The Effects of Cytosine Arabinoside upon Proliferating Epithelial Cells

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INTRODUCTION

Several studies have demonstrated that ara-C, a potent inhibitor of DNA synthesis (3-5, 7, 10, 12, 14, 16, 19, 25, 27, 35, 39-41, 43, 44, 47, 54, 55, 59, 62), induces severe damage in the intestinal crypts of both mice (19, 44) and rats (43, 47). In order to study this phenomenon in greater detail, we undertook an ultrastructural and autoradiographic investigation in the hope of obtaining some insight into the mechanism by which this antimetabolite might be exerting its necrogenic effect. It was also anticipated that this approach would provide the basis for future experiments designed to establish the means by which 2 inhibitors of protein synthesis, cycloheximide (cf. Ref. 73) and tenuazonic acid (58, 72), prevent the ara-C-induced intestinal damage (47).

Examination by electron microscopy of the mucosal lesions occurring in response to ara-C treatment revealed that the damage to the crypt epithelial cells, seen so early with light microscopy, was only apparent and that the target for the lethal cytotoxic effect of ara-C in vivo was actually the lymphocyte. The evidence supporting this conclusion is the subject of this communication. Subsequent papers in this series will be concerned with the similarities and differences between the effects of ara-C and of some alkylating agents on the structure and function of the intestinal mucosa.

MATERIALS AND METHODS

Male white Wistar rats (Carworth Farms Inc., New City, N. Y.), weighing between 160 and 200 g after a 16- to 18-hr period of fasting, were utilized in all experiments. ara-C (Cancer Chemotherapy Service Center of the National Cancer Institute, Bethesda, Md.), used in the hydrochloride form, was freshly prepared in distilled water (50 mg/ml), adjusted to pH 5.5 to 6.0 with a few drops of 10% NaOH, and injected i.p. in a dose of 250 mg/kg body weight. Control animals received corresponding volumes of 0.9% NaCl solution by a similar route. (Utilization of the 0.9% NaCl solution was considered appropriate, since ara-C was supplied as the hydrochloride.) All injections were given between 8:00 and 9:00 a.m. to minimize diurnal variations.

The animals were killed by decapitation at various intervals after the administration of the drug or 0.9% NaCl solution. Selected for study were tissues of the skin, tongue, esophagus, stomach, and small intestine, all of which consist in part of epithelium containing populations of continuously dividing cells (18). Fixation for light microscopic morphological studies was routinely carried out in Stieve's solution. For histological examination, sections of paraffin-embedded tissue were stained with H & E as well as by the Feulgen method for DNA. Mitotic activity, expressed as percentage of mitoses, was established in each animal by counting the number of dividing and nondividing nuclei in at least 30 longitudinally sectioned intestinal crypts. Comparative DNA synthesis, as measured by precursor incorporation and corrected for changes in the precursor pool, was determined as previously described (70).

For electron microscopic studies, 1 control and at least 2
The procedures used for the general selection and processing after the administration of either 0.9% NaCl solution or ara-C. Experimental animals were killed at 0.5, 1.5, 2.5, 3.5, and 8 hr after the administration of either 0.9% NaCl solution or ara-C. Ideally, it would have been best to examine cells known to be in each phase of the cell cycle. However, this can be done with a degree of specificity in vivo only with autoradiography and then only for cells in the S phase. For a study of cells in all phases of the cycle, tissue obtained from a minimum of 5 blocks from each ara-C-treated animal was examined at each predetermined time interval. This approach provided a sufficient number of sections to assure the inclusion of cells in different compartments of the life cycle in a random sampling.

Autoradiographs were prepared from tissue obtained from animals given injections of thymidine-2H, 1 μCi/g body weight (New England Nuclear, Boston, Mass), 6.76 × 10^3 mCi/mmole, 1000 μCi/ml. A 15-cm segment of proximal intestine was fixed in 10% formalin and embedded in paraffin. Four- to 5-μm sections were placed on glass slides and dipped into NTB3 liquid emulsion (Eastman Kodak Co., Rochester, N. Y.). After an exposure period of from 1 to 3 weeks at 4°C, the autoradiograms were developed with Kodak D-19 and stained with H & E. From each animal an average of approximately 100 mitotic figures, selected at random and without regard to their stage in division, were scored as being either labeled or unlabeled according to the criteria established by Pederson and Gelfant (53).

RESULTS

Effects of ara-C on DNA Synthesis. The 1st series of experiments was conducted to ascertain both the degree and duration of inhibition of DNA synthesis induced by ara-C under our experimental conditions. As can be seen in Chart 1, the uptake of the precursor fell to 2% of control levels within 0.5 hr following the injection of ara-C. After remaining at essentially this same low level for an additional 4.5 hr, the rate of DNA synthesis returned to about 75% of control values by 10 hr.

Light Microscopic Observations. For a study of the histological response of a variety of proliferating epithelial cells to a single injection of ara-C, 1 control and 2 experimental animals were killed at each of the following intervals: 0.5, 1.5, 2.5, 3.5, 8, 12, 14, 16, and 24 hr subsequent to the administration of the antimetabolite. Although actively engaged in both DNA replication and mitotic division (18), epithelial cells in the mucosa of the esophagus and stomach, as well as those in the basal layer of the epithelium of the tongue and stomach, revealed no light microscopic evidence indicative of a cytotoxic reaction to the ara-C treatment. This paucity of obvious degenerative changes in these particular tissues was in marked contrast to the striking alterations noted in the crypts of the small intestine. As reported previously in both mice (19, 44) and rats (43, 47), stigmata of apparent crypt cell damage were readily discernible within 2.5 to 3.5 hr after administration of ara-C. In H & E-stained sections, the lesion was manifested by the development of numerous intracellular eosinophilic inclusions.

These occasionally contained fragments of basophilic debris (Fig. 1) which, in some instances, yielded a strongly positive reaction when stained with the Feulgen method for DNA. The epithelial cell nuclei, however, appeared to be normal. By 8 hr only a few intracellular inclusions were still present. Recovery, as measured by the disappearance of intracellular debris, confirmed to progress so that beyond 16 hr no cytological differences could be detected between the control and ara-C-treated animals (47).

This apparent damage to the intestinal crypts was also accompanied by an interruption of cell division (Chart 2). Mitotic activity, markedly reduced at 1.5 hr, was completely abolished within 2.5 hr. Subsequently, mitoses began to reappear and were appreciable but were still below the normal value at 8 hr, i.e., at the time of disappearance of the intracellular inclusions. After this time they progressively increased in number, reaching a peak at 12 hr when they showed a 3-fold increase over control values. Cell division subsequently decreased and returned to the range of the controls by 16 to 24 hr (Chart 2).

Careful examination of the intestinal mucosa, especially of the crypts, revealed obvious necrotic cells immediately beneath the epithelial cells, as well as in the underlying lamina propria. Although these cells were possibly lymphocyte-like in appearance, their advanced nuclear and cytoplasmic degeneration precluded an exact identification by light microscopy. However, subsequent electron microscopic observation indicated the likelihood of their being lymphocytes (see below).

In agreement with our previous findings (47), severe necrosis was noted in the germinal centers of intestinal lymphoid follicles. The peripheral zone of small lymphocytes, however, was unaffected (Fig. 2).

Autoradiographic Studies. For establishment of the source of the mitotic intestinal epithelial cells noted during recovery from the inhibitory effects of ara-C, autoradiographic procedures were used.
Junctional complexes in their apical regions. Membrane-bound vacuoles (cytolysomes) which contain homogeneous dense Golgi complexes. The nucleus is situated in the basal portion of the cell and contains 1 or 2 nucleoli. The lateral cell membranes of adjacent cells are relatively straight and display mitochondrial modifications in the nonepithelial, lymphocyte-like cells were observed in only widely scattered epithelial cells. While the exact nature of these cells cannot be established unequivocally, their ultrastructural organization is compatible with their being intraepithelial lymphocytes.

In animals treated with ara-C, no definitive evidence of epithelial cell necrosis was observed at any of the intervals studied. Preservation of the structural integrity of all the stem cells was readily apparent even at 3.5 hr, the time at which karyorrhexis and other stigmata of cell death, as interpreted by conventional microscopy, were maximal (Fig. 9). The stem cells frequently contained a considerable number of membrane-bound vacuoles enclosing degenerating organelles. Especially prominent in these vacuoles were large and small pieces of fragmented nuclei (Figs. 9, 10, and 12), yet the epithelial cells in which these vacuoles were found uniformly failed to show any indication of lethal damage. The integrity of the epithelial cell nuclei was particularly obvious in that the nuclear chromatin was well dispersed, the nucleolar components retained their usual relationships, and the normal-appearing, double nuclear membranes remained intact (Figs. 9 and 10). These reproducible findings led us reluctantly to the tentative conclusion that the nuclear and cytoplasmic fragments found within the epithelial cell vacuoles were of exogenous origin, presumably arising as a result of the phagocytosis of dead or degenerating cells. Indeed, modifications in the nonepithelial, lymphocyte-like cells were readily seen. Although first detected at 1.5 hr after administration of ara-C, these alterations were much more dramatic at both the 2.5- and 3.5-hr intervals. An increase in the number of lymphocytes within the epithelial lining was a characteristic and consistent finding. In tangential sections, this phenomenon was manifested by numerous cytoplasmic, finger-like protrusions which could be seen lying within and causing a dilation of the intercellular spaces (Fig. 11). In some instances, the lymphocytes projected into the adjacent epithelial cell, resulting in an indentation of its nucleus. Quite commonly, the epithelial cell nucleus appeared to be partially wrapped around portions of the lymphocyte (Fig. 12). Clumping of chromatin along the lymphocytic nuclear membrane, as well as apparent nuclear fragmentation, was also frequently seen (Figs. 13 and 14). In addition, necrotic cells or portions thereof were noted deep within the lamina propria (Fig. 15), as well as adjacent to or passing through the basement membrane (Figs. 16 and 17). Despite the striking accumulation of cellular debris noted between 1.5 and 3.5 hr after the administration of ara-C, by 8 hr large cytolysomes were observed in only widely scattered epithelial cells. Moreover, as in the earlier intervals, the structural organization of the stem cells themselves showed no changes, compared with similar cells obtained from control animals.

In many instances it was impossible to ascertain whether the cytoplasmic vacuoles were really intracellular or were merely representative of extracellular material projecting into the adjacent epithelial cells. However, as demonstrated in Fig. 10, some of the inclusions were enclosed by a single membrane and contained readily identifiable nuclear material, even though the epithelial cell nucleus appeared to be normal. Thus, there

Chart 2. Effects of ara-C on mitotic activity in the intestinal crypts. Points in the experimental groups, mean of 2 animals; vertical bars, ranges.

On 2 separate occasions, 4 animals initially were given i.p. injections of thymidine-3H and, after a 1-hr incorporation period, ara-C. The rats were killed 12 hr after this latter injection, the interval at which recovery of cell division had reached its peak (Chart 2). Examination of the autoradiograms revealed the presence of silver grains over virtually 100% of the mitotic figures (Fig. 3).

Electron Microscopic Observation. The ultrastructural appearance of normal crypt undifferentiated epithelial cells has been described in detail by a number of investigators (63, 67), and our observations generally do not differ significantly from those earlier reports. Therefore, only a brief discussion of the cytotological features of these cells is included in this communication.

The hyaloplasm of the stem cell is of moderate electron density and contains numerous free ribosomes and mitochondria, as well as scattered dense bodies, strands of rough endoplasmic reticulum, and moderately well-developed Golgi complexes (Fig. 6). The nucleus is situated in the basal portion of the cell and contains 1 or 2 nucleoli. The lateral cell membranes of adjacent cells are relatively straight and display junctional complexes in their apical regions. Membrane-bound vacuoles (cytolysomes) which contain homogeneous dense material and cellular debris in varying stages of dissolution are occasionally found lying within the cytoplasm of the stem cell (Fig. 7). Also noted within the epithelial layer of the crypts are cells which are cytologically distinct from the stem cells. As shown in Fig. 8, these cells are considerably smaller than the epithelial cells, are roughly oval, and demonstrate a striking paucity of cytoplasmic organelles, except for the presence of numerous free ribosomes together with occasional mitochondria and solitary, irregular channels of endoplasmic reticulum. Junctional complexes were not present between these cells and the adjacent epithelial cells. While the exact nature of these cells cannot be established unequivocally, their ultrastructural organization is compatible with their being intraepithelial lymphocytes (1, 63, 64).
is some evidence that the epithelial cell had ingested exogenous cellular debris.

**Effects of Multiple Injections of ara-C.** ara-C has been reported to be rapidly metabolized and excreted (11, 15, 22, 38, 61, 62). Therefore, it seemed important to conduct studies in which sufficient levels of this agent were maintained to ensure a maximal inhibition of DNA synthesis over a period of time equivalent to the entire duration of the cycle of the intestinal crypts, i.e., 10.5 to 12 hr (8). In addition to the immediate effect on those cells already engaged in the replication of their DNA, this approach would also permit the eventual exposure to the drug of cells originally in G₂, M, or G₁ as they traversed the cell cycle and entered or approached the S phase.

Eight animals were given ara-C every 2 hr for 6 doses. Four additional rats received corresponding injections of 0.9% NaCl solution and served as controls. Two control and 4 experimental animals were killed either 24 or 48 hr after the initial injections of either ara-C or 0.9% NaCl solution. At the 24-hr interval, the intestines of the ara-C-treated rats revealed slightly atrophic crypts in which the goblet cells appeared to be more prominent than those in control animals. Only scattered mitotic figures and isolated damaged cells were observed (Fig. 4). By 48 hr, the crypts were histologically indistinguishable from those of the 0.9% NaCl solution-injected animals (Fig. 5). In addition, no cytological aberrations were noted at either interval in the epithelial components of skin, tongue, esophagus, or gastric mucosa.

**DISCUSSION**

It is evident from this study that ara-C is not necrogenic to epithelial cells of the intestinal crypts in vivo under conditions in which the antimetabolite inhibits DNA synthesis by over 95% for several hr and in which certain cells in the lymphoid follicles are readily destroyed. The evidence for this conclusion is both structural and functional and can be summarized as follows: (a) the absence of definitive ultrastructural manifestations of irreversible damage, particularly with respect to the nucleus; (b) the presence of labeled thymidine in virtually 100% of the mitotic figures at 12 hr after the administration of ara-C and 13 hr after the injection of thymidine-3H (this particular observation precludes the possibility of a selective destruction of S-phase epithelial cells by the analog (36, 76) or of a mobilization of reserve G₂ cells (23, 53, 56) for entry into mitosis); (c) the enhanced mitotic activity at 12 hr which closely followed the resumption of a considerable degree of DNA synthesis in the intestinal crypts; and (d) the failure to find any indication of intestinal epithelial cell death in animals killed 24 and 48 hr after the 1st of multiple injections of ara-C. This latter regimen was sufficient to inhibit DNA synthesis maximally and continuously for well over 12 hr, i.e., the length of the entire cell cycle (8). The slight atrophy of the crypts seen at 24 but not at 48 hr could be due to the fact that cell migration onto and cell extrusion from the villus proceeds independently of the rate of cell formation in the proliferative compartment of the intestinal mucosa (28). Thus, the uninterrupted maturation and movement of cells onto the villi in the presence of the block in cellular replication (as manifested by the pronounced depression of mitosis at 24 hr) could easily account for the transient atrophy noted at that time interval. However, our observations relative to the effects of multiple injections of ara-C in the rat contrast with those of Leach et al. (42). As described above, the regimen used in the present study (i.e., the administration of ara-C to rats every 2 hr over a 12-hr period) was not associated with necrogenic alterations of the crypt epithelial cells. On the other hand, Leach et al. (42) have demonstrated that mice treated with ara-C every 3 hr for 24, 72, or 96 hr developed severe karyorrhexis and distortion of the intestinal mucosa. While this disparity of results may be explained at least in part on the basis of species and/or dose differences, it also seems likely that the crypt damage noted in the studies of Leach et al. (42) represents a reflection of the development of cellular aberrations occurring in response to exposure to the antimetabolite for longer periods of time. Conceivably, the inhibition of DNA synthesis for extended periods might activate secondary responses which, in turn, lead to necrosis. Alternatively, certain aspects of intermediary metabolism of ara-C that are of minor importance in a 12-hr experiment may assume greater biological significance in experiments of longer duration and could lead to qualitatively new phenomena such as cell death.

Evidence that the conclusion concerning intestinal epithelial cell viability with ara-C might pertain to other tissues programmed for continuous replication, such as the epithelial components of the tongue, skin, esophagus, and stomach (18), is also presented. However, this aspect of the study is not as well documented and is based solely on histological observations.

The contention that ara-C is not necrogenic to proliferating epithelial cells is in agreement with that of at least 2 other groups of investigators. Hirschman et al. (27) have demonstrated that ara-C almost completely inhibited DNA synthesis in cultured 3T3 cells, and yet excellent recovery and growth were obtained even after an exposure to the drug of from 6 to 8 hr. Graham and Whitmore (25) found that concentrations of ara-C sufficient to inhibit DNA synthesis in mouse L-cells by more than 97% for nearly 2 generation times were without a significant effect on cell viability. Only much higher concentrations of the analog were lethal to those cells.

Although ara-C apparently is not necrogenic to crypt epithelial cells, little doubt remains that the drug is cytotoxic to certain other intestinal cells, including lymphocyte-like cells in the mucosa of the crypts. With conventional microscopy, numerous intracytoplasmic inclusion bodies were noted within the epithelial cells lining the Lieberkühn crypts. In many instances, these inclusions contained Feulgen-positive fragments, thus indicating the presence of nuclear material. Similar structures have been observed in the intestine following irradiation (24, 45, 47), as well as after the administration of alkylating agents (47), methotrexate (65), or aminopterin (49); and these apparently represent the karyolytic bodies originally described by Montagna and Wilson (51). Recent electron microscopic studies (24, 31, 33, 34, 49, 66) have revealed that these karyolytic bodies actually consist of highly complex structures composed of degenerating nuclear and cytoplasmic constituents. The demonstration of acid phosphatase (29) and thiamine pyrophosphatase activities (30) in these bodies indicates a relationship to cytolyomes
or autophagic vacuoles, both of which are involved in the process of intracellular digestion. After examining the ultrastructural appearance of mouse intestine at several intervals after exposure to irradiation, Hugon and Borgers (31) suggested 3 possible sources of the cellular debris found within these cytoplasmic vacuoles: focal cytoplasmic degradation of injured but still viable epithelial cells, phagocytosis of dead stem cells, or phagocytosis of dead lymphoid cells. We cannot totally rule out the possibility that at least some of the inclusions noted in the epithelial cells of ara-C-treated animals represent an attempt by the stem cells to segregate portions of injured cytoplasm and that these structures are therefore indicative of areas of focal cytoplasmic degradation. However, although many of the vacuoles in the crypt epithelial cells contained nuclear fragments, we were unable to detect any alterations in the epithelial cell nuclei (e.g., clumping of the chromatin) that would suggest injury to these particular cells. Therefore, it can be concluded that these inclusions are not formed as the result of the phagocytosis of necrotic epithelial cells. Accordingly, it seems most likely that the karyolytic bodies are produced by the incorporation of dead lymphocytes into adjacent crypt epithelial cells. A source of such cells was readily apparent in our study, since dead and dying lymphocytes were frequently present in both the crypt epithelial lining and the lamina propria of the intestines of animals treated with ara-C.

Although an occasional lymphocyte was noted between the epithelial cells in control rats, the frequency with which they were encountered was markedly increased in the ara-C-treated animals. While the cell membranes of the lymphocytes and neighboring epithelial cells remained intact, numerous lymphocyte pseudopods were seen distending the intercellular spaces, as well as projecting into the epithelial cell cytoplasm. In this latter location, they were frequently found in close proximity to and compressing the nucleus of the epithelial cells, thus imparting the impression of an early phagocytic process (Fig. 12). Pycnosis and karyorrhexis of the lymphocytic nuclei were also evident (Figs. 13 and 14). These cytological changes are comparable to those occurring in lymphocytes at the end of their life-span or in those killed by a variety of experimental procedures (69). Inasmuch as approximately 2 to 3% of the intraepithelial lymphocytes replicate their DNA in situ (21), it is conceivable that the nuclear alterations noted in the present study reflect an ara-C-induced disruption of nucleic acid synthesis within the intraepithelial lymphocytes. However, approximately 50 to 55% of the proliferating crypt epithelial cells are also actively engaged in synthesizing DNA at any given time (46). It therefore becomes difficult to explain the greater susceptibility of the lymphocyte to the ara-C-induced metabolic imbalance. In an attempt to explain this inherent vulnerability of lymphocytes, Trowell (68) has suggested that their poor resistance to a number of toxic agents, e.g., irradiation, cortisone, and nitrogen mustard, might be related to a disturbance of mitochondrial function. Since lymphocytes contain a paucity of these organelles, any interference with their physiochemical activities could result in the inability of these cells to provide the internal environment required for the maintenance of homeostasis. In this respect, it is interesting that the mitochondrial changes noted in the intestinal epithelial cells in animals exposed to irradiation (33) or methotrexate (66), both of which disrupt nucleic acid metabolism (48, 60) and induce regressive changes in the epithelial cells of the Lieberkühn crypts (31, 34, 47, 51, 65), were not observed in animals treated with ara-C.

An abundance of necrotic cells or portions thereof were also seen in the lamina propria, often adjacent to or in the process of passing through the basement membrane (Figs. 15, 16 and 17). The advanced state of degenerative changes of this material precludes the establishment of its origin with any degree of certainty, neither is it possible to determine the direction of movement from static micrographs. However, this study, as well as our earlier investigations (47), disclosed that ara-C caused extensive necrosis of the germinal centers of intestinal lymphoid follicles. Consequently, it is highly probable that most if not all of this cellular debris was derived from the necrotic germinal centers and that it subsequently migrated into the crypt epithelial cells where it underwent further degradation. Such an interpretation would suggest that the intestinal epithelium might play a major role in the disposal of lymphocytes. Some support for this contention can be derived from the studies of Bryant (6). Following the transfusion into isogenic, partially hepatectomized mice of cell suspensions of lymphocytes that had been previously exposed to thymidine-3H, the highest concentrations of the radioactive isotope were found in the epithelial cells of Lieberkühn's crypts. It was therefore concluded that the donor lymphocytes were broken down and their DNA realit on in association with the renewal of the intestinal epithelium of the recipient animal. While it is conceivable that this utilization of lymphocyte DNA by the crypt epithelial cells might account for the labeled mitotic figures observed in animals recovering from the effects of ara-C, certain parameters ascribed to the cell cycle argue against this possibility. Cairnie et al. (8) have established that the duration of S and G2 in Lieberkühn's crypts are 6.5 to 8 hr and 1 hr, respectively. Therefore, if S-phase cells were selectively killed by ara-C and if G1 cells eventually incorporated labeled nucleotides derived from the degenerating lymphocytes into their own genome, the least amount of time required for the detection of these G1 cells as labeled mitoses would be 7.5 hr after the reinitiation of DNA synthesis. Since the peak of mitotic recovery occurred 12 hr subsequent to the administration of ara-C (Chart 2), a significant restitution of DNA synthesis would have had to develop at the 4.5-hr interval. However, as shown in Chart 1, nucleic acid synthesis was still almost totally suppressed at that time period.

A number of other observations regarding the origin and fate of intestinal lymphocytes also support the idea that the intestinal crypt epithelial cells play a role in the disposal of lymphocytes. In untreated animals, cells morphologically indistinguishable from small lymphocytes are often found in considerable numbers in lamina propria (1). Many of these lymphoid cells pass through the basement membrane (1, 52, 63, 64), after which time they either occupy the intercellular spaces (1, 63, 64) or penetrate into the cytoplasm of the stem cells (1, 2) where they frequently undergo degeneration in large vacuoles (1, 2).
either migrate through the epithelium and are eventually shed into the lumen (75) or remain intact within the epithelium for indefinite periods of time (1, 26). Following irradiation, the number of lymphocytes migrating through intestinal epithelium increases considerably (45). Electron microscopic studies conducted in irradiated mice (31, 32) have demonstrated that many of the lymphoid cells present evidence of acute injury and that they are ultimately engulfed by the crypt epithelial cells. The observations derived from the present study suggest that a similar exaggeration of the normally occurring processes of migration and degradation of intestinal lymphoid elements might take place in animals treated with ara-C and that this phenomenon could be a reflection of the unusually high survival of some lymphocytes to interferences with many metabolic processes (20). The interpretations and conclusions derived from our results seem to be in conflict with those of Lenaz et al. (44) in mice treated with ara-FC, a compound similar in many of its effects to ara-C. By means of autoradiographic procedures, these investigators found that, following the administration of ara-FC, 97% of the epithelial cells of the intestinal crypts considered by light microscopy to be necrotic were labeled. However, parallel quantitative studies of the persistence of prelabeled DNA of the entire intestinal wall showed only a 50% decrease in radioactivity 16 hr after the injection of ara-FC. To explain this unanticipated finding, Lenaz et al. (44) suggested that there is reutilization of the labeled DNA products released from the degenerating epithelial cells.

An equally plausible explanation and one in basic accord with our observations is as follows. Certain lymphoid as well as crypt epithelial cells are labeled in vivo following the injection of radioactive thymidine. The destruction of the lymphoid cells by ara-C or ara-FC and their phagocytosis by the crypt epithelial cells would account for the large number of labeled, damaged cells noted in Lieberkühn’s crypts (44). Similarly, the ingestion of fragments of lymphoid cells would, on a light microscopic level, simulate actual karyorrhexis of the crypt epithelial cells. The observed 50% decrease in DNA radioactivity (44) could represent the loss of label from lymphoid cells as a result of digestion within and ultimate elimination from the crypt epithelial cells. Retention of the residual radioactivity (50%) could reflect the continued presence of labeled DNA within the intact, undamaged epithelial cells. Thus, in actuality, no discrepancy may exist between the results of our study and those of Lenaz et al. (44).

It has been demonstrated that the ara-C-induced inhibition of DNA synthesis is dependent upon the capacity of the target cell to convert the drug, via phosphorylation by deoxycytidine kinase, to its active derivatives, ara-C diphosphate and ara-C triphosphate (10, 11, 13, 38, 57). Since Kress (quoted in Ref. 61) found that 70% of the drug was excreted unchanged in the urine of the rat and since the antitumor activity in the rat was reported to be limited (74), it appeared that the drug was not activated in that species (61). However, more recent evidence has demonstrated that deoxycytidine kinase is indeed present in the rat, although its concentrations in various tissues varies considerably. Kessel et al. (37) demonstrated that the capacity for drug phosphorylation was highest in mature lymphocytes. Mitchell et al. (50) also found proportionally high levels of the activating enzyme in lymphoid tissue. Similarly, Durham and Ives (17) reported that lymph nodes, spleen, and thymus in the rat contained high levels of deoxycytidine kinase while other nonlymphoid tissues, including the intestine, contained much lower levels. However, the pronounced block in the incorporation of radioactive precursor into DNA noted in this study, as well as in other investigations (43, 47), clearly indicate that the intestine of the rat must contain concentrations of the activating enzyme that are high enough to permit the requisite conversion of ara-C into its metabolically active forms. Conversely, the subsequent enzymatic transformation of ara-C to physiological inert 1-β-D-arabinofuranosyluracil (9) by intestinal pyrimidine nucleoside deaminase activity (17) presumably accounts for the eventual recovery of DNA synthesis. This pattern of suppression and restitution of DNA synthesis results, first, in a blockage of cells that were in the S phase at the time of drug administration and, then, in a wave-like movement of these cells out of S, through G2 and, ultimately, into mitosis. The partial synchrony thus obtained is manifested morphologically by the mitotic overshoot of control levels observed 12 hr after the injection of ara-C. A similar phenomenon has been detected in vitro in HeLa cells (39), as well as in vivo in murine melanoma and Ehrlich tumors (5) and mouse intestine (44) treated with the antimetabolite. The rapidity with which mitotic activity initially disappeared and the subsequent abrupt increase in the number of dividing cells noted between 10 and 12 hr in the present study indicate that a block induced by ara-C occurs at or near the end of the S phase. The finding that the total interruption and eventual resumption of cell division in rat intestine occurred at time intervals different from those previously reported in mice (44) may be due to species differences and/or to the smaller dose of ara-C utilized by these latter investigators.

The results of our experiments pertaining to the resistance of epithelial cells to severe perturbations of DNA synthesis by ara-C raise a very important practical problem concerning the basis for use of the analog in the treatment of nonepithelial neoplasms. Is ara-C acting as a lethal cytotoxic agent or is its effect more subtle? For example, since it is often used with other chemotherapeutic drugs, could its action, at least in part, be due to some induced synchronization of the neoplastic cells? If this were the case, perhaps regimens designed to synchronize the target cells more efficiently might be explored.

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Fig. 1. Intestinal mucosa 3.5 hr after the administration of ara-C. Numerous eosinophilic inclusions, some of which contain fragments of basophilic debris (arrows), are present in the crypt epithelial cells. H & E, X 400.

Fig. 2. Intestinal lymphoid follicle 3.5 hr after the administration of ara-C. Note the extensive necrosis of cells in the germinal center (GC). H & E, X 400.

Fig. 3. Autoradiogram of intestinal crypts 12 hr after the administration of ara-C and 13 hr after the injection of thymidine-3H. Silver grains are present over every mitotic figure (arrows). H & E, X 900.

Fig. 4. Intestinal mucosa from a rat given injections of ara-C every 2 hr for a total of 6 doses. The animal was killed 24 hr after the initial injection. Note the prominence of the goblet cells within the somewhat atrophic crypts. Only an occasional mitotic figure is present (arrows). H & E, X 200.

Fig. 5. Intestinal mucosa from a rat given injections of ara-C every 2 hr for a total of 6 doses. The animal was killed 48 hr after the initial injection. Crypts reveal a normal histological appearance. H & E, X 125.
Fig. 6. Survey view of several adjacent crypt undifferentiated epithelial cells from a control rat. The hyaloplasm is of moderate electron density and contains numerous free ribosomes (R) and mitochondria (M), as well as scattered dense bodies (DB) and strands of rough endoplasmic reticulum (RER). The lateral cell membranes (CM) of adjacent cells are relatively straight and display junctional complexes (JC) in their apical regions. N, nucleus; NCL, nucleolus; G, Golgi apparatus; LP, lamina propria. X 7,600.

Fig. 7. A portion of a crypt stem cell from a control rat. Note the membrane-bound vacuole or cytolysome (arrow) containing cellular debris in varying stages of dissolution. N, epithelial cell nucleus; LP, lamina propria. X 8,000.

Fig. 8. An intraepithelial lymphocyte (L) from a control rat. Note the paucity of cytoplasmic organelles except for ribosomal particles (R), occasional mitochondria (M), and solitary, irregular channels of endoplasmic reticulum (ER). Note also the absence of junctional complexes between lymphocyte and adjacent epithelial cells (EC). LP, lamina propria. X 12,800.
Figs. 9 and 10. Intestinal crypt cells 3.5 hr after administration of ara-C. Note the preservation of the structural integrity of the epithelial cells (EC) and the normal chromatin pattern of their nuclei (N). Vacuoles (V) containing degenerating organelles and fragments of nuclear material (arrows) are present in the cytoplasm of the epithelial cells. Note also that the vacuole demonstrated in Fig. 10 is surrounded by a distinct single membrane. Fig. 9, × 6,100; Fig. 10, × 11,000.
Fig. 11. Intestinal crypt 2.5 hr after administration of ara-C. Numerous lymphocytic cytoplasmic protrusions (arrows) can be seen lying within the intercellular spaces. N, epithelial cell nuclei; LP, lamina propria. × 11,000.

Fig. 12. Intestinal crypts 3.5 hr after administration of ara-C. An indented epithelial cell nucleus (N) appears to be wrapped around a portion of a lymphocyte (L). Note also the nuclear material (arrow) contained within the membrane-bound vacuole (V). × 7,600.

Fig. 13. An intraepithelial lymphocyte (L) 1.5 hr after administration of ara-C. Note the clumping of chromatin (CH) along the nuclear membrane (arrows). EC, epithelial cells. × 6,700.

Fig. 14. Intraepithelial lymphocytes (L) 1.5 hr after administration of ara-C. Note the apparent nuclear fragmentation (arrows). LP, lamina propria. × 12,400.
Fig. 15. Intestinal mucosa 2.5 hr after administration of ara-C. Portions of several necrotic cells (arrows) are present deep within the lamina propria (LP). X 18,400.

Fig. 16. Intestinal mucosa 3.5 hr after administration of ara-C. A necrotic cell (arrow) can be seen lying adjacent to the basement membrane (BM). EC, epithelial cell. X 15,600.

Fig. 17. Intestinal mucosa 2.5 hr after administration of ara-C. A portion of a necrotic cell (arrow) appears to be passing through the basement membrane (BM). Additional necrotic debris is present in the lamina propria (LP) and within an epithelial cell cytoplasmic vacuole (V). X 13,400.
The Effects of Cytosine Arabinoside upon Proliferating Epithelial Cells


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