Induction of Adenocarcinomas of the Colon in Mice by Weekly Injections of 1,2-Dimethylhydrazine

N. Thurnherr, E. E. Deschner, E. H. Stonehill, and M. Lipkin

Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and the Cornell University Medical College, New York, New York 10021

SUMMARY

Thirty-four CF1 mice were given weekly s.c. injections of 20 mg of 1,2-dimethylhydrazine hydrochloride per kg; this treatment induced colonic carcinomas in more than 90% of the animals after 186 days. The earliest change was found to be a widening of the proliferative compartment within the colonic crypts; at a later stage, there was an increase of the tritiated thymidine-labeling index from 9.3% at Day 45 to 16.2% at Day 87. The first histological changes were focal hyperplastic changes and focal atypias on the tip of the folds of the distal colonic mucosa (Day 38) confined to single crypts. The colonic tumors were located in the distal colon and rectum, with a high incidence of squamous cell cancers originating from the anal canal. Saline extracts of colonic tumor material precipitated specific antiserum embryonic antigen. The colonie tumors were located in the distal colon and rectum, with a high incidence of squamous cell cancers originating from the anal canal. Saline extracts of colonic tumor material precipitated specific antiserum embryonic antigen. The colonie tumors were located in the distal colon and rectum, with a high incidence of squamous cell cancers originating from the anal canal. Saline extracts of colonic tumor material precipitated specific antiserum embryonic antigen.

INTRODUCTION

Although carcinoma of the colon is a very common neoplasm in man (22), it has been difficult to induce it to develop in animals by the use of either oncogenic viruses or carcinogens. Within the past few years, the development of carcinomas of the colon has been observed in rodents administered cfcad meal, its carcinogenic compound cycasin, and DMH. A potent carcinogenic effect on intestinal cells was first observed when cycad meal was fed to rats (14). Since that time, the group of 1,2-dialkylhydrazines and azoxyalkanes has been studied (8–12, 12). Studies in rats and mice have shown that s.c. injections of this group of dialkylhydrazines reproducibly induces tumors in the gastrointestinal tract of animals (10, 17–19, 24, 29, 30).

We performed this study in order to measure early changes in colonic cells occurring after administration of the chemical agent DMH. These changes include the appearance and distribution of tumor nodules in the intestine, early changes in DNA synthesis as revealed by microautoradiography, the activity of enzymes involved in the synthesis of nucleic acid precursor molecules, and the appearance of a tumor-specific embryonic antigen in the colonic mucosa of the mouse. The findings were compared with those observed in the colon of humans before and during the development of neoplasia.

MATERIALS AND METHODS

Among 56 female CF1 mice (Group 1) (Carworth Farms, New City, N. Y.) kept on a standard diet and water ad libitum, 34 were given weekly s.c. injections of 20 mg DMH per kg (Schuchardt, Munich, Germany) for up to 24 weeks. An equal number of control mice were used. Prior to injection, the DMH was dissolved in 0.001 M EDTA and was brought to pH 6.5 with sodium bicarbonate. The mice were sacrificed in groups of 3 at biweekly intervals for the first 8 weeks and then every 4 to 6 weeks. The 8 mice in Group 2 were given DMH for only 6 weeks; 3 and 5 mice (respectively) were sacrificed at Days 214 and 354 after the last injection. Animals used for enzyme assay had their last DMH injection 30 days prior to sacrifice.

Microautoradiographic Studies. In the microautoradiographic experiments, 1 hr before sacrifice the mice were given i.p. injections of 20 μCi of TdR-3H (New England Nuclear, Boston, Mass.) in 0.2 ml of 0.9% NaCl solution. The mice were sacrificed by heart puncture under ether anesthesia. At standard sites, specimens from the stomach, upper jejunum, terminal ileum, ascending colon, and distal colon were removed. For histology and autoradiography, the tissue was fixed in 10% neutral formalin. Hematoxylin and eosin stain and microautoradiographs were made as described previously (8).

Enzyme Assays. Ten DMH-treated animals (all but 1 of which had developed colonic cancer after a mean induction time of 230 days after the beginning of DMH treatment) and 10 control animals of the same age were assayed for TdR-kinase and for HPRT. In 1 experiment, 2 animals were processed for TdR-kinase assay.

The small intestine was opened in the cold room and rinsed
with cold Tris-HCl (pH 7.4), and a strip of upper jejunum was placed on an intestinal planing apparatus, described previously (11). With this planing apparatus, the upper one-third of the villus was removed (upper villus fraction). Then with a glass slide the remainder of the intestinal mucosa was removed (crypt fraction). This procedure was performed at 33°C in the cold room. The respective fractions of intestine were transferred into microhomogenizers containing ice-cold Tris-HCl (pH 7.4) and adjusted to a volume of 0.5 ml. The homogenates were centrifuged at 33°C at 15,000 X g for 15 min. The supernatants were used for enzyme assays as reported previously (11). The precipitates were assayed for DNA according to the method of Burton (3).

The assay of TdR-kinase was performed according to the method of Behki and Morgan (1) and the DEAE-paper method of Breitman (2). The 0.5 M Tris-HCl buffer (pH 8.0 at 37°C) contained 1 mg of NaF per ml.

In the HPRT assay, the reaction mixture contained 150 nmoles hypoxanthine-8-14C (New England Nuclear), 50 nmoles of 5-phospho-D-ribosepyrophosphate, 1 μmole MgCl2, 10 μmoles of Tris-HCl (pH 8.0), and 0.1 ml of supernatant containing enzymes in a total volume of 0.2 ml. The reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by immersion in boiling water for 3 min; then it was cooled and the precipitate was centrifuged at low speed for 10 min. An aliquot of supernatant (0.020 ml) was transferred on DEAE-paper (Whatman No. DE 81 paper) and processed according to the method of Breitman (2).

For fractionation of the small bowel mucosa, the planing apparatus described by Imondi et al. (11) was used. The supernatants for HPRT assay were carried out simultaneously after storage at -20°C for 3 weeks.

TSE-AG. Mouse TSE-AG was prepared as previously described (25, 26). Saline extracts of intestinal specimens of control and DMH-treated animals were used in an immunodiffusion technique with rabbit antimouse embryonic antigen. This rabbit antiserum had been absorbed with organ homogenates of control animals in this experiment. The assay detected TSE-AG in concentrations higher than 350 μg/ml.

RESULTS

The DMH-treated animals gained weight, as did the control animals (25 to 32 g). The animals were in good general condition until the late stage, when multiple tumors occurred. They did not have diarrhea, and the tumor carriers always had formed stool in the distal colon at the time they were sacrificed.

Gross Pathological Findings

At 135 days after the beginning of DMH injections, the 1st animal with tumor of the anal canal was found. Subsequently, 12 of 13 animals had 1 or more growths in the large bowel. At 210 days, all but 1 of the mice had 1 or as many as 40 tumor nodules in the distal large bowel. There was a predominance of tumor nodules in the distal colon (Chart 1). No tumors were found above the colon flexure in the ascending colon or cecum. Two animals had hemangioendotheliomas in the liver, and 1 had a mucus-producing cyst of 1 ovary. One animal developed a multifocal spindle-cell sarcoma of the lung. No gross histological changes were observed in the jejunum.

Histological Findings

The proximal colon and the upper jejunum 4 cm distal to the pylorus was examined in all animals.

Distal Colon. The animals sacrificed after 7 and 21 days did not show any histological changes. At Day 38 after the beginning of DMH injections, 1 animal had a focal hyperplastic change in a crypt (Fig. 1A) and one had a focal atypia (Fig. 1B). The distribution of these lesions and times of appearance are summarized in Chart 2.

At Day 87 after the beginning of DMH injection, all animals had microscopic abnormalities involving the colonic crypts. The glands were focally elongated, and there was local loss of mucus-producing cells, intersitial edema, and areas with increased inflammation. At Day 109, there was a diffuse disturbance of the colonic mucosa, and 2 of the 3 animals examined had several areas of focal atypias and 1 had an in situ carcinoma. At Day 135, 2 of 3 animals had multifocal in situ carcinomas and 1 had, in addition, a squamous cell carcinoma infiltrating and obstructing the distal rectum. At Day 186 and later, all animals had severely deranged mucosa of the distal colon with 1 or more adenocarcinomas. Adenomatous polyps and multiple carcinomas occurred in the same animals. Several animals developed squamous cell carcinomas infiltrating the pararectal tissue originating from the junction of the squamous and columnar cell epithelium at the anal orifice. Group 2 animals that received injections for only 6 weeks developed in situ carcinomas and infiltrating adenocarcinomas (3 of 8 mice; 7 of 8 mice had hyperplastic changes and adenomatous polyps).

Jejunum. The villi remained normal in size. No tumors developed in the small bowel during the entire 240-day experiment. There was no increase in inflammatory reaction in the DMH-treated animals, compared with controls.

Microautoradiography of the Rectosigmoid Mucosa

At 49 days after the start of DMH injections, when the histology was normal except for the focal changes mentioned above, abnormal microautoradiographic findings with tritium-
labeled thymidine were observed. Previous examinations of the colonic mucosa of rodents and humans (4, 15, 16), and this study, showed that epithelial cells proliferate in the lower two-thirds of the colonic crypts. These cells stop DNA synthesis and proliferative activity as they migrate to and reach the upper one-third of the cryptal columns (16). To evaluate numerically what happens during tumor induction with DMH, we counted tritiated thymidine-labeled cells to identify those synthesizing DNA, and evaluated their distribution in the crypts. Chart 3, A and B, shows the position of the tritium-labeled cells in the crypts in control and DMH-treated animals at Days 45 and 87. Only histologically normal crypts were scored. Fifty crypts per animal were analyzed. After 45 days of DMH treatment, more labeled cells were close to the luminal surface of the colonic crypts in treated than in control animals. After 45 days of treatment, the fraction of cells labeled (that is, the number of cells labeled compared to total epithelial cells) remained unchanged (labeling index, 9.3% in controls and 8.4% in the DMH group). After 87 days of treatment, both the position and the total number of cells labeled had increased (labeling index, 7.3% in controls and 16.2% in the DMH group). Furthermore, at the later interval, epithelial cells that were labeled 1 hr after injection with TdR-3H were present at the luminal surface of the colonic
Colon Cancer Induced in Mice

TIME OF APPEARANCE OF LESIONS

<table>
<thead>
<tr>
<th>TUMOR NODULES</th>
<th>0</th>
<th>40</th>
<th>80</th>
<th>120</th>
<th>160</th>
<th>200</th>
<th>240 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HISTOLOGY

<table>
<thead>
<tr>
<th>Inflammation</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal hyperplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Focal atypia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor specific embryonic antigen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chart 2. Time of appearance of gross pathological, histological, or immunological changes. *, positive pathological, histological, or immunological findings in 1 animal; 0, negative pathological finding for 1 animal.

crypts (Fig. 2). In some instances, cells incorporating thymidine at the surface of the mucosa appeared histologically normal.

Microautoradiography of the Jejunal Mucosa

Labeled cells in both groups were present only in the crypts below the villus-crypt junction, and no surface labeling of the villi could be demonstrated (in contrast to the colon).

Enzyme Assays

Ten control animals and 10 animals treated for 24 weeks with DMH were analyzed for TdR-kinase and for HPRT in the small bowel mucosa. Chart 4 compares the results of the upper villus fraction and the crypt fraction (including lower villus) in the DMH-treated and control mice. In the upper villus fraction, the control group had a mean TdR-kinase activity of 2.6 ± 0.65 nmoles/mg DNA/hr and that of the DMH group was 1.2 ± 0.62 nmoles/mg DNA/hr. The crypt activity of the controls was 16.0 ± 4.1 nmoles/mg DNA/hr compared to that of the DMH-treated group (22.4 ± 5.1 nmoles/mg DNA/hr). There was no significant difference between the DMH and control groups in crypt or upper villus fractions.

In the HPRT assay in controls, activities of 5.63 ± 0.45 μmoles in the upper villus fraction and of 2.98 ± 0.18 μmoles in the crypt fraction were present. In DMH-treated animals, HPRT activity of only 2.1 ± 0.21 μmoles/mg DNA/hr in the villus fraction and of 1.63 ± 0.26 μmoles/mg DNA/hr in the crypt fraction were found. HPRT enzyme activity in the villus fraction was significantly decreased in DMH-treated animals compared to controls (p < 0.001).

Tumor-specific Embryonic Antigen

With the immunodiffusion technique, only gross tumor material in 2 specimens gave immunoprecipitation with antibodies against embryonic antigens (Chart 2).

DISCUSSION

When the potent carcinogenic effect of cycasin and its aglucone methylazoxymethanol became known (9, 14), it made available a very reliable animal model for inducing a variety of tumors in the intestinal tract. The mechanism of activation of the extract of Cycas circinalis with cleaving of the β-glycoside binding by intestinal bacteria was elucidated by Laqueur (12). The group of 1,2-methylhydrazines and methylazoxymethanol are potent alkylating agents, and Shank and Magee (21) and Matsumoto and Higa (18) showed that their mode of action involves the purine base guanine of both DNA and RNA, which is methylated to 7-methylguanine. Methylazoxymethanol is the activated alkylating compound that undergoes oxidative demethylation (8). It also was demonstrated that methylazoxymethanol has a mutagenic effect on Salmonella typhimurium (23) and that cycasin has a potent radiomimetic effect and causes chromosomal damage in root-tip cells of Allium cepa (27). High doses of methylazoxymethanol induce acute inhibition of DNA, RNA, and protein synthesis within hours in rat liver and induce focal cell death...
in the crypt of the duodenum and colon (18, 32). The repair of this damage occurs within 1 week (34).

In recent years, with variations in doses and duration of treatment with this group of carcinogens, intestinal tumors have been induced in rats and mice (8, 9, 12, 19, 20, 24, 30). Some tumors have been found in the duodenum and, when DMH was given to young rats, it induced hepatomas. Administration of DMH p.o. induced increased numbers of liver tumors, and the early application of cycasin to neonatal mice induced nephroblastomas. Hemangioendotheliomas of the liver have been described (8).

Injections of DMH (s.c.) and of methylxoyazometanol (i.v.) have a systemic effect on the intestinal tract, as has been shown by Zedeck et al. (32) and also, extra intestinal tumors and cell damage have been documented on many occasions (32). It has been shown by Wittig et al. (31) that the distribution of the colonic tumors in rats is influenced by the fecal stream. Transverse colostomy increased the incidence of tumors at the site of the proximal stoma of the colostomy, but some tumors still occur distal to the colostomy independent of the fecal stream.

It is known that, in humans and animals, tritium-labeled thymidine is incorporated into proliferating epithelial cells normally in the lower colonic crypts. However, in human patients who develop neoplastic lesions of the colon, proliferating cells occupy an increased anatomic zone, and thymidine-labeled cells are found throughout the crypt and at the luminal surface (4–7).

The widening of the proliferative compartment in the crypt of the colonic mucosa and the tendency of the cells to go on synthesizing DNA, as they reach the upper one-third of their crypt in humans, are similar to findings with DMH-treated rodents. In addition, in the DMH model after 5 weeks of treatment, cells were found at the luminal surface of the crypts that were histologically normal and labeled with thymidine 1 hr after injection, similar to previous findings in man (4–7). Later, after 13 weeks of DMH treatment, a higher labeling index was found as a greater total number of labeled cells per crypt were present.

After 17 weeks, the arrangement of the colonic crypts became disturbed, and a numerical evaluation of the positional distribution of the labeled cells became inaccurate. In the later stages, as described above, in addition to the inflammation, the architecture of the glandular arrangements became disturbed and multifocal tumors occurred that ranged from adenomatus polyps to metaplasias and carcinomas. The distribution was such that the early focal lesions tended to be on the tips of the mucosal folds, as described by Wiebecke et al. (29).

We measured TdR-kinase and HPRT in the small bowel in order to study changes in enzyme activity in these cells during migration and cell differentiation. Imondi et al. (11) described distributions of enzymes involved in purine and pyrimidine synthesis in the intestinal cells which change as the cells move from the crypt to the surface of the jejunal villi. In neoplastic lesions of the colon in man, nucleic acid precursor enzyme
between the treated and untreated groups in this study, and a
normal gradient of activity from crypt to the villus was shown.
HPRT, however, was markedly decreased in the DMH group in
the villus cells in jejunum. This could indicate either a change
in the rate of synthesis as well as degradation of this
differentiation-specific enzyme in the cells or a more rapid
migration of immature cells, possibilities that can be analyzed
in separate studies.

This model of chemical carcinogenesis with DMH is similar
in many respects to the development of neoplasms in the
human colon. With this 1 compound, a variety of large bowel
tumors was observed in rodents, and the distribution of
tumors within the colon and rectum of rodents is similar to
that in humans. In addition, this carcinogenic agent induced
tumors in flat mucosa without polyp formation. The action of
the carcinogen induced an enlargement of the proliferative
zone in the colonic crypts, led to an increase in the total
number of labeled cells in the crypts, and decreased the
activity of the enzyme HPRT, all of which develop in human
colonic neoplasms.

REFERENCES

1. Behki, R. M., and Morgan, W. S. Studies of Phosphorylation of
Thymidine in Regenerating Liver. Acta Biochim. Biophys., 107:

2. Breitman, R. T. The Feedback Inhibition of Thymidine Kinase.

3. Burton, K. A Study of the Conditions and Mechanism of the
Diphenylamine Reaction for the Colorimetric Estimation of

4. Cole, J. W., and McKalen, A. Studies on the Morphogenesis of
Adenomatous Polyps in the Human Colon. Cancer, 16: 998–1002,
1963.

5. Deschner, E. E., Lewis, C. M., and Lipkin, M. In Vitro Study of
Human Rectal Epithelial Cells. I. A Typical Zone of H2-thymidine
Incorporation in Mucosa of Multiple Polyposis. J. Clin. Invest., 42:

Cells in Vitro. III. RNA, Protein and DNA Synthesis in Polyps and

Rectal Epithelial Cells in Vitro. II. H2-Thymidine Incorporation

8. Druckrey, H. Production of Colonic Carcinomas by 1,2-Dialkylhy-
drazines and Azoxyalkanes. In: W. J. Burdette (ed.), Carcinoma
of the Colon and Antecedent Epithelium. pp. 267–279. Springfield,
Ill.: Charles C Thomas, Publisher, 1970.

Selektive Erzeugung von Darmkrebs bei Ratten durch 1,2-

10. Druckrey, H., Steinhoff, D., Preusmann, R., and Ivanovic, S.
Erzeugung von Krebs durch eine einmalige Dosis von Methyl-
nitroso-Hamstoff und verschiedenen Dialkylaminosaminen an Ratten.

Activities Accompanying Differentiation of Intestinal Epithelial Cells.

12. Laqueur, G. L. The Induction of Intestinal Neoplasms in Rats with
Glucoside Cycasin and Its Aglucone. Virchows Arch. Abt. A

Induction in Germfree Rats with Methylazoxymethanol (MAM)
1967.


15. Lipkin, M., Bell, B., and Sherlock, P. Cell Proliferation Kinetics in
the Gastrointestinal Tract of Man. I. Cell Renewal in Colon and

16. Lipkin, M., and Quastler, H. Cell Population Kinetics in the Colon

17. Lohrs, U., Wiebecke, B., and Eder, M. Morphologische und
Autoradiographische Untersuchung der Darmschleimhautverander-
ungen nach einmaliger Injektion von 1,2-Dimethylhydrazin. Z. Ges.

18. Matsumoto, H., and Higa, H. H. Studies on Methylazoxymethanol,
the Aglucone of Cycasin. Methylation of Nucleic Acids in Vitro.

1,2-Dimethylhydrazin induzierten Adenocarcinomen des Ratten-

20. Schauer, A., Volltagel, T., and Wildanger, F. Carcinogenesis of the
Rattentumore durch 1,2-Dimethylhydrazin. Z. Ges. Expf. Med.,

21. Shank, R. C., and Magee, P. N. Similarities between the
Biochemical Actions of Cycasin and Dimethylnitrosamine. Bio-

Cancer Facts and Figures. New York: American Cancer Society,

23. Smith, D. W. Mutagenicity of Cycasin Aglucone (Methylazoxym-
ethanol), a Naturally Occurring Carcinogen. Science, 152:

1,2-Dimethylhydrazin-induzierten Dick- und Darmsarkoms der

25. Stonehill, E. H., and Bendich, A. Retrogenetic Expression: The
Reappearance of Embryonal Antigens in Cancer Cells. Nature,

26. Stonehill, E. H., Borenfreund, E., and Bendich, A. Anachronistic
Genetic Expression Recognized by the Production of Fetal
Antigens in Cancer Cells. Proceedings of the First Conference and
Workshop on Embryonic and Fetal Antigens in Cancer, May 1971,
Oak Ridge, Tenn., pp. 85–104.


28. Troncale, F., Hertz, R., and Lipkin, M. Nucleic Acid Metabolism in
Proliferating and Differentiating Colonic Cells of Man and in

tierexperimentelle und bioptische Untersuchungen zur Morpho-

Darmtumoren beim Mauen durch 1,2-Dimethylhydrazin. Z. Ges.

31. Wittig, Von G., Wildner, G. P., and Ziebarth, D. Der Einfluss der
Ingesta auf die Kanzerisierung des Rattentumors durch Dimethyl-

Biochemical and Pathological Effects of Methylazoxymethanol
Induction of Adenocarcinomas of the Colon in Mice by Weekly Injections of 1,2-Dimethylhydrazine


Cancer Res 1973;33:940-945.