

Allergens in Hymenoptera venom

XVII. Allergenic components of *Solenopsis invicta* (imported fire ant) venom

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Pure venom from *Solenopsis invicta* was collected by having the insects sting into a capillary tube. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that three major protein bands were present in the venom. A prototype commercial extract was compared and found to contain the three major venom proteins and additional components as well as high potency and specificity by RAST. Immunoblot studies were performed with sera from allergic individuals with blots prepared from denatured SDS-PAGE gels and from two types of nondenaturing gels. The nondenaturing systems demonstrated the presence of two major allergens and three other allergens, each reacting with a third of the sera. Three of the allergens detected in SDS-PAGE blots were of identical mobility to the three major proteins in the pure venom sample. IgE binding to the SDS-PAGE blots was significantly less than that to nondenaturing blots, suggesting that much of the allergenic activity is conformation dependent. (*J ALLERGY CLIN IMMUNOL* 1987;80:300-6.)

Venom from the imported fire ant, *S. invicta*, has not been extensively studied as the venoms from honeybees and vespid wasps have been.¹ Imported fire ants are relatively small insects, and their venom, unlike those of bees and wasps, is composed of about 95% water-insoluble piperidine alkaloids.²⁻⁴ In their carefully performed study of fire ant venom, Baer et al.⁵ demonstrated that venom from *S. invicta* contained about 0.1% protein by weight and that there were at least three proteins present in the aqueous phase, two of which possessed hyaluronidase and phospholipase activities. A small amount of venom was fractionated, and at least three fractions could bind IgE antibodies in sera from allergic patients.

The venom sac of each ant contains about 40 nl of venom.⁵ Venom can be collected either by direct stinging or by squeezing dissected venom sacs. Both methods are extremely laborious, and the amount of aqueous phase obtained is small. Almost all studies reported since those of Baer et al.⁵ have used some type of body or abdominal segment extract. These extracts are known to contain venom, but they are of

Abbreviations used

IEF:	Isoelectric focusing
IFAV:	Imported fire ant venom
PAGE:	Polyacrylamide gel electrophoresis
SDS:	Sodium dodecyl sulfate
TEMED:	<i>N,N,N',N'</i> -tetramethylethylenediamine
Tris:	Tris(hydroxymethyl)aminomethane
%T:	Total acrylamide concentration
%C:	Percent cross-linker in acrylamide gels

variable and unpredictable potency.⁵⁻⁹ In this study we examined *S. invicta* venom and compared it to a high potency prototype commercial extract prepared by electrical stimulation. The prototype extract is demonstrated to contain a number of fire ant allergens by immunoblotting analyses, and at least several of these allergens are identical to venom components.

MATERIAL AND METHODS

Venom from *S. invicta* was collected from Craven County, North Carolina. Each *S. invicta* worker was allowed to sting into a 5 μ l capillary tube held in a rubber stopper. The capillary tube led into a 6 by 50 mm test tube containing a few drops of distilled water. The test tube was placed in a serum bottle stopper inside a small filter flask. A slight vacuum of about 10 cm Hg was applied to the filter flask in order to pull the venom into the capillary. Collected venom was stored at -20° C. The alkaloid was removed before use by hexane extraction.

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Received for publication March 7, 1986.

Accepted for publication March 7, 1987.

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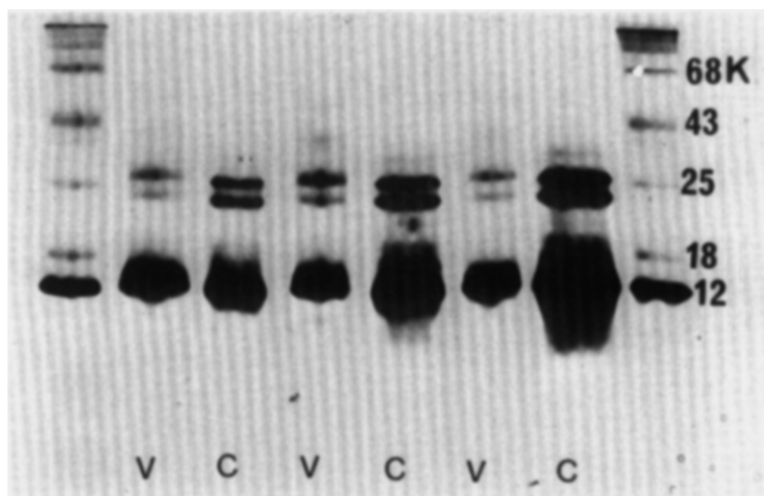


FIG. 1. SDS-PAGE of pure venom from *S. invicta* (v) and prototype commercial extract (c). The tracks on both sides are molecular weight standards. Samples were reduced with dithiothreitol before electrophoresis. Proteins are stained with Rapid-Ag stain. Sample loadings are 2, 4, and 8 μ g for the commercial extract. Calculated molecular weights of the major components were 27,000, 25,000, and 14,000. Minor components were observed at 34,000 and 55,000 with a complex at about 40,000.

Prototype extract

An extract of *S. invicta* prepared by electrical stimulation was purchased from Vespa Laboratories, Spring Mills, Pa. This extract was a white freeze-dried material, which did not appear to contain any alkaloid.

Patient sera

Sera from patients clinically allergic to *S. invicta* stings were obtained from North Carolina, Texas, Florida, and Alabama. All patients had definitive histories of systemic reactions after fire ant stings. The reaction was fatal in one case. Sera was stored at -20°C . Some patients were tested to other Hymenoptera venoms.

RAST

RAST was performed with cyanogen bromide-activated paper disks by the methods we have previously described.¹⁰

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed by the methods we have previously described.¹¹ Running gels were 10 %T and 2.8 %C, and stacking gels were 4 %T and 2.8 %C. Samples were reduced with dithiothreitol before electrophoresis.

Nonequilibrium isoelectric-focusing gels

Nonequilibrium pH gradient electrophoresis was performed in gels of 10 %T and 2.8 %C containing 5 mol/L of urea. Gels were run in a Hoefer (Hoefer Scientific Instruments, San Francisco, Calif.) "Mighty Small" apparatus. Each 7.5 ml of gel contained 0.175 ml of Servalyt 3-10 and 0.075 ml of Servalyt 9-11 (Serva Fine Biochemicals Inc., Westbury, N. Y.). The cathode solution of

0.02 N NaOH was placed at the bottom, and the anode was 0.01 mol/L of H_3PO_4 . The sample was dissolved in 2% Servalyt in 6 mol/L of urea. Electrophoresis was performed at 2.5 W until the voltage was stable at >600 V for 20 minutes.

Nondenaturing acid gels

Nondenaturing electrophoresis was performed in 8 by 10 cm of minigels. The separating gel was 11.1 %T and 2.8 %C, and the stacking gel was 3.7 %T and 2.8 %C. Separating gel buffer was prepared as an eight times concentrate from 48 ml 1 N of KOH, 17.2 ml of glacial acetic acid, and 4 ml of TEMED; this was adjusted to 100 ml and pH 4.4. Separating gels were polymerized with 0.16 ml of fresh 10% ammonium persulfate per 16 ml of gel solution. The stacking gel buffer eight times concentrated was prepared from 48 ml 1 N of KOH, 2.9 ml glacial acetic acid, and 0.46 ml of TEMED adjusted to 100 ml and pH 6.7. Stacking gels were polymerized with 0.16 ml of 10% ammonium persulfate and 0.1 ml of 1% riboflavin per 16 ml of solution. The tank buffer was 0.021 mol/L of β -alanine adjusted to pH 4.3 with acetic acid. Samples were applied in stacking gel buffer containing 4 mol/L of urea and methyl green tracking dye. Sample loadings were approximately 27 μ g of venom for the whole 8 cm width of a minigel or 1.6 μ g for a single well for electrophoretic transfers. The gels were run toward the cathode at 20 mA per gel with cooling.

Stains

Polyacrylamide gels were stained with either Bio-Rad silver stain (Bio-Rad Laboratories, Richmond, Calif.) or Rapid-Ag stain (ICN Radiochemicals, Irvine, Calif.). Ni-

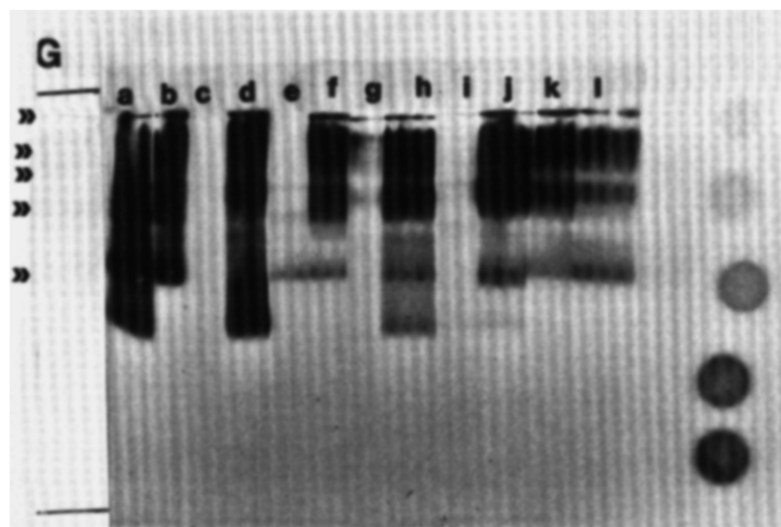


FIG. 2. Autoradiograph of IgE antibody binding from 12 sera from allergic patients to a non-denaturing acidic gel electrophoresis immunoblot to nitrocellulose of prototype commercial *S. invicta* venom. The pattern on the left is an immunoperoxidase stain of hyperimmune rabbit IgG binding to a strip from the same immunoblot used for human IgE studies. The arrow heads mark the bands visible on the peroxidase blot. The circles to the right are radioactivity standards.

trocellulose membranes were stained for protein by biotinylation and avidin-horse radish peroxidase detection (Bio-Rad Laboratories) or by immunodetection with hyperimmune rabbit antiserum generously donated by Dr. Brian Butcher.

Electrophoretic transfer blots

Transfers were made to Whatman 0.45 μ m nitrocellulose membranes type WCN (Curtin Matheson Scientific Inc., Marietta, Ga.). Membranes from three other manufacturers were tested and found to provide less binding and/or higher background. SDS-PAGE gels were transferred in a buffer composed of 0.025 mol/L of ethanolamine/glycine, pH 9.5, plus 20% methanol at 100 V for 1 hour toward the anode.¹² Transfers were performed in a Transphor apparatus (Hoefer Scientific Instruments) with cooling.

IEF gels were equilibrated for 10 minutes with 0.7% acetic acid and then transferred in 0.7% acetic acid at 100 V for 45 minutes toward the cathode. Nondenaturing gels were also transferred in 0.7% acetic acid toward the cathode. Most transfers from these gels were made to Immobilon PVDF transfer membrane (Millipore Corp., Bedford, Mass.). This membrane, which binds protein covalently, was superior to all nitrocellulose membranes tested.

After transfer, membranes were blocked with 5% bovine serum albumin in Tris-buffered saline, pH 7.4, for 45 minutes. Membranes could be stored at 4° C after blocking.

Immunodetection on nitrocellulose blots

All proteins were diluted in 0.5% bovine serum albumin in 0.02 mol/L of Tris HCl, pH 7.4, and 0.15 mol/L NaCl; 0.05% Tween 20 was included in the first two of each series of three wash solutions. For detection of human IgE antibodies, sera from allergic patients were diluted from 1:2 to

1:20; a 1:5 dilution was used in most experiments. A strip cut from the transfer blot was incubated with 1 ml of serum dilution in an Accutray (Schleicher & Schuell, Keene, N. H.). After rocking overnight, the strips were washed five times. The strips were incubated successively for immunoperoxidase detection with goat antihuman IgE at 1 μ g/ml (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Md.), biotinylated antigoat IgG at 1 μ g/ml (Bethesda Research Laboratories, Gaithersburg, Md.), and streptavidin-horse radish peroxidase (Bethesda Research Laboratories) at 1:500 or 1:1000, depending on the lot. Peroxidase activity was detected with 0.015% H₂O₂ and 0.05% of 4-chloro-1-naphthol in 20% methanol.

For autoradiographic detection, the strips were incubated in ¹²⁵I-labeled horse antihuman IgE used for RAST. The same concentration was used that was optimal for RAST, about 80 ng/ml. Strips were incubated for 4 hours and then were exhaustively washed in Tween-Tris-buffered saline. After two water washes, the strips were dried and taped to a card along with graded activity standards. Exposures of up to 6 days were made at room temperature with Kodak (Eastman Kodak Co., Rochester, N. Y.) X-omat AR film. A tenfold increase in sensitivity at the expense of some resolution could be obtained by exposure with a DuPont Cronex Hi-Plus intensifying screen (Chesapeake X-ray Products, Farmville, N. C.) at -70° C.

RESULTS

The prototype commercial *S. invicta* extract was coupled to cyanogen bromide-activated paper disks for use in the RAST. This extract was compared to freshly prepared abdominal end extract by RAST with a panel of 18 sera from fire ant-allergic patients. Six-

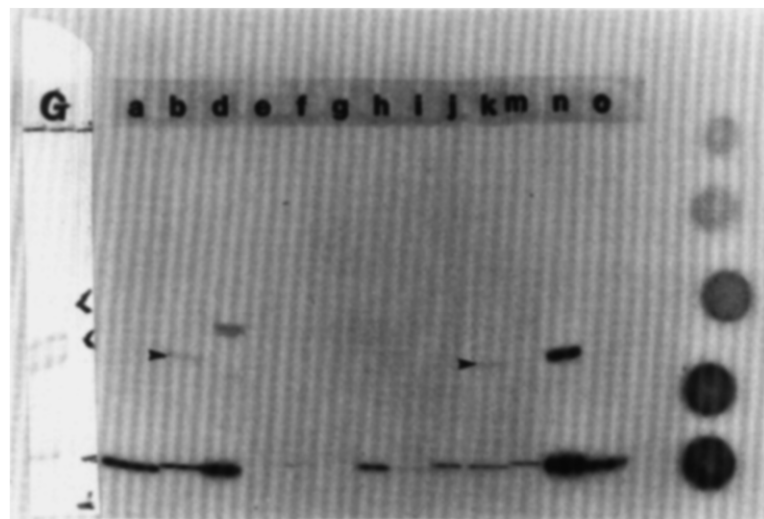


FIG. 3. Autoradiograph of human IgE binding from 13 sera from allergic patients to an immunoblot prepared from an SDS-PAGE gel of prototype commercial *S. invicta* venom transferred to nitrocellulose. A strip developed by immunoperoxidase with hyperimmune rabbit IgG is illustrated on the left. The positions of weak bands are marked with arrowheads. Sera identified with the same letters as those in Fig. 2 are identical. The circles to the right are radioactivity standards.

teen sera were positive to the prototype extract, and only six sera to abdominal end extract. Our laboratory received a total of 51 requests for fire ant-venom RAST. Of these requests, 41 sera elicited positive RAST to *S. invicta*, including all the cases with convincing histories of systemic reactions to fire ant stings within the preceding year. Of the 10 negative specimens, four were reactive with other Hymenoptera, four had improbable histories of reactions from fire ant stings, and no data was available for two cases. Of the 41 positive sera, 15 were also tested to bee, yellow jacket, yellow hornet, white-faced hornet, and paper wasp venoms. Sera from eight patients were negative to all the panel of five other Hymenoptera. Of the seven sera positive to other venoms, two were positive to honeybee and *S. invicta* and four were positive to yellow jacket and *S. invicta*. This suggests that the sensitivity to IFAV can be independent of prior stinging insect-venom sensitivity. RAST was not performed with the pure hand-collected fire ant venom.

Pure *S. invicta* venom was compared to the prototype commercial extract by SDS-PAGE. The pure venom contained three major bands, some silver-stainable material that moved with the front, and several minor bands that can be observed in Fig. 1. The prototype commercial extract contained these three major bands as well as the stained material at the front and a number of bands not visible in the pure venom. The finding of three major proteins in *S. invicta* venom is consistent with the original study of Baer et al.⁵

IgE antibody-binding activity was evaluated by im-

munoblot techniques with electrophoretic transfers onto nitrocellulose membranes. Individual sera from RAST positive fire ant-allergic individuals were used. The sera studied bound from three times background to 35% of added radioactivity in RASTs. The first system tried was SDS-PAGE with streptavidin-biotin amplification and horse radish peroxidase detection. The results were usable but are not demonstrated because of the difficulties encountered in producing high contrast photographs. Only four of the nine sera tested demonstrated detectable IgE binding to prototype extract in this assay, demonstrating from one to six stained bands. The same panel of nine sera was tested with nonequilibrium IEF for antigen separation. With this method, seven sera demonstrated IgE binding eliciting two to eight bands. More bands were observed in the IEF gels than in the SDS-PAGE for all of the positive sera. The IEF experiments demonstrated that the proteins in *S. invicta* venom were highly basic as they are in other Hymenoptera venoms; therefore, a nondenaturing acidic gel system was developed that could separate the native molecules. We tried 10 systems that had been published in the literature for the separation of basic proteins, but none of these methods was satisfactory.

The nondenaturing acidic PAGE system was tested with honeybee and *Vespula maculifrons* venoms and elicited satisfactory separations of both of these well-investigated venoms. Better contrast and sensitivity could be obtained by use of autoradiography rather than by immunoperoxidase detection, even with



FIG. 4. Autoradiograph of human IgE binding of 10 sera from fire ant-allergic patients to an immunoblot prepared by transfer of a nondenaturing electrophoresis of pure *S. invicta* aqueous phase venom to Immobilon PVDF. All sera demonstrate IgE binding to at least two bands. Binding is observed to at least five well-defined bands. The circles to the left are exposure standards.

the additional amplification provided by the biotin-streptavidin procedure. IgE antibody-binding activities from sera of 12 *S. invicta*-allergic individuals are presented along with a pattern stained with hyperimmune rabbit IgG of the prototype extract in Fig. 2. Binding was detected in 11 of the 12 sera, with all the positive sera eliciting multiple bands. The intensity generally correlated with RAST scores. Several of the IgE binding bands do not correspond to bands visible in the protein stain, e.g., the bottom band visible in sera, a, d, h, and j. There were two components that bound IgE in most of the sera, three components that bound IgE in about a third of the sera, and a few weakly binding minor components. Control strips incubated with horse serum and with sera from honeybee venom-allergic and yellow jacket venom-allergic individuals demonstrated either no binding or trace binding to a single band.

For comparison, most of the same sera were tested against blots prepared from an SDS-PAGE separation of the prototype extract. The autoradiograph results are illustrated in Fig. 3 along with a rabbit IgG-stained blot of the SDS-PAGE pattern. With the 13 sera from allergic patients tested, IgE binding was detectable in 12 sera, although it was very weak in three sera. Most of the IgE binding activity was directed against material that moved with the electrophoretic front. However, binding to the larger molecular weight bands was observed in four sera. IgE binding was observed to both of the two strongest bands visible in the rabbit

IgG antibody stain. As can be observed in Fig. 1, these two components are found in the pure *S. invicta* venom; thus, they represent allergens of venom origin.

The IgE binding patterns of 10 sera from fire ant-allergic patients to pure *S. invicta* aqueous phase venom are illustrated in Fig. 4. The venom was separated by nondenaturing acid gel electrophoresis and electrophoretically transferred to Immobilon PVDF membrane. The sera were chosen to represent a range of RAST binding from weakly to strongly positive. All sera demonstrated IgE binding to at least two components, and at least five well-defined IgE binding components were present in the venom. The patterns were similar to those observed with the prototype commercial extract, although the components were better separated in the venom, and an additional high-molecular-weight band was observed in the pure venom, as is illustrated in Fig. 5.

DISCUSSION

The prototype commercial *S. invicta* venom extract prepared by electrical stimulation was demonstrated to have a high potency by RAST. All sera tested from patients with recent definitive histories of systemic reactions after imported fire ant stings were positive to this extract by RAST. In more than half of the patients tested, no IgE antibodies reacting with other Hymenoptera venoms could be demonstrated. This prototype extract appeared to have at least four to more than 10 times the allergen content of a good IFAV

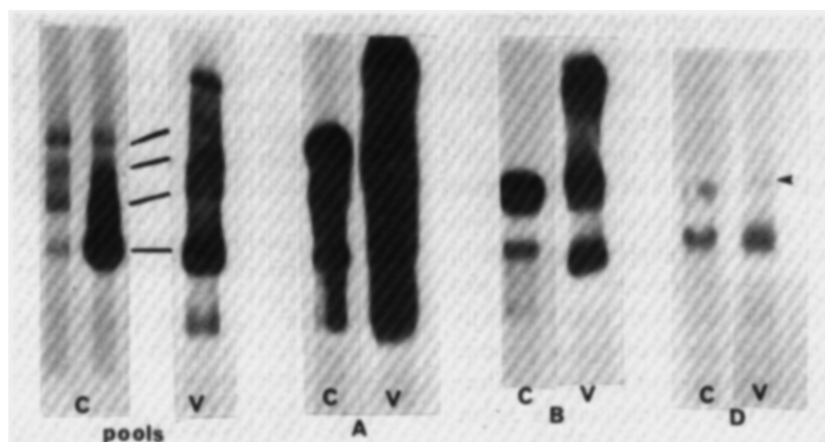


FIG. 5. Comparison of prototype commercial *S. invicta* venom (C) with pure aqueous phase venom (V) by immunoblotting of nondenaturing acidic buffer gels to Immobilon PVDF. The patterns to the left are from pooled sera from fire ant-allergic patients; the connecting lines correct for differences in magnification. The other three pairs of patterns (A, B, and C) demonstrate comparisons of patterns with individual sera from IFAV-allergic patients. The highest molecular weight band is not usually present in gels of commercial extract because it probably aggregates and does not enter the gel. The arrowhead marks a faint band.

whole body extract by direct RASTs.¹³ The relative potency was dependent on the serum used. The prototype commercial extract was found to contain the three major protein bands found in pure *S. invicta* venom by SDS-PAGE (Fig. 1).

Our preparation of pure *S. invicta* venom was collected by allowing live insects to sting into a capillary tube. Our results are compatible with the original studies of Baer et al.,⁵ who also described the presence of three major proteins in the aqueous phase. We found significant phospholipase A activity in our aqueous venom preparation, as was reported by Baer et al.⁵ We did not test our preparation for the presence of hyaluronidase.

The IgE binding activity of immunoblots prepared from SDS-PAGE separations was substantially less than that of immunoblots prepared from nondenaturing separations, both acidic buffer gels and nonequilibrium IEF gels. The initial studies were performed with a biotin-streptavidin amplified immunoperoxidase detection system, but autoradiography was found to be more sensitive and to elicit better defined results. In addition to the lower total binding, IgE antibodies bound mainly to smaller molecular weight materials in the SDS-PAGE immunoblots. Samples for SDS-PAGE were reduced with dithiothreitol and boiled in SDS; therefore, the native conformations of the molecules were destroyed. In the immunoblots prepared from nondenaturing gels, there may be some distortion of conformation caused by binding to the nitrocellulose membrane, but the molecules appear to be substantially in their native conformation, as dem-

onstrated by antibody binding. Immobilon PVDF binds the protein molecules covalently. These observations were further investigated with the more highly characterized *Apis mellifera* and *V. maculifrons* venoms, and similar results to those with *S. invicta* were obtained.¹⁶ The relatively poor binding of IgE antibodies to the denatured polypeptide chain determinants suggests that most allergenic determinants are topographical in nature rather than sequential. This concept of antigenicity has been discussed in detail by Benjamin et al.¹⁴

The nondenaturing acidic gel immunoblot studies indicated that *S. invicta* contains a number of allergens. There were two bands observed that would correspond to major allergens, consistent with the observations of Baer et al.⁵ There were at least three additional bands that bound IgE from a third of the allergic sera tested and a few weakly binding allergen bands. One of the allergen bands was not visible in blots stained for either total protein or with hyperimmune rabbit IgG. The SDS-PAGE blots revealed most binding at the front, but two additional IgE binding components were detected by four of the sera tested. These components corresponded to the two major bands visible in the protein-stained blot and silver-stained gel and were both found in the pure venom preparation. Immunoblot results with the pure aqueous phase venom and the prototype commercial extract were comparable.

Recently, several other investigators have attempted to investigate IFAV allergens. Nordvall et al.¹⁵ used whole body extract, which makes it difficult to com-

pare their results with studies performed with venom. Butcher et al.⁸ used crossed immunoelectrophoresis to compare whole body extracts and venom. They reported finding two antigenic components in IFAV, but the methods they used were not optimal for working with highly basic proteins. Paull et al.⁹ reported on the isolation of 21 peaks by Sephadex gel filtration of IFAV and the presence of multiple components in SDS-PAGE. This group also reported that the isoelectric points of the fractions with RAST activity were in the neutral range. These results are at variance with those reported here and those of Baer et al.⁵ Since Paull et al.⁹ reported that these 21 fractions were identical with substances found in IFA whole body extract, it is probable that they are mainly body proteins that may have some venom antigens associated with them.

The studies reported in this article demonstrate that a prototype commercial extract of *S. invicta* can be prepared that contains the venom protein components in native form. This extract is of high potency for diagnostic purposes and is specific for IFAV. It appears that this extract can be produced in quantity, since we had little difficulty acquiring about 40 mg. By contrast, it is extremely laborious to obtain even small amounts of pure venom from *S. invicta*, making it improbable that a commercial pure venom extract could be produced.

We thank Mr. Robert Jacobson for collecting the fire ants and milking the venom, and Ms. Elizabeth Kopelman for her technical assistance in developing the immunoblot protocols. Sera from many of the allergic patients were provided by Dr. Donald MacQueen and Dr. Ab Eisen. Dr. Brian Butcher of Tulane University made a generous gift of rabbit antiserum against the prototype commercial extract. Mr. Miles Guralnick of Vespa Laboratories generously provided his protocol for nondenaturing PAGE.

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