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PLGA-microencapsulation protects *Salmonella typhi* outer membrane proteins from acidic degradation and increases their mucosal immunogenicity

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ABSTRACT

Salmonella (*S.*) *enterica* infections are an important global health problem with more than 20 million individuals suffering from enteric fever annually and more than 200,000 lethal cases per year. Although enteric fever can be treated appropriately with antibiotics, an increasing number of antibiotic resistant *Salmonella* strains is detected. While two vaccines against typhoid fever are currently on the market, their availability in subtropical endemic areas is limited because these products need to be kept in uninterrupted cold chains. Hence, the development of a thermally stable vaccine that induces mucosal immune responses would greatly improve human health in endemic areas. Here, we have combined the high structural stability of *Salmonella typhi* outer membrane proteins (porins) with their microencapsulation into poly(lactic-co-glycolic acid) (PLGA) to generate an orally applicable vaccine. Encapsulated porins were protected from acidic degradation and exhibited enhanced immunogenicity following oral administration. In particular, the vaccine elicited strong *S. typhi*-specific B cell responses in Peyer's patches and mesenteric lymph nodes. In sum, PLGA microencapsulation substantially improved the efficacy of oral vaccination against *S. typhi*.

1. Introduction

Salmonella (*S.*) *enterica* infections remain an important global health problem. For example, global estimates for typhoid fever report more than 20 million annual cases and 200,000 deaths [1]. The prevalence of *Salmonella*-related illness is particularly high in developing countries; however there is an increasing incidence of *Salmonella typhi* and *Paratyphi A* and *B* typhoid and paratyphoid fevers in industrialized countries [2,3]. Moreover, it is estimated that the number of travellers entering *S. enterica* endemic regions will further increase in the future [4,5]. Although enteric fever is treatable with antibiotics, various *Salmonella* strains have developed resistance to such first line treatment. Therefore, vaccination seems to be the most suitable option for the prevention of *Salmonella* infections in both travellers and individuals residing in endemic regions [2–4].

Currently, only two *S. typhi* vaccines are licenced, while vaccines against other *S. enterica* serovars are not available. Vi polysaccharide, the capsular component of *S. typhi*, is formulated as a sterile solution for intramuscular delivery. Importantly, the use of Vi polysaccharide vaccines is limited due to the lack of immunogenicity in children younger than 2 years. [6,7] and the emergence of multidrug-resistant *S. typhi* Vi antigen-negative strains [8]. The live-attenuated Ty21a vaccine is a highly attenuated Vi-negative *S. typhi* strain, generated by random chemical mutagenesis [9]. The use of both vaccines in endemic areas is limited, since they have to be kept in uninterrupted cold chains [4]. Thus, it is

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particularly important to provide a cost-effective oral *Salmonella* vaccine that can be delivered to patients in endemic areas without the need to maintain complicated cold chains.

Antibodies are crucial in conferring immunity against *Salmonella* infection [10,11]. Outer membrane proteins (Omps), also known as porins, represent important targets of the protective response against *Salmonella* [12]. Interestingly, these β-barrel-shaped transport channels exhibit repetitive structural elements [13] that most likely increase their immunogenicity. Moreover, *S. typhi* porins can stimulate antigen presenting B cells via TLR-mediated activation [14] and prime T-helper cells to promote protective antibody responses [15]. Patients recovering from typhoid fever present with both IgG and IgM circulating antibodies against porins [16], and purified *S. typhi* porins OmpC and F induce long-lasting IgM and IgG bactericidal antibody responses in humans [17]. Likewise, during non-typhoid Salmonellosis, anti-porin antibodies have been shown to be critical for protection [18]. A porin-based vaccine candidate based on *S. typhi* OmpC and F has been tested in humans and found to be safe and immunogenic following subcutaneous application [19]. Since both typhoidal and non-typhoidal *Salmonella* strains spread via the oral route, a formulation of porins that allows for passage through the gastrointestinal tract without significant loss of their high immunogenicity would represent a promising vaccine candidate for oral vaccination.

Poly(lactic-co-glycolic acid) (PLGA) is widely used as biodegradable carrier for the delivery of different therapeutic agents such as proteins, peptides, DNA and other large and small molecular drugs [20]. Importantly, PLGA is approved as carrier by regulatory authorities, and its safety is well established [21]. In the field of vaccines, diverse formulations have been produced and tested in pre-clinical studies using PLGA micro- and nanoparticles containing viral and bacterial antigens [22–25]. Besides safety and biocompatibility aspects, the efficacy of PLGA particles in preclinical immunization has elicited increasing interest in the field of vaccines. The immunization efficacy of PLGA particle-based formulation has been ascribed to the controlled release of antigens in specific sites of vaccine administration, particle phagocytosis, and prolongation of antigen presentation by dendritic cells and macrophages [26,27]. Here, we show that *S. typhi* porins OmpC and F encapsulated in PLGA microparticles are protected from acidic degradation and, therefore, maintain their immunogenicity following oral administration. Moreover, the formulation enhanced induction of *S. typhi* porin-specific B cell responses in Peyer's Patches and mesenteric lymph nodes. We conclude that PLGA microparticles containing *S. typhi* porins may represent a versatile platform for mucosal vaccination against Salmonellosis.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice were purchased from Charles River Laboratories (Germany). Mice were maintained in individually ventilated cages and were used between 6 and 9 weeks of age. Experiments were performed in accordance with federal and cantonal guidelines (Tierschutzgesetz) under permission numbers SG09/15 and SG13/02 following review and approval by the Cantonal Veterinary Office (St. Gallen, Switzerland).

2.2. Porin production

Porins were purified from *S. typhi* ATCC 9993 as previously described [14,19]. Lipopolysaccharide (LPS) content was determined using the limulus amoebocyte lysate (LAL) assay (Charles River Endosafe Laboratories), and all batches were found to be negative by LAL assay (detection limit 0.2 ng LPS/mg protein). Western blot analysis using anti-LPS polyclonal sera confirmed that LPS was not detectable by these means.

2.3. Capture ELISA

High-binding 96-well polystyrene plates (Corning) were coated with 100 ng/ml of anti-porin IgG 48-11B monoclonal antibody [15]. Plates were incubated overnight at 4 °C and washed five times in PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) (Sigma–Aldrich). Non-specific binding was blocked with PBS containing 1% FCS for 1 h at 37 °C. After washing, serial dilutions of the standard (*S. typhi* porins) and samples were added to the wells, and plates were incubated for 2 h at RT, followed by five washes with PBS-T. Biotinylated detection antibody IgG 45-1D (1 μg/ml) was diluted 1:10,000 and added to the plate, followed by incubation at RT for 1 h. After washing, streptavidin-HRP (Mabtech) was added, and plates were incubated for 30 min at RT, followed by five washes with PBS-T. Substrate solution (Sigma fast OPD, Sigma) was added, and plates were incubated for 30 min at RT. The reaction was stopped with 1.25 M H_2SO_4, and optical density was measured at 492 nm using an automated ELISA reader (Tecan). Individual samples were tested in duplicates, and mean concentrations were calculated using values of the standard curve.

2.4. Microencapsulation process

Porin proteins (1 mg/ml) were dissolved in phosphate buffer (186 mM Na_2HPO_4, 13 mM NaH_2PO_4, pH 7.8) and subsequently emulsified in a 5:95 ratio (v/w) with a solution of PLGA in dichloromethane (50 mg/g) by ultrasonication for 15 s at an energy output of 150 W (Vibra Cell, Sonics & Materials). The particle dispersion was spray-dried (Mini Spray Dryer B-191 connected to at PTFE tubing pump, MasterFlex, Büchi) at 38 °C inlet and 34 °C outlet temperatures and a flow rate of 2 ml/min. The resulting microparticles were harvested from the collection vessel by rinsing the product with a 0.05% poloxamer 388 (Synperonic PE/F108; ICI) solution. The particles were collected on a 0.2 μm RC 58 membrane filter (Schleicher & Schuell) and dried at 40 mbar and room temperature for 18 h. To determine the concentration of porins per mg PLGA, microparticles were dissolved in dichloromethane and protein content was determined by fluorescence spectrometry (Cary Eclipse Fluoresence Spectrophotometer). To determine the structure of encapsulated porins, microparticles were dissolved in acetone, centrifuged for 2 min at 14,000 rpm, and the supernatant containing the dissolved polymer was discarded. Protein pellets were reconstituted in PBS-T, incubated at 37 °C overnight, and samples were analyzed by SDS–PAGE.

2.5. Immunization

Mice were immunized intraperitoneally (i.p.), intravenously (i.v.), intramuscularly (i.m.), or via the oral route by gavage (per os, p.o.) with 10 μg *S. typhi* porins in solution or in PLGA microparticles (PLGA-P). Sera and lymphocytes from Peyer's patches (PP) and mesenteric lymph nodes (mLNs) were collected at the indicated time points post immunization.

2.6. Detection of porin-specific antibodies and antibody-secreting cells

Antibody titers against porins in sera of immunized mice were determined as described previously [15]. Briefly, high-binding 96-well polystyrene plates (Corning) were coated with 10 μg/ml of porins in 0.1 M carbonate-bicarbonate buffer, pH 9.5. Plates were incubated for 1 h at 37 °C and then overnight at 4 °C. Before use, plates were washed three times in PBS containing 0.05%

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