Early Changes in the Dynamics of Crypt Cell Populations in Mouse Colon following Administration of 1,2-Dimethylhydrazine

Thomas C. Richards

Department of Anatomy, School of Medicine, University of Oregon Health Sciences Center, Portland, Oregon 97201

SUMMARY

The effects of the carcinogen, 1,2-dimethylhydrazine (DMH), on the proliferative characteristics of the crypt cell population of mouse colon were studied. DMH (20 mg/kg body weight) was injected s.c., weekly, for 2, 8, 16, 20, or 26 weeks. At the end of each treatment period, a group of animals was injected with [3H]thymidine and killed. After 2 weeks of DMH treatment, the crypts appeared normal histologically, but the total number of cells, the number of labeled cells, and the percentage of labeled cells per crypt column had increased. The relative distribution of labeled cells in crypt columns was not changed. DMH treatment did not affect the phases of the cell cycle of epithelial cells and the transit time of these cells through the crypt. None of the indices of crypt dynamics were altered further with the appearance of focal atypias (after 16 weeks of DMH). However, the total number of cells per crypt increased and the percentage of labeled cells decreased as adenocarcinomas developed in adjacent areas of the mucosa (after 20 to 26 weeks of DMH). The exact role of these early mucosal changes in the eventual development of malignant tumor has not been established. However, it appears that DMH carcinogenesis may involve two steps: (a) an initial increase in the number of mitotically active cells leading to an enlarged cell population; and (b) an eventual transformation of at least some of the crypt cells of the enlarged population.

INTRODUCTION

Several recent reports (5, 6, 20, 21) have shown that a high incidence of adenocarcinoma in distal colon can be induced by s.c. injection of DMH into mice or rats at weekly intervals for 20 to 30 weeks. However, because of different experimental approaches used by various investigators, it appears that DMH carcinogenesis may involve two steps: (a) an initial increase in the number of mitotically active cells leading to an enlarged cell population; and (b) an eventual transformation of at least some of the crypt cells of the enlarged population.

MATERIALS AND METHODS

Animals and Treatments

Female Swiss-Webster mice, at least 60 days old and 20 g in body weight, were housed 5 animals/cage and were given free access to food and water. The animals were given weekly injections of DMH-HCl (20 mg/kg body weight; American Scientific, Portland, Oreg.). DMH was dissolved in 0.001 M EDTA, adjusted to pH 6.5 with sodium bicarbonate, and injected s.c. Controls were given comparable volumes of 0.001 M EDTA.

Animals were divided into 5 groups with at least 30 experimental and 30 controls in each group. DMH treatment was initiated at the same time for all groups and was carried out for 2, 8, 16, 20, or 26 weeks. One week after cessation of treatment, the animals were killed and their colons were analyzed. Several animals (3 to 7) were used to determine changes in DNA-synthetic activity in cells of the crypt; each animal was given an i.p. injection of [3H]Tdr, 0.5 μCi/g...
body weight (specific activity, 20 Ci/mmol; New England Nuclear, Boston, Mass.), and killed by cervical dislocation 1 hr later. The remainder of the animals were used to determine the length of the cell cycle; they were given injection of [3H]TdR and killed at intervals of 1 hr from 2 to 12 hr later or intervals of 2 hr from 12 to 36 hr later. One group was killed at 48 hr.

**Tissue Handling**

A segment of distal colon at the level of the pelvic rim was excised and used for analysis because previous studies demonstrated that the descending colon was the most frequent site of tumor formation (5, 7). Care was taken not to distort the colon during removal. A small section was excised from the middle of the segment, washed thoroughly in ice-cold 0.9% NaCl solution, fixed in Carnoy's fluid for 2 hr, and postfixed in 10% neutral-buffered formalin for 24 hr. The tissue samples were embedded in paraffin and 5-μm sections were prepared. Selected slides were prepared for histological analysis and stained with hematoxylin and eosin.

For radioautographic processing, 2 slides from each colon were treated with 1 N HCl (60°) for 1 hr to hydrolyze the nucleic acids and to remove any free labeled nucleotide (18), immersed in Schiff's reagent, and rinsed in sodium bisulfite (9). The slides were then dipped in NTB3 emulsion (Eastman Kodak Co., Rochester, N. Y.), dried for 1 hr, exposed for 3 weeks, and developed in Dektol (Eastman) (15).

**Analyses of Crypt Columns**

Crypt columns, defined in these experiments as columns of epithelial cells along 1 side of a mucosal crypt, were selected for cell kinetic analysis if they were: (a) not distorted by the presence of abnormal cell lesions (focal atypias) or tumors, (b) sectioned longitudinally from top to bottom, and (c) in contact with the muscularis mucosae. The sections selected for examination were spaced so that each crypt column was encountered only once. Fifty crypt columns were analyzed for each animal.

**Ll.** Four indices of kinetics of crypt cells were measured. The length of the crypt column was determined by counting the numbers of labeled cells in quarters and segments of the crypts. Division of the crypts into quarters and segments was based on the relative lengths of the crypt column.

Crypts were divided into quarters, and each labeled cell was assigned to a specific quarter. An analysis of variance was used to test for differences in values within each quarter of the crypt. The Newman-Keuls multiple range test was used to determine differences in values between the quarters of the crypt. Differences between the means from control and DMH-treated animals for each quarter were determined by an analysis of variance.

The crypt was further divided into 10 equal segments to localize more accurately changes in the distribution of mitotically active cells; the percentage of labeled cells in each crypt segment was calculated to determine whether or not there was an increase in the proportion of cells that were dividing. These data were transformed for statistical purposes by the arc sine of the square root of the percentage, and differences in distribution of labeled cells between control and DMH-treated animals were determined by covariance analysis (19).

**Length of the Cell Cycle.** The phases of the cell cycle of crypt cells were determined from a curve that was generated by plotting the fraction of labeled mitoses against the time after injection with [3H]TdR (17). At least 50 mitoses from each animal were counted. The curves for the control and DMH-treated animals were fitted to the points by hand, and individual pairs of points along the curves were compared statistically by Student's t test.

**Rate of Migration In Crypt Columns.** The velocity at which cells moved upward in the crypt was deduced from the distribution of mitotic cells in the crypt. The number and position of all mitotic figures were recorded. It was assumed that the length of mitosis was 1 hr at all levels of the crypt. The rate of migration of cells at any position in the crypt was considered to be equal to the cumulative rate of proliferation up to that position. The rate of proliferation was determined by the mitotic index of each position (1).
weeks was an increased cellularity (compare Figs. 1 and 2). Although only the changes in the crypts were quantitated, there appeared to be an increase also in the number of cells in the lamina propria and submucosa. Crypt cell length initially increased by about 25% over control values (Table 1). Subsequent treatments caused no further change in the length of the crypts until after 26 weeks when the crypt length increased by 40% over controls. This marked increase in the total number of cells coincided with the appearance of frank tumor in adjacent areas of the mucosa.

Focal atypias were first observed in scattered crypts after 16 weeks of DMH treatment (Fig. 3). The atypias, defined as localized lesions of atypical cell structure and high mitotic activity (5), were usually located in the outer or luminal halves of the crypts. The lesions were irregular in shape and occupied areas of the lamina propria; they were not observed deep to the muscularis mucosae.

A few small but grossly visible tumors were observed in the colon from some animals after 20 weeks of DMH treatment. Multiple large lesions were observed throughout the distal colon of almost all animals treated for 26 weeks with DMH (Fig. 4). In many of the tumors examined histologically, cells in the base of the lesion had invaded the submucosa; this was interpreted as evidence of cancer. Large overgrowths of cells blocked the lumens of many colons examined.

LI. The mean number of labeled cells per crypt column and the LI increased after 2 weeks of DMH treatment (Table 1). The number of labeled cells per crypt column approximately doubled compared to control values, whereas the LI increased by a lesser amount. Neither of these values changed further with subsequent treatment until frank tumors began to develop after 20 to 26 weeks. In crypt columns adjacent to developing tumor, the crypt lengths increased markedly, whereas comparable increases in the number of labeled cells did not occur; hence, the LI decreased in these crypts.

### Absolute and Relative Distribution of Labeled Cells in Crypt Columns

The distribution of labeled cells in each quarter of the crypt column is shown in Chart 2. The absolute numbers of labeled cells in each quarter of the crypts were greater in DMH-treated animals than in controls ($p < 0.01$). Although there was some variation between the treatment periods as to the number of labeled cells within each of the quarters, analysis of variance revealed that these differences were not significant ($p > 0.05$). The analysis of variance and the Newman-Keuls multiple range test showed that there were significantly greater numbers of labeled cells in Quarter I compared to Quarter II ($p < 0.01$) and in Quarter III compared to Quarter IV ($p < 0.01$) for all durations of treatment.

The effects of treatment on LI in individual segments of the crypt columns are shown in Chart 3. Covariance analysis of data from control and DMH-treated animals indicated that the slopes of the 2 lines were the same but that there was an overall increase in LI in the experiments ($p < 0.01$). The distribution of labeled cells from animals treated for 26 weeks was different than that from animals in other treatment groups. The results revealed that (a) DMH treatment increased the number of labeled cells uniformly throughout the proliferative zone of the crypt; and (b) although the crypt columns lengthened initially, the relative distribution of the labeled cells in the crypts was not altered by DMH treatment until frank tumor development after 26 weeks. In these later animals, relatively fewer cells were seen at the base and relatively more cells were seen at the mouth of the crypt.

### Length of the Cell Cycle

The lengths of the cell cycle and S phase (DNA synthesis time) were measured in the crypt cell populations at selected times after DMH treatment (Chart 4). The values from animals treated with DMH for various durations were not different; therefore, the values

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### Table 1

**Effects of DMH treatment on cells of crypt columns from distal colon**

<table>
<thead>
<tr>
<th>Length of treatment (wk)</th>
<th>Treatment</th>
<th>Crypt length</th>
<th>Labeled cells/ crypt</th>
<th>LI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>DMH (4)</td>
<td>37.9 ± 1.7†</td>
<td>7.7 ± 0.7</td>
<td>20.5</td>
</tr>
<tr>
<td></td>
<td>Control (5)</td>
<td>31.3 ± 1.2</td>
<td>4.0 ± 0.6</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; 0.01$</td>
<td>$p &lt; 0.01$</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>DMH (4)</td>
<td>39.4 ± 1.8</td>
<td>8.5 ± 0.9</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td>Control (5)</td>
<td>28.3 ± 0.7</td>
<td>3.8 ± 0.9</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; 0.01$</td>
<td>$p &lt; 0.01$</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>DMH (3)</td>
<td>42.6 ± 1.8</td>
<td>9.0 ± 0.3</td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td>Control (3)</td>
<td>29.0 ± 1.4</td>
<td>4.4 ± 0.8</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; 0.01$</td>
<td>$p &lt; 0.01$</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>DMH (3)</td>
<td>42.1 ± 0.5</td>
<td>7.4 ± 0.3</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>Control (7)</td>
<td>31.8 ± 1.0</td>
<td>3.5 ± 0.2</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$p &lt; 0.005$</td>
<td>$p &lt; 0.01$</td>
<td></td>
</tr>
</tbody>
</table>

† Numbers in parentheses, number of animals.

‡ Mean ± S.E.

§ Controls in this group were mishandled and are not included.
showing that treatment of mice with DMH for 26 weeks induces a high incidence of adenocarcinoma in mucosa of distal colon. Small lesions, consisting of clusters of irregularly shaped crypt glands lined with atypical epithelial cells (focal atypias), appear on the mucosal folds of colon prior to the development of frank tumor. These lesions commonly occur between the 12th (5, 20) and 15th to 16th (13) weeks of DMH treatment, although a few scattered atypias were noted after 38 days of treatment (5, 20). In this study, atypias were first observed in colonic mucosa after 16 weeks of DMH treatment. In addition, the present results demonstrate that DMH leads to a marked increase in cellularity in colonic crypts several weeks before the appearance of atypias. The proportion of cells in the elongated crypts that were actively dividing, i.e., cells in mitosis or in the S phase of the cell cycle, also increased, although the relative distribution of dividing cells within the crypt was not affected. Further changes in the indices of cell kinetics of mucosal crypts were not observed until frank tumor had developed after 26 weeks of DMH treatment. These findings suggest that the mechanisms that regulate crypt dynamics are "reset" and that a larger crypt cell population is established long before the appearance of adenocarcinoma. It cannot be determined from the present results whether these initial changes are an integral part of the carcinogenic process or represent a less specific response to DMH.

Chan et al. (2) reported that, following a single i.r. instillation of DMH, the LI in distal colon increased by 2-fold after 24 hr and by 3-fold after 2 weeks. These changes in the LI occurred earlier and were greater in magnitude than those observed here. Such differences in latency and amplitude of response of DMH-sensitive cells in colon may be due to the route of administration and/or the concentration of DMH.

The eventual development of neoplastic growth in mucosa of colon may be dependent in part on disruptions in the normal distribution of dividing cells in the crypt. Epithelial cells located in the lower two-thirds of the colonic crypt (proliferative zone) undergo DNA synthesis and proliferative activity (4). These cells migrate toward the cryptal lumen and cease dividing as they reach the upper portion of the crypt (4, 12). Thunnhemr et al. (20) and Lipkin (10) reported that, in animals treated with DMH for 12 weeks, a greater number of dividing cells were located in cell positions further from the crypt base than in comparable controls. This alteration was interpreted as evidence for the expansion of the proliferative zone of the crypt. The results of the present study support this interpretation in that an absolute enlargement of the proliferative zone was observed; i.e., more cell positions in the crypt columns contained mitotically active cells. However, a relative enlargement of the proliferative zone was not demonstrated because the total number of cells in the crypt column also increased. Thus, the distance between the highest cells undergoing division and the mouth of the crypt remained fairly constant. Therefore, the action of DMH does not alter the relative distribution of labeled cells in crypts, at least not during the inductive period prior to the appearance of tumors. This difference in interpretation between the present study and the work of Thunnhemr et al. (20) and Lipkin (10) may be a reflection of the method by which the crypt cells were analyzed. In this study the LI was calculated from populations of cells in the

DISCUSSION

The results of the present experiments confirm the work of Thunnhemr et al. (20), Deschner (5), and Maskens (13) in
quarters and segments of crypt columns, and these populations were normalized to reflect fluctuations in the total number of cells in the crypt column. In contrast, Thurnherr et al. (20) and Lipkin (10) determined the LI of individual cell positions and did not take into account the crypt length.

The present results show that the crypt columns are enlarged by 2 weeks after initial DMH treatment. Chang and Nadler (4) suggested that the balance between cell production and cell loss is dependent in part on the maturity of the cells. An increased crypt length might be expected to result from an increase in cell division because less mature cells would be located higher in the crypts and would not be lost until they had had time to mature. Thus, lengthening of the crypt would continue until sufficient time for maturation had elapsed, at which point cell loss should again equal cell production (14). Under these conditions the population size would be reset at a higher level.

Kanagalingam and Balis (8) postulated that the development of tumor results from a reduced ability of cells to repair DNA damaged by chronic exposure to high doses of a carcinogen. Colonic mucosa consists of cells with a short G1 phase (10 hr) and a life-span of 100 to 120 hr (3, 4). In such a tissue errors in DNA could be incorporated into the next generation of cells if DNA repair is not completed before cell division occurs. In this way the errors would accumulate during chronic exposure to the carcinogen. Enlargement of the population may play a significant role in the carcinogenic response to DMH by increasing the number of cells at risk. Lipkin and Deschner (11) proposed that the inductive period represents the time necessary for enough generations of cells to pass until a population is reached that possesses the characteristics conducive to malignant growth. Under the conditions of the present study, the results suggest that 140 to 175 generations of cells, or somewhat less because all proliferating cells may not recycle immediately, are required before transformation occurs.

Neoplastic transformation in colon may be similar to the 2-stage induction of skin tumors. O'Brien et al. (16) showed in mouse skin that DNA and RNA synthesis is increased by "initiators" and that actual tumor formation is effected by specific "promoters" acting on the altered cells. That a similar mechanism may be involved in DMH-induced colon cancer is suggested by the finding that, after the initial changes in LI, no further alterations were observed in the crypt until the appearance of focal atypias after 16 weeks of treatment. Thus, the action of DMH may require 2 steps: (a) an initiating step that stimulates a pool of DMH-sensitive cells to divide and (b) a promoting step in which the enlarged cell population requires chronic insult in order to undergo alterations requisite for malignant changes.

The significance of the early mucosal changes to the oncogenic process cannot be determined from the present experiments. However, it appears that the DMH-induced transformation to tumor occurs in an altered population of crypt cells.

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REFERENCES

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