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Activities of polysaccharides obtained from *Grifola frondosa* on insulin-dependent diabetes mellitus induced by streptozotocin in mice

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In an experiment with C57BL/6J model mice having insulin-dependent diabetes mellitus (IDDM) induced by streptozotocin, the glucans obtained from Maitake fruit-body were found to suppress activation and proliferation of macrophages and consequently to inhibit the generation of nitric oxide (NO) and IL (interleukin)-1 β, which are β-cell destruction factors, suppressing their production to 0.17 and 0.33, respectively, relative to the control mice. In addition, the glucans caused proliferation and activation of B cells, as detected by flow cytometric analysis of whole spleen cell lymphocytes. Cytokine secretion from whole spleen cells was also investigated by ELISA, which showed that IFN-γ production was suppressed while IL-4 production increased. These glucans, acting in the diabetic mice, accelerate recovery of Th2 cell functions, regulate the balance between Th1 cells and Th2 cells, and consequently repress the cytotoxic reaction. In spite of the effect of the glucans on the immune reactions, IDDM itself does not show any favorable signs of recovery. For the result, some other factors are suggested to be acting in the complex immunity network in relation to IDDM generation.

Key Words——IDDM; polysaccharide; streptozotocin.

Various physiological activators have recently been extracted from fruit-bodies of Basidiomycetes in the Eumycote, and some are already in practical use in the clinic as BRMs (biological response modifiers). Our laboratory has already reported on the anti-tumor effect of the D-fraction, a polysaccharide extracted from Maitake (*Grifola frondosa* S. F. Gray) (Adachi et al., 1987; Hishida et al., 1988; Nanba et al., 1987). Tomoda et al. (1986) also reported a blood glucose-depressing effect of the polysaccharide obtained from Mannentake (*Ganoderma lucidum* (Leyss. ex Fr.) Karst.). In our laboratory, we have investigated the blood glucose-depressing effect of the polysaccharide from Maitake, which has β-1, 6 bonding glucan as main chain structure and α-1, 4 bonding as branch chains, and is designated as X-fraction. The X-fraction promotes the responsiveness of the insulin receptors and leads to some recovery from the insulin non-dependent diabetes, besides being a remedy for obesity (Kubo, 1990). The immune system has been recognized to be closely related to the onset of IDDM (insulin dependent diabetes mellitus; Sugita, 1990). The polysaccharide extracted from maitake was found to have a favorable effect in autoimmune hepatitis through its effect on the immune reaction (Kubo et al., 1998). We have extended our study to see if similar effect could be found for IDDM. IDDM is an autoimmune disease that is organ-specific. In IDDM, high blood glucose level is observed caused by the selective destruction of β-cells in the pancreas (Sugita, 1990). The present study examines whether an improving effect based on regulation of immunity as seen in the autoimmune hepatitis is observed also in IDDM, i.e., whether D-fraction activates killer T cells, which eliminate the liver cells that have caused the inflammation; and whether X-fraction acts on suppressor T cells, which inhibit excessive action of the killer T cells.

Helper T cells (Th cells) are classified into two subsets: Th1 cells, which produce IL-2 (interleukin-2) and IFN-γ (interferon-γ); and Th2 cells, which produce IL-4, IL-5 and IL-6 (interleukins-4, -5 and 6). Th1 and Th2 cells are known to inhibit each other activated by antigen-stimulation. The mode of the immune response of the organism seems to be influenced by the predominant type of cell. The balance of Th1 cells and Th2 cells has recently been reported to contribute appreciably to the cause of IDDM (Trembleau et al., 1995), and the reported mechanism is that the Th1 cells, which induce cellular immunity, over-react and become dominant over Th2 cells, which induce humoral immunity, and result in the destruction of pancreatic β-cells by autoimmune reaction. In this paper, we report that the polysaccharides from Maitake promote recovery of Th2 cell function, resulting in regulated balance of Th1 cells and Th2 cells, an ultimately suppress the cytotoxic reaction.

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Materials and Methods

Materials Dried powder made from the fruit-bodies of Maitake (Yukiguni Maitake Co.) was used. Rosewell Park Memorial Institute (RPMI)-1640 medium and phosphate-buffer saline [PBS (-)] were purchased from GIBCO Lab., Japan and Nissui Pharm. Co., Japan respectively. Enzyme-linked immunosorbent assay (ELISA) kits for mouse interleukin (IL)-1β, IL-4, and interferon (INF)-γ were purchased from Genzyme Techne Co., Japan. Streptozotocin (STZ) was obtained from Sigma-Aldrich Japan Co. Insulin Analysis Kit (Morinaga Pharm. Lab., Japan), Glucose B-test Wako (Wako Pure Industry Co., Japan), and Perm/Wash and Cytofix/Cytoperm Plus (with Goldi Stop) Kit (Permingen Co., Japan) were also used.

Animals Male ICR mice (4 wk) and male C57BL/6J mice (6 wk) were obtained from Clea Japan Inc. and given food and water ad libitum until use.

Antibodies Ten kinds of antibodies were purchased from Pharmingen Co., Japan: anti-CD16/CD32 monoclonal antibody (CD, cluster of differentiation), R-PE conjugated anti-CD4 monoclonal antibody (R-PE, R-phycoery thrin), FITC conjugated anti-CD8 monoclonal antibody (FITC, fluorescein isothiocyanate), R-PE conjugated anti-CD69 monoclonal antibody, FITC conjugated anti-CD69 monoclonal antibody, R-PE conjugated anti--CD19 monoclonal antibody, Cy-Chrom™ conjugated anti-CD3ε monoclonal antibody, FITC conjugated anti--IFN-γ monoclonal antibody, PE conjugated anti-IL-4 monoclonal antibody.

Preparation of D-fraction and X-fraction D-fraction and X-fraction were obtained from Maitake (*Grifola frondosa*) as shown in Fig. 1. Briefly, the hot-water-soluble fraction from the dried powder of Maitake was obtained, EtOH was added to a final concentration of 50%, and the mixture was centrifuged. The floating substance, named crude X-fraction, was collected and dissolved in deionized water. After collecting the floating substance, EtOH was added to the residue to a final concentration of 80%, and the mixture was centrifuged. The precipitate was named crude D-fraction. X-fraction and D-fraction were each purified from the crude preparations by DEAE cellulofine column chromatography using 12.5 mM Tris-HCl buffer (pH 7.25). Saccharide concentration was determined by the anthrone method (Dreywood, 1946).

Preparation of C-feed, D-feed, and X-feed C-feed, D-feed, and X-feed were made as follows: 4 g each of laminarin, D-fraction, and X-fraction were mixed with 1,500 g of CE-2 in 1,200 ml of deionized water to form a paste. Laminarin was used as a control for administration of D- or X-fraction. These food pastes were cut into small blocks of $3\times3\times1$ cm, dried at 80°C for 3 d, and stored at 4°C until use.

STZ-induced IDDM mice STZ was dissolved in 0.1 M citrate buffer (pH 4.6)-0.9% NaCl (1: 2), just before use. The STZ solution was injected into the tail vein of ICR mice deprived of food for 18-20 h in a single dose of 120 mg/kg (high-dose STZ). The STZ solution also was injected intraperitoneally (i. p.) to C57BL/6J mice in multiple low doses of 40 mg/kg/d for 5 consecutive d (low-dose STZ). Hyperglycemia was defined as a urine glucose level of 300-500 mg/dl. The concentration of glucose was measured with TES-TAPE (Shionogi Pharm. Co., Japan)

Dose of D-fraction or X-fraction To compare solid feed with liquid feed, laminarin, D- or X-fraction was administered orally to ICR mice (high-dose STZ mice) through a tube once a day at the same concentration as in the solid feed. Laminarin, D- or X-fraction also was administered i. p. to C57BL/6J mice (low-dose STZ mice) at the concentration of 8.7 mg/kg/d. Two things were employed for the start of the intraperitoneal injection: 1) with the onset of IDDM symptoms, 2) at the same time as the low dose of STZ. Plasma glucose level, insulin level, and body weight were measured weekly after STZ injection and intake amount was measured every 3 d. The average intake per day per mouse was calculated.

Chemical analysis Blood was collected from the retro orbital sinus using glass capillary tubes ($\phi0.7\times75$ mm) treated with heparin, and centrifuged at $8,750\times g$ for 4 min. The separated plasma was stored at −20°C until assayed. Plasma glucose level was determined using Glucose B-test Wako. Insulin Analysis Kit was used for plasma insulin.

Preparation of macrophages Mice were killed by cervical dislocation 2 wk after glucan administration. Mouse peritoneal macrophages were collected by lavage with cold PBS (−). The macrophages were washed with cold PBS (−) and suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS) (1×10^6 cells/ml). Cells were cultured at 37°C in 5% CO_2 atmosphere for 2 h. After removing the culture supernatant, the adherent cells were washed with 37°C PBS (−) and suspended in RPMI-1640 medium containing 10% FCS. Macrophages (1×10^5 cells) were seeded in a 96-well plate and cultured at 37°C in 5% CO_2 atmosphere for 20 h, then centrifuged at $300\times g$ for 5 min. The supernatant was collected and used for NO assay and IL-1β assay.

Preparation of whole spleen cells After 2 wk of glucan administration, mouse spleens were taken out and washed with PBS (−). Sterile spleen cell suspension was prepared by the following procedure. The mice spleens were passed through a cell strainer ($\phi70\mu$m, Falcon Co., Japan), washed with RPMI-1640 medium, and centrifuged at $300\times g$ for 5 min. The precipitates were collected, mixed with 5 ml of hemolytic buffer, centrifuged again, and washed with cold RPMI-1640 medium. The precipitate was mixed with 2 ml of RPMI-1640 medium containing 5% FCS and 5×10^{-5} M of 2-mercaptetanol. Whole spleen cells (1×10^6 cells/well) was seeded into a 96-well plate, Con A was added at the final concentration of 1μg/ml for IFN-γ assay, or 10 μg/ml for IL-4 assay, and the plate was incubated at 37°C in 5% CO_2 atmosphere for 24 h or 48 h. After centrifugation at $300\times g$ for 5 min, 100 μl of supernatant was collected and assayed for IL-1β, INF-γ, and IL-4 by ELISA.

Determination of NO One hundred microliter of mouse peritoneal macrophages (1×10^6 cells/ml)was applied to

a 96-well plate, and the plate was cultured at 37°C in 5% CO_2 atmosphere for 20 h. After the culture, 100 μl of supernatant was obtained by centrifugation at 300×*g* at 4°C for 5 min and reacted for 10 min at room temperature with an equal volume of Griess reagent (1% sulfanilamids, 0.1% naphthylethylenediamine hydrochloride, 2.5% phosphoric acid). The optical density of the mixture was measured at 550 nm (Ref. 630 nm).

Alamer Blue assay One hundred microliter of macrophages (1×10^6 cells/ml) or whole spleen cells (2×10^6 cells/ml) was applied to a 96-well plate. For immunocompetent cells, Con A (concanavalin A) was added to a final concentration of 10 μg/ml and the cells were cultured at 37°C in 5% CO_2 atmosphere for 44 h. Con A was not used for macrophages. After the culture, Alamer Blue (Srotec Ltd., Japan) was added to a the final concentration of 10%, and the culture was continued for 4 h. Stained cells were measured by use of Fluoromark (Bio-Rad Lab., Japan), with excitation at 540 nm and emission at 590 nm.

Flow cytometry analysis For cell-surface antigen, 50 μl of whole spleen cells (2×10^7 cells/ml) was mixed with 1 μl of CD16/CD32 Fc blocker in tubes and reacted at 4°C for 5 min. The cells were incubated with FITC-conjugated antibodies (anti-CD8, anti-CD69), R-PE-conjugated antibodies (anti-CD4, anti-CD19, anti-CD69), or Cy-Chrome™-conjugated anti-CD3ε antibody at 4°C for 20 min, washed with staining medium [SM, 0.05% NaN_2 and 3% FBS (fetal bovine serum) in PBS (−)], then suspended in 400 μl of SM and counted using FACScan™ (Beckton Dickinson Co., Japan)

For intracellular cytokines, 2 ml of whole spleen cells (2×10^6 cells/ml) was applied to a 24-well plate, ionomycin (1 mg/ml) and phorbol-12-myristate-13-acetate (50 ng/ml) were added to each well, and the plate was incubated with 0.7 μl of Goldi Stop at 37°C in 5% CO_2 atmosphere for 4 h. After the stimulation, 1 μl of CD16/CD32 Fc blocker was added and reacted at 4°C for 5 min. Then 2 μl each of Cy-Chrome™-conjugated anti-CD3ε antibody and FITC-conjugated anti-CD69 antibody were added and reacted at 4°C for 20 min. After the reaction, the cells were washed with SM and incubated with 100 μl of Cytofix/Cytoperm at 4°C for 20 min, then washed again with Perm/Wash. The stained cells were reacted with 2 μl each of FITC-conjugated anti-IFN-γ antibody and PE-conjugated anti-IL-4 antibody at 4°C for 30 min, then washed with Perm/Wash, suspended in 50μl of SM, and counted using FACScan™.

Statistical analysis The data were analyzed for significance by Scheffe's F-test. P values of less than 0.05 and 0.01 were considered statistically significant (*$p<0.05$, **$p<0.01$).

Results

Recovery of diabetic model mice treated with a single dose of STZ (high-dose STZ) We investigated the effect of the glucan on the regeneration and restoration of the damaged pancreatic β-cells. For this investigation, seriously diabetic model mice were prepared from ICR mice by giving a single injection of diabetes-inducing STZ to the tail vein, which completely destroyed the pancreatic β-cells. Groups of model mice were fed orally with solid feed (C-feed, D-feed, and X-feed) or force-fed with liquid feed (laminarin, D-fraction, and X-fraction) with tubes, and plasma glucose level and insulin level were determined. Prior to this investigation, we examined the food intake and body weight of mice under the glucan administration and found that food intake amount does not significantly affect the diabetic condition of the mice. Fig. 2a shows the plasma glucose level, and Fig. 2b shows the insulin level. For plasma glucose level and insulin level, no significant difference was observed among the three glucan groups with either method of administration, i.e., orally administered solid feed (Solid-Feed), or forcibly administered liquid feed (Liquid-Feed). These

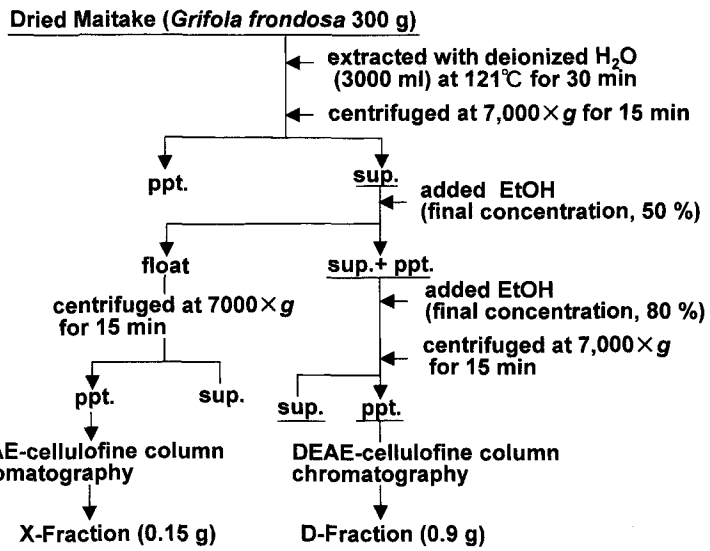

Fig. 1. Preparation of D-fraction and X-fraction from Maitake (*Grifola frondosa*).

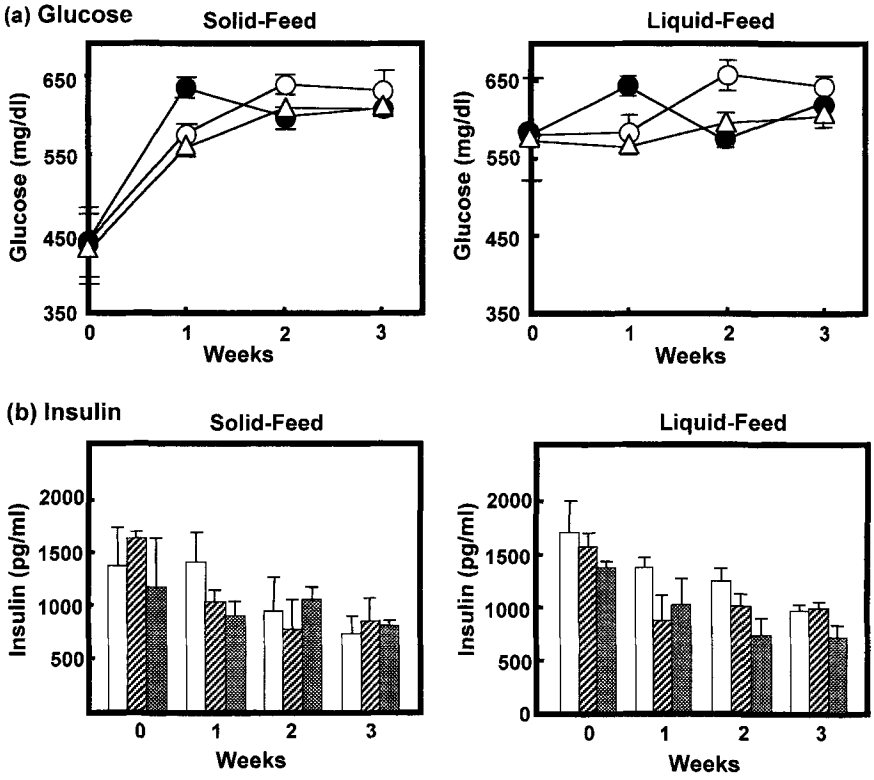

Fig. 2. Effects of glucans on plasma glucose and insulin level of high-dose STZ induced diabetic mice.
ICR diabetes mice treated with a single dose of STZ (120 mg/kg) were given solid feed or liquid feed each. In both groups, blood glucose level and insulin level were determined as described in Methods. Symbols for plasma glucose: ●, C-feed, laminarin; ○, X-feed, X-fraction; △, D-feed, D-fraction. Symbols for insulin: □, C-feed, laminarin; ▨, X-feed, X-fraction; ▩, D-feed, D-fraction.

results confirmed that neither the D-fraction nor the X-fraction exhibits a regenerating or restoring effect on pancreatic β-cell that have been completely destroyed by STZ.

Recovery of diabetic model mice treated with multiple low doses of STZ (low-dose STZ) With multiple low doses of STZ, pancreatic antigen is generated, and destruction of pancreatic β-cells is provoked by the induction of cytotoxic T cells and macrophages, and as a consequence autoimmune diabetes is generated (Yonemura et al., 1984). We investigated the effect of the glucan to regenerate and restore pancreatic β-cells, and to protect the β-cells from destruction. For this purpose, hereditarily Th1 cell-predominant model mice were prepared from C57BL/6J mice by administering STZ i. p. for 5 d consecutively, which evoked IDDM as the result of their autoimmune mechanism and the direct action of STZ. Plasma glucose level and insulin level were investigated in the model mice, which were fed with C-feed, D-feed, and X-feed (Figs. 3a, 4a). X-fraction, D-fraction, and laminarin in a dose equal to the oral dose as the total sugar quantity was injected into the mice. The intraperitoneal injection was started with the onset of IDDM symptoms to investigate the direct effect of the glucan on the β-cells (Figs. 3b, 4b), while administration of the glucan is started at the same time as the dose of STZ in order to investigate the protective effect of the glucan on the β-cells, which are destined to be destroyed by the immune reaction in the mice (Figs. 3c, 4c). Plasma glucose levels of each group of mice are shown in Fig. 3. In the orally administered groups, no significant difference was observed between the X-feed, D-feed, and C-feed groups (Fig. 3a). In the i. p. injected groups, though a significant difference was observed at 2 wk between X- or D-fraction group and laminarin group and at 3 wk between D-fraction group and laminarin group, the plasma glucose levels of the X-fraction group and D-fraction group were not significantly lower than those of laminarin group mice (Figs. 3b, 3c). The insulin level of X-feed group mice was 1.4 times of that of the control group mice at 2 wk, but at 3 wk it fell below than that of the C-feed group mice as shown in Fig. 4a. In Fig. 4b, a significant difference in insulin levels was observed between the D-fraction group and laminarin group at 3 wk. But the insulin level for the D-fraction group was lower than that of laminarin group mice. In Fig. 4c, no significant change was observed in the insulin level that could favorably affect the cure of the disease. As the result of these investigations, we confirmed that the glucan administration does not exhibit any significant changes in the plasma glucose level or insulin level that would promote recovery from IDDM in the low-dose STZ

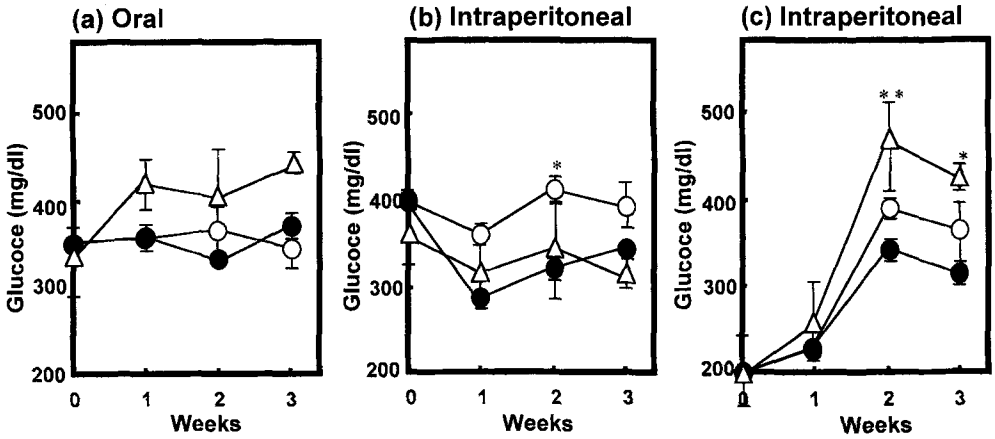

Fig. 3. Effects of glucans on plasma glucose of low-dose STZ induced diabetic mice by oral and intraperitoneal administration.
C57BL/6J diabetes mice treated with multiple low doses of STZ (40 mg/kg/d) for 5 consecutive d were given C-feed, D-feed, or X-feed (a), laminarin, D- or X- fraction at the concentration of 8.7 mg/kg/d was administered i. p. (b). C57BL/6J mice were treated with STZ and at the same time laminarin, D- or X- fraction was administered i. p. (c). Plasma glucose level was investigated in the model mice as described in Methods. Symbols: ●, C-feed, laminarin; ○, X-feed, X-fraction; △, D-feed, D-fraction. Significant difference was determined by Scheffe's F-test (*$p<0.05$, **$p<0.01$).

induced IDDM model mice.

Activity of immuno-competent cells in the diabetic model mice treated with multiple low doses of STZ (low-dose STZ) The effects of the glucans on formation of NO (nitric oxide), a β-cell-destroying factor, and of the IL-1, a putative effector molecule in immune cell-mediated β-cell damage, which seems to require generation of NO, were examined. We prepared model mice with slowly de-

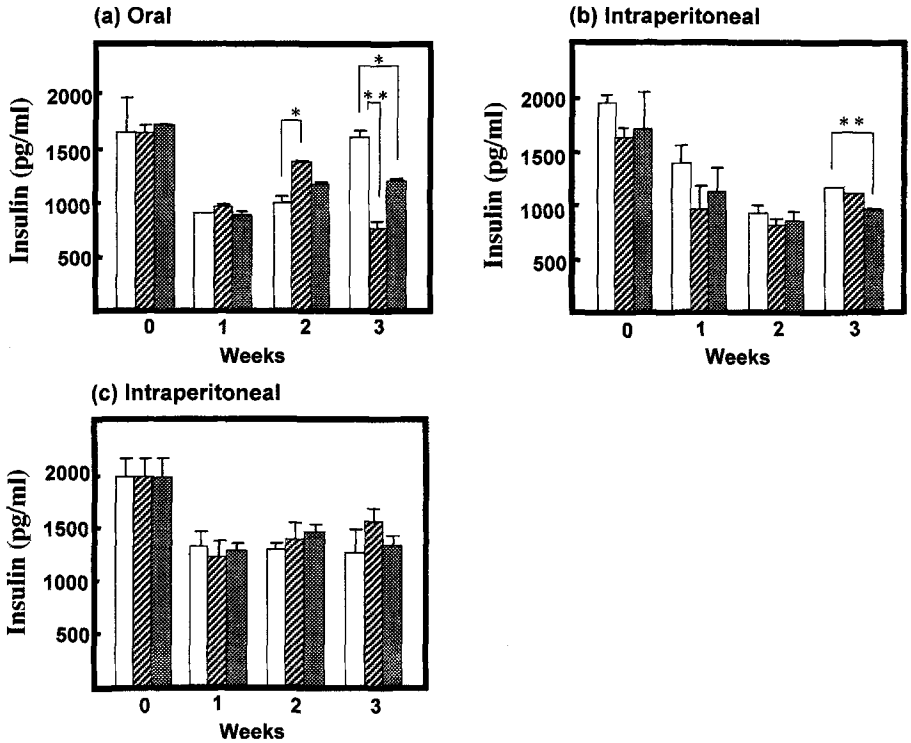

Fig. 4. Effects of glucan on plasma insulin level of low-dose STZ induced diabetic mice.
C57BL/6J diabetes mice treated with multiple low doses of STZ (40 mg/kg/d) for 5 consecutive d were given C-feed, D-feed, or X-feed (a), laminarin, D- or X- fraction at the concentration of 8.7 mg/kg/d was administered i. p. (b). C57BL/6J mice were treated with STZ and at the same time laminarin, D- or X- fraction was administered i. p. (c). Plasma insulin level was investigated in the model mice as described in Methods. Symbols: □, C-feed, laminarin; ▨, X-feed, X-fraction; ▩, D-feed, D-fraction. Significant difference was determined by Scheffe's F-test (*$p<0.05$, **$p<0.01$).

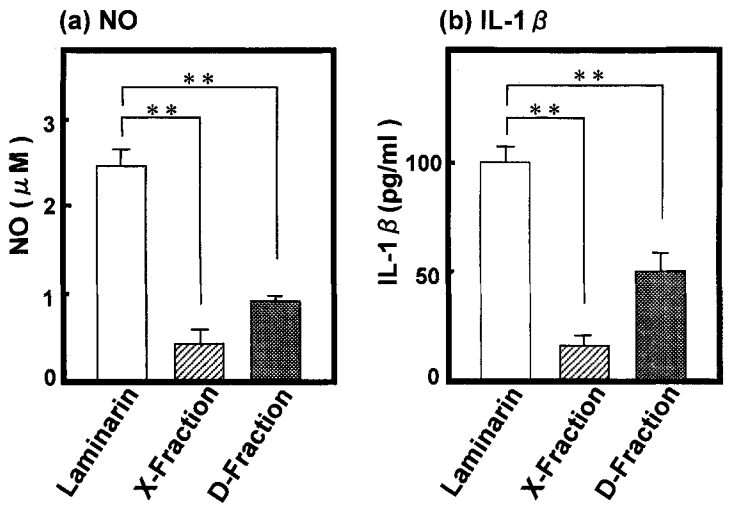

Fig. 5. Effects of glucans on NO and IL-1β level produced by peritoneal macrophages.
C57BL/6J diabetes mice were treated with multiple low doses of STZ (40 mg/kg/d) for 5 consecutive d, and at the same time three kinds of glucan were administered. After 2 wk of glucan administration, macrophages were collected from mice and their NO and IL-1β productivity were investigated as described in Methods. Symbols: □, laminarin; ▨, X-fraction; ▩, D-fraction. Significant difference was determined by Scheffe's F-test. (**$p<0.01$).

veloping diabetes caused by gradual destruction of the pancreatic β-cells by autoimmune response. Figure 5 shows the NO concentration and IL-1β concentration secreted from the macrophages obtained from the mice, which were treated with STZ and D-fraction or STZ and X-fraction administered simultaneously for 2 wk. The glucan-administered mice showed considerably inhibited NO production, which was 0.17 relative to laminarin-administered mice, while IL-βproduction was suppressed to 0.33 times the laminarin mice. The proliferation and activation of the peritoneal macrophages and whole spleen cells were also examined in mice treated simultaneously with STZ and the glucan for 2 wk. Alamer Blue assay was employed for the analysis. The result is shown in Fig. 6. For the whole spleen cells no difference was observed among the three groups, while for peritoneal macrophages, proliferation and activation were observed in the Maitake-administered groups. IDDM is understood to be associated with helper T cell expressing CD4 (CD4$^+$ T cell), cytotoxic and suppressor T cells (CD8$^+$ T cells) expressing CD8, B cells expressing CD19, and so on, in a complex manner. Based on this knowledge, we examined the proportion and the activation of sub-class lymphocytes in the whole spleen cells under influence of the glucan by flow cytometry. As shown in Table 1, neither CD4, CD8, nor their activated forms (i.e., CD4/CD69 and

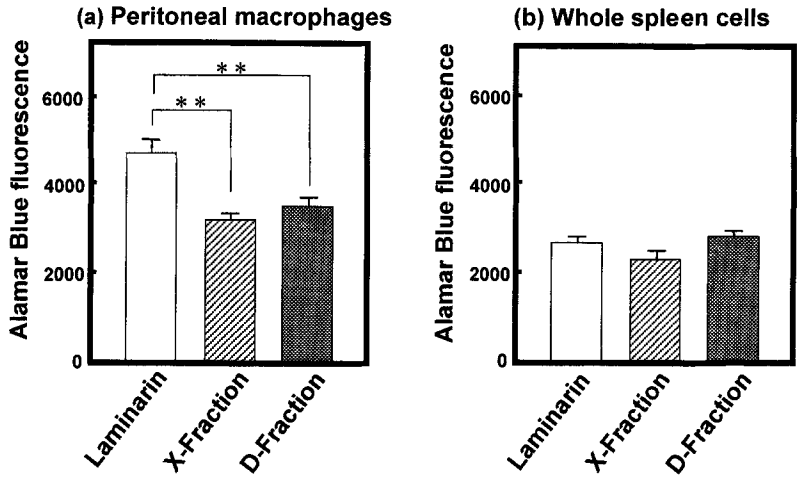

Fig. 6. Effects of glucans on the cell growth of peritoneal macrophages and whole spleen cells determined by Alamar Blue.
C57BL/6J diabetes mice were treated with multiple low doses of STZ (40 mg/kg/d) for 5 consecutive d, and at the same time three kinds of glucan were administered. After 2 wk of glucan administration, macrophages and whole spleen cells were collected from mice, and the growth was investigated as described in Methods. Symbols: □, laminarin; ▨, X-fraction; ▩, D-fraction. Significant difference was determined by Scheffe's F-test. (**$p<0.01$).

Table 1. Whole spleen cell surface antigen expression analysis.

CD antigens	Ratio (%)[a]		
	Laminarin	X-Fraction	D-Fraction
$CD3^+CD4^+$	53.97±3.06	53.77± 2.67	57.16±2.85
$CD3^+CD4^+CD69^+$	7.00±1.74	7.59± 2.75	4.91±0.35
$CD3^+CD8^+$	33.74±2.75	31.26± 0.86	29.11±2.81
$CD3^+CD8^+CD69^+$	2.90±1.20	2.44± 0.99	2.40±0.91
$CD19^+$	37.93±7.37	63.05± 1.17	51.16±6.37
$CD19^+CD69^+$	6.28±0.73	19.55±11.40	5.92±0.08

a) Percentage of CD antigen-positive cells to whole spleen cells was measured by FCM analysis.

CD8/CD69) showed any major difference in the D-fraction, X-fraction, and laminarin groups, but for the B cells, their marker CD19 showed a slight increase, and the CD19/CD69 (the activated form of CD19) showed a 3.1-fold increase for the X-fraction group compared with the laminarin group. These results support the assumption that the Th2 cell function must be resumed and B cell functions accelerated. The generation of IDDM is also known to be influenced strongly by the disturbed balance between Th1 cells and Th2 cells, especially by the depressed Th2 cell functions. The data in Table 1 suggest that glucan administration promotes recovery of Th2 cell function. The amounts of IFN-γ secreted from Th1 cells and IL-4 secreted from Th2 cells in the whole spleen cells of the mice after 2 wk of glucan administration were determined by ELISA. The results are shown in Fig. 7. Administration of X-fraction and D-fraction suppressed IFN-γ production and increased IL-4 production. The effect of the Maitake glucan was more significant in the X-administered group, in which IFN-γ and IL-4 production were respectively 0.4 times and 2.6 times the control level. At the same time, the IFN-γ and IL-4 secreted from the T cells uniquely existing in the whole spleen cells were also determined by flow cytometry. Although little difference was observed in the INF-γ production, IL-4 production was increased in the Maitake glucan-administered groups, especially the X-fraction group, where the production was 1.71 times the control level (data not shown). The result of these experiments confirms that the glucan, by activating the Th2 cell functions, recovers the regular balance between Th1 cells and Th2 cells, and consequently inhibits the cytotoxic reactions. This suggests that the glucans adjust the immunity which have been disrupted by the dominant Th1 cell function caused by IDDM.

Discussion

IDDM is a disease caused by autoimmune disorders, in which organs are damaged specifically, causing selective destruction of pancreatic β-cells, which results in hyperglycemia. On the other hand, in autoimmune hepatitis, the D-fraction is known to activate killer T cells, which destroy damaged liver cells, while the X-fraction is

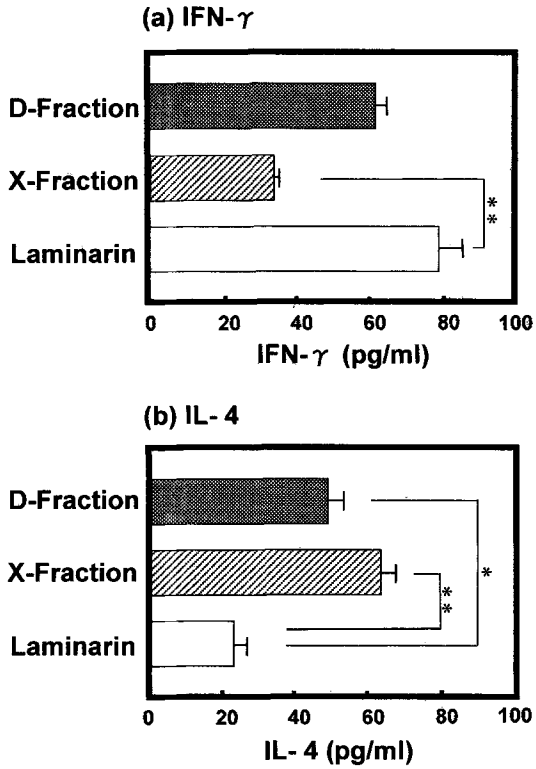

Fig. 7. Effects of glucans on IFN-$\gamma$and IL-4 produced by whole spleen cells.
C57BL/6J diabetes mice were treated with multiple low doses of STZ (40 mg/kg/d) for 5 consecutive d, and at the same time three kinds of glucan were administered. After of 2 wk of glucan administration, macrophages and whole spleen cells were collected from mice and their IFN-$\gamma$and IL-4 productivity were investigated as described in Methods. Symbols: □, laminarin; ▨, X-fraction; ■, D-fraction. Significant difference was determined by Scheffe's F-test. (*$p<0.05$, **$p<0.01$).

known to act on suppressor T cells to inhibit excessive reactions of killer T cells, and the glucans consequently adjust immunity. Based on this knowledge, we examined whether the glucan could similarly immunomodulate T cells in IDDM and possibly bring about a recovery from IDDM symptoms. The effect of the glucan in restoration and regeneration of the pancreatic β-cells as well as on the protection of these cells from destruction by autoimmunity mechanism was also investigated (Figs. 3, 4). For this purpose, model mice were prepared from Th1 cell-dominant C57BL/6J mice, in which the autoimmune mechanism and direct action of STZ induce IDDM. We confirmed that the glucan has no effect to lower plasma glucose level or raise insulin level in these mice, and consequently has insufficient effect to suppress diabetes. The cause of IDDM has hereditary factors in the background, and the development is started with insulitis and pancreatic β-cell destruction introduced by disrupted immunity. Macrophages are considered to be one of the effectors that cause the immunity disruption. NO, synthesized and secreted by macrophages, damages pancreatic β-cells not only directly but

also indirectly by producing IL-1β cells, which accelerate NO production, and IFN-γ, which affects pancreatic β-cells directly. In the investigation, however, NO and IL-1β production were found to be inhibited, and so we assumed that the glucan inhibits one of the β-cell damaging factors (Fig. 5). Proliferation and activation of whole spleen cells and of macrophages were investigated by Alamer Blue assay, and intraperitoneal macrophages were found to be significantly inhibited in their functions by the glucan (Fig. 6). These findings suggest that the inhibition of NO and IL-1β production result from the suppressed proliferation and activation of the macrophages. The process leading to IDDM is thought to involve $CD4^+$ T cells, $CD8^+$ T cells, and cytokine secreted from these cells. It has recently been reported that the balance between Th1 cells and Th2 cells is intimately involved in the development of IDDM (Trembleau et al., 1995). The dominance of Th1 cells over Th2 cells provokes autoimmunity and then destruction of pancreatic β-cells, when Th1 cells induce cellular immunity and Th2 cells induce humoral immunity.

Subclasses of lymphocytes were determined for CD4 and CD8, as they are the suface antigen markers for the helper T cells and cytotoxic T cells. CD69, the activation marker, was also employed in the determination. Increase of CD19, the B suface antigen marker, was confirmed in X-fraction-administered group (Table 1). This result suggests that Th2 cells resumed their function to induce humoral immunity, because the X-fraction activated the B cells. IFN-γ secreted from Th1 cells and IL-4 secreted from Th2 cells were examined by ELISA with the supernatant of the cultured whole spleen cells. With the administration of the glucan, IFN-γ production was suppressed, and IL-4 production was increased (Fig. 7). Lymphocytes indicate T cells, B cells, and various other cells, and so we performed flow cytometry and pinpointed cytokine production secreted from T cells alone. The IL-4 production increased 1.71 times in the X-administered-group (data not shown). We confirmed that the dominance of cytotoxic reactions was suppressed by the regulated balance between Th1 cells and Th2 cells, which was due to the accelerated recovery of Th2 cell function by the glucan. In spite of the effect of the glucan on the immune reactions, IDDM itself does not show any favorable signs of recovery. Other factors are suggested to participate in the immune network in relation to IDDM generation, such as the costimulator molecule, which relates signal transduction from antigen-presenting cells to T cells, destruction by natural killer cells, and destruction mechanism caused by apoptosis of β-cells, etc. Further investigation is required to clarify these factors.

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