

Biochemical and Immunological Characterization of Pollen-Derived β -Galactosidase Reveals a New Cross-Reactive Class of Allergens among Mediterranean Trees

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Key Words

Allergy · Cypress pollen · Olive pollen · Peptides · IgE, specific · T lymphocytes

Abstract

Background: The most potent allergens in the Spermatophytae family exhibit significant homology with enzymes. Some of these are thought to be involved in pectin metabolism, recognition of compatible stigma and delivery of sperm cells to the ovule. **Objective:** To test if glycohydrolase activities from some Mediterranean tree pollens could act as allergens in sensitized hosts. **Methods:** Freshly collected *Cupressus* and *Olea* pollens were investigated for their glycohydrolase activities by means of synthetic fluorogenic substrates and isoenzymes characterized by DEAE-cellulose ion-exchange chromatography. Binding of specific IgE was investigated by immunoblotting in 30 tree-sensitive subjects, as well as in 20 atopic non-tree-sensitive and 15 healthy controls. The enzymes were also adopted to stimulate proliferation of allergen-specific T cell clones. Finally, they were tested in vivo in a cutaneous immediate wheal and flare reaction.

Results: β -Galactosidase (β -GAL) is present with different isoenzymatic patterns on both pollen extracts, could be recognized by circulating IgE, as well as immunoprecipitated by sera from allergic subjects. The enzyme could stimulate the proliferation of T cells from allergic subjects, and favor the emergence of CD4⁺ T cell clones with specific in vitro reactivity to β -GAL. Finally, the enzyme induced in vivo a cutaneous wheal and flare reaction in clinically sensitive subjects. **Conclusions:** Despite different isoenzymatic patterns, *Olea*-derived β -GAL cross-reacted with that from cypress pollen, suggesting that these enzymatic glycoproteins may represent major native allergens among these Mediterranean trees.

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Introduction

Pollens of *Cupressus arizonica*, *Cupressus sempervirens* and *Olea europea* are a major cause of allergy in the Mediterranean area, where these species are widely found [1, 2]. Cypress tree inflorescence is usually from Decem-

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ber to March and generally causes seasonal allergic rhinitis or oculorhinitis [3, 4]. A recent epidemiological survey from a large cohort of young adults in central Italy showed that the prevalence of sensitization is around 8% of the general population [5]. Clinically an apparently heterogeneous group of subjects is affected, half of whom are monosensitive to cypress, whereas the other 50% display cutaneous sensitization, but less frequently respiratory symptoms, which, when present, seem to be easily related to *Parietaria*, *O. europea* and/or dust mites [5, 6].

There have been few attempts to quantify the real prevalence of seasonal (June) rhinitis and/or asthma due to *O. europea* allergy [7], but a cumulative percentage of about 5% of the general population has been suggested [8].

Therefore, the prevalence of clinical sensitization to Mediterranean pollens is low, especially if one considers the unusually higher amounts of grains released from these trees, which largely exceed those of other nonarboreal species, such as Graminaceae or *Parietaria* [9, 10]. The absence of small starch granules inside tree pollen grains may partially explain the lower incidence of asthma symptoms among patients sensitive to cypress or olive as compared to grasses [11, 12], and indicate that higher atmospheric counts are needed to induce airway sensitization [10]. In addition, patients allergic only to cypress pollen have normal or subnormal total serum IgE levels and lower specific IgE, as compared with polysensitized ones [13].

Current knowledge about the biochemical characterization of Mediterranean tree pollens is based on the identification within fresh grain extracts [14, 15] of proteic substances with defined molecular mass that identify major and minor allergens [16, 17]. IgE reactivity has been linked with sugar moieties of *C. arizonica* extract, presumably glycoproteins [18], and, in fact, many glycoproteic substances with enzymatic activities have been described among the commonest allergens [19]. Although it is tempting to think that these biochemical properties might contribute to allergenicity, as they are known to induce IgE reactivity in the host, their role as complete antigens has still not yet been demonstrated and there are no conclusive data on the enzymatic activity of plant allergens, enzyme distribution and cross-reactivity [20]. We, therefore, hypothesized that the presence of hydrolytic enzyme activities among the membranes of inaperturate pollens, which have relevant physiological functions in pollination processes in higher plants [21], could be the source of allergenic substances, both in terms of IgE and T cell reactivity.

In this paper we detected several acidic glycohydrolases in Mediterranean tree (*C. arizonica*, *C. sempervirens* and *O. europea*) pollen extracts and demonstrated that β -galactosidase (β -GAL) was the most prevalent. As its enzymatic activity is specifically recognized by T cells and it binds circulating IgE from sensitive subjects, it might be a new cross-reactive pan-allergen in the Spermatophytaceae family.

Materials and Methods

Patients and Controls

Sera were collected from 30 sensitive patients with clinical histories and positive skin prick test reactivity to our own as well as to commercial (Lofarma, Milano, Bracco-Allergopharma, Milano and Anallergo, Firenze, all in Italy), *C. arizonica*, *C. sempervirens* and *O. europea* extracts. Informed consent was obtained from all participants to the study and the in vitro procedures were preliminarily approved by the local ethical committee. All samples were screened for the presence of specific IgE by conventional solid-phase RAST (Pharmacia, Uppsala, Sweden). Clinically, 15 patients were monosensitive to cypress, 10 to both cypress and *O. europea*, and the other 5 to *Olea* only. Twenty atopic subjects with skin reactivity to at least four aeroallergens but no reactivity against cypress or *Olea* were also enrolled in the study. Fifteen nonatopic control sera were obtained from individuals with no history of allergic disease and no skin reactivity to a panel of 24 seasonal and perennial allergens. Peripheral blood mononuclear cells (PBMC) from 4/15 cypress-sensitive patients were used for in vitro culture experiments.

Pollen Collection and Preparation of Extracts

Samples of mature inflorescence from *C. sempervirens*, *C. arizonica* and *O. europea* trees were collected separately, according to the pollen calendar of central Italy. Pollens were collected on polished brown paper and then purified (up to 90%) by sequential sieving. Extracts were prepared as previously described [22]. Briefly, 10 g of pollens were defatted 3 times with 200 ml of diethyl ether and dried overnight at room temperature, then suspended in 300 ml of 125 mM NaHCO₃ and sonicated, on ice, with a 100-watt MSE ultrasonic disintegrator equipped with a 'microtip' at a 12-mW wave amplitude (three sonications; 5 min each). The homogenate was automatically stirred overnight at 4°C, then centrifuged at 22,000 g for 30 min. Supernatants were used as native crude extracts.

Enzyme Assays

Glycohydrolase activity was evaluated in pollen extracts as described [23], using 3 mM solutions of the appropriate synthetic substrates for 4-MU- α -mannoside, 4-MU- β -galactoside, 4-MU- β -glucuronoside, 4-MU- α -L-glucoside. Fluorescence of the released 4-methyl-umbelliferone was measured on a Perkin Elmer LS-3 fluorimeter (excitation 360 nm, emission 446 nm).

One unit of enzyme activity was defined as the amount of enzyme that hydrolyzes 1 mol of substrate/min at 37°C. Protein content was measured by the method of Bradford [24], using crystalline bovine γ -globulin as standard. Specific activity was expressed as U/mg of protein.

Cytochemical Localization of the Enzyme in Pollen Granules

Fresh cypress pollen grains were cytocentrifuged (Shandon-Lipshaw, Pittsburgh, Pa., USA) on chemically clean slides, air dried, fixed or not with 4% formaldehyde and then stained for β -GAL activity according to the simultaneous azo-coupling method of Hayaishi [25], with naphthol-AS-BI- β -N-acetylgalactosaminide as substrate and an incubation time of 60 min at 37°C. Sample staining without adding the substrate was used as control.

Ammonium Sulfate Fractionation

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to native pollen extracts (30 ml at time) to give a final saturation of 50%, and stirred gently at 4°C for 60 min. The precipitate, tested for the absence of β -GAL activity, was removed by centrifugation at 12,000 *g* for 30 min at 4°C and discarded. The supernatant was brought to 80% saturation with $(\text{NH}_4)_2\text{SO}_4$, stirred at 4°C for 7 h and the precipitate recovered by centrifugation as before, and after testing for the absence of β -GAL activity, the supernatant was discarded. In preparation for DEAE-cellulose chromatography, the precipitate was dissolved in, and dialyzed against, 0.01 *M* Na-phosphate buffer, 10 *mM* sodium chloride, 1 *mM* EDTA, pH 7.0.

DEAE-Cellulose Ion-Exchange Chromatography

DE52 DEAE-cellulose chromatography was performed according to Norden et al. [26]. Samples from extracts were loaded into the DEAE-cellulose column (1 ml) equilibrated with 0.01 *M* Na-phosphate buffer, 10 *mM* NaCl, 1 *mM* EDTA, pH 7.0. The column was washed with the same buffer until the absorbance at 280 nm reached zero, then developed with a linear gradient of NaCl (0.1–0.5 *M*) in 25 ml of buffer. The flow rate was 0.5 ml/min. Fractions were collected and assayed for their activity towards the 4-MU- β -D-galactoside substrate. Finally, the column was eluted with 1.0 *M* NaCl in the same buffer.

The enzymatically active peaks eluted within the gradient were collected, concentrated to 0.5 ml, and dialyzed against 0.01 *M* Na-phosphate buffer, pH 6.0 by vacuum dialysis (Sartorius apparatus, Sartorius, Göttingen, Germany), and sterilized by filtration on 0.22- μ m Millipore filters.

Immunoprecipitation of β -GAL by Sera of Allergic Subjects

IgG-depleted (Affi-Prep Protein A, Bio-Rad Laboratories, Milano, Italy) pooled sera (prepared separately) from cypress- or from olive-allergic subjects were mixed with increasing amounts (from 1 to 10 μ g/ml) of the crude *C. arizonica*, *C. sempervirens* and *O. europaea* extracts. After gentle shaking for 2 h at 4°C, aliquots of all mixtures were centrifuged at 200 *g* for 1 min on a microcentrifuge (Beckman), and the supernatant tested for β -GAL activity. The same experiments were repeated with fixed amounts of β -GAL (200–230 mU/ml) from DEAE-cellulose, and with the same amounts of commercial preparation of β -GAL from *Escherichia coli*, *Faseolus vulgaris* and bovine liver (Sigma Chemical Co., St. Louis, Mo., USA).

ELISA Detection of Specific IgE to β -GAL

Enzyme preparations from the DEAE-cellulose column were dialyzed and concentrated before use. Coated plates (20 μ g/ml of protein in 96-well U-bottom plates; Becton Dickinson France) were incubated with all IgG-depleted (Affi-Prep Protein A) serum samples and developed with an alkaline-phosphatase-conjugated antiserum to human IgE (polyclonal anti-Fc ϵ receptor, Sigma) diluted 1:500 in PBS. Mean absorbance was read at 450 nm. In some experiments, the

recovered fractions after enzyme immunoprecipitation were also used.

To test β -GAL extract inhibition of IgE reactivity, the more active fractions from DEAE-cellulose chromatography (with known specific activity) were serially diluted. Final volumes of 100 μ l were added to 250- μ l aliquots of pooled sera, incubated for 2 h, centrifuged, and the supernatant tested in an ELISA assay, as above. Human serum albumin served as a negative control.

Immunoblotting Detection of Specific IgE to Purified β -GAL

SDS-PAGE was performed using Laemmli's method [27], under reducing conditions, with a continuous gradient of 7.5–14% acrylamide on 0.75-mm-thick 'minigels' at 12 mA. Gels were then blotted on PVDF membrane (Bio-Rad) with a Bio-Rad transblot system. Transfer was carried out for 120 min at 400 V [28].

IgG-depleted serum from cypress- and olive-sensitive, atopic non-tree-sensitive and nonatopic subjects, diluted 1:2 in 0.5 ml PBS, was used as primary antibody. Polyclonal anti-human IgE (alkaline phosphatase or peroxidase-conjugated, all from Sigma) diluted 1:500 was used as secondary antibody. Membranes were finally incubated in alkaline phosphatase or peroxidase substrates.

Recognition of Cypress β -GAL by T Cells of Allergic Subjects

Native crude extracts of *C. arizonica* and *C. sempervirens* were used to induce the in vitro emergence of allergen-specific T cell lines [29]. Briefly, $0.5\text{--}1 \times 10^6$ nonadherent PBMC/ml from each sample were cultured in RPMI 1640 medium supplemented with 2 *mM* L-glutamine (Gibco), 20 μ M 2-mercaptoethanol, 100 U/ml penicillin, 50 μ g/ml streptomycin (complete medium), and 10% FCS with autologous dendritic cells in 24-well flat-bottomed culture plates (Costar, Cambridge, Mass., USA) in a final volume of 3 ml. Cells were exposed to native extracts without adding cytokines or growth factors and restimulated every 2 weeks with autologous feeder cells plus cypress extracts. PBMC suspensions of control health care workers were cultured as described above. Proliferating lines were further expanded with recombinant human interleukin-2 (rhIL-2, 50–100 U/ml; EuroClone) and then tested in vitro for antigen specificity. The specificity of T cell lines was assessed by incubating 2×10^4 T cell blasts in triplicate 200- μ l cultures in the presence of 10^5 irradiated autologous feeder cells plus purified or heat-inactivated (50°C for 1 h) [30] cypress-derived β -GAL (10 μ g/ml) in 96-well round-bottomed microtiter plates (Costar) for 72 h at 37°C in a humidified atmosphere of 5% CO₂. Parallel control cultures were settled by incubating the cells with tetanus toxoid (5 μ g/ml). After pulsing for 16 h with 0.5 μ Ci ³H-thymidine (Amersham Biosciences, Little Chalfont, UK), radionuclide uptake was measured by scintillation counting. Responses were considered as positive when the stimulation index (ratio between the mean cpm obtained in triplicate T cell cultures with autologous irradiated PBMC plus antigen and the mean cpm obtained in triplicate cultures containing autologous irradiated PBMC alone plus antigen) was >10.

β -GAL-specific T cell blasts were seeded at 0.5, 1 and 2 cells per well in 96-well round-bottomed plates (Nunc, Nunc, Denmark) with 10^5 irradiated (5,000 rad) autologous non-T PBMC as feeder cells, 1% (vol/vol) PHA and rIL-2 (20 U/ml) in complete medium supplemented with 10% heat-inactivated human AB serum. Growing microcultures were then expanded at weekly intervals with 10^5 irradiated feeder cells and rIL-2. To expand established clones, T cell blasts were restimulated every 3 weeks with 0.1% PHA-P and irradiated autologous feeder cells.

The immunophenotype of emerging clones was established by flow cytometry with the following mAbs: Cy-chrome-conjugated anti-CD4 (clone RPA-T4; PharMingen, San Diego, Calif., USA), fluorescein (FITC)-conjugated anti-CD8 (PharMingen), anti- $\alpha\beta$ (Serotec), and anti-pan $\gamma\delta$ (clone 5A6.E9; T Cell Diagnostics, Woburn, Mass., USA). The Lysis II program (Becton Dickinson) was used to optimize gating of lymphocytes, providing an objective means of excluding debris (noncellular events due to particulate matter) from the lymphocyte gate.

Emerging T cell clones were reassayed for their reactivity to β -GAL from different sources (including heat-inactivated enzymes) by means of ^3H -thymidine incorporation after 5 days' culture in the presence of 10^5 irradiated autologous non-T cells as APC. Control cultures were represented by autologous irradiated non-T cells plus antigen. Results of in vitro stimulation are expressed in cpm $\pm$ SD.

Phenotype and Cytokine Production of β -GAL-Specific T Cell Clones

Phenotypes and intracellular IL-4, IL-5 and IFN- γ were detected by flow cytometry using the method of Jung et al. [31] with partial modifications. Cells were stained with either phycoerythrin-conjugated anti-CD4 (PharMingen), anti-CD8 (PharMingen), anti- $\alpha\beta$ (Serotec, Marseille, France) mAbs or FITC-conjugated anti- $\gamma\delta$ (Serotec) mAb for surface phenotyping. For the double-color staining procedure, after permeabilization, intracellular cytokines were stained with FITC-labeled anti-IL-4, IL-5 and IFN- γ mAbs (Becton Dickinson, San Jose, Calif., USA). Cytokine production was also measured in the supernatants of β -GAL-specific CD4+ T cell clones after in vitro priming with enriched enzyme, by means of commercially available kits (IL-4 and IFN- γ ; Serotec) [23].

In vivo Testing of β -GAL Extracts

Cutaneous prick tests were performed with commercially available reagents (Anallergo, Florence, Italy; ALK-Abelló, Denmark) and with our enriched β -GAL extracts on all allergic and control subjects. Commercial reagents were used at the arbitrary dose of 1,000 BU/ml, whereas β -GAL from cypress and olive pollens was used at 0.5 $\mu\text{g}/\text{ml}$ protein concentration. We also tested other sources of β -GAL, such as those from *F. vulgaris*, bovine liver and *E. coli* (all from Sigma), all adjusted to 0.5 $\mu\text{g}/\text{ml}$ of protein content. The appearance of wheal and flare reactions was recorded and their diameter measured. Mean horizontal and vertical diameters are expressed in millimeters $\pm$ SD, as are the measurements of lesions 15 min after forearm puncture.

Results

Glycohydrolase Activities in Pollen Extracts

Total protein yield of pollen extraction was 49.5 mg for *C. arizonica* and 53.8 mg for *O. europea*. Pollen extracts hydrolyzed synthetic fluorogenic substrates for the acidic glycohydrolases: α -fucosidase, β -hexosaminidase, β -mannosidase, α -mannosidase, β -GAL and β -glucuronidase. Table 1 shows enzyme levels with a wide range of activities. β -GAL had the highest specific activity.

Figure 1 illustrates the β -GAL isoenzyme pattern of *C. arizonica* (fig. 1a) and *O. europea* (fig. 1b) pollen ex-

Table 1. Glycohydrolase-specific activity in crude pollen extracts

Substrate	<i>C. arizonica</i>	<i>C. sempervirens</i>	<i>O. europea</i>
β -Galactosidase	47.21 $\pm$ 3.12	44.68 $\pm$ 4.23	66.11 $\pm$ 1.8
β -Hexosaminidase	0.19 $\pm$ 0.06	0.28 $\pm$ 0.04	2.73 $\pm$ 0.9
α -Mannosidase	1.47 $\pm$ 0.12	2.05 $\pm$ 0.22	49.00 $\pm$ 1.5
β -Mannosidase	0.16 $\pm$ 0.02	0.19 $\pm$ 0.03	0.10 $\pm$ 0.1
α -Fucosidase	0.09 $\pm$ 0.03	0.07 $\pm$ 0.01	0.02 $\pm$ 0.05
β -Glucuronidase	n.d.	n.d.	0.16 $\pm$ 0.20

Specific activity: mean values (mU/mg of protein) $\pm$ SD represent ten independent experiments, all performed in triplicate. n.d. = Not detectable.

tracts. Two enzymatically active peaks were resolved upon application of the NaCl gradient to cypress extracts, accounting for 3.3% (peak I) and 2.2% (peak II) of the loaded activity. No further activity was eluted by washing with 1 M NaCl. *O. europea* pollen extract (fig. 1b) gave rise to a large enzymatically active peak that accounted for over 40% of the loaded activity.

Identical isoenzymatic patterns were also obtained with crude pollen extracts that had previously been fractionated with ammonium sulfate.

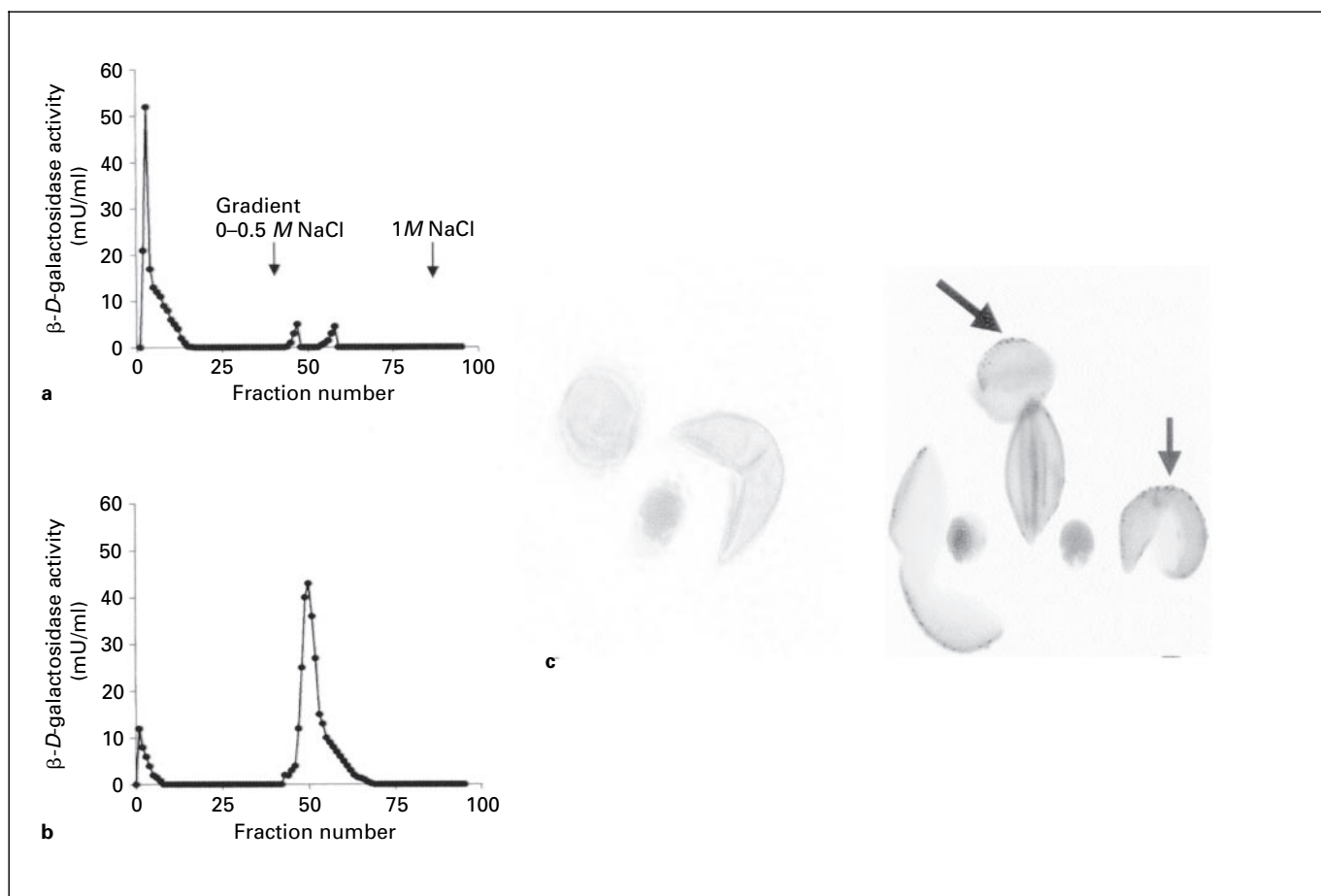
Localization of β -GAL Activity in Cypress Pollen Grains

Cytochemical staining for the enzyme substrate revealed that β -GAL activity was mainly located on the surface of the cypress pollen grain (fig. 1c, arrows). No membrane leaching occurred during the entire procedure, because the staining pattern was not affected whether samples were pretreated with 4% formaldehyde or not.

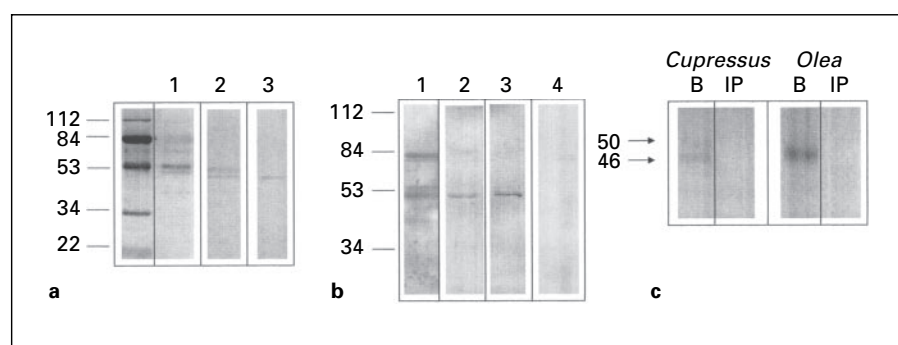
SDS-PAGE and Immunoblotting Analysis of Pollen-Derived β -GAL

SDS-PAGE performed on enzymatically active peaks derived from DEAE-cellulose chromatography indicated selected bands that were peculiar to β -GAL enriched from tree pollens (lanes 2 and 3), as compared with the pattern of whole cypress extract (lane 1). In particular, β -GAL from cypress resolved in two proteic bands of approximately 50 and 46 kDa, whereas olive pollen β -GAL showed an isolated 46-kDa proteic band.

Immunoblotting experiments (fig. 2b) demonstrated that circulating IgE from both cypress- and olive-allergic subjects was able to bind the 46-kDa polypeptides. In particular, 14 out of 15 patients monosensitive to cypress (as



1



2

Fig. 1. **a** DEAE-cellulose chromatography of cypress-derived β -GAL isoenzymes. Native cypress extract containing enzyme activity was fractionated upon NaCl gradient. Resolved peaks are depicted. **b** DEAE-cellulose chromatography of *Olea*-derived β -GAL isoenzymes. Native *O. europea* extract was processed as above. The profiles are representative of at least ten independent experiments. **c** Cytochemical staining of β -GAL activity on fresh cypress pollen grains. Left side represents pollen grains stained without adding enzyme substrate, right side shows a positive reaction (arrows on brown dots) after adding naphthol-AS-BI- β -N-acetyl-galactosaminide as enzyme substrate. Original magnification $\times 400$.

Fig. 2. **a** SDS-PAGE of enzymatically active fractions from cypress (lane 1: native extract; lane 2: fractions from 47 to 57) and olive

pollens (lane 3: fractions 49 to 53). The experiment is representative of four different extractive procedures performed on freshly collected pollen grains. The same amount of protein was loaded into each track. **b** Immunoblotting of native *C. arizonica* extract (lane 1) and enriched cypress (lane 2)- and olive-derived β -GAL (lane 3). A representative experiment of all performed in sensitive subjects ($n = 30$) is depicted. Lane 4 is a representative negative control sample. **c** SDS-PAGE of β -GAL extracts from cypress and olive pollens performed after immunoprecipitation with a serum pool from sensitive subjects. Samples assayed after immunoprecipitation (IP) showed the complete disappearance of proteic bands characteristic of the enzyme-enriched samples (B). A representative experiment of all those performed is depicted.

Table 2. Immunoprecipitation of enriched β -GAL

	<i>C. arizonica</i>	<i>C. sempervirens</i>	<i>O. europea</i>	<i>E. coli</i>	Bovine liver	<i>F. vulgaris</i>
Patients	36.2 $\pm$ 2.9*	34.0 $\pm$ 3.8*	40.2 $\pm$ 5.1*	80.7 $\pm$ 7.5	90.2 $\pm$ 3.5	78.3 $\pm$ 6.7
Atopics	89.2 $\pm$ 4.8	84.6 $\pm$ 5.7	90.3 $\pm$ 8.5	81.2 $\pm$ 4.8	86.5 $\pm$ 4.2	80.7 $\pm$ 4.5
NC	95.5 $\pm$ 3	96.3 $\pm$ 3.2	93.6 $\pm$ 5.2	88.6 $\pm$ 3.8	95.2 $\pm$ 2.7	91.5 $\pm$ 3.5

Enzyme contents in the supernatant are expressed as % ($\pm$ SD) of specific activity of reference samples incubated with normal human purified IgG fraction (from Immuno, Geneva, Switzerland) diluted in PBS. Reference extracts had known enzyme activity (200–230 mU/ml). IgG-depleted serum samples from cypress-sensitive (n = 15), olive-sensitive (n = 5), control atopic (n = 10) and normal subjects (NC, n = 10) were tested in three separate experiments performed in duplicate. * p < 0.005.

in lane 2) and 5 out of 5 monosensitive to olive pollen (as in lane 3) gave a result as that depicted in figure 2b. Nine out of ten tested samples from patients sensitive to both cypress and olive pollens had specific IgE to the same 46-kDa protein fraction from β -GAL of both pollen sources (data not shown). In the same manner, 12 out of 15 patients monosensitive to cypress had specific IgE to the 46-kDa fraction of the olive-derived β -GAL extract, and all subjects monosensitive to olive showed specific IgE to the 46-kDa fraction from cypress-derived β -GAL in the direct immunoblotting assay (data not shown). Negative results were always observed with sera from atopic non-tree-sensitive subjects (n = 20) and from 15 healthy controls, and are represented by lane 4 in figure 2b. For a comparison, figure 2b shows in lane 1 the spectrum of IgE reactivity to the whole cypress extract, which includes, together with that to the 46-kDa fraction, the specificities toward peptides (42, 53 and 82 kDa) other than those observed in experiments performed on the cypress β -GAL-enriched fraction as in lane 2.

Immunoprecipitation of β -GAL by Sera of Sensitive Subjects

Preliminary IgG depletion was employed in all samples tested including those of atopic control patients. Values of total IgG after Affi-Prep Protein A separation were less than 0.01 mg% for the 15 cypress-sensitive, 0.03 mg% for the 5 olive-sensitive and 0.02 mg% for the 10 control atopic subjects. IgG-depleted sera from allergic subjects immunoprecipitated β -GAL from tree extracts (table 2), as indicated by the significant fall in specific activity assayed in the supernatants. Incubation of purified *E. coli*-, *F. vulgaris*- or bovine- and liver-derived β -GAL with the same serum samples did not significantly decrease enzyme activity of the supernatants. Unchanged activity levels of

other glycohydrolases, such as α -mannosidase or β -hexosaminidase (data not shown), confirm the specificity of β -GAL immunoprecipitation. There were no appreciable changes in the enzymatic activity of the supernatants of samples incubated with normal control sera.

SDS-PAGE analysis of cypress and olive β -GAL fractions performed after enzyme immunoprecipitation with pooled patient sera showed 50- and 46-kDa proteic bands from cypress extract and 46 kDa from olive extract disappeared (fig 2c).

ELISA Detection of Specific IgE to β -GAL

Figure 3a illustrates the results of the ELISA assay for specific IgE detection for all cypress extracts. No reactivity was detected when crude cypress extract was matched with normal sera. Mean optical density for all patient samples was significantly higher for both crude or β -GAL-enriched extracts (p < 0.001 with respect to normal controls). After immunoprecipitation the same β -GAL-depleted fractions demonstrated a significant reduction in their IgE reactivity.

Since IgE from cypress- and olive-sensitive subjects binds a 46-kDa proteic fraction present on both β -GAL extracts, we looked for cross-reactivity among patients' sera adopting the same ELISA assay. Figure 3b shows that preincubation of sera from cypress- and olive-sensitive subjects with, respectively, *Olea*- or cypress-derived β -GAL cross-inhibited specific IgE reactivity and that a >50% inhibition of positive results occurred with purified enzyme fractions ranging from 10 to 100 nU/ml.

Allergen-Specific T Cell Lines and Clones

T cell reactivity was tested against undenatured whole cypress extract and β -GAL from the tree pollens, as well as from *E. coli*, *F. vulgaris* and bovine liver. Heat-inacti-

vated (50°C for 1 h) enzymes from all sources were used as a control of antigenic specificity [28]. In healthy control subjects there was no appreciable response to in vitro stimulation with either whole cypress extract or enriched β -GAL (data not shown). Figure 4a shows the proliferative response (cpm $\pm$ SD of incorporated ^3H -thymidine in experiments performed in triplicate) of all growing T cell lines from cypress-allergic subjects before cloning. Responding T cell lines were selected on the basis of their ^3H -thymidine incorporation following in vitro stimulation with β -GAL from cypress extract, as compared with the same cultures exposed to an irrelevant antigen (tetanus toxoid, 5 $\mu\text{g}/\text{ml}$). Heat-inactivated β -GAL gave comparable results in terms of ^3H -thymidine incorporation, as the active enzyme, thus ruling out the possibility of a direct functional effect of the enzymatic protein, not mediated by an MHC-restricted peptide presentation pathway. The two (LC12C and LC8B) with greatest in vitro specific reactivity (with respect to cpm from control cultures) were then cloned.

Among all the emerged clones (34 of CD4, 5 of CD8 and 2 of $\gamma\delta$ phenotype), 18 CD4+ and 2 CD8+ demonstrated a significant in vitro proliferation against both cypress- and olive pollen-derived β -GAL. They showed once again a slightly lower ^3H -thymidine incorporation when tested against heat-inactivated enzymes (data not shown). Another 16 CD4+, 3 CD8+ and 2 $\gamma\delta$ TcR+ T cell clones reacted specifically only with cypress-derived β -GAL. However, no clones reacted with other purified enzymes (fig. 4b). Functionally all CD4+ β -GAL-specific T cell clones were of the Th0 phenotype, as confirmed by intracellular cytokine staining with monoclonal antibodies against human IL-4, IL-5 and IFN- γ (fig. 4c). Indeed, the net cytokine secretion pattern (obtained by subtracting the pg/ml secreted by samples containing only APC plus antigen from those also containing T cells) of the 18 CD4+ T cell clones that reacted with β -GAL from both cypress and olive pollen extracts was confirmed by in vitro IL-4 and IFN- γ production (fig. 4c) after stimulation with the corresponding purified enzyme.

In vivo Testing of Cypress-Derived β -GAL Activity

Pollen-derived β -GAL fractions were tested in vivo for their ability to induce an immediate cutaneous reaction. Extracts derived from *E. coli*, *F. vulgaris* and bovine liver were also employed at the same proteic concentrations as pollen β -GAL (0.5 $\mu\text{g}/\text{ml}$). Table 3 shows the means of the wheal and flare reactions to each purified extract. The two pollen-derived β -GAL demonstrated a full reactivity when cross-tested in cypress- or *Olea*-sensitive patients.

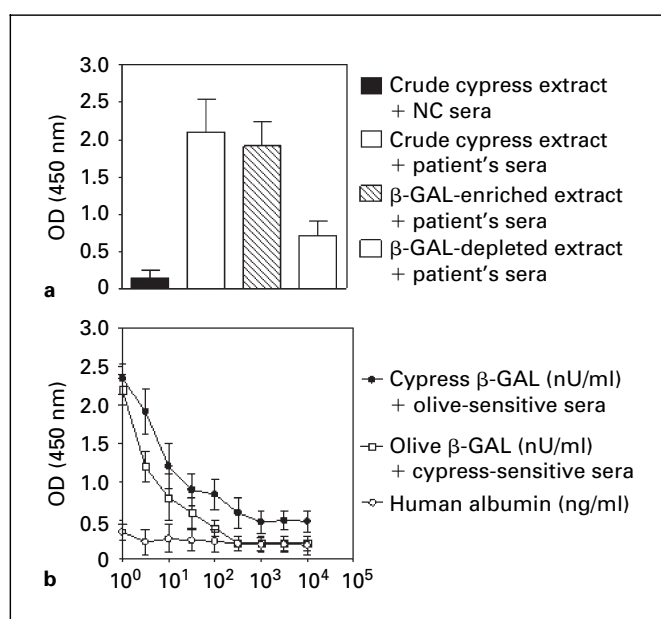


Fig. 3. **a** ELISA detection of specific IgE to different cypress extracts. Crude cypress extract and DEAE-cellulose fractions before (β -GAL enriched) and after immunoprecipitation (β -GAL depleted) were incubated with pooled sera of 15 cypress-sensitive patients and 15 control subjects (NC). The reaction was developed with an alkaline phosphatase-conjugated anti-human IgE antibody (Sigma). Mean optical densities (OD) $\pm$ SD of bound circulating IgE are reported. **b** ELISA cross-inhibition with cypress or *Olea*-derived β -GAL. Variable amounts of cypress-derived peak II extract were preincubated with pooled *Olea*-sensitive sera ($n = 5$), whereas *Olea*-derived β -GAL was preincubated with pooled cypress-sensitive sera ($n = 15$), all diluted 1:2.5 in PBS-Tween 1%. Human serum albumin served as control. Mean optical densities $\pm$ SD of bound circulating IgE to specific allergen after cross-adsorption are reported.

Among other enzyme sources employed, only *F. vulgaris*-derived β -GAL demonstrated a minimal cutaneous wheal reaction in tree-sensitive subjects, which however did not significantly differ from that of controls. *E. coli*- and bovine-derived enzymes always gave negative results.

Discussion

In the present study we identified unexpectedly high levels of β -GAL activity in crude extracts of pollens from *C. arizonica*, *C. sempervirens* and *O. europea* species. β -GAL was partially purified with ammonium sulfate precipitation and DEAE-cellulose chromatography. Interestingly, it possessed some typical allergenic properties: the ability to bind circulating specific IgE, in vitro induction

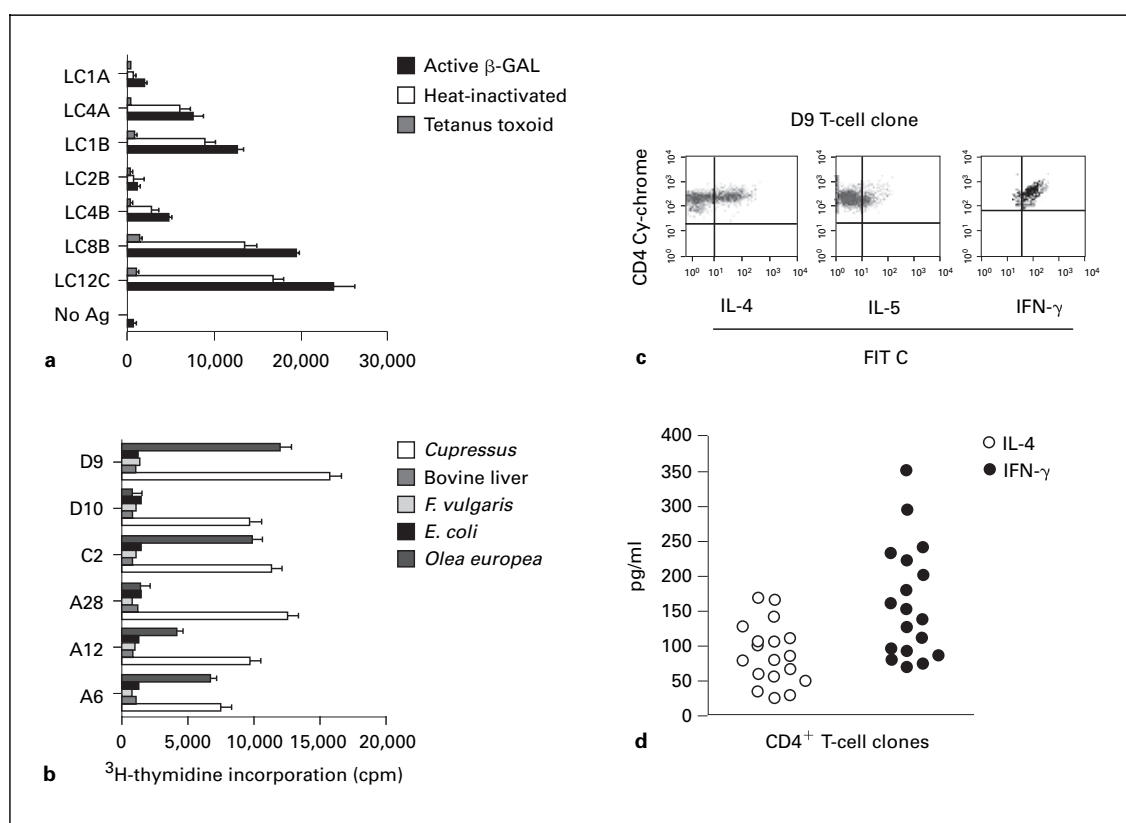


Fig. 4. Functional characteristics of β -GAL-specific T cells. **a** ^3H -thymidine incorporation (mean cpm $\pm$ SD) of T cell lines derived from 4 different cypress-sensitive subjects. Autologous irradiated non-T cells were employed as APC. All experiments were performed with active enzyme, heat-inactivated cypress-derived β -GAL or tetanus toxoid as control antigen. A 5- to 10-fold increase (above the basal values) in mean cpm after stimulation with cypress-derived enriched β -GAL was considered a reliable index of antigen specificity of responding cells. **b** In vitro proliferative response of six representative CD4 $^{+}$ T cell clones to β -GAL from *E. coli*, *F. vulgaris*, bovine

liver, *C. arizonica* and *O. europea* extracts. **c** A representative Th0 cytokine secretion pattern of a CD4 $^{+}$ T cell clone, as evidenced by intracellular staining with FITC-conjugated mAb directed toward human IL-4, IL-5 and IFN- γ . **d** Net cytokine production pattern of all 18 (β -GAL cross-reactive) CD4 $^{+}$ T cell clones. Supernatants were collected after 36 h of antigen stimulation and assayed for IL-4 and IFN- γ production. Net values (pg/ml) are calculated by subtracting cytokine levels derived from samples with APC alone plus antigen from those containing also β -GAL-specific T cells.

Table 3. Cutaneous skin prick tests with enriched β -GAL

Enzyme source	Cypress allergic (15 patients)	Olive allergic (5 patients)	Control atopic (20 patients)	Normal controls (15 patients)
<i>C. arizonica</i>	15.3 $\pm$ 3.1**	9.1 $\pm$ 2.5*	3.7 $\pm$ 1.5	2.9 $\pm$ 0.6
<i>O. europea</i>	8.4 $\pm$ 1.6*	14.8 $\pm$ 4.1**	2.9 $\pm$ 0.8	3.1 $\pm$ 1.1
<i>F. vulgaris</i>	5.4 $\pm$ 2.4	6.2 $\pm$ 2.8	3.0 $\pm$ 1.8	2.2 $\pm$ 0.9
<i>E. coli</i>	3.5 $\pm$ 1.6	2.7 $\pm$ 0.8	3.4 $\pm$ 1.2	3.2 $\pm$ 1.1
Bovine liver	2.8 $\pm$ 0.9	2.9 $\pm$ 0.6	3.1 $\pm$ 1.4	2.5 $\pm$ 0.5

Tree-allergic subjects (n = 20) were considered as monosensitive on the basis of the concordance of clinical symptoms with pollen calendars in our county. Values represent (in mm $\pm$ SD) mean diameters of cutaneous horizontal and vertical wheal and flare reactions. * p < 0.05; ** p < 0.005 with respect to controls.

of β -GAL-specific T cell clones from allergic subjects, and full IgE cross-reactivity. These properties were not observed in purified enzymes from other vegetal or mammalian species. In addition, in vivo skin prick testing demonstrated that only pollen-derived β -GAL induced wheal and flare reactions in sensitized subjects.

β -GAL (EC 3.2.1.23) catalyzes the hydrolysis of terminal β -galactoside linkage in ganglioside GM1, other carbohydrate-containing compounds such as glycolipids, glycoproteins and glycosaminoglycans, and several artificial substrates. Lysosomal β -GAL has been purified to apparent homogeneity from different sources and species [32]. In mammalian tissues most of the active enzyme is present in a high-molecular-weight aggregate, and only a small fraction is found as 64-kDa monomer polypeptide.

In pollen-derived β -GAL from both cypress and olive trees, the IgE-binding site is the 46-kDa peptide, which seems to possess cross-reactive properties. It could be immunoprecipitated by sera of sensitive subjects. Cypress-derived β -GAL showed an additional 50-kDa fraction that is not recognized by specific IgE of sensitive subjects, but can be coimmunoprecipitated together with the 46-kDa peptide. Therefore, it seems advisable to assume that these two fractions functionally aggregate in vivo in an higher molecular enzyme complex.

β -GAL has been isolated and cloned from apple and tomato where its surface location at the onset of ripening determined its definition as exo-(1 $\rightarrow$ 4)- β -D-galactanase. In a recent classification, class II–IV β -glucanases are extracellular acidic proteins without the C-terminal glycosylated extension, which are related to developmental and physiological processes in healthy plants [33]. In particular, the tomato-derived enzyme has the most active fraction within a protein band at 75 kDa on SDS-PAGE [34], whereas the apple-derived β -GAL shows many active fractions in the polypeptides of 32, 44 and 78.5 kDa [35]. Recently, Ole e 9 allergen from *O. europea* pollen has been shown to possess β -1,3-glucanase activity with a molecular mass of 46.4 kDa [36]. Further examples of β -GAL activity are those described in Brassica [37] and tobacco [38] pollens, in which the enzyme seems to be involved in pollen tube growth and/or fertilization.

Several other enzyme activities in pollen extracts from Taxodiaceae and Cupressaceae have been described. However, they were assumed on the basis of homology between cloned peptide fragments from the pollen extract and some pectate lyase and polymethylgalacturonase isoenzymes secreted by plant pathogenic bacteria [39]. Their role in inducing pollen allergy is only suspected for Jun a 1 [40], the major allergen from *Juniperus ashei* and

for Cup a 1 [41], a 37-kDa protein from *C. arizonica*, both with pectate lyase activity. Moreover, the 43-kDa fraction of *Platanus acerifolia* has been identified to possess polygalacturonase activity and some cross-reactivity with Cupressaceae pollen allergens [42]. Although only few experiments have been designed to directly investigate the allergenic potential of a previously purified glycohydrolase protein from tree pollens, it could be noted that this class of hydrolytic enzymes is particularly expressed both in plant pollens and foods, with which they share significant homology in amino-acidic sequences [42]. Thus, the coexistence of different enzymatic activities (e.g. pectate lyase, polymethylgalacturonase and glycohydrolase) at the surface of pollen grains from Mediterranean trees would influence their recognition and processing as allergens, given that mucosal surfaces are the primary sites of pollen exposure.

Our study confirmed that the enzyme protein complex was localized on the surface of cell wall, as in other pollens [32], fruits and vegetables. Furthermore, pollen-derived polypeptides from enriched β -GAL which bind circulating IgE from allergic subjects seem to be similar to active peptides from apple- or from tomato-derived β -GAL. Since this glycoprotein has a broad spectrum of activity stimulating the hydrolyses of glycolipids, regardless of glycosphingolipids or nonceramide glycolipids [43], the enzyme activity may facilitate the cell wall rupture during the pollens' reproductive season and penetration into mucosal epithelial barriers. Once penetrated, the pollen membrane with the active enzyme, as well as other protein, glycoprotein or lipid complexes interact with mucosal APC, thus initiating the local allergic immune response. Indirect evidence for this mechanism is the in vitro generation of β -GAL-specific T cell lines and clones after an immunization protocol adopting autologous dendritic cells as APC. Cross-reactivity of most T cell clones to enriched cypress- and olive-derived enzyme seems to be specific, since it was not observed with other vegetal or mammalian enzymes. Moreover, most reactive clones display the CD4⁺ phenotype with a Th0 subtype pattern of cytokine production, which is common to several allergen-specific T cell clones [44].

The fact that one of the major allergens in Mediterranean tree pollens is an enzyme may also explain the loss of biological activity [30, 45] in starved pollen grains, a finding we previously described for commercial cypress vaccine preparations [46]. This may have profound clinical implications in the management of the allergic patient. Most if not all pharmaceutical companies are supplied with pollen extracts collected, and probably stored for a

long time, by foreign manufacturers. Then, simplified extractive procedures for cost-restraining policies together with long-term storage of pollens may affect their biological characteristics, thus compromising their diagnostic accuracy and, perhaps, therapeutic efficacy.

Finally, the ubiquity of enzymes with hydrolase activity in higher plants suggests they could be panallergens that induce cross-reactivity between different vegetable sources (especially between pollens and fruits). They could be distinct from pathogenesis-related proteins [47], which are usually triggered by environmental factors. Cross-reactivity among whole olive pollen extracts and those from unrelated pollens, such as cypress, has been described [20]. Our results indicate that the β -GAL enzyme purified from both pollens contains proteic subunits that share common IgE-binding epitopes. However, because of the different isoenzymatic pattern, cross-reactivity did not always indicate coexisting clinical sensitization. In fact, although all subjects were positive to cutaneous skin prick tests with purified β -GAL obtained from both pollen sources, only one third reported defined seasonal respiratory symptoms (15/30 in winter and 5/30 in spring)

presumably related to only one plant-related clinical allergy. On the other hand, not all the cypress-specific T cell clones we tested in vitro cross-reacted with olive-derived β -GAL. Thus, the antigenic properties of diverse isoenzymes could substantially differ in their ability to either stimulate T cell reactivity or to bind specific IgE. Purified enzyme functions as complete antigen in stimulating the in vitro reactivity of T cell lines and clones. However, while the IgE reactivity is apparently restricted to the 46-kDa fraction in both cypress and olive extracts, an IgE unreactive 50-kDa fraction could be coimmunoprecipitated in β -GAL preparations from cypress pollen. This would suggest that conformational characteristics of undenatured cypress antigen may drive different humoral and cellular immune responses. Indeed, as opposed to that observed in circulating IgE reactivity, T cell clones generated from cypress-sensitive subjects were not fully cross-reactive against *Olea*-derived β -GAL. For these reasons, studies on the N-terminal sequence of pollen-derived β -GAL and cloning of the T cell receptor of β -GAL-specific T cells are now in progress in our laboratory.

References

- 1 European Community Respiratory Health Survey: Variations in the prevalence of respiratory symptoms, self-reported asthma attacks and use of asthma medication in the European Community Respiratory Health Survey (ECRHS). *Eur Respir J* 1998;9:887–895.
- 2 Ariano R, Panzani R, Chiappella M, Augeri G: Pollinosis in a Mediterranean area (Riviera Ligure, Italy): Ten years of pollen counts, correlation with clinical sensitization and meteorological data. *J Invest Allergol Clin Immunol* 1994;4:81–86.
- 3 Panzani R: L'allergie respiratoire aux pollens de conifères. *Rev Fr Allergol* 1962;2:164–170.
- 4 Mari A, Di Felice G, Afferni C, Barletta B, Tinghino R, Pini C: Cypress allergy: An underestimated pollinosis. *Allergy* 1997;52:355–356.
- 5 Agea E, Bistoni O, Russano A, Corazzi L, Minelli L, Bassotti G, de Benedictis FM, Bertotto A, Spinozzi F: The biology of Cupressaceae allergy. *Allergy* 2002;57:959–960.
- 6 Fiorina A, Scordamaglia A, Guerra L, Canonica GW, Passalacqua G: Prevalence of allergy to cypress. *Allergy* 2002;57:861–862.
- 7 Panzani RC, Mercier P, Delord Y, Riva G, Falagiani P, Revirion D, Auquier P: Prevalence of patent and latent atopy among a general normal adult population in the south east of France by RAST investigation and correlation with circulating total IgE levels. *Allergol Immunopathol* 1993;21:211–219.
- 8 Sorlano J, Antò JM, Sunyer J, et al: Risk of asthma in the general Spanish population attributable to specific immunoresponse. *Int J Epidemiol* 1999;20:728–734.
- 9 Corsico R, Falagiani P, Ariano R, et al: An epidemiological survey on the allergological importance of some emerging pollens in Italy. *Invest Allergol Clin Immunol* 2000;10:155–161.
- 10 Florido JF, Delgado PG, de San Pedro BS, Quirarte J, de Saavedra JM, Peralta V, Valenzuela LR: High levels of *Olea europea* pollen and relation with clinical findings. *Int Arch Allergy Immunol* 1999;119:133–137.
- 11 Suphioglu C, Singh MB, Taylor P, Bellomo R, Holmes P, Puy R, Knox RB: Mechanism of grass-pollen-induced asthma. *Lancet* 1992;339:569–572.
- 12 Currie AJ, Stewart GA, McWilliam AS: Alveolar macrophages bind and phagocytose allergen-containing pollen starch granules via C-type lectin and integrin receptors: Implications for airway inflammatory disease. *J Immunol* 2000;164:3878–3886.
- 13 Bousquet J, Knani J, Hejjoui A, Ferrando R, Cour P, Dhivert H, Michel FB: Heterogeneity of atopy. I. Clinical and immunologic characteristics of patients allergic to cypress pollen. *Allergy* 1993;48:183–188.
- 14 Cimignoli E, Broccucci L, Cernetti C, Gerli R, Spinozzi F: Isolation and partial characterization of *Cupressus sempervirens* allergens. *Aerobiologia* 1992;8:465–470.
- 15 Di Felice G, Caiaffa MF, Bariletto G, Afferni C, Di Paolo R, Mari A, et al: Allergens of Arizona cypress (*Cupressus arizonica*) pollen: Characterization of the pollen extract and identification of the allergenic components. *J Allergy Clin Immunol* 1994;94:547–555.
- 16 Spinozzi F, Cimignoli E, Cernetti C, Broccucci L, Bistoni O, Gerli R, Grignani F: Immunochemical analysis of Cupressaceae allergens. *Clin Exp Allergy* 1990;20(suppl 1):66.
- 17 Lauzurica P, Gurbindo C, Maturi N, et al: Olive (*Olea europea*) pollen allergens. II. Isolation and characterization of two major allergens. *Mol Immunol* 1988;25:337–344.
- 18 Afferni C, Iacovacci P, Barletta B, Di Felice G, Tinghino R, Mari A, Pini C: Role of carbohydrate moieties in IgE binding to allergenic components of *Cupressus arizonica* pollen extract. *Clin Exp Allergy* 1999;29:1087–1094.
- 19 Shakib F, Furmonaviciene R: The significance of enzymic and other biological activities of proteins in relation to their capacity to serve as allergens. *Clin Exp Allergy* 2000;30:1056–1057.
- 20 Gonzalez EM, Villalba M, Rodriguez R: Allergic cross-reactivity of olive pollen. *Allergy* 2000;55:658–663.

- 21 Hadfield KA, Bennet AB: Polygalacturonase: Many genes in search of a function. *Plant Physiol* 1998;117:337–343.
- 22 Mori T, Yokoyama M, Komiyama N, Okano M, Kino K: Purification, identification, and cDNA cloning of Cha o 2, the second major allergen of Japanese cypress pollen. *Biochem Biophys Res Commun* 1999;263/1:166–171.
- 23 Emiliani C, Falzetti F, Orlacchio A, Stirling JL: Treatment of HL60 cells with dimethyl sulphoxide inhibits the formation of L-N-acetyl-hexosaminidase S. *Biochem J* 1990;272:211–215.
- 24 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–254.
- 25 Hayashi M: Comments on the cytochemical techniques using naphthol AS-BI substrates and hexazonium pararosanilin for the demonstration of acid phosphatase, beta-glucuronidase and N-acetyl-beta-glucosaminidase activities. *J Histochem Cytochem* 1977;25:1021–1023.
- 26 Norden AG, Tennant LL, O'Brien JS: GM1 ganglioside beta-galactosidase. A. Purification and studies of the enzyme from human liver. *J Biol Chem* 1974;249:7969–7976.
- 27 Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970;227:680–685.
- 28 Towbin H, Stehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4354.
- 29 Spinozzi F, Agea E, Piattoni S, Bistoni O, Grignani F, Bertotto A: Co-stimulation-induced release of pro-inflammatory cytokine interleukin-8 by allergen-specific T cells. *Scand J Immunol* 1996;44:80–84.
- 30 Nguyen VT, Morange M, Bensaude O: Protein denaturation during heat shock and related stress. *Escherichia coli* β -galactosidase and *Photinus pyralis* luciferase inactivation in mouse cells. *J Biol Chem* 1989;264:10487–10492.
- 31 Jung T, Schauer U, Heusser C, Neumann C, Rieger C: Detection of intracellular cytokines by flow cytometry. *J Immunol Methods* 1993; 159:197–207.
- 32 Conzelmann E, Sandhoff K: Glycolipid and glycoprotein degradation. *Adv Enzymol Relat Areas Mol Biol* 1987;60:89–216.
- 33 Leubner-Metzger G, Meins F Jr: Pathogenesis-Related Proteins in Plants. Boca Raton, CRC Press, 1999, pp 49–75.
- 34 Smith DL, Starret DA, Gross KC: A gene encoding for tomato fruit β -galactosidase II is expressed during fruit ripening. *Plant Physiol* 1998;117:417–423.
- 35 Ross GS, Wegrzyn T, MacRae EA, Redgwell RJ: Apple beta-galactosidase activity against cell wall polysaccharides and characterization of a related cDNA clone. *Plant Physiol* 1994; 106:521–528.
- 36 Huecas S, Villalba M, Rodriguez R: Ole e 9, a major olive pollen allergen is a β -1,3-glucanase. Isolation, characterization, amino acid sequence and tissue specificity. *J Biol Chem* 2001;276:27959–27964.
- 37 Singh MB, Knox RB: Gene controlling β -galactosidase deficiency in pollen of oil seed rape. *J Hered* 1985;76:199–201.
- 38 Rogers HJ, Maund SL, Johnson LH: A β -galactosidase-like gene is expressed during tobacco pollen development. *J Exp Bot* 2001;52:67–75.
- 39 Di Felice G, Barletta B, Tinghino R, Pini C: Cupressaceae pollinosis: Identification, purification and cloning of relevant allergens. *Int Arch Allergy Immunol* 2001;125:280–289.
- 40 Midoro-Horiuti T, Goldblum RM, Kurosky A, Wood TG, Schein CH, Brooks EG: Molecular cloning of the mountain cedar (*Juniperus ashei*) pollen major allergen Jun a 1. *J Allergy Clin Immunol* 1999;104:613–617.
- 41 Aceituno E, Del Ponzo V, Minguez A, Arrieta I, Cortegano I, Cardaba B, Gallardo S, Rojo M, Palomino P, Lahoz C: Molecular cloning of major allergen from *Cupressus arizonica* pollen: Cup a 1. *Clin Exp Allergy* 2000;30:1750–1758.
- 42 Ibarrola I, Arilla C, Martinez C, Asturias JA: Identification of a polygalacturonase as a major allergen (Pla a 2) from *Platanus acerifolia* pollen. *J Allergy Clin Immunol* 2004;113:1185–1191.
- 43 Li SC, Sonnino S, Tettamanti G, Li YT: Characterization of a nonspecific activator protein for the enzymatic hydrolysis of glycolipids. *J Biol Chem* 1988;263:6588–6591.
- 44 Constant SL, Bottomly R: Induction of the Th1 and Th2 CD4+ T cell responses: Alternative approaches. *Annu Rev Immunol* 1997;15:297–322.
- 45 Goi G, Fabi A, Lombardo A, Burlina AB, Tiby V, Visciani A, Malesani L, Tettamanti G: Stability of enzymes of lysosomal origin in human cerebrospinal fluid. *Clin Chim Acta* 1987;163: 215–224.
- 46 Spinozzi F, Cimignoli E, Grignani F: Immunotherapy: What are we administering to the allergic patient? *Lancet* 1993;341:386.
- 47 Midoro-Horiuti T, Brooks EG, Goldblum RM: Pathogenesis-related proteins of plant allergens. *Ann Allergy Asthma Immunol* 2001;87: 261–271.