On the Mechanism of the Lethal Action of 5-Fluorouracil on Mouse L Cells

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SUMMARY

Dose-survival curves for mouse L cells in culture exposed to 5-fluorouracil for one hour during the log phase of growth were determined by the colony formation method of Puck et al. The effect of the presence of thymidine or uridine either during or following exposure to the drug was investigated. The survival of cells exposed to this agent was markedly increased by small quantities of thymidine, particularly when it was present following the exposure. The addition of thymidine following exposure eliminated the differential action of 5-fluorouracil on logarithmic as compared with stationary phase cells. Fetal calf serum may contain quantities of a dialyzable substance which behaves like thymidine in this system. Possible mechanisms for the thymidine-reversible and thymidine-irreversible modes of action of 5-fluorouracil are discussed.

INTRODUCTION

Since the demonstration of the inhibition of the growth of mammalian and of bacterial cells by 5-fluorouracil, two chief modes of action of this drug at the biochemical level have been demonstrated. First, 5-fluorouracil, like 5-fluorodeoxyuridine (FUDR) may be converted to 5-fluorodeoxyuridylic acid (FUDMP) which blocks the formation of thymidylate from deoxyuridylic acid by the enzyme thymidylate synthetase, thus inhibiting DNA synthesis (6–8, 10, 11). Second, 5-fluorouracil may be incorporated into RNA to give an aberrant form of one or more of the classes of RNA (2, 5, 9, 11, 12, 20). It has also been shown that high concentrations of 5-fluorouracil may interfere with the incorporation of uracil into RNA (7, 8). However, such biochemical events are characterized at the time of, or immediately following, incubation of a cell system with the drug. To correlate such events with a biologic end-point, such as the colony-forming ability of cells following treatment with the drug, is a difficult problem on account of the time required for cell proliferation. Kessel et al. (12) observed a correlation between the rate of incorporation of 5-fluorouracil into the RNA of certain tumor cells in vitro and the life span of animals bearing the same tumor cells treated with 5-fluorouracil in vivo.

The present experiments were designed as a first step toward evaluating the contribution of the two chief biochemical modes of action outlined above to the lethal action of 5-fluorouracil. The effect of the presence of thymidine or uridine either in the medium at the time of treatment or in the posttreatment medium in which surviving cells grew to form colonies in vitro was examined. It was postulated that a protection by thymidine would support the former mode of action and a protection by uridine, the latter.

MATERIALS AND METHODS

Mouse L60 cells were grown as suspension cultures in our standard medium CMRL 1066 (15) from which nucleosides and liver extract were omitted. The medium was supplemented with fetal calf serum (Flow Laboratories, Rockville, Maryland) at a concentration of 10% v/v. This serum was dialyzed three times against a phosphate-buffered saline solution, as previously described (14), in order to remove nucleosides present in the serum. To this standard medium, quantities of 5-fluorouracil, FUDR, thymidine, or uridine were added in certain experiments. 5-Fluoro deoxyuridyline and 5-fluorouracil were obtained from Hoffmann-LaRoche, Montreal, Quebec, thymidine from General Biochemicals, Cleveland, Ohio, and uridine from the Sigma Chemical Company, St. Louis, Missouri. They were dissolved in 10 ml of the medium and sterilized by filtration through a Millipore membrane before addition to medium stocks.

Dose-survival curves were determined by the method of Puck et al. (16). Cells, in suspension at a concentration of about 4.10^6/ml, were dispensed into each of a series of translucent plastic test tubes (Falcon Plastics, Los Angeles, California), 9.5 ml per tube. The tubes were placed in a roller wheel overnight at 37°C for the cells to enter the log phase of growth, or for five days to give stationary phase cultures. Graded doses of either 5-fluorouracil or FUDR were added in 0.5 ml of medium per tube to give a final concentration in the range of 0–50 µg/ml. In both cases the cells were incubated with the drug for 1 hour at 37°C in all the experiments to be described. They were then spun down in a clinical centrifuge, the medium decanted, and the cells resuspended in fresh medium containing serum. In experiments with FUDR, they were given a wash by centrifuging a second time and resuspending them in fresh medium. While for FUDR this extra wash was necessary to re-

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move trace amounts of the drug, for 5-fluorouracil this did not affect the survival of the cells on subsequent incubation and was therefore discarded from the procedure after preliminary experiments. Following appropriate dilutions the cells from each tube were plated into each of five tissue culture dishes (Falcon Plastics) which were incubated at 37°C for 14 days in a humidified atmosphere of 95% air, 5% CO2. The resultant cell colonies were stained with methylene blue and counted by eye. The plating efficiency of untreated log phase cells in these experiments lay between 70% and 100%, that of stationary phase cells between 50% and 80%. All survival curves shown below were repeated at least twice, yielding similar results in each case. In every experiment the survival curve for cells exposed to 5-fluorouracil in the absence of nucleosides was determined as a control.

RESULTS

The effects of thymidine and uridine on the survival of cells in the logarithmic phase exposed to 5-fluorouracil for one hour were examined in two series of experiments.

Posttreatment Reversal by Thymidine and Uridine. First, the effects of their presence following the one-hour exposure to 5-fluorouracil were examined. The results of this first type of experiment in which the exposed cells were plated in medium containing various concentrations of thymidine or uridine are illustrated in Chart 1. In the absence of thymidine, the survival curve was exponential in form and was characterized by a C10 (the concentration of the drug that reduces the survival of the cells by a factor of 10) of 4.5 μg/ml. Thymidine had no detectable effect on plating efficiency or colony size of untreated cells, but it protected a fraction of the cells treated with 5-fluorouracil. As low a concentration of thymidine as 0.05 μg/ml gave a considerable protection, though not as great as that given by either 4 or 5 μg/ml. Curves for cells plated into medium containing 0.1, 0.5, 1, 2, and 3 μg/ml of thymidine lay between the two curves shown in Chart 1 for the two extreme values of thymidine concentration. When the concentration of thymidine was raised from 5 to 10 μg/ml, no additional protection was observed.

Similar experiments were performed in which known quantities of uridine were added to the medium in which cells were plated. Concentrations of uridine of 5, 50, or 500 μg/ml yielded the same survival curve, illustrated in Chart 1 by the data for a uridine concentration of 500 μg/ml. While uridine did give some protection against the drug, even at a concentration of 500 μg/ml the effect is less than that of 0.05 μg/ml of thymidine.

The posttreatment effect of the addition of thymidine together with uridine was next examined. Chart 1 includes the survival curve for cells treated with 5-fluorouracil and plated into medium containing 5 μg/ml of thymidine plus 500 μg/ml of uridine. Curves for medium to which 5 or 50 μg/ml of uridine, in addition to the thymidine, were added lay between this curve and that for 5 μg/ml of thymidine alone. It is clear that the addition of 500 μg/ml of uridine in addition to thymidine in the posttreatment medium raises the surviving fraction by a factor of only two at high doses of 5-fluorouracil.

Effect of Thymidine or Uridine Present during Exposure of Cells to 5-Fluorouracil. In the second series of experiments, the effects of the presence of thymidine or uridine during the one-hour exposure to 5-fluorouracil were examined. Cells suspended in medium containing either 10 μg/ml of thymidine or 500 μg/ml of uridine were treated with 5-fluorouracil and plated in medium without nucleosides as described. The results are shown in Chart 2. In both cases exponential survival curves were obtained. The presence of uridine increased the C10 from 4.5 to 7 μg/ml, that of thymidine to 20 μg/ml. In neither case was a two-component survival curve obtained. The effect of the presence of these nucleosides during the incubation with 5-fluorouracil was clearly much less compared with their presence in the posttreatment medium.

Time Course of Posttreatment Reversal by Thymidine. Since the greatest reversal of the action of 5-fluorouracil was seen when thymidine was present in the posttreatment medium, experiments were performed to investigate the effect of the addition or removal of thymidine from cells as a function of time following treatment with 5-fluorouracil.
Chart 2. The effect of the presence of thymidine (10 μg/ml) or uridine (500 μg/ml) during the one-hour exposure to 5-fluorouracil. The cells (log phase) were plated in medium containing neither thymidine (T) nor uridine (U).

Cells in the logarithmic phase of growth treated for 1 hour with 10 μg/ml of 5-fluorouracil in medium lacking thymidine were resuspended in either medium containing 10 μg/ml of thymidine or in medium without thymidine and plated into a series of Petri dishes. At various times thereafter, the medium from the former dishes (containing thymidine) was removed by gentle aspiration and replaced with medium lacking thymidine. Similarly, the medium from the latter dishes (lacking thymidine) was removed and replaced with the thymidine-containing medium. A replicate set of dishes was plated with control cells not treated with the drug but subjected to the same manipulations. This control series showed that the colony counts were not complicated by any reseeding of the dishes as a result of the medium change. The results of the experiment with 5-fluorouracil-treated cells are shown in Chart 3. The abscissa is the time at which the media were changed, the ordinate the surviving fraction. Two curves are shown corresponding to the conditions when thymidine was added (T− → T+) and when thymidine was removed (T+ → T−). When the cells were plated directly into medium either lacking or containing thymidine and not subject to any medium change, the survival is as shown for t = 0. It can be seen that when cells were plated into medium lacking thymidine which was subsequently replaced with the thymidine-containing medium, the surviving fraction initially decreases exponentially with time and then asymptotically approaches a value for cells plated and left in medium lacking thymidine for the whole of the post-treatment time of incubation. It is evident that the action of the drug is still being expressed under these conditions 80 hours or more after the one-hour incubation with 5-fluorouracil. In contrast, when as a function of time, the medium was removed from cells plated in medium containing thymidine and replaced with medium lacking thymidine, there was no further effect on the surviving fraction. Cells left in thymidine-containing medium for 2 hours show the same surviving fraction as cells left in thymidine medium for greater lengths of time before the medium was changed to one lacking thymidine. No medium change was attempted in less than 2 hours, because it was necessary to allow the cells to adhere to the surface of the dishes before the medium could be changed.

Chart 3. The effect of the addition or removal of thymidine as a function of time following the exposure of log phase cells to 10 μg/ml of 5-fluorouracil for one hour. The dashed line labeled T+ → T− is for cells plated in medium containing 10 μg/ml of thymidine changed to a medium containing no thymidine at the time shown on the abscissa. The solid line labeled T− → T+ is for cells plated into medium containing no thymidine changed to a medium containing 10 μg/ml of thymidine at the times shown on the abscissa. The two points at zero time are for cells plated into medium containing either 10 μg/ml thymidine (○) or no thymidine (▲) with no further change of media.
Lethal Mechanism of 5-Fluorouracil

14) The first of these concerns the influence of the incubation conditions normally employed in the measurement of cell survival. The viability of cells is usually scored under conditions in which the treated cells are plated in medium supplemented with undialyzed serum. Is survival influenced by the presence of undialyzed serum?

In order to ascertain the effect of plating cells treated with 5-fluorouracil into medium supplemented with dialyzed as opposed to undialyzed serum, cells grown and treated in the logarithmic phase of the growth cycle in medium supplemented with dialyzed serum were plated into medium supplemented with either dialyzed or undialyzed serum. The results are shown in Chart 5. The survival curve for cells plated into medium supplemented with dialyzed serum is exponential as previously observed with a C10 of 4.5 µg/ml. However, the survival curve for cells plated into medium supplemented with undialyzed serum was biphasic in form. This shows that a low molecular weight substance present in the serum gave a very marked protection against the lethal action of 5-fluorouracil. Presumably, this substance was thymidine (cf. Charts 1 and 5). The presence of a small quantity of thymidine in fetal calf serum has previously been reported (19). This result may be contrasted with that from our own previous work where plating into undialyzed serum (from a different lot) yielded an exponential survival curve with a C10 of 6 µg/ml (14). Presumably, the

Survival of Cells Exposed to FUDR. The marked reversal by thymidine of the lethal action of 5-fluorouracil is consistent with the suggestion that 5-fluorouracil causes death of cells after its conversion to FUDMP. Since, unlike 5-fluorouracil, FUDR is an immediate precursor of FUDMP, it was of interest to examine the survival of cells treated in exactly the same way as described above, but with FUDR in place of 5-fluorouracil. Logarithmic phase cells treated with FUDR for 1 hour over the concentration range 0—50 µg/ml were given an extra wash as described in Materials and Methods. They were then plated into either medium lacking thymidine or medium containing 5 µg/ml of thymidine. The survival curves so obtained are shown in Chart 4 and may be contrasted with those for 5-fluorouracil under the same conditions (Chart 1). When the FUDR-treated cells were plated into medium lacking thymidine, the survival curve reached a plateau at about 45% survival. When the cells were plated into medium containing thymidine, the reversal was almost complete, 80% of the cells surviving. Clearly, the survival curves obtained for FUDR are different from those for 5-fluorouracil under similar conditions. They demonstrate a marked difference in action between 5-fluorouracil and FUDR.

Effect of Dialysis of Serum. The marked reversal of the lethal action of 5-fluorouracil by thymidine raises two questions which bear on our previous studies with 5-fluorouracil (3, 4, 14). The first of these concerns the influence of the incubation conditions normally employed in the measurement of cell survival. The viability of cells is usually scored under conditions in which the treated cells are plated in medium supplemented with undialyzed serum. Is survival influenced by the presence of undialyzed serum?

In order to ascertain the effect of plating cells treated with 5-fluorouracil into medium supplemented with dialyzed as opposed to undialyzed serum, cells grown and treated in the logarithmic phase of the growth cycle in medium supplemented with dialyzed serum were plated into medium supplemented with either dialyzed or undialyzed serum. The results are shown in Chart 5. The survival curve for cells plated into medium supplemented with dialyzed serum is exponential as previously observed with a C10 of 4.5 µg/ml. However, the survival curve for cells plated into medium supplemented with undialyzed serum was biphasic in form. This shows that a low molecular weight substance present in the serum gave a very marked protection against the lethal action of 5-fluorouracil. Presumably, this substance was thymidine (cf. Charts 1 and 5). The presence of a small quantity of thymidine in fetal calf serum has previously been reported (19). This result may be contrasted with that from our own previous work where plating into undialyzed serum (from a different lot) yielded an exponential survival curve with a C10 of 6 µg/ml (14). Presumably, the

Chart 4. The effect of the presence of thymidine (T) (5 µg/ml) during the posttreatment period on the survival of log phase cells exposed to 5-fluorodeoxyuridine for one hour.

Chart 5. The effect of dialysis of the serum supplement to the posttreatment medium on the survival of log phase cells exposed to 5-fluorouracil for one hour.
concentration of thymidine in serum varies greatly from lot to lot.

Stationary Phase Cells and Posttreatment with Medium Containing Thymidine. The second question raised by our previous work concerns the action of thymidine on the survival of stationary phase cells treated with 5-fluorouracil. We have shown that the survival curve for logarithmic phase cells differs markedly from that for stationary phase cells when cells were treated and plated in medium lacking thymidine (14). Is the differential still present when the cells are plated in medium containing thymidine? Stationary phase cells treated with 5-fluorouracil over the dose range 0—50 μg/ml were plated into medium either with or without thymidine (10 μg/ml). The same experiment was repeated with logarithmic phase cells to give a direct comparison. The survival curves under these conditions are shown in Chart 6. When plated in medium lacking thymidine, the marked difference in sensitivity to this drug between logarithmic and stationary phase cells was again observed. The initial slope of the exponential region of the curve for stationary phase cells yielded a C10 of 18 μg/ml, the same as previously reported (14). For 5-fluorouracil concentrations between 20 and 50 μg/ml, the surviving fraction of stationary phase cells reaches a plateau, thus enhancing the differential action of the drug between proliferating and nonproliferating cells. However, Chart 6 also clearly shows that when either logarithmic or stationary phase cells treated with 5-fluorouracil were plated into a thymidine-containing medium, they exhibit a common survival curve. Hence, the difference between the action of 5-fluorouracil on logarithmic and stationary phase cells is due to that part of the action of the drug which is reversible by the posttreatment presence of thymidine. The differential is lost when thymidine is present following the one-hour exposure to the drug.

DISCUSSION

It was found in these experiments that the survival of cells exposed to 5-fluorouracil was markedly influenced by small quantities of thymidine, as little as 0.05 μg/ml, in the posttreatment medium. In contrast, the presence of uridine under these conditions had relatively little effect on the survival, even at high concentrations. These results are consistent with those from earlier studies of the reversal of the growth-inhibitory effect of 5-fluorouracil on mammalian cells (1, 17, 18). It is thus possible to consider the lethal action of 5-fluorouracil on L cells in two parts. One part is reversible by thymidine after a short exposure to the drug, the second is not reversible by thymidine.

The thymidine-reversible mode of action may be explained in a number of ways. First, 5-fluorouracil or one of its metabolites may interfere with the production of thymidine, leading to a thymidine-less state which ends in cell death. It is of interest to note that Maaløe and Kjeldgaard (13) found that bacteria (E. coli) could be protected against thymine-less death by the presence of 0.05 μg/ml of thymine. The enzyme thymidylate synthetase is inhibited by FUDR after its conversion to FUDMP. Similarly, 5-fluorouracil may itself be converted to FUDMP. It is perhaps difficult to reconcile a similar biochemical mode of action for 5-fluorouracil and FUDR with the great discrepancies between the survival curves for the two agents (Charts 1, 4). However, this discrepancy may be accounted for if it is postulated that 5-fluorouracil is retained within a much larger intracellular pool than is FUDR. Such a pool would have to persist for a period at least approaching that of the generation time of these cells (about 16 hours) to cause a thymidine-less death (21). The observation that the thymidine-reversible action of 5-fluorouracil appears to continue long after a one-hour exposure is consistent with this postulate (Chart 3, squares). Second, the thymidine-reversible action may be explained if the presence of thymidine were to facilitate a mechanism for repairing the lethal damage effected during a one-hour exposure to 5-fluorouracil. This is perhaps unlikely since the presence of thymidine for two hours following treatment yields an effect the same as that when thymidine is present for a greater length of time (Chart 3, diamonds). Third, there is still the possibility that the thymidine-reversible mode of action is due to the incorporation of a derivative of 5-fluorouracil into a species of RNA and that a metabolite of thymidine may affect this process.

The action of 5-fluorouracil not reversible by thymidine is presumably due to the incorporation of 5-fluorouracil into an
aberrant form of RNA. The failure of uridine to markedly counteract such a mode of action suggests that such an incorporation is irreversible. This failure might also be due to differences between the rates of entry of uridine and 5-fluorouracil into the cell or to the distribution of uridine and 5-fluorouracil into separate intracellular pools.

The difference between the action of 5-fluorouracil on murine bone marrow and that on lymphoma cells (3, 4) has been compared with the difference between its action on logarithmic and on stationary phase cells (14). The finding that this latter differential action depends upon the absence of thymidine, together with the demonstration that serum may contain a dialyzable substance presumed to be thymidine, suggests that an important consideration in the clinical use of 5-fluorouracil must be the level of thymidine in a patient's serum.

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