

In situ Localization of Pollen Allergens by Immunogold Electron Microscopy: Allergens at Unexpected Sites

Monika Grote

Institute of Medical Physics and Biophysics, Münster University, Münster, Germany

Abstract

The windborne pollen grains of many trees and grasses contain a number of highly water-soluble (glyco)proteins which upon moistening, e.g. on the human respiratory mucosa, rapidly diffuse out of the pollen grain. In susceptible individuals, they may cause allergic reactions. Because of their rapid release from the pollen, pollen allergens were expected to be located in the outermost layers of the pollen grain, i.e. on the surface and in the wall (exine, intine). First attempts to localize allergens by microscopic methods in the pollen grain supported this view. However, since conventional preparation methods were used, artificial mobilization of allergens could not be excluded. Based on new, completely anhydrous preparation protocols and on the immunogold-labeling technique, pollen allergens were electron-microscopically shown to be located in the interior of the pollen grain, i.e. in the cytoplasm, often within ribosome-rich areas. This unexpected result was obtained in all strictly anhydrously prepared pollen species. The biological function of these allergens is largely unknown, however, clues as to possible cellular functions have been obtained for the birch pollen major allergen Bet v 1.

Introduction

Tree and grass pollen allergens belong to the most potential elicitors of atopic diseases all over the world [1]. Localization and visualization of the allergens in the pollen grain were first attempted by light-microscopical techniques and were governed by the idea that these (glyco)proteins, due to their rapid diffusion from the grain in a moist surrounding, should be located at the periphery of the pollen grain, i.e. on the surface and/or in the pollen wall [2, 3]. These early attempts to visualize pollen allergens in situ used routine preparation methods for microscopy which include aqueous fixation and dehydration techniques. It was found, however, that diffusible allergenic and antigenic proteins could not be found in pollen walls after routine aldehyde fixation [4]. Thus, alternative preparation techniques had to be developed. Grass pollen grains were fixed in absolute methanol [5] or a combination of freezing techniques and direct embedding into plastic was used [6, 7] thus avoiding aqueous fixatives and graded ethanol series. These studies localized pollen antigens and allergens in the wall and occasionally in the cytoplasm of the pollen grains. However, morphological preservation of these specimens was bad, especially at the ultrastructural level.

It was shown that the addition of precipitating agents such as cetylpyridinium chloride to the aqueous fixation fluid led to the retention of otherwise lost material on the surface of the birch pollen grain and in exine cavities [8, 9]

but, as demonstrated later on [10], this material did not represent pollen allergens.

The onset of truly in situ localizations of allergens in the dry pollen grain came with the development of novel, strictly anhydrous, chemical fixation and dehydration protocols. This, together with the production of highly specific mono- or polyclonal antibodies directed against individual pollen allergens and the overwhelming advance of the immunogold technique in biological electron microscopy, led to the first localization of rye grass allergen Lol p 1 [11] and birch allergen Bet v 1 [10] on an ultrastructural level.

The following methodological section is intended to give a short survey of the different techniques now routinely used for the in situ localization of pollen allergens.

Anhydrous Fixation: A Major Requirement to Trace Pollen Allergens in the Pollen Grain

Anhydrous fixation of pollen grains may be performed by vapor fixation or by fixation in anhydrous liquids. Vapor fixation techniques for pollen grains were adapted from existing preparation protocols for dry plant specimens (seeds, resurrection plants) and involve fixation in *p*-formaldehyde vapor [10], glutaraldehyde vapor [11] or acrolein vapor [12]. Residual water of the pollen grain is removed by absolute ethanol and/or by chemical conversion into acetone and methanol by dimethoxypropane (DMP).

Fixation in nonaqueous liquids is performed by dissolving aldehyde fixatives (*p*-formaldehyde and/or glutaraldehyde) in anhydrous liquids such as glycerol [13] or DMP [14]. This may be carried out at room temperature or, as freeze-substitution, at -70°C using shock-frozen pollen samples. A simple freeze-substitution protocol for the localization of allergens in mature rye grass pollen using a substitution mixture of *p*-formaldehyde, glutaraldehyde, uranyl acetate, acetone, methanol and DMP was presented [15]. The residual water of the pollen grain is chemically transformed into acetone and methanol by DMP.

After fixation and dehydration, pollen grains are infiltrated with a resin suitable for immunocytochemical labeling techniques. Lowicryl K4M at -35°C was used for this purpose [10, 12], others embed pollen samples in LR gold at -25°C [15]. In both cases, enormously prolonged infiltration periods of up to several weeks are required for obtaining sectionable pollen samples. Additionally, a graded series of absolute ethanol/resin may be helpful for a better infiltration.

The colloidal gold/antibody technique [16, 17] is in the method of choice for the localization of pollen allergens

with the electron microscope. Here as in all other fields of cell biology, colloidal gold has replaced ferritin [7, 18] as an electron-dense marker.

In the most common indirect labeling technique, the ultrathin section of the pollen grain is incubated with the primary antibody which is either a polyclonal or a monoclonal antibody directed against the allergen in question. Binding sites of this antibody are detected and visualized by the secondary antibody which is directed against the species in which the primary antibody was produced. This secondary antibody is coupled with one or more particles of colloidal gold of appropriate diameter, e.g. 10 or 20 nm. Due to their electron-scattering properties, gold particles appear as black dots in the electron micrograph of the pollen grain, thus indirectly indicating the binding sites of the antibody and the location of the labeled epitope.

Instead of animal antibodies, human IgE antibodies have been used for the detection of allergens in pollen grains [11, 19–21]. This involves the use of a bridge antibody (anti-human IgE antibody) which is coupled with a third antibody directed against the species in which the bridge antibody was raised. Both immunogold methods are sketched out in figure 1 and examples for allergen localization are shown in figures 3–5. For the matter of comparison, the result of allergen localization in an aqueously fixed pollen grain is given in figure 2.

The immunocytochemical resolution, i.e. the distance between the gold particle and the labeled epitope, may amount to about 20 nm in the two-step technique and to more than 20 nm in the three-step technique due to the fact that the antibodies will not keep standing upright on the epitope but fall aside during drying of the section [22].

It is obvious that the shortening of the detection system by use of F(ab) or by direct conjugation of the primary antibody or antibody fragment to the gold particle would much improve the preciseness of allergen localization. However, direct coupling of antibodies to gold particles requires expertise and technical equipment, and apart from that, sufficient amounts of antibodies.

Simultaneous localization of two or more allergens can be achieved by using detection antibodies conjugated with gold particles of different sizes, e.g. 10 and 20 nm. An example of the double localization of birch and grass pollen allergens is shown in figures 3 and 5, respectively.

Specific localization of pollen allergens presupposes the availability of poly- or monoclonal antibodies directed against allergen epitopes. Production of antibodies, in turn, depends on the purification of the allergen from the pollen extract or on the existence of recombinant allergens for immunization. For the first ultrastructural localization of Lol

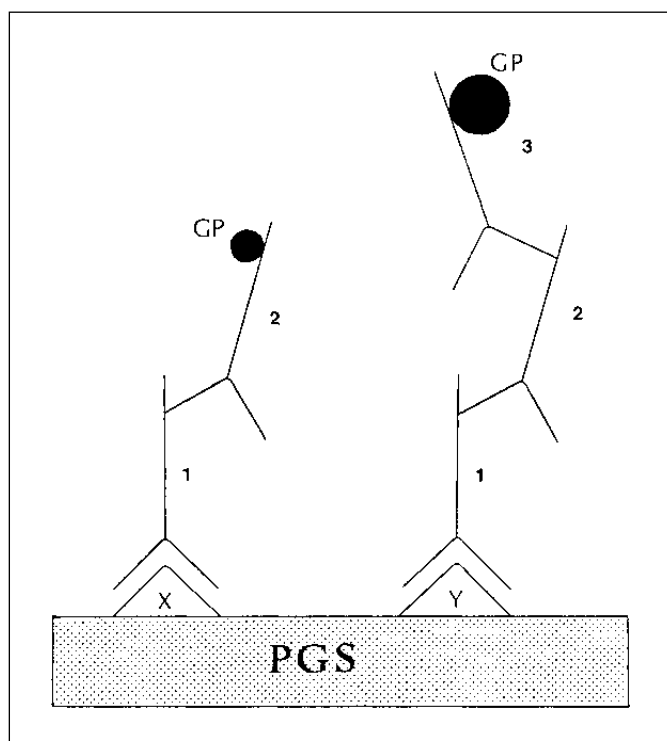


Fig. 1. Two common techniques for the detection of allergens in a pollen grain section (PGS). Allergen X is, e.g., detected by a rabbit (or mouse monoclonal) anti-X antibody (1) which is in turn coupled with a goat anti-rabbit (or anti-mouse) antibody (2) labeled with a colloidal gold particle (GP). On the transmission electron micrograph, colloidal gold particles appear as black dots due to their strong scattering of the electron beam. Allergen Y is, e.g., detected by a human anti-Y IgE antibody (1) which, in turn, is coupled with a rabbit anti-human IgE antibody (2). This is bound to a goat anti-rabbit antibody (3) labeled with a colloidal gold particle (GP). If allergens X and Y are to be detected simultaneously in the pollen grain section (= double labeling), gold particles must be of different sizes (e.g. 10 and 20 nm).

p 1 in anhydrously fixed rye grass pollen a mixture of monoclonal antibodies was used [11]. First immunoelectron-microscopic localization of Bet v 1 could be performed in anhydrously fixed birch pollen [10] after a monoclonal antibody directed against this birch pollen major allergen had been produced [23].

As mentioned above, specific human IgE antibodies have also been used in immunolocalization studies [11, 20, 21]. However, human IgE containing sera are rather problematic tools in immunocytochemical labeling since they have to be applied in a concentrated form due to the low content of specific IgE antibodies. This may cause enormous difficulties with background labeling. Purification of specific IgE by affinity purification [11] is time-consuming and troublesome.

Recently, Bet v 1 the major birch pollen allergen, was localized in birch pollen using monoclonal antibodies derived from an allergic individual [24]. They were obtained by establishing B-cell clones secreting Bet v 1-specific antibodies [25]. These human monoclonal antibodies exactly trace the allergenic structures recognized by the patient's immune system and thus constitute an attractive alternative to IgE antibodies in immunolocalization studies.

Where Do Allergens Come from?

In the following section the results of the localization studies of the most important allergens in tree, grass and weed pollens will be summarized. Most of this information was obtained following water-free preparation protocols in combination with immunogold techniques.

Tree Pollen Allergens

Birch. Bet v 1. The major birch pollen allergen was localized in numerous studies [10, 13, 24, 26, 27]. It was found exclusively in the cytoplasm of the pollen grain. Within the cytoplasm, Bet v 1 is associated with the matrix which in dry pollen is rich in closely packed ribosomes. The allergen is also found in the pollen nuclei. Bet v 2 (profilin). This panallergen [28, 29] was shown to be situated in the cytoplasmic matrix and the nuclei of birch and tobacco pollen [21, 26, 27]. 3A4-reactive allergen. It is a 60-kD cross-reactive allergen of pollen and plant-derived food [30] which was localized in the matrix of birch pollen cytoplasm, in pollen nuclei and within the mitochondria. This allergen was also found in mugwort pollen (see below), timothy grass pollen and in apple and celery tissues in identical sites [31].

Olive. Ole e I. The major olive pollen allergen was localized in the pollens of several Oleaceae species (olive, ash, privet, lilac and forsythia). It was found exclusively in the endoplasmic reticulum. In these studies, conventional aqueous preparation techniques were used [32–34].

Japanese Cedar. Cry j I. This allergen which is the main cause of Japanese cedar pollinosis was observed in several pollen wall layers and in the Golgi bodies in the cytoplasm. Results were mainly obtained using aqueous fixation techniques; however, a freeze-substitution technique was also employed [35].

Grass Pollen Allergens

Rye Grass and Timothy Grass. Rye grasspollen allergen Lol p 1 was detected in the cytoplasmic matrix and in the mitochondria. The pollen wall (exine) was also labeled

Fig. 2. Birch pollen grain after aqueous fixation in *p*-formaldehyde. Distinct signs of rehydration are visible (e.g. swelling of the cisternae of the endoplasmic reticulum). No Bet v 1 major allergen can be detected in this pollen grain. $\times 32,000$. Bar = 0.25 μm .

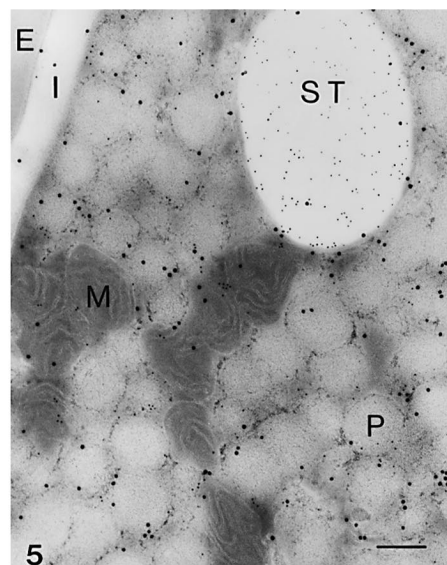
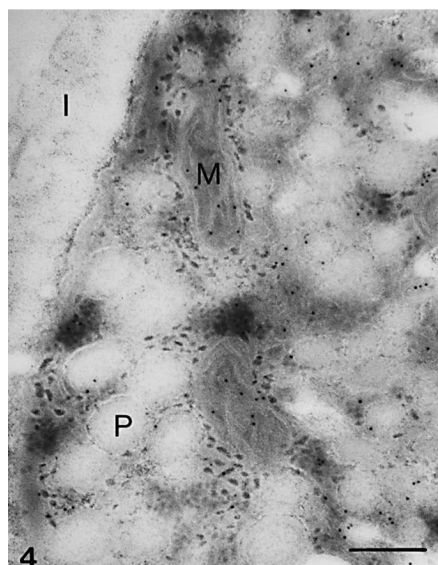
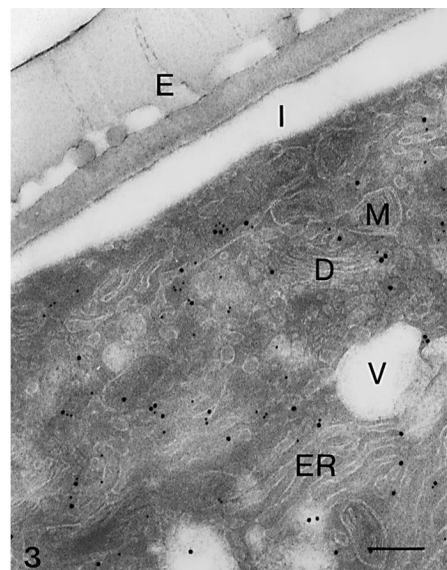
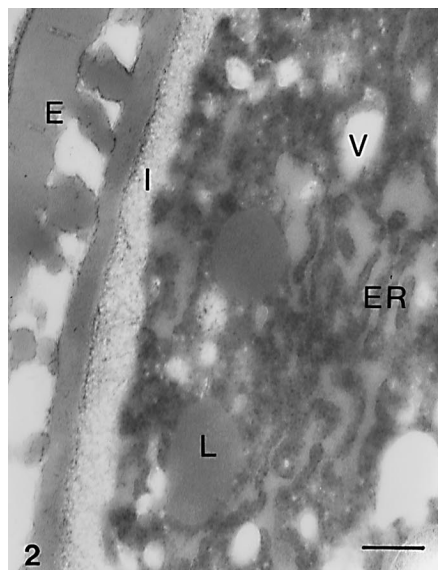
Fig. 3. Birch pollen grain after fixation in *p*-formaldehyde vapor. No rehydration took place. Cell organelles like mitochondria, dictyosomes or the endoplasmic reticulum are clearly delineated. Bet v 1 allergens (small gold particles) are detected predominantly in the cytoplasmic matrix by a first incubation of the section with a mouse monoclonal antibody. Human IgE antibodies which were applied secondly in this case detect epitopes different from that recognized by the anti-Bet v 1 antibody (large gold particles). Binding sites are equally located in the pollen cytoplasm. $\times 29,000$. Bar = 0.25 μm .

Fig. 4. Mugwort pollen grain after fixation in acrolein vapor. Mouse 3A4 antibody directed against Art v 1 mugwort pollen allergen binds to the mitochondria and the cytoplasmic matrix. $\times 39,000$. Bar = 0.25 μm .

Fig. 5. Timothy grass pollen after fixation in acrolein vapor. Group 1 allergen (Phl p 1, large gold particles) is detected by a rabbit antibody mainly in the cytoplasmic matrix, whereas group 5 allergen (Phl p 5, small gold particles) is localized mainly in the amyloplasts by a monoclonal antibody. $\times 25,000$. Bar = 0.25 μm .

Abbreviations used in figures 2-5.

D = Dictyosome; E = exine; ER = Endoplasmic reticulum; I = intine; L = lipid; M = mitochondrion; P = polysaccharide (p) particle; ST = starch grain (amyloplast); V = vacuole.



[11]. The homologous allergen in Phleum pollen, Phl p 1, was found in similar sites except for the mitochondria [12]. Phl p 4. This timothy grass pollen allergen was located in the pollen matrix and in the amyloplasts. Some allergenic activity was also found in the exine [36]. Lol p 5 in rye grass pollen and Phl p 5 in timothy grass pollen predominantly mapped to the amyloplasts which were heavily labeled. Fewer gold particles were detected in the cytoplasm. There was no labeling or sparse labeling of the wall [12, 14, 37]. Phl p 6. This allergen in timothy grass pollen was detected in the polysaccharide (p)-particles of the cytoplasm and in the cytoplasmic matrix. Little activity was observed in the exine [Vrtala et al., 1998, submitted].

Weed Pollen Allergens

Mugwort. Art v 1. The major mugwort pollen allergen Art v 1 is a highly cross-reactive allergen which was localized using the antibody 3A4 in mugwort pollen, but also in birch pollen, timothy pollen and in plant-derived food (see above). It is found in the cytoplasmic matrix and the pollen nuclei, but also in the mitochondria of mugwort pollen [31]. Allergenic proteins of *Parietaria judaica* (Urticaceae) were found predominantly in the intine of the pollen grains [38]. In this study pollen grains were investigated only after rehydration and by use of conventional aqueous preparation techniques.

The above presentation summarizing the results of the various studies on pollen allergen localization shows that – in contrast with the earlier concepts of the pollen allergens being harbored by the pollen wall layers – allergens of the dry pollen are found in the pollen cytoplasm, especially in the condensed, ribosome-rich matrix. Moreover, special cell components like mitochondria, nuclei, amyloplasts or polysaccharide (p)-particles may be labeled. Additional location of some allergens in the pollen wall is reported. This may be original, but care should be taken to exclude diffusion effects in the native pollen grain before fixation. Such diffusion of these highly mobile allergens may start when exposing pollen grains from the fridge to room air. The question of wall-bound allergens can only be solved by systematic reinvestigation of these pollen species strictly excluding any such accidental rehydration effects on the native pollen grain.

If pollen allergens are predominantly located in the cytoplasm, how do they manage to get out of the grain so quickly? A detailed investigation of this problem has not been carried out so far. However, there are reports [11] on an altered, 'leaky', configuration of the phospholipid bilayers in the membranes of dry plants as compared with the fully hydrated state of the membranes in these plants [39, 40]. Allergens could use this short gap between the dehydrated state of the membrane and the reestablishment of the normal membrane architecture to stream out of the pollen grain.

Biological Significance of Pollen Allergens

There is general agreement that diffusible pollen proteins should play a role in the process of fertilization (pollen-stigma interaction, compatibility/incompatibility recognition, etc.). Some efforts have been spent to more precisely elucidate the biological functions of these proteins, which – only in a kind of unwanted and unlucky accident – affect sensitized human beings.

Thus, for birch pollen major allergen Bet v 1, some information concerning a possible function in the cellular mechanism has been gathered. Thus, RNase-like activity of the Bet v 1 molecule is reported [41, 42]. This would be in good agreement with the observed ultrastructural localization pattern (ribosome-rich cytoplasm, nucleus). When investigating the three-dimensional structure of Bet v 1 by X-ray crystallography and nuclear magnetic resonance spectroscopy, the existence of a glycine-rich loop in the molecule similar to those found in nucleotide-binding proteins was detected [43]. Recently, sequence analysis of Bet v 1, homologous allergens and pathogenesis-related proteins revealed a highly conserved p-loop-like motif which is typically found in nucleotide-binding proteins [24]. Thus, a possible function for Bet v 1 as a nucleotide binding protein suggests itself. In any case, this would again be in good agreement with the immunolocalization data. It would also explain why many atopic individuals are cross-sensitized against these conserved proteins.

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