Changes in Immune Mediators in Mouse Lung Produced by Administration of Soluble (1→3)-β-D-Glucan

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In this study, we showed that systemic administration of SSG, a highly branched soluble (1→3)-β-D-glucan obtained from Sclerotinia sclerotiorum, induced immunological changes in the alveolar space of mice in vivo, assessed by analyzing some immune mediators in bronchoalveolar lavage (BAL) fluid. A single i.v. administration of SSG (250 μg/mouse) induced a rapid but transient leakage of the serum components, IgG and fibroinectin, into the alveolar space. This was apparent 12 h post-administration and reached a peak on day 2. Similar kinetic changes were found for lysosomal enzyme activities and interferon γ (IFNγ) concentrations in BAL which are markers of activated alveolar macrophages (AMs) or pulmonary T cells. BAL prepared from SSG-treated mice stimulated lysosomal enzyme release from AMs in vitro. However, SSG did not provoke the chronic accumulation of serum proteins in alveoli and did not induce the release of detectable amounts of nitric oxide and the inflammatory cytokines, IL-1, IL-6 and TNFα, into BAL. However, their mRNAs were detected in lung tissue using the reverse-transcriptase polymerase chain reaction (RT-PCR) technique. Similar results were observed for multiple i.v. administration (250 μg, once a day for 10 consecutive days), and there were little differences between single and multiple administration.

In summary, systemic administration of SSG induces immune responses, including activation of AMs and lymphocytes, but does not provoke chronic inflammation in the alveolar space when administered either as single or multiple doses. This finding is very important for the clinical application of SSG in Immunocompromised hosts as a biological response modifier (BRM) without toxic-side effects on lung tissue.

Keywords (1→3)-β-D-glucan; pulmonary immune response; alveolar macrophage; interferon γ; fibronectin

Recently, biological response modifiers (BRMs) have been applied in different combinations to compensate for the side-effects of various cancer therapies. In particular, BRMs which can increase the immune response are very useful because micrometastasis of cancer cells which have escaped surgery, chemotherapy and irradiation occurs more often in the lungs than in any other organs. It has also been reported that pulmonary infectious disease as a side-effect of chemotherapy and irradiation, is the major fatal complication in cancer patients. In spite of this, there have been few reports concerning the effects of BRMs on pulmonary immune responses on any detailed observations of kinetic changes involving immune mediators in the lungs.

(1→3)-β-D-Glucans with 1,3 and/or 1,6 linkages are ubiquitous in nature, as the major structural components of yeasts and fungi, and are well known to possess immunomodulating effects. However, the biological activities of (1→3)-β-D-glucans are not completely understood since recent studies by our research group have suggested that the biological effects of (1→3)-β-D-glucans are significantly affected by their chemical properties, such as side chain, molecular weight and ultrastructure. In addition, no detailed investigation of their modulating effects on the pulmonary immune system has been carried out. We have been studying the immunomodulating and antitumor activities of a soluble, highly branched (1→3)-β-D-glucan, SSG, obtained from a liquid-cultured filtrate of the fungus Sclerotinia sclerotiorum IFO 9395 belonging to Ascomycota. The physicochemical characteristics of SSG are as follows: (1) it branches at every other main chain glucosyl unit at position C-6, (2) it has an average molecular weight of > 5 x 10^6, and (3) its viscosity is higher than that of other (1→3)-β-D-glucans. SSG exhibits potent immunomodulating and antitumor activities when administered systemically, intravenously (i.v.), in murine models.

Alveolar macrophages (AMs) are a major cell type found in the bronchoalveolar space and they play an important role in preventing cancer metastasis and microbial infection in lung tissue. We have demonstrated that administration of SSG enhances several AM functions in vivo and inhibits the pulmonary metastasis of tumor cells mediated by AM activation in mice. These data indicate that SSG effectively increases the pulmonary immune responses and investigations of kinetic changes in the immune mediators in the lungs of mice given SSG would be of great value in increasing our understanding of the potential applications of this compound and in clarifying the in vivo mechanism by which AM is activated.

In this study, we have analysed immune mediators such as IgG, fibronectin, lysosomal enzymes, nitric oxide and some cytokines (interferon γ (IFNγ), interleukin 1α (IL-1α), IL-6 and tumor necrosis factor α (TNFα)) in bronchoalveolar lavage (BAL) fluid from CDF1 mice given i.v. SSG as either single or multiple doses. We also investigated the effect of BAL prepared from SSG-treated mice on AM stimulation in vitro.

MATERIALS AND METHODS

Animals Male CDF1 (BALB/c x DBA/2) mice bred under specific pathogen free conditions were purchased from Japan SLC, Inc. (Shizuoka) and used at 6 weeks of age.

Preparation of SSG SSG is composed of 100% carbohydrate and contains no protein and was prepared as previously described. Endotoxin contamination of this
preparation was less than 0.00001 % (wt/wt), as determined by Endospecy® (Endotoxin Specific Test; Seikagaku Kogyo Co., Tokyo).

BAL SSG was dissolved in physiological saline, sterilized by autoclaving and then aliquots (0.2 ml/mouse) of the solution were administered i.v. (via the tail vein) to CDF1 mice. A few days after SSG administration, BAL was collected from these mice and stored at -20°C until analysis. A method for the preparation of BAL has been described elsewhere. Mice were anesthetized by i.p. injection of 150 mg/kg sodium pentobarbital and exsanguinated by cutting the artery renalis. The thoracic cavity was opened and the lung removed together with the trachea. The whole lungs were then lavaged with Hank’s balanced salt solution (HBSS; Nissui Seiyaku Co., Tokyo) via a tracheal cannula. The bronchoalveolar fluid was alternatively flushed in and out of the trachea before finally transferring it to the syringe. A total of 1.0 ml of lavage fluid per mouse was used. Cells and cell debris were pelleted by centrifugation and the resulting supernatants were used as BAL. For the in vitro AM stimulation assay, HBSS was replaced by RPMI-1640 medium (Nissui).

AMs Alveolar cells were harvested from CDF1 mice by bronchial lavage using Ca²⁺ and Mg²⁺-free phosphate-buffered saline (PBS) containing 0.05% EDTA as described previously. At least 97% of these cells were AMs as judged by examining cell smears using the Diff-Quik Stain kit (Kokusai Shiyaku Co., Hyogo, Japan) or non-specific esterase stain. AMs (2 x 10⁵/well) were incubated for 2 h at 37°C in a CO₂ incubator using a 96-well flat-bottomed tissue culture plate and washed twice to remove any non-adherent cells.

Measurement of Protein Concentration The concentration of total protein in BAL was measured using BCA protein assay reagent (Pierce Co., Rockford, IL) with bovine serum albumin as a standard.

Measurement of IgG Immunoglobulin G (IgG) in BAL was quantitated by a double-sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, a 96-well plate was coated with goat anti-mouse IgG serum (Miles-Yeda Ltd., Israel) in a bicarbonate buffer (pH 9.6). Uncoupled binding sites in the wells were blocked with PBS containing 0.25% bovine serum albumin and 0.05% Tween 20 (PBBSPT). Wells contained 50 µl of sample and incubation was carried out for 40 min at 37°C, followed by exposure to peroxidase-labeled goat anti-mouse IgG serum (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). The plate was developed using peroxidase substrate (TMB microwell peroxidase substrate system; Kirkegaard & Perry), and aliquots of purified mouse IgG (Zymed Laboratories, Inc., San Francisco, CA) were used to construct a standard curve.

Measurement of Fibronectin Fibronectin in BAL was quantitated by ELISA. A 96-well plate was coated with samples diluted in a bicarbonate buffer. After blocking, incubation was carried out with rabbit anti-mouse fibronectin serum (UCB-Bioproducts S.A., Bergium), followed by development using peroxidase-labeled goat anti rabbit IgG, Fc fragment (Organon Teknika, Co., West Chester, PA) and peroxidase substrate. Aliquots of purified mouse fibronectin (Chemicon International, Inc., Temecula, CA) were used to construct a standard curve and the data were expressed as a percentage with reference to control BAL.

Measurement of Lysosomal Enzymes Lysosomal enzyme activities in BAL and AM culture supernatants were assayed by a method described in a previous paper using p-nitrophenyl phosphate or p-nitrophenyl-β-D-glucuronide (Sigma Chemical Co., St. Louis, MO) as a substrate for acid phosphatase or β-D-glucuronidase, respectively. Enzyme activity was expressed as the release of p-nitrophenol measured with reference to a standard curve.

Measurement of Nitric Oxide Nitric oxide (NO⁻) concentrations in BAL and AM culture supernatants were measured by the Griess reaction using a microassay described previously. Briefly, an equal volume of Griess reagent was incubated with samples for 10 min at room temperature and then the absorbance was measured at 550 nm using a 630 nm reference filter. NO⁻ concentrations were determined using NaN₃ as a standard.

Measurement of Cytokines IFNγ and IL-1α were quantitated by a double sandwich ELISA technique as described previously. Briefly, a 96-well plate was coated with hamster anti-rMu IFNγ or anti-rMu IL-1α MAb (Genzyme Co., Boston, MA) in a bicarbonate buffer. After blocking with PBBSPT, 50 µl samples were added to the wells and then exposed to rabbit polyclonal anti-rMu IFNγ (a gift from Daiichi Pharmaceutical Co., Tokyo) or anti-rMu IL-1α serum (Genzyme). The plate was developed using peroxidase-labeled anti rabbit IgG, Fc fragment and peroxidase substrate. Samples were analysed in triplicate and aliquots of rMu IFNγ or rMu IL-1α (Genzyme) were used to construct a standard curve.

IL-6 activity was determined by colorimetric assay using an IL-6-dependent murine hybridoma cell line, MH60. BSF2 (a gift from Sapporo Breweries Ltd., Shizuoka), as described elsewhere using standard rMu IL-6 (R & D Systems Co., Minneapolis, MN).

TNFα activity was measured by quantitating cytolytic activity against on L929 target cell line as described.

Determination of Cytokine mRNA from Lungs by a Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Technique Total cellular RNA was isolated from the lungs of SSG-treated mice using the acid guanidium thiocyanate/phenol/chloroform extraction method. RNA was reverse-transcribed into cDNA by the method described in a previous paper. An aliquot of cDNA was mixed with primer, and 25 cycles (for β-actin) or 30 cycles (for others) of PCR were performed to amplify the DNA. The resulting DNA fragment was detected by agarose gel electrophoresis after staining with ethidium bromide.

Statistics The statistical analysis of all the experiments as carried out using Student's t-test. A value of p < 0.05 was considered statistically significant.

RESULTS

Changes in Protein Concentration in BAL It has often been reported that some serum proteins leak into the alveolar space when an immune response occurs in the lung and it is likely that these proteins participate in the
immune response by modulating the effector activities of AMs. To determine whether serum proteins leaked into the alveolar space following SSG administration, we monitored the kinetic changes in protein concentration in BAL from SSG-treated mice. SSG was administered i.v. to CDF$_1$ mice at a dose of 250 µg, the optimum dose for activation of AMs in vivo, on day 0, and BAL was prepared several times from these mice until day 8. When multiple administrations were given, SSG (250 µg/mouse) was administered i.v. on days −9−0 (10 consecutive days), once daily, and BAL was prepared on day 1.

As shown in Fig. 1 A, a single administration of SSG induced a marked influx of serum protein into the alveolar space from 12h post-administration reaching a peak on day 2 (152.9 ±10.3 µg/ml), followed by a rapid decline by day 4. Multiple SSG administration also increased the protein concentration in BAL on day 1 (122.0 ±14.4 µg/ml).

Kinetic behavior similar to that exhibited by total protein was seen for two serum components, IgG and fibronectin. Following a single administration, the level of IgG increased to reach a peak on day 2 post-administration and then declined by day 4 (Fig. 1 B). The concentration of fibronectin was also elevated, starting from day 1 post-administration, and then declined after reaching a peak on day 2 to reach control levels on day 4 (Fig. 1 C). Following multiple administration, the IgG level in BAL increased, but the fibronectin level did not changed (Fig. 1 B and C).

Changes in Lysosomal Enzyme Activity and Nitric Oxide Production in BAL As markers of AM activation, we measured lysosomal enzyme activities and nitric oxide concentrations in BAL.

A single SSG administration increased the activity of two species of lysosomal enzymes, acid phosphatase and β-D-glucuronidase, but very little difference was observed.
in their kinetics. Acid phosphatase activity was increased from 12 h post-administration. The highest activity was shown on day 2 and it decreased to the control value by day 4. The activity was found again on day 8 (Fig. 1D). In contrast, the \( \beta \)-\( \delta \)-glucuronidase activity was increased from day 1 post-administration, reaching a peak on day 2. Thereafter, it decreased gradually to the control value (Fig. 1E). Multiple SSG administration also produced a slight increase in acid phosphatase activity in BAL (Fig. 1D), but there was no change in \( \beta \)-\( \delta \)-glucuronidase activity (Fig. 1E).

The nitric oxide concentration in BAL did not change following either single or multiple SSG administration (Fig. 1F).

Changes in Cytokine Levels in BAL Levels of IFN\( \gamma \) were determined using a double sandwich ELISA technique. Following single SSG administration, the cytokine increased slowly starting at 12 h post-administration and reaching a peak on day 2 (3.9 \pm 0.1 U/ml). Thereafter, it declined somewhat and remained significantly elevated until day 8 (2.8 \pm 0.1 U/ml) (Fig. 2). Multiple SSG administration also increased the IFN\( \gamma \) level on day 1.

However, only trace amounts (<2.0 pg/ml) of IL-1\( \alpha \) were detected during the course of both single and multiple SSG administrations (data not shown). We also failed to detect IL-6 (<0.5 pg/ml) and TNF\( \alpha \) (<100.0 pg/ml) using cell lines MH60.BSF2 and L929 which were responsive to IL-6 and TNF\( \alpha \) respectively (data not shown). Control experiments with authentic standards added to BAL samples indicated that failure to demonstrate cytokine activity in the bioassays was not due to recovery problems or errors in the analytical methods (data not shown).

Changes of Cytokine mRNA Expression in Lung Tissue The mRNA expression for IL-1\( \alpha \), IL-1\( \beta \), IL-6 and TNF\( \alpha \) by lung tissues from SSG-treated mice was examined by RT-PCR. Figure 3 shows the ethidium bromide-stained cytokine PCR products in the agarose gel. Spontaneous mRNA expressions were detected for IL-1\( \beta \) and TNF\( \alpha \) but not for IL-1\( \alpha \) and IL-6 under our experimental conditions. Following a single SSG administration, IL-1\( \beta \) mRNA was strongly induced within 3 h post-administration with a peak expression at 6 h and then it was declined to the control value at 24 h. IL-6 mRNA was first detected 3 h post-administration and persisted for at least 6 h. TNF\( \alpha \) mRNA was also elevated slightly with peak expression at 3 to 6 h, but no signal for IL-1\( \alpha \) was observed.

Multiple SSG administration increased mRNA levels for IL-1\( \beta \) and IL-6 but not for IL-1\( \alpha \) and TNF\( \alpha \), measured 6 h after the last administration.

Effects of BAL on AM Stimulation \textit{in Vitro} We investigated the effect of BAL prepared from SSG-treated mice on AM stimulation \textit{in vitro}. As shown in Fig. 4, BAL harvested from normal mice produced a slight increase in lysosomal acid phosphatase release from AMs, compared with medium alone. A more powerful effect was observed with BAL prepared from mice after a single SSG administration, reaching a peak at 12 h (about two-fold that seen with medium alone), and then falling to the control value at day 2. BAL obtained from mice after multiple SSG injections also exhibited a slight enhancement in enzyme release from AMs.
In contrast, nitric oxide release from AMs was unchanged in our experiments (data not shown).

DISCUSSION

In this study, we have shown by analysis of immune mediators in BAL that i.v. administration of SSG, a highly branched (1→3)-β-d-glucan obtained from *S. sclerotiorum*, induces immunological changes in the alveolar space. Our study presents the first comprehensive analysis of the mediators of pulmonary immune responses in an *in vivo* murine model following treatment with (1→3)-β-d-glucan.

Other workers have reported that some serum proteins leak into the alveolar space following immune responses in lung tissue. As shown in Fig. 1A, a single SSG administration induced a rapid, temporary influx of serum proteins into the alveolar space from 12 h post-administration and reached a peak on day 2. We previously demonstrated using isotope-labeled material, that i.v. administered (1→3)-β-d-glucan was immediately distributed to lung tissue (within 1 h) and in a recent study, we showed that SSG induced acute phase responses. These findings suggest that i.v. SSG is immediately distributed to lung tissue to produce temporary inflammatory-like responses in alveoli. It is probable that these inflammatory-like responses participate in the pulmonary immune responses by modulating the effector activities of AMs. In our preliminary experiments, a single i.v. administration of SSG increased several AM functions *in vivo*, and the highest AM activity appeared 12 h to 1 d post-administration (manuscript submitted). We also showed in this study that the lysosomal enzyme activities in BAL, which are markers of AM activation, are increased following SSG treatment 12 h to 2 d post-injection (Fig. 1D and E). Furthermore, BAL harvested from SSG-treated mice stimulated the release of acid phosphatase from AMs *in vitro* with a peak at 12 h after a single administration (Fig. 4). Taken together, this suggests that *in vivo* AM activation by SSG might be mediated by stimulation produced by serum components which leaked into the alveolar space in the early phase.

Recent evidence has documented that continuous accumulation of fibronectin in the alveoli is correlated with the development of chronic fibrosing lung diseases. Mulligan *et al.* noted that the intrapulmonary deposition of IgG immune complexes led to acute lung injury with damage to both vascular endothelial and alveolar epithelial cells. It has also often been reported that several inflammatory mediators secreted from activated AMs, such as IL-1, IL-6, TNFα and fibronectin, contribute to further pulmonary inflammation. The administration of insoluble micro-particulate (1→3)-β-d-glucans is known to be associated with such systemic chronic inflammation, including pulmonary granulomatosis. We have demonstrated in this study that the i.v. administration of SSG results in a temporary influx without causing chronic accumulation of IgG and fibronectin in the alveolar space (Fig. 1B and C) and it did not induce the release of detectable amounts of the inflammatory cytokines, IL-1, IL-6 and TNFα, from activated AMs (data not shown), although they were detected by mRNA levels in lung tissues using RT-PCR (Fig. 3). Furthermore, SSG produced no increase in the concentration of nitric oxide, one of the cell injury factors, in BAL (Fig. 1F). These results imply that the systemic administration of SSG induces only immune responses without provoking chronic inflammation in the alveolar space.

It has been reported that IFNγ is secreted from activated pulmonary T cells when immunological events occur in the alveolar space and plays a central role in controlling the pulmonary immune system. In this study, we demonstrated that a single i.v. administration of SSG increased IFNγ production in BAL, reaching a peak at 2 d (Fig. 2). This result indicates that SSG induces T cell activation in lung tissue. In immunocompromised hosts, including AIDS patients, T cell function is impaired in the alveolar space and subsequently fungal infections frequently appear in the lung tissue. Prior and Haslam reported that decreased IFNγ release from pulmonary T cells might be a potentiating factor in the pathogenesis of fibrosing lung diseases. Furthermore, IFNγ is believed to stimulate AMs which may have an important role as effector cells in the pulmonary immune system. Considering these facts, SSG treatment could be very useful for controlling the pulmonary immune system of immunocompromised patients mediated by increased expression of IFNγ in the alveoli.

We previously reported that multiple SSG administration was required to produce the most effective antitumor activity against solid tumors or pulmonary metastatic tumor cells in mice. In this study, we demonstrated that multiple i.v. administrations (once daily for 10 consecutive days) of SSG induce pulmonary immune responses on the first day after the last administration as in the case of a single administration. However, for some mediators there was very little difference between single and multiple administration. Multiple SSG injections caused an influx of IgG into the alveolar space, while the fibronectin concentration in BAL remained unchanged (Fig. 1B and C). Lysosomal acid phosphatase activity in BAL was also increased slightly, but β-d-glucuronidase activity was unaffected (Fig. 1D and E). Furthermore, although multiple SSG injections induced the mRNA expression of IL-1/β and IL-6 in lung tissue (Fig. 3), we failed to detect the activities of inflammatory cytokines in BAL (data not shown) and, as shown in Fig. 2, there was also a slight increase in IFNγ production in BAL. These data suggest that multiple SSG administration causes immunological events in the alveolar space by means of a different mechanism from that which operates after a single administration.

In conclusion, SSG administered i.v. increases immune responses, including activation of macrophages and lymphocytes, but without provoking chronic inflammation in the alveolar space by both single and multiple administration. This observation is very important for the clinical application of SSG in immunocompromised hosts as a BRM without toxic side-effects on lung tissue.

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