

## Effects of *Maitake* (*Grifola frondosa*) Polysaccharide on Collagen-Induced Arthritis in Mice

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**ABSTRACT**—We recently reported the anti-hepatitis effect of a polysaccharide, designated as the D-fraction, extracted from *maitake*. Its effect includes immuno-regulating activities. We investigated the effect of the glucan in collagen-induced arthritis (CIA). The D-fraction was administered to CIA mice for 30 consecutive days. Arthritis development was observed from the 4th day after the second immunization. The D-fraction did not have any influence on anti-type II collagen antibodies in blood serum or activated B cells. To determine how cellular immunity may be involved in the development of CIA, ratios of CD4<sup>+</sup> T cells and their activated form in the axillary and inguinal lymph node T cells were detected by flow cytometry analysis. The ratios were not different between the D-fraction group and the control group. However, interleukin-1 $\beta$ , granulocyte-macrophage colony-stimulating factor and tumor necrosis factor- $\alpha$  productions from splenic macrophages were significantly increased to 2.0, 4.7 and 1.9 times the control group level, respectively. The ratio of macrophages in the whole spleen cells was 2.3 times that of the control group, and their migrating ability was 1.9 times higher. Based on these results, we concluded that the arthritis development induced by D-fraction administration is attributable to the activation of splenic macrophages.

**Keywords:** Collagen-induced arthritis, *Maitake* polysaccharide, Splenic macrophage

Autoimmune diseases originate from a collapsed immunity system, and they are very often accompanied by responses to the patients own antigens, causing self-destructive damaging of the patients' tissues. It is well known that it is difficult to cure autoimmune diseases. For healthy individuals, under ordinary conditions, self-immunologic tolerance is established and prevents the immunological response to the auto-antigens. In the autoimmune diseases, there seems to be a collapse of the self-tolerance system. Rheumatoid arthritis (RA), one of the autoimmune diseases, is an overall bodily disorder whose major symptoms are the inflammatory changes of articular joints such as proliferation of the joint synovial membrane and destruction of cartilage and bone tissues. Hereditary factors, viruses and environmental factors are suggested to cause RA, but its crisis mechanism or the treatment methods have not been fully elucidated so far. Recently clinical immunotherapy has been recognized as one of the treatments for RA, because pharmaceutical therapy of RA has more side effects than the therapeutic effects.

The polysaccharides have recently been reported to exist in the fruit bodies of fungi belonging to the Basidiomycetes, and they affect various physiological functions of the organism. The high-molecular polysaccharides, such as crestin extracted from *kawaratake* (1), and lentinan from *shiitake* (2), have been recognized as one of biological response modifiers and have been approved for clinical use because they exert an immuno-competent cell-activating effect on tumors. Our laboratory has reported a high-molecular glucan extracted from *maitake* fruit body, named D-fraction, whose chemical structure has  $\beta$ -1,6 bonds as the main chain and  $\beta$ -1,3 bonds as side chains. The D-fraction exerts its anti-tumor effect through the activation of immuno-competent cells (3) and has a beneficial effect in autoimmune hepatitis through its immuno-regulating actions (4).

Recently, there was a report that immune complexes cause chronic arthritis in DBA/1 mouse (5). Therefore, we investigated if the *maitake* glucan exhibits immuno-regulating actions in model mice suffering collagen-induced arthritis (CIA), which is regarded as one of the experimental models of RA (6, 7).

From the present data, we concluded that in the RA de-

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velopment enhanced by the D-fraction in the CIA model mice, humoral immunity was not involved, and the activation of the cellular immunity, especially the activated splenic macrophages, is the main cause.

## MATERIALS AND METHODS

### Materials

The dried powder ( $\phi$  200 nm) made from the fruit body of *maitake* was purchased from Yukiguni Maitake Co. (Niigata). Eagle's minimal essential medium (MEM), Rosewell Park Memorial Institute (RPMI)-1640 medium, Hanks' solution and phosphate-buffered saline (PBS) were purchased from Nissui Seiyaku (Tokyo). Mouse interleukin (IL)-1 $\beta$ , granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor (TNF)- $\alpha$  enzyme-linked immunosorbent assay (ELISA) Kits were purchased from Genzyme Co. (Minneapolis, MN, USA). The mouse IgG type II collagen ELISA Kit was purchased from Chondrex Co. (Seattle, WA, USA).

### Antibodies

Six kinds of antibodies were purchased from Pharmingen Co. (San Diego, CA, USA): anti-CD16/CD32 monoclonal antibody (0.5 mg/ml), R-PE-conjugated anti-CD4 monoclonal antibody (0.2 mg/ml), FITC-conjugated anti-CD69 monoclonal antibody (0.5 mg/ml), R-PE-conjugated anti-CD19 monoclonal antibody (0.5 mg/ml) and Cy-Chrom<sup>TM</sup>-conjugated anti-CD3 $\epsilon$  monoclonal antibody (0.2 mg/ml).

### Animals

Male DBA/1J mice (6-week-old) were provided from Charles River Japan, Inc. (Yokohama). Food and water were given freely to these mice until they were used for the experiments.

### Fractionation of *maitake* extract

As shown in Fig. 1, the hot-water soluble fraction (WS-fraction) from the dried powder of *maitake* was obtained, and EtOH was added at the final concentration of 50%. After removing the floating substances, EtOH was added to the mixture of supernatant and pellet at the final concentration of 80% and centrifuged. The precipitate was purified by DEAE cellulofine column chromatography (Seikagaku Kogyo Co., Tokyo) using 12.5 mM Tris-HCl buffer (pH 7.25). The non-adherent fraction was recovered as the D-fraction. Saccharide concentration was determined by the anthrone method (8).

### Induction of CIA

According to a modification of the method of Kakimoto et al. (6), 2 mg of collagen type II (Collagen Technique Seminar, Tokyo) was solubilized in 1 ml of 20 mM Tris-150 mM NaCl and emulsified in 1 ml of complete Freund's adjuvant (Difco Lab., Detroit, MI, USA). Male DBA/1J mice were intracutaneously injected into the cauda radicular with 0.05 ml of the collagen emulsion. After 21 days, these mice were intracutaneously injected into the dorsal area. After second immunization, mice were examined daily for the onset and severity of arthritis.

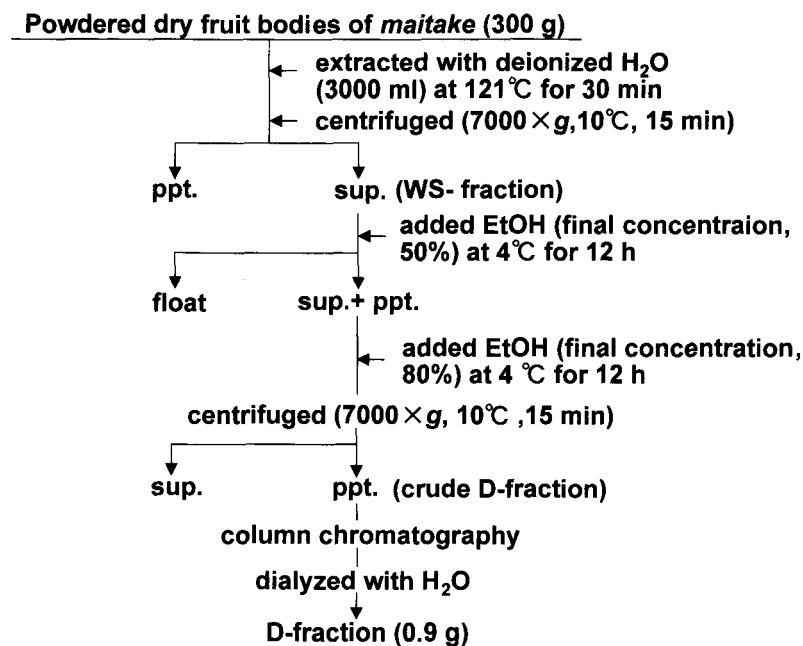


Fig. 1. Fractionation of *maitake* extract.

### *Clinical evaluation of arthritis*

The animals were graded for severity of arthritis by the method of Wood et al. (9). Lesions of the extremities were graded on a scale of 0 to 4, based on the number of joints involved and the degree of erythema and swelling. The sums of the scores for the limbs were calculated as an arthritic index, with a maximum possible score of 16 per mouse. Scores: 0, normal; 1, only one part of the joint of a limb had swelling and erythema; 2, more than two parts of a joint of the limb or wrist and ankle joint had swelling and erythema; 3, one hand or leg had swelled and exhibits erythema; 4, maximum of one hand or leg had swelling and erythema.

### *Dosage of WS-fraction or D-fraction*

After the second immunization, the mice were administered intraperitoneally with WS-fraction or D-fraction for 30 consecutive days at the concentration of 8.7 mg/kg per day.

### *Measurement of plasma anti-type II collagen IgG antibody*

After the second immunization, blood was collected from the retroorbital sinus using capillary tubes ( $\phi$  0.7  $\times$  75 mm) treated with heparin every 5th day; the collected blood was centrifuged at  $8,750 \times g$  for 4 min, and the separated plasma was stored at  $-80^\circ\text{C}$  until assayed. Plasma anti-type II collagen IgG antibody titer was determined by ELISA.

### *Preparation of splenic cells*

Mouse spleens were taken out on the 7th day after the second immunization and washed with PBS. Sterile splenic cell suspension was prepared by the following procedure: The mice spleens were passed through nylon mesh ( $\phi$  70  $\mu\text{m}$ ), washed with PBS and centrifuged at  $300 \times g$  for 5 min. The precipitates were collected and mixed with 40 ml of hemolytic buffer (17 mM Tris-NH<sub>4</sub>Cl, pH 7.65), centrifuged again, and then washed with Hanks' solution. The cells were suspended in 10 ml of PBS containing 0.09% NaN<sub>3</sub> and 1% fetal calf serum (FCS) (SM: staining medium) to a final concentration of  $1 \times 10^7$  cells/ml.

### *Preparation of lymph node T cells*

On the 7th day after the second immunization, mouse axillary and inguinal lymph node were taken out and passed through nylon mesh ( $\phi$  70  $\mu\text{m}$ ) and then washed with PBS by centrifugation at  $300 \times g$  for 5 min. The cells were collected and suspended in 100  $\mu\text{l}$  of SM at the final concentration of  $1 \times 10^7$  cells/ml.

CD4<sup>+</sup> T cells were separated from lymph node T cells by using a Mouse CD4<sup>+</sup> T cell column (Cytovax Biotechnologies, Inc., Edmonton, Alberta, Canada) and were suspended in RPMI-1640 medium containing 10% FCS at the

concentration of  $2 \times 10^6$  cells/ml.

### *Preparation of antigen-presenting cells (APC)*

The un-sensitized mice were killed by cervical dislocation. Mouse spleens were taken out and washed with PBS. The mice spleens were passed through nylon mesh ( $\phi$  70  $\mu\text{m}$ ), washed with PBS and centrifuged at  $300 \times g$  for 5 min. The precipitates were collected, mixed with 40 ml of hemolytic buffer, centrifuged again, and then washed with Hanks' solution. The cells were suspended in Hanks' solution containing 5% FCS at the final concentration of  $1 \times 10^7$  cells/ml. After the addition of mitomycin at the final concentration of 50  $\mu\text{g/ml}$ , the cells were incubated at  $37^\circ\text{C}$  in a 5% CO<sub>2</sub> atmosphere for 30 min. After the incubation, the cells were washed with Hanks' solution by centrifugation at  $300 \times g$  for 5 min and suspended in RPMI-1640 medium containing 10% FCS at the concentration of  $2 \times 10^6$  cells/ml.

### *Determination of DNA synthesis of CD4<sup>+</sup> T cells*

The specificity for type II collagen antigen of CD4<sup>+</sup> T cells was measured by the lymphocyte transformation method. Briefly, prepared CD4<sup>+</sup> T cells ( $1 \times 10^5$  cells/well) were mixed with APC ( $1 \times 10^5$  cells/well) in a 96-well plate and incubated at  $37^\circ\text{C}$  for 4 days in a 5% CO<sub>2</sub> atmosphere with concanavalin A (Con A) or antigens (collagen types I, II and III). The cells were incubated with 10  $\mu\text{l}$  of <sup>3</sup>H-thymidine (370 kBq/ml) the last 18 h of the culture. The cells were then harvested, and the incorporated <sup>3</sup>H-thymidine was counted using a liquid scintillation counter (LSC-700; Aloka Co., Tokyo).

### *Preparations of peritoneal and splenic macrophages*

On the 7th day after the second immunization, the mice were killed by cervical dislocation. Mouse peritoneal cells or splenic cells were washed with Hanks' solution by centrifugation at  $300 \times g$  for 5 min and suspended in RPMI-1640 medium containing 10% FCS ( $1 \times 10^7$  cells/ml). The cells were cultured at  $37^\circ\text{C}$  in a 5% CO<sub>2</sub> atmosphere for 2 h. After removing the culture medium, the adherent cells were washed with  $37^\circ\text{C}$  PBS, treated with trypsin, collected, and then suspended in RPMI-1640 medium containing 10% FCS, at a cell concentration of  $1 \times 10^6$  cells/ml.

### *Cytokine production of peritoneal and splenic macrophages*

Peritoneal macrophages or splenic macrophages ( $1 \times 10^5$  cells/well) were seeded in a 96-well plate and cultured at  $37^\circ\text{C}$  for 24 h in a 5% CO<sub>2</sub> atmosphere. After the culture, 100  $\mu\text{l}$  of the supernatant was collected by centrifugation at  $300 \times g$  for 5 min; and IL-1 $\beta$ , GM-CSF and TNF- $\alpha$  were determined by ELISA.

### Migration of peritoneal and splenic macrophages

According to a modification of the method of David et al. (10), the migration activity of the macrophages was measured by using a glass tube. A glass capillary tube ( $\phi$  0.7 × 75 mm) was filled with peritoneal or splenic macrophages ( $5 \times 10^8$  cells/ml). After the glass tube was centrifuged at  $55 \times g$  for 10 min, the cell suspension was separated into the supernatant fraction and the cell fraction. Then the tube was cut at the boundary line between the supernatant and the cells. The portion of the tube containing cells was fixed by vaseline to the base of the culture dish and cultured at 37°C in a 5% CO<sub>2</sub> atmosphere for 48 h. The distance to the most outer edge of the cells that did migrate from the cut section was measured after end of culture.

### Flow cytometry analysis (FCM)

For cell surface antigen, 100  $\mu$ l of spleen cells or axillary inguinal lymph node ( $1 \times 10^7$  cells/ml) were mixed with 2  $\mu$ l of CD16/CD32 Fc blocker and reacted at 4°C for 5 min. The cells were incubated with 2  $\mu$ l of FITC-conjugated CD69 antibody, R-PE-conjugated CD4, CD19 antibodies and Cy-Chrome™-conjugated CD3 antibody at 4°C for 20 min and washed with SM. The washed cells were suspended in 500  $\mu$ l of SM to the concentration of  $2 \times 10^6$  cells/ml and enumerated using a FACScan™ flow cytometer (Beckton Dickinson Co., Grenoble, France).

### Statistical analyses

The significance of the experimental data was determined by analysis of variance (ANOVA) and Dunnett's test. *P* values less than 0.05 were considered statistically significant.

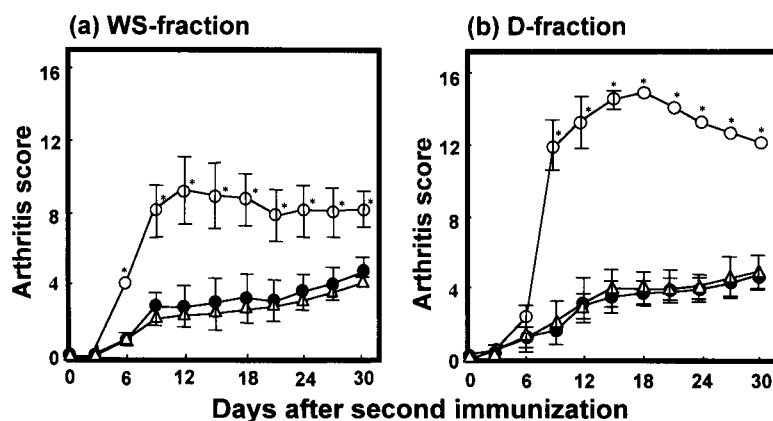
## RESULTS

### Effects of maitake glucans on CIA

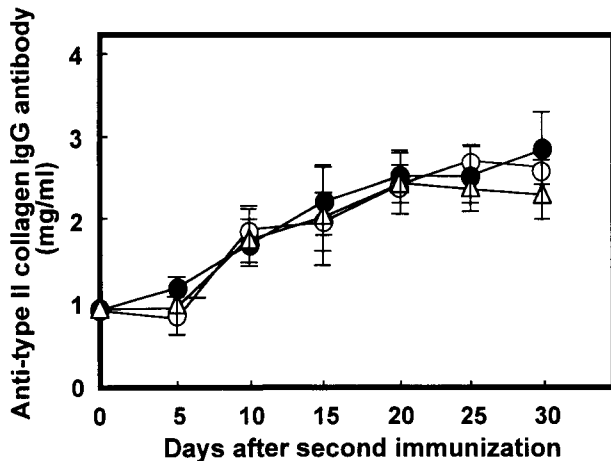
WS- and D-fractions were administered to CIA mice for 30 consecutive days, and the arthritis scores were recorded. The data are shown in Fig. 2. Saline and dextran were administered for the control. Arthritic symptoms were observed in all groups of mice from the 4th day after the second stimulation. Significantly high arthritis scores were indicated in the WS-fraction group as compared to the dextran-administered group and the control group (Fig. 2a), but heavier arthritic development was observed in the D-fraction group (Fig. 2b). These results suggest that to CIA mice, D-fraction facilitates more arthritis development by enhancing immunity-activating actions than expedites recovery from the disease by immuno-regulating actions brought in by the *maitake* glucans.

### Effects of D-fraction on humoral immunity

The cause of RA has not yet been elucidated, but humoral immunity is understood to have an important role (11, 12). In a study on CIA, an experimental model for RA, the auto-antibodies to type II collagen were suggested to be the direct cause of the onset of arthritis (13). Thus we investigated the effects of the D-fraction on humoral immunity. The D-fraction was administered to CIA mice, and the antibody titer for anti-type II collagen IgG antibody in blood serum was determined (Fig. 3). From the second stimulation, the antibody titer showed a gradual increase equally in all groups of mice, including the D-fraction, dextran and control groups. The antibody titer for the D-fraction group was a same level compared to the control group, even during the time of strong arthritic surges between the 5th day and the 10th day. The influence of the D-



**Fig. 2.** Clinical severity scores in CIA mice administered with WS-fraction (a) or D-fraction (b). WS- or D-fraction was administered to CIA mice for 30 consecutive days at the concentration of 8.7 mg/kg per day, and then the arthritis scores were recorded. Values are the means  $\pm$  S.E.M. of 6 mice. \**P* < 0.05, compared with the dextran group. ●, Saline; △, Dextran; ○, WS-fraction or D-fraction.



**Fig. 3.** Effects of D-fraction on anti-type II collagen IgG antibody titer. D-fraction (8.7 mg/kg per day) was administered to CIA mice, and the antibody titer for anti-type II collagen IgG antibody in the blood serum was investigated. Values are the means  $\pm$  S.E.M. of 6 mice. ●, Saline; △, Dextran; ○, D-fraction.

**Table 1.** Effects of D-fraction on activation of splenic B cells

Treatments	Splenic B cells	
	CD19 <sup>+</sup> (%) <sup>a</sup>	CD19 <sup>+</sup> CD69 <sup>+</sup> (%) <sup>a</sup>
Saline	47.5 $\pm$ 4.6	1.0 $\pm$ 0.6
Dextran	45.5 $\pm$ 5.8	1.4 $\pm$ 1.1
D-fraction	42.9 $\pm$ 3.3	1.0 $\pm$ 0.6

<sup>a</sup>% of positive cells was determined in saline-, dextran- or D-fraction-treated whole spleen cells. Values are the means  $\pm$  S.D. of 6 mice.

fraction on splenic B cells, the antibody generators, was also investigated by the FCM method, and the results are shown in Table 1. In the D-fraction-administered group, there was no significant difference in comparison with the other two groups with regards to the ratio of the B cells to the whole spleen cells and the ratio of the B cells expressing CD69, a marker for the activation of B cells, to the whole spleen cells.

#### Effect of D-fraction on cellular immunity

The influence of the D-fraction on axillary and inguinal lymph node T cells was investigated by the FCM method. As shown in Table 2, there was no significant difference in the ratio of CD4<sup>+</sup> T cells to axillary and inguinal lymph node cells nor was there any difference in the expressed amount of CD69, the marker for CD4<sup>+</sup> T cell activation, between the D-fraction group and the other two groups.

The effect of the D-fraction on axillary and inguinal lymph node T cells was also investigated by assessing DNA synthesis ability (Table 3). In the presence of Con A, one of the representative mitogens, the amount of <sup>3</sup>H-thymi-

**Table 2.** Effects of D-fraction on activation of lymph node T cells

Treatments	Lymph node T cells	
	CD3 <sup>+</sup> CD4 <sup>+</sup> (%) <sup>a</sup>	CD3 <sup>+</sup> CD4 <sup>+</sup> CD69 <sup>+</sup> (%) <sup>a</sup>
Saline	29.1 $\pm$ 1.4	6.2 $\pm$ 0.3
Dextran	29.9 $\pm$ 5.2	5.5 $\pm$ 0.6
D-fraction	28.9 $\pm$ 1.7	6.0 $\pm$ 0.3

<sup>a</sup>% of positive cells was determined in saline-, dextran- or D-fraction-treated lymph node cells. Values are the means  $\pm$  S.D. of 6 mice.

**Table 3.** Incorporation of <sup>3</sup>H-thymidine into CD4<sup>+</sup> T cells

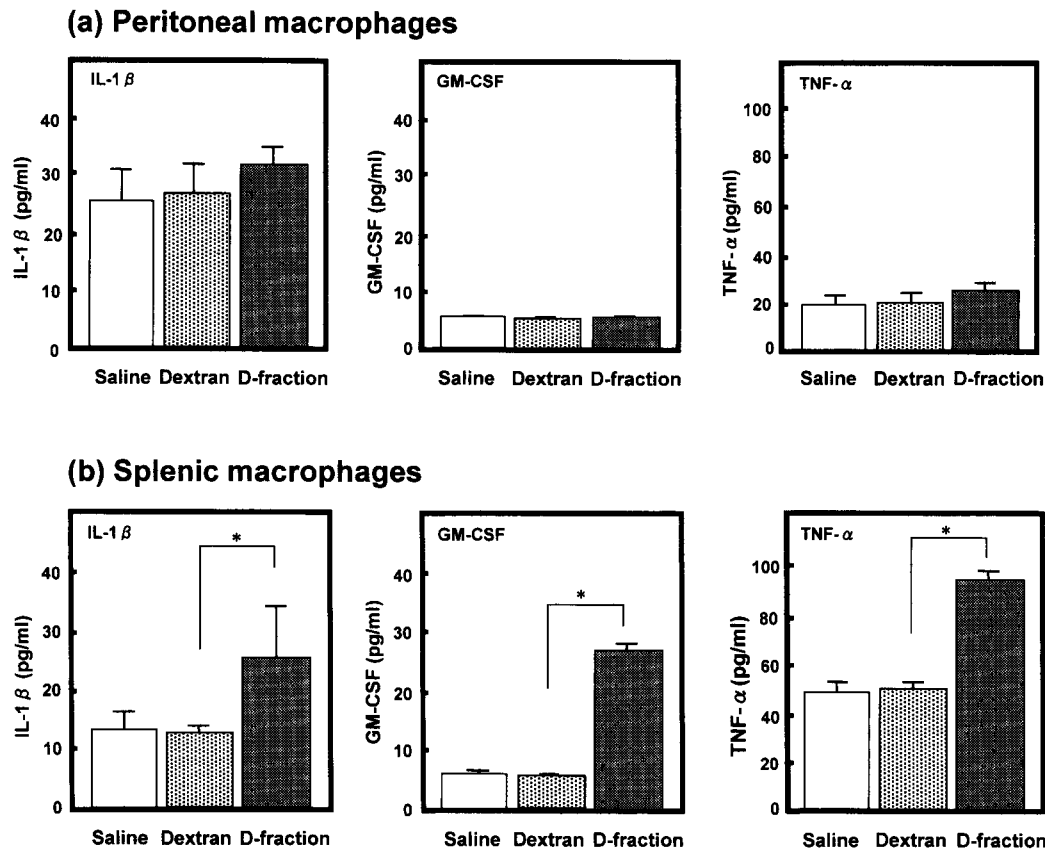
Antigens	Incorporation of <sup>3</sup> H-thymidine (cpm $\times$ 10 <sup>3</sup> )		
	Saline	Dextran	D-fraction
No antigen	0.69 $\pm$ 0.07	0.61 $\pm$ 0.08	0.82 $\pm$ 0.24
Collagen type I	0.40 $\pm$ 0.01	0.41 $\pm$ 0.10	0.40 $\pm$ 0.20
Collagen type II	1.80 $\pm$ 0.14	1.75 $\pm$ 0.21	1.80 $\pm$ 0.38
Collagen type III	0.40 $\pm$ 0.07	0.42 $\pm$ 0.14	0.42 $\pm$ 0.09
Con A	119 $\pm$ 2.96	118 $\pm$ 13.4	101 $\pm$ 5.35

Values are the means  $\pm$  S.D. of 6 mice. \**P* < 0.05.

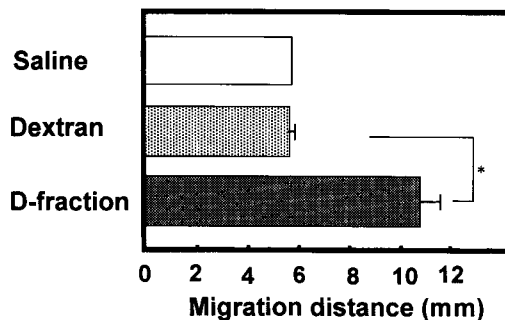
dine uptake by CD4<sup>+</sup> T cells in the D-fraction-administered group was lower than those in other two groups. The result shows that T cell reactivity for Con A was decreased by the D-fraction. The D-fraction did not enhance proliferation of lymph node T cells induced by any of the 3 types of collagen. Type I and III collagen stimulation in all three groups did not enhance proliferation of lymph node T cells, but type II collagen initiation strongly stimulated the proliferating response.

Effects of the D-fraction on peritoneal and splenic macrophages were also investigated. Peritoneal or splenic macrophages were cultured for 24 h; and IL-1 $\beta$ , GM-CSF and TNF- $\alpha$  concentrations were determined by the ELISA method. Small increases in IL-1 $\beta$  and TNF- $\alpha$  production by peritoneal macrophages was observed in the D-fraction group as shown in Fig. 4a. For the D-fraction group, the IL-1 $\beta$ , GM-CSF and TNF- $\alpha$  productions by splenic macrophages were significantly higher; i.e., 2.0, 4.7 and 1.9 times each the respective level in the control group (Fig. 4b). On the 7th day from the second stimulation, the weight of the spleen was 1.3 times more in the D-fraction group than that of the control group (data not shown).

Migrating activity of peritoneal macrophages was also investigated. The migrating ability of macrophages from sensitized mice was determined relative to that for the macrophages of un-sensitized mice, which was defined as zero. Migrating distance for the D-fraction group was 4.4  $\pm$  2.1 mm, which did not show great difference from the data obtained from the other 2 groups (data not shown).



**Fig. 4.** Effects of D-fraction on production of IL-1 $\beta$ , GM-CSF and TNF- $\alpha$  by peritoneal macrophages (a) or splenic macrophages (b). On the 7th day after the second immunization, peritoneal and splenic macrophages were prepared. Their cytokines productivity was investigated as described in Materials and Methods. Values are the means  $\pm$  S.D. of 6 mice. \* $P$ <0.05, compared with the dextran group.



**Fig. 5.** Effects of D-fraction on migration activities of splenic macrophages. On the 7th day after the second immunization, splenic macrophages were prepared. Their migration activities were investigated as described in Materials and Methods. Values are the means  $\pm$  S.D. of 6 mice. \* $P$ <0.05, compared with the dextran group.

Figure 5 shows the migrating ability of splenic macrophages, which was 1.9 times more activated in the D-fraction-administered mice than the control group mice.

The ratio of splenic macrophages to the whole spleen

**Table 4.** Effects of D-fraction on splenic macrophages

Treatments	Macrophages (%) <sup>a</sup>
Saline	6.4 $\pm$ 1.2
Dextran	6.2 $\pm$ 2.6
D-fraction	14.4 $\pm$ 6.0

<sup>a</sup>% of macrophages was determined in saline-, dextran- or D-fraction-treated whole spleen cells. Values are the means  $\pm$  S.D. of 6 mice. \* $P$ <0.05.

cells was analyzed with FCM method (Table 4). The ratio in the D-fraction group mice was 2.3 times higher than that of the control group.

## DISCUSSION

One of the interesting findings in the present experiments is that the WS-fraction enhanced rather than suppressed the development of arthritis. The result led us to the conclusion that *maitake* polysaccharides have no beneficial effect to help CIA mice recover from arthritis. The onset of

RA is known to be closely related with humoral immunity since anti-type II collagen antibodies (rheumatoid factors) exist in the patients' blood serum. The present studies were performed to clarify what substance in *maitake* fruit body promotes CIA. The D-fraction was found to enhance strongly the development of arthritis, raising the possibility that this fraction affected humoral immunity. However, the D-fraction apparently did not alter the amount of anti-type II collagen antibodies and CIA-induced activation of B cells. Therefore, it is suggested that humoral immunity is not involved in the development of arthritis enhanced by the D-fraction in CIA mice. As many RA patients exhibit CD4<sup>+</sup> T invasiveness and macrophage-like cell proliferation, it is presumed that cellular immunity participates in the onset of RA. Based on this presumption, we investigated the effect of the D-fraction on the axillary and inguinal lymph node CD4<sup>+</sup> T cells of CIA mice. The ratio of CD4<sup>+</sup> T cells / whole lymph node cells and the ratio of activated CD4<sup>+</sup> T cells / whole lymph node cells were investigated. No significant difference in the data was observed between the administered groups (Table 2). We found that the D-fraction had no influence on the proliferation or activation of CD4<sup>+</sup> T cells. DNA synthesis ability was evaluated to investigate the activation of CD4<sup>+</sup> T cells (Table 3). The result showed that T cell reactivity to Con A was decreased by the D-fraction. The low reactivity for the mitogen of a reactive joint integration T cell in RA has been reported (14). Therefore, the possibility that there was a decrease in the T cell activity of the lymph node was considered, when the arthritis symptom was developed in response to the D-fraction. On the other hand, DNA synthesis ability was very high in the presence of type II collagen, but in the presence of type I or type III collagen. The data showed almost the same tendency in all administration groups. From these results, we assumed that T cells are not involved in the arthritis development enhanced by the D-fraction.

Macrophages are said also to participate in the onset of RA (15, 16). The point of RA symptoms is the synovial membrane, which consists of the synovial A cells and the synovial B cells. The synovial A cells are also called macrophage-like cells. In RA, the macrophage-like cells that proliferate in the synovial membrane are mostly cells that have migrated from the blood and not cells generated in the synovial membrane itself (17). In RA patients' synovial membrane, there are more cytokines of macrophage origin than those of T cell origin (18). Based on this knowledge, we studied the peritoneal and splenic macrophages to determine the activities of the D-fraction toward these macrophages. The concentration of IL-1 $\beta$ , GM-CSF and TNF- $\alpha$  produced by peritoneal macrophages were not different between the D-fraction group and the control group (Fig. 4a). The migration ability test also gave the same result.

On the other hand, the productions of IL-1 $\beta$ , GM-CSF and TNF- $\alpha$  by splenic macrophages were significantly higher in the D-fraction group compared to the control group (Fig. 4b). The ratio of macrophages to the whole spleen cells increased (Table 4). Also the weight of spleens increased (data not shown). The migration activity of splenic macrophages was 1.9 times that of the control group (Fig. 5). These results suggest that the synovial membrane macrophage-like cells proliferating in parts of the joints originate from splenic macrophages.

Feldmann et al. pointed out that TNF- $\alpha$  is a key cytokine that accelerates auto-antigen presentation of macrophages, enhances onset of inflammatory responses, and also affects the productions of IL-1 $\beta$  and GM-CSF (19). The presence of TNF- $\alpha$  in RA is consistent with this idea. In the agreement with this report, we assume that the effects of the D-fraction are mediated through a mechanism where the D-fraction stimulates splenic macrophages to release an increased amount of TNF- $\alpha$ , which leads to increased productions of IL-1 $\beta$  and GM-CSF. The released cytokines generate autocrines and proliferate splenic macrophages, which migrate through blood vessels and become concentrated in the articular joints, which then proliferate and cause inflammatory changes at this point.

#### REFERENCES

- 1 Moss RW: Cancer Therapy, p 246, Equinox Press, New York (1992)
- 2 Chihara G, Hamuro J, Maeda Y, Arai Y and Fukuoka F: Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Res* **30**, 2776-2781 (1970)
- 3 Nanba H, Hamaguchi A and Kuroda H: The chemical structure of an antitumor polysaccharide in fruit bodies of *Grifola frondosa* (*Maitake*). *Chem Pharm Bull (Tokyo)* **35**, 1162-1168 (1987)
- 4 Kubo K and Nanba H: Modification of cellular immune responses in experimental autoimmune hepatitis in mice by *Maitake* (*Grifola frondosa*). *Mycoscience* **39**, 351-360 (1998)
- 5 Blom AB, Van Lent PL, Holthuysen AE and Van den Berg WB: Immune complexes, but not streptococcal cell walls or zymosan, cause chronic arthritis in mouse strains susceptible for collagen type II auto-immune arthritis. *Cytokine* **11**, 1046-1056 (1999)
- 6 Kakimoto K, Katsuki M, Hirofuji T, Iwata H and Koga T: Isolation of T cell line capable of protecting mice against collagen-induced arthritis. *J Immunol* **140**, 78-83 (1988)
- 7 Kobayashi Y, Shirahase H, Zhao HF, Kakizoe E and Okunishi H: Effect of orally available prodrug of cromaglycic acid on collagen-induced arthritis mice. *Folia Pharmacol Jpn (Nippon Yakurigaku Zasshi)* **114**, 154-158 (1999) (text in Japanese with English abstract)
- 8 Dreywood R: Qualitative test for carbohydrate material. *Ind Eng Chem Anal Ed* **18**, 499 (1946)
- 9 Wood FD, Pearson CM and Tanaka A: Capacity of mycobacterial wax D and its subfractions to induce adjuvant arthritis in rats.

- Int Arch Allergy Appl Immunol **35**, 456–467 (1969)
- 10 David JR, Askari AL and Thomas L: Delayed hypersensitivity in vitro. *J Immunol* **93**, 264–273 (1964)
  - 11 Stuart JM, Cremer MA, Townes AS and Kang AH: Type II collagen-induced arthritis in rats. Passive transfer with serum and evidence that IgG anticollagen antibodies can cause arthritis. *J Exp Med* **155**, 1–16 (1982)
  - 12 Kerwar SS, Englert ME, McReynolds RA, Landes MJ, Lloyd JM, Oronsky AL and Wilson FJ: Type II collagen-induced arthritis. Studies with purified anticollagen immunoglobulin. *Arthritis Rheum* **26**, 1120–1131 (1983)
  - 13 Staines NA, Ekong TA, Thompson HS, Isaacs AB, Loryman B, Major PJ, Hobbs SM and Devey ME: Low affinity antibodies against collagen type II are associated with pathology in collagen-induced arthritis in mice. *J Autoimmun* **3**, 643–657 (1990)
  - 14 Firestein GS, Alvaro-Gracia JM and Maki R: Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J Immunol* **144**, 3347–3353 (1990)
  - 15 Burmester GR, Stuhlmuller B, Keyszer G and Kinne RW: Mononuclear phagocytes and rheumatoid synovitis. Mastermind or workhorse in arthritis? *Arthritis Rheum* **40**, 5–18 (1997)
  - 16 van den Berg WB and van Lent PL: The role of macrophages in chronic arthritis. *Immunobiology* **195**, 614–623 (1996)
  - 17 Matuyama T and Nakazima N: Rheumatoid arthritis. In *The Clinical Immunity*, Edited by The Clinical Immunity Editing Committee, Vol **30**, pp 214–219, Kagaku Hyouron-sha, Tokyo (1998)
  - 18 Feldmann M, Brennan FM and Maini RN: Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol* **14**, 397–400 (1996)
  - 19 Feldmann M, Brennan FM, Chantry D, Haworth C, Turner M, Katsikis P, Londei M, Abney E, Buchan G and Barrett K: Cytokine assays: role in evaluation of the pathogenesis of autoimmunity. *Immunol Rev* **119**, 105–123 (1991)