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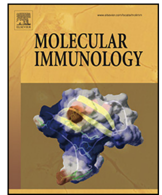

Lactobacillus buchneri S-layer as carrier for an Ara h 2-derived peptide for peanut allergen-specific immunotherapy

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ABSTRACT

Peanut allergy is an IgE-mediated severe hypersensitivity disorder. The lack of a treatment of this potentially fatal allergy has led to intensive research on vaccine development. Here, we describe the design and initial characterization of a carrier-bound peptide derived from the most potent peanut allergen, Ara h 2, as a candidate vaccine. Based on the adjuvant capability of bacterial surface (S-) layers, a fusion protein of the S-layer protein SlpB from *Lactobacillus buchneri* CD034 and the Ara h 2-derived peptide AH3a42 was produced. This peptide comprised immunodominant B-cell epitopes as well as one T cell epitope. The fusion protein SlpB-AH3a42 was expressed in *E. coli*, purified, and tested for its IgE binding capacity as well as for its ability to activate sensitized rat basophil leukemia (RBL) cells. The capacity of Ara h 2-specific IgG rabbit-antibodies raised against SlpB-AH3a42 or Ara h 2 to inhibit IgE-binding was determined by ELISA inhibition assays using sera of peanut allergic patients sensitized to Ara h 2. IgE specific to the SlpB-AH3a42 fusion protein was detected in 69% (25 of 36) of the sera. Despite the recognition by IgE, the SlpB-AH3a42 fusion protein was unable to induce β-hexosaminidase release from sensitized RBL cells at concentrations up to 100 ng per ml. The inhibition of IgE-binding to the natural allergen observed after pre-incubation of the 20 sera with rabbit anti-SlpB-AH3a42 IgG was more than 30% for four sera, more than 20% for eight sera, and below 10% for eight sera. In comparison, anti-Ara h 2 rabbit IgG antibodies inhibited binding to Ara h 2 by 48% ±13.5%. Our data provide evidence for the feasibility of this novel approach towards the development of a peanut allergen peptide-based carrier-bound vaccine. Our experiments further indicate that more than one allergen-peptide will be needed to induce a broader protection of patients allergic to Ara h 2.

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1. Introduction

Peanut seeds are widely utilized in the food industry due to their high protein and oil content but, at the same time, they are one of the most common causes for severe allergic reactions (Bock et al., 2001; Bock et al., 2007). Allergen sensitization develops early in life, but in contrast to other food allergies in childhood, such as cow's milk allergy, peanut allergy tends to persist into adulthood and thereby affects 0.6–0.8% of the adult population (Osborne et al., 2011; Skolnick et al., 2001; Soller et al., 2012). An efficient therapy is not available and therapeutic approaches that modify the immune response to peanut allergens and induce oral tolerance are being intensively investigated (reviewed in (Bublin and Breiteneder, 2014a; Commins et al., 2016; Kingwell, 2016)).

Several attempts have been made to design hypoallergenic variants of the major peanut allergens to avoid serious side effects, but still to generate tolerance or desensitization when used as vaccine components. Use of genetically modified peanut allergens with a highly reduced capacity to bind IgE but without compromising the ability of T cell stimulation is widely investigated (Burks et al., 1999; King et al., 2005; Rabjohn et al., 2002; Rolland et al., 2009; van

Abbreviations: CBB, colloidal Coomassie Brilliant Blue R, 250; GdHCl, guanidinium hydrochloride; GRAS, generally regarded as safe; OD, optical density; RBL cells, rat basophilic leukemia cells; S-layer, cell surface layer.

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Hoffen et al., 2014). Several immunotherapy trials were conducted with hypoallergenic peanut proteins and heat-killed bacteria as adjuvants (Li et al., 2003a,b; Wood et al., 2013). Another attractive approach is the use of short, linear peptides representing T cell epitopes of the major allergens that are able to modulate allergen-specific T cell responses without the ability to cross-link IgE and to activate effector cells (Sicherer and Sampson, 2007).

Bacterial surface (S-) layers, which are two-dimensional crystalline arrays of (glyco)protein subunits that make up the outermost layer of many bacteria, are excellent candidates to be used as antigen carriers in vaccine development (Sleytr et al., 2007; Sleytr et al., 2014). Proof-of-principle was accomplished by the translational fusion of the major birch pollen allergen Bet v 1 to the S-layer protein SbsC from *Geobacillus stearothermophilus* resulting in an *Escherichia coli*-produced fusion protein which showed reduced allergenicity and immunomodulatory capacity (Bohle et al., 2004; Gerstmayr et al., 2007; Gerstmayr et al., 2009). S-layers from lactic acid bacteria are particularly interesting candidates for carrier molecules in vaccine research because of their "generally regarded as safe" (GRAS) status. Currently, lactic acid bacteria are being intensively investigated as live vaccine delivery systems (Berlec et al., 2012; Trombert, 2015). Furthermore, their ability to withstand the passage through the gastrointestinal tract makes them an ideal oral antigen delivery system (Hynönen and Palva, 2013). In this context, the development of *Lactobacillus* strains carrying S-layers composed of tailored fusion proteins and, thus, displaying thousands of regularly arranged copies of antigenic peptides on the bacterial cell surface, is of great interest. Model peptides have already been fused to and displayed on each monomer of the S-layer of *Lactobacillus brevis* (Avall-Jääskeläinen et al., 2002) and *Lactobacillus acidophilus* (Smit et al., 2002) by chromosomal integration based on homologous recombination.

An important feature of B-cell epitope–based vaccines is that the use of a strongly immunogenic carrier allows induction of allergen-specific IgG antibodies also against allergens that intrinsically are poorly immunogenic and, thus, would induce a poor blocking IgG response when used as natural allergen. However, regarding food allergy, little is known until now about such vaccines based on an immunogenic carrier protein.

In this study, we investigated the S-layer protein SlpB from *Lactobacillus buchneri* CD034 as a carrier of a peptide derived from a major peanut allergen for peanut allergen-specific immunotherapy. Out of sixteen peanut allergens reported to date (www.allergen.org; reviewed in (Bublin and Breiteneder, 2014b)), Ara h 2 was described as the most dangerous one and was identified as a predictor of clinical reactivity to peanut (Nicolaou et al., 2010; Nicolaou et al., 2011; van Erp et al., 2016). Of ten identified Ara h 2 IgE-binding epitopes, three are immunodominant and located of the exposed and structurally flexible regions in the native protein (King et al., 2005; Stanley et al., 1997). Multiple T cell epitopes of Ara h 2, with three amino acid regions containing the majority of peptides that reacted with T cells from most patients, have been reported and the results hinted at the potential use of these peptides to treat peanut allergy (Glaspole et al., 2005; Prickett et al., 2011, 2013).

Here, we produced and characterized a carrier-bound Ara h 2-derived peptide. This fusion protein consisted of the recombinant S-layer protein SlpB from *Lactobacillus buchneri* CD034 and the Ara h 2-derived immunodominant peptide AH3a42. The recombinant fusion protein (His_6-SlpB-AH3a42) was expressed in *E. coli*, purified, and tested for its IgE-binding capacity as well as for the ability to release mediators from sensitized rat basophilic leukemia (RBL) cells. The recombinant construct without the Ara h 2-derived peptide served as control. Additionally, the generation of peanut allergen blocking IgG antibodies in rabbits after immunization with the SlpB-AH3a42 construct was investigated.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli DH5α (Life Technologies, Vienna, Austria) and *E. coli* BL21 (DE3) (Thermo Fisher Scientific, Life Technologies, Waltham, MA USA) were cultivated at 37 °C and 200 rpm in lysogeny broth (LB) medium supplemented with 50 μg kanamycin (Kan) per ml.

2.2. Cloning of the recombinant SlpB fusion constructs

The expression vector pET28a(+) (Novagen, Madison, WI, USA) was used for the production of N-terminally hexahistidyl-tagged SlpB and the Ara h 2-derived peptide fusion construct. The gene encoding SlpB devoid of its N-terminal signal sequence was PCR-amplified from genomic DNA of *L. buchneri* CD034. The construct His-SlpB was PCR amplified with the primer pairs (Thermo Fisher Scientific, Life Technologies, Waltham, MA USA) His_SlpB_NcoI_for/SlpB_XhoI_rev (Table 1). The construct His-SlpB-AH3a42 was in a first step PCR-amplified with primer pair SlpB_NheI_for/SlpB_AH3a42_1_rev and in a second step, with the primer pair SlpB_NheI_for/SlpB_AH3a42_2_XhoI_rev using the PCR product from step 1 as a template (Table 1). The final products His-SlpB and His-SlpB-AH3a42 were digested with NcoI/XhoI and NheI/XhoI and ligated into the NcoI/XhoI and NheI/XhoI linearized expression vector.

2.3. Expression and purification of the recombinant SlpB and SlpB-AH3a42 fusion protein

The resulting recombinant plasmids (pET28a-His-SlpB and pET28a-His-SlpB-AH3a42) were propagated into *E. coli* BL21 Star (DE3) cells for production of hexahistidyl-tagged proteins. The transformed strains were grown in 800 ml of LB medium, each, supplemented with kanamycin (50 μg/ml) at 37 °C and 200 rpm. At the mid-exponential growth phase ($OD_{600} \sim 0.6$), protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and cultivation was continued for 4.5 h at 37 °C. Cells were pelleted by centrifugation, washed with 0.9% NaCl, resuspended in lysis buffer (50 mM sodium citrate buffer, pH = 6.2, 0.1% Triton X-100) and, after addition of lysozyme (800 μg/ml; Sigma-Aldrich, St. Louis, MO, USA) and benzonase (50 U/ml; Sigma-Aldrich), incubated for 30 min at 37 °C. Bacteria were further lysed by ultrasonication (Branson Sonifier, duty cycle 50%; output 6) applying ten cycles of 10 pulses with 30 s breaks, each, and insoluble inclusion bodies containing the recombinant proteins were pelleted. The proteins were extracted from the pellets with binding buffer (50 mM sodium citrate buffer, pH = 5.5, 5 M GdHCl, 20 mM imidazol, 0.5 M NaCl) for 1 h at 4 °C. The extracts were centrifuged and membrane-filtered (0.45-μm pore size). The resulting protein samples were applied to a 1-ml HisTrap HP column (Thermo Fisher Scientific, Waltham, MA USA) and recombinant proteins were recovered with elution buffer (50 mM sodium citrate buffer, pH = 5.5, 5 M GdHCl, 1 M imidazol, 0.5 M NaCl). Recombinant SlpB and the SlpB-based fusion protein were dialyzed against PBS at 4 °C for 24 h and concentrated using Amicon ultra-15 centrifugal filter units (Merck Millipore, Darmstadt, Germany) with 30 kDa cut off. Precipitates were removed from soluble proteins by centrifugation. The absorption of the protein solutions at 280 nm was measured by a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) and protein concentrations were calculated. The aggregation profile of the recombinant proteins His-SlpB and

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