Mucosal and Systemic Immune Responses Induced by Recombinant Lactobacillus spp. Expressing the Hemagglutinin of the Avian Influenza Virus H5N1

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To develop a safe, effective, and convenient vaccine for the prevention of highly pathogenic avian influenza (HPAI), we have successfully constructed two recombinant lactobacillus strains (LA4356-pH and DLD17-pH) that express the foreign HPAI virus protein hemagglutinin 1 (HA1). The mucosal and systemic immune responses triggered by these two recombinant lactobacilli following oral administration to BALB/c mice were evaluated. The results showed that both LA4356-pH and DLD17-pH could significantly increase the specific anti-HA1 IgA antibody level in the mucosa and the anti-HA1 IgG level in serum, as well as stimulating the splenic lymphocyte proliferative reaction through increased expression of interleukin-4 (IL-4). Compared with LA4356-pH, DLD17-pH was more effective at inducing systemic and mucosal immune responses, with higher anti-HA1-specific IgA and IgG levels. Therefore, DLD17-pH could be a promising oral vaccine candidate against HPAI.

Highly pathogenic avian influenza (HPAI) virus (H5N1) is a threat to the world’s poultry industry. As a zoonotic agent, this virus also has the potential to cause a human pandemic (29). Current vaccination (by intramuscular immunization) against HPAI has succeeded in reducing morbidity and mortality in poultry. However, the intense stress caused by injection could hamper the animal’s growth (34). Thus, many investigators are pursuing more convenient and economical avenues for construction of new vaccine candidates, such as recombinant subunit vaccines using baculovirus (28), plasmid DNA (3), or replication-incompetent adenovirus (rAd) (26, 27) vectors. The H5N1 strain can infect animals through their respiratory and intestinal tracts (10). Because there are many mucosa-associated lymphoid tissues underneath the epithelia of the respiratory and intestinal tracts, protective HPAI virus antigens may induce an effective mucosal immune response to prevent the invasion of HPAI virus if they can be transported easily to these tissues. Thus, developing mucosal vaccine candidates based on these considerations is a feasible strategy.

Mucosal vaccines, which are administered mainly orally or intranasally, cause less stress in the animal and have been the subject of growing interest due to the advantages that they offer over conventional parenteral vaccines. The biggest advantage is the stimulation of mucosal immune responses (15, 24). Lactobacillus is a genus of Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria. These beneficial bacteria normally live in the digestive, urinary, and genital systems without causing disease. Their abilities to transit through the stomach intact and to associate closely with the intestinal epithelium, combined with their immunomodulatory properties, have made Lactobacillus spp. attractive candidates as live vehicles for the delivery of immunogens to the intestinal mucosa (6, 12). It was recently shown that specific Lactobacillus species may be selected (32). Specifically, Lactobacillus delbrueckii subsp. lactis D17 (DLD17), which is isolated from the chicken intestine, can colonize the digestive tract (36).

In this study, we constructed recombinant strains based on Lactobacillus acidophilus ATCC 4356 (LA4356-pH) and Lactobacillus delbrueckii subsp. lactis D17 (DLD17-pH), both expressing hemagglutinin 1 (HA1) of HPAI virus. Local mucosal and systemic immune responses were then tested and compared after oral administration of the two recombinant lactobacillus strains to mice.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** The *E. coli*–Lactobacillus shuttle vector pLEM415 was donated by Pascale Serror (23). The plasmid pRV85, containing the lactate dehydrogenase (LDH) promoter, was supplied by Monique Zagorec, and its GenBank accession number is AF054624 (9). The plasmid pCI, containing the HA1 genes of the HPAI H5N1 virus, was provided by the Harbin Veterinary Research Institute (HVRI), and its GenBank accession number is AF144305.1 (Table 1).

*L. delbrueckii* subsp. lactis D17 (DLD17) was isolated from the chicken intestine (36), and *Lactobacillus acidophilus* ATCC 4356 (LA4356) was purchased from the China General Microbiological Culture Collection Center (CGMCC). *Lactobacillus* was grown in MRS medium (Difco Laboratories, Detroit, MI) at 37°C. When necessary, antibiotics were added to the culture medium at the following concentrations: ampicillin at 100 µg/ml for *Escherichia coli* and erythromycin (EM) at 5 µg/ml for *Lactobacillus*. Chicken anti-HA-positive sera (H5 subtypes) were provided by the Harbin Veterinary Research Institute.

**Construction of recombinant pLEM415-PHA1, plasmid and lactobacillus strains LA4356-pH and DLD17-pH.** To insert the PLDH fragment (245 bp) into the KpnI and XhoI sites of the pLEM415 vector, PCR products were isolated, and the plasmid pLEM415-PHA1 was obtained. This plasmid was digested with KpnI and XhoI and ligated with the PCR fragment to construct the pLEM415-PHA1 vector. This vector was then transformed into *E. coli* DH5α, and the recombinant plasmids were isolated and sequenced. The LM415-PHA vector was used to transform *Lactobacillus* spp. to generate recombinant strains.

**Construction of recombinant lactobacillus strains LA4356-pH and DLD17-pH.** The plasmid pLEM415-PHA1 was transformed into *Lactobacillus* spp. using the heat shock method (4). The transformed cells were plated on MRS plates containing ampicillin and EM (100 µg/ml and 5 µg/ml, respectively) and selected for colonies that were kanamycin resistant. The recombinant plasmid was isolated from the selected clones and sequenced to confirm the correct insertion of the HA1 gene. The recombinant plasmids were then used to transform *Lactobacillus* spp. to generate recombinant strains.
was performed using pRV85 as a template. The P_{LDH} fragment was amplified using the following primers: 5'-TAAGGTACCTACTGAGAAGTTGCTC-3' (KpnI restriction endonuclease site is underlined) and 5'-TAACCTCGAGGCCGACGAGGATAACT-3' (XhoI restriction endonuclease site is underlined). To insert the HA1 gene (975 bp) of HPAI virus into the XhoI and EcoRV sites of the pLEM415-P vector, PCR was performed using pCI as a template. The HA1 fragment was amplified using the following primers: 5'-TAACCTCGAGGATCAGATTTGCATTGGTTACC-3' (XhoI restriction endonuclease site is underlined) and 5'-TAAGATATCTTACTACTCTCTCTGAGGGGTATTTC-3' (EcoRV restriction endonuclease site is underlined). The amplified HA1 gene was cloned into the XhoI and EcoRV sites of the pLEM415-P vector (Fig. 1). The recombinant plasmid was labeled pLEM415-PHA1 and sequenced to ensure that the HA1 open reading frame (ORF) was correct. Finally, the pLEM415 plasmid and the recombinant pLEM415-PHA1 plasmid were transformed into Lactobacillus acidophilus ATCC 4356 and Lactobacillus delbrueckii subsp. lactis D17 (Table 1). The recombinant plasmids were transformed into Lactobacillus by electroporation as previously described (8).

**Analysis of HA1 expression in recombinant lactobacilli.** To examine HA1 expression, the recombinant LA4356-pLEM415 and DLD17-pLEM415 were grown in MRS broth supplemented with 5 μg/ml EM, and cell fractionations and protein extractions were performed as previously described (17). For immunodetection of the heterologous HA1 protein by Western blotting, chicken anti-HA1-positive serum (1:200) (HVRI) and TABLE 1 Strains and plasmids used for this study

| Strain or plasmid | Relevant features | Source or reference |
|-------------------|------------------|---------------------|
| Plasmids          |                  |                     |
| pLEM415           | Escherichia coli-Lactobacillus shuttle vector | 23 |
| pRV85             | Expression vector containing the constitutive ldhL promoter | 9 |
| pCI               | Expression vector containing hemagglutinin (HA1) genes of HPAI H5N1 virus | Harbin Veterinary Research Institute |
| pLEM415-pHA1      | Expression vector containing the constitutive ldhL promoter and HA1 gene | This study |
| Bacterial strains |                  |                     |
| LA4356            | Lactobacillus acidophilus ATCC 4356 | CGMCC |
| DLD17             | Lactobacillus delbrueckii subsp. lactis D17 | 36 |
| LA4356-pLEM415    | Lactobacillus acidophilus ATCC 4356/pLEM415 | This study |
| LA4356-pH         | Lactobacillus acidophilus ATCC 4356/pLEM415-PHA1 | This study |
| DLD17-pLEM415     | Lactobacillus delbrueckii subsp. lactis D17/pLEM415A1 | This study |
| DLD17-pH          | Lactobacillus delbrueckii subsp. lactis D17/pLEM415-PHA1 | This study |

**FIG 1** Map of the constitutive pLEM415-PHA1, plasmid.
rabbit anti-chicken IgY (1:5,000) conjugated to horseradish peroxidase (HRP) (Sigma) were used. Finally, the blots were developed by enhanced chemiluminescence (ECL) using an ECL Plus detection kit (Thermo Scientific).

**Preparation of recombinant strains for immunization.** The recombinant lactobacillus strains (LA4356-pH and DLD17-pH) and corresponding empty plasmid control strains (LA4356-pLEM415 and DLD17-pLEM415) were grown in MRS. The bacteria were washed three times with sterile phosphate-buffered saline (PBS), and the final pellets were resuspended in sterile PBS supplemented with 1% sucrose for oral immunization at the appropriate concentration. Plate counts were performed for each inoculum to determine the number of CFU for administration.

**Mucosal immunizations and sample collection.** BALB/c mice were kept under specific-pathogen-free (SPF) conditions in individual ventilated cages, and all experiments were carried out under the guidelines of the National Animal Study Board. Six-week-old BALB/c mice (120 mice) were divided into four groups (30 mice/group). Immunizations were administered orally to the mice three times at 2-week intervals; each administration included three consecutive daily doses of 200 μl (10^10 CFU) of LA4356-pLEM415, LA4356-pH, DLD17-pLEM415, and DLD17-pH. The mice were killed and samples were collected 35, 49, and 63 days after the first immunization. Tracheal and intestinal lavage fluids were obtained by washing the respective organs three times with 0.5 ml of ice-cold PBS containing protease inhibitors. Samples were centrifuged at 10,000 × g for 20 min at 4°C, and the supernatants were stored at −20°C until they were analyzed.

**Detection of specific IgG and IgA antibodies by ELISA.** Specific secretory IgA antibody titers in the intestinal and tracheal lavage fluids were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (33). ELISA plates were coated with purified HA1 protein in carbonate buffer (pH 9.6) at 4°C overnight. The plates were washed five times with PBS containing 0.05% Tween 20 and saturated with PBS containing 5% skim milk at 37°C for 1 h. Intestinal and tracheal lavage fluids were added in duplicate and incubated for 1 h at 37°C. The plates were then washed five times with PBS containing 0.05% Tween 20. HRP-conjugated goat anti-mouse IgG or IgA was added to each well and incubated for an additional 1 h at 37°C. After another round of washing, a substrate solution containing 3,3’-diaminobenzidine (DAB) and H2O2 was added. The reaction was allowed to proceed for 15 min at room temperature before it was terminated by the addition of a stop solution. The absorbance was measured at 495 nm, using an automated ELISA reader (Molecular Devices). Endpoint titers were defined as the maximum dilution showing a reaction.

**HI assay.** Hemagglutination inhibition (HI) titers of sera were determined by a standard HI microtiter assay as previously described (11). The HI titer was defined as the highest serum dilution capable of preventing hemagglutination.

**T cell stimulatory index and analysis of cytokines.** BALB/c mice that were immunized with the recombinant lactobacilli were sacrificed 35, 49, and 63 days after the first immunization. Splenic lymphocytes were isolated from each mouse for analysis of their proliferative response and cytokine secretion. Lymphocytes were cultured in vitro following restimulation with purified HA1 protein. The purified HA1 protein was expressed in *E. coli* and then purified using a His protein pull-down kit (Promega). The splenic lymphocyte cultures were seeded into 96-well plates at 100 μl per well (1 × 10^6 cells) and then treated with purified recombinant HA1 (final concentration, 10 μg/ml) in vitro for 72 h. After incubation with 25 μl of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; 1 mg/ml] for 4 h at 37°C, the reaction was stopped by the addition of an equal volume of lysis buffer (30% dimethyl sulfoxide [DMSO] and 20% SDS, pH 7.4). The absorbance was read at 570 nm. The proliferative capacity is shown as the stimulatory index (SI), which was calculated according to the following equation: SI = cpm of stimulated cultures/ cpm of control cultures.

The lymphocytes isolated 49 days after the first immunization were then cultured at 37°C for 48 h in the presence or absence of purified recombinant HA1 (final concentration, 10 μg/ml). Gamma interferon (IFN-γ) and interleukin-4 (IL-4) levels in cell culture supernatants were determined by quantitative ELISA, using commercial kits from BD Pharmingen. The detailed procedure was performed as previously described (31).

**RESULTS**

**Constructive recombinant plasmid and recombinant lactobacilli.** To express the HA1 protein of HPAI virus in a lactobacillus delivery vehicle, the promoter (P; 245 bp) and subsequently the HA1 (975 bp) gene were inserted into the E. coli-Lactobacillus shuttle vector pLEM415. The recombinant plasmid pLEM415-HA1 was constructed and confirmed by restriction enzyme digestion with KpnI, XhoI, and EcoRV (Fig. 2). pLEM415-HA1 was transformed into LA4356 and DLD17, giving strains LA4356-pH and DLD17-pH, respectively (Table 1). The empty plasmid pLEM415 was transformed into LA4356 and DLD17, and the resulting strains served as negative controls.

**Cultured recombinant lactobacilli express the HA1 protein.** As shown in Fig. 3, the recombinant lactobacilli were able to express the 39-kDa HA1 protein, as expected. The results of Western blot analysis indicated that the HA1 protein expressed by the recombinant lactobacilli had effective antigenicity.

**Recombinant lactobacilli induce mucosal HA1-specific IgA antibodies in intestinal and tracheal lavage fluids in immunized mice.** For mice administered recombinant strain DLD17-pH, anti-HA1 IgA antibodies were detected in the intestinal and tracheal lavage fluids 49 days after the first immunization (Fig. 4), indicating that the recombinant *Lactobacillus* strain stimulated the mucosal immune response in the respiratory and digestive tracts. Anti-HA1 IgA antibodies were maintained at a high level until 63 days after the first immunization.

However, for mice administered the recombinant strain LA4356-pH, anti-HA1 IgA antibodies were detected only in the intestinal mucosa, from 35 days to 63 days after the first immunization (Fig. 4). Furthermore, the antibody level was significantly lower than that induced by DLD17-pH at 49 days (P < 0.05).

**Levels of HA1-specific IgG antibodies in the sera of mice immunized with recombinant lactobacilli.** To examine the systemic immune responses to orally administered recombinant lactobacilli, we measured the levels of anti-HA1 IgG antibodies in the sera of immunized mice. Serum samples were collected from the mice 35, 49, and 63 days after the first immunization. The HA1-specific
IgG titers of the LA4356-pH- and DLD17-pH-immunized groups increased over time, although these increases were not significant compared with those for the corresponding control groups until 49 days after the first immunization (Fig. 5). We also found that the HA₁-specific IgG titers induced by DLD17-pH were significantly higher than those induced by LA4356-pH from 49 days to 63 days after the first immunization. These results suggested that a significant HA₁-specific systemic immune response was induced by oral immunization with the recombinant lactobacilli and that the immune efficiency of DLD17-pH was better than that of LA4356-pH.

HI titers in sera of mice immunized with recombinant lactobacilli. The antibody titers in the sera 35, 49, and 63 days after the first immunization were detected by HI assay. As shown in Fig. 6, antibody titers were not detected after immunization with the negative-control recombinant lactobacilli, LA4356-pLEM415 and DLD17-pLEM415, whereas antibody titers could be detected and increased gradually after immunization with LA4356-pH and DLD17-pH. The antibody titers of mice immunized with DLD17-pH were higher than those of mice immunized with LA4356-pH.

Systemic cell-mediated immune responses to recombinant lactobacillus immunization. We evaluated the proliferative response and cytokine secretion of splenic lymphocytes primed with the HA₁ antigen in vitro to determine the systemic cell-mediated immune responses to recombinant lactobacilli. Spleen lymphocytes were isolated from each mouse 35, 49, and 63 days after the first immunization with recombinant lactobacilli. After challenging the isolated and cultured splenic lymphocytes with purified HA₁ antigen in vitro, the proliferative response was determined (Fig. 7). The SI of the LA4356-pH group was markedly higher than that of the DLD17-pH group at 49 days.

Cytokine detection kits were used to analyze the subset immune responses in mice. The results showed that the secretion of IL-4 was significantly higher after oral administration with the recombinant lactobacilli than after administration of the control plasmid ($P < 0.05$) 49 days after immunization (Fig. 7). The IL-4 level in mice immunized with DLD17-pH was also significantly higher than that in mice immunized with LA4356-pH ($P < 0.05$). However, IFN-γ production was not significantly different among the tested groups.

DISCUSSION

HA is the principal antigen target for neutralizing antibodies (25). The adhesion of a virus to the epithelium is essential for efficiently transferring avian influenza virus genes into cells, via a process that can be blocked by antibodies binding to HA proteins. HA is usually cleaved into HA₁ and HA₂ by a trypsin-like serine endoprotease targeting a specific site (20). HA₁ contains the most important protective epitope for stimulating the body to produce neutralizing antibodies (21). Thus, HA₁ is an important target for vaccine development. *Lactobacillus* is widely used as a live vaccine vehicle against various microbes (1, 7, 35). Accordingly, we constructed a recombinant plasmid, pLEM415-PHA₁, and recombinant lactobacillus strains, LA4356-pH and DLD17-pH, that produce the HPAI virus protective antigen HA₁. Oral immunization with the recombinant lactobacilli elicited HA₁-specific antibodies and Th1-type cellular immune responses. The experimental results showed that immunization with the recombinant strain...
DLD17-pH resulted in higher antibody levels than immunization with LA4356-pH.

It is generally assumed that IgA is the main antibody isotype and effector in the host defense at mucosal surfaces (14). Our results indicated that DLD17-pH, expressing HA1, triggered a mucosal immune response in both the respiratory and digestive tracts (Fig. 3), whereas the recombinant strain LA4356-pH induced a mucosal immune response only in the digestive tract. The different IgA antibody levels induced by recombinant LA4356-pH and DLD17-pH could be explained by the differences in Lactobacillus strain and origin. DLD17, which was isolated from the chicken gut, is well adapted to the living conditions of the intestine. Therefore, the recombinant strain DLD17-pH would likely survive better in the chicken intestine than in the respiratory tract and be able to stimulate the intestinal mucosal immune response persistently. LA4356 originated from the human pharynx, and thus the adhesion of LA4356 to the chicken intestinal epithelium may be weak, resulting in a lesser mucosal immune response. This hypothesis was confirmed in a previously published article (36).

The recombinant LA4356-pH and DLD17-pH bacteria also effectively triggered systemic immune responses against HA1, although the HA1-specific IgG and HI antibody titers were low. Our studies demonstrate that mucosal immunization with recombinant lactobacilli can elicit both mucosal IgA and circulating IgG and support the theory that mucosal immunization can provoke both mucosal and circulating antibody responses. These results are consistent with other observations (14). Furthermore, the HA1-specific IgG and HI antibody titers elicited by DLD17-pH were higher than those elicited by LA4356-pH, which could be attributed to the Lactobacillus strain and origin.

Although oral immunization with recombinant LA4356-pH and DLD17-pH induced HA1-specific serum IgG and mucosal IgA production, cell-mediated immune responses also played a crucial role in protecting the host from invading pathogens. Our results demonstrated that the purified HA1 protein obviously promoted the splenic lymphocyte proliferative reaction and cytokine secretion compared with those of controls (P < 0.05). Moreover, IL-4 was secreted at higher levels in the groups that were administered the recombinant lactobacilli than in the control groups. These results indicate that a specific Th2-like cell-mediated immune response was evoked through a common mucosal immune mechanism. In contrast, we found no significant differences in the secretion of IFN-γ among the different groups.

Oral immunization offers several advantages over intranasal vaccination. First, oral immunization induces more effective mucosal immune responses and protective rates (13). Second, oral immunization is easier to administer to animals, particularly poultry. Finally, Lactobacillus possesses the ability to adhere to and colonize the gastrointestinal tract as well as to perform adjuvant functions (5, 19).

In conclusion, we have demonstrated the feasibility of using Lactobacillus species as vehicles for an orally administered avian influenza virus vaccine candidate. Compared with LA4356-pH, DLD17-pH was more effective at inducing systemic and mucosal immune responses, with higher anti-HA1-specific IgA and IgG levels. This result implies that the acquired immune response is dependent on the strain of Lactobacillus. Thus, recombinant DLD17-pH could be used as an oral vaccine to induce protective immunity against HPAI.

ACKNOWLEDGMENTS

This work was supported by the Doctoral Program Research Fund of National Higher Education (grant 20060307013), by a Youth Science and Technology Innovation Grant of Nanjing Agricultural University (grant 090600321), and by a National Science Grant of China (grant 30871858).

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