

Immunochemical partial identity between two independently identified and isolated major allergens from *Alternaria alternata* (Alt-I and Ag 1)

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Partially purified preparations of Alt-I, the main allergenic fraction of Alternaria alternata isolated by Yunginger, and of Ag 1, shown in crossed radioimmuno-electrophoresis (CRIE) to be the dominating major allergen of A. alternata (Løwenstein, Nyholm), were compared by tandem crossed immunoelectrophoresis (CIE), RAST inhibition, and the CRIE-related technique, single radial radioimmunodiffusion (SRRID). The two allergen preparations showed reaction of identity in tandem-CIE and indistinguishable specific IgE binding in CRIE and SRRID, regardless of antibodies and serum pools used. In RAST inhibition, the relative potencies of the allergen preparations and of the crude extracts correlated well with their Alt-I/Ag 1 content as estimated by rocket immunoelectrophoresis. Moreover, all inhibition curves were parallel, confirming identical IgE binding by Alt-I and Ag 1 with the serum pools used. A second preparation of Alt-I, isolated from another strain of Alternaria, showed reaction of partial identity with Ag 1 in tandem-CIE, indicating that different variants of Alt-I (Ag 1) may exist in different strains of A. alternata. (J ALLERGY CLIN IMMUNOL 71:461, 1983.)

Yunginger et al.¹ have reported the isolation of a major allergenic fraction from *Alternaria alternata* (Alt-I), the purification procedure of which was monitored by RAST inhibition. Alt-I was found to have an apparent molecular weight of 25,000 to 50,000 daltons (Sephadex G-100) and a pI of 4.2. Independently, Løwenstein² and later Nyholm and Løwenstein³ have shown, using sera from different patient panels in CRIE, that among several important allergens of *Alternaria*, one allergen (Ag 1) was the dominating major allergen, since more than 50% of the patient sera contained IgE that bound strongly to this allergen in CRIE. Ag 1 had an apparent molecular weight of 60,000 daltons (ACA-34) and a pI of 4.0.³

We here present evidence that Alt-I and Ag 1 are the same allergen and that different varieties of this allergen may exist in different strains of *A. alternata*.

MATERIALS AND METHODS

Antigens

Alt-I was isolated from *A. alternata* powder from Greer Laboratories, Lenoir, N.C., as described previously.¹ Briefly, *A. alternata* from strains 6663 and 16086 (American Type Culture Collection, Rockville, Md.) were grown separately on modified Czapek's medium for 20 days, after which mold pellicles were harvested, homogenized, defatted, dried, and powdered. Crude extracts were prepared from these powders with Tris-HCl buffer: crude US(33) from *Alternaria* powder Lot 33, derived from strain 6663, and crude US(37) from *Alternaria* powder Lot 37, derived from strain 16086. Two Alt-I preparations, Alt-I(33) and Alt-I(37), were isolated from these crude extracts by ion-exchange chromatography, preparative isoelectrofocusing, and Sephadex G-100 gel filtration, as described previously.¹

Ag 1 was isolated from a crude extract of *A. alternata* (crude DK), prepared by Allergologisk Laboratorium, Copenhagen, Denmark (ALK). Source material was the biomass from a strain of *A. alternata* obtained from Centraalbureau voor Schimmelcultures, Baarn, Holland. As described in detail previously,⁴ the mold was grown on V-8 broth, a complex natural medium, harvested after 3 wk, freeze-dried, powdered, and extracted. Partly purified Ag 1 was prepared from this extract by a combination of cation-

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Abbreviations used

CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmuno-electrophoresis
RAST:	Radioallergosorbent test
SRID:	Single radial immunodiffusion
SRRID:	Single radial radioimmunodiffusion
Rocket-IE:	Rocket immunoelectrophoresis
CFA:	Complete Freund's adjuvant
IFA:	Incomplete Freund's adjuvant

exchange chromatography and gel filtration, as described in detail elsewhere.⁵ A supplementary preparation of Ag 1 was prepared as described above, omitting the gel filtration step, from the same strain of *A. alternata* grown on Czapek-Dox medium supplemented with vitamins. Since the Ag 1 in this preparation could not be distinguished from the Ag 1 in above-mentioned preparations in tandem-CIE, we have not specified which preparation was used throughout the study. The tandem-CIE shown in Fig. 2 was performed with the latter preparation (Czapek-Dox medium); all other reported experiments were performed with the V-8 medium.

Antibodies

Rabbit anti-Alt-I antibodies were produced by two subcutaneous injections, 1 wk apart, of 50 μ g of Alt-I(33) in CFA, followed by another subcutaneous injection of 50 μ g of Alt-I in IFA after 1 wk. Bleedings commenced thereafter, and after seven bleedings at 1 wk intervals, four additional booster doses of 50 μ g of Alt-I were given subcutaneously in IFA at 4 to 8 wk intervals over the next several months. Multiple bleedings were tested by immunoelectrophoresis, then pooled and stored at -20° C.

Rabbit anti-*Alternaria* antibodies, anti-crude US, were produced by two subcutaneous injections, 3 wk apart, of 1 mg of crude *Alternaria* extract (Lot 33) in CFA, followed by another intramuscular dose of 3 mg of antigen in IFA after 4 mo. Bleedings commenced thereafter, and after five bleedings at 1 to 2 wk intervals, six additional booster doses of 3.0 to 6.6 mg antigen were given subcutaneously in IFA at 4 to 8 wk intervals over the next several months. Bleedings obtained after each booster dose were tested for precipitating antibody by immunoelectrophoresis, then pooled and stored at -20° C.

Rabbit anti-Ag 1 antibodies were produced by weekly subcutaneous injections in the subscapular region with 20 μ g of Ag 1 in IFA. The animal was bled by the ear vein after 6 wk, and subsequently immunized and bled alternately every 2 wk for 6 mo. The immunoglobulin fraction of the antiserum was obtained as described by Harboe and Ingild.⁶ Rabbit purified antibodies against crude *A. alternata* extract, anti-crude DK, were long-term immunization antibodies available from ALK.

CIE, tandem CIE, and rocket-IE

These procedures were performed as described previously.⁷⁻⁹ The buffer consisted of 0.073M Tris, 0.024M barbital, and 0.003M sodium azide (pH 8.6, 25° C). The gel

consisted of 1% (w/v) agarose (type HSA; Litex, Glostrup, Denmark) in electrophoresis buffer. Electrophoresis was performed in 0.15 cm thick gel at 16° C. In CIE and tandem-CIE, first-dimension electrophoresis was performed at 10 V/cm for 25 min (Fig. 1, 35 min), and second-dimension electrophoresis was performed at 2 V/cm overnight. Rocket-IE was also performed at 2 V/cm overnight.

CRIE

CRIE⁷ was performed mainly as described previously.⁷ After CIE, the plates were washed, pressed, dried in cold air, and incubated overnight with serum (0.5 ml of serum in 8 ml of 0.067M phosphate buffer, pH 7.0) pooled from 30 *Alternaria*-CRIE-positive individuals (DK serum pool). After washing (0.05M phosphate buffer, pH 7.5, containing 0.3% [w/v] bovine serum albumin, 0.15M NaCl, 15 mM NaN_3 , 2.7 mM $\text{Na}_2\text{-EDTA}$, and 1% [v/v] Tween 20), the plates were incubated overnight with 125,000 counts per minute (counting efficiency approximately 40%) ^{125}I -rabbit anti-human IgE⁹ (specific activity approximately 7 $\mu\text{Ci}/\text{ml}$) in 8 mg of the above-mentioned washing buffer, washed in 0.9% NaCl, dried, and autoradiographed for 10 days. Two different negative controls were performed with CIE plates of crude DK against anti-crude DK: one plate was incubated with a 1000-donor normal serum pool, the other with a serum pool from mite-allergic patients. Neither of the controls showed any IgE binding to Ag 1.

SRRID

SRRID was performed as described by Peter Lind (personal communication). SRID was performed essentially as described by Mancini et al.¹⁰ with Alt-I(37) and Ag 1 against anti-Alt-I and anti-Ag 1. The antigens were allowed to diffuse only overnight to obtain circular areas of precipitate instead of the more ring-formed precipitates that may result from several days of diffusion. The SRID plates were then incubated with either a DK serum pool (see description of CRIE) or a US serum pool (see description of RAST), incubated with ^{125}I -anti-IgE and autoradiographed as described for the CRIE procedure.

RAST inhibition

A solid-phase Alt-I preparation was obtained by reacting Alt-I(33) (2 mg) with CNBr-activated microcrystalline cellulose particles (250 mg).¹¹ By measuring the spectrophotometric absorbance of the coupling supernatant and subsequent washings, we estimated that 47% of the Alt-I was bound to the cellulose. The allergenicities of crude US(33), crude US(37), Alt-I(37), crude DK, and Ag 1 were compared in a mini-RAST procedure¹² using the Alt-I solid phase described above and an IgE-containing serum pool comprising equal quantities of sera from seven *Alternaria*-sensitive individuals who had never received immunotherapy (US serum pool). Various quantities of test inhibitors were added in the first stage of the mini-RAST, after which the solid phase was washed three times, reacted with ^{125}I -anti-IgE (5 ng), incubated overnight, washed as before, and counted in a gamma-scintillation counter.

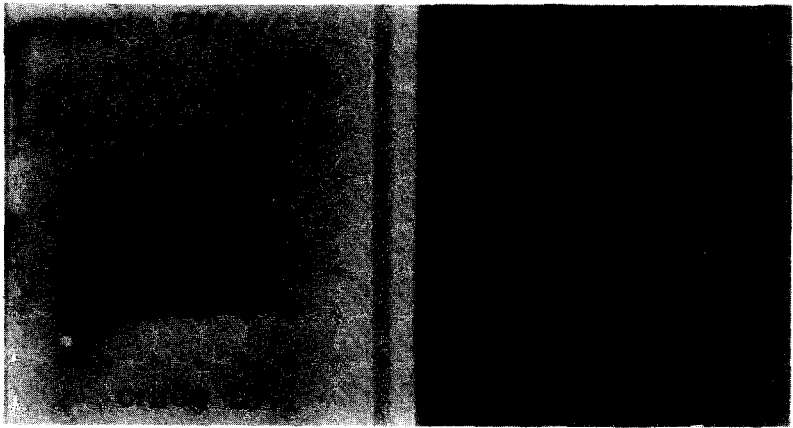


FIG. 1. CIE and CRIE of crude *A. alternata* extract. A, CIE of crude DK (160 μ g) against anti-crude DK (200 μ l). Arrow, Ag 1 precipitate. B, CRIE of the gel in A, showing Ag 1 and other allergens.

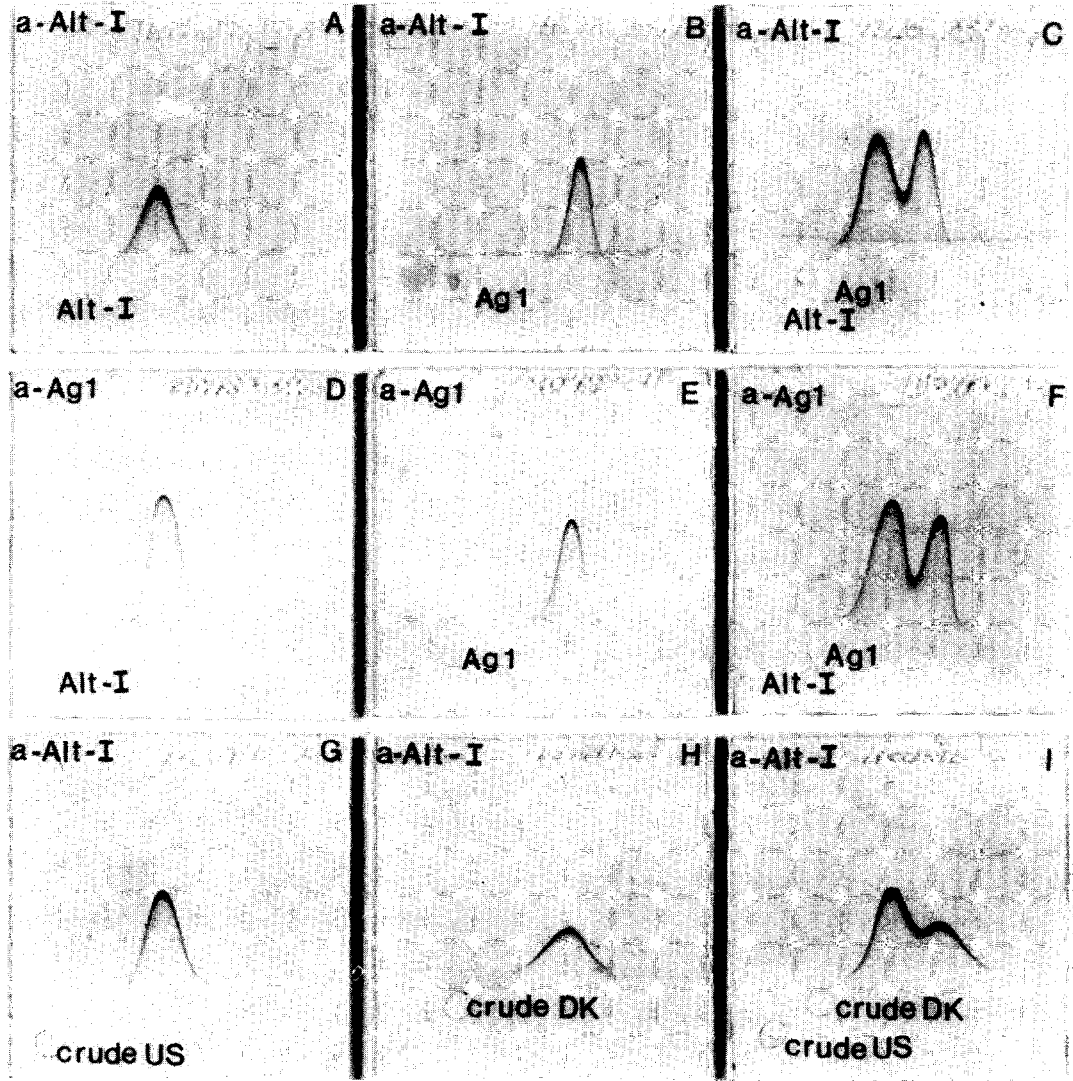


FIG. 2. Tandem-CIE of purified allergens and crude extracts. Antigens—A and D: left well, Alt-I(37), right well, buffer; B and E: left well, buffer, right well, Ag 1; C and F: left well, Alt-I(37), right well, Ag 1; G: left well, crude US(37), right well, buffer; H: left well, buffer, right well, crude DK; I: left well, crude US(37), right well, crude DK. Antibodies—A, B, C, G, H, I, anti-Alt-I; D, E, F, anti-Ag 1.

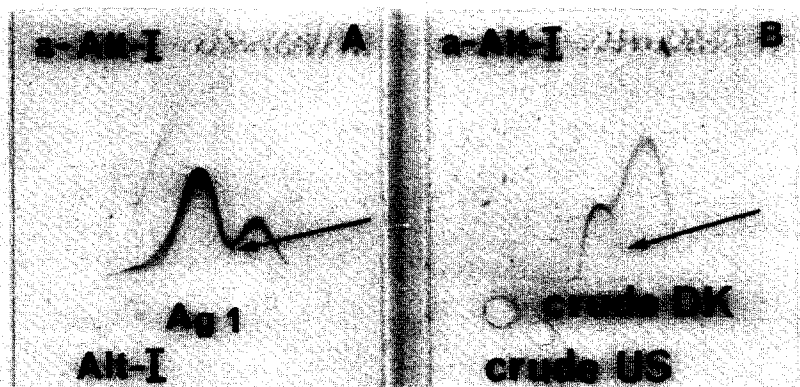


FIG. 3. Tandem-CIE of purified allergens and crude extracts. Antigens—A: left well, Alt-I(33), right well, Ag 1; B: left well, crude US(33), right well, crude DK. Antibodies—A and B: anti-Alt-I. Arrows, Spur, indicating partial identity.

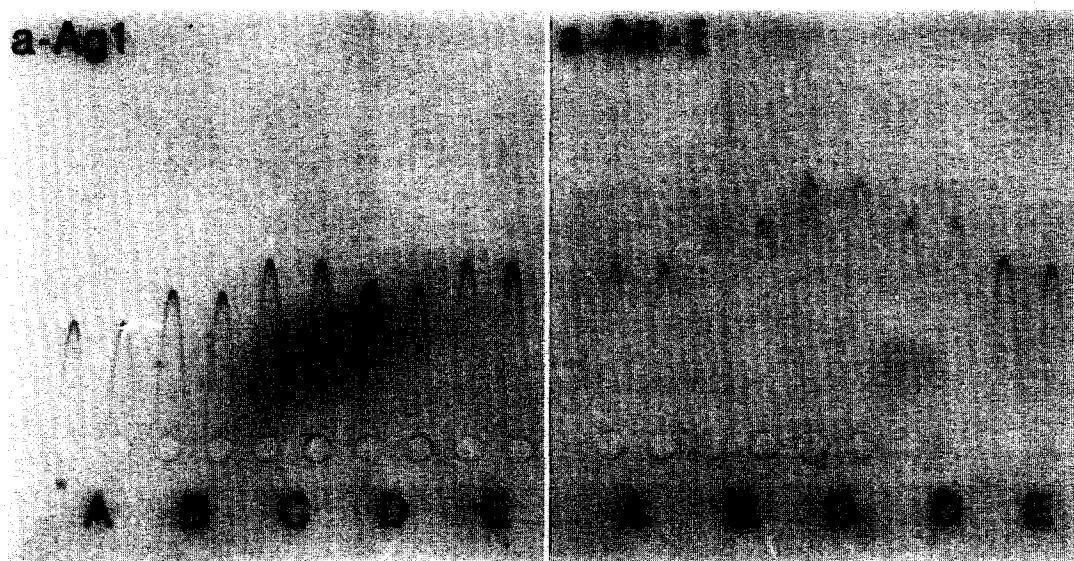


FIG. 4. Rocket-IE. Paired antigens—A, B, and C: 300, 400, and 500 µg crude DK; D: 0.5 µg Ag 1; E: 0.29 µg Alt-I(37). Antibodies—left: anti-Ag 1; right: anti-Alt-I.

RESULTS AND DISCUSSION

CIE of crude *Alternaria* extract against polyspecific antibodies to crude *Alternaria* extract shows the complexity of the extract (Fig. 1). One of the fastest-migrating antigens in the crude DK extract, Ag 1, represented by an almost invisible precipitate in the stained CIE plate but showing strong IgE binding in CRIE (Fig. 1), has been identified as a major allergen in earlier studies.^{2, 3} In introductory CRIE investigations of the two purified allergen preparations, Ag 1 and Alt-I(37), using anti-crude DK, the two allergen precipitates showed similar electrophoretic mobility and IgE binding, thus suggesting that Alt-I and Ag 1 might be the same allergen. It was also found that neither of the two allergen preparations were immunochemically completely pure, but none of the im-

purities bound specific IgE in CRIE, whether with anti-crude DK or anti-crude US.

That Ag 1 and Alt-I are indeed the same allergen is seen from the tandem-CIE shown in Fig. 2. The Alt-I and Ag 1 precipitates, whether originating from the purified allergen preparations or from crude extracts, fused completely, thus indicating immunochemical identity between Alt-I and Ag 1. The faint precipitate, visible, for instance, in Fig. 2, C, represents an impurity and should not be confused with a spur (see below). The identity of the Alt-I/Ag 1 precipitate was confirmed in CRIE, showing strong IgE binding to this precipitate.

Although Alt-I and Ag 1 are thus established to be the same allergen (which we propose be called Alt-I), some of our results indicate that this allergen may

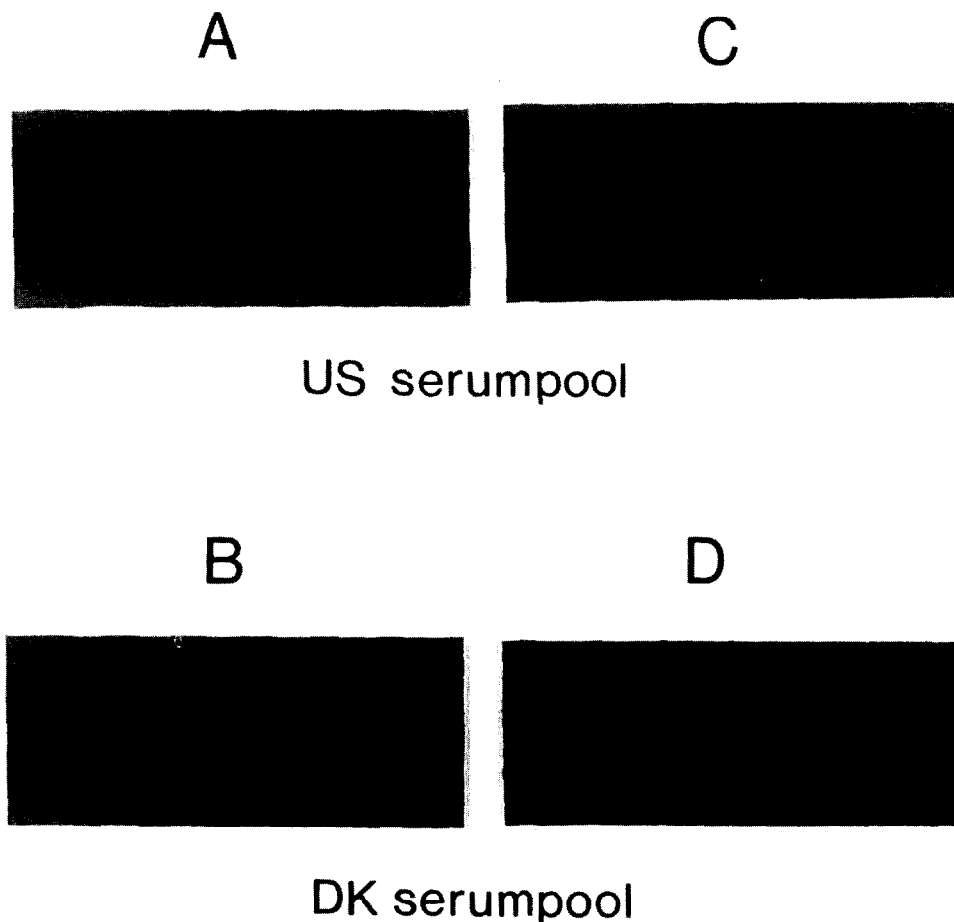


FIG. 5. SRRID. Antigens (left to right): 0.24 and 0.58 μ g Alt-I(37), 0.4, 0.8 μ g Ag 1. Antibodies—**A** and **B**: 20 μ l anti-Ag 1; **C** and **D**: 40 μ l anti-Alt-I. Serum pools—**A** and **C**: US; **B** and **D**: DK.

exist in several different varieties. Thus tandem-CIE of Alt-I and Ag 1, using Alt-I(33) instead of Alt-I(37), showed a faint spur between the fused precipitates, indicating immunochemical differences between the allergens (Fig. 3). Since the spur is already apparent in tandem-CIE of the crude parent extracts (Fig. 3), we may disregard the possibility that the observed difference was caused by changes induced in one or the other allergen preparation during the purification procedure. Even the apparently identical Alt-I(37) and Ag 1 (Fig. 2) behaved slightly differently in rocket-IE, with the relative rocket height depending on the antibodies used. As seen in Fig. 4, a given amount of Ag 1 or crude DK will give relatively smaller rockets in the homologous antibody than in the heterologous one when compared with Alt-I(37), thus indicating immunochemical differences between the two allergens. Since Alt-I(33), Alt-I(37), and Ag 1 were derived from different strains of *A. alternata*, it is tempting to hypothesize that different varieties of Alt-I may be present in different strains of this mold, in other *Alternaria* species, or even in closely related

genera. One of us has already partly addressed this question,¹³ and we are currently investigating the issue in Copenhagen.

Although Alt-I and Ag 1 were evidently very closely related as judged from tandem-CIE, the possibility remained that the two allergens might have different IgE binding capacities—either inherent, as part of their parent crude extracts, or because of changes induced by the purification procedure. We evaluated the IgE binding properties of Alt-I and Ag 1 in RAST inhibition and SRRID. Whereas the RAST inhibition allows a comparison between the allergenicities of purified allergens as well as crude extracts, the SRRID, by using purified allergens in combination with monospecific antibodies, minimizes the possible interference of allergenic impurities. Fig. 5 shows that with the various antibodies and serum pools used, the IgE binding of Alt-I(37) and Ag 1 is indistinguishable in SRRID. [Alt-I(33) was not tested in SRRID.] The purified allergen preparations Alt-I(37) and Ag 1, and the crude extracts crude US(33), crude US(37), and crude DK, were compared in

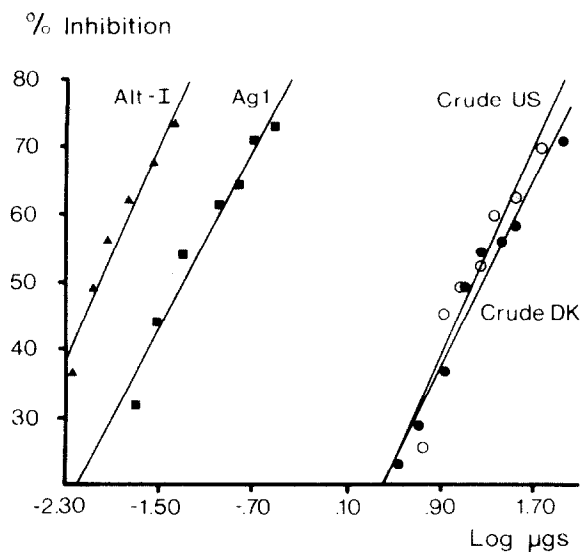


FIG. 6. RAST inhibition. Δ , Alt-I(37); $\blacksquare$, Ag 1; $\circ$, crude US(33); $\bullet$, crude DK. Analysis of covariance of slopes: $F_{3,20} = 0.56$, N.S.

TABLE I. Reciprocal RAST-inhibiting potencies of various *Alternaria* preparations

<i>Alternaria</i> preparation	50% Inhibition (μ g)
Crude US(37)	1.8
Crude US(33)	16.5
Crude DK	17.8
Alt-I(37)	0.01
Ag 1	0.05

RAST inhibition tests with Alt-I(33) in the solid phase in two experiments (the result of one experiment is shown in Fig. 6). The slopes of all inhibition curves were very similar as measured by analysis of covariance, suggesting that the same allergenic determinants were being measured in all preparations. In Table I is given the amount of material necessary to obtain 50% inhibition with the five above-mentioned preparations. The difference in RAST-inhibiting potencies between the Alt-I and the Ag 1 preparations is probably due to inaccuracies in absolute weight determinations, combined with loss of activity during shipment of Ag 1 to the United States (see below).

To be able to express the RAST-inhibiting potency per unit Alt-I/Ag 1, we have estimated the content of this allergen in the different preparations by rocket-IE. This required some caution in interpretation of results, since quantification of antigens by this method presupposes immunochemical identity between the antigens to be quantified. Thus, although the quantification of, for instance, Alt-I(37) relative to crude US(37) or Ag 1 relative to crude DK was straightforward, the attempted quantification of two

TABLE II. Alt-I/Ag 1 content compared with RAST-inhibiting potency

	Relative Alt-I/Ag 1 content (rocket-IE)	Relative RAST-inhibiting potency
Alt-I(37) vs. crude US(37)	130	180
Crude US(37) vs. crude US(33)	10	10
Crude US(33) vs. crude DK	1	1
Ag 1 vs. crude DK	840	360
Alt-I(37) vs. Ag 1	2	5

partially identical antigens as Alt-I(33) or Alt-I(37) relative to Ag 1 or crude DK gave divergent results, depending on the antibody used, as already mentioned. Nevertheless, we have used rocket-IE as the best method available for estimation of the Alt-I/Ag 1 content by taking the mean of the results obtained with the two monospecific antibodies. Taking the example in Fig. 4, 0.29 μ g of Alt-I(37) corresponded to 300 μ g of crude DK (or 0.37 μ g of Ag 1) with anti-Alt-I as antibodies, but this amount corresponded to 500 μ g of crude DK (or 0.58 of μ g Ag 1) using anti-Ag 1 as antibodies. Thus we concluded that 0.29 μ g Alt-I(37) contained the same amount of allergen as did 400 μ g of crude DK (or 0.48 μ g Ag 1). In Table II the relative RAST-inhibiting potencies of the different preparations are compared with their relative Alt-I (Ag 1) content as estimated by rocket-IE. It is evident that the RAST-inhibiting potency correlated quite closely to the Alt-I (Ag 1) content. For instance, the crude US(37) is 10 times more potent in RAST inhibition than crude US(33) and it also contains 10 times more Alt-I than the latter extract. Crude US(33) contains the same amount of allergen as does crude DK, and the two extracts are equally potent in RAST inhibition. The Ag 1 preparation is not as potent in RAST inhibition as should be expected from its allergen content. Since the SRRID showed that the IgE binding capacities of Alt-I and Ag 1 were indistinguishable, the obvious explanation of the unexpected low potency of Ag 1 in RAST is that the Ag 1 preparation (shipped as 0.1 mg/ml solution) lost some of its activity during shipment to the United States. Even with this minor discrepancy it is evident that the RAST-inhibiting potencies of the various preparations are due to the Alt-I (Ag 1) content only and that the IgE binding properties of the different allergen preparations cannot be distinguished in RAST inhibition using Alt-I(33) as allergosorbent. The possibility remains that the use of multiple individual sera instead of a serum pool in both RAST and

SRRID might be able to reveal differences in the IgE binding capacity of the investigated allergens or that other variants of Alt-I having different IgE binding properties may be discovered.

Although the crude *Alternaria* extracts varied 10-fold in their Alt-I content, they all contained very little Alt-I when compared with other allergenic extracts such as ragweed or mite, where the major allergen (AgE, Ag 42) constitutes approximately 6% (w/w) of the extract.^{14, 15} Thus, from the degree of purification (Table II) it can be calculated that the crude extract richest in Alt-I, crude US(37), contains less than 0.7% Alt-I based on dry weight determination (RAST, less than 0.55%; rocket-IE, less than 0.77%). This explains the low titer against Alt-I in both polyspecific antibodies. From our current investigations on *A. alternata*, in which we have estimated the Alt-I content in multiple extracts from a few strains, an Alt-I content of less than 0.7% does not seem atypical for extracts of this mold. Whether the use of other strains or species, or maybe other growing conditions, may result in extracts richer in Alt-I remains an open question.

CONCLUSION

In conclusion, two independent groups, using different methods and different raw materials, have identified the same allergen as the most important allergen of *A. alternata*. * Alt-I exists in at least three slightly different varieties, that are probably related to strain differences. These variants can be differentiated in tandem-CIE or rocket-IE but seem to bind specific IgE in vitro equally well. It remains to be investigated whether other variants of this allergen can be found in other *Alternaria* strains or species and whether all variants are equivalent as allergens and in diagnosis and treatment.

The assistance of Mr. Richard T. Jones and Mrs. Marian Bortolon is greatly appreciated, and we are grateful to Lotte Månsson for skillful technical assistance.

*We have recently found that the Ag8 isolated by Aukrust et al.¹⁶ is also a variant of Alt-I, since monospecific antibodies to Ag8 precipitate Alt-I (both the Alt-I and the Ag 1 preparation) and no other component in the crude DK *Alternaria* extract.

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