A Comparison of the Effects of the Hypcholesteremic Agents, Cholestyramine and Candididin, on the Induction of Intestinal Tumors in Rats by Azoxymethane

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SUMMARY

The effects of the ingestion of cholestyramine (2% in Purina rat chow) and candididin (0.04%) on intestinal carcinogenesis by azoxymethane was studied in male Sprague-Dawley rats. Groups of 20 animals were given 8 mg of azoxymethane per kg per week for 25 weeks when the study was terminated. Although each drug affects lipid metabolism differently, they increased the number of intestinal tumors over those of controls to the same degree, from an average of 5 to 7.5 tumors per rat. There were no tumors in animals not injected with azoxymethane. The cholestyramine-treated group of rats developed significantly more tumors in the large intestine than did the other groups, whereas candididin-fed rats had the greatest number of tumors in the distal small intestine. The feces of the cholestyramine-fed animals contained more total bile acids and a greater percentage of secondary bile acids than in normal animals. In the candididin-treated group, the feces had increased amounts of cholesterol and its metabolites and also a greater degree of cholesterol degradation. These results suggest that bile acids have a greater promoting effect on tumorigenesis in the large intestine, whereas the effect of cholesterol and/or its degradation products is greater in the distal small intestine. These findings support the concept that the carcinogenic process may vary in the different segments of the intestinal tract.

INTRODUCTION

Current emphasis on the importance of diet in the etiology of cancer has stimulated interest in the use of animal models for studying the effects of dietary factors on chemical carcinogenesis (5). Such studies show that the ingestion of large amounts of fat increases tumor incidence in the intestine (21). Although it is unclear how excessive fat intake influences the development of intestinal tumors, one possibility is that the effect is mediated through changes in lipid metabolism.

One aspect of this alteration that has been studied extensively is the effect of bile acids on intestinal carcinogenesis. Nigro et al. (13) found that the frequency of AOM-induced tumors was increased when rats were given cholestyramine. This nonabsorbable resin acts by binding bile acids in the intestine, causing an increase in their secretion from the liver of the rat (8, 23). The end result is increased fecal elimination of bile acids, the hepatic degradation products of cholesterol (2, 9). Chomchai et al. (4), observed a similar increase in tumor frequency when the fecal bile acid content was increased by diversion of bile to the lower small intestine. In other animal models, diets containing varying amounts of cholesterol and/or its degradation products are given prenatally to rats to increase the number of adenomas in the large intestine of rats induced by rectal injection of N-methyl-N'-nitro-N-nitrosoguanidine (12). A similar result was obtained by rectal instillation of sodium deoxycholate in germ-free animals (18).

Another drug that has been found to have a hypocholesteremic effect when given p.o. to animals is candididin, a polyene macrolide. This agent appears to act by inhibiting cholesterol absorption from the intestine (22). Therefore, its hypocholesteremic mechanism differs from that of cholestyramine in that it appears to involve cholesterol directly.

There is no experimental evidence relating hypocholesteremia to colon cancer, but cholestyramine and candididin both cause specific alterations in lipid metabolism affecting the intestinal luminal environment in a manner that may relate to tumor development. The purpose of this study is to compare the effects of these drugs on intestinal tumor frequency induced by AOM and attempt to correlate changes with lipid substances found in the feces.

MATERIALS AND METHODS

Chemicals. AOM was obtained from Ash Stevens Co., Detroit, Mich., and was prepared as an aqueous solution. Cholestyramine was purchased from Merck Sharpe and Dohme, West Point, Pa., and candididin from S. B. Penick and Co., New York, N. Y. Radioactively labeled steroids, [14C]cholic acid and [14C]cholesterol, were obtained from New England Nuclear, Boston, Mass. The radiochemical purity was 96 and 98% for cholic acid and cholesterol, respectively, as examined by thin-layer chromatography.

1 Supported by the Matilda R. Wilson Fund.
2 To whom requests for reprints should be addressed.
3 Present address: Food and Drug Administration, 200 C Street S.W., Washington, D. C. 20204.
4 The abbreviation used is: AOM, azoxymethane.
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Animals. Six groups of 10-week-old Sprague-Dawley rats weighing approximately 200 g from Sparton Research Animals, Inc., Haslett, Mich, were used in this study. Each animal group was composed of 20 rats that were labeled, fed, and injected as follows: Group A, Purina rat chow (normal), AOM; Group B, Purina rat chow + 2% cholestyramine, AOM; and Group C, Purina rat chow + 0.04% candicidin. AOM. Groups D, E, and F are respective dietary groups run as no carcinogen controls.

Diets containing drugs were prepared on a weight percentage basis, with the powdered drugs mixed with granular Purina rat chow. When fed to the animals in this form, such diets contain approximately 5% fat. Animals were acclimated to their particular diets over a 3-week period. Weekly increments of candicidin were in amounts from 0.01 to 0.02 to 0.04% of the diet. For cholestyramine, the weekly increments were 0.5, 1.0, and 2.0%. Control animals were fed granular Purina rat chow. The s.c. injection of AOM at 8 mg per kg body weight per week was begun after the animals were fed the highest level of drug for a 1-week period. The injections were continued for the duration of the experiment and the diets and water were given ad libitum. All animals were caged individually.

At the end of 25 weeks of AOM injections, the animals were killed and necropsies were performed on all rats. Abdominal and thoracic tissues were examined grossly for evidence of tumors. The number, size, and location of all intestinal tumors were recorded. Histological preparations were made from 30 representative intestinal tumors, 10 from each group of animals receiving AOM. Tissues were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin. Tumors from each of the 3 groups of animals were selected for comparison as follows: 5 from the distal half of both the small and large intestine, all about 0.5 cm in diameter.

Biochemical Assays. Fecal samples were collected at designated time points from 10 animals in each group every 24 hr for 3 days. The material from 2 animals was combined for each sample to be analyzed and frozen at -20°. Fecal bile acids, and neutral steroids were extracted by the methods previously described (15). The efficiency of extraction of bile acids and neutral steroids was determined using [14C24]cholic acid or [14C4]cholesterol, respectively.

Trimethylsilyl ethers of neutral steroids were prepared as previously described (15). Bile acid methyl esters were analyzed by gas chromatography as partial or full trimethylsilyl ethers, using bis(trimethylsilyl)trifluoroacetamide as silylating reagent and carbon disulfide as injection solvent. The use of partial derivatives greatly improves the separation of deoxycholic, chenodeoxycholic, hyodeoxycholic, and cholic acids on columns of trifluoropropyl, methyl silicone polymers. A detailed description of this analysis will be reported elsewhere.

All gas-liquid chromatographic analyses were performed on a Varian Aerograph 2700 gas chromatograph (Varian Instrument Co., Palo Alto, Calif.) equipped with a 6-ft glass column [2 mm (inside diameter) x 0.25 inch (outside diameter)] packed with trifluoropropyl, methyl silicone polymers on 100 to 120 mesh Gas Chrom Q (Applied Science, State College, Pa). Gas flows were maintained at 20 ml of N2 per min, 40 ml of H2 per min, and 300 ml of air per min. The flame ionization detector was set at 275° and injector at 250°. Column temperatures were 230° for analysis of both the bile acid and neutral steroid derivatives. Peaks were identified by relative retention time and peak area enhancement with the use of standard reference compounds. A Hewlett-Packard 3380A reporting integrator interfaced with the chromatograph was used to quantitate, with 5α-cholestan as an internal standard. The analysis of each sample was done in duplicate.

Statistical Analyses. All data were analyzed with Student's t test (3).

RESULTS

Weight gain. Average body weights of animals in each experimental group were obtained at 4-week intervals. Animals given cholestyramine (Group E) gained the most weight (p<0.025 after 9 weeks), whereas the animals given candicidin (Group F) gained less than the normal diet animals (Group D, p<0.025 after 5 weeks). In each case, animal groups receiving injections of AOM (Groups A to C) gained less weight than their respective controls, but only those fed candicidin (Group C versus F) differed significantly over a long period of time (p<0.01 between 9 and 20 weeks). These data are summarized in Chart 1.

Tumor Frequency. All animals killed at the end of the experiment that received AOM (Groups A to C) developed intestinal tumors. A total of 19 rats remained in each group and are included in the data. Group A animals given the normal Purina rat chow diet developed 5.1 ± 0.89 tumors/rat (total, 97). Those animals given 0.04% candicidin (Group C) developed 7.47 ± 0.95 tumors/rat (total, 142), whereas those given 2% cholesteramine developed 7.79 ± 0.87 tumors/rat (total, 149). These increases in tumor frequencies between the normal diet and the 2 drug-fed groups were significant (A versus B, p < 0.025; A versus C, p < 0.05). However, there was no significant difference between the candicidin- and cholestyramine-treated animals (B versus C, not significant). These results are summarized in Chart 2. There were no tumors in animals not given injections of AOM (Groups D to F).

The location of intestinal tumors was determined by dividing both the small and large bowels into proximal and distal...
segments. When tumor frequency was studied with respect to location, significant differences were observed between the animal groups as is shown in Chart 2. Normal diet animals (Group A) developed the greatest number of tumors in the proximal large bowel, which contained an average of 1.94 ± 0.50 tumors/rat. About 1 tumor/rat developed in each of other segments of the intestine.

Candicidin-treated animals (Group C) developed the greatest number of tumors in the distal small bowel, 3.52 ± 0.57 tumors/animal. This was a significant increase over that of normal diet animals (p < 0.005). On the other hand, a significant decrease (p < 0.025) from the normal tumor frequency was observed in the distal large bowel of these animals. In the proximal segments of both the small and large bowels, the tumor frequency in candicidin-treated animals was not significantly different from that of normal animals.

Animals fed 2% cholestyramine (Group B) had the highest tumor frequency in the large bowel. There were 2.94 ± 0.43 tumors/rat in the proximal segment and 2.36 ± 0.44 in the distal. These tumor frequencies in the large intestine were significantly greater than those of normal animals, p < 0.05 and < 0.025, respectively. In the small intestine, there was a slight but significant increase (p < 0.025) in tumor frequency over that of normal animals in the proximal part, but there was no difference in the distal segment.

There were metastatic lesions in all 3 AOM-treated animal groups. The most common were peritoneal metastases; there were 7 in Group A, 10 in Group B, and 9 in Group C. Lung metastases were present in all 3 groups, 4, 11, and 8, respectively. Liver metastases occurred in only 2 animals, both in Group A. Ear cancers occurred in 2 rats in Groups B and C. Histologically, all tumors reviewed were adenocarcinomas, and there were no differences between groups in the degree of anaplasia or in the extent of invasion of the bowel wall in the tumors reviewed, all of which were of comparable size.

**Fecal Bile Acids.** Bile acids were extracted from feces by ethanolic solvation. We did not use the special method of Manes and Schneider (11) to extract conjugated bile acids from feces containing cholestyramine by including 0.5 N HCl in the ethanol. It would seem unlikely that bile acids, tightly sequestered by the cholestyramine matrix and not removed by exhaustive extraction into ethanol, could be held responsible for the promotion of tumor development we observed in the cholestyramine group.

The major fecal bile acids identified by gas-liquid chromatographic analysis were lithocholic, deoxycholic, hyodeoxycholic, chenodeoxycholic, 12-ketolithocholic, and cholic acids. The sum of the concentrations of these bile acids as a function of time is shown in Chart 3. In the normal animals (Group D), the sum of these bile acids ranged from 1.2 ± 0.19 to 2.7 ± 0.16 mg/g of dry feces at the 20th week. On the other hand, the total bile acid concentration in feces excreted by animals given cholestyramine or cholestyramine and AOM (Groups E and B) was significantly increased over that of normal animals at both the 10- and 20-week time points (p < 0.0005). The concentration of fecal bile acids was highest at 10 weeks, with 5.43 ± 0.42 mg/g of dry feces in Group B and 7.77 ± 0.34 mg/g of dry feces in Group E. In contrast, the animal groups receiving AOM, candicidin, or AOM and candicidin (Groups A, F, and C) did not have elevated concentrations of fecal bile acids at any time point. This finding is somewhat different from the increase in fecal bile acid concentration we observed in a previous study in animals receiving AOM (14). This difference probably is due to the different extraction procedure and enzymatic analysis used in the earlier study as com-
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Table 1

Percentage of secondary fecal bile acids

<table>
<thead>
<tr>
<th>% bile acids</th>
<th>AOM-injected</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk</td>
<td>Normal Group A</td>
<td>Cholestyramine Group B</td>
</tr>
<tr>
<td>0</td>
<td>78.9 ± 1.0</td>
<td>78.9 ± 1.0</td>
</tr>
<tr>
<td>10</td>
<td>79.1 ± 0.8</td>
<td>86.0 ± 0.7</td>
</tr>
<tr>
<td>20</td>
<td>79.5 ± 1.3</td>
<td>85.1 ± 0.5</td>
</tr>
</tbody>
</table>

\* % = \(\text{deoxy + litho + 12-ketolitho + hyodeoxy} \times 100\)

\* Results are expressed as mean ± S.E. for 5 determinations; comparisons made between experimental groups and normal control (Group D) at same time point.

\* \(p < 0.01\).

\* \(p < 0.05\).

\* \(p < 0.05\).

\* \(p < 0.01\).

\* \(p < 0.005\).

\* \(p < 0.01\).

pared with the extensive purification and gas chromatographic analysis of bile acids used here.

The concentration of the individual fecal bile acids as a function of time for each animal group is shown in Chart 4. During the course of the experiment, animals given cholestyramine (Group E) or cholestyramine and AOM (Group B) showed elevated concentrations of all major fecal bile acids \((p < 0.05\) to \(< 0.0005)\) over those of normal animals (Group D). In comparison, the time profiles of fecal bile acid composition from candididin, AOM, or AOM and candididin (Groups F, A, and C) were similar to that of the normal group. Secondary bile acids represented a significantly greater percentage of the major fecal bile acids in the cholestyramine and candididin controls (Groups E and F), as well as the cholestyramine-AOM-treated animals (Group B) compared with the normal animals (Group D) at 10 and 20 weeks. These data are summarized in Table 1.

Fecal Neutral Steroids. The total neutral steroid concentration (cholesterol + coprostanol + coprostanone) of feces from normal animals (Group D) ranged from 2.00 ± 0.23 to 2.52 ± 0.30 mg/g of dry feces during the course of the experiment. Significant increases over this level were observed in the other animal groups (Chart 3). The largest concentration of neutral steroids \((7.99 ± 0.30)\) was found after 10 weeks in feces from animals treated with candididin (Group F; \(p < 0.0005\)). At the same time point, animals fed cholestyramine (Group E) excreted \(4.89 ± 0.17\) mg of neutral steroid per g of dry feces. This was significantly lower than the candididin group \((p < 0.0005)\) but higher than that of normal group \((p < 0.0005)\).

Animals given AOM and candididin (Group C) excreted lower levels of neutral steroids at 10 weeks than did the animals given candididin alone \((p < 0.05)\). After 20 weeks, these 2 animal groups excreted about the same level of neutral steroids. In contrast, AOM did not change the level of neutral steroid excretion in cholestyramine-fed animals (Group B) at 10 weeks but did at 20 weeks (Group B versus E; \(p < 0.01\)). AOM itself (Group A) caused an increase \((p < 0.0005)\) in neutral steroid levels over those of the normal animals (Group D) at 20 weeks.

The time profiles for fecal cholesterol, coprostanol, and coprostanone for each animal group are shown in Chart 5. Fecal cholesterol concentration remained unchanged in the normal diet animals (Group D). Elevated cholesterol excretion was observed at 10 and 20 weeks in animals given cholestyramine or candididin, with or without AOM (Group D versus B, C, E, and F; \(p < 0.05\)). Only at 20 weeks of treatment did AOM alone cause increased cholesterol excretion (Group D versus A; \(p < 0.05\)).

Animal groups fed candididin or cholestyramine, with or without AOM, had a significant increase in fecal coprostanol over that of normal animals throughout the experiment (Group D versus B, C, E, and F; \(p's < 0.0005\)). The highest level of fecal coprostanol was observed at 10 weeks in the candididin group \((5.75 ± 0.17\) mg/g of dry feces). A slightly lower amount was observed in the feces of animals given candididin-AOM (Group C). Cholestyramine did not elevate the fecal coprostanol level to the same extent as candididin but to a level significantly greater than that of normal animals \((p's < 0.0005)\). In normal diet animals given AOM (Group A), the level of fecal coprostanol was elevated above normal only at 20 weeks (Group D versus A; \(p < 0.005\)).

Table 2 indicates the percentage of cholesterol degrada-
The candididin-treated animals developed a marked increase in cancers in the various segments of the intestine. The frequency is increased from an average of about 5 tumors/animal in control animals fed the Purina rat chow normal diet developed tumors that were fairly well distributed throughout the intestinal tract. The rats fed 2% cholestyramine developed tumors at a slower rate than those given injections of AOM (Group B) showed a significant increase in cholesterol degradation. Animals fed the normal diet (Group D) had no change in cholesterol degradation.

**DISCUSSION**

The results of this study contribute to the increasing evidence that certain aspects of lipid metabolism are involved in the etiology of intestinal cancer. We have altered lipid metabolism in the rat by the use of 2 hypocholesteremic drugs, and have found that both enhance AOM tumorigenesis in the intestine. When cholestyramine or candididin is fed to rats given s.c. AOM injections, the intestinal tumor frequency is increased from an average of about 5 tumors/rat in the controls to about 7.5 in the groups treated with either drug.

However, there was a striking difference in the distribution of cancers in the various segments of the intestine. The control animals fed the Purina rat chow normal diet developed tumors that were fairly well distributed throughout the intestinal tract. The rats fed 2% cholestyramine developed tumors at a slower rate than those given injections of AOM (Group B) showed a significant increase in cholesterol degradation. Animals fed the normal diet (Group D) had no change in cholesterol degradation.

The alteration in tumor distribution is of particular interest since the 2 drugs used act primarily in the intestinal lumen, but alter lipid metabolism by a different mechanism. Cholestyramine tends to lower cholesterol levels by increasing the elimination of bile acids (9). We observed a dramatic increase in the concentration (mg/g dry feces) of fecal bile acids in animal groups treated with this drug, with or without the carcinogen. Secondary bile acids represented a greater percentage of these bile acids when compared with the normal animals. This finding supports the work where increased secondary bile acids are correlated with enhanced tumorigenesis (15, 20). However, cholestyramine has been shown by Asano et al. (1) to enhance intestinal tumor development by 1,2-dimethylhydrazine in germ-free animals, where secondary bile acids are not formed. Although a mechanism for tumor promotion by cholestyramine is not clear at this time, a study into the possible correlation between bile acid synthesis and intestinal tumorigenesis may be relevant.

On the other hand, candididin appears to inhibit cholesterol absorption in the small intestine. In studies with cockerels, cholesterol and bile acid absorption from the intestine was significantly decreased by candididin, and the excreta of these animals had increased levels of cholesterol and bile acids (7, 10). In our study we did not observe an increase in the concentration of fecal bile acids over the normal from rats treated with candididin, with or without the carcinogen. There was, however, a large increase in the concentration of fecal neutral steroids, cholesterol and bile acids (7, 10). In our study we did not observe an increase in the concentration of fecal neutral steroids, cholesterol and co-prostanol. In addition, the percentage of degradation of fecal cholesterol was significantly increased in animal groups treated with candididin but not with cholestyramine.

The findings of this study suggest that variations in the mixture of acid and neutral steroid concentrations in the intestinal lumen have a selective promoting effect on the carcinogenic process in this animal model. Increased concentrations of cholesterol and/or its metabolites had greater promoting activity in the distal small intestine, whereas increased concentrations of acid steroids had a greater promoting effect on the mucosa of the large intestines.
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These results support the concept suggested by epidemiological evidence that the carcinogenic process may vary somewhat in the different parts of the intestinal tract (6). A better understanding of the promotion of this process may result from studies correlating interluminal lipids and the function of mucosal cells at different levels of the intestinal tract.

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