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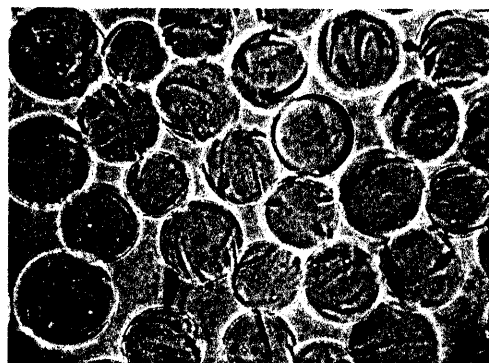
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(From D.W. Levine et al., p. 160.)

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Original Papers

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Studies on Alternaria Allergens

I. Isolation of Allergens from *Alternaria tenuis* and *Alternaria solani*¹

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Abstract. Extracts of *Alternaria tenuis* and *Alternaria solani* were separated into dialyzable (molecular weight <10,000) and non-dialyzable forms. The latter was further fractionated by gel filtration through Sephadex G-100 followed by ion-exchange chromatography on DEAE-cellulose. The dialyzable material was fractionated by gel filtration through Sephadex G-50. The allergenic activities of the fractions obtained from the *A. tenuis* extract were measured *in vitro* by the radioallergosorbent test assay and the allergenic potency was measured by radioallergosorbent test inhibition assay. Allergenic activity was detected in most of the non-dialyzable fractions, the majority of the activity being in the last G-100 fraction (MW ≈ 20,000) which was predominantly protein in nature. The same component may be responsible for the activity found in the dialyzate and its first G-50 fraction since the immunodiffusion studies indicated that the last G-100 fraction has antigenic components in common with those of the first G-50 fraction. In addition, cross-reactions between *A. tenuis* and *A. solani* extracts show that the two species share common antigenic determinants.

Introduction

The allergen preparations used in clinical allergy contain non-allergenic protein antigens and sometimes irritant components

as well as active allergenic proteins. The content of allergenic material and composition of the extracts prepared by different companies may vary considerably. For more rapid progress in the standardization of allergen extracts for clinical use, identification and purification of the allergenic constituents is of prime importance. The availability of isolated allergens will also facilitate quantitative studies of the patient's reaction to the individual allergens, and the nature of the immunological response of pa-

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tients who are only allergic to one allergen and hyposensitized with a mixture of other potential allergens might be elucidated. Furthermore, a detailed knowledge of the physicochemical structure and biological properties of the allergen molecules may be the key to a new understanding of the basic phenomenon giving rise to the allergic diseases.

Studies on the type and distribution of various allergenic fungi have shown that the Fungi Imperfecti are of greatest clinical significance and of these, various genera of the dematiaceous group predominate [7]. In particular, *Alternaria* sp., which have been detected frequently in routine atmospheric sampling in many countries [5, 15] and commonly found in soil [8], are known to be important causes of bronchospasm in a significant number of patients with bronchial asthma [1, 2, 6, 9, 13]. It has also been reported that hypersensitivity pneumonitis, a condition that has been linked with precipitating antibodies of the IgG class [10] may occur from sensitization to *Alternaria* sp. [12]. Therefore, it seems desirable to investigate the allergen composition of *Alternaria* sp.

In 1976, Yunginger *et al.* [17] reported that gel filtration through Sephadex G-25 of a fraction obtained from *Alternaria tenuis* showed that almost all allergenic activity was located in a fraction containing both protein and carbohydrate, eluting at the column's void volume. The present study describes a more elaborate fractionation of the allergenic components in commercial material from two members of the dematiaceous family, *A. tenuis* and *Alternaria solani*, by gel filtration and ion-exchange chromatography and the assessment of their allergenicity by *in vivo* and *in vitro* techniques.

Materials and Methods

Preparation of *A. tenuis* Extracts

50 g of *A. tenuis* powder (Hollister-Stier, Mississauga, Ont.) were defatted four times with 250 ml of acetone at room temperature. The resulting defatted material (38.0 g) was extracted twice with 190 ml of 0.05 M Tris buffer, pH 7.8, containing 0.15 M NaCl (Tris-saline) and stirred magnetically for 48 h at 4 °C. The suspension was centrifuged at 1,000 g at 4 °C for 30 min. The supernatant was centrifuged at 30,000 g at 4 °C for 20 min to remove trace-suspended particles. The resulting supernatant which we term *A. tenuis* crude extracts (AT-CE) was concentrated by ultrafiltration with an Amicon UM-2 membrane (Amicon Corp., Lexington, Mass.). The concentrated AT-CE was dialyzed for 48 h at 4 °C against three changes of Tris-saline buffer, using dialysis tubing with an approximately 10,000 MW cutoff. The combined dialyzates (AT-D) were concentrated and washed with water using an Amicon UM-05 membrane, then freeze-dried and stored at 4 °C. The non-dialyzable (AT-ND) material was further dialyzed exhaustively against water and freeze-dried.

Gel Filtration of AT-ND Extract

500 mg of AT-ND product were dissolved in 20 ml of 0.02 M phosphate buffer, pH 7.0, and subjected to gel filtration on a column (5 × 85 cm) of Sephadex G-100 (Pharmacia Laboratories, Montreal, Que.) at 4 °C. Carbohydrate content was assayed by the orcinol/sulphuric acid method using glucose as a standard [4]. The column was calibrated with bovine serum albumin (MW, 68,000), carbonic anhydrase (MW, 29,000), and lysozyme (MW, 14,300).

Ion-Exchange Chromatography

The fractions of AT-ND allergens obtained from the Sephadex G-100 gel filtration were further purified by ion-exchange chromatography on DEAE-cellulose (Whatman DE-52; Mandel Scientific Ltd., Montreal, Que.). Fraction G1 was concentrated to 20 ml and adsorbed onto a column (1.6 × 25 cm) of DEAE-cellulose equilibrated with 0.02 M phosphate buffer, pH 7.0, at 4 °C. After washing the column with the same buffer, a linear gradient made from 250 ml of 0.02 M phosphate buffer, pH 7.0, and 250 ml of phosphate

buffer containing 0.3 M NaCl was used to elute the material from the column. The eluted material was combined into four fractions, G1D1, G1D2, G1D3 and G1D4 which were dialyzed against water and freeze-dried.

Fraction G2 from the Sephadex G-100 fractionation step was similarly subfractionated by DEAE-cellulose chromatography.

Gel Filtration of *A. tenuis* Dialyzate

253 mg of AT-D were subjected to gel filtration on a column (2.5 × 90 cm) of Sephadex G-50 (Pharmacia Ltd.) using water as the eluent. Aliquots of 5 ml were collected and combined into three fractions, AT-DA, AT-DB and AT-DC.

Preparation of *A. solani* Extracts

The fractionation of commercial *A. solani* material (Meridian Bio-Medical Inc., Denver, Colo.) was carried out in the same way.

Antisera

Antisera were prepared to the various fractions obtained from the *A. tenuis* and *A. solani* extracts by immunizing New Zealand white rabbits (2-3 lbs) by subcutaneous injection into four foot pads, of an emulsion prepared from 1 ml of saline, containing 2 mg of *Alternaria* extract, and 1 ml complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). 2 weeks later, the animals received the same dose of antigen in 1 ml of emulsion. Thereafter for 7 weeks, trial bleeding and booster shots were carried out at weekly intervals.

Immunodiffusion

Immunodiffusion was performed in 1% agar prepared in 0.05 M barbital buffer, pH 8.2, containing 1% polyethyleneglycol (MW 6,000), for periods of 48-72 h at 4 °C. Samples of 50 µl of 2 mg/ml solutions of the antigens were applied to the wells.

Passive Cutaneous Anaphylaxis (PCA)

The PCA activity of rabbit antisera to the AT-ND fraction was measured in guinea pigs as described by Ovary [11]. All tests were done in duplicate animals. Intradermal injections of 0.1-ml quantities of test serum serially diluted in saline were made into the back of freshly shaved male Hartley strain albino guinea pigs (250-350 g). After 4-5 h, the animals were injected intravenously with either AT-ND or AT-DA or AT-DC extract

(2 mg in 0.5 ml saline) mixed with 0.5 ml of 2% Evans blue dye. The skin reactions were examined after 20 min. The antibody titres were determined in duplicate and are expressed as the reciprocal of the highest dilution giving a diameter greater than 5 mm in the blueing reaction.

In order to examine the ability of the dialyzate fractions to inhibit the PCA activity of AT-ND antibody, volumes of 0.2 ml of AT-ND antiserum were incubated either with 2 mg of AT-DA or 10 mg AT-DC at 37 °C for 1 h followed by standing at 4 °C for 12 h. The mixtures were then centrifuged at 12,100 g for 10 min. Quantities of 0.1 ml of the corresponding supernatants were intradermally injected into the recipient guinea pigs. 4 h later, the animals injected with AT-ND antiserum absorbed with AT-DA were challenged with AT-DA followed by a second intravenous injection of AT-ND. The animals injected with AT-ND antiserum absorbed with AT-DC were injected intravenously with AT-ND extract. The reduction in PCA titre of AT-ND antibody due to the pretreatment with the dialyzate fractions (AT-DA or AT-DC) was taken to represent the relative measure of the common antigens shared between the dialyzate fractions used for neutralization of PCA activity of AT-ND antibodies and the antigen (AT-ND) used for challenging the animals.

In another experiment, fraction AT-DC was evaluated for its ability to inhibit *in vivo* the PCA activity of AT-ND antibody. 4 h after sensitization of guinea pig skin by the intradermal injection of 0.1-ml volumes of serially diluted serum (as for the standard PCA reaction), the animals were injected intravenously with 8 mg of AT-DC. 20 min later, the animals were challenged by a second intravenous injection of AT-ND. Any reduction in PCA activity due to the intravenous injection of the antigen would be considered to represent the degree of *in vivo* neutralization of the PCA activity of AT-ND antiserum by common antigenic constituents present in AT-DC.

Direct Radioallergosorbent Test (RAST) and RAST Inhibition Studies

Allergenic potencies of AT-CE and its fractions obtained by Sephadex G-100 and DEAE-cellulose separations were tested *in vitro* by direct RAST and RAST inhibition techniques.

Filter paper discs activated with CNBr were coupled with AT-CE at concentrations ranging

from 1 μ g to 2 mg/disc [18]. Direct RAST assay was performed according to the method of *Yunginger et al.* [18] using reference sera and ^{125} I anti-human IgE (Pharmacia). A number of sera obtained from patients allergic to molds were screened by direct RAST using discs coupled with AT-CE. Equal amounts of four sera which gave 2+ to 3+ reaction in RAST were pooled together. None of these patients had been treated by hyposensitization therapy with *A. tenuis* allergens. The RAST assay was performed with the pooled serum using discs coupled with AT-CE or fractions obtained from it (100 μ g/disc). The percent of total count bound by normal serum control was subtracted from all of the tests. Results were expressed as the percentage of total radioactive counts bound to the discs.

RAST inhibition assays were performed in essentially the same way as the direct RAST with the exception that the pooled serum and solid phase allergen (disc coupled with 100 μ g of AT-CE) were incubated in the presence of inhibitor, i.e., AT-CE and its fractions, at concentrations ranging from 0.1 μ g to 100 μ g. The degree of inhibition of RAST reactivity was obtained by subtracting the mean percentage (average of duplicates) of residual radioactivity of the absorbed samples from 100%, i.e., the value given by a control sample containing the pooled serum alone but diluted to comparable volume with the buffer used in the above assay. Mean values of individual experiments were plotted on semilogarithmic paper from which results could be expressed as the

amount of absorbing allergen causing 50% inhibition of RAST.

Results

Isolation of Fractions from *A. tenuis* Material

Extraction of 38.0 g defatted *A. tenuis* yielded 1.8 g of water-soluble non-dialyzable (AT-ND) and 1.12 g of the dialyzable (AT-D) components. Gel filtration on Sephadex G-100 of 500 mg of AT-ND resulted in three fractions, AT-G1, AT-G2 and AT-G3 (163 mg), on the bases of 280 nm absorption and carbohydrate analysis (fig. 1). Figures 2A and B show the ion-exchange chromatography of fractions AT-G1 and AT-G2, respectively, on DEAE-cellulose. Gel filtration of AT-D on Sephadex G-50 gave three fractions AT-DA, AT-DB and AT-DC (fig. 3). The yields of the fractions from AT-ND obtained on Sephadex G-100 and DEAE-cellulose and of AT-D obtained on G-50 are given in table I. The total recovery of AT-ND and AT-D fractions was approximately 69 and 49%, respectively.

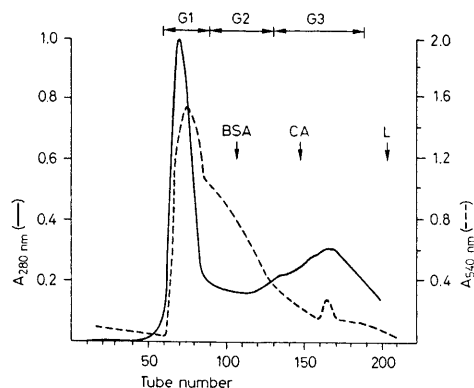


Fig. 1. Gel filtration of *A. tenuis* non-dialyzable fraction (AT-ND) on Sephadex G-100. Fractions (7 ml) were pooled as indicated. $A_{540\text{nm}}$ refers to readings in the carbohydrate assay. The arrows indicate elution positions of the proteins used to calibrate the column, bovine serum albumin (BSA), carbonic anhydrase (CA) and lysozyme (L).

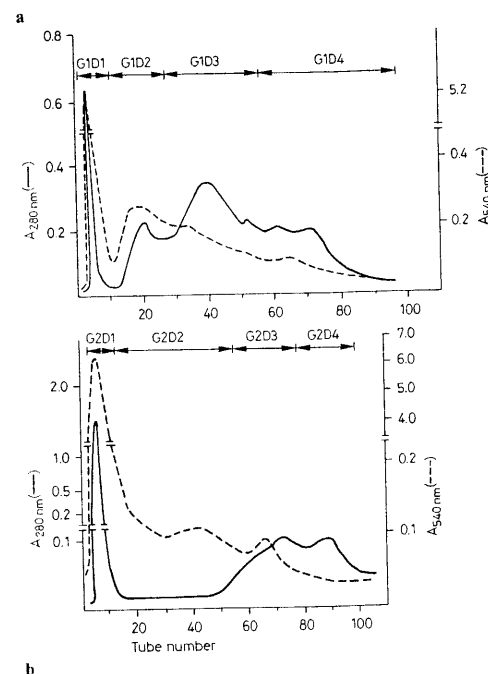


Fig. 2. Ion-exchange chromatography of G1 (a) and G2 (b) fractions of *A. tenuis* on DEAE-cellulose. Fractions (5 ml) were pooled as indicated.

Isolation of Fractions from *A. solani* Material

The purification of *A. solani* resembled that of *A. tenuis*; however, extracts of the latter contained more carbohydrate than the former. 50.0 g of *A. solani* was defatted and extracted, yielding 2.23 g of water-soluble non-dialyzable (AS-ND) and 1.57 g of the dialyzable (AS-D) products. Recovery of AS-ND material after gel filtration through Sephadex G-100 and chromatography of DEAE-cellulose was 66% and recovery of AS-D after Sephadex G-50 was 49%.

Immunodiffusion Studies

The antigenic composition of the fractions was tested by gel diffusion with rabbit antiserum to AT-ND. This antiserum dem-

onstrated three weak precipitin bands with AT-ND (fig. 4A). One band appeared common to AT-G1D1, G1D2 and G1D3, being strongest in G1D2. In addition, AT-G1D3 exhibited two extra bands, one of which was shared with AT-G1D4. The latter also showed one very strong band. Similarly, AT-G2D2, AT-G2D3 and AT-G2D4 had a precipitin band in common and AT-G2D3 had several components (fig. 4B), but AT-G2D4 was weakly reactive with the antiserum unlike AT-G1D4. Fractions AT-G1D1 and AT-G2D1 which contain the bulk of the carbohydrate, and AT-G3 were weakly reactive.

With the fractions derived from AT-D, it was found that the fraction AT-DA was able to produce a strong precipitin antibody

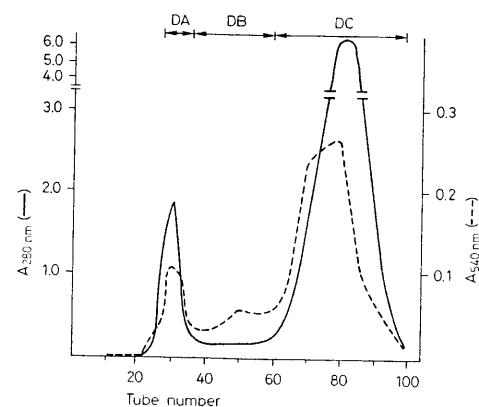


Fig. 3. Gel filtration of *A. tenuis* dialyzate (AT-D) on Sephadex G-50. Fractions (5 ml) were combined as indicated.

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

<i>A. tenuis</i> fraction	Yield, mg	RAST, % counts bound	RAST inhibition, % ¹ at inhibitor of				Amount required for 50% inhibition, μ g
			100 μ g	10 μ g	1 μ g	0.1 μ g	
AT-CE		12.93	74	50	23	9	10
AT-ND	1,800 ²	14.56	89	58	23	13	6
AT-G1D1	34.4 ³	3.70	18	7	8	9	> 100
AT-G1D2	7.3	4.90	19	11	2	0	> 100
AT-G1D3	23.8	3.87	36	14	8	5	> 100
AT-G1D4	16.7	4.38	35	11	5	10	> 100
AT-G2D1	38.4 ³	7.58	22	19	5	2	> 100
AT-G2D2	7.3	12.30	33	14	13	1	> 100
AT-G2D3	14.0	11.71	80	41	20	5	17
AT-G2D4	40.2	10.85	44	34	19	6	> 100
AT-G3	163.0 ³	18.4	89	71	38	13	3
AT-D	1,120 ²	3.14	50	19	13	7	> 100
AT-DA	14.4 ⁴	7.13	67	50	15	9	10
AT-DB	22.6	6.34	58	22	7	8	60
AT-DC	84.2	2.75	3	9	14	5	> 100

¹ Obtained by subtracting the mean percent bound (average of duplicates) from 100%.

² Obtained from 38.0 g defatted *A. tenuis* followed by extraction and dialysis with Tris-saline buffer.

³ Obtained from 500 mg of AT-ND by gel filtration through Sephadex G-100 followed by chromatography on DEAE-cellulose.

⁴ Obtained from 253 mg of AT-D by gel filtration through Sephadex G-50.

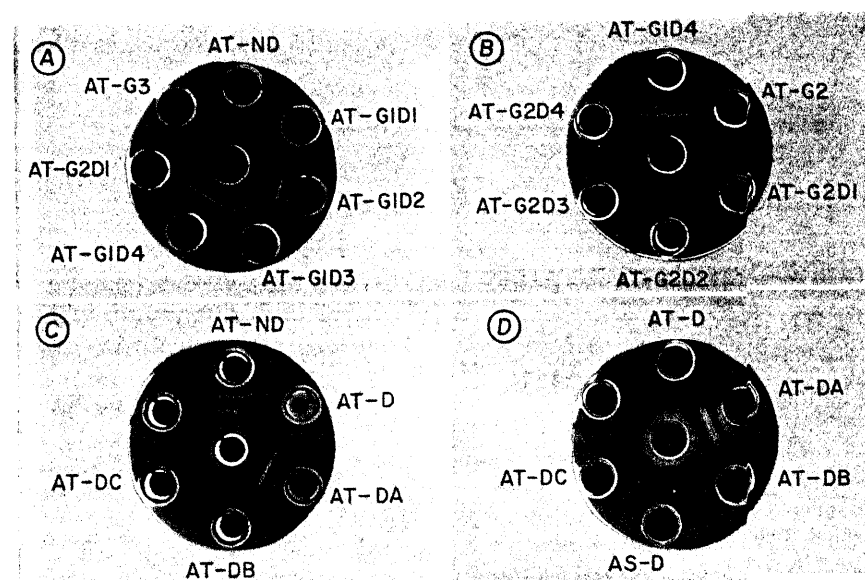


Fig. 4. Immunodiffusion tests of *Alternaria* fractions. A, B The centre wells contained rabbit antiserum to AT-ND fraction. C The centre well

contained rabbit antiserum to AT-CE. D The centre well contained rabbit antiserum to AT-D.

even at an immunizing dose of 200 μ g whereas fraction AT-DC failed to generate a detectable response at higher immunizing doses (1.4 and 2 mg). Tests with rabbit antiserum to AT-CE (fig. 4C) or to AT-D (fig. 4D) against AT-ND and AT-D and its fractions (AT-DA, DB and DC) obtained from Sephadex G-50 gel filtration gave one band of identity between AT-ND, AT-D and AT-DA fractions suggesting that one antigen partially passed through the dialysis bag and AT-DA also contained a second weaker antigen. Fractions AT-DB and DC gave no precipitin reactions.

In order to examine the cross-reactivity between *A. tenuis* and *A. solani*, rabbit antiserum to AS-ND was tested against

AS-ND and AT-ND and its fractions. Two precipitin bands were given by AT-G1D4 fraction as shown in figure 5A. Similarly, when rabbit anti-AT-CE was tested against AT-DA, DB and DC and AS-DA, DB and DC, two and three precipitin bands were obtained with AS-DA and AT-DA, respectively, as shown in figure 5B. These results suggest that AT-G1D4 and AT-DA contain antigens common to *A. tenuis* and *A. solani*. Rabbit antiserum to AT-ND extract was also tested against *Aspergillus fumigatus*, *Fusarium* sp., *Helminthosporium* sp., *Hormodendrum* sp., *Spondylocradium* sp. and *Chaetomium* sp. It was found that *A. fumigatus* gave a strong reaction, *Helminthosporium* and *Hormodendrum* species gave a

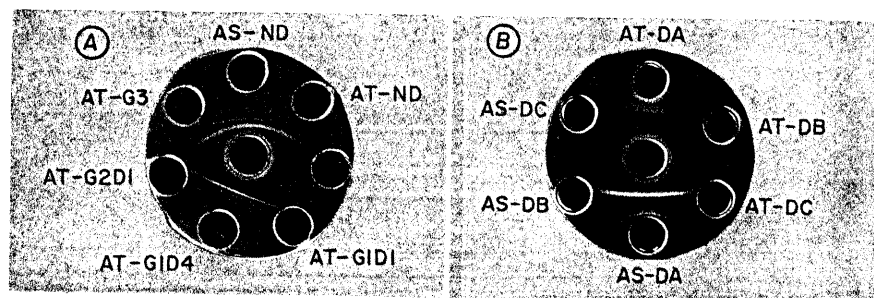


Fig. 5. Cross-reactivity between *A. tenuis* and *A. solani* fractions. **A** The centre well contained rabbit antiserum to *A. solani* non-dialyzable

very weak reaction and others failed to give any reaction.

PCA Studies

In order to examine whether the immunogenic (AT-DA) or non-immunogenic (AT-DC) fractions of AT-D could inhibit the PCA activity of rabbit anti-AT-ND serum, guinea pigs were sensitized with the latter. Table II shows that these animals when challenged with 2 mg of AT-ND or AT-DA fraction gave strong PCA titres of 2,048 and 1,024, respectively. On the other hand, attempts to elicit PCA reactions with concentrations of AT-DC fraction up to 8 mg were unsuccessful. Moreover, injection of 8 mg of AT-DC fraction 20 min prior to AT-ND challenge gave no reduction in PCA activity of AT-ND antibody (titre 1,024). These results were further confirmed by performing a separate experiment in which guinea pigs were sensitized with AT-ND antiserum absorbed with AT-DA or DC (0.2 ml antiserum absorbed with 2 mg AT-DA or 10 mg AT-DC). The guinea pigs sensitized with the antiserum absorbed with AT-DA gave no PCA reaction on challenge

(AS-ND) extract. **B** The centre well contained rabbit antiserum to AT-CE.

Table II. PCA titers of rabbit antiserum to fraction AT-ND using AT-ND, AT-DA and AT-DC as challenging antigens

Treatment of AT-ND antiserum	Challenging antigen	PCA titer
None	AT-ND	2,048
None	AT-DA	1,024
None	AT-DC	0
	(1st injection)	
	AT-ND	1,024
	(2nd injection)	
Absorbed with AT-DA	AT-DA	0
	(1st injection)	
	AT-ND	0
	(2nd injection)	
Absorbed with AT-DC	AT-ND	2,048

with a regular dose (2 mg) at AT-DA. Moreover, absorption of the antiserum with AT-DA also destroyed the PCA activity of AT-ND antibody (PCA titre from 2,048 to 0). Absorption of the antiserum with AT-DA also destroyed the PCA activity of AT-ND antibody when challenged with AT-ND (PCA titre 1,024). These results suggest that fraction AT-ND share common

antigenic determinants with fraction AT-DA and moreover AT-DA can inhibit all the PCA activity of AT-ND antibody. On the other hand, AT-DC appears to be immunologically inactive and devoid of hapten-like properties.

Direct RAST and RAST Inhibition Studies

The potency of the various *A. tenuis* fractions was also determined by direct RAST and RAST inhibition techniques. It can be seen from table I that AT-G3 is the most potent fraction in both assays followed by AT-DA. As would be anticipated, AT-CE and AT-ND were also potent. Fractions G1D1 and G2D1 which contain the bulk of carbohydrate gave little inhibition (19–22%) even at a concentration of 100 μ g. This is not unexpected since the protocol used for the RAST procedure would not measure the activity of carbohydrate moiety. Fraction AT-DC also appeared to be inactive. These results agree with those from agar gel immunodiffusion and PCA reactions in guinea pigs.

In order to examine if the activity of AT-DA fraction could be due, at least in part, to the same allergen as AT-G3, immunodiffusion tests with rabbit anti-AT-CE serum were made against AT-ND, AT-G3 and AT-DA fractions. Two precipitin bands were formed with AT-DA, one of which seemed identical with a band shown by AT-G3 (fig. 6). The latter showed another band which spurred over the lower band of the AT-DA fraction. These results suggest that the AT-DA fraction contains one identical antigen and another sharing common antigenic determinants with those of AT-G3 fraction.

From the yield data and the RAST inhi-

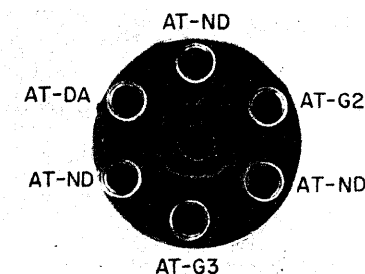


Fig. 6. Cross-reactivity between AT-G3 and AT-DA fractions. The centre well contained rabbit antiserum to AT-CE.

bition results (table I) on the basis of a unit of activity being the amount required for 50% inhibition of the RAST test, the following distribution of activity is obtained. The total activities of the AT-ND and AT-D fractions were 3×10^5 units and 1.1×10^4 units, respectively. Fractionation of 500 mg of AT-ND (8.3×10^4 units) yielded 163 mg of AT-G3 containing 5.4×10^4 units (65%). Fractionation of 253 mg of AT-D (2.53×10^3 units) yielded 14.4 mg of AT-DA containing 1.44×10^3 units (57%).

Discussion

From the battery of immunological tests applied to the various fractions obtained from the experiments, several antigenic components were located and the RAST experiments indicate one antigen, present in two of the fractions, may account for most of the allergenic activity. Gel diffusion analysis showed that fractions AT-G1D1, G2D1 and G2D2 which were rich in carbohydrate did not give any precipitin reaction with

AT-ND antiserum. The polysaccharide(s) produced by the organism are, therefore, not important as antigens nor are they allergenic. However, fractions AT-G1D3, G1D4, G2D3 and G2D4 seemed to be strongly antigenic and cross-reactive suggesting that some components of these fractions may be identical and others may share common antigenic determinants. The activities of these high molecular weight components ($> 50,000$) in RAST experiments were, however, low. The highest allergenic activities, as measured by direct RAST and RAST inhibition assays, were present in fractions AT-G3 (MW $\approx 20,000$) and AT-DA (table 1) with the bulk of the activity on a mass basis being in AT-G3. Yunginger and Jones [16] recently reported that a fraction with an apparent MW of $\approx 30,000$ daltons contained major allergens of *A. tenuis*. These results suggest that fractions AT-G3 and AT-DA possess essentially the allergenic activity of AT-ND and AT-D. The allergenicity of the two fractions may be due, at least in part, to a common allergen. Gel diffusion results (fig. 6) demonstrated that out of two bands shown by AT-G3 against AT-CE antiserum, one band appears to be identical with that of AT-DA fraction and the other band had a spur over that of the latter. Hence AT-DA has antigenic components in common with those of AT-G3 fraction. Since a molecular weight of the order of 20,000 is indicated by the gel filtration behaviour of AT-G3, it is not surprising that some of the allergenic component could pass through the dialysis bag and appear in fraction AT-DA while the retained material would appear in AT-G3. Furthermore, AT-DA fraction showed precipitin bands with antiserum to AT-ND or AS-ND, hence the allergenic protein could

also be present in *A. solani* extracts. There were also other cross-reactions between *A. tenuis* and *A. solani*; AT-G1D4 appeared to contain an antigen in common with a similar fraction from *A. solani*. In addition, AT-ND antiserum cross-reacted with *Aspergillus fumigatus* extracts. However, it failed to react with extracts from samples of *Helminthosporium* sp., *Hormodendrum* sp., *Fusarium* sp. and *Chaetomium* sp. These results are in agreement with those reported in the literature [3, 14].

In all the tests made, fraction AT-DC, which contains the lowest molecular weight material (down to 1,000 MW, the cutoff of the membrane used for ultrafiltration of AT-D) showed no antigenic or allergenic activity. Moreover, it did not inhibit the reactions of other fractions in the manner of a hapten. Hence, *Alternaria* extracts lack haptenic material which might inhibit *in vitro* tests for the potent components, or interfere in clinical use.

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