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Tumor Induction in Germ-free Rats Fed Bracken (*Pteridium aquilinum*)[1]

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ABSTRACT

This study indicated that there was no significant difference in the incidence of intestinal tumors between germ-free and conventional rats fed a diet containing bracken, suggesting that gut microflora did not play a definite role in bracken tumorigenesis. However, bracken induced exclusively sarcoma but no adenocarcinoma in germ-free rats, whereas it induced predominantly adenocarcinoma in conventional rats.

INTRODUCTION

It has been known that intestinal and urinary bladder neoplasms are induced by feeding bracken fern to rats and cattle (1, 10, 13, 16). Hirono *et al.* (6, 7) reported that unprocessed young bracken and processed bracken, which have long been used as a human food in Japan, also produced tumors in the ileum and urinary bladder of conventional rats. Hirono *et al.* (8) also reported that the carcinogen in bracken is extractable in boiling water and is most probably water soluble. However, the nature of the carcinogen in bracken has not yet been definitely elucidated. Pamukcu *et al.* (12) suggested that the carcinogenic substance in bracken is present in the acidic fraction of the urine of cattle fed bracken and that it is soluble in methanol (14). Although indanones (3, 4) and pterolactam (18) have also been isolated from bracken fern, there are no data to indicate that these chemicals are carcinogenic (6, 17). Evans and Osman (2) reported that shikimic acid was contained in bracken and that it was carcinogenic in mice. However, no tumors were induced in rats fed a diet containing shikimic acid in the experiment of Hirono *et al.* (5). Thus, it was considered that the carcinogenicity of bracken, at least in rats, is attributable to a substance(s) other than shikimic acid. Recently, Wang *et al.* (20) reported that tannin was isolated from bracken and that it induced bladder tumors when implanted intravesically in the mouse. However, neither intestinal nor bladder tumor was induced when the tannin-containing diet was fed to rats (15). Very recently, Pamukcu *et al.* (11) suggested that the quercetin may be one of the active ingredients in bracken fern.

This study was designed to investigate whether or not gut microflora plays any role in the induction of tumors by bracken carcinogen. For this purpose, germ-free rats were used.

MATERIALS AND METHODS

Germ-free and conventional Wistar female rats bred by the technique described previously (9) in the Laboratory of Germfree Animal Research, Kawashima, Japan, were used. The germ-free rats were maintained in a plastic isolator. Their germ-free status was checked once every 2 weeks as described by Wagner (19). The conventional rats were housed in a temperature- and humidity-controlled clean room. Bracken (*Pteridium aquilinum*) in the experiment was collected in Hokkaido in May and June. The fresh bracken was dried in a dryer equipped with a blower. This material was then milled and mixed with the rat basal diet CL-2 (CLEA Japan, Inc., Tokyo, Japan) in the proportion by weight of 1 part of bracken per 2 parts of the diet. The diet, packaged in a polyethylene bag, was irradiated with a 5-megarad dose of ^{60}Co γ-rays. The diet, sterilized in this way, was proved sterile on repeated bacteriological examination. Ten germ-free and 16 conventional rats weighing 80 to 90 g at the start of the experiment were fed the sterilized bracken diet until these animals were moribund or died from tumor. The diet intake per animal was checked every week. The average diet intake per animal per day was 19.7 ± 1.00 (S.D.) g in germ-free rats and 18.4 ± 1.07 g in conventional rats.

The treated germ-free and conventional rats were weighed once weekly during a 6-month pretumorous period in order to determine any toxic effect of bracken. However, there was no significant difference in the rate of body weight gain between both groups.

The experimental animals which were killed in moribund condition or had died were autopsied and examined for tumors on various organs, including the entire gastrointestinal tract. For light microscopy, tissues were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and by the Van Gieson method.

RESULTS

There was no remarkable difference in intestinal tumor incidence between germ-free and conventional rats fed bracken diet. Ninety % of 10 germ-free rats had tumors, while 94% of 16 conventional rats had tumors. These intestinal tumors were exclusively located in the distal portion of the ileum in both germ-free and conventional rats, with the exception of 3 cases with cecal tumor only in the germ-free rats. The average latent period of the tumors was 11.7 months in germ-free rats, the first tumor occurring in the 9th month and the last tumor occurring in the 14th month; whereas the average latent period of the tumors was 13.7 months in the conventional rats, the first tumor appearing in the 11th month and the last tumor appearing in the 16th month (Table 1). Regarding tumor multiplicity in the intestinal tract, there were 4.6 tumors/animal in the germ-free rats, whereas there were 4.4 tumors/animal in the conventional rats.

The results of observation on sites and histological types of tumors induced in both groups are given in Table 2. Sarcomas of the intestinal tract were found in 9 germ-free treated rats (90%) and in 5 conventional rats (31%). The sarcomas in both

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Table 1

Time at which germ-free and conventional rats fed bracken diet died or were killed and the corresponding incidence of tumors

Time after initiation of feeding (mos.)	Germ-free rats					Conventional rats				
	No. of deaths	No. of rats with tumors of				No. of deaths	No. of rats with tumors of			
		Ileum	Cecum	Bladder	Adrenal		Ileum	Cecum	Bladder	Adrenal
9–10	1	1								
10–11	1					1	1			
11–12	5	5	2		1	3	2			1
12–13	1	1				3	3			1
13–14	1	1				2	2			
14–15	1	1	1			2	2			
15–16						5	5		1	
Total	10	9	3	0	1	16	15	0	1	2

Table 2

Sites and histological types of tumors induced in each group

Tumor sites and histological types	No. of rats with tumors	
	Germ-free rats (10)	Conventional rats (16)
Ileum		
Adenoma	6	8
Adenocarcinoma[a]	0	10
Sarcoma[b]	9	5
Cecum		
Adenoma	3	0
Urinary bladder		
Papilloma	0	1
Adrenal		
Cortical adenoma	1	2

[a] $p < 0.01$
[b] $p < 0.02$

germ-free and conventional rats were almost nodular growths elevated on the serosa. Those of germ-free rats were, however, larger, more numerous, and more intensive in growth rate than those of conventional rats; the largest one found in a germ-free rat reached a diameter of 4.7 cm. The primary ileal sarcoma in a germ-free rat gave rise to a transcoelomic metastasis followed by infiltration into the liver, binding the abdominal viscera into an inseparable mass. Histologically, most of the sarcomas were composed of interlacing bundles of spindle-shaped cells which sometimes showed variable anaplasia, mixed with giant cells, there being no difference in such a histological pattern between germ-free and conventional rats. None of the germ-free rats had adenocarcinoma, while 10 of the conventional rats (63%) had adenocarcinoma. Histologically, the adenocarcinomas of the conventional rats had a glandular structure and sometimes infiltrated the submucosa, extending into the serosa. Regarding benign tumors in the intestinal tract, 7 of the germ-free treated rats (70%) had adenomas, while 8 of the conventional treated rats (50%) had adenomas. Their histological pattern of glandular structure was the same in germ-free rats as it was in conventional ones.

With regard to tumor in organs other than the intestinal tract, there was one case with adrenocortical adenoma in germ-free rats, whereas there were 2 with adrenocortical adenomas as well as one with urinary bladder papilloma in conventional rats.

DISCUSSION

The results of this study not only indicated that there was no difference in intestinal tumor incidence between germ-free and conventional rats but also revealed that the latent period of the tumor was shorter in germ-free rats than it was in conventional ones. Metastatic lesions were observed only in a germ-free rat, suggesting that gut microflora did not play any essential role in the tumor induction by bracken carcinogen. In the present study, it was noted that adenocarcinoma was never induced but that sarcoma was induced in a significantly higher incidence in germ-free rats than it was in conventional ones. Hirono *et al.* (6, 7) suggested that rats which received a diet containing a greater amount of bracken carcinogen were more likely to induce sarcoma than adenocarcinoma. Since the same amount of bracken diet (per rat per day) was ingested by both germ-free and conventional rats, a probable explanation for the high incidence of sarcoma in germ-free rats and adenocarcinoma in conventional rats would be that gut microflora might be involved in the degradation of bracken carcinogen in conventional rats.

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