**Objective and background:**

The aim of the project “Novel Vaccines for Allergen-specific immunotherapy of Ragweed pollen Allergy” is two-fold: (1) to develop the heterologous expression of hypoallergenic pectate lyases from ragweed and mugwort pollen and (2) to characterise the allergenic and immunogenic effect of these proteins for use as putative vaccine candidates for allergen-specific immunotherapy.

Ragweed is considered highly allergenic since very low pollen concentrations are sufficient to trigger allergic symptoms in sensitized individuals, including hay fever, asthma, contact dermatitis and anaphylaxis. Short ragweed or common ragweed *(Ambrosia artemisiifolia or* *Ambrosia elatior)* is widespread especially across the United States, Canada and Europe, where it is particularly abundant in France, North Italy, Austria, Hungary, Croatia and Bulgaria. Amb a 1 is the major allergen of ragweed pollen which cross-reacts with Art v 6 from mugwort pollen at the IgE and T cell level. Both proteins comprises of two chains, the alpha-chain featuring all the requirements of a hypoallergenic molecule i.e. low IgE reactivity and maintained T cell reactivity, and could therefore be considered a naturally occurring hypoallergen. However, plant derived members of this family are difficult to produce in a heterologous recombinant form and consequently are not available for therapy. Therefore, clinically relevant, well defined hypoallergenic variants are needed for the development of better reagents for immunotherapy with minimised risk of anaphylactic side effects and shorter regimens compared to the variable biological extracts.

**Results:**

Both alpha chains from Amb a 1 (from ragweed pollen) and its homologous in mugwort pollen Art v 6 and a hybrid molecule, consistent of a combination of the clinically most relevant T cell epitopes of both pectate lyases Amb a 1 and Art v 6 alpha chains, were designed. The individual proteins as well as the hybridwere cloned, expressed in a suitable *Escherichia coli* host strain and purified to homogeneity by standard chromatographic techniques. A detailed study of the physicochemical, allergenic and immunogenic features of these molecules as well as their *in vivo* immune responses in a murine model was carried out. The secondary structure of the purified proteins indicated a mixture of alpha-helical and beta-sheet structures as determined by circular dichroism (CD)-spectroscopy which is in accordance with the known structure of pectate lyase proteins.

Human allergen-specific IgE binding capacity recombinant allergens was decreased compared to that of the natural counterpart as assessed by ELISA experiments using ragweed and mugwort allergic patients. Since previous studies have pointed out that antigen resistance to endolysosomal proteolysis in vitro enhances immunogenicity and to gain insight into antigen processing by the antigen presenting cells and their potential immunogenicity, all the proteins were subjected to *in vitro* endolysosomal degradation assays for up to 48 h. Amb a 1 alpha chain and hybrid were less susceptible to endolysosomal degradation than Art v 6 alpha chain.

Proliferative responses of peripheral blood mononuclear cells from 10 ragweed pollen-allergic individuals stimulated with equimolar concentrations of each of the proteins were determined. The candidate vaccines induced lymphoproliferative responses comparable with those induced by the wild type allergens, indicating that T cell epitopes are retained in the sequence of the engineered hypoallergens. Moreover, T cell lines specific to nAmb a 1 and nArt v 6 were generated from PBMC of 13 ragweed/mugwort allergic patients.

*In vivo* immune responses to Amb a 1 and Art v 6 alpha chain and hybrid were evaluated and compared to the natural proteins in a murine model. Mice immunisation with the recombinant proteins induced IgG1 and IgG2a antibodies against themselves as well as Amb a 1 or Art v 6-specific IgG1 and IgG2a antibodies. Their allergenic activity was evaluated by *in vitro* mediator release assays using RBL-2H3 cells expressing the high-affinity receptor for murine IgE. While natural Amba a 1 and Art v 6 induced strong degranulation of RBL-cells with all the tested sera, the recombinant proteins induced significant less mediator release. Splenic lymphocytes were isolated from immunized mice and challenge with the vaccine candidates to determine the comparative T-cell response. The recombinant proteins showed a mixed Th1/Th2 response with the production of antigen-specific cytokines IL-4, IL-5 (TH 2), IFN-γ (TH 1), IL-10 and IL-13.

**Conclusions and socio economical impact**:

This is the first time that hypoallergenic variants Amb a 1 and Art v 6 alpha chains have been produced and purified as recombinant soluble proteins. A hybrid molecule of the ragweed and mugwort pollen pectate lyases Amb a 1 and Art v 6 alpha chains has been also successfully expressed in a heterologous host. Up to date, no other detailed studies of the allergenic and immunogenic features of these proteins have been performed. Both alpha chains and the hybrid molecule showed reduced IgE reactivity and retained antigenicity or T cell reactivity. These proteins might be considered as candidates for specific immunotherapy for both ragweed and mugwort allergic patients.

Detailed investigation on the immunogenic properties of a panel of structural variants of a major allergenic protein will influence international research on the development of more efficient allergy vaccines substituting the highly variable allergen extracts and to advance the potential of patient-tailored immunotherapy. These results will be of Biotechnologicalinterest to develop safer vaccines for pharmaceutical application.

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