A Delayed Cytotoxic Reaction for 6-Mercaptopurine


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SUMMARY

In this study of the cytotoxic action of 6-mercaptopurine (6MP), a delay was demonstrated between exposure of mouse tumor cells to 6MP and physical manifestations of toxicity by the cells. Proliferation of cells of the Ehrlich ascites carcinoma in vivo and of mouse lymphoma L5178Y in culture continued during and beyond the period of exposure to 6MP, although this treatment was ultimately lethal. In the case of the cultured lymphoma cells, the terminal phase of postexposure proliferation was characterized by unbalanced growth in which cell protein and size increased above normal values. Subsequently, division ceased and the enlarged cells disintegrated. Two effects of 6MP were distinguishable in cultures of the lymphoma cells, the delayed cytotoxic activity and an acute inhibitory effect on the rate of cell proliferation.

INTRODUCTION

Tomizawa and Aronow (11) reported that brief exposure to 6MP caused a "delayed" cytotoxicity in cultures of Earle's L strain of mouse fibroblast, and Bases (1) observed that 6MP-treated HeLa cells continued to divide when all but 1 or 2% were sterile. More recently, Paterson and Moriwaki (7) described a delay in the response of cultured cells of mouse lymphoma L5178Y during continuous exposure to a low concentration of 6MP. Similarly, Hirshaut et al. (5) found that, when human malignant leukocytes were cultured in the presence of moderate levels of 6MP, cell multiplication continued for several days before lethal effects were apparent.

In the studies reported here, toxic effects of 6MP on the EAC in vivo and on lymphoma L5178Y cells in culture were observed at a time when the drug was no longer available to the cells. Attempts to explain the antiproliferative activity of 6MP in biochemical terms must account for this delayed type of response. The present description of the delayed effect of 6MP in cultures of lymphoma L5178Y is part of a biochemical study of 6MP action in this system.

MATERIALS AND METHODS

Unless otherwise stated, cell numbers were determined with a Coulter Model F electronic particle counter. The same instrument, which incorporates a pulse height analyzer, was used to monitor cell volume distributions. The counter was calibrated for volume determinations with a suspension of ragweed pollen grains of known dimensions (Hollister-Stier Laboratories, Spokane, Wash.).

The EAC was passaged in female Ha/ICR mice (from our own colony) by weekly transplantation of $5 	imes 10^6$ cells. Mice bearing the L5178Y lymphoma were provided initially by Dr. G. A. Fischer, Brown University, Providence, R. I. The lymphoma has been maintained in male BDF1 mice (Microbiological Associates, Inc., Bethesda, Md.) by weekly i.p. transplantation of $10^7$ cells obtained from ascitic fluid. Each of the cell culture experiments described was initiated with a separate inoculum of lymphoma cells obtained from ascitic fluid of mice bearing the in vivo-passaged cell line. Cells were cultured at 37.5°C in stoppered, stationary tubes (16 x 100 mm) containing 6 ml of Fischer's medium (4) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with sodium bicarbonate (1.125 g/liter); 10% horse serum and antibiotics; streptomycin (100 μg/ml); and penicillin (100 units/ml). All cultures were prepared in duplicate at least.

Where larger culture volumes were required for sampling, as in cell volume and protein determinations, the cells were grown as 120-ml cultures in stoppered, soda glass bottles.

In the in vivo experiment, female Ha/ICR mice were implanted i.p. with $6 	imes 10^6$ EAC cells, and 6MP treatments were started 24 hr later. 6MP hydrate (Sigma Chemical Co., St. Louis, Mo.) was administered as a very finely ground suspension in 0.9% NaCl solution. The treated mice received 5 i.p. injections of 6MP (40 mg/kg body weight) at intervals of 24 hr, and control mice received i.p. injections of 0.9% NaCl solution on an identical schedule. Treated and control mice in groups of 5 were killed every 2nd day; from each, the ascitic fluid and NaCl solution rinsings of the peritoneal cavity were collected, and the cells were counted.

Exponentially proliferating L5178Y cells were treated with 6MP in the following manner. 6MP solution, 2 mM in 0.9% NaCl solution, was added to cultures to achieve the 6MP concentrations specified; control cultures received identical volumes of 0.9% NaCl solution. Cell densities did not exceed 100,000/ml when 6MP was added. 6MP treatment was terminated by changing the medium. The cells were collected by centrifugation, washed once in warmed, drug-free medium, and then resuspended in the latter. During subsequent
incubation, exhaustion of the medium was avoided by the dilution of measured volumes of suspended cells with fresh, warmed medium. Control cultures were diluted once daily throughout the experiment. Treated cultures were diluted once or twice during the period of growth between removal of the drug and onset of the delayed response; further dilution was not necessary until the surviving population became appreciable. Cell densities reported in the charts are hypothetical values derived from actual cell counts and cumulative dilution factors.

Total protein in lymphoma cell cultures was assayed by the procedure of Lowry et al. (6). Intact lymphoma cells were recognized by their ability to exclude trypan blue stain (0.4% in Hanks’ balanced salt solution; Grand Island Biological Co.). Nonstaining cells were counted in a hemocytometer.

6MP-8-14C, specific activity, 24 mCi/mmmole (Schwarz BioResearch, Inc., Orangeburg, N. Y.), was used to measure the disappearance of 6MP from the culture medium in which the initial cell density was 100,000 cells/ml.

RESULTS

Effect of 6MP on the Proliferation of EAC Cells in Vivo. The time course of EAC cell proliferation was followed by counting cells in peritoneal rinsings from groups of 5 mice originally derived from a large group of mice that had received identical implants of the tumor cells. Chart 1 shows the effect of 6MP (i.p. injection) on the proliferation of these cells. The ascitic population continued to multiply throughout the period of treatment, although the rate at which cell numbers increased was lower than that in untreated control animals. In this experiment, a posttreatment interval of 4 to 5 days elapsed before tumor cells stopped proliferating; during this period, the tumor cell population approximately doubled. Following this, cell numbers in the peritoneal cavity declined rapidly until the relatively constant value of about $4 \times 10^6$ cells/mouse was reached. Similar cell numbers were found in peritoneal rinsings from normal female Ha/ICR mice that had not been implanted; thus, 6MP treatment reduced the peritoneal tumor cell population to below the level of the normal ascitic cell population. As an integral part of this experiment, survival times were determined for groups of treated and untreated mice (EAC cell implants were identical with those of mice that yielded the data in Chart 1). The mean survival time (±S.D.) for a group of 20 untreated, EAC-bearing mice was 17 (±3) days. Of 20 such mice, treated with 6MP on the schedule specified, 17 were alive and without evident tumor on the 50th day after implantation. Thus, the 6MP regimen reduced tumor cell numbers, at least to the extent that host defenses were effective.

Effects of Brief Exposure to 6MP on Cultured Lymphoma Cells. Cultures of L5178Y cells which were derived separately, each from a different explant of the in vivo-passaged lymphoma, exhibited moderate differences in sensitivity to 6MP. However, in spite of such differences, a constant feature of these experiments was the delayed response to timed exposures to 6MP.

The delayed response to 6MP of the lymphoma cells became apparent when the cells, proliferating exponentially in culture, were exposed to the drug for definite periods and then
transferred to fresh, drug-free medium. Cell division in such cultures continued in the presence of 6MP and, after removal of the drug, a further 1 to 2 doublings occurred before the cytotoxic action of the drug was manifested. This behavior was observed when cells were exposed to 20 to 100 µM 6MP for periods up to 25 hr. The delayed cytotoxic effect was elicited by these conditions of 6MP treatment. This was characterized by an increase in cell volume, division arrest, and eventual cell lysis. In a representative experiment (Chart 2), the cell population doubled during the interval between removal of 6MP and decline of cell numbers. In other experiments, following shorter periods of 6MP exposure, cell numbers increased by as much as 2 doublings after removal of the drug. In the experiment shown in Chart 2, the median cell volume gradually increased after removal of the drug and was maximal at about the time that cell numbers began to decline. In this experiment, cell concentrations were determined both by means of an electronic particle counter and by counting trypan blue-excluding cells in a hemocytometer. During the period of limited growth which followed 6MP treatment, the 2 methods of enumeration agreed closely (Chart 2) and, therefore, the electronic particle counter was used to assay cell number in subsequent experiments. Determinations of cell numbers by the 2 methods diverged after cell division had stopped, evidently because trypan blue-staining cells and cell debris accounted for a substantial portion of the counts recorded by the electronic particle counter. At the time cell numbers began to fall, large, nonstaining cells were predominant, and globular cell fragments (which stained with trypan blue) became abundant in the cultures as cell disintegration proceeded. Cell fragments registered by the electronic particle counter were responsible for the progressive decrease in the apparent median cell volume to below that of control cells (Chart 2).

Surviving cells eventually repopulated 6MP-treated cultures and established exponential proliferation rates which did not differ significantly from those of untreated controls. In other words, the exponential portions of survivor growth curves (log cumulative cell number versus time) paralleled the exponential, straight-line plots for the corresponding control cultures. The displacement (measured in the ordinate direction) of the exponential portion of the survivor curve from the control plot was used as a measure of the fraction of cells that survived the drug treatment. In so doing, the simplifying assumption is made that the generation time of cells destined to survive was unaltered during recovery from drug exposure. This provides only a minimum estimate of the surviving fraction. The real circumstances are undoubtedly complex, and the true surviving fraction may well be larger. Similar assumptions have been used in estimating the fraction of tumor cell populations killed in vivo by chemotherapeutic treatment (9); the data yielded are qualitatively similar to those obtained by clonal methods (2). The fraction of L5178Y lymphoma cells that survived 6MP treatment, as estimated by the above method, was dependent upon the duration of the exposure to 6MP and upon the concentration of 6MP present during exposure. Estimates of minimal surviving fraction are plotted against the corresponding periods of exposure in Chart 3A. The data indicate that a constant fraction of tumor cells were killed during consecutive equal periods of exposure to a fixed concentration of 6MP. This response occurred over a logarithmic range of approximately 3 and at several concentrations of 6MP. Kill kinetics of this sort has been described as empirically 1st order with respect to concentration of cells (10). The following experiment indicated that 6MP concentrations in the medium did not decline appreciably during the period of cell treatment; a 60 µM medium concentration of 6MP-8-14C was not significantly

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**Chart 3.** A, exposure-survival curves for L5178Y cells treated with 6MP. Surviving fractions are minimal values derived from exponential survivor growth curves. 6MP concentrations: ○, 48 µM; ▵, 77 µM; ●, 109 µM. Curves, data obtained with different explants of the in vivo-passaged lymphoma. B, dose-survival curve for L5178Y cells exposed to 6MP. Duration of exposure was 13 hr. Surviving fractions are minimal values derived from exponential survivor growth curves. All cultures were derived from the same explant of the in vivo-passaged lymphoma.
reduced in a 25-hr incubation during which cell density increased from 100,000 to 370,000 cells/ml.

Chart 3B presents dose-response data for a fixed period (13 hr) of exposure to 6MP and demonstrates that the rate of cell kill was not 1st order with respect to the concentration of the drug. Successive equal increments in drug concentration contributed progressively less to the overall fractional kill. Results qualitatively similar to those illustrated in Chart 3B have been obtained in preliminary experiments with the use of a cloning assay in which surviving cells form colonies in a soft agar medium.

Cell populations that survived exposure to 6MP were substantially less sensitive to further challenge with the drug; this is exemplified in the data of Chart 4, in which it is seen that cells that survived a 25-hr exposure to 200 µM 6MP were less sensitive to 50 µM 6MP than were untreated cells from the same explant. It appears that the 25-hr exposure to 200 µM 6MP significantly enriched the population with resistant cells.

The increase in the doubling time of cells during exposure to 6MP seen in Charts 2 and 4 was not always observed. The decreased rate of proliferation might have an explanation in a partial inhibition of cellular metabolic processes by 6MP metabolites or could possibly be due to the acute death of a portion of the cells during 6MP exposure. The latter possibility would still be compatible with the absence of trypan blue-staining cells at this stage (Chart 2). Damaged cells might persist and exclude trypan blue for some time after they had lost the capacity to divide. However, the experiment of Chart 5 suggests that acute cell death was not primarily responsible for increased doubling times in the presence of 6MP; it is apparent that the blockade of cell division by 6MP was not immediately lethal.

Cell populations that had become static in the presence of 6MP (Chart 5) remained so for 7 hr and then, upon removal of the drug, resumed division. Chart 5, lower curve, shows changes in cell number that occurred between 0 and 25 hr in the presence of 200 µM 6MP. Cell multiplication ceased 6 hr after 6MP was added, and cell numbers did not change significantly during the remaining 19 hr of exposure. However, when the drug was removed at 6, 11, or 13 hr, proliferation resumed. The delayed cytotoxic effects of 6MP were manifested in these cultures about 40 hr after 6MP removal.
Cells did not resume proliferation after 25 hr of 6MP exposure but did increase in volume after the drug was removed. It would seem that different mechanisms were responsible for this acute inhibition of proliferation and for the delayed lethal activity of 6MP.

The increase in cell volume which is characteristic of the delayed effect was suggestive of the condition of “unbalanced growth;” the experiment of Chart 6 bears on this question. Chart 6 demonstrates that, in untreated cultures of the lymphoma cells, the relationship between cell numbers and the total cellular protein did not change with time. In contrast, the relationship between these parameters was not constant in cultures that had been treated with 6MP; these data indicate that the cellular protein content increased during expression of the delayed toxicity of 6MP. As a further part of this experiment (data not shown), cell volume measurements were made at intervals before and throughout the period shown in Chart 6.

The volume distribution of exponentially proliferating lymphoma cells in control cultures was logarithmic normal. This type of distribution yields a straight line when the data are plotted on logarithmic probability graph paper. Rosenberg and Gregg (8) have used this type of representation to detect small changes in cell volume distribution and have referred to distribution plots on logarithmic probability paper as volume spectra. In the experiment of Chart 6, the volume spectra (not shown) of treated cells were identical with those of control cells both during the period of exposure and for as long as 14 hr after the drug was removed. Thereafter, the spectrum of the treated cells shifted progressively to higher volumes and became biphasic; the higher volume portion of the plot remained parallel to the control cell line. The biphasic spectrum probably reflects the presence of 2 populations: one population in which cells have become blocked in the cell cycle and are growing unnaturally large and another in which cells have not reached this stage and are still dividing. The median cell volume increased to approximately 160% of the control value before the accumulation of debris obscured the spectrum.

DISCUSSION

Cells that have been exposed to 6MP are able to undergo abortive proliferation before they disintegrate. The high incidence (>70%) of 50-day survivors indicated that chemotherapy with 6MP was an effective treatment for EAC-bearing mice, and yet tumor cell proliferation continued for more than 4 days after the course of therapy was terminated. Cultured mouse lymphoma L5178Y cells exhibited a similar delayed reaction to brief exposure with 6MP. In this case, cell populations underwent 1 to 2 doublings after the drug was removed.

The reason for this type of drug effect is not clear. The culture medium used in experiments with the lymphoma provided no content of purines and pyrimidines (apart from that which might be present in serum) and, accordingly, the lymphoma cells were entirely dependent upon their own capacity to synthesize these compounds. It might therefore be reasoned that these cells would be very sensitive to inhibitions at the level of purine nucleotide biosynthesis and their interconversions [see review by Elion (3)]. However, this type of mechanism would be expected to produce a rapid effect on cell multiplication. Inhibitions at the level of intermediary purine nucleotide metabolism are probably responsible for the acute, reversible blockade of cell proliferation that we have observed at high concentrations of 6MP (Chart 5). Removal of the drug allowed cell multiplication to resume temporarily before delayed toxicity was exhibited. Increased doubling times during exposure to lower concentrations of 6MP may represent minor manifestations of this same effect.

Effects of 6MP at the macromolecular level would offer a more likely possibility to explain the delay in expression of 6MP lethality. However, the occurrence of unbalanced growth as an integral part of the delayed reaction indicates that at least the gross capacity for protein synthesis is not impaired at this time.

Attention should also be drawn to the dose-response data for a fixed period of exposure (Chart 3B). The response of the lymphoma approached a maximal value as the 6MP concentration was increased, and further cell kill was achieved only by increasing the length of the exposure period. This type of behavior has been associated with agents that are toxic to cells only in a particular phase of the cell cycle (2).

The present work has partly characterized the delayed reaction to 6MP of EAC cells in vivo and lymphoma L5178Y in culture. The in vivo data do not demonstrate that 6MP is directly involved in the delayed effect on EAC cell proliferation. Other factors might possibly be invoked. For example, it might be argued that the delayed effect represents a successful host reaction against the tumor which was facilitated by preceding chemotherapy with 6MP. However, it seems more likely that the delayed effect on EAC cell proliferation in vivo may be related to the delayed effect of 6MP on cultures of L5178Y cells. Furthermore, a drug mechanism at the macromolecular level would provide a more satisfactory explanation for delayed cytolysis than would acute inhibitions of purine nucleotide biosynthesis and interconversions.

REFERENCES

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