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Specificity Study to Evaluate Induction of Aberrant Crypts in Murine Colons

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ABSTRACT

We have previously reported on our findings of aberrant crypts (AC) in the colons of rodents treated with a colon carcinogen. In this report, the specificity of AC formation was assessed by testing a variety of agents for their ability to induce AC in the colons of CF_1 and C57BL/6J mice. In addition, the ability of each of the agents to induce nuclear aberrations (NA) was assessed and compared with the AC data. The test agents included hydrazines, polycyclic aromatic hydrocarbons, aromatic amines, and nitrosocompounds. The colons were assessed for AC 2 or 4 weeks following a single treatment with the test agent. Of the seven agents that induced AC formation, five were colon carcinogens and the other two were agents believed to be carcinogenic to organs other than the colon. None of the five agents believed to be noncarcinogens induced AC whereas three of them did induce NA in at least one of the strains of mice tested. Comparison of AC and NA induction for each test agent showed that all agents that induced AC also induced NA and that the converse was not true. The findings of the present study indicate that AC are induced specifically in response to colon carcinogens and support our contention that AC are preneoplastic lesions.

INTRODUCTION

Previous work done in our laboratory on AC[2] has suggested that AC may be a significant biological lesion in the development of colon cancer (1). In the murine system, we have observed that AC can be induced in 2 weeks by a very low dosage of a colon carcinogen; they are induced in a dose-dependent manner, their growth is affected by dietary modifications, and they are largely located in the distal colon. In addition, we have shown that the morphology of AC, as determined by viewing histological sections, is atypical. We hypothesize that AC represent preneoplastic lesions.

At the present time there is not an *in vivo* system for quantification of preneoplastic lesions in the colon. Therefore, research aimed at collecting data to support or refute the hypothesis that AC could be preneoplastic lesions is needed. However, before we put our efforts into the long term studies necessary to characterize the progressional morphology of AC, it was important to determine whether or not these lesions are specifically formed in response to colon carcinogens. To do this we closely followed the protocol used by Wargovich *et al.* (2) in assessing the specificity of the induction of NA. NA are degenerative changes induced in the proliferative compartments of the colonic crypts in response to genotoxic agents. It is purported that induction of NA can be used as an *in vivo* assay to detect colon carcinogens (3). In the present study specificity of AC formation in response to various carcinogens (colon and noncolon) and noncarcinogens was investigated and compared with that of the induction of NA in two strains of mice.

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[2] The abbreviations used are: AC, aberrant crypts; NA, nuclear aberrations; 2AB, 2-aminobiphenyl; 4AB, 4-aminobiphenyl; AOM, azoxymethane; BaP, benzo(*a*)pyrene; BeP, benzo(*e*)pyrene; DMAB, 3,2′-dimethyl-4-aminobiphenyl; DMBA, 7,12-dimethylbenz(*a*)anthracene; 1,1-DMH, 1,1-dimethylhydrazine; 1,2-DMH, 1,2-dimethylhydrazine; HS, hydrazine sulfate; MCA, 3-methylcholanthrene; MNU, *N*-nitroso-*N*-methylurea; MU, methylurea; MTD, maximum tolerated dose.

MATERIALS AND METHODS

Animals. For all experiments, 7-week-old inbred C57BL/6J (C57) female mice (The Jackson Laboratory, Bar Harbor, Maine) and outbred CF_1 female mice (Charles River Canada Inc., Montreal, Quebec, Canada) were used. They were housed in plastic cages with wire tops and sawdust bedding with a 12-h light-dark cycle. The animals were fed AIN pellets (4) *ad libitum* and had free access to chlorinated water (5–10 ppm).

Chemicals. 1,2-DMH, DMAB, 4AB, MCA, BaP, BeP, MNU, and cholchicine were purchased from Sigma Chemical Co., St. Louis, MO. 1,1-DMH, HS, 2AB, and MU were purchased from Aldrich Chemical Co., Milwaukee, WI. AOM was purchased from Ash-Stevens Inc., Detroit, MI. DMBA was purchased from Eastman Kodak, Rochester, NY.

Radiation. Groups of 10 mice were exposed to whole body γ-radiation from a ^{137}Cs irradiator at the Ontario Cancer Institute, Toronto, Ontario (5).

Administration of Chemicals. Except for MNU and MU, all chemical compounds were administered as an oral intubation with the use of a 21-gauge ball-tipped syringe. The gavage volume was 0.2 ml. MNU and MU were administered intrarectally using the same 21-gauge ball-tipped syringe with a gavage volume of 0.1 ml. The polycyclic aromatic hydrocarbons and the aromatic amines were dissolved in medium-chain triglyceride oil (Mead Johnson and Co., Belleville, Ontario). All other compounds were dissolved in saline.

Selection of Dose Levels. Identical dosages of each agent were used for induction of AC and NA. Approximately one-half the MTD of each agent was used unless otherwise stated (2). In the case of MNU one-eighth of the MTD was selected because at one-half the MTD 70% mortality occurred within 7 days. Since AC require more than 7 days to form, this dose could not be used. The dosage of 1,2-DMH used in this study is similar to the dosage used in our and other laboratories to induce tumors in C57 and CF_1 mice using multiple injections (6). The dosage of DMBA administered was above the level normally given to rats to induce mammary tumors (7). For colchicine the dose used corresponded to the level used to arrest cells in metaphase (8). In the case of the radiation treatment two doses, 200 and 100 rads, were selected. Dose levels of all agents tested are listed in Table 1.

Induction of Colonic NA. A group of mice (five per treatment group) were treated with various carcinogens and noncarcinogens. All animals were terminated by cervical dislocation 24 h after treatment. Their colons were removed, flushed with Kreb's Ringer, slit open from cecum to anus, and fixed in 10% buffered formalin and processed. Histological sections stained with hematoxylin & eosin were evaluated for NA (micronuclei, karyorrhexis, and pyknosis). Scoring was done starting from the anal end and was restricted to longitudinal sections of complete crypts. For each colon 10–20 crypts were scored. The detailed methodology has been published (3). Although the usual procedure for identifying NA is to stain with Feulgen with a fast green counterstain (3), in this study it was decided to stain the tissue sections with hematoxylin & eosin. This decision was based on the results of comparative studies undertaken in our laboratory which showed that the NA incidences determined by using the two staining procedures were found to be similar.[3] Also, hematoxylin & eosin staining is more convenient than Feulgen staining because unlike the latter, the former staining is routinely carried out in histology laboratories by an automated procedure. Statistical analysis of the data was performed by Student's *t* test. A probability of less than 5% ($P < 0.05$) was considered significant.

Induction of AC. 10 CF_1 and 10 C57 mice were used per chemical or radiation dosage. The CF_1 mice were terminated 2 weeks following

[3] Unpublished observation.

Table 1 *Induction of colonic AC and NA in CF_1 and C57BL/6J mice by various test agents*

Groups of 15 CF_1 and 15 CF57BL/6J mice were given a single treatment with one of the test agents. 24 h later five mice from each group were terminated and their colons were scored for NA. 2 or 4 weeks following treatment 10 CF_1 mice or C57 mice, respectively, from each group were terminated and their colons scored for AC.

Test agent[a]	Dose[b]	Assay result[c]			
		CF_1		C57	
		AC[f]	NA	AC	NA
Hydrazines					
1,2-DMH cc	20	5.50 ± 1.07	4.10 ± 1.72[e]	4.10 ± 1.28	3.81 ± 1.32[e]
AOM cc	5	3.20 ± 0.59	4.04 ± 0.99[e]	2.50 ± 0.40	2.80 ± 0.99[e]
1,1-DMH c	50		0.14 ± 0.80		0.21 ± 0.07
HS nc	50		0.37 ± 0.21[e]		0.18 ± 0.10
Aromatic amines					
DMAB cc	100		0.33 ± 0.09		0.61 ± 0.36[d]
DMAB cc	50		0.36 ± 0.37		0.57 ± 0.29[d]
4AB cc	50	0.22 ± 0.15	1.09 ± 0.37[d]		2.19 ± 0.71[d]
2AB nc	50		0.29 ± 0.18		0.19 ± 0.18
Polycyclic aromatic hydrocarbons					
MCA cc	100	3.25 ± 0.53	0.94 ± 0.31[d]	3.56 ± 1.20	0.84 ± 0.33[d]
BaP c	100	1.29 ± 0.64	1.67 ± 0.74[d]	1.10 ± 0.35	1.47 ± 0.46[d]
DMBA c	50	0.1 ± 0.1	1.08 ± 0.55[d]	0.80 ± 0.29	3.20 ± 1.01[d]
BeP nc	100		0.33 ± 0.18		0.22 ± 0.05[d]
Nitrosocompounds					
MNU cc	25	5.0 ± 0.54	1.70 ± 1.43[e]	1.50 ± 0.37	3.69 ± 2.21[e]
MU nc	500		0.09 ± 0.07		0.14 ± 0.13
Radiation c	100 rads		2.51 ± 1.44[d]		3.83 ± 0.45[d]
Radiation c	200 rads		2.36 ± 0.67[d]		4.27 ± 0.45[d]
Colchicine nc	1.0		0.71 ± 0.55[d]		1.24 ± 0.19[d]
Control (MCT oil)	0.2 ml		0.35 ± 0.28		0.05 ± 0.05
Control (saline)	0.2 ml		0.07 ± 0.07		0.12 ± 0.10

[a] Test agents classified as: cc, colon carcinogen; c, noncolon carcinogen; nc, noncarcinogen.
[b] In mg/kg body weight unless stated otherwise.
[c] NA, no. of NA per crypt section; AC, no. of AC foci per 5 cm of colon; mean ± SE.
[d] Significantly greater ($P < 0.05$) than respective medium-chain triglyceride oil (MCT oil) control.
[e] Significantly greater ($P < 0.05$) than respective saline control.
[f] No value given if test agent failed to induce AC.

treatment and the C57 mice were terminated 4 weeks following treatment. The length of time between treatment and termination is based on findings of a previous study which showed that the maximum number of AC formed following administration of the colon carcinogen, AOM, was 2 weeks for CF_1 mice and 4 weeks for C57 mice (1). Therefore, it was reasoned that in order to assure detection of AC in the C57 mice it would be necessary to allow 4 weeks between treatment with the test agents and assessment, instead of the 2 weeks for CF_1 mice. There is no *a priori* reason to suspect that this time discrepancy will introduce any error into the AC data. All animals were terminated by cervical dislocation. Immediately following termination of the animal, the colon was removed, flushed with Kreb's Ringer, slit open from cecum to anus, and fixed flat in 10% buffered formalin. Following the protocol described by Bird (9), the fixed colons were placed in buffered saline containing 0.2% methylene blue for 15–30 min. The stained colons were then placed on a glass slide with the luminal side up. By viewing the stained colons with the light microscope at a magnification of 40×, the colons were assessed for the presence of AC.

RESULTS

Classification of Test Agents. The classification of the test agents is shown in Table 1. We chose to study the colon carcinogens from four chemical classes: the hydrazines (1,2-DMH), the direct-acting nitrosocompound (MNU), the aromatic amines (DMAB and 4AB), and the polycyclic aromatic hydrocarbons (MCA). The two structural analogues of each of these agents that are considered to be either carcinogenic for an organ other than the colon or are considered noncarcinogens, were also selected (2). It should be noted that our classification of 4AB as a colon carcinogen differs from Wargovich *et al.* (2) as they classified it as a noncolon carcinogen. Our classification is based on the work of Walpole *et al.* (10), who showed that 4AB induced tumors in the rat colon. γ-Radiation was used as a nonspecific agent (11). In addition, we included the known breast carcinogen DMBA (7) and the metaphase arrest agent colchicine in our study (8).

Induction of AC and NA. The numerical results of the induction of AC and NA by each of the agents tested is shown in Table 1 and is summarized in Table 2. Of the six colon carcin-

Table 2 *Induction of colonic AC and NA in CF_1 and C57BL/6J mice by various test agents*

Classification of test agents	Assay result[a]			
	CF_1		C57BL/6J	
	AC	NA	AC	NA
Colon carcinogens				
1,2-DMH	+	+	+	+
AOM	+	+	+	+
DMAB	−	−	−	+
4AB	+	+	−	+
MNU	+	+	+	+
MCA	+	+	+	+
Noncolon carcinogens				
1,1-DMH	−	−	−	−
BaP	+	+	+	+
DMBA	+	+	+	+
Noncarcinogens				
HS	−	+	−	−
2AB	−	−	−	−
BeP	−	−	−	+
MU	−	−	−	−
Colchicine	−	+	−	+
Nonspecific carcinogen				
γ-Irradiation	−	+	−	+

[a] +, positive result; −, negative result.

ogens tested all produced AC in both strains of mice, except for 4AB which did not produce any in C57 mice and DMAB which did not produce any in either strain. Of the three noncolon carcinogens tested 1,1-DMH was the only one that failed to produce any AC. None of the five noncarcinogens tested produced any AC. Radiation also failed to induce AC formation.

Five of the six colon carcinogens tested induced NA in both strains of mice. DMAB failed to induce NA in CF_1 mice. As was seen with AC induction, of the three noncolon carcinogens tested, 1,1-DMH was the only one that failed to induce NA. Of the five agents selected as noncarcinogens, colchicine caused an increase in NA in both strains of mice, HS in CF_1 and BeP in C57 mice. The results for NA induction generally confirmed the findings of Wargovich *et al.* (2), with the exception of DMAB which was found to be only a weak inducer of NA in C57 mice.

DISCUSSION

The findings of this study would appear to indicate that AC formation is specific for exposure to colon carcinogens. None of the noncarcinogens tested induced AC formation. With the exception of 4AB in C57 mice and DMAB in both strains of mice, all the colon carcinogens tested induced AC formation. DMAB and 4AB have been shown to be weak colon carcinogens in rats (10). However, to the best of our knowledge, these compounds have not been demonstrated to be carcinogenic to the murine colon. Therefore, the partial failure of 4AB, and the complete failure of DMAB, to induce AC in the present study may be because these compounds are only weakly carcinogenic to the murine colon. Perhaps multiple treatments and/or higher doses of these compounds are required to induce AC. The ability of 4AB to induce AC in CF_1 mice and not in C57 mice may be due to strain differences (12).

In the present study, the two low doses of whole-body irradiation tested failed to induce AC. The evidence for radiation inducing neoplasms in the colons of rodents has come from studies in which a high, localized dosage of radiation (approximately 2500–6500 rads) was given to the colon (13). However, observations of the effects of whole-body irradiation at doses less than 1000 rads suggest that in these instances radiation may be exerting its effect more as a promoter than as an initiator (11). If AC are preneoplastic lesions then they should be induced only in response to an initiating dose of an agent. Therefore, the absence of AC induction may be accounted for by the possibility that the doses of radiation tested in the present study were not high enough to initiate the cancer process. Further studies, following protocols that have reported radiation induced colon cancer, are needed to assess this possibility.

The induction of AC by BaP and DMBA, two compounds considered to be carcinogenic for organs other than the colon, may be due to the fact that these compounds are only weakly carcinogenic to the colon and strongly carcinogenic to other organs. Hence, tumors arise in the mammary tissue or forestomach in the case of DMBA (7, 14) and the lung, skin, mammary tissue, or forestomach in the case of BaP (15), long before they arise in the colon. It should be noted that both these compounds caused a significant increase in NA. Hence, we know that these compounds are reaching the proliferating cells in the crypts and thus can easily be genotoxic to the colonic epithelial cells.

It is generally believed that the ability of carcinogens to initiate cells is due to their interaction with the cell's DNA. A logical extension of this is the assumption that any agent which is genotoxic to cells may also be capable of interacting with cellular DNA without causing cell death and hence be capable of initiating cells. It is along this line of reasoning that the theory behind the NA assay as a measure of the genotoxicity and hence, carcinogenicity of agents to the colon is based (3). The NA assay measures the number of cell lethal events in the colon which presumably are attributed to damage to the cell's DNA.

By comparing the induction of AC and NA by each test agent we observed that (*a*) AC were only formed in response to genotoxic agents (*i.e.*, all agents that induced AC also induced NA) and (*b*) the property of a compound with respect to its toxicity to epithelial nuclei does not predict AC formation (*i.e.*, not all agents that induced NA also induced AC). Based on these observations it would appear that the NA assay is more sensitive than the AC assay for detecting colon-specific, genotoxic and hence possibly carcinogenic, agents. For example, DMAB and 4AB, two known colon carcinogens in rats, were picked up by the NA assay but only partially by the AC assay. Also, the known colon carcinogen γ-radiation was not detected by the AC assay but was by the NA assay. Further evidence of the greater sensitivity of the NA assay is that it picked up more false positives than the AC assay which had none. Therefore, a valid interpretation of the data is that the NA assay is a more sensitive test for the identification of colon-specific, genotoxic and hence possibly carcinogenic, agents than the AC assay.

A further and still valid interpretation of the data is that the AC assay is able to distinguish between genotoxic agents that are initiators at a certain dose and those that are not, whereas the NA assay is unable to do this. For example, unlike NA, AC were not induced by the low doses of γ-radiation considered to be promoting but not initiating doses. In addition, the absence of and very low levels of AC induction in response to a single treatment with DMAB and 4AB, respectively, may reflect the very low potency of these compounds to induce cancer in the murine system. The strongest evidence in support of this latter interpretation of the data would be if AC represent preneoplastic lesions. This is because an assay which employs preneoplastic lesions as its endpoint is by definition a more specific assay for the identification of initiating agents than is an assay with genotoxicity as its endpoint.

It is the opinion of the authors that the findings from the present study combined with our findings presented elsewhere (1), provide strong evidence supporting our hypothesis that AC represent preneoplastic lesions. We therefore propose that the AC assay, in conjunction with the NA assay, could be used to screen for compounds which are capable of inducing colon cancer. The NA assay is more sensitive than the AC assay for detecting colon-specific, genotoxic agents. Therefore, the NA assay could be employed as the initial test in the screening system. The AC assay could then be used to determine whether or not the compounds identified by the NA assay are capable of initiating the cancer process. Furthermore, we would expect that the induction of AC would depend on the same variables known to affect tumor yield, *e.g.*, dose of carcinogen, route of administration, species, strain, sex, and age of the animal. Therefore, in addition to identifying the carcinogenic compounds, the AC assay could also provide information as to the dose of the compound required to initiate colon cancer, as well as the species, strain, sex, and age most sensitive to the carcinogenic actions of the compound.

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