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Colon Carcinogenesis with Azoxymethane and Dimethylhydrazine in Germ-free Rats[1]

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SUMMARY

The effect of intestinal microflora on the sensitivity of the colon to the carcinogenic effect of azoxymethane and a large dose of 1,2-dimethylhydrazine was studied using germ-free and conventional female Fischer rats. Injection s.c. of 1,2-dimethylhydrazine induced tumors of the ear duct, kidney, and small intestine of conventional rats but none in germ-free animals. Only 20% of germ-free rats showed 1,2-dimethylhydrazine-induced colonic tumors, whereas 93% of conventional rats developed multiple colonic tumors. Intrarectal instillation of azoxymethane appreciably increased the multiplicity of colonic tumors in germ-free rats and in gnotobiotic rats contaminated with *Clostridium perfringens*, as compared to conventional controls. None of the germ-free rats showed ear duct tumors. The incidence of kidney tumors was lower in germ-free rats than in other groups. It is concluded that the intestinal microbial populations alter the effect of carcinogens in the large intestine.

INTRODUCTION

Epidemiological studies suggest that diet, particularly as it relates to combined fat and animal protein, may be among the most important causal factors associated with colon cancer in man (1, 2, 4, 21, 24). These dietary effects may be mediated through changes in the composition of endogenous compounds secreted into the gut as well as the composition of intestinal microflora, which could convert dietary components and endogenous secretions into cocarcinogens and/or carcinogens (5, 6, 12, 20, 22, 23).

Within the past few years an experimental animal model has been developed to study colon carcinogenesis by the administration of DMH,[2] AOM, and MNNG (3, 9, 19). Adenomatous polyps and carcinomas of the colon induced by these chemicals in rats were similar to those observed in man (14, 17, 19).

The importance of intestinal bacteria for liberating active key intermediates with this type of chemical carcinogen to induce colon tumors in experimental animals stems from the observations of Laqueur (8). We are currently engaged in studies on the metabolism and mode of action of DMH and related compounds. Our recent data demonstrate that the intestinal microflora accelerated, or was indeed instrumental, in colon tumor production by DMH administered s.c. but that it slightly inhibited colon tumor formation by a direct-acting carcinogen (MNNG) infused i.v. (11). It is thought that DMH yields AOM which is hydroxylated to an active metabolite, and intestinal bacteria have been shown to play a modifying role in colon carcinogenesis induced by various chemicals. Thus, we have now extended our studies to elucidate the effect of the intestinal microflora on the carcinogenicity of AOM on the colon. We have also investigated the action of increased doses of DMH on the induction of colon carcinoma in germ-free rats, as a continuation of our earlier studies (11).

MATERIALS AND METHODS

Weanling female Fischer germ-free and conventional rats were obtained from Charles River Breeding Laboratory, Wilmington, Mass., and fed *ad libitum* a steam-sterilized Purina laboratory chow, 5010C. Germ-free rats were housed in the Trexler flexible plastic isolater, and the conventional rats were kept in a temperature- and humidity-controlled clean room (13). In 1 series of experiments weanling germ-free rats were monocontaminated with *Clostridim perfringens*, kept in the germ-free isolators, and reared under germ-free conditions. The germ-free or monocontaminated status of the animals was determined at biweekly intervals and also at the termination of the experiment (18). Identification of *C. perfringens* was done as described by Holdeman and Moore (7).

At 50 days of age germ-free, monocontaminated, and conventional rats (except controls) were given 20 weekly i.r. instillations of AOM at a dose level of 10 mg per kg of body weight per week. In another series, germ-free and conventional rats (except controls) were given s.c. injections of DMH at a dose level of 20 mg per kg of body weight per week for 20 weeks. Before injection, DMH dihydrochloride or AOM were dissolved in 0.9% NaCl solution and brought to pH 6.5. For injections into germ-free and monocontaminated animals, the AOM or DMH solution was sterilized by Millipore filtration and transferred into the isolators. Controls were given an equal volume of 0.9% NaCl solution.

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[2] The abbreviations used are: DMH, 1,2-dimethylhydrazine; AOM, azoxymethane; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; i.r., intrarectal.

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All animals were autopsied 15 weeks after the last injection for a total experimental period of 35 weeks. All organs, including the intestines, were examined grossly and histologically for the number and type of tumors. The dimensions and location of intestinal tumors were recorded. Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin, and the sections were stained with hematoxylin and eosin.

RESULTS

Tumor Induction by AOM. Table 1 summarizes the tumor incidence in germ-free, monocontaminated, and conventional rats treated i.r. with AOM. None of the germ-free rats showed tumors of the ear duct, whereas nearly one-half of the monocontaminated and conventional animals developed squamous cell carcinomas of the external auditory canal. There was no difference in the incidence of small intestinal tumors, mainly localized 2 to 4 cm from the gastroduodenal junction in all groups. In addition to duodenal tumors 12% of the conventional rats developed tumors in the proximal jejunum, but none of the germ-free or monoassociated animals did.

Multiple tumors were induced in the colon of germ-free, monoassociated, and conventional rats. The number of rats with colon tumors were increased in monocontaminated and germ-free groups as compared to the number in conventional controls. The tumors occurred throughout the colon but were found more frequently in the distal half. The diameter of colon tumors was 0.5 to 3.0 cm in monocontaminated rats, whereas they ranged from 0.1 to 1.5 cm in germ-free and conventional rats. The number of colon tumors per rat were higher in germ-free and monocontaminated rats than in their conventional controls. Monocontaminated rats developed more adenocarcinomas than the other groups did. Aside from those noted, none of the other organs showed any tumors.

Tumor Induction by DMH. DMH, injected s.c., induced tumors of the ear duct, kidney, and small intestine in conventional rats but none in germ-free animals (Table 2). Tumors of the ear duct were squamous cell carcinomas, and the kidney tumors were of mesenchymal type. Tumors of the small intestine predominated in the duodenum (14 tumors in 11 rats), 1 to 4 cm from the pylorus, but 2 animals showed 2 jejunal tumors. About 36% of the duodenal and all jejunal tumors were adenocarcinomas.

Neoplasms of the colon were observed in 5 of 24 germ-free rats and in 14 of 15 conventional animals. In line with our earlier observations (12) not only did germ-free status have a significant influence on the number of rats in which colon tumors developed, but there were also fewer neoplasms per tumor-bearing rat. The diameter of colon tumors varied from 0.1 to 0.3 cm in germ-free rats, and 0.4 to 1.4 cm in conventional animals. The percentage of distribution of adenocarcinomas and adenomas in the colon was similar in both germ-free and conventional rats.

Table 1

Tumor incidence in germ-free, monocontaminated, and conventional rats treated with AOM

Status	Body wt (g)	Animals with tumors: Ear canal No.	Ear canal %	Kidney No.	Kidney %	Small intestine No.	Small intestine %	Colon No.	Colon %	Classification and multiplicity of colon tumors (per rat): Total tumors	Adenocarcinoma	Adenoma
Germ-free (15)[a]	188	0	0	2	13	8	53	14	93	4.3 ± 0.3[b]	1.8 ± 0.3	2.5 ± 0.4
Monocontaminated (7)[c]	187	3	43	3	43	3	43	7	100	6.6 ± 1.1	3.3 ± 0.7	3.3 ± 0.9
Conventional (25)	191	12	48	12	48	12	48	15	60	2.4 ± 0.4	1.2 ± 0.2	1.2 ± 0.3

[a] Numbers in parentheses, number of rats.
[b] Mean ± S.E.
[c] Seven of 14 monocontaminated rats died 10 weeks before the last injection of carcinogen due to possible toxemia.

Table 2

Tumor incidence in germ-free and conventional rats with DMH

Status	Body wt (g)	Animals with tumors: Ear canal No.	Ear canal %	Kidney No.	Kidney %	Small intestine No.	Small intestine %	Colon No.	Colon %	Classification and multiplicity of colon tumors (per rat): Total tumors	Adenocarcinoma	Adenoma
Germ-free (24)[a]	198	0	0	0	0	0	0	5	21	0.2 ± 0.1[b, c]	0.1 ± 0.1[c]	0.1 ± 0.1[c]
Conventional (15)	179	13	87	3	20	12	80	14	93	2.1 ± 0.4	1.2 ± 0.2	0.9 ± 0.3

[a] Numbers in parentheses, number of rats.
[b] Mean ± S.E.
[c] Significantly different from conventional group; $p < 0.01$.

The histopathology of neoplasms of the colon and small intestine in rats treated with AOM was similar to those given DMH. Duodenal and jejunal carcinomas were well-differentiated, tubular or papillary adenocarcinomas, and in a few cases, signet-ring cell carcinomas invading extensively into the lamina propria or into the serosa. Adenomatous polyps of the large intestine were pedunculated and sessile types. The majority of the carcinomas of the colon were well-differentiated, tubular adenocarcinomas, and signet-ring cell carcinomas and were confined to the cecum and proximal colon.

DISCUSSION

Several groups of investigators (3, 19) have shown that AOM, administered s.c. or p.o., is an effective carcinogen for the intestine. In these experiments it was possible to induce colonic tumors by i.r. instillation of AOM in rats. The observation that i.r. administration of AOM produced tumors in the kidney, ear duct, small intestine, and large intestine of conventional animals can mean that the compound reaches the liver after absorption; possibly it is transported via bile and blood stream to various sites.

The present study confirmed our earlier findings (11) that the intestinal microflora played a modifying role in accelerating colon tumor production by DMH. Our data also demonstrate that DMH, even in high doses, had only a limited effect in germ-free rats, whereas it induced multiple colonic tumors in conventional animals.

The induction of ear duct tumors by DMH and AOM in conventional rats, but not in germ-free animals, seems to suggest that these compounds are metabolized by the intestinal microflora liberating reactive metabolites which probably reach the ear duct after absorption. However, additional considerations involve the possible contribution of microbial elements in the ear duct in a more direct way. Many years ago Rudali *et al.* (15) and Skoryna *et al.* (16) discussed the involvement of otitis media in ear duct carcinogenesis induced by *N*-2-fluorenylacetamide. Our findings open the door to further investigations of the participation of local and intestinal microflora in cancer induction at this site.

Of particular interest is the presence of neoplasms in the small intestine of conventional but not germ-free rats with DMH. From the localization of these lesions it would appear that a carcinogenic metabolite is delivered to that region in fairly concentrated form, probably with bile. Investigations on the metabolism of DMH are now proceeding in our laboratories to develop specific information on this point. In an earlier experiment, where the dose of DMH administered was lower, a lesser response in the small intestine and also the large bowel was noted (11).

In contrast to DMH carcinogenesis, the resident microflora seems to have some inhibiting effect on colonic tumor production by i.r. instillation of AOM. The mechanism is not clear. Although the uptake of this compound after i.r. instillation was not measured, the available evidence indicates that the absorption of a variety of compounds from the intestinal tract was substantially higher in the germ-free animal (10). Thus, in germ-free rats there may be an increased absorption of AOM leading to an altered metabolism in the liver and supplying increased amounts of cocarcinogen and/or carcinogen to the target organ. The route of compound administration may also be of importance. Apparent physical differences in the rectal contents between the germ-free and conventional rats might also affect the dosimetry. We are currently studying all of these questions.

The carcinogenic effect of AOM could be increased by monoassociation of germ-free rats with *C. perfringens* which has a strong β-glucuronidase activity. Although the mechanism requires elucidation, the potential usefulness of individual strains of microorganisms to alter the effects of chemical carcinogens has been demonstrated. This also suggests that, in the search for causes of the differences in colon cancer risk in man in various geographical areas, it is important to consider not only dietary variations but also diet-mediated changes of intestinal microbial population that might alter the carcinogenic and/or cocarcinogenic activity of chemicals and metabolites in the large intestine.

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