Foci of Aberrant Crypts in the Colons of Mice and Rats Exposed to Carcinogens Associated with Foods

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ABSTRACT

Aberrant crypt foci can be identified in the colons of rodents treated 3 wk earlier with azoxymethane, a known colon carcinogen. These crypts can easily be visualized in the unsectioned methylene blue-stained colons under light microscopy, where they are distinguished by their increased size, more prominent epithelial cells, and pericryptal space. They occur as single aberrant crypts or as two, three, or four aberrant crypts in a cluster. We compared the reported ability of carcinogens associated with the human diet to induce colon cancer with the measured rate of induction of aberrant crypts in female CFI mice and Sprague-Dawley rats. The carcinogens used were 1,2-dimethylhydrazine, methyl nitrosourea, N-nitrosodimethylamine, benzo(a)pyrene, aflatoxin B1, 2-amino-6-methyl-dipyridyl(1,2-c2, 3',2'-d'jimidazole, 2-amino-3-methylimidazo(4,5-p)quinoline, 2-amino-3,4-dimethylimidazo(4,5-p)quinoline, and 3-amino-1-methyl-5H-pyrido(4,3-b)indole. Graded doses of these compounds were given to the animals by gavage twice with a 4-day interval, and the animals were terminated 3 wk later. All colon carcinogens induced aberrant crypts in a dose-related fashion. N-Nitrosodimethylamine and 3-amino-1-methyl-5H-pyrido[4,3-b]indole, carcinogenic compounds that do not induce colon cancer, did not induce them. The ability of the studied compounds to induce aberrant crypts was species specific; e.g., aflatoxin B1, and 2-amino-3,4-dimethylimidazo(4,5-p)quinoline induce about 20 times more in rats than mice. This relationship was consistent with their reported ability to induce colon cancer in these species. Results of the present study support the use of the aberrant crypt assays to screen colon-specific carcinogens and to study the process of colon carcinogenesis.

INTRODUCTION

The early events leading to colorectal cancer are not well understood. Cancer appears to originate from the neoplastic transformation of stem cells in Lieberkühn crypts and by the repopulation of the normal crypts with atypical cells (1). Dysplastic crypts can be seen in sections of colons of both experimental animals within 7 to 9 wk of repeated carcinogen treatment (1, 2) and of humans predisposed to colon cancer with either familial polyposis or long-standing ulcerative colitis (3). The methods that have been used up to now to display these early lesions, however, require either multiple serial sections or scanning electron microscopy. They do not lend themselves to the quantitative study of early events in colon carcinogenesis.

Bird (4) has recently described a method for directly visualizing altered crypts induced by carcinogens in the intact colon. When the colon is stained with methylene blue, these "aberrant crypts" can be identified by their increased size, thicker epithelial lining, and increased pericryptal zone (Fig. 1). Many observations suggest that aberrant crypts are early, preneoplastic lesions in the colon: they are not present in untreated animals; they appear as soon as 2 wk after carcinogen treatment and persist; they reveal histological changes from mild atypia to dysplasia; and these changes increase with time in response to a high-fat diet, a known colon tumor promoter (6).

This paper has two related aims: to assess the application of the aberrant crypt assay in the detection of food-related carcinogens, and to determine whether this assessment provides further support for the role of aberrant crypts in colon carcinogenesis. Nine known carcinogens were administered p.o. at graded doses to CFI mice and Sprague-Dawley rats to test for their ability to induce aberrant crypts. The carcinogens chosen were selected on the basis of their differential ability to induce colon tumors in rats and mice as detailed in Table 1 and because they are, or are associated with, chemicals humans eat. 1,2-Dimethylhydrazine is related to cyscin which is present in the cyscin nut and consists of the β-glucoside of methylazoxymethanol, a metabolite of 1,2-dimethylhydrazine (7). N-Nitrosodimethylamine and methylnitrosourea are examples of nitrosamines and nitrosamides (8) that can occur in foods or can be formed endogenously (9). Benzo(a)pyrene is the most prevalent carcinogenic polycyclic aromatic hydrocarbon present in foods, either as a result of cooking method (e.g., charcoal grilling) or by contamination prior to food processing (10). Aflatoxin B1, a metabolite of Aspergillus flavus and A. parasiticus, has been associated with hepatic cancer in Africa and the Far East (11) but is also a colon carcinogen. Glu-P-14 and Trp-P-2 are examples of heterocyclic aromatic amines formed in cooking as a result of amino acid pyrolysis (9). IQ and MelQ are examples of compounds formed by the condensation of creatinine, sugar, and amino acid moieties (9). In addition to the nine carcinogens, we have carried out of preliminary study of the effect of a mutagen recently isolated from fried beef (12), PhIP. PhIP is known to be a weak mutagen for Salmonella TA-1538, but a very potent inducer of mutations at the hprt locus, sister chromatid exchanges, and chromosomal aberrations in mammalian cells in vitro (13). The carcinogenic properties of PhIP are not known.

MATERIALS AND METHODS

Chemicals. Citric acid, DMH, B(a)P, and AFB1 were from Sigma (St. Louis, MO); IQ, MelQ, Glu-P-1, and Trp-P-2 were from Toronto Research Chemicals (Toronto, Canada), purity being judged to be greater than 98% on the basis of thin-layer chromatography and NMR analyses; MNU and NDMA were a gift of Dr. M. Archer (Ontario Cancer Institute); PhIP was initially kindly supplied by Dr. J. Felton (Lawrence Livermore National Laboratory) and was subsequently purchased from Toronto Research Chemicals, both samples being judged to be greater than 98% pure.

Animals. In all studies 27- to 31-day-old female CFI mice (Charles River, St. Constant, Quebec, Canada) and 21-day-old female Sprague-Dawley rats [Crl:CD(SD);BR; Charles River] were used. The animals

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The abbreviations used are: Glu-P-1, 2-amino-6-methyl[dipyridyl(1,2-c2, 3',2'-d'jimidazole; AFB1, aflatoxin B1; B(a)P, benzo(a)pyrene; DMH, 1,2-dimethylhydrazine; IQ, 2-amino-3-methylimidazo(4,5-p)quinoline; MelQ, 2-amino-3,4-dimethylimidazo(4,5-p)quinoline; MNU, methyl nitrosourea; NDMA, N-nitrosodimethylamine; PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-b]indole.
Fig. 1. Aberrant crypt foci in the mouse (a, b, c) and rat (d, e, f) colon showing round (a, d) and elongated (b, e) single aberrant crypts and clusters of aberrant crypts (c, f). The colons were opened, stained with methylene blue, and observed on a glass slide with a x100 microscope objective.

Table 1 Reported ability of the carcinogens to induce colon cancer in mice and rats when administered p.o.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mice</th>
<th>Rats</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMH</td>
<td>+</td>
<td>+</td>
<td>2, 20</td>
</tr>
<tr>
<td>MNU</td>
<td>+</td>
<td>+</td>
<td>21</td>
</tr>
<tr>
<td>NDMA</td>
<td>-</td>
<td>-</td>
<td>22</td>
</tr>
<tr>
<td>B(a)P</td>
<td>-</td>
<td>+</td>
<td>23–25</td>
</tr>
<tr>
<td>AFB1</td>
<td>-</td>
<td>-</td>
<td>26–29</td>
</tr>
<tr>
<td>Glu-P-1</td>
<td>-</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>IQ</td>
<td>-</td>
<td>+</td>
<td>30</td>
</tr>
<tr>
<td>MelQ</td>
<td>-</td>
<td>+</td>
<td>31, 32</td>
</tr>
<tr>
<td>Trp-P-2</td>
<td>-</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>PhIP</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

* Established on basis of intrarectal administration in mice by p.o. administration in rats.

were housed in plastic cages with hardwood chip bedding (Sani-Chips; P. F. Murphy Forest Products, Montville, NJ) and wire tops and fed chow diet containing 6% fat (Teklad, 6%, Madison, WI) and water ad libitum. The temperature in the animal room ranged from 21–24°C, and a 12-h light-dark cycle was maintained.

Carcinogen Treatment. Groups of mice and rats with 10 or more animals per group were treated with the same dose of carcinogen per kg of body weight by gavage. The treatment was repeated twice, 4 days apart, the total dose given below being the sum of the two carcinogen treatments. This schedule was used as pilot studies suggested that it permitted higher dosages and introduced less variability than single treatments. Two or more dose levels and a solvent control were used in each study, the high dose being chosen to be approximately one-half of the 50% lethal dose for the compound. The following solvents were used to dissolve the carcinogens: 0.9% NaCl solution for DMH, AFB1, Trp-P-2, and NDMA; 55% ethanol in 0.9% NaCl solution for IQ, MelQ, and Glu-P-1; medium chain triglycerides (Mead Johnson, Belleville, Ontario, Canada) for B(a)P and PhIP, and 1% citric acid (pH 3) for MNU. The gavage volumes were from 0.1 to 0.3 ml. No serious short-term effects were observed. The mice and rats were kept on their normal diet for the 21 days after their first gavage.

Analysis of Ablignant Crypts. Aberrant crypts were scored 21 days following the first carcinogen treatment using the procedure described by Bird (5). The animals were killed by cervical dislocation, and their colons were removed and flushed with Krebs' Ringer salt solution. They were then cut open along the longitudinal median axis, fixed flat between filter paper in 10% formalin, and stained with methylene blue (0.2% in Krebs' Ringer) for 30 to 60 min in order to visualize crypt outlines. The colons were placed on microscope slides with the mucosal side up, and aberrant crypts were scored under the light microscope at a magnification of 40 or x100. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, significantly increased distance from the luminal to basal surfaces of cells, and the easily discernible pericryptal zone. Control studies showed that scoring involved little subjectivity. However, crypts overlying lymphoid follicles were excluded from the score since normal crypts in this area can occasionally be confused with aberrant crypts. The number of aberrant crypt foci observed per colon, the number of aberrant crypts observed in each focus, and the shape and location of each of the foci (see Fig.
were recorded. Average values reported were based on all animals examined. All colons were scored by one observer. Several groups were coded and rescored blindly. The repeated scores yielded very similar values. Scoring is easiest, however, shortly after staining.

RESULTS

Examples of aberrant crypts in mice and rat colons 3 wk after carcinogen treatment are presented in Fig. 1. Although they were usually found as single aberrant crypts (Fig. 1, a and d), two, three, or four aberrant crypts were sometimes found together as a cluster (Fig. 1, c and f). In this case, they were considered to be one lesion and scored as one focus, as McLellan and Bird (7) had shown that single crypt foci may be replaced with multicrypt foci during the period from 2 to 4 wk following carcinogen. The average numbers of aberrant crypts seen in each aberrant crypt focus are presented in Table 2. There was perhaps a tendency for a focus to contain more than a single aberrant crypt with higher doses of carcinogen, though the effect is not strong or consistent. The crypts were also found to be in several shapes (i.e., Fig. 1, b and e). Data relating to the shape of the crypts did not show any relationship to chemical compound or dose (data not shown). The dose-response data are therefore presented as foci of aberrant crypts per colon without reference to crypt size or shape.

Fig. 2 shows the dose-responses for aberrant crypt foci induced by the carcinogens in the two rodent species, female CF1 mice and female Sprague-Dawley rats. None of the vehicle control groups yielded any aberrant crypts in a total of 90 mice and 90 rats (0 dose points in the figure). There was a marked difference in the slopes of the curves. At the highest doses tested, large numbers of aberrant crypts were seen with some carcinogens (DMH and MNU), none was seen with others (NDMA and Trp-P-2), and smaller numbers for the remainder. There was also a marked difference in the sensitivity of rats and mice to some of the carcinogens. B(a)P produced 15 times the number of aberrant crypts in mice as in rats, while MelQ produced 20 times as many aberrant crypts in rats than in mice.

The distribution of aberrant crypts between cecal, mid-colon, and rectal segments is shown in Fig. 3. The aberrant crypts were seen primarily in the middle colon and rectum except in the case of IQ and B(a)P in mice, where they were more frequent in the cecal end.

Table 2 Carcinogen dose and size of aberrant crypt foci in CF1 mice and Sprague-Dawley rat colons

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>No. of aberrant crypts/focus</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mice</td>
</tr>
<tr>
<td>DMH</td>
<td>30</td>
<td>1.27 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.64 ± 0.08</td>
</tr>
<tr>
<td>MNU</td>
<td>40</td>
<td>1.35 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>1.21 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>180</td>
<td>1.61 ± 0.10</td>
</tr>
<tr>
<td>B(a)P</td>
<td>100</td>
<td>1.17 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td>AFB1</td>
<td>3</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Glu-P-1</td>
<td>70</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>IQ</td>
<td>200</td>
<td>1.10 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>1.05 ± 0.04</td>
</tr>
<tr>
<td>MetIQ</td>
<td>200</td>
<td>1.30 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>1.30 ± 0.17</td>
</tr>
</tbody>
</table>

* None of the vehicle control animals had aberrant crypts.
* Average ± SE.

DISCUSSION

The nine carcinogens tested were found to induce aberrant crypts in experimental animals in 3 wk in a manner that paralleled the activity of the compounds in long-term carcinogenesis studies. The correlations cannot be considered con-
exclusive as the carcinogenesis and aberrant crypt focus assays were carried out under different conditions with different sexes and strains of animals in some cases. However, parallels were seen in both the relative potency of the agents to induce aberrant crypts and colon cancer and the selectivity of the agents with relation to the mouse and rat species. The results thus add support to the evidence linking the aberrant crypt in the sequence of events leading to colon cancer.

The dose-response curves for the induction of aberrant crypts by carcinogens (Fig. 2) are remarkable in their differences in slopes. DMH and MNU induced 20 to 80 aberrant crypts at their maximum doses, while NDMA and Trp-P-2 produced none in over 40 animals. This is a difference of over 2 orders of magnitude that parallels the known carcinogenicity of these pairs of compounds in long-term carcinogenicity studies (Table 1). The remaining 5 compounds induced 0.2 to 5 aberrant crypts at the maximum dosages. Four of these 5 compounds are known to lead to colon cancer in long-term studies. The one exception, B(a)P, has not been reported as a colon carcinogen when administered p.o. to either mice or rats (14), but did induce aberrant crypts in mice and in small numbers in rats. Interestingly, B(a)P is activated by colonic cells (15), leads to colonic nuclear toxicity by both intrarectal (16) and p.o. administration (17), and at high doses has been shown to induce colon cancer in hamsters (14). Furthermore, the doses used in the earlier negative carcinogenicity data were low (10 and 30 mg/kg), doses that do not induce aberrant crypts in mice. Carcinogenicity studies are in progress to determine whether B(a)P induces colon cancer in mice when it is administered at higher doses.

The slopes of the dose-response curves for mice and rats are very different for many of the chemicals studied. The sensitivity of the rat exceeds the mouse with DMH, AFB1, Glu-P1, IQ, and MeIQ, the differences being in the most extreme a ratio of about 20-fold for AFB1 and MeIQ. The sensitivity of the mouse exceeded that of the rat in the case of B(a)P, the ratio in this case being about 15-fold. The differential effect for the induction of aberrant crypts can be compared with the selectivity observed in the animal carcinogenicity studies (Table 1). Again there is good concordance though, as noted earlier, the carcinogenicity data for B(a)P are incomplete. Interestingly, the slopes of the dose-response curves often differ markedly from linearity. This is most marked with MNU where apparent thresholds are seen in both mice and rats.

The evidence presented here and in earlier publications indicates that aberrant crypts are lesions involved directly in the development of colon cancer. It seems likely they will provide a model of colon carcinogenesis similar to the focus, nodule, cancer model for liver carcinogenesis. Here Farber, Piot, and their coworkers (18, 19) have shown that only a fraction of preneoplastic nodules persist and develop into cancer, while the remainder remodel or redifferentiate back to normal appearing liver. A similar phenomenon may occur in the colon. The lack of exact congruence between the low frequency of aberrant crypts in this study and the absence of tumors in carcinogenesis studies with AFB1, Glu-P-1, IQ, and MeIQ in mice suggest that this may be the case. Although we do not know what the quantitative ratio of preneoplastic to neoplastic events in the colon will be, it seems likely that only a fraction of aberrant crypts, the small, very early stage of the disease, will develop into colon cancer.

The study with PhIP demonstrates the possibility of using the aberrant crypt assay with compounds with unknown carcinogenic activity. PhIP is perhaps the aromatic amine pyrrolysine product in greatest abundance in cooked meat (12). As many other aromatic amines from meat induce aberrant crypts, it was expected to show a similar activity. In fact, in mice it was similar in its effects to IQ and MeIQ. We might thus expect that it will be found to be carcinogenic. The aberrant crypt assay might well be used in the assay of less purified components of foods. For instance, it might be possible to examine fractions of cooked meats, such as the basic organic soluble fraction, for total activity, rather than the purified and identified structures. In this way it may be possible to define the components of the human diet that could be responsible for the origin of colon cancer.

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