Detection of Nuclear Anomalies in the Colonic Epithelium of the Mouse

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ABSTRACT

Colon carcinogens produce a variety of nuclear anomalies including pyknotic, karyorrhectic, and micronucleated cells in the colonic epithelium within a few hr. Two model carcinogens, 1,2-dimethylhydrazine and γ-rays, have been used to determine appropriate techniques, conditions, and scoring criteria for detecting such nuclear anomalies most efficiently. The results show that a rapid and sensitive assay for nuclear anomalies can be conducted with a variety of preparation techniques. We anticipate that the assay will be useful as a screen for potential colon carcinogens in the diet or elsewhere. The assay as recommended by us requires at least five animals per group and takes about 1 hr to analyze. A single sample at 6 or 24 hr after treatment should detect most carcinogens.

INTRODUCTION

Cancer of the colon is one of the most common forms of cancer in many “Western” countries (6). Epidemiological studies of migrants indicate that environmental factors, possibly dietary carcinogens, may be more important than genetic factors in the etiology of colon cancer (11). Unfortunately, the identification of environmental carcinogens with conventional carcinogenicity studies is time consuming and expensive. Short-term in vitro assays for mutagenicity, such as the Salmonella/mammalian microsome test (1), greatly reduce both the time and cost of screening but do not give information as to organ specificity and do not reflect in vivo pharmacokinetics. Short-term in vivo assays do reflect the complex pharmacokinetics involved in the uptake, metabolism, and distribution of a carcinogen, but the established in vivo short-term assays, such as the bone marrow micronucleus assay (12) or the sperm abnormality assay (2), are performed on tissues of convenience rather than on the tissue of interest. Here, we present an in vivo assay for carcinogens in the colonic epithelial cells.

Chromosome assays would provide a quantitative measure of genotoxicity, but the difficulty in obtaining good metaphase chromosome preparations from the colonic epithelium has made chromosomal analysis impractical so far, in spite of one encouraging report (18). Chromosomal breakage can, however, be measured indirectly by enumerating micronuclei which arise fromacentric fragments that have been excluded from the daughter nuclei following cell division (13). Typical micronuclei have the staining intensity, shape, and internal structure of normal nuclei but are less than ½ to ¼ the diameter. Although not all types of chromosomal aberrations become micronuclei, the number of micronuclei in a cell population provides a sensitive measure of chromosome breakage.

We began to examine the production of micronuclei in the colonic epithelium in γ-ray- or DMH-treated mice (10, 12). Early in our studies, however, it became clear that micronuclei that had a typical appearance were less frequent than pyknotic and karyorrhectic nuclei. Pyknosis and karyorrhexis (nuclear fragmentation) are characteristic of one process of cell death known as apoptosis (15, 22). Apoptosis begins with nuclear condensation leading to pyknosis, followed by nuclear fragmentation, cellular fragmentation, and phagocytosis by other cells. In previous reports, one or another stage of this process has been used as an index of cellular damage. DMH and other carcinogens elevate the karyorrhectic index in the epithelial cells of the colon (17) and induce apoptosis in the small intestine (14, 19). Because it is difficult and time consuming to distinguish between nuclear fragments produced by different processes, we have chosen to combine micronuclei and apoptotic figures into a single category to be referred to as nuclear anomalies. In the present study, we used 2 model carcinogens (DMH and γ-rays) to determine the conditions under which nuclear anomalies might be used as an assay for colon carcinogens.

MATERIALS AND METHODS

Animals

The animals used in all experiments were 6- to 10-week-old C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). The mice were given food and water ad libitum and were housed under constant temperature and humidity with alternating 12-hr intervals of light and dark.

Treatment

Radiation. For dose response experiments, 5 animals were irradiated at each dose in a 137Cs γ-irradiator (4) and sacrificed 24 hr later. To determine the most suitable time for analysis, 3 to 5 animals were sacrificed at various times after 100-rad γ-radiation. The average of the untreated control values is shown as the t = 0 value.

sym-DMH (Sigma). Animals were given injections i.p. of DMH dissolved in 1 mw EDTA (Sigma) solution the pH of which had been adjusted to 6.5 with n NaOH (Sigma). Doses of DMH (0, 1, 5, 10, or 20 mg/kg) were given to groups of 3 animals which were sacrificed 24 hr following treatment. Change in the frequency of nuclear anomalies as a function of time after treatment with DMH was studied by treating 10 animals with DMH (20 mg/kg) (as above) and sacrificing the animals at various times following treatment.

Sample Preparation

Three methods of preparation were used in this study. Colonic cells were examined in histological sections, in preparations made from single cell suspensions, or in squashes made from small pieces of the colon. Histological sections were made from colons dissected out of the abdomen and rinsed with Ca2+ and Mg2+-free Hanks’ balanced salt solution.
were embedded in paraffin, sectioned, cleared, stained by the Feulgen
Swiss roll (9), and then fixed in 10% buffered formalin. Later, the samples
were embedded in paraffin, sectioned, cleared, stained by the Feulgen
reaction, and counterstained with Fast green (5).

Cell suspensions were made by evert ing the colon and placing it in
0.075 M KCl containing 20 mM EDTA for 20 to 60 min. To separate the
crypts from the muscle layer, all but approximately 2 ml of the KC1:EDTA
solution was discarded, and the colon was sucked in and out of a
needleless tuberculin syringe until a minimum of resistance was encoun-
tered. The resulting suspension of crypts was then passed through a
21-gauge needle. Approximately 5 ml of 3:1 ethanol:acetic acid fixative
were added to each cell suspension during vortexing to avoid the
aggregation of cells. The fixative was changed twice by centrifuging at
1000 rpm for 5 min, discarding the supernatant, and adding fresh fixative.
Finally, cells were resuspended in fresh fixative, dropped onto a clean
glass microscope slide, allowed to air dry, and then stained by the
Feulgen:Fast green method.

Squashes were made by fixing the colons in 3:1 ethanol:acetic acid
overnight, rehydrating small pieces of the colon in distilled H2O, and then
hydrolyzing with HCl for 10 min at 60° prior to staining for 15 to 60
min with the Feulgen method. Each stained sample of the colon was
macerated in a drop of 45% acetic acid, and squashed between the coverslip
and the slide. Preparations could be made permanent using the dry-ice method of Conger and Fairchild (3).

Analysis

Sections. Scoring was restricted to those crypts in which a single
continuous row of epithelial cells could be discerned from the proximal
end of the crypt adjacent to the muscle layer to the distal end of the
crypt at the mucosal surface. The distal end of the crypt can be positively
identified only if the opening to the lumen of the colon is seen, so only
crypts showing such an opening were scored. In this way, even an agent
which damages only a specific region of the crypt will be detected, and
the number of cells scored from each region will be similar in each crypt
scored. This is important because the location of damaged cells within
the crypts of the small intestine is not the same for all agents (14).

In preliminary experiments, each type of nuclear anomaly (micronu-
cleated, pyknotic, and karyorrhectic) was recorded, and the number of
each aberrant type was expressed in relation to the number of cells
counted. For quick routine analysis, cells were scored as either abnormal
or normal, and only the number of abnormal cells observed was recorded
for each crypt. Swiss-rolled preparations were always scored starting
from the anal end of the colon and continuing towards the caecal end.

Cell Suspensions. The nuclear anomalies evident in single cell sus-
pensions included both micronuclei (Fig. 1) and apoptotic bodies, al-
though pyknotic nuclei were difficult to recognize. Damage was scored
as the number of micronuclei in a sample population of 1000 cells for
each animal.

Cell Squashes. In the colon squashes, crypts could be distinguished if
the amount of pressure applied during maceration and squashing was
controlled carefully (Fig. 2a). Single and multiple fragments as well as
pyknotic nuclei could be observed readily (Fig. 2b). Damage could be
quantified by counting the number of abnormal nuclei per crypt.

RESULTS

The nuclear anomalies induced by γ-radiation and DMH in the
colic epithelium included several types of Feulgen-stained
structures. There were nuclear fragments which conformed to the
criteria established for micronuclei (i.e., not larger than \( \frac{1}{4} \) to
\( \frac{1}{2} \) the diameter of the primary nucleus; Fig. 3a), large pyknotic
nuclei which were darkly stained with no discernible internal structure
(Fig. 3b), and darkly stained bodies which were usually
arranged in clusters and often contained within vacuoles (Fig. 3,
c and d). Like pyknotic nuclei, these bodies had less internal
structure than the normal nucleus. Pyknotic and karyorrhectic
nuclei as well as typical micronuclei were observed primarily in the
proximal third of the crypt, especially in treated animals
(Chart 1). The colonic squash preparations illustrated in Fig. 2, a
and b, could be carried out rapidly and provided information
regarding the amount and location of damage in the crypt. Under
ideal circumstances, the number of anomalies in 25 crypts could
be assessed in about 10 min, but in heavily damaged crypts, the
amount of damage was difficult to quantify due to the overlapping
cells.

In cell suspensions (Fig. 1), 1000 cells could be scored in
about 10 min. The type of damage scored was easily restricted
to micronuclei (Fig. 1) or karyorrhectic nuclei; pyknosis was
difficult to recognize. Although cell suspensions could be ana-
alyzed quickly, the time and amount of handling required to prepare
each sample limited the number of samples that could be
collected at one time and increased the interval between samples.
While this would be a serious difficulty when samples are closely spaced or when the exact timing is important, it
should not be a major problem in routine sampling.

Histological sections provided the most information. The po-
sition of the damage within the crypt could be determined
accurately (Chart 1), and when the colon was rolled longitudinally
to form a Swiss roll (9), the location of damaged crypts along

![Chart 1. Distribution of micronuclei (C), pyknotic nuclei (B), and karyorrhectic
nuclei (A) within the crypt detected using histological sections. Position 1 represents
the bottom of the crypt section. The number of nuclear anomalies detected in 3
cell regions of the crypt is shown: a, untreated controls; b, 3 hr; and c, 24 hr
following treatment with 100-rad γ-radiation.](chart1.png)
NUCLEAR ANOMALIES IN MURINE COLONIC EPITHELIUM

the colon was also evident. When the damage was classified as to type and expressed as a fraction of the total number of cells, 10 crypts took up to 30 min to score. However, sections could be scored rapidly by counting the number of damaged cells per crypt. Using this method, the time required to score 10 crypts was reduced to 5 to 10 min.

To determine the most suitable time to measure nuclear anomalies, we irradiated mice with 100-rad γ-radiation or gave them injections of DMH (20 mg/kg) i.p. and sampled at several times after treatment. The mean values for several experiments are shown in Chart 2. Three to 5 animals were examined at each time in each experiment. For both agents, the level of nuclear damage increased rapidly and remained elevated for about 24 hr, returning to controls levels by 96 hr.

The frequency of radiation-induced nuclear anomalies 24 hr postirradiation is shown in Chart 3a. The relationship is linear at low doses, but at 100 and 150 rads, we observed fewer nuclear anomalies than predicted, assuming linearity. The increases in nuclear abnormalities detected after radiation doses of 10 rads and above were significant (for 10 rads, \( t = 2.86; \) d.f. = 8; \( p < 0.025 \)). When varying doses of DMH were administered, the nuclear anomalies present 24 hr after treatment increased in a dose-related manner (Chart 3b). Significant increases in the level of damage were observed at doses of 10 and 20 mg/kg (for 10 mg/kg, \( t = 0.933; \) d.f. = 3; \( p < 0.01 \)).

To estimate the variability in the number of nuclear anomalies per crypt measured 24 hr following irradiation, groups of 5 animals were irradiated at the same time of day on 4 different days and sacrificed 24 hr later. The results presented in Table 1 indicate that there is no significant variability (analysis of variance:

\[ F = 1.288; p < 0.05 \]) between groups, but there is significant variability (analysis of variance: \( F = 2.543; p > 0.01 \)) between animals in each group. In addition to mouse-to-mouse variation, there is crypt-to-crypt variation. The distribution of damage between crypts is not significantly different from the distribution of damage predicted by a Poisson distribution (\( p > 0.05 \)) at each dose level tested in the radiation-dose response. This implies that much of the variation between crypts is due to chance alone.

In our studies, dose- and time-response curves were obtained by scoring the damage in 10 crypts in each colon with 5 animals in each treatment group. However, in view of the mouse-to-mouse variation, it may be possible to improve the power of this assay to detect low-level induced effects if more than 5 animals are included in each treatment group.

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Chart 2. Time course of appearance of nuclear anomalies following treatment with 100-rad γ-radiation (a) and DMH (b). The data shown for DMH represent 2 studies reported elsewhere and reproduced with the permission of the authors: O, Ronen et al. (20); and M, Wargovich et al. (21). Bars, S.E.

Chart 3. Dose response measured as anomalies per crypt 24 hr following treatment with increasing doses of γ-radiation (a) and DMH (b). Bars, S.E.

Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Animal</th>
<th>Damaged cells/crypt</th>
<th>Group mean</th>
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<tr>
<td>Irradiated animals</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
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<td>1</td>
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<td>0.4</td>
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<td>1.5</td>
<td></td>
</tr>
<tr>
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<td>1.2</td>
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<tr>
<td>5</td>
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CANCER RESEARCH VOL. 45 JANUARY 1985

244

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DISCUSSION

The purpose of the study was to develop a short-term assay relevant to carcinogenicity in the colon. Previous studies have revealed that changes in the histology and cell kinetics of the colon can be detected following treatment with the colon carcinogen DMH (7). Unfortunately, these are relatively late events, and it was not clear which changes, if any, were produced only by colon carcinogens and not by other toxic agents.

The rapid turnover of colonic cells should make the colonic epithelium suitable for the measurement of DNA damage, particularly micronuclei. Our preliminary studies to determine the influence of γ-radiation on micronucleus frequency revealed that, in the colonic epithelium, micronuclei were outnumbered and obscured by apoptotic bodies. Apoptotic figures appeared within 3 hr after treatment and therefore represented one of the earliest detectable changes in the colon. Similarly, in the rat colon (16) and the small intestine of the mouse (19), treatment with carcinogenic agents induced a rapid increase in apoptotic figures. For both DMH and γ-radiation, the time-related induction of micronuclei and apoptotic bodies was the same (Chart 2). For the purposes of this study, we have chosen to combine these effects into a single index of nuclear damage, called nuclear anomalies.

The detection of nuclear anomalies appears to provide a rapid and inexpensive assay for carcinogen-induced damage. Although there may be agents which induce either of these phenomena exclusively, our experience to date with a variety of carcinogenic and noncarcinogenic agents has provided no indication that micronuclei and apoptotic bodies are induced independently in the colonic epithelium (20, 21).

To characterize the assay and maximize its effectiveness, it was necessary to determine: (a) the preparation method most suitable for easy, rapid analysis; (b) the time at which animals should be sacrificed following treatment; (c) the magnitude and source of variability between samples; and (d) the sensitivity of the assay.

Three methods of sample preparation were compared at 24 hr posttreatment. Each method offers some advantages with respect to the ease of preparation, speed of scoring, or information provided (Table 2). Nuclear anomalies are classified most easily in histological sections. Furthermore, in sections of colons rolled end to end, the location of the damage within the crypt as well as the location of the crypt within the colon can be determined. Since histological sections provide the most information regarding induced nuclear anomalies, we collected the majority of data presented in this paper from sections of Swiss-rolled colons, and we recommend this method of sample preparation. However, if the expertise and facilities required to embed and section the material are not available, then nuclear anomalies can be analyzed in cell suspensions and colon squashes.

Direct comparisons between the absolute amount of damage detected using each technique are complicated by the measurement of different anomalies in sections and suspensions. Nuclear anomalies were scored as the number of damaged cells per crypt in sections or the number of micronuclei per 1000 cells in cell suspensions. Micronuclei were scored selectively in cell suspensions, because we believed that the micronucleus frequency would provide an accurate and selective indication of chromosome breakage, and because micronuclei were easily identified in cell suspensions. Subsequent studies have shown that, even after exposure to ionizing radiation, one of the most potent chromosome-breaking agents, most nuclear anomalies do not arise from chromosomal fragments lost during mitosis and, hence, are not typical micronuclei (1). We therefore recommend that, in future work, all types of nuclear fragments should be counted as nuclear anomalies in cell suspensions.

To determine the most suitable sampling time, samples were collected at various intervals following treatment with γ-radiation or DMH (Chart 2). The induction of nuclear anomalies by γ-radiation shows a time course characterized by a rapid increase in damage reaching a maximum at about 6 hr after treatment. The level of damage remains elevated for about 24 hr. This curve obtained from sectioned material is similar to that obtained from single cell suspensions (12), as expected. We have chosen to collect samples 24 hr following treatment, but from the time course of induction of nuclear anomalies by γ-radiation and DMH, which have different mechanisms of action, there were 2 peaks for induced anomalies, one at 6 hr and the other at 24 hr following treatment. Sampling at either 6 or 24 hr after treatment is suitable for both γ-radiation and DMH. In order to allow time for uptake, distribution, and metabolism of carcinogens, we regard 24 hr as the better of the 2 times. It may be, however, that some agents, such as 1-β-D-arabinofuranosylcytosine, that act very rapidly in the small intestine (16) would be detectable at 6 hr but not at 24 hr. Indeed, no increase in nuclear anomalies was detected in the colon 24 hr following treatment with 1-β-D-arabinofuranosylcytosine (21). If the maximum induction of nuclear anomalies is to be detected, then a time course experiment should be performed.

The epithelial cells of the crypt normally migrate from the proximal base to the distal end of the crypt, but damaged cells were not found distally at later times nor were they still frequent at the proximal end (Chart 1). There are several possible reasons

Table 2

<table>
<thead>
<tr>
<th>Aberrant types scored</th>
<th>Prefixation sampling time (min)</th>
<th>Time to score (min)</th>
<th>Damage location within crypt</th>
<th>Background level</th>
<th>Increase induced by 100 rads (4-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squashes</td>
<td>All</td>
<td>2±</td>
<td>10</td>
<td>±</td>
<td>6.2/1000 cells</td>
</tr>
<tr>
<td>Suspensions</td>
<td>Micronuclei-type aberrants</td>
<td>20-80</td>
<td>10</td>
<td>±</td>
<td>2.59/1000 cells</td>
</tr>
<tr>
<td>Sections</td>
<td>Nuclear fragments not in vacuoles</td>
<td>2-5</td>
<td>30</td>
<td>+</td>
<td>0.68/crypt section</td>
</tr>
<tr>
<td>Sections</td>
<td>All</td>
<td>2-5</td>
<td>5-10</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* Apparent location of damage within crypt may be affected when crypts are squashed.
* Although each sample requires 20- to 60-min treatment prior to fixation, samples can be collected sequentially so that 5 to 10 samples can be collected in 1 hr.
* Each cell in 100 crypts counted and damage types classified in detail.
* Cells classified as normal or abnormal and number of abnormal cells per crypt determined for 10 crypts.
* We have now accumulated additional data which indicate that the background level is closer to 0.20/crypt section.

CANCER RESEARCH VOL. 45 JANUARY 1985
245
for this observation: (a) the damaged cells may have been sloughed and lost; (b) they may have been digested by other cells during the phagocytic stage of apoptosis; or (c) they may have extruded their nuclear fragments.

An important factor in determining sample size is the variability of the index. Studies with both DMH and γ-radiation showed a large quantitative variation in response between experiments. This variation may be due to the circadian effects (8). The variation may also reflect interanimal differences. In order to assess sample variability, groups of 5 animals were irradiated and sampled at the same time each day for 4 days. The results of this study (Table 1) indicate that, even under carefully controlled circumstances, there is still a large, although not significant, variation between groups of animals. There is, however, a significant difference between animals within each group.

The sensitivity of the nuclear anomaly assay was determined by examining the dose relationship for γ-radiation and DMH. This assay can detect doses of carcinogen far lower than the doses required for tumorgenesis. DMH-induced colon tumors are evident only following injections at doses of 20 mg/kg (7), but a significant increase in nuclear anomalies was detected 24 hr after treatment with DMH (10 mg/kg) or 10-rad γ-radiation. In other studies, we have detected increased levels of nuclear anomalies at still lower doses of DMH (21).

Nuclear anomalies were observed only in the basal regions of the crypts where cell division occurs. Hence, proliferative activity would appear to be a prerequisite for carcinogen-induced apoptosis. Since only a limited number of cells are at risk, the effect of very potent agents may be underestimated because of this limiting factor. The dose response for γ-radiation, in which the slope is reduced at doses above 50 rads, illustrates this phenomenon.

A strong correlation between the induction of nuclear anomalies and the carcinogenicity in the colon has been demonstrated for several colon carcinogens and noncarcinogens using the nuclear anomalies assay (21). Furthermore, the site specificity of both DMH, which induces cancer primarily in the descending colon, and methyl nitrosourea, which induces cancer primarily in the forestomach, is reflected in their ability to induce nuclear anomalies at these sites (20). The nuclear anomalies assay has also been used to demonstrate the ability of protective agents to reduce the amount of nuclear damage induced by DMH in the colon (12, 21). However, like many short-term assays, the colonic nuclear anomaly assay must be regarded not as a definitive indicator of carcinogenic action, but rather as a screening method which can be used to identify agents which may have carcinogenic potential in the colon. The assay may be adaptable to a number of other tissues; it has been used successfully in several other tissues in the gastrointestinal tract (20).

REFERENCES

Fig. 1. In single cell suspensions, cell overlapping is reduced, and nuclear anomalies are detected early. Micronuclei are easily identified in these preparations.
Fig. 2. Individual crypts (a) and nuclear anomalies (arrowhead) (b) are evident in preparations made by squashing small pieces of Feulgen-stained colon.
Fig. 3. Nuclear anomalies (arrowheads) detected in histological sections of the colonic epithelium stained with Feulgen and Fast green. a, micronucleus; b, Feulgen-stained bodies within vacuole; c, pyknotic nucleus; and d, karyorrhectic nucleus.
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