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Evaluation of commercial imported fire ant extracts by crossed immunoelectrophoresis and radioallergosorbent test

Brian T. Butcher, PhD, and Margaret A. Reed, MS *New Orleans, La.*

Imported fire ant whole-body extracts (IFAWBE) from three major U.S. commercial suppliers and a reference IFAWBE and imported fire ant venom (IFAV) preparation were compared by crossed immunoelectrophoresis and RAST-inhibition testing. Results of crossed immunoelectrophoretic studies showed major differences in antigen content between commercial preparations. IFAWBE and IFAV RAST-inhibition testing showed that no commercial extract had equivalent inhibitory activity to the reference IFAWBE. Reference IFAWBE reached 50% inhibition of IFAWBE and IFAV RAST at 52 and 492 µg/ml, respectively. IFAV gave 50% inhibition of IFAWBE and IFAV RAST at 5.9 and 58 µg/ml, respectively. Of the commercial extracts, only those from one supplier were able to reach 50% inhibition of the reference IFAWBE RAST, and no commercial extract from any supplier was able to reach 50% inhibition of IFAV RAST. Analysis of inhibition slopes and intercepts confirmed that there were both qualitative and quantitative differences among the commercial extracts and the reference IFAWBE and IFAV. Our findings suggest possible reasons why some individuals fail to respond to testing or immunotherapy with IFAWBE and show the urgent need for standardization of allergenic extracts for diagnosis and treatment of imported fire ant sensitivity. (J ALLERGY CLIN IMMUNOL 1988;82:770-7.)

Allergic reactions after sting by the two species of IFA, *Solenopsis richteri* and *S. invicta*, are a major medical problem in the southern United States.¹⁻⁴

S. invicta, the dominant species of IFA, is responsible for the majority of allergic reactions seen in the United States.⁵ It is reported that as many as 58% of individuals living in infested areas experience at least one sting each year,⁶ and that 5% of those stung experience some form of allergic reaction requiring physician management.³ Severe anaphylactic reactions are reported to occur in 0.6% of stung individuals,¹ and some deaths have been reported. Although there is no information available on the number of patients receiving treatment, generally, standard medical practice is to provide immunotherapy to those experiencing severe allergic reactions after IFA sting.⁷⁻⁹

From the Clinical Immunology Section, Department of Medicine, Tulane University Medical Center.

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Reprint requests: Brian T. Butcher, PhD, Clinical Immunology Section, Department of Medicine, Tulane University Medical Center, 1700 Perdido St., New Orleans, LA 70112.

Abbreviations used

CIE:	Crossed immunoelectrophoresis
IFA:	Imported fire ant
IFAWBE:	Imported fire ant whole-body extract
IFAV:	Imported fire ant venom
PBS:	Phosphate-buffered saline
CL:	Center Laboratories
GL:	Greer Laboratories, Inc.
HS:	Hollister-Stier

Because it is not currently possible to obtain IFAV in quantity, only IFAWBEs are available commercially for skin testing and immunotherapy. Depending on the supplier, these extracts range from species-specific to mixed-species preparations. At present, there are no guidelines governing preparation of IFA extracts, and commercial extracts used in diagnosis and immunotherapy are not standardized.

In this report we describe CIE and RAST-inhibition studies evaluating IFAWBE preparations from three major U.S. commercial suppliers. Our findings of marked qualitative and quantitative differences in antigenic composition and allergenic potency suggest possible reasons why some individuals fail to react to testing or immunotherapy with IFAWBE. These results show an urgent need for standardization of allergenic extracts for diagnostic and immunotherapeutic use.

MATERIAL AND METHODS

Reference preparations

With the use of methods developed in our laboratory,¹⁰ reference IFAWBE was prepared from 12 separate *S. invicta* colonies collected from the New Orleans area. Randomly selected IFAs from each colony were identified by an entomologist before pooling for extraction.

Briefly, reference IFAWBE was prepared by homogenization and extraction of IFA in PBS and centrifugation to remove particulate material, followed by concentration on an Amicon YC-05 ultrafilter (exclusion limit 500 daltons). The extract dry weight was 54.5 mg/ml and protein concentration, as determined by a Biuret method,¹¹ was 28.9 mg/ml. This reference IFAWBE 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. The reported dry weight of this preparation was 400 $\mu\text{g}/\text{vial}$.

Commercial IFAWBE preparations

Commercial nonglycerinated IFAWBE was purchased from three major U.S. extract suppliers: Center Laboratories, Port Washington, N.Y., fire ant mix (*S. invicta* plus *S. richteri*); Greer Laboratories, Inc., Lenoir, N.C., *S. invicta* and *S. richteri*; and Hollister-Stier, West Haven,

Conn., *S. invicta* and *S. richteri*, and fire ant mix (*S. invicta* and *S. richteri*). Protein concentration was determined on each of these commercial IFAWBE preparations by a Biuret method,¹¹ and final protein concentrations of all extracts were standardized before CIE and RAST-inhibition studies.

Hyperimmunization of rabbits with IFAWBE and IFAV

Hyperimmune antisera to reference IFAWBE and IFAV were produced in two groups of New Zealand white rabbits.¹² Before immunization, a serum sample was obtained from each animal as a control to exclude nonspecific precipitation and preexisting antibodies.

Briefly, for immunization, animals received initial injections of antigen emulsified with an equal volume of complete Freund's adjuvant in the toe pads and on the back. Thereafter, weekly booster injections of antigen in an equal volume of incomplete Freund's adjuvant were given in the back only. After 9 weeks of immunization, serum from each rabbit was tested by an Ouchterlony technique¹³ to confirm the presence of specific antibodies. Then, multiple serum samples, collected over a 2-week period, were pooled, concentrated by ammonium sulfate precipitation, aliquoted, and stored at -70°C until used.

In conducting the research described in this report, 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.

CIE to determine IFAV and IFAWBE antigens in commercial IFAWBE

The CIE method currently in use in our laboratory¹⁰ was utilized. Briefly, 50 μl of 1 mg protein/ml PBS concentration of commercial or reference IFAWBE, or 50 μl of 0.1 mg/ml concentration of IFAV, were 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 an optimal dilution of concentrated hyperimmune rabbit antiserum to IFAWBE (250 μl in the top section, 100 μl in the bottom section) or IFAV (200 μl in the top section, 100 μl in the bottom section) was poured on the remaining area of the plate. Electrophoresis was performed at 10°C overnight at 2 to 3 V/cm, so that the antigen migrated into the concentrated hyperimmune antiserum (i.e., at 90 degrees to the original direction). Plates were stained with 0.5% Coomassie brilliant blue and examined on a light box for precipitin arcs.

RAST-inhibition testing with commercial IFAWBE preparations

The RAST-inhibition method has been described previously.^{12, 14} Briefly, a reference IFAWBE-coupled or IFAV-coupled disk and 50 μl of a pool of 18 IFA RAST positive serum samples (22% of patients were receiving immunotherapy) (reference IFAWBE RAST percent binding = 14.7%; IFAV RAST percent binding = 29.4%)

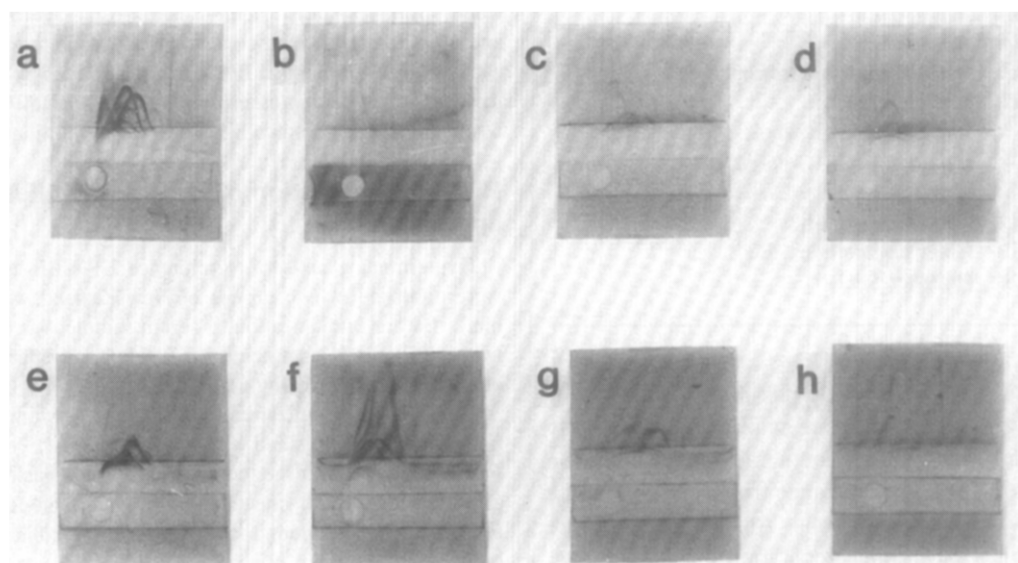


FIG. 1. CIE of IFAV, reference IFAWBE, and commercial IFAWBE preparations with anti-IFAWBE. *a*, reference IFAWBE; *b*, IFAV; *c*, GL *S. invicta*; *d*, GL *S. richteri*; *e*, CL IFA mix; *f*, HS *S. invicta*; *g*, HS *S. richteri*; *h*, HS IFA mix.

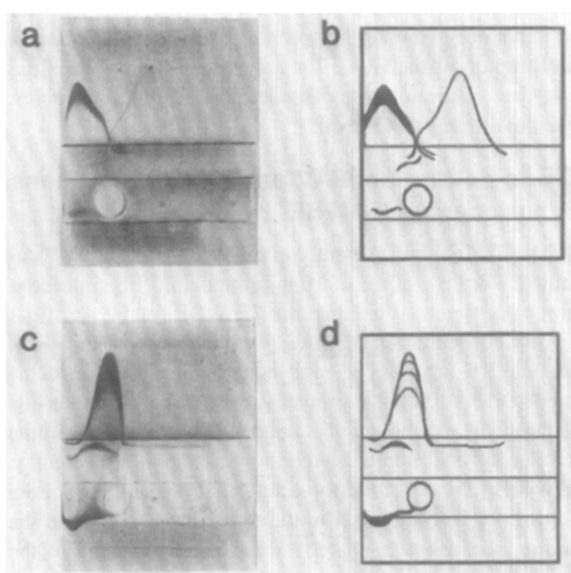


FIG. 2. CIE of reference IFAWBE and IFAV with anti-IFAV. *a* and *b*, Reference IFAWBE; *c* and *d*, IFAV.

were coincubated overnight with 50 μ l of dilutions of commercial IFAWBE preparations, reference IFAWBE, IFAV, or PBS, in duplicate. Commercial IFAWBE concentrations for inhibitions were the undiluted material "as purchased," with subsequent dilutions adjusted until all extract dilutions were in a standard range. Two extracts from two different suppliers that contained <1 mg/ml of protein were concentrated

on an Amicon YC-05 filter (molecular weight cutoff 500 daltons) to increase the starting concentration. Inhibiting concentrations of reference IFAWBE and IFAV were 5 to 0.0032 mg/ml and 0.6 to 0.003 mg/ml, respectively. After washing to remove unreacted serum and inhibiting extract, disks were incubated overnight with 100 μ l ^{125} I-labeled anti-IgE antiserum and washed to remove unreacted material. Total activity added was determined and all disks were counted in a gamma counter.

After log transformation, the percent inhibition of reference IFAWBE or IFAV RAST by the different dilutions of inhibitor preparations was calculated and results were plotted. Linear regression was performed on the linear ascending portion of the inhibition curve, and slopes and intercepts were determined. The 50% inhibition concentration was calculated from the linear regression equations.

Statistical analyses

Individual linear regressions were performed with a computerized statistical program. Multiple linear regression was performed on an IBM-XT with the STATISTIX interactive statistical analysis program for microcomputers (NE Analytical Software, St. Paul, Minn.).

RESULTS

Protein concentrations of commercial IFAWBE preparations

The protein concentration of the reference IFAWBE preparation was 28.9 mg/ml. Protein concentration of the CL IFA mix was 0.81 mg/ml. For GL preparations, protein concentrations of the *S. invicta* and

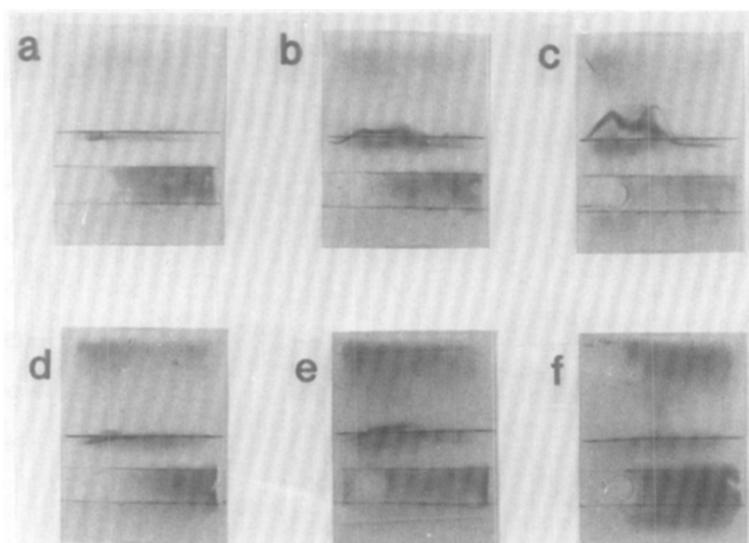


FIG. 3. CIE of commercial IFAWBE preparations with anti-IFAV. a, CL IFA mix; b, GL *S. invicta*; c, GL *S. richteri*; d, HS *S. invicta*; e, HS *S. richteri*; f, HS IFA mix.

S. richteri extracts were 5.64 and 4.12 mg/ml, respectively. For HS extracts, protein concentrations were 0.81 mg/ml for *S. invicta*, 3.58 mg/ml for *S. richteri*, and 2.96 mg/ml for the IFA species mix. All preparations were diluted to 1.0 mg/ml protein concentration for CIE. For RAST-inhibition assays, extracts were used at a range of dilutions, the highest being undiluted material, "as purchased," from the supplier. In addition, two of the extracts (CL IFA mix and HS *S. invicta*) containing <1 mg/ml "as purchased" were concentrated on an Amicon YC-05 filter to 1.7 and 2.2 mg protein/ml, respectively.

CIE with anti-IFAWBE serum

CIE peaks obtained with IFV and with reference IFAWBE at the same concentration as the commercial IFAWBE extracts contained 1 and 24 precipitin arcs, respectively. The number of peaks in the commercial IFA preparations were as follows: CL fire ant mix, 15; GL *S. invicta* and *S. richteri*, 11 and 6, respectively; and HS *S. invicta*, *S. richteri*, and fire ant mix, 18, 8, and 5, respectively. The precipitin patterns of reference and commercial IFA preparations are shown in Fig. 1.

CIE with anti-IFV serum

There were three and four CIE peaks obtained with IFV and reference IFAWBE, respectively (Fig. 2). The precipitin peaks obtained with the commercial IFAWBE are shown in Fig. 3. CL fire ant mix had 1 peak; GL *S. invicta* and *S. richteri* each had three

peaks; and HS *S. invicta*, *S. richteri*, and fire ant mix had two, one, and no peaks, respectively.

IFAWBE and IFV RAST-inhibition tests

For the reference IFAWBE, a plateau of inhibition of IFAWBE RAST was reached at 2 mg protein/ml concentration. For IFV, the plateau for inhibition of IFAWBE RAST was reached at 60 μ g/ml. No commercial extract reached a plateau of inhibition of IFAWBE RAST, even at the highest concentrations used. Regression lines of inhibition testing of reference IFAWBE RAST are shown in Fig. 4.

The 50% inhibiting concentration of reference IFAWBE RAST by reference IFAWBE was 52 μ g protein/ml, and by IFV it was 5.9 μ g/ml. For the CL IFA mix, 50% inhibition was not reached by the undiluted material. For GL preparations, the 50% inhibition concentrations were 3.2 mg protein/ml for *S. invicta* and 786 μ g/ml for *S. richteri*. None of the HS preparations attained 50% inhibition, even at the highest concentrations. These data are summarized in Table I.

Inhibition regression lines of IFV RAST are shown in Fig. 5. For IFV RAST, the plateau of inhibition was not reached at the maximum concentration of reference IFAWBE used (5 mg/ml); 50% inhibition was attained with 492 μ g protein/ml. For IFV, the inhibition plateau was reached at 300 μ g/ml and the 50% inhibiting concentration was 58 μ g/ml. Even at the highest concentrations used, no commercial extract reached 50% inhibition of IFV RAST: only GL

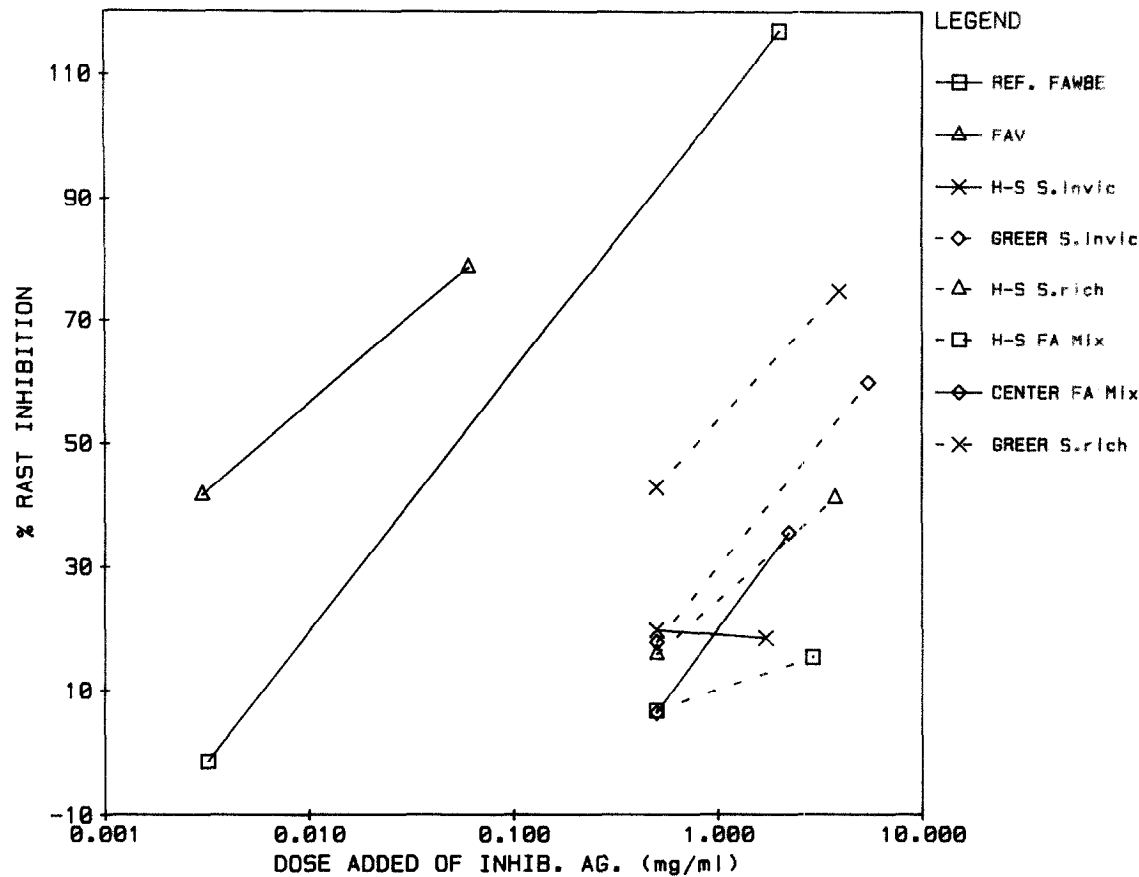


FIG. 4. Linear regression of inhibition of reference IFAWBE RAST by commercial IFA preparations.

TABLE I. IFAWBE RAST-inhibition studies

Inhibiting antigen	Plateau concentration (mg/ml)	Highest inhibition achieved	50% Inhibitory concentration (mg/ml)	Correlation coefficient (r)
Reference IFAWBE	2.0	90%	0.052	0.97
IFAV	0.06	84%	0.0059	0.97
CL mix	—	37%	—	0.96
GL	—	—	—	—
<i>S. invicta</i>	—	60%	3.2	0.98
<i>S. richteri</i>	—	71%	0.786	0.95
HS	—	—	—	—
<i>S. invicta</i>	—	20%	—	0.44
<i>S. richteri</i>	—	42%	—	0.98
Mix	—	19%	—	0.57

S. richteri, at the highest concentration, approached this level of inhibition (48%). These data are summarized in Table II.

Statistical analysis of slopes of IFAWBE RAST inhibition by the different extracts showed that inhibition slopes for reference IFAWBE, IFAV, GL *S. invicta* and *S. richteri*, and HS *S. richteri* had statistically

significant slopes and were statistically equivalent ($p > 0.05$). The remaining commercial extracts did not have statistically significant slopes. These equal and significant slopes indicate that the extracts were qualitatively equivalent in allergen content. Statistical evaluation of intercepts showed that only reference IFAWBE, IFAV, and GL *S. richteri* had equal inter-

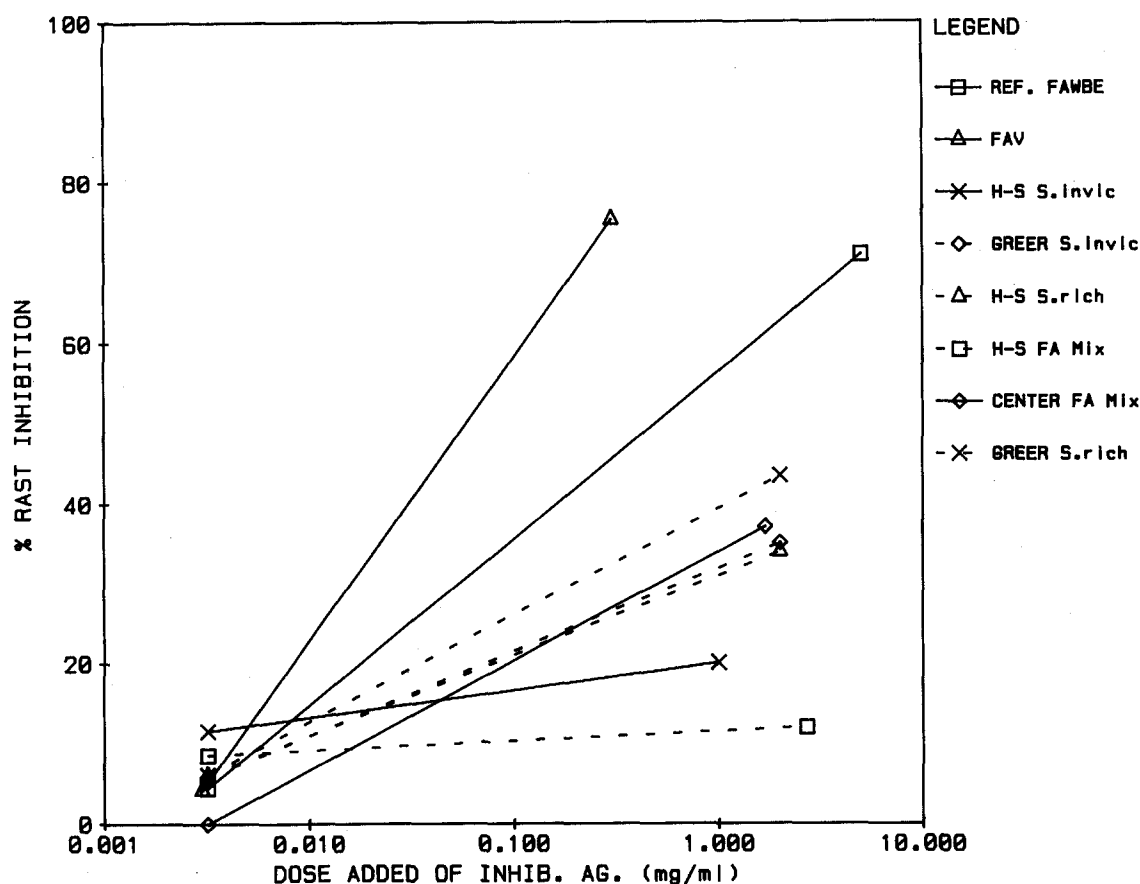


FIG. 5. Linear regression of inhibition of reference IFAV RAST by commercial IFA preparations.

TABLE II. IFAV-RAST inhibition studies

Inhibiting antigen	Plateau concentration (mg/ml)	Highest inhibition achieved	50% Inhibitory concentration (mg/ml)	Correlation coefficient (r)
Reference IFAWBE	—	74%	0.492	0.95
IFAV	0.3	85%	0.058	0.98
CL mix	—	40%	—	0.90
GL	—	—	—	—
<i>S. invicta</i>	—	42%	—	0.90
<i>S. richteri</i>	—	48%	—	0.88
HS	—	—	—	—
<i>S. invicta</i>	—	21%	—	0.86
<i>S. richteri</i>	—	40%	—	0.91
Mix	—	15%	—	0.30

cepts, indicating that the extracts are quantitatively similar.

For IFAV RAST inhibition, there was no statistically significant difference among the slopes for reference IFAWBE, IFAV, CL IFA mix, and GL *S. richteri*. The slopes for all other preparations were statistically different. HS *S. invicta* and IFA mix extracts did not

have significant slopes. Only reference IFAWBE and IFAV had equal intercepts.

DISCUSSION

These studies demonstrate that commercial IFAWBEs vary markedly in antigenic content and potency. Comparison of commercial extracts with ref-

erence IFAWBE, after standardizing protein content, showed a range from 5 to 18 total antigens in commercial extracts compared with 24 in the reference IFAWBE. Although the sensitivity and specificity of the CIE technique are limited by the ability of rabbits to recognize antigens that may be antigenic or allergenic in humans, CIE remains a useful technique for comparison of similar extracts.

Previous studies have shown that IFA allergenic activity resides in IFAV.^{10, 14-16} Thus, a more appropriate indication of the allergenic activity of IFAWBE is the number of IFAV antigens in an IFAWBE extract. Reference IFAWBE had four IFAV-specific antigens, whereas in commercial preparations the number of IFA-specific antigens range from three to zero. Therefore, all commercial extracts lacked at least one IFA-specific antigen component.

The demonstration of only one peak in the reaction between IFAV and anti-IFAWBE, but four peaks in the reverse experiment in which IFAWBE was electrophoresed into anti-IFAV, probably reflects the response of rabbits of immunization. Because the concentration of venom in IFAWBE is low, it is likely that the IFAV-specific response of rabbits to immunization with IFAWBE would be minimal, whereas immunization with "pure" IFAV is more likely to lead to production of more potent anti-IFAV antibodies that can detect the small amounts of venom components in IFAWBE.

Our demonstration of an additional peak in IFAWBE detectable by anti-IFAV that was not demonstrable when IFAV was electrophoresed against the same anti-IFAV is difficult to explain. It is possible that venom precursors are present in higher concentrations in IFAWBE than in IFAV or that conjugates of venom component may form with IFAWBE material, which would confer different electrophoretic mobility in CIE but not alter the antigenic specificity of the component.

Because CIE results are indicative only of antigen content, RAST-inhibition studies were performed as a measure of the allergenic potency of extracts. There were marked qualitative and quantitative differences in inhibitory activity of all commercial extracts. Only two commercial preparations were able to attain 50% inhibition of IFAWBE RAST, but concentrations required were at least 10-fold greater than for reference IFAWBE. No commercial extract reached 50% inhibition of IFAV RAST, although one approached this level (48%).

Analysis of RAST-inhibition slopes and intercepts confirmed qualitative and quantitative differences between extracts. Statistical analysis of slopes confirmed the qualitative similarity between some commercial

extracts and reference IFAWBE and IFAV. Some extracts differed both qualitatively and quantitatively from reference IFAWBE and IFAV.

Commercial extract preparations are commonly used for both diagnosis and treatment of IgE-mediated allergic disease. Currently, there is little or no effort to standardize these preparations or even to ensure that extracts have appropriate antigen content or allergenic activity. For a notable few, such standards have been proposed,¹⁷ but these are mainly cases in which specific allergens have been isolated in sufficient quantity for characterization and purification. Most commercial extracts are not well characterized and consist of crude preparations of materials that are relatively easy to obtain. Although the methods used to prepare these extracts commercially are proprietary, they are probably not standardized and may well vary widely from one supplier to another.

Our findings not only showed marked variability between content and activity of IFAWBEs from different commercial suppliers, but also demonstrated that some extracts lack appropriate antigens and have little or no allergenic activity. This may explain why some individuals with definitive history of IFA allergy do not react to skin testing with IFAWBE or their sera give negative results by *in vitro* testing.^{9, 12, 15, 16} Our findings may also explain why immunotherapy fails to confer protection in some patients. Clearly, for individuals experiencing life-threatening reactions after an IFA sting, it is vital that extracts used for diagnosis and immunotherapy contain the appropriate allergens to identify those with sensitivity and confer protection from further allergic reactions.

The nature of the allergens in IFAV remain to be determined. We have shown that allergenic activity resides in the IFAV^{9, 12, 14, 15} but is not a property of the alkaloid, which comprises $\geq 95\%$ of IFAV. It has been suggested that the active components of IFAV may be phospholipase A and *N*-acetyl- β -glucosaminidase,¹³ but this has not been confirmed. For other *Hymenoptera*, the major allergenic component of venoms has been identified as phospholipase A, and venom is now used routinely for immunotherapy of sensitive individuals.¹⁹ However, for IFA, because of the great difficulty of obtaining venom, such extracts are not available and only IFAWBE is currently manufactured commercially. Until IFAV can be prepared commercially, IFAWBE remains the only extract available for diagnosis and immunotherapy of IFA-sensitive individuals. Our ability to prepare more potent extracts is probably a reflection of our IFA collection technique, which ensures that only fresh IFA is used for extract preparation. The superiority of our extract over commercial preparations suggests that

IFAWBE prepared by our method may be appropriate for diagnosis and treatment of IFA allergy. However, our studies do show an urgent need for methods to standardize IFA extracts and indicate that immunoprecipitation and RAST inhibition testing may be useful for this purpose.

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