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Localisation of a carbohydrate epitope recognised by human IgE in pollen of Cupressaceae

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Abstract The objectives of the present study were: (1) to localise, at the subcellular level, the allergens in pollen of Cupressaceae species, using a monoclonal antibody (mAb 5E6) that is specific for carbohydrate epitopes of allergenic components of *Cupressus arizonica* pollen extract; (2) to determine whether the glycidic epitope recognised by mAb 5E6 was present in pollen of allergenic species taxonomically unrelated to Cupressaceae; and (3) to determine whether human IgE purified from monosensitive patients recognises the same epitope as mAb 5E6 in Cupressaceae pollen. Immunogold labelling of mAb 5E6 showed a high density of gold particles on the orbicules, supporting the hypothesis that they are important vectors of allergens. A high density was also found on the exine and in the cytoplasm, with the latter finding confirming that fragments of pollen ruptured under humid conditions can represent a vector. The glycidic epitope recognised by mAb 5E6 was detected in all of the species taxonomically unrelated to Cupressaceae, although with varying density. Human IgE recognised the same epitope as mAb 5E6. These findings are consistent with observations of diffuse allergenic cross-reactivity among various allergens. The in situ localisation of a common epitope recognised by both a monoclonal antibody and human IgE could be of importance in immunotherapy.

Key words Carbohydrate epitope · Cupressaceae · Freeze substitution · Immunogold labelling · Pollen allergens

Introduction

Cupressaceae pollen is an important cause of respiratory allergic reactions (Bousquet et al. 1993), constituting an extensive health problem in many areas of the world, given that the various species of this family release a great amount of pollen during anthesis (Panzani et al. 1986; Bousquet et al. 1993; Grilli Caiola et al. 2002). For example, in Japan, *Cryptomeria japonica* is responsible for Japanese cedar pollinosis, which has become a serious public-health concern (Miki-Hirosige et al. 1994). In the Mediterranean, the most common species of Cupressaceae are those belonging to *Cupressus* and *Juniperus*, which are characteristic of Mediterranean forests. In this area, the use of *Cupressus sempervirens* and *C. arizonica* for ornamental purposes (Krüssman 1995) and as wind and noise barriers has become an increasingly common practice, which, together with air pollution, has resulted in increasing attention being focussed on allergic reactions (Knox and Suphioglu 1996). A recent epidemiological study conducted in the area of Rome, Italy, reported that the concentrations of Cupressaceae and Taxaceae pollen were high and that 15% of the study population was allergic to this pollen (Grilli Caiola et al. 2002).

Pollen extracts from the species *Cupressus sempervirens* and *C. arizonica* have been shown to be low in protein content and high in carbohydrate content (Di Felice et al. 1994; Barletta et al. 1996). This is consistent with the findings of ultrastructural studies on *C. sempervirens* (Bortenschlager 1990; Milanese and Cresti 1997), which have shown that the pollen of this species has a thin exine with numerous orbicules, a thick intine, and a cytoplasm with a great number of callose-containing vesicles and osmophilic bodies (Grilli Caiola et al. 2000).

In *Cupressus arizonica*, the major allergen is Cup a 1, a glycoprotein of 43 kDa (Alisi et al. 2001), and this allergen has been shown to have a high degree of sequence identity with allergens of other conifers (Açetuno et al. 2000). The presence and nature of cross-reacting epitopes shared by various components of *C. arizonica* pollen extract have also

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been evaluated, and monoclonal antibodies specific for carbohydrate epitopes (mAb 5E6) and for proteinic epitopes (mAb 4A6), both of which are induced by immunisation with *C. arizonica* pollen extract, have been produced (Barletta et al. 1998). It has also been shown that carbohydrate epitopes of allergenic components of *C. arizonica* pollen extract are involved in the binding of IgE from persons who are allergic to Cupressaceae (Barletta et al. 1998; Afferni et al. 1999; Iacovacci et al. 2001, 2002).

In general, if pollen were to remain whole, as occurs in dry conditions, it would be too large to be inhaled and the allergens would only be able to induce an allergic reaction in the upper respiratory tract (Ong et al. 1995). However, conditions of high relative air humidity, such as those normally found on the mucosal surface of the upper respiratory tract, cause pollen to rupture, with the consequent release of micronic fragments, which can be inhaled and thus cause allergic reactions (Ong et al. 1995). In *Cupressus sempervirens*, over 50% of pollen grains have been shown to rupture in water (Grilli Caiola et al. 2000).

In the present study, we localised, at the subcellular level, the carbohydrate epitope of pollen of *Cupressus arizonica*, *C. sempervirens* f. *sempervirens* and f. *horizontalis*, and *Juniperus* sp., using mAb 5E6 previously characterised by Barletta et al. (1998). We also determined whether the glycidic epitope recognised by mAb 5E6 was present in pollen of other allergenic species that are not taxonomically related to Cupressaceae. Finally, we localised human IgE purified from monosensitive patients in pollen of *C. sempervirens* and demonstrated at the subcellular level that the IgE recognised the same epitope as mAb 5E6.

Materials and methods

Pollen

Mature pollen was collected at anthesis from cones of *Cupressus arizonica* Greene, *C. sempervirens* L. f. *sempervirens* and f. *horizontalis* (Mill.) Voss., *Juniperus* sp. and from anthers of the angiosperms *Ailanthus altissima* (Miller) Swingle, *Brassica nigra* Koch, *Chamaerops humilis* L., *Parietaria judaica* Auct., *Olea europaea* L., *Platanus acerifolia* L., *Artemisia vulgaris* L., *Robinia pseudoacacia* L., *Eucalyptus* sp., *Dactylis glomerata* L., and *Corylus avellana* L.

Monoclonal antibodies

The monoclonal antibodies mAb 5E6 and mAb 4A6, which recognise, respectively, a periodate-sensitive epitope and a periodate-resistant epitope of *Cupressus arizonica* pollen extracts, were prepared in accordance with the methodology of Barletta et al. (1998). Pollen (10 g) collected from the cones was extracted in 160 ml of 125 mM NH_4HCO_3 at

4°C for 12 h with slow stirring followed by centrifugation (13,000 g for 1 h at 4°C), filtering, and dialysis.

IgE serum fractions

Human serum was obtained from one monosensitive patient who was allergic to *Cupressus* pollen, as demonstrated by clinical history, skin-prick test, and the in vitro detection of specific IgE [CAP System (Pharmacia Upjohn, Uppsala, Sweden)]. The serum was separated into an IgE (IgG-depleted fraction) fraction and an IgG (IgE-depleted fraction) fraction by protein G affinity column [Protein G Sepharose 4 Fast Flow (Sigma)], as reported in Iacovacci et al. (2001). IgE reactivity was evaluated on enzyme-linked immunosorbent assay (ELISA) plate-adsorbed antigens, as described by Iacovacci et al. (2001). The best results were obtained with the 1:10 dilution of the IgE fraction; thus this dilution was used for immunogold labelling.

Immunogold electron microscopy

For all species, pollen grains were fixed in 3% paraformaldehyde and 0.5% glutaraldehyde in 0.2 M phosphate buffer, pH 7.2, for 2 h; they were then dehydrated in ethanol and embedded in Epon 812, according to Grilli Caiola et al. (1993). Immunogold labelling was carried out on ultrathin sections, according to Canini et al. (1992). Grids were first incubated with 5% BSA and then with one of the two primary antibodies (mAb 5E6 or mAb 4A6 at 1:100 dilution); they were then incubated with a secondary antibody (goat anti-mouse conjugated with 10-nm gold particles at 1:20 dilution). Other grids, after being incubated with 5% BSA, were incubated overnight with the IgE fraction (at 1:10 dilution), then for 1 h with a secondary antibody (rabbit anti-human IgE at 1:20 dilution), and finally with a tertiary antibody (goat anti-rabbit conjugated with 10-nm gold particles at 1:20 dilution). Controls were performed using pre-immune sera (at 1:100 dilution) instead of mAb 5E6 or mAb 4A6.

Pollen of *Cupressus arizonica* and *C. sempervirens* f. *sempervirens*, in addition to the above experiments, was also subjected to rapid freeze substitution using an electron microscopy freeze substitution (EM FS) apparatus (Leica), following the manufacturer's instructions, and then to immunogold labelling with mAb 5E6. In brief, pollen was first cryoprotected with 15% sucrose and then rapidly frozen using a slammer [Reichert EMM80 (Leica)] loaded with liquid nitrogen. The frozen pollen was then inserted into the EM FS apparatus, programmed at -90°C, and stained with 0.5% uranyl acetate in methanol. The temperature was progressively increased from -90°C to -50°C, and the uranyl acetate was substituted with absolute methanol. The specimens were then embedded in HM20 resin at -50°C. Polymerisation was carried out by exposure to a UV lamp for 24 h.

Table 1 Immunogold labelling by monoclonal antibody (mAb) 5E6 in Cupressaceae: mean density of gold particles per μm^2 by pollen site and species

Species	Mean number ^a ($\pm$ standard deviation) of gold particles per μm^2 at each site			
	Orbicules	Exine	Intine	Cytoplasm
<i>Cupressus arizonica</i>	3 $\pm$ 0.2	3 $\pm$ 0.1	2 $\pm$ 0.1	4 $\pm$ 0.2
<i>C. sempervirens</i> f. <i>sempervirens</i>	15 $\pm$ 1.5	15 $\pm$ 1.3	1 $\pm$ 0.1	12 $\pm$ 1
<i>C. sempervirens</i> f. <i>sempervirens</i> ^b	13 $\pm$ 1.0	16 $\pm$ 0.5	2 $\pm$ 0.3	10 $\pm$ 2
<i>C. sempervirens</i> f. <i>horizontalis</i>	11 $\pm$ 1.0	13 $\pm$ 1.1	1 $\pm$ 0.1	25 $\pm$ 2.3
<i>Juniperus</i> sp.	4 $\pm$ 0.2	10 $\pm$ 0.9	1.5 $\pm$ 0.1	20 $\pm$ 0.0

^aMeasurements carried out on 15 different micrographs obtained using Epon-embedded pollen

^bMeasurements following the fixation of pollen by rapid freeze substitution

All of the observations were carried out with a Zeiss CEM 902 electron microscope at 80 kV. The density of gold particles was estimated based on the analysis of 10–20 micrographs for each sample.

Results

Localisation of mAb 5E6 and 4A6 in pollen of *Cupressus arizonica*, *C. sempervirens*, and *Juniperus* sp.

Table 1 shows the mean density of gold particles per μm^2 in Cupressaceae and *Juniperus* sp., by site and species, obtained with immunogold labelling of mAb 5E6. For *C. arizonica* pollen, gold particles indicating the presence of glycidic epitopes recognised by mAb 5E6 were detected on the orbicules (Fig. 1A), exine, and intine, and in the cytoplasm (Fig. 1B); for all four sites, the density was low. For pollen of *Cupressus sempervirens* f. *horizontalis* and f. *sempervirens*, the density on the orbicules and exine and in the cytoplasm was higher than that for *C. arizonica* (Fig. 1C, D). However, differently from *C. arizonica*, the density of labelling in the nucleus of *C. sempervirens* f. *sempervirens* pollen was high (data not shown). When comparing *C. sempervirens* f. *sempervirens* and f. *horizontalis*, the densities found for the orbicules, exine, intine and nucleus were similar. For *Juniperus* sp., gold particles were prevalently located on the orbicules and exine and in the cytoplasm (Fig. 2A, B).

The immunogold labelling of mAb 4A6 (data not shown) revealed a low density of gold particles in the cytoplasm and on the exine and intine of *Cupressus arizonica* and *C. sempervirens*. No particles were detected on the orbicules.

No gold particles were detected in the control specimens.

Following the fixation of pollen by rapid freeze-substitution, in *Cupressus sempervirens* f. *sempervirens*, the density of gold particles decreased in the nucleus (i.e., 3 particles per μm^2 compared to 25 particles per μm^2 obtained without rapid freeze-substitution) (Fig. 2C). On the orbicules (Fig. 2C) and exine and in the cytoplasm, the gold particles were more clearly defined than those revealed without rapid freeze-substitution, although the densities were similar. In the callose vesicles, no particles were detected by either of the methods.

Table 2 Immunogold labelling by mAb 5E6 in allergenic species not taxonomically related to Cupressaceae: mean density of gold particles per μm^2 by pollen site and species

Species	Mean number ^a ($\pm$ standard deviation) of gold particles per μm^2 at each site		
	Cell wall	Cytoplasm	Starch
<i>Ailanthus altissima</i>	7 $\pm$ 0.5	10 $\pm$ 1	–
<i>Brassica nigra</i>	8 $\pm$ 0.3	8 $\pm$ 0.5	4 $\pm$ 0.2
<i>Pinus pinea</i>	8 $\pm$ 1.0	7 $\pm$ 0.3	–
<i>Chamaerops humilis</i>	4 $\pm$ 0.2	6 $\pm$ 0.5	–
<i>Parietaria judaica</i>	4 $\pm$ 0.3	4.5 $\pm$ 0.6	2 $\pm$ 0.1
<i>Olea europaea</i>	3 $\pm$ 0.7	4.5 $\pm$ 0.4	–
<i>Platanus acerifolia</i>	2.7 $\pm$ 0.2	3.8 $\pm$ 0.5	–
<i>Artemisia vulgaris</i>	2.5 $\pm$ 0.1	3.5 $\pm$ 0.1	2.2 $\pm$ 0.2
<i>Robinia pseudoacacia</i>	2 $\pm$ 0.1	3.2 $\pm$ 0.2	–
<i>Eucalyptus</i> sp.	1 $\pm$ 0.1	3.2 $\pm$ 0.2	–
<i>Corylus avellana</i>	1 $\pm$ 0.2	3 $\pm$ 0.1	–

^aMeasurements carried out on 15 different micrographs obtained using Epon-embedded pollen

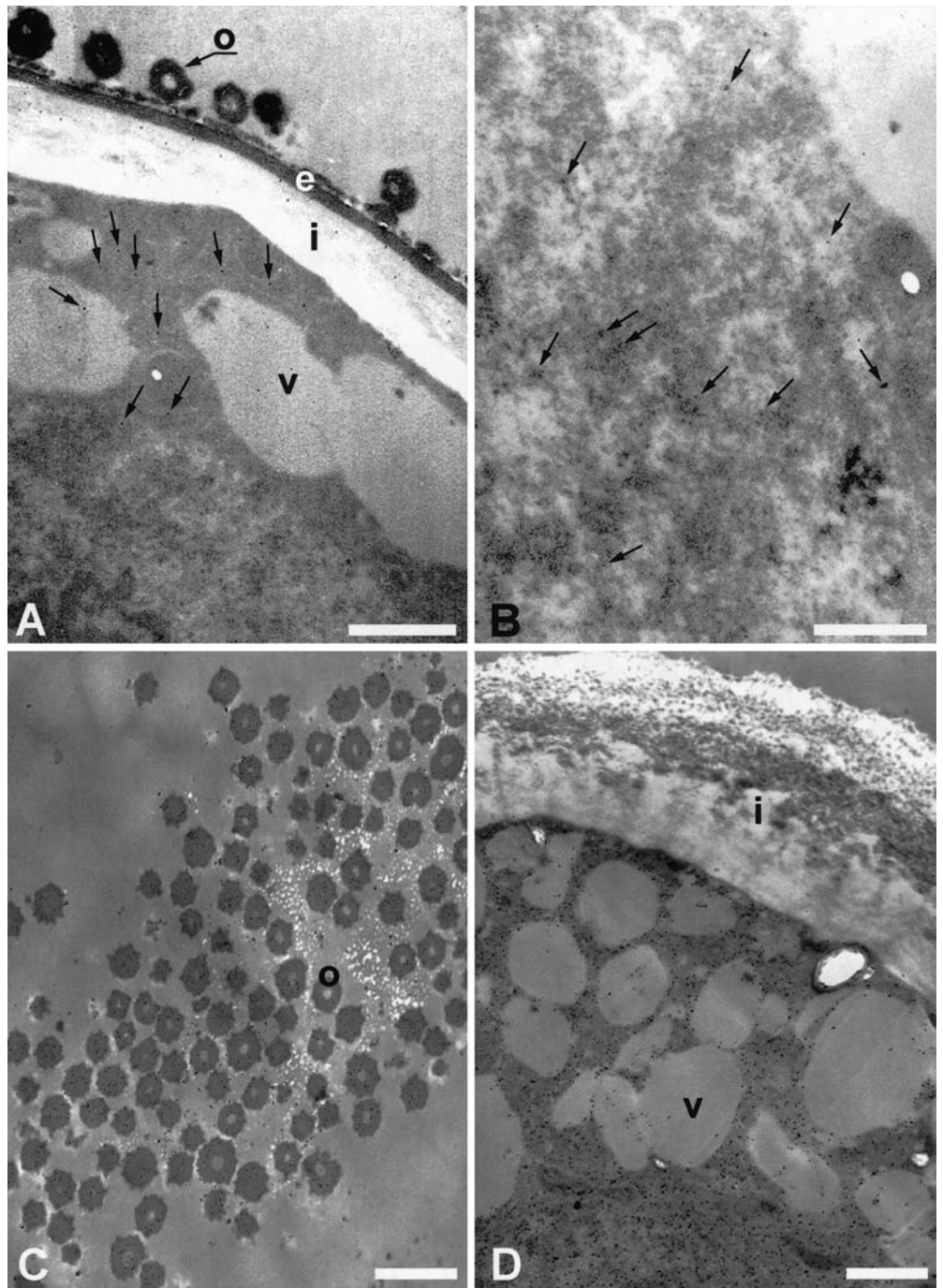
Immunogold labelling of mAb 5E6 for other allergenic plants

To determine, at the subcellular level, whether the glycidic epitope recognised by mAb 5E6 was present in pollen of other allergenic species taxonomically unrelated to Cupressaceae, immunogold labelling was performed on allergenic pollen previously investigated by ELISA (Iacovacci et al. 2001) (Table 2). The glycidic epitope was detected in all of the species examined, although with varying density. Specifically, the density was high on the cell wall and in the cytoplasm of the pollen of *Ailanthus altissima*, *Brassica nigra*, and *Pinus pinea*; it was intermediate in the pollen of *Chamaerops humilis*, *Parietaria judaica*, *Olea europaea*, *Platanus acerifolia*, and *Artemisia vulgaris* and very low in *Robinia pseudoacacia*, *Eucalyptus* sp., and *Corylus avellana*. Gold particles were also detected on starch granules of *B. nigra* (Fig. 3A, B), *P. judaica*, and *A. vulgaris*.

Immunogold labelling of human IgE purified fraction

The immunogold labelling using purified fraction of human IgE from monosensitive patients, performed for *Cupressus*

Fig. 1A–D Localisation of monoclonal antibody (mAb) 5E6 in *Cupressus* pollen. **A** *Cupressus arizonica*: gold particles (indicated by arrows) located on the orbicules (*o*), exine (*e*), intine (*i*), and in the cytoplasm and callose vesicle (*v*). Bar = 1 μ m. **B** Detail of cytoplasm of *C. arizonica* with gold particles (arrows). Bar = 0.5 μ m. **C** *C. sempervirens* f. *horizontalis*: gold particles on orbicules (*o*). Bar = 1 μ m. **D** *C. sempervirens* f. *horizontalis*: callose vesicles (*v*) and intine (*i*) with no gold particles. Bar = 1 μ m



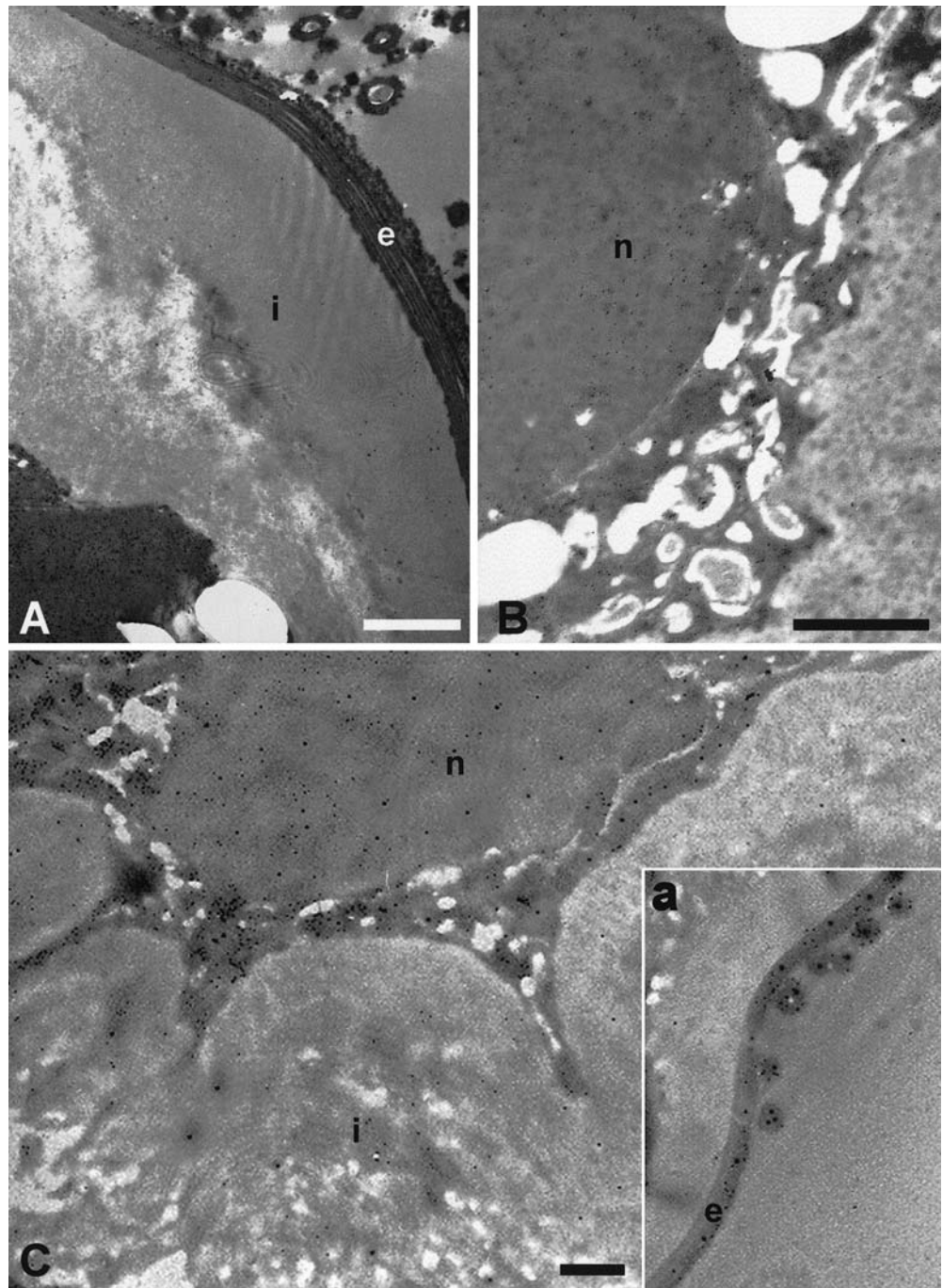
sempervirens pollen, revealed gold particles on the orbicules, exine, and intine (Fig. 4A) and in the cytoplasm (Fig. 4B). The pattern of labelling was similar to that obtained with mAb 5E6. To determine whether the IgE fraction recognised the same site as mAb 5E6, thus competing with the binding of mAb 5E6, ELISA was performed (Fig. 5). When extracts of *C. arizonica* were pre-incubated with mAb 5E6 before incubation with human IgE, the absorbance decreased to the value obtained with the control phosphate buffer. When pre-incubated with mAb 4A6, no significant variation with respect to human IgE alone was observed (Fig. 5).

When sections were pre-incubated with mAb 5E6 before incubation with the purified IgE fraction, there was a total lack of gold particles in the various pollen sites (Fig. 6).

Discussion

The results of this study show that the carbohydrate epitope recognised by mAb 5E6 is located in various pollen sites of *Cupressus arizonica*, *C. sempervirens*, and *Juniperus* and that the density of gold particles is high on the orbicules

Fig. 2A–C Localisation of mAb 5E6 in pollen. Bar = 1 μ m. **A** *Juniperus* sp.: gold particles located on the orbicules, exine (*e*), and intine (*i*). **B** *Juniperus* sp.: gold particles located in the cytoplasm and nucleus (*n*). **C** *Cupressus sempervirens* f. *sempervirens* subjected to rapid freeze-substitution before immunogold labelling: gold particles located in the nucleus (*n*) and on the intine (*i*); inset *a* detail of the exine (*e*) and orbicules



and exine and in the cytoplasm of *C. sempervirens* pollen. In *C. sempervirens* f. *sempervirens*, gold particles were also present in the nucleus, although the density was reduced when using rapid freeze-substitution.

The finding of glycidid components in the orbicules, which is consistent with studies on *Cryptomeria japonica* (Miki-Hirosige et al. 1994), is of particular importance, in that orbicules are part of the micronic fraction and, because of their size, can be inhaled. Moreover, a recent study (Vinckier and Smets 2001) found that orbicules were attached to the pollen exine and/or free in the anther of many allergenic plants, and the authors hypothesised that orbicules are important vectors of allergens. This hypothesis is supported

by a study that showed that the orbicules of *Cupressus* are recognised by Cry j1 mAb (Suárez-Cervera et al. 2003). Our results are also consistent with this hypothesis.

The finding of gold particles in the cytoplasm confirms that fragments of pollen ruptured under humid conditions can represent a vector of allergens. In contrast, the finding that the callose vesicles did not have gold particles indicates that they do not behave like p-particles or starch granules, which in some species have been shown to possess strong allergenic activity (Grote et al. 1994; El-Ghazaly et al. 1999).

The finding that, at the subcellular level, the epitopes recognised by IgE compete with those recognised by mAb

Fig. 3A,B Localisation of mAb 5E6 in *Brassica nigra* pollen. **A** Gold particles on the exine (*e*), intine (*i*), and in the cytoplasm (*c*). *Bar* = 0.5 μ m. **B** Detail of cytoplasm. *Bar* = 1 μ m

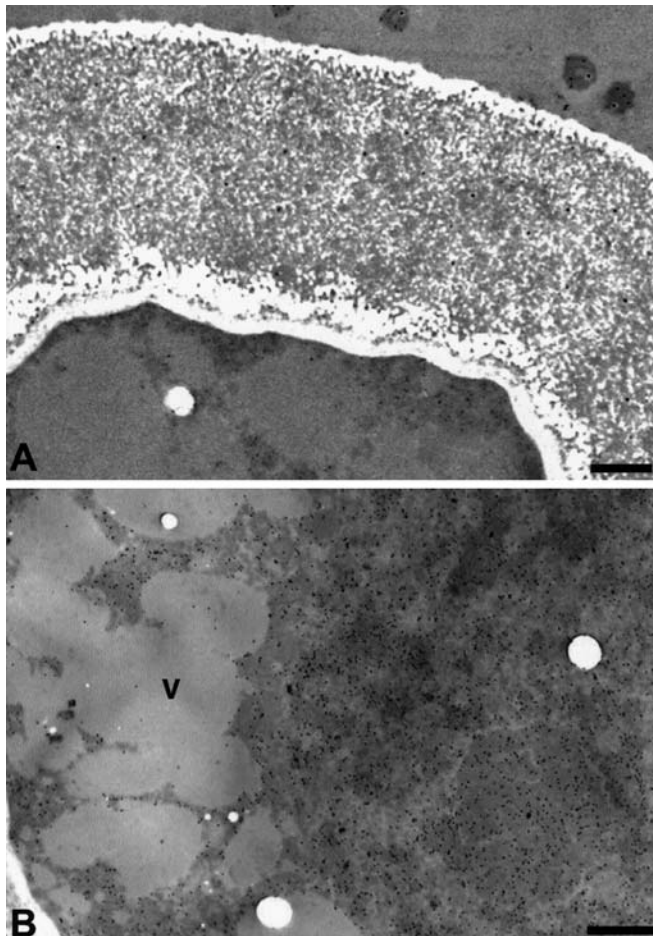
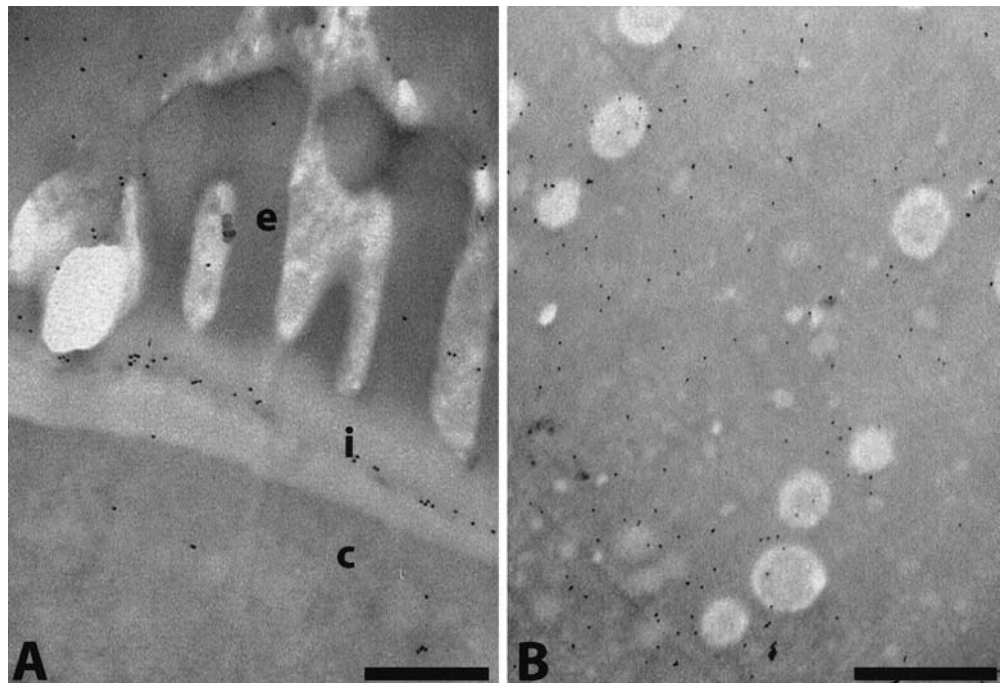


Fig. 4A,B Localisation of purified fraction of human IgE in *Cupressus sempervirens* f. *horizontalis* pollen. *Bar* = 1 μ m. **A** Gold particles on the orbicules, exine and intine. **B** Gold particles in the cytoplasm and callose vesicle (*v*)

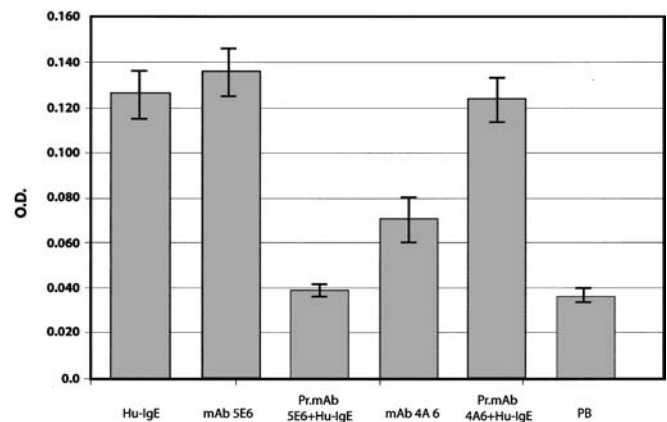


Fig. 5 Enzyme-linked immunosorbent assay of extract from *Cupressus arizonica* pollen incubated with human IgE (*Hu-IgE*) alone, or with mAb 5E6 alone (*mAb 5E6*), or pre-incubated with mAb 5E6 before *Hu-IgE* (*Pr. mAb 5E6+Hu-IgE*), or incubated with mAb 4A6 alone (*mAb 4A6*), or pre-incubated with mAb 4A6 before *Hu-IgE* (*Pr. mAb 4A6+Hu-IgE*), or incubated with phosphate buffer (*PB*) alone

5E6, as suggested by Iacovacci et al. (2001) based on the analysis of pollen extracts, demonstrates that carbohydrate epitopes in *Cupressus* pollen are involved in the binding of IgE. This, together with the finding that the epitope is present in a variety of taxonomically unrelated species and that it is thus widespread, is consistent with the fact that a single individual can be allergic to a diverse range of plants. It is known that mAb 5E6 recognises a carbohydrate epitope composed of a branched chain of seven sugars, two *N*-acetyl-D-glucosamine residues, three mannose residues, one fucose residue, and one xylose residue (Iacovacci et al. 2001). The presence of similar oligosaccharide structures on different allergens, such as Ole e 1 (Batanero et al. 1996), Fel d 1 (Duffort et al. 1991), and plant glycoproteins

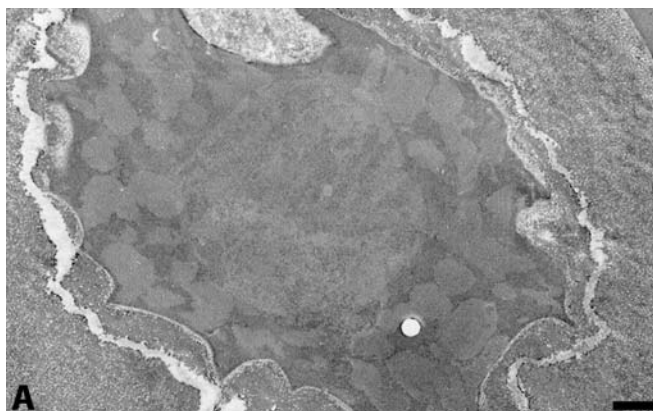


Fig. 6 Pollen section pre-incubated with mAb 5E6 before incubation with human IgE; no gold particles detected. Bar = 1 μ m

(El-Ghazaly et al. 1999), has recently been demonstrated and may explain the diffuse allergenic cross-reactivity among various allergens.

Finally, the in situ localisation of a common epitope recognised by both a monoclonal antibody and human IgE could be of importance in immunotherapy, even if a recently developed keyhole lampet hemocyanin-conjugated decapeptide vaccine was shown to be ineffective in treating allergies to cypress pollen (Demoly et al. 2002).

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