Th1/Th2-Balancing Immunomodulating Activity of Gel-Forming (1→3)-β-Glucans from Fungi

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The immunomodulating effects of various gel-forming (1→3)-β-glucans, grifolan (GRN), SSG, sonifilan (SPG) and alkaline-treated SPG (SPG-OH), on balancing helper T cell activity were examined in a murine model. Plasma from mice that were injected with GRN or SPG-OH and trinitrophenyl ovalbumin (TNP-OVA) contained TNP-specific antibodies of both IgG1 and IgG2a isotypes. Administration of SSG and TNP-OVA significantly augmented the synthesis of IgG2a antibodies, while the synthesis of IgG1 was reduced. However, SPG did not enhance the antibody response. In the culture supernatants of splenocytes obtained from GRN- or SPG-OH-administered mice, high levels of IgG1 and low levels of IgG2a and IFN-γ were detected. In contrast, high levels of IgG2a and IFN-γ and low levels of IgG1 were detected in the case of administration of SSG. Furthermore, it was shown by intracellular cytokine staining that the proportion of IFN-γ/CD4+ double-positive cells among the CD4+ cells from mice administered SSG was most strongly increased by addition of PMA and A23187. On the other hand, the expression of IL-12 p40 mRNA was more markedly elevated in splenocytes after combined administration of TNP-OVA plus SSG than after administration of TNP-OVA alone. The highest IFN-γ production was observed when adherent cells of mice administered TNP-OVA and SSG were cultured with TNP-primed lymphocytes. This effect of administration of SSG on IFN-γ production was completely inhibited by addition of anti-IL-12 mAb. In conclusion, our study showed that β-glucans have various effects on the Th1 or Th2-dependent antibody subclasses, in particular, SSG induces the development of Th1 cells via the IL-12 pathway.

Key words (1→3)-β-D-glucans; immunomodulating activity; Th1 adjuvant; IL-12

(1→6)-branched (1→3)-β-D-glucans (β-glucans) from fungi are known to enhance various immunopharmaceutical activities such as antitumor activity.1) Some of them, including lentinan2) and sonifilan (SPG),3) have been used clinically for cancer therapy in Japan. We have been investigating various immunopharmaceutical effects of β-glucans, such as grifolan (GRN) from Grifola frondosa4) and sclerotinia sclerotiorum glucan (SSG)5,6) from Sclerotinia sclerotiorum IFO 9395, which were isolated originally by our group. GRN has a similar primary structure to SPG,4) but SSG consists of a (1→3)-polyglucose backbone with every second residue substituted with mono-glucosyl branches.5,6) The ultrastructure of GRN and SSG is distinct from that of SPG.7) GRN and SSG contain a mixture of single and triple helix conformers, whereas SPG is composed of triple helices only.8,9) The molecular weight of a single chain of SPG is 15 kDa, which is much lower than those of GRN (50 kDa) and SSG (200 kDa).5,6,10) Our accumulated data suggest that the molecular weight, degree of branching, number of substituents, as well as ultrastructure, including the presence of single and triple helices, significantly affects the biological activities of β-glucans.11–14) For instance, it was demonstrated that GRN and SSG could more markedly induce various activities of immunological cells such as cytotoxic T cells and macrophages than SPG, although the antitumor effects of these two glucans were similar to those of SPG.15) Using SPG and SPG-OH, which is a single helical conformer prepared by alkaline treatment of SPG, we also found previously that the biological activities of β-glucans, i.e., blood clearance,16) reactivity of limulus factor G activation,17) and NO synthesis in vitro18) and in vivo,19) are strongly associated with their conformation.8,19) However, we do not yet know the details of activities of various β-glucans on helper T cell modulation.

T cell responses have been divided in two subclasses, Th1 and Th2, according to the profile of the lymphokines produced, i.e., IFN-γ and IL-2 vs. IL-4, IL-5, and IL-10, respectively.20) Evidence is accumulating that cytokines play a role during T helper cell differentiation. IL-4 has a direct effect on the induction of IL-4-producing Th2 cells21) and IFN-γ causes the preferential outgrowth of Th1.22) In addition, IL-4 appears to inhibit the induction of Th1 cells in vivo and in vitro.23,24) Antigen-presenting cells such as macrophages and dendritic cells also play a major role in T helper cell differentiation. IL-12 derived from antigen-presenting cells stimulates IFN-γ production from T cells and NK cells, thereby favoring a Th1-pattern of response.25,26) There is clear evidence that IL-12 can suppress IL-4 mRNA induction, both directly and indirectly, through induction of IFN-γ.27,28) In the antibody response, IgG2a responses are induced by IFN-γ and suppressed by IL-4.29) IFN-γ promotes isotype switching to IgG2a.30,31) IL-12 also stimulates the production of IgG2a, presumably through induction of IFN-γ from T cells and NK cells.32) Switching to IgG1 and IgE antibodies is regulated by IL-4 and inhibited by IFN-γ.33) Thus, Th1 cells are involved in the differentiation of B lymphocytes and production of the IgG2a isotype.33) Th2 cells help antibody-producing cells to induce class-switching of IgG1 and IgE, thus resulting in generation of immediate hypersensitivity responses.25) Polarized Th1 and Th2 phenotypes play a central role in the course of autoimmune and infectious diseases.25)

Although there are some reports showing an adjuvant effect of β-glucans,33–35) most of those studies were performed using particulate or triple helical β-glucans. In our previous studies, we proved that GRN has an adjuvant effect,36) but we have not examined possible differential effects of these β-glucans on Th1- and Th2-dependent antibody production. In this study, we examined in detail the adjuvant effects of several β-glucans, GRN, SSG, SPG and SPG-OH, on antibody
production, with respect to the patterns of antibody subclass and T cell-mediated cytokine production.

MATERIALS AND METHODS

Animals AKR/N male mice between 5 and 7 weeks of age were purchased from Japan SLC (Hamamatsu, Japan) and maintained under specific pathogen free conditions. We had verified in preliminary tests that AKR/N mice were most effective among various mouse strains, including BALB/c, C57BL/6, DBA/2, and C3H/HeN, for antibody production induced with β-glucans as adjuvants.16)

β-Glucans GRN from Grifola frondosa31 and SSG5) from Sclerotinia sclerotiorum IFO 9395 were prepared as described previously. SPG was provided by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Alkaline-treated SPG (SPG-OH) was prepared by dissolving SPG in 0.5 N NaOH, followed by dialysis against pyrogen-free saline (Otsuka, Japan) for 2 d.

Administration of Antigen and β-Glucans TNP-OVA (10 μg/0.2 ml) prepared as described previously36) and β-glucan (GRN, SSG, SPG or SPG-OH, 250 μg/0.2 ml) dissolved in pyrogen-free saline (Otsuka, Japan) were injected intraperitoneally into mice two or four times at 1-week intervals, respectively. The control mice were injected with TNP-OVA (10 μg/0.2 ml) and pyrogen-free saline (Otsuka, Japan) under the same conditions as the β-glucan-treated mice.

Isolation of Spleen Cells The mice were sacrificed on day 5 after the last administration of antigen and β-glucan. The spleen was excised and treated with 10% fetal calf serum (FCS) in RPMI 1640 medium (FCS-RPMI) containing collagenase (200 U/ml) in order to obtain a single cell suspension. The spleen cells were washed twice with Eagle’s minimum essential medium (EMEM) by centrifugation at 300×g for 5 min and resuspended in FCS-RPMI. The cells were cultured in a 48-well culture plate (SUMILON, Japan) at the concentration of 2.5×106 per well at 37°C in the presence or absence of 25 μg/ml TNP-OVA in a humidified 5% CO2 incubator.

ELISA for Antibody Titration Quantification of the anti-TNP-specific total IgG, IgG1 and IgG2a in peripheral blood plasma or culture supernatants was performed by ELISA using TNP-conjugated bovine serum albumin (TNP-BSA) as solid phase antigen. Microtiter ELISA plates (NUNC) were coated overnight at 4°C with TNP-BSA at 0.5 μg/well. The plasma (diluted 1000-fold) or the supernatant samples were added to the plate (50 μl/well). The plasma (diluted 1 : 1000) or the supernatant samples were added to the plate (50 μl/well). The bound antibody was detected using an anti-mouse IgG polyclonal antibody conjugated with horseradish peroxidase (Wako, Japan), or a biotinylated anti-mouse IgG1 or IgG2a monoclonal antibody (Pharmingen) diluted 1 : 5000, 1 : 1000 and 1 : 3000, and streptavidin–peroxidase (Zymed Laboratories Inc.) diluted 1 : 5000. The plates were developed using 50 μl/well of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Inc.). The reaction was stopped by the addition of 50 μl 1 N H3PO4/well, and reaction products were measured using a microplate reader (MTP-32, Corona Electric Co., Ltd.) at 450 nm. Statistical significance was determined using Student’s t-test.

ELISA for Cytokines A microtiter ELISA plate (NUNC) was coated overnight at 4°C with capture antibodies rat anti-mouse IFN-γ mAb (Pharmingen) diluted 1 : 2000 or anti-mouse IL-4 mAb at 0.25 μg/well. rm-IFN-γ or rm-IL-4 (Pharmingen) were used as standards. Fifty microliters of the supernatant samples obtained as described above were added to the plate. Biotinylated rat anti-mouse IFN-γ mAb or biotinylated rat anti-mouse IL-4 mAb (Pharmingen) diluted 1 : 2000 or 1 : 500, respectively, was used as detection antibody, respectively. After incubation and washing the plate, wells were further incubated with streptavidin-conjugated peroxidase (Zymed Laboratories Inc.) diluted 1 : 5000. The plates were developed using 50 μl/well of tetramethylbenzidine (Kirkegaard and Perry Laboratories, Inc.). The reaction was stopped by the addition of 50 μl 1 N H3PO4/well, and reaction products were measured using a microplate reader (MTP-32, Corona Electric Co., Ltd.) at 450 nm. Statistical significance was determined using Student’s t-test.

Preparation of Adherent and Non-adherent Cells The single cell suspension of spleen cells was cultured in a plastic dish (φ90×20 mm, SUMILON) at the concentration of 1.5×106 cells per dish. After incubation for 90 min, the dish was shaken gently and non-adherent cells in the culture supernatant were obtained. The dish was washed five times with warmed RPMI 1640 medium, and then cooled RPMI 1640 containing 0.5% lidocaine hydrochloride (Astra Japan, Ltd., Tokyo, Japan) was added. After incubation for 15 min, the remaining adherent cells were scraped from the dish with a pasteur pipette. Adherent and non-adherent cells were washed and resuspended in FCS-RPMI. Each type of cell was then adjusted to a concentration of 2×106 cells/ml and 50 μl of each cell suspension was dispensed per well of a 96-well culture plate (SUMILON) and incubated at 37°C for 5 d.

RT-PCR Total cellular RNA was extracted from the spleen cell suspension using Isogen. The RNA fraction was dissolved in 20 μl of DEPC-treated water. cDNA was prepared using random hexamers and Moloney–Murine leukemia virus reverse transcriptase (M-MLV RTase) (United States Biochemical Corporation). Samples were stored at 4°C until use. Samples of cDNA were amplified in 500 μl microcentrifuge tubes in the presence of 0.28 μM sense primer, 0.28 μM antisense primer, and 1.075 units of Taq DNA polymerase in PCR buffer. PCR was done in a DNA Thermal Cycler (Perkin-Elmer Cetus, CT, U.S.A.) for 30 to 40 cycles, with each cycle consisting of 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 3 min of extension at 74°C. Reaction products were visualized by electrophoresis of 10 μl of the reaction mixture at 100 V for 20 min in a 1.2% agarose gel containing 0.5 μg/ml of ethidium bromide. The primers used for this study were: β-actin: 5′-GGCATG-GATGACGATATCGCT-3′ and 5′-GTATGAGGTAGTCTG-TCAGTCT-3′; IL-12 (p40): 5′-CGTGTCTAGTTGGTGGC-AAAG-3′ and 5′-CTTCTACCTCCATTGAGTGGG-3′.

Intracellular Cytokine Staining The intracellular production of the cytokines IFN-γ and IL-4 was examined by using IC Screen (BioSource, CA, U.S.A.). Briefly, the single cell suspension of spleen cells was cultured in 6-well culture plates (SUMILON) at the concentration of 4×106 cells per well in the presence or absence of 50 ng/ml PMA and 250
ng/ml ionophore A23187. To prevent the release of cytokines from the cell, IC BLOCK was added to both unstimulated and stimulated cells. After incubation at 37 °C for 5 h in a humidified 5% CO2 incubator, cells were collected and washed twice with PBS by centrifugation at 300×g for 5 min. Then cells were resuspended to give a density of 1×10⁶ cells/50 µl in PBS, and 0.5 µg of FITC-conjugated anti-CD4 mAb (Sigma) was added to the samples. Following incubation for 30 min at 4 °C, cells were washed with PBS, supplemented with IC FIX (1 ml paraformaldehyde) and incubated for 10 min at 4 °C. Cells were washed three times with 1 ml of PBS, and then 0.5 ml of IC Perm was added to all samples and cells were incubated for 2—3 min at room temperature. After centrifugation, R-PE conjugated rat anti-mouse IFN-γ or R-PE conjugated isotype control mAb was added. Following incubation for 30 min at 4 °C, 1 ml of IC Perm was added to each sample. Cells were washed three times with 1 ml of IC Perm and resuspended in 0.5 ml of PBS for flow cytometry.

RESULTS

Effect of Several β-Glucans on Total Anti-TNP IgG Production in AKR/N Mice

To examine the effects of several β-glucans on the production of antibody against exogenous antigen, groups of four mice each were injected four times at weekly intervals with TNP-OVA (10 µg/0.2 ml/mouse) plus β-glucan (GRN, SSG, SPG, or SPG-OH, 250 µg/0.2 ml/mouse). Plasma was tested for the level of total TNP-specific IgG antibodies by ELISA using TNP-BSA coated plates on the days indicated. As shown in Fig. 1, in the plasma of mice treated with TNP-OVA and GRN, the total IgG antibody titer was increased most significantly from day 13 to 24. Administration of SSG or SPG-OH also caused a significantly larger increase of antibody titer than administration of antigen alone. However, SPG did not affect the antibody response.

Effect of Several β-Glucans on Production of Anti-TNP IgG subclasses in AKR/N Mice

The effects of several β-glucans on the IgG subclass antibody production was examined using the methods described above, since Th1 or Th2-type dominated immunity is associated with antibodies of the IgG2a or IgG1/IgE isotype, respectively. Figure 2 shows that administration of GRN strongly augmented production of both TNP-specific IgG1 and IgG2a isotypes. Although SPG-OH was not yet as effective as GRN, administration of SPG-OH caused a significant increase in production of both IgG1 and IgG2a isotypes. On the other hand, administration of SSG strongly augmented the synthesis of IgG2a antibodies but only slightly enhanced the synthesis of the IgG1 subclass. In the plasma of mice treated with TNP-OVA plus SPG, antibodies of both the IgG1 and IgG2a subclasses were produced in small amounts, which was in agreement with the data described above, indicating that the synthesis of total IgG was not significantly affected. These data suggest that various β-glucans have different effects on the Th1- or Th2-dependent class-switching of antibodies in vivo.

Production of Anti-TNP IgG1/IgG2a Subclasses of Antibody by Murine Splenocytes after Administration of TNP-OVA Plus β-Glucan

To investigate the effects of several β-glucans on induction of anti-TNP-specific IgG sub-
classes in the early stage, the spleen cells were isolated 5 d after the second immunization from mice which were immunized with TNP-OVA plus β-glucan on days 0 and 7, and TNP-specific antibody production was examined in the supernatants of spleen cells that were cultured with or without TNP-OVA. As shown in Fig. 3, when spleen cells of mice administered TNP-OVA and each of the β-glucans were cultured without TNP-OVA, IgG1 antibody was not detected. When spleen cells of mice administered TNP-OVA plus SSG or GRN were cultured with antigen, a relatively high level of IgG1 antibody was detected. However, administration of SSG or SPG did not affect the production of IgG1 antibody by splenocytes. In contrast to IgG1 production, significant synthesis of Th1 type antibody IgG2a was detected by specific ELISA in supernatants of spleen cells of mice administered TNP-OVA plus SSG.

IFN γ and IL-4 Cytokine Production by Murine Splenocytes after Administration of TNP-OVA Plus β-Glucan

It has been reported that cytokines play an important role in the antibody response, that is to say, isotype switching to IgG2a is promoted by IFN-γ and switching to IgG1 and IgE antibodies is induced by IL-4. Therefore, we examined production of IFN γ and IL-4 by murine splenocytes after administration of TNP-OVA plus β-glucan. As shown in Fig. 4, in the presence of TNP-OVA, IFN γ production by splenocytes from mice administered SSG was higher after 2—5 d culture than production stimulated by any other β-glucan. However, SPG-OH and GRN were less effective in inducing IFN-γ than SSG. SPG did not significantly affect IFN γ production in vitro.

On the other hand, although the IL-4 production by splenocytes administered TNP-OVA plus GRN or SPG-OH was lower than that of control, the kinetics of IL-4 production were similar to those of control mice. However, the splenocytes of mice administrered SSG showed a tendency to produce decreased amounts of IL-4 as the incubation time increased. The IL-4 response in SPG-treated mice was similar to that in control mice.

IFN γ Production in Mixed Cultures of Non-adherent Cells Administered TNP-OVA Plus β-Glucan and Adherent Cells Administered TNP-OVA Alone

The above findings suggested that β-glucans have different effects on induction of Th1- or Th2-dependent antibody subclasses not only in vivo but also in vitro. Furthermore, the pattern of in vitro antibody subclass production by splenocytes treated with each β-glucan tended to agree with the pattern of cytokine
production. This was especially true of SSG-up-regulated production of the Th1-dependent IgG2a antibody and IFN-γ. To investigate in detail these effects of β-glucans, we separated spleen cells into adherent and non-adherent cells. To study the effects of β-glucans on non-adherent cells, non-adherent spleen cells from mice administered TNP-OVA and each β-glucan, and adherent spleen cells of mice administered TNP-OVA alone were prepared 5 d after the second immunization. Non-adherent cells and adherent cells were mixed at a ratio of 1:1 and cultured with or without TNP-OVA. IFN-γ production in the culture supernatants was determined after 5 d of culturing. As shown in Fig. 5, in the absence of TNP-OVA, IFN-γ production was not significantly induced. In the presence of TNP-OVA, IFN-γ production was most greatly induced when non-adherent cells of mice administered TNP-OVA plus SSG were cultured with adherent cells of mice administered TNP-OVA alone. In the mixed-culture supernatant of non-adherent cells of mice administered TNP-OVA plus GRN or SPG-OH, relatively high levels of IFN-γ were detected as compared to controls which consisted of mixtures of non-adherent cells and adherent cells of mice administered TNP-OVA alone.

**Effect of Several β-Glucans on Intracellular IFN-γ Produced by CD4⁺ Cells** The above data indicated that the effects of various β-glucans on non-adherent cells were different. Since it was considered that CD4⁺ Th1 or Th2 cells probably were included among these non-adherent cells, the IFN-γ-producing cells among the CD4⁺ cells were identified by intracellular cytokine staining. The spleen cells were isolated on day 12 from mice immunized with TNP-OVA plus β-glucan on days 0 and 7, and stimulated with PMA and ionophore A 23187 to produce cytokines. Stimulation was carried out for 5 h in the presence of the transport inhibitor monensin. The stimulated or unstimulated cells were stained with FITC-tagged anti-CD4 monoclonal antibody and fixed with paraformaldehyde. Following this, cells were stained with R-PE-labeled anti-IFN-γ antibody in the presence of a permeabilizing agent. The stained cells were then analyzed by flow cytometry. Figure 6 shows that the proportion of IFN-γ⁺ CD4⁺ double-positive cells from mice administered SSG plus TNP-OVA was markedly increased by addition of PMA/A23187. There was also a slightly increased percent-
One of the major effects of IL-12 is the augmentation of IFN-γ production by macrophages, and promotes the development of Th1 cells. IL-12 is produced by antigen-presenting cells (APC) such as dendritic cells and macrophages. It has been reported that IL-12 production is stimulated by administration of SSG or GRN. No significant effect was observed when mice were administered TNP-OVA plus SPG or SPG-OH. Since preliminary examinations using GRN to determine the maximum antibody production revealed that intraperitoneal injection of 10 μg/mouse of TNP-OVA plus GRN at 250 μg/mouse could induce the highest activity, the same experimental conditions were also applied for the other β-glucans. In the plasma of mice administered TNP-OVA plus SSG, the percent-age of CD4+ cells producing IFN-γ among the CD4+ cells from mice administered GRN plus TNP-OVA. In the mice administered TNP-OVA plus SPG or SPG-OH, the percent-age of CD4+ cells producing IFN-γ was not increased compared with control mice in spite of PMA/A23187 stimulation. The TNP-OVA plus β-glucan and adherent cells administered TNP-OVA alone were adjusted to a concentration of 2×10^6 cells/ml. Fifty microliters of each cell suspension was dispensed per well of 96-well culture plates. The cells were incubated at 37°C for 5 d with or without TNP-OVA. Culture supernatants were collected and used to determine IFN-γ production. Data represent means±S.D. Significantly different from control, *p<0.05, **p<0.01.

**DISCUSSION**

Fungal (1→3)-β-d-glucans are known to act as immunostimulants enhancing host-mediated antitumor activities. Administration of β-glucan does not show direct cellular toxicity or extreme abnormality by histological assessment. Although various immunopharmacological activities of β-glucans have been examined extensively, there are only a limited number of studies on the adjuvant effects of β-glucans on antibody production. The biological effects on antibody production of gel-forming β-glucans possessing various physicochemical properties are not fully understood. Therefore, we investigated the adjuvant effects of various gel-forming β-glucans, GRN, SSG and SPG, using mice immunized with the soluble antigen TNP-OVA. The relationship between the ultrastructures of β-glucans and effects on antibody production was examined by comparing the effects of the single helical conformer of SPG (SPG-OH) and the triple helical conformer, SPG.

Since preliminary examinations using GRN to determine the maximum antibody production revealed that intraperitoneal injection of 10 μg/mouse of TNP-OVA plus GRN at 250 μg/mouse could induce the highest activity, the same experimental conditions were also applied for the other β-glucans. In the plasma of mice administered TNP-OVA plus GRN, SSG or SPG-OH, the total IgG titer was increased significantly compared to the titer in control mice administered TNP-OVA alone. However, SPG did not cause an enhancement of the antibody response. We previously demonstrated that SPG and SPG-OH had different immunopharmacological properties, including blood clearance, reactivity of limulus factor G, and effects on NO synthesis by macrophages in vivo and in vitro. Regarding these ac-

![Graph showing IFN-γ Production in Culture Supernatants](image)
activities, SPG was generally less effective than SPG-OH, suggesting that the single helical conformer is more effective in activation of host defense. Other β-glucans, GRN and SSG, are also reported to contain a single helical moiety. Considering this evidence, it is suggested that the single helical conformation is required for adjuvant activity, at least in our system using TNP-OVA as an antigen.

There is no doubt that cytokines are important for enhancing antibody production. We have reported that β-glucans can activate macrophages to produce the proinflammatory cytokines IL-1, TNF-α, and IL-6. These cytokines may act as stimulatory factors to enhance antibody production. The different abilities of various β-glucans to affect antibody production might be related to the effects of the β-glucans on cytokine production. In addition to the proinflammatory cytokines, IFN-γ and IL-4 are also known to affect antibody production by affecting isotype switching of B lymphocytes. IFN-γ substantially enhances IgG2a production and decreases IgG1 secretion. Administration of GRN and SPG-OH augmented production of both TNP-specific IgG1 and IgG2a isotypes, whereas SSG enhanced the production of IgG2a but not IgG1 in vivo. Splenocytes from SSG-administered mice showed an increased IgG2a response and reduced IgG1 production in vitro. Furthermore, IFN-γ production by the splenocytes from SSG-administered mice was maximal, and IL-4 was decreased in accordance with the increasing level of IFN-γ in the culture supernatants. Thus, the pattern of IgG subclass production in SSG-administered mice and in splenocyte cultures was consistent with that of cytokine production in vitro. On the other hand, relatively higher levels of the IgG1 response were detected in culture supernatants of splenocytes from mice administered GRN or SPG-OH, although there were lower levels of the IgG2a response, which is different from the results in plasma. For this reason, spleen
cells isolated on day 12 seemed to be at the earlier stage of immunization, since there is no significant difference in antibody production in plasma induced by β-glucans on day 12. Furthermore, in contrast to the elevated IgG1 production in vitro, administration of SPG-OH or GRN was less effective in inducing IgG2a production than administration of SSG, and also in enhancing IFN-γ production by splenocytes and suppressing IL-4 production relative to controls. In the supernatants of splenocytes isolated from GRN- or SPG-OH-administered mice on day 24 after the first immunization, production of both IFN-γ and IL-4 was significantly increased as compared with control mice (data not shown). This evidence suggests that IFN-γ may play a more dominant role than IL-4 during this early antibody response induced by GRN and SPG-OH.

We then investigated these effects of β-glucans in more detail. IFN-γ production was most greatly enhanced when non-adherent cells of mice administered TNP-OVA plus SSG were cultured with adherent cells of mice administered TNP-OVA alone. The proportion of IFN-γ+CD4+ double-positive cells in CD4+ cells from SSG-administered mice was also most dramatically increased by stimulation with PMA plus IL-4. Thus, these results strongly suggest that SSG preferentially induces Th1 cells producing IFN-γ. On the other hand, IFN-γ production was highest when adherent cells from SSG-treated mice and non-adherent cells from control mice were co-cultured. We could not identify the adherent cells which were contributing to this phenomenon, although it was likely that they mainly contained antigen-presenting cells such as macrophages and dendritic cells. This fact at least indicates, however, that adherent cells from SSG-treated mice have the capability of preferentially activating the Th1 population. Administration of GRN was less effective than administration of SSG, which also enhanced the percentage of CD4+ cells producing IFN-γ as well as IFN-γ production in supernatants of mixed cultures of adherent or non-adherent cells from GRN-treated mice. However, Th2 parameters such as intracellular IL-4 production were not detected by flow cytometry using clone BVD4-1D11 as a detection antibody (data not shown).

IL-12 has been shown to be a primary determinant of Th1 cell differentiation. Moreover, since IL-12 has been shown to prevent development of the type 2 immune response in several murine models of immune activation and infection, we sought to determine its role in the preferential development of Th1 cells caused by administration of SSG. The level of IL-12 p40 mRNA of spleen cells from SSG-administered mice was elevated relative to the level in control mice. In addition, neutralization of IL-12 in vitro completely inhibited the promotion of IFN-γ production by SSG. This result indicates that SSG can significantly activate splenocytes to produce IL-12 protein. In another report, we demonstrated that SSG was most effective among the tested gel-forming β-glucans in inducing generation of cytotoxic T lymphocytes in mixed lymphocyte reactions. SSG significantly induced the mRNA expression of IL-12 and IL-15 in the previous study. We observe similar results here indicating that SSG is able to induce IL-12 production in splenocytes which were sensitized with soluble antigen. Physicochemical characteristics suggest that SSG possesses a large molecular mass and single helical conformation, which are likely to be important in
activating leukocytes to induce cytokine expression. There are some reports suggesting multiple recognition molecules specific for β-glucans, such as CD11b, lactosylceramide, and an unidentified 20kDa protein subunit on monocytes. Although it is unclear how β-glucans activate macrophages at the molecular level, long (1→3)-β-glucosyl chains may be advantageous for recognition by several recognition units. The strong activity of SSG may result from its higher molecular weight compared to those of other soluble (1→3)-β-glucans.

In conclusion, this study has shown that β-glucans have different effects on induction of Th1- or Th2-dependent antibody subclasses, and that SSG especially promotes development of Th1 cells by inducing an IL-12 pathway. It is thought that polarized Th1 and Th2 phenotypes play a central role in the course of autoimmune and infectious diseases. Taken together, the present data suggest that β-glucans should be selected and used properly for modulation of immune responses.

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