

# Crossed immunoelectrophoretic studies of whole body extracts and venom from the imported fire ant *Solenopsis invicta*

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*Although allergic reactions occur after imported fire ant (IFA) sting, currently, only IFA whole body extract (IFAWBE) is available for diagnosis and immunotherapy of IFA-sensitive individuals. We report our crossed immunoelectrophoretic studies comparing antigenicity and allergenicity of Solenopsis invicta IFAWBE and IFA venom (IFAV). Rabbits were hyperimmunized with IFAWBE prepared from S. invicta or with IFAV obtained from S. invicta by an electric shock method. Crossed immunoelectrophoresis with anti-IFAWBE detected at least 29 precipitin lines in IFAWBE and three lines in IFAV, but none in a synthetic venom, transpiperidine. Anti-IFAV detected six precipitin lines in IFAV and five lines in IFAWBE, but no lines were detected with transpiperidine. Crossed-line immunoelectrophoresis confirmed the IFAV origin of at least three of the peaks in IFAWBE. Crossed radioimmunoelectrophoresis with 11 IFAWBE RAST-positive sera elicited radiostaining with five antigens in IFAWBE that were probably IFAV associated. One of these allergens was recognized by all sera; the other allergens were recognized by 8, 7, 5, and 4, respectively, of the 11 sera. Four of the antigens present in IFAV preparations had allergenicity. These findings indicate that IFA allergens probably originate in IFAV, but that transpiperidine, a major constituent of IFAV, does not appear to be immunogenic. The results suggest that IFAV may be a more appropriate reagent than IFAWBE for laboratory testing and for clinical diagnosis and immunotherapy of IFA-sensitive individuals. (J ALLERGY CLIN ALLERGY IMMUNOL 1988;81:33-40.)*

The two species of IFA, *S. invicta* and *S. richteri*, introduced from South America into the United States via Mobile, Ala., approximately five decades ago,<sup>1</sup> have successfully adapted to conditions in North America. In 1978, *S. invicta*, which is more prevalent, was estimated to occupy 160 million acres in 13 south-eastern states.<sup>2</sup>

IFA are aggressive and pugnacious. They attack up to 13.8% of the exposed population per year; 5% of those subjects that are stung require physician management for allergic reactions, which range from mild, local toxic reactions to life-threatening anaphylaxis.<sup>3</sup> Some studies report that as many as 16% of patients exhibit signs of generalized allergic reactions after IFA sting, with anaphylactic-type reactions occurring in 0.6% of victims.<sup>4</sup>

## Abbreviations used

|       |                                    |
|-------|------------------------------------|
| IFA:  | Imported fire ant                  |
| WBE:  | Whole body extract                 |
| IFAV: | Imported fire ant venom            |
| PBS:  | Phosphate-buffered saline          |
| CIE:  | Crossed immunoelectrophoresis      |
| CRIE: | Crossed radioimmunoelectrophoresis |
| CLIE: | Crossed-line immunoelectrophoresis |

The use of IFAWBE for diagnosis and treatment of sensitive individuals was first instituted in 1973.<sup>5</sup> Success rates as high as 90% for immunotherapy with IFAWBE have been reported.<sup>6,7</sup> Although these data appear impressive, however, some untreated IFA-sensitive individuals exhibit reactions of less severity when they are subsequently re-stung.<sup>7</sup>

Because of the difficulty in obtaining sufficient quantities of IFAV, little is known about its antigenic and allergenic properties. Unlike venom from other Hymenoptera, 70% of which consists of polypeptides,<sup>8</sup> IFAV appears to be a mixture consisting mainly of 2,6 di-substituted piperidines (probably trans-2-

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Supported by National Institutes of Health Grant AI 21552.  
Received for publication Jan. 12, 1987.  
Accepted for publication July 11, 1987.  
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methyl 6-n-undecylpiperidine or its enantiomer),<sup>9-11</sup> with only 1% to 5% proteinaceous material.<sup>12-14</sup>

Venoms have been demonstrated to be superior to WBEs for diagnosis and treatment of hypersensitivity to other Hymenoptera. When strict criteria were applied in a recent study of 34 IFA-sensitive individuals in which skin test reactions to IFAV and IFAWBE were compared, 91% of the individuals with history of immediate hypersensitivity to IFA demonstrated wheal-and-flare skin reactivity to IFAV, whereas only 53% reacted to IFAWBE. No significant differences were observed when relaxed criteria were used.<sup>15</sup> Different IFAWBE preparations demonstrated marked variability in local skin reactions, indicating the need for standard, well-characterized preparations for testing and desensitization of hypersensitive patients. Parallel laboratory studies demonstrated a component common to both IFAV and IFAWBE, suggesting the presence of at least one venom component in IFAWBE.

These findings raise a number of questions regarding the nature of the allergen(s) in IFA sensitivity and point out the need for careful evaluation of the components in IFAWBE and their relationship to IFAV. The studies described in this article begin to answer these questions.

## MATERIAL AND METHODS

### IFA antigens

Reference IFAWBE was prepared by a method developed in our laboratory. Twelve separate *S. invicta* colonies, collected from the New Orleans area, were stored at 4° C. A suction apparatus was developed to obtain 50 gm of live IFA from these colonies, which were stored at -70° C until they were extracted. IFAs collected from each colony were identified by an entomologist, Mr. M. Andis, New Orleans Mosquito Control, before pooling for extraction.

IFAWBE was prepared by homogenizing 5 gm of IFA in 100 ml of endotoxin-free PBS (pH 7.4) in a Tissuemizer (Heat Systems Ultrasonics, Inc., Farmingdale, N.Y.) with three 1-minute bursts. The mixture was extracted overnight at 4° C, centrifuged at 10,000 g for 20 minutes to remove particulate material, and concentrated on an Amicon YM-2 ultrafilter (Amicon Corp., Danvers, Mass.) (exclusion limit, 1000 daltons) to a final volume of approximately 5 ml. Extracts were pooled, the dry weight was determined, the final concentration was adjusted to 10 mg/ml, and the material was sterile filtered, aliquoted, and stored at -70° C until use.

IFAV from *S. invicta*, collected by an electric shock method, was a generous gift of Mr. Miles Guralnick, Vespa Laboratories, Spring Mills, Pa. Transpiperidine was prepared by Dr. T. Jones, William and Mary College, Williamsburg, Va. For comparative purposes, we performed CIE with an IFAV obtained from IFA venom sacs that was a kind gift of Dr. D. R. Hoffman, East Carolina University, Greenville, N.C.

### Hyperimmunization of rabbits with IFAWBE and IFAV

Hyperimmune IFAWBE and IFAV antisera were produced in two groups (three each) of New Zealand white rabbits. Before immunization, a 50-ml blood sample was collected, and serum was separated, aliquoted, and stored at -70° C. This sample was used as a control to exclude nonspecific precipitation and preexisting antibodies.

For immunization, the animals received initial 1-ml injections in the rear toe pads and at two sites on the back, with either 10 mg/ml of IFAWBE or 0.1 mg/ml of IFAV in sterile saline, emulsified with an equal volume of complete Freund's adjuvant. Thereafter, the animals were administered weekly 1-ml booster injections of the same concentrations of antigen in incomplete Freund's adjuvant at two sites in the back.

After 9 weeks of immunization, blood was obtained, and serum from each rabbit was tested according to the Ouchterlony technique<sup>16</sup> to confirm the presence of specific antibodies and to evaluate cross-reactivity of IFAWBE and IFAV. Multiple serum samples from rabbits, in each respective group, were collected, pooled, aliquoted, and stored at -70° C.

In conducting the research described in this article, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

### Concentration of hyperimmune rabbit antisera

After immunologic activity of rabbit antisera was confirmed by the Ouchterlony technique, specific pooled antisera from each group of rabbits were concentrated by ammonium sulfate precipitation. Filtered 100% ammonium sulfate (prepared by addition of 767 gm of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 1 L of water, heated with stirring until it was dissolved, and then allowed to cool in an ice bath until crystals formed) was added dropwise to each serum pool to reach a final 40% concentration, and the mixture was stirred overnight at 4° C. The resulting solution was centrifuged at 44,500 g for 30 minutes at 4° C, and the supernatant was discarded.

The pellet was resuspended in 0.01 mol/L of PBS, pH 7.2, and dialyzed to exhaustion against 20 changes of PBS. The retentate was centrifuged at 79,000 g for 30 minutes at 4° C, the pellet was discarded, and the supernatant volume was adjusted to the desired final concentration. Sera were aliquoted and stored at -70° C until time for use.

### CIE to determine the number of antigenic components in IFAV and IFAWBE

The CIE method currently in use in our laboratory<sup>17</sup> was used. Briefly, 20 µl of 10 mg/ml concentration of IFAWBE or 50 µl of 0.1 mg/ml concentration of IFAV was introduced into wells cut into 1% agarose gel and electrophoresed toward the anode at 10 V/cm for 20 minutes. For the second dimension electrophoresis, molten 1% agarose, containing

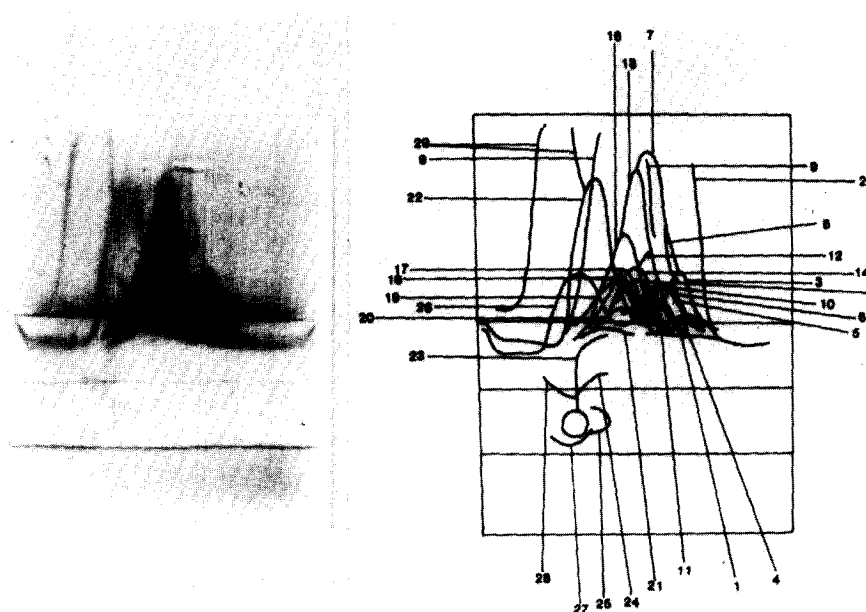


FIG. 1. CIE pattern obtained when IFAWBE was run into anti-IFAWBE.

an optimal dilution of concentrated hyperimmune rabbit antiserum to IFAWBE or IFV, was poured on the remaining area of the plate and electrophoresed at 10° C overnight at 2 to 3 V/cm, so that the antigen migrated into the concentrated hyperimmune antiserum (ie, at 90 degrees to the original direction). Plates stained with 0.5% Coomassie brilliant blue dye were examined on a light box for precipitin arcs. Precipitin peaks were assigned a number for comparative purposes.

## CLIE

For studies of cross-reactivity, CLIE was performed. Agarose, containing 100 µl of antigen different from that used in the first dimension electrophoresis (10 mg/ml of IFAWBE; 0.1 mg/ml of IFV), was poured as the middle gel between the test antigen and the rabbit hyperimmune antiserum to that antigen before the second electrophoresis stage. The second stage electrophoresis was run as described for CIE.

## CRIE to determine which of the peaks identified by CIE had allergenic properties

CRIE<sup>18</sup> was performed with sera from 11 patients having a positive IFAWBE RAST (percent binding 6.3% to 23.0%) and a positive IFV RAST (percent binding 17.6% to 35.4%). Briefly, IFAWBE or IFV CIE plates that had been processed as above, but without staining, were flooded with 400 µl of patients' sera in 6.6 ml of phosphate buffer and incubated overnight at room temperature. After incubation, plates were washed four times with saline, flooded with phosphate buffer, containing sufficient <sup>125</sup>I-labeled anti-IgE antiserum (Kallestad Laboratories, Inc., Austin, Texas) to elicit 250,000 cpm of radioactivity, and incubated overnight. After washing to remove unreacted radiolabeled anti-

IgE, autoradiography was performed to determine which antigenic precipitin arcs had allergenic properties. A measure of allergic potency was obtained by determining the CRIE class<sup>18</sup> of each allergenic precipitin arc with the different patient sera with radioactive reference disks as indicators of autoradiographic staining intensity.

## RESULTS

### Ouchterlony

All three rabbits immunized with IFAWBE exhibited at least one strong precipitin line to the extract. In two rabbits, two precipitin lines were observed.

Two of the three rabbits immunized with IFV exhibited one strong precipitin line to the venom. The other rabbit exhibited two strong precipitin lines.

Concentrated anti-IFV antiserum exhibited a strong clear line of identity between IFAWBE and IFV preparations. There were no discernible lines of non-identity or partial identity. IFV was not identified by concentrated anti-IFAWBE antiserum.

### CIE

The CIE pattern obtained when IFAWBE was electrophoresed into anti-IFAWBE is illustrated in Fig. 1. At least 29 precipitin arcs were demonstrable, one of which (No. 27) demonstrated cathodic migration.

When the IFAWBE was electrophoresed into anti-IFV, five distinct precipitin arcs were detected. One of these peaks (No. 5) demonstrated cathodic migration (Fig. 2). The peaks identified in this CIE appeared to correspond to peaks identified in the IFAWBE/anti-

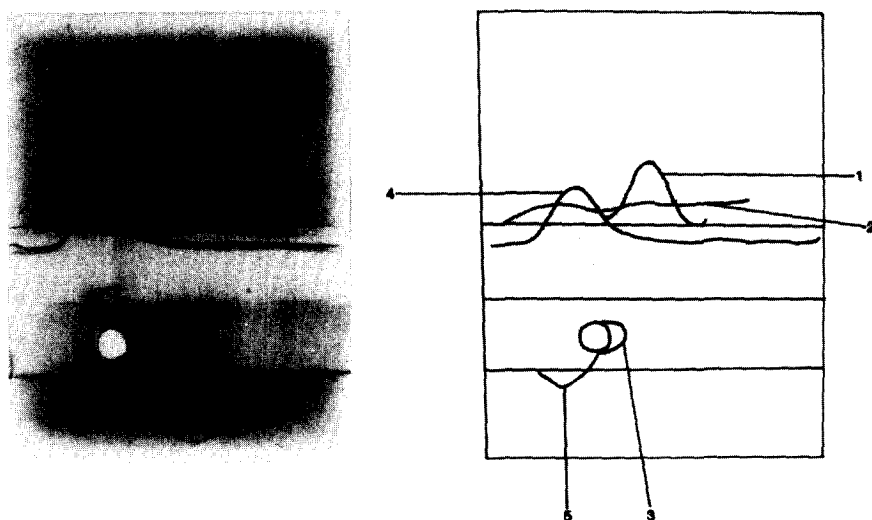


FIG. 2. CIE pattern obtained when IFAWBE was run into anti-IFAV.

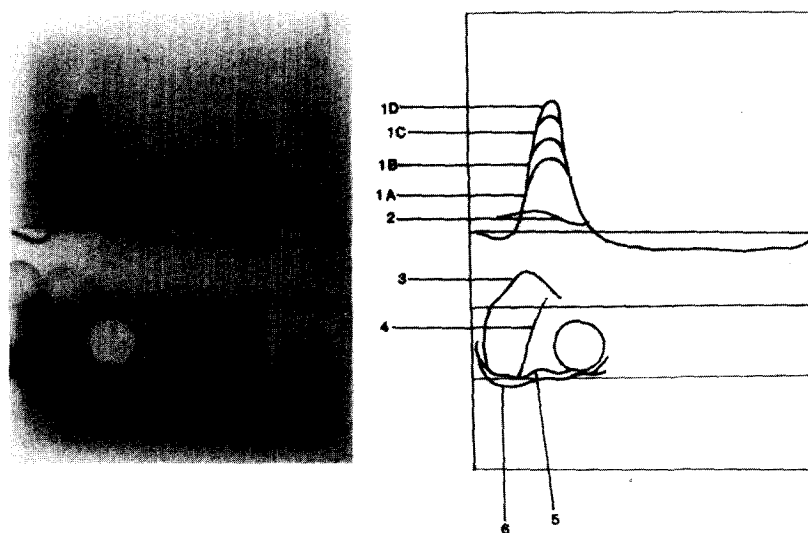


FIG. 3. CIE pattern obtained when IFAV was run into anti-IFAV.

IFAWBE CIE (Nos. 1 to 16, 2 to 26, 3 to 25, 4 to 29, and 5 to 27).

The CIE pattern obtained when IFAV was run into anti-IFAV is illustrated in Fig. 3. A total of six precipitin arcs were detected. For one peak (No. 1) of an antigen having an isoelectric point close to 8.6, there were three apparent precipitin arcs within that peak; however, these arcs were probably due either to antibody complexes or to change in mobility of the antigen during the second phase electrophoresis and are not considered to be separate antigenic peaks.

When IFAV was run into anti-IFAWBE, three precipitin arcs were detected. One of these peaks (No. 3) demonstrated cathodic migration (Fig. 4). Based on electrophoretic mobility, one of these peaks (No. 1) appeared to correspond to peak No. 29 in the IFAWBE/anti-IFAWBE CIE, and peak No. 3 appeared to correspond to peak No. 27.

No precipitin lines were obtained when the transpiperidine preparation was run against either anti-IFAWBE or anti-IFAV.

These results demonstrate that IFAWBE prepara-

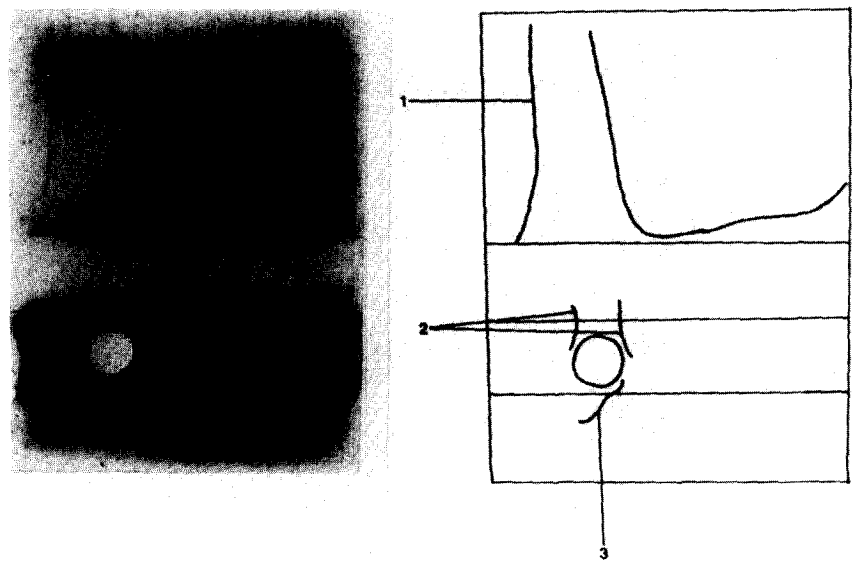


FIG. 4. CIE pattern obtained when IFAV was run into anti-IFAWBE.

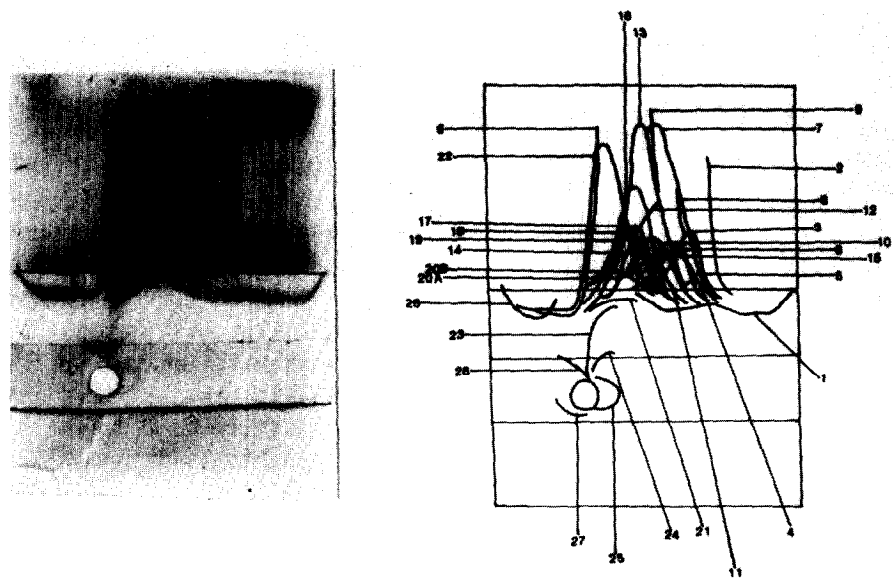


FIG. 5. CLIE pattern obtained with IFAV in intermediate gel and IFAWBE electrophoresed into anti-IFAWBE.

tions contain some, but not all, IFAV antigens and that these IFAV antigens are present in sufficient concentration to be immunogenic.

CIE with IFAV collected by expressing IFA venom sacs (a kind gift of Dr. D. Hoffman) demonstrated peaks with identical electrophoretic mobility to peaks obtained with IFAV collected by the electric shock method.

CLIE

CLIE, with 100  $\mu$ l of 0.1 mg/ml of IFAV in the intermediate gel, and IFAWBE run against anti-IFAWBE, completely eliminated peak No. 29 and significantly reduced peak No. 26. These two peaks appear to originate from IFAV. The appearance of an extra peak in the area around peak No. 20 (labeled No. 20 B in Fig. 5) was also noted. This new peak

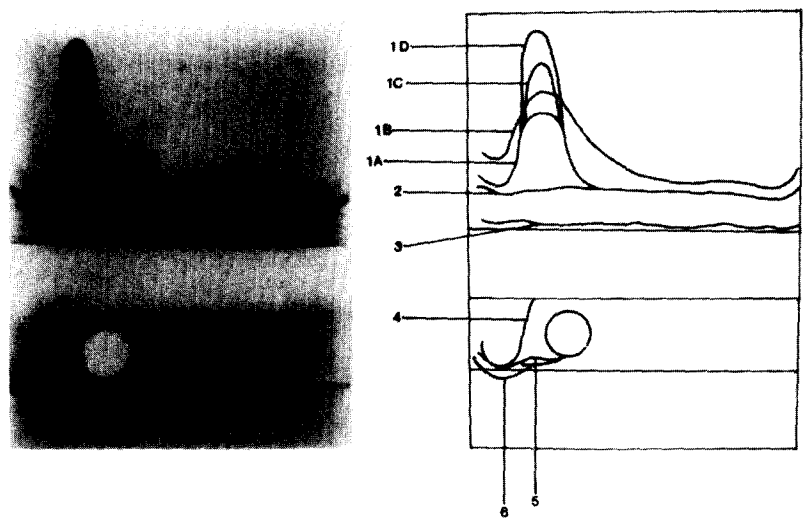


FIG. 6. CLIE pattern obtained with IFAWBE in intermediate gel and IFAV electrophoresed into anti-IFAV.

TABLE I. Radiostaining peaks and CRIE classes\* obtained with 11 IFA RAST-positive serum samples on IFAWBE anti-IFA plates

| Patient | IFAWBE<br>RAST<br>(% binding) | Well | Peak No. |    |    |    |
|---------|-------------------------------|------|----------|----|----|----|
|         |                               |      | 16       | 26 | 27 | 29 |
| 1       | 10.2                          | C    | —        | —  | D  | D  |
| 2       | 8.6                           | D    | —        | —  | E  | —  |
| 3       | 16.0                          | C    | E        | D  | —  | C  |
| 4       | 6.3                           | D    | —        | —  | E  | —  |
| 5       | 11.5                          | C    | E        | —  | —  | C  |
| 6       | 19.2                          | B    | —        | B  | C  | D  |
| 7       | 21.4                          | B    | —        | —  | D  | C  |
| 8       | 8.4                           | C    | —        | D  | D  | D  |
| 9       | 23.0                          | B    | —        | D  | D  | D  |
| 10      | 21.5                          | C    | E        | D  | —  | C  |
| 11      | 15.0                          | D    | C        | —  | —  | —  |

\*Classes graded A to E in decreasing order of density of radiostaining.

probably reflects increased resolution of precipitin arcs on this particular slide.

When CLIE was performed with 100  $\mu$ l of 10 mg/ml IFAWBE in the intermediate gel and 50  $\mu$ l of 0.1 mg/ml IFAV run against anti-IFAV, lines of identity were obtained with at least three peaks (Nos. 1B, 2, and 3 in Fig. 6).

These results confirm the presence of IFAV components in the IFAWBE preparation, although not all IFAV components were demonstrable.

CRIE

Serum from the 11 patients with IFAWBE-positive RAST elicited variable autoradiographic staining (see CRIE classes in Table I and representative autoradiographs in Fig. 7). CRIE plates with all 11 serum samples exhibited an area of radiostaining around the well. In addition, eight sera also demonstrated radiostaining against peak No. 29, seven sera demonstrated radiostaining to peak No. 27, five sera demonstrated radiostaining to peak No. 26, and four sera demonstrated radiostaining to peak No. 16.

When sera were subjected to CRIE with the IFAV/anti-IFAV preparations, radiostaining around the well was observed with all but one serum, and for peak Nos. 1, 3, and 5 with six, seven, and three sera, respectively (Table II).

DISCUSSION

These studies demonstrate that there are at least 29 antigenic components in IFAWBE. Furthermore, six immunogenic components were demonstrable in IFAV, and precipitin arcs associated with most of these were also demonstrated to be present in IFAWBE. Allergic properties were demonstrable by CRIE for five antigens demonstrable in IFAWBE, which are probably of IFAV origin, and with three of the antigens in IFAV, although they did not stimulate production of specific IgE antibodies in serum of all individuals. Our findings begin to characterize the allergens responsible for IFA hypersensitivity. The results not only demonstrate that a fresh IFAWBE preparation contained most, but not all, antigens associated with

**TABLE II.** Radiostaining peaks and CRIE classes\* obtained with 11 IFA RAST-positive serum samples on IFAV anti-IFA plates

| Patient | IFAV<br>RAST<br>(% binding) | Well | Peak No. |   |   |
|---------|-----------------------------|------|----------|---|---|
|         |                             |      | 1        | 3 | 5 |
| 1       | 18.9                        | E    | D        | D | D |
| 2       | 27.0                        | C    | —        | D | — |
| 3       | 31.8                        | C    | C        | C | C |
| 4       | 26.6                        | E    | —        | — | — |
| 5       | 23.0                        | D    | D        | D | — |
| 6       | 32.4                        | B    | B        | C | — |
| 7       | 33.1                        | D    | —        | C | — |
| 8       | 17.6                        | —    | D        | — | — |
| 9       | 35.4                        | D    | E        | E | — |
| 10      | 24.5                        | E    | —        | — | D |
| 11      | 20.6                        | E    | —        | — | — |

\*Classes graded A to E in decreasing order of density of radio-staining.

IFAV but also demonstrate that IFAWBE had a number of immunogenic substances that are not involved in the allergic reactions that can result from IFA sting.

These CIE findings generally agree with findings of Baer et al.,<sup>12</sup> Nordvall et al.,<sup>19</sup> and Hannan et al.,<sup>20</sup> who also found multiple antigenic and allergenic components in IFA preparations. Our results do not, however, agree with the findings of Paull et al.,<sup>13</sup> who, after Sephadex gel filtration, reported 21 antigenic peaks from IFAV.

Currently, diagnosis and treatment of IFA sensitivity involve the use of IFAWBE,<sup>2, 5, 21</sup> primarily because IFAV is difficult to obtain for any large-scale use, and also because few studies have been performed to characterize the antigens and allergens responsible for the IFA sensitivity. Little is known about the nature of IFAWBE preparations, and no studies to compare the efficacy of IFAV for treatment have been performed. Our findings that not all allergens demonstrable in IFAV are detectable in IFAWBE preparations indicate that this extract may not be ideal for diagnosis and treatment of IFA allergies. Furthermore, our demonstration that IFAWBE preparations have only low concentration of IFAV antigens confirms that IFAWBE is not an appropriate reagent.

Results from our immunoelectrophoretic studies may explain the findings by Strom et al.<sup>15</sup> of marked variability in local skin reactivity with various preparations of IFAWBE and that almost twice the number of patients tested reacted to IFAV than the number reacting to IFAWBE when strict criteria were applied.

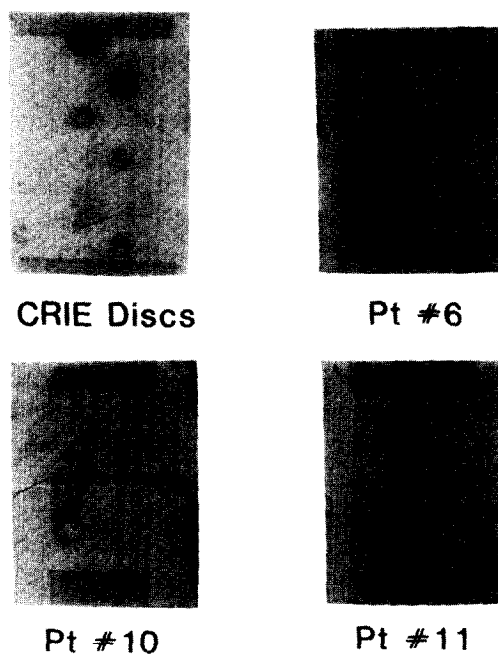


**FIG. 7.** Representative IFAWBE CRIE radiographs and control disks.

Since IFAWBE does not contain all IFAV antigens, the failure of approximately one half of the IFA-allergic individuals to react to skin test with IFAWBE could be accounted for by low or absent IFAV allergens in the IFAWBE preparation. Thus, a logical extension of these observations is that IFAV would appear to be a more appropriate reagent for skin testing and laboratory testing of IFA-sensitive individuals and, as has been demonstrated to be the case with other Hymenoptera,<sup>22</sup> may also prove to be a better material to use for desensitization. Our preliminary RAST-inhibition testing indicates that IFAV is a more appropriate antigen than IFAWBE for diagnostic testing.<sup>23</sup>

Currently, only IFAWBE is used for desensitization. There are a number of studies of lack of efficacy of IFAWBE as a desensitizing reagent.<sup>6, 15, 24</sup> This material contains large amounts of nonspecific components<sup>25</sup> that may be needlessly introduced into individuals receiving extended treatment of frequent injections of IFAWBE. Use of IFAV for desensitization regimens would not only ensure specificity of the protective effects of the treatment but would also reduce unnecessary exposure to the other components in IFAWBE.

Before IFAV is accepted for routine use in diagnosis and treatment of IFA allergies, however, further studies need to be performed to confirm the specificity of

the allergens in IFAV and begin to identify the nature of these allergens and to compare the use of IFAV, IFAWBE, or placebo as desensitizing regimens. Our preliminary observations that a synthetic venom alkaloid, transpiperidine, does not have antigenic or allergenic activities, suggest that these immunogenic properties of IFAV reside in other components, such as the small amount of protein known to be present. This may be phospholipase that was recently reported to correlate with levels of IgE antibodies in serum of IFA-allergic patients.<sup>20</sup> Thus, as allergenic components of IFA become identified, it may be possible to further purify IFAV preparations for use in diagnostic testing and treatment regimens. Further studies also need to be performed to determine whether IFAV is a suitable reagent for in vitro RAST or ELISA testing and to evaluate the specificity of any results to in vivo measurements of IFA allergy. Such studies are currently in progress in our laboratory.

We thank Mr. M. Guralnick, Vespa Laboratories, Spring Mills, Pa., for the generous donation of IFAV, Mr. M. Andis, New Orleans Mosquito Control, New Orleans, La., for identification of IFA species, Dr. T. Jones, William and Mary College, Williamsburg, Va., for supplying transpiperidine samples, and Dr. D. R. Hoffman, East Carolina University, Greenville, N.C., for a gift of IFAV obtained from IFA venom sacs.

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