Studies on Unbalanced Growth in Synchronized HeLa Cells

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SUMMARY

Unbalanced growth leading to a loss of colony-forming ability is induced by exposing mitotically synchronized HeLa cells to inhibitors of DNA synthesis. A sharply reduced cell survival (colony-forming ability) is observed following exposure of cells in the G1-S transition phase to 1-fluoroarabinosylcytosine or hydroxyurea for a period longer than 6–8 hr, a period corresponding to about the duration of the DNA synthetic phase (S) in HeLa cells. A decrease in cell survival occurs when the cells in mitosis are exposed to the inhibitors of DNA synthesis for a period longer than 16 hr, a period corresponding to about the duration of the G1 and S phases. Thus, it is concluded that the minimum time period needed for the induction of unbalanced growth leading to a loss of cell viability following exposure of HeLa cells to an inhibitor of DNA synthesis would be equal to the duration of DNA synthesis.

The use of cycloheximide, an inhibitor of protein synthesis, reverses the lethal effect of the inhibition of DNA synthesis. This finding indicates that the synthesis of protein may be one of the requisite steps in the induction of the unbalanced growth syndrome.

INTRODUCTION

Cohen and Barner (2) described a type of unbalanced growth in a “thymineless” state in bacterial systems which appeared to be correlated with a substantial decrease in cell viability. This phenomenon, “unbalanced growth,” has been further investigated in mammalian cells using several specific inhibitors of DNA synthesis (1, 3–5, 9–11, 13, 17, 19). Such studies suggested that exposure of mammalian cells in culture to inhibitors of DNA synthesis resulted in an “unbalanced growth” syndrome analogous to the “thymineless” system of bacterial cells. The syndrome includes the sharply reduced cell viability following exposure of HeLa cells to l-fluoroarabinosylcytosine (ara-C), excess thymidine, or hydroxyurea (9–11). This communication describes further analysis of unbalanced growth of cells leading to a loss of ability to form colonies in a synchronized culture treated with inhibitors of DNA synthesis. Studies were also carried out on the partial reversal of the lethal effect of ara-C by cycloheximide, a potent inhibitor of protein synthesis.

MATERIALS AND METHODS

Experiments were carried out with HeLa cells of the S-3 subline grown in Eagle’s medium supplemented with 10% calf serum. Culture conditions, scoring cell survival by colony formation, maintenance, and media composition have been described elsewhere (9, 10).

Synchronous cultures were initiated by plating mitotic cells which had been selectively removed by controlled shaking from a monolayer of cells in calcium deficient medium (12, 19). Labeling procedure, autoradiography, and determination of nucleic acids and protein have been described in detail elsewhere (11).

ara-C or hydroxyurea was added directly to the medium from a X 50 stock solution to yield the desired final concentration. The drug was removed by aspirating the old medium, and plates were rinsed twice with medium before fresh prewarmed medium was added. Control plates were treated in a similar manner. The plates were incubated in a humidified chamber at 37°C in an atmosphere of carbon dioxide to maintain the pH of the culture medium at 7.4.

Cell-plating efficiencies (defined here as the ratio of total colonies to the number of single cells initially inoculated per plate) were generally 0.5–0.6 for the mitotically synchronized HeLa S-3 cells.

RESULTS

Effect of ara-C or Hydroxyurea on Asynchronously Growing Cell Populations as a Function of Time

To determine details of the kinetics of cell killing by the inhibitors of DNA synthesis, an asynchronously growing population was exposed to ara-C or hydroxyurea for varying times. The monolayers were then suspended by standard trypsinization procedures and replated in regular culture medium. Chart 1 shows the percent survival as a function of time. There was 10–15% reduction in the survival at 6 hr after exposure to the

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drugs. It can be seen from Chart 3 that the shape of the survival curves exhibits a shoulder region followed by an exponential reduction, except the 10-hr cells treated by hydroxyurea which will be discussed later. Exposure of cells in the G1-S transition phase (i.e., 8-hr cells) to ara-C or hydroxyurea produced a sharply reduced cell survival after a period longer than 6–8 hr, a period corresponding to about the duration of the S phase in HeLa cells. When the cells in mitosis (i.e., 0-hr cells) were exposed to the inhibitors, a loss of cell viability occurred for a period longer than 16 hr, a period corresponding to about the duration of the G1 and the S phases. Cells in the synthetic (S) phase (i.e., 12.5-hr cells) require a longer exposure to the ara-C to lose their viability than those in the G1-S phase. This period appears to correspond to the duration of the complete S phase plus the time these cells spent in the S phase at the time of drug addition.

Chart 1. The loss of colony-forming ability following exposure of asynchronously growing HeLa cells to 1-β-D-arabinofuranosylcytosine (ara-C) or hydroxyurea. Single-cell suspensions were incubated for 12 days and macroscopic colonies were counted. All points represent the average of two experiments performed in 6 replicate plates. The plating efficiency was 65%. •, ara-C (0.5 μg/ml); Δ, hydroxyurea (10⁻³M).

inhibitor and this level was maintained for the following 10 hr. It then decreased exponentially to about 40% after 24-hr exposure. The concentration of the inhibitors employed in this and subsequent experiments has been previously shown to effect an immediate and substantial reduction of DNA synthesis in HeLa S-3 cells under our culture conditions (9, 10).

**Cell Survival in a Synchronously Growing Population**

The observation that 10–15% of the asynchronously growing population lost their reproductive capacity following exposure to ara-C or hydroxyurea from 6 to 16 hr suggested that the effect of exposure to the inhibitors may depend on the phase of a cell in its division cycle. Cells in the different phases of the cycle were exposed to ara-C or hydroxyurea for 15-hr periods. The percentage of surviving cells was then determined as described above and plotted in Chart 2 against the age of the cell in the division cycle (upper curve). It is seen in Chart 2 that the survival starts to decrease sharply about 6 hr after mitosis. The maximal number of cells in DNA synthesis is observed at 12 hr after mitosis.

Chart 2 shows survival curves of the different phases of the mitotically synchronized cells as a function of time of exposure to ara-C or hydroxyurea. Survival curves for ara-C were taken at 0, 8, 10, and 12.5 hours after incubation of mitotic cells. For hydroxyurea, survival curves were taken at 0, 8, 10, and 20 hours after incubation of mitotic cells. Thus, 0-hour cells represent those in mitosis at the time of exposure to the inhibitor and this level was maintained for the following 10 hr. It then decreased exponentially to about 40% after 24-hr exposure. The concentration of the inhibitors employed in this and subsequent experiments has been previously shown to effect an immediate and substantial reduction of DNA synthesis in HeLa S-3 cells under our culture conditions (9, 10).

Chart 2. The % survival of HeLa cells following 15-hour exposure to ara-C or hydroxyurea during the division cycle. Mitotically synchronized cells were exposed to ara-C (0.5 μg/ml) or hydroxyurea (10⁻³M) for 15 hr at different times after mitosis and the % survival was determined. The lower curve shows the number of cells labeled with tritiated thymidine during the division cycle. ara-C, 1-β-D-arabinofuranosylcytosine.
Regarding the survival curve of the 10-hr cells after hydroxyurea treatment, we believe that the more rapid killing of these cells by hydroxyurea compared to that by ara-C treatment may be due to an additional cytotoxic effect of hydroxyurea on the cells in the S phase. The following findings indicate that the anomalous shape of the hydroxyurea survival curve may be due to an extraordinary sensitivity of S cells (a) About 70% of the population displayed a rapid killing by hydroxyurea, as indicated by the break in the exponential killing curve; at the same time, about 70% of the cells were engaged in the DNA synthesis, as measured by the labeling index with tritiated thymidine. (b) It has been previously shown that 10⁻² M hydroxyurea had an immediate and direct killing effect on cells in the S phase (Chart 4 of Ref. 10).

Table 1 shows the DNA, RNA, and protein contents following exposure of cells to ara-C for 16 hr. The results show that RNA and protein continued to be synthesized during the period DNA synthesis was inhibited by ara-C and that the ratio of RNA to DNA, or of protein to DNA of synchronized cells, may be the maximal biochemical unbalance with which cells can retain their reproductive capacity.

**Effect of Cycloheximide on the Unbalanced Growth Induced by an Inhibition of DNA Synthesis**

The involvement of macromolecular synthesis in the induction of unbalanced growth leading to cell death has been investigated in several bacterial systems using a specific metabolic inhibitor (7, 18). In this series of experiments, we have employed cycloheximide (21), a potent inhibitor of protein synthesis, to determine the role of protein synthesis in the unbalanced growth syndrome. Table 2 shows that 0.4 μg/ml of cycloheximide after one-hour treatment reduces the rate of protein synthesis by 60%. Chart 4 shows that the addition of cycloheximide 4 hours after exposure of cells to ara-C partially prevents the loss of cell viability induced by ara-C. A higher concentration of cycloheximide (1.0 μg/ml) is too toxic to carry out meaningful reversal studies on the ara-C-treated cells.

**DISCUSSION**

The present study shows that exposure of HeLa cells to ara-C or hydroxyurea induces unbalanced growth leading to a loss of cell viability (defined here as a colony-forming ability). Both ara-C and hydroxyurea have previously been shown to be effective inhibitors of DNA synthesis in HeLa cells (9, 10). Measurements of the DNA, RNA, and protein content in the presence of ara-C (Table 1) show a state of unbalanced growth analogous to that induced by 5-fluorodeoxyuridine, excess thymidine, or amethopterin (1, 4, 11, 17, 20).

Since DNA synthesis in mammalian cells is a discrete event occurring during the division cycle, one aspect of the effects of
Hours after cycloheximide treatment

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Effect of cycloheximide on the incorporation of valine-3H into the protein. Eighteen hours after plating $10^5$ trypanized randomly dividing cells, cycloheximide was added, and at times indicated following the drug addition, a 20-min pulse was given to cells, which were then processed and their radioactivity measured as outlined in Materials and Methods. Data are % of the control.

inhibition of DNA synthesis in asynchronous culture is to prevent a progression of cells into the DNA synthetic phase, thus changing the population distribution within the division cycle. Synchronous cultures permit an easier analysis of the effect of inhibition of DNA synthesis on cells in the different phases of the cycle. Thus, the minimum time periods needed for the induction of unbalanced growth leading to a loss of cell viability in relation to the normal time parameters of various phases of the division cycle have been determined: (a) Exposure of cells in the G1-S transition phase to the inhibitor of DNA synthesis sharply reduces cell survival after a period longer than 6–8 hr, a period corresponding to about the duration of DNA S phase in HeLa cells. (b) When the cells in mitosis (M) are exposed to the inhibitor of DNA synthesis, a loss of cell viability occurs after a period longer than 16 hr, a period corresponding to about the duration of the G1 plus the S phase. Therefore, it can be assumed from the above data that the minimum time to induce a loss of cell viability would be equal to the duration of DNA synthesis in HeLa cells. Cells in either M or G2 would not be affected until they reach the boundary between G1 and S. Cells in the S phase require a longer exposure to be inactivated than those in the G1-S phase (Chart 3).

Chemical measurements of the DNA, RNA, and protein contents following exposure of cells to ara-C may indicate that the maximal macromolecular unbalance which cells can tolerate without losing their reproductive integrity would be the ratio of RNA and protein to DNA of synchronized cells treated by ara-C in Table 1. Recent studies by Lambert and Studzinski (13), however, showed that cells could accumulate unusually large amounts of RNA and protein without losing their capacity of cell division following exposure of HeLa cells to high concentrations of thymidine. This effect is contrasted to that achieved by other inhibitors of DNA synthesis (1, 9, 10). The much longer periods of reversible unbalanced growth seen after exposure of cells to excess thymidine, compared to other inhibitors of DNA synthesis, may be due to less effective inhibition of DNA synthesis or due to fewer side effects of thymidine nucleoside.

Little can be said at the moment about the mechanism by which unbalanced growth in mammalian cells leads to a loss of reproductive capacity. Several hypotheses have been advanced to explain "thymineless death" in bacterial system. Some of them are: (a) an accumulation of some substance(s) that can cause death after reaching a critical concentration; (b) chemical and physical changes in the essential component, such as nuclear damage (7), single-stranded nucleolytic scissions in DNA (15, 16), and loss of susceptibility to methylation (6, 15); and (c) induction of prophage or colicin (8, 14). As yet, there has been no experiment to show whether the observed
physical or chemical changes are the cause of cell death or merely expressive of thymineless death. Freifelder and Maaloe (6) have shown an energy requirement for all stages of thymineless death in E. coli. More recently, Rolfe (18) has shown that thymineless death involves two distinct steps, only the first of which can be blocked by actinomycin D. Our studies on the mechanism of unbalanced growth leading to cell death indicate that the synthesis of protein is a necessary step, since the inhibition of protein synthesis by cycloheximidine can partially prevent cell death in HeLa cells (Chart 4). Studies on a requirement for RNA synthesis in the unbalanced growth syndrome are presently hampered by lack of any suitable inhibitor of RNA synthesis, since actinomycin D in HeLa cells is toxic even at the level of 0.005 μg/ml.

It is hoped that further experimentation with suitable metabolic inhibitors in synchronized systems would produce a better understanding of unbalanced growth leading to cell death.

REFERENCES


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