In situ imaging of honeybee (*Apis mellifera*) venom components from aqueous and aluminum hydroxide–adsorbed venom immunotherapy preparations

Ulla Seppälä, PhD,[a]* Simona Francese, PhD,[b]* Stefano Turillazzi, Prof,[c] Gloriano Moneti, Prof,[c] Malcolm Clench, Prof,[b] and Domingo Barber, PhD[a] *Hørsholm, Denmark, Madrid, Spain, Sheffield, United Kingdom, and Florence, Italy*

Background: Treatment with aqueous and aluminum hydroxide ($Al[OH]_3$)–adsorbed purified honeybee *(Apis mellifera)* venom (HBV) preparations can reduce the incidence of side effects associated with venom immunotherapy.
Objective: The aim of the present study was to assess these purified HBV immunotherapy preparations *in situ*.
Methods: Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) was used to visualize the distribution of HBV components. The preparations were administered on the back legs of naive Wistar rats. The rats were killed, and cryosectioned tissue sections were subjected to hematoxylin and eosin staining and MALDI-MSI analyses.
Results: Low-density maps of tissue distribution of HBV peptides, such as secapin, mast cell degranulating peptide, and melittin (Api m 4) were detected in the tissue after administration of HBV immunotherapy preparations. In addition, release of biogenic amines, cytokines, and leukotrienes was observed, and the distribution of HBV allergens, such as Api m 1 and Api m 2, was shown. At the 24-hour time point, the major HBV allergen Api m 1 was still detected at the site of $Al(OH)_3$-adsorbed HVB injection, whereas in the case of aqueous HBV preparation, all the allergens, as well as most of the biogenic amines, were cleared at the 24-hour time point.
Conclusion: The present study shows that the majority of low-molecular-weight HBV components are rapidly removed from the site of venom immunotherapy administration. Furthermore, $Al(OH)_3$-adsorbed HBV preparation demonstrated a depot effect, prolonging the availability of bee venom allergens at the site of administration. (J Allergy Clin Immunol 2012;129:1314-20.)

Key words: *Allergen, inflammation, imaging,* in situ*, honeybee, venom, immunotherapy, subcutaneous*

Abbreviations used

$Al(OH)_3$:	Aluminum hydroxide
HBV:	Honeybee venom
H&E:	Hematoxylin and eosin
HSA:	Human serum albumin
LT:	Leukotriene
MALDI-MSI:	Matrix-assisted laser desorption ionization mass spectrometry imaging
MCD:	Mast cell degranulating peptide
mPEG:	Monomethoxy polyethylene glycol
PMF:	Peptide mass fingerprinting
VIT:	Venom immunotherapy

Honeybee venom (HBV) is a mixture of proteins and other pharmacologically active molecules that diffuse into the tissue on a bee sting.[1] These molecules are responsible for nonallergic reactions, such as local pain, itching, and inflammation, as well as severe allergic reactions, which can become life-threatening.[2] The majority of HBV allergens are active enzymes that are capable of inducing strong immune responses.[3,4] Phospholipase A_2 (Api m 1) is the major allergen and a valuable diagnostic marker for HBV allergy.[5] Introduction of Api m 1 to cell membranes triggers cleavage of membrane phospholipids, inducing pore formation and cell lysis. Another well-known HBV allergen, hyaluronidase (Api m 2), is capable of hydrolyzing hyaluronic acid in the target tissue. By breaking the local cell matrix, it enhances the penetration of venom components through the tissue fluid, and for this reason, hyaluronidases are also termed spreading factors.[1,3,4] More recently, a number of other enzymatic active HBV allergens have been expressed and characterized,[6-8] and currently, a total of 10 HBV allergens are known. The majority of dry-weight HBV is comprised of low-molecular-weight peptides and biogenic amines.[1,4] Melittin (Api m 4), a 26 amino acid–long basic peptide, is the most abundant molecule in the bee venom, comprising more than 50% of the dry weight. It is stored as a tetramer in the venom sac, but when released and dissolved, it dissociates into monomers that are capable of binding on cell membranes. Melittin can trigger lysis of a wide range of cells, such as muscle cells, leukocytes, and mast cells, which can lead, for example, to release of histamine and other intracellular components into surrounding tissue. Interestingly, the cell lysis is further enhanced when it is presented together with phospholipase A_2 (Api m 1).[1,4,9] Other small peptides, such as apamin, mast cell degranulating peptide (MCD), tertiapin, and secapin, are also present in HBV in modest quantities. However, these

From [a]Markets and Products Support, Global Research, ALK-Abelló, Hørsholm and Madrid; [b]Sheffield Hallam University, Sheffield; and [c]CISM, University of Florence.
*These authors contributed equally to this work.
Supported by ALK-Abelló, Hørsholm, Denmark.
Disclosure of potential conflict of interest: S. Francese, G. Moneti, and M. Clench have received research support from ALK-Abelló. The rest of the authors declare that they have no relevant conflicts of interest.
Received for publication July 1, 2011; revised October 13, 2011; accepted for publication October 14, 2011.
Available online November 21, 2011.
Corresponding author: Ulla Seppälä, PhD, Markets and Products Support, Global Research, Bøge Allé 3, ALK-Abelló 2970, Denmark. E-mail: USedk@alk-abello.com.
0091-6749/$36.00

doi:10.1016/j.jaci.2011.10.014

molecules, like many other neurotoxins, have high receptor specificity possessing rather low toxicity against mammalian cells.[1,4]

The therapeutic potential of HBV is well known across the medical field, and venom immunotherapy (VIT) is the choice of treatment for patients with honeybee allergy.[10,11] VIT is administered subcutaneously; however, factors influencing the uptake of the venom components and the local cellular events at the site of administration have not been elucidated in detail. In the study by Dreborg and Åkerblom,[12] migration of iodine 125–labelled monomethoxy polyethylene glycol (mPEG)–modified and native HBV were compared in time-course experiments at the site of subcutaneous administration. In this study the migration rate of the mPEG-modified HBV was shown to be slower when compared with that of native HBV. In general, larger proteins are considered to travel from the subcutaneous injection site through the tissue fluid into the lymphatic system, whereas small molecules (<16 kd) primarily diffuse through the vessel walls into the blood capillaries.[13] In particular, the imperative role of skin-residing antigen-presenting cells and their interactions with epidermal and dermal cells, such as keratinocytes, mast cells, and fibroblasts, after subcutaneous VIT administration are of interest and need to be further investigated.[14,15]

VIT is divided into 2 phases: the updosing phase and the maintenance phase. Numerous VIT protocols are available, but essentially, 2 different ways of arriving at the maintenance dose are being used: by using rush VIT, the maintenance dose is reached in 4 to 5 days increasing the dosage at short intervals, whereas in conventional VIT the injections are administered at longer intervals for several weeks or months.[2,10,11,16] In Europe formulated and lyophilized whole venom, purified aqueous venom, and aluminum hydroxide (Al[OH]$_3$)–adsorbed depot preparations are available, whereas in the United States only formulated whole-venom preparations are approved for VIT.[2] Purified aqueous and aluminum-adsorbed depot preparations, in which the amount of low-molecular-weight components is reduced, have been reported to diminish the frequency of early local side effects during VIT. However, the efficacy of VIT is considered to be comparable when either purified, nonpurified, or both types of preparations are used.[2,16]

In the present study a cutting-edge mass spectrometry technique, namely matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI), has been used to evaluate HBV-derived VIT preparations *in situ*. MALDI-MSI, which was first reported in 1997, enables images of the distribution of a variety of molecules, such as drugs, lipids, peptides, and proteins, to be obtained within intact cryosections of biological materials (eg, organs, biopsy specimens, and/or whole animal bodies) without resorting to any molecular or radioactive probes.[17] This technology has proved very informative in a variety of life sciences fields, including pharmaceutical research.[18,19] Furthermore, it has been successfully used by Francese et al[20] to map the diffusion and distribution of HBV allergens and toxins in a tissue on a honeybee sting. By use of MALDI-MSI, pharmacokinetics of HBV components, as well as the appearance of HBV-stimulated immunologic markers, in nonsensitized animals were assessed. Histopathologic evaluation of the tissue samples was performed in parallel and, where possible, on the same sections subjected to MALDI-MSI.

METHODS

Experimental animals

Fourteen naive Wistar rats (Harlan Nossan, Milan, Italy) 3 months of age with weights ranging from 340 to 410 g were used for the study. The study was approved by the ethics committee at the University of Florence, and all animal handling was carried out according to the European Community guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC).

In vivo experiments

The HBV used for commercial preparations and for purification of HBV hyaluronidase (Api m 2) was delivered by ALK Source Materials Laboratories, Inc (Spring Mills, Pa). Two purified HBV preparations, the aqueous preparation (10,000 SQ *Apis mellifera*, Aquagen SQ, dissolved in ALK diluent, ALK-Abelló, Hørsholm, Denmark) and the Al(OH)$_3$-adsorbed HBV (depot) formulation (10,000 SQ *Apis mellifera*, Alutard SQ, ALK-Abelló),[21] and 50 μL (2.5 μg) of purified non–human serum albumin (HSA)–formulated native HBV hyaluronidase (Api m 2) dissolved in sterile saline were administered subcutaneously in the back leg of a rat. The injections were individualized, and all the HBV preparations were investigated in a duplicate manner. The rats received a single injection of the aqueous, depot, or Api m 2 preparation. Saline and placebo depot formulations were used as negative controls and were also administered in a duplicate manner. The injection site or sites were marked with a permanent color pen. After administration, rats were killed by means of decapitation at the 30-minute and 24-hour time points (see Table E1 in this article's Online Repository at www.jacionline.org). The back legs were dissected, snap-frozen in liquid nitrogen, and stored at −80°C until use.

Cryosectioning and tissue staining

The tissue samples were then immediately mounted with optimal cutting temperature polymer (Leica Microsystems, Milan, Italy) and sectioned 10 μm stepwise at −25°C in a Leica CM 1950 Microsystems cryostat (Leica Microsystems). Six sections were obtained for each sample: 2 were destined for protein MALDI-MSI (and mounted on indium tin oxide glass slides), 2 to small-molecule MALDI-MSI (and mounted on indium tin oxide glass slides), and 2 to *in situ* tissue digestion followed by MALDI-MSI (and mounted on aluminum sheets). Sections were kept frozen at −80°C. The tissue sections were stained, as previously described, when possible after mass spectrometry imaging analyses with Harris-modified hematoxylin and eosin (H&E) solution (Sigma-Aldrich, St Louis, Mo; see Table E1).[20]

Sample preparation for MALDI-MSI

Cryosections destined for small-molecule imaging were directly spray coated with 5 mg/mL α-cyano-4 hydroxycinnamic acid (Sigma-Aldrich, Poole, United Kingdom) in acetonitrile, water, and trifluoroacetic acid (1:1:0.1 in volume) containing an equimolar amount of aniline (2.4 μL; Sigma-Aldrich, Dorset, United Kingdom). The matrix solution was deposited with the autosprayer. Sections to be subjected to protein imaging (m/z 6,000-70,000) were washed and prepared for spray coating, as previously described.[19] The matrix, 5 mg/mL sinapinic acid (Sigma-Aldrich) in 50% acetonitrile (Fisher Scientific, Loughborough, United Kingdom) with 0.1% trifluoroacetic acid (Sigma-Aldrich), was applied with the SunCollect autosprayer (SunchromGmbH, Friedrichsdorf, Germany). Cryosections destined for *in situ* digestion were spray coated with a solution of sequencing grade modified trypsin (Promega, Southampton, United Kingdom; 20 μg/mL in 50 mmol/L ammonium bicarbonate) containing 0.5% of β-octyl glucoside (Sigma-Aldrich). The sections were then incubated overnight at 37°C in a humid chamber in a 5% CO_2 atmosphere. Digested sections were spray coated with a solution of 5 mg/mL α-cyano-4 hydroxycinnamic acid in acetonitrile, water, and trifluoroacetic acid (1:1:0.1 in volume) containing an equimolar amount of aniline by using the autosprayer, as described above.

Data acquisition and processing

Protein and small-molecule MALDI-MSI analyses in the range of m/z 6,000 to 70,000 and m/z 100 to 3,000, respectively, were conducted by using