Promoter Function of Carrageenan on Development of Colonic Tumors Induced by 1,2-Dimethylhydrazine in Rats

Shoichi ARAKAWA,¹,* Masao ITO,¹ and Setsuzo TEJIMA²

¹ Division of Biology, Aichi Prefectural Institute of Public Health, Kita-ku, Nagoya 462, Japan
² Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467, Japan

(Received May 19, 1988)

Summary In order to understand the function of carrageenan, an indigestible polysaccharide, as a promoter of colonic tumors induced by 1,2-dimethylhydrazine (DMH), molecular weight distribution of fecal carrageenan and amounts of fecal bile acids in rats given carrageenan and DMH treatment were examined. Gel filtration pattern on Sephacryl S-300 of fecal carrageenan was very similar to that of feeding carrageenan, and carrageenan ingested was quantitatively excreted in feces. Hexafluoroisopropyl ester-trifluoroacetyl derivatives of fecal bile acids were analyzed by gas chromatography on QF-1. Although there was a decreased concentration of deoxycholic acid and total bile acids in carrageenan-fed rats compared to control rats, no difference in the daily output was found because carrageenan ingestion increases fecal output. Significant increased concentration and daily output of lithocholic acid, a tumor-promoter, by feeding carrageenan were found. Thus, it was suggested that the promoting effect of carrageenan on colon tumorigenesis by DMH may be mediated by increased excretion of lithocholic acid and may not participate in degradation of carrageenan ingested.

Key Words colonic tumorigenesis, 1,2-dimethylhydrazine, carrageenan, molecular weight distribution, bile acid

There have been a number of animal models for the relationship between indigestible polysaccharides and chemically induced colon cancer (1, 2). It has been considered that the polysaccharides are protective against the development of colonic tumors, by diluting the intestinal contents and decreasing transit time (3).
However, we previously indicated an enhancing effect of carrageenan, an indigestible polysaccharide, on colonic tumorigenesis by 1,2-dimethylhydrazine (DMH) in rats fed a diet containing an ordinary fat, in spite of increased fecal bulk (4). This result confirms that of Watanabe et al. (5), who demonstrated that native carrageenan produced the enhancing effect on colorectal carcinogenesis by azoxymethane or methyl nitrosourea in rats fed a high dietary fat and that carrageenan itself did not produce colonic tumor, suggesting that this polysaccharide appears to be a tumor promoter.

Although indigestible polysaccharides are resistant to the action of human intestinal tract enzymes, they are susceptible to degradation by bacterial enzymes. For example, cellulose and pectin had been shown to be degraded during passage through the gut (6, 7). In animals, degraded carrageenans, molecular weight of 100,000 or less, were detected in feces of rats fed native carrageenans (8). On studies related to the effect of degraded carrageenan on colon carcinogenesis and on enhancement of colon cancer induced by DMH in rats (9, 10), the molecular weight of degraded carrageenan used was in the range 20,000–40,000.

It has been considered that bile acids are important etiological factors in colon cancer and act as tumor promoters rather than as direct carcinogens in animal experiments (11, 12). Carrageenan has been shown to increase bile acids in rat feces (13). Thus, for understanding the function of carrageenan as a promoter of colonic tumors induced by DMH, we examined the molecular weight distribution of fecal carrageenan and the amounts of fecal bile acids in rats given carrageenan and DMH treatment.

**METHODS**

1. **Animals and Maintenance.** Twenty-four male F344 rats were divided into four groups, housed individually in stainless steel wire-bottomed cages in a temperature- and humidity-controlled room. When animals reached 7 weeks of age, group 1 was given weekly subcutaneous injections of DMH (Aldrich Chemical Co., USA) at a dosage of 20 mg/kg body weight for 3 weeks and received a carrageenan diet for 3 weeks. Group 2 received the same DMH treatments and was fed a control diet for the same period as group 1. Group 3 received a carrageenan diet without carcinogen treatment. Group 4 received a control diet. The composition of the diets was described previously (4). Rats had free access to food and water. Daily food consumption and fecal output were measured for the last 4 days of the experiment. The fecal samples were frozen at −20°C until analysis.

2. **Molecular weight distribution.** Feces from individual rats were freeze-dried and ground in a homogenizer. One hundred mg of feces in groups 1 or 3 was added to 40 ml of 0.2 M lithium chloride, warmed for 15 min at 65–70°C. After cooling to room temperature, the mixture was centrifuged at 6,000 rpm for 15 min. Five ml of the supernatant was applied to a water-jacketed chromatographic column containing Sephacryl S-300 (Pharmacia Fine Chemicals, Sweden). The procedure
was carried out at 30°C, because carrageenan showed a transition of relative viscosity at 23°C (14). A Shodex standard P-82 kit (Showa Denko Co., Tokyo) was used as a standard for molecular weight. Aliquots of fractions were measured by phenol-sulfuric acid method (15), and the supernatants were also measured to determine the amount of carrageenan in feces.

3. Fecal bile acids. Feces were mainly processed by the method of Ferguson et al. (16) with some modifications, omission of thin-layer chromatography, and a Baker 10 Amino column (J. T. Baker Chemical Co., USA) for purification instead of a column of PHP Sephadex LH-20. Our preliminary experiment revealed that there were little conjugated bile acids in rat feces. Using the Amino column, free bile acids were eluted with 0.1 M acetic acid in 90% ethanol (10 ml), and extracted with chloroform/methanol by the method of Grundy et al. (17). Hexafluoroisopropyl ester-trifluoroacetyl derivatives of the acid samples prepared as described by Imai et al. (18) were analyzed by gas chromatography on QF-1, and were identified by gas chromatography-mass spectrometry. The data were expressed as means ± SE and analyzed statistically by Student’s t-test.

RESULTS

As shown in Table 1, no significant differences were found in body weight and food intake among the groups, and in carrageenan intake between groups 1 and 3. The rats fed the carrageenan diet excreted more feces than those fed the control diet. There were no significant differences in the amount of fecal carrageenan and the proportion of fecal carrageenan to ingested carrageenan between groups 1 and 3. The proportion in group 1 was 97.3%. As shown in Fig. 1, the molecular weight distribution of this polymer in feces in group 1 by Sephacryl S-300 elution pattern was over 100,000, and was very similar to that of the feeding carrageenan. After hydrolysis of each fraction in fecal samples by trifluoroacetic acid, gas chromatographic analysis of the trimethylsilyl derivatives showed that there was no monosaccharide except galactose.

For estimation of recovery of bile acids, 0.5 mg of each bile acid standard was added to 0.5 g portion of feces. Table 2 summarizes recovery and relative retention time of each bile acid. Although hexafluoroisopropyl ester-trifluoroacetyl derivatives have several advantages, such as simplicity of preparation, absence of artifacts, stability during storage, and resolution, compared to methyl-trifluoroacetyl derivatives (19), the derivative of hyodeoxycholic acid (HDCA) could not be separated from that of ursodeoxycholic acid (UDCA). In general HDCA is a major component and UDCA is a trace in rat feces. Thus, we regarded the substance, which had the same retention time on chromatogram as HDCA in fecal samples, as hyodeoxycholic acid-like substance. However, we did not analyze HDCA and UDCA quantitatively, because these bile acids have not been noted to associate with colon cancer. Fecal excretion of free bile acids is shown in Table 3. Significant increased concentration of lithocholic acid (LCA) and decreased

Vol. 34, No. 6, 1988
Table 1. Body weight, and intake and fecal excretion of CAR. \(^a\)

| Group | Treatment | Body weight (g) | Food intake (g/day) | CAR intake (g/day) | Fecal output (g/day) | CAR in feces (g/day) |
|-------|-----------|----------------|---------------------|--------------------|----------------------|----------------------|
|       |           | Initial        | Final               |                    |                      |                      |
| 1     | DMH + CAR | 138.0 ± 3.0\(^b\) | 214.5 ± 4.1 | 13.9 ± 0.3          | 0.83 ± 0.02          | 1.88 ± 0.06\(^c\)   | 0.81 ± 0.02          |
| 2     | DMH       | 138.6 ± 3.1    | 226.7 ± 4.9        | 14.6 ± 0.2         | —                    | 0.96 ± 0.03          | (97.3 ± 1.1)\(^d\)   |
| 3     | CAR       | 137.6 ± 2.9    | 223.7 ± 4.0        | 14.9 ± 0.4         | 0.89 ± 0.02          | 2.08 ± 0.08\(^e\)   | 0.88 ± 0.03          |
| 4     | —         | 136.0 ± 3.7    | 222.3 ± 5.2        | 14.2 ± 0.3         | —                    | 0.92 ± 0.03          | (98.2 ± 2.2)         |

\(^a\) CAR, carrageenan. \(^b\) Mean ± SE of 6 rats. \(^c\) \(p<0.05\) between groups 1 and 2. \(^d\) Values in parentheses represent percentage of ingested carrageenan. \(^e\) \(p<0.05\) between groups 3 and 4.
Fig. 1. Elution pattern of native carrageenan and typical fecal carrageenan from rats given carrageenan and 1,2-dimethylhydrazine treatment. Column, Sephacryl S-300, 2.6 × 90 cm; eluent, 0.2 M LiCl; flow rate, 48 ml/h; fraction volume, 6.4 ml. Arrows represent standard molecule size: a, 400 kDa; b, 200 kDa; c, 100 kDa; d, 50 kDa; e, 30 kDa.

Table 2. Recovery and relative retention time of each bile acid.

| Bile acid             | Recovery (%) | Relative retention time |
|-----------------------|--------------|-------------------------|
| Cholic acid           | 89.1<sup>a</sup> | 2.21<sup>b</sup>        |
| Chenodeoxycholic acid | 90.6         | 1.29                    |
| Deoxycholic acid      | 93.3         | 1.00                    |
| Lithocholic acid      | 93.4         | 0.61                    |
| Hyodeoxycholic acid   | 94.5         | 1.48                    |

<sup>a</sup> Values are average of duplicates. <sup>b</sup> Relative retention times are referred to deoxycholic acid (actual time = 10.0 min). GC conditions: column, 1% QF-1 on Chromosorb W (1 m × 3 mm); column temp., 220°C; N₂, 35 ml/min.

concentration of total bile acids as well as deoxycholic acid (DCA) and hyodeoxycholic acid-like substance were found in group 1 given carrageenan and DMH treatment compared to group 2 given DMH treatment. A significant increase in the concentration of LCA in rats fed the carrageenan diet and of DCA in rats fed the control diet was caused by DMH treatment. The daily output of LCA was significantly higher in group 1 than it was in group 2. However, no differences in the excretion of DCA, hyodeoxycholic acid-like substance, and total acids were found between groups 1 and 2.
Table 3. Free bile acid excretion in rat feces.

| Group | Treatment     | CA\(^a\)  | CDCA     | DCA       | LCA       | HDCA-S    | Total     |
|-------|---------------|------------|----------|-----------|-----------|-----------|-----------|
|       | (mg/g of dry feces) |            |          |           |           |           |           |
| 1     | DMH + CAR     | 0.01 ± 0.01\(^b\) | 0.06 ± 0.01 | 0.23 ± 0.03\(^c\) | 0.26 ± 0.01\(^cd\) | 1.38 ± 0.09\(^c\) | 1.91 ± 0.12\(^c\) |
| 2     | DMH           | 0.05 ± 0.02  | 0.08 ± 0.01 | 0.38 ± 0.02\(^e\) | 0.16 ± 0.01  | 2.80 ± 0.28 | 3.47 ± 0.31 |
| 3     | CAR           | 0.02 ± 0.01  | 0.06 ± 0.01 | 0.21 ± 0.03  | 0.22 ± 0.01\(^f\) | 1.28 ± 0.12\(^f\) | 1.79 ± 0.17\(^f\) |
| 4     | —             | 0.03 ± 0.02  | 0.07 ± 0.01 | 0.24 ± 0.01  | 0.16 ± 0.02  | 2.42 ± 0.20 | 2.92 ± 0.22 |

|       | (mg/kg body weight/day) |            |          |           |           |           |           |
|-------|------------------------|------------|----------|-----------|-----------|-----------|-----------|
| 1     | DMH + CAR              | 0.12 ± 0.13 | 0.48 ± 0.04 | 1.98 ± 0.27 | 2.23 ± 0.08\(^c\) | 11.9 ± 0.66 | 16.2 ± 0.84 |
| 2     | DMH                    | 0.23 ± 0.11 | 0.36 ± 0.04 | 1.60 ± 0.08\(^e\) | 0.68 ± 0.03  | 12.0 ± 1.15 | 14.8 ± 1.26 |
| 3     | CAR                    | 0.17 ± 0.08 | 0.62 ± 0.06\(^f\) | 2.04 ± 0.31\(^f\) | 2.14 ± 0.13\(^f\) | 12.6 ± 1.29 | 17.6 ± 1.78\(^f\) |
| 4     | —                      | 0.13 ± 0.06 | 0.31 ± 0.04 | 1.00 ± 0.04  | 0.66 ± 0.06  | 10.1 ± 0.88 | 12.2 ± 0.96 |

\(^a\)CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; HDCA-S, hyodeoxycholic acid-like substance; CAR, carrageenan; DMH, 1,2-dimethylhydrazine. \(^b\)Mean ± SE of 6 rats. \(^c\)p < 0.05 between groups 1 and 2. \(^d\)p < 0.05 between groups 1 and 3. \(^e\)p < 0.05 between groups 2 and 4. \(^f\)p < 0.05 between groups 3 and 4.
DISCUSSION

There were increased concentrations of fecal LCA, a secondary bile acid, in group 1 given DMH and the carrageenan diet compared to that in group 2 given DMH and the control diet and even to that in group 3 given the carrageenan diet. The daily output of LCA was also increased in group 1 compared to that in group 2. It is generally accepted that increased excretion of fecal bile acids is due to the ability of indigestible polysaccharides to adsorb bile acids. However, this may not be true for water-soluble materials. Kiriyama et al. (20) showed that water-soluble konjac mannan failed to adsorb bile acids in a dialysis experiment. Thus, increased excretion of fecal bile acid by water-soluble carrageenan, with water-holding and gel-forming abilities (21), would be due to the participation of water in carrageenan molecules rather than the adsorption.

There is a good deal of study on the effects of bile acids on chemically induced colon tumors in rats. Bile acids have been shown to enhance intrarectally induced colon cancer in both conventional and germfree rats and to have no carcinogenicity (12, 22), suggesting that bile acids act as colon tumor-promoters. Especially secondary bile acids, LCA and DCA, were shown to have a strong tumor-promoting activity, which may be associated with the participation of ornithine decarboxylase and prostaglandin E2 in the colonic epithelium (23, 24). Significant increased concentration and daily output of LCA were observed by carrageenan and DMH treatment. Thus, the above findings serve to focus attention on the possibility that the promoting effect of carrageenan on colon tumorigenesis by DMH is mediated by increased excretion of the secondary bile acid LCA.

As shown in Fig. 1, elution pattern of fecal carrageenan in group 1 was very similar to that of original polymer in the diet, and no loss of carrageenan ingested was found. Thus, the present data shows that carrageenan ingested had not been degraded in the gut of rats treated with DMH. This result was in agreement with that of Hawkins and Yaphe (25) who indicated that native carrageenan ingested was quantitatively excreted in rat feces, but not in agreement with observations of Pittman et al. (8) who showed that degradation of this polymer had occurred in rats. The reason for these differing observations is not known, but it may be partially explained in terms of the composition of the diet containing carrageenan, because it is likely that the diets may alter the physicochemical environment in rat intestine. Degradation of carrageenan is of interest in connection with safety of this polysaccharide. Further studies are needed to determine the conditions which influence the degradation.

The authors wish to thank Dr. H. Oka and Mr. Y. Ikai for their kind help in gas chromatography-mass spectrometry of bile acids.
REFERENCES

1) Bauer, H. G., Asp, N.-G., Öste, R., Dahlqvist, A., and Fredlund, P. E. (1979): Effect of dietary fiber on the induction of colorectal tumors and fecal β-glucuronidase activity in the rat. Cancer Res., 39, 3752–3756.

2) Freeman, H. J., Spiller, G. A., and Kim, Y. S. (1980): A double-blind study on the effects of differing purified cellulose and pectin fiber diets on 1,2-dimethylhydrazine-induced rat colonic neoplasia. Cancer Res., 40, 2661–2665.

3) Barbolt, T. A., and Abraham, R. (1978): The effect of bran on dimethylhydrazine-induced colon carcinogenesis in the rat. Proc. Soc. Exp. Biol. Med., 157, 656–659.

4) Arakawa, S., Okumura, M., Yamada, S., Ito, M., and Tejima, S. (1986): Enhancing effect of carrageenan on the induction of rat colonic tumors by 1,2-dimethylhydrazine and its relation to β-glucuronidase activities in feces and other tissues. J. Nutr. Sci. Vitaminol., 32, 481–485.

5) Watanabe, K., Reddy, B. S., Wong, C. Q., and Weisburger, J. H. (1978): Effect of dietary undegraded carrageenan on colon carcinogenesis in F344 rats treated with azoxymethane or methylxanthine. Cancer Res., 38, 4427–4430.

6) Bryant, M. P. (1978): Cellulose digesting bacteria from human feces. Am. J. Clin. Nutr., 31, S113–S115.

7) Cummings, J. H., Southgate, D. A. T., Branch, W. J., Wiggins, H. S., Houston, H., Jenkins, D. J. A., Jivraj, T., and Hill, M. J. (1979): The digestion of pectin in the human gut and its effect on calcium absorption and large bowel function. Br. J. Nutr., 41, 477–485.

8) Pittman, K. A., Golberg, L., and Coulston, F. (1976): Carrageenan: The effect of molecular weight and polymer type on its uptake, excretion and degradation in animals. Fd. Cosmet. Toxicol., 14, 85–93.

9) Wakabayashi, K., Inagaki, T., Fujimoto, Y., and Fukuda, Y. (1978): Induction by degraded carrageenan of colorectal tumors in rats. Cancer Lett., 4, 171–176.

10) Iatropoulos, M. J., Golberg, L., and Coulston, F. (1975): Intestinal carcinogenesis in rats using 1,2-dimethylhydrazine with or without degraded carrageenan. Exp. Mol. Pathol., 23, 386–401.

11) Hill, M. J., Crowther, J. S., Drasar, B. S., Hawksworth, G., Aries, V., and Williams, R. E. O. (1971): Bacteria and etiology of cancer of the large bowel. Lancet, 1, 95–100.

12) Narisawa, T., Magadia, N., Weisburger, J. H., and Wynder, E. L. (1974): Promoting effect of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl- N'-nitro-N-nitrosoguanidine in rats. J. Natl. Cancer Inst., 53, 1093–1097.

13) Reddy, B. S., Watanabe, K., and Sheinfil, A. (1980): Effect of dietary wheat bran, alfalfa, pectin and carrageenan on plasma cholesterol and fecal bile acid and neutral sterol excretion in rats. J. Nutr., 110, 1247–1254.

14) Ekström, L.-G., Kuivinen, J., and Johansson, G. (1983): Molecular weight distribution and hydrolysis behaviour of carrageenans. Carbohydr. Res., 116, 89–94.

15) Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956): Colorimetric method for determination of sugars and related substances. Anal. Chem., 28, 350–356.

16) Ferguson, L. R., Rewcastle, G. W., Lello, J. M., Alley, P. G., and Seelye, R. N. (1984): Quantitation of free and conjugated bile acids in human feces using a high-pressure liquid chromatography counterion method. Anal. Biochem., 143, 325–332.
17) Grundy, S. M., Ahrens, E. H., Jr., and Miettinen, T. A. (1965): Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. J. Lipid Res., 6, 397–410.

18) Imai, K., Tamura, Z., Mashige, F., and Osuga, T. (1976): Gas chromatography of bile acids as their hexafluoroisopropyl ester-trifluoroacetyl derivatives. J. Chromatogr., 120, 181–186.

19) Edenharder, R., and Slemr, J. (1981): Gas chromatographic and mass spectrometric analysis of bile acids as trifluoroacetyl-hexafluoroisopropyl and heptafluorobutyryl derivatives. J. Chromatogr., 220, 1–12.

20) Kiriyama, S., Enishi, A., and Yura, K. (1974): Inhibitory effect of konjac mannan on bile acid transport in the everted sacs from rat ileum. J. Nutr., 104, 69–78.

21) Stephen, A. M., and Cummings, J. H. (1979): Water-holding by dietary fibre in vitro and its relationship to faecal output in man. Gut, 20, 722–729.

22) Reddy, B. S., Narisawa, T., Weisburger, J. H., and Wynder, E. L. (1976): Promoting effect of sodium deoxycholate on colon adenocarcinomas in germfree rats. J. Natl. Cancer Inst., 56, 441–442.

23) Takano, S., Akagi, M., and Bryan, G. T. (1984): Stimulation of ornithine decarboxylase activity and DNA synthesis by phorbol esters or bile acids in rat colon. Gann, 75, 29–35.

24) Narisawa, T., Sato, M., Tani, M., Kudo, T., Takahashi, T., and Goto, A. (1981): Inhibition of development of methylnitrosourea-induced rat colon tumors by indomethacin treatment. Cancer Res., 41, 1954–1957.

25) Hawkins, W. W., and Yaphe, W. (1965): Carrageenan as a dietary constituent for the rat: Faecal excretion, nitrogen absorption, and growth. Can. J. Biochem., 43, 479–484.