Major Allergens of Hinoki- and Sugi-Pollen Exist on Their Grain Wall

Yuzo TAKAHASHI[1], Takeshi IDE[2], Tuneo ASHIDA[3], Shiro TABATA[2] and Tsuneji ARAKI[1]

1) *Department of Parasitology, Nara Medical University Shijo 840, Kashihara, Nara 634, Japan.*
2) *Department of Chemistry, Nara Medical University Shijo 840, Kashihara, Nara 634, Japan.*
3) *Ashida ENT Clinic Kosaka 3-4-51, Higashiosaka 577, Japan.*

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We investigated the *in situ* localization of major allergen in two kinds of pollens, *Chamaecyparis obtusa* Endl. (Hinoki, false cypress) and *Cryptomeria japonica* D. Don (Sugi, Japanese cedar), by means of immunostaining with anti-HMA antibody, anti-*Cry j* I antibody or sera from patients with Sugi pollinosis. Anti-HMA antibody and anti-*Cry j* I antibody predominantly immunostained the Ubish body, sexine and nexine of respective pollens. Patients' sera also stained the same structures. Thus the major allergen was shown to exist on the external wall of the pollen grains. Cross-reactivity between HMA and *Cry j* I was revealed by the cross immunostaining results that anti-HMA antibody stained the surface of Sugi pollen as well, and anti-*Cry j* I antibody stained Hinoki pollen.

Key Words : Allergen, Pollen, Sugi pollinosis, *Chamaecyparis obtusa*, *Cryptomeria japonica*, Immunohistochemistry.

Introduction

Among various kinds of air-borne pollen, the pollen of *Cryptomeria japonica* D. Don (Sugi, Japanese cedar) is dominant during the Spring in Japan. Sugi pollinosis remains one of the major clinical problems because of the increasing numbers of patients. The disease has been extensively studied from etiologic, pathologic and clinical points of view[1]. However, the pollen of *Chamaecyparis obtusa* Endl. (Hinoki, false cypress) has received little attention although its involvement in Sugi pollinosis has been suggested. For example most of Sugi pollinosis patients develop the symptoms even after so called "Sugi pollen season", corresponding to the time when Hinoki pollen is being dispersed into the air.

Previous studies have identified, purified and partially characterized the major allergen responsible for the disease, namely HMA of Hinoki pollen[2] and *Cry j* I of Sugi pollen[3]. HMA is a heterodimer of glycoproteins with molecular mass of 45/50 KDa and pl 6.8 and *Cry j* I is composed of 4 kinds of glycoproteins with molecular mass of 41 KDa (pl 8.9, 9.2) and 46 KDa

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Some questions about pollen antigens, however, are still to be answered, which include the way of antigen presentation to the human immune system ; the best way to mass-isolate major pollen antigens ; and cross-reactivity between Sugi and Hinoki pollen antigens. The existance of cross reactivity has been suggested by some authors[4-8], which is very important in understanding the etiology of pollinosis. To address these questions, anatomical localizaion of major allergens should be determined. An earlier author has suggested *Cry j* I is inside pollen grains based on rather indirect evidences[9]. In this contribution we employed a postembedding immunogold method to detect HMA and *Cry j* I at the subcellular level.

Materials and Methods

Pollens of Hinoki and Sugi were obtained from respective twigs by our method reported previously by Ide *et al.*[10]. Rabbit antibodies were prepared against HMA and *Cry j* I by subcutaneous injection of HMA and *Cry j* I , respectively.

Patients' sera were obtained from three immunologically-untreated patients with typical clinical pictures of Sugi pollinosis during the pollination season, positive skin tests and positive RAST. Sera were separately frozen at −80℃ until use.

For immunoelectron microscopy, pollen grains were chemically fixed with 4 % paraformaldehyde and 2.5% glutaraldehyde for 30 minutes at 4 ℃, dehydrated with ascending series of alcohol, and embedded in LR White resin according to the producer's instruction. Ultrathin sections were made by means of an ultramicrotome and mounted on nickel grids.

Immunostaining was performed to detect HMA or *Cry j* I on the pollen at room temperature allocating 30 minutes for each process. The ultrathin sections were reacted with anti-HMA or anti-*Cry j* I rabbit antisera (1 : 50 dilution) and subsequently with protein A-colloidal gold complex according to the established method[11]. For controls, identical staining was performed using normal rabbit sera.

To detect pollen grain antigens recognized by patients' IgG, in other words to detect pollen antigens responsible for production of IgG, ultrathin sections were reacted with three layers of reagents including (1) patients' sera, (2) biotinated anti-IgG antibody (3) and avidine-colloidal gold complex according to the established manner[12], and observed under an electron microscope. For controls identical staining was performed using sera from healthy volunteers without any known allergy.

To examine if antigenic cross-reactivity exists between HMA and *Cry j* I , Hinoki pollen grain was immunostained with anti-*Cry j* I antibody and Sugi pollen grain was immunostained with anti-HMA antibody, and processed for observation.

Results

Immunocytochemical staining with anti-HMA or anti-*Cry j* I antibody revealed intrapollen localization of HMA or *Cry j* I , respectively. Control sera failed to stain any structures confirming specificity of the immunostain (Fig. 2). Positive stainings for HMA were predominantly on the Ubish body, sexine and nexine (Fig. 1) of Hinoki pollen grain. No structures inside the pollen grain were effectively stained with the immunized sera which acted as a built-in control (Fig. 3). Identical results were obtained as to Sugi pollen grain (Fig. 5) largely confirming

previous study by Nakamura *et al.*[13], with only one contradiction that the interphase between nexine and intine was negative in our results but positive in their results.

Anti-HMA antibody positively stained the surface of Sugi pollen grain (Fig. 7) in the same pattern as described for Hinoki counterparts but staining intensity was much less. The same was true for anti-*Cry j* I antibody ; it reacted with the surface of Hinoki pollen grain (Fig. 8).

Antigens recognized by IgG from patient's sera were also localized by the postembedding immunogold staining as shown in Figs. 4 (Hinoki pollen) and 6 (Sugi pollen). Positive staining was predominantly on the Ubish body, sexine and nexine. The three sera gave the same results. Control sera did not stain any structures, confirming the specificity of the method.

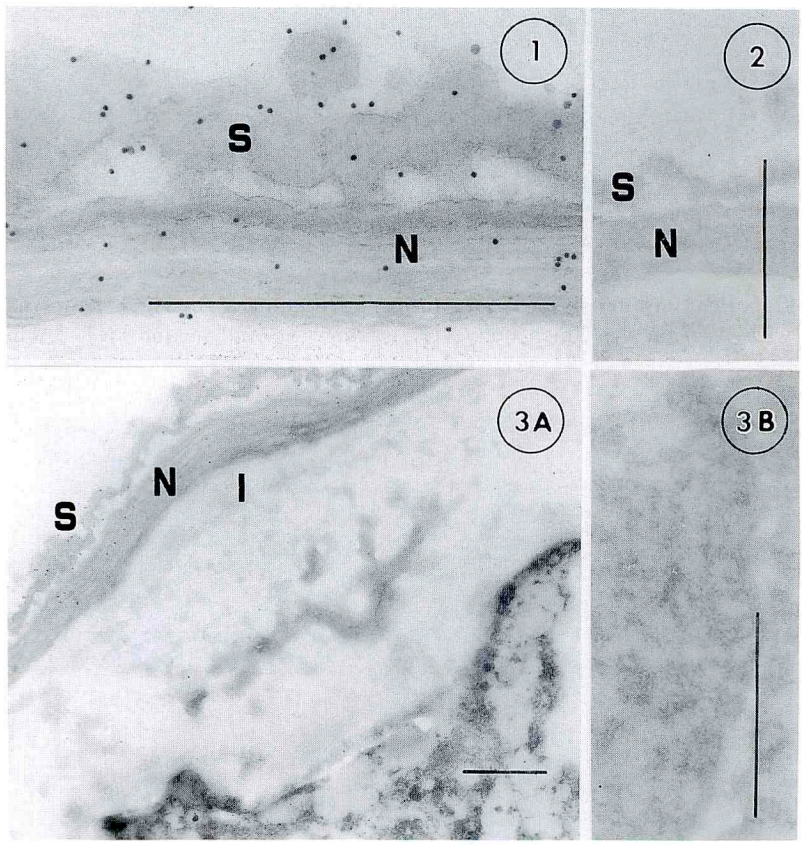

Fig. 1. Hinoki pollen immunostained with anti-HMA. Gold particles are seen on sexine (S) and nexine (N). A bar = 1 μm

Fig. 2. Hinoki pollen immunostained with control sera. No effective staining was observed. S, sexine. N, nexine. A bar = 1 μm

Fig. 3A. Hinoki pollen immunostained with anti-HMA. No effective staining was seen in the intine (I) and cytoplasm although sexine (S) and nexine (N) were positive. A bar = 1 μm

Fig. 3B. Hinoki pollen immunostained with anti-HMA. No effective staining was seen in the cytoplasm including rough endoplasmic reticulum. A bar = 1 μm

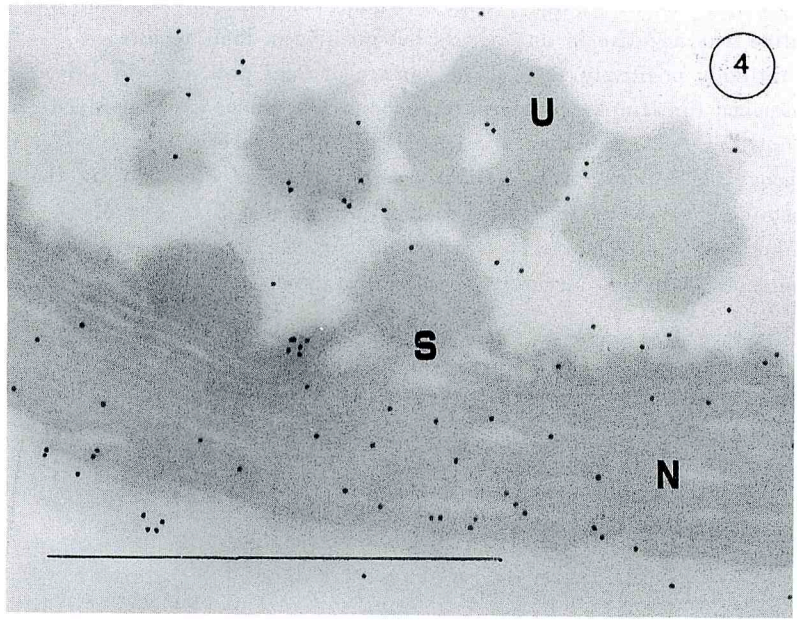

Fig. 4. Hinoki pollen immunostained for antigens recognized by IgG from patients' sera. Gold particles were seen on the Ubish body (U), sexine (S) and nexine (N). A bar = 1 μm

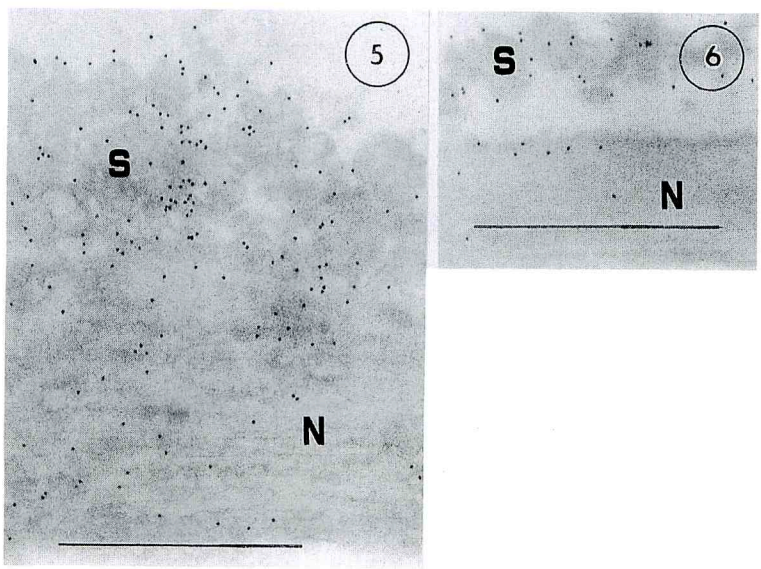

Fig. 5. Sugi pollen immunostained with anti-*Cry j* I. Gold particles were seen on sexine (S) and nexine (N). A bar = 1 μm

Fig. 6. Sugi pollen immunostained for antigens recognized by IgG from patients' sera. Gold particles are seen on sexine (S) and nexine (N). A bar = 1 μm

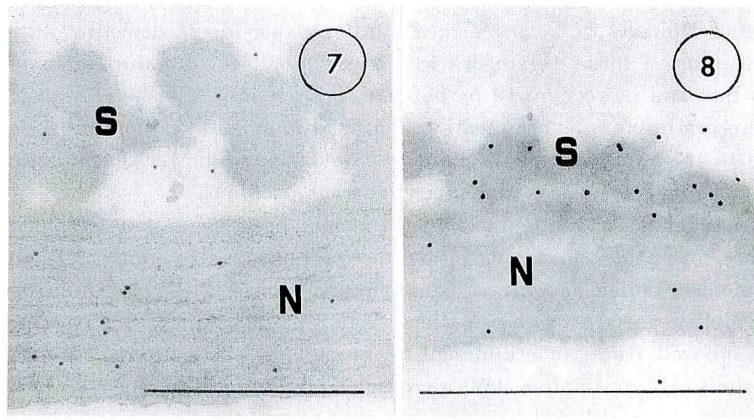

Fig. 7. Sugi pollen immunostained with anti-HMA. Staining pattern was identical to those in Fig. 5 but with less stain intensity. S, sexine. N, nexine. A bar = 1 μm

Fig. 8. Hinoki pollen immunostained with anti-*Cry j* I. Staining pattern was identical to those in Fig. 1 but with less stain intensity. S, sexine. N, nexine. A bar = 1 μm

Discussion

It has been long believed that major allergens of Sugi- and Hinoki-pollen are associated with internal structures of pollen grains and hence the immune system of patients could be triggered after rupture of inhaled pollen grains. This thesis came from the observation that higher yields of allergen is achieved after rupture of pollen.

As far as sticking to this thesis, however, one cannot explain why patients develop symptoms immediately after inhalation of pollen. There should be time lag for major allergen to be released. Recent observations made by Takahashi *et al.*[10], provided another enegma : they collected paricles smaller than pollen from the air, surprisingly the air-borne particles also had allergenic activity.

This enegma may be solved by our present results ; the pollen surface has a pool of major allergen, whose antigenicity was recognizable by patients. This location likely ensures immediate availability of allergen for activation of the host immune system which results in an allergic reaction.

Major allergen is also located on the Ubish bodies which are easily detached by mechanical force from pollen grains of Sugi and Hinoki. If such detached Ubish bodies are flying in the air, Ubish bodies may account for the air-borne paricles with allergenic activity.

Interestingly major allergen was localized on the surface of external layers and Ubish bodies, which raises the question whether allergen is derived from the anther or synthesized in pollen grains. The fact, pollen is formed in the tapetum of anther which is responsible for providing nutrients to developing pollen grains[9], supports the thesis that the allergen has tapetum origin. However, one cannot deny the possibility that the allergen is secreted from pollen grain and adsorbed onto the surface. To address this, it will be necessary to follow the occurrence of allergen during the pollen formation.

Grote *et al.*[11], employed similar methods to detect antigenic proteins of pollens of different

species from ours. They detected antigens in the cytoplasm, which is in sharp contrast to our results. Knox[15] and Knox *et al.*[16], performed similar experiments detecting antigens in intine. Immunological meaning of these differences would be the subject of future investigations.

Pollen antigens that can be recognized by patients' IgE are more likely relevant to pathogenecity of pollinosis. Our preliminary experiments failed to immunostain pollen antigens with patients' IgE probably due to its minute amounts compared to IgG and IgM. Our laboratories are currently improving the reagents to overcome such inherent problem.

The cross-reactivity between HMA and *Cry j* I has been suggested from immunochemical evidences[4-8]. This is further strengthened by the present result ; anti-HMA antibody reacted with the surface of Sugi pollen as well as Hinoki pollen, and anti-*Cry j* I antibody reacted the surface of Hinoki pollen as well as Sugi pollen. This antigen sharing by two major pollens in Japan cannot be ignored from an etiological point of view, because Hinoki pollinosis can be inducted by not only Hinoki pollen but also Sugi pollen, and *vice versa*. Therefore Sugi pollinosis patients are recommended to avoid Hinoki pollen as well.

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ヒノキおよびスギ花粉の主要抗原は花粉壁に存在する

高橋優三[1)]，井手　武[2)]，芦田恒雄[3)]，田端司郎[2)]，荒木恒治[1)]
[1)]奈良県立医科大学　寄生虫学教室，〒634　橿原市四条町840
[2)]奈良県立医科大学　化学教室，〒634　橿原市四条町840
[3)]芦田耳鼻咽喉科医院，〒577　東大阪市小阪3 - 4 -51

スギの主要抗原（*Cry j* I）とヒノキの主要抗原（HMA）の花粉に於ける局在性をコロイド金による免疫電顕法で検索した．ウサギを免疫して作製した抗 *Cry j* I 抗体，抗 HMA 抗体による免疫染色陽性部位はいずれもユービシュ体，sexine，nexine であった．細胞質は免疫染色陰性であった．コントロール血清も免疫染色陰性であり染色の特異性が裏づけられた．故に，スギとヒノキ花粉の主要抗原は花粉壁に存在するものと判断された．この抗原性はスギ花粉症の患者血清によっても認識された．また従来スギとヒノキ花粉について報告されていた抗原性の交差が免疫電顕法によっても確認された．すなわち抗 *Cry j* I 抗体によりヒノキ花粉壁が免疫染色され，抗 HMA 抗体によりスギ花粉壁が同じく免疫染色された．