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Different role of serum components and cytokines on alveolar macrophage activation by soluble fungal (1 → 3)-β-D-glucan

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Abstract

In this study, we investigated the mechanism of alveolar macrophage activation by systemic administration of SSG, a soluble highly branched (1 → 3)-β-D-glucan obtained from a fungus *Sclerotinia sclerotiorum* IFO 9395. Multiple i.v. administration (10 mg/kg; once daily for 10 consecutive days) of SSG enhanced some functions of alveolar macrophages, such as lysosomal enzyme activity and nitric oxide secretion, on day 1 after the last administration, and it also elevated the concentrations of serum protein, interferon γ and SSG in bronchoalveolar lavage fluid on the same day. On the in vitro assay system, stimulation by SSG alone (500 μg/ml) slightly augmented the lysosomal enzyme activity of alveolar macrophages, but it had no effect on nitric oxide production of cells. Stimulation by serum (1 or 10% mouse serum) or serum components, such as fibronectin (25 μg/ml) and albumin (500 μg/ml), alone strongly augmented only the lysosomal enzyme activity of alveolar macrophages, but it had no effect on nitric oxide secretion from cells, and no synergism or additive-like effect was observed between serum components and SSG. In contrast, stimulation by crude lymphokine (5%) or recombinant murine interferon γ (100 U/ml) alone did not induce augmentation of lysosomal enzyme activity and nitric oxide production of alveolar macrophages in vitro, but when cells were incubated together with crude lymphokine or recombinant murine interferon γ and SSG (500 μg/ml), a significant combined effect was observed on both functions of alveolar macrophages. In addition, pretreatment of crude lymphokine or recombinant murine interferon γ enhanced the expression of β-D-glucan specific binding sites on the alveolar macrophage surface in vitro though pretreatment by serum components had no effect. Based on these findings, the enhancement of alveolar macrophage functions by systemic administration of SSG appears to be mediated, at least in part, by both the simple effect of serum components including fibronectin and albumin leaked from pulmonary peripheral blood into the alveoli and the synergistic effect between lymphokines released from activated pulmonary T cells and SSG itself entering the alveoli after SSG injection *via* the priming effect of lymphokines which enhances the expression of β-D-glucan specific binding sites on the alveolar macrophage surface.

Keywords: β-D-glucan; Alveolar macrophage; Serum component; Interferon γ; Nitric oxide (NO); β-D-glucan receptor

1. Introduction

Recently, biological response modifiers have been applied in different combinations to compensate for the adverse reaction to other cancer therapies. In particular, biological response modifiers which can increase the pulmonary immune system are very useful because micrometastasis of cancer cells which have escaped surgery, chemotherapy and irradiation occurs more often in the lung than in any other organs (Poste and Fidler, 1980). It has also been reported that pulmonary infectious diseases are the major fatal complication in cancer patients as an adverse reaction of chemotherapy and irradiation (Meitner et al., 1990). Alveolar macrophages play an important role in controlling the pulmonary immune system by their inhibitory actions against metastatic tumor cells and infectious microorganisms (Nickerson and Jakab, 1990). Alveolar macrophage activation by biological response modifiers

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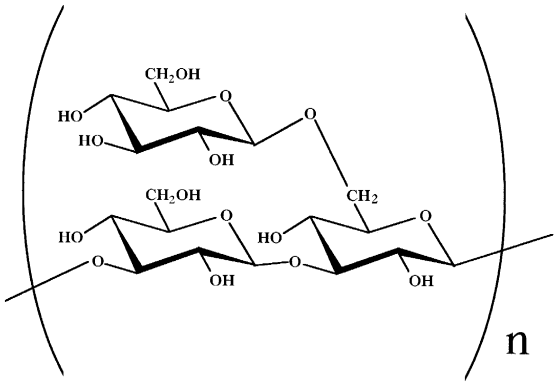

Fig. 1. Primary structure of SSG.

is considered to be very important for their antimetastatic and antimicrobial effects in the lung.

β-D-Glucans with 1,3 and/or 1,6 linkages are ubiquitous in nature as major structural components of yeasts and fungi (Mullins, 1990) and are known to possess antitumor activity. We have been studying the antitumor activities of a soluble highly branched (1 → 3)-β-D-glucan, SSG, obtained from liquid-cultured filtrate of a fungus, *Sclerotinia sclerotiorum* IFO 9395 belonging to Ascomycotina. Characteristic chemical and physical properties of SSG are as follows: (1) SSG branches at every other main chain glucosyl unit at position C-6 (see Fig. 1) (Ohno et al., 1986); (2) SSG has an average molecular weight of $> 5 \times 10^6$; and (3) the viscosity of SSG is high in comparison with that of other β-D-glucans (Ohno and Yadomae, 1987). We also mentioned previously that the administration of SSG enhanced some functions of alveolar macrophages including cytolytic activity against cancer and microbial cells in vivo and inhibited experimental pulmonary metastasis of tumor cells in murine models (Sakurai et al., 1991, 1992, 1995; Suzuki et al., 1991). However, it is not clear why this β-D-glucan augmented the functions of alveolar macrophages in vivo, and little is known about the mechanism of alveolar macrophage activation by other kinds of biological response modifiers.

We previously demonstrated that intravenously (i.v.) administered SSG elevated the concentrations of serum protein and interferon γ in bronchoalveolar lavage fluid in a murine model, and bronchoalveolar lavage fluid prepared from SSG treated mice enhanced the function of alveolar macrophages in vitro (Sakurai et al., 1994). Considering these facts, it is likely that alveolar macrophage activation in vivo by SSG treatment was mediated by serum components leaked from pulmonary peripheral blood and lymphokines secreted from pulmonary-activated T cells after SSG administration. In this study, we examined alveolar macrophage activation by SSG in vitro and confirmed that serum components and lymphokines, such as interferon γ, which leaked into the alveolar space after the SSG injection, play key roles in alveolar macrophage activation via different mechanisms.

2. Materials and methods

2.1. Mice

Specific-pathogen-free male C57BL/6, CDF_1 (BALB/c × DBA/2) and ICR mice were purchased from Japan SLC (Shizuoka). These mice were used at 4–8 weeks of age and were bred under specific pathogen free conditions.

2.2. Reagents

The preparation method of SSG has been described previously (Ohno et al., 1986). SSG contains no protein and 100% carbohydrate, and lipopolysaccharide contamination of this preparation was less than 0.00001% (wt/wt) determined by endotoxin specific limulus test. Mouse fibronectin and mouse serum albumin were obtained from Chemicon International (Temecula, CA) or Inter-cell technologies (Hopewell, NJ), respectively. The purity of these proteins were $> 95\%$ determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the lipopolysaccharide contaminations were less than 0.00005% (wt/wt). Recombinant murine interferon γ was purchased from Genzyme (Boston, MA) and lipopolysaccharide was not detected.

2.3. Alveolar macrophages

Mice were anesthetized by intraperitoneal (i.p.) injection of 150 mg/kg sodium pentobarbital and exsanguinated by cutting the arteria renalis. The thoracic cavity was opened and the lung removed together with trachea. Alveolar cells were harvested by bronchial lavage using Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.05% ethylenediamine tetraacetate as described previously (Akagawa and Tokunaga, 1985). A total of 6.0 ml of lavage fluid per mouse was used. Cells were collected by centrifugation and resuspended in ice-cold RPMI-1640 medium (Nissui). At least 97% of these cells were macrophages as judged by using cell smears of Diff-Quik Stain kit (Kokusai Shiyaku, Hyogo) or nonspecific esterase stain. Cells were incubated for 2 h at 37°C in a CO_2 incubator and were washed twice with warmed RPMI-1640 medium to remove any nonadherent cells.

2.4. Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid was prepared from anesthetized mice by bronchial lavage using Hank's balanced salt solution (HBSS; Nissui Seiyaku, Tokyo) via a tracheal cannula. Bronchoalveolar lavage fluid was alternatively flushed in out of the trachea before finally transferring into 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 bronchoalveolar lavage fluid.

2.5. Crude lymphokine

Crude lymphokine was prepared by the method described in previous paper with slight modification (Badger et al., 1974; Fidler et al., 1976; Sone and Fidler, 1980; Akagawa and Tokunaga, 1985). Briefly, 1×10^7/ml spleen cells obtained from ICR mice were cultured in RPMI-1640 medium containing 2.5 μg/ml of concanavalin A (Sigma Chemical, St. Louis, MO) and 10% of heat-inactivated fetal calf serum (Boehringer Mannheim, Mannheim, lipopolysaccharide was not detected (10 pg/ml)) for 48 h at 37°C. After incubation, culture supernatants were harvested by centrifugation and passed Sephadex G-10 column (Pharmacia LKB Biotechnology AB, Uppsala), then filtrated through a 0.20 μm filter and stored at -80°C.

2.6. Measurement of interferon γ in crude lymphokine

Interferon γ in crude lymphokine was quantitated by a double sandwich enzyme-linked immunosorbent assay (ELISA) technique. A 96-well plate was coated with hamster anti-recombinant murine interferon γ monoclonal antibody (Genzyme) in a biocarbonate 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. Wells contained 50 μl of sample and incubation was carried out for 40 min at 37°, followed by exposure to rabbit polyclonal anti-recombinant murine interferon γ (a gift from Daiichi Pharmaceutical, Tokyo). The plate was developed using peroxidase-labeled goat anti rabbit IgG, Fc fragment (Organon Teknika, West Chester, PA) and peroxidase substrate (TMB microwell peroxidase substrate system; Kirkegaad and Perry Laboratories, Gaithersburg, MD). Samples were analyzed in triplicate and aliquats of recombinant murine interferon γ (Genzyme) was used to construct a standard curve.

2.7. Administration of samples

SSG was dissolved in physiological saline, sterilized by autoclaving, and aliquots (0.2 ml/mouse) of the solution were administered intravenously (i.v.) to mice via the tail vein.

2.8. Measurement of β-D-glucan concentration in bronchoalveolar lavage fluid

The concentration of β-D-glucan in bronchoalveolar lavage fluid was measured using β-D-glucan specific reagent, Gluspecy® (Seikagaku Kogyo, Tokyo).

2.9. Assay for proliferative response of alveolar macrophages

Alveolar macrophage monolayers on 96-well culture plate (2×10^5 alveolar macrophages/well) were incubated for 30 h at 37° in RPMI-1640 medium containing 10% of heat-inactivated fetal calf serum, and cultures were pulse labeled with ^{3}H-thymidine (0.5 μCi/well) for the final 1 h of incubation period. At the end of the period, the cells were harvested and the radio activity of the cells was measured in a liquid scintillation counter.

2.10. Assay for cellular lysosomal enzyme activity of alveolar macrophages

Cellular lysosomal enzyme (acid phosphatase) activities in alveolar macrophage culture supernatants or lysates (cells were solubilized by 0.1% Triton X-100) were assayed by a method described elsewhere using *p*-nitrophenyl phosphate (Sigma) as a substrate (Sakurai et al., 1992). Enzyme activity was expressed as % control determined by the release of *p*-nitrophenol.

2.11. Measurement of nitric oxide production of alveolar macrophages

Culture supernatants of alveolar macrophages were assayed for NO_2^- by the Griess reaction according to described microassay (Corradin and Mauel, 1991). Briefly, an equal volume of Griess reagent was incubated with alveolar macrophage supernatants for 10 min at room temperature and absorbance was measured at 550 nm using a 630 nm reference filter. NO_2^- concentration was determined using $NaNO_2$ as a standard.

2.12. Measurement of spreading ability of alveolar macrophages

Alveolar macrophage monolayers on 96-well culture plates were incubated for 48 h at 37°C. After incubation, the number of spreading alveolar macrophages exhibiting pseudopods was counted, and spreading ability were expressed as percentage of spreading alveolar macrophages in total 300 alveolar macrophages.

2.13. Measurement of binding of β-glucan on the alveolar macrophage surface

Fluorescein conjugated particulate β-glucan was prepared from zymosan A (Sigma) using fluorescein isothiocyanate (FITC) by a method described elsewhere (De Belder and Granath, 1973). The binding of FITC-zymosan on the alveolar macrophage surface was measured with fluorescence spectrophotometer (microplate reader MTP-32, Corona Electric, Tokyo) as described in previous our paper (Suzuki et al., 1988b). Alveolar macrophage monolayer (1×10^5) in 96-well tissue culture plate was prestimulated with samples in RPMI-medium for 20 h at 37°C, and were further incubated with FITC-zymosan (1×10^7/well) in HBSS in the presence of 0.1% NaN_3 for 1 h at 37°C. After incubation, alveolar macrophages were

washed three times by warmed PBS and solubilized by 100 μl of 50 mM sodium cholate, and fluorescence intensity in wells was measured with an excitation wave length of 490 nm and an emission wave length of 530 nm. The binding ratio of FITC-zymosan on alveolar macrophage surface was calculated using the following formula: Binding (%) = fluorescein intensity of alveolar macrophage solution/fluorescein intensity of total FITC-zymosan × 100.

2.14. Statistics

Statistical evaluations were performed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of administration of SSG on alveolar macrophage functions in vivo

Table 1 shows the effect of i.v. administration of SSG on some alveolar macrophage functions. SSG (10 mg/kg) was administered i.v. to mice via the tail vein on days −9–0 (10 consecutive days), and alveolar macrophage functions were assessed on day 1. As a result, SSG enhanced some alveolar macrophage functions, such as proliferative response, lysosomal enzyme activity, nitric oxide production and spreading ability, but it did not influence the numbers of alveolar macrophages (Table 1).

3.2. Effect of administration of SSG on β-D-glucan concentration in bronchoalveolar lavage fluid

Fig. 2 shows the change in concentration of β-D-glucan leaking into the alveolar space after i.v. administration of SSG. SSG (10 mg/kg) was administered i.v. to mice via the tail vein on days −9–0 (10 consecutive days), bronchoalveolar lavage fluid was prepared on day 1 and the concentration of β-D-glucan was measured. As a result, SSG administration induced a marked influx of β-D-glucan, SSG, into the alveolar space.

Table 1
Effect of SSG on alveolar macrophage functions in vivo

	Control	SSG
Alveolar macrophage number [a] ($\times 10^5$/mouse)	6.9 ± 0.7	7.4 ± 2.7
Proliferative response (cpm) [b]	441.0 ± 28	934.0 ± 68 [c]
Lysosomal enzyme activity (% control) [b]	100.0 ± 14.9	125.7 ± 4.8 [c]
Nitric oxide production (pmol) [b]	nd [d]	210.2 ± 0.0 [c]
Spreading ability (%) [b]	11.7 ± 1.8	36.0 ± 11.1 [c]

SSG (10 mg/kg) was administered i.v. on days −9–0 (10 consecutive days) to three/group C57BL/6 mice. Alveolar macrophages were prepared from these mice on day 1 and functions were assessed as in Section 2. Proliferative response, Uptake of ^{3}H-TdR; Lysosomal enzyme activity, Acid phosphatase activity in alveolar macrophage lysates; Nitric oxide production and spreading ability of alveolar macrophages were assayed after incubation of cells in RPMI-1640 medium containing 10% fetal calf serum for 48 h at 37°C; Nitric oxide, pmol/1×10^5 cells.

[a] Results are expressed as arithmetic mean ± standard deviation (S.D.) of three mice.

[b] Results are expressed as arithmetic mean ± S.D. of three separated experiments performed in triplicate out of six similar experiments.

[c] $P < 0.01$ comparison with control.

[d] Not detected.

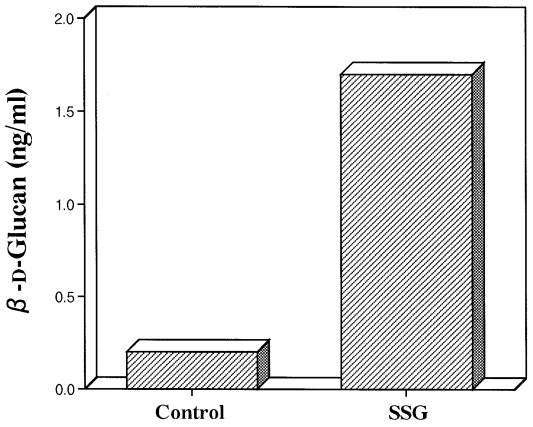

Fig. 2. Effect of administration of SSG on β-D-glucan concentration in bronchoalveolar lavage fluid. SSG (10 mg/kg) was administered i.v. to CDF_1 mice via the tail vein on days −9–0 (10 consecutive days), bronchoalveolar lavage fluid was prepared on day 1 and the concentration of β-D-glucan was measured using β-D-glucan specific reagent, Gluspecy®. Data are expressed as the arithmetic mean of the results of analysis from three mice performed in triplicate, and S.D. was less than 2%.

3.3. Effect of SSG and serum components on alveolar macrophage functions in vitro

Table 2 shows the effect of SSG and serum components on alveolar macrophage functions in vitro. Alveolar macrophages were incubated with samples for 24 h, and lysosomal enzyme activity and nitric oxide concentration in the culture supernatants were assessed.

SSG (500 μg/ml) alone slightly augmented the lysosomal enzyme activity of alveolar macrophages, and mouse serum (1 or 10%) markedly enhanced the activity in a dose dependent manner. However, no synergism or additive-like effect was observed between SSG and serum. Similar results were observed when alveolar macrophages were cultured with heat-inactivated mouse serum and mouse standard complement serum (guinea pig serum) in the presence or absence of SSG (data not shown). Some serum components, such as fibronectin (25 μg/ml) or albumin (500 μg/ml) which are known to be markers of pulmonary immune responses originating from peripheral blood (Watts and Bruce, 1992; Watts et al., 1992) alone did not affect the activity of alveolar macrophages, but when the cells were incubated with fibronectin (25 μg/ml) and albumin (500 μg/ml) simultaneously, the activity was strongly augmented. No synergism or additive-like effect was observed between these serum components and SSG.

SSG, serum or serum components alone did not induce nitric oxide secretion from alveolar macrophages at all,

Table 2
Effect of SSG and serum components on alveolar macrophage functions in vitro

SSG (500 μg/ml)	Serum components	Lysosomal enzyme activity (% control)	Nitric oxide secretion (pmol/1 × 10^5 AMs)
−	−	100.0 ± 10.8	nd [c]
+	−	118.4 ± 9.4 [a]	nd
−	Serum (1%)	207.7 ± 22.1 [b]	nd
−	Serum (10%)	345.0 ± 33.4 [b]	nd
+	Serum (1%)	227.5 ± 3.5 [b]	nd
+	Serum (10%)	365.0 ± 49.5 [b]	nd
−	Fibronectin (25 μg/ml)	109.9 ± 9.2	nd
+	Fibronectin (25 μg/ml)	124.1 ± 88.6	nd
−	Albumin (500 μg/ml)	99.5 ± 24.3	nd
+	Albumin (500 μg/ml)	102.6 ± 17.4	nd
−	Fibronectin (25 μg/ml) + Albumin (500 μg/ml)	236.8 ± 30.6 [b]	nd
+	Fibronectin (25 μg/ml) + Albumin (500 μg/ml)	247.9 ± 91.8 [b]	nd

Alveolar macrophages isolated from ICR mice were incubated with SSG (500 μg/ml) and/or serum components for 24 h, and functions of AMs were assessed as in Section 2. Lysosomal enzyme activity, acid phosphatase activity in alveolar macrophage culture supernatants. Results are expressed as arithmetic mean ± S.D. of three separated experiments performed in triplicate out of six similar experiments.
[a] $P < 0.01$ in comparison with alveolar macrophages incubated with medium alone.
[b] $P < 0.001$ in comparison with alveolar macrophages incubated with medium alone.
[c] Not detected.

and no combined effect was observed between SSG and serum or serum components (Table 2).

Spreading abilities of alveolar macrophages were all about 15–20% (abilities of control alveolar macrophages and alveolar macrophages stimulated by SSG alone were both about 3%) when cells were incubated with serum or serum components in the presence or absence of SSG for 48 h (data not shown).

3.4. Effect of SSG and lymphokines on alveolar macrophage functions in vitro

Monolayers of alveolar macrophages were incubated with lymphokines with or without SSG (500 μg/ml) in the absence of serum for 24 h, and lysosomal enzyme activity and nitric oxide concentration in the culture supernatants were assayed. The spreading ability of alveolar macrophages was also observed after 48 h incubation with samples. As shown in Table 3, crude lymphokine (5%); culture supernatants of concanavalin A stimulated splenocytes, alone did not augment the lysosomal enzyme activity and nitric oxide production of alveolar macrophages at all. However, in the presence of SSG, these functions were significantly augmented. In contrast, the spreading ability of alveolar macrophages was enhanced by crude lymphokine alone, and no combined effect was observed between crude lymphokine and SSG. Concanavalin A (2.5 μg/ml contained in crude lymphokine) alone did not

Table 3
Effect of SSG and lymphokines on alveolar macrophage functions in vitro

SSG (500 μg/ml)	Lymphokines [a]	Anti-interferon γ antibody	Lysosomal enzyme activity (% control)	Nitric oxide secretion (pmol/1 × 10^5 cells)	Spreading ability (%)
−	−	−	100.0 ± 9.8	nd [f]	3.4 ± 1.9
+	−	−	120.0 ± 16.1 [b]	nd	5.9 ± 2.9
−	Crude lymphokine	−	97.3 ± 14.0	nd	22.0 + 8.0 [c]
−	Crude lymphokine	+	−	−	20.5 ± 8.3 [c]
+	Crude lymphokine	−	173.3 ± 29.3 [c,d]	222.8 ± 49.0 [c,d]	31.0 ± 9.6 [c]
+	Crude lymphokine	+	110.8 ± 15.1 [e]	185.0 ± 29.1 [c]	23.7 ± 5.1 [c]
−	Interferon γ	−	85.1 ± 17.9	nd	8.2 ± 4.3 [b]
+	Interferon γ	−	146.0 ± 11.3 [c,d]	126.1 ± 35.7 [c,d]	8.1 ± 1.0 [b]

Alveolar macrophages isolated from ICR mice were incubated with SSG (500 μg/ml) and/or lymphokines for 24 h, and functions of alveolar macrophages were assessed as in Section 2. Lysosomal enzyme activity, acid phosphatase activity in alveolar macrophage culture supernatants. Results are expressed as arithmetic mean ± S.D. of three separated experiments performed in triplicate out of six similar experiments.
[a] In the presence or absence of crude lymphokine (5%) or recombinant murine interferon γ (100 U/ml).
[b] $p < 0.01$ in comparison with cells incubated with medium alone.
[c] $p < 0.001$ in comparison with cells incubated with medium alone.
[d] $p < 0.01$ in comparison with cells incubated with lymphokines in the absence of SSG.
[e] $p < 0.001$ in comparison with cells incubated with SSG and lymphokines in the absence of anti-interferon monoclonal antibody.
[f] Not detected.

affect lysosomal enzyme activity, nitric oxide production and spreading ability of alveolar macrophages at all with or without SSG as a control experiment (data not shown).

Akagawa and Tokunaga (1985) noted that the active molecule as a macrophage activating factor in crude lymphokine was interferon γ, and therefore, crude lymphokine was incubated with hamster anti-recombinant interferon γ monoclonal antibody (IgG; Genzyme). The interferon γ titer of 5% crude lymphokine was 2.6 U/ml as determined by the double sandwich ELISA technique, and 1 μg of monoclonal antibody was able to neutralize 140 U of interferon γ. Crude lymphokine (50 μl) was preincubated with 10 μg of anti-interferon γ monoclonal antibody for 1 h at room temperature, and alveolar macrophages were incubated with either of these preincubated crude lymphokine (5%) with or without SSG (500 μg/ml); alveolar macrophage functions were then assessed. As a result, monoclonal antibody completely neutralized the effect of crude lymphokine on the enzyme release from alveolar macrophages in the presence of SSG, but it had no inhibitory effect on nitric oxide secretion and spreading ability of alveolar macrophages induced by crude lymphokine with or without SSG (Table 3). An isotype control hamster IgG (Genzyme) did not neutralize the effects of crude lymphokine on alveolar macrophage functions at all (data not shown)

Based on these results, we examined the effect of recombinant murine interferon γ (100 U/ml) and SSG (500 μg/ml) on the functions of alveolar macrophages. As a result, interferon γ alone did not influence the lysosomal enzyme release and nitric oxide secretion, although it slightly enhanced the spreading ability of alveolar macrophages. When the cells were incubated with interferon γ and SSG simultaneously, lysosomal enzyme activity and nitric oxide production of alveolar macrophages were markedly augmented, but there was no combined effect on the spreading ability of alveolar macrophages (Table 3).

3.5. Priming effect of serum components and lymphokines on binding of β-D-glucan on alveolar macrophages

Fig. 3 shows the priming effect of serum components or lymphokines on the binding of β-D-glucan on the alveolar macrophage surface. Alveolar macrophage monolayers were preincubated with fibronectin (50 μg/ml), albumin (500 μg/ml), fibronectin plus albumin, crude lymphokine (5%), recombinant murine interferon γ (100 U/ml) or medium alone for 20 h at 37°C, and the binding ratio of FITC-labeled particulate β-D-glucan (FITC-zymosan) on the alveolar macrophage surface was measured using a fluorescence spectrometer. As a result, lymphokines, crude lymphokine and recombinant murine interferon γ, enhanced the binding ratio of FITC-zymosan on the alveolar macrophage surface. Unstimulated alveolar macrophages showed slight binding with FITC-zymosan on their surface (about 20% of added FITC-zymosan), but about twice the binding was observed after pretreatment with lymphokines. It is likely that this increased binding by lymphokines was specific to the β-D-glucan structure because binding of FITC-zymosan was inhibited completely to the control level by the addition of the unlabeled soluble β-D-glucan, SSG (Fig. 3). In contrast, pretreatment of serum component, fibronectin, albumin or fibronectin plus albumin, did

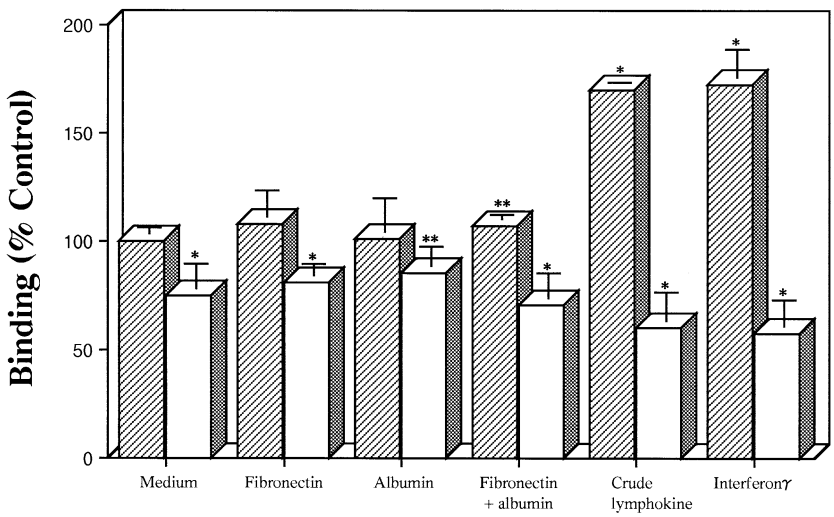

Fig. 3. Priming effect of serum components and lymphokines on binding of β-D-glucan on alveolar macrophages. Alveolar macrophages isolated from ICR mice were preincubated with fibronectin (50 μg/ml), albumin (500 μg/ml), fibronectin (50 μg/ml) plus albumin (500 μg/ml), crude lymphokine (5%), recombinant murine interferon γ (100 U/ml) or medium alone for 20 h at 37°C. After preincubation, alveolar macrophages were washed twice with warmed PBS and incubated with (open bars) or without (checked bars) 500 μg/ml of the unlabeled soluble β-D-glucan, SSG, for 1 h. Cells were then washed twice again and further incubated with FITC-zymosan for 1 h at 37°C. The binding ratio of FITC- zymosan on the alveolar macrophage surface was measured using a fluorescence spectrophotometer as in Section 2. Results are expressed as the arithmetic mean ± S.D. of triplicate dishes. $^{*}P < 0.001$ in comparison with control. $^{**}P < 0.01$.

not influence the binding of β-D-glucan on the alveolar macrophage surface.

4. Discussion

Multiple i.v. administration (once daily for 10 consecutive days) of a soluble highly branched β-D-glucan, SSG, enhanced some functions of alveolar macrophages in vivo on day 1 after the last administration (Table 1), and it also elevated the concentrations of protein, interferon γ (Sakurai et al., 1994) and SSG (Fig. 2) in bronchoalveolar lavage fluid on the same day. It was likely that this protein leaked into the alveoli originated from peripheral blood because it has often been reported that some serum proteins leak into the alveolar space when an immune response occurs in the lung (Watts and Bruce, 1992; Zhou et al., 1992). We previously demonstrated that functions of T cells were enhanced by multiple i.v. injection of SSG at a systemic immune site, the spleen (Suzuki et al., 1988a), and we also showed using isotope-labeled material that i.v. administered soluble β-D-glucan was immediately distributed to lung tissue (Takeyama et al., 1988). In addition, we reported previously that bronchoalveolar lavage fluid harvested from SSG treated mice enhanced the lysosomal enzyme activity of alveolar macrophages in vitro (Sakurai et al., 1994). These data imply that the alveolar macrophage activation in vivo by SSG treatment was mediated, at least in part, by serum components leaked from pulmonary peripheral blood, lymphokines secreted from pulmonary-activated T cells and SSG itself entering into the alveolar space after SSG administration.

As shown in Tables 2 and 3, stimulation by SSG alone (500 μg/ml) slightly augmented only the lysosomal enzyme activity of alveolar macrophages in vitro, but it had no effect on nitric oxide production and the spreading ability of alveolar macrophages. Mouse serum (1 or 10%) alone markedly enhanced the lysosomal enzyme activity of alveolar macrophages; about two to four times the control activities were observed, but it did not induce nitric oxide secretion at all, and no synergism or additive-like effect was expressed between serum and SSG on both functions of alveolar macrophages (Table 2). Similar results were observed for serum components, fibronectin and albumin, which are major markers of pulmonary immune responses (Watts and Bruce, 1992; Watts et al., 1992). Stimulation by these components separately was ineffective on all functions of alveolar macrophages, but when alveolar macrophages were incubated with both components simultaneously, only the lysosomal enzyme activity of alveolar macrophages was enhanced, although nitric oxide secretion was not induced at all, and no synergism or additive-like effect was observed between SSG and these serum components. Taken together, it is likely that serum components leaking into the alveoli from pulmonary peripheral blood due to SSG injection contribute strongly to the augmentation of some alveolar macrophage functions without any combined effect with SSG in vivo. This suggestion was supported by an experiment showing that pretreatment by serum components did not influence the binding of particulate β-D-glucan on the alveolar macrophage surface (Fig. 3).

In contrast, a significant combined effect of SSG and lymphokines appeared on both lysosomal enzyme activity and nitric oxide production of alveolar macrophages (Table 3). Crude lymphokine is very useful as a multiple cytokine source reflecting in vivo T cell activation (Badger et al., 1974; Fidler et al., 1976; Sone and Fidler, 1980; Akagawa and Tokunaga, 1985). Stimulation by crude lymphokine alone enhanced only the spreading ability of alveolar macrophages, but it had no effect on lysosomal enzyme activity and nitric oxide production of alveolar macrophages. However, when alveolar macrophages were incubated together with crude lymphokine and SSG, a strong combined effect was observed on both of these functions of alveolar macrophages. It was suggested that the effect of crude lymphokine on alveolar macrophage activation in the presence of SSG was partly dependent on interferon γ because anti-interferon γ monoclonal antibody completely neutralized the combined effect of crude lymphokine and SSG on the lysosomal enzyme activity of alveolar macrophages, and recombinant murine interferon γ showed a similar effect with crude lymphokine on both lysosomal enzyme activity and nitric oxide production of alveolar macrophages in the presence of SSG (Table 3). However, anti-interferon γ monoclonal antibody had no effect on nitric oxide secretion from alveolar macrophages stimulated together with crude lymphokine and SSG, and crude lymphokine alone induced strong spreading ability of alveolar macrophages although the effect of recombinant murine interferon γ was very weak. These data imply that the active molecule of crude lymphokine which activates the alveolar macrophage functions with SSG is not dependent only on interferon γ but also on other factors which were produced by activated pulmonary T cells. Further experiments are needed to clarify the other active molecules contained in crude lymphokine. However, in our preliminary experiment, interleukin-2 and granulocyte–macrophage-colony stimulating factor, which are macrophage activating factors contained in crude lymphokine, had no activating effect on murine alveolar macrophage functions in vitro (unpublished data).

Pretreatment of alveolar macrophages with lymphokines increased the binding ratio of FITC-labeled particulate β-D-glucan on the alveolar macrophage surface, and the increased binding was returned completely to the control level by the addition of unlabeled soluble β-D-glucan. These results taken together showed that lymphokines enhanced the expression of β-D-glucan binding sites on the alveolar macrophage surface, resulting in augmentation of the alveolar macrophage response to SSG. Szabo et al. (1995) reported that β-glucan specific receptors are pre-

sent on mammalian monocytes. These receptors initiate binding and phagocytosis of particulate yeast β-D-glucans, such as heat-killed *Candida* and zymosan particles, but the binding could be inhibited by soluble β-D-glucan (Kadish et al., 1986; Janusz et al., 1988; Adachi et al., 1993: Szabo et al., 1995). It was possible that the β-D-glucan specific binding sites on the alveolar macrophage surface shown in Fig. 3 might be these β-D-glucan specific receptors.

It has been reported that the response of alveolar macrophages to bacterial products is significantly different from that of other macrophage populations. Akagawa and Tokunaga (1985) noted that mouse resident alveolar macrophages were unresponsive to bacterial lipopolysaccharide but alveolar macrophages became responsive to lipopolysaccharide after treatment with interferon γ. A similar result was obtained for muramyl dipeptide, the minimal structure of *Mycobacterium*, using rat alveolar macrophages (Jorens et al., 1993). We demonstrated in this study that this mechanism also conforms to the response of alveolar macrophages to a β-D-glucan, a fungal component. From these findings, it appears that the alveolar macrophage response to microbial products is limited in the normal lung, but when immune and/or inflammatory events occur in the alveolar space, lymphokines are released from pulmonary T cells and stimulate the response of alveolar macrophages to microbial products. It is likely that this mechanism also plays an important role in the activation of the alveolar macrophage functions by the injection of a fungal biological response modifier, soluble β-D-glucan.

In conclusion, we demonstrated in this study that the systemic administration of SSG, a fungal soluble β-D-glucan, augmented the functions of alveolar macrophages in vivo mediated, at least in part, by both simple activation by serum components, such as fibronectin and/or albumin, leaked from pulmonary peripheral blood and the synergism between lymphokines including interferon γ released from activated pulmonary T cells and SSG entering the alveolar space via the priming effect of lymphokines which enhances the expression of β-D-glucan binding sites on the alveolar macrophage surface. Prior and Haslam (1992) reported that interferon γ released from activated pulmonary T cells contribute to the prevention of phathogenesis of fibrosing lung diseases caused by influx of serum components into the alveolar space. It is likely that SSG will be very useful clinically as a biological response modifier to activate alveolar macrophages without provoking chronic inflammation in the alveolar space.

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