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Immunocytochemical localization of the allergenic proteins in the pollen of *Cryptomeria japonica*

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Abstract Applying an immunocytochemical method, a localization of the protein *Cry j I* in the *Cryptomeria japonica* pollen, which is the major allergen responsible for Japanese cedar pollinosis, is investigated with the monoclonal and polyclonal antibodies produced from the protein. The protein that reacts to the polyclonal antibody localizes on the sexine, nexine, between nexine and intine layers, orbicules, cell wall of a generative cell, Golgi body and Golgi vesicles. The allergenic protein contained in the exine and orbicules of Japanese cedar pollen can diffuse or dissolve easily from there into the mucus covering of the eye and nose, causing a response in less than 1 min after exposure. Since the orbicules have a diameter of about 430 nm, they can pass easily through the pores of most protective masks to reach the sensitive tissues of the patient. The proteins react to the monoclonal antibodies (J1BO1 and J1BO7) and localize on the Golgi body, sexine, nexine and orbicules (but not between the nexine and intine layers), and on the generative cell wall. In the young pollen grain, numerous allergenic protein particles contained in the orbicules and sexine layer, but there is only a small amount of the protein between the nexine and intine layers, since the intine layer is not yet complete at this stage. More will be accumulated there during developmental maturation. The allergenic protein is also found on the tapetal materials remaining in the young anther. Since the materials forming the exine layer and orbicules come from tapetal tissue, it is assumed that some of the allergenic protein is produced in the tapetum and localized in the orbicules and pollen wall during maturation, and that the

rest of the allergenic protein is produced in the Golgi body in the mature pollen grain.

Key words Allergic symptoms · Allergenic protein *Cryptomeria japonica* · *Cry j I*
Immunocytochemistry

Introduction

Cryptomeria japonica in Gymnospermae is wind-pollinated or anemophilous, and its pollen grains are produced as a yellow-colored dust which can cause both eye and nose mucous-membrane irritations in susceptible individuals. Proteins released from the pollen grains function as an allergen on both eye surface tissues and nose mucous membranes. A susceptible individual shows symptoms of pollinosis by sneezing or tear production or both and the reaction is very swift. Recently, Japanese cedar pollinosis has increased to the extent that it has become a medical problem in Japan. The allergenic symptoms caused by *Cryptomeria japonica* pollen were investigated by Horiguchi and Saito (1964), and since then, other investigators (Saito 1988; Sasazuki et al. 1983; Sasazuki et al. 1984; Shibamura and Nishimura 1991) have reported on the mechanism of the disease, its medical treatment and prevention, and gene-level investigations. Staff et al. (1990) reported on the allergenic proteins in rye-grass (*Lolium perenne*), which causes a pollinosis in southeastern Australia. This allergen is localized in the pollen exine and mitochondria. Grote (1992) also reported on a technique of allergen detection in birch pollen. Until now, however, localization of the allergenic proteins in Japanese cedar pollen has not been reported. Applying an immunocytochemical method, we investigated in the pollen of *Cryptomeria japonica* the protein *Cry j I*, which is the major allergen responsible for Japanese cedar pollinosis and a heat-sensitive basic glycoprotein of approximately 40000 molecular weight (Yasueda et al. 1983). Our work involved the monoclonal and polyclonal antibodies produced from the protein.

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Materials and methods

Young pollen cones of *Cryptomeria japonica* were collected in September, and mature ones were collected in February 1991 in the suburbs of Kamakura, Japan, and stored at 4°C until used.

For ultrastructural observations, the materials for TEM were fixed with osmium vapor overnight, postfixed with 3% glutaraldehyde for 3 h and 1% osmic tetroxide for 1 h, and then treated by the usual procedures for TEM. The materials for SEM were prepared in the usual routine way.

For electron microscopic immunocytochemistry, the materials were fixed by two different methods (A and B) and the results were compared. (A) Materials were fixed with 1.5% paraformaldehyde and 1.5% glutaraldehyde containing 0.2% picric acid and buffered by 0.05 M sodium cacodylate at pH 7.5 (Maldonado et al. 1986), dehydrated, embedded in Spurr's resin, and sectioned by routine procedures. As the fixation for immunocytochemistry of pollen cytoplasm is very difficult, we used the microwave irradiation (MWI) method (Mizuhira et al. 1990). Ultrathin sections were mounted on 200 mesh nickel grids. (B) A rapid freezing and freeze substitution technique was applied to freeze the materials rapidly with liquid nitrogen, which was subsequently substituted by 2% osmium tetroxide-acetone solution; the materials were then embedded, and sectioned. The method of Probert et al. (1981) was followed for the post-embedding, immuno-gold staining technique. For control staining, and to demonstrate the specificity of immuno-gold labeling, the antiserum was replaced with normal rabbit serum.

Rabbit polyclonal antibody against *Cry j I* was isolated from anti-*Cry j I* antiserum (Yasueda et al. 1983) by affinity chromatography on an immunosorbent column consisting of purified *Cry j I* coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden).

In mouse monoclonal antibodies against *Cry j I*, the two *Cry j I* specific monoclonal antibodies, J1B01 and J1B07 used in this study were derived from a single fusion using Sp2/0-Ag14 myeloma cells and spleen cells from BALB/c mice which had been immunized with purified *Cry j I* in complete Freund's adjuvant. Both were isotype IgG1 and were directed against different epitopes on the *Cry j I* molecule. Ascites were produced by injecting 10⁷ hybridoma cells i.p. into pristane-treated BALB/c mice. The monoclonal antibodies were purified from ascites fluid by affinity chromatography on a protein A column (Ampure PA kit, Amersham Japan, Tokyo Japan).

Results

A pollen grain of *Cryptomeria japonica* is spherical with a spiny projection and covered by a grainy surface and orbicules (Fig. 1a). A pollen grain surface is usually covered with abundant orbicules, but the pollen grain in Fig. 1a has small amount of orbicules because they were washed off by the organic solvent, used in fixation. A sectioned pollen grain that has absorbed water shows a thick cell wall with one exine (sexine and nexine) and four intine layers (Fig. 1b). When the mature pollen grain is treated with polyclonal antibody (*Cry j I*) in the immunocytochemical procedure, gold particles can be seen on the sexine and nexine, between nexine and intine layers, and in the peripheral zones of the orbicules (Fig. 2). Following fixation of the mature pollen grain by the rapid freeze substitution method (method B, Fig. 3), many gold particles on the exine layer and orbicules are observed more clearly than after fixation by method A. In a protoplasm of the mature pollen grain, gold parti-

cles are seen on the Golgi body and Golgi vesicles (Fig. 4a) and the cell wall of the generative cell (Fig. 4b). On the control specimen, the gold particles are not shown on the exine layer, orbicules, between nexine and intine layers (Fig. 5a), generative cell wall (Fig. 5b), and Golgi body and Golgi vesicles (Fig. 5c).

When mature pollen grains are treated with monoclonal antibodies (J1B01 and J1B07), gold particles can be seen on the Golgi body, orbicle, sexine and nexine, but not between the nexine and intine layers of the cell wall (Figs. 6a and b) or on the generative cell wall. A comparison of the localization of the allergenic proteins by the two monoclonal antibodies shows little difference between them.

Young pollen grains, 6 months before being shed, when treated with the same polyclonal antibody (anti *Cry j I*), show gold particles with the same distribution as those in mature pollen wall except that there are fewer particles between nexine and intine layers (Fig. 7a). In this young stage, the intine is not yet complete. Gold particles can be seen associated with residual tapetal materials of immature microsporangia and surface of the orbicules (Fig. 7b), but are not seen as yet on Golgi bodies. The orbicules and sexine layers of the pollen increase their thickness with the attachment of residual tapetal materials to their surfaces during maturation.

Discussion

From this investigation, it is apparent that the allergenic protein is located in the sexine, nexine, between nexine and intine layers, orbicules, generative cell wall, Golgi bodies and Golgi vesicles of the mature pollen grains of *Cryptomeria japonica* (Figs. 2–4). Staff et al. (1990) reported that in rye grass (*Lolium perenne*) the allergen is labeled in mature pollen grains in the exine, electron opaque areas of the cytoplasm and especially the mitochondria. They found the allergen to be absent from the intine layers, polysaccharide particles, amyloplasts, Golgi bodies and endoplasmic reticulum. Our work shows that Japanese cedar pollen has the allergenic protein not only in the exine layers, but also in the generative cell wall, Golgi bodies and Golgi vesicles. Both kinds of pollen cause symptoms in susceptible individuals, suggesting that these proteins can diffuse or dissolve easily from the exine into the mucous coverings of the eye and nose and cause a response in less than 1 min after exposure. Simon (1974) reported that when pollen grains are placed in contact with water, soluble cell constituents leak out for up to several minutes, until membrane integrity is reestablished. A difference in localization of the allergenic proteins between the pollen examined by Simon and Japanese cedar pollen is assumed. The orbicules which cover the Japanese cedar pollen surface in abundance (Fig. 1a) also contain allergenic protein (Figs. 2, 3), and since the orbicules have a diameter of about 430 nm, they can pass easily through the pores of most protective masks to reach the sensitive

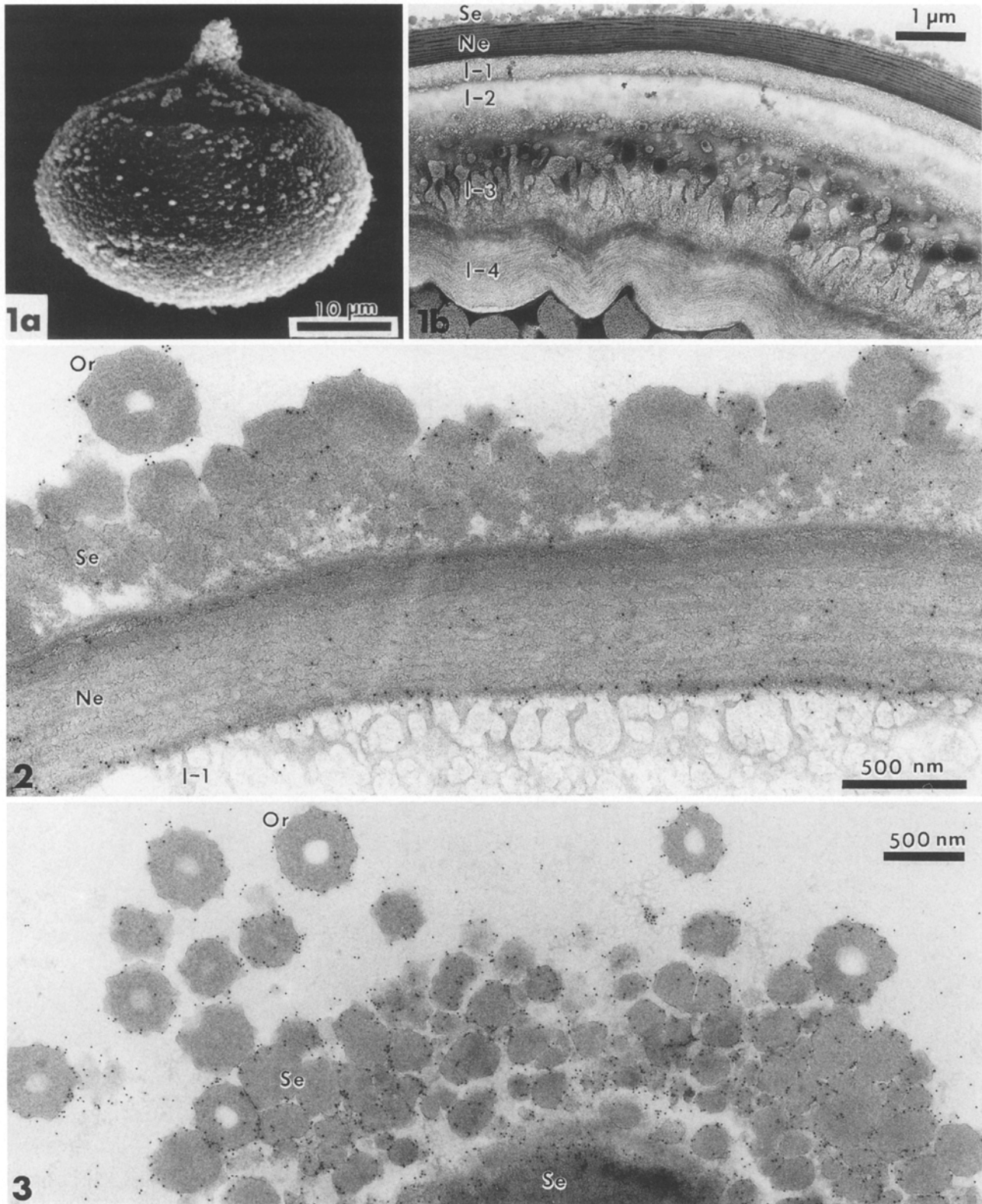


Fig. 1 **a** A mature pollen grain of *Cryptomeria japonica* is spherical with a grainy surface having a spiny projection and covered with orbicules. $\times 1600$ **b** A sectioned mature pollen grain that has absorbed water shows a thick cell wall with exine (sexine and nexine) and intine (*I*), which is divided into four layers. *Se*, Sexine; *Ne*, nexine. $\times 12000$

Fig. 2 A mature pollen grain treated with the polyclonal antibody, and fixed by method A. Numerous gold particles are shown on sexine, nexine, particularly between nexine and intine layers, and orbicules (*Or*). $\times 42000$

Fig. 3 A tangentially sectioned cell wall of a mature pollen grain fixed by the rapid freeze substitution technique (method B), and treated with polyclonal antibody. Many gold particles can be seen on the orbicules and exine layer. $\times 28000$

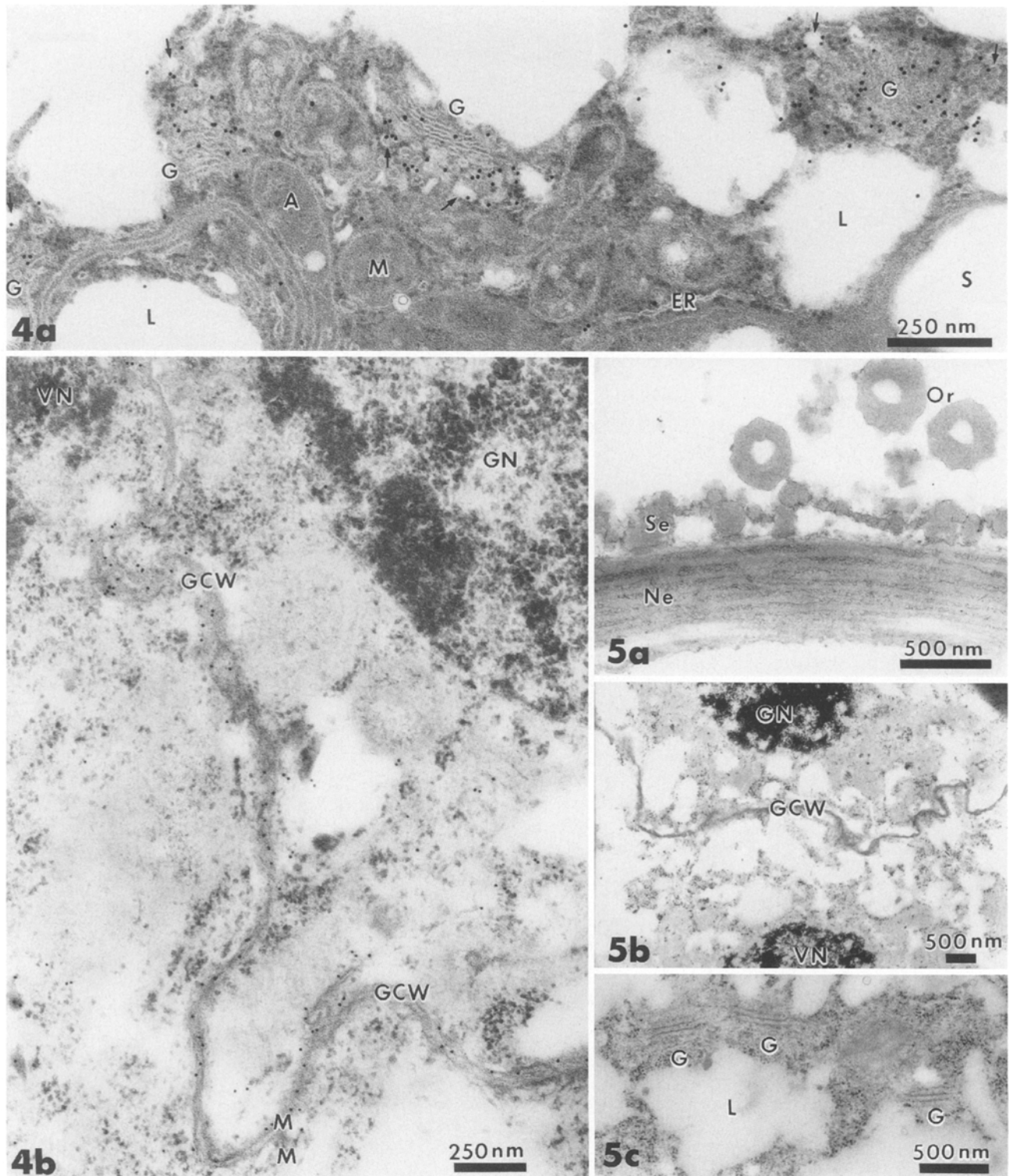


Fig. 4a, b The protoplasm of a mature pollen grain treated with polyclonal antibody (MWI method). **a** Numerous gold particles are shown on Golgi bodies (G) and golgi vesicles (arrows) in the pollen grain. $\times 70000$ **b** Many gold particles can be seen on a generative cell wall (GCW) in the pollen grain. GN, Generative nucleus; VN, vegetative nucleus; A, amyloplast; M, mitochondrion; S, starch; L, lipid body; ER, endoplasmic reticulum. $\times 48000$

Fig. 5a–b Control pictures of the treatment of *Cry j* I antibody on the Japanese cedar pollen. There are no gold particles on the exine, orbicles between nexine and intine layers (**a**; $\times 30000$), generative cell wall (**b**; NWI method, $\times 10000$), or Golgi body and Golgi vesicles (**c** NWI method, $\times 23000$)

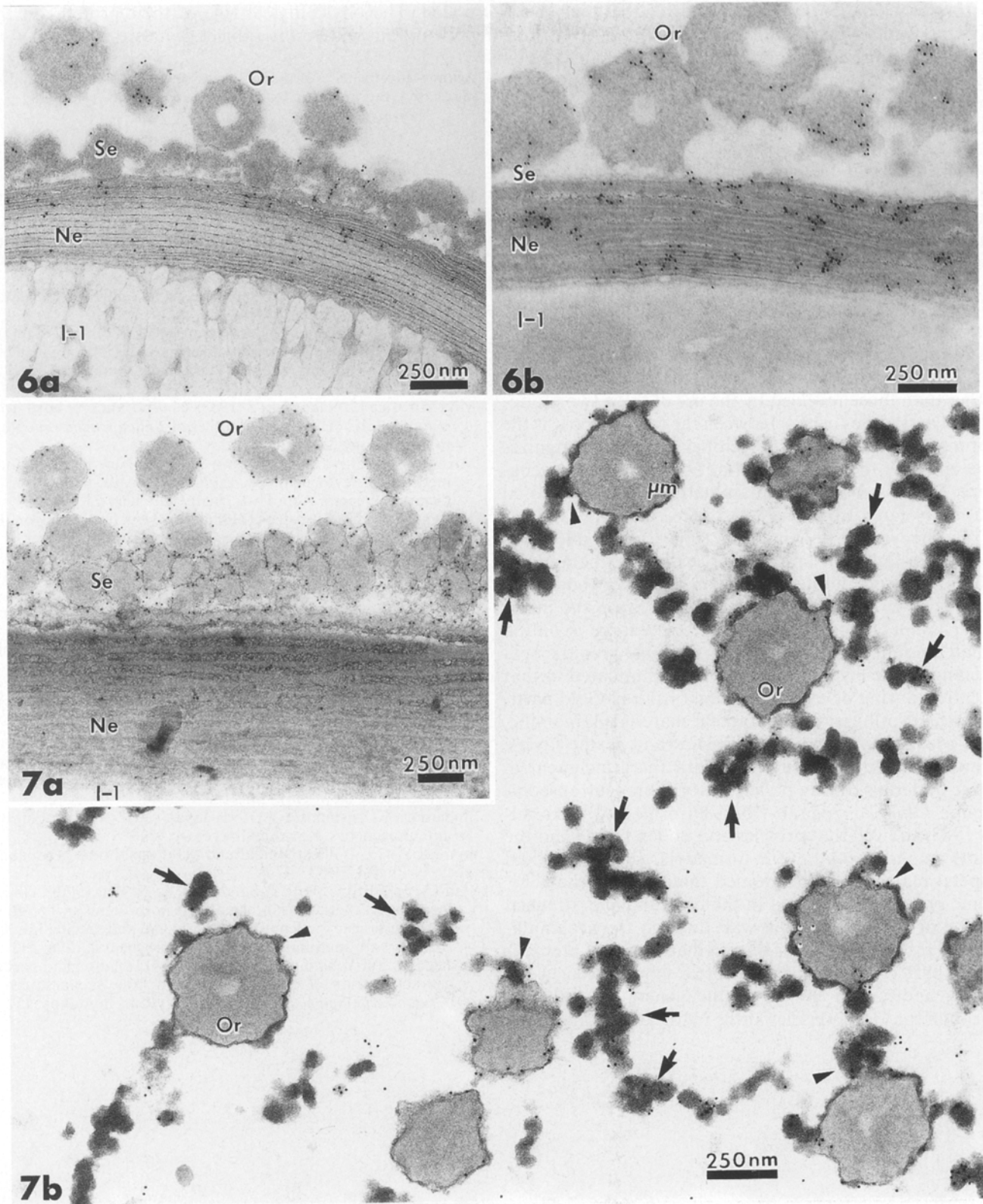


Fig. 6 Mature pollen grains treated with monoclonal antibodies **a** J1BO1 and **b** J1BO7 Gold particles can be seen on the Golgi body, sexine, nexine and orbicles, but not between nexine and intine layers. No substantial difference in gold particle localization was seen with the different techniques

Fig. 7a, b A young male flower 6 months before anthesis, treated with the polyclonal antibody, and fixed by method A. **a** Numerous gold particles are seen on the sexine, nexine and orbicles, and a small number between nexine and intine layers. $\times 31\,500$ **b** By the time the anther is developed, the tapetum has disappeared, but the tapetal materials remaining (arrows) are marked with numerous gold particles in the young anther. The materials attach to the surfaces of orbicles (arrow heads) and sexine and increase their thickness. $\times 48\,000$

tissues of the patient. We have reported that the generative cell wall is built with Golgi vesicles derived from Golgi body in *Lilium longiflorum* (Nakamura and Miki-Hirosige 1985); therefore, it is assumed that the Golgi vesicles that contain the allergenic protein derived from the Golgi body have many gold particles built up in the generative cell wall.

The material fixed by method B provided much clearer immunocytochemical results. Since the allergenic protein may be water soluble, the rapid freeze substitution method probably did not remove as much the protein as method A.

In the technique using the monoclonal antibodies (J1BO1 and J1BO7), gold particles can be seen on the orbicles, exine (Fig. 6), or Golgi vesicles, but no proteins were localized on the generative cell wall and between the nexine and intine layers. It is possible that a protein other than those revealed by the use of these two monoclonal antibodies occurs between the two layers. It is the future problem why the polyclonal antibody recognizes the epitopes the monoclonal antibodies do not recognize. The possibility of contamination in the polyclonal antibody cannot be considered here.

In the young pollen grain (Fig. 7), the allergenic protein is found in and on the sexine, nexine and orbicles, and to a limited extent on the Golgi body and between the nexine and intine layers. Since the intine layer is not yet complete at this stage, there is only a small amount of the allergenic protein between the nexine and intine layers. More will be accumulated in that location during developmental maturation. Gold particles are also found on the tapetal materials remaining, and on the surfaces of the orbicles and sexine layers, which increase their thickness with the attachment of these materials during pollen maturation. Our observation in *Lilium longiflorum* (Miki-Hirosige and Nakamura 1983) showed that orbicles and pollen sexine had the same materials and were formed largely of residual tapetal materials. It is assumed that some of the allergenic protein is produced in the tapetum, and attached to the orbicles and pollen wall through the accumulation of residual materials during maturation. Later, another part of the allergenic protein is produced in the Golgi bodies and moves to the generative cell wall through the Golgi vesicles at the maturation stage of the

pollen. The physiological role of their proteins in the pollen grain will be the subject of future research.

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