Phase Specificity of 5-Azacytidine against Mammalian Cells in Tissue Culture

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SUMMARY

5-Azacytidine (1 to 5 µg/ml) significantly inhibited mitosis of L1210 cells in culture only after 2 hr of incubation. The inhibition of mitosis was correlated to the inhibition of DNA synthesis.

With asynchronous DON and L1210 cells, the cell-kill (inhibition of proliferative capacity) reached a saturation value at high doses of the drug. Any further increase in drug concentration did not result in a corresponding increase in percentage of cell-kill. This indicated that 5-azacytidine kills cells in certain phases of the cell cycle. With synchronous DON cells, it was shown that the drug was lethal predominantly in the S phase.

5-Azacytidine caused considerable chromosome damage when L1210 cells in culture were exposed to the drug at 5 µg/ml for 2 hr at 37°C.

INTRODUCTION

Some antitumor agents have selective biological and/or biochemical effects (1, 8, 9, 11–13, 16, 21) at certain phases of the cell cycle. Cytosar [cytarabine, (The Upjohn Co., Kalamazoo, Mich.) cytosine arabinoside, ara-C] and hydroxyurea inhibit cells primarily in the S phase, while vinblastine is effective only in the mitotic stage. Several agents, such as γ-radiation and nitrogen mustard, lack this phase specificity and kill cells in all phases of the cell cycle. Such information, for instance, was used by Skipper et al. (18) in designing effective dosage schedules for ara-C.

Since 5-azaCR2 inhibited DNA synthesis more than RNA synthesis (10), the action of the drug on asynchronous L1210 cells and asynchronous and synchronous DON cells was studied to determine whether the drug specifically inhibited a certain phase of the cell cycle. The effect of the drug on the chromosomal integrity and the inhibition of mitosis of L1210 cells is also reported.

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2The abbreviations used are: 5-azaCR, 5-azacytidine; TdR, thymidine; MI, mitotic index.

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inhibited to a greater extent than RNA synthesis at all doses. This is shown in Chart 1 and indicates that DNA synthesis was inhibited more than RNA synthesis.

**RESULTS**

**Determination of Percentage of Survivors after Exposure of L1210 Cells to 5-AzaCR.** L1210 cells were exposed to 5-azaCR in a series of concentrations for varying periods. The cells were centrifuged and resuspended in fresh medium, and 10^6 cells were injected i.p. into female BDF1 mice (10 to 20 mice/group). The control (not drug-treated) cells were injected into mice at concentrations ranging from 10^3 to 10^5 cells. The median day of death was determined according to protocols established by Chemotherapy, National Cancer Institute (4). The percentage of cell-kill was determined by comparing the life-span (median day of death) of animals given injections of 5-azaCR-treated cells to a standard curve showing the relationship between injected untreated cells and the life-span of the corresponding animals. The BDF1 [(C57BL/6 × DBA/2) F1] mice (20 g ±2) were obtained from Jackson Memorial Laboratory, Bar Harbor, Maine.

The percentage of survival of drug-exposed cells was also determined by the cloning methods of Himmelfarb et al. (7).

**Determination of Phase Specificity of 5-AzaCR in Both Asynchronous and Synchronous DON Cells.** A synchronous culture of DON cells was prepared from mitotic cells harvested by the procedure described by Stubblefield et al. (19). Cells were grown in 32-oz. prescription bottles and mitotic cells were accumulated by exposure to 0.06 µg/ml Colcemid for 3 hr. Mitotic cells were harvested by shaking the bottles for 1 min (reciprocating shaker, 88 strokes/min, 2 inch/stroke) with 40 ml of cold 0.125 mg/ml trypsin, and the mitotic cells were accumulated by centrifugation. About 10^6 cells were planted in 3-oz. bottles (Duraglas, Owens-Illinois Co., Toledo, Ohio) in 10 ml of fresh medium and briefly gassed with a 5% CO2-95% air mixture. The cells were then exposed to 5-azaCR for 2 hr at different times after planting to expose cells in different parts of the cell cycle.

For experiments with asynchronous cells, 10^6 cells in logarithmic growth were planted in 3-oz. prescription bottles. After a 24-hr incubation, the monolayer cultures were exposed to different concentrations of 5-azaCR for 3 or 5 hr. After exposure to 5-azaCR, the medium was poured off, and the cells were detached with trypsin, centrifuged, and resuspended in fresh medium. The cells were diluted in warm medium, and about 40 cells were plated in plastic Petri plates (Linbro Chemical Co., New Haven, Conn.) and incubated in an atmosphere of 8% CO2-92% air for 7 days (37°). Then the medium was removed by suction, and the colonies were stained with 0.2% methylene blue in 70% ethanol and counted with a Quebec Colony counter (Spencer Lens Co., Buffalo, N. Y.). The plating efficiencies were about 40 to 50% for synchronous cells and 60 to 80% for asynchronous cells.

**RESULTS**

**Effect of 5-AzaCR on Macromolecule Synthesis.** The effect of 5-azaCR on polynucleotide synthesis in intact L1210 cells is shown in Chart 1 and indicates that DNA synthesis was inhibited to a greater extent than RNA synthesis at all doses.
Chart 2. Inhibition of polynucleotide synthesis of DON cells in culture by 5-azaCR. Cells (circa 2 X 10^6 cells/8-oz. prescription bottle) were incubated with 5-azaCR and labeled metabolite (Tdr-3H or Ur-3H, 6.4 μCi/4.6 μg/ml of reaction system) at 37° for 2 to 8 hr. After incubation, the radioactive medium was poured off, and cells (attached to the glass) were washed with fresh medium containing a high concentration (10 mg/ml) of either nonlabeled TdR or uridine. After a quick rinse with 2 ml of trypsin (1 mg/ml), cells were detached from the glass by incubation with 0.5 ml of trypsin (1 mg/ml) for circa 5 min and then suspended in 10 ml of fresh medium. Acid-insoluble fractions were obtained by extracting the washed cell pellet twice with 1 ml of 0.5 N perchloric acid (70°C) and used for the determination of radioactivity and nucleic acid content.

Firmed when synchronously growing DON cells were exposed to 5-azaCR (Chart 5). The results clearly indicate that the percentage of cell-kill is minimal during M and G1 phases and increases as the cells progress into S phase.

**DISCUSSION**

Fučík et al. (5) reported that 5-azaCR caused chromosomal mutation in the root meristem of *Vicia faba* and assumed the mutagenic activity of this compound was associated with its incorporation into DNA. The drug inhibited both DNA and RNA synthesis, was incorporated into the polynucleotides in L1210 cells (10), and caused sticky chromosomes, achromatic gaps, and chromosome fragmentation in these cells (Fig. 1).

Our results (Chart 1) showed that the inhibition of DNA synthesis (but not of RNA synthesis) parallels the inhibition of mitosis of L1210 cells; RNA synthesis is much less...
days incubation, the colonies were stained and counted. The percentage of surviving colonies in drug-treated samples to those in control samples. The cloning efficiency in the control (no drug) samples was about 20% of the mean value.

inhibited by this agent. Since the length of G1, S, G2, and M phases of L1210 cells in culture are, respectively, 1.2, 8.2, 1.2, and 0.6 hr (B. K. Bhuyan, unpublished data), these results indicated that cells previously in late S phase (2 hr prior to M phase) are blocked from proceeding to mitosis.

Bruce et al. (2, 3) have shown that with phase-specific agents, once the inhibitory concentration is reached, the percentage of cell-kill reached a constant saturation value for that period of exposure. With a longer period of exposure, more cells enter the sensitive phase and are killed, resulting in a higher saturation value for the percentage of cell-kill. Such data were obtained with presumably phase-specific agents such as Cytosar by Karon and Shirakawa (9) and with vinblastine, TdR-3H, and hydroxyurea by Bruce et al. (2, 3). With phase-nonspecific agents, however, an exponential cell-kill pattern is seen, and a saturation value for the percentage of cell-kill will not be obtained, as observed by Bruce et al. (2) with γ-radiation and nitrogen mustard.

Chart 3 illustrates the relationship between 5-azaCR dose and percentage of cell survival of asynchronous L1210 and DON cells, respectively. A saturation value of the percentage of cell-kill was obtained in both cell lines studied. In L1210 cells, a plateau of cell-kill was seen in both cloning and animal experiments when 5 μg/ml or higher concentrations of 5-azaCR were used. It must be realized, however, that results from the animal experiments are based on the assumptions that the drug-induced increase in host life-span results from the death of a high percentage of the L1210 cells and not from an increase in either the generation time or the lag phase of drug-treated L1210 cells. These possibilities have been appropriately discussed by Skipper et al. (17). In DON cells, the percentage of cell-kill becomes constant (about 90%) when the cells are incubated with 30 μg/ml 5-azaCR or more for 3 to 5 hr (37°). These results are in agreement with the previous findings, which suggest 5-azaCR acts more or less as a phase-specific agent, killing cells predominantly in a certain phase of the cell cycles of both cell lines studied.

The percentage of cell-kill of a synchronous DON cell population is minimal during M and G1 phases and increases as the cells progress into S phase. In the synchronous culture, there was a burst of mitosis about 8 hr after the mitotic cells were planted. When the synchronous culture was pulse-labeled with TdR-3H the percentage of labeled cells was 0, 72, 85, 90, 62, and 44% at 1, 2, 3, 4, 6, 8, and 10 hr, respectively, after planting mitotic cells. This indicates that, while synchrony was maintained during G1 and early S phases, the cells in G2 phase are contaminated with a large percentage of S phase cells. Therefore, although it seems that percentage of cell survival decreases during G2 phase, it cannot be so stated. It is fairly obvious that 5-azaCR acts as an S phase-specific agent against DON cells, presumably through its greater inhibition of DNA synthesis (Chart 2). The shape of these curves (Chart 5) is similar to those obtained for Cytosar, a known S phase-specific agent (B. K. Bhuyan, unpublished data).

Work is in progress to determine the cell-cycle specificity of 5-azaCR upon a synchronous culture of L1210 cells. However, by carefully comparing the results of L1210 cells (Charts 1 and 3) with those obtained from DON cells (Charts 2, 4, and 5), it is reasonable to assume that 5-azaCR acts principally as an S phase-specific agent against leukemic L1210 cells in culture. Although this antimetabolite inhibits both DNA and RNA synthesis in leukemia L1210 systems, presumably via its incorporation into the respective molecules (10), the marked effect on DNA molecules...
appears to be one of the primary determinants of cytotoxicity.

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REFERENCES


Fig. 1. Chromosome aberrations in 5-azaCR-treated cells. L1210 cells were exposed to 5 μg/ml 5-azaCR for 2 to 3 hr. The cells were centrifuged and resuspended in warm medium containing Colcemid for 1 hr, and then slides were prepared. (A) Control (no drug) cells; (B) note achromatic gaps; (C) and (D) sticky and elongated chromosomes; and (E) fragmented chromosomes.
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