

Allergic bronchopulmonary aspergillosis: Reactivity of IgE and IgG antibodies with antigenic components of *Aspergillus fumigatus* (IgE/IgG antigen complexes)

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Sera of patients with ABPA were tested by XRIE tests incorporating their own serum (self-XRIE) to detect the presence of IgG/IgE antigen complexes to a "reference" Aspergillus fumigatus preparation. Of the 32 sera studied, 29 (90%) had visible precipitin (IgG) peaks, and 27 of these 29 as well as the three apparently precipitin-negative sera, i.e., 30 (94%), showed binding of specific IgE by autoradiography. The two precipitin-positive sera that did not show IgE binding were also skin test negative and RAST negative to this A. fumigatus antigen. Specific IgG as determined in ELISA correlated well with the grading of the XIE precipitin peaks ($p < 0.05$). There was also a highly significant correlation between specific IgE by RAST and grading the radioactive uptake seen in the autoradiograph ($p < 0.001$) indicating, for each serum, the presence of IgG antibodies to most of the components to which there was specific IgE. In the self-XRIE tests there was considerable variation of reactivity from serum to serum, in numbers of antigen/antibody peaks observed, in relative peak heights, and in the intensity of the respective staining. By comparing each test to a "reference" pattern developed with the use of an ABPA serum pool, the antigenic components of A. fumigatus were found to be of two main types: (1) antigens that appeared to be poorly precipitating (possibly low-molecular-weight components) but showed strong IgE binding (these were apparently major allergenic components and with one exception proved to be the faster migrating components) and (2) antigens that produced the strongest precipitin reactions with only weak binding of specific IgE and therefore minor allergenic components. (J ALLERGY CLIN IMMUNOL 72:668-669, 1983.)

Most patients with ABPA have in their serum both specific IgE and IgG (precipitating) antibodies to *Aspergillus fumigatus*. Levels of these antibodies are known to fluctuate and have been associated with the patient's episodes of pulmonary eosinophilia.¹⁻⁴

Relatively little is known about the physical or chemical properties of the specific antigens or allergens that may be involved. In earlier studies, the main protein/glycoprotein components were shown to be the most important diagnostically both for detec-

tion of specific precipitating antibody and for their skin test reactivity,¹⁻⁵ although there was no indication as to whether the same or different antigenic/allergenic components were involved. Dessaint et al.⁶ reported the presence of an allergen distinct from the *A. fumigatus* species-specific antigenic component, which possessed chymotryptic activity and reacted with 53% of sera from their patients with aspergillosis. More recently an antigen precipitating with 75% of sera of patients with aspergilloma and ABPA was characterized as an acidic glycopeptide,⁷ but no information regarding its allergenicity was available.

There is now, however, evidence that several of the antigenic components do react with both types of antibody, i.e., formation in vitro of IgE/IgG antigen complexes.^{8,9} The method used was an XRIE test in which antigen-specific rabbit antiserum was replaced by the patient's own serum, i.e., self-XRIE, and the specificity of these tests has been demonstrated.⁸ The aim therefore of the present investigation was

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Abbreviations used

ABPA:	Allergic bronchopulmonary aspergillosis
XRIE:	Crossed radioimmunoelectrophoresis
ELISA:	Enzyme-linked immunosorbent assay
XIE:	Crossed immunoelectrophoresis
RAST:	Radioallergosorbent test
SPT:	Skin prick test
PBS:	Phosphate-buffered saline
APC:	Allergen particle complex
Af:	<i>Aspergillus fumigatus</i>
S-TS:	Short-term sensitivity

threefold: (1) to determine the incidence of these self-XRIE types of reactions for the sera of 32 patients with ABPA, (2) to assess their relative reactivity (i.e., precipitins by stained XIE plate and IgE by autoradiography) and compare these reactivities to the levels obtained by conventional methods (IgG by ELISA¹⁰ and IgE by RAST), and (3) to identify the antigenic components that were most frequently involved.

MATERIAL AND METHODS

Sera

Sera were obtained from 32 patients with chronic ABPA, all of whom fulfilled the necessary immunologic and radiologic criteria, viz., all were skin test positive to *A. fumigatus* and had had recurrent episodes of asthma associated with pulmonary eosinophilia.^{2, 11} In routine double diffusion tests (kindly performed by Mr. H. Wingfield, Mycology Laboratory, Brompton Hospital), 17 (53%) were precipitin positive with neat serum and 24 (75%) with a threefold concentration of serum.

A "reference" ABPA serum pool was also prepared from equal aliquots of eight sera that showed the highest self-XRIE activity.

A. fumigatus antigen

This was a freeze-dried, dialyzed, culture-filtrate extract from a 4 to 5 wk surface culture grown in a synthetic medium. It was the same reference preparation (B27) as used previously.^{8, 12, 13}

In 22 of the 32 patients with ABPA, SPTs were performed with this antigen at a concentration of 1 mg/ml.

¹²⁵I-labeled anti-human IgE

Anti-IgE Fc specific kindly supplied by the late Dr. C. E. Arbesman, Buffalo, New York,¹⁴ was labeled with ¹²⁵I by the chloramine-T method and used in the self-XRIE tests at 20,000 cpm/100 μ l and in RAST at 180,000 cpm per test.

Self-XRIE tests

XIE tests, as described previously,^{12, 15} were performed for each serum sample. Briefly, 1.5 μ l *A. fumigatus* (B27) antigen at either 12 or 60 mg/ml was electrophoresed in the

first dimension (4.5 v/cm, 80 min) and 25 μ l patient's serum incorporated per square centimeter in the antibody containing gel for the second-dimension electrophoresis (1.5 v/cm, overnight).

The plates were washed and dried, and then 3 ml of a 1:10 dilution (in PBS, pH 7.3) of the same serum as previously incorporated in the gel was overlaid and allowed to react overnight (18 to 20 hr).⁸ After four or five washes with PBS, there was a further overnight incubation with 4 ml ¹²⁵I-anti-IgE.

The washed and dried plates were then exposed to x-ray film (NS-2T; Eastman Kodak Ltd. Rochester, N. Y.) for 5 to 7 days after which the films were developed according to manufacturer's instructions, and the glass slides were stained for protein in 0.5% Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, Mo.). Each stained XIE plate was then compared with its corresponding autoradiograph to determine which precipitated components also showed binding of IgE antibody.

Before the results of RAST or ELISA were known, both XIE plate and autoradiograph were assessed independently by two observers and graded 0 to 4, taking into account both the number of peaks and relative intensity of staining (protein/radioactivity, Fig. 1). There was >95% agreement between the results of the two observers.

Af-RAST

APC was prepared by coupling the *A. fumigatus* antigen (50 mg) to CNBr-activated Sepharose 4B (5 gm), as recommended by the manufacturer (Pharmacia Fine Chemicals, Piscataway, N. J.), and tests used 100 μ l APC (18.75%) with 50 μ l patient's serum followed, after washing, with 50 μ l ¹²⁵I-anti-IgE. The optimal concentration of APC was determined by an initial titration with sera from three patients with ABPA and positive skin tests. Results are expressed as percent uptake ¹²⁵I-anti-IgE.

ELISA

The method previously described for IgG antibodies to *A. fumigatus* was used.¹⁰ A 25 μ g/ml concentration of antigen for coating the wells of the microhemagglutination plates, a 1:100 dilution of serum, and a 1:2000 dilution of the anti-IgG alkaline phosphatase conjugate (Miles Laboratories Inc., Elkhart, Ind.) were found by checkerboard titration to give the optimal conditions for the assay.

Total IgE

Serum IgE determinations were kindly carried out by Mr. M. Kemp, Department of Pathology, Brompton Hospital, with the commercially available Phadebas-PRIST kits (Pharmacia Diagnostics, Piscataway, N. J.).

Statistics

Analysis of data was by linear regression analysis.

RESULTS

Results of the various tests carried out on the 32 ABPA patients are listed in Table I.

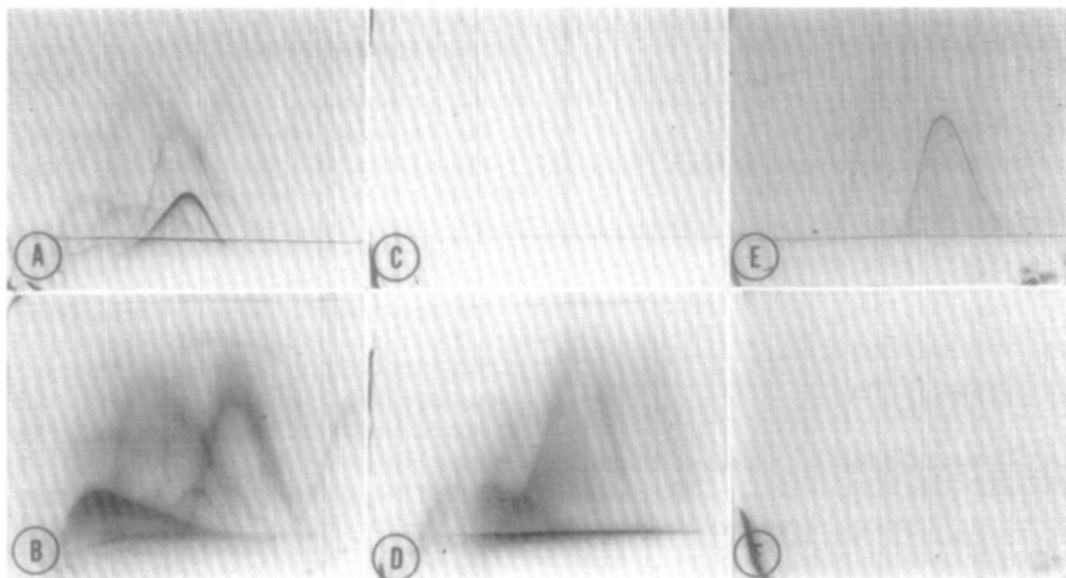


FIG. 1. Self-XRIE tests of three patients with ABPA. **A-B**, Serum of patient No. 5 shows Grade 4 reactions in XIE (**A**) and in autoradiograph (**B**). **C-D**, Serum of patient No. 10 shows no precipitins XIE (**C**) but positive reaction on autoradiograph (**D**). **E-F**, Serum of patient No. 8 shows precipitins XIE (**E**) but no radioactive uptake (**F**). For each test $1.5 \mu\text{l}$ *A. fumigatus* antigen B27 at 12 mg/ml was used with $25 \mu\text{l}$ serum/cm² gel. The anode is to the *right* for the first-dimension electrophoresis and to the *top* for the second-dimension electrophoresis.

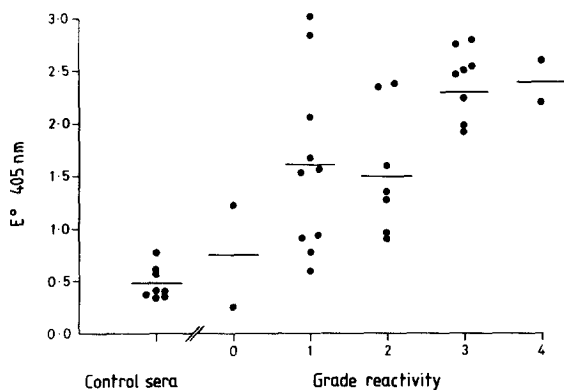


FIG. 2. Comparison of specific IgG by ELISA ($E^\circ 405 \text{ nm}$) with grade of reactivity of precipitins (XIE) for 29 ABPA sera. Values for a group of eight control sera are also included. (Mean ELISA value for each grade is indicated.)

Self-XRIE tests

In these tests, not all the stained precipitin peaks that were seen on the XIE plate showed corresponding uptake of the anti-IgE tracer, and these were frequently the sharp, most strongly stained protein peaks, whereas some peaks that showed intense radioactive uptake were frequently poorly stained or even "invisible" in the XIE plate.

Considerable variation was observed between the tests on the individual sera; however, most (27, 84%) demonstrated some positivity on both XIE and the corresponding autoradiograph. Some sera, e.g., Nos. 5, 11, 13, and 25, were strongly positive in both tests, i.e., as judged by the multiplicity of peaks obtained and the relative intensity of protein/radioactive staining, and for others there was a degree of variation between the tests. There was a definite overall tendency to attribute a higher grading to the radioactive uptake, and this is further reflected in the three sera (Nos. 9, 10, and 32) that were apparently precipitin negative but positive on autoradiography. A total therefore of 94% (30/32) of sera were positive on autoradiography.

By contrast, two sera (Nos. 3 and 8) were precipitin positive but negative in their autoradiographs. Both of these sera were also negative by RAST and in SPTs when tested with the same antigen (1 mg/ml) as used for the *in vitro* tests. (When tested with 10 mg/ml antigen, one patient developed a minimal 3 mm wheal, and the other developed a doubtful reaction.)

The one other serum sample (No. 21) negative by RAST was found weakly positive on autoradiography and did develop a minimal SPT response.

Examples of these self-XRIE tests are shown in Fig. 1, and the overall results summarized in Table II.

TABLE I. Results of the various immunologic tests performed for the 32 allergic patients with bronchopulmonary aspergillosis (ABPA)

Patient No.	Skin test Af 1 mg/ml (mm wheal diameter)	Total IgE (IU/ml)	IgE-RAST (% ¹²⁵ I-labeled uptake)	Self-XRIE*		IgG-ELISA (E° 405 nm)
				IgE	Precipitins	
1	9	700	9.12	2	1	3.18
2	nt.	300	7.28	1	1	1.56
3	—	820	2.4†	0	2	1.66
4	nt.	3200	13.59	4	3	1.86
5	3	1640	15.15	4	4	2.64
6	7	750	11.05	3	2	2.36
7	6	480	11.04	3	1	1.60
8	—	330	2.12†	0	2	1.32
9	15	620	9.38	2	0	1.22
10	6	350	7.78	2	0	0.24
11	5	5200	15.04	4	4	2.22
12	nt.	210	3.88	1	2	0.92
13	nt.	1300	12.17	4	3	2.52
14	10	2120	11.83	4	3	1.98
15	4	900	9.12	2	1	0.60
16	8	280	7.25	2	1	0.86
17	5	380	6.77	1	3	2.54
18	5	300	8.85	3	2	2.36
19	5	3200	13.15	4	2	1.28
20	3	1500	12.28	3	1	2.12
21	2	60	2.10†	1	1	1.68
22	nt.	160	4.45	1	1	0.84
23	nt.	270	4.63	2	2	0.86
24	3	645	8.40	2	3	2.48
25	5	2000	11.17	4	3	2.76
26	4	1320	10.44	3	3	2.58
27	10	600	10.58	3	1	0.78
28	2	900	12.83	2	3	2.24
29	nt.	620	7.13	1	1	2.86
30	nt.	900	6.73	2	1	nt.
31	nt.	880	8.98	3	2	nt.
32	nt.	nt.	4.63	1	0	nt.

Nt. = not tested.

*Grade of reactivity as assessed by two independent observers.

†These sera regarded as RAST negative (mean + 2 SD for control sera 2.12%).

Relationships between the immunologic tests

Precipitins (XIE) and specific IgG antibody by ELISA (Fig. 2). There was a definite correlation ($p < 0.01$, $n = 29$, $r = 0.52$) between the grading of precipitin activity (XIE) and the values obtained for specific IgG antibody by ELISA. When compared to a control group of sera (from nonatopic subjects), there were only three sera (i.e., one serum sample grade 0 and two sera grade 1) below the mean + 2 SD value of 0.79 E° 405 nm.

Specific IgE uptake in self-XRIE (by autoradiography) and specific IgE by RAST (Fig. 3). The values

for specific IgE antibody by RAST showed a highly significant correlation with the grading of the radioactive uptake ($p < 0.001$, $n = 32$, $r = 0.89$). With the exception of sera Nos. 3, 8, and 21, all sera were well above 2.12% ¹²⁵I-anti-IgE uptake, the mean + 2 SD value of the 10 control sera (five nonatopic and five atopic rat allergic subjects).

Specific IgG (ELISA) and specific IgE (RAST) to A. fumigatus. For the 29 sera for which both tests were carried out, there was a definite correlation ($0.02 < p < 0.05$) between the levels of both classes of antibodies.

Total and specific IgE. There was also a significant

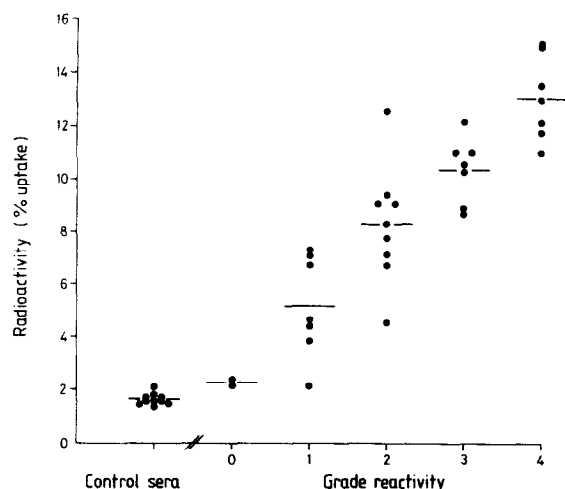


FIG. 3. Comparison of specific IgE by RAST (percent uptake of ^{125}I -anti-IgE) with grade of reactivity (autoradiograph) for the 32 ABPA sera. Values for a group of 10 control sera are also included. (Mean percent uptake for each grade is indicated.)

correlation ($p < 0.001$, $n = 32$, $r = 0.70$) between levels of total and specific IgE. Only five of the sera had levels >2000 IU/ml; the mean level for this group of patients with ABPA was 1062 IU/ml. For this study, neither the patient's steroid therapy nor the interval between taking the blood samples and the last episode of pulmonary eosinophilia (i.e., the acute phase when high levels of IgE are a feature) has been specifically documented.

Antigenic profile of reactivity of individual sera

Analysis of the reactions, both IgG and IgE, of the sera to the various antigenic components of *A. fumigatus* was achieved by comparison to "reference" patterns of reactivity (Fig. 4) (precipitin and radioactivity) obtained in self-XRIE with the ABPA serum pool. The self-XRIE test of each individual serum sample (XIE and autoradiograph) was compared to the reference pattern, and reactions to the antigenic components as numbered were noted, i.e., peaks appearing in the individual test that correspond to those numbered in the reference pattern. No attempt, however, was made to grade the individual reactions either by measurement of corresponding peak heights (an indication of antibody titer to a particular component) or by relative intensity of respective staining, although both these parameters did vary considerably from antigen to antigen and sample to sample. Therefore the results shown in the histogram (Fig. 5) are expressed merely as the numbers of sera

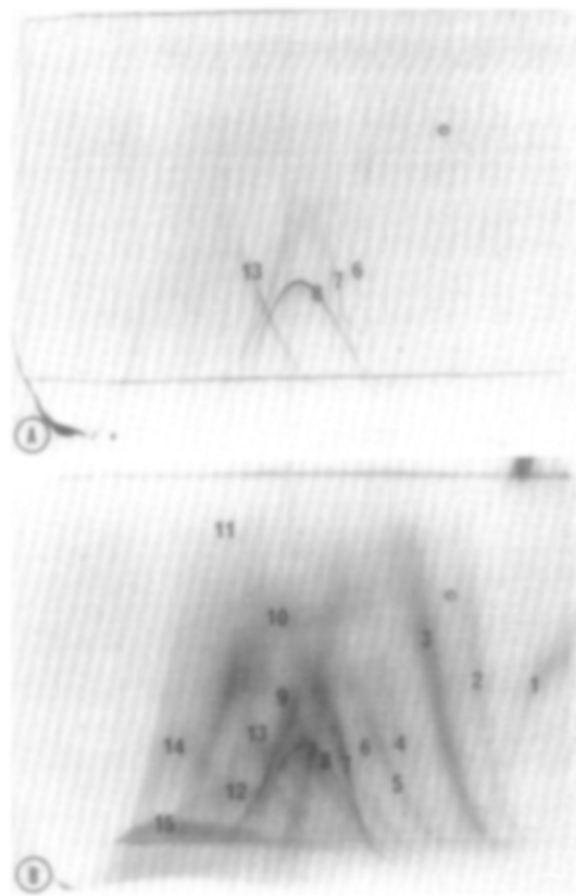


FIG. 4. Self-XRIE test of pooled ABPA reference sera with $1.5 \mu\text{l}$ *A. fumigatus* antigen B27 at 20 mg/ml and $25 \mu\text{l}$ pooled sera incorporated per square centimeter of gel. **A**, Protein stained XIE; **B**, corresponding autoradiograph. Peaks have been arbitrarily numbered from the anode (electrophoresis directions as in Fig. 1).

reacting IgG and IgE to the individual components. From these results it is apparent that the antigens fall into two main categories: those that are apparently poorly precipitating (only weakly protein stained) but show strong IgE binding, viz., antigens 1, 2, 3, 5, and 15, and those that show the stronger, sharper precipitin peaks and bind much lower amounts of IgE, viz., 6, 7, 8, and 13.

DISCUSSION

This study not only confirms previous findings of the close association between IgE and IgG antibodies in the sera of patients with ABPA¹⁻⁴ but also demonstrates that for the vast majority of such sera (30/32), there is evidence that both IgG (precipitating) and IgE antibodies are reacting with one or more of the antigenic components of *A. fumigatus* (Tables I and II). No such combined reactivity has been found with

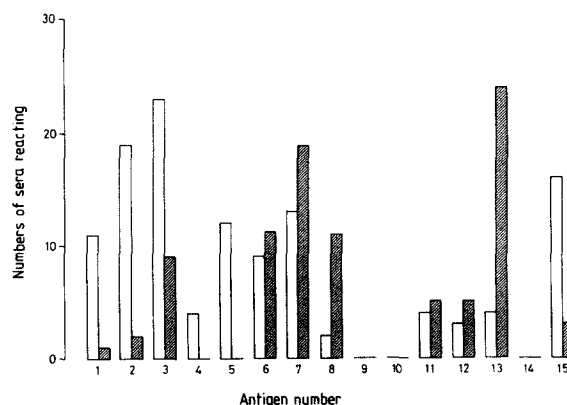
TABLE II. Summary of data for the 32 ABPA sera: Results of autoradiography compared to presence or absence of precipitins and to RAST

ABPA sera	Radioactive uptake XRIE		Totals
	+ve	-ve	
Precipitins XIE			
+ve	27	2	29
-ve	3	0	3
Totals	30	2	32
RAST			
+ve	29	0	29
-ve	1	2	3
Totals	30	2	32

control sera, which included sera from *A. fumigatus* skin test-positive patients and a patient with uncomplicated aspergilloma and a negative skin test with multiple precipitins, or in a rat allergen system.⁸

The two sera that were precipitin positive (XIE) but showed no IgE binding were also negative by RAST (i.e., < mean + 2 SD value for control sera) and came from patients that were SPT negative at 1 mg/ml to this *A. fumigatus* antigen (Table I). This apparent absence of *A. fumigatus*-specific IgE may result from this antigen lacking a specific, albeit minor, allergenic component, since it is well known that there is wide antigenic (allergenic) variation from batch to batch.^{1, 16, 17} Weak SPT reactions were elicited with a higher antigen concentration, 10 mg/ml, which may indicate that another class of antibody, e.g., IgG S-TS, is mediating the immediate reactions.¹⁸

By comparing each immunoprecipitate with its corresponding autoradiograph, varied patterns of reactivity were seen—precipitin peaks with and without associated radioactivity as well as some peaks seemingly “invisible” on XIE and appearing only in the autoradiograph, frequently with intense radiostaining. Such visualized precipitates still remained in tests incorporating IgE-depleted serum in the gel with the recovered IgE fraction in the overlay,⁸ and the presence of IgG has also been shown with ¹²⁵I-anti-IgG as the radioactive tracer (personal observation). Self-XRIE therefore resulted in an increased detection of precipitating antibodies for those sera that also contained specific IgE to the same antigenic components; in fact, except for autoradiography, three sera would have been regarded as precipitin negative. Somewhat similar findings have been noted on XIE in which disappearance of precipitates in a reference rabbit antiserum pattern did not always result in corresponding

**FIG. 5** Assessment of individual self-XRIE tests of the 32 ABPA patients expressed as numbers of sera-reacting IgG (precipitin XIE) (hatched bars) and IgE (radioactivity auto-radiograph) (open bars) to the antigenic peaks as numbered in the reference patterns in Fig. 4. Note that although peaks 9, 10, and 14 were apparent in the reference patterns, no reactions could be attributed to these antigens in the individual tests.

precipitates appearing in the intermediate gel containing patient's serum.¹⁹

Although this absence of visible precipitates is a reflection of the relative insensitivity of the protein (Coomassie blue) stain,²⁰ these reactions possibly also go undetected in routine double diffusion tests and may otherwise be regarded as caused by “non-precipitating” antibodies. Such antibodies would be detected by the more sensitive primary binding assays such as ELISA¹⁰ and radioimmunoassay^{3, 4, 21, 22} and may account for some of the discrepancies reported between results of these different methods.²³ However, as discussed later, the nature of these precipitin reactions probably depends largely on the physicochemical properties of the antigens involved.

With use of a much reduced antigen concentration and incorporation of an increased amount of serum in the gel, as compared to that required for strongly precipitating aspergilloma sera¹² (i.e., 18 µg to 204 µg and 25 µl/cm³ to 15 µl/cm³, respectively), the sensitivity of the XIE method was considerably increased for sera of patients with ABPA compared to that previously reported.¹³ It also resulted in the precipitin reactions mostly forming discrete peaks within the serum-containing gel, whereas previously with stronger antigens, frequently only very faint and “open” peaks could be detected. The overall incidence of precipitins by XIE (i.e., protein stained plates) was considerably higher (91%) than by routine double diffusion tests with neat sera (53%) or after threefold concentration (75%), although some of this discrep-

ancy in positivity may be due to the use in double diffusion of different *A. fumigatus* antigens. There was, however, good correlation ($p < 0.01$) between ELISA for IgG and precipitins.¹⁰ Even with this increased sensitivity of XIE and its still further increase by autoradiography, it is difficult to recommend these rather complex methods as routine for antibody detection. Nevertheless, their use may be borne in mind when the clinical picture and other criteria are suggestive of ABPA, and when routine precipitin tests are negative. It is possible, however, that the ability to demonstrate the formation of such complexes in vitro may be a criterion more indicative of ABPA than merely the independent determination of both specific IgE and IgG antibodies, since in this study more than 90% of sera showed this phenomenon.

Unlike RAST and ELISA, in which the allergen is chemically or physically bound to an insoluble support phase so that all proteins of a complex system, whether or not they are allergenic, are bound and antibody binding sites may be masked and unlike the variety of methods with radiolabeled allergens that assume that neither the radiolabel nor the labeling process has altered significantly the antigenicity of the molecule, this self-XRIE test, although not a test for total IgE antibody, possibly resembles the in vivo situation with only unaltered antigen coming into contact with the patient's antibodies.

Where antigen is diffusing from spores and mycelium after inhalation or growth in the lung, the formation of such complement-fixing IgE/IgG antigen complexes may be responsible for inducing the dual type responses characteristic of these patients²⁴ by triggering antigen-specific IgE-sensitized mast cells, type I reaction, with activation of complement and the late type III response, hence the chronic inflammatory changes seen in the bronchi at the site at which mucous plugs containing fungal mycelium have blocked the bronchi. In patients with uncomplicated aspergilloma (i.e., without skin reactivity) in whom the fungus is also actively growing, the absence of IgE in immune complexes may explain why such reactions do not occur, although these patients have very high levels of specific IgG antibody to many of the same *A. fumigatus* antigens as in ABPA. Other patients with aspergilloma and skin reactivity frequently have associated clinical features of ABPA. The possibility of a particular subclass of IgG antibody being specifically involved is also being investigated.

Since the development of XRIE, it has been possible to identify allergenic components within complex antigenic mixtures.²⁰ This method relies not only on all the relevant allergens being immunogenic for rab-

bits but also after fixation of the allergen in the gel as an immune precipitate, on sufficient sites being available for reaction with the patient's specific IgE. In the test used here, although the latter condition still applies, the specific IgE visualized by autoradiography is only to those allergenic components to which the patient has his or her own IgG antibody, and IgE antibody to other potential allergenic components, if present, would not be detected. The highly significant correlation ($p < 0.001$) between the grading of radioactivity visualized (both numbers of peaks and their intensity being taken into consideration) and the RAST results (Fig. 3) as found in conventional XRIE^{20, 25} suggests that for each of these patients most of IgE antibody is to the same, although not necessarily all, antigenic components to which there is also IgG-precipitating antibody.

All the immunoprecipitated antigens appeared able to bind specific IgE to some degree, as deduced both from the reference pattern with the pooled ABPA serum (Fig. 4) and from the analysis of self-XRIE tests for the individual sera (Fig. 5). However, they can clearly be divided into two main types. First are the group of six components that appear to be poorly precipitating yet show high binding of IgE as evidenced by their strong diffuse radiostaining (as previously discussed) and that were in most instances IgE reactive for 11 or more of the sera tested. These components (Fig. 4) were with one exception (antigen 15) the electrophoretically faster migrating components (antigens 1 to 5), which from the nature of their reactions are most likely to be smaller molecular protein/glycoprotein components (i.e., less immunogenic²⁰). Antigens 2, 3, and 15, which show IgE binding with >50% of sera tested, probably represent the major allergenic components. The second group of antigens (viz., antigens 6 to 8 and 11 to 13) were characterized by producing most of the visible precipitin reactions with fewer sera that showed associated IgE binding. Some of this IgE binding may be nonspecific as a result of coprecipitation of allergenic components in the denser precipitates as indicated by the sharper nature of the radioactive peaks.^{20, 25} It is possible that for most of the sera, these precipitates are saturated with IgG antibodies thereby blocking any IgE binding or, alternatively, they may consist of components of higher molecular weight more likely to be immunogenic with respect to IgG antibody production but with few binding sites for specific IgE. These components therefore represent only minor allergens of *A. fumigatus*.

Hence it would appear that the main antigens detected by precipitin tests are not the major allergens of

A. fumigatus, although separation and characterization of these various components will be necessary to confirm this.

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