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In Vitro Biochemical and Cytotoxicity Studies with 1-β-D-Arabinofuranosylcytosine and 5-Azacytidine in Combination[1]

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SUMMARY

The effect of 1-β-D-arabinofuranosylcytosine (ara-C) and 5-azacytidine (5-aza-C), alone and in combination, on DNA synthesis and cytotoxicity in hamster fibrosarcoma cells has been studied. After a 2-hr exposure of S-phase cells to ara-C at concentrations of 2 to 200 μM, the cells required about 4 to 6 hr to recover from inhibition of DNA synthesis. When 2 exposures to ara-C were used, maximal cytotoxicity occurred when the 2nd dose of ara-C was administered at the time when the cells recovered from the inhibition of DNA synthesis. When the S-phase cells were exposed to ara-C, the maximal killing effect of 5-aza-C occurred when this agent was administered 6 hr later, at the time when the cells had recovered from the inhibition of DNA synthesis. When S-phase cells were exposed to 5-aza-C, the maximal cell kill produced by ara-C also occurred 5 to 6 hr later. When the S-phase cells were exposed simultaneously to both ara-C and 5-aza-C, significant antagonism with respect to cytotoxicity was observed between these 2 agents. When cells in G_1 were exposed to 5-aza-C, the cytotoxicity produced by ara-C on these cells when they entered S phase was additive with respect to the cytotoxicity produced by 5-aza-C exposure alone.

INTRODUCTION

5-aza-C,[3] a nucleoside analog, is a new active agent for the treatment of acute myelogenous leukemia (8, 15). *In vitro* studies have shown that 5-aza-C is an agent very potently cytotoxic to mammalian cells (14). Although 5-aza-C is active through the cell cycle, this analog exerts its greatest cytotoxic action on S-phase cells (13). The biochemical mode of action of 5-aza-C is complex; this nucleoside analog produces some inhibition of pyrimidine biosynthesis (24), protein, RNA, and DNA syntheses (12, 21). 5-aza-C also produces breakdown of polyribosomes (11) and blocks the induction of certain enzymes by steroids (4).

The biochemical mode of action of ara-C, a nucleoside analog cytotoxic to S-phase cells (7, 27) which is active against acute myelogenous leukemia (5), is intimately involved with DNA replication (17). It is of interest to study the antileukemic activity of the combination of 5-aza-C and ara-C, 2 antimetabolites that have different modes of action. In a preliminary study Neil *et al.* (19) have shown that the time interval between administration of ara-C and 5-aza-C to leukemic mice markedly influenced the antileukemic activity of this drug combination. In this report we studied the cytotoxic activity of this drug combination *in vitro* against synchronized cells in order to understand more fully the interaction between these 2 drugs.

MATERIALS AND METHODS

Cell System and Mitotic Harvest. Transformed hamster fibroblasts cells, the $A(T_1)Cl$-3 line generously donated and previously described by Benedict (2), were maintained in suspension culture in minimum essential medium (F_{14}) or McCoy's spinner medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum (Flow Laboratories, Rockville, Md.). The cells have a doubling time of between 9 and 11 hr. On the day before harvesting, the cells were seeded in plastic 250-ml tissue culture flasks at about 6×10^6 cells/flask. The cells were allowed 90 min to adhere to the bottom of the flask at 37°. The medium was then replaced with McCoy's Medium 5A (Flow Laboratories); only McCoy's was used in subsequent procedures. Synchronized cells were harvested by the technique of mitotic detachment (22). Two preliminary shakes at 10-min intervals were followed by 2 shakes at 30-min intervals. Only cells from the last shake were used. Yields were between 20,000 and 30,000 cells/ml in a volume of 10 ml/flask. The yield of mitotic cells was greater than 80%. Radioautographic analyses showed that the [^{3}H]thymidine labeling indexes of the G_1 and S-phase cells were 10 and 80%, respectively.

Drugs and Drug Exposure. ara-C and 5-aza-C were obtained from the National Cancer Institute. The latter drug was always dissolved in water or media immediately prior to use. Cells in G_1 were exposed to drug the 1st 2 hr after mitotic harvest, early-S-phase cells were exposed 3 to 5 hr after harvest, and mid-S-phase cells were exposed 5 to 7 hr after harvest. Dishes and plates were always rinsed once with drug-free medium following drug exposure. Medium during drug exposure, including controls, con-

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[3] The abbreviations used are: 5-aza-C, 5-azacytidine; ara-C, 1-β-D-arabinofuranosylcytosine.

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tained 5% fetal calf serum; otherwise the medium contained 10% serum.

[³H]Thymidine Incorporation Assay. Upon mitotic harvest, cells were seeded on duplicate 35-mm plastic dishes in a volume of 2 ml and placed in an air-CO_2 incubator. At the time of assay, 2.0 ml of fresh medium containing 10% normal or dialyzed serum were added and 1 μCi of [³H]thymidine (New England Nuclear, Boston, Mass.), 6.7 or 20 Ci/mmole, was added to each dish. After a 30-min pulse, the radioactivity-containing medium was aspirated. The cells were treated with trypsin-EDTA (Grand Island Biological) for 10 min and the suspension was poured onto 2.4-cm Whatman GF/C glass fiber filters. Filters were rinsed with 0.9% NaCl solution, 5% cold trichloroacetic acid, and absolute ethanol. They were dried at 80° for 1 hr, placed in vials containing scintillation fluid, and counted in a Packard Tri-Carb liquid scintillation spectrophotometer. Each graph point represents the mean of duplicate samples; duplicates agreed within 15%.

Chemotherapy Assay. Mitotic cells were diluted to 25 cells/ml and seeded onto plastic 60-mm plastic dishes at 5 ml/dish. Four dishes were used for each point. Cells were exposed to drug as indicated in the charts and placed in the air-CO_2 incubator for 5 days. The plates were then rinsed rapidly with 0.9% NaCl solution, fixed with absolute methanol, and stained with Giemsa. The colonies were counted electronically with an automatic colony counter (New Brunswick Scientific Co. Inc., New Brunswick, N. J.). The plating efficiency was about 50%. Each experiment was repeated at least once and gave comparable results.

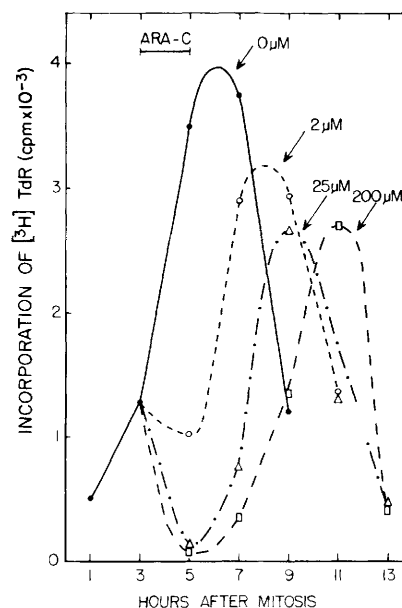

Chart 1. Time required by S-phase hamster fibrosarcoma cells to recover from inhibition of DNA synthesis after exposure to different concentrations of ara-C. Mitotic cells were harvested by mechanical detachment from monolayers growing in McCoy's Medium 5A containing 10% dialyzed serum. About 3 × 10⁴ mitotic cells were placed in plastic Petri dishes and exposed to the indicated concentrations of ara-C from 3rd to 5th hr after mitosis. The cells were washed with fresh medium and then incubated in medium containing 10 μM deoxycytidine. At the times indicated the cells were pulsed with 1 μCi of [³H]thymidine (20 Ci/mmole) for 30 min, and the amount of radioactivity incorporated into the acid-insoluble fraction was measured as described under "Materials and Methods."

RESULTS

The effect of different concentrations of ara-C on the time required for S-phase fibrosarcoma cells to recover maximally from inhibition of DNA synthesis is shown in Chart 1. Depending on the concentration of ara-C the cells required from 4 to 6 hr to recover from the inhibition of DNA synthesis. The addition of deoxycytidine (10 μM) to the medium after exposure of cells to ara-C did not increase the rate of recovery of DNA synthesis (R. L. Momparler and J. Goodman, unpublished observation).

Chart 2 shows the cytotoxic effect of 2-hr exposures to 200 μM ara-C administered to cells at different times before, during, and after a 2-hr exposure to 5 μM ara-C in early S phase. When the cells received no 5 μM ara-C exposure, the maximal cytotoxicity produced by 200 μM exposure to ara-C occurred in late S phase. When the S-phase cells were exposed to 5 μM ara-C from 3 to 5 hr, maximal cytotoxicity induced by the 200 μM exposure to ara-C occurred when this dose was administered at the time when the cells had recovered from the inhibition of DNA synthesis (7 to 11 hr).

The cytotoxic effects of the combination of ara-C and 5-aza-C on S-phase fibrosarcoma cells are shown in Chart 3. In cells not exposed to ara-C, 5-aza-C produced maximal cytotoxicity when administered in mid-S phase. When the cells were exposed to 2 μM ara-C from 3 to 5 hr, 5-aza-C produced maximal cytotoxicity when administered at the time the cells had recovered from inhibition of DNA synthesis (7 to 11 hr). During the interval when DNA synthesis was markedly inhibited by ara-C, the cytotoxic action of 5-aza-C was reduced compared to cultures not exposed to ara-C.

Chart 4 shows the effect of 5-aza-C treatment of S-phase cells on cytotoxicity induced by ara-C at different time intervals before, during, and after exposure to 5-aza-C. When the interval between 5-aza-C and ara-C was short, the cell kill was less than the summation of the kill produced by each agent separately. However, when the interval between 5-aza-C and ara-C was greater than 5 hr, there was enhancement of the ara-C-induced cytotoxicity. ara-C produced its maximal cytotoxic action when the 5-aza-C-exposed cells were exposed to ara-C in late S phase.

The effect of ara-C on S-phase cells that were previously exposed to 5-aza-C during G_1 phase is shown in Chart 5. Exposure to G_1 cells to 10 μM 5-aza-C from 3 to 5 hr produced a slight reduction in DNA synthesis of these cells when they entered S phase. The cytotoxicity induced by ara-C in these S-phase cells previously exposed to 5-aza-C was additive with respect to the cytotoxicity produced by 5-aza-C alone.

DISCUSSION

Since the nucleoside analogs 5-aza-C and ara-C each have unique activity for the treatment of acute myelogenous leukemia (5, 8, 15), it is of interest to study the chemothera-

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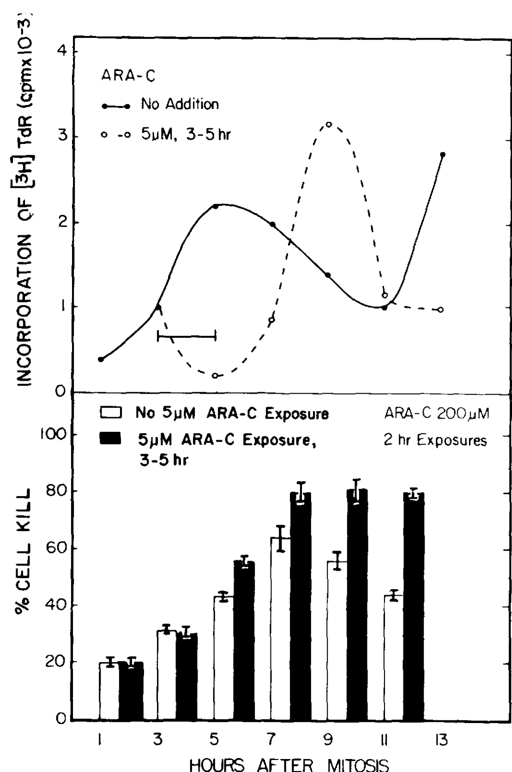

Chart 2. Effect of low-dose ara-C exposure of S-phase hamster fibrosarcoma cells on DNA synthesis and cytotoxicity induced by a 2nd high dose of ara-C. Mitotic cells were harvested by mechanical detachment from monolayers growing in McCoy's Medium 5A containing 10% fetal calf serum. For DNA synthesis and ara-C cytotoxicity measurements, 3 × 10^4 and 250 cells, respectively, were plated onto plastic Petri dishes. *Horizontal bar*, cells exposed to 5 μM ara-C from 3rd to 5th hr after mitotic harvest. *Upper*, cells pulsed with 1 μCi [^{3}H]thymidine (20 Ci/mmole) for 30 min at the times indicated and the amount of radioactivity incorporated into the acid-insoluble fraction determined as described under "Materials and Methods." *Lower*, cells exposed once to 200 μM ara-C for 2 hr at the times indicated. Cells then placed in fresh medium containing 5 μM deoxycytidine and number of colonies counted 5 days later as described under "Materials and Methods." There was no detectable kill observed with cells treated with only 5 μM ara-C from 3rd to 5th hr. The cytotoxicity data are expressed as mean ± S.D.

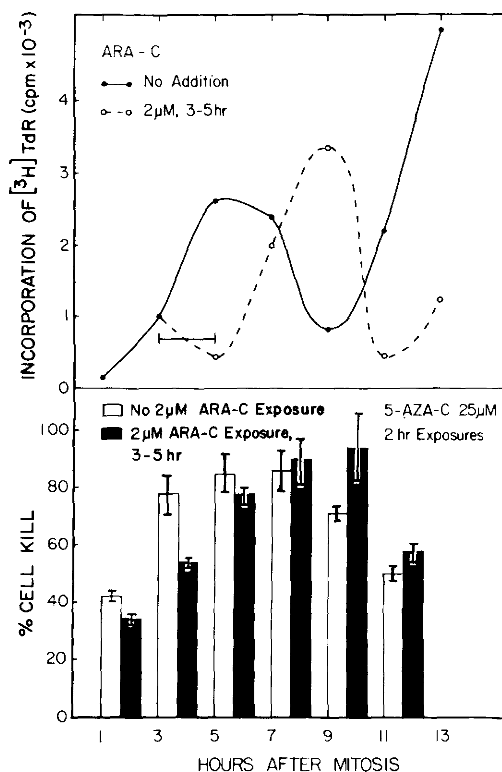

Chart 3. Effect of low-dose ara-C exposure of S-phase fibrosarcoma cells on DNA synthesis and on 5-aza-C-induced cytotoxicity. The experimental conditions were the same as described in Chart 2 except that in *lower* section the cells were exposed once to 25 μM 5-aza-C for 2 hr at the times indicated and then placed in fresh medium. There was 5% kill observed with cells exposed to only 2 μM ara-C from 3rd to 5th hr. The cytotoxicity data are expressed as mean ± S.D.

peutic potential of these 2 drugs when used in combination for the treatment of this disease. The use of this drug combination is also of theoretical interest because the phosphorylation of these nucleoside analogs to active agents is catalyzed by different enzymes, uridine-cytidine kinase (10) and deoxycytidine kinase (18). Some ara-C-resistant cells have been shown to have a decrease in levels of deoxycytidine kinase (9), whereas some 5-aza-C-resistant cells have been demonstrated to have a decreased uridine kinase activity (25, 26). Theoretically, drug-resistant leukemic cells that lack either one of these enzymes should still be sensitive to the cytotoxic action of the 5-aza-C and ara-C combination.

Prior to studying in detail the schedule of a drug combination, it is important to determine the optimal schedule for each agent when used alone. Recently, Bhuyan *et al.* (3) and Neil and Homan (20) have published interesting data on the importance of the schedule of ara-C in the therapy of L1210 leukemia. Using *in vitro* and *in vivo* studies, these authors demonstrated that the chemotherapeutic effectiveness against L1210 cells of 2 separate doses of ara-C was greatest when this analog was administered about 8 hr apart, the time required for the cell population to recover from inhibition of DNA synthesis after the 1st dose of ara-C. The interpretation of these results is difficult because part of the enhanced cytotoxic effects produced by the 2nd dose of ara-C may have been due to the progression of G_1 cells into S phase during the interval between the ara-C doses. In order to overcome this problem on the interpretation of drug effects on an asynchronous cell population where cell cycle progression is an important factor, we have studied the effects of ara-C on synchronized S-phase cells. After exposure of S cells to low and high concentrations of ara-C, the cells recover from inhibition of DNA synthesis in 4 to 6 hr (Chart 1). These data are useful in determining the time that should be used between 2 doses of ara-C. When the S-phase cells were exposed to 2 doses of ara-C, maximal cytotoxicity occurred when the 2nd dose was administered at the time when the cells had recovered from the inhibition of DNA synthesis. Perhaps the optimal scheduling of 2 doses of ara-C brings about greater cell kill by producing damage at multiple chromosomal loci (17).

In an analysis of the cytotoxic activity of the drug combination ara-C and 5-aza-C, 2 questions can be asked.

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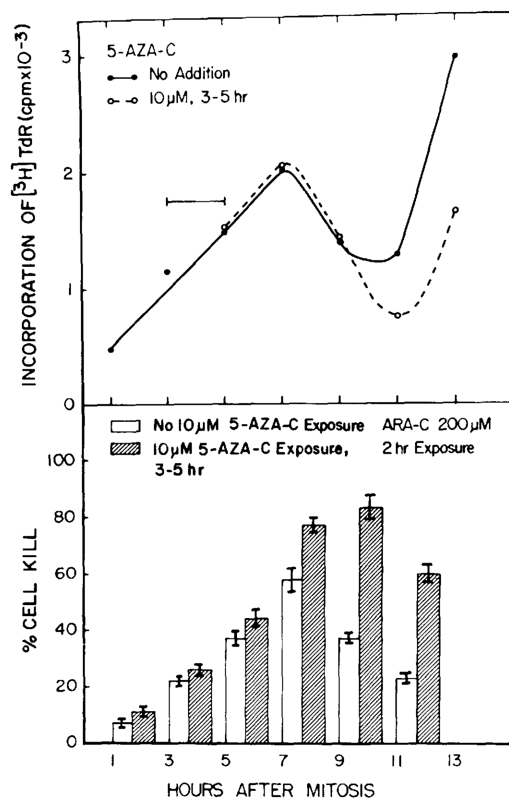

Chart 4. Effect of 5-aza-C exposure of S-phase hamster fibrosarcoma cells on DNA synthesis and ara-C-induced cytotoxicity. The experimental conditions were the same as described in Chart 2 except that the S-phase cells were exposed to 10 μM 5-aza-C from 3rd to 5th hr after mitosis. *Upper*, 3.5 × 10^4 cells in medium containing 10% dialyzed serum cells pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mmole) for 30 min. *Lower*, cells exposed once to 200 μM ara-C for 2 hr at the times indicated. The cells were placed in fresh medium containing 10 μM deoxycytidine. There was 26% kill with cells exposed to only 10 μM 5-aza-C from 3rd to 5th hr. The cytotoxicity data are expressed as mean ± S.D.

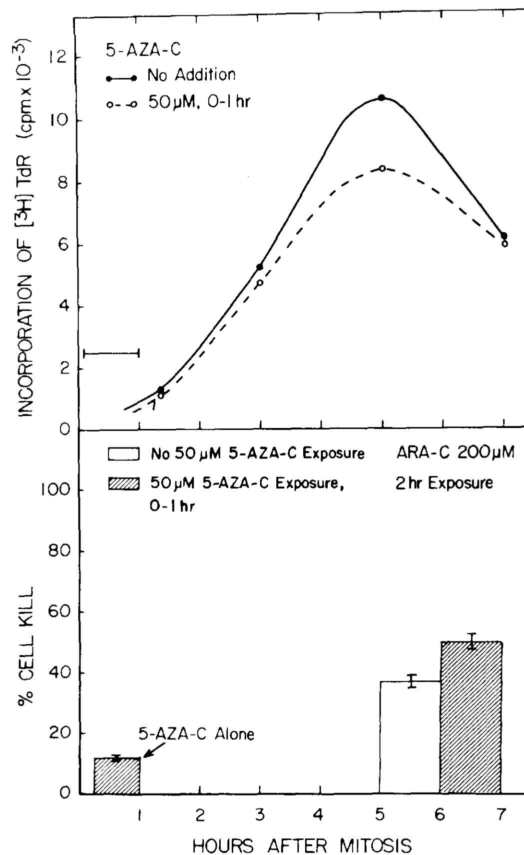

Chart 5. Effect of 5-aza-C exposure of G_1 hamster fibrosarcoma cells on DNA synthesis and ara-C-induced cytotoxocity. Mitotic cells were harvested by mechanical detachment from monolayers growing in McCoy's Medium 5A containing 10% dialyzed fetal calf serum. For measurement of DNA synthesis and ara-C-induced cytoxicity, 9 × 10^4 and 150 cells, respectively, were plated onto plastic Petri dishes. *Horizontal bar*, cells exposed to 50 μM 5-aza-C from 0 to 1st hr after mitotic harvest. *Upper*, cells pulsed for 30 min at times indicated with 1 μCi of [^{3}H]thymidine (20 Ci/mmole) and incorporation of radioactivity into acid-insoluble material measured as described under "Materials and Methods." *Lower*, cultures exposed once to 200 μM ara-C from 5th to 7th hr after mitotic harvest. The cells were placed in fresh medium containing 10 μM deoxycytidine, and 5 days later the number of colonies were counted as described under "Materials and Methods." There was 12% kill observed with G_1 cells exposed to only 50 μM of 5-aza-C from 0 to 1st hr after mitosis. The cytotoxicity data are expressed as mean ± S.D.

Does ara-C block the cytotoxic action of 5-aza-C and does 5-aza-C block the cytotoxic action of ara-C? In the case of ara-C, the possibility of drug antagonism occurring between this analog and 5-aza-C should be investigated carefully since several agents have been shown to antagonize the inhibitory action of ara-C (6, 16). In a preliminary report, Benedict *et al.* (1) found that when asynchronous fibrosarcoma cells were exposed to ara-C and 5-aza-C simultaneously *in vitro*, there was an antagonism with respect to the cytotoxic activity of these 2 analogs. In another preliminary report, Neil *et al.* (19) found that, when ara-C and 5-aza-C were administered simultaneously to mice with L1210 leukemia, significant antagonism was observed with respect to increase in survival time between these 2 agents. Since a study of the ara-C and 5-aza-C combination on asynchronous cells is difficult to analyze in depth, we have studied the effect of this drug combination on synchronous cells. The simultaneous exposure of S-phase-fibrosarcoma cells to ara-C and 5-aza-C also resulted in an antagonism of the cytotoxic activity between these 2 agents (Chart 3). The maximal cytotoxic action produced by 5-aza-C occurred when the cells were exposed to this analog at the time when the cells had recovered from the ara-C-induced inhibition of DNA synthesis. These studies suggest that the cytotoxic action of 5-aza-C is related to DNA synthesis. It is possible that the incorporation of 5-aza-C after conversion to its deoxynucleotide form into DNA may be responsible for the major cytotoxic effects produced by this analog or that the lethal action of 5-aza-C involves those events in RNA or protein synthesis directly associated with DNA replication.

Since low cytotoxic concentrations of 5-aza-C do not produce a rapid and profound inhibition of DNA synthesis (12, 21), one might expect *a priori* that pretreatment of S-phase cells with 5-aza-C would not antagonize the cytotoxic action of ara-C by blocking its incorporation into DNA. As expected, when the S-phase cells were pretreated with 5-aza-C, there was no significant antagonism of the cytotoxic action of ara-C (Chart 4). On the other hand,

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if 5-aza-C blocks the progression of G_1 cells into S phase, as reported by Tobey (23), one might expect *a priori* that treatment of asynchronous cells with 5-aza-C would block part of the cytotoxic action of ara-C by preventing some of the cells from entering S phase. However, under the conditions used in our experiments, 5-aza-C treatment of G_1 cells did not appear to block the cytotoxic action of ara-C when these cells were exposed to this analog during S phase (Chart 4). These results suggest that 5-aza-C, under the conditions used here, did not produce a significant block in the progression of G_1 cells into S phase. The reason for the difference between Tobey's and our data on the effect of 5-aza-C on cell cycle progression is not known and may be related to the different methods used to synchronize the cells.

The biochemical and cytotoxic interactions that occur between 2 chemotherapeutic agents *in vitro* can be very complex. The ara-C and 5-aza-C combination is currently being investigated further *in vivo* in experimental animals with leukemia.

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