SUPPLEMENTARY MATERIAL

Materials and Methods

Natural HBV and VIT-products

31 different batches of four purified aqueous HBV VIT products (A, B, C, D) by three different manufacturers were analyzed for the presence of Api m 10. The batches of each particular product differed in terms of the time of their manufacture and submission for state batch testing. They included 18 out of 19 batches that had been previously analyzed with IgG-immunoblotting and were reported with partly contradicting results [Frick M et al 2016; Blank S et al. 2016]. The lyophilized VIT products were stored until use at 4 °C and freshly reconstituted with ultrapure water to a concentration of 1 mg/mL immediately prior to the respective analyses. Crude HBV (Latoxan, Portes lès Valence, France) and rApi m 10 (expressed in E. coli with an N-terminal His tag) was used as control for IgG Immunoblotting experiments.

SDS-PAGE

To determine the presence of Api m 10 in the individual VIT-batches, 5 or 25 µg, respectively, of reconstituted HBV was incubated for 5 min at 95 °C in Lämmli buffer and analyzed on a 13% SDS-PAGE (VWR® Perfect Blue TwinS, VWR International GmbH, Darmstadt Germany)), followed by Semi-Dry blotting onto a 0.45 µm nitrocellulose membranes (GE Healthcare, Freiburg, Germany). For transfer control, Western blot membranes were stained for 5 minutes with 0.1% Ponceau S solution in 5% acetic acid. After documentation, membranes were destained with ultrapure water.

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IgG-Immunoblotting

Detection of Api m 10 was performed using a polyclonal rabbit antibody against Api m 10 (1:500) and a HRP conjugated goat anti-rabbit IgG secondary antibody (1:20.000; Sigma A0545). Blocking was carried out with TBS + 0.1 % Tween20™ + 4 % milk powder, washing steps with TBS + 0.1 % Tween20™. For visualization, membranes were incubated with ECL (SuperSignal™ West Pico Chemiluminescent Substrate, Thermo Fisher #34077).

High Definition mass spectrometry (HDMS^E)

HDMS^E was done as previously described [Spiric J et 2l. 2017] with the following modifications compared to the reference: The samples were reconstituted according to the manufacturer's instructions and 30 µl of this solution was diluted to a volume of 60 µl. We used a Synapt G2si for data acquisition and PLGS 3.03 for raw data processing by utilizing a UniProt database restricted to reviewed entries of *Apis mellifera.* Statistical significance of the results was reflected by total protein PLGS scores [Li GZ et al. 2009] between 370 and 9674 (Table E1)

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SUPPLEMENTARY RESULTS

Table E1: Accession no. and total protein PLGS scores of mass spectrometric analyses of 31 batches of four VIT products

The PLGS score [Li GZ et al. 2009] is the vendor's proprietary measure of the probability of error of the result. The higher the score, the lower the probability of error. In this study always less than 5%.

Manufacturer	Product	Sample	Acc. No.	Description	PLGS Score
1	A	Batch 1	Q5EF78	Icarapin	5663
		Batch 2	Q5EF78	Icarapin	2946
		Batch 3	Q5EF78	Icarapin	2784
		Batch 4	Q5EF78	Icarapin	2298
		Batch 5	Q5EF78	Icarapin	7583
		Batch 6	Q5EF78	Icarapin	6646
		Batch 7	Q5EF78	Icarapin	8774
		Batch 8	Q5EF78	Icarapin	9674
2	B	Batch 1	Q5EF78	Icarapin	554
		Batch 2	Q5EF78	Icarapin	1799
		Batch 3	Q5EF78	Icarapin	2821
		Batch 4	Q5EF78	Icarapin	3782
		Batch 5	Q5EF78	Icarapin	3113
		Batch 6	Q5EF78	Icarapin	2207
		Batch 7	Q5EF78	Icarapin	995

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		Batch 8	Q5EF78	Icarapin	714
3	C	Batch 1	Q5EF78	Icarapin	370
		Batch 2	Q5EF78	Icarapin	681
		Batch 3	Q5EF78	Icarapin	934
		Batch 4	Q5EF78	Icarapin	1205
		Batch 5	Q5EF78	Icarapin	4196
		Batch 6	Q5EF78	Icarapin	2551
		Batch 7	Q5EF78	Icarapin	646
	D	Batch 1	Q5EF78	Icarapin	2922
		Batch 2	Q5EF78	Icarapin	2403
		Batch 3	Q5EF78	Icarapin	1642
		Batch 4	Q5EF78	Icarapin	893
		Batch 5	Q5EF78	Icarapin	1773
		Batch 6	Q5EF78	Icarapin	982
		Batch 7	Q5EF78	Icarapin	905
		Batch 8	Q5EF78	Icarapin	1665

SUPPLEMENTARY DISCUSSION

Systemic-allergic sting reactions to HBV have been reported in up to 5% of the population and up to 32% in beekeepers [Ludman SW & Boyle RJ 2015]. The therapeutic efficacy of different VIT-treatment protocols did not differ [Ruëff et al. 2004]. Identification of the mechanism of successful immunotherapy in general and, in consequence, therapy guiding

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biomarkers is still in its infancy [Zissler UM & Schmidt-Weber CB 2020]. IgG_4 is currently the only generally accepted biomarker of allergen immunotherapy that demonstrates that the patient has received the therapeutic antigen [Zissler UM & Schmidt-Weber CB 2020]. The potential of IgG_4 in relation to IgE to bind to an allergen has been investigated intensively as a tolerance biomarker; however, its relationship to clinical symptoms is only visible in larger cohorts [Sturm GJ et al. 2018; Zissler UM & Schmidt-Weber CB 2020]. In a small clinical cohort of well characterized patients with HBV anaphylaxis, sensitized both to Api m 1 and Api m 10, VIT with a HBV-product of unknown quantity of Api m 10 reduced sIgE to both components but $sIgG_4$ levels were increased exclusively for Api m 1 [Pereira Santos MC et al. 2020]. Five patients (two in the Api m 10 predominant group) were re-stung without anaphylaxis [Pereira Santos MC et al. 2020]. This could point to the conclusion that - despite predominance of Api m 10 specific IgE - Api m 10 sensitization is not clinically relevant in some patients or other mechanisms independent from venom-specific IgG_4 may play a role in the protective immune response in Api m 10 sensitization. Further prospective studies are encouraged to investigate immunologic and clinical efficacy of HBV VIT in patients with different sensitization profiles.

SUPPLEMENTARY REFERENCES

Blank S, Etzold S, Darsow U, Schiener M, Eberlein B, Russkamp D, et al. Component-resolved evaluation of the content of major allergens in therapeutic extracts for specific immunotherapy of honeybee venom allergy. Hum Vaccin Immunother. 2017;13:2482-2489.

Frick M, Fischer J, Helbling A, Rueff F, Wieczorek D, Ollert M, et al. Predominant Api m 10 sensitization as risk factor for treatment failure in honey bee venom immunotherapy. J Allergy Clin Immunol. 2016;138:1663-1671.

Li GZ, Vissers JP, Silva JC, Golick D, Gorenstein MV, Geromanos SJ, et al. Database searching and accounting of multiplexed precursor and product ion spectra from the data

doi: 10.18176/jiaci.1093

independent analysis of simple and complex peptide mixtures. Proteomics 2009;9:1696-1719.

Ludman SW, Boyle RJ. Stinging insect allergy: current perspectives on venom immunotherapy. J Asthma Allergy 2015;8:75-86.

Pereira Santos MC, Lourenco T, Pereira Barbosa M, Branco Ferreira M. Evolution of Api m10 Specific IgE and IgG4 After One Year of Bee Venom Immunotherapy. Eur Ann Allergy Clin Immunol. 2020;52:175-181.

Ruëff F, Wolf H, Schnitker J, Ring J, Przybilla B. Specific immunotherapy in honeybee venom allergy: a comparative study using aqueous and aluminium hydroxide adsorbed preparations. Allergy 2004;59:589-595.

Spiric J, Schulenborg T, Engin AM, Karas M, Reuter A. Model for Quality Control of Allergen Products with Mass Spectrometry. J Proteome Res 2017;16:3852-3862.

Sturm GJ, Varga EM, Roberts G, Mosbech H, Bilò MB, Akdis CA, et al. EAACI guidelines on allergen immunotherapy: Hymenoptera venom allergy. Allergy 2018;73:744-764.

Zissler UM, Schmidt-Weber CB. Predicting Success of Allergen-Specific Immunotherapy Front Immunol. 2020 Aug 25;11:1826.

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