

Immunochemical and physicochemical characterization of commercial *Alternaria* extracts: a model for standardization of mold allergen extracts

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To develop a model for mold allergen extract standardization, we studied eight commercial Alternaria extracts from various suppliers by a variety of immunochemical and physicochemical techniques, including measurement of Alt-I, a purified allergenic fraction of Alternaria. Wide variations were noted in the allergenic and antigenic potencies of these extracts. Estimates of Alt-I content measured by Alt-I RAST inhibition and by radioimmunoassay correlated significantly ($p < 0.05$), but Alt-I activity by either method could not be correlated with allergenic potency as measured by RAST inhibition using solid-phase Alternaria. Each test extract produced unique and differing patterns of Coomassie blue-stained bands in isoelectrofocusing gels and in crossed immunoelectrophoresis gels using rabbit antibodies to Alternaria. The optimal method for mold allergen standardization involves a combination of RAST inhibition, isoelectrofocusing, and crossed immunoelectrophoresis techniques, and, if possible, quantitation of individual allergens. (J ALLERGY CLIN IMMUNOL 70:432, 1982.)

Both the diagnostic efficiency and the therapeutic efficacy of allergen extracts depend on the allergen content in these extracts. Each allergen extract contains a number of biochemical components of different molecular weights and properties, and these may differ in stability.¹ There may be variations in relative concentrations of allergenic components in commercial extracts when different extraction procedures are used. In addition, there may be gross changes in the allergenic activity of the commercial allergen extracts during their processing, shipment, and storage.² Hence, various immunochemical and biochemical tests have been used for the characterization of differ-

ent allergen extracts such as skin tests, leukocyte histamine release, direct RAST, RAST inhibition, and CIE. However, well-accepted criteria for quality control and standardization of allergen extracts have yet to be developed.² A generally acceptable method for standardization may be to measure the purified major allergens in the crude extracts, as has been done previously for AgE in short ragweed pollen extracts³ and for group I allergens in grass pollen extracts.⁴ With a view to developing a model for allergen extract standardization for molds, we analyzed various commercial *Alternaria* extracts by different physicochemical and immunochemical tests, including measurement of Alt-I,⁵ the purified major allergenic fraction of *Alternaria*.

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Supported by a grant from the National Institute of Allergy and Infectious Diseases (AI-16791) and by Mayo Foundation.

Received for publication May 21, 1982.

Accepted for publication July 1, 1982.

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Presented in part at the 38th Annual Meeting of the American Academy of Allergy, March 1982.

Abbreviations used:

CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
DARIA:	Double-antibody radioimmunoassay
PNU:	Protein nitrogen unit
TLIEF:	Thin-layer isoelectrofocusing
TNU:	Total nitrogen unit

TABLE I. Commercial *Alternaria* extracts studied*

Extract No.	Manufacturer	Label	Expiration date	Concentration (w : v)	Lot No.
1	Allergy Laboratories of Ohio, Inc.	A	Feb. 1984	1:20	B011159A
2	Allergy Laboratories, Inc. Oklahoma	At	Jan. 1982	1:20	3215
3	Barry Laboratories, Inc.	At	Jan. 1984	1:33	09-15
4	Berkeley Biologicals	At	Feb. 1984	1:10	376266
5	Center Laboratories	A	Feb. 1984	1:20	816424g
6	Greer Laboratories, Inc.	At	Feb. 1984	1:20	GM1-122B-1A2
7	Hollister-Stier Laboratories	A	Feb. 1982	1:10 regular	130997436
8	Hollister-Stier Laboratories	A	Feb. 1982	1:100 MMP†	1229360

A = *Alternaria*; At = *Alternaria tenuis*.

*All were supplied in 50% glycerine.

†MMP is the manufacturer's code for a particular extraction procedure.

MATERIALS AND METHODS

Allergens

For these studies, eight *Alternaria* extracts were purchased from different suppliers (Table I). These extracts were labeled as *Alternaria* or *Alternaria tenuis*. However, all extracts were reported to be prepared from *A. tenuis*. *A. alternata* is a newer taxonomic designation that is synonymous with *A. tenuis*.⁶ A reference crude *Alternaria* extract was also prepared in our laboratory.⁵ Alt-I was prepared from the latter extract by sequential ammonium sulfate precipitation, ion-exchange chromatography, preparative isoelectrofocusing, and gel filtration, as previously described.⁵ All extracts were stored at 4° C, and assays were performed between February and September 1981, well before the labeled expiration dates of the commercial extracts.

Antibodies

Rabbit anti-*Alternaria* antibodies were purchased from Allergologisk Laboratories A/S (Alk), Copenhagen, Denmark. Anti-Alt-I antibodies were raised in rabbits by methods described elsewhere.⁷

RAST inhibition studies

Allergenic activity of *Alternaria* and Alt-I in all commercial extracts was assayed in mini-RAST inhibition assays⁸ using *Alternaria* (70% ammonium sulfate-precipitable fraction) and Alt-I allergens linked to cyanogen bromide-activated microcrystalline cellulose.⁵ The IgE antibody preparation was prepared by mixing equal volumes of defibrinated plasma from seven patients highly allergic to *Alternaria*; none of these patients had received previous immunotherapy with *Alternaria*. In each assay we included a reference preparation of either crude *Alternaria* extract or Alt-I prepared in our laboratory.

DARIA for Alt-I

The Alt-I content of all commercial *Alternaria* extracts was measured by DARIA.⁹ Alt-I fraction was radiolabeled with ¹³¹I (5.5 μ Ci/ μ g) by the chloramine-T method.¹⁰

Rabbit anti-Alt-I serum (1:20 v/v, 0.1 ml), radiolabeled Alt-I (10 ng, 0.1 ml), and various amounts of unlabeled Alt-I and different *Alternaria* extracts were incubated overnight at 4° C, followed by addition of normal rabbit serum (1:20 v/v, 0.05 ml) and an excess of burro anti-rabbit IgG (0.2 ml) to precipitate all rabbit IgG. Dose-response curves of each extract were plotted in a logit-log fashion¹¹ using a programmable Hewlett Packard 9845B computer.

CIE

CIE studies were undertaken to study the number and relative quantity of antigenic determinants¹² in each of the eight extracts by means of the commercial rabbit anti-*Alternaria* antibodies. CIE gels were prepared from 1% agarose (Indubiose agarose, L'Industrie Biologique Francaise, Clichy, France) in 0.024M barbital buffer (pH 8.6). First- and second-dimension electrophoreses were performed in 0.15 cm-thick gel at 10 V/cm for 25 min and 2 V/cm for 16 hr, respectively, at 10° C. Commercial rabbit anti-*Alternaria* antibodies (75 μ l) were incorporated into the anodic gel during the second-dimension electrophoresis. The plates were washed, pressed, dried, and stained with Coomassie Brilliant Blue.

TLIEF in polyacrylamide gel

Proteins in each *Alternaria* extract and Alt-I were separated by TLIEF (pH 2.5 to 6.0) by the method of Awdeh et al.¹³ Gels were fixed and stained with Coomassie Brilliant Blue.

RESULTS

RAST inhibition studies

All eight commercial *Alternaria* extracts produced dose-related RAST inhibition with solid-phase *Alternaria*, indicating the presence of *Alternaria* allergens in all extracts (Fig. 1). In this and in the following two figures the volumes of test extracts shown have been corrected for differences in weight:volume labeling.

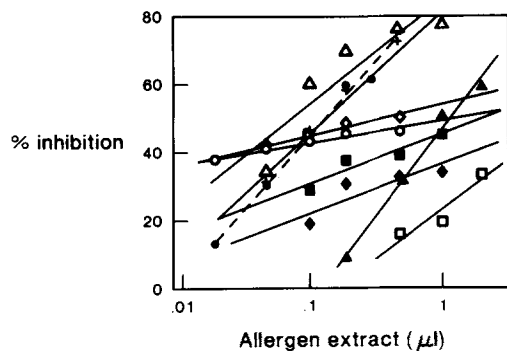


FIG. 1. RAST inhibition curves produced by different *Alternaria* extracts using solid-phase *Alternaria* extract. ●, Reference *Alternaria* extract (dashed line); ○, Ohio; ▲, Oklahoma; △, Barry; ■, Berkeley; □, Center; +, Greer; ◆, Hollister-Stier regular; ◇, Hollister-Stier MMP.

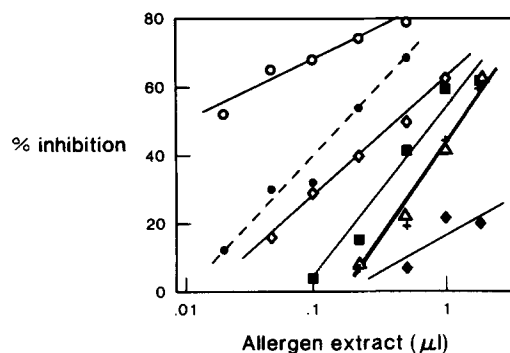


FIG. 2. RAST inhibition curves produced by different *Alternaria* extracts using solid-phase Alt-I reference. ●, Alt-I reference (dashed line); ○, Ohio; △, Barry; ■, Berkeley; +, Greer; ◆, Hollister-Stier regular; ◇, Hollister-Stier MMP.

There were wide variations in the allergenic potency of different extracts; the slopes of dose response curves were different, indicating not only the presence of variable allergen content but also difference in the nature of *Alternaria* allergenic determinants. Therefore quantitation of allergen activity based on the reference extract was not possible. It was also not possible to achieve 50% inhibition of binding with several of the test extracts. Nevertheless, 50% inhibition points were estimated for all extracts by extrapolation of inhibition curves to permit establishment of rank orders of potency.

In the RAST inhibition with solid-phase Alt-I, six of the eight extracts tested produced significant dose-related inhibition (Fig. 2). Extracts 2 and 5 (Oklahoma and Center) did not show significant inhibition at test volumes up to 20 μ l of 1:200 w:v. Again, the slopes of the dose-response curves with different extracts were not all parallel to the Alt-I standard curve. Hence, absolute quantities of Alt-I could not be calcu-

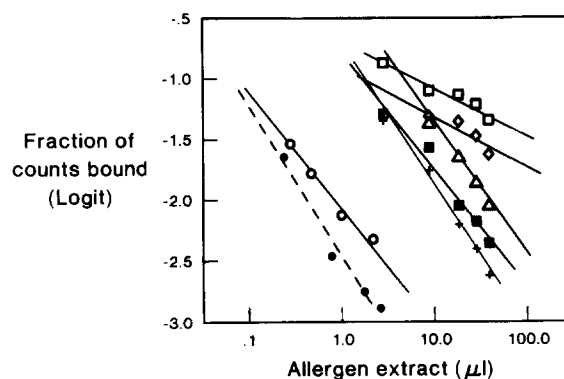


FIG. 3. Dose-response curves produced by different *Alternaria* extracts in DARIA for Alt-I. ●, Alt-I; ○, Ohio; △, Barry; ■, Berkeley; □, Center; +, Greer; ◇, Hollister-Stier MMP.

lated from the standard curve. However, an estimate of Alt-I content in each extract was again made on the basis of the volume of extract required for 50% inhibition in Alt-I RAST. These results showed wide variations in Alt-I activity in various extracts.

DARIA for Alt-I

Only six of the *Alternaria* extracts tested showed significant Alt-I activity by DARIA (Fig. 3). Extracts 2 and 7 (Oklahoma and Hollister-Stier regular) showed no measurable Alt-I activity. As observed in the *Alternaria* and Alt-I RAST inhibition studies, wide variations in the antigenic activity were observed in these experiments. Here again, the slopes of the dose-response curves for the different extracts did not all parallel that of the Alt-I standard. Consequently, absolute quantities of Alt-I could not be calculated for every extract. An estimate of Alt-I activity was made by calculating the volume of each extract required for 50% inhibition in DARIA. The rank orders of Alt-I potency, estimated by Alt-I RAST inhibition and DARIA for Alt-I, were significantly correlated (Spearman's $r = 0.8$, $n = 8$; $p < 0.05$). However, Alt-I content, measured either by RAST inhibition or DARIA, did not correlate with the total allergenic potency of the extracts as measured by RAST inhibition with solid-phase *Alternaria* (Spearman's $r = 0.46$ and 0.51 , $n = 8$; $p > 0.2$, respectively).

CIE

In CIE experiments using rabbit anti-*Alternaria* antibodies, seven commercial *Alternaria* extracts produced a varying number and pattern of Coomassie blue-stained precipitation bands (Fig. 4). Extract 5 (Center) failed to show any precipitin bands in these experiments.

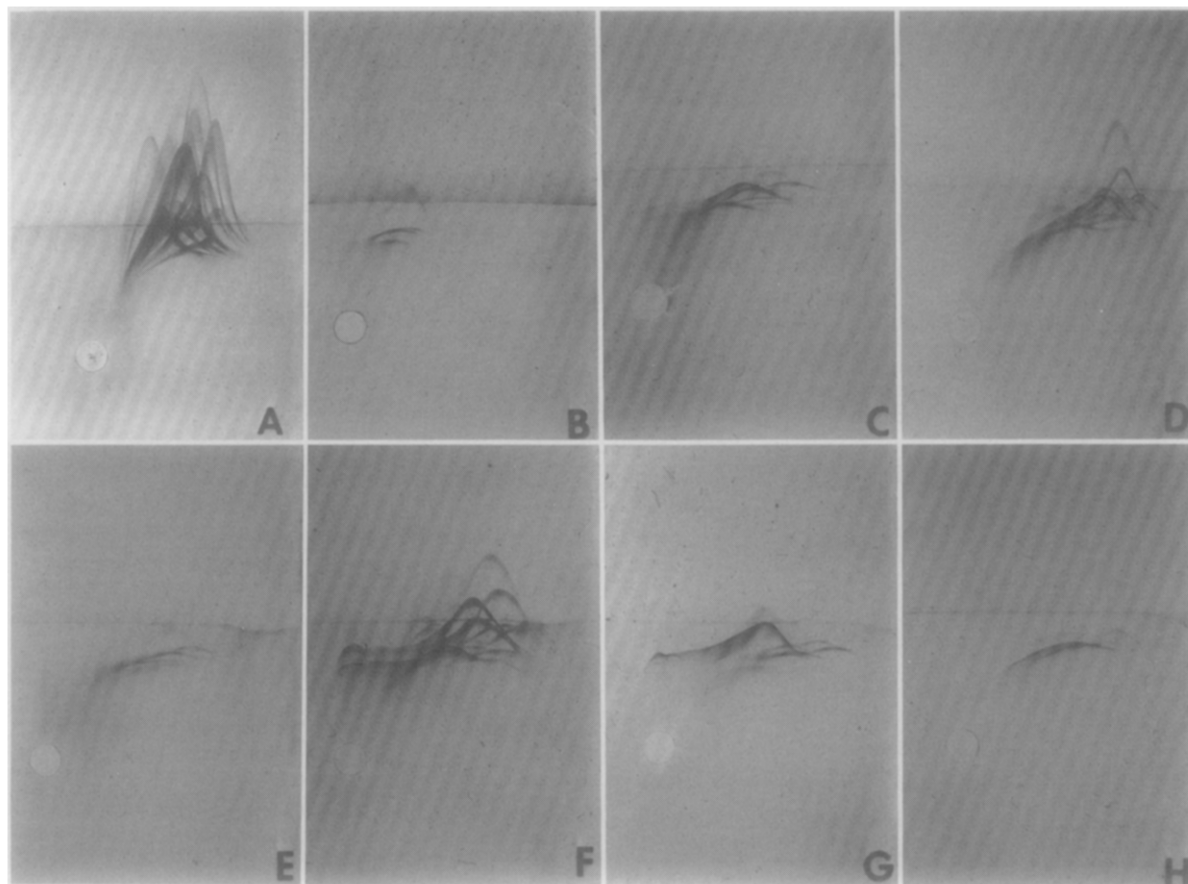


FIG. 4. CIE patterns obtained with: **A**, *Alternaria* reference extract (8 mg/ml) and, **B** to **H**, commercial *Alternaria* extracts 1, 2, 3, 4, 6, 7, and 8, respectively (1: 20 w: v). Extracts labeled other than 1: 20 w: v concentration were diluted or concentrated accordingly. The anode is to the right for the first-dimension electrophoresis and to the top for the second-dimension electrophoresis. See Table I for identification numbers of commercial extracts.

TLIEF

On TLIEF analysis, each *Alternaria* extract produced a distinctive pattern of protein bands in different pI regions (Fig. 5). Once again, extract 5 (Center) failed to show any protein bands in the TLIEF.

DISCUSSION

Although the need for allergen standardization has been recognized by allergists for several decades, it has been only during the past 10 yr that sophisticated biochemical and immunologic tests have been developed to facilitate standardization. During the 1920s and 1930s attempts were made to standardize allergen extracts on the basis of TNU or PNU, or even simple ratio of weight to volume.¹⁴ However, TNU or PNU estimations do not necessarily correlate with the allergenic activities, since the extracts also contain various nonallergenic proteins.¹⁵⁻¹⁷ Recently, RAST inhibition assays have been suggested as a practical way

to standardize the extracts. This test, however, needs reliable solid-phase allergens, containing all the allergenic determinants present in the fluid extract. Moreover, one needs a suitable serum pool containing a representative sample of IgE antibodies directed toward both major and minor allergenic determinants in the test extracts.

Recently, it has been widely agreed that quantitation of purified major allergens by various procedures may be an ideal approach to standardize allergens.³ Single radial immunodiffusion or DARIA may be used for measurement of individual antigens within crude extracts, but these tests require the isolation and purification of individual allergens, the subsequent production of high-titered animal antibodies, and, in the case of DARIA, the radiolabeling of the purified antigen. CIE and TLIEF have also been used for the analysis of proteins in allergen extracts. TLIEF provides high resolution of allergen extracts into multiple

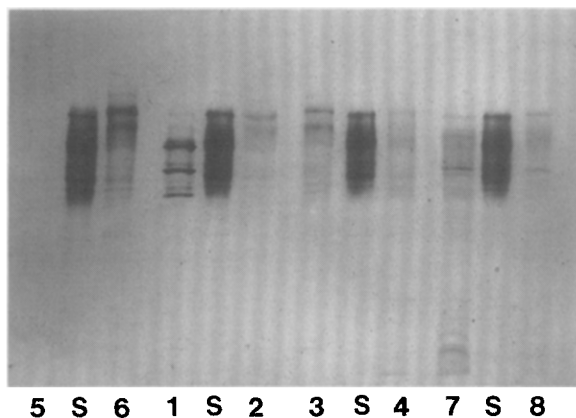


FIG. 5. TLIEF analysis (pH 2.5 to 6.0) of Alt-I standard and various commercial *Alternaria* extracts; the anode is to the top. S, Alt-I standard; see Table I for identification numbers of commercial extracts.

components, but this test provides no information regarding the allergenic activity. Similarly, with CIE one separates and identifies the antigenic components in each extract, but information as to which antigens are allergenic is not provided. Although CRIE gives quantitative and qualitative information regarding components that are both allergenic as well as antigenic, an allergen extract may theoretically contain allergenic components that may not be antigenic to animals. In general, each test procedure has its own limitations. It would seem that a combination of tests may be optimal for developing proper criteria for allergen standardization.¹⁸

We have elected to apply these approaches to study commercial *Alternaria* extracts, not only because of our previous studies of *Alternaria* extracts¹⁷ but also because prior allergen standardization efforts have been directed to pollen, venom, or dander allergens and not to molds. Our results showed wide variations in the allergenic and antigenic activity in different extracts. We found in Alt-I RAST and DARIA that the slopes of the dose-response curves with different extracts were not parallel, and absolute quantitation of Alt-I was not possible in every case. The Alt-I content estimated by either method did not show any statistically significant correlation with the allergenic activity determined by RAST inhibition with solid-phase *Alternaria*. The results show that *Alternaria* extracts vary both quantitatively and qualitatively with respect to allergen content and also that measurement of a single purified allergen in a crude allergen mixture may not adequately reflect the total allergenicity of the crude allergen mixtures. However, the combination of test procedures, although not quantitative, was certainly adequate to separate grossly deficient ex-

tracts from the other extracts. In summary, we believe that a combination of the RAST inhibition, CIE, TLIEF, and, if possible, DARIA procedures is a practical way to standardize mold allergen extracts.

We acknowledge with gratitude the technical assistance of Mark C. Swanson, the editorial assistance of Cheryl Adolphson, and the secretarial assistance of Marian Bortolon in preparing the manuscript.

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