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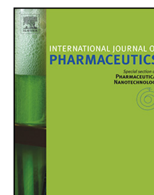

Pharmaceutical Nanotechnology

An allergen-polymeric nanoaggregate as a new tool for allergy vaccination

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ABSTRACT

A recombinant hybrid composed of the two major allergens of the *Parietaria* pollen Par j 1 and Par j 2 has been generated by DNA recombinant technology (PjED). This hybrid was produced in *E. coli* at high levels of purity. Then, the engineered derivative has been combined with a synthetic polyaminoacidic derivative having a poly(hydroxyethyl)aspartamide (PHEA) backbone and bearing both butyryl groups (C4) and succinyl (S) moieties in the side chain (PHEA-C4-S). The allergen-copolymer nanoaggregate was characterized by means of DLS, zeta potential, electrophoretic mobility and atom force microscopy analysis displaying the formation of a stable complex. Its safety has been proved *in vitro* on a murine cell line, human erythrocytes and basophils. Moreover, the formation of the complex did not alter the ability of the allergens to cross-link surface bound specific IgE demonstrating that the combination of an engineered hybrid with a copolymer did not interfere with its biological activity suggesting its employment as potential vaccine against *Parietaria*-induced allergies.

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1. Introduction

IgE-mediated allergy is one of the most common immunological diseases affecting an increasing percentage of people living in the industrialized countries (Holgate and Broide, 2003).

The symptoms of allergic reactions include several local symptoms such as rhinitis and conjunctivitis, but systemic life-threatening reactions including anaphylactic shock were reported as well. Furthermore, it has been observed that if not properly diagnosed and treated, allergy can progress to a severe and chronic disabling disease such as asthma. The only treatment able to modify the natural outcome of the disease restoring a normal immunity against allergens is specific immunotherapy (SIT) (Bousquet et al., 1998).

So far, immunotherapy is performed by s.c. injection or mucosal administration of a mixture of proteins from natural sources which are not easy to standardize and without taking care of the individual sensitization profile of the patient (Brunetto et al., 2010; Focke et al., 2008, 2009). In the last years, a few papers demonstrated that clinical immunotherapy trials with recombinant wild-type allergens were safe and effective and can replace allergen extract-based vaccines (Jutel and Cromwell, 2006; Pauli et al., 2008). In addition, it has been reported that allergens can be produced as hybrid molecules incorporating the epitopes of several allergens with the advantage to facilitate vaccine production and to increase immunogenicity (Linhart et al., 2002, 2005).

Parietaria judaica (Pj) pollen allergens are proteins from dicotyledonous weeds of the Urticaceae family. Immunologically, this family represents the most relevant species since its pollen represents one of the main outdoor sources of allergens in the Mediterranean area (D'Amato et al., 2007). In particular, almost 30% of all the allergic subjects in the Southern Mediterranean present a Skin Prick Test (SPT) reactivity toward the Pj pollen and more than 50% of these subjects have experienced bronchial asthma with high levels of bronchial hyper-responsiveness (D'Amato, 2000).

The composition of the allergenic extracts of the Pj pollen has been studied and, by means of molecular cloning, it has been demonstrated that this pollen contains at least 3 major allergens belonging to the Lipid Transfer Proteins (LTP) (Amoresano et al., 2003). In particular, the family of the Par j 1 allergen is

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composed of the Par j 1.0101, a protein of 139 amino acids (Costa et al., 1994), and a shorter variant, Par j 1.0201, composed of 102 amino acids (Duro et al., 1997). The coding regions of the Par j 1.0102 and Par j 1.0201 isoforms show a 95% identity at the amino-acid level within the first 97 amino acids. The two isoforms differs for the presence of a 37 amino acids COOH-terminal tail in the Par j 1.0101 allergen which has been shown to contain a LPS binding region with immunomodulatory activity (Bonura et al., 2013). The Par j 2 allergen is a 102 amino acid long protein with a deduced Mw of 11,344 Da. Sequence similarity, 3D modeling (Colombo et al., 1998) and enzymatic digestion (Amoresano et al., 2003) have shown that major Pj allergens attain a three-dimensional structure consistent with that of the Lipid Transfer Proteins (LTP). Par j 1 and Par j 2 allergens are the species-specific allergens (Stumvoll et al., 2003) and represent the major allergens of this pollen (Costa et al., 1994; Duro et al., 1996).

Following this line of evidence, our research group developed several strategies to design new pharmacological compositions as new tool to cure Pj allergic patients (Bondi et al., 2011; Bonura et al., 2001, 2007; Orlandi et al., 2004). In particular, a hybrid molecule comprising the Par j 1 and Par j 2 allergens was evaluated *in vitro* and *in vivo* showing that heterodimers present improved immunological features (Bonura et al., 2007, 2012).

In this paper, firstly we described the design of a head–tail hybrid molecule expressing two isoforms of the major allergens of the Pj pollen, Par j 1.0201 and Par j 2.0101. This hybrid was expressed in *E. coli* as a His-tagged recombinant protein. Then the engineered derivative has been combined with a properly synthesized biocompatible multifunctional copolymer in order to produce a polymeric nanoaggregate of the allergens. This copolymer is a synthetic polyaminoacidic derivative having a poly(hydroxyethyl)aspartamide (PHEA) backbone and bearing both butyryl groups (C4) and succinyl (S) moieties in the side chain (PHEA-C4-S). Recently, it reported the ability of this copolymer to other complex protein molecules such as insulin and protect this protein against chemical and enzymatic degradation (Licciardi et al., 2013b).

Experimental data reported herein demonstrated that the combination of this copolymer with the engineered hybrid expressing the allergens of the Pj pollen retain its biological activity suggesting its employment as potential vaccine against *Parietaria*-induced allergies.

2. Experimental

2.1. Cloning, expression and purification of a recombinant Par j 2/Par j 1 hybrid

A head-to-tail hybrid expressing the wild type Par j 1 and Par j 2 allergens was generated by PCR (named PjED). Briefly, a DNA fragment containing the full-length coding region of the wild type Par j 2 sequence (EMBL accession number #X95865) was generated by PCR using the Pj2 for (5′-att**GGATCC**CAAGAAACCTGCGGGACTATG-3′) and Pj2rev (5′-cgc**GGATCC**ATAGTAACCTCTGAAAATAGT-3′) oligonucleotides. Following the same strategy, a DNA fragment containing the full-length coding region of the Par j 1.0201 allergen (EMBL accession number #X85012) (Duro et al., 1997) was generated using the Parj1for (5′-att**GGATCC**TGAAGAAACTTGCGG-3′ and the Parj1rev (5′-cgc**GGATCC**CTAATTTCCTTTGTAGTG-3′) oligonucleotides. Bold letters indicate the restriction enzyme sites (Bam H1) introduced for cloning and lower case letters the nucleotides inserted to improve restriction enzyme cut efficiency. A head-to-tail dimer (Parj2–Parj1) containing the two cDNAs was prepared cloning the

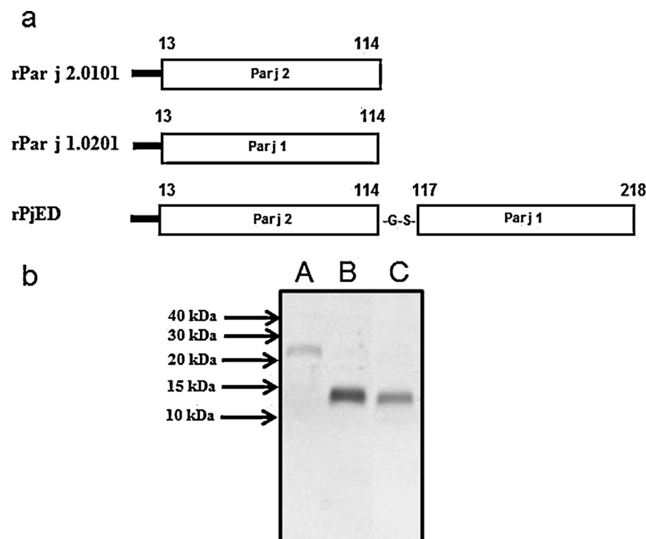

Fig. 1. Structure and Coomassie brilliant blue stained SDS-PAGE of the purified rPjED hybrid. (Panel a) Schematic representation of the Par j 1, Par j 2 and engineered hybrid PjED. Solid bars indicate the amino-terminal tag; open boxes the coding regions of the Par j 2 and Par j 1 allergens. Numbers show the size of the proteins. (Panel b) Coomassie brilliant blue stained SDS-PAGE of the purified recombinant proteins used for the assays. Molecular weights are indicated on the left side. Lane A shows the PjED hybrid, lane B and C the rPar j 1 and rPar j 2 allergens, respectively.

Par j 1 and Par j 2 BamH1 restricted fragments within the BamH1 restriction site of the pQE30 vector (Qiagen, Milan). The correct orientation of the two cDNA was checked by sequencing. Engineered hybrid was transfected for expression into *E. coli* M15 strain (Qiagen, Milan, Italy). The recombinant derivative expresses a fusion protein of 218 AA containing a 12 amino-terminal fusion peptide, a hexa-histidine tag and two additional amino acids (G and S) in position 115 and 116 as a consequence of the cloning procedures (sequence of the Bam H1 cloning site) (see Fig. 1 panel a for details). Engineered hybrid was purified as previously described (Bonura et al., 2007). Briefly, recombinant clones were grown over night at 37 °C in 2YT broth (Bacto-tryptone 16 g/l, Bacto-yeast 10 g/l, NaCl 5 g/l, pH 7.0). A 1:40 dilution was made and the culture was grown for 2 h at 37 °C and, after that, induced with isopropyl-D-thiogalactopyranoside for 2 h at 37 °C. Then cells were harvested, resuspended in a buffer containing 20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 8 M urea and lysed with mild sonication. Cell debris was removed by centrifugation at 10,000 rpm for 30′ at 4 °C. The supernatant was filtered using a 8 μm disk and then loaded on a HisTrap column (GE, Uppsala, Sweden) following the manufacturer's instructions. The recombinant proteins were eluted using a buffer containing 20 mM phosphate buffer pH 7.4, 0.5 M NaCl, 8 M urea and 500 mM imidazole. Then, the fractions containing the recombinant PjED were reload on a HisTrap column for a second run of purification and eluted as above described. The collected fractions were analyzed by 16% SDS-PAGE and Coomassie brilliant blue staining. Fractions containing the recombinant hybrid were dialyzed against a buffer containing 20 mM phosphate buffer pH 7.4, 0.5 M NaCl to allow refolding of the proteins. Buffer exchange was performed using a Sephadex G-25 column (GE, Uppsala, Sweden) in 1× PBS. Purity and concentration were determined by Coomassie brilliant blue staining and densitometric analysis. Recombinant proteins for the cell assays were further purified using a Detoxi-gel endotoxin removing gel (Pierce, USA) and tested for the endotoxin content using the Multi-test Limulus Amebocyte Lysate (LAL) pyrogen plus test (Bio-Whittaker, USA).

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