

Immunological studies on *Alternaria* sensitivity use of crossed radioimmuno-electrophoresis, precipitins and enzyme-linked immunosorbent assay

R. K. BUSH, M. J. VOSS, J. JONES and D. K. FLAHERTY

Allergy Section, William S. Middleton Memorial Veterans Hospital and Department of Medicine, University of Wisconsin-Madison, Madison, Wisconsin, U.S.A.

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Summary

Characterization of the immunological response to *Alternaria* in sensitive subjects is not complete. We used crossed radioimmuno-electrophoresis (CRIE) to identify antigens in *Alternaria* extracts reacting with IgE antibody in five patients with *Alternaria*-sensitive asthma, four with *Alternaria*-induced rhinitis, three non-allergic asthmatics, and three normal controls. All five *Alternaria*-asthma patients and three of four *Alternaria*-rhinitis patients showed IgE binding to a single antigen peak in the CRIE. One patient exhibited specific radio-staining to a second peak, and two other patients had IgE binding to a third antigen. These results suggest an analogy of *Alternaria* antigens with that found in ragweed pollen extracts, i.e. that IgE antibody is directed against more than one antigen. Using the enzyme-linked immunosorbent assay (ELISA), we found a significant difference ($P < 0.05$, unpaired Student's *t*-test) in IgG binding between *Alternaria*-sensitive asthmatics and normal controls. There was no apparent difference in IgG binding between untreated *Alternaria*-sensitive asthmatics and those receiving high-dose immunotherapy.

Introduction

Sensitivity to the dematiaceous mould *Alternaria*, was first reported as a cause of asthma over 50 years ago (Hopkins, Benham & Kesten, 1930). Using the radioallergo-sorbent test (RAST), several investigators recently identified major *Alternaria* antigens that may trigger episodes of asthma and rhinitis (Vijay *et al.*, 1979; Yunginger *et al.*, 1980). However, final characterization of these antigens is incomplete. And, although immunotherapy with *Alternaria* extracts has been used in many atopic patients, the immune responses produced by this treatment also are not yet understood.

Flaherty *et al.* (1980) recently examined serologically defined HLA types in *Alternaria*-sensitive rhinitis and asthma patients and found that the majority of *Alternaria*-sensitive patients had reactive airway disease (asthma) with or without rhinitis and few had rhinitis alone. This raised the question of whether IgE antibodies of *Alternaria*-sensitive asthmatics are directed against the same or different antigens

Correspondence: Dr R. K. Bush, William S. Middleton Memorial Veterans Hospital, 2500 Overlook Terrace, Madison, WI 53705 U.S.A.

than in patients with rhinitis alone. Previous studies also indicated that *Alternaria* IgG precipitins occur frequently in patients receiving immunotherapy, particularly in those with adverse reactions to that therapy (Busse *et al.*, 1976). These observations prompted us to ask what role IgG antibodies play in allergic reactions to *Alternaria*.

We used crossed radioimmuno-electrophoresis (CRIE) as developed by Weeke & Lowenstein (1973) to study IgE antibodies. This method had been used to study allergens in the mould, *Cladisporium* (Aukrust, 1979) and in grass pollen (Weeke, Lowenstein & Nielsen, 1974). The role of immunoglobulin classes other than IgE in *Alternaria* sensitivity were examined by the Ouchterlony gel immunodiffusion technique. The enzyme-linked immunosorbent assay (ELISA) provided a practical means to assay serum IgG antibodies in response to immunotherapy with *Alternaria* extracts.

Materials and methods

Patient population

Alternaria-sensitive asthmatics were defined by positive immediate skin tests to *Alternaria*, clinical symptoms of asthma between July and November for 2 consecutive years, and reversible airway obstruction documented by pulmonary function testing.

Alternaria-sensitive rhinitis patients were defined by positive immediate skin test reactivity to *Alternaria* extracts, clinical symptoms of nasal pruritus, congestion, and rhinorrhea between July and November, and absence of reactive airway disease as witnessed by a negative methacholine challenge.

Non-allergic asthmatics were defined by negative skin test reactions to a battery of common inhalant antigens (ragweed, grass, tree pollens, animal danders and house dust), negative RAST to multiple antigens, and reversible airway obstruction on pulmonary function testing.

Normal individuals were defined by a negative personal or family history of atopic disease, negative immediate skin tests to the same battery of common inhalant antigens, and negative RAST to multiple antigens.

Antigen and antisera preparation

For the CRIE experiments, commercially available, dry, defatted *Alternaria* powder (Greer Laboratories, Lenoir, NC, U.S.A.) was extracted in 0.05 M Tris-HCl buffer pH 8.0, in a 1:100 w/v concentration. This extract was mixed 50:50 with fortified (3 mg *Mycobacterium tuberculosis*/ml) Freund's complete adjuvant and used to immunize 3 kg adult white rabbits (Klubertanz, Stoughton, WI, U.S.A.). Rabbits were immunized subcutaneously with 1 ml aliquots in multiple injection sites at weekly intervals for 6 weeks. They were then bled.

For the gel immunodiffusion technique, a culture filtrate was prepared from an *Alternaria alternata* strain obtained from the Wisconsin State Laboratory of Hygiene (Madison, WI, U.S.A.) by growing the mold on Czepak's Dox broth for 21 days. The culture filtrate was lyophilized and resuspended in saline at 1:20 w/v for testing of serum specimens. Preliminary experiments indicated that this preparation gave positive reactions comparable to commercially obtained extracts.

This same *Alternaria alternata* strain was also grown and extracted according to the method of Yunginger *et al.* (1980). The crude extract was lyophilized, stored at -20°C , and later used to devise the ELISA that detected IgG antibody.

CRIE

We conducted CRIE on fifteen subjects (*Alternaria*-induced asthma, five; *Alternaria* rhinitis, four; non-allergic asthmatics, three; and controls, three). CRIE was performed basically as described by Aukrust (1979). Antigens in complex mixtures were first separated primarily according to charge by electrophoresis in one dimension. Electrophoresis in a dimension perpendicular to the original electrophoresis into a gel containing rabbit anti-*Alternaria* serum (as described above) yielded precipitin arcs. Upon incubation with the gel, the *Alternaria*-specific IgE antibodies in a given patient's serum, bound to the appropriate antigens in the crossed immunoprecipitates and were subsequently detected by adding radiolabelled anti-human IgE and conducting autoradiography.

In these experiments, first dimension immunoelectrophoresis of the *Alternaria* extract (10 μ l of a 1:10 w/v extract) was performed in a 1% agarose (type I, low EEO, Sigma Chemical, St. Louis, MO, U.S.A.) gel prepared in an 0.073 M Tris, 0.024 M barbital, 0.006 M calcium lactate buffer, pH 8.6 on a 10 $\times$ 10 cm glass plate at 10 volts/cm for 25 min. Gel strips (1 $\times$ 5 cm) were transferred to 5 $\times$ 7 cm glass plates and placed 1 cm from and parallel to one end of the plate. A brass bar was placed 1 cm above the gel strip and 1 ml of the agarose solution were pipetted onto the plate to act as a spacer gel. The brass bar was then cut free of the gel. Two ml of the agarose solution containing 400 μ l of rabbit antisera were poured onto the remainder of the plate to act as the second dimension reference gel. One ml of agarose containing 100 μ l of rabbit antisera was pipetted onto the 1 $\times$ 5 cm area below the first dimension gel in order to detect cathodally-migrating antigens. Second dimension immunoelectrophoresis was performed at 2 volts/cm for 18 hr.

The gels were incubated overnight with 400 μ l of patient's sera in 7 ml of 0.07 M sodium phosphate, 15 mM sodium azide buffer, pH 7.0 in 7 $\times$ 5 cm plastic boxes and then washed three times with 0.15 M sodium chloride. Next, the plates were incubated overnight with ^{125} I-labelled anti-human IgE (Pharmacia, Piscataway, NJ, U.S.A.) at 250 000 c.p.m./plate in 7 ml of 0.05 M sodium phosphate, pH 7.5, which contained 0.3% bovine serum albumin, 0.15 M sodium chloride, 15 mM sodium azide, and 2.7 mM disodium EDTA. The plates were then washed with 0.15 M sodium chloride, dried, and exposed to Kodak XRP-1 film (Kodak, Rochester, NY, U.S.A.), in a light-proof film box for three weeks at -20°C . The film was then developed, and the gel plates were stained with Coomassie brilliant blue. The antigens identified by the Coomassie brilliant blue staining were numbered beginning with the most anodally migrating one.

Gel immunodiffusion

Sera from 107 subjects were examined by gel immunodiffusion (forty-two *Alternaria*-sensitive asthmatics, twenty *Alternaria* rhinitis patients, twenty-one non-allergic asthmatics, and twenty-four normal controls). The double immunodiffusion gel technique of Flaherty *et al.* (1974) was used to examine sera for precipitating antibodies against *Alternaria*. A 1:20 w/v concentration of the extract described above was used for the testing. The plates were incubated under high humidity at room temperature for 48 hr.

ELISA for IgG antibodies

An ELISA method similar to that described by Sepulveda, Longbottom & Pepys (1979) was used to compare serum samples from untreated *Alternaria*-sensitive

asthmatics, *Alternaria*-sensitive asthmatics who had received immunotherapy, and normal controls, for the presence of IgG antibodies. Each well of plastic microtitre plates (Flow Laboratories, Hamden, CT, U.S.A.) was coated with 100 μ l of *Alternaria* antigen (100 μ g/ml) in 0.05 M NaCO₃, 0.01% sodium azide, pH 9.0 and incubated overnight at 37°C. Wells were washed with a sodium phosphate buffer (0.55 M phosphate, 0.14 M NaCl that contained 0.2% bovine serum albumin, 0.05% Tween 20, pH 7.5) and aspirated dry. One hundred microtitres of a 1:20 dilution of patient sera in PBS, pH 7.5, containing 0.05% Tween 20, were added to the wells and the plates incubated at 37°C for 60 min. The serum was aspirated from the wells and the plates washed three times. Peroxidase-conjugated rabbit anti-human IgG (100 μ l, diluted 1/2000 in PBS with 5% BSA) was added to the wells and incubated at 37°C for 30 min. A 100 μ l aliquot of 2,2'-azino-di-3 ethyl-benzthiazoline sulphonic acid, (ABTS; Sigma, St. Louis, MO, U.S.A.) enzyme substrate solution (0.4 M ABTS and 0.5 M H₂O₂ in 0.05 M sodium citrate, pH 4.0) was then added, and the reaction was allowed to proceed to good colour development (30 min) at room temperature. It was stopped by the addition of 100 μ l of 0.001 M edetic acid in 0.1 M hydrofluoric acid. The optical density of the samples was then determined at 414 nm in a micro-sampling spectrophotometer (Guilford, Oberlin, OH, U.S.A.). We studied sera from eight untreated *Alternaria*-sensitive asthmatics, eight *Alternaria*-sensitive asthmatics who had received a mean of 45 843 PNU (range 14 885–67 188 PNU) of *Alternaria* antigen immunotherapy in the year prior to the study, and seven normal individuals.

Results

Demonstration of a major antigen recognized by IgE antibody using CRIE

Coomassie brilliant blue staining of the two-dimensional immunoelectrophoresis gels is shown in Fig. 1A. We were able to identify twenty-one antigens in the *Alternaria* extract by varying the concentration of antigen in the first dimension of the immunoelectrophoresis gel and the concentration of rabbit antisera in the second dimension reference gel of the CRIE. A composite drawing based on these studies is shown in Fig. 1B.

The autoradiographs obtained when sera from normal persons or non-allergic asthmatics were applied to the plates following electrophoresis and incubation with radiolabelled anti-human IgE showed no radiostaining for IgE antibodies against *Alternaria*.

A single major antigen reacted with IgE in three of the four *Alternaria*-sensitive rhinitis patients as seen by the peak in Fig. 2A. This major peak corresponded with antigen number 6 in the Coomassie brilliant blue stained reference plate. Another slight reaction with IgE exhibited by one patient corresponded to antigen number 20 in the reference gel (Figure 2A). One patient failed to show any specific radiostaining in the autoradiography. This patient had received high-dose immunotherapy which may have produced blocking antibodies that interfered with the CRIE. The specific IgE may also have been too low to detect by this method.

Autoradiographs from all five *Alternaria*-sensitive asthmatics produced a single major peak, again corresponding to antigen number 6 in the reference pattern. We also identified fainter staining peaks in two patients which corresponded to antigen number 12 in the reference gel (Fig. 2B).

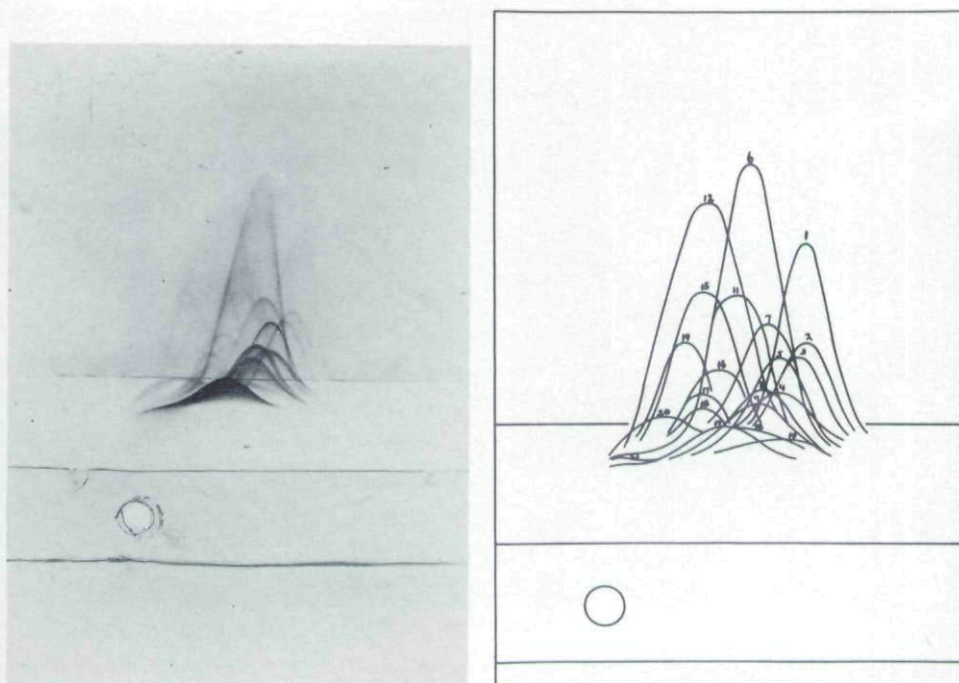


Figure 1A (left) Two-dimensional immunoelectrophoresis plate stained with Coomassie brilliant blue. Conditions of the electrophoresis were: 10 μ l of *Alternaria* antigen 1:10 w/v applied to the first dimension gel well; 400 μ l of rabbit sera in the upper antibody gel; 100 μ l of rabbit antisera in the lower gel. Note that no cathodally migrating antigens were detected.

Figure 1B (right) Composite drawing of two-dimensional immunoelectrophoresis precipitin arcs. The drawing was based on preliminary experiments in which the amount of antigen applied to the first dimension gel well and the quantity of rabbit sera in the antibody gel were varied. Twenty-one precipitins were identified.

Detection of precipitins against Alternaria antigens by double immunodiffusion.

Only one of 107 serum samples studied showed a positive precipitin reaction to *Alternaria*. This patient was an *Alternaria*-sensitive asthmatic who had received immunotherapy for a number of years without adverse effect.

Quantitation of anti-Alternaria IgG antibodies by ELISA.

ELISA results are summarized in Table 1. Untreated *Alternaria*-sensitive asthmatics

Table 1. Comparison of anti-*Alternaria* IgG responses in untreated and treated asthmatics

Subjects	n	Mean absorbance units at OD ₄₁₄	s.e.mean
Normals	7	1.749	± 0.331
Untreated <i>Alternaria</i> -sensitive asthmatics	8	2.784	± 0.227
<i>Alternaria</i> -sensitive asthmatics receiving immunotherapy	8	2.791	± 0.258

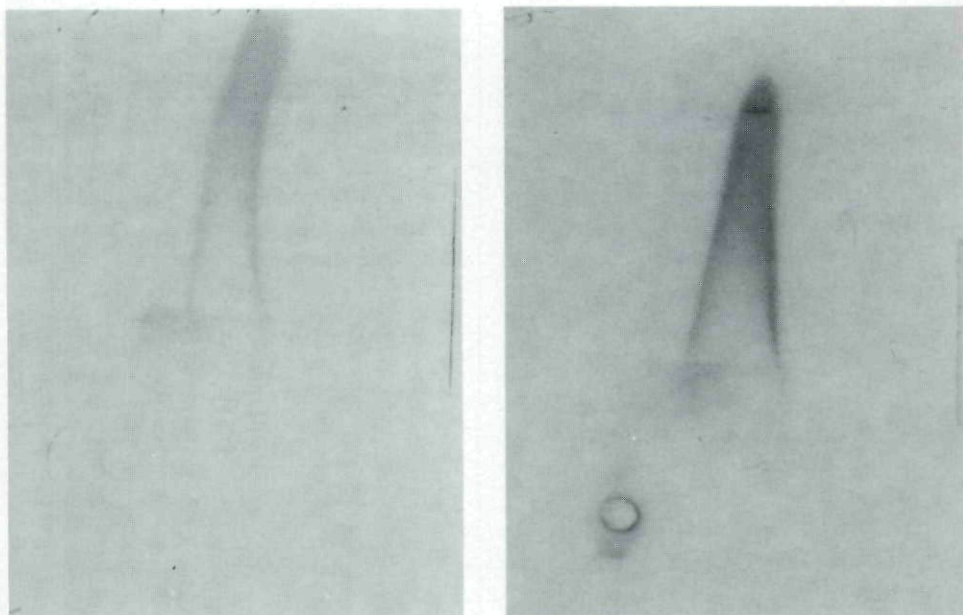


Figure 2A (left). Autoradiograph of CRIE plate. CRIE plate was incubated with 400 μ l of patient sera followed by ^{125} I-labelled anti-human IgE (250 000 cpm) and subsequently exposed to X-ray film for 21 days. Specific IgE binding to antigen 6 in the gel and fainter binding to antigen 20 were detected in this patient's sera.

Figure 2B (right). Similar autoradiograph from another patient shows specific IgE binding to antigen 6 and to antigen 12.

and those who had received immunotherapy had significantly higher IgG binding to antigens that adhered to plastic in the ELISA assay than did the normal controls ($P < 0.05$, unpaired Student's *t*-test). There was no significant difference between the untreated *Alternaria*-sensitive asthmatics and those who had received immunotherapy.

Discussion

The CRIE technique has been used to identify IgE-binding antigens in a variety of complex mixtures, including extracts of moulds (Lowenstein, Aukrust & Gravesen, 1977), pollens (Weeke *et al.*, 1974), and animal danders (Blands, Lowenstein & Weeke, 1977). Our studies indicate that CRIE is useful in identifying *Alternaria* antigens which react with IgE. Although we could not correlate a specific antigen with a specific disease state such as rhinitis or asthma, we did find that nine of ten *Alternaria*-sensitive patients had IgE antibodies directed against a major antigen. In addition, we identified two other antigens which also bind to IgE. Whether the major antigen seen in the CRIE is the same as the ALT-1 described by Yunginger (1980) is unknown. We are exploring this possibility in further studies. Based on the results of the present study, we feel that the IgE-reacting antigens in *Alternaria* are analogous to ragweed antigens E, Ra3, Ra5, and K.

Contrary to the observations of Busse *et al.* (1976), we did not find a high incidence

of precipitating antibodies against *Alternaria* and could not correlate the presence of precipitins with a specific disease state. The use of a more sensitive method such as counterimmunoelectrophoresis may have yielded more positive results. Kawai *et al.* (1973) found a high percentage of IgG precipitins against *Alternaria* by using counterimmunoelectrophoresis; however, there was no difference in the percentage of positive precipitins among normal persons (47.1%), atopic asthmatics (34.6%) or asthmatics (45.2%) receiving immunotherapy.

In the ELISA experiments, we demonstrated a significant difference between IgG antibody levels directed against *Alternaria* antigens which bound to plastic surfaces in sera from normal controls and those from *Alternaria*-sensitive asthmatics. High-dose immunotherapy had no effect on these levels. There are a number of possibilities that might account for these findings. First, IgG antibody may have been directed against antigens other than those recognized by IgE. Secondly, the antigens recognized by IgE might not have bound to the plastic plate. Lastly, the antigens recognized by IgE may have constituted only a small portion of the total antigen binding to the plate. Therefore, the group which received immunotherapy might have IgG antibody against major *Alternaria* antigens recognized by IgE but it is not being detected by ELISA. We are currently planning CRIE experiments to examine IgG binding components using sera from untreated and treated *Alternaria*-sensitive asthmatics.

In summary, our studies demonstrate the presence of specific IgE antibodies directed at a single antigen in the sera of a majority of patients with clinically defined *Alternaria* sensitivity. We also identified two other minor antigens which bind IgE. Further studies on larger populations will be necessary to confirm these observations and to determine the clinical significance of the individual antigens. In addition to a specific IgE response to *Alternaria*, sera from sensitive patients contain IgG antibody that could be quantitated by the ELISA. The role of anti-*Alternaria* IgG antibodies in the host response to *Alternaria* remains to be determined.

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