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STUDIES ON THE MECHANISM OF ANTIFOLATE-INDUCED INHIBITION ON HeLa CELL GROWTH

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Abstract—Aminopterin, methotrexate and dibromoaminopterin inhibited HeLa cell growth by 50 per cent at concentrations of $3{\cdot}5 \times 10^{-9}$, $2{\cdot}5 \times 10^{-7}$ and 10^{-6} M, respectively. However, all three antifolates inhibited crude folate reductase, isolated from HeLa cells, to the same extent when added at equimolar concentrations. Antifolate levels in the cells 24 hr after contact did not correlate with the inhibition of folate reductase or with inhibition of HeLa cell growth. The level of aminopterin 24 hr after contact was approximately four times higher than that of dibromoaminopterin and twice that of methotrexate. This ratio remained constant throughout the whole population doubling time. Maximum levels of antifolates, N^5[methyl-^{14}C]tetrahydropteroylglutamic acid and [^{14}C]thymidine occurred at the logarithmical phase of growth. However, the activity of [^{14}C]glucose rose steadily during the whole cycle and was at its maximum during the stationary phase.

THE PENETRATION of antifolates as powerful cytostatics has been studied in relation to cell resistance or sensitivity to chemotherapy;[1,2] attention being focused on the transport of methotrexate, which seems to be a mediated process. This process is temperature-dependent and exhibits Michaelis–Menten kinetics with competitive inhibition by structural analogues.[3,4] The role of folate reductase, as a target enzyme for the action of antifolates has been previously suggested as a possible factor influencing the rate of antifolate penetration.[5] However, after the cytostatic effect of antifolates has occurred, some discrepancies have been found between the effectiveness of the drugs and the inhibition of folate reductase.[6] This paper investigates the uptake of the structurally related antifolates aminopterin, methotrexate and dibromoaminopterin into HeLa cells. Penetration of all the antifolates increased during the growth of cultured cells and dropped in the stationary phase. Differences in the uptake of individual antifolates could not be correlated with the inhibitory effect on folate reductase or the growth of HeLa cells.

MATERIALS AND METHODS

Chemicals. Aminopterin (4-aminopteroylglutamic acid) and methotrexate (4-amino-N^{10}-methylpteroylglutamic acid) were purchased from Lederle, England. Dibromoaminopterin (3,5′-dibromo-4-aminopteroylglutamic acid) was prepared in this laboratory. Folic acid was obtained from Lepetite, Milan. NADPH was purchased from CalBiochem, U.S.A. N^5[methyl-^{14}C]tetrahydropteroylglutamic acid Ba salt (61 mCi/mmole) was purchased from the Radiochemical Centre, Amersham, England. [^{14}C]Thymidine (30·7 mCi/mmole) and [^{14}C]glucose (9·463 mCi/mmole) were purchased from the Institute for Research, Production and Utilization of Radioisotopes,

Prague, Czechoslovakia. Scintillation fluid SLD 31 was purchased from Spolana Neratovice, Czechoslovakia.

Tissue culture cells. Seven-day-old HeLa cells, cultured in a 300 ml Rous flask were harvested in a Dulbecco phosphate buffer, pH 7·4, containing 0·025 per cent trypsin. After washing with phosphate buffer the cells were resuspended in Eagle's medium, supplemented with 20 per cent human serum, and contained 100 units of penicillin and 200 μg of streptomycin/ml. The cells required for each experiment were diluted as required and used as an inoculum.

Radioactive labelling experiments. Labelling experiments were carried out in the cultures of HeLa cells grown in Eagle's medium. Incorporation was terminated by chilling the monolayer in an ice-bath. The medium was discarded and the cells washed three times with 10 ml of ice-cold Dulbecco phosphate buffer (pH 7·4). The washed cells were then scraped from the glass and collected in a test tube. After centrifugation at 150 *g* for 5 min, the cells were washed in 4 ml of ice-cold phosphate buffer and centrifuged at 150 *g*. The pellet was resuspended in 1 ml of 25 per cent saponine solution. Eleven ml of scintillation fluid was added and the samples were counted in a ABAC SL 40 Intertechnique liquid scintillation counter.

Folate reductase assay. 2×10^8 HeLa cells, cultivated for 48 hr in Eagle's medium, were washed with isotonic 0·85 per cent NaCl solution, collected by centrifugation and resuspended in 2 ml of 0·1 M citrate buffer (pH 5·5). The cells were disrupted by three times repeated freezing and thawing. Folic acid reductase activity was determined in the 20,000 *g* supernatant of centrifuged cell homogenate by the method described by Souček.[7] Estimation of the inhibitory effect was always performed on the day of isolation.

Determination of antifolates. The determination of aminopterin, methotrexate and dibromoaminopterin in HeLa cell suspension was based on the observation of the "stoichiometric" binding of all three antifolates by folic acid reductase. Cells were isolated as in the labelling experiments except that the antifolates were extracted from the final pellet three times with 1 ml of 0·1 M citrate buffer (pH 5·5) in a boiling water bath for 5 min. The antifolates were determined in combined extracts according to Werkheiser[8] using partially purified folate reductase from chicken liver (30–60 per cent ammonium sulfate fraction).

RESULTS

The effect of aminopterin, methotrexate and dibromoaminopterin on the growth of HeLa cells cultured in monolayer was tested (Table 1). The strongest inhibitor was aminopterin, which was one-hundred times more effective than methotrexate. Dibromoaminopterin was the weakest inhibitor, having only a quarter the activity of methotrexate. However, similar activity of folate reductase was estimated by titration of cell free extracts from HeLa cells with equimolar concentrations of these three antifolates calculated per 10^6 cells. The penetration of antifolates into HeLa cells in various growth phases was measured to find the cause of the discrepancies between the identical inhibition of folate reductase by antifolates *in vitro* and their different cytostatic effects. These studies showed that the penetration of antifolates is strictly dependent on the age of culture. It was necessary to cultivate the cells in 300 ml flasks to obtain enough cells for the determination of intracellular unlabelled antifolates (Fig. 1). Antifolate concentration in the cells after 24 hr of incubation was calculated

TABLE 1. THE INHIBITORY EFFECT OF ANTIFOLATES ON HeLa CELLS *in vivo* AND *in vitro*

	Half maximal growth inhibition* (M)	Moles of folate-reductase per 10^6 cells†
Aminopterin	$3{\cdot}5 \times 10^{-9}$	$2{\cdot}19 \times 10^{-12}$
Methotrexate	$2{\cdot}5 \times 10^{-7}$	$1{\cdot}46 \times 10^{-12}$
Dibromoaminopterin	$1{\cdot}0 \times 10^{-6}$	$2{\cdot}30 \times 10^{-12}$

* 2×10^5 cells were inoculated into 16 × 160 mm test tubes containing 2 ml Eagle's medium. After 24 hr of cultivation at 37° the medium was removed and the culture was overlaid with medium containing antifolates in concentrations from 10^{-9} to 10^{-4} M. This medium was replaced after 24 hr and the experiment was terminated at 48 hr by washing the cells with ice-cold phosphate buffer. Cell proliferation was evaluated according to Sanford *et al.*[10]

† Folate reductase, present in the cell free extract, was calculated from the titration curves obtained with aminopterin, methotrexate and dibromoaminopterin.

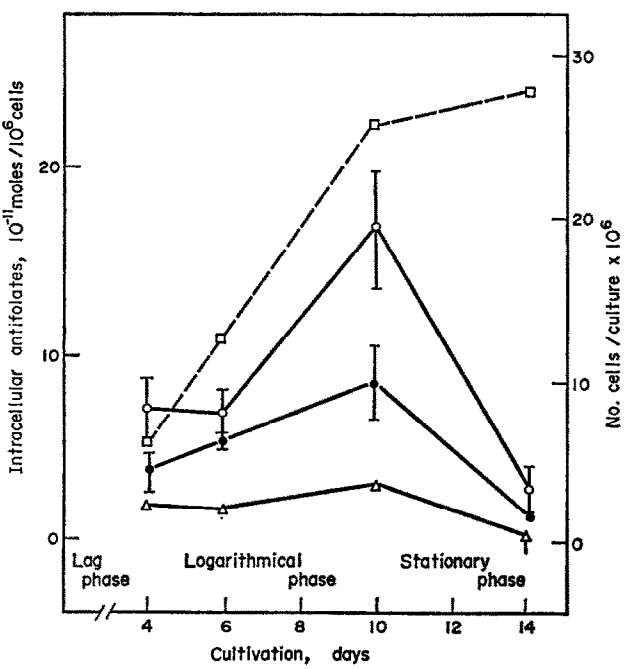

FIG. 1. The effect of the culture age on the accumulation of antifolates in HeLa cells. Three-hundred ml Roux flasks containing 20 ml of Eagle's medium were inoculated with 5×10^6 HeLa cells. The experiment was continued for the population doubling time, i.e. during the logarithmic and stationary phases. Twenty-four hr before the time indicated on the abscissa the old culture-medium was removed and replaced by fresh medium enriched with antifolates and incubated at 37°. The experiments were terminated by washing the cells with cold phosphate buffer. The extracellular concentration of antifolates was $1{\cdot}34 \times 10^{-5}$ M. Number of cells per culture (□); concn of aminopterin (○); methotrexate (●); and dibromoaminopterin (△) expressed as 10^{-11} moles per 10^6 cells. Each point represents the average and S.D. of three determinations from a representative experiment. In some cases the S.D. was too small to be shown.

per 10^6 cells and plotted against the days of growth. It was evident that the cells growing in the logarithmic phase accumulated greater amounts of drugs than cells growing at the beginning of this phase or in the stationary phase. Although the antifolates were added at the same concentration, aminopterin was twice the concentration of methotrexate and four times that of dibromoaminopterin in the cells. This ratio remained almost constant throughout the cell culture cycle. Antifolates added to the cell free homogenate were totally recovered after 50 min of incubation, indicating that none of them was metabolized. The penetration of a metabolizable analogue of

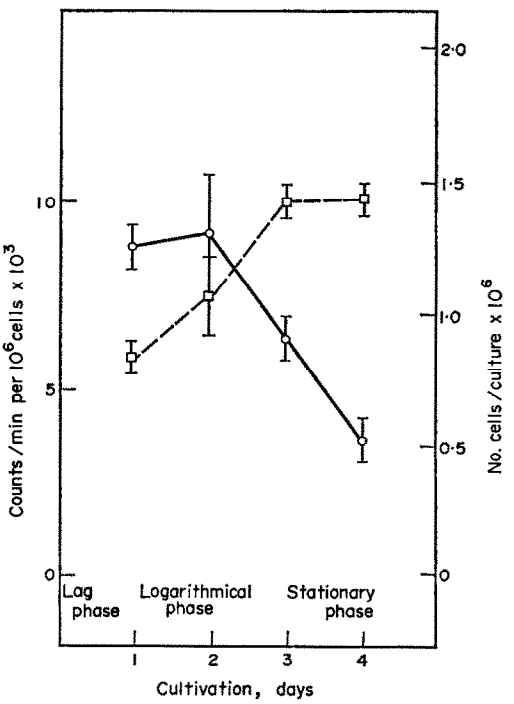

FIG. 2. The effect of the culture age on the uptake of N^5[methyl-^{14}C]tetrahydropteroylglutamic acid into HeLa cells 5 × 10^5 cells were inoculated into test tubes (16 × 160 mm) in 3 ml of Eagle's medium. As the time indicated on the abscissa the cultures were overlaid with fresh medium containing 0·2 μCi of N^5[methyl-^{14}C]tetrahydropteroylglutamic acid and were incubated at 37° for 5 hr. Number of cell per culture (□); cpm of N^5[methyl-^{14}C]tetrahydropteroylglutamic acid per 10^6 cells (○). Each point represents the average and S.D. of three determinations from a representative experiment.

folic acid, i.e. N^5-methyltetrahydrofolic acid was studied (Fig. 2). The course of growth with labelled substrate was shorter than in the previous experiments, the stationary phase appearing around the third day of cultivation in contrast to the tenth day in the experiments with non-labelled compounds. After 5 hr incubation of cells with N^5[methyl-^{14}C]tetrahydrofolic acid, the peak of the radioactivity was found in the logarithmic phase and decreased rapidly in the stationary phase similar to the results obtained with non-labelled antifolates.

[^{14}C]Thymidine and [^{14}C]glucose were studied to see if the transport of metabolically important substances was influenced by the age of the cells. Thymidine was

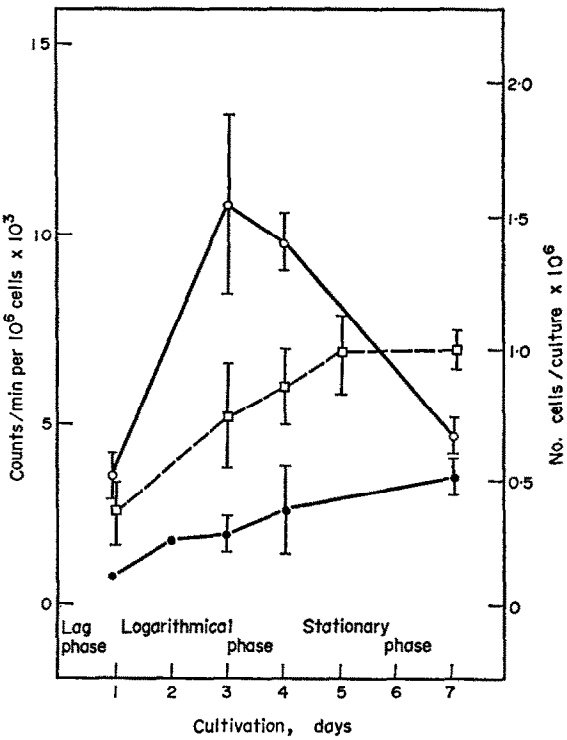

FIG. 3. The effect of the culture age on the uptake of [^{14}C]thymidine and [^{14}C]glucose into HeLa cells. 5 × 10^5 cells were inoculated into test tubes (16 × 160 mm) in 3 ml of Eagle's medium. At the time indicated on the abscissa the cultures were overlaid with the fresh medium containing 0·2 μC [^{14}C]thymidine or 0·5 μCi [^{14}C]glucose. The cultures were incubated for 60 min with thymidine and for 120 min with glucose. Number of cells per culture (□); cpm of [^{14}C]glucose (●); and cpm of [^{14}C]thymidine (○). Each point represents the average and S.D. of nine determinations from three independent experiments. In two cases the S.D. was too small to be shown.

used because tetrahydrofolate takes place in endogenous thymidylate biosynthesis and glucose was taken for comparison as the metabolite involved in general energy metabolism (Fig. 3). Cells were cultured to prolong the logarithmic phase of growth. Cells were incubated with [^{14}C]thymidine for 60 min and with [^{14}C]glucose for 120 min. Using thymidine the accumulation of radioactivity increased reaching the maximum in the second half of the logarithmic phase and then it decreased. These results contrasted with those obtained with glucose where radioactivity accumulated gradually during the whole cell cycle and did not reach a maximum even in the stationary phase.

DISCUSSION

The three antifolates, when added in equimolar concentrations to the external medium, affaired different intracellular levels after 24 hr of incubation. Aminopterin was twice the concentration of methotrexate and four times that of dibromoaminopterin. This ratio was maintained during the whole growth period, but did not correlate quantitatively with cell growth inhibition caused by the three antifolates. To reach the

same inhibition of cell growth it was necessary to use a 1000-fold amount of dibromoaminopterin and a 100-fold amount of methotrexate to that of aminopterin.

Aminopterin has been found to be transported by the same carrier system as the folates because they compete in the uptake.[4] In our experiments the folates present in the incubation medium were transported into the cells continuously. At least two mechanisms could be involved: competition of folates with antifolates in the uptake and heteroexchange diffusion of antifolates with naturally occurring folates in the cells. Each of these compounds tested could act in another way with different final effect on the cell proliferation. Therefore relatively small differences in their uptake might strongly influence the intracellular concentration of tetrahydrofolate coenzymes and in the consequence also the DNA synthesis during the cell growth. In the case of dibromoaminopterin the bromine atoms in position 3′ and 5′ could enhance the hydrophobic interaction of the aromatic nucleus with the lipidic component of the membrane. The binding of the antifolates to the intracellular folate reductase does not seem to be critical because this enzyme is inhibited *in vitro* approximately to the same extent by the three antifolates (Table 1).

The maximum uptake of antifolates observed in the logarithmic phase of growth of the cells correlates with the elevated DNA synthesis in this phase.[9] Because both thymidine and thymidilate are essential for DNA synthesis in HeLa cells, there is an increased uptake of thymidine and antifolates transporting instead of folates. The continuous glucose uptake during the whole cell cycle is because glucose is a source of energy.

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