Synchronization of Cell Division in Vivo through the Combined Use of Cytosine Arabinoside and Colcemid

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Summary

Mitotic activity in Lieberkühn's crypts increased from the 3 to 5% levels normally occurring in 0.9% NaCl solution-injected rats to peak values of 15% in animals treated with a single injection of either ara-C or Colcemid. In animals treated with ara-C followed by Colcemid, 33 to 38% of the crypt epithelial components could be identified as dividing cells, thus indicating a synchronization of at least two-thirds of the proliferating epithelial cells. Of additional significance was the finding that these regimens were not necrogenic to the intestinal epithelium. Further studies utilizing a second injection of Colcemid indicated that synchrony was lost once traversal through the cell cycle was reinitiated.

Introduction

Partial synchronization of cell division in vitro has been obtained with considerable success through the implementation of a variety of techniques (25, 35, 37, 38, 40, 50–52, 57, 60, 61, 65). One method generally used is the exposure of randomly proliferating cells to agents that interfere with specific biosynthetic activities, e.g., DNA replication. The cells thus blocked in the S phase are subsequently released by simply being washed in control media, followed in some instances by the addition of exogenous nucleotides, thereby permitting their simultaneous wave-like movement into succeeding segments of the cell cycle (1, 5, 14, 15, 17, 18, 23, 29, 36, 39, 44, 47, 48, 53, 55, 59, 62, 63, 66). However, the utilization of antimetabolites to duplicate this phenomenon in vivo presents a much more complex situation. In the intact animal, intricate enzymatic reactions determine not only the rapidity by which the inhibitory agent exerts its influence but also the rapidity by which it is catabolized. This latter factor is obviously a prerequisite both for the resumption of movement of the previously arrested cells and for establishing the degree to which synchronization is attained. Consequently, this approach necessitates the careful selection of suitable inhibitors with structural configurations and chemical characteristics that render them susceptible to the intrinsic intracellular regulatory mechanisms. In an attempt to identify such a compound, we have been examining the effects of various antimetabolites on the crypts of the small intestine, since these structures provide a readily accessible in vivo source of continuously dividing cells. Thus far, the agent that seemed to possess the greatest potential for meeting the metabolic criteria outlined above was ara-C, a fraudulent nucleoside and potent inhibitor of DNA synthesis (2–4, 8, 10–13, 16, 19, 20, 22, 24, 26, 27, 31, 32, 34, 43, 45, 46, 58). This tentative conclusion was formulated on the basis of the following data. (a) An almost complete inhibition of DNA synthesis occurs in the Lieberkühn crypts within 10 to 15 min (34). In apparent contrast to the findings of other investigators (16, 31, 32, 34), this interruption of the formation of this species of nucleic acid is not associated with the concomitant development of epithelial cell death (64). (b) The subsequent restitution of DNA replication in this tissue occurs within a reasonable period of time (31, 32, 34).

A number of studies have also demonstrated that ara-C inhibits mitotic activity in mammalian cells both in vitro (24) and in vivo (4, 32). More recent experiments conducted in this laboratory (64) revealed a similar effect in the proliferative compartment of rat intestinal mucosa and, in addition, disclosed that recovery from the initial interference with cell division was characterized by a pronounced rebound 12 hr after the administration of the antimetabolite. At this particular time interval, the population of dividing cells in the intestinal crypts increased from the 3 to 5% levels normally occurring in 0.9% NaCl solution-injected animals to approximately 15% in rats treated with ara-C. Additional data obtained from autoradiograms indicated that the cells contributing to this rebound were those originally blocked in the S phase of the cell cycle (64).

These observations raised the possibility that an even greater accumulation of proliferating cells and, consequently, a considerable degree of synchrony could be achieved by temporarily preventing the subsequent passage of these particular cells through mitosis. Such an approach has been made feasible by the availability of agents such as colchicine and CCM, both of which arrest mitotic cells in metaphase (cf. Ref. 7). The utilization of the former compound was deemed impractical because it induces epithelial cell degeneration in Lieberkühn's crypts (21). Since CCM has been reported to be less cytotoxic (54), this agent was used in the current investigations. The results of experiments designed to explore...
the concept outlined above are presented in this communication.

MATERIALS AND METHODS

Male white Wistar rats (Carworth Farms, Inc., New City, N. Y.), weighing 165 to 200 g after a 16- to 18-hr period of fasting, were utilized in all experiments. Freshly prepared solutions of the following compounds were injected i.p.: (a) ara-C (250 mg/kg) (Cancer Chemotherapy Service Center of the National Cancer Institute, Bethesda, Md.), as the hydrochloride, was made up in distilled water (50 mg/ml) and adjusted to pH 5.5 to 6.0 with a few drops of 10% NaOH; and (b) CCM (0.05 mg/100 g) (Ciba Pharmaceutical Company, Summit, N. J.) in a concentration of 1 mg/ml (CCM, 0.01 g; 95% ethanol, 0.65 ml; propylene glycol, 1.0 ml; Na₂HPO₄·7H₂O, 0.015 g; NaH₂PO₄·H₂O, 0.0133 g; and distilled water, a sufficient quantity up to 10 ml). Control animals received either vehicle or 0.9% NaCl solution by a similar route.

The rats were sacrificed by decapitation, and the first 15-cm segment of the small intestine was removed and fixed in Stieve's solution. For histological examination, sections of paraffin-embedded tissue were stained with hematoxylin and eosin. Mitotic activity, expressed as percentage of mitoses, was established in each animal by count of the number of dividing and nondividing nuclei in at least 30 longitudinally sectioned, intestinal crypts.

RESULTS

Effects of a Single Injection of CCM. The 1st series of experiments was designed to establish: (a) the sequential pattern of mitotic activity and (b) the presence or absence of necrosis in the intestinal crypts of animals given a single injection of CCM. As shown in Table 1, there were no significant differences in mitotic activity in control animals treated with either 0.9% NaCl solution or vehicle. The number of dividing cells in these 2 groups of rats ranged from 3 to 4.9%. In contrast, mitoses increased substantially in those animals treated with CCM. This augmentation was apparent within 1 hr, when 9.2% of the crypt cells were in mitosis. After reaching a peak value of 15.6% at the 2-hr interval, mitotic activity progressively declined, returning to control levels within 4 to 6 hr after the administration of CCM. Also, there was no evidence of cell damage at any of the intervals studied. The histological appearance of the intestinal crypts obtained from a control animal and from rats killed 2 and 6 hr after the administration of CCM is demonstrated in Figs. 1 to 3. These observations confirm those of Kleinfeld and Sisken (28) which indicated that, under certain conditions, cells exposed to CCM are capable of re-forming a functional spindle and completing mitosis.

Mitotic Response to the Combined Administration of ara-C and CCM. On the basis of the data derived from the preceding experiments, it seemed reasonable to consider that a synergistic effect on mitotic activity might be obtained through the utilization of ara-C in conjunction with CCM. For a test of this hypothesis, ara-C was administered at zero time and CCM was administered 12 hr thereafter. Additional rats that served as controls received the following combinations of injections at comparable times: NaCl-NaCl, NaCl-vehicle, NaCl-CCM, ara-C-NaCl, or ara-C-vehicle. All animals were killed 2 hr after the 2nd injections. Chart 1 summarizes data obtained from 3 separate experiments; findings in each of the 3 independent studies were quite similar and reproducible. The mean mitotic activity in the NaCl-NaCl or NaCl-vehicle-treated animals ranged between 4.7 and 5.1%, while mitoses increased to approximately 15% in rats receiving 0.9% NaCl solution followed by CCM. In those animals treated with ara-C-NaCl or ara-C-vehicle, approximately 8.0 to 8.5% of the crypt cells were in mitosis. However, in rats given injections of both ara-C and CCM, 38% of the crypt cells were undergoing division. Of additional significance was the finding that this latter regimen was not necrogenic to the intestinal epithelium (Fig. 4).

Maintenance of Synchronization. To determine the degree to which this synchronization of cell division was maintained, animals were given either 0.9% NaCl solution or ara-C at zero time and CCM 12 hr later. Groups of rats were then killed at 2-hr intervals beginning at 14 hr and terminating at 24 hr after the initial injections. Table 2 shows that mitotic activity in animals treated with NaCl-CCM followed a pattern analogous to that found in our initial studies with this agent (Table 1). Additionally, Table 2 reveals a pronounced increase in mitotic cells in ara-C-treated animals that were killed 2 hr after the subsequent administration of CCM. In these latter rats, 33% of the crypt cells were in mitosis at this particular interval. This value is consistent with the earlier findings reported above. However, after this initial augmentation, the percentage of mitotic cells in ara-C-CCM-treated animals killed 4 hr after the administration of CCM dropped abruptly to 2.5% indicating a
release from the blockade. Mitosis then returned to the 3 to 5% levels observed previously in control rats treated with either 0.9% NaCl solution or vehicle.

For a further examination of the kinetics of cellular proliferation induced by the concomitant administration of ara-C and CCM, additional studies that extended over a longer period of time and utilized a 2nd injection of CCM were conducted. The design of and results obtained from these experiments are outlined in Table 3. In control animals receiving NaCl-vehicle-vehicle, from 4.0 to 4.7% of the crypt cells were in mitosis. No significant differences in the percentage of mitoses were noted at any of the intervals in animals receiving either NaCl-CCM-CCM or ara-C-CCM-CCM. In both instances the values were essentially the same as those observed in animals killed 2 hr after the injection of CCM alone. Measurements of DNA synthesis (E. Farber and H. Liang, unpublished data) in the total intestinal wall of ara-C-CCM-treated animals showed a pronounced decrease (80%) in the incorporation of labeled thymidine into this species of nucleic acid at 12, 14, and 18 hr after the initial injection of the analog. DNA synthesis continued to be markedly depressed at 20 hr even though the percentage of mitoses at that particular time had fallen to within the control range (Table 2). Thus it appears that the initial synchronization of cell division induced by the combined utilization of ara-C and CCM is lost once traversal through the cell cycle is reinstituted. A similar dispersion or decay of synchrony has also been noted in many different cell types in vitro (e.g., Refs. 41 and 62).

**DISCUSSION**

Inherent to a more rational approach to the treatment of neoplastic disorders is a greater understanding of the mechanisms by which certain antimetabolites interfere with the functional activities of replicating cells. Requisite to the accumulation of such information is the ability to study the effects of these agents at specific loci within the cell cycle. Such an approach can be achieved most effectively by utilizing synchronous cell populations in vivo. Efforts to induce cell synchronization in the intact animal have, for the most part, been hindered by at least 2 important factors: (a) the inability to collect sufficiently large numbers of cells in any given compartment of the cell cycle and (b) the induction of irreversible damage in those cells exposed to the inhibitor. That these difficulties are not insurmountable, however, has recently been demonstrated by the findings of Rajewsky (49) and Nitze et al. (42) which disclosed that a considerable degree of synchronization of cell division, without a concomitant loss of cell viability, could be achieved in vivo in rodent or human malignant neoplasms exposed to hydroxyurea or 5-fluorouracil. Moreover, Bertalanffy and Gibson (4) have recently reported synchronization of proliferating cell populations in murine melanoma and Ehrlich ascites tumors treated with ara-C.

It is now apparent from the data obtained from the present study that similar phenomena can be successfully reproduced in normal tissues comprised of cells programmed for continuous replication, i.e., the crypts of the small intestine of the rat. The procedure used to accomplish these results involves the induction of sequential blocks in 2 different stages of the cell cycle by the administration of ara-C followed, at an appropriate time, by the injection of CCM. The former compound, by virtue of its ability to interrupt DNA synthesis,
institutes an S-phase block. The latter agent prevents progression through actual mitosis by impeding migration of the mitotic centrioles, thereby interfering with the organization of the mitotic spindle (6, 7). The efficiency of this regimen is readily apparent; treatment with either ara-C (64) or CCM results in peak mitotic values of approximately 15%. When compared with controls, this represents a 3- to 5-fold increase in the number of mitoses. When these compounds are used sequentially, however, an even more substantial elevation of the mitotic index occurs. Under these particular conditions, from 33 to 38% of the crypt epithelial components can be identified as dividing cells, thus reflecting a considerable degree of synchrony. The magnitude of the synchronization can best be appreciated by examining the various parameters ascribed to the cell cycle of the intestinal crypts. In the rat, the total generation time of the proliferating cells has been reported to be approximately 12 hr, with the mitotic phase occupying 1 hr (9). Our observations as well as those of other investigators (30, 33) indicate that, in control animals, from 3 to 5% of the epithelial cells are in mitosis at any given time. Consequently, these latter values would represent one-twelfth of the population acutally traversing the cell cycle. Therefore, if total synchronization were achieved, one would expect this to be reflected by a 12-fold increase in the number of dividing cells, in which case from 36 to 60% of the crypt cells would be in mitosis. The higher of these 2 estimated values is in good agreement with that determined by Lesher and Bauman (33). Therefore, the 33 to 38% values obtained by treatment with ara-C and CCM indicate synchronization of at least two-thirds of the proliferating epithelial cells in the intestinal mucosa. However, our data also indicate that this synchronization is dissipated once the cells originally arrested in mitosis pass into subsequent stages of the cell cycle. Under our experimental conditions, this appears to occur once the cells enter into G1, presumably as a result of the variability and complexity of the regulatory mechanisms operative during this stage of the cycle (46). Nevertheless, by first treating animals with ara-C-CCM and then injecting other metabolic inhibitors at 10, 14, or 16 hr after the initial administration of ara-C, it may be possible to determine the effects of these other agents on cells in G2, M, or G1, respectively. Consequently, the system described in this communication may potentially provide a unique model that can be utilized to ascertain the response of proliferating cells in the intact animal to disturbances in their biosynthetic activities during different, predetermined stages of the cell cycle.

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Fig. 1. Mucosa of the small intestine of a 0.9% NaCl solution-injected rat. Several mitotic figures are present in the Lieberkühn crypts. H & E, × 400.

Fig. 2. Mucosa of the small intestine 2 hr after the i.p. administration of CCM (0.05 mg/100 g). Note the numerous mitotic figures and the absence of necrosis in the crypts. H & E, × 400.

Fig. 3. Mucosa of the small intestine 6 hr after the i.p. administration of CCM (0.05 mg/100 g). Mitotic activity has returned to control levels, and no evidence of cell damage is apparent. H & E, × 400.

Fig. 4. Mucosa of the small intestine of a rat given i.p. injections of ara-C (250 mg/kg) at zero time and at 12 hr with CCM (0.05 g/100 g). The animal was killed 14 hr after the initial injection. The number of mitoses in the crypts exceeds that noted in animals given injections of either 0.9% NaCl solution (Fig. 1) or CCM (Fig. 2) alone. Note also the absence of crypt necrosis. H & E, × 400.
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