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A novel antigen-carrier system: The *Mycobacterium tuberculosis* Acr protein carried by raw starch microparticles

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ABSTRACT

Microparticles have been used as promising carriers for in vivo vaccine delivery. However, the processes for immobilizing peptides or proteins on microparticles usually require the use of undesirable compounds and complex protocols. In this work, we propose a new immobilization and delivery system with raw starch microparticles and a starch binding domain (SBD) tag fusion protein. The heat shock protein alpha crystallin from *Mycobacterium tuberculosis* was used as model. The immunogenicity of the system was investigated in BALB/c mice inoculated with purified Acr-SBD_{tag} protein (pAcr-SBD_{tag}) and starch immobilized Acr-SBD_{tag} protein (μAcr-SBD_{tag}) by oral and intranasal routes. We demonstrated mucosal immunization with the μAcr-SBD_{tag} protein induced systemic antibodies that were predominantly immunoglobulin G2a (IgG2a). An analysis of the cytokines from spleen cells and lung homogenates revealed that loaded microparticles induced the secretion of interferon-γ (INF-γ), suggesting an adjuvant effect from the immobilization. The immune responses induced by immobilized protein were primarily affected by the route of administration. These results demonstrate that the system exhibits the necessary characteristics to improve antigen release and presentation to antigen presenting cells (APCs) in the mucosae. Because no extra adjuvants were used, we posit that the system may be suitable for delivery and presentation to the field of subunit vaccine development.

1. Introduction

The association of proteins or peptides with polymeric microparticulated systems is a successful strategy to carry and deliver antigens in the mucosae. Not only do the systems retain activity and prevent enzymatic degradation, but they also induce strong immune responses against many antigens such as protein subunits, peptides or naked DNA, which are poorly immunogenic when administered without adjuvants (Perrie et al., 2008).

Biocompatible polyester, in the form of poly-lactide-co-glycolide (PLG) microparticles, is the most commonly used to adsorb or encapsulate a variety of agents. This material has been used in humans for many years as resorbable suture material and as a controlled release and delivery system (Elamanchili et al., 2004; Waeckerle-Men and Groettrup, 2005). These microparticles are used to induce enhanced responses, especially for cases in which strong compartmentalized immune responses are needed (Csaba et al., 2008; Malyala et al., 2009). Similarly, chitosan (Amidi et al., 2007) or polyacryl starch microparticles (Heritage et al., 1996; Wikingsson and Sjöholm, 2002), which are theoretically biodegradable and have mucoadhesive properties, have shown adjuvant potential and favored the induction of enhanced immune responses in mucosal tissues (Rydell and Sjöholm, 2005; Balasse et al., 2008). However, regardless of the support material, a chemical process and the cross-linking of organic compounds are usually necessary for allowing covalent interactions between the support and the protein or peptide of interest, which is a process that can affect the protein during preparation (van de Weert et al., 2000).

An advantageous strategy to immobilized peptides or proteins on polymeric raw substrates without the need for covalent binding is to use systems comprising carbohydrate binding

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modules (CBMs) (Nilsson et al., 1997). These systems permit the simultaneous purification and immobilization of recombinant proteins fused with the selected affinity tag, and they offer significant benefits for the research and pharmaceutical industry because immobilization can be performed on abundant and cheap matrices that are already in particulate form. In this work, we use a Starch Binding Domain as a tag to attach a fusion protein on raw starch microparticles and for its later mucosal administration. This SBD has been successfully used as an affinity tag for the adsorption of several proteins to raw starch and for protein purification by affinity chromatography (Guillén et al., 2013). Even in previous studies, a potential was demonstrated for using this non-covalent binding system to confer stability to recombinant immobilized proteins under harsh conditions, such as those of the gastrointestinal tract (Moreno-Mendieta et al., 2014, submitted).

The carrier and adjuvant potential of the SBD_{tag}–starch microparticle system was tested by using the protein alpha crystallin (Acr) from *Mycobacterium tuberculosis.* Acr (also called Acr1, HspX, Hsp16.3, or Rv2031c) is a heat shock protein and an ATP-independent chaperone of low molecular weight that is induced by hypoxia (Rosenkrands et al., 2002). It is important in the extracellular phase of the pathogen and contributes to replication during the initial infection and growth of mycobacteria in the macrophage (Yuan et al., 1998). However, the most important role of this protein seems to be the viability maintenance of long-term bacilli survival and adaptation to the environment during latent infection (Monahan et al., 2001). The possibility of using this protein as a component in subunit vaccines that were designed to replace or reinforce protection with BCG was documented some years ago (Roupie et al., 2007; Zvi et al., 2008) and recently mentioned (Singh et al., 2013). Its participation in the lag phase, the fact that the serum of patients with latent tuberculosis is enriched with antibodies that strongly recognize this protein (Demissie et al., 2006), and its recent use in multistage subunit vaccines (Niu et al., 2011) or immune complexes (Pepponi et al., 2013) demonstrate its potential in differential diagnosis and as a vaccine component.

Here, we used BALB/c mice to evaluate the ability of starch microparticles to facilitate Acr-SBD_{tag} protein uptake, transport and presentation after oral (*o.*) and intranasal (*i.n.*) immunization without the need for extra adjuvants. We tested the capacity of the loaded microparticles (μAcr-SBD_{tag}) to enhance the antibody response against Acr protein and its efficiency in inducing interferon gamma (IFN-γ) production.

2. Materials and methods

2.1. The production of $AcrSBD_{tag}$ and Acr proteins

The plasmid pQAcr-SBD_{tag} that expressed the fusion protein was constructed and produced as previously described. In brief, the alpha crystallin coding region from *M. tuberculosis* (GB M76712) was PCR-amplified from a pCR4Topo-Acr template, which was kindly donated by Dr. C. Espitia, using synthetic oligonucleotide primers containing the *BamH*I sequence. The amplified fragment was purified and ligated to the pGEM-T-*Easy* vector following the manufacturer's instructions and then transformed into *E. coli* DH5α by electroporation (Sambrook et al., 1989). The resulting pG-Acr construct was verified by PCR and sequencing (Laragen, Inc., Los Angeles, CA). The fragment released by digestion was cloned into the pQE31-SBD_{tag} vector containing a His_{tag} at the N-terminus and the SBD_{tag} at the C-terminus. The recombinant Acr-SBD_{tag} protein was purified from the clarified extracts by affinity chromatography using an ÄKTA Prime system (GE Healthcare, Chalfont St. Giles, UK) and a β-cyclodextrin-epoxy-activated Sepharose 6B column (16 by 35 mm) (Guillén et al., 2013).

To obtain Acr protein as a control, the pET-Acr vector (Dr. C. Espitia), was used to express 6xHis-tagged-Acr protein under the control of the phage T7 promoter. The vector was transformed into *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA), and clones of the strain were grown at 29 °C in LB medium with 1% glycerol supplemented with ampicillin (100 μg/mL). Protein expression was induced by the addition of IPTG (0.1 mM). The resulting cultures were harvested after 4 h, the cells were disrupted, and the recombinant protein was purified from the clarified extracts by metal affinity chromatography using an ÄKTA Prime system (GE Healthcare, Chalfont St. Giles, UK) and a HisTrapTM 15HP column. The proteins concentration was determined by measuring the absorbance at 280 nm with the theoretical molar extinction coefficients (Acr-SBD_{tag} ε = 104170 L/mol cm; Acr ε = 4470 L/mol cm) obtained for each protein with the software ProtParam (ExPASy). Purity of proteins was analyzed by SDS-PAGE (Laemmli, 1970).

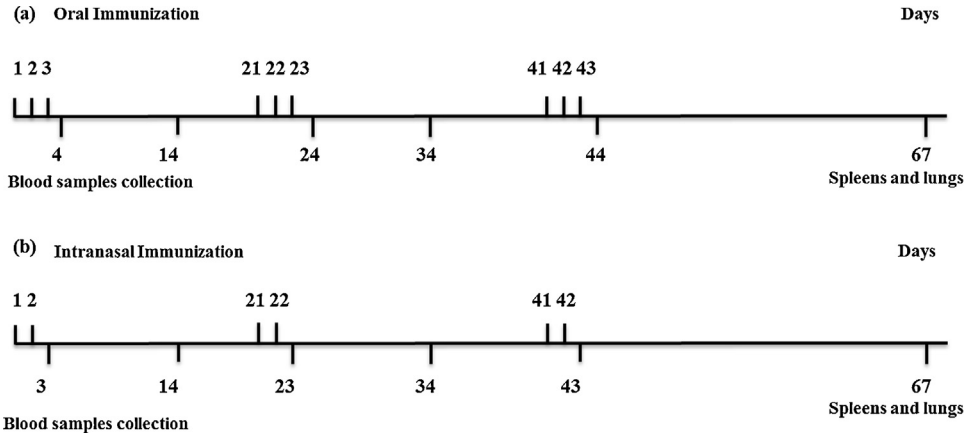

Fig. 1. Immunization protocols and sample collection schemes. The schemes of consecutive doses were performed without the use of adjuvants. Each dose for oral administration contained 50 or 100 μg of immobilized or free Acr-SBD_{tag} Booster doses were given in the same manner on days 21–23 and 41–43. Mice immunized intranasally received 25 or 50 μg of immobilized or free Acr-SBD_{tag} protein in two consecutive days (1–2). Booster doses were given in the same manner on days 21–22 and 41–42. Control mice were treated under the same immunization protocols. Positive control mice were administered with Acr protein at the same doses. Mice administered with 1 mg of starch microparticles were used as carrier control and untreated mice were used as negative controls. Pre-immune or negative serum was obtained from mice before immunization.