

The 31 kd major allergen, *ALT a I₁₅₆₃*, of *Alternaria alternata*

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A component of Alternaria extract, previously identified as the major allergen, Alt a I₁₅₆₃, was purified to homogeneity from Alternaria mycelium by means of acetone precipitation and ion-exchange chromatography. The homogeneity of Alt a I₁₅₆₃ was assessed by one single band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, by one single radiolabeled band after transfer to nitrocellulose, and by one single peak after size-exclusion chromatography by high-performance liquid chromatography. Alt a I₁₅₆₃ was isolated as a heat-stable acidic glycoprotein (carbohydrate content, 20%; pI, 4.0 to 4.5; 31 kd). The role of the carbohydrate moiety in the allergenicity is suggested. This major allergen is located in the cytoplasm of mycelium and spore. (J ALLERGY CLIN IMMUNOL 1991;88:902-8.)

Key words: *Alternaria*, allergens, glycoproteins, purification

In fungal allergy, *Alternaria* is considered as one of the most important fungi responsible for allergic rhinitis and/or asthma in humans.¹ Several allergens from *Alternaria* have been detected by crossed radioimmuno-electrophoresis and immunoblotting.²⁻⁵ A single allergen elicited the most intense reaction in immunoblotting analysis with ¹²⁵I-labeled anti-IgE antisera. Moreover, this allergen was recognized by 85% to 90% of the sera of patients allergic to *Alternaria*.^{6,7} This major allergen has been characterized under the names *Alt I*, *Ag 1*, *Ag 8*, and *G2D3-4*, depending on the laboratory.^{6,9} Its isolation was undertaken by Yunginger et al.⁶ and Vijay et al.⁹ by liquid-chromatography techniques. However, these authors were not able to purify this major allergen to homogeneity.

The present study describes the purification of a 31 kd *Ag* isolated from *A. alternata*. Previous studies have demonstrated that this 31 kd protein is one of the four major allergens identified in *Alternaria* and

Abbreviations used

SDS:	Sodium dodecyl sulfate
PAGE:	Polyacrylamide gel electrophoresis
CHAPS:	3[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
TC:	Tris-CHAPS buffer
pI:	Isoelectric point
IEF:	Isoelectric focusing
HPLC:	High-performance liquid chromatography
F-ELISA:	Fluorescent ELISA
PBS:	Phosphate-buffered saline
BSA:	Bovine serum albumin
Ab:	Antibody
Ag:	Antigen
UV:	Ultraviolet
MW:	Molecular weight

demonstrates the highest amount of IgE binding as well as the highest frequency of IgE binding. On the basis of allergen nomenclature,¹⁰ this 31 kd allergen could be named *Alt a I₁₅₆₃*.¹¹

In pollens, allergens are present in the wall and cytoplasm and are instantly released on contact with the mucosa or with the extraction buffer.^{12,13} With *Alternaria*, the localization of the allergens has not been attained until now. This study will also demonstrate the localization of *Alt a I₁₅₆₃* with im-

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munoelectron microscopy techniques based on anti-*Alt a* 1₅₆₃ monospecific antiserum.

MATERIAL AND METHODS

Culture and extraction

Spores of *A. alternata* (Fr.) Keissler, strain 1563 (Institut Pasteur, Paris), from a 2% malt agar slant were used to inoculate flasks containing liquid Sabouraud medium (peptone, 10 gm; glucose, 20 gm; H₂O liter). Two flasks (100 ml medium) were used to inoculate a 2 L fermenter (Biolafitte, St. Germain en Laye, France) containing 1.2 L of medium. After 3 days of growth at 21° C, the culture was inoculated into a 20 L fermenter (Chemap, Volkswil, Switzerland) containing 15 L of medium. The fermentation conditions were 500 rpm, 21° C, and 7.5 L of air per minute. After 2 days of growth, the mycelium was separated from the medium by filtration on Supra 250 (Seitz, Badkreuznach, Germany) filter under vacuum and washed three times with saline.

Mycelium was suspended at a 1:10 ratio (wt/vol) in a carbonate buffer (50 mmol/L of NaHCO₃ and 0.15 mol/L of NaCl) containing 1 mmol/L of phenylmethylsulfonyl fluoride, 5 mmol/L of ethylenediaminetetraacetic acid, 5 mmol/L of thiourea, and 25% (wt/vol) of insoluble polyvinylpyrrolidone. After a 24-hour incubation at 4° C, the mycelium was removed by filtration on a filter paper (Supra SKS, Seitz). The filtrate was precipitated with 4 vol of cold acetone and stored at -20° C until use. After removal of the acetone by centrifugation, the precipitate was dissolved in 25 mmol/L of Tris HCl buffer, pH 7.5, containing 1 mg/ml of CHAPS (TC). The resulting solution was dialyzed and concentrated with a Centricon ultrafiltration cell (Amicon Corp., Danvers, Mass.) equipped with a molecular cutoff limit of 30 kd. This extract was labeled AP 80.

Biochemical analysis

Protein content was determined according to the method of Bradford¹⁴ with BSA as a standard. Total carbohydrate content was estimated by the phenol-sulphuric acid method according to Dubois et al.¹⁵ with glucose as a standard.

SDS-PAGE. SDS-PAGE was performed as previously described¹⁶ with the discontinuous buffer system of Laemmli.¹⁷

IEF. Samples were subjected to IEF on preformed ampholine-PAG plates (LKB, Bromma, Sweden) (pH 3 to 10). Electrofocusing, fixing, staining, and destaining was performed as described in the LKB instruction manual; pI was determined with Bio-Rad (Bio-Rad Laboratories, Richmond, Calif.) calibration proteins.

Immunologic analysis

Patient sera. The IgE Ab pool was derived from 20 patients who had documented positive clinical histories of mold sensitivity, positive *Alternaria* skin tests, and with elevated serum IgE Abs to *Alternaria* (Phadebas RAST class 2 to 4). As an indication, this pool produced, on Pharmacia disks (Pharmacia Diagnostics, Uppsala, Sweden), 15.4%

of total count added compared to 23.3% with our crude *Alternaria* mycelial extract (12.5 to 0.6 µg of protein) coupled to CNBr-activated paper. A pool of normal human sera having negative skin test and RAST toward *Alternaria* was included in the study as a nonallergic control.

F-ELISA. F-ELISA was performed on Terasaki plates (Greiner GmbH, Nürtingen, Germany), provided by Ribotek (Paris, France), as described by Labrousse and Avrameas¹⁸ and Labrousse et al.¹⁹ with the following modifications: Each well was coated with 2 µl of extract (100 µg of protein per milliliter diluted tenfold with 50 mmol/L of carbonate buffer, pH 9.6). The plates were incubated at 36° C for 1 hour and at 4° C for 18 hours. Plates were washed three times with PBS-Tween 0.1%. Two milliliters of patient serum diluted tenfold with PBS-Tween was added to each well and kept overnight at 4° C. Plates were washed again three times with PBS-Tween. The β-galactosidase-labeled anti-IgE¹⁹ was added and incubated for 24 hours at room temperature. After an additional wash, β-umbelliferyl galactoside was added. The color reaction was allowed to develop for 15 minutes and stopped by the addition of 50 ml of 2 mol/L of Na₂CO₃ solution. Fluorometric determinations were made at 405 nm with a Titertek fluoroskan (Flow Laboratories, McLean, Va.).

Immunoblotting. Immunoblotting was performed as previously described¹⁴ according to the method of Towbin et al.²⁰

Dot-blot. One microliter of eluted fractions was spotted on a nitrocellulose sheet that had been presoaked in water. This sheet was then dried before immunoblotting.

Isolation and characterization of the 31 kd

Anion-exchange chromatography. AP 80 (5 mg) was loaded on a prepacked anion-exchanger column (Mono Q HR 5/5, Pharmacia, Uppsala, Sweden) equilibrated with TC buffer. The bound material was eluted with a two-step linear salt gradient (LKB, 2249 HPLC pump, 0 → 0.3 mol/L → 1 mol/L of NaCl) with a flow rate of 0.8 ml/min. The eluted fractions (0.8 ml) were monitored by measuring the UV absorbance with a spectral detector between 190 and 360 nm (RSD-2140, LKB) and analyzed by F-ELISA and/or dot-blot immunoassay to test their allergenic activity.

Size-exclusion chromatography. The fraction eluted with 0.45 mol/L of NaCl (0.45 M fraction) was dialyzed and concentrated with a Centricon and loaded on to a Superose 12 column (Pharmacia). Elution was performed with 25 mmol/L of Tris HCl buffer, pH 7.5 (flow rate, 0.4 ml/min). Fractions were monitored as described above.

Heat stability. The 31 kd allergen was heated at 100° C in a water bath for 5, 10, 15, and 30 minutes. Aliquots were taken from the solution and analyzed by immunoblotting.

Murine Ab. A preparative gel was run with AP 80 (1 mg of protein). The gel was blotted, and a lane was used to localize the 31 kd band in the central preparative blot. The 31 kd strip was cut from the blot into small pieces and sonicated in PBS with an ultrasonication probe for 10 seconds several times until an homogenous suspension was

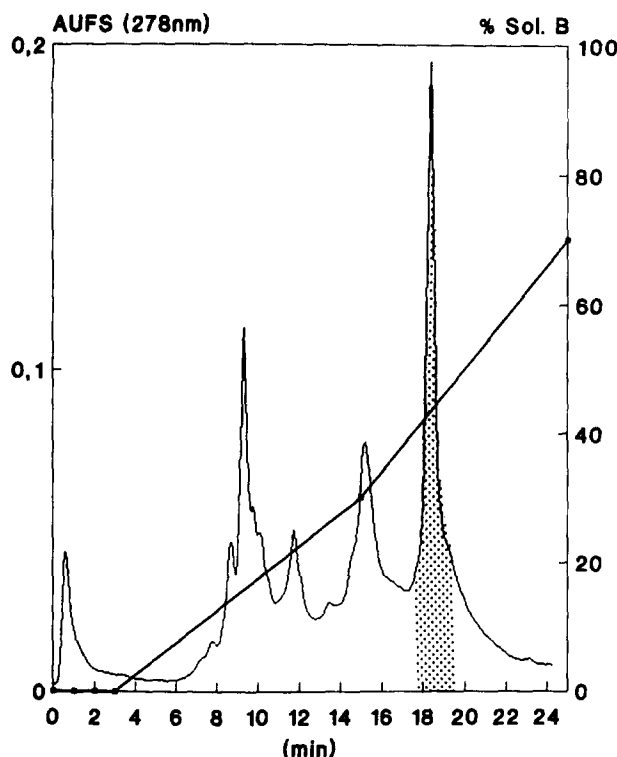


FIG. 1. Anion-exchange chromatography of AP 80 fraction (80% acetone precipitate). **Bold line** illustrates NaCl gradient. Allergenic activity determined by F-ELISA in collected fractions (1 minute) is illustrated by the **shaded area**; operating conditions: column, Mono Q HR 5/5, Pharmacia; mobile phase A: TC buffer; phase B: TC buffer with 1 mol/L of NaCl; flow rate, 0.8 ml/min; detection: UV absorption at 278 nm; AUFS: absorption units full scale.

obtained. BALB/c mice were inoculated intracutaneously with 200 μ l of the nitrocellulose suspension. Three boosts were performed 2, 4, and 6 weeks after the first injection. The immunization was followed by immunoblot analysis with a peroxidase-conjugated antimouse IgG. Mice were killed after blots were positive at 1:1000 serum dilution.

Immunoelectron microscopy

Germinating spores of *A. alternata* were fixed for 1 hour in 2.5% *p*-formaldehyde and 0.1% glutaraldehyde in PBS. After fixed spores were washed, they were incubated overnight at 4°C in PBS containing 10 mmol/L of ammonium chloride. Fungal material was dehydrated with increasing concentration of ethanol and embedded in Lowicryl (Link System, Evry, France) K4M, as described by Carlemalm et al.²¹

Ultrathin sections recovered on Formvar-coated grids were incubated for 1 hour at room temperature in the anti-31 kd antiserum diluted 1:1000 in PBS containing 1% BSA. Three washes were performed in PBS containing 0.1% BSA. Grids were then incubated with antimouse IgG colloidal gold (5 nm of conjugate) (Janssen Pharmaceutica, Beerse, Belgium). After 1-hour incubation at room temperature, sections were washed three times with PBS/0.1%

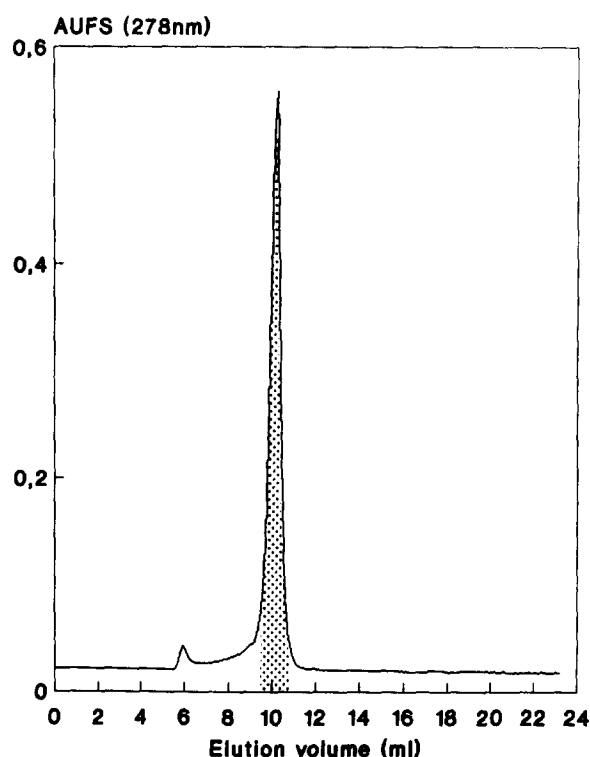


FIG. 2. Size-exclusion chromatography of allergenic fraction separated on anion-exchange chromatography. Operating conditions: column, Superose 12 HR 10/30 (Pharmacia); mobile phase, 25 mmol/L of Tris HCl, pH 7.5; flow rate, 0.4 ml/min; detection, UV absorption at 278 nm; AUFS: absorption units full scale.

BSA, twice with PBS, and five times with water. Controls included preimmune mouse serum diluted at 1:1000. Sections were examined in a Philips (Philips Electronics Instruments, Inc., Mahwah, N.J.) CM12 electron microscope at 80 kV.

RESULTS

For 1 gm of fresh mycelium (200 mg of dry weight), a yield of approximately 1.5 to 2 mg of acetone precipitate was produced that elicited between 100 to 150 μ g of protein of AP 80.

Purification of *Alt a* I₁₅₆₃

A typical elution profile of AP 80 on an anion-exchange column is illustrated in Fig. 1. The column adequately separated components of the starting material. F-ELISA demonstrated that IgE-binding material was recovered when the salt concentration reached 0.45 mol/L of NaCl. Immunoblotting analysis demonstrated that the 0.45 M fraction contained the 31 kd *Alt a* I₁₅₆₃ allergen.

The homogeneity of the 0.45 M fraction was demonstrated by chromatography on a size-exclusion Superose 12 column (Fig. 2). Except for one minor peak

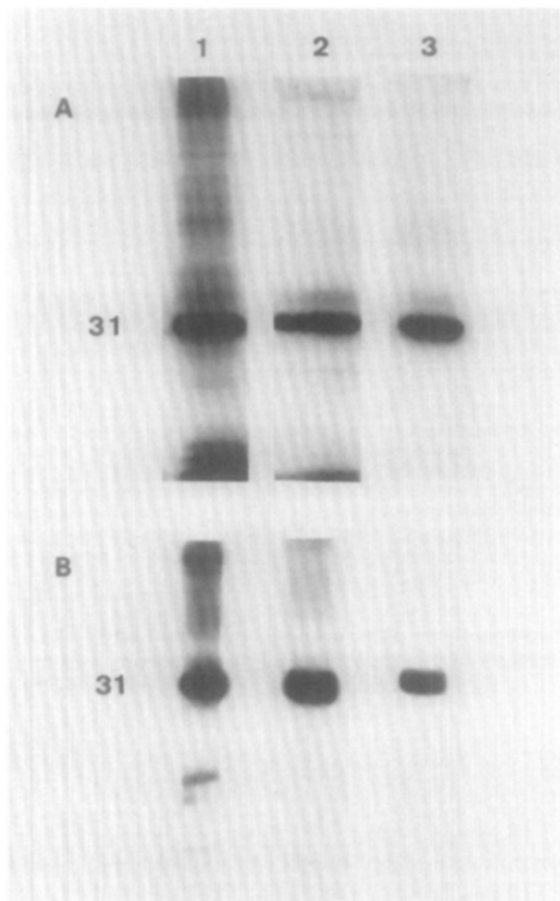


FIG. 3. A, SDS-PAGE (Coomassie blue staining) and **B,** Western immunoblotting of the products obtained during the purification of *Alt a* I₁₅₆₃; lane 1, crude extract; lane 2, AP 80; lane 3, 0.45 M fraction; 31, 31 kd.

with no IgE-binding activity, the 0.45 M fraction elicited one major symmetrical peak with an elution volume of 7.5 ml, corresponding to 30 kd MW. This fraction demonstrated a strong IgE-binding activity. Immunoblot analysis confirmed that this fraction contained the pure 31 kd allergen (Fig. 3). IgE binding to *Alt a* I₁₅₆₃ blot was inhibited by *Alt a* I₁₅₆₃ at a dose of 4 µg/ml of the pool of sera (data not presented).

Characterization of *Alt a* I₁₅₆₃

The UV absorption spectrum of the 31 kd allergen, scanned from 190 to 300 nm, demonstrated a typical spectral profile with two λ maximum at 217 and 278 nm (Fig. 4). One major band with pI 4.2 was visualized after IEF. Two other variants with pI 4.25 and 4.35 were also identified (Fig. 5). *Alt a* I₁₅₆₃ is a heat-stable protein. Heating of *Alt a* I₁₅₆₃ at 100° C for up to 30 minutes did not alter its binding properties as tested by Western blotting. *Alt a* I₁₅₆₃ is a glycoprotein containing 20% sugar.

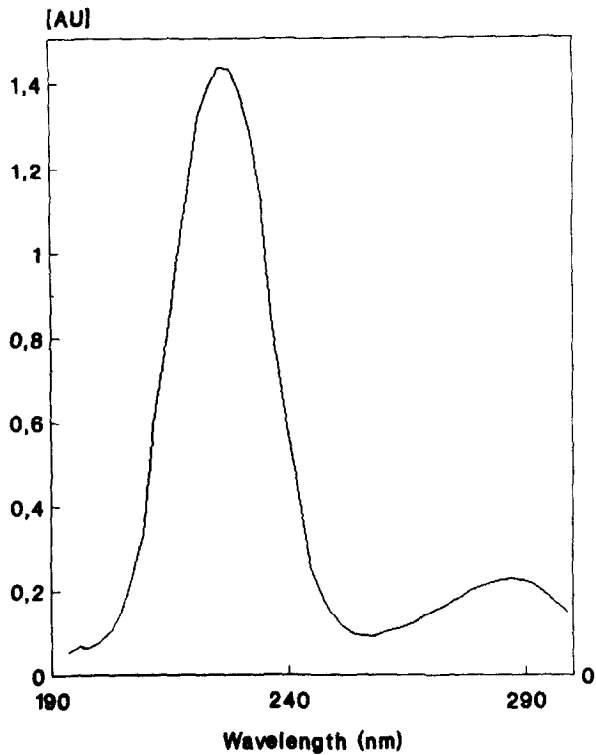


FIG. 4. UV absorption spectrum of the 31 kd allergen scanned from 190 to 300 nm, corresponding to the peak (shaded area) illustrated in Fig. 2; AU, absorption units.



FIG. 5. Thin-layer electrofocusing of the 0.45 M fraction; pH gradient (3.5 to 9.5) determined by pH (*Alt a* I₁₅₆₃, arrow).

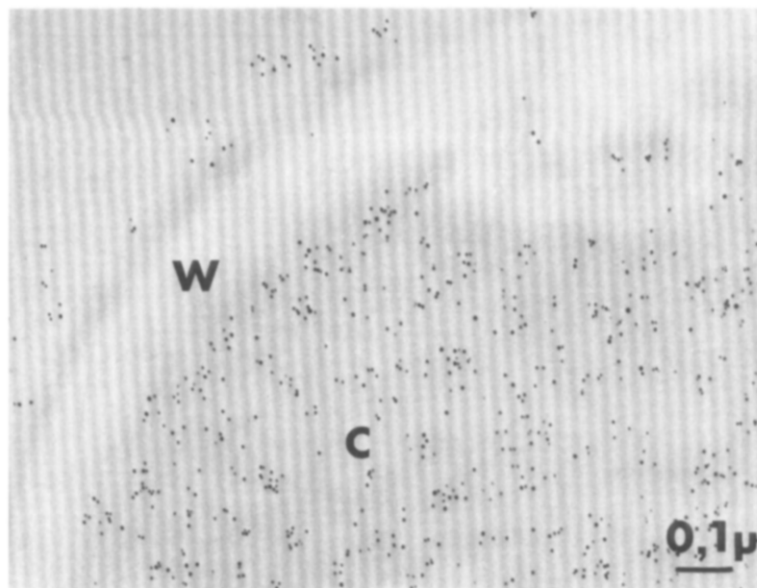


FIG. 6. Labeling of *A. alternata* mycelium thin sections with an anti-31 kd antiserum and an antimouse IgG (H plus L) antiserum conjugated to 5 nm colloidal gold; W, cell wall; C, cytoplasm. (Original magnification $\times 70,000$.)

The monospecific anti-31 kd antiserum bound predominantly to cytoplasmic compartments, whereas the cell wall was devoid of *Alt a* I₁₅₆₃ (Fig. 6). This localization is identical in spore and mycelium.

DISCUSSION

Initial experiments performed with a crude *Alternaria* extract loaded directly on the Mono Q column led to poor resolution and clogging of the column. Important concentrations of pigments in this extract were responsible for low resolution during chromatographic separation. The role of pigments in interfering with purification of allergens by liquid chromatography has already been documented by Haavik et al.²² and Nilsen and Paulsen²³ with pollen extract and by Folgert et al.²⁴ with *Alternaria* extract. All efforts during initial preparation of crude extracts have been made to minimize the presence of pigments in the *Alternaria* fraction. Cultures on different media demonstrated that the yield of *Alt a* I₁₅₆₃ was greatly increased when mycelium was grown on rich nondefined medium, such as Sabouraud, in which growth is rapid without pigmentation. When nutrient-poor media (e.g., 2% malt extract) were used, pigmented mycelium was always obtained. Pigments could be partly removed by polyvinylpyrrolidone treatment of mycelial extracts. However, pigments were concentrated during acetone precipitation and precipitated *Alt a* I₁₅₆₃. Although Yunginger et al.⁶ and Vijay et al.⁹ obtained good results with acetone-defatted matts, with our conditions, fresh mycelium was more suit-

able for fractionation experiments than lyophilized and/or defatted (acetone or ether) mycelium.

The localization of *Alt a* I₁₅₆₃ in the cytoplasmic compartment of the fungal cell explains why this allergen can be recovered after breakage of the mycelium. However, in spite of its intracellular localization, *Alt a* I₁₅₆₃ can be easily recovered after incubation of intact allergenic cells in carbonate buffer.^{12, 13, 16} This nondestructive incubation of the mycelium in an extraction buffer was chosen for *Alt a* I₁₅₆₃ purification because this procedure extracted fewer allergens besides *Alt a* I₁₅₆₃. Thiourea, a phenoloxidase inhibitor,²⁵ added to the extraction buffer, improved the yield on *Alt a* I₁₅₆₃ yield. Among the different concentration protocols used (acetone, (NH₄)₂SO₄, or polyethyleneglycol precipitations), acetone precipitation elicited the best results, probably by eliminating lipids or lipoproteins. CHAPS, a nondenaturing detergent used for solubilization of concentrated protein solution in IEF,²⁶ proved to be very efficient for protein purification during HPLC.

The biochemical characteristics of *Alt a* I₁₅₆₃ described in the present study (MW, 31 kd; pI, 4.0 to 4.5; carbohydrate content, 20%) are in agreement with the values obtained for the major allergens, *Alt I* and G₂D₃₋₄, by Yunginger et al.⁶ and Vijay et al.⁹ (MW, 25 to 50 kd; pI, 4.0 to 4.5; carbohydrate content, 33%; and MW, 30 to 40 kd, respectively). However, no direct comparison has been done between *Alt I* and *Alt a* I₁₅₆₃. We only know that our crude extract contained a panel of allergens similar to an international

standard containing *Alt I* (Yunginger JW. Personal communication). The major allergen of *Alternaria* was thus found to exist in multiple "isoallergenic" forms that are variants that appear to be immunologically distinct in the case of *Alt I* and Ag 8 or similar in the case of *Alt I* and Ag 1.^{7, 8}

Many allergens have been identified as glycoproteins, but only a limited number of studies have been performed to elucidate the importance of the carbohydrate moiety in the IgE binding. So far, the only allergen found to loose its IgE binding after deglycosylation was the pollen allergen, *Xan VIa* of *Xanthium strumarium*.²⁷ Removal of the carbohydrate moiety of *Alt a I*₅₆₃ by the trifluoromethanesulfonic acid technique abolished all the IgE-binding ability of the molecule (data not presented), suggesting the role of the carbohydrate moiety. This finding is in agreement with studies on crude *Alternaria* extract in which (1) allergenic activity could not be correlated with the protein content of the extract,²⁸ and (2) removal of carbohydrate by the trifluoromethanesulfonic acid method abolished entirely the allergenic activity of the crude extracts.²⁹ The carbohydrate moiety could also be responsible for the remarkable heat stability of *Alt I*. Variations in the amount of glycosylation of *Alt a I*₅₆₃ have been observed with culture medium (data not presented). Variations may also occur depending on the strain or the propagule (spore or mycelium) studied. Studies are currently undertaken to investigate the chemical composition of the carbohydrate moiety of *Alt a I*₅₆₃.

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Acute consumption of C1 inhibitor in a patient with acquired C1-inhibitor deficiency syndrome

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Acquired C1-inhibitor (C1 INH) deficiency is usually found in association with an underlying disease that is believed to be responsible for increased C1 INH catabolism, ultimately leading to the development of C1 INH deficiency. We report a remarkable patient with acquired C1 INH deficiency in whom a unique progression of complement- and contact-system abnormalities has been observed. S. G. suffers from recurrent episodes of angioedema and hypotension. Results of repeated complement studies were initially normal, and the patient was diagnosed as having idiopathic anaphylaxis. Two years later, the patient was found to develop acute consumption of C1 INH with activation of the complement and contact systems during episodes of angioedema. The patient continued to have normal C1 INH levels and to have no evidence for complement- or contact-system activation between attacks of angioedema. One year later, her course evolved into a more typical course for acquired C1 INH deficiency consisting of continuously low functional C1 INH levels with evidence of activation of the complement and contact systems. S. G. provides a unique insight into the development of acquired C1 INH deficiency. (J ALLERGY CLIN IMMUNOL 1991;88:908-18.)

Key words: C1 inhibitor, acquired, deficiency, angioedema, anaphylaxis, complement, kallikrein, kininogen, autoantibody

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Abbreviations used

C1 INH:	C1 inhibitor
EACA:	Epsilon aminocaproic acid
HMWK:	High-molecular-weight kininogen
PBS:	Phosphate-buffered saline
RT:	Room temperature
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The syndrome of acquired C1 INH deficiency was first described in a patient with lymphoma, recurrent angioedema, and C1 INH deficiency.¹ Subsequently,