Soluble Branched β-(1,4)Glucans from Acetobacter Species Show Strong Activities to Induce Interleukin-12 in Vitro and Inhibit T-helper 2 Cellular Response with Immunoglobulin E Production in Vivo*

Kimika Saito‡, Toshiki Yajima‡‡, Hitoshi Nishimura‡, Keiko Aiba‡, Ryotaro Ishimitsu‡, Tetsuya Matsuguchi‡, Yoshihumi Ohshima†, Yoshinori Tsukamoto†, and Yasunobu Yoshikai‡

From the ‡Division of Host Defense, Research Center of Preventive of Infectious Disease, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, the †Department of Host Defense, Nagoya University School of Medicine, Nagoya 466-8550, and the ¶Centeral Research Institute, Mitsukan Group Co., Ltd., Honda 475-8585, Japan

An extracellular polysaccharide, AC-1, produced by Acetobacter polysaccharogenes, is composed of β-(1,4)glucan with branches of glucosyl residues. We found that AC-1 showed a strong activity to induce production of interleukin-12 p40 and tumor necrosis factor-α by macrophage cell lines in vitro. Cellulase treatment completely abolished the activity of AC-1 to induce tumor necrosis factor-α production by macrophages, whereas treatment of AC-1 with polymyxin B or proteinase did not affect the activity. Results of experiments using Toll-like receptor (TLR) 4-deficient mice and TLR4-transfected human cell line indicated that TLR4 is involved in pattern recognition of AC-1. In vivo administration of AC-1 significantly reduced the serum levels of ovalbumin (OVA)-specific IgE and interleukin-4 production by T cells in responses to OVA in mice immunized with OVA. AC-1, a soluble branched β-(1,4)glucan may be useful in prevention and treatment of allergic disorders with IgE production.

Toll was first identified as a protein that controls dorsoventral pattern formation in the early stage of development of Drosophila (1) and was shown to participate in antimicrobial immune responses (2). Over the years, 10 mammalian Toll homologues, called Toll-like receptor (TLRs),1 have been identified and shown to play important roles in the recognition of various microbial components (3). TLR4, one of the identified TLRs, has been reported to function as a receptor for lipopolysaccharide (LPS), an integral component of the outer membrane of Gram-negative bacteria (4–7). TLR2 reportedly specifies in the recognition of lipoprotein from diverse species of bacteria, including Mycobacterium tuberculosis, Mycoplasma fermentans, Treponema pallidum, and Borrelia burgdorfer (8–15). TLR6 in combination with TLR2 recognizes zymosan and peptidoglycan (16). TLR9 and TLR5 have been shown to recognize bacterially derived CpG DNA (17) and flagellin (18), respectively. TLR3 and TLR7 have been reported to recognize double-stranded RNA and imidazoquinolines, respectively (19, 20). Thus, TLRs have been identified as ancient receptors that confer specificity to the host innate immune system allowing the recognition of pathogen-associated molecular patterns.

β-(1,3)Glucans are major structural components of fungal cell walls that modulate innate immunity in part by macrophage activation in mammals. There have been many reports in which antitumor activities of lentinan (21), schizophyllan (22), and krestin (23), all of which contain branched β-(1,3)glucan, are described. Zymosan, which consists of yeast cell particles, is also one of the strong macrophage activators containing β-(1,3)glucans (24–26). Zymosan has been reported to bind to membrane components such as complement receptor 3, a scavenger receptor, lactosylceramide, and dectin-1 (27, 28). It has been suggested that signals from zymosan are transmitted through heterodimers of TLR2 and TLR6 (15, 16), although zymosan is thought to contain multiple stimulators for macrophages in addition to β-(1,3)glucans. It has recently been reported that curduran, a linear β-(1,3)glucan, stimulates the binding macrophages to pattern-recognition receptors using MyD88 for its signal transduction, although the responsible receptors includes TLRs have not been identified (29). Cellulose β-(1,4)glucan is the predominant polysaccharide in plant cell walls but is also produced by fungi and bacteria such as Acetobacter species (30–32). Hemicellulose derived from soybean hull, presumably containing β-(1,3)-(1,4)glucan, has been reported to stimulate macrophages to produce nitric oxide and interleukin (IL)-1β (33). However, there is no report concerning the immunostimulatory activity of β-(1,4)glucan.

Allergic asthma is a chronic inflammatory disease associated with a predominant T-helper 2 (Th2) cellular response, IgE synthesis, airway infiltration by eosinophils, and bronchial hyperreactivity (34, 35). Naive CD4+ T cells initially stimulated with an allergen in the presence of IL-4 tend to develop into CD4+ T cells that secrete IL-4, IL-5, IL-6, and IL-13 for IgE isotype switching. Th1 cells, into which naive CD4+ T cells preferentially differentiate in the presence of IL-12, IL-18, and interferon-γ (IFN-γ), secrete IFN-γ and TNF-α not only for induction of cell-mediated immunity but also for inhibition of Th2 responses (36–38). Therefore, cytokines involved in Th1-biased response are thought to regulate Th2-mediated allergic response.

Received for publication, May 12, 2003, and in revised form, June 4, 2003
Published, JBC Papers in Press, June 10, 2003, DOI 10.1074/jbc.M304948200
In the present study, we found that a bacterial cellulose AC-1 derived from Acetobacter species was a potent inducer of IL-12 production by macrophages in vitro. AC-1, a β-glucan composed of β-(1,4)glucan with branches of glucosyl residues, has a molecular mass of $1 \times 10^6$ daltons. Polyoxymycin B and proteinase-treated AC-1 stimulated spleen-adherent cells to produce TNF-α, whereas such cytokine production was not observed using proteinase-treated AC-1. Results of experiments using TLR4-deficient mice and TLR4-transfected human cell line indicated that TLR4 is involved in pattern recognition of AC-1. When oral administration of AC-1 was begun immediately after ovalbumin (OVA) immunization, significant decreases in the serum levels of OVA-specific IgE and IgG, accompanied by increased IFN-γ production occurred. These results suggest that AC-1, a potent IL-12 and TNF-α inducer, suppresses allergic inflammation with IgE production, thus offering an approach for the treatment of allergic disorders.

**EXPERIMENTAL PROCEDURES**

**Animals—**C3H/HeN mice, C3H/HeJ mice and BALB/c mice (SLC, Shizuoka, Japan) were used in the experiments at 6–10 weeks of age. Mutant mice (F2 interbred from 129/Ola x C57BL6) with a deficiency in TLR2 gene were obtained by gene targeting by S. Nara (Osaka University, Japan) (4). Age- and sex-matched groups of TLR2-positive (+) mice and their littermate (TLR2−) mice were used for the experiments. These mice were bred in our Institute under specific pathogen-free conditions.

**Purification of AC—**The polysaccharide AC series of the Acetobacter species used in this study were prepared and purified by the method reported in a previous paper (30). Briefly, five strains of polysaccharide-producing Acetobacter species were cultivated in a shaking flask at 30 °C for 5 days, and the cells were removed by both centrifugation (10,000g) of diluted broth and filtration with celite. The cell-free polysaccharides were precipitated by the addition of isopropyl alcohol, and the precipitate was dissolved in water. The insoluble acidic polysaccharide-cetyltrimethylammonium bromide (CTAB) solution was added until no more precipitation was formed. The insoluble acidic polysaccharide-cetyltrimethylammonium bromide complex was collected by centrifugation and redissolved in 20% sodium chloride solution. After dialysis against distilled water and lyophilization, we named these polysaccharides AC series (as follows): AC-1, Acetobacter polysaccharogenes MT-11-2; AC-2, A. polysaccharogenes 1007; AC-3, A. polysaccharogenes 1011; AC-4, Acetobacter xylinum MH-1597; AC-5, A. xylinum 1053). The 10-mg sample was mixed with a final concentration 2N HCl and hydrolyzed at 110 °C for 24 h. LPS was treated in the same manner.

**Analysis with High-performance Anion-exchange Chromatography Coupled with a Pulsed Amperometric Detection—**The polysaccharides from Acetobacter species were subjected to acid hydrolysis using trifluoroacetic acid and high-pH anion-exchange chromatography for removal of protein. AC-1 (5 mg) was incubated with 1 mg of β-(1,4)glucanase (Wako) in 0.05 M acetate buffer (2.5 ml) at pH 5.0 and 37 °C for 24 h. LPS was treated in the same manner. The 10-mg sample was mixed with a final concentration 2 N trifluoroacetic acid containing 10 ml/gucose as an internal standard in a glass tube sealed with a screw cap. After 18 h at 100 °C, solutions were filtered through a stream of nitrogen and the supernatant was dissolved in water and the hydrolysate solutions transferred to autosampler vials. The hydrolysates were stable in water at −20 °C for at least 1 month. Samples were analyzed by high-performance anion-exchange chromatography on a Dionex 500 system (Dionex Corp.) supplied with a pulsed amperometric detector. The system was equipped with a Carbopack A column (packed silica appropriate for C4, C8, C18, and oligosaccharide analysis). Sodium hydroxide solution (NaOH, 250 mM in water) was used as the eluant. Analyses were performed in the isocratic mode (% water% NaOH = 48:52). Flow rate was set to 0.6 ml/min. To minimize carbonate formation in the system, which leads to a dramatic reduction of the retention times, a small amount of NaCl (150 mM) was added to the alkaline eluent. Samples were collected and analyzed on computers equipped with the Dionex PeakNet software.

**Cell Preparation—**All cell lines were grown in tissue culture flasks at 37 °C in 5% CO2, 95% air and passed every 2 or 3 days to maintain logarithmic growth. Two mouse macrophage cell lines, J744.1 and RAW264.7, and a human embryonic kidney cell line, HEK 293 (human embryonic kidney 293), were obtained from the Institute of Physical and Chemical Research Cell Bank (Tsukuba, Japan) and maintained in Dulbecco’s minimum essential medium with 10% fetal bovine serum (JRH Bioscience). Adherent cells from the peritoneal cavity of C3H/HeN, C3H/HeJ, TLR2−/−, or TLR2+ mice were used as a source of peritoneal macrophages. Briefly, peritoneal exudate cells suspended in RPMI 1640 containing 10% fetal bovine serum were plated on plastic plates for 2 h at 37 °C. After nonadherent cells had been removed, fresh complete medium was added to the adherent cells with or without AC-1 or control reagents in the presence of IFN-γ (30 units/ml).

**Isolation of the Full-length cDNA Clone Encoding Mouse TLRs—**Messenger RNA was isolated from RAW264.7 cell using a Quick Prep Micro mRNA Purification kit (Amersham Biosciences). mRNA (1 μg) was treated with DNase prior to reverse transcription-PCR using a Superscript 2 premultiplication system. The resulting templates were subjected to PCR reactions with murine each TLR-specific primers. The primers used were as follows: TLR1 sense, 5′-TGGCGGCGACATTGACTAAACAAAAATCTC-3′; TLR1 antisense, 5′-CGGATCTTCTGTTGTTACACCTCCTCT-3′; TLR2 sense, 5′-TGGCTGAGCCACCTTGAAGGA-3′; TLR2 antisense, 5′-GGACTTTTGTAGTCTCAG-3′; TLR3 sense, 5′-TGGCGGGGCGTCTCAGTTTGACCTC-3′; TLR3 antisense, 5′-CGGATCCTGAGGCTAAATGTCGCTAC-3′; TLR5 sense, 5′-TGCTGGGCAATGCTGAAATGTCTTCCTG-3′; TLR5 antisense, 5′-CAGCCATATTATTATTTTTCTTCTAATG-3′; TLR9 sense, 5′-AGGTTCGACCTGGTCGACGC-3′; TLR9 antisense, 5′-GGACGGAGATCCACCAACAC-3′. Each PCR product was electrophoresed and transferred to and hybridized with murine TLR cDNA as a probe. DNA sequence analysis was performed on these plasmids using a dye-terminating DNA sequencing kit (373A) and a Thermo Sequenase cycle sequencing kit (PE Biosystems).

**Plasmids—**Murine TLR tagged with p3XFLAG at the carboxyl terminus was generated by PCR and ligated into the expression plasmid CMV14 (Sigma). All TLR plasmids used in transfections were purified using an Endo-free plasmid kit (Qiagen). A mouse CD14 expression plasmid, pMD2-NF-B-Luc, has been described previously (39). A carboxyl-terminal FLAG-tagged each TLRs expression plasmid and mouse MD2 expression plasmid were inserted into the whole mTLR and MD-2 coding region cDNA into the p3XFLAG-CMV14 vector.

**TLR Expression by Western Blotting—**Cells were plated in isosin buffer (50 mM Hepes (pH 7.0), 150 mM NaCl, 10 mM glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 100 mM NaF, 10 mM NaPPi, 1 mM NaVO3, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) at a 1 × 106 cells/ml. The lysates were separated in SDS-polyacrylamide gels and then electrotransferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h in 5% nonfat milk-TBST (20 mM Tris-HCl pH 7.6, 0.15 mM sodium chloride, 0.1% Tween 20), incubated with anti-FLAG monoclonal antibody M2 (Sigma) in TBST for 1 h, washed three times with TBST, and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit Ig (Amersham Biosciences) diluted 1:5000 in 5% nonfat milk-TBST. After three washes in TBST, the blots were enhanced with a chemiluminescence detection system (Amersham Biosciences) and analyzed by a lumino-image analyzer (LAS-1000plus, Fujiﬁlm).

**Luciferase Assay—**HEK 293 cells were seeded in 6-well plates at a density of 1 × 105 cells/well 1 day before transfection. HEK 293 cells were transiently transfected with 0.4 μg of pGL3-NF-B-Luc (a luciferase reporter plasmid) and 0.4 μg of pSv-β-galactosidase as an internal control (Promega, Madison, WI), and then 1.0 μg of mTLR plasmid using LipofectAMINE (Invitrogen). At 48 h after transfection, the HEK 293 cells were stimulated with AC-1 for 5 h. The cells were harvested, washed, and lysed in 200 μl of lysis buffer, and the luciferase activity was measured using a luciferase assay kit (Promega/Luciferase Reporter Assay System (Toyo Ink, Tokyo, Japan) or Steady-Glo Luciferase Reporter Assay System (Promega)) according to the manufacturer’s instructions. Background luciferase activity was subtracted.

**Cytokine Measurement—**J744.1 or RAW264.7 cells (5 × 105 cells/ml)
were stimulated with the AC series, and the levels of IL-12 p40 and TNF-α production were determined by using an enzyme-linked immunosorbent assay (ELISA) 24 h after incubation. Adherent cells of PEC from C3H/HeN and C3H/HeJ or TLR2−/− mice were stimulated with AC-1, LPS, or PBS and left overnight and then incubated with peroxidase-conjugated monoclonal antibody. T cells (5 × 10⁵) were cultured in 96-well cell culture plates (Falcon, BD Bioscience) with 0.15 M NaCl, pH 8.6, 100 μg/mL of OVA in 100 μl of PBS and stored at −20°C until analysis.

Measurement of Cytokine Production of Spleen Cells—Spleen cells were incubated on a nylon wool column at 37°C in 5% CO₂ for 60 min. The cell population eluted from the column contained >90% T cells as determined by flow cytometric analysis with anti-CD3 mouse IgE antibody. T cells (5 × 10⁵) and mitomycin C-treated naive spleen cells (5 × 10⁵) were cultured in 96-well cell culture plates (Falcon, BD Biosciences) with 200 μg/mL of OVA. After 48 h of culture, the cytokines produced were determined by ELISA. Commercial ELISA kits were used to measure the levels of IL-4 and IFN-γ (Genzyme (Cambridge, MA), as well as the number of secreting IL-4 and IFN-γ in the supernatants were determined by ELISA. ELISA kits for IL-12 p40 and TNF-α were performed using commercially available kits from Genzyme (Cambridge, MA).

Measurement of OVA-specific IgE, IgG1, and IgG2a—Levels of OVA-specific IgE, IgG1, and IgG2a were determined by ELISA. Sample wells of an ELISA plate were coated with OVA and left overnight and then blocked with 1% bovine serum albumin in borate-buffered saline (0.05 M borate, 0.15 M NaCl, pH 8.6, 100 μM Cu²⁺) for 30 min. Diluted samples (100 μg/well) were incubated for 90 min at room temperature (Samples for IgE, IgG1, and IgG2a were diluted 1:1000, 1:10000, and 1:100000, respectively). The plates were washed with borate-buffered saline with 0.05% Tween 20 and incubated with peroxidase-conjugated anti-rat IgE (GAM/IgE(Fe) PO, Nordic), IgG1 (peroxidase rabbit anti-mouse IgG1, yl-specific, Zymed Laboratories Inc.), or IgG2a (peroxidase rabbit anti-mouse IgG2a, yβ-specific, Zymed Laboratories Inc.) for 90 min at room temperature. After further washing, plates were incubated for 20 min at room temperature with 100 μM of o-phenylenediamine solution (1 μg/ml with 3% H₂O₂), and the OD was read at 492 nm.

Statistical Analysis—The statistical significance of the data was determined by Student’s t test. A p value of less than 0.05 was taken as significant.

RESULTS

Structure of the Polysaccharide AC Series—Five strains of Acetobacter species that produce a new type of extracellular soluble polysaccharide were isolated, and the composition of their carbohydrate components was analyzed using high-performance anion-exchange chromatography with a pulsed amperometric detector. A representative polysaccharide, AC-1, isolated from the culture filtrate of A. polysaccharogenes MT-11-21005 is composed of β-glucose, β-galactose, β-mannose, and β-glucuronic acid in the molar ratio of 3.0:1.0:1.1:1.5. The composition of the carbohydrate components in each sample is shown in Table I. NMR spectroscopy indicated that the dominant β-glycosidic linkages must be in β-configuration (30). To obtain information on the mode of glycosidic linkages, both native and carboxyl-reduced polysaccharides were methylated, and the partially methylated sugars in the acid hydrolysate were analyzed by gas liquid chromatography. The identities and proportions of the cleavage fragments of the native and carboxyl-reduced polysaccharides indicate that polysaccharide AC-1 has a highly branched structure with a repeating unit of 11 sugar residues. It contains a backbone chain of β(1,4)-linked β-glucose, residues and two of the four β-glucose residues are branched at the O-3 position. There are two kinds of side chains; one is terminated with β-glucose residues and the other with β-glucuronic acid residues, as indicated by the increase in approximately 1 mol of tetra-O-methyl-β-glucose in the methylated, carboxyl-reduced polysaccharide. In addition, the polysaccharide contains (1,6)-linked β-glucose, (1,6)-linked β-galactose, and (1,2)-linked β-mannose residues. They are most probably located in the side chains, as revealed by the fragmentation analysis (31). Similar analysis was performed on AC-4 derived from A. xylinum MH-1597. The structural feature of polysaccharide AC-4 resembles that of polysaccharide AC-1 except for the sugar arrangement in the side chains. Thus, AC is a β-glucan composed of β(1,4)-glucan with branches of glucosyl residues (Fig. 1).

Bacterial Cellulose Derived from Acetobacter Species Stimulated Mouse Macrophages to Produce IL-12 p40 and TNF-α—We screened soluble β(1,4)glucans derived from Acetobacter species by measuring the levels of cytokine production in the mouse macrophage cell lines J774.1 and RAW264.7. Among the various preparations, AC-1, -2, -3, -4, and -5, all of which are derived from A. polysaccharogenes, stimulated J774.1 to produce IL-12 p40 (Fig. 2A). AC-1, derived from Acetobacter polysaccharogenes MT-11-2, at a final concentration of 100 μg/ml induced the maximal level of TNF-α production in both mouse macrophage cell lines among the preparations (Fig. 2B and C). RAW264.7 cells did not produce IL-12 p40 in response to AC-1 or LPS (data not shown). AC-1 was used in the following experiments.

Next, an experiment was carried out to determine whether AC-1 induces production of IL-12 p40 and TNF-α by primary culture of macrophages from naive mice. As shown in Fig. 3A and B, AC-1 induced significantly high levels of IL-12 p40 and TNF-α production by peritoneal adherent cells from BALB/c mice. To further purify the polysaccharides from AC-1, we treated AC-1 with protease and subjected them to ion exchange chromatography. Protease-treated products of AC-1 exhibited almost the same level of activity to induce TNF-α production as that of nontreated AC-1 (Fig. 3A, A and B). Thus, the protein moiety of AC-1 is not required for the activity of AC-1. Because Acetobacter species are Gram-negative bacteria containing LPS, we examined the effect of treatment of AC-1 with polymyxin B, which neutralizes LPS activities, on the production of TNF-α by macrophages. As shown in Fig. 3C, polymyxin B inhibited the activity of LPS (100 ng/ml) but not the activity of AC-1. Thus, the possibility that induction of the activity of AC-1 is due to contamination by LPS is ruled out.

Backbone Chain of β(1,4)Glucan Is Important for AC-1 Activity—As shown in Fig. 1, AC-1 has a branched structure containing a backbone chain of β(1,4)-linked β-glucose, two of

| Strain | Glc | Gal | Fuc | Man | Rha | GlcA | ManA | GlcNAc | GalNAc |
|--------|-----|-----|-----|-----|-----|------|------|--------|--------|
| AC-1   | 3.0 | 1.0 | 1.1 | 1.5 | 0.1 | -    | +    | -      | -      |
| AC-2   | 3.0 | 0.5 | 0.5 | 0.3 | -   | -    | +    | -      | -      |
| AC-3   | 3.0 | 1.1 | 1.9 | 0.7 | 0.1 | -    | -    | -      | -      |
| AC-5   | 3.0 | -   | 2.2 | 0.8 | 0.6 | 0.1  | -    | -      | -      |

| Molar ratio of carbohydrate components of polysaccharides AC series |
|---------------------------------------------------------------|
| Rha, rhamnose; GlcA, glucuronic acid; ManA, mannose A. +, detectable; −, undetectable. |

**Table I**

**Immunostimulatory Activities of β(1,4)Glucans**

38573
every four glucose residues being substituted at the O-3 positions to form two kinds of branches. To determine the involvement of β-(1,4)glucose linkage of AC-1 in stimulation of macrophages, AC-1 was treated with β-(1,4)endoglucanase, which degrades polysaccharides possessing β-(1,4)glucan backbones. As shown in Fig. 4A, the ability of AC-1 to induce production of TNF-α by macrophages was abolished by cellulase treatment, whereas the ability of LPS to induce production of TNF-α by macrophages was not affected by the same treatment. We further examined the effect of laminarin, a β-(1,3)glucan antagonist, which partially inhibits binding of β-(1,3)glucan to receptors (40), on the activity of AC-1. When RAW264.7 cells were stimulated with AC-1 in the presence of laminarin, TNF-α production was not inhibited by any dose of laminarin (Fig. 4B). These results suggest that β-(1,4)glucan is required for the stimulatory activity of AC-1.

**TLR4 Is Involved in TNF-α Production by PEC-adherent Cells Stimulated with AC-1**—TLRs have been identified as ancient receptors that confer specificity to the host innate immune system, enabling the recognition of pathogen-associated molecular patterns. We examined the involvement of TLR in the recognition of AC-1 by using TLR-deficient mice. The peritoneal adherent cells from C3H/HeN mice with normal TLR4 (TLR4+/+) were cultured with AC-1, and TNF-α levels in the supernatant were determined by ELISA. The results indicated that the adherent cells produced TNF-α in response to in vitro stimulation with AC-1 in a dose-dependent manner (Fig. 5A and B). We then examined TNF-α production by the peritoneal adherent cells of C3H/HeJ mice with mutated TLR4 (TLR4−/−) to determine whether TLR4 is involved in TNF-α production induced by AC-1. As shown in Fig. 5A, the cells from TLR4−/− mice responded to lipoprotein, a ligand for TLR2, but did not respond to synthetic lipid A or AC-1 to produce a high level of TNF-α, indicating that TLR4 is involved in AC-1-mediated TNF-α production by the adherent cells. We next examined the production of TNF-α in the supernatants of the peritoneal adherent cells from TLR2−/− or TLR2−/− mice co-cultured with AC-1. As shown in Fig. 5B, the cells from TLR2−/− mice did not respond to lipoprotein to produce TNF-α, whereas those from TLR2−/− mice produced TNF-α in response...
to AC-1 as well as lipid A, although to a lesser degree compared with those from TLR2 mice. These data suggest that TLR2 is not involved mainly in AC-1-mediated cellular activation.  

Expression of Mouse TLR4 Conferred AC-1-mediated NF-κB Activation in a Human Cell Line—To confirm the involvement of TLR in AC-1-mediated cellular activation, we inserted the coding region of mTLR cDNA into a mammalian expression plasmid, CMV14, with a carboxyl-terminal FLAG tag. The FLAG-tagged mTLR was transiently expressed in a HEK 293 cell line. The expression of the FLAG-tagged mTLR was confirmed by means of Western blotting. Cells were transiently transfected with 0.1 g of pGL3-NF-κB/Luc. Forty-eight hours after the transfection, cells were stimulated with AC-1, and 8 h later cells were lysed, and the luciferase activity was measured. As shown in Fig. 6A, transfection of the control vector alone did not mediate the induction of AC-1 NF-κB activity, indicating that the parental HEK 293 cells were hyporesponsive to AC-1. When mTLR4 was expressed, AC-1 significantly induced NF-κB activation, whereas the cells expressing other TLRs including 1, 2, 6, 9, 12, or 26 did not respond to AC-1. Coexpression of TLR2 with TLR6 activates NF-κB when stimulated not with AC-1 but with zymosan (Fig. 6B). Polymyxin B-treated AC-1 also induced NF-κB activation in TLR4-transfected HEK 293 cells, again confirming that contamination of LPS is not involved in the AC-1 activities (Fig. 6C).

Effects of Oral Administration of AC-1 on Th2 Response with IgE Production to OVA—To investigate the role of AC-1 in the modulation of immune response in vivo, we examined the effect of oral administration on antibody production in mice immunized with OVA. BALB/c mice were sensitized intraperitoneally with OVA/alum on day 0 and day 12. Mice were orally administered AC-1 or PBS once every 5 days from day 1 to day 14 (Fig. 7A). The serum level of OVA-specific IgE, IgG1, or IgG2a on day 29 after immunization with OVA/alum was measured. The AC-1-treated mice had lower OVA-specific IgE and IgG1 levels in serum than did PBS-treated mice after OVA sensitization. On the other hand, the level of OVA-specific IgG2a was significantly higher in AC-1-treated mice than in PBS-treated mice sensitized with OVA (Fig. 7B).

We next separated T cells from the spleens of mice treated with AC-1 or PBS on day 29 after OVA immunization. As shown in Fig. 7C, CD3+ T cells from PBS-treated mice sensitized with OVA produced IL-4 in response to OVA, whereas the level of IL-4 production was significantly reduced in AC-1-treated mice sensitized with OVA. The level of IFN-γ production in response to OVA was increased in the culture supernatant of spleen T cells from AC-1-treated mice sensitized with OVA.
Immunostimulatory Activities of β-(1,4)Glcans

Fig. 5. AC-1-induced production of TNF-α by macrophages from the PEC of TLR4- or TLR2-deficient mice. Adherent cells from the PEC of C3H/HeN (TLR4+/−) or C3H/HeJ (TLR4−/−) mice (A) or TLR2−/− or TLR2−/− mice (1 × 10^6 cells/ml) (B) were stimulated with AC-1 (10, 50, or 100 μg/ml), lipoprotein (L.P.) (0.1 μg/ml) and lipid A (L.A.) (0.1 μg/ml) in the presence of IFN-γ (30 units/ml), and the culture supernatants were then collected. TNF-α levels in the culture supernatants were determined by means of ELISA. Three independent experiments showed similar results; data are expressed as means ± S.D. of triplicate cultures in a representative experiment. *, **, and ***, significantly different from the value for control mice (p < 0.05, p < 0.005, and p < 0.001, respectively).

Fig. 6. Reconstitution of responses of HEK 293 cells to AC-1 after transfection with murine TLR4. A, HEK 293 cells were co-transfected with NF-κB-luciferase and β-galactosidase reporter plasmids together with the indicated TLR expression constructs; 48 h later transfectants were treated with AC-1 (100 μg/ml) for an additional 8 h, and luciferase activity was measured. B, HEK 293 cells were co-transfected with NF-κB-luciferase and β-galactosidase reporter plasmids together with TLR2 alone or with a complex of TLR2 and TLR6 expression constructs and stimulated with AC-1, zymosan, or lipoprotein (L.P.), and luciferase activity was measured. C, TLR4-transfectants were stimulated with AC-1 or LPS pretreated with polymyxin B (10 μg/ml), and luciferase activity was measured. Three independent experiments showed similar results; data are shown from a representative experiment. *, p < 0.05.

DISCUSSION

We have identified an active substance derived from A. poly saccharogenes that shows activity to induce production of TNF-α and IL-12 p40 by macrophages. AC-1 is a soluble β-glucan composed of β-(1,4)glucan with branches of glucosyl residues and has a molecular mass of 1 × 10^6 daltons. Our results suggest that the β-(1,4)glucan backbone of AC-1 is important for the activation of macrophages. The production of TNF-α by peritoneal adherent cells from TLR4-deficient mice in response to AC-1 was significantly impaired, and the biological response of HEK 293 cells to AC-1 was reconstituted by transfection with murine TLR4. These results suggest that TLR4 is at least partly involved in the cellular response to AC-1 containing soluble β-(1,4)glucan with branches of glucosyl residues produced by Acetobacter species.

Toll was first identified as a protein that controls dorsal-ventral pattern formation in the early stage of development of Drosophila (1) and was shown to participate in antimicrobial immune responses (2). Recently, several mammalian Toll homologues have been identified and have been shown to play important roles in the recognition of various bacterial components (3). Among them, TLR4 has been shown to recognize LPS derived from Gram-negative bacteria, whereas TLR2 recognizes peptidoglycan and lipopeptide derived from Gram-positive bacteria and zymosan containing β-(1,3)glucan produced by yeast (4–10,16). TLR9 has recently been shown to play a critical role in the recognition of bacteria-derived CpG DNA (17). Although the functions of other TLRs are not known, it has been shown that TLR6 or TLR1 can form a signaling complex with TLR2 (41). The results of the present study showed that production of TNF-α in response to AC-1 normally occurs in spleen adherent cells from TLR2-deficient mice. Furthermore, HEK 293 cells transfected with TLR2 alone or TLR2 together with TLR1 or TLR6 did not respond to AC-1. The activity of AC-1 was not affected by treatment with protease, thus ruling out the possibility of contamination by the biologically active protein moiety in AC-1. Furthermore, the cellular response to AC-1 is not due to LPS contamination because polymyxin B did not inhibit the activity of AC-1 but did inhibit the activity of LPS in a dose-dependent manner. Taken together, TLR4 is a receptor for pattern recognition of AC-1.

β-Glucans are major structural components of fungal cell walls that modulate innate immunity in part by macropage activation in mammarys. There have many reports in which immunostimulation of fungal cell walls that contain β-(1,3)glucan has been described (21–23). Zymosan, which consists of β-(1,3)glucans (25, 26, 43), has been reported to bind to membrane components such as complement receptor 3, a scavenger receptor, lactosylceramide, and dectin-1 (27, 28). It has been suggested that zymosan transmits signals through heterodimers of TLR2 and TLR6 (15, 16), although zymosan is thought to contain multiple stimulators for macrophages in
Three independent experiments showed similar results; data are expressed as means ± S.D. of five mice in a representative experiment. ** and *** are significantly different from the value for PBS (p < 0.01, p < 0.001, respectively). T cells of spleens from mice orally given AC-1 or PBS were cultured in 96-well culture plates with 200 μg of OVA for 48 h. The levels of IL-4 and IFN-γ in the culture supernatant were measured by ELISA. Three independent experiments showed similar results; data are expressed as means ± S.D. of five mice in a representative experiment. ***, significantly different from the value for PBS (p < 0.001).

In the present study, we found that oral administration of AC-1 to mice significantly inhibited the production of IgE and IgG1, whereas it augmented IgG2a production following OVA immunization. The inhibition of IgE and IgG1 production is mainly due to insufficient induction of Th2 response, whereas the augmentation of IgG2a production is because of increased Th1 response producing IFN-γ. Naïve CD4+ T cells initially stimulated in the presence of IL-12 tend to develop into CD4+ Th1 cells that produce IFN-γ, which are not only for induction of cell-mediated immunity against intracellular parasites and tumor but are also for inhibition of Th2 responses causing allergic diseases (36–38). Therefore, AC-1-induced cytokines involved in Th1-biased response are thought to regulate Th2-mediated allergic response. Induction of IL-12 production by macrophages/dendritic cells via TLR4 signaling may be a major mechanism by which AC-1 promotes Th1-biased response to OVA. AC-1 derived from Acetobacter species may have prophylactic and therapeutic applications not only for controlling allergic diseases with dominant Th2 responses but also for preventing tumor development and infection of which protective mechanisms mainly depend on Th1 responses.

In conclusion, we have found that soluble branched β-(1,4)glucans from A. polysaccharogenes show strong activities to induce the production of IL-12 p40 and TNF-α in vitro and inhibit Th2 response with IgE production in vivo. TLR4 is required for cellular responses to AC-1. AC-1 may be useful for the prevention of allergy responses.

Acknowledgments—We thank Dr. S. Akira (Institute for Microbial Disease, Osaka University) for kindly providing TLR2−/− mice, Keiko Itano, Ayumi Nishikawa, and Eriko Nagasawa for technical assistance, Yasuko Yoshikawa for advice, and Ono Pharmaceuticals Co. Ltd. for providing synthetic lipid A (Ono-4007).

REFERENCES

1. Belvin, M. P., and Anderson, K. V. (1996) Annu. Rev. Cell Dev. Biol. 12, 393–416
2. Lammers, B., Nicolas, E., Michaut, L., Reichhart, J. M., and Hoffmann, J. A. (1996) Cell 86, 973–983
3. Rock, F. L., Hardiman, G., Timans, J. C., Kastelein, R. A., and Bazan, J. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 588–593
4. Takeuchi, O., Hoshino, K., Kawai, T., Sanjo, H., Ogawa, T., Takeda, K., and Akira, S. (1999) Immunol. 11, 443–451
5. Poloruka, A., Ho, X., Smirnova, I., Liu, M., Huffel, C. V., Du, X., Birdwell, D., Ajees, K., Silva, M., Galanos, C., Freudenberg, M., Castagnoli, P., Layton, B., and Beutler, B. (1998) Science 282, 2085–2088
6. Poloruka, A., Ricciardi-Castagnoli, P., Citterio, S., and Beutler, B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 2163–2167
7. Hoshino, K., Takeuchi, O., Kawai, T., Ogawa, T., Takeda, K., and Akira, S. (1999) J. Immunol. 162, 3749–3752
8. Brightwell, D. H., Drobny, D., Hultkrantz, S. R., Yang, R. B., Belisle, J. T., Blehrarski, J. R., Maitland, M., Norgard, M. V., Pleym, S. E., Smale, S. T., Brennan, P. J., Bloom, B. R., Godowski, P. J., and Modlin, R. L. (1999) Science 285, 732–736
9. Michel, T., Reichhart, J. M., Hoffmann, J. A., and Royet, J. (2001) Nature 414, 756–759
10. Lien, E., Sellati, T. J., Yoshimura, A., Flo, T. H., Rawadi, G., Finberg, R. W., Carroll, J. D., Espevik, T., Davis, R. B., Radolf, J. D., and Golenbock, D. T. (1999) J. Biol. Chem. 274, 33419–33425
11. Thoma-Uasyinski, S., Stenger, S., Takeuchi, O., Ochoa, M. T., Engele, M., Sieling, P. A., Barnes, P. F., Rollinghoff, M., Bolecki, P. L., Wagner, M., Akira, S., Norgard, M. V., Belisle, J. T., Godowski, P. J., Bloom, B. R., and Modlin, R. L. (1999) Science 285, 1544–1547
12. Nishiguchi, M., Matsumoto, M., Takao, T., Hoshino, M., Shimonishi, Y., Toyoshima, K., and Seya, T. (2000) J. Immunol. 164, 554–557
13. Takeuchi, O., Kaufmann, A., Grote, K., Kawai, T., Hoshino, K., Mohr, M., Muhlradt, P. F., and Akira, S. (2000) J. Immunol. 164, 2382–2386
14. Hirschfeld, M., Kirschning, C. J., Schwendner, R., Weehe, H., Weis, J. H., Wooten, R. M., and Weis, J. J. (1999) J. Immunol. 163, 2382–2386

Immunostimulatory Activities of β-(1,4)Glucans

Fig. 7. Immunization and challenge. A, immunization protocol. BALB/c mice were sensitized intraperitoneally with OVA/alum on day 0 and day 12. Mice were administered AC-1 or PBS orally once every 5 days from day 1 to day 14. Then the levels of OVA-specific IgE, IgG1, and IgG2a production in the serum and Th1/Th2 responses of spleen T cells were determined on day 29 after immunization with OVA/alum. Serum and IgG2a production in the serum and Th1/Th2 responses of spleen T cells were determined on day 29 after immunization with OVA/alum. Serum and IgG2a production in the serum and Th1/Th2 responses of spleen T cells were determined on day 29 after immunization with OVA/alum.

B. Analysis of OVA-specific IgE, IgG1, and IgG2a in serum of mice orally given AC-1 (black bars) or PBS (white bars) followed by intraperitoneal immunization with OVA in alum. Serum samples were obtained from five mice/group and tested individually. Three independent experiments showed similar results; data are expressed as means ± S.D. of five mice in a representative experiment. ** and *** are significantly different from the value for PBS (p < 0.01, p < 0.001, respectively). T cells of spleens from mice orally given AC-1 or PBS were cultured in 96-well culture plates with 200 μg of OVA for 48 h. The levels of IL-4 and IFN-γ in the culture supernatant were measured by ELISA. Three independent experiments showed similar results; data are expressed as means ± S.D. of five mice in a representative experiment. ***, significantly different from the value for PBS (p < 0.001).

C. IL-4 and IFN-γ production following OVA immunization. The inhibition of IgE and IgG1 production is mainly due to insufficient induction of Th2 response, whereas the augmentation of IgG2a production is because of increased Th1 response producing IFN-γ. Naïve CD4+ T cells initially stimulated in the presence of IL-12 tend to develop into CD4+ Th1 cells that produce IFN-γ, which are not only for induction of cell-mediated immunity against intracellular parasites and tumor but are also for inhibition of Th2 responses causing allergic diseases (36–38). Therefore, AC-1-induced cytokines involved in Th1-biased response are thought to regulate Th2-mediated allergic response. Induction of IL-12 production by macrophages/dendritic cells via TLR4 signaling may be a major mechanism by which AC-1 promotes Th1-biased response to OVA. AC-1 derived from Acetobacter species may have prophylactic and therapeutic applications not only for controlling allergic diseases with dominant Th2 responses but also for preventing tumor development and infection of which protective mechanisms mainly depend on Th1 responses.

In conclusion, we have found that soluble branched β-(1,4)glucans from A. polysaccharogenes show strong activities to induce the production of IL-12 p40 and TNF-α in vitro and inhibit Th2 response with IgE production in vivo. TLR4 is required for cellular responses to AC-1. AC-1 may be useful for the prevention of allergy responses.

Acknowledgments—We thank Dr. S. Akira (Institute for Microbial Disease, Osaka University) for kindly providing TLR2−/− mice, Keiko Itano, Ayumi Nishikawa, and Eriko Nagasawa for technical assistance, Yasuko Yoshikawa for advice, and Ono Pharmaceuticals Co. Ltd. for providing synthetic lipid A (Ono-4007).
