Lignin-rich Enzyme Lignin (LREL), a Cellulase-treated Lignin-Carbohydrate Derived from Plants, Activates Myeloid Dendritic Cells via Toll-like Receptor 4 (TLR4)

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Background: Although lignins are the second major biomass, their biological activities are little known.

Results: Certain barley husk-derived lignin-carbohydrate fractions strongly activated dendritic cells via the receptor protein TLR4.

Conclusion: Composition of the lignin-carbohydrate, especially the characteristic distribution of neutral sugars, is essential for the immunostimulatory activity.

Significance: Immunostimulatory lignin-carbohydrates could be potentially used as anti-infectious agents and as vaccine adjuvants.

Lignin-carbohydrates, one of the major cell wall components, are believed to be the structures that form chemical linkage between lignin and cell wall polysaccharides. Due to the molecular complexity of lignin-containing substances, their isolation and the assignment of their biological activities have so far remained a difficult task. Here, we extracted two lignin-containing carbohydrates, lignin-rich enzyme lignin (LREL) and pure enzyme lignin (PEL), from barley husk and demonstrated that they act as immune stimulators of dendritic cells (DCs), which are particularly important in linking innate and adaptive immunity. Thioacidolysis, acid hydrolysis, and mild alkali hydrolysis of both LREL and PEL revealed that their immunostimulatory activities depended on the lignin structure and/or content, neutral sugar content (especially the characteristic distribution of galactose and mannose), and presence of an ester bond. Furthermore, we showed that the immunostimulatory potency of the lignin-carbohydrate depended on its molecular weight and degree of polymerization. We also demonstrated that the LREL-induced activation of DCs was mediated via TLR4. Thus, LREL-induced increases in the expression levels of several cell surface marker proteins, production of inflammatory cytokines IL-12p40 and TNF-α, and activation and nuclear translocation of transcription factors, as was observed in the WT DCs, were completely abrogated in DCs derived from the TLR4−/− mice but not in DCs derived from the TLR2−/−, TLR7−/−, and TLR9−/− mice. We further demonstrated that LRELs isolated from other plant tissues also activated DCs. These immunostimulatory activities of lignin-carbohydrates, extracted from edible plant tissues, could have potential relevance in anti-infectious immunity and vaccine adjuvants.

Lignin is a major class of natural products found in various plants by phenolic oxidative coupling processes (1). It has been proposed earlier that lignin macromolecules are formed by random dehydrogenative polymerization of the three major phenylpropane units, known as guaiacyl (G)3 (derived from coniferyl alcohol), syringyl (S) (derived from syringyl alcohol), and p-hydroxyphenyl (H) (derived from p-coumaryl alcohol) (2). The amount and composition of lignins vary among taxa, cell types, and individual layers of the cell wall, and both are also influenced by developmental and environmental cues (3). Although there are some exceptions, lignins from dicotyledonous plants are principally composed of G and S units and traces of H units, whereas lignins from monocotyledonous plants contain comparable levels of G and S units and have more H units than dicotyledonous plants (4). Some polysaccharides in the cell walls are linked to lignin through hydroxycinammic acids by forming ester-ether bridges to form structurally complex lignin-carbohydrates (5–7). Because of the molecular complexity of lignin-carbohydrates, stable preparation of lignin has proven to be difficult, and as a result, very little attention has been paid to the biological activities of lignins or lignin-containing substances. Although several earlier reports indicated a link between lignin and immunostimulation (8–10), analytical procedures used in these studies were not very clear and remained controversial as well. Additionally, lignin-containing substances used in these studies probably did not occur naturally because they were either extracted using harsh chemical methods (such as strong alkaline extraction) or were extracted from something unusual. In this report, we have succeeded in

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fractionating analytically obvious lignin-containing substances by enzyme treatment, which is a naturally occurring phenomenon during the action of digestive enzymes, and then we determined their biological activity and mechanism. As our primary source for isolating lignin, we used barley husk, which is a major byproduct of the malting and brewing industry. In the barley seed, husk is the only tissue that is highly lignified, and it is mainly composed of hemicellulose (about 30%), cellulose (about 27.7%), lignin (about 23%), and a small amount of ash and proteins (11).

Dendritic cells (DCs) are particularly important immune cells linking innate and adaptive immunity due to their deployment at barrier sites of antigen encounter, trafficking to central lymphoid sites of T cell recruitment, and exquisite tissue and environmental regulation of their antigen-presenting traits (12, 13). Upon activation, expressions of MHC proteins and costimulatory molecules, such as CD86, on the cell surface of DCs are up-regulated, and secretions of various cytokines, especially those of inflammatory cytokines, from these cells are increased. As a result of DC activation, the acquired immune system, such as cell-mediated immunity and humoral immunity, is activated (14, 15).

Toll-like receptors (TLRs), which constitute a family of pattern recognition receptors, recognize specific molecular patterns in pathogenic microbial components and also in some endogenous ligands, and they act as the first line of defense against many bacteria and play an important role in the innate immunity (16–19). It is well established that TLR stimulation leads to DC activation by activation and nuclear translocation of transcription factors, such as nuclear factor-κB (NF-κB), interferon regulatory factor 3/7 (IRF3/7), and/or activator protein-1 (AP-1) either via the MyD88-dependent pathway or via the TIR-domain containing adapter protein inducing interferon beta (TRIF)-dependent pathway (20–22). The signaling cascade varies according to the TLR type, and TLR4 is the only TLR that activates both MyD88-dependent and TRIF-dependent pathways (20).

Because we always ingest lignins through dietary consumption of vegetables and other plant products, we hypothesized that the naturally extracted lignins, and not the chemically extracted ones, might have innate immunomodulating activities. We also hypothesized that the immunomodulating activity of lignin is mediated via the TLRs, which are the major receptors related to innate immunity. In the present study, we showed that lignin-rich enzyme lignin (LREL) and pure enzyme lignin (PEL) stimulated DCs and were analytically obvious lignin-carbohydrates. We also provided evidence that the immunostimulatory activity of LREL was indeed induced via the TLR4 signaling pathway. These results provided some new insights into the immunologic profile of the host response to lignin-carbohydrates, and they might be pertinent to lignin’s utility in host defense against microbial or viral infection and also against cancer.

EXPERIMENTAL PROCEDURES

Plant Materials—Barley husk was a kind gift from KIRIN Brewing Co. Ltd. (Tokyo, Japan). Wheat bran was purchased from Nisshin Seifun Co. Ltd. (Tokyo, Japan). Rice husk, cinnamon bark, sesame seeds, green tea leaf, and turmeric rhizome were purchased from various commercial sources in Japan.

Lignin Extraction—Lignin was extracted from plant samples following a previously published protocol with some modifications (23). The extraction method is schematically described in Fig. 1. Briefly, the air-dried samples were incubated overnight in toluene/ethanol (2:1, v/v) to remove wax residues. The dewaxed samples were crushed into powders using a multibead shocker (Yasui Kikai, Tokyo, Japan) and then passed through a 200-μm size screen. The ground samples were extracted twice (12 h each time) with a dioxane/water mixture (90:10, v/v), followed by another extraction with a dioxane/water mixture (50:50, v/v) for 12 h. All three extractions were carried out at room temperature in the dark. Liquids obtained from these three extractions were combined into one composite sample, and dioxane was removed by using a rotary vacuum evaporator (EYELA, Tokyo, Japan). The hemicellulose-rich milled lignin (HRML) was obtained from the leftover liquid by precipitation with 4 volumes of ethanol. The pure milled lignin (PML) was obtained by lyophilizing the supernatant from ethanol precipitation. After the extraction of HRML and PML, the dioxane/water-extracted residues were washed with water more than once and then treated with a mixture of same volume of Aspergillus niger cellulase (Sigma) and A. niger hemicelulase (Sigma) (32 mg/ml each enzyme) dissolved in 0.2 mM sodium acetate buffer (pH 4.8) at 37 °C for 72 h. After filtration through a nylon cloth, the insoluble residues were washed with a large amount of water, and the LREL and PEL samples were obtained by successive extractions with 90 and 50% dioxane/water as before. Klasen lignin content in each fraction was determined by measuring the yield of insoluble residues after treating with sulfuric acid according to the standard procedure with slight modification as described previously (24). The sulfuric acid-soluble fraction was used for determining the neutral sugar composition (see below). Milled wood lignin (MWL) and the lignin-carbohydrate complex (LCC) were prepared from beech (Fagus crenata) using their extractive-free wood meals according to the conventional method (25, 26).

Mice—6–10-week-old female C57BL/6N wild-type mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). TLR1−/−, TLR2−/−, TLR4−/−, TLR6−/−, TLR7−/−, TLR9−/−, and MyD88−/− mice with the C57BL/6N genetic background were obtained from Oriental Bioservice (Kyoto, Japan). All animal experiments were performed in accordance with the guidelines for care and use of laboratory animals of KIRIN Co. Ltd. (Tokyo, Japan).

Culture of Bone Marrow (BM)-derived DCs and Measurement of Immunostimulatory Activity—Flt-3L-induced BM-derived DCs (BM-DCs) were generated as described previously (27). In brief, BM cells were isolated from C57BL/6J, and erythrocytes were removed by a short exposure to 0.168 M NH4Cl. Cells were cultured at a density of 5 × 105 cells/ml for 7 days in RPMI1640 medium supplemented with 1 mM sodium pyruvate (Invitrogen), 2.5 mM HEPES (Invitrogen), 50 units/ml penicillin/streptomycin (Invitrogen), 50 μM β-mercaptoethanol (Invitrogen), 10% FCS, and 100 ng/ml Flt-3L (R&D Systems, Minneapolis, MN) at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Lignin was added at a concentration of 1
ng/ml to 100 μg/ml, and cultures were maintained in the incubator for another 24 h. The immunostimulatory activities of lignin substances were measured by following the expression levels of cell surface markers by FACS (BD Biosciences) and by measuring the concentration of inflammatory cytokines in the cell culture supernatant by ELISA (see below). As positive controls, we used cells treated with 10 ng/ml LPS (Sigma).

Antibodies—The following fluorescent dye-conjugated anti-mouse mAbs were purchased from eBioscience (San Diego, CA): CD11c-PE-Cy7 (N418), CD40-FITC (HM40-3), CD80-APC (16-10A1), CD86-APC (GL1), PD-L1-PE (M1H5), ICOS-LPE (HK5.3), and MHC class II-FITC (M5/114.15.2). Fluorescent dye-conjugated anti-mouse mAb CD11b-APC-Cy7 (M1/70) was purchased from BD Pharmingen. For the receptor inhibition assay, monoclonal anti-mannose receptor (clone 15-2) antibody was purchased from Abcam (Cambridge, UK). Anti-mouse dectin-1 monoclonal (clone 2A-11) antibody was purchased from AbD Serotec (Oxford, UK).

FACS Analysis—Cells for FACS analysis were stained with the appropriate fluorescent dye-conjugated Abs (FITC, PE, PerCP, APC, PE-Cy7, and APC-Cy7). After staining, the cells were washed twice with FACS buffer (0.5% BSA in PBS buffer) and suspended in 2% paraformaldehyde for FACS analysis. Data were collected by using a FACS Canto II cell analyzer (BD Biosciences), and the collected data were analyzed by the FCS Express software (De Novo Software, Los Angeles, CA). Cells gated on CD11c+ and CD11b+ were defined as Flt-3L induced BM-derived myeloid DCs (mDCs).

ELISA—The concentration of cytokines in cell culture supernatant was measured by using commercially available enzyme-linked immunosorbert assay (ELISA) kits. For this purpose, the OptEIA™ mouse IL-12 (p40) ELISA kit was purchased from BD Pharmingen, and the mouse TNF-α and mouse IL-4 ReadySET-GO kits were purchased from eBioscience.

Lignin Analysis by Thioacidolysis—Monolignol analysis by thioacidolysis was performed according to a method described previously (28). The resulting products were trimethylsilylated and analyzed with an Agilent 7890A gas chromatograph (Agilent Technologies, Santa Clara, CA). The yields of monolignols were determined by comparing the areas of the peaks of the monolignols with those of the corresponding standard monolignols.

Activation of Transcription Factors by LREL—mDCs from Flt-3L-induced BM-derived DCs were sorted by a FACS Aria cell sorter (BD Biosciences). The purity of sorted mDCs was more than 95%. Purified mDCs were stimulated with by LREL or LPS for 3 or 8 h. Nuclear extracts of mDCs were prepared by using NE-PER nuclear and cytoplasmic extraction reagent (Pierce) following the manufacturer's instructions. Activated transcription factors (NF-κB, AP-1, IRF-3, and IRF-7) were quantified by ELISA using the TransAM transcription factor ELISA kit (Active Motif, Carlsbad, CA).

RESULTS

Immunostimulatory Activity of Lignin Fractions—First, we prepared four different lignin fractions, named here as HRML, PML, LREL, and PEL, from barley husk by a slightly modified version of Sun’s method (23) as illustrated in Fig. 1. Approximately yields of HRML, PML, LREL, and PEL were 2.5, 6, 0.13, and 3%, respectively. Total lignin contents in HRML, PML, LREL, and PEL, as determined by Klason lignin analysis, were 37.3, 50.8, 55.2, and 62.8%, respectively. The immunostimulatory activity of each one of these lignin fractions on innate immunity was then determined using Flt-3L-induced BM-DCs. We found that only LREL and PEL had immunostimulatory activity (Fig. 2, A and B). We also tested the immune stimulatory abilities of MWL and LCC, both of which are extracted...
using methods different from the one described here, and found that neither of them could activate BM-DCs (data not shown). Next, to determine the specific stimulatory effect of each lignin, we compared the expression levels of marker proteins MHC class II, CD40, CD80, and CD86 on the cell surface and concentrations of IL-12p40, TNF-α, and IL-4 in the cell culture supernatant after treating independent cultures of BM-DCs either with a 100 ng/ml concentration of one of the lignin fractions or with 10 ng/ml LPS. Strikingly, stimulation with LREL led to large increases in the expression levels of all of the cell surface marker proteins: 10-fold increase in CD40, 4-fold increase in CD80, 10-fold increase in CD86, and 4-fold increase in MHCII (Fig. 3A). Additionally, there were 5- and 20-fold increases in the concentrations of IL-12p40 and TNF-α, respectively, in the culture supernatant (Fig. 3B). IL-4 was not detected from any samples (data not shown). Although stimulation with PEL also led to significant increases in the expression levels of marker proteins on the cell surface and concentrations of cytokines in the culture supernatant, the stimulatory effect of PEL was much weaker than that of the LREL (Fig. 3). In contrast, HRML and PML did not activate the DCs. Therefore, LREL and PEL, both of which were obtained by cellulase and hemicellulase digestion, are immunostimulators. Interestingly, the biological activity of LREL was much higher than that of PEL. Thus, we focused on LREL and PEL for further analysis.

**Determination of Thioacidolysis Monomeric Reaction Products in LREL and PEL**—We next performed thioacidolysis of lignin fractions to further characterize them. Thioacidolysis, an acid-catalyzed reaction that causes depolymerization of lignins (29), proceeds by cleaving only the β-O-4 linkage, which is actually the most frequent type of bond found in lignins. Therefore, thioacidolysis is a useful method for detecting lignins and comparing the relative amounts of monomeric building blocks of lignin, known as G unit, S unit, and H unit (30). We found that the yield of each monomeric building block of lignin found in LREL was about 4 times more than that in PEL (Table 1), and the ratios of S unit content and H unit content to G unit content in LREL and PEL were comparable. Thus, lignin could be extracted from barley husk after successive extraction with dioxane/water and then enzyme (cellulase and hemicellulase) treatment.

**Determination of Neutral Sugars and Uronic Acids in LREL and PEL**—Next, we performed solvent acid hydrolysis of LREL and PEL and analyzed the hydrolyzed products by ion chromatography to determine the composition of neutral sugars and uronic acids. As summarized in Table 2, the amounts of neutral sugars and uronic acids in LREL were about 3 times greater than those in PEL, whereas the ratio of each molecule in LREL and PEL was largely similar. Xylose and arabinose were the most and second most neutral sugars, respectively, found in...
each lignin fraction. Glucose, galactose, and mannose were found at levels comparable with that of arabinose, and each fraction contained only a small amount of uronic acids.

Role of Ester Bonds in Immunostimulatory Activity of LREL—It is well known that lignin substances are etherified with hydroxycinnamic acids and esterified with either polysaccharides or lignins through etherified hydroxycinnamic acids (5–7). To determine whether the ester bonds have any functional importance, DC stimulation experiments were performed using mild alkali-hydrolyzed LREL. As shown in Fig. 4, the immunostimulatory activity of LREL became weaker as the time of alkali treatment got longer, and after more than 3 h of alkali treatment, its immunostimulatory activity was completely lost. Thus, these results suggested that the ester bonds of LREL play an essential role in the immunostimulatory activity of LREL.

Evaluation of Molecular Weights of Lignin Fractions—To understand whether there is any relationship between the molecular weight and the biological activity, molecular weight ($M_r$) of each lignin fraction was determined by gel permeation chromatography. The estimated weight-average molecular weights of LREL and PEL were 70,640 and 23,922, respectively (i.e. both over 20,000). $M_r$ of LREL was 3 times greater than that of PEL. Because the observed biological activity of LREL was more than that of PEL, this result suggested a correlation between the molecular weight and immunostimulatory activity.

Taken together, our results suggested that LREL and PEL were analytically obvious lignin-containing immunostimu-
Because LREL is a better immunostimulator than PEL, we next focused our attention on LREL and examined the immunostimulatory mechanism of LREL.

**LREL-induced DC Activation Is Mediated via the TLR4 Signaling Pathway**—Pattern recognition receptors play a crucial role in host defense. TLRs, which are members of the pattern recognition receptor family, are essential for recognition of bacteria by DCs. In order to identify the responsible TLR through which LREL activates DCs, the degree of activation was compared using BM-DCs derived from various TLRs/MyD88-deficient mice. As shown in Fig. 5 (A and C), LREL-induced increases in the expression level of CD86 and production of IL-12p40 were completely abrogated in DCs derived from TLR4/−/− mice and partially abrogated in DCs derived from MyD88/−/− mice but remained unchanged in DCs derived from TLR2/−/−, TLR7/−/−, and TLR9/−/− mice (compared with WT mice). In this experiment, we used LPS as a positive TLR4L control and confirmed that the DCs stimulated with LPS responded in a similar manner as the DCs stimulated with LREL (Figs. 5, B and C). These data suggested that, like LPS, LREL-induced activation of DCs is also mediated via TLR4.

TLR4 has long been considered as a bacterial sensor based on its specificity for LPS, a characteristic molecule found on the cell wall of Gram-negative bacteria. Therefore, it is possible that the observed activation of DCs by LREL via the TLR4 signaling pathway might be due to endotoxin contamination. To exclude this possibility, we performed the following two experiments. In the first experiment, polymyxin B, a polycationic antibiotic, which is known to block many biological effects of LPS by binding to the lipid A portion of the molecule (31), was added to the DC culture during stimulation with LREL. As shown in Fig. 6A, although a slight decrease in CD86 expression was observed upon the addition of polymyxin B, LREL still caused significant and steady activation of BM-DCs. On the other hand, the addition of polymyxin B completely blocked the activation of DCs induced by LPS. In the second experiment, we performed GC/MS analysis of alkali-hydrolyzed LREL and searched for fatty acids, which are one of the major and integral components of LPS. However, we failed to detect any fatty acid molecule in the hydrolysate (data not shown). These results suggested that although the structures of LREL and LPS were completely dif-

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**TABLE 1**

| Sample | G unit (μmol/g) | S unit (μmol/g) | H unit (μmol/g) | S/G ratio |
|--------|----------------|----------------|----------------|-----------|
| LREL   | 63.93          | 45.63          | 9.66           | 0.71      |
| PEL    | 16.15          | 10.02          | 2.81           | 0.62      |

**TABLE 2**

Neutral sugars and uronic acids (percentage, w/w) in LREL and PEL fractions

| Sugar                  | LREL   | PEL    |
|------------------------|--------|--------|
| Arabinose              | 3.22 (18.4) | 1.00 (19.1) |
| Galactose              | 2.38 (13.6) | 0.96 (18.4) |
| Glucose                | 3.18 (18.1) | 0.90 (17.2) |
| Xylose                 | 6.43 (36.7) | 1.62 (31.0) |
| Mannose                | 2.24 (12.8) | 0.65 (12.4) |
| Galacturonic acid      | 0.02 (0.11) | 0.03 (0.57) |
| Glucuronic acid        | 0.06 (0.34) | 0.08 (1.53) |
| Total                  | 17.94 (100) | 5.23 (100) |

**FIGURE 4.** Effect of alkaline hydrolysis on LREL activity. LREL was first hydrolyzed by treating with 1 N NaOH for different time periods, and excess alkali was neutralized with 1 N HCl. Flt-3L-induced BM-DCs were then incubated with the indicated concentrations of alkali-hydrolyzed LREL. Untreated LREL was included as a positive control. After 24 h, cells were analyzed by flow cytometry (gated on CD11b+/CD11c+ DCs) to determine the cell surface expression level of CD86, and cell supernatants were analyzed by ELISA to determine the concentration of IL-12p40. A, expression levels of CD86 (expressed as median fluorescence intensity) on the cell surface of DCs stimulated with the indicated concentrations of alkali-hydrolyzed LREL. Untreated LREL was included as a positive control. After 24 h, cells were analyzed by flow cytometry (gated on CD11b+/CD11c+ DCs) to determine the cell surface expression level of CD86, and cell supernatants were analyzed by ELISA to determine the concentration of IL-12p40. A, expression levels of CD86 (expressed as median fluorescence intensity) on the cell surface of DCs stimulated with the indicated concentrations of untreated and NaOH-treated (as indicated) LREL. B, culture supernatants of untreated and NaOH-treated (as indicated) cells were assayed for IL-12p40 and TNF-α production by ELISA. Each bar represents the mean concentration of each cytokine ± S.D. (error bars) (n = 3). The immunostimulatory activity of LREL became weaker as the time of alkali treatment grew longer. *, p < 0.05; **, p < 0.01 compared with control (Ctrl).
Lignin-Carbohydrate Activates Dendritic Cells via TLR4

(A) CD86 expression

(B) CD86 expression

(C) IL-12p40

|          | WT        | TLR2-/-   | TLR4-/-   |
|----------|-----------|-----------|-----------|
| CD86     | 415.2     | 561.3     | 456.5     |
| TLR7-/-  | 1035.7    | 1322.9    | 536.1     |
| TLR9-/-  | 28463     | 27668     | 117461    |
| MyD88-/- | 27921     | 17526     | 114558    |

|          | WT        | TLR2-/-   | TLR4-/-   |
|----------|-----------|-----------|-----------|
| CD86     | 415.2     | 561.3     | 456.5     |
| TLR7-/-  | 1035.7    | 1322.9    | 536.1     |
| TLR9-/-  | 28463     | 27668     | 117461    |
| MyD88-/- | 27921     | 17526     | 114558    |

|          | WT        | TLR2-/-   | TLR4-/-   |
|----------|-----------|-----------|-----------|
| IL-12p40 | 8         | 7         | 8         |

LREL | LPS
different, the LREL structure might contain some element that is similar to that of LPS. Therefore, we believe that the observed immunostimulatory activity of LREL was not due to endotoxin contamination but due to a novel TLR4 ligand.

Next, we examined whether there is any relationship between phagocytosis and stimulatory activity of LREL. As shown in Fig. 6B, inhibiting phagocytosis by treating with cytochalasin D had no effect on the LREL-induced responses in DCs, suggesting that the endosomal receptors are not involved in the activation process.

Because polysaccharides are one of the main components of LREL, we next examined whether other plasma membrane receptors, such as mannose receptor and C-type lectin receptor, are involved in activation of DCs by LREL stimulation. To test this possibility, we performed a receptor inhibition assay using blocking antibodies directed toward each receptor. As shown in Fig. 7, LREL-induced activation of DCs was not inhibited by pretreatment with blocking antibodies directed to each receptor.

**Figure 5.** Immunostimulatory activity of LREL is mediated via TLR4. Flt-3L-induced BM-DCs were prepared from wild-type (WT), TLR2−/−, TLR4−/−, TLR7−/−, TLR9−/−, and MyD88−/− mice. These cells were subsequently cultured in the presence of 100 ng/ml LREL for 24 h. A, comparison of CD86 expression levels on LREL-stimulated DCs by flow cytometry (gated on CD11c+ CD11b− DCs). Gray graph, medium (control); white graph, LREL. B, comparison of CD86 expression levels on LPS-stimulated DCs by flow cytometry. Gray graph, medium (control); white graph, LPS. LREL- and LPS-induced increase in the CD86 expression was completely abrogated in TLR4−/− DCs and significantly reduced in MyD88−/− DCs. Each bar represents mean ± S.D. (error bars) (n = 3). LREL-induced activation of DCs was not inhibited by pretreatment with blocking antibodies directed toward each receptor.

**Figure 6.** Effect of polymyxin B and cytochalasin D on LREL-induced activation of DCs. Flt-3L-induced BM-DCs were treated with LPS inhibitor polymyxin B (A) and phagocytosis inhibitor cytochalasin D (B) and then incubated for 24 h with 100 ng/ml LREL (top) or 10 ng/ml LPS (bottom). Untreated BM-DCs were used as a control (Ctrl). The cell surface expression level of CD86 was determined by flow cytometry (gated on CD11b+ CD11c− DCs). A, BM-DCs treated with 5 mg/ml polymyxin B prior to LREL or LPS stimulation. Gray graph, medium (control); white graph, LREL (top) or LPS (bottom). Polymyxin B completely abrogated the LPS-induced increase in the cell surface expression of CD86 but only partially inhibited the LREL-induced increase in the cell surface expression of CD86. Data shown are representative results obtained from an experiment performed in triplicate. B, BM-DCs treated with 2 μM cytochalasin D prior to LREL and LPS stimulation. This experiment was performed in triplicate. Each bar represents mean CD86 expression ± S.D. (error bars) (n = 3). LREL-induced activation of DCs was not inhibited by cytochalasin D (Cyto D).

**Figure 7.** Effect of pattern recognition receptor-blocking antibodies on LREL-induced activation of DCs. Flt-3L-induced BM-DCs were treated with the blocking antibody (Ab) directed toward mannose receptor, blocking antibody directed toward dectin-1, or isotype-nonspecific control antibody (Ctrl) and then incubated with 100 ng/ml LREL for 24 h. Cells were analyzed by flow cytometry (gated on CD11b+ CD11c− DCs) to determine the cell surface expression level of CD86 (expressed as median fluorescence intensity) (A), and cell supernatants were analyzed by ELISA to determine the concentration of IL-12p40 (B). Each bar represents mean value ± S.D. (error bars) (n = 3). LREL-induced activation of DCs was not inhibited by pretreatment with blocking antibodies directed to each receptor.
was not inhibited by these blocking antibodies, suggesting that neither of them is involved in the activation process.

Activation of Intracellular Transcription Factors by LREL—Because TLR stimulation triggers activation and nuclear translocation of transcription factors, some of which are involved in cytokine production (22), we next examined the DNA binding levels of activated NF-κBp65, c-Jun, and IRF-3 in the nuclear extracts of stimulated mDCs for 3 and 8 h. DNA binding levels of activated IRF-7 in the nuclear extracts of stimulated mDCs for 3 and 8 h were measured by ELISA. This experiment was performed using three independent cell cultures. Each bar represents mean ± S.D. (error bars) (n = 3). The nuclear levels of NF-κB and c-Jun were significantly up-regulated, and although the nuclear level of IRF-3 was consistently found to be higher than that in the control, the increase was not statistically significant (p < 0.1) for short term stimulation. The nuclear level of IRF-7 was significantly up-regulated only for long term stimulation. *, p < 0.05; **, p < 0.01 compared with control.

Comparison of the Activity of LREL Prepared from Various Plants—We next examined the immunostimulatory activity of LRELs prepared from other grain husks and various tissues of edible plants. For this purpose, we chose rice husk and wheat bran as grain husks, sesame as seeds, cinnamon as barks, green tea as leaves, and turmeric as rhizomes. As before, the activity of LREL was measured by monitoring the expression level of CD86 on the cell surface of DCs and the concentration of IL-12p40 in the cell culture supernatant. Results shown in Fig. 8 were performed using three independent cell cultures. Each bar represents mean ± S.D. (error bars) (n = 3). The nuclear levels of NF-κB and c-Jun were significantly up-regulated, and although the nuclear level of IRF-3 was consistently found to be higher than that in the control, the increase was not statistically significant (p < 0.1) for short term stimulation. The nuclear level of IRF-7 was significantly up-regulated only for long term stimulation. *, p < 0.05; **, p < 0.01 compared with control.

DISCUSSION

Barley grain is one of the raw materials for the malting and brewing industry, and a large amount of barley husk is produced as a by-product. The present study is the first report demonstrating that an analytically obvious lignin-carbohydrate, extracted from barley husk, is a potent immunostimulator of dendritic cells, and this lignin-carbohydrate acts via TLR4.
Lignin-Carbohydrate Activates Dendritic Cells via TLR4

It was intriguing that BM-DCs were activated by only LREL and PEL, which were obtained by treating barley husks with cellulase/hemicellulase. Surprisingly, LREL stimulated DCs at a quite low concentration, whereas HRML and PML, both of which were extracted without the cellulase/hemicellulase treatment, could not activate DCs. These results strongly suggested that cellulase/hemicellulase treatment was essential for preparing the immunostimulatory fractions.

Our results also suggested that four important material features were responsible for the high immunostimulatory activities of LREL and PEL. The first important feature was the lignin content. As shown in Table 1, both LREL and PEL were found to contain thioacidolysis monomeric reaction products (G, S, and H units). The ratios of S and H unit contents to G unit in LREL and PEL were comparable. Thus, it was analytically obvious that LREL and PEL contained lignin, and the chemical structures of their lignins were probably similar. In addition, because LREL contained much more monomeric building blocks of lignin than PEL, the lignin content might have some relationship to immunostimulatory activity.

The second important feature was the content and composition of neutral sugars. Although LREL was found to contain more neutral sugars and uronic acids than PEL, the compositions of neutral sugars in LREL and PEL were comparable. Thus, we believe that, just like the lignin, the molecular structure of polysaccharides in LREL was similar to that of PEL, and the higher content of neutral sugars in LREL might be contributing to its higher activity. As reported previously, the major types of dietary fiber present in the barley husk were β-glucan and arabinoxylan (32, 33). Thus, barley husk contained mainly xylose, arabinose, and glucose and only minor amounts of galactose, mannose, and uronic acids. Characteristically, LREL and PEL were rich not only in xylose and arabinose but also in galactose and mannose. The galactose and mannose residues could have originated from the presence of arabinoxylan and glucomannan (34, 35). As reported previously, arabinoxylan from larch woods showed immunostimulatory activity both in vitro and in vivo (36, 37). Furthermore, Leung et al. (38) demonstrated that the immunopotency of aloe polysaccharides increased as their mannose content increased. Taken together, these results suggested that the enrichment of galactose and mannose residues in LREL and PEL might be important for their immunostimulatory activity.

The third important feature was the presence of ester bonds. Hydroxycinnamic acids are covalently linked to lignin by ester and ether bonds and to polysaccharides mainly by ester bonds (5–7). The ester bonds in LCCs are known to be cleaved by mild alkali treatment (39). As described above, we found that mild alkali-treated LREL could not stimulate DCs. Thus, our results suggested that the ester bonds between lignin and polysaccharides play an important role in the immunostimulatory activity. The molecular weights of lignin monomeric units and neutral sugars are approximately between 150 and 200. Therefore, considering the observed high $M_w$ of LREL and PEL, they may be regarded as highly polymerized biopolymers, the so-called “lignin-carbohydrates.” Because the $M_w$ of LREL was much higher than that of PEL, our results suggested a correlation between the activity and $M_w$. Thus, for the immunostimulatory activity, it is important that the lignin-containing substances contain neutral sugars (especially enriched with galactose and mannose) and ester bonds (linking the lignin molecular to polysaccharides), and it might be also important that the lignin units and neutral sugars are highly polymerized. As described above, MWL and LCC from beech could not activate DCs. MWL is known to be widely used for the structural studies of lignin and to contain mainly lignin and a few carbohydrates (40). In general, LCC is obtained by extracting the solid residue that remained after the extraction of MWL and harbors chemical linkages (such as ester, ether, and glucoside linkages) between the lignin and polysaccharides (41). Thus, the results on the activities of MWL and LCC suggested that only lignin-carbohydrates, extracted by a specific method, such as cellulase/hemicellulase treatment, could stimulate DCs.

Interestingly, LRELs extracted from barley husk as well from the edible parts of various other plants could stimulate DCs. Thus, potent immunostimulators could be extracted from plant sources by cellulase/hemicellulase treatment. Among these LRELs, the gramineae husk LREL exhibited the strongest activity. Because the amount and composition of lignin units vary among taxa and cell types (3), it is possible that the ratio of monomeric building blocks of lignin may have some relevance to the immunostimulatory potency. By analyzing our results on lignin fractions, we identified four important features that are responsible for the activity. We therefore believe that similar analysis of other LRELs from various plant sources could lead to the identification of additional important activity-related features.

The present study is the first report that demonstrated LREL is a novel ligand for TLR4 by using $tlr$-deficient mice. It has been shown earlier that TLR4 uses both MyD88-dependent and TRIF-dependent signaling cascades (19, 20). This may explain why we observed partial abrogation of LREL-induced activation of MyD88$^{-/-}$ DCs. There is also a possibility that not only TLR4 but also other pattern recognition receptors might be involved in mediating the stimulator effect of LREL. We have also excluded the possibility that phagocytosis and endosomal receptors, such as mannose receptor and C-type lectin, were involved in the stimulatory action of LREL. In addition, our results showed that LREL and LPS were different molecules, and our LREL preparation was free from bacterial contamination. We have also shown in Fig. 8 that short term LREL stimulation led to the activation of transcription factors NF-κBp65, c-Jun, and IRF-3, and long term stimulation led to the activation of IRF-7. Activation of these transcription factors was known to be induced by stimulation of TLRs (22). As reported previously, TLR4-mediated IFN-β expression is induced via mainly IRF-3 activation (42, 43). Once the initial activation of IFN genes has occurred, the positive feedback regulation becomes operational, and IRF-7 would be induced by IFN signaling (44). Our observation that short term and long term stimulation by LREL resulted in IRF-3 and IRF-7 activation, respectively, strongly supports the idea noted above. Taken together, we concluded from these results that LREL is a novel TLR4 ligand.
Lignin-Carbohydrate Activates Dendritic Cells via TLR4

Several previous studies have identified novel TLR4Ls from plants. For example, dioscorin, a glycoprotein isolated from Dioscorea alata, was reported to activate macrophages via TLR4 (45). ALLb-A, a 55-kDa protein isolated from Aegirina indica L., was reported to induce anti-tumor immunity via TLR4 (46). These TLR4Ls might be useful as anti-tumor reagents and vaccine adjuvants. However, because these TLR4Ls normally contained proteins, their activities might be lost when they are orally administrated. In this paper, we successfully identified a lignin-carbohydrate as a novel TLR4L. Because lignins hardly get digested in the digestive tract (47), we believe that LREL might be a strong candidate as an oral immunostimulatory reagent.

There are several recent reports describing the physiological activities of lignins. For example, Sakagami et al. (9, 10) investigated the biological activities of lignin-carbohydrate complexes, obtained from the pine cones of Pinus parviflora Sieb et Zucc by extracting with hot water and alkali. They showed that the alkali-extracted fraction had various biological activities. Another study from this research group reported that the alkali-extracted fraction from pine cone contained a lignin-like polyphenolic skeleton and polysaccharides (48). In addition, the alkali-extracted fraction from Lentinus edodes mycelia was shown to induce macrophage activation via dectin-2 (49) but not via TLR4 (50). Because the alkali-extracted fraction from L. edodes mycelia was found to contain lignin structure (51), lignin was believed to be required for the activity. These authors claimed that their lignin-carbohydrate complexes were not related to LPS because they did not act via the TLR4 signaling pathway (52). The following results strongly suggested that their “lignin-carbohydrate complexes” are totally different from LREL, because (i) the activity of LREL was found to be mediated via TLR4 signaling pathway, and (ii) the activity of LREL was completely diminished by mild alkaline treatment.

In conclusion, we demonstrated that the cellulase-treated lignin-carbohydrate, called LREL, is a novel TLR4 ligand. We suggest that LREL could be used as an antibacterial, anticancer reagent and also as a vaccine adjuvant. It is also well known that cellulosytic bacteria are present among the ruminant rumen bacteria (53–55). Thus, barley husk, which is a by-product of the malting and brewing industry, could be utilized as an immunostimulatory feed for the ruminant without any cellulase treatment or any extraction step. We are currently exploring this possibility.

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