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Adjuvant Effects for Oral Immunization Provided by Recombinant *Lactobacillus casei* Secreting Biologically Active Murine Interleukin-1β[▽]

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Vaccine delivery systems using lactic acid bacteria are under development, but their efficiency is insufficient. Autologous cytokines, such as interleukin-1β (IL-1β), are potential adjuvants for mucosal vaccines and can be provided by recombinant lactic acid bacteria. The aim of this study was the construction and evaluation of recombinant *Lactobacillus casei* producing IL-1β as an adjuvant delivery agent. The recombinant strain was constructed using an expression/secretion vector plasmid, including a mature IL-1β gene from mouse. The biological activity of the cytokine was confirmed by IL-8 production from Caco-2 cells. In response to the recombinant *L. casei* secreting IL-1β, expression of IL-6 was detected *in vivo* using a ligated-intestinal-loop assay. The release of IL-6 from Peyer's patch cells was also detected *in vitro*. Intragastric immunization with heat-killed *Salmonella enterica* serovar Enteritidis (SE) in combination with IL-1β-secreting lactobacilli resulted in relatively high SE-specific antibody production. In this study, it was demonstrated that recombinant *L. casei* secreting bioactive murine IL-1β provided adjuvant effects for intragastric immunization.

At present, a number of vaccines based on lactic acid bacteria (LAB vaccines) are under development (31). These commensals, including *Lactococcus lactis* and *Lactobacillus* strains, have been used as vaccine delivery vectors. Several LAB vaccines, such as tetanus toxin fragment C (TTFC)-producing LAB, exhibit high immunogenicity and can induce both mucosal and systemic immune responses, as well as protective immunity (11, 22). However, most LAB vaccines administered via the mucosal route, especially the oral or intragastric (i.g.) route, exhibit relatively low efficiency. As expected, a high dosage is required to elicit effective immunity. It is usually required that mice receive more than 10^9 CFU of bacteria on three or more consecutive days with two or more boosts (4, 5, 16). In several cases, LAB vaccines were administered in combination with a supplemental adjuvant to induce sufficient immune responses (3, 32). In order to improve the efficiency of vaccination, several kinds of additional factors that assist LAB vaccines have been investigated. Steidler et al. carried out a pioneering study that demonstrated the adjuvant effect provided by *L. lactis* expressing TTFC intracellularly and also secreting either murine interleukin-2 (mIL-2) or mIL-6 (28). In another report, a single-chain murine IL-12 was produced by *L. lactis* and enhanced antigen-specific Th1 cytokine production (2). Recently, a unique strategy to accelerate bacterial uptake by dendritic cells (DCs) was developed by Mohamadzadeh et al. (18). They achieved effective oral vaccination using recombinant *Lactobacillus acidophilus* secreting a protective antigen of *Bacillus anthracis* in combination with a DC-targeted peptide. Although all these adjuvant molecules are promising, there are still only a few options for their use as mucosal adjuvants at present. Hence, exploration of other mucosal adjuvants that are applicable to LAB vaccines is important.

The present study investigated IL-1β as a mucosal vaccine. IL-1β is produced by activated monocytes and macrophages, etc., as a precursor that is proteolytically processed into a mature form by IL-1β-converting enzyme (ICE), also known as caspase 1 (6). This proinflammatory cytokine and other IL-1 family cytokines play important roles in modulating the adaptive immune response (7). In fact, a deficiency of IL-1β impairs T-cell-mediated cellular immune responses (26, 29). It was reported previously that IL-1β exhibited adjuvant effects in both mucosal and systemic immunization (27). In addition, Nakae et al. demonstrated that IL-1 enhanced T-cell-dependent antibody production (19). This evidence suggests that IL-1β may be a promising adjuvant for mucosal immunization if it is produced by recombinant lactic acid bacteria. Unlike other mucosal adjuvants, such as cholera toxin, IL-1β is an autologous protein and therefore nonimmunogenic. This may be a preferable feature, because adaptive immunity against adjuvant proteins induced by repeated doses may reduce the adjuvant effect in further immunizations. The aim of this study was to construct an IL-1β-secreting *Lactobacillus* and to evaluate its adjuvant effect.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Lactobacillus casei* IGM393 and a nonexpressing control strain carrying pLPEmpty (LCN) were grown in de Mann-Rogosa-Sharpe (MRS) medium (Difco) at 37°C (14). Erythromycin (5 μg/ml) was used as a supplement for the culture of recombinant lactobacilli. Bicarbonate buffer (50 mM; pH 7.0 to 8.0) was also added to the bacterial culture in some experiments to control the pH of the media. *Salmonella enterica* serovar Enteritidis (SE) no. 40 was grown in Luria-Bertani (LB) broth (Difco) at 37°C under aerobic conditions (1).

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TABLE 1. Cytokine-specific primer pair sequences used in PCR

Gene	Sense primer	Antisense primer	Size[a]	Reference
IL-1β	TCATGGGATGATGATGATAACCTGCT	CCCATACTTTAGGAAGACACGGATT	502	30
IL-2	TGATGGACCTACAGGAGCTCCTGAG	GAGTCAAATCCAGAACATGCCGCAG	167	30
IL-4	CGAAGAACACCACAGAGAGTGAGCT	GACTCATTCATGGTGCAGCTTATCG	180	30
IL-5	GTGAAAGAGACCTTGACACAGCTG	CACACCAAGGAACTCTTGCAGGTA	290	10
IL-6	CTGGTGACAACCACGGCCTTCCCTA	ATGCTTAGGCATAACGCACTAGGTT	600	30
IL-10	ACCTGGTAGAAGTGATGCCCCAGGCA	CTATGCAGTTGATGAAGATGTCAAA	237	30
IL-12 p35	GCAAGAGACACAGTCCTGGG	TGCATCAGCTCATCGATGGC	618	30
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG	243	30
TNF-α	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG	307	30
TGF-β	TGGACCGCAACAACGCCATCTATGAG	TGGAGCTGAAGCAATAGTTGGTATCCAG	502	23
MIP-2	GCCAGTGAGCTGCGCTGTCAGTGC	GTTAGCCTTGCCTTTGTTCAGTATG	221	33
β-Actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG	348	30

[a] Predicted size of the PCR product (base).

Plasmid construction and transformation. For expression of murine IL-1β in *L. casei*, a plasmid was constructed from pIGM2, which was established in our previous study (13). Although the plasmid already included a promoter from *Lactobacillus brevis slpA* (*slpAp*) and a secretion signal of *L. casei prtP* (SS*prtP*) for heterologous expression and secretion, minor modification of the sequence was required in order to express the proper size of IL-1β. DNA fragments consisting of *slpAp*, SS*prtP*, and the IL-1β gene were generated by an overlap PCR technique. Total RNA was prepared from the spleen of a BALB/c mouse, followed by reverse transcription (RT) using Retroscript (Ambion). The IL-1β fragment was amplified from the total cDNA using primers IGM617 (GCG AAA TCC AAG CAA AGG CGG TTC CCA TTA GAC AAC TGC A) and IGM603 (CCC CCT CGA GCC GGC TTA GGA AGA CAC GGA TTC C). A DNA segment from *slpAp* to SS*prtP* was prepared by PCR with pIGM2 as the template and with primers IGM289 (CCC AAG CTT AGA TCT GAT TAC AAA GGC TTT AAG CAG G) and IGM618 (TGC AGT TGT CTA ATG GGA ACC GCC TTT GCT TGG ATT TCG C). The two resulting fragments were then connected by PCR with IGM289 and IGM603. The expression cassette of IL-1β was digested with BglII and XhoI and inserted into the same restriction site of pIGM2. Transformation of *L. casei* was performed by the method described previously (21).

Immunoblotting. Overnight cultures of recombinant lactobacilli were separated into bacterial cells and culture supernatants by centrifugation. The bacterial cells were washed with 50 mM Tris (pH 8.0) and treated with 5 mg/ml lysozyme and 20 U/ml of mutanolysin for 30 min. The spheroplasts were washed with 0.3 M sucrose in 50 mM Tris buffer, dissolved in Laemmli sample buffer, and boiled for 10 min. The culture supernatants were concentrated 50-fold using trichloroacetic acid, dissolved in Laemmli sample buffer, and boiled for 5 min. These samples were applied for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene fluoride filter. The blot was conjugated with anti-mouse IL-1β (Peprotech Inc.) and horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Sigma). IL-1β-specific bands were visualized with ECL-plus (GE Healthcare) using a ChemiDoc system (Bio-Rad).

Quantitative determination of mIL-1β. Prewarmed MRS medium supplemented with erythromycin and bicarbonate buffer (MRSCE) (final pH, 7.0, 7.5, or 8.0) was inoculated with washed cells from 1/10 volume of overnight culture of recombinant lactobacilli. After 5 h of incubation, the culture supernatants were collected by centrifugation and sterilized using a 0.45-μm-pore filter (Millipore). The concentration of IL-1β in each culture supernatant was determined using an mIL-1β enzyme-linked immunosorbent assay (ELISA) kit (Biosource) in accordance with the manufacturer's instructions.

Caco-2 cell culture and stimulation with IL-1β. Caco-2 cells, purchased from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 incubator. A single-cell suspension was prepared by trypsin-EDTA treatment and seeded in a 98-well culture plate (2×10^4 cells/well). After overnight incubation, the culture supernatant of recombinant lactobacilli or purified recombinant murine IL-1β (Peprotech) was added to the Caco-2 culture, followed by an additional 16 h of incubation. The culture supernatants were then collected, and the amount of human IL-8 was determined using an OptEIA ELISA kit (BD Biosciences).

Mice. Female BALB/c mice and C3H/HeJ mice (8 to 10 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The care and use of experimental animals complied with the Principal Law on Animal Experimentation of the National Institute of Health Sciences of Japan.

Ligated-intestinal-loop assay. BALB/c mice starved overnight were injected intraperitoneally (i.p.) with 0.1 ml of pentobarbital (80 mg/kg of body weight). The abdomen was incised, and the ileum was ligated with surgical thread approximately 0.5 cm each side of one Peyer's patch (PP). Recombinant lactobacilli (1×10^9 CFU/ml) suspended in MRSCE medium (pH 7.0) were injected into the ligated intestinal loop containing a single PP. The abdomen was then closed by sewing, and the mice were kept for 4 h before sacrifice. The loop was collected and washed extensively with phosphate-buffered saline (PBS). The single PP and a small piece of non-PP lamina propria (LP) in the loop were isolated and transferred to lysis buffer for RNA preparation. Total RNA was isolated using an SV Total RNA isolation system (Promega) in accordance with the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop ND-1000.

RT-PCR. Total cDNA was prepared from 1 μg of RNA using Retroscript. PCR was then performed to detect IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p35, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), macrophage inflammatory protein 2 (MIP-2), and β-actin. The sequences of primers are shown in Table 1. The reaction conditions were 30 cycles of 94°C for 25 s, 50°C for 15 s, and 72°C for 40 s. The PCR products were applied to 2% agarose gel electrophoresis and visualized with ethidium bromide using a ChemiDoc gel documentation system.

Preparation and stimulation of PP cells. A single-cell suspension of PP was prepared from BALB/c mice in accordance with a protocol described previously (24). PP cells were prepared using collagenase type 1 (Sigma-Aldrich). The PP cells were then separated into $CD11c^+$ and $CD11c^-$ cells using a Magnetic Cell Separation (MACS) system (Miltenyi Biotec) in accordance with the manufacturer's instructions. The $CD11c^+$ and $CD11c^-$ cells were suspended in RPMI 1640 medium (Sigma) supplemented with 10% FCS and seeded in a 96-well microplate. The stimulants, which were recombinant *L. casei* cells and cell-free MRS culture supernatants, were added to the wells and incubated for 24 h. The culture supernatants of the PP cell cultures were collected and analyzed by ELISA. The quantity of IL-6 was measured using a mouse IL-6 ELISA kit (Biosource).

Immunization of mice. C3H/HeJ mice were previously optimized for immunization against SE (14). The mice were immunized i.g. with a mixture of 5×10^9 CFU/mouse of heat-killed SE (HKSE) and 5×10^9 CFU/mouse of viable recombinant *L. casei*. HKSE was prepared from an overnight culture. SE cells were collected, washed gently, and suspended in distilled water. The suspension was incubated at 60°C for 30 min and then freeze-dried. Complete inactivation of SE was confirmed by LB plate culture for a week. Recombinant *L. casei* for administration was prepared from overnight cultures. Prior to immunization, the lactobacilli were incubated in MRSCE (pH 8.0) for 1 h to enhance mIL-1β expression and then resuspended in fresh MRS supplemented with bicarbonate buffer (MRSC) (pH 8.0). A mixture of HKSE and recombinant lactobacilli was prepared by simple mixing of both suspensions in MRSC (2.5×10^{10} CFU/ml each; 0.2 ml/dose). Three consecutive daily doses were performed 3 times at 2-week intervals. The mice were sacrificed 2 weeks after the last immunization, and blood and feces were collected. Sera were prepared from blood samples by centrifugation. Fecal extracts were prepared as cleared supernatants of suspended feces in PBS (1:10). Both samples were stored at −20°C until they were used.

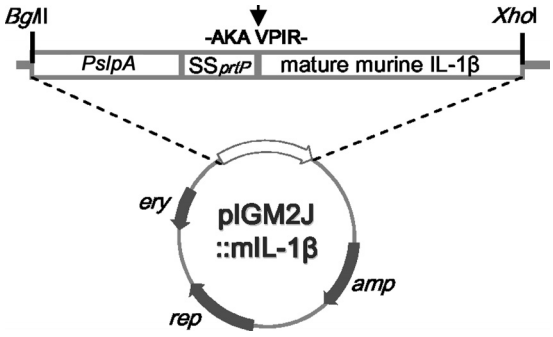

FIG. 1. Feature map of pIGM2J::mIL-1β. The structure, expression cassette, restriction sites, and cleavage point of the fusion protein (arrow) are shown. Genes: *amp*, ampicillin resistance; *rep*, replication protein; *ery*, erythromycin resistance.

Detection of SE-specific antibodies. SE-specific IgG in serum and IgA in fecal extracts were detected by ELISA. HKSE cells were homogenized in PBS using a FastPrep bead beater (Bio 101), and the cleared lysate was collected by centrifugation. The concentration of protein in the HKSE lysate was determined by a Bradford protein assay (Bio-Rad). A microtiter plate was coated overnight with 5 μg/ml (as a protein solution) of HKSE lysate. Properly diluted samples were then added to each well and incubated for 2 h. HRP-conjugated anti-mouse IgG (or IgA) was added and incubated for 1 h. A TMB Substrate Reagent Set (BD Biosciences) was used for color development, and 2 N H_2SO_4 was added to stop the reaction. The optical density at 450 nm was measured using a microplate reader.

Statistical analysis. Statistical significance ($P < 0.05$) was determined by Tukey's multiple-analysis test.

RESULTS

Secretion of IL-1β from recombinant *L. casei*. The ligated plasmid pIGM2J::m IL-1β, shown in Fig. 1, was introduced directly into *L. casei* by electroporation. The production of murine IL-1β by recombinant *L. casei* was confirmed by immunoblotting. As shown in Fig. 2, IL-1β-specific bands were detected in both cell extracts and the culture supernatants of recombinant *L. casei* (KJ725). A single band corresponding to the size of IL-1β fused to a signal peptide appeared in the lane of the cell extract of KJ725, while two bands were observed in the lane of the culture supernatant. The smaller band seemed to be the same size as purified IL-1β. No signal was detected from the control strain. The influence of the pH on IL-1β secretion was also determined. Approximately 1 μg/ml of IL-1β was secreted if the initial pH of the medium was between 7.0 and 8.0 (Table 2). However, only one-third of the IL-1β was detected without pH control.

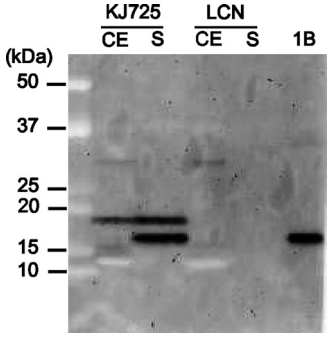

FIG. 2. Detection of IL-1β-specific bands by immunoblotting. Lysozyme-treated cell extracts (CE), concentrated culture supernatants (S), and 10 ng of purified IL-1β (1B) were applied to SDS-PAGE and immunoblotting. Anti-IL-1β antibody was used at 1:5,000, and HRP-conjugated anti-rabbit IgG was used at 1:50,000. Molecular masses are shown on the left.

TABLE 2. Yield of IL-1β in KJ725 culture with and without buffer

Culture condition	IL-1β yield (μg/ml)[a]
No buffer (pH 6.3)	0.326
Buffer (pH 7.0)	1.032
Buffer (pH 7.5)	0.966
Buffer (pH 8.0)	1.116

[a] Mean value of triplicate cultures.

Biological activity of the recombinant IL-1β. The biological activity of the recombinant IL-1β produced by KJ725 was determined by stimulation of the Caco-2 cells. Dose-dependent IL-8 release induced by IL-1β was observed in a Caco-2 cell culture stimulated with the supernatant of KJ725 (Fig. 3). Despite the same amount of IL-1β being used, the release of IL-8 by stimulation with KJ725 was less than that with purified IL-1β. No IL-8-inducing activity was detected by stimulation of Caco-2 cells with the culture supernatant of LCN.

Detection of cytokine expression by ligated-intestinal-loop assay. In order to evaluate the immunological impact of KJ725 *in vivo*, a ligated-intestinal-loop assay was performed. Preliminary experiments indicated that over 10^9 CFU/ml of bacterial suspension induced cytokine expression stably, and thus, it was applied in this study. The same experiment was repeated to normalize the results. Eleven kinds of cytokine expression in PP and LP were analyzed by RT-PCR. Specific bands of IL-1β, IL-6, IL-12, TNF-α, and TGF-β, with β-actin as an internal standard, were constantly detected in PP cells, while no bands, except for the band of β-actin, were detected in LP cells (Fig. 4).

Production of IL-6 by PP CD11c$^+$ cells. PP cells were separated into CD11c$^+$ cells and CD11c$^-$ cells using MACS. Each cell type was stimulated with the KJ725 cell or culture supernatant. As shown in Fig. 5, IL-6 was efficiently produced by

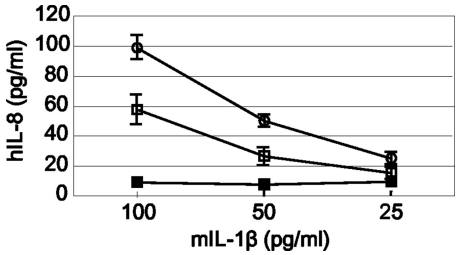

FIG. 3. Biological activity of recombinant murine IL-1β produced by KJ725. Human IL-8 released from Caco-2 cells was measured after 24 h of stimulation with culture supernatant of KJ725 (empty squares) or LCN (filled squares) or with purified murine IL-1β standard (empty circles). The concentration of total murine IL-1β in the culture supernatant of KJ725 (in MRSEC medium) was determined by ELISA and then adjusted to 100, 50, and 25 pg/ml by dilution in complete RPMI medium. The corresponding volume of culture supernatant of LCN was added to the Caco-2 cell culture as a negative control. The result shown is representative of three separate experiments; the values are the means ± standard deviations (SD) of duplicate samples.

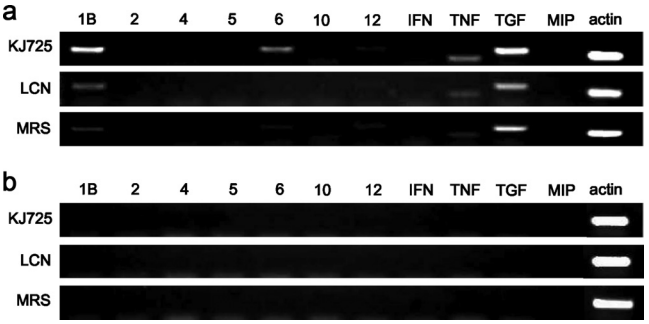

FIG. 4. Cytokine expression induced by ligated-intestinal-loop assay using RT-PCR. PCR products were applied to 2% agarose gels. Eleven kinds of cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p35 [lanes 1B to 12]; IFN-γ [IFN]; TNF-α [TNF]; TGF-β [TGF]; and MIP-2 [MIP]) expressed in PP cells (a) and LP cells (b) were detected by RT-PCR using specific primer pairs. As an internal control, a DNA fragment of β-actin (actin) was also amplified. The data represent at least three separate experiments.

CD11c$^+$ cells in response to both KJ725 cells and culture supernatant. Meanwhile, a low level of IL-6 was induced by stimulation with LCN cells and culture supernatant compared with KJ725 cultures (Fig. 6).

Enhancement of antibody production by IL-1β-producing *L. casei*. To evaluate the adjuvant effect of KJ725, i.g. immunization of mice was performed. HKSE was used as an immunogen and administered in combination with either KJ725 or LCN. As shown in Fig. 7, SE-specific antibodies were induced by i.g. immunization with HKSE. In the group that received HKSE and KJ725, the highest level of SE-specific IgG ($P < 0.05$ compared with the HKSE and LCN group or the MRS group) and IgA ($P < 0.05$ compared with the MRS group) was detected in their sera and feces.

DISCUSSION

The delivery of vaccine antigens by live bacterial carriers, both pathogenic and commensal bacteria, has been studied (17). Compared with vaccine delivery systems using attenuated pathogens, vaccines based on lactic acid bacteria require high doses, a high frequency of immunization, and highly immunogenic antigens for successful induction of protective immunity. For instance, *Lactobacillus plantarum* expressing *Borrelia burgdorferi* OspA was administered at 4×10^{10} cells in four consecutive daily doses with additional priming and two boosts (total, 16 days; 6.4×10^{11} cells), while an attenuated *Salmonella enterica* serovar Typhimurium producing OspA was inoculated at 10^8 organisms with a total of five weekly doses (total, 5 days; 5×10^8 cells) (5, 8). In this context, it is important to provide supplemental adjuvant effects for the development of vaccine using lactic acid bacteria. For this purpose, recombinant *L. casei* secreting mature murine IL-1β was constructed. Using the secretion system developed in our previous study, recombinant IL-1β conjugated with signal peptide was produced intracellularly, and a mature form of IL-1β was then secreted extracellularly. The results of immunoblotting indicated that the secretion system worked properly. At the same time, a considerable part of the extracellular IL-1β remained

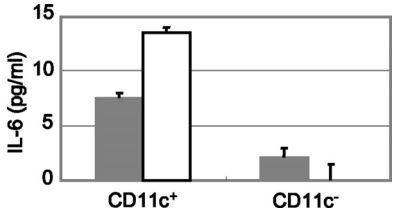

FIG. 5. IL-6 release by PP CD11c$^+$ or CD11c$^-$ cells. MACS-separated CD11c$^+$ or CD11c$^-$ cells (5×10^4 cells/well) were stimulated with KJ725 cells (solid bars; 5×10^5 CFU/well) or culture supernatant (open bars; 0.5%). After 24 h of incubation, samples were collected and released IL-6 was detected by ELISA. The results shown are representative of three separate experiments; the values are means and SD.

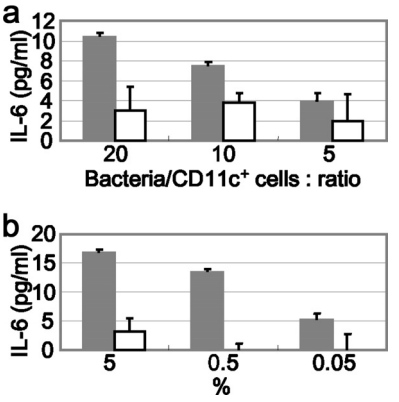

FIG. 6. IL-6 released from PP CD11c$^+$ cells. MACS-separated CD11c$^+$ cells (5×10^4 cells/well) were stimulated with recombinant *L. casei* cells (bacteria/CD11c$^+$ cell ratios, 20, 10, and 5) (a) or culture supernatant (5%, 0.5%, and 0.05% diluted in RPMI 1640 medium) (b). After 24 h of incubation, samples were collected and released IL-6 was detected by ELISA. The results are representative of three separate experiments; the values are means and SD. Solid bars, KJ725 cells/supernatants; open bars, LCN cells/supernatants.

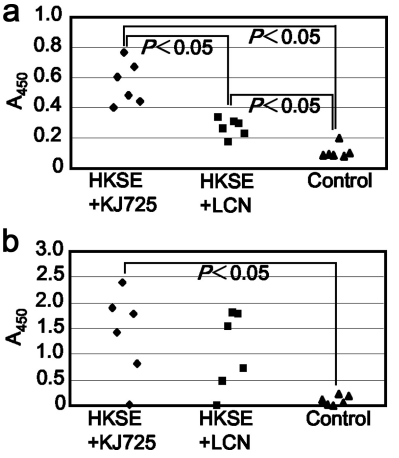

FIG. 7. SE-specific antibody production induced by HKSE with either KJ725 or LCN. Anti-SE IgG in 100-fold-diluted serum (a) and anti-SE IgA in 50-fold-diluted cecum lavage fluid (b) were detected by ELISA. Each value represents the absorbance at 450 nm (A_{450}). The immunization groups are shown at the bottom. Statistical significance was accepted at $P < 0.05$. The control was a group that received MRSC medium alone.

as a fusion protein. This protein seemed to be leaked or secreted without the involvement of signal peptidase. Further investigations are required to explain the phenomenon. The amounts of IL-1β production were measured under different pH conditions, because IL-10 production using the same system was found to be pH dependent in the previous study (13). The results showed that an approximately 3-fold-larger amount of the protein was produced when carbonate buffer was added to the culture. Considering this characteristic, MRS medium was supplemented with the buffer when the recombinant strains were applied to the *in vivo* assay. The biological activity of IL-1β was determined by the stimulation of Caco-2 cells as described previously (9, 25). In response to IL-1β produced by the recombinant *L. casei*, IL-8 was released from the cell culture. The activity of IL-1β from recombinant bacteria was approximately 60% of that of purified IL-1β. Because the amino acid sequence of the N terminus of IL-1β is critical for its biological activity, extra peptides at the N terminus drastically impaired the activity (12). In fact, the biological activity was lost by adding only 5 additional amino acids (DTNSD) at the N terminus of IL-1β (data not shown). As described above, part of IL-1β secreted by recombinant *L. casei* was conjugated with the signal peptide. Hence, contamination by inactive IL-1β resulted in lower biological activity.

The immunological properties of the recombinant *L. casei* producing IL-1β were analyzed *in vivo* using a ligated-intestinal-loop assay. In this assay, the expression of cytokine genes was detected only in the PP. The result of cytokine profiling by RT-PCR showed that the expression of IL-6 seemed to be upregulated. In order to confirm the induction of IL-6, PP cells were prepared and stimulated with IL-1β-secreting lactobacilli. The result showed that IL-6 was efficiently released from $CD11c^+$ cells by stimulation with both IL-1β-secreting bacteria and the culture supernatants in a dose-dependent manner. IL-6 is produced by monocytes, macrophages, dendritic cells, etc., and enhances antibody production from B cells (15, 20). Moreover, it was reported that $CD11c^+/B220^-/CD11b^+$ Peyer's patch DCs secrete IL-6 and induce antibody production from naïve B cells (24). These findings suggested that IL-1β-secreting lactobacilli could be applied in vaccination as an adjuvant that increases antibody production. In order to evaluate this effect, the immunization of mice was performed using HKSE in combination with the recombinant *L. casei* strains. The result showed that comparatively large amounts of SE-specific antibodies, both IgG and IgA, were achieved by immunization with HKSE plus IL-1β-secreting lactobacilli. This result suggested that the recombinant strain had adjuvant effects *in vivo*. If purified IL-1β was administered orally or intragastrically, it could be degraded by the digestive process in the gastrointestinal (GI) tract. Meanwhile, IL-1β released by the recombinant *L. casei* could be delivered directly to the GI tract with less exposure to gastric acid, bile, and digestive enzymes. This is an advantageous feature of adjuvant delivery using recombinant lactobacilli. Because IL-1β is a proinflammatory cytokine, there may be a risk of side effects. In this study, however, apparent side effects, such as weight loss and diarrhea, were not observed.

Several kinds of cytokines have been produced by lactic acid bacteria to complement vaccine efficacy (2, 28). The present study demonstrated adjuvant effects of recombinant *L. casei* secreting biologically active IL-1β. Although it is known that IL-1β can be used as a mucosal adjuvant, it has not been reported previously that intragastrically administered recombinant lactobacilli secreting cytokines exhibited an adjuvant effect. The recombinant strain developed in this study could be a powerful tool to improve the efficacy of vaccines based on lactic acid bacteria or other delivery agents that have relatively weak immunogenicity.

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