that the 'exponential' shape of the dose-response curve of 5-FU-treated cells could be changed to a biphasic shape by adding thymidine to the Petri dishes in which surviving cells grew to colonies.

In our experiments, 5-FU-treated 9L cells produced dose-response curves with either steep or biphasic slopes, depending on the number of feeder cells added during colony formation. A biphasic shape resulted from addition of $2 \times 10^4$ feeder cells but could also be obtained by adding thymidine and uridine or, to a lesser extent, uridine alone to the Petri dishes. We conclude that feeder cells provide these nucleotides to plated cells after treatment with 5-FU and partly rescue them from 5-FU interference with DNA and RNA metabolism (5, 8). The steep, 'exponential' survival curve for 5-FU-treated cells is probably related to the interference of 5-FU with RNA synthesis, because uridine alone reverses the curve to a biphasic form.

Cell survival after treatment with MTX also depends on the environment in which the cells are assayed. If dialyzed FBS with nondialyzed FBS used for the Petri dishes; solid lines, culture medium supplemented with dialyzed FBS used for the Petri dishes. Dashed lines, culture medium supplemented with dialyzed FBS used for the Petri dishes; bars, S.E. of 2 cultures from which cells were seeded into 10 Petri dishes each.
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xanthine, all substrates depleted by the action of MTX (1, 6), could increase the SF as much as feeder cells.

If antimetabolites are evaluated by cell survival in a clonogenic cell assay, the assay should be carefully optimized for effects of feeder cells and the serum component, which may vary from batch to batch, of the culture medium. Dialyzable substances released from feeder cells or present in FBS can interfere with the action of the drugs on cells growing to colonies and bias the measured SF's.

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