

Carrier-bound, nonallergenic Ole e 1 peptides for vaccination against olive pollen allergy

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Background: Trees of the family Oleaceae (olive and ash) are important allergen sources in Mediterranean countries, Northern and Central Europe, and North America. The major olive pollen allergen Ole e 1 represents the majority of allergenic epitopes in olive pollen and cross-reacts with Fra e 1, the major ash pollen allergen.

Objective: We sought to develop a safe vaccine for the treatment of Oleaceae pollen allergy.

Methods: We synthesized 5 peptides ranging from 32 to 36 amino acids, which covered the whole sequence of Ole e 1. The IgE and T-cell reactivity of the peptides was compared with that of Ole e 1 by means of dot blot experiments, as well as ELISA, and in proliferation assays. Rabbits were immunized with non-IgE-reactive, keyhole limpet hemocyanin-coupled peptides or Ole e 1. The reactivity of the IgG antibodies with Ole e 1 and their ability to inhibit IgE binding to nOle e 1 was evaluated by means of ELISA.

Results: Only the C-terminal Ole e 1 peptide showed IgE binding, whereas the other peptides were nonallergenic.

Immunization of rabbits with Ole e 1-derived peptides bound to the carrier molecule keyhole limpet hemocyanin induced in rabbits the production of Ole e 1-specific IgG antibodies, which cross-reacted with Fra e 1, and inhibited olive and ash pollen-sensitized patients' IgE binding to Ole e 1.

Conclusion: Two non-IgE-binding peptides with low T-cell reactivity from the N-terminus of Ole e 1 were identified that might represent safe vaccine candidates for immunotherapy of Oleaceae pollen allergy. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Olive pollen allergy, Ole e 1, specific immunotherapy, peptides, epitopes

In Mediterranean countries and some parts of Australia and North America, the pollen of the olive tree constitutes a main allergen source.^{1,2} Ole e 1 is the major allergen in olive pollen, and its content in olive pollen extract is directly related to the allergenic activity of the whole extract.³ Ole e 1 occurs in several closely related polypeptide chain isoforms of 145 amino acids. Depending on the presence of carbohydrates, the apparent molecular weight of Ole e 1 varies between 18 and 20 kDa.⁴⁻⁶ Because Ole e 1 shows a high degree of sequence homology and IgE cross-reactivity to allergens in other plant pollens (eg, ash, lilac, and privet), it can be used as a marker allergen to detect genuine sensitization to the family Oleaceae and in particular to ash, which is a relevant allergen source in areas without olive trees, such as Middle Europe and North America.⁷⁻¹¹ Because of the high sequence identity (ie, 89%) and IgE cross-reactivity with the major allergen of ash (Fra e 1), Ole e 1 can be also used for diagnosis and specific immunotherapy (SIT) of ash pollen allergy.^{12,13} Therefore several attempts have been made to characterize IgE epitopes and T-cell epitopes of Ole e 1 for the development of a molecule-based therapeutic strategy.^{7,14,15} With the aim of reducing IgE-mediated side effects during SIT, 2 approaches have thus far been pursued. One approach was based on the use of T cell-reactive Ole e 1 peptides¹⁶ without IgE for inducing T-cell tolerance.¹⁷⁻¹⁹ By using the strategy of producing recombinant hypoallergenic derivatives with reduced IgE reactivity and preserved allergen-specific T-cell epitopes,²⁰ Ole e 1 mutant proteins were engineered.²¹ Here we present another approach for the development of a safe vaccine for olive pollen allergy, which uses nonallergenic peptides derived from surface-exposed areas of Ole e 1. Nonallergenic Ole e 1 peptides with low T-cell reactivity were identified, coupled to an unrelated carrier protein, and tested for their ability to induce allergen-specific IgG antibodies, which inhibited the binding of IgE from patients with olive and ash pollen allergy to Ole e 1. These Ole e 1 peptides lack IgE reactivity, as well as the majority of Ole e 1-specific T-cell epitopes, and therefore should induce neither IgE- nor T cell-mediated side effects when used for SIT of olive pollen allergy.

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METHODS

Characterization of patients with Oleaceae allergy

Patients with olive pollen allergy from Italy and Spain and patients with ash pollen allergy from Austria with allergic rhinitis, conjunctivitis, and/or asthma were characterized by the measurement of olive pollen-specific and/or ash

Abbreviations used

KLH: Keyhole limpet hemocyanin
SIT: Specific immunotherapy

pollen-specific and/or Ole e 1-specific IgE levels by means of CAP-FEIA or Immuno Solid-phase Allergen Chip (ISAC). Tables E1 to E3 (available in this article's Online Repository at www.jacionline.org) summarize demographic, clinical, and serologic data, as well as information regarding additional sensitizations. In total, sera from 67 Oleaceae-sensitized patients and serum from a nonallergic subject were included in the study. Anonymized serum samples were used with approval of the ethics commission of the Vienna General Hospital.

Cloning, expression, and purification of rOle e 1 and purification of nOle e 1

rOle e 1 was expressed and purified as described in the [Methods](#) section of this article's Online Repository at www.jacionline.org. The purification of nOle e 1 is also described in the [Methods](#) section of this article's Online Repository.

Peptide synthesis and characterization

Five peptides of a length between 32 and 36 amino acids covering the sequence of Ole e 1 were synthesized (see [Table E4](#) in this article's Online Repository at www.jacionline.org).^{4,22} Putative solvent-exposed regions and surface exposure of amino acids were identified with the ProtScale bioinformatic tool made available by the EXPASY server.²³ Peptides were identified by means of mass spectrometry and purified to greater than 90% purity by means of preparative HPLC (PiChem, Graz, Austria).

Circular dichroism measurements of the 5 Ole e 1-derived peptides were carried out on a Jasco J-810 spectropolarimeter (JASCO Corp, Tokyo, Japan).²⁴

IgE and T-cell reactivity of Ole e 1 and of Ole e 1-derived peptides

IgE antibody reactivity of nOle e 1 and the 5 synthetic peptides was determined in nondenaturing, RAST-based dot blot experiments and ELISAs (see the [Methods](#) section in this article's Online Repository). T-cell reactivity was assessed in lymphoproliferative assays by using PBMCs from allergic patients (see the [Methods](#) section in this article's Online Repository).

Generation and measurement of allergen- and peptide-specific antibodies

Ole e 1-derived peptides were coupled to keyhole limpet hemocyanin (KLH; Pierce, Rockford, Ill) according to the manufacturer's instructions and purified with a Conjugation Kit (Sigma, St Louis, Mo). Rabbits were immunized with 200 µg of the KLH-conjugated peptides or of rOle e 1 per injection by using Freund's complete and incomplete adjuvant (Charles River, Kisslegg, Germany). Serum samples were obtained before immunization and at 4-week intervals after immunization.

Rabbit immune responses were analyzed by means of ELISA. For the measurement of specific rabbit IgG antibodies, ELISA plates were coated overnight with 5 µg/mL nOle e 1. After blocking, the plates were incubated overnight with serial dilutions of the rabbit anti-peptide antisera, the anti-rOle e 1 antiserum, or the corresponding preimmune sera (1:1,000, 1:5,000, 1:10,000, and 1:50,000). Bound rabbit IgG antibodies were detected with a 1:1,000 diluted horseradish peroxidase-labeled donkey anti-rabbit IgG antiserum (Amersham Biosciences, Little Chalfont, United Kingdom; for details, see the [Methods](#) section in this article's Online Repository).

Immunoblot analysis of cross-reactivity of rabbit anti-Ole e 1 and anti-peptide antisera with the major ash pollen allergen Fra e 1

Ash (*Fraxinus excelsior*) pollen extract¹³ was separated by means of 15% preparative SDS-PAGE²⁵ and blotted onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Nitrocellulose strips were blocked in PBST (PBS containing 0.5% vol/vol Tween 20) and then incubated overnight with 1:5000 diluted rabbit anti-rOle e 1, rabbit anti-peptide antisera, and, for control purposes, the corresponding preimmune sera or rabbit anti-KLH antibodies. Bound rabbit antibodies were detected with a 1:3000 diluted iodine 125-labeled goat anti-rabbit antibody (Perkin Elmer Life Science, Boston, Mass) and visualized by means of autoradiography.

ELISA competition assay for analyzing the inhibition of human IgE binding to nOle e 1 by peptide-specific IgG antibodies

The ability of peptide-induced rabbit antibodies to inhibit the binding of Oleaceae pollen-sensitized patients' IgE to nOle e 1 was examined in ELISA competition assays, as described previously (see the [Methods](#) section in this article's Online Repository).²⁶

RESULTS

Characterization of Ole e 1-derived peptides

Four Ole e 1-derived peptides (P2-P5) with a length of 32 to 36 amino acids were selected to contain surface-exposed residues according to prediction programs ([Fig 1](#)).²⁷ P1, representing the 35 N-terminal amino acids, comprised a region of Ole e 1 with predicted low surface accessibility and was included so that P1 to P5 covered the complete sequence of Ole e 1 (Swiss-Prot accession no. P19963).⁴ Additional cysteines were added to the N-terminal peptides 3, 4, and 5 and to the C-terminus of peptides 1 and 2 to facilitate coupling to KLH by means of the formation of disulfide bridges and to keep the terminal ends in the same orientation as in Ole e 1. Peptides 4 and 5 contained previously described T-cell epitopes of Ole e 1 ([Fig 1](#)).¹⁴ The molecular weights of the peptides ranged from 3511.9 Da (peptide 2) to 4069.6 Da (peptide 1). Peptides 1, 2, and 3 were acidic, with calculated isoelectric points of less than 5.0, whereas peptides 4 and 5 were basic, with isoelectric points of greater than 9.0 ([Table E4](#)).

Analysis of the peptides by means of far-UV circular dichroism showed spectra with minima at 200 nm or less, demonstrating the absence of any secondary structure elements (data not shown).

Identification of Ole e 1-derived peptides that lack IgE-binding capacity and show low T-cell reactivity

The 5 synthetic Ole e 1-derived peptides were compared with nOle e 1 regarding their IgE-binding capacity in nondenaturing RAST-based dot blot experiments. Sera from 23 olive pollen-sensitized patients (10 from Spain and 13 from Italy) and 7 ash pollen-sensitized patients (Austria) showed IgE binding to nOle e 1 of varying intensity, but none of the patients displayed IgE reactivity to peptides 1, 2, 3, and 4. Ten (33.3%) of the 30 sera tested (4 from Spain, 3 from Italy, and 3 from Austria) recognized peptide 5 ([Fig 2, A](#)).

A more detailed analysis of the IgE-binding capacity of the Ole e 1-derived peptides was performed by means of ELISA with sera from 63 Oleaceae-sensitized patients (Spain, n = 22; Italy, n = 27; and Austria, n = 14; [Fig 2, B](#)). Olive pollen-sensitized (Spain and Italy) and ash pollen-sensitized patients (Austria) showed pronounced IgE reactivity to nOle e 1. None of the patients

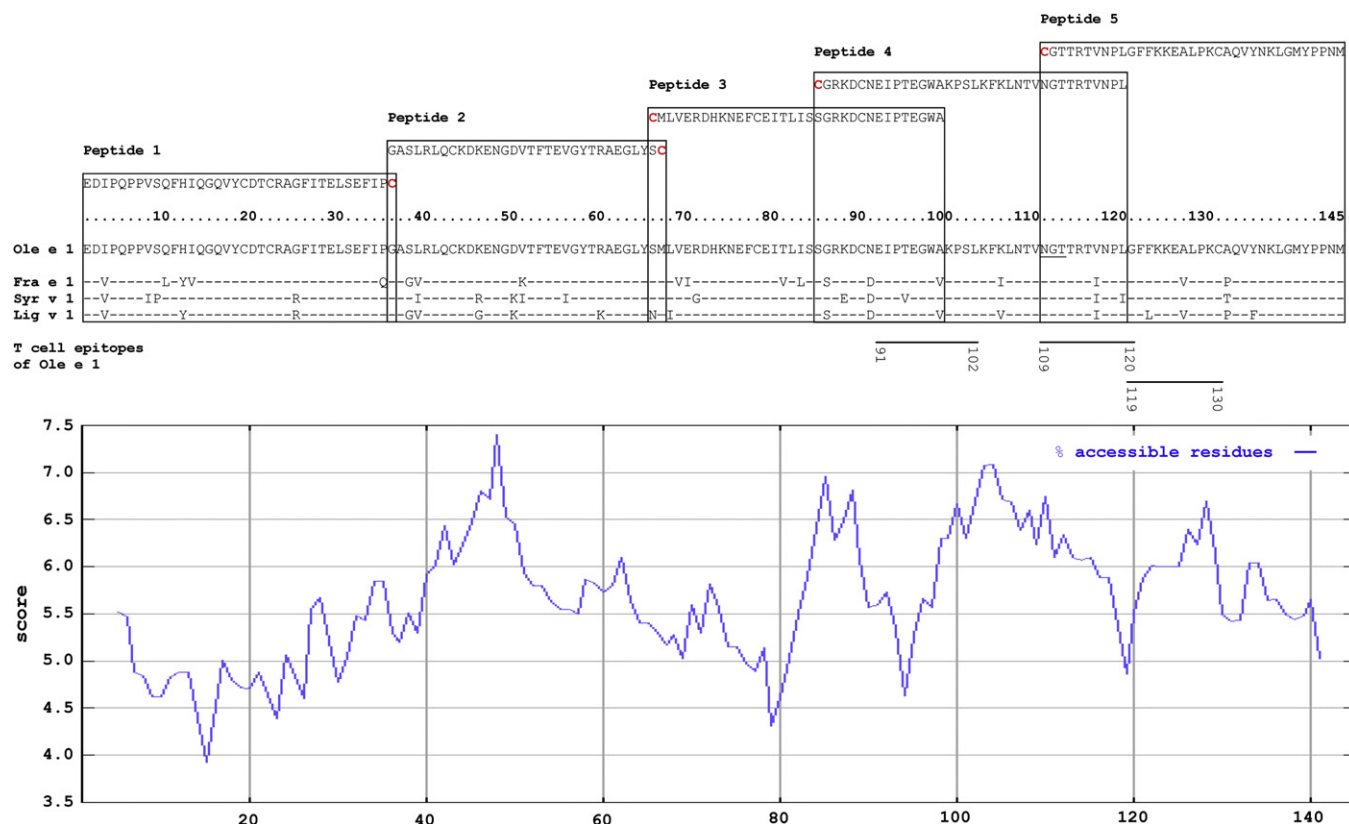


FIG 1. Amino acid sequence alignment of Ole e 1, Fra e 1 (ash), Lig v 1 (privet), and Syr v 1 (lilac). The N-glycosylation motif of Ole e 1 (*underlined*) and identical amino acids (*dashes*) are shown. Synthetic Ole e 1-derived peptides are shown in *boxes*. Bars indicate major T-cell epitopes of Ole e 1. A surface accessibility plot of Ole e 1 is shown at the *bottom*. Regions (*x-axis*) with high surface accessibility show scores of greater than 5.5 (*y-axis*).

displayed any relevant reaction to peptides 1 and 4, 1 patient (patient 2 in Table E2) reacted weakly with peptide 2 and 1 (patient 7 in Table E2) with peptide 3. Five patients (patients 7 and 20 in Table E1, patients 3 and 11 in Table E2, and patient 11 in Table E3) showed IgE reactivity to peptide 5. Because of the fact that peptide 5 exhibited IgE reactivity, it was not further evaluated as a vaccine candidate.

Results from the lymphoproliferation experiments performed with PBMCs from 6 Ole e 1-sensitized patients showed that the mean stimulation indices obtained with nOle e 1 and with a mix of the 5 peptides were greater than 2, whereas those of the individual peptides were much lower (Fig 3).

Immunization of rabbits with Ole e 1-derived peptides induces IgG antibodies that recognize Ole e 1 and Fra e 1

Next, rabbits were immunized with KLH-coupled peptides (P1-P4) or rOle e 1. The levels of Ole e 1-specific IgG antibodies induced by immunization with Ole e 1 or peptides were investigated by means of ELISA (see Fig E1 in this article's Online Repository at www.jacionline.org). Each of the peptides (P1-P4), as well as rOle e 1, induced anti-Ole e 1 antibody responses. The highest Ole e 1-specific antibody levels were obtained by immunization with peptides 1 or 2 and exceeded even those induced by immunization with rOle e 1. Peptides 3 and 4 induced significantly lower Ole e 1-specific IgG antibody levels than the other peptides.

We further analyzed whether antibodies induced by immunization of rabbits with Ole e 1 peptides or rOle e 1 cross-react with Fra e 1, the Ole e 1 homologous major allergen from ash pollen. Immunoblot experiments showed that all 4 peptide antisera (P1 = P2 > P3 > P4) and the anti-rOle e 1 antiserum recognized natural Fra e 1 in ash pollen extract (Fig 4), whereas no reactivity was observed with rabbit anti-KLH antibodies (data not shown).

Peptide-specific IgG antibodies inhibit allergic patients' IgE binding to Ole e 1

The capacity of the rabbit anti-peptide antibodies and anti-rOle e 1 antibodies to inhibit patients' IgE binding to nOle e 1 was investigated by means of ELISA competition experiments. In a first experiment the ability of the antisera to block patients' IgE reactivity to Ole e 1 was investigated for several serum dilutions ranging from 1:50 to 1:50,000. As exemplified in Fig E2 (available in this article's Online Repository at www.jacionline.org) for patient 1 (from Table E3), anti-peptide 3 and anti-peptide 4 antibodies competed only weakly with the patient's IgE binding, even when the rabbit sera were applied in high concentrations (1:50 dilution). In contrast, anti-peptide 1 and anti-peptide 2 antibodies, as well as the anti-rOle e 1 antibodies, strongly inhibited patients' IgE binding up to serum dilutions of 1:100.

Using sera from 14 Oleaceae-sensitized patients (Fig 5), we found that antibodies raised against peptides 1 and 2 inhibited

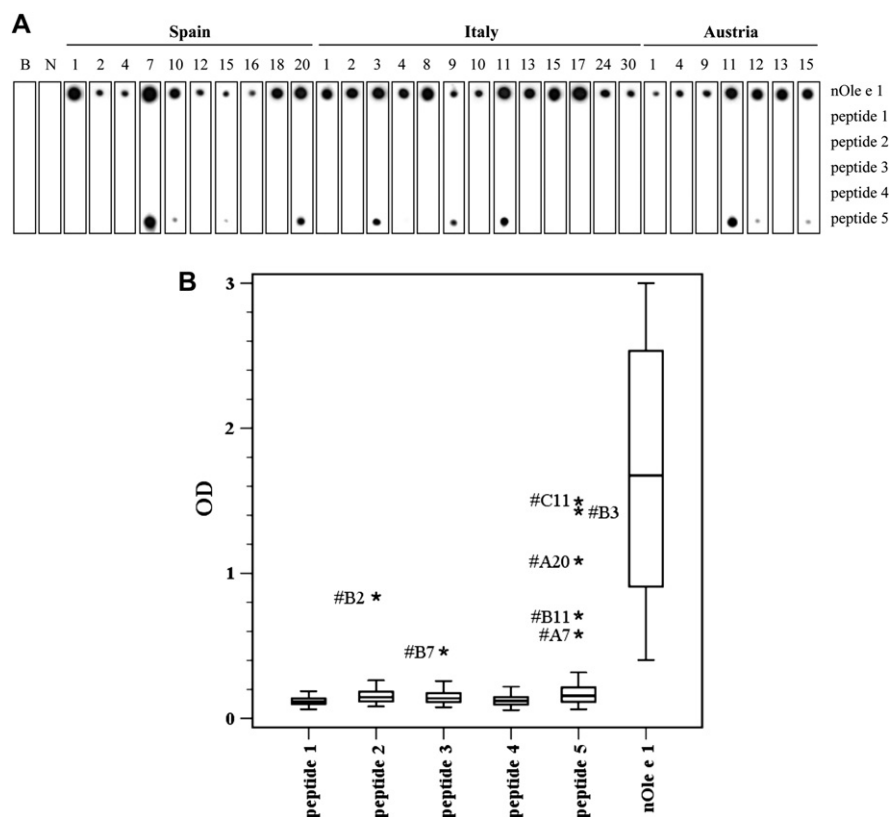


FIG 2. IgE reactivity of nOle e 1 and Ole e 1-derived peptides. **A**, Dotted nOle e 1 and peptides probed with sera from allergic patients, a nonatopic subject (*N*), or buffer (*B*). **B**, Box plot of OD values corresponding to bound IgE from 63 allergic patients. Patients are identified as in [Tables E1 to E3](#). Horizontal bars, Median values; asterisks, extreme values.

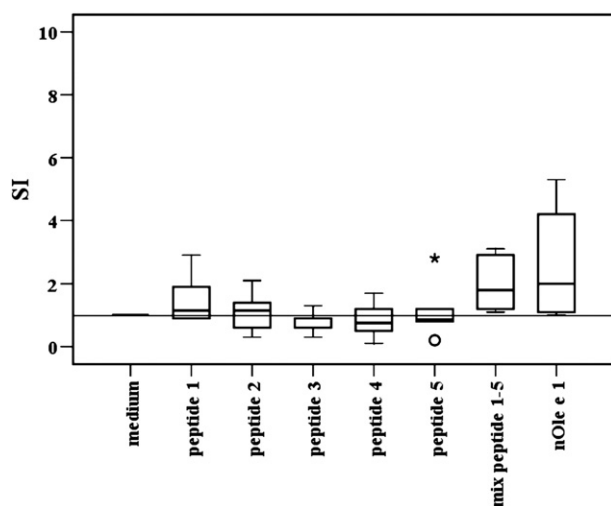


FIG 3. Lymphoproliferative responses to nOle e 1 and Ole e 1-derived peptides. Stimulation indices (*SI*; *y*-axis) are shown as box plots (horizontal bars, median values; circles and asterisks, outliers and extreme values, respectively) for PBMCs from 6 allergic patients for medium, peptides, and nOle e 1 (*x*-axis).

patients' IgE binding to Ole e 1 well (mean inhibition: anti-peptide 1, 41%; anti-peptide 2, 39%). With anti-peptide 3 and 4 antisera, only low inhibition rates with mean inhibitions of less than 5% were detected. Interestingly, mixtures of the

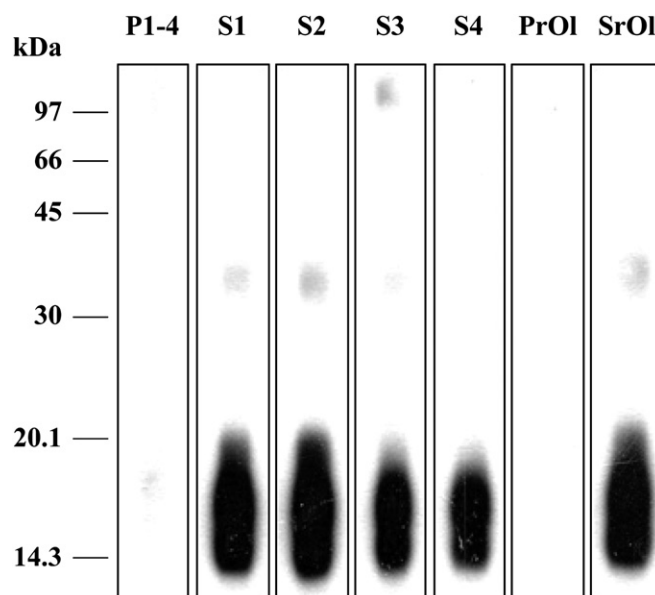


FIG 4. Anti-peptide and anti-rOle e 1 antibodies cross-react with nFra e 1. Nitrocellulose-blotted ash pollen extract was incubated with the anti-peptide antisera (*S1-S4*, anti-peptide 1 to anti-peptide 4), the anti-rOle e 1 antiserum (*SrOI*), and, for control purposes, a mixture of the anti-peptide preimmune sera (*P1-4*) or the anti-rOle e 1 pre-immune serum (*PrOI*). Molecular weights are indicated in the left margin.

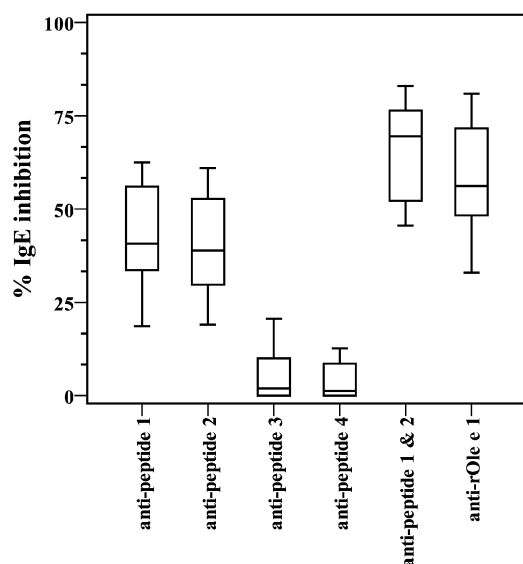


FIG 5. Rabbit anti-peptide and anti-rOle e 1 antibodies inhibit Oleaceae-sensitized patients' IgE binding to Ole e 1. Percentage inhibitions of IgE binding to Ole e 1 by Ole e 1- or peptide-specific antibodies for sera of 14 Oleaceae-sensitized patients are shown as box plots. Horizontal bars, Median values.

anti-peptide 1 and anti-peptide 2 antisera could increase the inhibition capacities of the single antisera and resulted in inhibition rates (mean inhibitions of 68%) that were greater than those obtained with the anti-rOle e 1 antiserum, which inhibited patients' IgE binding in the range of 45% to 70% (mean, 55%).

DISCUSSION

In this study we used a strategy for the development of an olive and ash pollen vaccine, which should strongly reduce or even eliminate IgE- and T cell-mediated side effects.²⁸ In fact, it has been noted in clinical immunotherapy studies that non-IgE-reactive T-cell peptides and recombinant hypoallergenic allergen derivatives lacking IgE reactivity induced T cell-dependent late-phase side effects.^{18,19,29}

The vaccine designed in this study therefore consists of non-IgE-reactive allergen-derived peptides that are bound to an unrelated carrier molecule. We chose KLH and chemically coupled allergen-derived peptides for our study because KLH has been used already for vaccination of patients.³⁰ According to the hapten-carrier principle, as described by Benacerraf and colleagues, carrier-bound peptides induce on immunization a robust antibody response against the peptide and corresponding antigen with T-cell help from the carrier.^{31,32} The carrier therefore provides T-cell help but does not activate allergen-specific T cells, which are responsible for late-phase reactions. The allergen-derived peptides should induce IgG antibodies, which block IgE binding to the allergen. We therefore searched first for Ole e 1 peptides with no or strongly reduced IgE and T-cell reactivity. Four Ole e 1 peptides of approximately 30 amino acids that were rich in solvent-exposed amino acids were identified by using programs for the prediction of surface accessibility based on the allergen sequence. An additional N-terminal peptide was included to cover the complete Ole e 1 sequence, although it was predicted to contain fewer surface-exposed amino acids. When

tested for IgE reactivity with sera from 67 patients with olive and ash pollen allergy, 4 of the 5 peptides showed very little or no IgE binding. Only the C-terminal peptide 5 showed IgE reactivity. This finding was in accordance with a previous study from González et al,¹⁵ who described an IgE-binding region at the C-terminus (amino acids 114-145) of Ole e 1. Because of the lack or reduction of IgE reactivity, peptides 1 to 4 appeared to be possible candidates for the development of a hypoallergenic olive pollen vaccine.

Several studies performed in the last years confirmed earlier findings that the induction of allergen-specific IgG is important for the success of SIT. Therapy-induced allergen-specific IgG antibodies inhibit (1) allergen-induced basophil degranulation and are associated with reduced skin and nasal sensitivity,³³⁻³⁷ (2) IgE-facilitated antigen presentation,³⁸⁻⁴¹ and (3) the allergen-induced boost of IgE production.^{33,34,42} There is also the possibility that co-cross-linking of FcεRI and FcγRIIB IgG antibodies, as has been described in murine models,⁴³ might play a role in SIT, although this is a matter of debate because thus far no evidence for a role for this mechanism in SIT has been found.^{44,45} Therefore we searched for non-IgE-reactive peptides that, on immunization, would give rise to the induction of IgG antibodies recognizing natural Ole e 1. IgG antibodies raised by immunization with KLH-coupled peptides 1 to 4 were studied for their ability to recognize Ole e 1 and to block allergic patients' IgE binding to Ole e 1. A combination of antibodies against peptides 1 and 2 inhibited allergic patients' IgE binding to Ole e 1 (mean inhibition, 68%) even more than IgG antibodies obtained by immunization with the complete Ole e 1 allergen (mean inhibition, 55%) at the dilutions tested. Immunization with peptides 3 and 4 did not induce IgG, which inhibited IgE binding to Ole e 1. The strong blocking capacity of anti-peptide 1 antibodies was unexpected because a relatively low surface probability had been predicted for peptide 1, and thus far no major IgE epitopes have been described for the N-terminus of Ole e 1. Until now, there have been no available x-ray crystallographic data for the identification of conformational epitopes of Ole e 1, and therefore only sequential epitopes (ie, IgE-binding assays with short overlapping peptides) have been determined.¹⁵ We have used the peptide-specific IgG antibodies for competitive epitope mapping, an approach that has been described earlier as a possible strategy for the mapping of conformational epitopes.⁴⁶ The fact that IgG antibodies specific for the N-terminal peptides of Ole e 1 strongly inhibited IgE binding indeed revealed a hitherto unrecognized major IgE reactive area on the Ole e 1 N-terminus. The strong inhibition of IgE reactivity to conformational epitopes on Ole e 1 by the peptide-induced IgG antibodies might be explained by the fact that they recognize amino acids that are part of conformational epitopes or bind in close vicinity and thus inhibit IgE binding by means of steric hindrance.

Because of the high degree of sequence identity between Ole e 1 and the major allergens from ash (Fra e 1), lilac (Syr v 1), and privet (Lig v 1) (Fig 1), it was not surprising that the antibodies induced by immunization with the Ole e 1 peptides showed strong cross-reactivity with Fra e 1. It might thus be expected that a vaccine based on peptides 1 and 2 will be also useful for the treatment of allergy to ash pollen and to pollen from other related Oleaceae plants.

Our approach of using carrier-bound nonallergenic Ole e 1 peptides for SIT is different from the approach of using recombinant hypoallergenic mutants/variants of Ole e 1.²¹ The

mutants also induced blocking IgG antibodies on immunization of mice, exhibited reduced allergenic activity, but preserved T-cell reactivity and hence activated allergen-specific T cells. In contrast, we have removed the majority of Ole e 1-specific T-cell epitopes described for the C-terminus of Ole e 1.¹⁴ Peptides 1 and 2 induced much lower lymphoproliferative responses than the Ole e 1 allergen when tested with PBMCs from allergic patients. It might thus be expected that the carrier-bound Ole e 1 vaccine will not activate Ole e 1-specific T cells, and thus it should be possible to avoid not only IgE- but also T cell-mediated side effects during SIT. Nevertheless, there is evidence that T-cell modulation by peptides containing T-cell epitopes plays a role in SIT,^{19,47} and it will therefore be interesting to compare the efficacy of therapy with T cell-reactive peptides with the mainly antibody-based approach suggested by us in clinical studies for efficacy.

It has been recently shown for the major grass pollen allergen Phl p 1 that viral proteins can be used as carrier molecules for nonallergenic peptide vaccines.⁴⁸ A similar strategy might also be used for the Ole e 1 peptides described by us. It would facilitate the production of the vaccine and allow us to augment/modulate the immune response and also to obtain antiviral immunity. Vaccines based on carrier-bound Ole e 1-derived peptides might represent safe vaccines for the therapy and eventually prophylaxis of Oleaceae pollen allergies.

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Clinical implications: We have investigated a possible new approach for SIT against olive pollen allergy based on nonallergenic carrier-bound Ole e 1 peptides.

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METHODS

Cloning, expression, and purification of rOle e 1

For isolation of a cDNA coding for Ole e 1, total RNA from mature olive pollen (Allergon, Ängelholm, Sweden) was extracted and purified with the NucleoSpin RNA L Kit (Macherey-Nagel, Düren, Germany). Then the olive pollen RNA was converted into single-stranded cDNA, followed by PCR reactions with the SuperScript One-Step RT-PCR System (Invitrogen Ltd, Paisley, United Kingdom) and Ole e 1-specific^{E1} primers (MWG Biotech, Ebersberg, Germany): Ole e 1 forward, 5'-GCG GAT CCA CAT ATG GAG GAT GTT CCG CAA CCT CCA G-3' (the *NdeI* restriction site containing the methionine-encoding ATG is underlined); Ole e 1 reverse, 5'-AT CCC GGG GAA TTC TTA GTG ATG ATG ATG ATG CAT GTT GGG CGG GTA CAT GCC CAG C-3' (the *EcoRI* site is underlined, and a DNA segment introducing 6 histidine residues in front of the stop codon is given in italics). The RT-PCR product was cloned into the pSTBlue-1 AccepTor Vector (pSTBlue-1 AccepTor Vector Kit; Novagen, Madison, Wis) and then cut with *NdeI* and *EcoRI* and subcloned into the *NdeI/EcoRI* site of the expression vector pET17b (Novagen, Cambridge, United Kingdom). The DNA sequence of the expression plasmid, designated Ole e 1-pET17b, was confirmed by means of automated sequencing (VBC Genomics, Vienna, Austria).

For expression of recombinant Ole e 1, BL21-CodonPlus (DE3)-RIPL competent cells (Stratagene, La Jolla, Calif) were transformed with the Ole e 1-pET17b construct and grown in liquid cultures containing 100 mg/L ampicillin and 32 mg/L chloramphenicol to an OD (600 nm) of 0.6. Protein expression was induced by addition of isopropyl- β -D-thiogalactoside (1 mmol/L). The majority of the histidine-tagged recombinant protein accumulated in the inclusion body fraction. Therefore bacterial cells were lysed in urea buffer (8 mol/L urea, 0.1 mol/L NaH₂PO₄, and 0.01 mol/L Tris-HCl, pH 8). Solubilized proteins were purified by means of Ni²⁺-affinity chromatography (Qiagen, Hilden, Germany). Fractions containing recombinant Ole e 1 were pooled, and the buffer was exchanged to 10 mmol/L Tris (pH 7.5) by using a PD-10 column (GE Healthcare Biosciences AB, Uppsala, Sweden). The purity of the protein sample was evaluated by means of analysis on Coomassie brilliant blue R-250 (Bio-Rad, Richmond, Calif)-stained SDS-PAGE.^{E2} The protein concentration was determined with the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, Ill) by using BSA as a standard. The purified recombinant protein was stored at -20°C.

Purification of nOle e 1

For purification of nOle e 1, olive pollen was extracted overnight at 4°C in 10 mmol/L NaH₂PO₄, pH 7.5 (1 g of pollen/10 mL). After centrifugation at 20,000g for 30 minutes at 4°C, the extract was sterile filtered (Acrodisc, 0.2 μ m; Gelman Sciences, Lana Cove, Australia) and applied on a HiPrep 26/60 Sephacryl S-100 High Resolution gel filtration column (Amersham Biosciences, Uppsala, Sweden) equilibrated in 10 mmol/L NaH₂PO₄. Ole e 1-containing fractions were pooled, dialyzed against distilled water, lyophilized, dissolved in 20 mmol/L NaH₂PO₄ (pH 7.5), and loaded on a HiTrap QFF anion-exchange column (Amersham Biosciences). Fractions containing purified nOle e 1 were eluted with a linear salt gradient (0-0.5 mol/L NaCl in 20 mmol/L NaH₂PO₄) and dialyzed against 10 mmol/L NaH₂PO₄. The purity of nOle e 1 and the protein concentration were assessed as described for rOle e 1 (see the "Cloning, expression, and purification of rOle e 1" section).

IgE and T-cell reactivity of Ole e 1 and Ole e 1-derived peptides

For dot blot experiments, 1 μ g per dot of purified nOle e 1 or equimolar amounts of the Ole e 1-derived peptides (0.2 μ g per dot) were dotted onto nitrocellulose membranes (Protran Nitrocellulose Transfer Membrane, Schleicher & Schuell). Membranes were incubated with sera diluted 1:10 in PBST (PBS and 0.5% vol/vol Tween 20, pH 7.4) from Oleaceae-sensitized patients and, for control purposes, with the serum from a nonatopic subject or with PBST. Bound IgE antibodies were detected with 1:15 diluted iodine 125-labeled anti-human IgE antibodies (IBL, Hamburg, Germany) and were visualized by means of autoradiography with Kodak XOMAT films and intensifying screens (Kodak, Heidelberg, Germany).

For ELISA experiments, ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight at 4°C with 5 μ g/mL nOle e 1 or equimolar amounts of the Ole e 1-derived peptides in 100 mmol/L sodium bicarbonate buffer (pH 9.6). After washing with TBST (Tris-buffered saline with 0.05% vol/vol Tween 20, pH 8) and blocking with 1% wt/vol BSA in TBST for 2.5 hours at 37°C, plates were incubated overnight with sera (diluted 1:5 in TBST and 0.5% wt/vol BSA) from Oleaceae-sensitized patients and, for control purposes, with serum from a nonallergic subject. Bound IgE antibodies were detected with horseradish peroxidase-coupled goat anti-human IgE antibodies diluted 1:2500 in TBST and 0.5% wt/vol BSA (Kirkegaard & Perry, Gaithersburg, Md). The color reaction was started by adding 1.7 mmol/L 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), which was obtained from Sigma-Aldrich, in 60 mmol/L citric acid, 77 mmol/L Na₂HPO₄·2H₂O, and 3 mmol/L H₂O₂. ODs were measured in an ELISA reader (Dynatech, Denkendorf, Germany) at 405 nm. All determinations were conducted as duplicates, and results were expressed as mean values with a variance of less than 5%.

For lymphoproliferation assays, nOle e 1 was purified from endotoxins by means of polymyxin B affinity chromatography (Affi-Prep; Bio-Rad Laboratories, Hercules, Calif). Endotoxin levels in the nOle e 1 and peptide preparations were assessed with the QCL-1000 Endpoint Chromogenic LAL Assay (Lonza, Basel, Switzerland) and found to be less than the detection limit of the assay.

PBMCs were isolated by means of Ficoll density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden) from heparinized blood of 6 Ole e 1-reactive allergic patients. Cells were washed 3 times with DPBS (Gibco, Carlsbad, Calif) and diluted with Ultra Culture medium (BioWhittaker, Rockland, Me) containing 2 mmol/L L-glutamine (Sigma, St Louis, Mo), 50 mmol/L β -mercaptoethanol (Sigma), and 0.1 mg/mL gentamicin (Sigma) to a concentration of 2×10^6 /mL. One-hundred-microliter aliquots of PBMCs were cultured in triplicates in 96-well round-bottom plates (Nunc; Nalgen Nunc International, Roskilde, Denmark) and were stimulated with 100 μ L of nOle e 1 (5 μ g/mL), peptides 1 to 5 (1 μ g/mL), an equimolar mixture of peptides 1 to 5 (5 μ g/mL in total), IL-2 (4 units), or Ultra Culture medium alone. After 6 days of culture at 37°C and 5% CO₂ in humidified air, 0.5 mCi of tritiated thymidine (Amersham) was added to each well, and its incorporation was measured after 16 hours by means of liquid scintillation counting with a microbeta scintillation counter (Wallac ADL, Freiburg, Germany). From the resulting counts per minute (cpm), the stimulation index (SI) was calculated as follows:

SI = cpm (mean of triplicates) of antigen-stimulated cells/cpm (mean of triplicates) of medium-treated cells.

ELISA competition assay for analyzing the inhibition of human IgE binding to nOle e 1 by peptide-specific IgG antibodies

ELISA plates (Nunc) coated overnight with 1 μ g/mL nOle e 1 were preincubated for 24 hours with different dilutions of each of the anti-peptide antisera, of mixtures containing equal amounts of the anti-peptide antisera, of anti-rOle e 1 antiserum, or, for control purposes, of the corresponding preimmune sera. After overnight incubation with sera from Oleaceae-sensitized patients (diluted 1:5), bound IgE antibodies were detected with the horseradish peroxidase-labeled goat anti-human IgE (Kirkegaard & Perry) antibodies, as described in the "IgE and T-cell reactivity of Ole e 1 and Ole e 1-derived peptides" section. The percentage reduction of IgE binding achieved by means of preincubation with rabbit antisera was calculated as follows:

$$\text{Percentage inhibition of IgE binding} = 100 - (\text{OD}_I / \text{OD}_P) \times 100,$$

where OD_I and OD_P represent optical density values after preincubation with the rabbit immune serum or preimmune serum, respectively.

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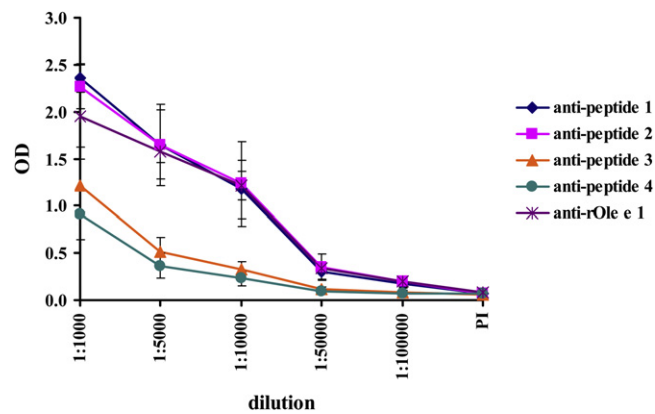


FIG E1. Induction of Ole e 1-specific IgG antibodies in rabbits. Rabbits were immunized with rOle e 1 or with the KLH-coupled Ole e 1-derived peptides. IgG reactivities of the antisera were measured by means of ELISA. Four different serum dilutions (*x-axis*: 1:1,000, 1:5,000, 1:10,000, and 1:50,000) are displayed as mean OD values (*y-axis*).

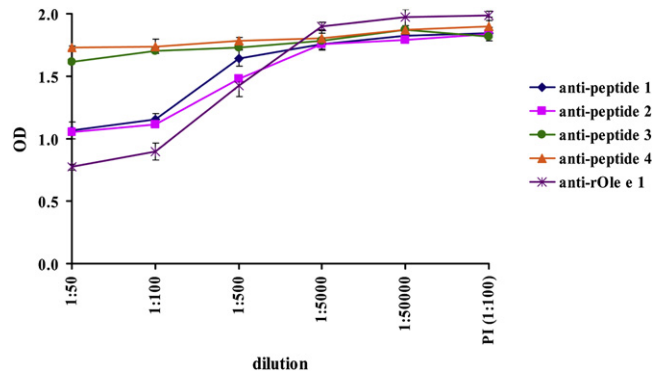


FIG E2. Rabbit anti-peptide and anti-rOle e 1 antibodies inhibit Oleaceae-sensitized patients' IgE binding to Ole e 1. nOle e 1 preincubated with increasing dilutions (*x-axis*) of the antisera or with the corresponding pre-immune sera was incubated with serum IgE from an Oleaceae-sensitized patient. IgE reactivity is displayed as OD values.

TABLE E1. Demographic, clinical, and serologic features of Oleaceae-sensitized patients from Spain

Patient no.	Sex	Age (y)	CAP (kU/L)			Symptoms	Other allergies	
			Total IgE	Olive	nOle e 1		Respiratory	Food
1	M	16	1641	33.5	44	rc	g, t, hdm, ae	sf
2	M	27	892	12.9	12	rc	g, ae	—
3	F	21	140	3.0	4	rc	g, t, mo	—
4	M	26	202	7.2	4	rc	g, t, l	me
5	F	37	2381	33.2	5	rc, a	g, hdm	—
6	M	39	119	3.4	3	rc	g, t, w, ae	—
7	F	30	403	34.2	44	rc	g, ae	—
8	F	23	1696	6.8	8	rc, a	g, hdm, ae	—
9	F	46	90.6	10.2	9	rc	—	—
10	M	25	258	8.6	9	rc	hdm, l	—
11	F	44	567	13	5	rc, a	g, t, ae	—
12	F	52	492	14.5	6	rc	g, t	—
13	M	19	1063	10.6	8	rc, a	g, t, l	n, fr
14	M	18	678	2.2	2	rc, a	g, t	—
15	M	34	1262	75.1	19	rc, a	g, ae	—
16	M	16	1401	21.3	8	rc, a	g, t	—
17	F	39	652	4.8	4	rc, a	g, t	—
18	F	20	2279	60.9	61	rc, a	g, t, ae	e, cm
19	M	34	1202	15.4	5	rc	g, w	s
20	F	50	259	47.8	62	rc, a	g, ae	—
21	F	37	112	5.8	2	rc, a	g, w	—
22	F	26	157	3.1	3	rc	g, t	—

a, Asthma; *ae*, animal epithelia; *cm*, chicken meat; *e*, egg; *F*, female; *fr*, fruits from the Rosacea family; *g*, grass pollen; *l*, latex; *M*, male; *me*, melon; *mo*, mold; *n*, nuts; *rc*, rhinoconjunctivitis; *s*, soy; *sf*, seafood; *t*, tree pollen; *w*, weed.

TABLE E2. Demographic, clinical, and serologic features of Oleaceae-sensitized patients from Italy

Patient	Sex	Age (y)	CAP (kU/L) Total IgE	ISAC (kU/L) nOle e 1	Symptoms	Other allergies	
						Respiratory	Food
1	M	20	ND	61.8	rc, a	g, t, mo, ae, l	wh
2	M	10	ND	14.2	r, a	g, t, w, mo	—
3	F	23	ND	54.0	r, a	g, t	fr, s
4	F	8	ND	7.6	r	t, hdm, ae	—
5	F	40	ND	11.6	rc, a	g, w, hdm	—
6	F	34	ND	5.3	r	g, t, w, mo, hdm	—
7	M	18	ND	8.8	r, a	g, t, w, mo, hdm, ae	—
8	F	16	ND	16.5	r	g, hdm, ae	—
9	F	12	ND	79.3	r	g, t, w, hdm	—
10	F	9	ND	7.5	r, a	g, t	—
11	F	36	ND	44.1	rc, a	g, hdm, ae	—
12	M	10	ND	11.8	r, a	g, t, hdm	—
13	F	31	1298	5.1	r	g, t, ae	—
14	M	15	ND	2.0	r, a	g, hdm	—
15	F	13	ND	19.0	r	g, t, mo, hdm, ae	—
16	M	43	ND	7.6	r, a	g, hdm	—
17	F	21	ND	22.0	rc	g, hdm, ae	—
18	M	16	106	8.1	r, a	t	—
19	F	39	ND	2.2	—	g, t, w	—
20	M	27	448	5.2	r, a	g, t, hdm, ae	—
21	F	7	ND	1.33	r	t	—
22	M	22	ND	7.7	r	g, t, w, hdm, ae	—
23	F	36	ND	1.0	—	—	—
24	F	13	ND	16.8	rc, a	g, t, hdm, ae	—
25	F	40	ND	12.7	r	t	—
26	M	39	ND	12.8	r, a	t, w	—
27	F	11	ND	13.5	rc, a	g, t, w, mo, hdm, ae	fr
28	M	37	ND	5.5	r	g, t, mo	sf
29	M	19	ND	10.5	r, a	g, t, mo, hdm, a	—
30	F	74	ND	7.8	r	g, t, w, ae	—

a, Asthma; ae, animal epithelia; c, conjunctivitis; F, female; fr, fruits from the Rosacea family; g, grass pollen; ISAC, Immuno Solid-phase Allergen Chip; l, latex; M, male; mo, mold; ND, not determined; rc, rhinoconjunctivitis; r, rhinitis; s, soy; sf, seafood; t, tree pollen; w, weed; wh, wheat.

TABLE E3. Demographic, clinical, and serologic features of Oleaceae-sensitized patients from Austria

Patient	Sex	Age (y)	CAP (kU/L)				Symptoms	Other allergies	
			Total IgE	Olive	Ash	nOle e 1		Respiratory	Food
1	M	17	945	ND	22.8	ND	rc, a	g, t, hdm, mo	n
2	F	44	ND	ND	ND	5.7	rc	t, hdm, ae	—
3	M	24	ND	ND	ND	9.9	rc, a	g, t, w, hdm, ae	fr
4	F	32	599	ND	27.1	ND	rc, a	g, t, w, hdm, ae	—
5	M	46	ND	ND	ND	6.0	c	t	n, fi
6	M	50	459	ND	ND	20	rc, a	g, t, w	n, s
7	M	29	258	ND	ND	7.1	rc	t, hdm	n
8	M	28	ND	ND	ND	3.1	rc, a	t, ae	fr, n
9	M	42	ND	6.2	4.6	ND	rc, a	g, t, mo	wh
10	F	30	291	11.4	6.9	ND	rc, a	g, t, w, l	—
11	M	32	3432	ND	ND	>100	rc, a, ad	g, t, w, hdm, ae	—
12	M	31	221	ND	ND	13.9	rc, a	g, t, hdm, ae	—
13	F	30	>5000	ND	ND	13.3	rc	g, t, hdm, ae	—
14	M	35	544	ND	ND	5.8	rc	g, t	—
15	F	38	55	ND	ND	7.9	rc	t, hdm, ae	—

a, Asthma; *ad*, atopic dermatitis; *ae*, animal epithelia; *c*, conjunctivitis; *e*, egg; *F*, female; *fi*, fig; *fr*, fruits from the Rosacea family; *g*, grass pollen; *l*, latex; *M*, male; *mo*, mold; *n*, nuts; *ND*, not determined; *rc*, rhinoconjunctivitis; *s*, soy; *t*, tree pollen; *w*, weed; *wh*, wheat.

TABLE E4. Characterization of Ole e 1–derived synthetic peptides

	Position	No. of amino acids	Sequence	Molecular weight	Isoelectric point
Peptide 1	1-35	36	EDIPQPPVSQFHIQQQVYCDTCRAGFITELSEFIPC	4069.6	4.2
Peptide 2	36-66	32	GASLRLQCKDKENGDVTFTEVGYTRAEGLYSC	3511.9	4.9
Peptide 3	67-99	34	CMLVERDHKNEFCEITLISSGRKDCNEIPTEGWA	3925.5	4.8
Peptide 4	86-120	36	CGRKDCNEIPTEGWAKPSLKFKLNTVNGTTTRTVNPL	3989.6	9.3
Peptide 5	112-145	35	CGTTRTVNPLGFFKKEALPKCAQVYNKLGMYPPNM	3918.7	9.5

Position, number of amino acids, sequence, molecular weight (in Da), and isoelectric point of Ole e 1–derived peptides are shown. Peptides 1 and 2 contain an additional cysteine (marked in boldface) at the C-terminus, and peptides 3, 4 and 5 contain an additional cysteine (marked in boldface) at the N-terminus.