Differences in the Acute Response of the Various Segments of Rat Intestine to Treatment with the Intestinal Carcinogen, Methylazoxymethanol Acetate

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SUMMARY

Previous work has shown that single injections of methylazoxymethanol acetate in rats induce tumors predominantly in the colon, occasionally in the duodenum, and rarely in the jejunum and ileum. These studies describe the acute pathological and biochemical alterations induced by this carcinogen in the different segments of rat small intestine and colon. Karyorrhexis was found in crypts of duodenum, cecum, and all segments of colon at 6 hr after treatment. Much of the cellular debris was removed by 24 hr, although mitoses did not return to normal levels until the third day after treatment. No pathological alterations were found in jejunum or ileum, even as late as 24 hr after treatment. Studies of DNA synthesis at 24 hr after treatment indicated that jejunum and ileum were much less affected than were duodenum, cecum, or colon. In contrast, 5-fluorouracil and nitrogen mustard, agents that can inhibit proliferating cells but are not known to be intestinal carcinogens, affected all of the segments equally. The results indicate that a correlation exists between those segments of intestine acutely affected by methylazoxymethanol acetate and the sites of eventual tumor development. The level of deacetylase activity in the various intestinal segments did not correlate with sensitivity to methylazoxymethanol acetate-induced inhibition of DNA synthesis. We also found that methylazoxymethanol acetate inhibited DNA synthesis in the duodenum and colon in rats with cannulated bile ducts. These data indicate that the carcinogen does not require biliary transport to the intestinal lumen to exert its biological effects. Mechanisms that might account for the observed selectivity in action of methylazoxymethanol acetate in the various rat intestinal segments are discussed.

INTRODUCTION

There are numerous reports citing the greater sensitivity of colon than of small intestine to the tumor-inducing properties of agents such as MAM, dimethylhydrazine, and azoxymethane (see Ref. 15). One explanation to account for the difference between colon and small intestine is the fact that the intestinal flora (1, 5, 12) and the bile acids (1, 2, 8, 9) can influence the incidence of colonic tumors. Colonic tumors can be induced, however, in germ-free rats (1, 5, 12) and in segments of rat intestine distal to a colostomy (14). It appears, therefore, that part of the difference in response of these 2 tissues to the tumorigenic effects of chemicals is due to factors inherent within the colonic epithelium itself. Experiments conducted by Gennaro et al. (3) support this suggestion. They transposed segments of mid-small intestine to the colon and segments of colon to the mid-small intestine. Rats treated with azoxymethane developed tumors of the colon, regardless of its location, while no tumors appeared in the segments of small intestine exposed to the milieu of the colon.

The above findings led us to investigate whether the different segments of small intestine and colon would also respond selectively to the acute effects of carcinogens. A carcinogen suitable for such a study is MAM acetate, which induces significant numbers of colon tumors in rats following a single treatment, with only a few tumors appearing in the small intestine (15). We report herein that, in fact, there appears to exist a relationship between those segments of intestine affected acutely and the sites of tumor formation.

MATERIALS AND METHODS

Weanling male Sprague-Dawley rats (CD line) were purchased from Charles River Breeding Laboratories, Brookline, Mass.; these were given Purina laboratory chow and water ad libitum and were used when 4 weeks old. MAM acetate was purchased from Schwarz/Mann, Orangeburg, N. Y.; horse serum cholinesterase (EC 3.1.1.8) and eserine (physostigmine) sulfate were from Sigma Chemical Co., St. Louis, Mo.; [methyl-3H]thymidine (2 Ci/m mole) was from New England Nuclear, Boston, Mass.; 5-fluorouracil and nitrogen mustard were taken from stock material kept in the Laboratory of Pharmacology at Sloan-Kettering Institute. All solutions for injection were made in 0.9% NaCl solution and, except where noted, were injected via the tail vein in unanesthetized rats in a volume of 10 ml/kg. Except for the isotopically labeled precursor, all solutions were prepared immediately before use. Animals given 0.9% NaCl solution served as controls in all experiments.
Protein and DNA content and incorporation of [methyl-\(^{3}H\)]thymidine into DNA were determined as previously described (17), except that the following liquid scintillator mixture was used for acid-containing fractions: 16.5 g PPO, 0.3 g dimethyl POPOP, 1000 ml Triton X-100, and 2000 ml toluene. For microscopic study, tissues were fixed in Bouin's solution; sections were stained with hematoxylin and eosin.

The effect of MAM acetate on incorporation of [methyl-\(^{3}H\)]thymidine into intestinal DNA of rats following cannulation of their bile ducts was also determined. For these experiments, 8-week-old rats were first anesthetized with sodium pentobarbital, 30 mg/kg i.p. Polyethylene tubing (Intramedic PE 10; Clay-Adams, Inc., New York, N. Y.) was inserted into their bile ducts and the rats were placed on an inclined board and covered to prevent loss of body heat. Solutions of 0.9\% NaCl solution or MAM acetate, 35 mg/kg, were given i.v. via the femoral vein. After 3 hr, the rats were given [methyl-\(^{3}H\)]thymidine, 50 \(\mu\)Ci/2 \(\mu\)moles/kg i.v., via the femoral vein and were killed 10 min later. The segments of intestine were removed and the incorporation of isotopic precursor into DNA was determined as described above. Additional anesthetic was administered as required during the 3-hr period.

Free MAM was prepared by incubating 50 mg MAM acetate with 40 mg horse serum cholinesterase in 20 ml buffer consisting of 20 mM sodium phosphate, 154 mM NaCl, pH 7.8, for 3 hr at 25\(^\circ\). The amount of acetate released during the incubation was determined by monitoring the decrease in pH with m-nitrophenol as indicator in a manner similar to that described by Rappaport et al. (11). To avoid having m-nitrophenol in the solution of MAM, aliquots of the reaction mixture were added to solutions of nitrophenol phosphate at intervals during incubation. Following the 3-hr incubation, when all the MAM acetate was deacetylated, the mixture was filtered through Amicon UM-iO Diaflo ultrafilters under 60 psi \(N_2\) gas. The ultrafiltrate was stored at \(-20\)°. For these studies, control rats received an ultrafiltrate of the cholinesterase solution without MAM acetate.

Deacetylase activity of rat tissues with MAM acetate as substrate was determined as follows. Tissues were homogenized with a Teflon pestle in buffer consisting of 20 mM triethanolamine and 154 mM NaCl, pH 7.8. The homogenates were filtered through gauze and the filtrate was diluted to 2.5 \(A_{280}\) units/ml buffer. Whole blood was collected via the aorta into a heparinized syringe and diluted to 40 \(A_{280}\) units/ml. The mixtures were equilibrated for 3 min at 35\(^\circ\). To 1 ml of the homogenates or dilutions of blood were added 3 \(\mu\)moles of MAM acetate, and the rate of deacetylation was determined by measuring the decrease in pH with time with a radiometer pH meter having a single electrode and expanded scale. Standard curves of pH versus acetate concentration were prepared with the use of acetic acid.

RESULTS

Microscopic Studies. The number of rats studied for histological alterations in the duodenum and descending colon at each interval after receiving MAM acetate is given in Table 1. The effect of MAM acetate, 35 mg/kg i.v., on mitosis in rat duodenum and descending colon. Rats were given either 0.9\% NaCl solution (\(\bullet\)) or MAM acetate (\(\bigcirc\)) and were sacrificed at various intervals thereafter. Sections of duodenum and descending colon were fixed and slides were prepared as described in "Materials and Methods." Numbers in parentheses, number of rats studied at each interval. The data are expressed as the mean ± S.E.

<table>
<thead>
<tr>
<th>Intestinal segment</th>
<th>% of control in Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum*</td>
<td>41.7 37.0 39.6</td>
</tr>
<tr>
<td>Jejunum</td>
<td>69.3 55.3 66.5</td>
</tr>
<tr>
<td>Ileum</td>
<td>68.7 78.0 72.6</td>
</tr>
<tr>
<td>Cecum</td>
<td>37.4 26.4 37.5</td>
</tr>
<tr>
<td>Ascending colon</td>
<td>34.9 40.3 20.5</td>
</tr>
<tr>
<td>Transverse colon</td>
<td>28.0 41.3 14.8</td>
</tr>
<tr>
<td>Descending colon</td>
<td>34.1 34.9 28.7</td>
</tr>
</tbody>
</table>

* In each of 3 experiments, the segments of 3 control rats were pooled, as were those of 3 treated rats. The results of each experiment are given.

\* The first several cm of intestine from the pylorus, with the bile duct at the mid-point of each sample, is denoted duodenum; the remainder of the small intestine was cut in half to give jejunum and ileum. The colon between cecum and anus was divided into 3 equal segments.

Chart 1. The effect of MAM acetate, 35 mg/kg i.v., on mitosis in rat duodenum and descending colon. At 24 hr after treatment with either 0.9\% NaCl solution or MAM acetate, 35 mg/kg i.v., weanling rats were given [methyl-\(^{3}H\)]thymidine (50 \(\mu\)Ci/2 \(\mu\)moles/kg) i.v., and were killed 10 min later. The tissues were homogenized and the DNA was extracted as described in "Materials and Methods."

Chart 1. In addition, at both 6 and 24 hr after treatment, 6 rats were studied for microscopic alterations in each segment of intestine (Table 1). The results obtained in the duodenum and descending colon at 6 and 24 hr after treatment are in agreement with those reported previously (17).
At 6 hr after rats received a carcinogenic dose of MAM acetate, 35 mg/kg (15), severe karyorrhexis was evident in the duodenum and in the transverse and descending colon; the change was more marked in the duodenum. The ascending colon and cecum were less affected. By 24 hr, much of the cellular debris was removed, although uneven alignment, enlargement of crypt nuclei, and some karyorrhexis were still evident. No pathological alterations were observed, however, in jejunum or ileum as late as 24 hr after treatment. Unlike the duodenum, at 72 hr, edema, dilated crypts, and mucosal atrophy could still be found in the descending colon. At 7 days, there were occasionally increased numbers of inflammatory cells in the lamina propria in both duodenum and colon. Except for a rare karyorrhectic cell observed in a few rats at 24 hr after receiving MAM acetate, the Peyer's patches of the small intestine and the lymphoid follicles of the colon appeared normal at each of the times studied.

The data in Chart 1, in agreement with the observed karyorrhexis, indicate that MAM acetate induced significant decreases in the number of mitotic figures as early as 6 hr in both duodenum and descending colon. The effect was greater in descending colon than in duodenum. The MAM acetate-induced inhibition of mitoses was long lasting and, in each tissue, the number of mitoses did not reach control values until the 3rd day after treatment.

DNA Synthesis. A reduction of the incorporation of [methyl-3H]thymidine into DNA can be due to either direct inhibition of DNA synthesis or a reduction of the number of proliferating cells. Either result represents an alteration induced by the agent under study.

The effect of MAM acetate, 35 mg/kg, on DNA synthesis was determined in whole small intestine and colon at various intervals after treatment (Chart 2). Inhibition occurred rapidly in colon, and at 6 hr DNA synthesis was decreased by 75%. This effect persisted for about 24 hr, after which DNA synthesis began to recover and returned to normal levels at the 3rd day. By contrast and in agreement with our earlier findings (17), the effect of MAM acetate on DNA synthesis in whole small intestine was minimal and short-lived.

Since much karyorrhexis and a significant inhibition of mitoses occurred in the duodenum (see above), it is probable that changes in DNA synthesis in the duodenum were masked by including in the analysis the jejunum and ileum which were relatively unaffected and which represent the major portion of the small intestine. DNA synthesis was determined, therefore, in each segment of the small intestine and, in addition, in the cecum and in the various segments of the colon. The data in Table 1 indicate that, in small intestine, DNA synthesis was inhibited in duodenum to a greater extent than in jejunal or ileum, and that cecum and each segment of the colon were affected similarly.

In another experiment using tissues pooled from 3 animals, DNA synthesis was determined at 6 hr after a lethal dose of MAM acetate, 70 mg/kg. The percentages of DNA synthesis in each segment relative to controls were as follows: duodenum, 7.6%; jejunal, 41.3%; ileum, 57.0%; cecum, 18.0%; ascending colon, 9.1%; transverse colon, 3.3%; descending colon, 6.3%.

As positive controls, we tested 5-FU and HN2, chemotherapeutic agents not considered intestinal carcinogens but having the ability to inhibit proliferating cells (4). At 24 hr after treatment with 5-FU, 50 mg/kg i.v., the percentages of DNA synthesis in the entire small intestine and colon were 17.2 and 10.4%, respectively. Decreasing the dose of 5-FU to a level that was no longer inhibitory to the small intestine, 25 mg/kg, resulted in the loss of inhibition in the colon as well. The percentages of DNA synthesis in the entire small intestine and colon of rats at 24 hr after receiving HN2, 2 mg/kg i.v., were 19.0 and 18.7%, respectively. Thus, there was no difference between small intestine and colon in sensitivity to the effects of either 5-FU or HN2. In contrast (Chart 2), at 24 hr after treatment with MAM acetate, the percentages of DNA synthesis in the entire small intestine and colon were 82.6 and 33.3%, respectively.

In order to determine the role played by biliary excretion in the transport of carcinogen to the intestinal epithelium, we measured DNA synthesis in adult rats that had their common bile duct cannulated prior to receiving MAM acetate, 35 mg/kg i.v. The results shown in Table 2 are similar to those obtained in intact rats (Table 1). The duodenum, cecum, and each segment of colon were more affected by MAM acetate than was the jejunum or ileum. MAM acetate had no effect on bile flow during the 3-hr period; control and carcigen-treated rats excreted, respectively, 5.1 and 5.3 ml in Experiment 1 and, respectively, 4.0 and 4.3 ml in Experiment 2.

The effects of free MAM were also studied. At 24 hr after receiving a dose of MAM equivalent to 35 mg/kg MAM acetate, rats were given a pulse dose of [methyl-3H]thymidine (see Table 1). The percentages of DNA synthesis in each of the intestinal segments (segments were pooled from 3 control and 3 treated rats) relative to controls were as follows: duodenum, 51.9%; jejunal, 84.5%; ileum,
The bile ducts were cannulated and the rats were treated as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Intestinal segment</th>
<th>% of control in Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>14.5</td>
</tr>
<tr>
<td>Jejunum</td>
<td>48.9</td>
</tr>
<tr>
<td>Ileum</td>
<td>65.4</td>
</tr>
<tr>
<td>Cecum</td>
<td>23.3</td>
</tr>
<tr>
<td>Ascending colon</td>
<td>23.3</td>
</tr>
<tr>
<td>Transverse colon</td>
<td>14.0</td>
</tr>
<tr>
<td>Descending colon</td>
<td>29.1</td>
</tr>
</tbody>
</table>

a In each of 2 experiments, DNA synthesis was determined in a control and in a carcinogen-treated rat. The results of each experiment are given.

b See Table 1, Footnote b.

### Table 3

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Deacetylase activity (units/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>329.0</td>
</tr>
<tr>
<td>Liver plus physostigmine sulfate</td>
<td>102.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>176.0</td>
</tr>
<tr>
<td>Duodenum</td>
<td>144.5</td>
</tr>
<tr>
<td>Jejunum</td>
<td>103.1</td>
</tr>
<tr>
<td>Ileum</td>
<td>78.4</td>
</tr>
<tr>
<td>Cecum</td>
<td>41.3</td>
</tr>
<tr>
<td>Colon</td>
<td>35.1</td>
</tr>
<tr>
<td>Blood</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a One unit of enzymatic activity can hydrolyze 1 nmole of MAM acetate per min at 35°.

79.3%; cecum, 34.2%; ascending colon, 23.8%; transverse colon, 19.1%; descending colon, 26.2%.

**Deacetylation of MAM Acetate.** Since free MAM is the biologically active material (6), it was conceivable that the differences in response described above might have been due to differences in the deacetylation of MAM acetate by the various intestinal segments. Deacetylation was therefore determined in homogenates of various tissues and in blood. The results shown in Table 3 indicate that blood was less active than other tissues in deacetylase activity and that colon, while being exceptionally sensitive to the acute effects of MAM acetate, had less activity than the other segments of the rat intestine. Liver and kidney were more active than any of the intestinal segments.

The enzyme responsible for deacetylation of MAM acetate is presumably cholinesterase, since the cholinesterase inhibitor, physostigmine, can prevent the biological effects of the carcinogen in vitro (10). The deacetylation of MAM acetate as determined in these studies was reduced by the addition of 30 μM physostigmine sulfate to homogenates of liver just prior to the addition of carcinogen.

### DISCUSSION

The acute actions of MAM acetate are minimal in jejunum and ileum in contrast to the effects of the agent on colon and duodenum. Since the agent induces few tumors in jejunum and ileum, these results suggest that a correlation exists between the acute effects of MAM acetate and tumor induction. A main point of interest is the resistance of jejunum and ileum to the actions of the carcinogen. The studies presented herein indicate that the relative resistance is not due to a lack of deacetylase activity and is not the result of differences in blood flow, since HN2 and 5-FU induced significant inhibitions of DNA synthesis throughout the intestines. It seems unlikely that the jejunum and ileum are less permeable to this agent than is the remainder of the intestines.

Some differences have been seen in the acute effects induced by MAM acetate in duodenum and colon. The inhibition of mitoses is greater in the descending colon and, at 72 hr after treatment, when the duodenum appears relatively normal, the colon is still altered histologically. The mechanism responsible for the more prolonged effect in colon may be related to that causing initiation of a greater number of tumors relative to duodenum. In addition, the intestinal flora and the bile acids, although not essential for induction of tumors (1, 5, 12, 14), may act as promoters of tumor formation in colon.

The fact that MAM acetate inhibits DNA synthesis in the duodenum and colon of rats with cannulated bile ducts indicates that the carcinogen can exert its biological effects upon the intestinal epithelium via the circulation rather than via the intestinal lumen. These data also suggest that the mechanism by which this carcinogen induces a greater incidence of colonic tumors than of duodenal tumors is not related to activation by colonic flora of biliary-excreted carcinogenic material.

We suppose that the differences observed acutely in this study could be due to selective enhancement of the conversion of MAM to its ultimate reactive form. Although MAM degrades spontaneously to liberate an alkylating moiety (7), it is conceivable that the activation of MAM could be enhanced enzymatically. In this regard, Schoental (13) has suggested that MAM might be activated by alcohol dehydrogenase. We found in preliminary experiments that the alcohol dehydrogenase activity of 169,000 × g supernatant fractions of rat tissues was highest in colon, duodenum, and cecum, with little activity in jejunum and ileum. We also found that MAM, like ethanol, can act as substrate in NAD-dependent reactions. We are currently attempting to determine whether those tissues sensitive to the acute effects of MAM acetate are able to activate the carcinogen to a greater extent than those tissues which are relatively unaffected.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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