

Assay for detecting IgE and IgG antibodies against *Candida albicans* cell-wall mannan

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In the present study, we assayed mannan-specific IgE and IgG antibodies in samples of serum isolated from blood collected from adult patients with bronchial asthma, using a liquid-phase method with a polysaccharide, mannan (Mn), purified from *Candida albicans* (*C. alb*), and investigated the relationships of allergenicity among a crude extract of *C. alb*, purified Mn, and acid protease (AP). The correlations between the titers of anti-Mn A and anti-Mn B IgE and IgG were very strong, and the levels of inhibition of anti-Mn A IgE and IgG reactions by Mn A and Mn B were almost identical. Although no common allergenicity was observed between Mn A and AP because there was no correlation between the titers of anti-Mn A and anti-AP IgE, and no inhibition of the anti-Mn A IgE reaction by AP, both antigens were found to exist in crude *C. alb*. The level of inhibition of anti-crude *C. alb* IgG reaction by Mn A or Mn B was about 60%.

Approximately 70% inhibition of the anti-Mn A IgE reaction was observed for eight different fungal allergen extracts, but no inhibition was observed for 11 of the other fungal allergen extracts tested. The above results indicate that common antigenicity was observed between Mn A and Mn B in the human IgE and IgG antibody production system, and the cross-allergenicity observed among some fungi was considered to be the result of the common antigenicity of Mn isoforms.

In addition to mites and pollen, fungi are considered to be major sources of allergens in allergic disorders such as bronchial asthma; however, the relationship between the allergenicities of fungi and the pathophysiology of allergic disorders is unclear in comparison to mites. *Candida albicans* (*C. alb*) is a yeast-like fungus found in the human oral cavity, gastrointestinal tract, and vaginal mucosa as part of the normal flora. We have reported the significance of an acid protease (AP) as a major allergen in mucosal atopic reactions (1, 2). *C. alb* exists in the host mucosa and secretes enzymes, such as AP and a phospholipase, which degrade secretory IgA and IgG – these immunoglobulins attack *C. alb* as part of the host's immune defense system. The production of IgE antibodies against these enzymes leads to atopic reactions.

Mannan (Mn), a component of cell-wall polysaccharides, is known to be a fungal antigen; in particular, in terms of the serotyping of *C. alb*. A number of studies have been carried out on polysaccharides as fungal antigens (3–7). However,

there have been few reports on Mn as an atopic allergen due to its reactivity to IgE, because of the difficulty of detecting IgE antibodies against this compound.

In this study, we developed an assay to detect IgE and IgG antibodies against Mn, using a liquid-phase method with Mn antigens purified from *C. alb* to investigate the role of Mn in IgE-mediated allergy.

Material and methods

Subjects

The subjects were adult patients with bronchial asthma who attended the allergy section, National Sagami Hospital, and tested positive in the immediate hypersensitivity skin reaction test against crude *C. alb* antigen extract (Torii Pharmaceutical, Japan). Sera obtained from 21 nonatopic healthy subjects who tested negative in this test were used as controls. Serum samples were stored at –20°C before use.

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C. alb mannan (Mn)

Two purified mannans, *C. alb* NIH A-207 (serotype A) mannan (Mn A) and *C. alb* NIH B-792 (serotype B) mannan (Mn B) (gifts from Prof. S. Suzuki, Tohoku Pharmacology University), were used (8–10). Mn A consists of 95% mannose, 1–2% protein, and 0.7–0.8% phosphate, and has a specific rotation of +41.2° (8). Mn B consists of 89.7% mannose, 3.1% protein, and 1.48% phosphate, and has a specific rotation of +63.1°C (9).

C. alb acid protease (AP)

The AP used did not have a saccharide side-chain and was purified from *C. alb* by the method of Yamamoto et al. (11, 12). Briefly, production of AP was induced in YCB-BSA medium, and incubation was continued until the proteolytic activity of the culture medium reached a maximum level. Acid protease was purified from the proteases secreted by *C. alb* strain no. 114, which was obtained from the Research Center for Medical Mycology, Teikyo University School of Medicine, Tokyo, Japan. The cell-free broth was loaded onto a DEAE Sephacel column and eluted. Fractions containing proteolytic activity were pooled, and further purification was carried out by the simplified method of Ruchel et al. (13). Approximately 15 mg of AP was recovered from 2 l of culture. The molecular weight of this protease was approximately 44 kDa. Its isoelectric point was pH 4.2 and optimal pH for enzymatic activity was 3.2. It was stable below pH 7.0 and lost its activity above pH 8.0 upon incubation at 37°C for 60 min. AP was stored lyophilized at –80°C before use.

IgE and IgG antibody assay

An enzyme immunoassay method (AlaSTAT method) with liquid-phase polysaccharide allergens was used to assay IgE and IgG antibodies (14). Briefly, 150 nM of matrix-OH (soluble dextran or soluble Ficoll) was dissolved in 4.0 ml of 0.3 M sodium hydroxide containing 0.26 mM sodium borohydride and reacted with 2.7 mM 1,4-butanedioldiglycidyl ether for 4 h at room temperature. The activated matrix was then adjusted to pH 8.5. Lyophilized allergen extract was directly coupled to the activated matrix, and the amount of allergen added to the reaction was adjusted to ensure the availability of oxirane groups for the subsequent coupling of polylysine biotin. Ligand-coated tubes were prepared by immobilizing biotinylated protein on 12×75 mm polystyrene tubes by a modification of the method of Engvall & Perlmann (15). A volume of 50 µl of a patient's serum sample was

first incubated in a ligand-coated tube for 1 h with 100 µl of an individual allergen. During this liquid-phase reaction, IgE (IgG) specific for the given allergen bound to the allergen on the liquid matrix. The allergen-IgE (IgG) complex thus formed in the liquid phase was then reacted for 1 h with 50 µl of avidin, which, during this second incubation period, bound to the biotin-matrix-allergen-IgE (IgG) complex and also to the biotin on the coated tube, thereby forming a bridge between the solid phase and the reacted IgE (IgG). The amount of specific IgE (IgG) that was immobilized on the tube was quantitated by adding 200 µl of horseradish peroxidase-conjugated monoclonal anti-IgE (IgG) antibody. This reaction was allowed to proceed for 1 h, and then excess enzyme was washed away. Enzyme substrate (500 µl) was added and allowed to react with the bound enzyme for 15 min, producing an intensity of color directly proportional to the amount of endogenous IgE (IgG) specific for the allergen.

Inhibition of IgE antibody reaction

A volume of 50 µl of serum (from patient no. 73, who exhibited an anti-Mn A IgE antibody titer of 4.96) was incubated with 10 µl of inhibitors (Mn A, Mn B, crude *C. alb* extract, and AP) at various concentrations, and anti-Mn A IgE antibody was assayed. Mn A or Mn B (1.67 mg/ml) was used as inhibitor at ratios of 1:1, 1:2, 1:10, and 1:50. AP (0.24 mg/ml) and 0.74 mg/ml of crude *Candida* extract were used at ratios of inhibitor to Mn A in the anti-Mn A IgE antibody assay of 1:1, 1:10, and 1:50.

Inhibition of IgG antibody reaction

Volumes of 50 µl of serum samples (nos. 106, 111, 151, and 161), which showed high titers of anti-crude *C. alb* extract and anti-Mn A IgG, were incubated with 10 µl of inhibitors (Mn A and Mn B) at 10 mg/ml, and anti-crude *C. alb* extract and anti-Mn A IgG antibodies were assayed.

Inhibition of IgE antibody reaction with antigens pretreated with pronase K

To determine whether the protein component of Mn affects the results of the Mn-specific IgE antibody assay, we inhibited the IgE antibody reaction with Mn pretreated with pronase. Volumes of 50 µl of Mn A (10 mg/ml) and AP (1.3 mg/ml) were incubated with 40 µg/ml of pronase K (Boehringer Mannheim, Germany) at 37°C for 5 h and then at 100°C for 5 min before use as inhibitors. The serum samples positive for anti-Mn A IgE (nos. 3, 5, 10, and 489) or anti-AP IgE (no. 22) were

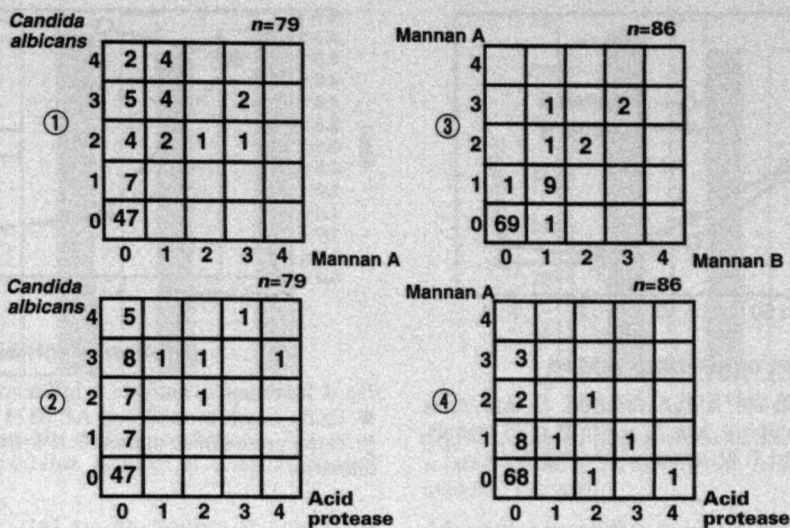


Fig. 1. Correlation between IgE antibody titers: 1) crude *C. alb* vs Mn A; 2) crude *C. alb* vs AP; 3) Mn A vs Mn B; 4) Mn A vs AP. Numerals on vertical and horizontal axes represent scores of IgE antibody titers.

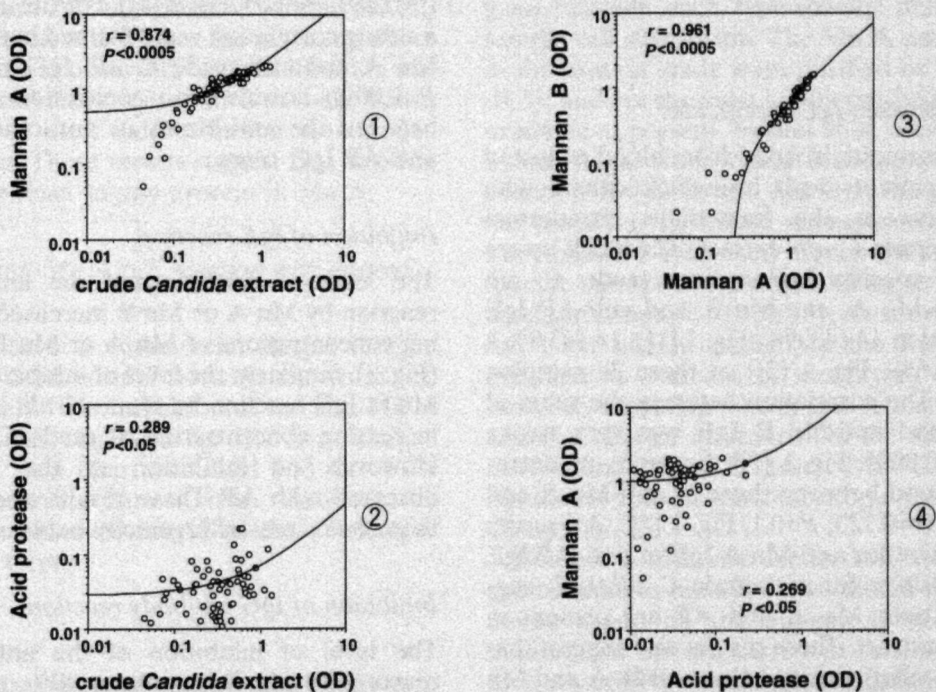


Fig. 2. Correlation between IgG antibody titers: 1) crude *C. alb* vs Mn A; 2) crude *C. alb* vs AP; 3) Mn A vs Mn B; 4) Mn A vs AP. Numerals on vertical and horizontal axes represent optical density as measure of IgG antibody titers.

used in the anti-Mn A IgE assay and anti-AP IgE assay, respectively.

Inhibition of IgE antibody reaction with several fungal allergens

To determine the cross-reactivity of *C. alb* Mn among fungal allergens, we used various fungal allergens as inhibitors. Sera from four individuals

(nos. 28, 71, 79, and 86) who were positive for anti-crude *C. alb* IgE (13.38, 1.91, 0.86, and 57.35 IU/ml, respectively) and anti-Mn A IgE (4.61, 4.00, 2.33, and 1.52 IU/ml, respectively) were pooled. A volume of 50 μ l of pooled serum and 10 μ l of various fungal allergen extracts were incubated at 4°C overnight, after which anti-Mn A IgE was assayed. The inhibitors used in the study were fungal allergens extracted from *C. alb*, *Aspergillus*

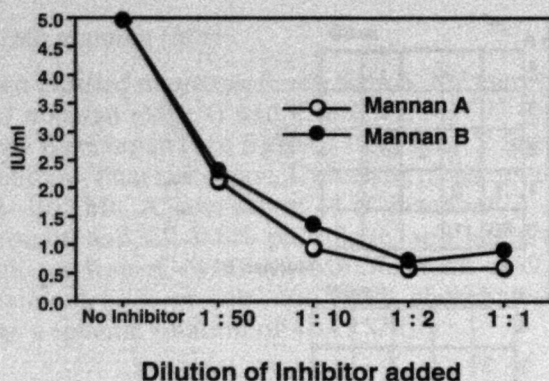


Fig. 3. Inhibition of anti-Mn A IgE reaction. ○: Mn A as inhibitor; ●: Mn B as inhibitor. Mn A or Mn B (1.67 mg/ml), diluted 1:1, 1:2, 1:10, and 1:50, was used as inhibitor.

fumigatus, *Penicillium luteum*, *Alternaria kikuchi*, and *Cladosporium cladosporioides* (Torii Pharmaceutical, Japan) and 17 other fungal allergens (Greer, USA).

Results

Correlation between IgE specificities

Of 79 serum samples isolated from blood collected from adult patients with bronchial asthma who tested positive in the immediate intradermal response to crude *C. alb* extract, 32 (40.5%) were positive for specific IgE against crude *C. alb* extract. Anti-Mn A, anti-Mn B, and anti-AP IgE were detected in 14 (43.8%, Fig. 1 [1]), 14 (43.8%), and five (15.6%, Fig. 1 [2]) of these 32 samples, respectively. The correlation between the titers of anti-Mn A and anti-Mn B IgE was very strong ($r=0.914$, $P<0.0005$, Fig. 1 [3]); however, no correlation was found between those of anti-Mn A and anti-AP IgE ($r=0.123$, $P>0.1$, Fig. 1 [4]). All serum samples positive for anti-Mn A IgE or anti-AP IgE were also positive for anti-crude *C. alb* IgE, suggesting that both Mn A and AP are present in crude *C. alb* extract. These results also suggest that there is cross-allergenicity between Mn A and Mn B, but no cross-allergenicity between Mn A and AP. For the 21 control sera, the titers of anti-MnA, anti-AP, and anti-crude *C. alb* IgE were extremely low, less than 0.3 IU/ml.

Correlation between IgG specificities

Fig. 2 shows the correlation between titers of IgG against various antigens (crude *C. alb*, Mn A, Mn B, and AP) for 65 serum samples. Strong correlation was observed between the titers of anti-Mn A and anti-crude *C. alb* IgG ($r=0.874$, $P<0.0005$, Fig. 2 [1]) and between those of anti-Mn A and anti-Mn B IgG

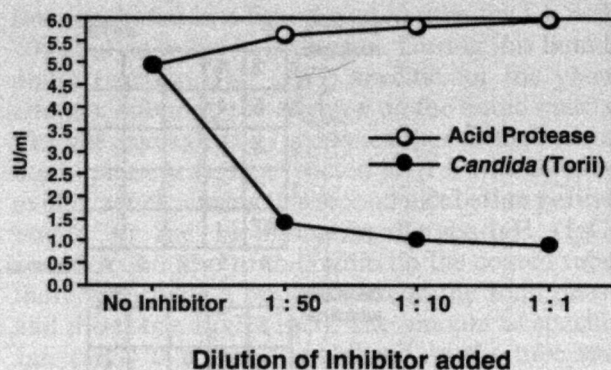


Fig. 4. Inhibition of anti-Mn A IgE reaction. ○: AP as inhibitor; ●: crude *C. alb* as inhibitor. AP (0.24 mg/ml) or crude *C. alb* (0.73 mg protein/ml), diluted 1:1, 1:10, and 1:50, was used as inhibitor.

($r=0.961$, $P<0.0005$, Fig. 2 [3]); however, no correlation was found between those of anti-AP and anti-crude *C. alb* IgG ($r=0.289$, $P<0.05$, Fig. 2 [2]) or between those of anti-AP and anti-Mn A IgG ($r=0.269$, $P<0.05$, Fig. 2 [4]). For the 21 control sera, a strong correlation was observed between the anti-Mn A and anti-crude *C. alb* IgG titers ($r=0.939$, $P<0.001$); however, no correlation was observed between the anti-Mn A or anti-crude *C. alb* and anti-AP IgG titers.

Inhibition of IgE reaction

The level of inhibition of the anti-Mn A IgE reaction by Mn A or Mn B increased with increasing concentrations of Mn A or Mn B, respectively (Fig. 3). Similarly, the level of inhibition of the anti-Mn A IgE reaction by crude *C. alb* increased with increasing concentration of crude *C. alb* (Fig. 4). However, no inhibition of the reaction was observed with AP. These results also suggest that there is no cross-allergenicity between Mn and AP.

Inhibition of IgG antibody reaction

The level of inhibition of the anti-Mn A IgG reaction by Mn B was about 90%, similar to that by Mn A (Fig. 5). The level of inhibition of the anti-crude *C. alb* IgG reaction by Mn A or Mn B was about 60% (Fig. 6).

Inhibition of IgE reactions with antigens pretreated with pronase K

For determination of the effect of any protein present as a contaminant in Mn A on the IgE assay system, Mn A was pretreated with pronase K and then used as an inhibitor in the anti-Mn A IgE assay. For serum samples which were positive for anti-Mn A IgE (nos. 3, 5, 10, and 489) (Table 1),

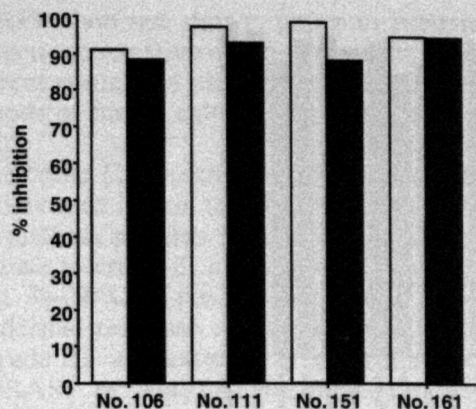


Fig. 5. Inhibition of anti-Mn A IgG reaction. □: Mn A as inhibitor; ■: Mn B as inhibitor. Amount of 10 mg/ml of each inhibitor was used.

the level of inhibition by Mn A pretreated with pronase K was similar to that by Mn A pretreated with PBS. In contrast, when the assay was performed with anti-AP IgE-positive serum (no. 22) and AP pretreated with PBS as the inhibitor, about 80% inhibition was observed, but no inhibition was observed when AP pretreated with pronase K was used. Pronase K itself did not inhibit the anti-Mn A IgE reaction. These results suggest that anti-Mn A IgE did not react to any protein in Mn A.

Inhibition of anti-Mn A IgE reaction with various fungal allergens

In this assay, several commercial fungal allergen extracts were used as inhibitors. As shown in Fig. 7, approximately 70% inhibition of the anti-Mn A IgE reaction was observed for the five allergen extracts from Torii Pharmaceutical and six of the 17 allergen extracts from Greer. However, no inhibition was observed for 11 of the 17 allergen extracts from Greer.

Discussion

We showed that assay for specific IgE (IgG) antibodies against polysaccharides such as Mn is possible by using the liquid-phase *in vitro* allergen-specific IgE (IgG) assay (AlaSTAT) outlined in this paper.

Hasenclever & Mitchell first reported that *C. alb* is serologically heterogeneous (3–5). They found that *C. alb* may be classified serologically into types A and B, and that the main *C. alb* antigen is a water-soluble polysaccharide, Mn, present on the cell wall and formed by polymerization of D-mannose. Since Mn shows serologic specificity according to the fungal strain, analysis of Mn is useful for classifying and identifying yeasts. Serotype A *C. alb* is distin-

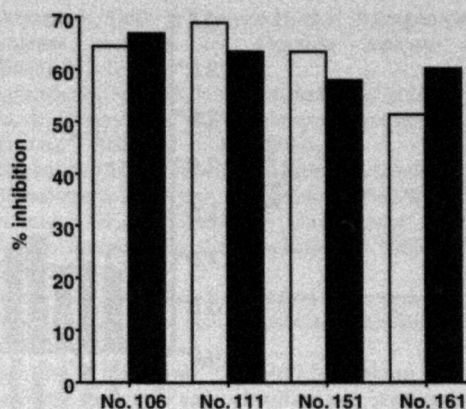


Fig. 6. Inhibition of anti-crude *C. alb* IgG reaction. □: Mn A as inhibitor; ■: Mn B as inhibitor. Amount of 10 mg/ml of each inhibitor was used.

guished from serotype B by a β -1,2-linked branch (factor 6). Determination of the serotype is based on the results of an agglutination reaction on a glass glass between each type-specific antibody and a sample cell suspension. The Mn A and Mn B used in the present study were purified by Suzuki et al. (8, 9), and are the most highly purified types of Mn available at present. Suzuki et al. conducted many immunologic studies and found that a structure common to Mn A and Mn B is the inner part of the branch, α -1,2-linked mannotetraosyl, and that Mn A has a β -1,2-linked branch which is a type A specific epitope at the nonreducing terminal of this common structure (8–10, 16). As shown above, Mn has been widely studied in the field of *C. alb* infection as a means of identifying causal fungi; however, there have been few reports of Mn as a cause of allergic disorders. On the other hand, we have found that AP secreted by *C. alb* plays a significant role in mucosal atopic reactions as an allergen (1, 2).

Because a cellulose carrier activated by cyanogen bromide is used in conventional IgE assays, an amino group on the antigen is necessary as a binding site for the carrier in such assays. Therefore, a purified polysaccharide cannot bind to the carrier, making it impossible to detect an IgE antibody against a purified polysaccharide. Since the *in vitro* liquid-phase, allergen-specific IgE assay (AlaSTAT) involves use of a liquid matrix and a coupling agent, a purified polysaccharide can bind to the matrix, enabling assay for an antibody against the purified polysaccharide (14).

As mentioned above, the Mn types used as allergens in this study are the most highly purified available; however, the possibility of their contamination by protein cannot be excluded. The inhibitory activity of Mn A in the anti-Mn A IgE assay was not decreased even when the Mn A was

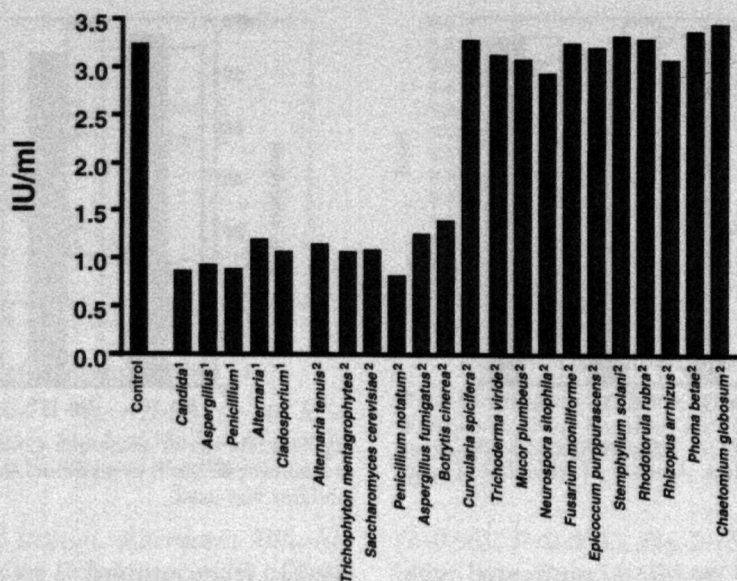


Fig. 7. Inhibition of anti-Mn A IgE reaction by various fungal antigens. 1) Commercial extracts purchased from Torii Pharmaceutical; 2) commercial extracts purchased from Greer.

Table 1. Results of IgE assay performed after pretreatment of antigens with protease

Inhibitor	(IU/ml)				
	No. 3	No. 489	No. 5	No. 10	No. 22
Mannan A + PBS	0.33	0.77	0.93	0.55	
Mannan A + pronase K	0.25	0.01	1.03	0.49	
H ₂ O + PBS	1.17	4.97	8.71	17.32	13.24
H ₂ O + pronase K	1.13	4.93	8.71	16.44	13.39
Acid protease + PBS					2.96
Acid protease + pronase K					14.09

Nos. 3, 489, 5, and 10: serum samples positive for anti-Mn A IgE; no. 22: serum sample positive for anti-AP IgE.

pretreated with pronase K, a protein degradation enzyme, as shown in Table 1. Thus, it is clear that we detected specific IgE against Mn A. In contrast, the inhibitory activity of the AP decreased significantly when it was pretreated with pronase K, suggesting that specific IgE against AP was detected in the anti-AP IgE assay. Our results show that the correlations between the titers of anti-Mn A and anti-Mn B IgE and IgG were very strong (Fig. 1 [3], Fig. 2 [3]), Mn B inhibiting anti-Mn A IgE (Fig. 3) and IgG (Fig. 5) reactions, and suggest that a structure common to Mn A and Mn B was recognized as the main epitope by IgE and IgG. No correlation was found between the titers of anti-Mn A and anti-AP IgE (Fig. 1 [4]) and IgG (Fig. 2 [4]). Mn A and AP clearly have completely different allergenicity/antigenicity. All of the serum samples positive for anti-Mn A IgE or anti-AP IgE were also positive for anti-crude *C. alb* IgE (Fig. 1

[1] and [2]). In addition, the results of the inhibition of the anti-Mn A IgE reaction by crude *C. alb* (Fig. 4) and our previous findings (2) indicate that both Mn A and AP are present in crude *C. alb* extract.

Some of our patients tested positive in the immediate intradermal response to several fungal allergens. To investigate the degree of cross-allergenicity among fungal allergens, we studied inhibition of the anti-Mn A IgE reaction with various commercial fungal allergen extracts as inhibitors. Similar inhibition to that observed with crude *C. alb* extract was found for five different fungal allergen extracts from Torii Pharmaceutical and six different fungal allergen extracts from Greer, as shown in Fig. 7. This result indicated that the cross-allergenicity among these different fungal allergens is due to the allergenicity of Mn. However, the other fungal allergen extracts from Greer such as those of *Curvularia spicifera*, *Trichoderma viride*, *Mucor plumbeus*, *Neurospora sitophila*, *Fusarium moniliforme*, *Epicoccum purpurascens*, *Stemphylium solani*, *Rhodotorula rubra*, *Rhizopus arrhizus*, *Phoma betae*, and *Chaetomium globosum* did not inhibit the anti-Mn A IgE reaction; identification of fungal species producing cross-allergenic mannan requires future study. The results of a mouse passive cutaneous anaphylaxis inhibition assay have confirmed that there is no common antigenicity among AP and other fungal allergens (data not shown; paper in preparation). We also compared the composition of AP in crude *C. alb* extracts from different suppliers such as Torii Pharmaceutical, Greer, and Hollister-Stier and showed that there is a marked difference between

the APs (data not shown; paper in preparation). Allergen extracts from different suppliers might have different content of allergens due to the different methods of fungal culture and allergen extraction used.

The crude *C. alb* extract of Torii Pharmaceutical is widely used in skin tests in Japan. About 50% of adult asthma patients show a positive immediate response to crude *C. alb* extract, but usually only about 30–50% of positive patients in immediate intradermal response exhibit serum IgE antibody to crude *C. alb* extract, as determined by the CAPRAST or AlaSTAT methods. On the other hand, the response to house-dust-mite or Japanese cedar-pollen allergens in the intradermal skin test and serum IgE antibody level are highly correlated even if the allergen extracts used are different. Thus, the reason that only 40% (32/79) of skin-positive subjects had serum IgE antibody to crude *C. alb* extract in this study is not yet known. One reason may be the higher sensitivity of the intradermal skin test than the serum IgE antibody assay or the occurrence of a non-IgE-mediated response to fungi. The significance of Mn as a causal allergen in allergic disorders has not been clarified; thus, further study is required.

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