Venoms of Neotropical wasps lack cross-reactive carbohydrate determinants enabling reliable protein-based specific IgE determination

To the Editor:

Insect venom hypersensitivity represents an important health problem in Neotropical regions. In Latin America, Hymenoptera stinging is reported to be 1 of the 3 major causes (14%) of severe allergic reactions.[1] In Europe and Northern America, venoms and allergens of clinically relevant species, honeybee and yellow jacket, have been extensively characterized. Allergy testing using venoms and recombinant components can yield precise information on sensitization profiles and even potential risk factors for venom immunotherapy.[2] In contrast, little is known about the venoms from Neotropical species.

Clinically relevant Hymenoptera species in South America include honeybee, fire ants, and importantly various wasps endemic in different regions of the continent. Systematic studies showed that in contrast to Europe and Northern America, the most commonly found wasp species in Neotropical regions belong to the Epiponini tribe of the Polistinae subfamily including, for example, the genera *Polybia*, *Apoica*, and *Agelaia*.[3] Despite the high incidence, the severity of sting events, and the broad diversity of clinically relevant Hymenoptera identified in this geographical zone, venom allergy diagnosis is essentially based on either commercial venom extracts from European and North American species, or noncommercial venoms from endemic species. The use of extracts from other than the relevant species and the potentially limited cross-reactivity might result in lower sensitivity. Although the use of components in Hymenoptera venom allergy is now state-of-the-art, important molecular allergens from endemic species are not available for molecular diagnosis.[4]

Extract-based diagnostics however are often prone to high levels of cross-reactivity due to molecular similarity of the venoms and the presence of the cross-reactive carbohydrate determinants (CCDs).[2] Insect venom CCDs are defined by an alpha-1,3-linked fucose residue at the innermost *N*-acetylglucosamine of the N-glycan of mostly oligomannosidic type.[5] Extensive CCD-based cross-reactivity of honeybee venom (HBV) and yellow jacket venom (YJV), and protein-based cross-reactivity between allergens from Polistes and YJV are the most relevant problems in Hymenoptera venom allergy diagnostics in Europe and Northern America, respectively.[6] Irrespective of a potential clinical relevance of CCD, carbohydrate-related cross-reactivity causes up to 69% to 75% of double-positive test results to HBV and YJV.[7] Failure in the identification of the culprit venom due to CCD-related cross-reactivity often leads to the inclusion of multiple venoms in the immunotherapy, which results in increasing risks of *de novo* sensitizations.

Recently, we reported that the venoms of European and North American *Polistes* species are devoid of CCDs.[8] So far, we interpreted the lack of alpha-1,3 core fucosylation primarily as a biological peculiarity because CCDs are found within various species ranging from plants to helmiths and in other Hymenoptera such as honeybee and yellow jackets. The presence of CCDs and therefore the molecular basis for cross-reactivity among venoms of Neotropical insects remained largely unexplored.

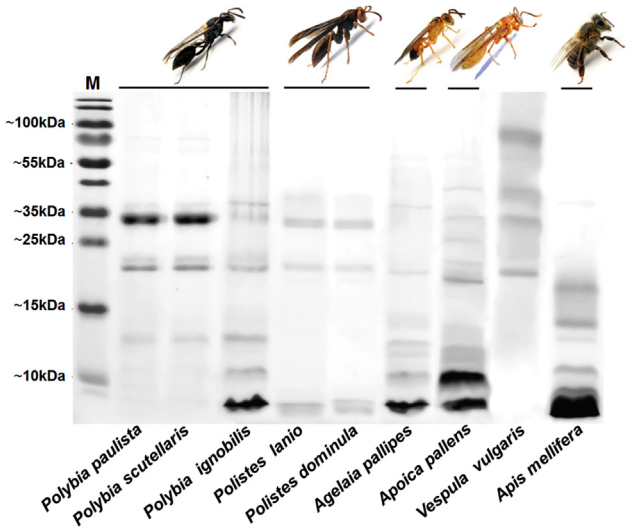

FIG 1. SDS-PAGE analysis of the venoms manually collected from Brazilian wasps and clinically relevant European insects. Photos were kindly provided by Professor Mario S. Palma, UNESP, Rio Claro, SP, Brazil.

Here, we addressed the CCD-based IgE reactivity of 6 venoms of Neotropical wasps as compared with HBV, and 2 venoms of common European wasps: *Vespula vulgaris* (yellow jacket) and *Polistes dominula* (Mediterranean wasp). After harvesting of the nests, venoms were collected manually using 1000 venom glands of each species, and the quality and composition were analyzed by SDS-PAGE (Fig 1; see the Methods section in this article's Online Repository at www.jacionline.org). Notably, the proteins within one genus appear similar although pronounced variations in size and relative intensity were found for different genera.

The general presence of oligomannosidic N-linked glycans was evaluated using biotinylated Concanavalin A, a lectin that recognizes α-linked mannose residues in the predominantly high mannose-type glycans found on insect venom allergens. Presence of α1,3-core fucosylation and therefore CCDs was detected using an anti–horseradish peroxidase (HRP) serum. HBV (i1), YJV (i3), *P dominula* venom (i4), and CCD-positive rVes v 3 were used as controls.

Immunoblotting analyses revealed the presence of several glycoproteins in all the venoms tested (Fig 2, *A*; see also data in this article's Online Repository). Presence of CCDs was detected for HBV, YJV, and rVes v 3. In contrast and similar to *P dominula* venom, none of the venom extracts of Neotropical wasps was recognized by the anti-HRP antibodies. ELISA analyses under nondenaturing conditions (Fig 2, *B*; see also data in this article's Online Repository) corroborated the immunoblotting results.

These data suggest that the venom components of all the Neotropical wasps analyzed here lack α1,3-core fucosylation and therefore presence of CCDs (Fig 2, *A*). To verify the lack of CCD-reactivity, we selected sera of allergic patients with elevated specific IgE (sIgE) level to CCDs. As shown in Fig 2, *C*, none of the Neotropical wasp venoms was recognized by the patients' sera in ELISA while pronounced signals were obtained for the CCD-positive HBV, YJV, and rVes v 3.

Our finding supports previous results showing that venoms of 6 *Polistes* species from Europe and Northern America are devoid of

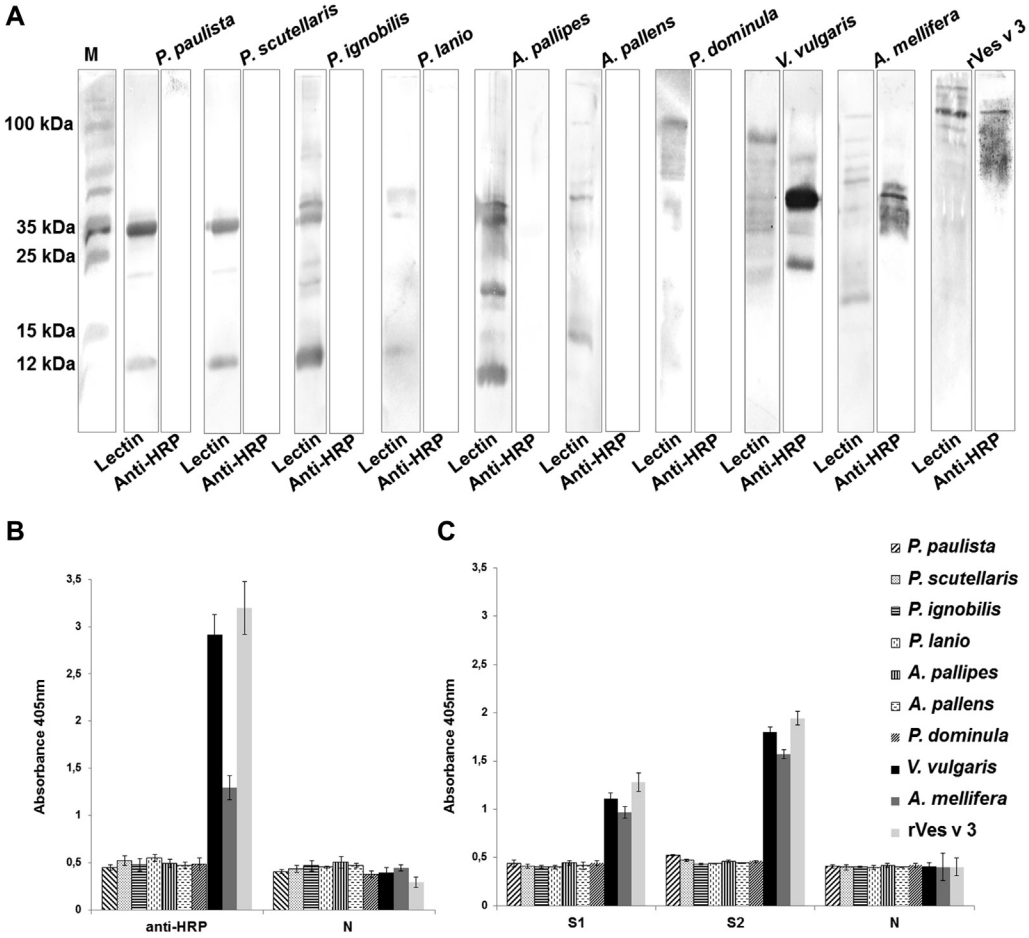

FIG 2. Glycosylation and CCDs of Neotropical wasp venoms and European insects analyzed by immunoblotting **(A)** and ELISA (**B** and **C**). Immunoblotting was performed using the lectin Concanavalin A for detection of glycosylation and anti-HRP serum for specific detection of CCDs. In ELISA, detection was performed using anti-HRP and nonimmune serum as well as human sera without sIgE to venom components but elevated levels of sIgE to CCDs **(C)**.

CCDs.[8] Taxonomically, the Neotropical wasps assessed here belong to a different tribe of the *Polistinae* subfamily. Together the absence of CCDs found for all members of this subfamily suggests a general absence in all Polistinae and provides a new perspective on the biology of CCDs. Recent findings suggest developmental implications of the α1,3-core fucosylation in plants, supporting also the neural relevance of the epitope in insects.[9] Against this background not the absence of CCDs but their presence on HBV and YJV might represent the biological peculiarity.

The lack of CCDs in all the clinically relevant wasps from Southern America has major implications for the procedures currently used for allergy diagnosis. While allergen components remain unavailable, crude venoms from these species allow for reliable determination of CCD-independent sensitization. Therefore, the differentiation of sensitization to HBV or wasp venom is significantly facilitated in particular in those patients having sIgE to CCDs. Because of frequent lack of information, positive sIgE to these venoms now can be interpreted as a purely venom-based sensitization in patients with and without sIgE to CCDs. Moreover, the quantitative determination of sIgE levels to venoms of wasps from different tribes might allow the identification of culprit insects even within taxonomically related species. The rational design of strategies for differential allergy diagnosis involving carefully selected venoms and recombinant components will contribute to dissect the complex picture of wasp venom allergy in Neotropical regions worldwide.

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Increased GATA-3 and T-bet expression in eosinophilic esophagitis versus gastroesophageal reflux disease

To the Editor:

Eosinophilic esophagitis (EoE) is a clinicopathological condition characterized by symptoms of esophageal dysfunction and dense eosinophil infiltration of the esophageal epithelium. The current diagnostic metric requires 15 eosinophils (eos) per hpf in at least 1 mucosal biopsy specimen following 6 to 8 weeks of treatment with high-dose proton pump inhibitor (PPI).[1] Although this histologic threshold distinguishes most subjects with EoE, several shortcomings exist including the following: (1) eosinophilia may underestimate the extent of eosinophil activity; (2) some patients with gastroesophageal reflux disease (GERD) may have distal esophageal eosinophilia exceeding 15 eos/hpf; and (3) patients often require more than 1 biopsy to determine the underlying diagnosis and establish appropriate treatment. Recent evidence suggests that EoE and proton pump inhibitor-responsive esophageal eosinophilia (PPI-REE) have a similar transcriptome[2] and are phenotypically indistinguishable.[3] Novel histologic biomarkers may further aid in distinguishing causes of esophageal eosinophilia.

Characterizations of mucosal inflammatory responses in EoE and PPI-REE describe a T_H2-predominant pattern with overexpression of T_H2-associated genes.[4] In EoE, IL-5 and IL-13 levels are associated with eotaxin-3 expression, which likely drives eosinophil recruitment. In contrast, esophageal biopsies from patients with GERD are more commonly associated with a T_H1 phenotype defined by increased mRNA expression of IL-1β, IL-8, and IFN-γ.[5]

T-bet and GATA-3 are transcriptional regulators that drive differentiation of T_H0 $CD4^+$ lymphocytes to T_H1 and T_H2 lineages, respectively. We previously demonstrated the utility of characterizing tissue-specific immune polarization using immunohistochemistry-based assessments of GATA-3 and T-bet in bladder cancer.[6] Given its role in T_H2-associated inflammation, we hypothesized that GATA-3 expression would be increased in EoE and PPI-REE and that the ratio of GATA-3/T-bet expression would differentiate these individuals from subjects with GERD.

We performed a retrospective, case-control study of children characterized clinically as having EoE (n = 24), PPI-REE (n = 10), or GERD (n = 28) and as controls (n = 32). Subjects diagnosed with EoE were treated with an elimination diet (n = 7), swallowed topical steroids (n = 12), or a combination of elimination diet and swallowed topical steroids (n = 5) and those with PPI-REE were treated with high-dose PPI (2 mg/kg/d). All subjects with EoE and PPI-REE demonstrated histologic resolution of esophageal eosinophilia (<15 eos/hpf) after 6 to 8 weeks of treatment, respectively.

Tissue sections from active and matched posttreatment biopsies were assessed with hematoxylin and eosin, and immunohistochemical staining for T-bet and GATA-3 was performed. Slides stained for T-bet and GATA-3 were digitized and staining of the epithelial layer was quantified (see Fig E1 in this article's Online Repository at www.jacionline.org). A nuclear algorithm was used to identify T-bet+ and GATA-3+ cells. The number of positive cells was divided by the total area of esophageal epithelium analyzed in order to normalize the number of T-bet+ and GATA-3+ cells/mm.^{2} Polarization of the immune microenvironment was assessed by the GATA-3+ cells/mm^2/T-bet+ cells/mm^2 (G/T) ratio. Details regarding the study population, methods for T-bet/GATA-3 staining/quantification, and statistical analysis are detailed in this article's Online Repository at jacionline.org.

Table E1 in this article's Online Repository at www.jacionline.org details the demographic and clinical characteristics of the study population. Staining for GATA-3 and T-bet by immunohistochemistry is shown in Fig 1. In comparison to GERD, subjects with active EoE demonstrated increased GATA-3+ cells/mm^2 (median, 2.81 vs 8.46 cells/mm^2; $P < .0001$; area under the curve, 0.88; 95% CI, 0.78-0.97) and T-bet+ cells/mm^2 (median, 7.12 vs 12.01 cells/mm^2; $P < .0001$; area under the curve, 0.79; 95% CI, 0.66-0.93) (see Fig 2, *B*, *C*, and *F*). No statistical differences in GATA-3 and T-bet expression were found between subjects with active EoE and PPI-REE. GATA-3 and T-bet expression was also significantly elevated in subjects with active EoE compared with healthy controls. Following treatment, GATA-3 and T-bet expression decreased significantly in subjects with

METHODS

Collection of venoms from Neotropical insect species

Nests from Neotropical wasps were captured around or within the campus of Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Rio Claro, SP, Brazil (licensed by Sisbio, No. 58500). The wasps were immediately anesthetized at low temperature (−80°C). The venom glands of 1000 insects were extracted using sterile tweezers, washed with sterile ultrapure water, and suspended in a protease inhibitor mix (2 mM leupeptine and 1 mM phenylmethylsulfonyl fluoride; Sigma-Aldrich, St Louis, Mo). Then, the gland suspensions were macerated, washed 3 times with the protease inhibitor mix, and centrifuged at 10,000*g* for 15 minutes at 4°C. The resulting supernatants were collected and stored at −20°C until use.

HBV (i1), YJV (i3), and *P dominula* venom (i4) were obtained from Euroimmun (Lübeck, Germany). Recombinant Ves v 3 was produced in HighFive insect cells, which are known to establish CCDs.

SDS-PAGE analysis and detection of N-glycans in immunoblotting

SDS-PAGE was performed using standard protocols according to Laemmli,[E1] using a Mini-Protean Tetra Cell System (BioRad, Sao Paulo, Brazil). After running, the gels were stained with Coomassie Brilliant Blue R-250 (CBB) (Sigma Aldrich, Sao Paulo, Brazil). For immunoblotting analysis for detection of glycosylation and CCDs, the venoms were subjected to (12%) SDS-PAGE and then transferred to a 0.22-μm nitrocellulose membrane using a semi-dry system (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad). For HRP detection the membranes were blocked for 30 minutes at room temperature with 4% nonfat dry milk powder (AppliChem, Darmstadt, Germany) in TBS buffer (20 mM Tris-HCl and 150 mM NaCl; pH 7.4). The membrane was then incubated overnight (4°C, 150 rpm) with rabbit anti-HRP serum (1:5000) diluted in TBS buffer with 2% nonfat dried milk powder. After washing 3 times with TBS buffer containing 0.5% Tween, the membrane was incubated for 1 hour at room temperature with goat anti-rabbit IgG (Sigma-Aldrich, Copenhagen, Denmark) (1:20,000) conjugated with alkaline phosphatase. Bound antibodies were visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) as substrate (Sigma, Copenhagen, Denmark).

For the detection of glycosylation, the membranes containing the venoms were blocked using a Blocking Solution (Vector Labs, Peterborough, United Kingdom) (1 hour at room temperature). After washing with TBS-T, 2 μg/mL of biotinylated Concanavalin A (Vector Labs), a jack bean lectin that binds internal and nonreducing terminal α-D-mannosyl and α-D-glucosyl groups in glycans, was added. After incubation for 30 minutes at room temperature, the membranes were washed and then incubated with streptavidin conjugated to alkaline phosphatase (1:70,000) (Sigma) for 30 minutes at room temperature. Bound lectin then was visualized as above.

Detection of CCDs in ELISA

The patient sera were selected for a high level of sIgE to CCDs (class 5) and negative sIgE to HBV and YJV components as determined by Euroline assay measurements.

For detection of CCDs by the rabbit anti-HRP serum and sIgE in patient sera, 384-well microtiter plates (Nunc, Thermo Fisher Scientific, Ulm, Germany) were coated with 20 ng of the venoms (4°C, overnight). Coated plates were then blocked with 4% nonfat dry milk powder (AppliChem, Darmstadt, Germany) in TBS at room temperature for 1 hour. After washing with TBS, either rabbit anti-HRP serum (1:5000) or patient sera (1:2) (diluted in TBS and 2% nonfat dry milk powder) were added to the plates and incubated overnight at 4°C. A goat anti-rabbit IgG (1:20,000) (Sigma-Aldrich) and a goat anti-human IgE (1:20,000) (Biozol, Eching, Germany), both conjugated to alkaline phosphatase, were added for detection of sIgE to HRP, or insect CCDs. After incubation for 1 hour with gentle shaking at room temperature, the detection was performed by adding 50 μL of the substrate solution per well (5 mg/mL 4-nitrophenylphosphate; AppliChem, Darmstadt, Germany). The absorbance was read at 405 nm. A signal that duplicates the means of the negative control was interpreted as positive result. Three independent experiments for each type of antibodies were conducted.[E2]

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