

Allergenecity of acid protease secreted by *Candida albicans*

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We have previously reported the cases of *Candida albicans* (*C. alb*) acid protease (CAAP)-induced atopic asthma. In this study, the allergenicity of the released enzyme CAAP was examined among asthmatic patients with positive immediate skin response to crude *C. alb* antigen. Among 49 patients with positive skin response to crude *C. alb*, anti-crude *C. alb* IgE antibodies were detected in 40 and anti-CAAP IgE antibodies were detected in 18. Moreover, anticrude *C. alb* IgE antibodies were detected in all of the patients in whom anti-CAAP IgE antibodies were detected. No correlations between IgG antibodies to both antigens or between IgE and IgG antibodies to CAAP were observed. CAAP induced significant T-cell proliferation in 20/28 patients showing positive T-cell proliferation response to crude *C. alb* antigen. Most of the patients showing positive conjunctival response to crude *C. alb* antigen also showed positive response to CAAP. Most of the patients showing high levels of serum IgE antibody and positive histamine-release response of peripheral blood leukocytes to CAAP showed positive conjunctival response. The results indicate that CAAP is an important allergen in *C. alb*-related mucosal allergy.

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The yeast *Candida albicans* (*C. alb*) is a common commensal of the human oral and vaginal mucosae and gastrointestinal tract, where it is present as part of the normal flora. *C. alb* has been reported to secrete acid protease (CAAP), which cleaves human defensive proteins such as secretory IgA, leading to the persistence of yeasts colonizing mucous membranes (1). CAAP has been investigated from the aspect of virulence in opportunistic infection and dermatology.

C. alb is also known as an important allergen in cases of atopic asthma (2, 3). In Japan, *C. alb* antigen is widely used in allergy skin tests. There have been several reports describing the allergenic components of *C. alb* which react with IgE (4–8).

We have reported the cases of two atopic asthma patients allergic to CAAP (9). Here we report that CAAP is an important *C. alb*-related allergen inducing IgE-mediated mucosal allergy in man.

Material and methods

Study population

The patients included in this study were bronchial asthmatics who showed positive immediate skin reaction to crude *C. alb* antigen. Sera collected from these patients were stored at –20°C before use.

Antigen preparation

C. albicans strain no. 114 was obtained from the Research Center for Medical Mycology, Teikyo University School of Medicine, Tokyo, Japan. Production of CAAP was induced with YCB-BSA medium, and incubation was continued until the proteolytic activity of the culture medium reached a maximum level. CAAP was purified from the protease secreted by *C. alb* strain no. 114 by the

method of Yamamoto et al. (10). 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 a simplified method of Ruchel et al. (1). Approximately 15 mg of CAAP 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 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. This acid protease was secreted by 140/140 strains of *C. alb* tested, 13/18 strains of *C. tropicalis*, 11/18 strains of *C. parapsilosis*, 1/3 strains of *C. zelanoides*, 1/5 strains of *C. lisitaniae*, and 1/12 strains of *C. guilliermondii*. No other *Candida* species or yeasts tested secreted acid protease. CAAP used in this study migrated as a single 44-kDa protein band in SDS-PAGE (Fig. 1). CAAP was stored as lyophilized at -80°C before clinical use. Crude *C. alb* antigen was supplied by Torii Pharmaceutical Co. (Tokyo, Japan).

CAAP has been termed "SAP" (secreted aspartyl proteinase) since the meeting on "Candida and candidiasis" held by the American Society for Microbiology in 1993 (11). However, in this paper, we use the term "CAAP" instead of "SAP", because we have been using the former term since we started this series of our work in 1990.

Measurement of IgE antibody

Anti-crude *C. alb* and anti-CAAP IgE antibodies were measured by the radioallergosorbent test (RAST), as described previously (12). Solid-phase antigens were prepared by linking either 50 µg/ml of purified CAAP or 2 mg/ml of crude *C. alb* antigen to 200 cyanogen bromide-activated paper disks in 10 ml of borate-buffered saline (pH 8.5). Radiolabeled anti-IgE was obtained from Pharmacia Diagnostics (Phadebas RAST kit, Uppsala, Sweden). RAST results were expressed as percentage of total counts (percentage binding), which was calculated by dividing the counts bound by the total counts added. A percentage of binding of more than twice the mean value of three healthy controls (1.2% for crude *C. alb* and 1.0% for CAAP) was scored as positive.

Measurement of IgG antibody

Measurement of IgG antibody was performed by a protein A solid-phase radioimmunoassay (RIA) (13) with a slight modification (14). Briefly, an antigen-coupled glass rod was incubated with 100 µl of diluted serum for 4 h at room temperature. The glass rod was then washed, and 10 ng of

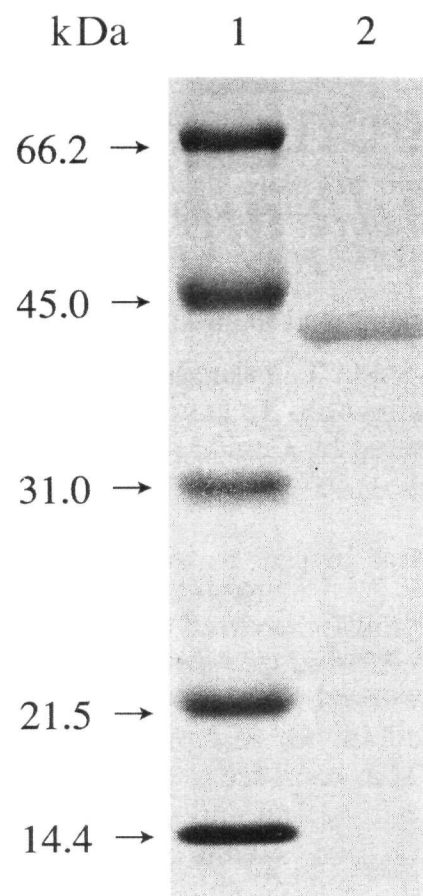


Fig. 1. Purity of CAAP on SDS-PAGE. CAAP used in this study migrated as single 44-kDa protein band on SDS-PAGE.

¹²⁵I-labeled protein A (approximately 100 000 cpm, Amersham, Buckinghamshire, UK) in 100 µl was added. After overnight incubation at room temperature, the glass rod was washed, and bound radioactivity was quantitated in a gamma counter. A pool of five sera with high IgG antibody activity for ovalbumin (OVA) was arbitrarily defined as containing 100 units per milliliter (U/ml) of anti-OVA IgG. Serial dilutions of this anti-OVA reference serum with OVA-coupled glass rods were run in parallel with each assay. The specific IgG antibody titers were calculated by interpolation from the dilution curve of the reference serum.

Histamine release from peripheral blood leukocytes

Mixed leukocytes were prepared from citrated blood of the patients by the dextran sedimentation procedure, as described previously (15). These mixed leukocytes were challenged with a series of concentrations of crude *C. alb*, CAAP, or anti-IgE as positive control *in vitro*. A release of histamine of more than 10% of total histamine content after subtraction of spontaneous histamine release was judged positive. CAAP and crude *C. alb* were found not to induce histamine release from the peripheral blood leukocytes of normal control subjects.

Lymphocyte stimulation test

Patients' peripheral blood mononuclear cells were stimulated with 1–100 µg/ml of crude *C. alb*, CAAP, or 10 µg/ml of PHA-P as positive control. ³H-labeled TdR uptake was measured after 4-day culture, and an uptake of at least 2.0 times that of the control medium was scored as positive.

Conjunctival provocation test (CPT)

A volume of 100 µg/ml of CAAP and 1 : 400 w/v crude *C. alb* were used for CPT. One conjunctiva was used as a control and was challenged with diluent, and the other was challenged with crude *C. alb*. After the response to crude *C. alb* was judged, the control side was challenged with CAAP. These antigens were tested on several normal controls and elicited no conjunctival response. The results of this test were judged positive when two or more of three signs and symptoms, namely, conjunctival itching, hyperemia, and edema, appeared within 15 min after the antigen challenge of the conjunctiva. The results of CPT have been shown to be strongly correlated with the results of the bronchial provocation test with *C. alb* (2, 3). The CPT was approved by the ethics committee of National Sagamihara Hospital.

Results

Conjunctival provocation test (CPT)

The CPT was performed on 68 patients with positive skin response to crude *C. alb*. Among these patients, eight (11.8%) showed a positive response to crude *C. alb* and six of these (75%) also showed positive response to CAAP. One out of the 60 patients showing a negative response to crude *C. alb* (1.7%) showed a positive response to CAAP (Table 1).

Table 1. Results of conjunctival provocation test

	Crude <i>C. albicans</i> extract		Total
	+	–	
<i>C. albicans</i> acid protease			
+	6*	1**	7
–	2	59	61
Total	8	60	68

*Six of eight (75%) patients showing positive CPT response to crude *C. albicans* showed positive CPT response to CAAP.

**Only 1/60 (1.7%) patients showing negative CPT response to crude *C. albicans* showed positive CPT response to CAAP.

CAAP-specific IgE and IgG antibodies

Among 49 patients with positive skin response to crude *C. alb*, anti-crude-*C. alb* IgE antibodies were detected in 40 and anti-CAAP IgE antibodies were detected in 18. Moreover, anti-crude-*C. alb* IgE antibodies were detected in all of the patients in whom anti-CAAP IgE antibodies were detected. These results suggest that CAAP is the component of crude *C. alb* which induces IgE antibody production (Fig. 2).

The specific IgG antibodies to crude *C. alb* and CAAP were also measured in the same sera (Fig. 3). A normal value or cutoff point of IgG antibodies was not determined because most patients are probably sensitized to *C. alb*, which is a common commensal among the normal flora in the human body. The horizontal and vertical lines in Fig. 3 show mean values of three normal controls as a reference. No correlations between IgG antibodies to both antigens or between IgE and IgG antibodies to CAAP were observed.

Presence of CAAP-reactive T cells

Stimulation of patients' peripheral blood mononuclear cells with crude *C. alb* induced significant T-cell proliferation in all 28 patients tested, 20 of whom showed a similar response to CAAP. This result suggests that CAAP-reactive T cells are present in *C. alb*-allergy patients (Fig. 4).

Histamine release from peripheral blood leukocytes

Peripheral blood leukocytes were challenged with a series of concentrations of crude *C. alb*, CAAP, and anti-IgE. A release of histamine of more than 10% of total histamine content after subtraction of spontaneous histamine release was judged positive. The leukocytes from most of the patients with positive CPT showed release of large amounts of histamine upon stimulation with crude *C. alb* or CAAP (Fig. 5).

Discussion

C. albicans is part of the endogenous flora of mucocutaneous regions in human and animal hosts. It usually colonizes the digestive tract, and may be present in other anatomic regions, such as the larynx, upper airways, or vagina. Depending upon the host immune condition, the fungus may act as an opportunistic pathogen (16). *C. alb* is known to produce cytolytic enzymes such as proteases (1, 17) and phospholipases (18). The acid protease of *C. alb* cleaves IgA1, IgA2, and secretory IgA, all of which play a role in mucosal immunity (1). It is

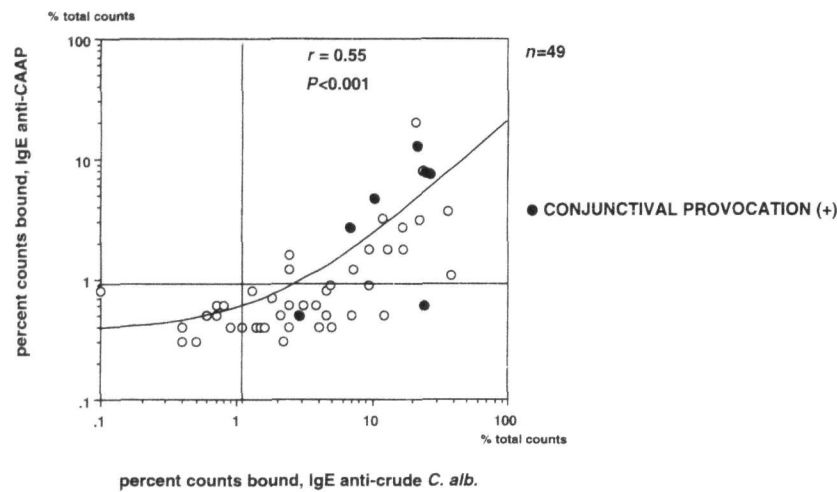


Fig. 2. IgE antibody to crude *Candida albicans* (*C. alb*) and *C. albicans* acid protease (CAAP). Horizontal and vertical lines show twice mean values of three normal controls. Values greater than these were scored as positive. Closed circles indicate patients scored as positive on conjunctival provocation test.

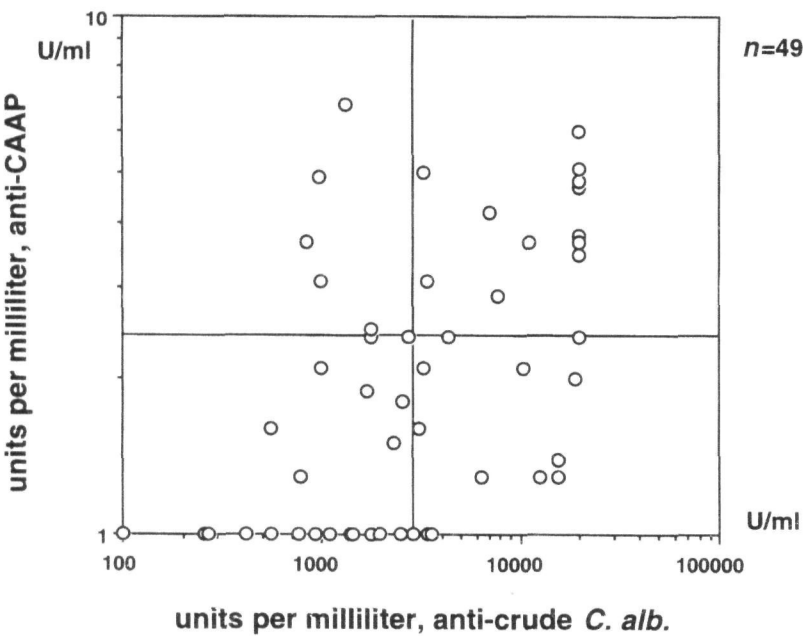


Fig. 3. IgG antibody to crude *Candida albicans* (*C. alb*) and *C. albicans* acid protease (CAAP). Horizontal and vertical lines show mean values of three normal controls.

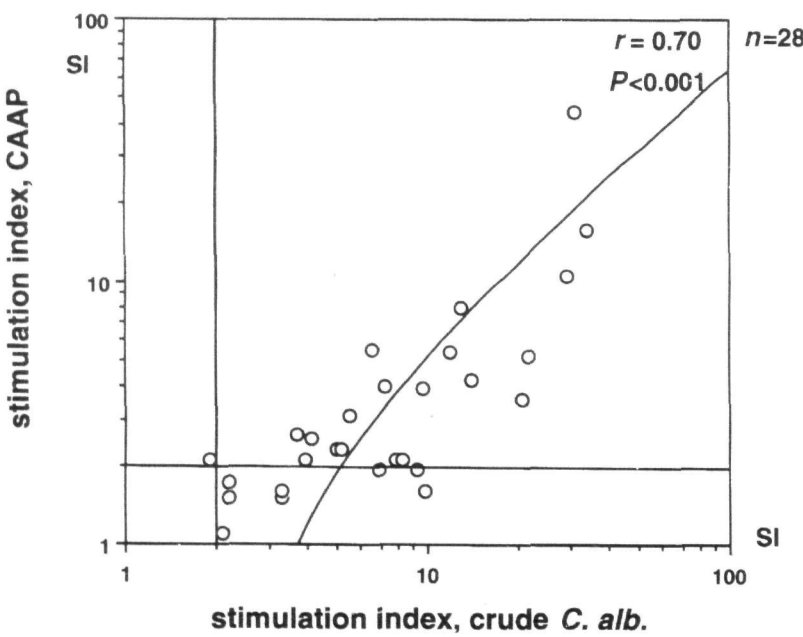


Fig. 4. Results of lymphocyte stimulation test.

possible that CAAP produced by *C. alb* destroys immunoglobulins by cleavage and hence improves

conditions for its localized colonization or eventual systemic infection (1). These *C. alb*-related enzymes have been widely investigated for their role in the pathogenicity of fungal infections (1, 17, 18). However, we have been unable to find any published report describing the relationship between these enzymes and allergy.

Immunochemical studies on *Candida* species have mainly aimed at classifying and identifying these yeasts for taxonomic and diagnostic purposes. Although the members of the genus *Candida* have a complex array of cell-wall and cytoplasmic antigens, species-specific immunodominants have been identified by various immunochemical techniques. Mannan is one of the major components of the yeast cell wall and is also the immunodominant structure on that wall. Cytoplasmic antigens of 40–50 kDa have been proposed as major *Candida* antigens, but mostly from the viewpoint of *Candida* infection (19–21). There have been few reports on the allergenic components of *C. albicans* (4–8), and most of these reports focused on cytoplasmic fractions as the allergen sources. However, in the host-parasite relationship, an enzyme such as CAAP, released from *C. alb* when the yeast begins to colonize or invade host mucocutaneous regions, might be an important allergen in inducing the host atopic response as a defense mechanism.

We reported the cases of two asthma patients allergic to CAAP (9). Both had high levels of serum IgE and specific IgE antibodies to both CAAP and crude *C. alb*, and both showed positive CPT to CAAP as well as the positive response of peripheral leukocyte histamine release when challenged with CAAP or *C. alb*. Since both of them showed a positive immediate bronchial response to CAAP, we diagnosed these patients as atopic asthmatics allergic to CAAP.

In this paper we examined the allergenicity of CAAP among asthmatic patients who showed a

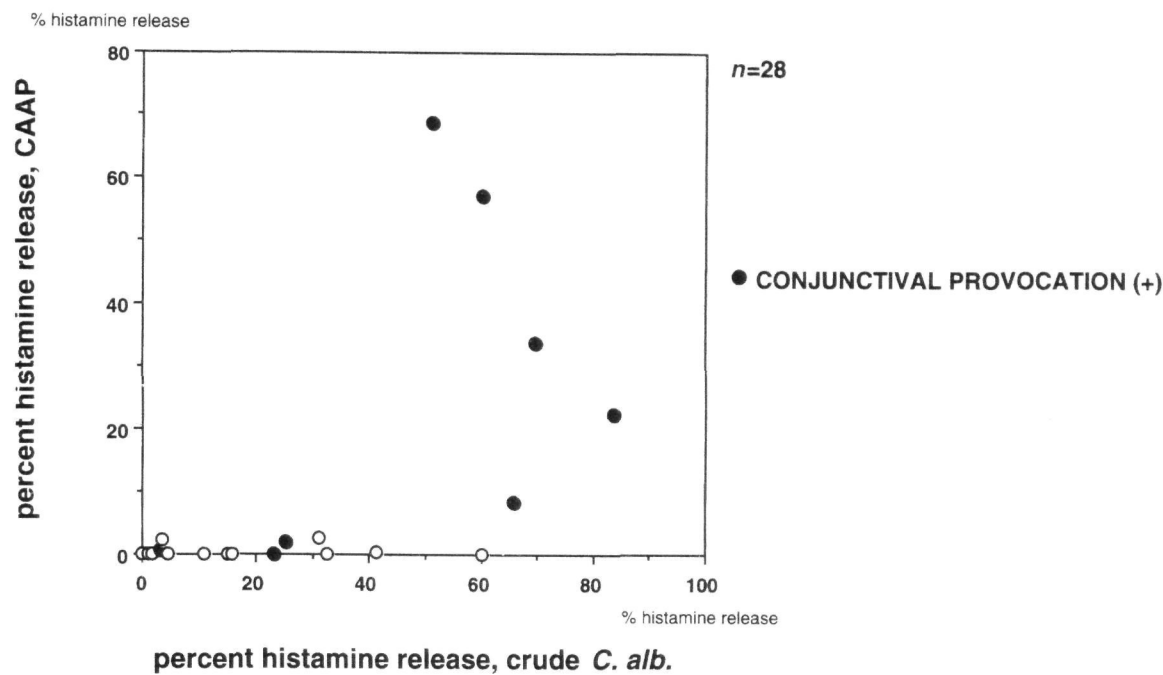


Fig. 5. Histamine release from peripheral blood leukocytes. Closed circles indicate patients scored as positive on conjunctival test.

positive immediate skin reaction to crude *C. alb.* The results of CPT showed that 6/8 patients (75%) showing a positive CPT response to crude *C. alb.* showed a positive response to CAAP, and that only 1/60 patients (1.7%) showing a negative CPT response to crude *C. alb.* showed a positive response to CAAP. Most of the CPT-positive patients had high levels of IgE antibody activity in their sera and showed a positive histamine-release response of their peripheral blood leukocytes to CAAP. These results suggest that CAAP is important as an allergen in mucosal allergy. The patients who showed a positive histamine-release response to crude *C. alb.* but no response to CAAP might have had mucosal allergy to other secreted enzymes such as phospholipase.

In conclusion, CAAP, which is an enzyme secreted by *C. alb.*, is not an allergen that commonly induces IgE antibody production, but it has an important role as a causative factor in *C. alb.*-related mucosal allergy.

The role of secreted enzymes as allergens might be relevant in cases of allergy to other microorganisms that are constituents of the normal microflora.

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