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A bacterial protease inhibitor protects antigens delivered in oral vaccines from digestion while triggering specific mucosal immune responses

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ABSTRACT

We report here that a bacterial protease inhibitor from *Brucella* spp. called U-Omp19 behaves as an ideal constituent for a vaccine formulation against infectious diseases. When co-administered orally with an antigen (Ag), U-Omp19: i) can bypass the harsh environment of the gastrointestinal tract by inhibiting stomach and intestine proteases and consequently increases the half-life of the co-administered Ag at immune inductive sites: Peyer's patches and mesenteric lymph nodes while ii) it induces the recruitment and activation of antigen presenting cells (APCs) and increases the amount of intracellular Ag inside APCs. Therefore, mucosal as well as systemic Ag-specific immune responses, antibodies, Th1, Th17 and $CD8^+$ T cells are enhanced when U-Omp19 is co-administered with the Ag orally. Finally, this bacterial protease inhibitor in an oral vaccine formulation confers mucosal protection and reduces parasite loads after oral challenge with virulent *Toxoplasma gondii*.

1. Introduction

Among different mucosal routes, oral delivery is the natural choice not only for drugs, but also for vaccines, by virtue of its ease of administration and cost. Although oral delivery of vaccines has been the Holy Grail for generations of vaccinologists [1], oral vaccination has been historically thought to be largely ineffective at providing effective mucosal and/or systemic immunity. This is mainly due to the fact that antigens (Ags) undergo proteolytic degradation in the stomach and intestine. Another barrier that would be bypassed by inducing an appropriate inflammatory response is the immune tolerance resulting from Ag feeding [2]. Consequently, to reliably immunize orally with peptide- or protein-based vaccines, Ags must be protected, uptake enhanced and the immune tolerance properly controlled.

Induction of immune responses following mucosal immunization with non-live vaccines is usually dependent upon the co-administration of appropriate adjuvants that can initiate and support the transition from innate to adaptive immunity [3]. Currently, to induce mucosal adaptive immune responses — in mice — two powerful adjuvants are used: cholera toxin (CT) from *Vibrio cholera* and labile enterotoxin (LT) from *Escherichia coli* [4]. In humans, however, they are responsible for the cholera and "Travelers diarrhea" and therefore cannot be used. In attempt to avoid the enterotoxic effects caused mainly by the A subunit of the cholera toxin, subunit B (CTB) was expressed

Abbreviations: Ab, antibody; Ag, antigen; ALT, alanine aminotransferase; APCs, antigen presenting cells; AST, aspartate aminotransferase; a.u, arbitrary units; *B. abortus*, *Brucella abortus*; BSA, bovine serum albumin; CD, circular dichroism; CFSE, Carboxyfluorescein succinimidyl ester; CT, cholera toxin; CTB, cholera toxin subunit B; DCs, dendritic cells; dmLT, double mutant labile enterotoxin; DTH, delayed type hypersensitivity response; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FI, fluorescence intensity; FITC, fluorescein isothiocyanate; HKS, heat killed extract from *Salmonella typhimurium*; IFN-γ, gamma interferon; Ig, immunoglobulin; IL-, interleukin; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; LT, labile enterotoxin; MHC, major histocompatibility complex; MLNs, mesenteric lymph nodes; OD, optical density; OVA, ovalbumin; PBS, phosphate-buffered saline; PPs, Peyer patches; SEM, standard error of the mean; ST, Shiga toxin; Th, T helper; TLR4, Toll Like Receptor 4; U-Omp19, Unlipidated Outer membrane protein 19; U-Omp16, Unlipidated Outer membrane protein 16.

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and is now being used in a human vaccine (Dukoral®) [5]. Double-mutant LT (dmLT), with reduced enterotoxicity was developed and shown to increase immune responses [6]. Different strategies to increase Ag delivery like antigen targeting to M cells, nanoparticle-releasing vaccines at the colon, *etc*. for enhancing the efficacy of mucosal vaccines have been and are currently being investigated [7–10]. Meanwhile different new approaches need to be explored to develop novel oral adjuvants and delivery systems [11], especially there is a need of those that can induce T helper (Th)1 and $CD8^+$ T cell responses that can prevent infectious diseases related to intracellular pathogens.

In our laboratory we have been working on the use of a *Brucella* spp. protein devoid of its lipid moiety called U-Omp19 (Unlipidated Outer membrane protein 19) as an antigen for a vaccine against brucellosis. This protein is soluble and easy to express and purify at high scales [12,13]. Oral immunization with U-Omp19 produced either in *E. coli* or in plants without adjuvants conferred significant protection against oral *B. abortus* infection and induced a mixed Th1-17 immune response independent of TLR4. U-Omp19 also induced the maturation of murine dendritic cells (DCs) *in vivo* [14].

Of note, BLAST, Pfam and MEROPS sequence analysis report that U-Omp19 has sequence identity with other bacterial protease inhibitors, particularly with those of the protease inhibitor family inh from *Erwinia chrysanthemi* (family I38) [15,16]. This family of proteins would interact with specific proteases released by plant, insect and animal pathogens [15,17]. Thus, we hypothesize that U-Omp19 would inhibit protease activity at mucosal tissues and would increase the half-life of the co-administered Ag thus increasing its immunogenicity.

In this work we demonstrate that this bacterial protease inhibitor protects antigens delivered in oral vaccines from digestion while triggers specific mucosal immune responses. To our knowledge there are no reports in the literature describing the use of bacterial protease inhibitors in oral vaccine formulations. Therefore, this is an unconventional bacterial molecular pattern never explored before.

2. Materials and methods

2.1. Ethics statement

All experimental protocols of this study were conducted in strict accordance with international ethical standards for animal experimentation (Helsinki Declaration and its amendments, Amsterdam Protocol of welfare and animal protection and National Institutes of Health, USA NIH, guidelines: Guide for the Care and Use of Laboratory Animals). The protocols of this study were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) from the University of Buenos Aires (Permit Number: 2079) and CICUAE from University of San Martin (Permit Number: 052,014), Buenos Aires, Argentina.

2.2. Animals

Eight week old female BALB/c, C57BL/6 or C3H/HeN mice were purchased from University of La Plata (La Plata, Argentina) or from University of San Martín (UNSAM) and housed in the animal resources facility of University of Buenos Aires (UBA) or UNSAM (Buenos Aires, Argentina). Animals were devoid of food but with free access to water 2 h previous and 2 h after any administration/immunization.

2.3. Ags and adjuvants

Chicken egg OVA grade V (Sigma) was used as a model Ag. Recombinant unlipidated (U)-Omp19 was obtained as previously described [13]. LPS contamination from U-Omp19 was adsorbed with Sepharose-polymyxin B (Sigma). Endotoxin determination was performed with Limulus amoebocyte chromogenic assay (LONZA). All U-Omp19 preparations used contained <0.1 endotoxin units per mg of protein. CT and aprotinin were purchased from Sigma.

2.4. Determination of protease inhibitor activity in vitro and in vivo

Protease activity was determined using a casein fluorimetric kit (EnzChek, Invitrogen). This kit has casein-BODIPY-FL, whose fluorescence is quenched. Protease-catalyzed hydrolysis relieves this quenching, yielding bright green fluorescent peptides. The increase in fluorescence emission is proportional to casein digestion and protease activity. Pepsin (0.483 μM, Sigma), pancreatic Elastase (0.965 μM, Sigma), Trypsin (0.965 μM, Sigma), α-Chymotrypsin (0.965 μM, Sigma), Carboxypeptidase A (0.160 μM, Sigma) and B (0.065 μM, Sigma) were incubated with U-Omp19 at different molar ratios protease:U-Omp19 (1:1, 1:5, 1:10 and 1:50 — only for pepsin). As positive control a mammalian protease inhibitor cocktail was used. Bovine serum albumin (BSA) and U-Omp16 – a *Brucella* spp. protein with a similar molecular weight to U-Omp19 and expressed and purified in the same way – were used as negative controls. Each reaction mix was incubated at room temperature (RT) for 1 h and then substrate (casein-BODIPY-FL, 1 μg/ml) was added. Fluorescence was measured with a fluorescence plate reader (FilterMaxF5 Molecular devices).

To study pH stability of the inhibitory activity of U-Omp19, U-Omp19 was pre-incubated at RT for 1 h at different pH (2, 5, 7.5 and 8) buffers containing 10 mM sodium phosphate and 50 mM NaCl and then adjusted to the optimum pH for α-Chymotrypsin (pH = 7.8). Microcon 3 (Biopore, Germany) was then used to exchange buffers. Residual inhibitor activity was assessed using casein-BODIPY (1 μg/ml) and α-chymotrypsin (0.965 μM) as model protease in a molar ratio protease: U-Omp19 of 1:10. Thermal stability of the inhibitory activity of U-Omp19 was assessed by incubating different tubes containing U-Omp19 at different temperatures (25–100 °C) for 1 h and subsequently adjusting them at RT. Residual inhibitor activity was assessed as above described for pH stability experiments using casein-BODIPY-FL and α-chymotrypsin or elastase (0.965 μM) as model proteases. Far-UV circular dichroism (CD) spectra of U-Omp19 under different pHs and temperatures were conducted (see supp. Materials and methods).

To further study the protease inhibitory mechanism of U-Omp19, the kinetic of trypsin, α-chymotrypsin and pancreatic elastase inhibition was performed (see supp. Materials and methods).

To evaluate if U-Omp19 inhibits proteolytic activity of stomach and intestine extracts, stomach or intestine extracts from mice were pre-incubated with buffer, different amounts of U-Omp19, Inhibitor cocktail or BSA as negative control. Then, the mixtures were incubated with casein BODIPY-FL for 1 h or with OVADQ (quenched protein that releases fluorescence upon digestion) for 4 h and the fluorescence increment was determined.

To study protease inhibitor activity *in vivo* BALB/c mice were orally — intragastrically (i.g.) — administered with casein-BODIPY alone (100 μg), plus U-Omp19 (150 μg) or aprotinin (1 μg). Stomachs were removed 15 min and 1 h after oral delivery and extracts of the organs were obtained. Afterwards Ag digestion was determined by fluorescence emission. The dose of aprotinin used in this work was chosen because it did not produce adverse effects on treated animals and also induced proteolysis inhibition of the antigen similar to U-Omp19 (data presented in this manuscript and not shown).

To study Ag fate after delivery BALB/c mice were i.g. administered with PBS, OVA-FITC (100 μg) alone or OVAFITC (100 μg) plus U-Omp19 (150 μg). Stomachs and small intestines were removed 90 min after oral delivery and extracts of the organs were obtained. Afterwards Ag presence was determined by fluorescence emission.

2.5. Determination of Ag fate and proteolysis in vivo

To study Ag proteolysis *in vivo* Casein-BODIPY-FL or OVADQ were used. To evaluate Ag fate and internalization *in vivo* OVA-AlexaFluor647 or OVA-FITC were used. If Ags (OVA-FITC or casein-BODIPY-FL) were used alone, mice were fed Ag (100 μg) plus i) buffer,