

Studies on *Alternaria* Allergens

II. Presence of Two Related Antigens with Contrasting Allergenic Properties in *Alternaria tenuis* Extracts¹

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Abstract. Earlier, it was reported that most of the allergenic activity of *Alternaria tenuis* seemed to reside in the last Sephadex G-100 fraction (G3, molecular weight ~ 20,000). These conclusions were based on results obtained by the in vitro techniques of radioallergosorbent tests (RAST) and RAST inhibition. In the present studies, the allergenic activity of *A. tenuis* fractions was measured in vivo. It was found that fraction G3 was neither capable of inducing reaginic (IgE) antibodies in rats nor eliciting a skin reaction in rats sensitized with reagins against *A. tenuis* non-dialyzable fraction (MW > 12,000). On the other hand, subfractions G2D3 and G2D4 obtained from the middle fraction of G-100 (G2, MW ~ 30,000-40,000) followed by ion-exchange chromatography on DEAE-cellulose, showed the greatest allergenic activity in vivo, giving high titrated reaginic antibodies in rats. When compared by RAST inhibition, the activities of fractions G2D3 and G2D4 were slightly lower than that of G3. Fraction G3 was immunogenic in rabbits. In immunodiffusion tests, G2D3, G2D4 and G3 appeared to be identical and they were cross-inhibiting in RAST inhibition tests. It was concluded that the sizes and multivalent properties of G2 and G3 antigens exerted a strong influence on their allergenic activities in rats (IgE antibody production) but this factor was of little importance for their antigenic activity in rabbits (precipitin antibody production) and their behaviour in the in vitro tests.

Introduction

A previous report from these laboratories described the isolation and fractionation of allergenic components in commercial preparations of *Alternaria tenuis* by gel-fil-

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tration and ion-exchange chromatography [3]. In subsequent radioallergosorbent tests (RAST) and RAST inhibition analyses, activity was detected in most of the non-dialyzable fractions (MW > 12,000) and the majority of activity was shown to reside in the last fraction (G3, MW ~ 20,000) obtained on Sephadex G-100.

In order to determine if G3 is a major allergen of *A. tenuis*, the present study was designed to examine the allergenicity of all the *A. tenuis* fractions. Laboratory animals were immunized with various *A. tenuis* fractions to produce reaginic (IgE) antibodies, the strength of which was subsequently measured by passive cutaneous anaphylaxis (PCA) tests. Fraction G3 was also further fractionated to obtain the major antigen in higher purity.

Materials and Methods

Antigens

Preparation of the *A. tenuis* crude extract (AT-CE) and *A. tenuis* non-dialyzable (AT-ND) extract and the fractions obtained by gel-filtration of AT-ND on Sephadex G-100 (AT-G1, G2 and G3) and their subfractions obtained by ion-exchange chromatography on DEAE-cellulose (AT-G1D1, G1D2, G1D3, G1D4, G2D1, G2D2, G2D3 and G2D4) used in this study was reported previously [3].

Passive Cutaneous Anaphylaxis

Eleven groups, each consisting of male inbred Wistar Furth rats weighing 225-250 g (Microbiological Associates, Bethesda, Md.) were immunized. Animals in each group were given a single intraperitoneal injection of 1.0 ml containing 200 µg of 1 of the 11 fractions (AT-CE, AT-ND, AT-G1D1, AT-G1D2, AT-G1D3, AT-G1D4, AT-G2D1, AT-G2D2, AT-G2D3, AT-G2D4 and AT-G3) precipitated with 30 mg of aluminum hydroxide (Amphojel, Wyeth Ltd., Toronto, Ont., Canada). The animals were given halothane anaesthesia and bled by cardiac puncture at days 11, 14, 18, 21 and 24 postimmunization. The serum samples were stored at -70 °C until used in PCA reactions.

The level of specific IgE antibodies in these antisera was determined by the PCA test [2] using a sensitization period of 24 h. Briefly, 0.1 ml quantities of various dilutions of test sera were injected intradermally into the skin of 2 different rats. The animals were subsequently challenged intravenously with either 2 mg of AT-ND or 1 mg

of the immunizing antigen in 0.5 ml saline, mixed with 0.5 ml of 2% Evans blue dye. The skin reactions were examined after 30 min. Duplicate antibody titers were expressed as the reciprocal of the highest dilution giving a bluing diameter greater than 5 mm. Heat-stable homocytotropic antibody (IgGa) was measured by performing 2- to 4-hour PCA tests with the antisera which had been heated at 56 °C for 4 h.

Other Immunological Assays

Direct RAST and RAST inhibition assays for measurement of allergenic potency of *A. tenuis* fractions were carried out as described earlier [3]. In the present studies, a pool of sera used contained equal amounts of five sera which gave a 3+ reaction in RAST using discs coupled with AT-CE. The pool of sera used previously consisted of 4 sera giving 2+ to 3+ reactions in RAST.

Antisera to the various fractions were produced in New Zealand white rabbits and subsequent immunodiffusion analyses were also carried out as described previously [3].

Ion-Exchange Chromatography

AT-G3, the last fraction eluting from Sephadex G-100 [3] was subfractionated by DEAE-cellulose chromatography in the same manner as reported earlier for fractions G1 and G2 [3]. Briefly, AT-G3 (80.6 mg) was adsorbed onto a column (1.6 × 25 cm) of Whatman DE-52 cellulose in 0.02 M phosphate buffer pH 7.0 and eluted with a gradient formed from 250 ml each of the buffer and 0.3 M NaCl in buffer, followed by a wash with 1 M NaCl. The eluate was analysed for protein and carbohydrate and tested by immunodiffusion in gel with rabbit antisera to AT-G3. The eluates were combined into 8 fractions (AT-G3D1, G3D2, G3D3, G3D4, G3D5, G3D6, G3D7 and G3D8) which were dialyzed against distilled water and freeze-dried.

Results

Radioallergosorbent Tests

Earlier, it was reported that the highest activities, as measured by the direct RAST and RAST inhibition assays, were present in fraction AT-G3 (MW ~ 20,000). The

Table I. Potency of *A. tenuis* fractions in direct RAST and RAST inhibition assays

<i>A. tenuis</i> fraction	Yield, mg ¹	RAST, % counts bound ²	Amount required for 50% RAST inhibition, µg
AT-CE	-	29.4	2.2
AT-ND	500	31.1	1.4
AT-G1D1	34.4	20.6	> 500
AT-G1D2	7.3	21.5	168.0
AT-G1D3	23.8	23.8	5.2
AT-G1D4	16.7	27.9	3.0
AT-G2D1	38.4	19.5	112.0
AT-G2D2	7.3	15.8	100.0
AT-G2D3	14.0	27.9	1.2
AT-G2D4	40.2	28.9	1.0
AT-G3	163.0	31.4	0.8

¹ From 500 mg of AT-ND.² Total count in 50 µl (8 ng) of ¹²⁵I anti-human IgE was 24,781, using discs coupled with AT-CE.**Table II.** Mean homocytotropic (IgE) antibody titers in rats immunized with various fractions of *A. tenuis*: PCA titers on challenge with AT-ND fraction

Immunizing antigen	Reciprocal PCA titers days after immunization				
	11	14	18	21	24
AT-CE	8(8/10)	16(1/10)	0(0/9)	2(1/9)	0(0/9)
AT-ND	16(13/18)	4(4/18)	8(2/18)	2(2/18)	0(0/18)
AT-G1D1	0(0/9)	0(0/9)	0(0/9)	0(0/9)	0(0/9)
AT-G1D2	0(0/9)	2(1/9)	0(0/8)	0(0/9)	0(0/7)
AT-G1D3	8(8/9)	0(0/8)	0(0/8)	0(0/8)	0(0/8)
AT-G1D4	4(3/9)	8(9/9)	8(9/9)	2(2/9)	2(3/9)
AT-G2D1	2(2/9)	8(1/9)	4(1/9)	2(2/9)	2(1/9)
AT-G2D2	16(4/5)	16(4/5)	8(3/5)	0(0/5)	0(0/3)
AT-G2D3	32(9/9)	32(9/9)	8(9/9)	0(0/9)	2(1/9)
AT-G2D4	64(9/9)	64(9/9)	32(9/9)	8(8/9)	2(3/9)
AT-G3	0(0/9)	8(1/9)	0(0/9)	0(0/9)	0(0/9)

In each group, positive sera were pooled.

Number of rats showing a positive PCA reaction is shown in parentheses.

RAST assays were repeated several times using a fresh pool of serum from 5 individuals allergic to *A. tenuis*. It was consistently found that in both RAST and RAST inhibition assays, AT-G3 was the most potent fraction followed by AT-G2D3 and AT-G2D4 (table I).

In the RAST tests, in which AT-CE was coupled to the discs, all reagenic activity could be inhibited by G2D3, G2D4 and G3. This indicates that the antigenic determinants being recognized by the patient's IgE antibodies are relatively few in number and that all determinants are present in each of the three fractions.

Reagenic Antibody Response

In order to determine the allergenic potency of the *A. tenuis* fractions in vivo, groups of male inbred Wistar-Furth rats were immunized with the various fractions.

Table III. Mean homocytotropic (IgE) antibody titers in rats immunized with various fractions of *A. tenuis*: PCA titers on challenge with homologous antigen

Immunizing and challenging antigen	Day of bleeding	Number of rats positive to AT-ND challenge	Reciprocal PCA titer on challenge with	
			homologous antigen	AT-ND
AT-CE	11	8/10	16	8
AT-ND	11	13/18	16	16
AT-G1D1	11	0/9	0	0
AT-G1D2	14	1/9	4	2
AT-G1D3	11	8/9	32	8
AT-G1D4	14	9/9	64	8
AT-G2D2	11	4/5	4	16
AT-G2D3	11	9/9	64	32
AT-G2D4	11	9/9	64	64
AT-G3	14	1/9	2	8
AT-ND (immunizing antigen), AT-G3 (challenging antigen)	11	13/18	0	16

In each group, positive sera were pooled.

It is evident from table II that the reagenic antibodies of highest response, i.e., PCA titer of 32–64, were present in the sera of rats immunized with either G2D3 or G2D4 fractions. Moreover, 100 of rats tested with these fractions (i.e., 9/9 rats) were positive. In contrast, sera of the rats immunized with fraction G3 failed to elicit PCA reactions. Out of 9 rats, only 1 showed a positive PCA reaction and that of a low titer. These results were further confirmed by performing (a) PCA reactions in which the homologous antigen was used as a challenging antigen, and (b) PCA reactions in which AT-ND was used as a sensitizing antigen and AT-G3 as the challenging antigen. The results are summarized in table III. The antisera to G2D3 and G2D4 on challenge with their

homologous antigen gave PCA titers of 64 which was similar to that obtained with AT-ND as a challenging antigen. On the other hand, sera from the rats immunized with AT-G3 even on challenge with homologous antigen failed to show any positive PCA reaction. Serum from 1 positive rat gave a PCA titer of only 2 which may be compared with a titer of 8 when AT-ND was the challenging antigen. It should be noted that fraction G3 also did not elicit skin reactions in rats sensitized with reagents against AT-ND. These results suggest that G3 whose molecular weight is the order of 20,000 and which appeared to be the most potent fraction in RAST assays was neither capable of inducing reagenic antibodies in rats nor eliciting skin reactions in rats sensi-

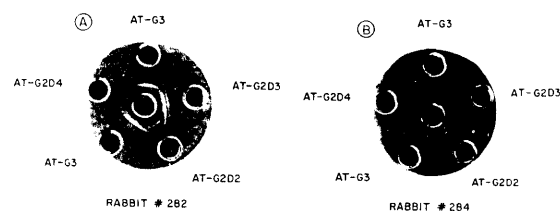


Fig. 1. Immunodiffusion of G2 and G3 fractions of *A. tenuis*. The centre well contained in A and B. No. 282 and 284 rabbit antiserum to G3, respectively.

tized with reagents against AT-ND. On the other hand, G2D3 and G2D4 which have molecular weights of the order of 30,000–40,000 and are the most potent fractions after AT-G3 in RAST assays exerted the greatest allergenic activity (IgE antibody response) in vivo. None of the antisera heated at 56 °C for 4 h elicited any PCA reactions at 2 and 4 h indicating a lack of IgGa antibody.

Immunodiffusion Studies

In order to examine whether the AT-G3 fraction was antigenic, i.e., could induce precipitin antibodies, rabbits were immunized with AT-G3 and other fractions of *A. tenuis*. Serum from rabbit 284 gave only one precipitin band against AT-G3 and G2D3 and G2D4 (fig. 1B) indicating common antigenic determinants. Serum from rabbit 282 gave two precipitin bands with AT-G3 which were also given by G2D3 and G2D4 fractions (fig. 1A). The pattern of one line was similar to that given by rabbit 284. These results show that AT-G3 though nonallergenic, being incapable of inducing IgE antibody in rats, is a good antigen in rabbits.

Purification of AT-G3

Fraction AT-G3 was further purified by DEAE-cellulose chromatography. The separation is shown in figure 2. The eluates

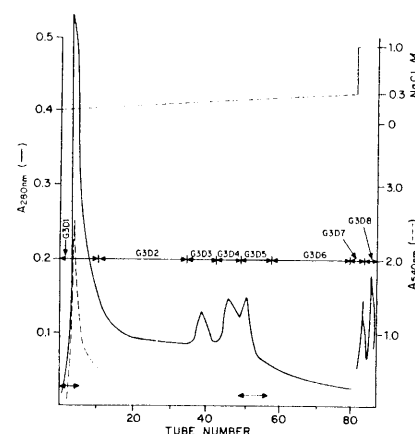


Fig. 2. Ion-exchange chromatography of AT-G3 on DEAE-cellulose. A_{280nm} refers to readings in the carbohydrate assay. Fractions of 6.25 ml were collected and combined into 8 pools (G3D1–G3D8) as indicated, on the basis of immunodiffusion tests and UV absorbances. The arrows at the bottom indicate regions where positive immunodiffusion tests were obtained and the dashed line, the sodium chloride gradient.

were tested by immunodiffusion with the two rabbit antisera described above. The tubes were combined to form eight fractions, on the basis of their antigenic properties and UV absorbances. The yields of the eight fractions are shown in table IV, together with their RAST inhibition results in test using discs coupled with AT-CE. The

Table IV. Properties of AT-G3 fractions

AT-G3 fractions	Yield, mg ¹	Amount required for 50% RAST inhibition, µg	Immunodiffusion ²	
			rabbit No. 282	rabbit No. 284
AT-G3	80.6	0.8	positive	positive
AT-G3D1	42.1	> 500	positive	negative
AT-G3D2	6.16	3.8	negative	negative
AT-G3D3	8.88	13.1	negative	negative
AT-G3D4	1.0	2.5	negative	negative
AT-G3D5	0.36	0.7	positive	positive
AT-G3D6	1.19	1.4	negative	negative
AT-G3D7	7.4	113.3	negative	negative
AT-G3D8	7.2	500.0	negative	negative

¹ From 80.6 mg of AT-G3.

² Tests of anti-G3 antiserum against AT-G3 fractions obtained on DEAE-cellulose.

overall recovery of activity from the separation was poor, approximately 4%. The results indicated that fraction G3D5 contained the most active material, though its specific activity was only of the level of the starting material G3. This fraction also was positive in immunodiffusion tests with both antisera. This behaviour suggests that G3D5 is the antigen of major importance, but lost its activity during the purification process or on freeze-drying.

Fraction G3D1, while the largest fraction by weight, and containing carbohydrate, gave virtually no RAST inhibition, and reacted with only one antiserum, No. 282.

Discussion

Previously, based on in vitro methods (RAST and RAST inhibition), G3 (MW ~ 20,000) was reported to contain the majority of the activity of *A. tenuis* [3]. Results of the present study, utilizing in vivo systems for the measurement of the allergenic

potency of G3 and other fractions of *A. tenuis* extract revealed that fraction G3 was capable of producing precipitin antibodies in rabbits but failed to induce reaginic (IgE) antibodies in rats or to elicit PCA reactions in rats sensitized with anti-AT-ND reagents. On the other hand, fractions G2D3 and G2D4 (MW ~ 30,000–40,000) which were the second most potent fractions after G3 by RAST assays exerted the greatest allergenic activity in vivo, producing a high-titered reaginic antiserum in rats.

Immunodiffusion studies demonstrated that the fractions AT-G3, AT-G2D3 and AT-G2D4 are antigenically related. In the RAST tests, these fractions were also cross-reactive. Of the 8 fractions of AT-G3, two (G3D1 and G3D5) were antigenic and one (G3D5) was the most potent fraction in RAST inhibition tests. G3D5 emerged from the DEAE-cellulose column at a sodium chloride concentration of 0.19 M which compares with 0.15–0.22 M for G2D3 and G2D4 [3].

It is quite possible that the sizes of the

fractions may be an important factor in their allergenic behaviour in the in vivo and in vitro tests. Fraction G3 being lower molecular weight than G2D3 and G2D4 may have a lower antigenic valency. In the RAST tests, the competitive effect of soluble antigens in this in vitro system is exerted on the primary interaction between IgE antibody and solid phase antigen, hence the antigenic valency of the inhibitor is probably of little importance in RAST. However, it may be of major importance in vivo behaviour of the antigens. Similar conclusions have been drawn by Lynch and Turner [1] in a study of rye grass pollen allergens. These authors suggested that allergens with a small number of determinants per molecule may be much less reactive in vivo than in vitro.

Although, among in vitro methods, RAST inhibition is a superior method for the measurement of potency of allergen extracts, this method does not directly reflect the biological properties of the allergens. Therefore, in addition to RAST, it is desirable to standardize the potency of allergen extracts by a suitable in vivo method.

The present results also explain the differences between our earlier work identifying G3 as a major *A. tenuis* antigen and that of Yunginger and Jones [4] who had reported the major allergen of *A. tenuis* to be a 30,000 MW species. It is probable that G2D3 and G2D4 contain the allergen studied by these workers.

In view of the observations that fraction AT-G3 was unable to induce reaginic antibodies or to elicit skin reactions against anti-AT-ND reagents while capable of inhibiting the binding of IgE antibodies to ATCE in RAST assays and also retaining the ability to induce precipitin antibodies in rabbits,

it is suggested that this fraction may prove to be an ideal antigen for the immunotherapy of patients allergic to *A. tenuis* allergens. The behaviour of AT-G3 in rabbits showed that it contained more than one antigen. Of the eight fractions derived from AT-G3, G3D5 had the most recognizable antigenic determinants by the atopic human sera. This fraction may possess the appropriate characteristics required for a therapeutic agent capable of desensitizing *A. tenuis* allergic patients without unleashing anaphylaxis. However, to verify this, more detailed clinical and other immunological studies of this fraction are required.

Acknowledgements

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