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Immunogenicity and protective efficacy of orally or intranasally administered recombinant *Lactobacillus casei* expressing ETEC K99

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ABSTRACT

In an effort to develop a safe and effective vaccine for the prevention of enterotoxigenic *Escherichia coli* (ETEC) K99 infections, we have developed a surface antigen display system using pgsA (poly-γ-glutamate synthetase A) as an anchoring matrix. The recombinant fusion proteins comprised of pgsA and fimbriae protein of ETEC K99 were stably expressed on *Lactobacillus casei*. Surface localization of the fusion protein was verified by immunoblotting, immunofluorescence microscopy and flow cytometry. Specific Pathogen Free (SPF) BALB/c mice orally or intranasally vaccinated with recombinant *L. casei* resulted in high levels of serum immunoglobulin G (IgG) and mucosal IgA against ETEC K99, as demonstrated by enzyme-linked immunosorbent assays using purified fimbriae peptides. The serum antibody isotypes elicited were predominantly IgG1 and IgG2a. Vaccinated SPF BALB/c mice were evaluated by oral challenge with standard-type ETEC C83912 after the last booster immunization. More than 80% of immunized mice survived regardless of the immune route. The antibody titers elicited following oral immunization were lower than those following intranasal immunization but the protective efficacy was in the same order of magnitude. These results indicate that mucosal immunization with recombinant *L. casei* expressing ETEC K99 fimbriae protein on its surface provides an effective means for eliciting a protective immune response against the ETEC K99.

1. Introduction

Enterotoxigenic Escherichia coli (ETEC) strains can produce fatal diarrhea in neonatal calves. These organisms possess at least two known virulence factors: production of enterotoxins, which produce diarrhea by a mechanism of villous hypersecretion [1], and surface antigens such as pili or fimbrial adhesins, which facilitate colonization of the small intestine. The K99 pilus antigen is one of the major adherence factors found on ETEC of neonatal calves [2,3]. Conventional vaccines against bovine ETEC have been shown to provide varied immunity, and the effectiveness of these vaccines has been described only in anecdotal reports [4]. Limited protection with purified K99 fimbriae or formalin-inactivated ETEC has been demonstrated [5–7], but the need for an efficacious vaccine against bovine ETEC still exists [4]. The development of effective strategies for the mucosal delivery of vaccine antigens has received considerable attention over the past decade, because this route of administration has the potential to elicit local immune responses at mucosal surfaces, the major portals of entry to the body for many pathogens [8]. The key effector molecule of the mucosal immune response is secretory immunoglobulin A (sIgA), which can play a key role in protecting against infection by inhibiting viral infectivity and bacterial colonization and by neutralizing the activity of microbial toxins [9–12]. For mucosal immunization, lactic acid bacteria (LAB) are more attractive as delivery vehicles than other live-vaccine vectors (e.g., *Shigella*, *Salmonella*, and *Listeria*) [13–16] because LAB are considered safe, they exhibit adjuvant properties, and they are weakly immunogenic [17–20]. In addition, extracellularly accessible antigens expressed on the surfaces of bacteria are better recognized by the immune system than those that are intracellular [14].

For surface display of antigens on LAB, we have developed an expression vector using the pgsA gene product as an anchoring matrix. The pgsA is a synthetase complex (PGS system) of *Bacillus subtilis* [21], and functions as a fusion partner for expression of heterologous antigens on the surface of *L. casei* [22]. The K99 fimbriae

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were inserted into the vector pLA, then expressed on the surface of *L. casei*. Intranasal and oral vaccination of mice with the live recombinant *L. casei* elicited high levels of systemic serum antibodies and local mucosal immunity against the antigen K99 fimbriae. The results of this study suggest a potential use for the surface expression system to construct ETEC K99 and against other diarrhea pathogens and respiratory system diseases that are transmitted mucosally.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. coli C83912 isolated from a calf with intestinal infection in Japan was purchased from China Center of Veterinary Culture Collection (CVCC), Beijing, China. *E. coli* XL1-Blue was used for construction of the expression vectors. The bacteria was cultivated in Luria-Bertani medium or on Luria-Bertani agar plates and grown at 37 °C. *L. casei* was grown at 37 °C in MRS broth (Difco Laboratories, Detroit, Mich.) where appropriate antibiotics were added. For *L. casei*, chloramphenicol was used at final concentrations of 10 μg/ml, in case of *E. coli*, ampicillin, 100 μg/ml.

2.2. Plasmids and transformation

The minimal surface display plasmid, pLA-K99 were constructed. The 498 bp DNA fragment encoding the fimbriae protein of K99 was amplified with 5′CGCGGATCCATGAAAAAGACTCTGA3′ and 5′CGCAAGCTTTTACATATAAGTGACT3′, digested by BamHI and HindIII, and inserted into the vector pLA which mainly includes HCE constitutive promoter and pgsA-tag gene. The resulting plasmid, in which the fusion protein of the pgsA-K99 was expressed as described previously [23], was designated pLA-K99.

L. casei isolated from Chinese food. Transformation of *L. casei* was performed by electroporation. The sample was subjected to a 2.2-kV, 200-Ω, 25-μF electric pulse in a 0.2-cm cuvette, using a Gene Pulser (Bio-Rad, Richmond, Calif.). As a negative control, *L. casei* was transformed with an empty shuttle vector to generate pLA/*L. casei*.

2.3. Immunoblotting, immunofluorescence microscopy and flow cytometry

The recombinant *L. casei* cells were grown at 37 °C. Protein extractions were performed as previously described [5]. For immunodetection of fusion proteins, mouse anti-pgsA (1:1000) and mouse anti-K99 (1:800) were used. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) was used as a secondary antibody. After washing the membranes with PBS containing 0.05% Tween 20 (PBS-T), the membranes were treated with Streptavidin-HRP complex (Vectastain ABC Kit, Vector Lab, USA) following the manufacturer's instructions. Visualization of immunobinding was carried out with diaminobenzidine (DAB) solution (Vector Lab, USA). For immunofluorescence microscopy, cells labeled with anti-K99 polyclonal antibodies and FITC conjugated anti-mouse antibodies were examined using a Carl Zeiss Axioskop 2 fluorescence microscope. Photographs were taken with an Axiocam high-resolution camera using identical exposure times. For flow cytometry, *L. casei* cells were cultured in MRS broth (Difco) overnight at 37 °C. The cell pellets were sequentially incubated with mouse anti-K99 polyclonal antibodies (1:800) and FITC-conjugated anti-mouse IgG secondary antibodies (1:5000; Sigma, St. Louis, MO). Finally, 3×10^4 cells were analyzed with FACS Calibur (Becton Dickinson, Oxnard, CA) equipped with CellQuest software.

2.4. Immunization of mice

SPF mice (BALB/c, female, five weeks old) were obtained from Vital River Laboratories, Beijing, China. These animals were raised and used according the animal protocols approved by the Institutional Animal Care and Use Committee (IACUC). Animals were placed in individual cages with autoclaved food and water available *ad libitum*. All animals were housed in asepsis room. Mice were acclimated to the new environment for one week after arrival prior to immunization. To study the possibility of the surface-displayed ETEC K99 proteins to induce the mucosal immunity in mice, the method of Jong-Soo Lee [22] was employed.

BALB/c mice (45 per group in 4 groups) were immunized orally or intranasally with an equal amount of live *L. casei* that express recombinant K99 protein from plasmid pLA-K99. *L. casei* harboring the parental plasmid pLA was used as a negative control. For the oral route, 5×10^9 pLA-K99/*L. casei* cells in 100 μl suspension were administered daily via intragastric lavage on days 0–4, 7–11, 21–25 and 49–53. For the intranasal route, 2×10^9 pLA-K99/*L. casei* cells in 20 μl suspension were administered into nostrils of lightly anesthetized mice on days 0–2, 7–9, 21 and 49.

2.5. Sampling

Blood samples were collected from the tail vein on days 0 (pre-immune), 14, 28, 42, 56, 70 and 84. Sera were prepared from the blood and stored at −20 °C until they were analyzed.

Fecal samples were collected every week. Fecal pellets (100 mg) were suspended in 0.5 ml PBS. After centrifugation at 15,000 × *g* for 5 min, the supernatants were collected and tested for IgA by ELISA.

To obtain intestinal lavage samples, 7 mice were sacrificed on days 56, 70 or 84. Following the method of Wu and Russell [24], gut lavage fluids were obtained by flushing the excised small intestine with 3 ml of PBS containing 50 mM EDTA and 0.1 mg/ml of soybean trypsin–chymotrypsin inhibitor (Sigma). The contents were collected and retained on ice for processing, whereupon the fluids were vortexed and centrifuged at 650 × *g* for 10 min at 4 °C. A 30 μl volume of 100 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) was added to the supernatants before they were vortexed and spun at 27,000 × *g* for 20 min at 4 °C. A further 20 μl of PMSF, 100 μl of fetal bovine serum (FBS), and 20 μl of 1% sodium azide (Sigma) were added to the supernatants before they were dispensed into aliquots and frozen.

Lung lavage fluids were obtained post mortem by inserting a nylon cannulain to the exposed trachea, which was tied in place. A hypodermic needle and syringe were attached and used to inject and withdraw 0.7 ml of 2 mM PMSF in PBS three times. The fluid samples were retained on ice before centrifugation at 27,000 × *g* for 20 min at 4 °C, and the supernatants were then stored in aliquots at −20 °C.

The vaginal fluids were obtained by washing the vagina three times with 0.5 ml of ice-cold saline containing protease inhibitors. Samples were centrifuged at 2500 × *g* for 20 min at 4 °C, and the supernatants were stored at −20 °C until they were analyzed.

2.6. ELISA

Antibody titers in serum, fecal samples, lung lavage, intestinal lavage and vaginal fluids were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described [22].

ELISA was performed three times for each serum sample. End point titers were defined as the maximum dilutions giving an A_{450} measurement of 0.1. This cutoff value represents the mean optical density plus 2 standard deviations of 10 normal mouse serum samples tested at 1:50 dilution. Statistical comparison was made using the Mann–Whitney *U*-test.