Macrophage Activation *in Vitro* by Chemically Cross-Linked (1→3)-β-D-Glucans

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The chemical cross-linking of soluble (1→3)-β-D-glucans having molecular weights of 21000 (CL 3 h) and 6400 (CL 6 h), and laminarin (CL LAMI), which showed negligible biological activity, by epichlorohydrin provided rigid particles. These particles showed no gel-to-sol transition upon the addition of sodium hydroxide. We compared the effects of chemical cross-linking on the biological activities of glucans. The alternative complement pathway was not activated by any of the cross-linked glucans. Glucose consumption, lysosomal enzyme release, and interleukin-1 production by mouse resident peritoneal macrophages incubated *in vitro* were strongly induced by CL 3 h, CL 6 h and CL LAMI. However, cross-linked dextran, Sephadex, did not exhibit any of these biological activities. These results suggested that chemical cross-linking of (1→3)-β-D-glucans enhances macrophage activities without opsonization by complement components.

Keywords *Grifola frondosa*; (1→6)-branched (1→3)-β-D-glucan; molecular weight; macrophage; complement; epichlorohydrin; cross-linking

High molecular weights (MW) of (1→3)-β-D-glucans such as lentinan, schizophyllan, TAK, and grifolan are important for antitumor activity, because if the molecular weight is less than about 10000, the molecules have no significant activity.[1] In a previous report we have described the effect of molecular weight of (1→3)-β-D-glucans on activation of macrophages and of the alternative complement pathway. The results of this investigation led to the following conclusions. 1) The activation of macrophage activities such as glucose consumption, lysosomal enzyme production, and interleukin-1 (IL-1) production decreased with the reduction of the molecular weight. 2) The molecular weight of glucans required for activation of the alternative complement pathway (>MW 250000) was higher than that for the macrophage activation (>MW 21000) (submitted for publication). It was reported that chemical cross-linking of laminarin by cyanogen bromide enhanced macrophage functions, *i.e.* spreading or incorporation of ^{14}C-glucosamine.[2] Recently, Seljelid *et al.* studied the stimulatory effects of (1→3)-β-D-glucan-derivatized plastic beads on macrophages. These reports suggested that immobilization of (1→3)-β-D-glucan augmented the effects on macrophage functions.[3] Antitumor (1→3)-β-D-glucans possess gel-forming ability which was due to physical cross-linking by hydrogen-bonding between hydroxyl groups at C-2 of glucose residues.[4] It was reported that gel-forming ability declined with decreasing molecular weight and it was proposed that a significant length of glucan chain was required for stable cross-linking of helical (1→3)-β-D-glucans.[5] These results led to the concept that cross-linking of (1→3)-β-D-glucan is important for the manifestation of biological activities. One approach to confirm this would be to use chemical cross-linking of low-molecular-weight glucans. In this paper, we aimed to investigate the effect of chemical cross-linking of low-molecular-weight glucans (MW 21000 and 6400) by using epichlorohydrin, which is a well-known reagent used to prepare dextran gel-beads, on macrophage-activating activity.

Materials and Methods

Mice Male 6- to 8-week-old ICR mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. The mice were placed under specific-pathogen-free conditions.

Reagents Zymosan A (zymosan), *p*-nitrophenyl-β-D-glucuronide and ethylene glycol bis-β-amino ethylether tetraacetate (EGTA) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Sephadex G-10 fine and dextran T-500 were obtained from Pharmacia Fine Chemicals. Laminarin from *Eisenia araborea* was purchased from Tokyo Kasei Kogyo Co., Ltd. Ethylenediamine tetraacetate (EDTA) was obtained from Dojin Laboratories, Kumamoto. Phytohemagglutinin-P (PHA) was purchased from Seikagaku Kogyo Co., Ltd., Tokyo. Epichlorohydrin, glucose B-test Wako, and β-D-glucuronidase from calf liver (EC 3.2.1.31) were purchased from Wako Pure Chemical Industry Ltd.

Heat-Treatment of LELFD LELFD, which is a (1→6)-branched (1→3)-β-D-glucan prepared from culture filtrate of *Grifola frondosa*,[6] was suspended in distilled water (2.5 mg/ml, 10 ml) and heated for various times at 150 °C using a glass tube with a screw cap in an aluminum block heater. The products heated for 0, 3 h, and 6 h were named LE (MW 800000), HD-LE 3 h (MW 21000), and HD-LE 6 h (MW 6400), respectively.

Cross-Linking of Glucans Each glucan (HD-LE 3 h, HD-LE 6 h, and laminarin) dissolved in 6 N NaOH (100 mg/ml) was mixed with the same volume of epichlorohydrin, and incubated for 3 min at 50 °C with vigorous stirring. After the incubation, the mixture was neutralized with AcOH, and dialyzed against tap water and then distilled water for 3 d. The yields of products of HD-LE 3 h (CL 3 h), HD-LE 6 h (CL 6 h), and laminarin (CL LAMI) were 69%, 41%, and 180%, respectively.

Activation of Alternative Complement Pathway Freshly prepared samples of human whole serum provided sources of complement. For assay, samples were dissolved in gelatin veronal buffer (GVB) containing 0.05 M EGTA and 0.01 M magnesium (EGTA–GVB–Mg^{2+}). Then, 0.1 ml of the sample was incubated with the complement solution (0.4 ml) at 37 °C for 1 h, and diluted with 0.01 M EGTA–GVB–Mg^{2+}. The solution (0.8 ml) was mixed with 0.2 ml of rabbit red blood cells (rabbit-RBC, 5×10^7/ml), and incubated at 37 °C for 1 h. A 2.0 ml portion of GVB containing 0.01 M EDTA (EDTA–GVB) was added to the solution to block further complement activation. After centrifugation, the optical density of the supernatant was read at 414 nm.

Macrophage Culture Resident peritoneal macrophages were isolated from the peritoneal cavity by lavage with 2 washes of 5—6 ml of Hanks' balanced salt solution (HBSS) (Nissui Seiyaku Co., Ltd., Tokyo Japan). The recovered lavage fluid was centrifuged at $300 \times g$ for 5 min to pellet the cells. The pellet was washed twice and resuspended in RPMI 1640 (Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing 1% heat-inactivated fetal calf serum (FCS) (Boehringer Mannheim, FRG), 5 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) (Sigma Chemical Co., St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan). The cell was cultured in 24- well or 96-well flat-bottomed plates (Sumitomo Bakelite Co., Ltd., Japan) at 2×10^6 per well (24 well) or 2×10^5 per well (96 well) in 1000 μl or 100 μl of culture medium, respectively. To ensure adherence, cells were cultured for at least 2 h before washing, and washed twice with 500 μl/well or 50 μl/well of fresh medium. Obtained macrophages were cultured for 48 or 72 h in a humidified 5% CO_2 incubator at 37 °C with 500 μg/ml of glucans in RPMI

1640 medium containing 5% FCS, 5 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. For IL-1 assay, culture supernatant from a 24-well culture was filtered through a syringe filter unit (0.20 μm, Corning), and macrophage lysate was prepared by repeated freeze and thawing (3 times) and filtered through the syringe filter unit.

Glucose Consumption Activity Glucose content remaining in the macrophage culture supernatant was measured by use of Glucose B-test Wako. The supernatants (10 μl) obtained from the macrophage culture with samples for 72 h at 37 °C were incubated with 1.5 ml of color reagent containing 0.1% phenol, 0.01% 4-aminoantipyrine, and 6.64 units/ml of glucose oxidase for 20 min at 37 °C. The optical density at 505 nm of the solution was measured and remaining glucose was determined from a calibration plot obtained with standard glucose solutions. The results were expressed as percent glucose consumption, which was calculated from the following formula: [1 − (glucose content in culture medium cultured with macrophages/glucose content in culture medium without macrophages)] × 100.

Lysosomal Enzyme Assay The activity of the β-D-glucuronidase in the culture supernatant or cell lysate was measured by using *p*-nitrophenyl-β-D-glucuronide as a substrate. The culture was carried out for 72 h at 37 °C in 200 μl/well of RPMI 1640 containing 5% FCS. After the incubation, 100 μl of the culture supernatant was mixed with 200 μl of 6 mM *p*-nitrophenyl-β-D-glucuronide in 0.1 M citrate buffer (pH 5.0), and incubated for 2 h at 37 °C. The reaction was terminated by adding 1 ml of 0.2 M borate buffer (pH 9.8). The *p*-nitrophenol released by the enzyme-dependent hydrolysis of the substrate was quantified spectrophotometrically by measuring the optical density at 405 nm. The enzyme activities were expressed as units assessed by a simultaneous experiment with standard β-D-glucuronidase from calf liver.

IL-1 Assay The IL-1 activity of supernatants or macrophage lysates was assayed in terms of their ability to stimulate murine thymocyte proliferation. Thymuses from C3H/HeJ mice were gently teased and filtered through a wire mesh to remove debris and aggregates. The cells were resuspended in RPMI 1640 with 5% FCS and with or without various additives as indicated. Thymocytes (1×10^6) were cultured for 72 h in flat-bottomed 96- well culture plates. Cultures were exposed for the final 12 h of incubation to 0.5 μCi of ^{3}H-thymidine (TdR) (ICN Biomedicals Inc.). The cells were collected on glass fiber filters with an automatic harvester. The filters were dried, and added to scintillation fluid, and the radioactivity was counted in a scintillation counter. The results are expressed in mean cpm of ^{3}H-TdR taken up by triplicate cultures.

Other Methods Preparation of LELFD and carbon-13 nuclear magnetic resonance (^{13}C-NMR) analysis were described previously.[6]

Results

Physicochemical Analysis of Cross-Linked Glucans Samples tested in this study were prepared by chemical cross-linking of glucans with epichlorohydrin, and were as follows: (1→6)-branched (1→3)-β-D-glucan (HD-LE 3 h, MW 21000; HD-LE 6 h, MW 6400) and linear (1→6)-β-D- and (1→3)-β-D-glucan (laminarin). Figure 1 shows ^{13}C-NMR spectra of laminarin and dextran and their cross-linked products dissolved or suspended in H_2O. After cross-linking of laminarin with epichlorohydrin, the C-1 (104 ppm) and C-3 (86 ppm) signals almost disappeared. This suggested a severe restriction of flexibility of the glucan chains by this treatment. The spectrum of CL 3 h in water (Fig. 2B) showed a similar signal peak to that of Sephadex G-10 (Fig. 1), suggesting similar cross-linking of Sephadex G-10. Since helical (1→3)-β-D-glucans are known to show gel-to-sol transition upon the addition of alkali as assessed by ^{13}C-NMR spectroscopy (Fig. 2A),[7] we examined whether similar gel-to-sol transition of cross-linked HD-LE 3 h (CL 3 h) could be observed. In the case of physically cross-linked glucans, sharp signals increased with the addition of alkali; the C-1 and C-3 signals appeared at about 104 and 88 ppm in 0.1 N NaOH, shifting to 103 and 85 ppm, respectively, with further addition of alkali. The transition of the chemical shift is thought to be

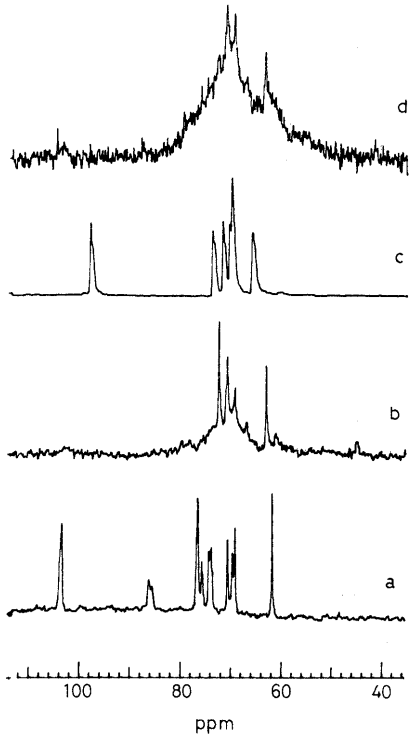

Fig. 1. ^{13}C-NMR Spectra of Glucans

Each glucan (40 mg) was dissolved or suspended in distilled water (2 ml), and the ^{13}C-NMR spectrum was measured. a, laminarin; b, CL LAMI; c, dextran T-500; d, Sephadex G-10.

due to conformational change, from single helix to random coil. No C-1 and C-3 signal peaks of CL 3 h appeared even in 0.25 N NaOH (Fig. 2B). These results indicated that molecular movement of the main glucosyl chain of CL 3 h was highly restricted. Benesi *et al.* found that the molecular motion of Sephadex G-75 was relatively free, as was that of dextran T-40, *i.e.* the C-1 and C-6 signals showed comparable chemical shift and line width to those of dextran T-40 even in water.[8] However, Sephadex G-10 showed broadened peaks (Fig. 1), and the C-1 signal could not be observed. These differences might be related to the number of cross-links present in Sephadex gels. It was also suggested that the degrees of cross-linking of CL LAMI and CL 3 h are similar to that of Sephadex G-10. The above results suggest that chemically cross-linked (1→6)-branched (1→3)-β-D-glucans possess few or no physical cross-links which can be observed by ^{13}C-NMR analysis.

Examination of the Ability of Glucans to Activate Alternative Complement Pathway The ability of cross-linked glucans to trigger the alternative complement pathway was compared with that of the parent glucans using unsensitized rabbit-RBC and human whole serum as a complement. As shown in Fig. 3, preincubation of the serum with 5 or 10 mg/ml of HD-LE 0 h (LE), laminarin, and zymosan decreased the residual hemolytic activity. In contrast, pretreatment of the serum with HD-LE 3 h, HD-LE 6 h or dextran did not inhibit the hemolysis significantly. Cross-linked glucans also failed to decrease hemolytic activity, although laminarin could reduce the activity. These results indicated that chemical cross-linking of lower-molecular-weight glucans would not augment the activity of the alternative complement pathway under these conditions. It is generally thought that C3b fragment reacts

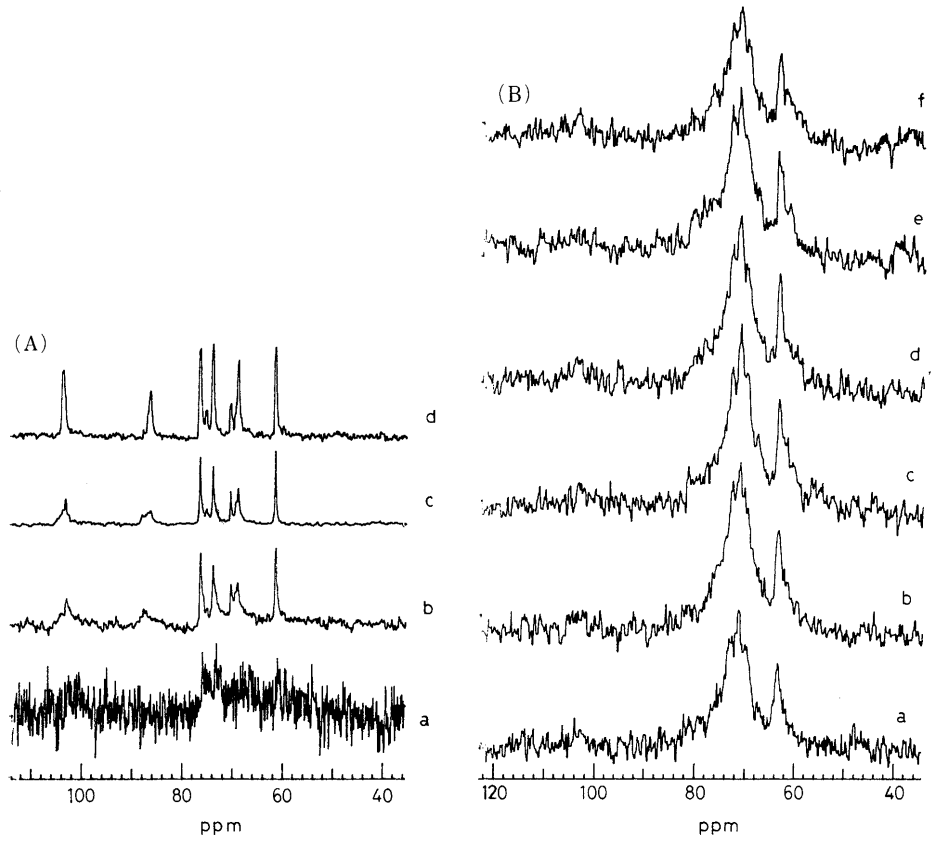

Fig. 2. Gel-to-Sol Transition of LE (A) and CL 3 h (B) in Sodium Hydroxide Solution Measured by ^{13}C-NMR Spectroscopy

Each glucan (40 or 20 mg) was dissolved or suspended in distilled water (2 or 1 ml), then sodium hydroxide (4 or 5 N) was added to give the indicated normality, and the ^{13}C-NMR spectrum was measured. (A) a, 0 N; b, 0.1 N; c, 0.2 N; d, 0.25 N; (B) a, 0 N; b, 0.05 N; c, 0.1 N; d, 0.15 N; e, 0.2 N; f, 0.25 N.

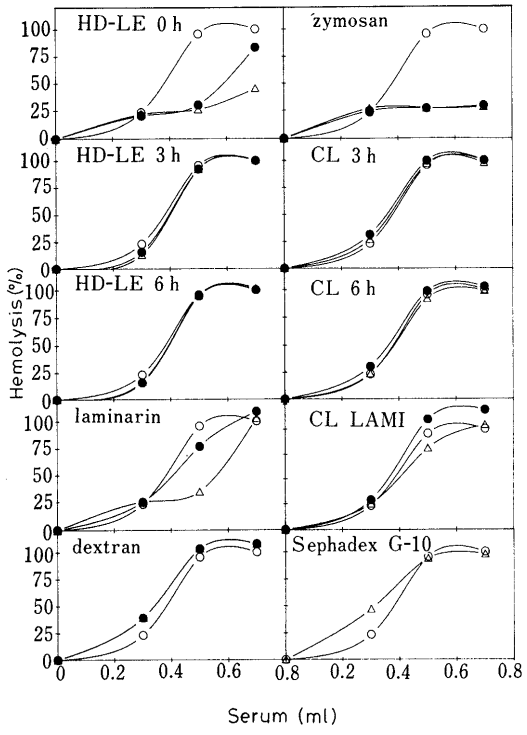

Fig. 3. Activation of the Alternative Complement Pathway by Glucans

Human sera pretreated with the indicated samples at the dose of 0 mg/ml (○), 5 mg/ml (●) and 10 mg/ml (△) were diluted with 0.01 M EGTA–GVB–Mg^{2+} and added to rabbit-RBC. The hemolysis by the treated sera were compared by measuring the absorbance of the supernatant at 414 nm.

TABLE I. Effect of Cross-Linking of Glucans on Glucose Consumption by Macrophages[a]

Sample (500 μg/ml)	Consumption (%, mean ± S.D.)
HD-LE 3 h	38.8 ± 0.5[c]
HD-LE 6 h	31.7 ± 0.6[c]
Laminarin	40.2 ± 1.8[c]
Dextran T-500	35.2 ± 1.7[c]
CL 3 h	68.1 ± 1.6[c,e]
CL 6 h	65.5 ± 0.6[c,e]
CL LAMI	37.9 ± 2.0[c]
Sephadex G-10	27.8 ± 0.7[b,d]
LE	52.3 ± 1.3[c]
Zymosan (1×10^7/ml)	47.4 ± 0.03[c]
Nil	18.8 ± 2.5

a) The glucose content in culture supernatant obtained from the wells after incubation for 72 h at 37 °C with samples was assessed by a Glucose B-test Wako. The macrophages (2×10^5/well) were cultured in 200 μl of medium with various additives. *b*) $p < 0.01$ compared with untreated macrophages. *c*) $p < 0.001$ compared with untreated macrophages. *d*) $p < 0.01$ compared with macrophages treated with the parent glucan. *e*) $p < 0.001$ compared with macrophages treated with the parent glucan.

with a hydroxyl group on the surface of the complement activator.[9] Because epichlorohydrin would randomly react with hydroxyl groups of glucosyl residues, this chemical modification might critically alter the localization and/or quantity of hydroxyl groups. Further, insolubilization could enclose significant quantities of hydroxyl groups inside particles, making them inaccessible to complement component. Considering these facts, it could be speculated that chemically cross-linked glucans have a significantly lower binding capacity for C3 component.

Examination of the Ability to Activate Macrophage in *Vitro* Since 500 μg/ml of grifolan LE,[6] sufficiently en-

hanced macrophage functions such as glucose consumption, lysosomal enzyme production, IL-1 production, and H_2O_2 induction triggered by phorbol myristate acetate in preliminary experiments (unpublished data), we used this concentration for the assay. In this study, we examined the changes of glucose consumption, lysosomal enzyme secretion, and IL-1 production induced by culture with cross-linked glucans. The macrophage-activating effects of various glucans were first evaluated by measuring the glucose consumption by resident peritoneal macrophages stimulated *in vitro* with glucans (500 μg/ml). As shown in Table I, all of the macrophages stimulated with samples used in this assay consumed larger amounts of glucose than did unstimulated macrophages. The extents of glucose consumption in the presence of LE, HD-LE 3 h, and 6 h were 52%, 39%, and 32%, respectively. Cross-linked HD-LE 3 h and HD-LE 6 h (CL 3 h and CL 6 h) resulted in significantly higher consumptions of glucose (68% and 66%, respectively). Cross-linking of laminarin and dextran did not augment the glucose consumption by macrophages.

TABLE II. Lysosomal Enzyme Production by Macrophages Incubated with Cross-Linked Glucans[a]

Sample (500 μg/ml)	β-D-Glucuronidase (unit, mean ± S.D.)
HD-LE 3 h	43 ± 5
HD-LE 6 h	49 ± 0.1
Laminarin	33 ± 1[b]
Dextran T-500	37 ± 7[c]
CL 3 h	245 ± 7[d,f]
CL 6 h	197 ± 11[d,e]
CL LAMI	68 ± 3[d,f]
Sephadex G-10	45 ± 4
LE	58 ± 3[c]
Zymosan (1×10^7/ml)	190 ± 7[d]
Nil	43 ± 2

a) The production of β-D-glucuronidase by macrophages cultured for 72 h at 37 °C with samples was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucuronide in culture supernatant. The culture conditions were the same as in Table I (footnote). *b*) $p<0.05$ compared with untreated macrophages. *c*) $p<0.01$ compared with untreated macrophages. *d*) $p<0.001$ compared with untreated macrophages. *e*) $p<0.05$ compared with macrophages treated with the parent glucan. *f*) $p<0.001$ compared with macrophages treated with the parent glucan.

TABLE III. IL-1 Activity of Macrophages Stimulated with Cross-Linked Glucans[a]

Sample (500 μg/ml)	^{3}H-TdR incorporation (cpm, mean ± S.D.)	
	Supernatant	Lysate
HD-LE 3 h	1238 ± 54[d]	632 ± 85[b]
HD-LE 6 h	380 ± 85[c]	785 ± 215
Laminarin	494 ± 149	537 ± 261
Dextran T-500	567 ± 47[c]	330 ± 59
CL 3 h	2871 ± 858	2150 ± 439[c,f]
CL 6 h	1593 ± 518[e]	2292 ± 1158
CL LAMI	3699 ± 1551	816 ± 97[c]
Sephadex G-10	464 ± 111[c]	351 ± 5
LE	2816 ± 126[c]	4038 ± 476[d]
Nil	765 ± 18	427 ± 87

a) IL-1 activity in culture supernatant and cell lysate was assessed by measuring the incorporation of ^{3}H-TdR into thymocytes cultured with several preparations and PHA. Macrophages (2×10^6/ml, 1 ml) were incubated with various glucans at the concentration of 500 μg/ml. The thymocyte culture was carried out for 72 h and ^{3}H-TdR was added 12 h before the termination. *b*) $p<0.05$ compared with untreated macrophages. *c*) $p<0.01$ compared with untreated macrophages. *d*) $p<0.001$ compared with untreated macrophages. *e*) $p<0.05$ compared with macrophages treated with the parent glucan. *f*) $p<0.01$ compared with macrophages treated with the parent glucan.

The effect of incubation with glucans on lysosomal enzyme release was examined by using resident peritoneal macrophages. The macrophages were cultured with 500 μg/ml of samples under the conditions as used in the study on glucose consumption. After the incubation, the β-D-glucuronidase activity in culture supernatants was measured by using *p*-nitrophenyl-β-D-glucuronide as a substrate. LE and zymosan enhanced the release of the enzyme (Table II). However, the enzyme activity induced by LE was less than that by zymosan. CL 3 h and CL 6 h markedly stimulated the enzyme release to extents comparable to that by zymosan. CL LAMI also elevated the level of enzyme release over that observed with laminarin. The enzyme activity induced with Sephadex G-10 was similar to that of untreated macrophages. These results suggested that the (1→3)-β-D-glucosyl linkage is important for triggering the lysosomal enzyme release in the case of cross-linked glucans.

The effect of cross-linked glucans on the production of IL-1 by resident peritoneal macrophages *in vitro* was also evaluated (Table III). IL-1 production in the culture supernatant was observed by macrophages treated with LE and HD-LE 3 h. The extent of IL-1 activity of LE was higher than that of HD-LE 3 h, suggesting a molecular weight dependency of the IL-1 production. Cross-linking of HD-LE 3 h (CL 3 h) augmented IL-1 release. In the case of HD-LE 6 h and laminarin, IL-1 production was observed only after cross-linking, and the value was comparable to that with LE. Incubation with Sephadex G-10 did not stimulate IL-1 production in culture supernatant or cell lysate. These results indicated that chemical cross-linking of (1→3)-β-D-glucans can induce IL-1 production.

Discussion

As described above, chemical cross-linking of (1→3)-β-D-glucans formed an insoluble rigid gel structure. Insoluble glucans, *e.g.* zymosan and yeast glucan, are known to be activators of complement system and phagocytes.[10] Therefore, we expected that the cross-linked product of soluble (1→3)-β-D-glucans would activate such functions. Since glucan-opsonization with complement is a candidate for the first interaction of glucans and host components, we examined the ability of cross-linked glucans to activate the alternative complement pathway. Although glucans with high molecular weight were required to activate the alternative complement pathway, cross-linked glucans such as CL 3 h, CL 6 h, CL LAMI, and Sephadex G-10 could not reduced hemolytic activity. However, serum pretreated with laminarin exhibited reduced hemolysis. This result suggested that cross-linking with epichlorohydrin inhibits the ability to activate the alternative complement pathway. This might be a result of substitution of hydroxyl groups, which may be the binding site of glucans,[9] or changes of ultrastructure. These findings suggested that insoluble glucans are not always activators of the alternative complement pathway, and that chemically cross-linked glucans are hardly recognized by phagocytes through the third component of complement.

Glucose consumption by macrophages treated with CL 3h and CL 6h was higher than those with the parent glucans or LELFD. The cross-linking of HD-LEs and laminarin produced a stimulatory effect on lysosomal enzyme release. Cross-linked products of (1→3)-β-D-glucans produce IL-1 as well as LELFD. However, a similarly cross-linked product, Sephadex G-10, had no such abilities under the present experimental conditions. The intensities of some of the biological activities of CL LAMI, CL 3h, and CL 6h, such as glucose consumption and lysosomal enzyme secretion, were different from each other. The reason for this is unclear. The segmental structure or gel structure in each cross-linked glucan might be different, because of differences in the primary structure of parent glucans. In the case of chemical cross-linking of polysaccharides, (1→3)-β-D-glucosyl linkages are suggested to be important to activate macrophage functions.

Recently, many reports have approached concerning β-glucan receptor which recognizes the (1→3)-β-D-glucosyl unit.[11] The receptor is closely related to the phagocytosis of particulate (1→3)-β-D-glucans and to lysosomal enzyme release triggered by zymosan or yeast glucan.[11] Although low-molecular-weight (1→3)-β-D-glucans could interfere with the receptor-mediated biological functions, these glucans failed to show activities such as induction of lysosomal enzyme secretion. It is generally accepted that biological activities mediated by certain receptors necessitate cross-linking of the receptors. This suggests the hypothesis that a sufficient length of (1→3)-β-D-glucosyl units or rigid structure is required for the β-glucan receptor-mediated activation mechanisms. If CL 3h and CL 6h are recognized through the β-glucan receptor, the cross-linking of lower-molecular-weight (1→3)-β-D-glucans might satisfy the requirements of the β-glucan-receptor activation mechanisms. As described above, phagocytes have a β-glucan-specific receptor which recognizes (1→3)-β-D-glucans without adhesion of complement components. Chemically cross-linked glucans failed to activate the alternative complement pathway, whereas only (1→3)-β-D-glucans could activate macrophages after chemical cross-linking. These data might imply the participation of β-glucan receptor in macrophage activation.

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