

Allergens in Hymenoptera venoms XXIII. Venom content of imported fire ant whole body extracts

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31279

A two-site immunoassay using monoclonal antibodies has been developed to measure the imported fire ant venom allergen Sol i III. The assay is specific for Solenopsis invicta venom, since one of the monoclonal antibodies does not react with Sol r III. A commercial imported fire ant whole body extract, known from RAST and skin test data to be potent, was found to contain 10.3 µg of Sol i III per milliliter. This was comparable to the content of an extract freshly prepared in our laboratory. The immunoassay detected Sol i III in an extract of fire ant abdomens, but not in head and thorax extract. The amount of imported fire ant venom in a potent whole body extract appears to be sufficient to provide possible protection for patients receiving it as immunotherapy, although this can only be verified by controlled clinical trials with intentional sting challenges.

INTRODUCTION

Since the first report of immunotherapy for imported fire ant stings with whole body extract by Triplett,¹ most investigators have found that imported fire ant whole body extracts contain significant amounts of venom proteins.²⁻¹¹ The presence of venom allergens in whole body extracts has been demonstrated by immunochemical means⁵⁻⁸ and by reports of successful use of whole body extracts in immunotherapy.^{1,9,10} The isolation and characterization of the venom allergens from imported fire ants¹²⁻¹⁵ has allowed the production of monospecific antibodies that allow direct measurement in mass units of imported fire ant venom allergens. Previously, we have described the purification of four allergens from *Solenopsis invicta*

venom, the cross-reactive allergen Sol i I, which is about 4% of the venom protein; phospholipase or Sol i II, which is about 66%; Sol i III, which comprises about 20%; and the *invicta* specific allergen Sol i IV, which makes up about 9% of the venom protein. In this paper we describe a two-site monoclonal antibody enzyme linked immunoassay (ELISA) to measure *Solenopsis invicta* venom allergen Sol i III, and use it to determine the allergen content of whole body extracts and individual fire ant workers.

MATERIALS AND METHODS

Purified Allergen

Sol i III was prepared from commercial venom as described previously.¹² Its purity was demonstrated by absorption to ELISA wells and testing with monoclonal antibodies against Sol i II, III, and IV. The preparation contained less than 0.1% Sol i II and IV.

Monoclonal Antibodies

Mouse antibodies were raised in Balb/c mice. Species specific antibodies to the surface determinants were produced by immunization with Ribi adjuvant (Ribi, Inc., Hamilton, Montana). Antibodies cross-reactive with *S. richteri*, which

reacted with the core determinants, were produced by hyperimmunization with antigen emulsified in Freund's complete adjuvant. Fusion and cloning utilized standard techniques.¹⁴ The antibodies for the two-site assay were chosen by testing in inhibition ELISA. The species specific antibody AMS32 was IgG1 and the cross-reactive antibody MS2 was IgG2a. The antibodies were produced as ascites and were purified using recombinant protein G bound to agarose purchased from Pierce Chemical Company, Rockford, Illinois. Antibody purification was carried out following the manufacturer's instructions.

The care and use of animals followed "Principles of Laboratory Animal Care" and "Guide for the Care and Use of Laboratory Animals." The protocol was approved by the Animal Care Committee of East Carolina University School of Medicine.

Enzyme Linked Immunoassay

The ELISA assay was performed using Immulon 2 microplates purchased from Dynatech, Chantilly, Va. The wells were coated with 10 µg/mL of purified monoclonal antibody AMS32 in carbonate buffer.¹⁶ The wells were then

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blocked with 1% bovine serum albumin. Purified monoclonal antibody, MS2, was labelled with biotin using sulfosuccinimidyl 6-(biotin-amido) hexanoate (NHS-LC-biotin), purchased from Pierce Chemical Company. The biotinylation was performed at a ratio of 700 $\mu\text{g}/\text{mg}$ of antibody in ice. Excess reagent was removed by diafiltration using an Amicon (Danvers, MA) YM-10 membrane. The optimal titer was determined to be 1:1000 by checkerboard titration. Samples and standards were diluted in 1% bovine serum albumin in 0.05 M Tris-HCl 0.9% saline, pH 7.4, and 100- μL amounts added to the microplate wells in quadruplicate. The antigen was removed after overnight incubation at room temperature and the wells were washed. Biotinylated MS2 antibody was added for a 3-hour incubation. After washing, a 1:1000 dilution of streptavidin-horse radish peroxidase purchased from BRL Laboratories, Gaithersburg, MD, was added for a 1-hour incubation. Peroxidase activity was detected by a 1 mg/mL solution of 2,2'-azinobis(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ABTS) and 0.03% hydrogen peroxide in 0.1 M sodium citrate buffer, pH 4.2. Assays were read in an automated microplate reader at 405 nm. Wells that did not contain antigen were used as blanks.

Extracts

Solenopsis invicta whole body extracts were purchased from Hollister-Stier, Spokane, WA. These extracts were shown to be potent by RAST studies.^{4,11} Experimental extracts were prepared from ants freshly collected in Craven and Pitt Counties, North Carolina. The ants were frozen and then crushed in a teflon pestle tissue grinder in ten times their weight of Tris-saline buffer. The extracts were then filtered and centrifuged. The weight of 250 *S. invicta* workers was 0.249 g. Head and thorax extract and abdomen extract were prepared by counting an equivalent number of

ants to those in the whole body extract rather than by weight.

RESULTS

After testing a number of monoclonal antibody pairs, antibodies AMS32 and MS2 were found to give the best results in a two-site ELISA. The monoclonal antibodies were purified and aliquots were labelled with biotin. Unlabelled antibody was coated on microtiter wells, which were then incubated with antigen, washed and then incubated with biotin-labelled antibody, followed by streptavidin-horse radish peroxidase. The pair of antibodies giving the highest binding was selected for the assay. The optimal concentrations of reagents were then determined by titration. The assay gave a dose dependent response from 0.1 to 50 $\mu\text{g}/\text{mL}$ of allergen *Sol i* III. A typical standard curve for the mid-range of 0.3 to 10 $\mu\text{g}/\text{mL}$ is shown in Figure 1.

Two commercial whole body extracts were tested at dilutions of 1:10 and 1:20 to eliminate effects from the 50% glycerin present. In five separate runs the commercial ex-

tracts were found to contain 10.3 ± 3.5 (SD) $\mu\text{g}/\text{mL}$ of *Sol i* III. Whole body extract made freshly in our laboratory contained 12.5 $\mu\text{g}/\text{mL}$ and abdominal extract contained 14.5 $\mu\text{g}/\text{mL}$. No *Sol i* III could be detected in head/thorax extract. Each worker ant weighed an average of 1 mg and contained 250 ng of *Sol i* III. In previously reported studies¹² of hand milked venom 120 ng of venom protein were collected per ant. The concentration of *Sol i* III varies from 20% of 40% of venom protein, so each ant contains the equivalent of about seven stings of venom protein.

DISCUSSION

As has been suggested by numerous reports in the literature,¹⁻¹¹ imported fire ant whole body extracts can contain significant amounts of venom proteins. *Sol i* III appears to be stable in whole body extracts, since the amount found in a 2-year old commercial extract was only slightly less than in a freshly prepared extract. This contrasts with the instability of venom proteins in whole body extracts of bees and ves-

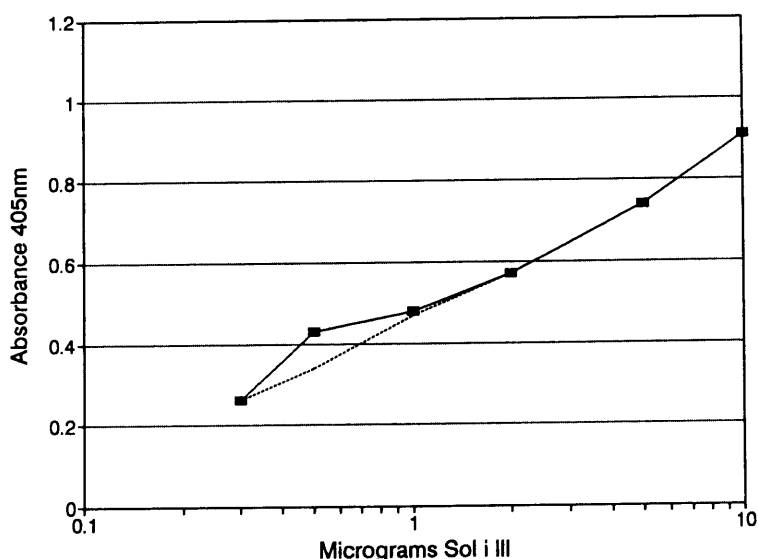


Figure 1. Standard curve from a single experiment for two-site monoclonal antibody ELISA for *Sol i* III. The dotted line indicates the best fit curve and the solid line directly connects the measured points. Measurements were all made in this region of the curve, which was closest to linear. The total range of the assay was from 0.1 to 50 $\mu\text{g}/\text{mL}$ of *Sol i* III.

pid wasps.¹⁷ If one extrapolates from the content of *Sol i* III to total venom protein, approximately 30 to 45 $\mu\text{g}/\text{mL}$ are present in good 1:10 whole body extracts. If the standard maintenance dose is 0.5 mL of concentrate, this would be 15 to 22 μg , as compared to the 100- μg dose used for other venoms. Since a fire ant sting is less than 100 ng of venom protein¹² as compared with bee and vespid stings of from 2 to 50 μg of venom protein,¹⁸ this dose may be sufficient to provide the protection observed in sting challenge studies.^{9,10}

Sol i III was chosen as the allergen to use in this assay for several reasons. Although there is usually more *Sol i* II, it is less stable because of an internal cleavage site and may exist in several forms.¹² *Sol i* IV is stable, but is only present in a small amount, and there is no parallel allergen in *Solenopsis richteri* venom.¹⁴ The cross-reactive allergen *Sol i* I is not suitable for assay because of its heterogeneity and low concentration, and because it may be related to other antigens found in the body but outside of the venom apparatus.¹³ It was relatively easy to raise monoclonal antibodies against *Sol i* III that recognized non-overlapping antigenic sites needed for a two-site immunoassay. The assay we report on here is very similar to the ones developed for the house dust mite allergens *Der p* I and *Der f* I by Luczynska et al.¹⁹ This group has used the mite allergen assays to determine the amounts of allergen in extracts prepared from dust collected in different houses.

This study demonstrates that *Solenopsis invicta* (imported fire ant) whole body extracts can contain a significant amount of venom protein, which explains the previous reports of their successful use in diagnosis and immunotherapy of imported fire ant venom allergy.

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