Morphometric Study of the Rat Duodenal Epithelium during the Initial Six Months of 1,2-Dimethylhydrazine Carcinogenesis

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ABSTRACT

Male Wistar rats weighing 100 g received 1,2-dimethylhydrazine (25 mg/kg s.c.) twice a week for 2 months and once a week thereafter for an additional 4 months. Groups of four to six rats were sacrificed monthly. Paraffin sections of duodenum were prepared and stained with periodic acid-Schiff and hematoxylin for cell counts and with toluidine blue for measuring nucleolar area. As an index of villus size and crypt size, the mean number of epithelial cells per representative sections of villi and crypts were used. Mitotic activity was assessed by counting the mean number of mitotic figures per representative crypt section. Nucleolar area was assessed from the analysis of drawn (camera lucida) images of nucleoli of columnar cells at six levels of the epithelium: lower, mid, and upper parts of the crypts and villi. Villus size increased progressively during the 6-month treatment, from 272 ± 2 (S.E.) to 349 ± 8. Crypt size increased from 118 epithelium: lower, mid, and upper parts of the crypts and villi. Mitotic number displayed a similar pattern of increase so that the percentage of mitotic figures in the crypts (mitotic index) remained constant (about 5%) in control and experimental animals. Nucleolar area in the controls decreased with age from 4.2 ± 0.08 sq μm in lower crypt at 1 month to 2.8 ± 0.04 sq μm at 6 months. During 1,2-dimethylhydrazine treatment, lower crypt nucleoli increased to 4.5 ± 0.12 sq μm after 3 months and decreased slightly thereafter, reaching 4.0 ± 0.14 sq μm by the sixth month. The nucleoli furthermore decreased gradually along epithelium (nucleolar compaction) by an average of 0.23% per cell position in control as well as treated animals. It appeared, then, that the main effect of 1,2-dimethylhydrazine was the enlarging of the four parameters measured. This effect seemed to relate in some manner to tumor formation as all the enlarge-ments were attenuated in intestinal tissue adjacent to tumors.

INTRODUCTION

Cancer of the colon and rectum accounts for approximately 15% of all malignant neoplasms in North America (37). World cancer statistics show that small intestinal cancer is more frequently found in countries that are known to also have a high incidence of colon cancer (28). In 1967, a reliable production of intestinal cancer in rats was established by Druckrey (18) using DMH2. This chemical carcinogen was found to induce tumors in the colon and the small intestine of rats (33, 44) showing a striking similarity to human carcinomas. Furthermore, DMH was found to affect the colon and, to a lesser extent, the small intestine. In the small bowel, the tumors formed primarily in the duodenum (38, 43, 44). The duodenum was also found to be the site of spontaneous small intestinal cancer most often seen in humans (28).

In our laboratory, the renewal of the small intestinal epithelium in the rat has been investigated, and some of the factors involved in the control of this renewal have also been identified (8). The aim of the present study was to extend these investigations to the epithelium that has been subjected to a specific carcinogenic influence. In a preliminary study, 4 parameters were found to respond rather markedly to DMH tretment. These were villus size, crypt size, mitotic number, and nucleolar size. These parameters were then analyzed systematically in samples taken from 5 regions along the small intestine. Most changes were observed in the epithelium of the duodenum. In the present article, these latter changes will be reported. Since these 4 parameters were previously used to evaluate certain aspects of renewal (6, 11), it was possible to use the present data to assess, to some extent, the effects of DMH on epithelial renewal.

MATERIALS AND METHODS

Animal Techniques. Young male Wistar rats (Charles River Canada Inc., St-Constant, Quebec) approximately 4 weeks old and weighing 100 to 150 g were used in the experiments. The experimental animals were given s.c. injections with a solution of 1,2-dimethylhydrazine dihydrochloride (Matheson Coleman and Bell Manufacturing Chemists, Norwood, Ohio) at a dose of 25 mg/kg body weight. The stock solution used for injections comprised 1000 mg of DMH dissolved in 200 ml of distilled water containing 37 mg of EDTA. The pH of this solution was adjusted to 6.5 using 1 N sodium hydroxide. The control animals were injected with an EDTA-0.9% NaCl solution only (44). Both experimental and control animals received injections twice weekly during the initial 2 months and only once a week thereafter. The experimental period extended to the end of 6 months. All animals were fed Purina laboratory chow and were allowed tap water ad libitum. They were kept in cages in groups of 4 to 6 and were maintained at a constant temperature (21°) and a 12-hour-night-light cycle.

In preparation for the injections, 4 to 6 animals were put in a plastic...
chamber which had an easily removable, clear plexiglass top. One to 2 ml of methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N. J.), a mild inhalation anesthetic, was then placed onto the bottom of the chamber and allowed to evaporate. At the first signs of anesthesia, the animals were removed, weighed, and then given s.c. injections with the appropriate amount of DMH (experimental group) or the EDTA 0.9% NaCl solution (control group).

Groups consisting of 4 to 6 DMH-treated rats were sacrificed monthly. The same number of control rats were also sacrificed at the same time. One week was allowed from the last DMH injection to the time of killing in order to allow recovery from any immediate toxic effect of the chemical (39). All animals were killed at approximately the same time of day (9 to 11 a.m.) in order to minimize the effects of diurnal variation (4).

Histological Techniques. All animals were killed by an overdose of chloroform. The small intestine was immediately removed, and a 1- to 2-cm piece of the upper duodenum was taken. This sample was cut open, flattened on a piece of cardboard, and then immersed into Carnoy’s fixative for 5 to 6 hr. It was then placed in 70% alcohol, cleared in chloroform, and embedded in paraffin.

The paraffin sections used for cell counts and for general histological observations were cut at 5 μm, stained by the periodic acid-Schiff technique, and counterstained in hematoxylin.

The paraffin sections used for measuring nucleolar size were cut at 10 μm. The sections were then stained with toluidine blue as described by Shea and Leblond (36). Before staining, the slides were exposed to a 5 × 10^-4% solution of DNase I (Sigma Chemical Company, St. Louis, Mo.) for 15 min at 37°C. This treatment enhanced the selective staining of the nucleolus without removing all chromatin.

Cell Counts. In well-oriented portions of the longitudinal sections of the duodenum, 3 parameters were determined by cell counts. These parameters were the number of epithelial cells per representative villus (“villus size” or “villus size index”), the number of epithelial cells per representative crypt (“crypt size” or “crypt size index”), and the number of mitotic cells per representative crypt (“mitotic number”).

A villus was called representative when the section passed along or nearly along the longitudinal central axis of the villus structure. These villi appeared relatively tall and finger-shaped with a single layer of columnar epithelium.

A crypt was called representative when the section passed along most (at least two-thirds) of its lumen. These crypts extended from the muscularis mucosa to the base of the villi. They showed a single layer of columnar epithelium on either side of the lumen.

The number of mitotic cells were estimated also in representative crypts. Since mitosis is known to be restricted to the lower two-thirds of the crypts (13), sections which contained only this lower portion of the crypts were also included in the counts.

The representative structures were located under low-power magnification (×100), while the cells were counted under high-power magnification (×1000). The crypt-villus junction was defined as the area adjacent to the lowermost points of the intervillus spaces. The intervillus spaces were recognized by the intense periodic acid-Schiff staining of their mucous content. Proceeding from the intervillus space, all epithelial nuclei were enumerated in villi as well as in crypts. All epithelial cell types (i.e., columnar, goblet, enteroendocrine, and Paneth) were included in the cell counts. As long as a normal distribution of cell types was seen in the sections, it was assumed that the columnar cells constituted the majority (about 90%) of the epithelial cells. In the assessment of mitotic number, all mitotic figures per crypt section were counted. These figures were well within the visible stages of mitosis which meant the presence of distinct chromosome accumulations. Most of these accumulations could be easily located since they were usually closer to the crypt lumen than the nonmitotic nuclei.

In the determination of the villus size index, 8 to 12 representative villi were counted per section of the duodenum. Similarly, for the crypt size index, 8 to 12 representative crypts were counted per section. In the assessment of mitotic number, 25 to 30 crypts were used per section. The counts for the villi, the crypts, and mitosis were then separately averaged to give values for that particular duodenal section. The minimal necessary number of villi and crypts to be measured in each animal was calculated by using a sample size which would yield a standard error less than 5% of the mean. The variation of villus size and crypt size was found to be small so that the necessary number of villi and crypts to be counted per duodenal section was approximately 4. However, mitotic number was more variable from crypt to crypt, and the minimal number of crypts to be used for measurements was 15. After the average values for villus size, crypt size, and mitotic number for each animal were determined, the mean for each entire group (i.e., 4 to 6 rats) was calculated along with the standard error of the mean.

Measurement of Nucleolar Size. The 10-μm-thick sections stained with toluidine blue were used to measure nucleolar size. In these sections, the nucleolus appeared dark blue, having a more or less rounded outline. The surrounding heterochromatin was less intensely stained, since it was partially digested by the brief exposure to DNase. The chromatin clumps were usually smaller than the nucleolus and of irregular outline. Also, the perinucleolar chromatin was easily distinguished from the nucleolus.

The sections were thick enough to include outlines of entire nucleoli which could usually be made out after proper focusing.

In well-oriented portions of the longitudinally cut duodenal sections, the crypts and the villi were each subdivided into lower, mid, and upper thirds under a microscope (Leitz, Wetzlar, Germany) equipped with a drawing tube (camera lucida). In each of these levels, the largest nucleoli were selected randomly and drawn on a white sheet of paper, at a constant magnification (×1800). In every section, 30 to 36 such nucleoli were drawn for each of the 6 levels along the epithelium. The area of each nucleolus drawn was then quantified with the use of a Videoplan Image Analyzer (Zeiss, Oberkochem, West Germany). The areas for each level were then averaged and the standard deviations were also calculated. Very little variation of area was found from nucleolus to nucleolus at a given level, so that the minimal number of nucleoli to be measured per level was usually not more than 5. Many of the cells contained more than one nucleolus, but always the one with the largest area was measured. The columnar cells at all the epithelial levels in the rat intestine were previously shown to contain an average of 3 to 3.5 nucleoli which did not fuse together or fragment during the migration of these cells (11).

Measurements in Tissue Adjacent to Tumors. The villus size index, crypt size index, mitotic number, and nucleolar area were all measured in the small intestinal mucosa adjacent to the site of the tumors found. Three tumors were used for these measurements. These included 2 adenocarcinomas that were found in the mid duodenum and the jejenum after 3 and 4 months of DMH treatment, respectively. The other tumor used was a microadenoma found in the lower duodenum of a rat after 3 months of treatment.

The 4 parameters were measured in 3 subsequent areas of tissue adjacent to each tumor. Using an ocular micrometer, the areas (measured from the side of the tumor) were designated as: Area 1 (0.0 to 0.41 mm); Area 2 (0.41 to 1.09 mm); and Area 3 (1.09 to 2.44 mm). In addition, measurements of nucleolar area were made in the cells within the tumors (Area 0).

RESULTS

Incidence and Pathology of Tumors. At the end of 6 months of DMH treatment, colonic tumors were found in all of the 6 animals examined. In 2 cases, 2 tumors were found per animal. The tumors were solitary adenocarcinomas, showing some invasion through the muscularis mucosa. In addition, 2 colonic tumors were found in the rats killed at 5 months and one tumor was found in a rat killed at 4 months. Of the 11 total tumors found, 6 were seen in the ascending colon, 3 in the transverse colon, and 2 in the descending colon. The tumors were often associated with marked lymphocyte aggregations either within or near the affected tissue.
Three small intestinal tumors were also found. In one animal, after 3 months of DMH treatment, a microadenoma was found in the mid duodenum (Fig. 1). One adenocarcinoma was found in the jejunum and lower duodenum in rats after 3 and 4 months of DMH treatment, respectively (Fig. 2).

The adenocarcinomas seen in the small intestine were composed mainly of epithelial cells forming gland- or crypt-like structures separated by acellular stroma. Also, within the tumor tissue, prominent nucleoli and a relative lack of goblet cells was observed.

Lymphocyte accumulations, which were usually prominent only in the ileum in the control animals, were often seen in the duodenum and jejunum of the treated animals (Fig. 3). These were mainly located in the submucosa and the lamina propria near the crypt base (Fig. 4). Also seen in the affected tissue were dilated lymphatic and blood vessels, especially in and around the tumors (Fig. 4).

Aside from obvious hyperplasia (Figs. 5 and 6) and lymphocyte aggregations, most of the tissue during the DMH treatment appeared to be normal under the light microscope. However, differences could be discerned in the size of the nuclei (Figs. 7 and 8). On the other hand, the quantitative measurements showed that the tissue was markedly affected soon after the initiation of the treatment.

**Villus Size Index.** The villus size index in the control animals showed no significant change during the entire 6-month period. The index remained close to an average value of 274.0 (Table 1). However, in the DMH-treated animals, a progressive increase in the villus size index was observed (Table 1). In the initial 3 months, this increase amounted to 9.2 to 9.5% above controls. A further increase was seen in the treated rats following each month until an average index of 348.5 (27% above controls) was reached by the end of the sixth month.

**Crypt Size Index.** The crypt size index in the control animals showed an apparent slight increasing trend with age. The index was only 127.1 (3.8% above controls) at 3 months. However, at 4 months, no difference between control and treated animals was observed. During the fifth and sixth months of treatment, an increase was again observed reaching 7.0, that is, 12.9%, above the control values in the 6th month.

**Mitotic Number.** The mitotic number (i.e., number of mitotic figures per crypt) averaged 6.0 in control animals (Table 1). It showed an apparent slight increasing trend with age. The index showed a definite increase in the treated animals (Table 1). The pattern was similar to that in crypt size index. During the initial 3 months, the increase was progressive, reaching 7.2, that is, 22% above controls, at 3 months. However, at 4 months, no difference between control and treated animals was observed. During the fifth and sixth months of treatment, an increase was again observed reaching 7.0, that is, 12.9%, above the control values in the 6th month.

**Mitotic Index.** The mitotic index expresses the proportion or percentage of crypt cells seen in mitosis at any given time (31). The mitotic index \( (M) \) was derived using the following equation:

\[
M = \frac{\text{Mitotic number}}{\text{Crypt size index}} \times 100
\]

The mitotic index in control animals was found to remain about the same throughout the entire 6-month period having an average value of 5.0% (Table 1). The same average value was found in the DMH-treated animals (Table 1).

**Nucleolar Area.** The nucleolus in the present study was found to decrease in size in relation to age and to increase in size during DMH treatment. Furthermore, a gradual decrease in nucleolar area was observed along the epithelium, that is, at the subsequent levels examined from the lower crypt to the upper villus (Chart 1). Thus, as the epithelial cells migrated from the crypt to the villus tip, a gradual diminution of their nucleoli took place. This latter phenomenon has recently been described (11) and will be referred to as nucleolar compaction. This nucleolar compaction was observed in both control and experimental animals. For example, in the control group sacrificed at the beginning (0 months), the percentage of crypt cells seen in mitosis was 6.8% (Table 1). The same average value was found in the DMH-treated animals (Table 1).
Duodenal Response to DMH

NUCLEOLAR COMPACTION IN THE RAT DUODENAL EPITHELIUM

Chart 1. Nucleolar area (sq µm) plotted against the level of the duodenal epithelium. Each column represents the average nucleolar area calculated from the group of 4 to 6 animals at 0 months (beginning of treatment). A continual and gradual decrease of nucleolar area can be seen between the lower crypt and upper villus. Bars, S.E.; LC, lower crypt; MC, mid crypt; UC, upper crypt; LV, lower villus; MV, mid villus; UV, upper villus.

Month) of the treatment, a 40% decrease in nucleolar area between the lower crypt and upper villus was observed (Table 2).

The nucleoli in the cells of the lower crypt were the largest seen in every age group whether control or experimental. A marked decrease of these nucleoli took place in the control animals with increasing age (Table 2; Chart 2). For example, the nucleolar area of the lower crypt in control animals decreased from 4.614 sq µm seen at 0 months to 2.850 sq µm seen at 6 months.

In contrast, in the DMH-treated rats, the nucleoli showed only a slight decrease with age (Table 2; Chart 3). For example, the lower crypt nucleoli measured 4.282 sq µm after 1 month of treatment and 3.973 sq µm after 6 months, a decrease of only 7%. However, during the first 3 months of treatment, a progressive increase took place at all 5 levels of the epithelium, not including the upper villus. At the fourth month, a decrease in all 6 levels began to take place (Table 2).

The difference in nucleolar area between the control and the experimental groups, especially at the level of the lower crypt, increased markedly with progressing treatment (Chart 3). These differences were also increased, but to a lesser extent, at the other 5 levels (Chart 3). The least difference was present at the level of the upper villus.

Data from Tissue Adjacent to Tumors. The villus size index, crypt size index, mitotic number, and nucleolar area were all measured in 3 adjacent areas next to each of the 3 small intestinal tumors (Table 3). All 4 parameters showed an increase in the region (Area 1) directly adjacent to the tumors. The values were lower but still elevated in the second region (Area 2), that is, slightly further away from the tumor tissue. The third region (Area 3), which was furthest away from the tumor site, was regarded as the base line. Here the values of 4 parameters were the lowest and were quite close to those measured in the tumor-free duodenum of DMH-treated rats.

Of all the parameters measured, the most marked elevations were found in the nucleolar area, especially in Area 1, that is,
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DECREASE OF NUCLEOLAR SIZE WITH AGE IN CONTROLS

Chart 2. Nucleolar area (sq μm) is plotted against time of treatment. At 0 months of the treatment, the animals were 1 month of age. The progressive decrease of nucleolar area with age in the control groups can be seen at all 6 levels of the duodenal epithelium. Points, mean from a group of 4 to 6 control animals; bars, S.E. Regression analysis showed a significant linear decrease with age at each level of the epithelium with negative slopes of 0.27, 0.25, 0.23, 0.22, 0.24, and 0.19, at each of the respective levels from lower crypt (LC) to upper villus (UV). MC, mid crypt; UC, upper crypt; LV, lower villus; MV, mid villus.

AGE-RELATED CHANGES OF NUCLEOLAR SIZE IN CONTROL and DMH-TREATED RATS

Chart 3. Nucleolar area (sq μm) is plotted against time of treatment, comparing control and treated groups. The upper portion of the graph shows changes of the lower crypt (LC) nucleoli over the 6-month period. The control values decrease linearly. The treated values show an increase during the first 3 months and a decrease thereafter.

The lower portion of the graph shows changes of the upper villus (UV) nucleoli. t tests showed no significant difference between the control and treated values at any month. Regression analysis showed a significant linear decrease in both sets of values, the control ones having a slope of -0.13, whereas the treated ones showed a slope of -0.10.

The middle portion of the graph shows changes in the average (AV) nucleolar area pooled from the 6 levels of the epithelium. An overall decrease with age is shown in the control groups, whereas the treated groups show an initial increase during the first 3 months and a slow gradual decrease thereafter. It can be seen that it is the difference between control and treated groups that progressively increases with time.

Table 3
Measurements in tissue adjacent to tumor

Cell counts and nucleolar measurements in tissue adjacent to each of 3 tumors found in the small intestine. All cell counts and nucleolar measurements were made in 3 consecutive adjacent areas starting from the tumor margin.

<table>
<thead>
<tr>
<th>Tumor 1st (mid duodenum)</th>
<th>Villus size index</th>
<th>Crypt size index</th>
<th>Mitotic index (%)</th>
<th>Nucleolar area (sq μm)</th>
<th>Nucleolar area in cells of the 3 tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area 1</td>
<td>236.5 ± 10.2 *</td>
<td>140.5 ± 5.5</td>
<td>8.0 ± 1.1</td>
<td>6.03 ± 0.2</td>
<td>7.034 ± 0.31</td>
</tr>
<tr>
<td>Area 2</td>
<td>235.0 ± 3.9</td>
<td>122.3 ± 4.3</td>
<td>6.9 ± 0.6</td>
<td>5.56 ± 0.16</td>
<td>5.21 ± 0.16</td>
</tr>
<tr>
<td>Area 3</td>
<td>211.8 ± 3.6</td>
<td>122.8 ± 3.5</td>
<td>6.8 ± 0.4</td>
<td>5.19 ± 0.17</td>
<td>4.69 ± 0.13</td>
</tr>
<tr>
<td>DMH-treated</td>
<td>301</td>
<td>132</td>
<td>7.2</td>
<td>4.49 ± 0.12</td>
<td>3.98 ± 0.16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumor 2 (lower duodenum)</th>
<th>Villus size index</th>
<th>Crypt size index</th>
<th>Mitotic index (%)</th>
<th>Nucleolar area (sq μm)</th>
<th>Nucleolar area in cells of the 3 tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area 1</td>
<td>346.9 ± 26.9</td>
<td>148.0 ± 10.0</td>
<td>6.6 ± 0.5</td>
<td>5.19 ± 0.22</td>
<td>6.470 ± 0.14</td>
</tr>
<tr>
<td>Area 2</td>
<td>294.8 ± 15.4</td>
<td>148.0 ± 10.0</td>
<td>6.6 ± 0.5</td>
<td>4.67 ± 0.21</td>
<td>4.77 ± 0.20</td>
</tr>
<tr>
<td>Area 3</td>
<td>275.1 ± 11.5</td>
<td>116.8 ± 6.9</td>
<td>5.3 ± 0.3</td>
<td>4.17 ± 0.18</td>
<td>3.98 ± 0.16</td>
</tr>
<tr>
<td>DMH-treated</td>
<td></td>
<td></td>
<td></td>
<td>3.06 ± 0.10</td>
<td>2.70 ± 0.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tumor 3 (jejunum)</th>
<th>Villus size index</th>
<th>Crypt size index</th>
<th>Mitotic index (%)</th>
<th>Nucleolar area (sq μm)</th>
<th>Nucleolar area in cells of the 3 tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area 1</td>
<td>315.6 ± 12.9</td>
<td>168.5 ± 25.5</td>
<td>5.8 ± 0.3</td>
<td>5.98 ± 0.41</td>
<td>6.551 ± 0.20</td>
</tr>
<tr>
<td>Area 2</td>
<td>280.6 ± 15.3</td>
<td>90.7 ± 2.3</td>
<td>5.8 ± 0.3</td>
<td>4.84 ± 0.16</td>
<td>3.45 ± 0.18</td>
</tr>
<tr>
<td>Area 3</td>
<td>240.6 ± 5.2</td>
<td>80.3 ± 2.6</td>
<td>5.1 ± 0.2</td>
<td>3.75 ± 0.14</td>
<td>2.77 ± 0.10</td>
</tr>
</tbody>
</table>

* Tumor 1, adenoma from mid duodenum after 3 months of treatment; Area 1, 0 to 0.41 mm from tumor; Area 2, 0.41 to 1.09 mm from tumor; Area 3, 1.09 to 2.44 mm from tumor; Tumor 2, adenocarcinoma from lower duodenum after 3 months of treatment; Tumor 3, adenocarcinoma from jejunum after 4 months of treatment.

* Mean ± S.E.
directly adjacent to the tumors. In Area 2, the nucleoli were still large but less than those in Area 1. The largest nucleoli were found within the tumors themselves (Area 0). While nucleolar compaction was evident in all the tissues near the tumors, the nucleoli were enlarged at all 6 levels of the epithelium in comparison to the same levels in the tumor-free duodenum.

**DISCUSSION**

The various investigators of DMH carcinogenesis found a high incidence of tumors in the colon, usually after 5 to 7 months of treatment (29,31,43). As in the present report, the small intestine also contained tumors but with a lower frequency (18,21,39,43,44). The incidence of small intestinal tumors was found to be dependent, to a great extent, on the dosage of DMH used (14,39). Furthermore, the duodenum was the most common site of the small intestinal tumors induced (33,39,43,44). In humans, spontaneous small intestinal tumors are known to occur mainly in the duodenum (28).

Aside from descriptions of induced tumors, other morphological effects of DMH on the small intestinal mucosa have also been reported in the literature. Wiebecke et al. (44) found localized areas of mucosal hyperplasia where villus enlargement and some fusion of the villi were noticed. In contrast, Haase et al. (21) did not observe villus enlargement in the mouse small intestine after 22 weeks of treatment. Crypt hyperplasia was demonstrated by Sunter et al. (39) in the rat jejunal mucosa after 6 months of treatment. These authors confined the measurements to the crypt enlargements and to the associated mitotic activity; villus size was not measured.

Villus size, crypt size, and mitotic activity have been examined in our laboratory in detail in the normal rat, and the relation of these parameters to epithelial renewal has been established (8,10). It was only recently that nucleolar size was found to be a parameter related to renewal (11). On the basis of the present data and our previous experience, we may now examine the possible significance of the various changes due to DMH and how these changes may relate to renewal.

**Villus Size.** The villus epithelial cells arise from the relatively undifferentiated cells of the crypt, where mitoses also take place. Cells eventually arising from this mitotic pool first become unable to cycle and gradually acquire features of differentiated absorptive cells (24). This process of differentiation is largely completed as the cells migrate through the upper crypt and reach the lower villus. Migration of the cells along the villus continues where further maturation takes place. After reaching the villus tip, the cells are lost by extrusion into the intestinal lumen (25). The size of the villus epithelium depends on the rate at which the cells from the crypts arrive and also on the rate at which these cells mature into the extruding cells of the villus tip (rate of maturation). An approximate estimate of this latter rate would be the turnover time of the villus epithelium, which is about 41 hr in the adult rat (9).

The size of the intestinal villi has been found to be proportional to the size of the villus epithelium and as in the present study, the number of villus epithelial cells per representative villus section has been accepted as an index of villus size (10). Furthermore, a villus size gradient was found to be maintained along the rat small intestine by villus size-controlling factors present in the intestinal chyme (5,10). Although these factors influence villus size, the villus structure is maintained by influences from within the tissue (10). One of the most important of these influences was shown to be the rate of protein synthesis within the villus epithelial cells (7,8). The rate of maturation of the cells appeared to be governed by the rate of protein synthesis.

In the present work, we found that DMH caused a progressive increase in the villus size index. During the same period of treatment, mitotic number increased to a lesser degree and in a different pattern. It follows that an increase in the output of cells from the crypts was not the sole cause of the progressive increase of villus size. It is likely that the rate of maturation of villus epithelial cells was slowed down by an increase in protein synthesis. DMH probably stimulated protein synthesis in some manner, and this effect appeared to be cumulative, that is, proportional to the time of treatment.

Turnover time of the villus epithelium may be used as an approximate measure of the rate of maturation of cells between their emergence on the villus and extrusion. The length of this time would be proportional to the time needed for maturation. Turnover time may be estimated by dividing the villus size index by the mitotic number and multiplying this value by a proportion factor. This factor may be the same for the control and experimental groups as long as the crypt frequency per villus is not changed by the experiment. In this case, the proportion factor can be omitted, and the ratios may be used as provisional turnover times for comparing the groups (6). In the present study, we estimated crypt frequency per villus in controls and in DMH-treated animals at 6 months. This frequency was 2.02 ± 0.07 (S.E.) in the control group and 2.05 ± 0.08 in the treated group. Thus, no significant difference due to DMH was present, and therefore the provisional turnover times were calculated for comparative purposes only. The average provisional villus turnover time for all control groups was 45 ± 0.4 hr. The turnover time for the treated groups was close to that of the controls during the initial 3 months. At 4, 5, and 6 months of DMH treatment, however, the average turnover time became elongated (about 5 to 6 hr longer) compared to controls. If the increase in the villus size index in the experimental groups would be caused by a proportional increase in mitotic number, there would be no change in the villus turnover time. If mitotic number would further increase above this proportion, then the turnover time would become shorter. However, if the villus size index increases without a proportional increase in mitotic number, then the turnover time would become longer. This latter event took place in 5 out of the 6 experimental groups. It appeared, then, that a mechanism was in operation which tended to maintain villus turnover at a relatively high level. The progressive increase of villus size may have been a morphological expression of this mechanism.

**Crypt Size Index.** While the crypt size index in the control animals remained fairly constant during the period of treatment, in the treated animals it showed a specific pattern of increase. A progressive increase was seen reaching a maximum at 3 months; then a decrease to near control level took place in the fourth month and a further progressive increase followed up to the sixth month. The sudden drop at 4 months merits some attention since this may be a result of factors counteracting the action of DMH. A progressive crypt hyperplasia in the large intestine with increasing duration of DMH treatment has been shown (34,35,40). For the small intestine, Sunter et al. (39) found crypt hyperplasia in the jejunal of DMH-treated rats. They found a sustained
increase (about 13%) in the mean number of cells of the crypts up to 24 weeks, when a more abrupt elongation (28 to 87%) took place which coincided with the first appearance of tumors.

The crypts in the small intestine contain mainly columnar cells aside from a relatively low proportion of goblet, enteroendocrine, and Paneth cells (32). The columnar cells go through 3 different stages of their life span while in the crypt: stem cell stage, proliferating (progenitor) stage, and early differentiation stage. The predominant location of these stages is the lower, mid, and upper crypt, respectively. The zone of early differentiation can be recognized as the mitosis-free zone of the upper crypt. This zone showed no increase in the treated animals, in fact in some locations it decreased. It thus appears that the crypt size increase by DMH took place by increasing the number of cycling cells. This could have taken place either in the progenitor stage or in the stem cell stage or in both stages.Unlike the villus turnover time, there was no significant change in the crypt turnover time after DMH treatment (about 20 hr in both control and treated groups). This would mean that the crypt size index changed in proportion with the changes in mitotic number. In other words, there was no change in the relative proportion of resting and dividing cells. This would mean that there was no change in the length of the mitotic phase and resting phase in the cycling cells; consequently, cell cycle time remained unchanged.

Mitotic Number and Mitotic Index. The increase in mitotic number followed the same pattern as that of the crypt size index (Table 1). This indicated that crypt size index and mitotic number were interrelated. Furthermore, since the increase of the villus size index followed a different pattern (no decrease was found), this latter parameter was probably independent of both mitotic number and crypt size index. Other lines of evidence have also shown this independence of the villus size index (6, 8).

The mitotic number depicts the overall number of mitotic figures per crypt whereas the mitotic index shows the proportion (or percentage) of mitotic figures in the crypts. Since mitotic number and crypt size index followed parallel patterns during the period of treatment, there were no major changes or fluctuations in the mitotic index. The mitotic index remained around an average value of 5.0% in all control and DMH-treated groups.

An increase in the mitotic number may be due either to an increase in the number of cycling cells, an increase in mitotic time, or a decrease in the cell cycle time. Each of these possible events would lead to an increased number of mitoses recorded. With an increased mitotic time or a shortened cell cycle time, mitotic index would increase. The fact that mitotic index remained unchanged, therefore, implies that it was the number of cycling cells which increased during the treatment. Similarly, Sunter et al. (39) found that the length of the S phase and the cell cycle remained more or less unchanged in the jejunum of DMH-treated rats using thymidine labeling. Tutton and Barkla (42) found no apparent cell cycle time differences in the crypts of the descending colon of control and DMH-treated rats, using the method of mitotic inhibition by vinblastine sulfate. Similarly, Richards (34) found no changes in the length of the cell cycle and S phase in the crypt cells of the distal colon of DMH-treated mice, using thymidine labeling.

An increase in the number of cycling cells may be accomplished either by an increase in the number of active stem cells and/or by an increase in the population size of the progenitor cells. The first possibility usually is accomplished through stimulating some of the reserve (G0) stem cells to enter cycling (23). To the best of our knowledge, however, no reserve stem cells are present in the small intestine (15). If, then, the number of active stem cells as well as the cycling time of these cells remains unchanged, the size of the progenitor cell population may be increased by an increase in the number of cycles before differentiation begins. This, then, is the probable event during DMH treatment.

Nucleolar Area. The most marked changes during DMH treatment were found in the nucleolar area. The importance of this parameter in intestinal morphology has only recently been realized when the migration and maturation of the columnar epithelial cells in the rat jejunum were shown to include a gradual compaction of the nucleolus. Also, specific changes were observed in the electron microscopic structure of the compacting nucleolus (11).

The present light microscopic technique for the analysis of nucleolar area and nucleolar compaction has certain advantages over the electron microscopic method, as large areas of tissue at well-defined levels can be scanned, and a large number of measurements can be made. However, this technique does not provide information on the ultrastructural events.

The present results have provided the first quantitative evidence of an age-related decrease in the nucleolar area at all the 6 levels of the duodenal epithelium over the 6-month period. The amount of this decrease appeared to be quite considerable even within a period of a month. Such a decrease with age was implied by Adamstone and Taylor (1-3) in studies on jejunum, kidney, and liver. It is probable that such a decline of nucleolar size may be associated with a decline in rRNA synthesis and protein synthesis. However, this is a subject for further studies.

The effect of DMH on nucleolar area should be viewed in comparison to the age-related decrease of nucleolar area in controls. That is, while the nucleoli in control animals decreased in size, there was no comparable decrease in the nucleoli of the treated rats. Nucleolar area in the treated animals increased progressively above initial values during the first 3 months of treatment and this was followed by some decrease in the last 3 months of the treatment. It is probable that during the latter 3 months, the age-related effects prevailed to some degree over the enlarging influence of DMH. The largest differences in nucleolar area between control and experimental groups were seen at the level of the lower crypt. It is probable that these nucleoli were affected primarily either by age or by DMH. It is likely that, as the cells migrated from the lower crypt, the nucleoli further decreased by the normal process of compaction. The nucleolar enlargement, initially caused by DMH, has also been observed in other studies of chemical carcinogenesis (20, 22). Nucleolar enlargement is believed to be associated with an increase in protein synthesis. Cancer cells are known to contain large nucleoli and, in addition, they engage actively in protein synthesis (12). In addition, nucleolar enlargement has been correlated in certain tumors with the grade of malignancy (19, 30). Drugs such as phytohemagglutinin and hormones, including estrogens, androgens, and growth hormone, have also been found to cause increases in nucleolar size (12).

The degree of nucleolar compaction may be estimated by the amount of nucleolar area lost. We estimated the total loss of nucleolar area between the lower crypt and the upper villus

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4 Unpublished observations.
Table 4

Amount of loss of nucleolar area

This rate is measured as the loss of nuclear area between lower crypt and upper villus in Columns 2 and 3. The approximate number of cell positions between the lower crypt and upper villus are given in Columns 4 and 5. The average rate of nucleolar compaction per cell position is given as the absolute loss of nucleolar area per cell position in Columns 6 and 7. This average rate can be expressed as the percentage of loss of nucleolar area, taking the lower crypt nucleoli as 100% (Columns 8 and 9).

<table>
<thead>
<tr>
<th>Time of treatment (mos)</th>
<th>Control</th>
<th>DMH-treated</th>
<th>Control</th>
<th>DMH-treated</th>
<th>Control</th>
<th>DMH-treated</th>
<th>Control</th>
<th>DMH-treated</th>
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<td>0</td>
<td>1.8</td>
<td>192.2</td>
<td>9.1</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.9</td>
<td>193.1</td>
<td>208.7</td>
<td>9.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.8</td>
<td>194.9</td>
<td>210.7</td>
<td>9.4</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>3</td>
<td>1.8</td>
<td>194.8</td>
<td>216.8</td>
<td>9.1</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.6</td>
<td>194.4</td>
<td>215.7</td>
<td>8.3</td>
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<tr>
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<td>195.2</td>
<td>229.4</td>
<td>8.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1.2</td>
<td>196.5</td>
<td>242.3</td>
<td>6.3</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Av.</td>
<td>1.7</td>
<td>194.5</td>
<td>220.6</td>
<td>8.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Near tumors:

| Area 1 | 2.5 | 223.0 | 11.2 | 0.19 |
| Area 2 | 2.3 | 196.9 | 11.7 | 0.22 |
| Area 3 | 1.9 | 174.4 | 10.9 | 0.25 |

* Sum of crypt size index and villus size index divided by 2.
* Amount of loss between lower crypt and upper villus divided by the number of cell positions.
* Percentage of loss from nucleolar area of the lower crypt divided by the number of cell positions.
* The values given are averaged from the 3 tumors examined.

Possible Significance of Changes Seen Near the Tumors.

The various results did not resolve whether the changes in the parameters were truly precancerous or represented a nonspecific influence of the carcinogen. This problem was approached by examining the small intestinal tumors themselves and the tissue adjacent to these tumors. Exact control tissue was not available since the tumors did not arise exactly in the region of duodenum examined regularly. However, the area (Area 3) farthest away from the tumors could be used as a base line in which values were close to the tumor-free tissue.

All changes observed as effects of DMH in the tumor-free tissue were present in an attenuated form near the tumors. It is then likely that the tumor secreted some substances which had an influence similar to DMH. Conversely, there is the alternate possibility that the tissue was most affected by DMH at and near the tumor and that, out of this very affected tissue, the tumor arose. At the present time, we have not resolved which of these 2 possibilities was effective. It seems to be certain, however, that the changes reported as effects of DMH were closely related to cancer and were not nonspecific influences. Furthermore, it also seems that DMH does not affect only localized areas, but it also has generalized effects on the duodenal mucosa.

REFERENCES


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Fig. 1. Adenoma in the mid duodenum of an animal after 3 months of DMH treatment. Adenoma is distinguished from adjacent normal epithelium by its altered structure and hyperchromatic staining. Periodic acid-Schiff-hematoxylin, × 139.

Fig. 2. Adenocarcinoma in the lower duodenum of an animal after 3 months of DMH treatment. Periodic acid-Schiff-hematoxylin, × 54.
Fig. 3. Accumulation of lymph nodes and lymphatic tissue is shown in the submucosa of upper duodenum of an animal after 3 months of DMH treatment. Such heavy accumulation was not seen in any of the control animals in this region of the intestine. Periodic acid-Schiff-hematoxylin, × 67.

Fig. 4. Lymphatic infiltration in the submucosa in the jejunum of an animal after 3 months of DMH treatment. Note dilation of vessels (arrows). Periodic acid-Schiff and hematoxylin, × 116.
Fig. 5. General histological appearance of the duodenum of a control animal at 3 months. Horizontal bar, crypt-villus junction. Periodic acid-Schiff-hematoxylin, x 86.

Fig. 6. General histological appearance of the duodenum of an animal after 3 months of DMH treatment. Increase in villus and crypt size in comparison to controls (Fig. 5) can be seen. Horizontal bar, crypt-villus junction. Periodic acid-Schiff and hematoxylin, x 86.

Fig. 7. Section through the lower crypt in the duodenum of a control animal after 6 months. These are thick (10 μm) sections. Prominent nucleoli can be seen (arrow). Toluidine blue, x 1618.

Fig. 8. Section through the lower crypt in the duodenum of an animal after 6 months of DMH treatment. Prominent nucleoli can be seen (arrow). The average size of the nucleoli is larger than that in controls (Fig. 7). For nucleolar measurements, the largest nucleoli seen were drawn to ensure that the section would include the entire nucleolus. Toluidine blue, x 1618.
Morphometric Study of the Rat Duodenal Epithelium during the Initial Six Months of 1,2-Dimethylhydrazine Carcinogenesis

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