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Allergens of the imported fire ant

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*A whole body extract (WBE) was prepared by saline extraction of ground imported fire ants (IFA). A serum pool from rabbits immunized with the WBE elicited 31 precipitates on crossed immunoelectrophoresis. Crossed radioimmuno-electrophoresis was performed with sera from 26 RAST IFA-positive subjects, 24 of whom were known for clinical hypersensitivity to IFA and two subjects, known to be hypersensitive to yellow jacket. Radiostaining was obtained to 14 precipitates, some of which were judged to be partially identical. Thus, six apparently different allergen groups could be identified. Two of these fulfilled arbitrary criteria of major allergens. Three of the IFA WBE allergens were easily identified in crossed immunoelectrophoresis of a commercially obtained IFA-venom preparation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the IFA WBE demonstrated protein bands with molecular weight ranging from 10,000 to 200,000 daltons. An immunoblotting technique with nitrocellulose paper strips revealed binding of human IgE from hypersensitive subjects to four different bands (either single bands or clusters of proteins of similar molecular weight) with molecular weights ranging from 13,000 to 42,000 daltons. It is a notable finding that these insect allergens thus were of a similar size as important aeroallergens. (*J ALLERGY CLIN IMMUNOL* 1988;82:567-76.)*

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Hypersensitivity to Hymenoptera stings occurs worldwide, and many different species evoke reactions. The ant family Formicidae comprises >10,000 species, but IgE-mediated allergy is only known to be caused by ants of the genera *Solenopsis* and *Pogonomyrmex*. The largest number and the most serious reactions are caused by the sting of the IFA, *S. invicta* or *S. richteri*, which inhabits the southeast and south-central parts of the United States and portions of South America.

The composition of the venom of the IFA is unique

Abbreviations used

IFA:	Imported fire ant
WBE:	Whole body extract
CIE:	Crossed immunoelectrophoresis
CRIE:	Crossed radioimmunoelectrophoresis
PDT:	Phosphate-dextrane-Tween
MW:	Molecular weight
PRU:	Phadebas RAST unit
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

among the Hymenoptera, with an extremely low protein content (<0.1%) and a predominance of toxic alkaloids.¹ It has been demonstrated that IFA WBE contains venom proteins, including the allergens.² On more clinical grounds, the WBE has been demonstrated to function in the clinical practice.³ A high incidence of skin test and RAST cross-reactions between IFA- and other Hymenoptera-hypersensitive subjects has been reported⁴ and attributed to similar antigenicity of the respective proteins.

This article reports on further characterization of the antigens and allergens of IFA WBE by CIE, CRIE, SDS-PAGE, and immunoblotting techniques with the sera from human hypersensitive subjects and probing for IgE. Furthermore, a comparison of the CIE pattern of a commercially obtained IFA-venom preparation with that of WBE was performed.

MATERIAL AND METHODS**IFA antigens and rabbit antiserum**

WBE of the IFA (*S. invicta*) was prepared by grinding and extracting whole ants. One gram of freeze-dried ants was rotated overnight in 10 ml of buffer solution (0.15 mol/L of NaCl with 0.05 mol/L of Na acetate, pH 4.2), centrifuged, and decanted. The protein content of the extract, determined by the Lowry technique, was adjusted to 5 mg/ml before use for immunization and immunochemical studies. A *S. invicta* venom-enriched extract was obtained from Vespa Laboratories, Spring Mills, Pa., lot No. 1123831. This extract held 0.29 mg of lyophilized substance per vial.

Three New Zealand white rabbits were immunized with the WBE and bled repeatedly. Their antisera were pooled.⁵

RAST

The extract of IFA was coupled to CNBr-activated paper disks.^{6,7} Briefly, portions of 20 CNBr-activated paper disks were simultaneously incubated in 1.5 ml of diluted IFA-allergen extract (200 µl of the allergen extract plus 1.3 ml of 0.2 mol/L NaHCO₃ in 0.3 mol/L of NaCl for 1 hour). After that, the disks were aspirated dry, incubated in 0.1 mol/L of ethanolamine in 0.2 mol/L of NaHCO₃ for 30 minutes, and aspirated dry again. The disks were then in-

TABLE I. Results in RAST expressed as PRU per milliliter with the respective patient serum

Patient serum	PRU/ml
1	7.6
2	1.3
3	0.8
4	8.4
5	4.7
6	2.9
7	7.1
8	1.1
9	2.0
10	7.9
11	1.6
12	150
13	8.0
14	1.8
15	1.8
16	0.6
17	1.1
18	0.8
19	3.3
20	1.1
21	3.2
22	0.41
23	1.7
24	6.6
25	5.1
26	5.9
27 (control serum)	<0.35
28 (control serum)	<0.35
29 (negative serum pool)	<0.35

Patients 1 and 2 are Swedish yellow jacket-hypersensitive subjects; patients 3 to 26 are American IFA-hypersensitive patients.

cubated in 50 mmol/L of Tris with 0.2 mol/L of glycine, pH 8, for 30 minutes and washed again for 10 minutes with Tris-glycine buffer of this same composition. Another wash was performed at acid pH with 0.1 mol/L of sodium acetate, pH 4, for 30 minutes; thereafter, washes were performed twice in a pH 7.4 phosphate buffer (50 mmol/L of phosphate, 0.15 mol/L of NaCl, 0.2% of bovine serum albumin, and 0.02% of sodium azide). The disks were then stored in this latter buffer solution. Commercially available IFA disks (fire ant, i 70, Pharmacia Diagnostics AB, Uppsala, Sweden) were also used.

The allergen-coated paper disks were incubated with undiluted patient serum for 3 hours, washed three times in approximately 2.5 ml of saline containing 0.1% Tween 20, and aspirated dry; after that, disks were incubated with 100 µl of ¹²⁵I-labeled D₂-specific rabbit anti-IgE⁸ (50 to 70,000 cpm per test tube) over night. The disks were counted in a gamma scintillation counter after repeat washing as above. The counts were related to the Phadebas RAST reference System (Pharmacia Diagnostics AB) and expressed in PRU

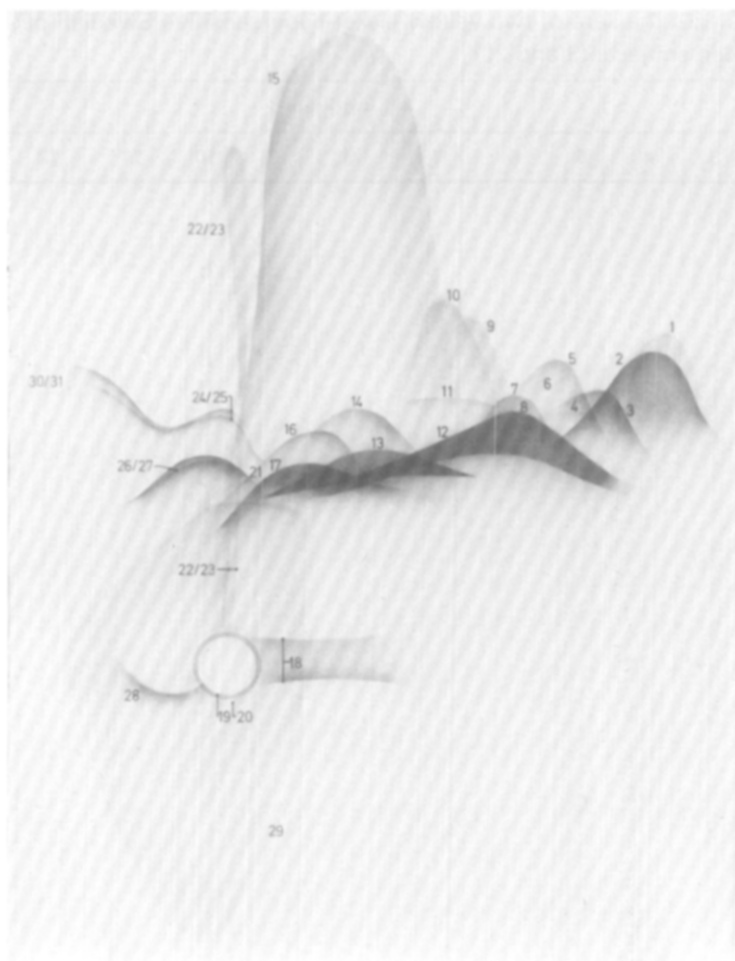


FIG. 1. CIE pattern of antigens of IFA WBE. The precipitates have been assigned arbitrary numbers. All precipitates except No. 29 were made visible after ordinary protein staining. Precipitate 29 was visualized only in the CRIE experiments. Anode to the right (*first dimension*) and up (*second dimension*).

per milliliter. No false positive (<0.35 U/ml) RAST results were obtained with sera having an IgE level <3000 kU/L.

Patient sera

Studies were made with sera from 26 patients with a documented case history of systemic reactions to IFA sting and positive RAST tests. In addition, the sera from two yellow jacket-hypersensitive Swedish inhabitants were analysed. These patients had no known contact with IFA but were IFA-positive in RAST.

As negative controls, two sera from Swedish patients with pollen hypersensitivity were used, as well as a serum pool of subjects with IgE <2.5 kU/L, and also the buffer solution alone.

CIE

CIE was performed essentially as described by Weeke⁹ with 1% agarose A (Pharmacia Fine Chemicals) and a Tris buffer, pH 8.6. Ten microliters of the IFA WBE was applied in 4 mm holes and electrophoresed for 45 minutes at 10

V/cm. Gel slices (10 by 50 mm) containing the separated proteins were cut and laid on gel bond film for the second perpendicular electrophoresis. The anodic field consisted of an intermediate gel free of antiserum (10 by 50 mm) and a 40 by 50 mm field cast with 2.7 ml of agarose solution mixed with 0.3 ml of the rabbit antiserum pool. The cathodic field (10 by 50 mm) was cast with 0.9 ml of agarose solution added to 0.1 ml of rabbit antiserum. The second dimension electrophoresis ran overnight at 2 V/cm. After washes, the plates were dried and kept cool ($+4^{\circ}$ C). Protein staining was performed with Coomassie brilliant blue stain, and the precipitates were assigned arbitrary numbers. Before this procedure was established, extensive control experiments had been performed with short periods of first-dimension electrophoresis (10 minutes) to rule out the possibility that rapidly moving proteins would be lost from the gel. These experiments included CIE and protein staining, as well as CRIE with the three patient sera with the highest IFA-specific IgE levels.

The lyophilized venom-enriched extract was reconsti-

TABLE II. CRIE results with the sera of two yellow jacket-hypersensitive Swedish patients (1 and 2) and 24 IFA-allergic American subjects (3 and 26)

Pt	Antigen														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1															
2															
3										E					
4															
5															
6										E					
7										D					
8															
9															
10										C					
11										F					
12										B					
13										B					
14															
15															
16															
17															
18															
19															
20															
21					G										
22															
23															
24															
25															
26															
27		(H)					(G)	(H)				(G)	(G)		
28		(H)					(G)	(H)				(G)	(G)		
29		(H)					(G)	(H)				(G)	(G)		
30		(H)					(G)	(H)				(G)	(G)		

Pt = patient.

Negative control sera. The CIE plates were incubated with a low IgE serum pool (27), the sera of two pollen-hypersensitive subjects (28 to 29), and the buffer solution alone (30).

tuted with 120 μ l of distilled water and 10 μ l as the solution was applied for the CIE assays, which were otherwise performed as described above in detail.

CRIE

In CRIE, the CIE plates were incubated in small plastic boxes overnight with the patient sera diluted 1/17 in a PDT buffer (pH 7.4) containing 0.1% Tween 20. Total volume of diluted serum used in these experiments was 8.75 ml. The serum was aspirated after incubation, and the CIE plate was washed three times with 10% PDT buffer in saline-added 0.1% Tween 20. The second incubation was performed overnight again in 8.75 ml of PDT buffer solution with ^{125} I-labeled anti-IgE added (72,000 cpm/ml), and the plates were then washed three times as before. Autoradiography used the rapid method previously described.¹⁰ The degree of radiostaining was graded semiquantitatively in CRIE classes (A to H) as previously described in detail.¹¹

SDS-PAGE and immunoblotting

SDS-PAGE used a discontinuous buffer system, as previously described.¹² Seventy-five microliters of nonreduced IFA WBE was layered onto a stacking gel applied to a 14 cm polyacrylamide running gel. MW markers (Bio-Rad Laboratories, Richmond, Calif.) were used as standards on each gel. An optimal gel density for good separation was determined with gradient polyacrylamide gels (5% to 25%) and immunoblots during pilot experiments. Thus, the final experiments could be performed with improved resolution in a 15% homogeneous polyacrylamide gel with certainty that low MW allergens were not lost into the buffer during electrophoresis. Electrophoresis was performed at 50 mA constant current and extended for 1 hour after the bromphenol blue dye front has migrated out the gel. The gel was cut into strips, and at least one was stained with Comassie brilliant blue together with the MW standards.

Thirty fairly well-separated precipitates could be made visible with protein staining of the CIE plates (Fig. 1). In CRIE, one more precipitate was made visible as positive radiostaining, No. 29 in the arbitrary numbering. Most antigens moved against the anode, whereas some few only migrated cathodically. The precipitate groups 24/25 and 30/31, which appeared partly identical, and 26/27, respectively, moved slightly cathodically in the first-dimension electrophoresis but anodically in the second dimension. The resolution of the precipitates formed around the application well^{7, 15, 16} was not quite complete. Antigens 22/23 emanating from this region had an

TABLE III. Radiostaining in CRIE classes of the individual patients to the six different IFA WBE allergens

Patient	Allergen precipitate					
	I (10)	II (17)	III (18-20)	IV (22/23)	V (24/25,26/27,30/31)	VI (29)
1		C	E			
2						
3	E		E	F		E
4			C	C		C
5			D		E	B
6	E		F	F	F	
7	D		D	E		
8				D		F
9			E	D		E
10	C		B	B	E	E
11	F		F			F
12	B		B	E	B	C
13	B		C	D		
14			D			D
15			E		F	F
16			E			
17			D		F	D
18			E		E	F
19			C	E	E	D
20			D	E	E	E
21			B	B	F	C
22			E	G		
23			D		D	D
24			E	E		E
25			E	E	F	E
26			E	F	F	F
Controls						
27	—	—	—	—	—	—
28	—	—	—	—	—	—
29	—	—	—	—	—	—
30	—	—	—	—	—	—

unusual appearance, atypical for an immunoprecipitate.

The RAST data are summarized in Table I. Generally, the IFA-specific IgE levels were fairly low, 16 of the patients' sera had <3.5 PRU/ml (Phadebas RAST class II), and the other sera ranged from 4.7 to 150 PRU/ml.

In CRIE (Table II), some nonspecific binding of low degree (classes G to H) was registered to the precipitates with the heaviest protein staining. Radiostaining higher than classes G to H was considered specific. Careful interpretation of the results of the autoradiography elicited radiostaining of as many as 14 precipitates, i.e., precipitates 10, 17, 18, 19, 20, 22, 23, 24, 25, 26, 27, 29, 30, and 31. This seem-

ingly high resolution was probably caused by the occurrence of groups of similar allergens. Thus, to interpret the data further, apparently associated allergens were incorporated to allergen groups (Table III).

Allergen I (precipitate 10). This allergen appeared as a typical single protein immunoprecipitate.

Allergen group II (precipitate 17). This precipitate was also a well-defined precipitate. Radiostaining was only observed with the serum of one of the yellow jacket-hypersensitive subjects, but no radiostaining was obtained with sera from the IFA-hypersensitive subjects.

Allergen group III (precipitates 18 to 20). The radiostaining was noted in close proximity to the

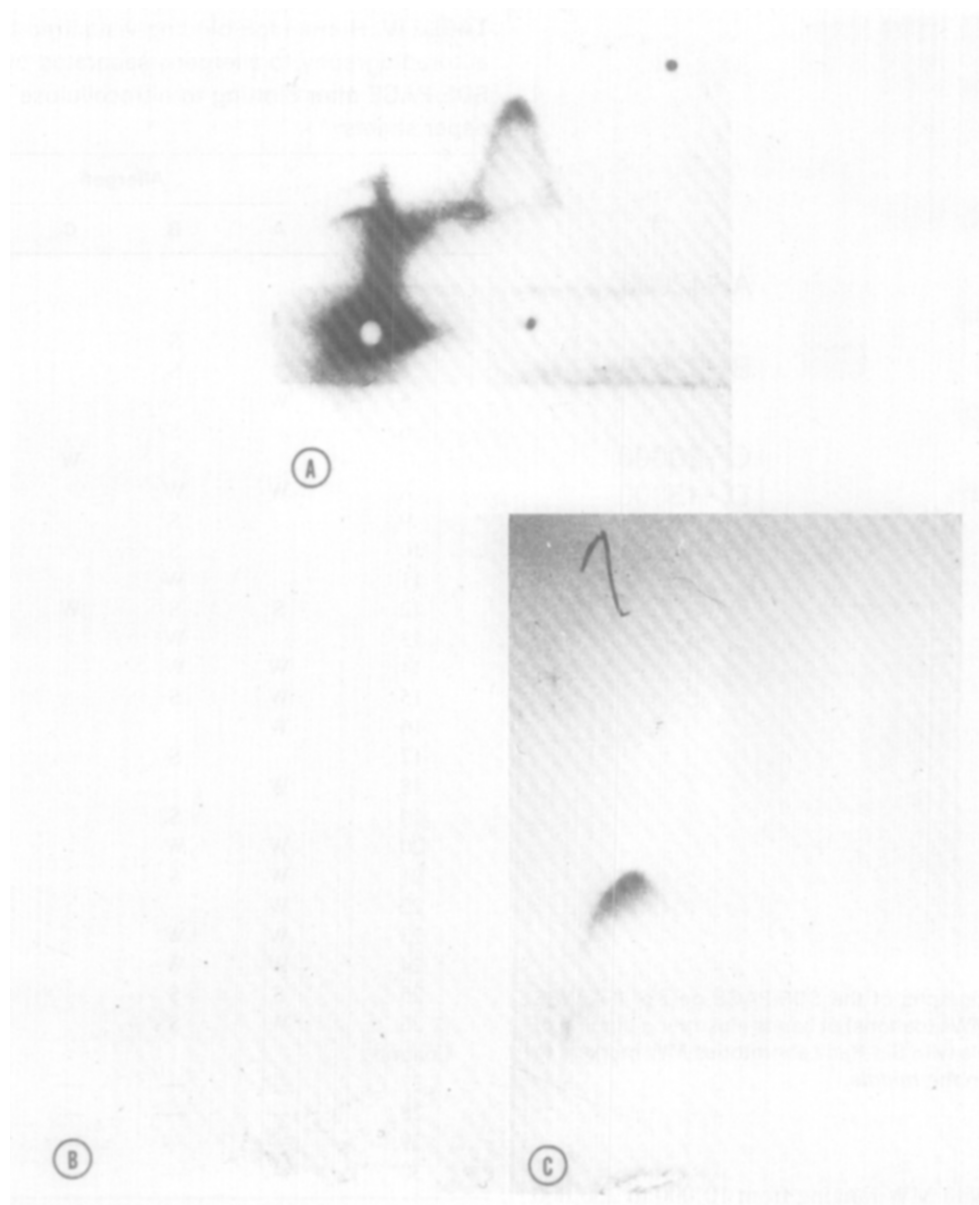


FIG. 2. Photographs of CRIE autoradiographs corresponding to CRIE class E with the serum of a patient with high IFA-specific IgE level (**A**), one patient demonstrating IgE binding to antigen 17 (*allergen II*) alone (**B**) and with a control serum without IFA-specific IgE (**C**).

application well, and it was occasionally difficult to determine which precipitates contributed to this binding.

Allergen group IV (precipitates 22 and 23). These partially identical precipitates were morphologically unusual with their origin from the application well and exhibited heavy radiostaining with several of the IFA-hypersensitive sera.

Allergen group V (precipitates 24/25, 26/27, and 30/31). These partially identical precipitate pairs had similar electrophoretic mobility and exhibited closely

correlating IgE binding, as visualized by radiostaining in CRIE.

Allergen group VI (precipitate 29). The radiostaining of antigen 29 appeared important in many of the patients.

Photographs of CRIE exposures corresponding to CRIE class E with one typical patient serum and the patient serum exhibiting binding to precipitate 17 (*allergen II*) alone and with one of the control sera is illustrated in Fig. 2.

SDS-PAGE resolved the IFA WBE into several pro-

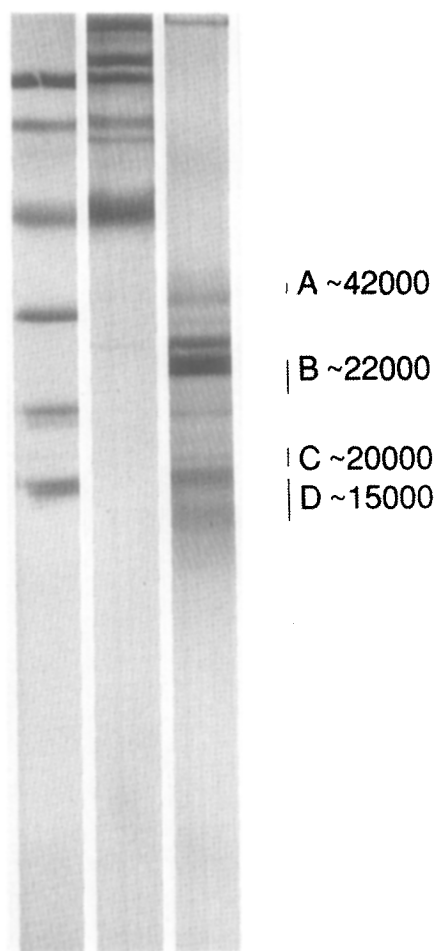


FIG. 3. Photographs of the SDS-PAGE gels of IFA WBE (right) with MWs (daltons) of bands illustrating binding of IgE (A-D). Gels with Bio-Rad Laboratories MW markers to the left and in the middle.

tein bands with MW ranging from 10,000 to 200,000 daltons (Fig. 3). IgE binding was detected by radiostaining of at least four of the protein bands or complexes of two to three closely situated bands. Allergen A, MW 42,000 daltons, appeared as a single well-defined band that elicited weak but clear-cut radiostaining of the immunoblots with most sera. Allergens B, C, and D each appeared to be complexes of two or three proteins with similar MW. The IgE binding of an individual serum was present or absent to all of the components of the respective complex, and the mutual degree of radiostaining of each of the bands was approximately equal when serum was positive. Thus, the respective components of the B, C, and D allergen complexes appeared to be antigenically related. Complex B consisted of at least two bands, MW 22,000 to 25,000 daltons; complex C consisted of two or three bands, MW 18,000 to 25,000 daltons;

TABLE IV. Human IgE binding visualized by autoradiography to allergens separated by SDS-PAGE after blotting to nitrocellulose paper sheets

Patient	Allergen			
	A	B	C	D
1				
2				
3	W	S		S
4		S		S
5	W	S		S
6		S		W
7		S	W	S
8	W	W		W
9		S		
10		S		S
11		W		W
12	S	S	W	S
13		W		S
14	W	W		W
15	W	S		S
16	W			
17		S		
18	W			W
19		S		
20	W	W		
21	W	S		S
22	W			W
23	W	W		
24	W	W		W
25	S	S		S
26	W	S		W
Controls				
27	—	—	—	—
28	—	—	—	—
29	—	—	—	—
30	—	—	—	—

W = weak radiostaining; S = strong radiostaining; for further explanations, see text.

complex D with the lowest MW consisted of three indistinct bands with MW 13,000 to 18,000 daltons. No radiostaining was observed with the control sera and the incubation buffer. These data are compiled in Table IV.

CIE examination of the IFA venom-enriched preparation elicited a sparse precipitate pattern with the pooled antiserum to IFA WBE (Fig. 4). None of the anodically migrating precipitates could be registered. Precipitates with the typical position and appearance of allergen IV and allergen V were easy to identify. It was also possible to identify allergen III as the rings around the application well (precipitates 19 to 20) but

also as an antigen not clearly represented in CIE of the WBE, similar to antigen 18 but moving in the opposite direction. Thus, at least allergens III, IV, and V were probably venom proteins.

DISCUSSION

Much literature is now available on characterization of allergens of different sources, but only little knowledge has been gained on the IFA allergens. This is obviously because of the limited availability of sufficient volumes of the relevant venom. We have chosen first to examine and characterize the allergen content of an IFA WBE, the intent being to try to compare the data with those obtained with pure venom. This appeared a feasible approach, since venom and WBE are known to share much or most of their allergenic activity.²

An allergen extract made from ground whole ants is a complex mixture of venom and body antigens. The techniques applied to the characterization of such crude preparations must have the highest possible power of resolution. CIE and CRIE have now been used in this field during the past decade^{9, 16} and have proven successful, although the techniques are laborious and require high-quality antisera.

A complexity of antigens was found in the CIE pattern of IFA WBE. Significant CRIE radiostaining was registered to 14 precipitates, some of which appeared cross-reactive and apparently represented isomeric forms of one allergen. Thus, it is possible to reduce the number of detected allergens to six. Arbitrary CRIE criteria for major allergens¹⁷ were fulfilled by two of the CIE/CRIE antigen groups, No. III and No. IV (antigen 29). The precipitates of allergens I, IV, and V fulfilled the criteria of intermediate allergens.

The existence of cross-reacting antigens among different Hymenoptera species, including IFA, has been previously demonstrated.⁴ Allergen group II (precipitate 17) was detected only by the serum of one Swedish yellow jacket-hypersensitive subject, and it is not known whether this subject is clinically hypersensitive to IFA. It is presumed that he has had no previous contact with IFA. Allergen group II appears to be irrelevant for IFA allergy, since none of the sera from IFA-hypersensitive Americans gave significant radiostaining to this precipitate. An allergen has been described common to Hymenoptera venom, grass pollen, and certain foods, primarily buckwheat.¹⁸ It appears likely that the antigenic determinants of this ubiquitous allergen may be shared by those of allergen group II of this extract, since binding of serum from the Swedish subject to an IFA RAST disk was partially inhibited by buckwheat extract (data not presented).

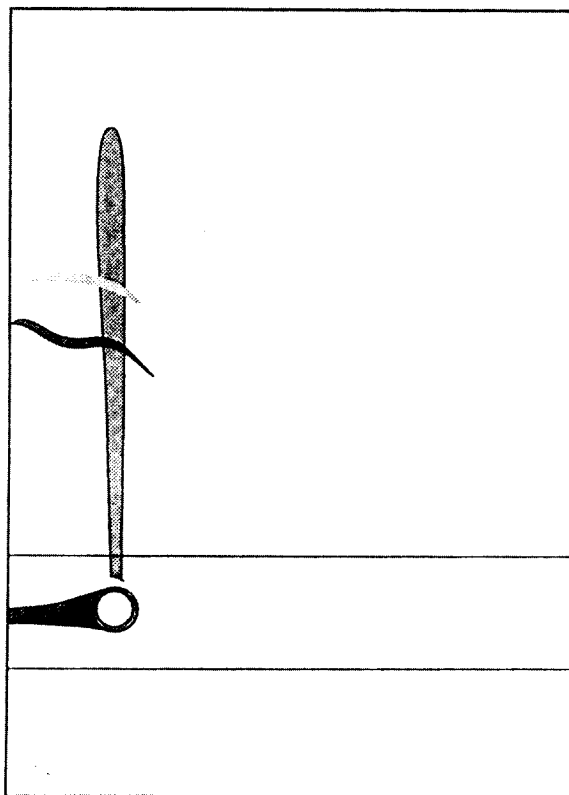


FIG. 4. CIE pattern of antigens of IFA venom-enriched preparation; for interpretation, see text.

The MW distribution of the IFA allergens, as indicated by the SDS-PAGE experiments, are of special interest. IFA allergens resided in the MW region of 13 to 42,000 daltons, which is typical for common aeroallergens, as well as for other Hymenoptera allergens. Previous data have indicated that IFA venom allergenic activity is present in proteins of considerably lower MW than 13,000 daltons.¹ This difference may result from disintegration of proteins in the venom used for the previous work, since proteolysis commonly occurs in protein solutions as dilute as IFA venom. The sparse occurrence of inhalant allergens >65,000 daltons has been attributed to mucosal impenetrability to molecules that are larger.^{19, 20} If naturally occurring sting allergens are also of this typical aeroallergen size, other explanations for the upper size limit of allergens should be considered.

Three of the WBE allergens could be identified in the CIE investigation of the commercially available venom-enriched preparation. Further experiments, including CRIE and separations with other analytical techniques, may very well reveal the presence in venom of other WBE antigens, including the important antigen 29, which was not made visible by protein

staining of the CIE plates of the WBE. Most IFA WBE antigens migrated toward the anode. These do not appear to be important for clinical hypersensitivity because IgE binding to these precipitates was not registered and also because CIE of the IFA venom did not exhibit these precipitates. Thus, these precipitates most likely represent antigenic body parts of the IFA. A further penetration of these problems will focus on a more detailed comparison of the venom and WBE antigens and allergens.

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