Proportion of Murine Cytotoxic T Cells is Increased by High Molecular-Weight Fucoidan Extracted from *Okinawa mozuku* (*Cladosiphon okamuranus*)

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Male BALB/c mice were administered three fucoidans with different molecular weights prepared from *Okinawa mozuku* (*Cladosiphon okamuranus*). The proportion of $CD8^+$ cells in the spleens of mice fed with high molecular-weight fucoidan significantly increased compared with those in mice fed a control diet. In addition, the $CD4^+/CD8^+$ ratio tended to decrease and the proportion of $CD11b^+$ cells to increase. These results suggest that high molecular-weight fucoidan promotes an increase in the proportion of murine cytotoxic T cells.

Key words —— fucoidan, cytotoxic T cell, lymphocyte, flow cytometry, mouse

INTRODUCTION

Seaweed is a popular foodstuff in Japan and a main source of dietary fiber (DF), because it is abundant in indigestible saccharides. The intraperitoneal administration of hot-water extract of seaweeds has antitumor activity in mice inoculated with L-1210 leukemia and sarcoma-180 cells.[1] Brown algae such as *mozuku*, *konbu* and *mekabu* contain abundant fucoidan and have particularly high antitumor activities.[2] Fucoidan is a sulfated polysaccharide with a mean molecular weight of 2×10^5 and the main structure consists of alpha-(1→2)-linked L-fucose. *Okinawa mozuku* (*Cladosiphon okamuranus*) has historically been utilized as a foodstuff in the Okinawa region. Fucoidan extracted from this *mozuku* has a high L-fucose content. Fucoidan is not degraded by human digestive enzymes and is minimally utilized by intestinal bacteria.[3]

Maruyama *et al*.[4] reported that one mechanism of the antitumor action of intraperitoneal *mekabu* fucoidan is the elevation of natural killer (NK) cell activity. On the other hand, oral DF changes the ratio of $CD4^+$ to $CD8^+$ of T cells in the mesenteric lymph node and spleen.[5,6] CD4 or CD8 on the mature T cell surface plays an important role in the stimulation of T cells as coreceptors in antigen recognition of the T cell receptor/CD3 complex.

The present study examines the effects of orally administrated fucoidan extracted from *Okinawa mozuku* on the expression of CD4, CD8, CD3, and CD11b (NK cell surface antigens) in murine splenocytes. We also examined the effects of fucoidans of various molecular weights.

MATERIALS AND METHODS

Preparation of Fucoidan —— The molecular weight of commercial fucoidan extracted from *Okinawa mozuku* (Fucoidan YSK-NB, Yaizu Suisankagaku Industry, Shizuoka, Japan) determined by gel permeation chromatography[7] was $2–3 \times 10^5$ (high molecular-weight fucoidan, HF; Table 1). The HF was hydrolyzed in HCl 0.05 or 0.5 M at 80°C for 30 min and then neutralized with sodium hydroxide. After removing salt by electrodialysis, the hydrolysates were lyophilized *in vacuo*. The mean molecular weights of these products were $8–9 \times 10^3$ (middle molecular-weight fucoidan, MF) and $0.5–1.0 \times 10^3$ (low molecular-weight fucoidan, LF), respectively.

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Table 1. Composition of Fucoidan Prepared from *Okinawa Mozuku* (*Cladosiphon okamuranus*) (%)

Sample	L-fucose	Glucuronic acid	Other sugars[a)]	Ester sulfate
HF	39.6	9.9	15.6	16.9

a) Without L-fucose. HF, high molecular-weight fucoidan.

Table 2. Effects of Fucoidan on Weight of Body and Spleen of Mice Fed Experimental Diets[a)]

	CP	LF	MF	HF
No. of mice	7	8	7	8
Body weight (g)	32.3 ± 1.2 a	31.2 ± 0.9 ab	28.6 ± 0.7 b	28.8 ± 0.5 b
Spleen (mg)	133 ± 10	135 ± 9	135 ± 9	115 ± 7
(mg/g body weight)	4.18 ± 0.42	4.30 ± 0.21	4.73 ± 0.32	4.03 ± 0.24

a) Mean ± SEM: values in a row not sharing a common letter differ significantly according to Duncan's multiple-range test at $p < 0.05$. CP, cellulose powder; LF, low molecular-weight fucoidan; MF, middle molecular-weight fucoidan; HF, high molecular-weight fucoidan.

Animals and Diets —— Six-week-old specific pathogen-free BALB/c male mice (Tokyo Experimental Animals, Tokyo, Japan) were housed in an environment-controlled room (temperature, 23 ± 1°C; relative humidity, 50%) maintained under a 12-hr light/dark cycle (light, 08:00–20:00). After acclimation to a commercially available diet (Oriental MF, Oriental Yeast, Co. Ltd., Tokyo, Japan) for 1 week, the mice were randomly divided into four groups and fed an experimental diet. The composition (g/kg) of the experimental diet was casein, 200; DL-methionine, 3; corn starch, 200; sucrose, 400; lard, 100; mineral mixture (AIN-76), 35; vitamin mixture (AIN-76), 10; choline bitartrate, 2; and test materials, 50. Cellulose powder (CP, Oriental Yeast Co., Ltd.) was used in the control diet. Food and deionized water were available *ad libitum*. The present study followed the Japanese governmental legislation guidelines regarding the proper care and use of laboratory animals (No. 6, March 27, 1980).

Flow Cytometry —— Ten weeks after administering the experimental diets, spleens were removed under diethyl ether anesthesia and passed through stainless wire mesh (#200). Splenocytes were obtained using Lympholyte-Mouse (Cedarlane Laboratories, Hornby, Ontario, Canada),[8)] and red blood cells were removed by hypotonic lysis in ammonium chloride. Lymphocytes were incubated in the dark at 4°C for 30 min with fluorescein isothiocyanate or R-phycoerythrin-labeled monoclonal antibody (anti-CD4, anti-CD8, anti-CD3, anti-CD11b, PharMingen, San Diego, CA, U.S.A.). Labeled cells were analyzed with flow cytometry using EXPO32 software (EPICS XL cytometer, Beckman Coulter, Miami, FL, U.S.A.).

Statistical Analysis —— Results are shown as mean ± SEM. Significance among diets was analyzed using Duncan's multiple-range test. Results were statistically analyzed using SPSS 9.0 for Windows (SPSS Japan, Tokyo, Japan).

RESULTS

Table 1 shows that the composition of the fucoidan (L-fucose + ester sulfate + neutral sugars + uronic acid) from *Okinawa mozuku*. The amount of F-fucose in this fucoidan was about 2.5-fold higher than that of fucoidan from *mekabu* (15.6%).[4)]

Table 2 shows the final weights of the body and spleen of the mice. The MF and HF groups weighed significantly less than the CP group. The spleens of the HF group tended to have lower weights. The ratio of the spleen to body weight was the lowest in the HF group, but the difference was not statistically significant.

Table 3 shows the surface molecules expressed on murine splenocytes as determined by flow cytometry. In the HF group, the proportion of $CD4^+$ and $CD8^+$ increased, whereas the ratio of $CD4^+/CD8^+$ tended to be lower than those in the CP, LF, and MF groups. The ratio of $CD3^+$ in the CP and HF groups were significantly higher than that in the LF group. The ratio of $CD11b^+$ in the HF group was significantly increased compared with that in the MF group.

Table 3. Effects of Fucoidan on Ratio of Lymphocytes Presenting CD4, CD8, CD3, and CD11b in Splenocytes from Mice Fed Experimental Diets[a)]

Item	CP	LF	MF	HF
No. of mice	7	8	7	8
Lymphocytes				
$CD4^+$ (%)	19.7 ± 1.3 ab	18.4 ± 0.3 a	21.5 ± 1.3 ab	21.9 ± 0.8 b
$CD8^+$ (%)	5.1 ± 0.3 a	5.3 ± 1.0 a	5.8 ± 0.7 ab	7.8 ± 0.7 b
$CD4^+/CD8^+$	4.0 ± 0.5	4.2 ± 0.6	4.0 ± 0.4	2.9 ± 0.2
$CD3^+$ (%)	31.1 ± 1.3 a	26.0 ± 1.3 b	29.5 ± 1.6 ab	31.7 ± 1.5 a
$CD11b^+$ (%)	3.7 ± 0.3 ab	3.9 ± 0.3 ab	3.6 ± 0.2 a	4.6 ± 0.4 b

a) Mean ± SEM: values in a row not sharing a common letter differ significantly according to Duncan's multiple-range test at $p < 0.05$. CP, cellulose powder; LF, low molecular-weight fucoidan; MF, middle molecular-weight fucoidan; HF, high molecular-weight fucoidan.

DISCUSSION

The results of this experiment indicated that higher molecular-weight fucoidan increases the ratio of cytotoxic lymphocytes. Some high-molecular DF influences antitumor activity and immune function.[9,10)] However, the method of administration was intraperitoneal injection. Some reports described the effects of oral DF on immune function. Lim *et al*.[5)] reported that pectin significantly increased the ratio of $CD4^+/CD8^+$ in rat lymphocytes from the mesenteric lymph nodes. Kudoh *et al*.[6)] reported that the ratio of $CD3^+$ cells in splenocytes was significantly decreased in rats fed gum arabic, as compared with cellulose powder. In addition, the oral administration of easily fermented oligosaccharides such as short-chain fructooligosaccharide and oligofructose affects T cell status and tumor induction.[11,12)] Those results suggest that the molecular weight of indigestible saccharides administered by orally can influence immune function.

With respect to the mechanism by which DF affects immune function, DF fermentation in the digestive tract might be relevant.[13)] However, fucoidan is not generally degraded by human intestinal bacteria.[3)] In addition, it has been not reported that intestinal bacteria can degrade fucoidan in mice. Therefore the effect of fucoidan on immune function is not dependent on its fermentability. Maruyama *et al*.[4)] showed that the intraperitoneal administration of fucoidan elevated NK cell activity in a study of mice inoculated with P-388 leukemia cells. We found that the ratio of cells presenting CD11b, which is an NK cell-surface antigen and which is also expressed in monocytes and macrophages, was elevated in the HF group as compared with that in the LF and MF groups. The ratio of lymphocytes presenting CD16 or CD56, specific antigens for NK cell, was not determined in this experiment, but HF may elevate the number of NK cells.

Elsenhans and Caspary reported that polyethylene glycol (MW 4000) is adsorbed from the intestine, although in trace amounts.[14)] Similarly, HF might be partially adsorbed by the same mechanism and thus affect immune function. The mechanism through which HF increases the ratio of $CD8^+$ cells much more than LF and MF remains unknown. $CD8^+$ T cells, which are major histocompatibility complex class I restricted killer T cells, are cytotoxic when activated. Intestinal intraepithelial T lymphocytes (IELs) located under the mucous layer play an important role in enteric immunity. Most IELs located under the mucous membrane layer are $CD8^+$.[15)] Fucoidan might have stimulated IELs through contact under the mucous membrane.

The results of this experiment indicate that the difference in the molecular weight of fucoidan is associated with immune function in mice. In conclusion, high molecular-weight fucoidan extracted from *Okinawa mozuku* changes the ratio of $CD4^+/CD8^+$ and increases the ratio of cytotoxic T cells in mice splenocytes.

REFERENCES

1) Yamamoto, I., Maruyama, H., Takahashi, M. and Komiyama, K. (1986) The effect of dietary or intraperitoneally injected seaweed preparation on the growth of Sarcoma-180 cells subcutaneously implanted into mice. *Cancer Lett.*, **30**, 125–131.
2) Yamamoto, I., Takahashi, M., Suzuki, T., Seino, H. and Mori, H. (1984) Antitumor effect of seaweeds. IV. Enhancement of antitumor activity by sulfation

of a crude fucoidan fraction from Sargassum kjellmanianum. *Jpn. J. Exp. Med*., **54**, 143–151.
3) Salyers, A. A., Vercellotti, J. R., West, S. H. E. and Wilkins, T. D. (1977) Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. *Appl. Environ. Microbiol*., **33**, 319–322.
4) Maruyama, H., Tamauchi, H., Hashimoto, M. and Nakano, T. (2003) Antitumor activity and immune response of Mekabu fucoidan extracted from *Sporophyll of Undaria pinnatifida*. *In Vivo*, **17**, 245–249.
5) Lim, B. O., Yamada, K., Nonaka, M., Kuramoto, Y., Hung, P. and Sugano, M. (1997) Dietary fibers modulate indices of intestinal immune function in rats. *J. Nutr*., **127**, 663–667.
6) Kudoh, K., Shimizu, J., Wada, M., Takita, T., Kanke, Y. and Innami, S. (1998) Effect of indigestible saccharides on B lymphocyte response of intestinal mucosa and cecal fermentation in rats. *J. Nutr. Sci. Vitaminol*., **44**, 103–112.
7) Rice, K. G., Kim, Y. S., Grant, A. C., Merchant, Z. M. and Linhardt, R. J. (1985) High-performance liquid chromatographic separation of heparin-derived oligosaccharides. *Anal. Biochem*., **1150**, 325–331.
8) Gallucci, R. M. and Meadows, G. G. (1995) Ethanol consumption reduces the cytolytic activity of lymphokine-activated killer cells. *Alcohol Clin. Exp. Res*., **19**, 402–409.
9) Nono, I., Ohno, N., Ohsawa, M., Oikawa, S. and Yadomae, T. (1989) Modulation of antitumor activity of grifolan by subsequent administration of 1,3-beta-D-glucanase in vivo. *J. Pharmacobiodyn*., **12**, 581–588.
10) Hishinuma, K., Hosono, A., Inaba, H. and Kimura, S. (1990) Effects of intraperitoneally administered dietary fibers on superoxide generation from peritoneal exudate macrophages in mice. *Int. J. Vitam. Nutr. Res*., **60**, 288–293.
11) Pierre, F., Perrin, P., Bassonga, E., Bornet, F., Meflah, K. and Menanteau, J. (1999) T cell status influences colon tumor occurrence in min mice fed short chain fructo-oligosaccharides as a diet supplement. *Carcinogenesis*, **20**, 1953–1956.
12) Buddington, R. K., Kelly-Quagliana, K., Buddington, K. K. and Kimura, Y. (2002) Non-digestible oligosaccharides and defense functions: lessons learned from animal models. *Br. J. Nutr*., **87**, S231–S239.
13) Ishizuka, S. and Tanaka, S. (2002) Modulation of CD8+ intraepithelial lymphocyte distribution by dietary fiber in the rat large intestine. *Exp. Biol. Med*., **222**, 1017–1021.
14) Elsenhans, B. and Caspary, W. F. (1989) Differential changes in the urinary excretion of two orally administered polyethylene glycol markers (PEG 900 and PEG 4000) in rats after feeding various carbohydrate gelling agents. *J. Nutr*., **119**, 380–387.
15) Guy-Grand, D., Cerf-Bensussan, N., Malissen, B., Malassis-Seris, M., Briottet, C. and Vassalli, P. (1991) Two gut intraepithelial CD8+ lymphocyte populations with different T cell receptors: a role for the gut epithelium in T cell differentiation. *J. Exp. Med*., **173**, 471–481.