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Effects of Ammonium Acetate and Sodium Cholate on *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine-induced Colon Carcinogenesis of Rats[1]

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ABSTRACT

This study was conducted to determine the effects of ammonium acetate alone or in combination with sodium cholate upon *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced colon carcinogenesis in rats. Ammonia, acetate, and deconjugated bile acids are produced by microbial enzymes in the gastrointestinal lumen. One hundred twenty male Sprague-Dawley rats, weighing 196 ± 2 g at 8 wk of age, were given four intrarectal doses of MNNG (2 mg/dose) over 2 wk. They were then randomly assigned among four treatment groups, each containing 30 rats. The groups were arranged in a 2 × 2 factorial design and given intrarectal infusions of the agents under study in 0.3 ml of double-distilled water 3 times weekly for 52 wk beginning 4 wk after the initial MNNG treatment. The experimental treatments were: double-distilled water as control; ammonium acetate (24.8 mg of ammonia); sodium cholate (2 mg of cholic acid); and a combination of ammonium acetate and sodium cholate. Ammonium acetate treatment increased the number of rats with fecal blood 4-fold after 56 wk, and this was associated with a higher incidence of adenocarcinomas with a polypoid morphology. The incidence and total number of carcinomas *in situ* (high grade dysplasia) increased with ammonium acetate treatment. Ammonium acetate increased the total number of adenocarcinomas. Sodium cholate had no significant main effects on the incidence or morphology of colon lesions. The data support the conclusion that ammonium acetate treatment acted as a promoting agent in MNNG-induced colon carcinogenesis.

INTRODUCTION

Epidemiological data suggest that the incidence of colon cancer is associated with the consumption of diets high in fat and protein (1, 2). A series of studies in rodents support a role for dietary fat in colon tumorigenesis (3), while others have failed to confirm such findings (4, 5). The mechanisms whereby dietary fat may influence colon carcinogenesis remain speculative. One hypothesis is that dietary fat changes the amount and type of microbial metabolites derived from bile acids and cholesterol which reach the lumen of the large bowel and act as promoting agents or alter the susceptibility of the mucosa to carcinogens (3, 6). Studies in rodents show that bile acids such as taurodeoxycholic acid (7), lithocholic acid (7, 8), deoxycholic acid (9), chenodeoxycholic acid (10, 11), and cholic acid (10, 12) are promoters of experimentally induced colon cancer.

The role of dietary protein in experimental colon carcinogenesis has been less extensively studied. Topping and Visek observed a significant increase in DMH[4]-induced colon, small-intestinal, and ear duct tumors in rats fed 15% and 22.5% protein compared to those fed 7.5% (13). However, the mechanisms whereby protein affects colon cancer remain speculative. Kari *et al.* reported that mice fed a low protein diet show decreased metabolism of the colon carcinogen DMH to its ultimate mutagenic metabolite (14). Other studies showed that increasing dietary protein produced a greater excretion of lipids in the feces of mice, suggesting that dietary protein may raise the intestinal concentration of bile acids and their metabolites (15, 16). Digestion products from dietary protein are stimuli for gastrointestinal secretions, including bile acids which may undergo microbial degradation to promoting agents in the gastrointestinal lumen (17, 18).

The present study examined the possibility that ammonia[5] participates in promoting experimental colon carcinogenesis. Ammonia formation from the catabolism of amino acids and other nitrogenous substrates within tissues and from microbial enzymes acting upon nitrogenous substrates within the gastrointestinal lumen is proportional to dietary protein intake. Urea, synthesized in the liver, is the main detoxication product of ammonia in mammals. About 25% of the urea excreted daily reaches the gastrointestinal tract in secretions where it is hydrolyzed by bacterial urease(s) to ammonia and CO_2. Ammonia released in the gastrointestinal lumen may be excreted in feces, synthesized into microbial protein, or absorbed and synthesized into nitrogenous substances, including urea. Ammonia elicits a number of biological responses which suggest its participation in colon tumorigenesis (19, 20). In an effort to gain further understanding of the role of naturally occurring metabolites in colon cancer, we have compared the effects of ammonium acetate and sodium cholate administration on MNNG-induced colon carcinogenesis in rats. A 2 × 2 factorial design allowed the evaluation of individual effects of the test chemicals and their possible synergistic or antagonistic interactions. Preliminary results of these studies have been reported (21, 22).

MATERIALS AND METHODS

Animals and Diets. One hundred twenty male Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN) with an initial average weight of 196 ± 2 g at 8 wk of age were individually housed in stainless steel wire-bottomed cages in rooms maintained at 22 ± 1°C with 14 h of fluorescent lighting/24 h. All were *ad libitum* fed the AIN 76A (23, 24) diet (Teklad, Madison, WI) in powdered form. Individual body weights and feed intakes were recorded throughout the study.

Carcinogen Treatment. All rats were adapted to the diet for 1 wk before receiving 4 intrarectal doses of MNNG (Sigma Chemical Co., St. Louis, MO) during the subsequent 2 wk. Each dose, containing 2 mg of MNNG dissolved in 0.3 ml of dimethyl sulfoxide, was administered via an 18 gauge, straight, ball-tipped feeding tube placed 6.5 cm into the colon via the rectum.

Administration of Compounds Investigated. Thirty rats, randomly assigned to each of 4 treatment groups after MNNG administration, subsequently received the putative promoting substances in DDW 3 times per wk for 52 wk beginning 1 mo after the initial MNNG dosing. All dosing volumes of 0.3 ml were administered as described for MNNG. Group 1 (controls) received DDW; Group 2, 24.8 mg of

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[4] The abbreviations used are: DMH, 1,2-dimethylhydrazine; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DDW, double-distilled water.

[5] "Ammonia" as used here signifies the sum of NH_3 and NH_4^+.

ammonia as ammonium acetate (Sigma); Group 3, 2.0 mg of cholic acid (Sigma) as the sodium salt; and Group 4 received the two combined in the same volume of DDW. The solutions were deposited at a preselected distance of 6.5 cm from the anus because at this site fecal material passing through the colon has been well formed into mucous-coated pellets, easily by-passed with the ball-tipped feeding tube. Preliminary studies with solutions of a red dye showed virtually no penetration or disintegration of the pellets with 0.3 ml of the solutions administered.

Necropsy and Histopathological Procedures. The animals were observed daily, the appearance of fecal blood was recorded, and moribund rats were necropsied. All survivors were euthanized and necropsied at 56 wk after the initial dose of MNNG. The animals and specimens were marked according to a coded system to conceal the identity of their treatment groups until all of the gross and histopathological studies were completed. At necropsy, the entire colon was removed, split longitudinally for gross examination, and fixed in 10% neutral buffered formalin. The location, appearance, and dimensions of all suspicious lesions were recorded following examination by two individuals (S. K. C., D. G. B.). Specimens from each lesion were fixed in 10% neutral formalin, embedded in paraffin, cut serially into 5-μm sections, and stained with hematoxylin and eosin. The following histological features were recorded: degree of dysplasia (25); degree of differentiation; stage of invasiveness (26); and presence of lymphoid aggregates. Mucin stains were used to assist in evaluation of selected histological sections.

Statistical Procedures. Statistical analyses of body weight, food intake, and tumor frequency used a 2-way analysis of variance to evaluate main effects and interactions. Tumor incidence data were subjected to χ^2 analysis (27). The computer programs PROC GLM and PROC FUNCAT of the Statistical Analysis Systems Institute were used (28).

RESULTS

All treatment groups showed similar growth (Table 1), but the ammonium acetate-treated rats averaged 4% less in final body weight than those not given this compound ($P < 0.05$). Neither ammonium acetate nor sodium cholate treatment changed food intake. Blood in the feces was first noted 22 wk after MNNG administration (Fig. 1). Fecal blood was observed in 48% of the ammonium acetate-treated rats by 56 wk compared to 12% of the non-ammonium acetate-treated groups ($P < 0.05$).

Table 2 shows the histological findings and prevalence of tumors. Well-formed lymphoid aggregates with or without germinal centers were present within the colonic mucosa in the majority of rats, and the incidence was not influenced by either of the agents administered after carcinogen treatment. Morphologically similar in all treatment groups, these lymphoid aggregates between the mucosa and muscle wall appeared as raised plaques ranging from 2 to 20 mm in diameter. Lymphoid aggregates were occasionally found in close association with colon adenocarcinomas and contained malignant epithelial cells. Only by histopathological evaluation was it possible to distinguish lymphoid aggregates from benign or malignant epithelial lesions. Incidence and frequency of tubular adenomas, designated as low-grade dysplasia (Table 2), were not influenced by treatment. Ammonium acetate increased the total number ($P < 0.003$) of adenocarcinomas, but we observed no effects of cholate at the dosage administered. Rats treated with ammonium acetate showed a significantly higher incidence ($P < 0.03$) and a greater number of lesions ($P < 0.003$) categorized as high-grade dysplasia (carcinoma *in situ*) when compared to rats not given ammonium acetate. Cholate alone showed no main effect and no interaction with ammonium acetate. There was no effect of either treatment on the incidence or frequency of adenocarcinomas limited to the mucosa. Although only 10% of all rats had adenocarcinomas extending through the mucosa, involving lymph nodes, or having metastatic sites, the results showed a significantly greater number of these lesions in rats given ammonium acetate ($P < 0.02$). A significant interaction between ammonium acetate and cholate was noted. Rats treated with ammonium acetate alone showed a 27% incidence of invasive carcinomas compared to 7% for rats treated with both ammonium acetate and cholic acid. Of the 80 adenocarcinomas found, 46% had polypoid morphology, with the remainder being sessile (Table 3). Ammonium acetate-treated rats showed a greater incidence ($P < 0.003$) and number ($P < 0.001$) of polypoid adenocarcinomas. The incidence of sessile lesions was unaffected by treatment with ammonium acetate or sodium cholate.

Table 1 *Mean body weights, food consumption, and survival of MNNG-treated rats fed the AIN-76A diet and given intrarectal infusions of ammonium acetate or sodium cholate alone or in combination*

Treatment	n[a]	Initial body wt (g)[b]	Final body wt (g)[c]	Food intake (kcal/day)	Survival (%)[d]
Control	27	202 ± 2[e]	535 ± 9	69 ± 1	82
Ammonium acetate	26	198 ± 2	519 ± 8	69 ± 1	65
Sodium cholate	27	203 ± 2	538 ± 10	69 ± 1	89
Ammonium acetate and sodium cholate	27	199 ± 2	517 ± 7	67 ± 1	89
Statistical analysis[f]					
Ammonium acetate	NS[g]	NS	$P < 0.05$	NS	NS
Sodium cholate	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	NS

[a] Thirty rats were initially assigned to each treatment group. Rats were eliminated from the analysis if they died before 30 wk following MNNG treatment. None of these rats had colon tumors.
[b] Body weight at the time of randomization to treatment following the final dose of MNNG.
[c] Data based upon rats surviving 56 wk after MMNG treatment.
[d] Percentage of rats (n) surviving 56 wk after MNNG treatment.
[e] Mean ± SE.
[f] Statistical evaluation expressed as main effects of ammonium acetate or sodium cholate or their interactions.
[g] NS, not significant at $P < 0.05$.

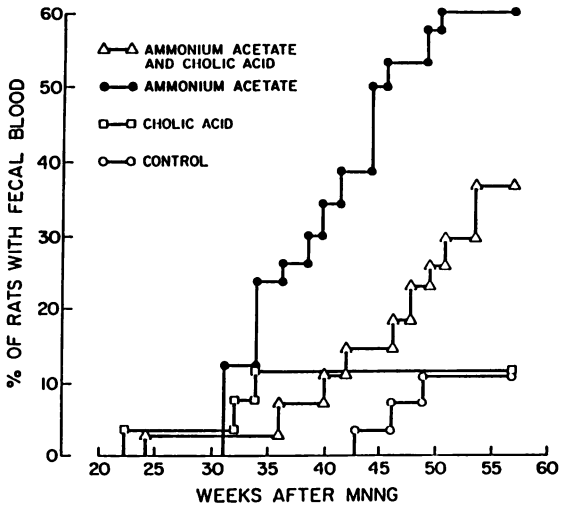

Fig. 1. Cumulative percentage of MNNG-treated rats showing fecal blood when given intrarectal infusions of ammonium acetate and sodium cholate singly or in combination 3 times per week for a total of 52 wk. Rats given ammonium acetate showed a significantly higher incidence of fecal blood ($P < 0.05$). There were no statistically significant main effects of cholate or interactions observed.

DISCUSSION

Ammonium acetate, given during the promotion phase of MNNG-induced colon carcinogenesis, shortened the latency

Table 2 *Histopathological characteristics and prevalence of MNNG-induced colonic lesions in rats given intrarectal infusions of ammonium acetate and sodium cholate alone or in combination*

Treatment	Lymphoid aggregates		Low-grade dysplasia		Adenocarcinomas							
					Total		Carcinoma-in-situ[a]		Limited to mucosa[b]		Invasive[c]	
	Incidence[d]	No.[e]	Incidence[d]	No.[e]	Incidence[d]	No.[e]	Incidence[d]	No.[e]	Incidence[d]	No.[e]	Incidence[d]	No.[e]
Controls	78	35	28	7	40	12	11	3	33	9	0	0
Ammonium acetate	69	26	29	7	69	28	50	14	23	6	27	8
Sodium cholate	81	32	19	5	52	16	30	8	22	6	7	2
Ammonium acetate and sodium cholate	81	34	19	5	56	24	44	17	22	6	7	2
Statistical analysis[f]												
Ammonium acetate	NS[g]	NS	NS	NS	NS	$P < 0.003$	$P < 0.003$	$P < 0.003$	NS	NS	NS	$P < 0.002$
Sodium cholate	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Interaction	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	$P < 0.02$

[a] Equivalent to high-grade dysplasia.
[b] Adenocarcinomas limited to the mucosa correspond to Dukes' Stage A (25, 26).
[c] Invasive adenocarcinomas include those extending through the mucosa, involving lymph nodes, or with metastatic sites. This category includes lesions classified as B, C, and D by the Dukes system (25, 26).
[d] Percentage of rats with at least one lesion.
[e] Total number of lesions observed.
[f] Statistical evaluation expressed as main effects of ammonium acetate or sodium cholate or their interaction.
[g] NS, not significant at $P < 0.05$.

Table 3 *Summary of morphology and size of MNNG-induced colon adenocarcinomas in rats given intrarectal infusions of ammonium acetate and sodium cholate alone or in combination*

Treatment	Polypoid[a]			Sessile[a]		
	Incidence[b]	No.[c]	Size	Incidence[b]	No.[c]	Size
Controls	11	3	0.42 ± 0.11[d]	33	9	1.34 ± 0.77
Ammonium acetate	54	15	0.51 ± 0.21	39	13	0.83 ± 0.19
Sodium cholate	22	6	1.06 ± 0.72	33	10	0.38 ± 0.15
Ammonium acetate and sodium cholate	33	13	0.92 ± 0.62	37	11	0.38 ± 0.11
Statistics[e]						
Ammonium acetate	$P < 0.003$	$P < 0.001$	NS[f]	NS	NS	NS
Sodium cholate	NS	NS	NS	NS	NS	$P < 0.05$
Interaction	NS	NS	NS	NS	NS	NS

[a] Limited to adenocarcinomas.
[b] Percentage of rats with at least one lesion.
[c] Total number of lesions.
[d] Mean ± SEM for the largest diameter (cm).
[e] Statistical evaluation expressed as main effects of ammonium acetate or sodium cholate or their interaction.
[f] NS, not significant at $P < 0.05$.

period for the appearance of fecal blood and increased the frequency of adenocarcinomas, specifically those with a polypoid morphology. The known biological effects of ammonia suggested that it may have a promoting role in colon carcinogenesis. Ammonia reduces colonic epithelial cell life span, alters DNA synthesis, disrupts intermediary metabolism, and increases mucosal cell turnover rates, and its highest concentrations occur in segments of the colon in humans where colon cancer incidence is highest (19, 29). At current intakes of protein, 3 to 4 g of ammonia nitrogen are released daily by microbial urease(s) within the gastrointestinal lumen of human adults eating "Western diets" (19, 20). Animal studies show that measures which reduce colonic bacterial ammonia production, such as germ-free conditions, dietary antibacterial agents, or induction of urease immunity significantly reduce gastrointestinal tissue mass (19, 20, 30). Thymidine incorporation into the colon mucosa was reduced 20% in mice immunized against urease to inhibit colonic ammonia release by urea hydrolysis (31). Germ-free animals show less intestinal tissue, slower mucosal cell regeneration, and less mucosal surface area. These characteristics correlate with lower colonic and portal blood ammonia than in conventional animals (19, 20, 32). Feeding of antibiotics, surgical diversion of the intestinal flow, and germ-free conditions all reduce chemically induced colon carcinogenesis in rodents, suggesting that bacterial metabolites, including ammonia, are involved in tumor production (33, 34). Ammonia affects cultured 3T3 fibroblasts more adversely than their simian virus 40-transformed counterparts, suggesting that ammonia confers a selective growth advantage upon transformed cells (35). In our previous study, showing an increase in DMH-induced colon cancer as dietary protein was raised from 7.5% to 15%, we found that colon ammonia concentrations increased in concert with protein intake. In the same study, groups of rats fed urea in an attempt to raise intestinal ammonia showed no effect on intestinal ammonia concentrations or tumor incidence (13).

The dose of ammonia administered in the present study (24.8 mg per infusion) was approximately 25% of the amount released daily in the intestinal tracts of rats (19, 20). However, the concentration of ammonia in the administered solution greatly exceeded the 6 to 10 mM concentrations in the intestinal fluid of rats (36) or the 3 to 43 mM concentrations of humans (37). It is also important to note that the ammonia was administered as ammonium acetate. Acetic acid is one of the metabolites produced during fermentation by intestinal microbes which may alter carcinogenesis by its trophic effects upon the colonic mucosa (38, 39). Thus, studies utilizing different ammonium salts at different concentrations and dosages are needed to characterize the role of ammonia in experimental colon tumorigenesis.

A number of studies suggest that one of the factors underlying the association of high fats diets with increased colon cancer incidence in humans is the greater concentration of bile acids and their metabolites in the feces of those consuming high fat diets. Alterations in bile acid metabolism by cholestyramine feeding (40), ileal resection, ileal bypass, or implanting of the bile duct to the distal small intestine (33) have all increased the incidence of chemically induced colon tumors in rodents. Increased biliary secretion of bile acids, including cholic acid, has been reported in rats fed a high fat diet (41). The fecal excretion of bile acids and their metabolites is also increased in rats fed diets high in fat (8, 42). Intrarectal administration or feeding of primary or secondary bile acids has been shown to promote colon cancer induced by different agents (3–12). Feeding of cholic acid at 0.2% of the diet increased *N*-methyl-*N*-nitrosurea-induced colon tumorigenesis (12). Previous studies using intrarectal administration of cholate used 20-mg doses 3 times per week (9). This exceeds total daily bile acid excretion which ranges from 10 to 18 mg/kg/day or 4 to 9 mg/day for a 400-g rat. The usual daily excretion of cholic acid approximates 0.6 to 0.9 mg/kg/day or less than 0.5 mg per day for a 400-g rat (38, 39). We administered a dose of 2 mg 3 times per week and failed to see promotional effects. In addition, sodium cholate did not enhance the effects of ammonium acetate. In fact, rats given ammonium acetate combined with sodium cholate tended to show fewer tumors than those given ammonium acetate alone. In retrospect, administering a salt of a secondary bile acid might have shown promotion. However, in studies by others, cholic acid enhanced tumor yield following intrarectal administration at higher dosages (10) or following feeding (12). Sodium cholate was chosen because our preliminary studies revealed that, unlike other bile acids, it would remain in aqueous solutions with ammonium acetate at the concentrations used. Although it is not known how cholic acid acts as a promoter, it can be degraded by intestinal bacteria to deoxycholate and lithocholic acids, both known to have promoting activity (3, 6). Additional studies using a variety of ammonium salts with different bile acids and their metabolites within the range of physiological concentrations are needed to define how these substances interact in the complex processes leading to experimental colon cancer.

Our experience in these studies agrees with that of Nauss *et al.* (4, 5, 43) in emphasizing the need for histopathological examination of all suspected colonic lesions. Discriminating between lymphoid aggregates, benign epithelial lesions, and adenocarcinomas by gross appearance or size is impossible and is likely to lead to errors in assessment of cancer incidence. Organized lymphoid aggregates were noted in approximately 75% of the rats. We also observed a number of sessile adenocarcinomas within or intermixed with lymphoid aggregates. Furthermore, lymphoid aggregates were frequently located in the distal colon where most of the tumors were found. Nauss *et al.* also observed that sessile DMH-induced colon tumors were more frequent at sites along the colon where lymphoid aggregates were more common. However, they reported no significant difference in the number, size, and histological characteristics of the lymphoid aggregates between DMH- and saline-treated rats (43).

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