PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 168, 105–109 (1981)

Enhancement of Methotrexate Activity by Theophylline in HeLa Cell Cultures (41241)

CHARLES M. BENDAS AND LAWRENCE E. D'ANTONIO

Department of Microbiology, Philadelphia College of Osteopathic Medicine, Philadelphia, Pennsylvania 19131

Abstract. Treatment of HeLa cells with 1.0 m*M* theophylline significantly reduced the growth rate of the cell cultures without affecting cellular viability. When methotrexate was added to the theophylline-treated cultures at a concentration of 0.5 μg/ml, the cytotoxic activity of methotrexate was enhanced. The rate of cell loss in these cultures was over 2.5 times greater than in those cultures receiving only methotrexate. This enhancement of methotrexate activity was also observed when the concentration of methotrexate was reduced to 0.1 μg/ml. It was concluded that treatment of cells with an agent which prolongs the cell cycle, such as theophylline, may aid in the development of more effective chemotherapy.

Transformed mammalian cells in culture have been demonstrated to respond to the addition of theophylline (1,3-dimethylxanthine) to the culture medium by a reduction in their growth rate (1–3). The proposed mechanism for this phenomenon is an increase in intracellular levels of cyclic AMP, since theophylline has been demonstrated to be a potent inhibitor of the cyclic AMP phosphodiesterase of mammalian cells (4). It has been postulated that the increase of intracellular levels of cyclic AMP induces this effect by prolonging, or arresting the cells in, the G_1 and G_2 phases of the cell cycle (5, 6), additionally the S phase of the cell cycle may be affected (7, 8).

Theophylline has been used in a variety of experimental systems in the study of cyclic AMP (1–3, 5, 9) and in clinical situations as a vasodilator, smooth muscle relaxant, and diuretic (10). In the studies reported here, the decrease in cellular growth rate induced by theophylline has been employed to enhance the cytotoxic activity of an antimetabolite. The antimetabolite chosen for these studies was methotrexate.

Methotrexate (amethopterin or 4-[^{10}N]-aminomethylpteroylglutamic acid) has been employed successfully in the treatment of acute leukemia and choriocarcinoma. It binds with a very high affinity to the enzyme dihydrofolate reductase and, in so doing, functions as an antimetabolite by blocking the reduction of folic acid to folinic acid (11). Folate antagonists, such as methotrexate, are most effective on cells in the log phase of growth and primarily kill during the S phase of the cell cycle. The G_1–S junction of the cell cycle has also been shown to be sensitive to the cytotoxic activity of methotrexate (12).

If the cell cycle can be prolonged by addition of theophylline to the culture medium and if those phases of the cell cycle affected by theophylline coincide with those phases during which methotrexate exerts its effect, then an enhancement of the cytotoxic effect of methotrexate is possible. The increase in intracellular cyclic AMP levels induced by theophylline seems to affect cells during the G_1, G_2, and S phases of the cell cycle (5–8) and methotrexate exerts its effects primarily during the S phase or G_1–S junction of the cell cycle (12), therefore, a prolongation of the S phase or G_1–S junction may make it possible to enhance methotrexate activity. A series of experiments were undertaken designed to determine if the addition of theophylline to the medium of cultured cells had any enhancing effect upon the activity of methotrexate.

Materials and Methods. The JJH strain of HeLa cells employed during the course of these experiments was originally obtained from Dr. R. L. Crowell, Hahnemann Medical College. Growth medium for the

0037-9727/81/090105-05$01.00/0

cells consisted of minimal essential medium (MEM) in Hanks' salt solution supplemented with 10% calf serum (Gibco, Grand Island, N.Y.) and penicillin and streptomycin at 100 units and 100 μg/ml, respectively. All cell cultures were propagated in 16 × 125-mm glass test tubes containing 1.5 ml of growth medium.

Stock solutions of theophylline were prepared at a concentration of 100 m*M*, sterilized by filtration, and diluted to the appropriate concentrations in complete growth medium. Methotrexate (Lederle, Carolina, Puerto Rico) was prepared as a sterile stock solution at a concentration of 50 μg/ml and diluted with complete growth medium or growth medium containing 1.0 m*M* theophylline according to the experimental design. All stock solutions were stored at 4°. Cell cultures were incubated at 37° in a stationary position.

Enumeration of viable cells was achieved by the following method. The cell cultures were washed twice in phosphate-buffered saline (PBS), dispersed with versene (0.02% tetrasodium EDTA in PBS), centrifuged at 800*g* for 10 min, resuspended in PBS, and diluted into a final concentration of 0.04% trypan blue. A hemocytometer chamber was loaded with the cell suspension and the cells excluding the stain after 2 min were counted as viable upon observation in the light microscope. In all instances duplicate samples were counted and averaged for each of the points in an experiment. All mathematical operations were performed on a programmable laboratory calculator.

Results. As reported by other investigators (1–3, 13), preliminary studies demonstrated that the growth rate of HeLa cells was significantly inhibited by the addition of 1.0 m*M* theophylline to the culture medium. A series of experiments were performed to determine if the observed reduction in cellular growth rate was due to a theophylline toxicity. Viable and total cell counts were done on the HeLa cell cultures in the log phase of growth utilizing trypan blue exclusion and the percentage viability of each experimental group compared. The data obtained from these experiments are presented in Table I and demonstrate that there was no significant difference in viability between cells grown in 1.0 m*M* theophylline and those grown in growth medium alone. These results confirm that the addition of theophylline to the medium of cultured cells slowed the growth rate without adversely affecting cellular viability.

To determine if the addition of theophylline to the culture medium would enhance the effect of an antimetabolite such as methotrexate, the following experiments were performed. HeLa cell cultures were established in glass test tubes as previously described. Half of the cultures received growth medium containing 1.0 m*M* theophylline while the remaining cultures received growth medium alone. The cultures were incubated at 37° for 24 hr. At the end of this incubation period duplicate cultures from each group were harvested and the number of viable cells per culture at Day 0 determined. At this time the remaining cultures were further divided into four groups. Half of each of the original groups received 0.5 μg/ml methotrexate in their respective growth media. Duplicate cultures from each of

TABLE I. THE EFFECT OF THEOPHYLLINE ON THE VIABILITY OF HELA CELLS GROWN IN CULTURE

Day	Percentage viable[a] (untreated)	No. of observations	Percentage viable[a] (1.0 m*M* theophylline)	No. of observations	Probability[b]
0	82.2 ± 4.6[c]	9	83.7 ± 5.7	6	$p > 0.5$
1	84.9 ± 6.2	9	82.9 ± 6.0	6	$p > 0.5$
2	85.2 ± 7.8	9	83.6 ± 5.1	6	$p > 0.6$
3	82.7 ± 8.2	7	82.4 ± 6.2	5	$p > 0.9$
Overall[d]	84.1 ± 6.5	37	83.3 ± 5.8	24	$p > 0.6$

[a] Determined by trypan blue exclusion.
[b] Determined by Fisher *t* test.
[c] ±SD.
[d] Days 0–4 inclusive.

these groups were harvested at 24-hr intervals and the number of viable cells per culture determined.

Figure 1A presents the results obtained in this experiment. The values displayed represent the averages of five different experiments. When a regression analysis of the data was performed, the growth curves shown in Fig. 1B were obtained. The comparison of the slopes generated from the regression analysis is presented in Table II. The value of the slope is expressed as change in log viable cells per day and the index value is the ratio of the slope of the experimental group as compared to the control. The negative signs seen in these values reflect a net decrease in the number of viable cells over time for each group. The control cultures increased at a rate of 2.9×10^5 cells per day as compared to an increase of 1.4×10^5 cells per day for the theophylline-treated cultures. The cultures receiving methotrexate alone lost 2.9×10^4 cells per day while the cells receiving the combination of theophylline and methotrexate lost 7.4×10^4 cells per day. The rate of cell loss in the cultures treated with the combination of theophylline and methotrexate was over 2.5 times greater than the loss of viable cells in the cultures receiving only methotrexate. This observed enhancement of the cytotoxic effect of methotrexate by theophylline was in agreement with the basic principles put forward previously.

To further test the hypothesis that theophylline could enhance the cytotoxic

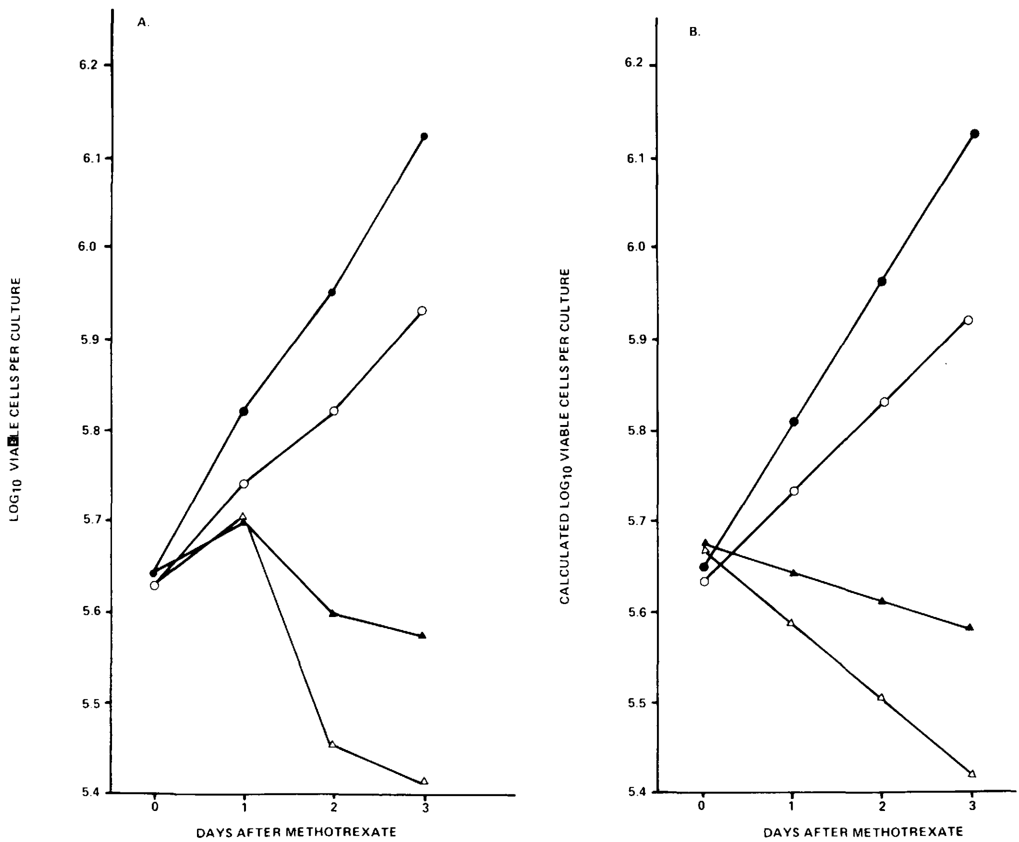

FIG. 1. Effect of 1.0 m*M* theophylline and 0.5 μg/ml methotrexate on the growth of HeLa cells. (A). Untreated cultures (●); 1.0 m*M* theophylline (○); 0.5 μg/ml methotrexate (▲); 1.0 m*M* theophylline + 0.5 μg/ml methotrexate (△). (B) Regression curves generated from the values presented in A. Symbols are the same as in A.

TABLE II. THE EFFECT OF THEOPHYLLINE AND METHOTREXATE ON THE GROWTH OF HELA CELLS IN CULTURE

Culture	Slope[a]	Index[b]
Control	0.1573	1.00
1.0 m*M* Theophylline	0.0979	0.62
0.5 μg/ml Methotrexate	−0.0297	−0.19
1.0 m*M* Theophylline + 0.5 μg/ml methotrexate	−0.0890	−0.57

[a] Slopes are expressed as the change in log viable cells per day.

[b] Index is the ratio of the slope of the experimental group as compared to the control group.

effect of methotrexate, a similar series of experiments were performed, however, in this instance the dose of methotrexate was reduced to 0.1 μg/ml. The results obtained from these experiments are presented in Fig. 2A and the regression curves generated from the data are shown in Fig. 2B. The comparisons of the slopes can be seen in Table III. At this low concentration of methotrexate the HeLa cells continued to grow at a very slow rate. This net increase in viable cell numbers suggested that the toxic effect of 0.1 μg/ml methotrexate alone was insufficient to overcome the growth rate of the cells. The cultures receiving theophylline and methotrexate also showed a net increase in viable cells over time, however, this increase was much smaller than the cultures receiving only methotrexate. These results support the previous observation that the growth-inhibiting effect of methotrexate, even at this relatively

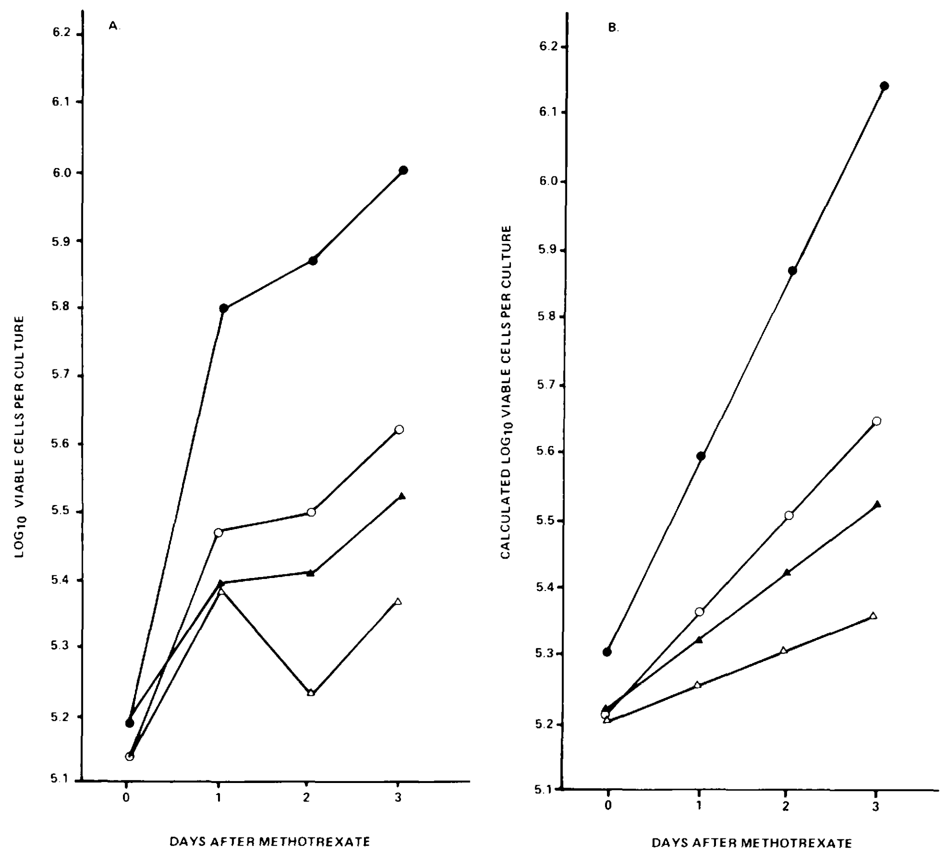

FIG. 2. Effect of 1.0 m*M* theophylline and 0.1 μg/ml methotrexate on the growth of HeLa cells. (A) Untreated cultures (●); 1.0 m*M* theophylline (○); 0.1 μg/ml methotrexate (▲); 1.0 m*M* theophylline + 0.1 μg/ml methotrexate (△). (B) Regression curves generated from the values presented in A. Symbols are the same as in A.

TABLE III. THE EFFECT OF THEOPHYLLINE AND METHOTREXATE ON THE GROWTH OF HELA CELLS IN CULTURE

Culture	Slope[a]	Index[b]
Control	0.2755	1.00
1.0 m*M* Theophylline	0.1457	0.53
0.1 μg/ml Methotrexate	0.1048	0.38
1.0 m*M* Theophylline + 0.1 μg/ml methotrexate	0.0533	0.19

[a] Slopes are expressed as the change in log viable cells per day.
[b] Index is the ratio of the slope of the experimental group as compared to the control group.

low dose, can be enhanced by the addition of 1.0 m*M* theophylline.

Discussion. The data presented in this report have demonstrated that the cytotoxic activity of methotrexate upon HeLa cells could be enhanced by the addition of theophylline. The reduction in cellular growth rate by the addition of theophylline was independent of the action of methotrexate and theophylline at a concentration of 1.0 m*M* did not exhibit any cytotoxicity of its own. Thus, the observed enhancement of methotrexate activity cannot be considered just an additive effect of two cytotoxic agents, but rather a combination of two separate effects which yield as their net result an increase in the cytotoxicity of the antimetabolite.

In previous studies employing the L1210 cell line it was suggested that increased intracellular levels of cyclic AMP decreased the rate of methotrexate transport into the cells (14). If this were occurring in the system reported here, then the effectiveness of methotrexate would be decreased since less of the drug would be transported into the cells. Another report (15) has suggested that the transport of methotrexate into Ehrlich ascites cells is unaffected by changes in cyclic AMP levels. The observations reported here which demonstrate an enhancement of methotrexate activity are possible reflections of a similar mechanism occurring in HeLa cells.

The concentration of theophylline employed in these studies was above the usual therapeutic levels of the drug. Whether or not the addition of theophylline at lower concentrations also enhances the cytotoxicity of methotrexate is a question which merits further examination. Other questions which merit further examination are whether the reported phenomena are unique to HeLa cells or can be applied to other cell systems and if this observed enhancement of cytotoxicity can be extended to other cytotoxic agents.

The results presented in this report describe a system in which two agents with different activities combine their effects and enhance the activity of one. This system can be employed as a model to test other agents known to affect the cell cycle and their interactions with various cytotoxic agents as a method for studying more effective means of chemotherapy.

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Received March 27, 1981. P.S.E.B.M. 1981, Vol. 168.