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Short communication

Protection against *Yersinia pseudotuberculosis* infection conferred by a *Lactococcus lactis* mucosal delivery vector secreting LcrV

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ABSTRACT

Herein, we sought to evaluate the potential of a recombinant *Lactococcus lactis* strain secreting the *Yersinia pseudotuberculosis* low-calcium response V (LcrV) antigen for mucosal vaccination against *Yersinia* infections. We showed that the recombinant strain induced specific systemic and mucosal antibody and cellular immune responses after intranasal immunization and protected mice against both oral and systemic *Y. pseudotuberculosis* infections. This constitutes the first proof of principle for a novel anti-*Yersinia* mucosal vaccination strategy using recombinant lactic acid bacteria.

1. Introduction

The genus *Yersinia* consists of 14 species, of which three (*Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*) are pathogenic for animals and humans. *Y. pestis* is responsible for plague, a serious world-wide public health threat now further aggravated by the emergence of multi-antibiotic-resistant strains [1]. *Y. pseudotuberculosis* (the evolutionary progenitor of the plague bacillus) and *Y. enterocolitica* are both food-/water-borne enteropathogens that induce mainly ileitis and mesenteric lymphadenitis. Outbreaks caused by each species have been reported in livestock, wild animals and humans. Vaccination is the most effective approach in the prevention and control of yersiniosis and has prompted a focus on the development of *Yersinia* vaccines [1,2]. A promising candidate is the virulence V antigen (termed LcrV), a 37-kDa multifunctional protein common to the three pathogenic *Yersinia* and encoded by a 70-kb plasmid-borne gene [3]. LcrV is a major bacterial pathogenicity determinant that circumvents host innate immunity by inducing the production of the anti-inflammatory cytokine interleukin (IL)-10 and by participating in the secretion and translocation of *Yersinia* outer protein toxins into phagocytes [4].

Very few studies have shown that mucosal immunization with recombinant live vectors producing LcrV is effective against *Yersinia*; furthermore, all of these studies used attenuated pathogens – raising safety issues for human administration [5–8]. Lactic acid bacteria are more attractive antigen delivery vehicles than other live-vaccine vectors because they are safe and weakly immunogenic and exhibit adjuvant properties [9]. The non-invasive, non-colonizing species *Lactococcus lactis* is the best studied so far for use as a vaccine vector. Several heterologous bacterial or viral antigens have been expressed in this bacterium and mucosal immunization with recombinant lactococci has been shown to elicit specific antibody and cellular responses and protective immunity [9]. In previous work, we constructed a recombinant *L. lactis* strain that secretes the *Y. pseudotuberculosis* LcrV [10]. The aims of the present study were to (i) characterize both the specific mucosal and systemic immune responses after intranasal or oral administration of the recombinant lactococcal strain to mice and (ii) evaluate the strain's ability to protect against *Yersinia* infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

L. lactis MG1363 Ll (containing the expression plasmid pNZYR) and Ll-LcrV (harboring the pNZYR derivative pMEC237, which bears the PCR-generated *Y. pseudotuberculosis lcrV* gene [nucleotide 1–984, GenBank accession EU616676] fused to the secretion signal

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of the lactococcal Usp45 protein and located downstream of the lactococcal Usp45 promoter [10]) were used in this study. pNZYR is a high-copy-number vector in *L. lactis*, with about 50 plasmid copies per chromosome equivalent [11]. Lactococcal strains were grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose and 10 μg/ml chloramphenicol (Sigma–Aldrich). *Y. pseudotuberculosis* IP32777 and *Y. pestis* 195/P biotype Orientalis were also employed and *Yersinia* were cultured in Luria-Bertani (LB) broth at 28 °C (for strain IP32777) or 21 °C (for strain 195/P).

2.2. Mice immunization

Seven-week-old, female BALB/c mice (Charles River) were kept in an accredited establishment in accordance with European guidelines. All animal protocols were approved by the local institutional review board. Groups of 10 mice were immunized intranasally or intragastrically with lactococci cultures harvested at $OD_{600} \approx 1.5–2$. Intranasal immunization was performed with mice that had been lightly anesthetized by intraperitoneal injection of 100 μl of a cocktail containing 20% Imalgene 1000 (Merial), 0.5 mg of Valium (Roche) and 62.5 μg of atropine (Aguettant Laboratories) per ml [12]. Doses of 10^9 CFU of Ll or Ll-LcrV (resuspended in 40 μl of sterile, phosphate-buffered saline (PBS)) were administered to the mice's nostrils on days 1, 2, 22, 23, 43 and 44. For intragastric immunization, mice were fed via a gavage tube with 10^9 CFU of Ll or Ll-LcrV in 100 μl of gavage buffer (0.2 M sodium bicarbonate, 5% casein hydrolysate and 0.5% glucose) on days 1, 2, 3, 22, 23, 24, 43, 44 and 45.

2.3. LcrV-specific antibody titer assays

Blood and bronchoalveolar and intestinal lavages were collected from immunized animals on day 53 as described elsewhere [12] and stored at −20 °C prior to analysis. LcrV-specific antibody levels in sera (IgG, IgG1 and IgG2a) and bronchoalveolar and intestinal lavages (IgA) were determined by ELISA. Microtiter well plates (Nunc-Immuno Plate) were coated with purified recombinant *Y. pestis* LcrV in PBS (pH 7.2) at 50 ng per well. Following overnight incubation, wells were blocked in PBS with 0.05% Tween 20, 3% bovine serum albumin (BSA). Blood samples were tested using twofold serial dilutions (from 1:50 and 1:2 dilutions for intranasally Ll-LcrV and Ll-immunized animals, respectively, and from a 1:2 dilution for intragastrically Ll and Ll-LcrV-immunized animals) in PBS with 0.05% Tween 20, 1% BSA, whereas lavage fluids were applied undiluted for detection of IgA. Specific antibody binding to LcrV was detected using horseradish peroxidase-conjugated rat anti-mouse IgG and anti-mouse IgG1, IgG2a and IgA (BD Biosciences), as previously described [12].

2.4. Splenocyte isolation and cytokine assays

The spleen from each immunized mouse was aseptically removed on day 53 and splenocytes were extracted by mechanical dissociation, followed by passage of the cell suspension through a nylon cell strainer (Becton Dickinson). After PBS washings and incubation at room temperature in a red blood cell lysis buffer, splenocytes were resuspended in complete RPMI 1640 medium (Gibco) containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, 8 μg/ml gentamycin and 0.05 M 2-mercaptoethanol. The splenocytes were then cultured at 37 °C at a density of 5×10^6 cells per ml in the presence or absence of purified recombinant *Y. pestis* LcrV (final concentration: 7.5 μg/ml) for 48 h. Murine Interferon (IFN)-γ, IL-2 and IL-10 levels in cell culture supernatants were determined by quantitative ELISA using commercial kits from BD Pharmingen.

2.5. Mice infection

Experimental *Yersinia* infection complied with Biosafety Level 2 and 3 procedures. The 50% lethal doses was assessed previously as $<10^2$ and $10^{7.3}$ bacteria via the intravenous and intragastric routes, respectively, for *Y. pseudotuberculosis* IP32777 [13] and as approximately 10 bacteria via the intradermal route for *Y. pestis* 195/P [14]. Two weeks after the last intranasal or intragastric immunization with Ll or Ll-LcrV, groups of 10 animals were challenged with *Y. pseudotuberculosis* intragastrically (4×10^8 CFU in sterile, distilled water) or intravenously (10^3 CFU in sterile PBS). Using the same general protocol, mice that had been immunized intranasally were challenged with *Y. pestis* intradermally (5×10^2 and 5×10^3 in sterile PBS). Bacterial inocula were prepared from overnight cultures. Mortality was monitored daily. Mice infected with *Y. pseudotuberculosis* were sacrificed and organs were aseptically removed, weighed and homogenized in PBS. Spleens and mesenteric lymph nodes were collected after intragastric administration, whereas spleens and livers were harvested after intravenous administration. Bacterial growth *in vivo* was assessed

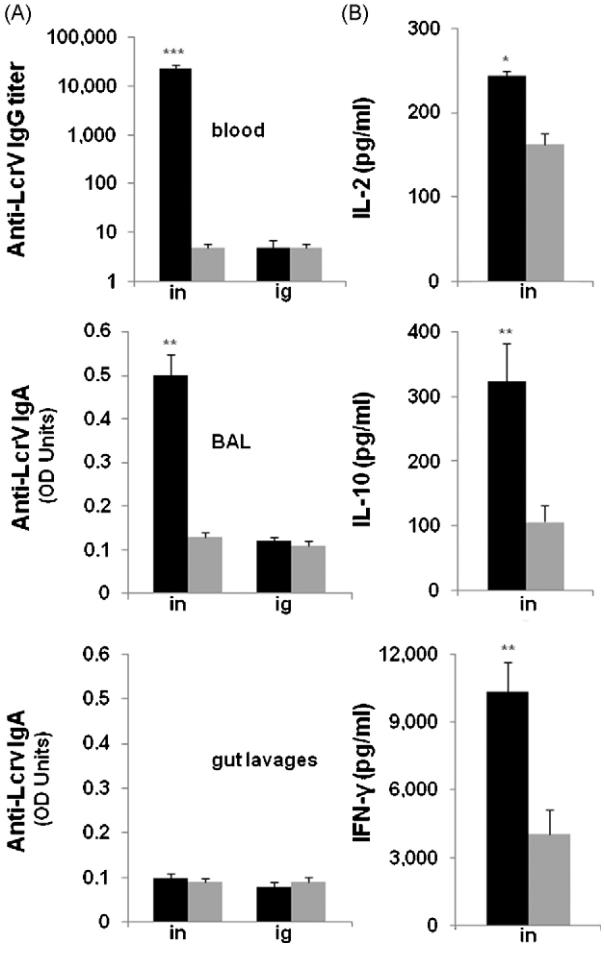

Fig. 1. LcrV-secreting *L. lactis* induces specific systemic and mucosal antibody and cellular responses after intranasal immunization of mice. (A) Anti-LcrV antibody levels in blood (measurements and calculation of IgG titers were performed as previously described [12]) and bronchoalveolar (BAL) and gut lavages (IgA levels were expressed in optical density units) on day 53 following intranasal (in) and intragastric (ig) immunization of mice with recombinant lactococci. Bars represent mean values for 10 animals ± S.E.M. (B) IL-2, IL-10 and IFN-γ levels in culture supernatants of spleen cells from lactococci-immunized mice after in vitro stimulation for 48 h with LcrV (7.5 μg/ml). Values are means ± S.E.M. for 10 individuals. Symbols: *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; *** $p < 0.001$; (■) Ll-LcrV immunized mice, (□) Ll-immunized mice.