

Immunotherapy with keyhole lampet hemocyanin-conjugated decapeptide vaccine in cypress pollen allergy

P. Demoly, L. Persi*, H. Dhivert, M. Delire† and J. Bousquet

Maladies Respiratoires and *Med-Bio-Med, Hôpital Arnaud de Villeneuve, Montpellier, France, and †SmithKline Beecham, Biologicals Immunotherapeutics sa, Rixensart, Belgium

Summary

Background Cupressaceae pollen allergy is a world-wide pollinosis but immunotherapy has rarely been tested. Immunotherapy is usually allergen-specific but new forms may be targeted towards IgE. **Objectives** A randomized, double-blind, placebo-controlled trial was carried out to assess the efficacy of a vaccine made of keyhole lampet hemocyanin (KLH)-conjugated decapeptide from the Fcε4 domain of the IgE in cypress pollinosis.

Methods Sixty patients with cypress pollen allergy were studied. They were included on a suggestive clinical history, positive skin tests and nasal challenge to cypress pollen extract. Three intramuscular injections of the vaccine (250 µg) or placebo were administered monthly with a booster injection 5 to 8 weeks later. The primary end-point criterion was the threshold dose inducing a positive nasal challenge. The secondary end-point was the symptom-medication scores measured when cypress pollen grains were over 50 grains/m³.

Results Nasal challenge before treatment was non-significantly different between the placebo and vaccine groups. After treatment there was no significant difference between the two groups. Pollen counts were over 50 grains/m³ for 8 weeks during the trial. There was no significant difference in total symptom scores between the placebo and vaccine groups. The vaccine was safe.

Conclusions The KLH-conjugated decapeptide vaccine was not effective in cypress pollen allergy.

Keywords allergy, cypress pollen, non-specific immunotherapy

Submitted 15 August 2001; revised 17 December 2001; accepted 3 January 2002

Introduction

Cupressaceae pollen allergy is a world-wide pollinosis caused by several species [1]. Members of the Cupressaceae family are important pollen producing trees in the Mediterranean area (*Cupressus sempervirens*, common cypress, *Cupressus arizonica*, Arizona cypress and *Juniperus communis* or *oxyedrus*, juniper) [2–7], North America (*Juniperus ashei*, mountain cedar) [8, 9] and Japan (*Cryptomeria japonica*, Japanese cedar) [10]. The amounts of Cupressaceae pollens released into the atmosphere are often far greater than those of grass pollens [3] and the prevalence of allergy to Cupressaceae pollens has increased dramatically within the past three decades throughout the world [1, 11, 12].

Allergen-specific immunotherapy has rarely been studied in Cupressaceae pollen allergy and there is one double-blind, placebo-controlled randomized clinical trial (RCT) available showing efficacy in mountain cedar pollen allergy [13].

New forms of non-specific immunotherapy have been proposed. In view of the pivotal role of IgE in the pathogenesis of allergic diseases, inhibition of the IgE response at either the level of IgE synthesis or effector phase may be of potential

therapeutic value. Among them, immunization of sensitized rats with a decapeptide from the Fcε4 domain of the IgE reduced IgE antibody formation and serum histamine concentration and abolished systemic anaphylactic reactions in response to allergen challenge. This peptide might form the basis of a vaccine in a new approach to the immunotherapy of atopic disease [14]. Although interesting, this approach has never been tested in humans.

A double-blind, placebo-controlled RCT was carried out in 60 patients allergic to cypress pollen using a keyhole lampet hemocyanin (KLH)-conjugated decapeptide vaccine. The efficacy of this new form of immunotherapy was studied using nasal challenge and symptom-medication scores during the season. The safety of this vaccine was studied carefully and the antibody response to vaccine was measured serially.

Materials and methods

Patients

Sixty-five cypress pollen-allergic patients (19 to 64 years) were included and 60 were randomized in the study using the following criteria [15]. All patients had rhinitis between January and March during the peak of the cypress pollen season for at least two years, a positive skin prick test with a cypress pollen extract and a positive nasal challenge with cypress pollen

Correspondence: Jean Bousquet, Hôpital Arnaud de Villeneuve, 34295 Montpellier CEDEX 5, France. E-mail: bousquet@montp.inserm.fr

extract, as described below. All patients had a positive Basotest® to cypress pollen [15]. Patients were also tested with standardized extracts of a battery of allergens as previously published [16] (Stallergènes SA, Antony, France) and the cypress pollen extract. Patients were sensitized to cypress pollen alone or to other pollen species. None of the patients had received allergen-specific immunotherapy to cypress pollen extracts.

Patients were excluded if they had a known allergy to molluscs as KLH is prepared from keyhole limpet. Other exclusion criteria included perennial rhinitis and/or asthma, any vaccination procedure within the past month, and any anti-allergic treatment within the past month.

Patients were informed and had given their written informed consent.

Cypress pollen allergen extract

The cypress pollen extract was not standardized because it seems that the proteins released by *Cupressus sempervirens*, the major cypress tree of the Montpellier area, are not in sufficient amount to make a reproducible standardization. As it is of paramount importance to use reproducible materials for nasal challenge and skin tests, we used lyophilized vials from the same batch. Such an approach, however, would not be acceptable for immunotherapy. Non-standardized extracts of cypress pollen (*Cupressus sempervirens*) (1:10 weight-by-volume) were prepared according to the proposals of the Allergen Subcommittee of the International Union of Immunology Societies, as previously described [17]. The same extract was lyophilized in different batches, which were reconstituted each test day.

KHL-conjugated decapeptide vaccine

The decapeptide vaccine, corresponding to the peptide used by Stanworth et al. [14] was a milky suspension, frozen at -70°C , and provided as a monodose in 5-mL vials reconstituted with $\text{Al}(\text{OH})_3$ solution prior to the injection. The placebo was a 5% sucrose solution stored and reconstituted similarly to the decapeptide vaccine. Vaccine was obtained through conjugation of decapeptide on KLH, a copper-containing respiratory protein of the marine gastropod *Megathura crenulata*, which is widely used in experimental immunology and clinically as an immunotherapeutic agent [18]. KLH and synthetic decapeptide are brought into solution in sterile water, at 2 g/L and 4 g/L, respectively, and sterile filtered. An equal volume of each solution is added and mixed gently at 4°C . Glutaraldehyde (25%) is added to decapeptide/KLH mixture to 0.2% (v/v). The preparation is gently mixed and stirred on a magnetic stirrer for 2 h at 4°C . Free peptide is removed by diafiltration. Conjugation and diafiltration are performed under aseptic conditions (under Class IIb cabinet/Class 100 suite conditions) to maintain sterility.

Vaccination

Subjects received, at 28-day intervals, three IM injections of either 250 µg of the KLH-conjugated decapeptide or placebo followed by a booster injection after 5 to 8 weeks following the third injection.

Nasal provocation test

We used a method previously described in detail [15]. Briefly, a new vial of the lyophilized cypress pollen extract was

reconstituted every test day and threefold increasing concentrations of the extract (0.014 to 10%) were insufflated into a nostril using a pump every 15 min until a cumulative symptom score of at least five was obtained [19, 20].

Skin prick tests

Skin prick test endpoint titration was carried out with the pollen cypress extract using five threefold increasing concentrations ranging from 0.014 to 10% (w/v) as previously described in detail [15, 17]. Mean weal and flare sizes were measured using computed planimetry. The area under the curve (AUC) of the dose-response was analysed.

Symptom-medication scores

Symptom-medication scores were filled in once a day from December 1998 to the end of March 1999. During this period, subjects recorded in the diary the consumption of a standardized set of medication (Table 1). A symptom-medication score was performed according to Table 2.

Total serum IgE

Total serum IgE was measured using the PRIST (Pharmacia Upjohn, Uppsala, Sweden) and following the manufacturer's recommendations.

Assessment of safety

After each injection, patients were followed for up to 6 h in the clinic to monitor the development of any local or systemic reaction.

Patients were also followed-up for 3 months to assess the development of serum antibodies against the vaccine. Blood samples for analysis of anti-decapeptide antibodies (IgG) and anti-KLH antibodies (IgE and IgG) were taken before each injection and at the end of the trial. IgG specific for decapeptide or KLH has been measured in human sera using a highly

Table 1. Medications used by the patients

Mild nasal symptoms: loratadine 10 mg OD
Moderate nasal symptoms: add beclomethasone 50 µg nasal spray in each nostril BID
Severe nasal symptoms: add prednisolone 20 mg tablet, OD
Eye symptoms: intraocular nedocromil BID

Table 2. Symptom-medication scores used during the cypress pollen season

No symptom	0
Sneezing	1
Nasal blockage	1
Rhinorrhea	1
Pruritus of the nose	1
No medication	0
Intraocular nedocromil	1
Loratadine	2
Beclomethasone	3
Prednisolone	4

sensitive time-resolved fluorescence-based assay. IgE specific for KLH has been measured using a 'capture' ELISA.

An intradermal skin test to KLH was performed at the beginning of the study. Two concentrations were injected (2 and 20 µg in 0.02 mL). None of the patients had any positive skin test to KLH.

Anti-decapeptide IgG assay

Briefly, serial dilutions of human sera (twofold dilution starting 1/5, incubated for 1½ h at 37°C) are added to streptavidin 96-well microtitre plates coated with biotinylated IgE decapeptide (1.4 µg/mL, incubated overnight at 4°C). Captured IgG are detected with europium-labelled anti-human IgG (Wallac Delfia 1244–330, 200 ng/mL incubated for 1 h at 37°C) and measured by time-resolved fluorescence following cleavage of europium with enhancement reagent (Wallac Delfia 1244–105). Plates were measured on time-resolved fluorescence on a Wallac 1420 Victor Multilabel counter.

IgG specific for KLH assay

Serial dilutions of human sera (twofold dilution starting 1/20, incubated for 1½ h at 37°C) are added to 96-well microtitre plates coated with KLH (10 µg/mL, incubated overnight at 4°C). Captured IgG are detected with europium-labelled antihuman IgG (Wallac Delfia 1244–330, 100 µL, 200 ng/mL incubated for 1 h at 37°C) and measured by time-resolved fluorescence following cleavage of europium with enhancement reagent (Wallac Delfia 1244–105). Plates were measured on time-resolved fluorescence on a Wallac 1420 Victor Multilabel counter.

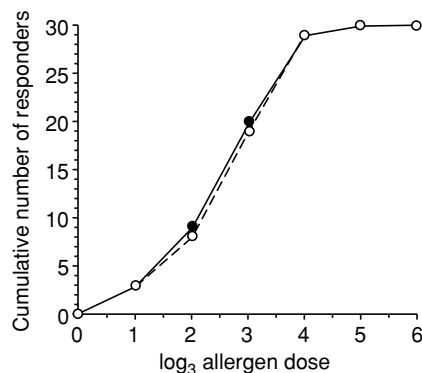


Fig. 1. Threshold dose inducing a positive nasal challenge at baseline. ●: vaccine; ○: placebo.

Table 3. Demographic parameters

	ITT population		PP population	
	Vaccine	Placebo	Vaccine	Placebo
Number	30	29	25	25
Males	14	13	12	11
Age (yrs)	34.2 ± 12.1	39.2 ± 11.3	34.2 ± 11.6	38.7 ± 11.7
Monosensitized patients (%)	10	10	12	12

Anti-KLH IgE assay

A capture ELISA has been set up to measure IgE specific for KLH. Serial dilutions of human sera (twofold dilution starting 1/5, incubated overnight at 4°C) are added to microtitre 96-well plates coated with a monoclonal antibody specific for human IgE (Sigma I-6510–1/7000, incubated for 2 h at 37°C). IgE captured are first incubated with soluble KLH (Byosyn, 1 µg/mL, incubated for 1½ h at 37°C) and then with rabbit anti-KLH polyclonal antibodies (Sigma H0892–1/1000, incubated for 1½ h at 37°C). Anti-KLH-specific IgE are detected with peroxidase-labelled anti-rabbit Ig (Sigma A05545–1/2500, incubated for 1½ h at 37°C) and ortho-phenylenediamine solution (Sigma, incubated for 15 min at room temperature in the dark); measured on microplate reader Bio-Rad at 490 nm–620 nm.

Pollen counts

Pollen counts were measured weekly.

Design of the study and statistical analysis of the data

All subjects were enrolled 6 to 7 months after the cypress pollen season in September 1998. As this was a proof-of-concept study, we decided to use an objective parameter for the primary endpoint and we assessed the change in threshold concentration during the nasal challenge with the cypress pollen extract carried out before any treatment and after the three first injections of vaccine or placebo (Fig. 1). Secondary endpoints included (i) the symptom and medication scores calculated daily during the weeks when the number of Cupressaceae pollens was over 50 grains/m³, (ii) skin test weals and flares and (iii) total serum IgE.

All randomized subjects with at least one vaccination were included in the intention-to-treat analysis. All subjects who had the first three vaccinations and who performed visits one to six without major protocol violation were included in the per-protocol population (PP).

The descriptive analysis used conventional parameters: means and standard deviations. Comparisons between groups were performed using Mann–Whitney test and Wilcoxon signed rank test. The level of significance was set with an alpha risk of 0.05.

Results

Demographic characteristics of the patients

There was no significant difference in the demographic analyses between the two groups of patients (Tables 3, 4 and 5).

Table 4. AUC for skin tests with cypress pollen extract (ITT analysis)

	Weal		Flare	
	Vaccine	Placebo	Vaccine	Placebo
Baseline	520 ± 143	511 ± 120	2430 ± 1377	1972 ± 1170
After treatment	474 ± 125	503 ± 106	2283 ± 1074	2180 ± 882

Results are expressed in mean ± SD.

Table 5. Total symptom score during the pollen season

	Vaccine		Placebo	
	n	Mean ± SD	n	Mean ± SD
ITT	29	2.34 ± 1.78	30	1.98 ± 1.35
PP	25	2.33 ± 1.86	25	1.99 ± 1.44

Patients studied

Sixty-five subjects were screened and 60 patients were randomized. All of them were analysed for ITT and 50 patients were analysed for PP. There was no drop-out due to treatment side-effects. Four patients in the active group and five in the placebo group were excluded from the symptom-medication analysis during the pollen season because of the intake of non-authorized drugs required by a virus infection of the upper airways.

Nasal challenge

The threshold dose inducing a positive nasal challenge before treatment was non-significantly different between the placebo and vaccine groups (Fig. 2). After treatment, there was no significant difference between the two groups (Fig. 3).

Skin tests

Mean skin tests AUC for weals and flares before treatment were non-significantly different between the placebo and vaccine groups (Table 4). After treatment, there was no significant difference between the two groups (Table 4).

Symptom-medication score

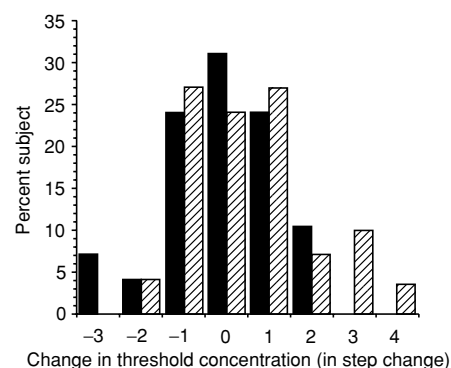
Pollen counts were over 50/m³ for 8 weeks during the trial. There was no significant difference between the placebo and vaccine groups in total symptom scores (Table 5 and Fig. 3).

Total serum IgE

Geometric means of total serum IgE were similar in both groups at baseline and were non-significantly reduced after vaccine or placebo treatment (Table 6).

Anti-decapeptide and anti-KLH serum antibodies

The anti-decapeptide IgG levels were unmeasurable in all patients before treatment and remained at this level in the placebo group during treatment. In the vaccine-treated group IgG were measurable in 28 patients at a low but significant level (geometric mean 1.80). There was no correlation between the levels of antibodies against decapeptide and any of the three outcome

**Fig. 2.** Change in the threshold dose inducing a positive nasal challenge between baseline and post-vaccination. Filled bar: vaccine; hatched bar: placebo.**Table 6.** Total serum IgE (ITT analysis)

	Vaccine	Placebo
Baseline	129	97
After treatment	95	77

Results are expressed in geometric means of KU/L.

measures. Anti-KLH serum antibodies could not be detected in any subject.

Safety

No subject had any anaphylactic-type reaction during vaccination. All subjects in both groups experienced at least one mild adverse event. One patient in the placebo group and four patients in the vaccine group had serious adverse events (headache, myalgia, tiredness).

Discussion

In the present study, highly selected patients with cypress pollen allergy were studied and it was found that a vaccine made of KLH-conjugated decapeptide from the Fcε4 domain of IgE was ineffective to overcome seasonal symptoms in these patients. This clinical study did not support the experimental study of Stanworth et al. in animals.

Because, in the Montpellier area, cypress pollen is the only winter allergen with ash pollen, and ash pollinosis is not clinically significant, we did not exclude patients allergic to other pollen species. However, we excluded patients with perennial symptoms. Moreover, we studied both patients mono- and polysensitized, as patients only allergic to cypress pollen have a different IgE immune response than those allergic to many pollen species [3, 21].

Nasal challenge was carried out according to our previous experience [19, 20] with two modifications. First, we used cypress pollen as the allergen instead of grass pollens, and secondly we used allergen extracts instead of pollen grains. However, the results obtained were similar to those previously published, as the reproducibility between and within groups is

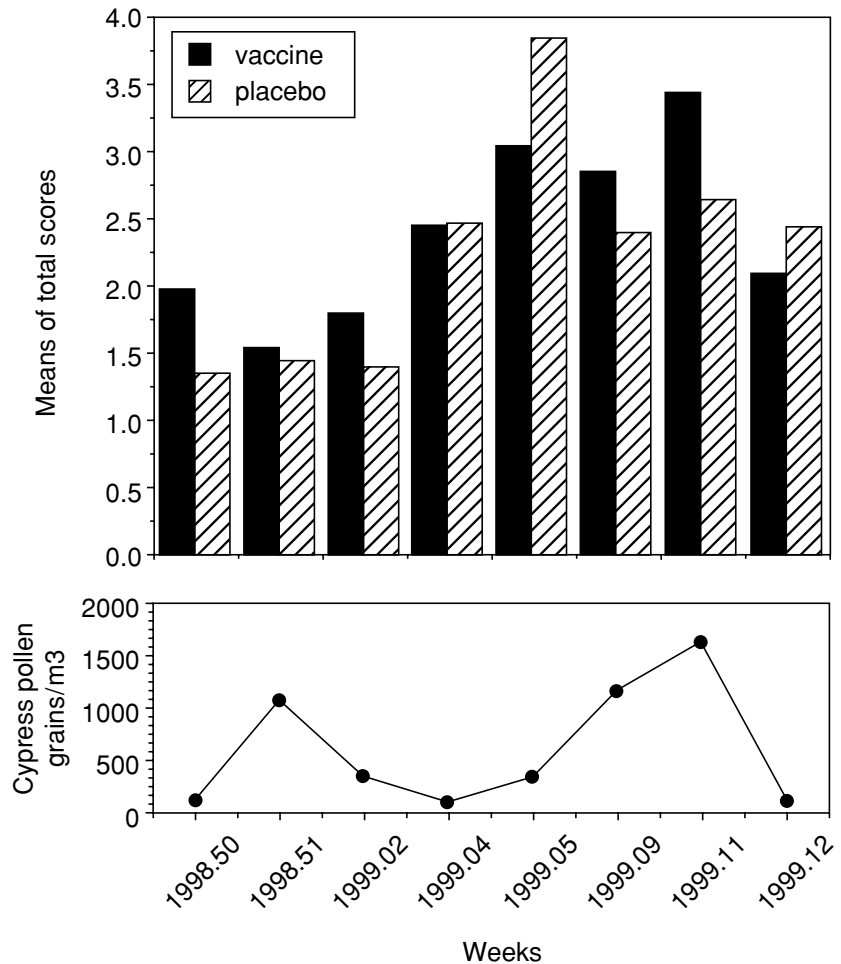


Fig. 3. Cypress pollen counts and total symptom scores in the ITT analysis.

high and there was a concordance between the lack of efficacy of the vaccine as measured with nasal challenge, symptom-medication scores and skin tests. It is interesting to note the concordance between nasal challenge and symptom-medication scores as previously reported [22–25].

No differences in symptom-medication scores were observed. As this was a secondary objective, we used a simple method to construct a combined score. However, the medications were given in a standardized manner in order to assess the potential severity of the season. We only analysed weeks for which a sufficient pollen count was reached as, in our area, cypress pollen-allergic patients experience bursts of a few days of symptoms over a 2- to 3-month period rather than continuous symptoms over the pollen season. These features are related to cypress pollinosis in the winter time in our area. The vaccine was strictly not effective when mean results were studied. We then decided not to study the response rate of the two treatments.

In the experimental study, it was found that IgE levels were reduced [14]. Other pre-clinical experiments performed in rabbits have clearly demonstrated the antigenicity and immunogenicity of the KLH-peptide, which was able to elicit a specific IgG antibody response in immunized animals. A phase I clinical trial has also been performed in human

healthy volunteers who have been injected with KLH-peptide. This vaccination was very well tolerated and induced in all subjects a specific IgG response against the conjugated peptide (data not published). *In vitro* tests have been unable to demonstrate a cross-reactivity of the anti-KLH peptide IgGs with human circulating IgE, and this observation led to the hypothesis that the mode of action of such anti-conjugated peptide antibodies could possibly be their recognition of a particular epitope on IgEs bound to their receptors on cells (data not published). However, in the present study, although there was some reduction in total serum IgE levels, the same decrement was found in the two groups (vaccine and placebo).

The lack of effectiveness of the vaccine was not related to the inability to mount an immune response against the decapeptide, as 28 out of 30 patients had an IgG response against it and there was no correlation between the antibody levels and the efficacy of the treatment. It may be proposed that a higher decapeptide dose or another adjuvant would have been more effective, but the dose used was already large and KLH was used effectively in the animal studies [14] from which the current study was based. Thus, the proof-of-concept of the decapeptide from the Fcε4 domain of the IgE [14] was not confirmed in humans using a model of IgE-mediated allergic response.

References

- 1 Mari A, Di-Felice G, Afferni C et al. Cypress allergy: an underestimated pollinosis. *Allergy* 1997; 52 (3):355–6.
- 2 Bousquet J, Cour P, Guerin B, Michel FB. Allergy in the Mediterranean area. I. Pollen counts and pollinosis of Montpellier. *Clin Allergy* 1984; 14 (3):249–58.
- 3 Bousquet J, Knani J, Hejjaoui A et al. Heterogeneity of atopy. I. Clinical and immunologic characteristics of patients allergic to cypress pollen. *Allergy* 1993; 48 (3):183–8.
- 4 Iacovacci P, Afferni C, Barletta B et al. *Juniperus oxycedrus*: a new allergenic pollen from the Cupressaceae family. *J Allergy Clin Immunol* 1998; 101 (6, part 1):755–61.
- 5 Pham NH, Baldo BA. Allergenic relationship between taxonomically diverse pollens. *Clin Exp Allergy* 1995; 25 (7):599–606.
- 6 Barletta B, Afferni C, Tinghino R et al. Cross-reactivity between *Cupressus arizonica* and *Cupressus sempervirens* pollen extracts. *J Allergy Clin Immunol* 1996; 98 (4):797–804.
- 7 Geller-Bernstein C, Waisel Y, Lahoz C. Environment and sensitization to cypress in Israel. *Allerg Immunol (Paris)* 2000; 32 (3):92–3.
- 8 Ramirez DA. The natural history of mountain cedar pollinosis. *J Allergy Clin Immunol* 1984; 73 (1, part 1):88–93.
- 9 Reid MJ, Schwietz LA, Whisman BA, Moss RB. Mountain cedar pollinosis: can it occur in non-atopics? *N Engl J Allergy Proc* 1988; 9 (3):225–32.
- 10 Hashimoto M, Nigi H, Sakaguchi M et al. Sensitivity to two major allergens (Cry j I and Cry j II) in patients with Japanese cedar (*Cryptomeria japonica*) pollinosis. *Clin Exp Allergy* 1995; 25 (9):848–52.
- 11 Panzani R, Yasueda H, Shimizu T, Shida T. Cross-reactivity between the pollens of *Cupressus sempervirens* (common cypress) and of *Cryptomeria japonica* (Japanese cedar). *Ann Allergy* 1986; 57 (1):26–30.
- 12 Ishizaki T, Koizumi K, Ikemori R, Ishiyama Y, Kushibiki E. Studies of prevalence of Japanese cedar pollinosis among the residents in a densely cultivated area. *Ann Allergy* 1987; 58 (4):265–70.
- 13 Pence H, Mitchell D, Greenly R, Updegraff B, Selfridge H. Immunotherapy for mountain cedar pollinosis. A double-blind controlled study. *J Allergy Clin Immunol* 1976; 58:39–50.
- 14 Stanworth DR, Jones VM, Lewin IV, Nayyar S. Allergy treatment with a peptide vaccine. *Lancet* 1990; 336:1279–81.
- 15 Paris-Kohler A, Demoly P, Persi L et al. In vitro diagnosis of cypress pollen allergy by using cytofluorimetric analysis of basophils (Basotest) [in process citation]. *J Allergy Clin Immunol* 2000; 105 (2, part 1):339–45.
- 16 Onorato J, Merland N, Terral C, Michel FB, Bousquet J. Placebo-controlled double-blind food challenge in asthma. *J Allergy Clin Immunol* 1986; 78 (6):1139–46.
- 17 Bousquet J, Djoukadar F, Hewitt B, Guerin B, Michel FB. Comparison of the stability of a mite and a pollen extract stored in normal conditions of use. *Clin Allergy* 1985; 15 (1):29–35.
- 18 Harris JR, Markl J. Keyhole limpet hemocyanin (KLH): a biomedical review. *Micron* 1999; 30 (6):597–623.
- 19 Lebel B, Bousquet J, Morel A et al. Correlation between symptoms and the threshold for release of mediators in nasal secretions during nasal challenge with grass-pollen grains. *J Allergy Clin Immunol* 1988; 82 (5, part 1):869–77.
- 20 Bousquet J, Hejjaoui A, Becker WM et al. Clinical and immunologic reactivity of patients allergic to grass pollens and to multiple pollen species. I. Clinical and immunologic characteristics. *J Allergy Clin Immunol* 1991; 87 (3):737–46.
- 21 Lagier B, Pons N, Rivier A et al. Seasonal variations of interleukin-4 and interferon-gamma release by peripheral blood mononuclear cells from atopic subjects stimulated by polyclonal activators. *J Allergy Clin Immunol* 1995; 96 (6, part 1):932–40.
- 22 Bousquet J, Maasch H, Martinot B, Hejjaoui A, Wahl R, Michel FB. Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. II. Comparison between parameters assessing the efficacy of immunotherapy. *J Allergy Clin Immunol* 1988; 82 (3, part 1):439–46.
- 23 Bousquet J, Maasch HJ, Hejjaoui A et al. Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. III. Efficacy and safety of unfractionated and high-molecular-weight preparations in rhinoconjunctivitis and asthma. *J Allergy Clin Immunol* 1989; 84 (4, part 1):546–56.
- 24 Bousquet J, Hejjaoui A, Soussana M, Michel FB. Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. IV. Comparison of the safety and efficacy of two dosages of a high-molecular-weight allergoid. *J Allergy Clin Immunol* 1990; 85 (2):490–7.
- 25 Bousquet J, Becker WM, Hejjaoui A et al. Differences in clinical and immunologic reactivity of patients allergic to grass pollens and to multiple-pollen species. II. Efficacy of a double-blind, placebo-controlled, specific immunotherapy with standardized extracts. *J Allergy Clin Immunol* 1991; 88 (1):43–53.