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Effects of Cholestyramine on 1,2-Dimethylhydrazine-Induced Enteric Carcinoma in Germfree Rats[1] (39124)

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In the etiology of human colo-rectal cancer, the importance of diet has been emphasized from epidemiological observations among various ethnic groups (1). In addition to a possible intake of carcinogens in the diet, attention has been focused on chemical transformation of carcinogens by activities of the microbial flora in the intestine (2). Recently, a cocarcinogenic role has been ascribed to secondary bile acids, such as lithocholic acid, which are derived from primary bile acids by the action of microbial agents (3). The use of germfree animals in the elucidation of colo-rectal carcinogenesis has been rewarding because ot the unequivocal absence of microorganisms and of secondary bile acids from the gastrointestinal tract. Lithocholic acid and taurodeoxycholic acid instilled intrarectally were shown to promote colonic carcinogenesis induced by a single dose of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in germfree rats (4).

An interesting publication by Nigro *et al.* (5) reported that the addition of cholestyramine resin in the diet enhanced and accelerated the incidence of colon tumors following administrations of 1,2-dimethylhydrazine to conventional rats. Since cholestyramine sequesters bile acids, the observation is contrary to the purported role of bile acids as cocarcinogens.

This study reports on (1) the susceptibility of germfree rats to DMH-induced colonic tumors, (2) the effect of dietary cholestyramine on carcinogenesis in the gut of germfree rats, and (3) the concentration of bile acids in the intestinal content with and without cholestyramine in the diet.

Methods. Germfree Sprague-Dawley (S-D) rats of both sexes were housed in plastic germfree isolators and fed autoclaved diet (Tek Lad L-485) and water *ad lib.* Standard operating procedures established at Lobund were used for maintenance of germfree rats. Conventional control rats were transferred from germfree stock to a conventional animal room in which they were treated in the identical manner except for germfree status.

The schedule of carcinogen administration was similar to that used by Newberne and Rogers (6); 1,2-dimethylhydrazine dihydrochloride (DMH) (Aldrich) was dissolved in physiological saline at 15 mg/ml. The solution was filter-sterilized (Millipore filter 0.22 μm), and administered per os at the dose of 30 mg/kg once a week for 10 weeks.

Cholestyramine resin, MKE 54 (Mead Johnson), was mixed at 2% by weight with powdered diet L-485, which was then pelletized. The latter pellets and regular diet pellets were sterilized in the autoclave. At 20 weeks after the onset of DMH treatments, the rats were sacrificed and examined by gross observation for tumors in the intestinal canal and for metastasis to other sites in the body. Individual tumors were counted at various locations and listed as sessile or pedunculated in appearance. Intestinal tumors, lymph nodes, and other organs were excised and fixed with Bouin's solution and paraffin-embedded sections thereof were stained with hematoxylin-eosin for histological examinations. Individual tumors were excised, minced with Medium 199, and transplanted subcutaneously into newborn conventional S-D rats. They were observed thereafter for development of tumors that were then examined histologically.

Determinations were made for bile acids in the cecal contents of DMH-treated germfree rats with and without cholestyramine. At autopsy the cecal content was separated *in toto* and centrifuged at 1500*g* at 4°C for 20

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TABLE I. INTESTINAL NEOPLASMS INDUCED BY DIMETHYLHYDRAZINE IN GERMFREE SPRAGUE–DAWLEY RATS[a]

Number	Sex	Body wt/g	Tumors					
						Colon		
			Duodenum	Jejunum	Cecum	1/2	1/2	Rectum
1	M	334					5	
2	M	327	3				5	
3	M	355	4	1			12[b]	1
4	M	367					8	
5	M	350	2				13	
6	F	185	2				2	
7	F	195	1				4	
8	F	203	2				9	
Total			14	1			58	1 = 74 9.3/rat

[a] Rats at age 2 months were fed DMH (30 mg/kg) once/week/10 weeks. Diet: Tek-Lad (L-485). Rats were killed for examinations at 20 weeks after onset of experiment.

[b] Metastatic-tumor in lymph node.

min. The supernatant fluid as well as the precipitate were examined for bile acids by a modification of the procedure described by Grundy *et al.* (7). For purposes of additional "clean-up" of the sample, as well as fractionation of the bile acids, a third step of thin-layer chromatography was carried out, using a third thin layer plate and a solvent system of chloroform/acetone/methanol (70/25/5). After elution from the silica gel, the bile acid methyl esters were analyzed as the trimethylsilyl ethers on gas liquid chromatographic columns of 3% QF-1 or 1% SE-30. Taurocholate-^{14}C was routinely added to the sample before analysis, and the final product was divided into aliquots for gas liquid chromatography and for scintillation counting, the latter to determine recovery factors.

Results. Examinations of many germfree rats, including the S–D strain, have not revealed in them spontaneous neoplasms of the gastrointestinal tract (8).

In response to orally-administered DMH, germfree S–D rats developed many neoplasms in the intestinal tract; and the numbers were significantly increased in the rats for which the diet had a supplement of 2% cholestyramine (Tables I and II). The spectrum of intestinal neoplasms in both groups ranged from small benign polyps on the surface of the mucosa to extensive malignant adenocarcinomas that penetrated through the wall into the serosal membrane (Fig. 1). Some of the tumors were large tissue masses in the lumen and attached by a slender stalk to the gut wall. Others were flat and invasive to the gut wall and more extensively protruding like a bunch of grapes into the serosal membrane. Intussusception was observed especially in the descending colon and rectum; and in some rats the rectal tumor was visible protruding through the anus. The neoplasms were most numerous in the descending colon, the duodenum, and the rectum. In addition, the rats on the cholestyramine-supplemented diet had significant numbers of tumors in the rectum, the jejunum, the cecum, and the colon, at an average of 16.5 tumors per rat, compared with 9.3 tumors per rat in those without cholestyramine in the diet.

Metastatic lesions were observed in the lymph nodes of two rats; the affected lymph nodes were swollen, cystic, and had part of the lymphatic tissue displaced by foci of carcinomatous cells with few mitotic figures (Fig. 2). In many of the rats, the livers carried many cysts.

Three tumors were minced and transplanted subcutaneously to litters of newborn Sprague–Dawley rats. All of them

TABLE II. INTESTINAL NEOPLASMS INDUCED BY DIMETHYLHYDRAZINE IN GERMFREE SPRAGUE–DAWLEY RATS[a] (CHOLESTYRAMINE)

			Tumors					
						Colon		
Number	Sex	Body wt/g	Duodenum	Jejunum	Cecum	1/2	1/2	Rectum
1	M	378					1	5[b]
2	M	342	6		1	3	6	3
3	M	368	9	2			11	
4	M	320	8	1		2	16	
5	F	200	3		1	2	2	
6	F	199	2				10	2
7	F	183	5	1	1		11	2
Total			33	4	3	7	57	12 = 116
								16.5/rat

[a] Rats at age 2 months were fed DMH (30 mg/kg) once/week/10 weeks. Diet: 2% cholestyramine. Tek-Lad (L-485). Rats were killed for examinations at 20 weeks after onset of experiment.

[b] Metastatic tumor in lymph node.

FIG. 1. Carcinoma induced in intestine of germfree Sprague–Dawley rat by orally-administered dimethylhydrazine. Note extension of neoplasm into serosa. × 8.

developed localized adenocarcinomas at the inoculated site within 50 days, which increased in size during the next 4 weeks. One tumor has been passaged to a second litter of S–D rats.

Table III shows the amount and concentration of bile acids in the supernatant and the precipitate of cecal content for individual rats with and without dietary cholestyramine resin. Despite the wide scattering

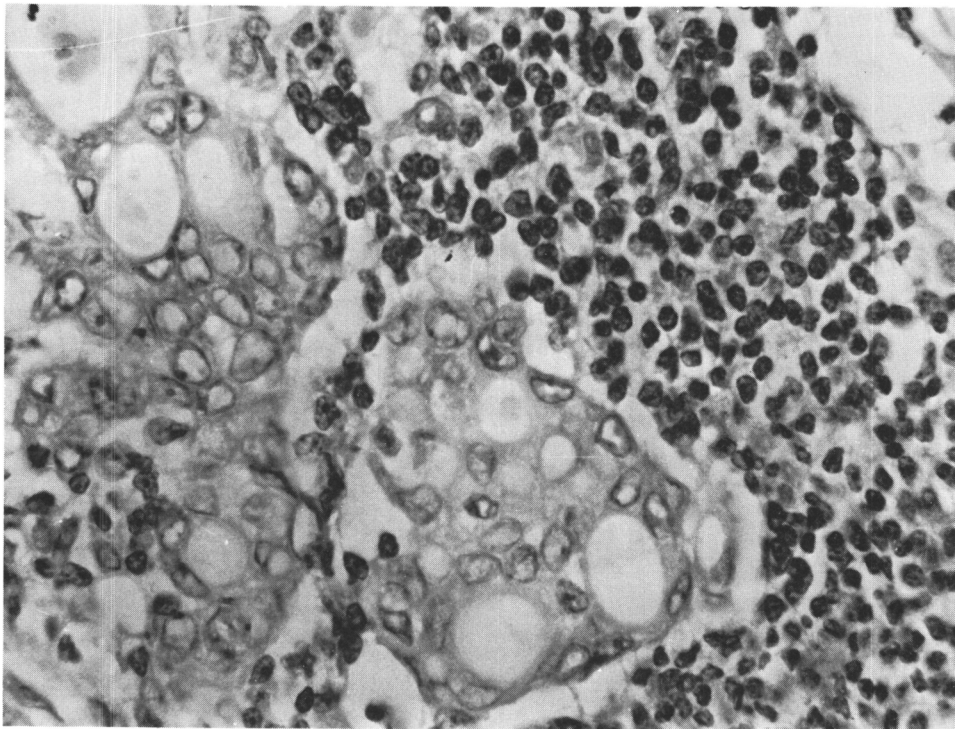

FIG. 2. Metastasis of carcinoma cells (left) from intestine into lymph node (right). × 120.

TABLE III. BILE ACID IN CECAL CONTENT

Rat number	Resin	Sex	Body wt (g)	Total amount of bile acids (mg)	Amount in supernatant (mg)	Concentration in supernatant (mg/ml)	Amount in precipitate (mg)	Concentration in precipitate (mg/ml)
C-1	+	M	378	15.11	1.93	0.19	13.18	1.29
C-5	+	F	200	16.57	1.12	0.16	15.45	1.88
C-6	+	F	199	12.20	1.91	0.27	10.29	1.25
C-7	+	F	183	11.29	1.28	0.25	10.01	1.88
D-2	−	M	328	4.66	2.26	0.19	2.40	0.25
D-3	−	M	355	10.00	4.55	0.43	5.45	0.52
D-4	−	M	367	3.78	2.01	0.23	1.77	0.18
D-8	−	F	203	5.57	2.88	0.33	2.69	0.34

of data, it is clear that the total bile acid content in the cecum is higher in the resin-fed group of rats than in the control group, and the difference is in the high content in the precipitate fraction. This evidence is comparable to the increased bile acid excretion observed previously (9) and can be interpreted as resulting from the binding of bile acid to the resin. The bile acid content and concentration in the supernatant of the cecal contents of the resin-fed group were only slightly lower than the controls. This might reflect an efficient negative feedback response of the enterohepatic circulation to compensate for the increased fecal loss of bile acid through binding to the resin.

Table IV shows the mean concentrations of individual bile acids in the supernatant as well as in the precipitate. The most salient feature is the increased concentration of all the components in the precipitate of the resin-fed group, a finding to be expected

TABLE IV. BILE ACID CONCENTRATIONS IN CECAL CONTENT

	Supernatant (mg/ml)	Precipitate (mg/ml)
With resin[a]		
Cholic acid	0.159	0.954
β-muricholic acid	0.031	0.290
Ketones	0.010	0.049
Chenodeoxycholic acid	0.013	0.288
R[b]	5.13	3.29
Without resin[a]		
Cholic acid	0.120	0.139
β-muricholic acid	0.126	0.138
Ketones	0.030	0.025
Chenodeoxycholic acid	0.016	0.023
R[b]	0.95	1.01

[a] The number of rats was four each.

[b] Ratio between cholic and β-muricholic acid concentration.

from the binding to the resin. It is of interest to compare the ratio of cholic to muricholic acids between the two groups. In the control group the ratio is close to one in the supernatant as well as in the precipitate. A similar value was reported for the intestinal contents of germfree rats by Wostmann (10), who stressed this low value, compared to the value of 5 to 6 in the conventional counterparts, as characteristic of germfree status. In order to account for the low ratio or a relative preponderance of muricholic acid in germfree rats, Kellogg (11) speculated that in germfree rats the greater enterohepatic circulation, because of the lack of bacterial destruction of bile acids, inhibits cholic acid synthesis more than that of muricholic acid, hence resulting in the relative predominance of the latter. In this study on the resin-fed group of rats, the ratio is close to that in conventional rats despite the fact that the rats were germfree. It is unclear at present whether the inhibition of enterohepatic circulation by the resin induced a relative predominance of cholic acid synthesis resulting in the higher ratio as observed.

Discussion. The results in this study did not substantiate the thesis that secondary bile acids act as cocarcinogens (3). GF rats which lack secondary bile acids developed very significant numbers of tumors in the intestinal tract in response to orally-administered DMH. The efficacy of a chemical carcinogen and the contribution of a cocarcinogen must be evaluated in regard to the dosage and the mode of administration. The regimen of DMH administration adopted in this study may have been excessive (6), so that the role of secondary bile acids as cocarcinogens may have been obscured. However, the effect of cholestyramine resin in increasing the tumor incidence and in accelerating malignant transformation in germfree rats indicates that the process of carcinogenesis was not saturated and was promoted by the resin. Aside from the remote possibility of contaminants in the resin preparation, the effect must be attributed to an alteration in bile acid metabolism.

Weisburger *et al.* (12) indicate that intrarectal administrations of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine induced twice as many colon adenomas in germfree Fischer rats as in conventional counterparts; however, there was no difference in numbers of induced adenocarcinomas. However, Reddy *et al* (13) indicated that the carcinogenic effect of DMH required metabolic activation by the microbial flora, and emphasized a strong relationship of carcinoegenesis to microbial activity. This was not confirmed in the results reported here with orally-administered DMH in germfree S–D rats. Actually, the numbers and types of tumors indicate clearly that microbial agents appear to have little role in DMH-induced intestinal neoplasms and that factors influencing bile acid metabolism are of importance to the carcinogenic process.

Three parameters of bile acid metabolism were shown to be altered by the cholestyramine resin and could be used as a possible link to the effects on carcinogenesis. The concentration of bile acids in the precipitate showed the highest rise over the control. However, the bile acids in this fraction are sequestered in the resin matrix and can hardly be expected to exert any influence on the cellular process in the intestinal mucosa. The bile acid concentrations in the supernatant must be the determining factor in its contact with cells in the mucosal membrane.

In the resin-fed group of rats the concentration of bile salts in the supernatant was close to and slightly lower than in the control rats, thereby minimizing its responsibility for promotion of carcinogenesis. The ratio of cholic to muricholic acid was higher in the resin-fed group of rats, which is of uncertain interpretation at this time.

Therefore, it appears that some aspect of the dynamic status of bile acid metabolism has a direct relation to intestinal carcinogenesis.

Summary. Oral administration of 1,2-dimethylhydrazine (DMH) induced intestinal neoplasms in germfree rats. A supplement of 2% cholestyramine resin in the diet increased the frequency of DMH-induced intestinal tumors and accelerated malignant transformation. Bile acids in the cecal content were determined with and without cholestyramine in order to obtain a correlation between the bile acid metabolism and the enteric carcinogenesis.

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