

Review article

Sublingual immunotherapy: from biological extracts to recombinant allergens

Sublingual vaccines based on biological extracts from various natural allergen sources are effective in the treatment of respiratory allergies. These vaccines comprise a complex mixture of proteins and glycoproteins that require dedicated standardization procedures to ensure batch-to-batch consistency. Because of the lack of correlation between the potency of an allergen extract and the quantity of major allergen content, standardization is achieved predominantly by determining the global IgE binding capacity of the extract *in vitro*. New proteomic technologies can be used to further characterize the most abundant proteins present in an extract. Second-generation sublingual vaccines based on recombinant allergens are under development. The aim is to produce molecularly defined vaccines that exhibit superior efficacy, while allowing for simplified immunization schedules. In this approach, recombinant DNA technology is used to express highly purified allergens in their native (i.e. wild-type) conformation. The recombinant allergens are then formulated with *ad hoc* adjuvants and/or mucoadhesive galenic excipients so that they specifically target oral Langerhans cells and induce allergen-specific regulatory T cells.

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Key words: allergen standardization; recombinant allergens; sublingual immunotherapy; therapeutic vaccine.

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Allergen-specific sublingual immunotherapy (SLIT) is widely considered as a valid, noninvasive alternative to subcutaneous desensitization (1–3). Nevertheless, vaccines for SLIT have particular requirements related to their route of administration and mode of action, which differs from that of subcutaneous vaccines. High doses of allergens (at least 50- to 100-fold more than for subcutaneous immunotherapy) are administered orally and held under the tongue for 1–2 min prior to swallowing. For SLIT to be effective, the allergens should be captured within the oral mucosa by Langerhans-like dendritic cells. These cells subsequently migrate to the proximal lymph nodes of the cervical/submaxillary chain where, they stimulate naïve T cells (4).

Currently, the vaccines administered by the sublingual route are based on aqueous biological extracts from natural allergen sources. These vaccines have been extensively administered as both drops (in glycerosaline solution) or fast-dissolving tablets. More than 500 million doses have been administered to humans to date, which demonstrates both the safety and clinical efficacy of SLIT.

Recent advances in our understanding of oral mucosal immunity, together with new proteomic techniques, are likely to influence the type of allergens used in the future. In this article, I discuss the characteristics and relative merits of the various types of vaccines used or under development for SLIT (Table 1 and Fig. 1).

The quality of sublingual vaccines

Sublingual vaccines based on aqueous allergen extracts are currently prepared from raw allergenic materials, such as grass or tree pollens, cat or dog epithelia, or cultures of common house dust mites (1–6). Quality control of raw materials includes both macroscopic and microscopic examination to determine protein content, electrophoretic (isoelectrofocusing) profiling, and evaluations of bacteriological load and biological activity.

Allergens are commonly extracted at 4°C with an ammonium bicarbonate solution, and the protein extract is ultrafiltrated to remove small (i.e. < 1 kDa) molecules. The resulting extract contains all the water-soluble allergens and proteins from the raw material. These include active ingredients (major or minor allergens) as well as nonbioactive components, such as other proteins, glycoproteins and carbohydrates.

In few instances, investigators have chemically modified allergens to produce allergoids, which have reduced allergenicity compared with the parent compound because of the destruction of the conformational B cell epitopes (7, 8). The modification involves formaldehyde or glutaraldehyde treatment to cross-link the protein. The formation of intra- as well as inter-molecular bonds results in high molecular weight polymers (200–2000 kDa). To produce monomeric allergoids appropriate for SLIT, carbamylation of the ϵ -aminogroups in

Table 1. Characteristics of biological extracts vs recombinant allergens for sublingual vaccines

Allergens	Advantages	Limitations/improvements
Biological extracts (e.g. aqueous extracts from house dust mites, tree or grass pollens) – Used in native form – Treated with glutaraldehyde (allergoids)	• Currently available to patients • Well-established safety and efficacy profiles, with more than 34 double-blind, placebo-controlled, clinical studies • Easy to manufacture • Quality control includes microscopic and macroscopic examination, protein content, electrophoretic profiling, determination of bioassay, biological activity [IgE binding by radioallergosorbent test or enzyme-linked immunosorbent assay (ELISA) inhibition] and measurement of selected major allergens (ELISA)	• Difficult to standardize because of inherent biological variability, and lack of harmonization of activity units used by the various manufacturers • Most extracts are currently given as drops or tablets without adjuvants. I-tyrosine and monophosphoryl lipid A (MPL) adjuvants are being evaluated with potential grass-pollen sublingual vaccines (currently in phase I trials)
Recombinant allergens (e.g. Der p 1, Der p 2 and Bet v 1) Proteins in a native configuration preferred for sublingual immunotherapy	• Recombinant proteins are highly purified, and should be available with specifications in terms of protein content, host cell protein/DNA content and potency (biological activity) • Vaccines based on a limited number of allergens will facilitate immunomonitoring.	• Unproven efficacy for the sublingual route • Indevelopment (all recombinant vaccines tested in humans have been used subcutaneously) • Likely to require an adjuvant and/or a galenic formulation.

lysine residues is often used, which maintains native allergen molecular size and confers resistance to gastro-enteric enzymes (8). However, based on our understanding of the oral immune system, treatments altering the native structure of allergens are likely to be inappropriate for SLIT (see below).

Over the past decade the major allergens of most important allergen sources have been identified, purified and characterized (9–12). The characterization of these complex biological extracts has been aided by new technologies that provide detailed profiles of the proteins present. When combined with analytical mass spectroscopy, proteomic techniques such as, surface enhanced laser desorption ionization-time of flight (SELDI-tof), two-dimensional fluorescence-difference gel electrophoresis (2D DIGE) and two-dimensional protein fractionation (PF2D), provide sophisticated means to separate proteins based on size, charge, pI value and affinity. However, these methods are not quantitative and, as such, cannot be used as a basis for establishing standardization or quality control of pharmaceutical batches (Fig. 1).

Standardization of sublingual allergens

Both the 1998 WHO position paper and the more recent ARIA workshop recommend the use of standardized vaccines of known potency and shelf-life whenever possible (1, 2). The standardization procedure ensures batch-to-batch consistency, and this translates into dependable quality of the allergen, which is critical for effective treatment.

Each manufacturer has its own in-house reference preparation (IHRP) to determine the immunological activity of an allergen preparation. The IHRP is subsequently used as a reference to adjust the allergenic activity of each commercial batch *in vitro*. To enable comparison of allergens from different manufacturers, in-house references should be calibrated by skin testing, as described in the Nordic guidelines and van Ree et al. (5, 13).

Current barriers to achieving standardization include the use of global IgE binding, by radioallergosorbent test or enzyme-linked immunosorbent assay (ELISA), using pooled sera from allergic individuals as a measure of total extract potency (5, 13). IgE binding assays are unable to detect the differences in individual major allergen quantities or ratios between major allergens for products with multiple component activities, such as grass pollens and house dust mites. In addition, ELISA assays commonly in use rely on antibodies and biological standards that have not been validated across laboratories (5, 13, 14).

To overcome these drawbacks, the European Commission has been funding a large, multidisciplinary project to improve allergen standardization in Europe. The CRE-ATE project aims to develop candidate reference materials consisting of purified natural or recombinant major

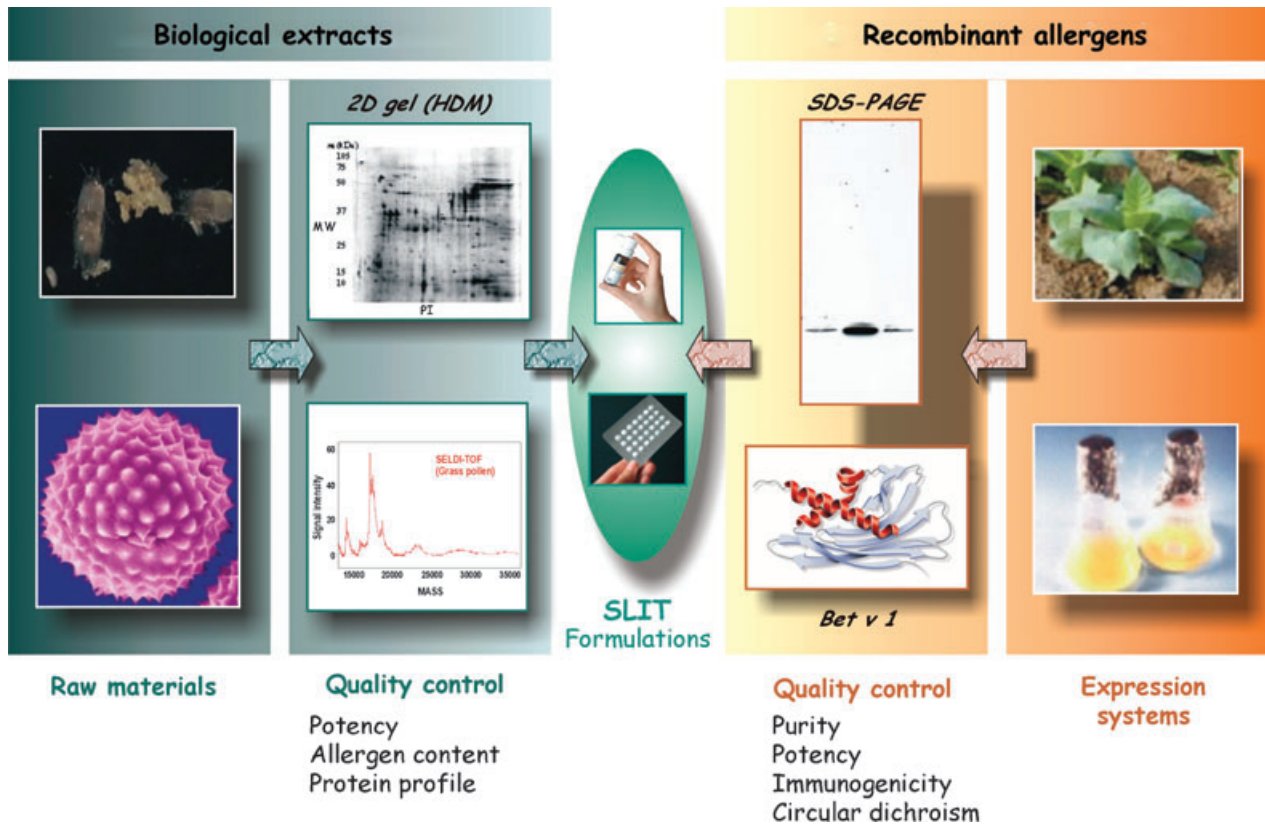


Figure 1. Use of biological extracts and recombinant allergens in sublingual immunotherapy. Biological extracts are prepared from raw materials (e.g. house dust mites or pollen) using ammonium bicarbonate. Extracts are analysed for potency (biological activity) by measuring global IgE binding, by RAST or ELISA inhibition, and major allergen content (ELISA). Protein profiles obtained by 2D gel electrophoresis of a *Dermatophagoides pteronyssinus* extract or Surface Enhanced Laser Desorption Ionisation / mass spectrometry analysis of a grass pollen extract are shown. Recombinant allergens are produced by inserting the corresponding gene in a heterologous expression system (e.g. tobacco plant, yeast or *Escherichia coli*). The recombinant allergens are purified, and their immunoreactivity is confirmed through patients' IgE binding, whereas correct folding is assessed by circular dichroism.

allergens and to compare and validate ELISA assays for the measurement of major allergens (10). Meanwhile, until such validated methods and reagents are available, extract potency is still measured in terms of overall IgE binding capacity as opposed to major allergen content in mass units.

Recombinant sublingual allergen vaccines

To date, several hundred allergens have been cloned and expressed as recombinant proteins. In this approach, the allergen cDNA is inserted into a heterologous expression host cell system (e.g. bacterium, yeast, mammalian or plant) to produce highly purified proteins in gram or kilogram quantities (see Fig. 1) (15–17). Tests of subcutaneous immunotherapy using recombinant vaccines based on wild-type or modified forms of the birch (*Betula verrucosa*) pollen major allergen Bet v 1, and a combination of five, recombinant, timothy-grass (*Phleum pratense*) pollen allergens (Phl p 1, Phl p 2, Phl p 5a, Phl p 5b, Phl p 6)

have yielded promising results (15, 18–20). Nevertheless, despite considerable interest, at the time of writing, no recombinant allergen sublingual vaccine has been yet tested in humans.

Recombinant allergens have several advantages over crude extracts. They are molecularly defined and are therefore easier, more reproducible and traceable to produce. Recombinant allergens can also be easily modified to create truncated or fusion proteins (15, 19). For instance, hypoallergenic forms of recombinant allergens, in which the three-dimensional structure has been altered to preclude IgE recognition, have also been produced. These hypoallergenic forms retain their capacity to elicit an IgG response and activate T cells but lack the ability to induce mast cell degranulation or basophils (15).

While hypoallergenic forms should, in theory, lead to safer vaccines for the parenteral route, their value in SLIT remains unclear. Oral Langerhans cells are known to express constitutively both the high (FcεRI) and low (CD23) affinity Fc receptors for IgE. Therefore, it is now thought that IgE binding to allergens produced in a

wild-type (i.e. native) conformation may facilitate capture by these Langerhans cells. Following binding at the high and low affinity receptors, sublingual Langerhans cells produce interleukin 10 (IL10) and transforming growth factor (TGF) β , and upregulate indoleamine deoxygenase, which suggests that native recombinant allergens could also potentially provide the appropriate signals to induce allergen-specific regulatory T cells (4).

With the above in mind we have two approaches in place to develop recombinant sublingual vaccines:

Identification of suitable production systems for pharmaceutical-grade recombinant allergens

In our hands, we have successfully used a variety of expression systems, including *Escherichia coli*, yeast (*Pichia pastoris*) and tobacco plants, to express recombinant allergens in their native configuration. Our expression systems produce good yields of the house dust mite [*Dermatophagoides pteronyssinus* (Der p)] allergens Der p 1 and Der p 2 and birch pollen allergen, Bet v 1 molecules (18). These recombinant molecules bind patients' IgE, as well as specific murine monoclonal antibodies. They also stimulate T-cell proliferation in a manner comparable with that of purified natural allergens, which suggests that the proteins are folded correctly.

Expression of Der p1 represents a particular challenge because this molecule has specific features that may contribute to molecular heterogeneity. These features include: two potential *N*-glycosylation sites, cysteine protease activity (which possibly contributes to its allergenicity), and a propeptide form (subsequently cleaved to yield the active allergen). Nevertheless, we have overcome these challenges to produce, in tobacco plants, forms of pro-Der p 1 (with or without the enzymatic activity and the glycosylation sites), which results in correctly folded molecules resembling purified natural Der p 1 allergen.

Evaluation of adjuvants and formulations adapted for the sublingual route

Highly purified proteins often exhibit low immunogenicity and require an adjuvant to stimulate an enhanced

immune response. A variety of candidate adjuvants acting on dendritic cells and/or T lymphocytes to stimulate IL10 and TGF β are being screened *in vitro* in cultured human immune cells. Our results suggest that a combination of 1,25-dihydroxyvitamin D3 with dexamethasone or selected bacteria, such as *Lactobacillus plantarum*, can induce strong IL10 production in human T lymphocytes. The IL10-inducing adjuvants significantly enhanced clinical vaccine efficacy, assessed by whole body plethysmography, when co-administered with the antigen via the sublingual route in mice sensitized with ovalbumin.

Allergen formulations other than drops or fast dissolving tablets (e.g. powder, biofilms or mucoadhesive tablets) should be tested in SLIT, particularly with the aim of prolonging mucosal contact and facilitating allergen capture by immune cells.

Conclusion

Sublingual vaccines based on biological extracts have been successfully used for the past 15 years, demonstrating both safety and clinical efficacy in clinical trials. Well-established standardization procedures that comply with guidelines from the regulatory authorities should achieve manufacturing consistency and ensure treatment safety and efficacy. Proteomic methods providing qualitative information on extract composition are likely to further improve extract characterization. Second-generation vaccines based on recombinant allergens are under development for treatment of house dust mite and birch pollen allergies. Given the particular mechanisms underlying immunization by the sublingual route, recombinant allergens in their native configuration are preferred over modified hypoallergenic forms because the native forms more efficiently target the Fc ϵ RI⁺ CD23⁺ Langerhans cells. In such second-generation vaccines, inclusion of regulatory T-cell adjuvant and/or use of mucoadhesive formulations of the recombinant allergen may ensure optimal allergen bioavailability at the mucosal surface.

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