

# Selective Destruction of Cultured Tumor Cells with Uncontrolled Nuclear Division by Cytochalasin B and Cytosine Arabinoside<sup>1</sup>

Frank J. O'Neill

*Departments of Microbiology and Pathology, University of Utah Medical Center, Salt Lake City, Utah 84112, and Veterans Administration Hospital, Department of Research, Salt Lake City, Utah 84113*

## SUMMARY

Cultures of normal human and hamster and malignant human and hamster cells respond to cytochalasin B (CB) differently. The neoplastic cells become highly multinucleated with continuous nuclear division, while cytoplasmic division is prevented. These cells exhibit uncontrolled nuclear division. The normal cells show control of nuclear division, since CB treatment results in only binucleation, although cytoplasmic division is prevented. The CB-treated normal cells also show reduced incorporation of [<sup>3</sup>H]thymidine. When rapidly growing normal or neoplastic cells of either species are treated with cytosine arabinoside (ara-C), all cells are killed within 10 to 20 days. If the normal cells are treated with ara-C in the presence of CB, the cells survive for at least 35 to 40 days, suggesting that CB can protect normal cells from the destructive effects of ara-C. However, if malignant cells are treated with ara-C in the presence of CB, all cells are destroyed in 25 days. Although CB affords some protection to tumor cells, it is relatively small and appears only to effect a delay in ultimate cell death. Treatment of any of the cell lines with CB alone results in some loss of viability, but the drug is reversible although less so for the malignant cells.

In additional experiments, ara-C- plus CB-treated normal and neoplastic cells were reversed from the drugs and propagated in normal medium at various periods after the initiation of ara-C treatment. ara-C- plus CB-treated normal cells could be freely reversed at any time after the start of ara-C treatment, up to at least 35 days. After reversal, cell growth resumed, and the total number of cells returned to that present prior to drug treatment. The tumor cells could not be freely reversed from ara-C plus CB, even when the ara-C was present for only 5 days. In this case the cells seemed to be destroyed at the same rate as in cultures where both drugs were kept in the medium. These results suggest that CB can protect normal but not malignant cells from the toxic effects of ara-C and offer a method to specifically destroy tumor cells.

## INTRODUCTION

CB<sup>2</sup> is a mold metabolite that prevents cytoplasmic cleavage when used at low concentrations (1 to 5 μg/ml) (2, 5). Previous studies have shown that CB-treated tumor cells and virus-transformed cells become highly multinucleated (9, 10, 14-18). It is suggested that CB-treated tumor cells and some virus-transformed cells show uncontrolled or unlimited nuclear division (15-17). In contrast, CB-treated normal cells show controlled or limited nuclear division, since nuclear division occurs usually just one (9, 10, 14-18). It has also been shown that DNA synthesis is markedly inhibited in CB-treated normal cells but continues in CB-treated transformed cells (9, 10, 15).

These observations suggested to us a method to specifically kill tumor cells without destroying normal cells. Since DNA synthesis and nuclear division are greatly reduced in CB-treated normal cells, the addition of a drug that is toxic to cells synthesizing DNA should not kill normal cells. Conversely, the addition of such a drug to CB-treated tumor cells should result in killing. This report shows that cytosine arabinoside destroys CB-treated tumor cells without adversely affecting CB-treated normal cells.

## MATERIALS AND METHODS

**Cell Cultures.** RD is a human rhabdomyosarcoma cell line which shows uncontrolled nuclear division in the presence of CB (14). HFF is a diploid cell strain of skin fibroblasts which shows normal control of nuclear division. BHK21/C13 is a line of syrian hamster cells obtained from the American Type Culture Collection which shows normal control of nuclear division (4, 14). BHK/IV<sup>3</sup> is a hamster cell line derived from a s.c. tumor produced by inoculation of a continuous BHK21 cell line.<sup>3</sup> All the hamster cells were propagated in basal Eagle's medium with 10% calf serum while the human cells were cultured in BME with 10% fetal bovine serum.

**Chemicals.** CB was purchased from Aldrich Chemical Co., Milwaukee, Wis., and dissolved in dimethyl sulfoxide at 2 mg/ml. Prior to use, it was added to culture medium

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<sup>2</sup>The abbreviations used are: CB, cytochalasin B; HFF, human fetal skin; ara-C, cytosine arabinoside.

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to make a final concentration of 1.5 to 2  $\mu\text{g/ml}$ . ara-C was purchased from Sigma Chemical Co., St. Louis, Mo., and dissolved in Tris buffer at 10 mg/ml. Prior to use, it was added to media at a final concentration of 10  $\mu\text{g/ml}$ .

**Cell Counting.** Thirty to 50 replicate cultures of each cell type were set up in 1-oz prescription bottles and allowed to reach partial confluency ( $0.5$  to  $2 \times 10^6$  cells/bottle). Following treatment with CB, CB + ara-C, or ara-C, replicate samples were harvested periodically (usually every 5 days), and the cells were counted in a hemocytometer. The cells were harvested by detaching with 0.25% trypsin-0.4% Versene. The cells were stained with trypan blue prior to counting.

**RESULTS**

**Effect of ara-C on CB-treated RD and HFF cells.** Thirty to 50 subconfluent replicate monolayers of RD or HFF cells ( $0.5$  to  $2 \times 10^6$  cells/bottle) were treated with CB (1.5  $\mu\text{g/ml}$ ) or ara-C (10  $\mu\text{g/ml}$ ) or CB + ara-C. This medium was changed twice weekly. With the normal human HFF cells, a smaller number of cells per culture bottle was used at the beginning of each experiment. This was done because normal human fibroblasts become confluent and contact inhibited at low concentration densities, resulting in resistance to ara-C. In cultures receiving both CB and ara-C, the ara-C was not added until 5 days after the beginning of CB treatment. This was done to ensure that there was sufficient time for the CB to effect inhibition of DNA synthesis. However, it now seems that the CB pretreatment period can be reduced to less than 5 days. Charts 1 and 2 show the results of the various treatment schedules. Following the addition of CB, CB + ara-C, or ara-C, growth of all cultures was halted since there was no increase in cell

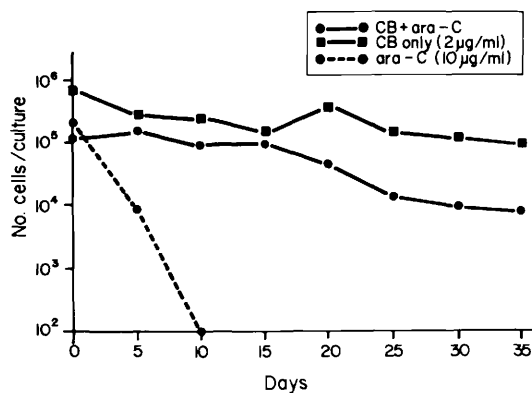


Chart 1. Survival curves of HFF in the presence of ara-C (10  $\mu\text{g/ml}$ ), CB (1.5 to 2  $\mu\text{g/ml}$ ) or ara-C + CB. Approximately 4 to  $8 \times 10^5$  HFF cells per replicate culture were used at the start of the experiments. In CB + ara-C experiments, the cells were CB treated 5 days prior to the addition of ara-C. Therefore, the curve begins at the time of ara-C introduction. Cell counts were made every 5 days; the cells were stained with trypan blue and counted in a hemocytometer. Since we could not count less than 100 cells accurately, the ordinate depicts only as few as 100 cells. However, the counts do go to zero in every case in which they go down to  $10^2$ , since no viable cells can be observed when entire culture surfaces are examined microscopically.

number. Treatment with ara-C alone caused a rapid detachment and killing of both the normal (HFF) and the neoplastic (RD) cells. Within 10 days all of the cells were destroyed. Treatment with CB alone resulted in a significant decrease in cell number, but this occurred over extended periods. The curves show that CB was effective in reducing the cell counts for both the RD and hamster tumor cells about 90%. When CB was used in combination with ara-C, there was again some decline in the cell counts for HFF cells, and this was not significantly greater than in cultures treated with CB alone (Chart 1). This suggests that CB serves to protect HFF cells from killing by ara-C. In contrast, when RD cells received both CB and ara-C, all replicate cultures were completely destroyed within 25 days (Chart 2). CB did, however, provide RD cells some protection against ara-C. In replicate cultures receiving no drug, the cell monolayers became confluent in a few days and this concentration density of cells was maintained. Table 1 shows the contraction densities reached and maintained by HFF, RD, BHK21/C13, and BHK/IV<sup>3</sup>.

In further experiments, replicate cultures receiving both drugs were periodically reversed to normal medium to determine the time required for ara-C to effect killing. This was done also to determine whether cells could grow after being treated with ara-C and CB. These results appear in Chart 3. HFF cells could be successfully reversed from the combined treatment even after 35 days, and the cells then grew rapidly as they did prior to drug treatment. In contrast, RD cells could not grow when reversed from the combined treatment as early as 5 days after the addition of ara-C (Chart 3). When these CB + ara-C-treated cultures were returned to normal medium, the cells continued to die

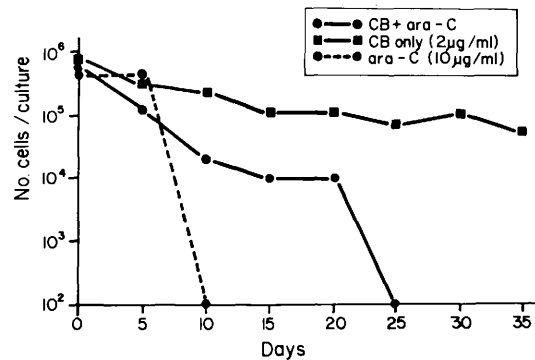


Chart 2. Survival curves of human RD cells in the presence of ara-C, CB, or ara-C + CB. These experiments were carried out as described in Chart 1.

Table 1  
Concentration densities achieved by normal and neoplastic cell lines

Cell line or strain	Concentration density (cells/culture)
HFF	$6 \times 10^5$
RD	$2 \times 10^6$
BHK21/C13	$3.7 \times 10^6$
BHK/IV <sup>3</sup>	$5 \times 10^6$

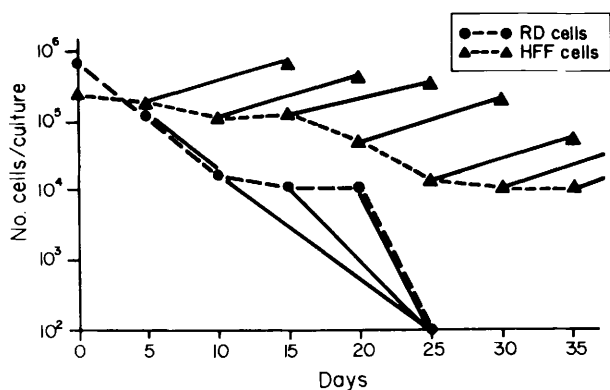


Chart 3. Survival of RD and HFF cells in the presence of ara-C + CB and after reversal to normal medium. The *dashed lines* are growth curves of either cell type during continuous treatment with ara-C + CB. The *solid lines* represent growth curves following reversal to normal medium. Replicate cultures were reversed every 5 days, fed with fresh medium twice weekly, and counted 10 days after reversal and then again on the last day, Day 25 for tumor cells. For the tumor cells only the counts for Day 25 are shown. After about 20 days reversal of HFF to normal medium, cells did not always restore themselves to their original population within 10 days, although growth was markedly increased, as shown. *ara-C*, 10  $\mu\text{g/ml}$ ; *CB*, 1.5  $\mu\text{g/ml}$ .

and at the same rate as those treated continuously with both drugs.

**Effects of ara-C on CB-treated BHK21/C13 and Hamster Tumor Cells.** In these experiments, BHK21/C13 cells rather than normal hamster embryo fibroblasts were compared with hamster tumor cells. This was done since CB was found to be toxic to both hamster embryo fibroblasts and kidney cells. The fibroblasts and kidney cells did not become multinucleated but, rather, detached from the culture substrate. This was unusual, since embryonic fibroblasts of human, mouse, rat, and chick origin did not show this effect (17). Thus we have used BHK21/C13 cells, a continuous line (12) that responds to CB with only binucleation (4, 14). It should be pointed out that BHK21/C13 cells can produce tumors but that all tumor cells become very highly multinucleated when treated with CB.<sup>3</sup> The results with the BHK21/C13 and the BHK tumor cells were very similar to those obtained with the normal and neoplastic human cells. Chart 4 shows that BHK21/C13 cells are killed in a short period when exposed to ara-C, but when CB is added there is protection against ara-C. Chart 5 shows that the tumor cells are killed by ara-C whether CB is or is not present. However, as in the human culture system, CB does afford some increase in the time required for ara-C to produce killing.

In cells treated with CB alone, as shown in Charts 1, 2, 4, and 5, there is a slow but continuous reduction in viability. In the case of both "normal" cell types, this reduction in viability shows some leveling off. Normal cells treated with CB can be maintained for more than 6 weeks without further loss of viability. However, with both tumor cell lines, viability begins to decrease more sharply after about 5 weeks and, by 8 weeks, nearly all the cells detach (data not shown). These cells appear no longer to be viable. Chart 6 shows that normal BHK21/C13 cells treated with CB +

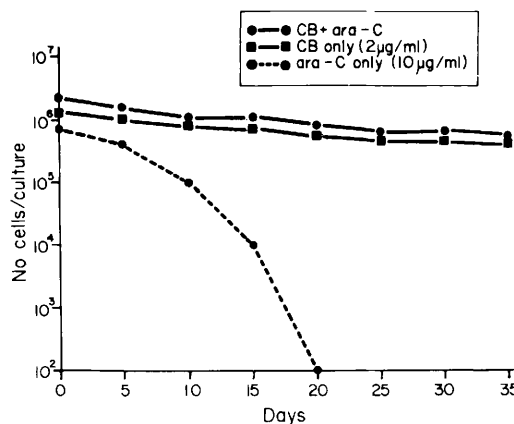


Chart 4. Survival curves of BHK21/C13 cells treated with ara-C (10  $\mu\text{g/ml}$ ), CB (2  $\mu\text{g/ml}$ ), or ara-C + CB. Experiments were performed as described in the legend to Chart 1, except that experiments were begun with about  $0.8$  to  $2 \times 10^6$  cells/replicate culture. Even with ara-C alone, 20 rather than 10 days were required for all cells to be destroyed.

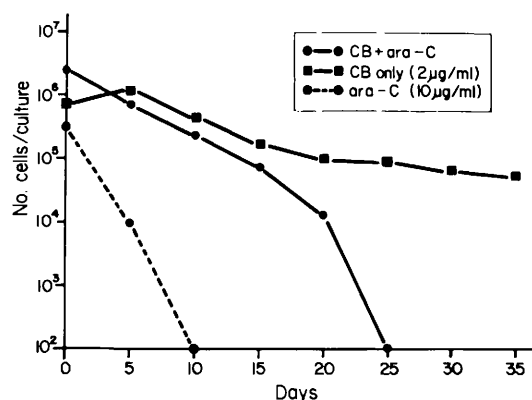


Chart 5. Survival of BHK tumor cells treated with ara-C, CB, or ara-C + CB. With ara-C alone, all the cells were destroyed in 10 days, but this experiment was begun with fewer cells than that in Chart 4.

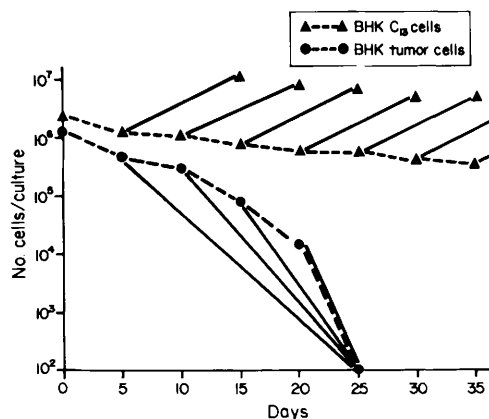


Chart 6. Survival of BHK21/C13 and BHK tumor cells in the presence of ara-C (10  $\mu\text{g/ml}$ ) + CB (2  $\mu\text{g/ml}$ ) and after reversal to normal medium. See legend to Chart 3.

ara-C can be successfully reversed as late as 30 days after the beginning of the combined treatment. However, as with RD, the BHK tumor cells cannot be successfully reversed as early as 5 days after the start of the combined treatment.

## DISCUSSION

Previous studies have shown that tumor cells (14–17) and cells transformed by DNA viruses (9, 10, 17, 18) show uncontrolled nuclear division and DNA synthesis in the presence of CB. Normal cells of human, hamster, rat, mouse, and chick origin show controlled nuclear division and reduced DNA synthesis in the presence of CB (9, 10, 17, 18). Cells such as 3T3 and BHK21, which show controlled nuclear division, are established lines (4, 10, 15). This differential response to CB appears to allow for the selective destruction of cells, with uncontrolled nuclear division, by ara-C. Since CB inhibits DNA synthesis and continued nuclear division in normal cells, it is suggested that ara-C, therefore, cannot effect cell destruction. However, it is unclear how normal cells remain viable in the presence of CB, since the drug does inhibit DNA synthesis. ara-C also inhibits DNA synthesis but very effectively destroys cells. CB has been shown to reduce transport of nucleosides, glucose, 2-deoxyglucose, and glucosamine in some cell lines (3, 6, 11, 13, 20), although its exact mechanism of action is unknown. It thus seems possible that CB may act by preventing transport of substances required for DNA synthesis rather than specifically interfering with their metabolism. ara-C, however, has been shown to inhibit DNA synthesis by interfering with metabolic events (7, 8). It seems that it strongly inhibits DNA polymerase (7, 8). Along these lines, of course, more work is required to define the action of CB. It is of interest that, thus far, 5-bromodeoxyuridine at 50  $\mu\text{g}/\text{ml}$  does not effectively kill CB-treated tumor cells when used in place of ara-C.

The data in Charts 3 and 6 also show that CB-treated tumor cells need not be exposed to ara-C continuously to achieve complete cell destruction, but an exposure of as little as 5 days can be sufficient. Therefore, in this system, ara-C is not reversible. This suggests that whatever damage produced by ara-C remains with the cells but the actual destruction of the cells often takes place later. We have not attempted to show whether the toxic effects of ara-C are reversible in cells treated only with ara-C. There is, however, one report describing ara-C reversibility (19). Evidently, this ara-C-induced damage does not appear in CB-treated normal cells, since they can be successfully reversed even after prolonged treatment. Bhuyon and Fraser (1) have shown antagonism between inhibitors of DNA and inhibitors of protein synthesis, but the mechanism appears somewhat different from that shown here, since inhibitors of protein synthesis would be expected to be effective on both normal and neoplastic cells. However, it seems possible that CB may inhibit protein synthesis of normal cells, perhaps by reducing transport.

These observations may suggest a possible method for the selective destruction of tumor cells *in vivo*. However, in preliminary studies, i.p. treatment of 8-week-old hamsters with as much as 1 mg of CB did not result in the formation of even binucleated bone marrow cells. The drug may therefore be rapidly metabolized or excreted. Higher doses administered more frequently may be required, but this treatment has not yet been attempted. Moreover, as dis-

cussed in a previous report (17), some kinds of cultured tumor cells, like human glioblastoma, do not show uncontrolled nuclear division. Also, cells transformed by RNA tumor viruses do not show uncontrolled nuclear division (17), although it is not as yet clear that CB inhibits DNA synthesis in these cells as it does in normal cells. It would thus appear that the CB + ara-C treatment schedule may not be effective against all kinds of transformed cells, although that remains to be determined.

The applicability of this combined treatment for *in vivo* use is presently unknown. First, a method must be found to maintain high levels of active CB in tissues. Second, will all kinds of transformed cells be killed by ara-C + CB, whether they do or do not show controlled nuclear division? Third, will some normal cells *in vivo* be adversely affected by the combined treatment? These areas are being investigated.

## ACKNOWLEDGMENTS

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