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Seasonal variation in antigens of the imported fire ant *Solenopsis invicta*

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Eight sequentially collected lots of aqueous extracts of imported fire ant (IFA) front end and abdominal end segments were assayed for phospholipase A (PLA), N-acetyl- β -glucosaminidase (NAG), and hyaluronidase. Relative potency of each extract lot and pooled venom was measured by RAST inhibition against a venom standard. More than a hundredfold difference in PLA activity was observed. Early summer collections had the highest activity. The May to June collection had more than twice the PLA activity of the next most potent lot. Discordancy in enzyme patterns was noted only in AE extract. NAG levels peaked earlier in the spring and summer and fluctuated less widely. Front end extract had lower activity levels for both enzymes, with no seasonal fluctuation in NAG and a single elevation in PLA activity in the April to May collection. RAST inhibition varied directly with PLA activity ($p < .05$) but not with NAG nor hyaluronidase activities. Fifty-one percent of systemic allergic reactions to IFA stings occurred in summer, and 19% occurred in spring. A reported demographic survey¹ demonstrated a higher incidence of IFA stings in the spring (39.9%) with a lower attack rate in the summer (31.9%). These findings suggest that the rate of systemic reactions to stings of the IFA may be related to seasonal variations in allergenic potency, as measured by PLA and RAST inhibition, rather than the sting attack rate. (J ALLERGY CLIN IMMUNOL 78:331-6, 1986.)

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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.

Abbreviations used

IFA: Imported fire ant
WBE: Whole body extract
FE: Front end
AE: Abdominal end
NAG: N-acetyl- β -glucosaminidase
PLA: Phospholipase A
AQV: Aqueous venom

Venoms of the winged Hymenoptera have proved to be superior to WBE in the diagnosis and treatment of allergic sting reactions.² Winged Hymenoptera venoms have been well characterized with regard to enzyme content and measurement of potency of the ma-

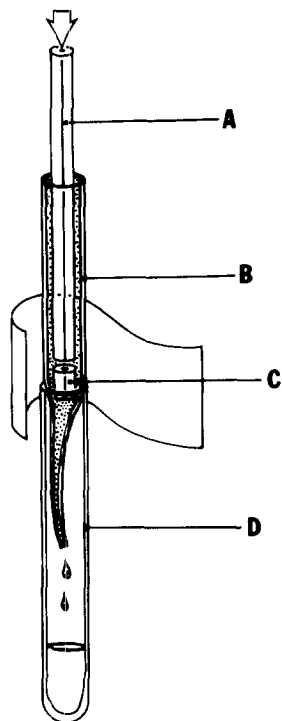


FIG. 1. Venom from the IFA was pooled as it was collected in 1 μ l capillary tubes (A). The device was constructed from a piece of heat-drawn polyethylene tubing (B) that served as a funnel. A 1 mm length of polyethylene 10 tubing (C) was inserted into this funnel to prevent the capillary from wedging into the collecting tube (D). The collecting tube was prepared from a length of nonheparinized hematocrit tubing that was heat sealed at one end. A graduation was marked on the collection tube that corresponded to a 20 μ l volume. The entire assembly was placed in a hematocrit centrifuge and spun for 5 minutes in the cold as necessary. When 20 μ l aliquots of venom were obtained, the tube was sealed with Parafilm and stored at -80° C.

for allergens.³ However, IFA antigens have not been as well studied. The small volume of venom recoverable from each fire ant specimen and the tedious method for its collection⁴ have led to many challenging technical problems that have made commercial production of purified IFA venom impractical. IFA WBE remains the only commercially available product for diagnosis and treatment of fire ant allergy. Although it has been proved to contain clinically relevant antigens that cross-react with venom, IFA WBE is less sensitive than venom. Strom et al.⁵ reported variability in responsiveness of sensitive patients to different WBE preparations. Extracts most predictive of IFA sensitivity by skin testing were also found to be the most potent as measured by histamine release and radioimmunoassay. Continued confidence in the clinical reliability of WBEs, however, will require standardization of their potency.⁵

IFA venom protein chemistry has had very limited study. Baer et al.⁶ demonstrated antigenic activity in three of four major protein fractions and tentatively identified two of these as phospholipase and hyaluronidase. In the same study this group also reported components common to both commercially available WBE and *Splenopsis invicta* venom. Recently, Paull et al.⁷ demonstrated 21 peaks in both IFA venom and IFA WBE; all inhibited an IFA venom RAST against a pool of sera from IFA-allergic patients. This article documents the complexity of IFA antigens and suggests an explanation for the variability noted in clinical reactivity.

Although WBE is reported to be efficacious in diagnosis and treatment,⁸⁻¹¹ it was found to be a poor predictor of IFA sensitivity when it was compared to IFA venom.¹² Paull et al.¹³ examined the relationship between graded, naturally occurring IFA sting reactivity and RAST against IFA venom and WBE, as well as measurements of cross-reactivity between them. These authors concluded that skin test and RAST with venom or IFA WBE are reliable for evaluating allergenicity. They hypothesized that other investigators' inconsistent results with IFA WBE may be due to variability in different IFA WBE preparations. Strom et al.⁵ demonstrated by immunodiffusion a substance common to both IFA WBE and IFE venom that precipitated with venom antisera, but this finding was inconsistent with their observation that specific IgG could not be detected against venom or WBE in a group of nine patients receiving WBE immunotherapy.

In an effort to characterize further IFA antigens for subsequent use in clinical studies, we prepared aqueous phase venom and extracts of IFA body segments for in vitro analysis. Each antigen lot was assayed for activity of the enzymes PLA, hyaluronidase, and NAG. Potency of each lot was assessed by IFA venom RAST inhibition.

MATERIAL AND METHODS

Antigen preparations

Three antigen preparations of IFA (*S. invicta*) were produced locally under the auspices of an approved investigational new drug protocol. Laboratory methods met or exceeded standard specifications under title 21 of Code of Federal Regulations, section 610.10-610.13. Ants were collected between June 1981 and November 1982 from specially designated mounds on the Fort Gordon Military Reservation. The mounds were documented by Dr. William Buren to contain colonies of *S. invicta*. By use of forceps, live ants were divided at the petiole into FE and AE segments. The AE was milked for whole venom into a 1 μ l pipette as described by Blum et al.⁴ and pooled with the device illustrated in Fig. 1. The pooled venom and the AE and FE body segments were kept in separate collection tubes

after milking each day and stored at -80°C . Each AE and FE extract lot was prepared from 2 gm of consecutively collected body segments that were maintained at 4°C after thawing and homogenized in a blender followed by extraction for 12 hours with 20 ml of Coca's alkaline extracting fluid with the phenol deleted.¹⁴ The $0.2\text{ }\mu\text{m}$ filter-sterilized product was lyophilized and capped under one atmosphere of nitrogen and stored at 4°C until it was reconstituted with sterile diluent for use. Concentration of AE and FE extracts were 1:10 w/v after reconstitution. After centrifugation, AQV was separated by breaking the capillary tubes at the phase interface and crushing the tubes in extraction solution. AQV was also sterilized by Millipore (Millipore Corp., Bedford, Mass.) filtration, lyophilized, capped under one atmosphere of nitrogen, and stored at 4°C until it was reconstituted with sterile diluent for use. AQV concentration after reconstitution was 1:1000 v/v.

In accordance with FDA quality control standards of Food and Drug Administration for investigational new drugs, each antigen lot was analyzed for sterility, general safety, purity, and potency.

RAST inhibition

Potencies of all extracts and venom were measured by a reduced reagent disc RAST-inhibition assay by the allergenic products staff, Bureau of Biologics, Food and Drug Administration. Aqueous phase venom was covalently bound to filter paper discs (Schleicher & Schuell, Inc., Keene, N. H., No. 595) by use of the CNBr activation. Extracts and venom were evaluated for potency by their competition for IgE binding from a pool of hyperimmune serum (1:10). Results are expressed as units of relative potency compared to a venom standard with an assigned value of 1 unit. Correlations between variables were assessed with Spearman rank correlation coefficients (r).

Each lot of AE and FE extract was also assayed for activity of the enzymes PLA, NAG, and hyaluronidase.

Phospholipase assay

Measurement of a cleared zone surrounding punched wells containing allergen preparation or standard PLA (from honeybee, Sigma Chemical Co., St. Louis, Mo.) in agarose egg plates (type I, low electroendosmosis agarose) incubated at 30°C was used to estimate phospholipase activity as described by Habermann and Hardt.¹⁵ Each lot of allergen was assayed in duplicate on two to three different agarose plates.

NAG assay

The spectrophotometric assay described by Cami et al.¹⁶ was used to evaluate NAG in each lot of extract or venom. Fire ant NAG activity is reported as millimoles of *p*-nitrophenol released from the substrate (*p*-nitrophenyl-*N*-acetyl- β -glucosaminide, Sigma Chemical Co.) per milliliter of reconstituted allergen.

Hyaluronidase assay

This is also an agarose plate assay in which visualized zones of digested hyaluronic acid (grade I, Sigma Chemical

Co.) are measured for quantitation by use of a method modified from Richman and Baer.¹⁷ Five microliters of reconstituted allergen was dispensed into the 3 mm diameter sample wells in the agarose plate. Hyaluronidase (type V, 1100 National Formulary units per milliliter, Sigma Chemical Co.) was used to produce the standard curve. To obtain activity from crude extracts, the hyaluronidase activator 1,10-diaminodecane was added to the agarose in the concentration of 1 mg/ml.¹⁸

RESULTS

Sterility of each lot was proved by lack of aerobic growth on soybean-casein digest media and lack of anaerobic growth in thioglycolate broth.

General safety was demonstrated by lack of signs of toxicity or weight loss after intraperitoneal injection of 0.5 ml of each lot of antigen into two guinea pigs weighing $<400\text{ gm}$ and two mice weighing $<22\text{ gm}$.

Purity was proved by lack of residual moisture as demonstrated by absence of product weight change after exposure to phosphorus pentoxide in a desiccator.

Enzyme analysis

The variation in PLA activity found in AE extracts of the IFA collected at different times of the year is represented in Fig. 2. Collection periods include variable portions of each month. The first bar primarily represents winter collection. The next four bars represent spring collection. The sixth and seventh bar legend represents collection in the summer, and the last bar primarily represents collection in the fall. More than a hundredfold difference in enzyme activity was found among the eight lots. The early summer IFAs contained the most PLA activity with the May to June collection having almost twice the activity of the next most potent lot.

Much lower PLA activity was present in all the lots of FE preparations. PLA activity in seven of the eight FE lots was less than one tenth of that measured in the least active AE lot. The enzyme activity in the most active FE extract lot (April to May) was approximately the same as that measured in the winter and fall lots of AE extract.

Results of the NAG assay on AE preparations are also presented in Fig. 2. The distribution of NAG activity differed significantly from that for PLA in AE extract ($r = 0.11$) ($p > 0.05$). NAG activity was noted to be greater in spring than in summer months. This distinct distribution of activities among lots for NAG and PLA suggests that specific shifts in constituent enzymes may have occurred rather than non-specific changes in total protein. The activity of NAG in FE extracts demonstrated uniformity from season to season. In contrast to the PLA results, the range of NAG activity differed within only a twofold order

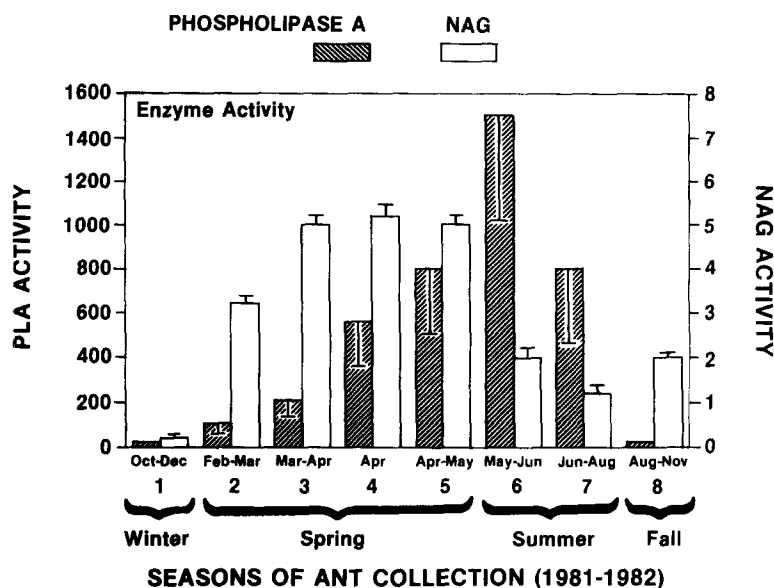


FIG. 2. *Solenopsis invicta* AE extract concentrations of PLA and NAG for the eight lots. Enzyme activities are expressed as nanograms per milliliter and millimoles of *p*-nitrophenol per milliliter, respectively. The first pair of bars represent winter, the next four pairs of bars represent spring, the next two pairs of bars represent summer, and the last pair of bars represents fall.

of magnitude among the FE extracts and a fivefold order of magnitude among the AE extract lots.

The presence of hyaluronidase was demonstrated in all AE preparations and three of the eight FE preparations. The activities for the AE lots preceding from the winter to the fall lot were 21, 12, 32, 13, 11, 27, 13, and 6 National Formulary units per milliliter, respectively.

RAST inhibition

The RAST-inhibition assay of AE extracts (Fig. 3) revealed the greatest potency in the same summer lot having peak PLA activity (Fig. 2). The distribution of RAST inhibition was also similar to that for PLA activity ($r = 0.81$; $p < 0.05$) except for the early spring lot that demonstrated a discordant relationship. Correlation coefficients comparing RAST inhibition and NAG or hyaluronidase activities demonstrated no significant differences. With pooled lots of AE and FE extracts, overall RAST-inhibition potency of FE extract was found to be approximately 47% that of the AE extract.

DISCUSSION

Earlier articles of the reliability of WBE^{8, 11} for diagnostic skin testing contrast with more recent articles that document differences in the relative value of IFA WBE versus IFA venom for diagnosis. Variations in methods of patient selection, criteria for positive skin test reactivity, and antigen composition may

contribute to the differences noted. James et al.¹⁹ used a diluent controlled wheal when patients with a history of IFA-induced systemic reactions were evaluated. In that study 14 of 18 patients (78%) were positive to WBE (*S. invicta*) with 15 of 18 patients (83%) positive to the IFA venom. This is similar to the observations of Strom et al.⁵ in patients with a history of large local or systemic reactions when the diluent standard was used (29 of 41 patients, 71%, positive with WBE of mixed *S. invicta* and *S. richteri*). In that study histamine skin test controls were also used, resulting in a similar incidence of positive responses to IFA venom (34 of 41 responses, 83%), but a much lower rate of positive responses to WBE (18 of 41 responses, 44%). These authors also found reduced potency of glycerinated WBE. Inspection of the graphs presented in the article of Paull et al.¹³ reveals that, based on a histamine standard, a high degree of concordance was found between skin test response rate to WBE (19 of 31 responses, 61%) and IFA venom (18 of 26 responses, 69%) in his patients with a history of large local and systemic reactions. The need to characterize better or standardize antigen preparations was expressed in each of the above three studies; however, no studies have yet appeared that compare lots of IFA extracts prepared from different collections of IFA to examine this variability in lieu of standardization.

The eight lots of FE and AE IFA extract in the present study were collected during the course of a year and identified by the season in which they were

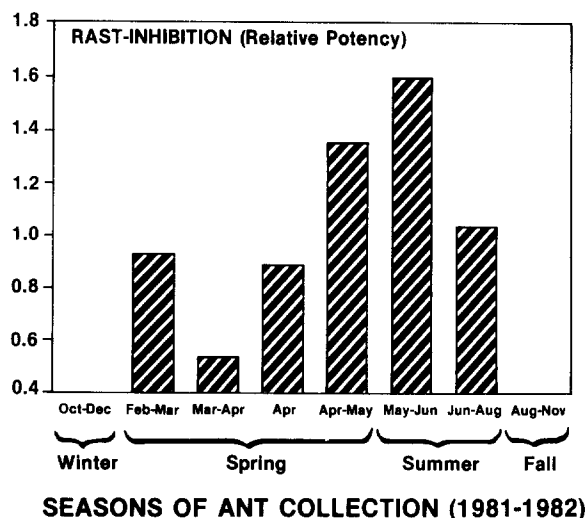


FIG. 3. RAST inhibition of AE *Solenopsis invicta* extracts. Relative potency is expressed as a unit value compared to a venom standard.

collected. These lots were obtained from the same limited geographic area, but not necessarily always from the same colony. Because mounds will be moved by the colony, especially when they are disturbed by sampling, some of the variability noted in these IFA extracts may be due to intercolony specificity as well as seasonal factors. Furthermore, this study did not make the somewhat difficult distinction between worker and soldier ants, the relative proportion of which may affect extract composition as indicated in the report by Brand et al.²⁰ of different amounts of venom alkaloid isomers in these castes. Heterogeneity in biochemical composition and/or concentration within a single colony may be related to the presence of more than a single functional queen.²¹ The seasonal correlations noted are not unprecedented. Similar seasonal variation has been reported in constituents of the Dufour's gland of *Anoplolepis custodiens*.²²

A significant correlation of PLA activity with RAST inhibition was demonstrated in the present study. The imperfect concordance noted may be related to the multiplicity of RAST positive proteins demonstrated in IFA venom.⁷ PLA has been determined to be the major allergen in honeybee venom, and some cross sensitivity between the IFA and other Hymenoptera has been reported.¹⁹ Mueller et al.²³ found considerable variation (two fold to fivefold) in phospholipase and hyaluronidase content of vespid venoms that were "suggested" to be related to RAST potency.

Seasonal variation in severity of IFA stings was also suggested by a retrospective review of clinical records of 37 patients who experienced systemic reactions to IFA stings during a 3-year period. Most reactions (19

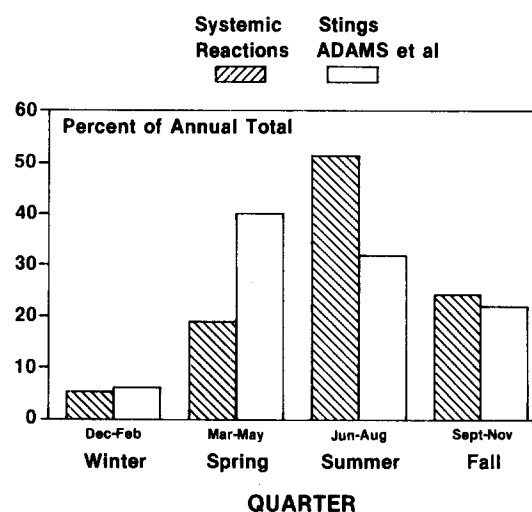


FIG. 4. Comparison of quarterly incidence of IFA stings and systemic allergic reactions (adapted data').

reactions, 51%) occurred during the summer, whereas much fewer (7 reactions, 19%) occurred in the spring. The possibility that this difference may have been due to venom potency rather than sting incidence is supported by results of a survey of the IFA seasonal sting attack rate conducted in Sumter County, Georgia, by Adams and Lofgren.¹ Among 213 IFA stings, the peak attack rate occurred during the spring (39.9%) with a lower attack rate in the summer (31.9%). These results contrast with the seasonal variation we noted in the IFA sting systemic reaction rate (corrected chi-square $p = 0.01283$)(Fig. 4). These results suggest that the higher incidence of systemic reactions of IFA stings in the summer may be related to greater venom potency rather than the sting attack rate.

The demonstrated seasonal heterogeneity in PLA activity and potency of *S. invicta* extracts as measured by RAST inhibition may explain the variable and conflicting clinical results that have been reported in skin test reactivity and response to immunotherapy with IFA WBE. Greater clinical response to IFA venom compared to WBE noted in some controlled clinical studies may be due to reduced potency of allergens in some WBE preparations rather than to differences in antigenic composition. Seasonal variability is an additional variable that needs to be controlled in future investigative studies with IFA antigens.

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