

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

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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 HBV 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 2011;■■■:■■■-■■■.)

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 A₂ (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 A₂ (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

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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 up dosing 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 al²⁰ 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-K-Abelló, Hørsholm, Denmark) and the Al(OH)₃-adsorbed HBV (depot) formulation (10,000 SQ *Apis mellifera*, Alutard SQ, ALK-Abelló),²¹ and 50 μ L (2.5 μ g) 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 μ m 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 μ L; 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 (Sunchrom GmbH, Friedrichsdorf, Germany). Cryosections destined for *in situ* digestion were spray coated with a solution of sequencing grade modified trypsin (Promega, Southampton, United Kingdom; 20 μ g/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% CO₂ 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

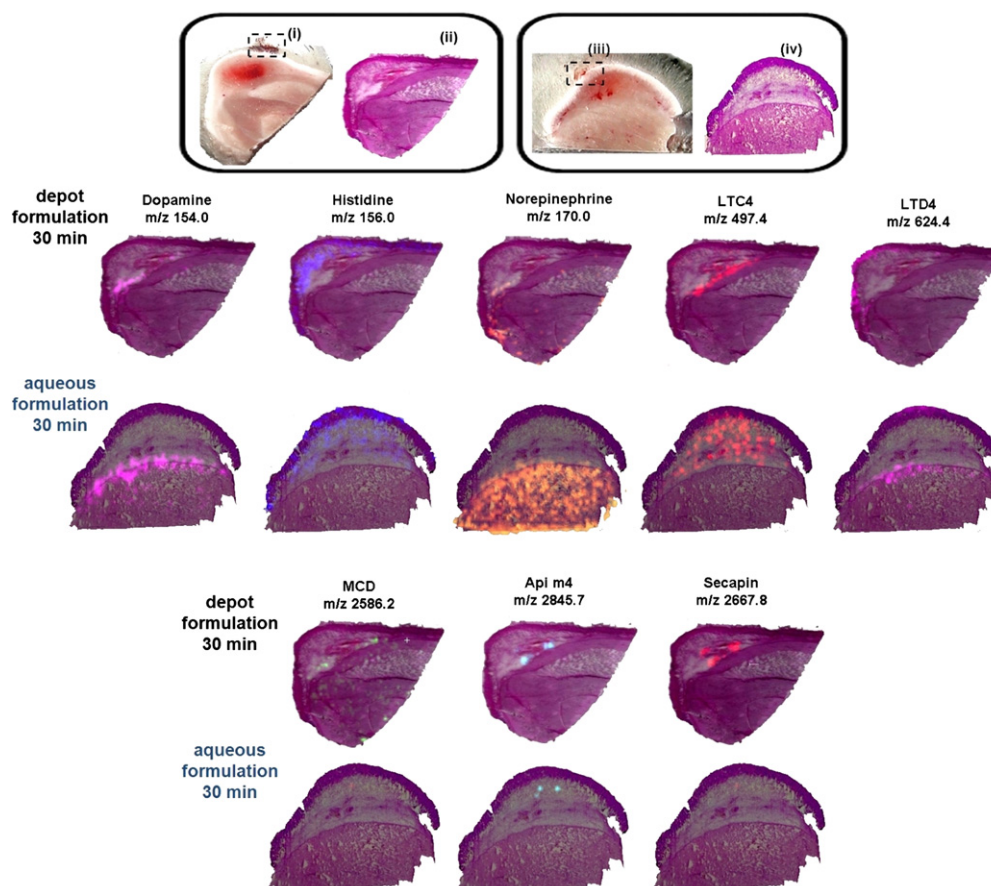


FIG 1. MALDI-MSI comparative analysis of leg muscle cryosections from rats administered with the depot (*i* and *ii*) and aqueous preparation (*iii* and *iv*) after 30 minutes. Panels *i* and *ii* show the rat muscle specimen (framing the injection area [*ii*]) and a corresponding cryosection stained with H&E. The same display is reported for the specimens obtained from the rat administered the aqueous preparation in panels *iii* and *iv*.

an Ultraflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) incorporating the Smartbeam Laser operating at a frequency of 200 Hz. This laser system is a frequency-tripled Nd:YAG laser at 355 nm. For protein imaging, the instrument was operated in positive linear mode, and the spatial resolution was set to 250 μm , setting the Smartbeam to “medium.” Ion sources 1 and 2 were set to 25,000 and 21,500 V, respectively, with the delay time at 200 ns. Small-molecule imaging data were acquired by operating the instrument in reflectron mode and decreasing the delay time to 60 ns. The instrument was calibrated by using a mixture of standard peptides and proteins (according to the type of analysis) at mass ranges of 1 to 3 kD and 5 to 66 kD, respectively, and recalibrated every 4 analyses. Mass spectra acquisition on the Ultraflex III MALDI TOF/TOF was performed with the Flex Imaging 2.0 and Flex Control 3.0 software (Bruker Daltonics), and data were processed through Flex Imaging 2.0, Flex Analysis 3.0, and Biomap 3.7.5 (Novartis, Basel, Switzerland) software. Images were normalized against total ion current.

Mass spectrometric imaging analysis of the peptides resulting from *in situ* digestion with trypsin were conducted by using a modified Applied Biosystems API Q-Star Pulsar i hybrid quadrupole time-of-flight instrument (Concord, Ottawa, Ontario, Canada). The orthogonal MALDI source has been modified to incorporate a SPOT 10-kHz Nd:YVO₄ solid-state laser (El-forlight Ltd, Daventry, United Kingdom) with a wavelength of 355 nm and a pulse duration of 1.5 ns and producing an elliptical spot size of 100 $\times$ 150 μm . Images were acquired by using oMALDI Server 5.1 software supplied by MDS Sciex (Concord). Acquisition parameter data processing was performed with BioMap 3.7.5 software (see Table E1).

RESULTS

Detection of small molecules *in situ*

Release of small molecules and endogenous biogenic amines, such as dopamine (m/z 154), histidine (m/z 156), and norepinephrine (m/z, 170) *in situ* were demonstrated in all the cryosections after administration of HBV preparations at 30 minutes (Fig 1). Similar to the HBV preparations, after administration of purified Api m 2, local release of dopamine, norepinephrine, and histidine was detected (Fig 2); in this case dopamine and histidine were found in the epidermal region, whereas norepinephrine was invasively present in the dermis. The biogenic amines were absent in both controls, the saline-derived cryosections (data not shown) and the depot placebo-derived cryosections (see Fig E1, A'-C', in this article's Online Repository at www.jacionline.org), indicating that the species can only derive from the residual presence of the formulated HBV preparation. In particular, at the 30-minute time point, the biogenic amines were present in both formulations, with greater distribution in the aqueous HBV-derived preparation (Fig 1). Both the cryosections derived from the injection of the 2 formulations exhibited the presence of sulfidoleukotrienes, such as leukotriene (LT) C₄ (m/z 497.5) and LTD₄ (m/z 624.7). These 2 species are again not detected in the placebo-derived cryosections (see Fig E1, D' and E'), showing that no inflammatory

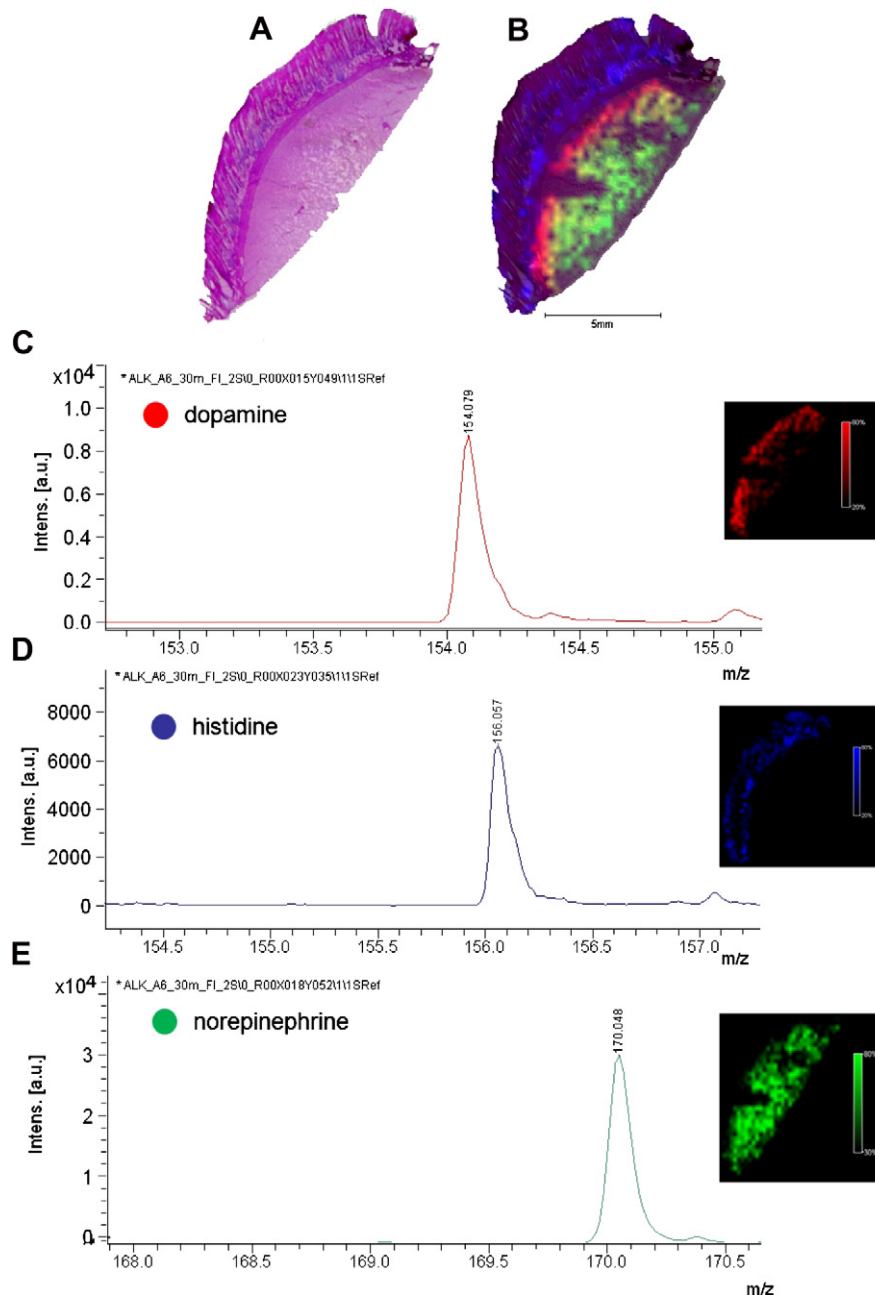


FIG 2. MALDI-MSI analysis of a leg muscle cryosection from a rat administered with purified Api m 2 at 30 minutes. **A** and **B**, H&E-stained tissue section image (Fig 2, A) and superimposed image demonstrating the tissue distribution of dopamine (red), histidine (blue) and norepinephrine (green; Fig 2, B). **C-E**, Mass spectrometry image of distribution of dopamine, histidine, and norepinephrine, respectively.

activity is triggered by the adjuvant only. A substantial reduction of levels of the biogenic amines and sulfidoleukotrienes detected is observed at the 24-hour time point for the aqueous formulation (Fig 3). The purification protocol or protocols eliminate HBV-derived peptides in large part. However, low-intensity ion signals for melittin (Api m 4, m/z 2845.7), MCD (m/z 2586.2), and secapin (m/z 2667.8) were still detected in the depot- and aqueous preparation-derived cryosections, although in the latter the density map was much lower (Fig 1), with no detection at 24 hours. This suggests a clearance from the tissue over the 24-hour period

(Fig 3). As expected, these peptides were not present in the placebo-derived cryosections (see Fig E1, H').

Detection and imaging of HBV- and formulation-derived proteins

The presence of HBV-derived (m/z 6,000-70,000) allergens was investigated by means of direct MALDI-MSI and *in situ* trypsin digestion (ie, by mean of peptide mass fingerprinting (PMF; m/z 100-3,000). Colocalization of proteins and their tryptic

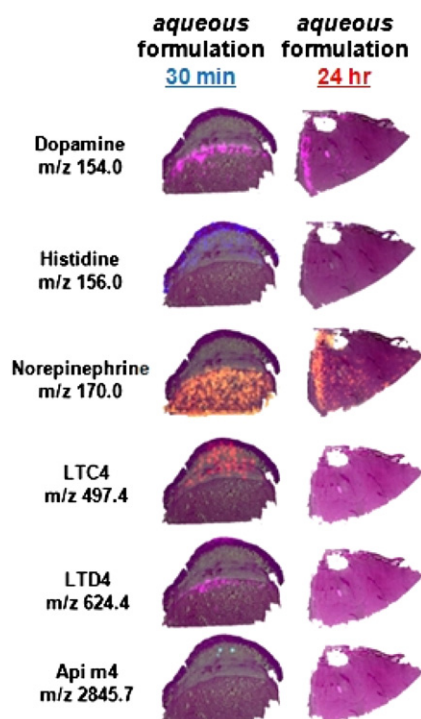


FIG 3. MALDI-MSI comparative analysis of leg muscle cryosections from rats administered with aqueous HBV preparation at 30 minutes and 24 hours. Sulfidoleukotriene levels were induced at 30 minutes and reduced or absent at 24 hours. The Api m 4 HBV-derived peptide present in the vaccine and visible at 30 minutes after administration is no longer detected after 24 hours.

peptides was used as a main strategy for the identification of larger species (see Fig E2 in this article's Online Repository at www.jacionline.org). For the depot preparation, Api m 1 (accession no. P00630) and HSA (accession no. P02768) were visualized both at 30 minutes and 24 hours. In particular, Api m 1 was highly localized at 30 minutes and more widely distributed, with a slightly lower density map within the tissue at 24 hours. Depot formulation–derived HSA was detected as localized both at 30 minutes and 24 hours. Api m 2 (accession no. Q08169) was only detected at 30 minutes (Fig 4, A). These molecules were detected through their tryptic peptides at m/z 1194.6 (amino acid positions 81-91), 508.2 (amino acid positions 230-233), and 862.4 (amino acid positions 306-312), respectively. Neither these proteins nor the abovementioned derived peptides were present in the control specimen (data not shown). For the aqueous HBV preparation, Api m 1 and Api m 2 were detected at the 30-minute time point through their tryptic peptides at 1194.6 (amino acid positions 81-91) and 862.4 (amino acid positions 306-312), respectively; however, as with the depot preparation, whereas Api m 1 was still detected at 24 hours (not localized but more distributed within the tissue), Api m 2 was no longer observed (data not shown).

In situ–derived inflammatory molecules

Inflammatory molecules, such as cytokines, were also putatively identified *in situ*, and their presence was interpreted as the biological response to the administration of the formulated HBV preparations. At 30 minutes, cytokines, such as IL-4 (accession no. P20096), IL-1 β (accession no. Q63264), and IL-13 (accession

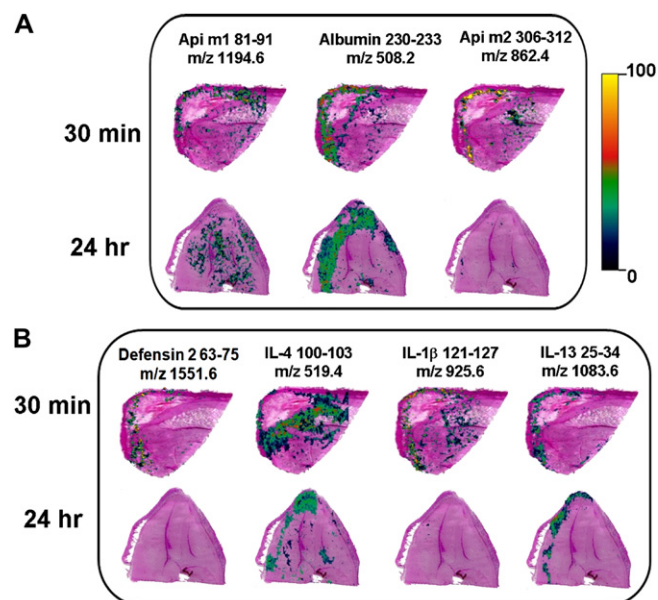


FIG 4. MALDI-MSI of peptides generated by means of *in situ* trypsin digestion of tissue sections from rats treated with the depot preparation at 30 minutes and 24 hours. **A**, A characteristic peptide from a sample of vaccine-derived proteins. **B**, A characteristic peptide from each of the proteins tentatively identified as present in response to the administration of the depot HBV preparation.

no. P42203), were detected through their tryptic peptides at m/z 519.4 (amino acid positions 100-103), 925.6 (amino acid positions 121-127), and 1083.6 (amino acid positions 25-34; Fig 4, B). Neither these proteins nor the abovementioned derived peptides were present in the control specimen (data not shown). With the exception of IL-13, which was found to have a similar extent of distribution at 30 minutes and 24 hours, all the other proteins exhibited a much lower density map at 24 hours when compared with that at 30 minutes. The peptides at m/z 519.4 (amino acid positions 100-103), 925.6 (amino acid positions 121-127), and 1083.6 (amino acid positions 25-34) were absent at both 30 minutes and 24 hours after administration of the aqueous formulation, suggesting that detectable levels of IL-4, IL-1 β , and IL-13 were not released after administration of the aqueous preparation. Interestingly, defensin-2, a small antimicrobial peptide (accession no. Q62715), was detected through its tryptic peptides at m/z 1551.6 (amino acid positions 63-75) and distributed in part on the administration site. This species was still detected at both 30 minutes and 24 hours after administration of the aqueous formulation (data not shown) but absent at 24 hours after depot administration.

Standard histopathologic analyses of the H&E-stained cryosections showed no signs of inflammatory cells or necrotic tissue at the site or sites of vaccine administration. Moreover, the analyses did not provide any additional information, for example, on which cells in the tissue cryosections would be responsible for the release of the chemical mediators, inflammatory molecules, or both.

DISCUSSION

VIT is considered the most effective form of specific immunotherapy protecting allergic patients from systemic anaphylactic

reactions in case of an in-field sting. VIT is administrated subcutaneously; however, factors influencing the kinetics of HBV components and the local responses at the site of administration have not been elucidated. Here, a cutting-edge mass spectrometric imaging technique named MALDI-MSI was applied to assess the distribution of HBV components *in situ* after subcutaneous administration of purified aqueous and depot preparations at 30 minutes and 24 hours. The release of small molecules, such as biogenic amines and sulfoleukotrienes, was found to be very similar in both preparations at the 30-minute time point. However, the release of dopamine, histidine, and norepinephrine, as well as LTC₄ and LTD₄, seemed to be more intense and widely spread with the aqueous preparation (Figs 1-3). It is of interest that these chemical mediators were released and localized in different layers of the skin *in situ*. In the previous study by Francese et al,²⁰ release of these species was observed as soon as 1 minute after a rat was stung by a live honeybee, suggesting that skin-residing cells, such as keratinocytes, mast cells, and/or dendritic cells, might have important roles in the modulation of immune responses.²² Interestingly, defensin-2 was localized at the site of administration. Defensins can be produced by the number of epithelial cells, including epidermal keratinocytes, and they play a pivotal role in cutaneous immune defense and wound repair.²³ In addition, they probably play a key role in the awakening of the adaptive immune system, thus providing a link between innate and adaptive immune responses. However, their role in immune defense, regulation, or both after an insect sting, VIT, or both is not known and needs to be further investigated.

HBV-derived toxic peptides, such as Api m 4, MCD, and secapin, were also visualized at the site of the administration (Fig 1). However, compared with the previous study by Francese et al²⁰ with the live honeybee sting, much lower-density maps were observed, particularly for Api m 4. In addition, the majority of the small molecules, including the endogenous biogenic amines and the HBV-derived peptides, were reduced, absent, or both from the site of administration at the 24-hour time point (Fig 3). Our results are in line with the clinical studies and reports showing that reduction of these molecules can reduce the incidence of local side effects associated with VIT and furthermore improve compliance with VIT.^{10,16}

The MALDI-MSI detection of high-molecular-weight species (>20 kD), such as intact proteins, might have sensitivity issues.^{18,19} Therefore for the imaging analysis of higher-molecular-weight species, an alternative experimental strategy was adopted in which 1 tissue section was directly analyzed at the *m/z* range of 6,000 to 70,000, whereas the other section was preliminarily digested *in situ* by using trypsin and then analyzed at the *m/z* range of 100 to 3,000 to detect and image the distribution of the peptides generated by the *in situ* hydrolysis. PMF was used to identify proteins from the peptides generated. PMF and protein/peptide colocalization were used for protein identification, results of which were reported for proteins exhibiting the presence of a number of peptides greater than 2 (see Fig E2). However, in many instances the protein signals, although present in the extracted spectra, were too weak to be satisfactorily imaged (eg, because of signal suppression or low ionization yield because of heavy glycosylation). In some other cases, expected proteins did not show any mass signal in the spectra, likely because of low sensitivity (very high-molecular-weight species, presence of posttranslational modifications, or very low protein abundance). In these circumstances images of peptides

were generated from *in situ*-digested sections, and protein identities were tentatively assigned through PMF. With this strategy, species deriving from the formulations injected were detected and mapped. In particular, Api m 1 and HSA were observed at both 30 minutes and 24 hours after administration by using the depot formulation. Api m 2 was also detected; however, it was only present at 30 minutes after administration of the depot formulation and no longer detected at 24 hours (Fig 4). This was also observed in case of the aqueous formulation.

Early signs of inflammation could also be observed from the local release of cytokines, such as IL-1 β , IL-4, and IL-13. These findings are in line with the previous study in which an instant release of IL-4 and IL-13 from the 1-minute to 120-minute time point was shown.²⁰ A very-low-intensity signal for IL-4 was detected at 30 minutes after the administration of purified Api m 2. These data would need confirmation by means of *in situ* tandem mass spectrometry. Should it be confirmed, it would allow the suggestion that purified Api m 2 alone can induce T_H2-mediated inflammatory responses.

In pharmaceutical research essential information can be obtained from knowing the uptake of the active substances, their metabolic pathway processes, and/or the site or sites at which they occur after administration.¹⁹ In the present study proof of concept investigating the distribution of HBV allergens and formulation-derived components after administration of VIT preparations by means of MALDI-MSI was demonstrated. In addition, the biological response of the purified honeybee VIT preparations, inducing local release of chemical mediators and other inflammatory molecules, was demonstrated. Further studies combining, for example, MALDI-MSI and advanced immunohistochemistry are needed to further elucidate the action of the VIT preparations *in situ*. The *in situ* imaging provides an opportunity to understand organisms' responses to VIT because this information is necessary to fully understand the efficacy and safety and thus the viability of the active substances.

Key messages

- Biogenic amines and HBV-derived peptides were rapidly removed from the site of VIT administration.
- The Al(OH)₃-adsorbed HBV preparation demonstrated a depot effect, prolonging the availability of bee venom allergens at the site of administration.
- The present study shows that MALDI-MSI is a sensitive tool that can be used to investigate pharmacokinetics and the organism's response *in situ* to the administration of VIT preparations.

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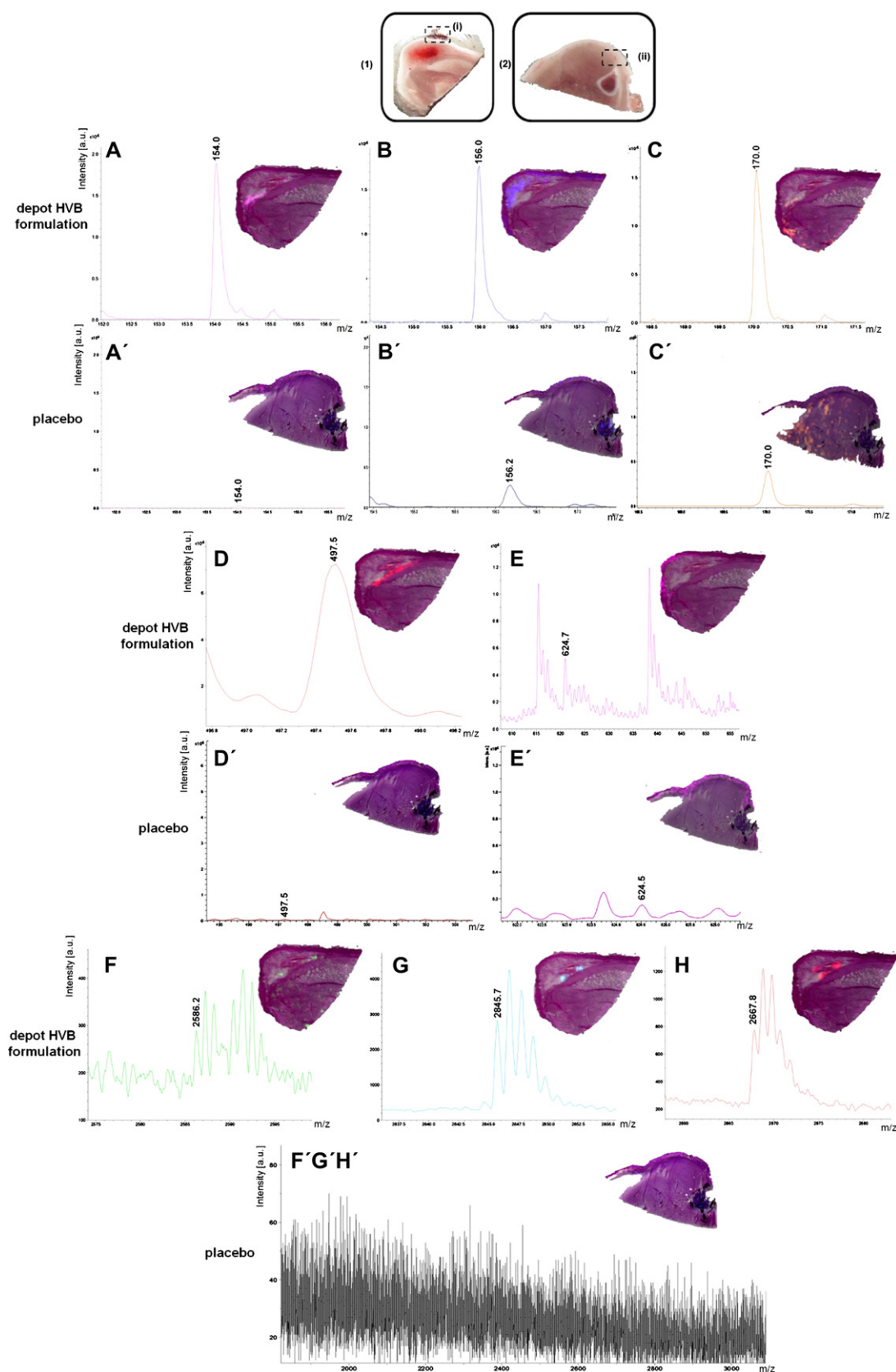


FIG E1. MALDI-MSI analysis of muscle cryosections from rats administered the $\text{Al}(\text{OH})_3$ -adsorbed HVB preparation (*depot*, 1) and the $\text{Al}(\text{OH})_3$ -adsorbed placebo (*placebo*, 2) at 30 minutes (with *panels i* and *ii* framing the injection area). **A** through **H** and **A'** through **H'** show the distribution of interesting endogenous species within the H&E-stained cryosections on administration of the depot and placebo, respectively.

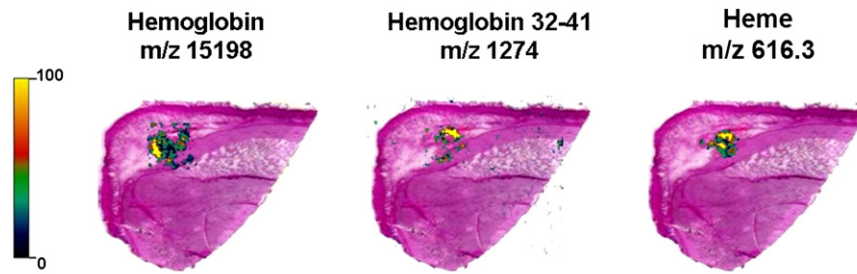


FIG E2. MALDI-MSI of hemoglobin in rat tissue administered with the depot preparation at 30 minutes. The distribution map of intact hemoglobin (m/z 15198) is shown alongside the distribution map of its peptide at m/z 1274 (resulting from the tissue digestion with trypsin) and of the heme group at m/z 617.

TABLE E1. Summary of MALDI MSI experiments and preparations

Study preparations	Time point	No. of animals/ study preparation	Summary of MALDI-MSI experiments	Detection range
Al(OH) ₃ -adsorbed HBV preparations	30 min	2	Direct MALDI-MSI Direct MALDI-MSI <i>In situ</i> digestion/MALDI -MSI	Small molecules/peptide range: 100-3,000 d Protein range: 6,000-70,000 d Peptide range: (100-3,000)
Al(OH) ₃ -adsorbed HBV preparations	24 h	2	Direct MALDI-MSI Direct MALDI-MSI <i>In situ</i> digestion/MALDI-MSI	Small molecules/peptide range: 100-3,000 d Protein range: 6,000-70,000 d Peptide range: (100-3,000)
Aqueous HBV preparations	30 min	2	Direct MALDI-MSI Direct MALDI-MSI <i>In situ</i> digestion/MALDI-MSI	Small molecules/peptide range: 100-3,000 d Protein range: 6,000-70,000 d Peptide range: (100-3,000)
Aqueous HBV preparations	24 h	2	Direct MALDI-MSI Direct MALDI-MSI <i>In situ</i> digestion/MALDI-MSI	Small molecules/peptide range: 100-3,000 d Protein range: 6,000-70,000 d Peptide range: (100-3,000)
Al(OH) ₃ -adsorbed placebo	30 min	2	Direct MALDI-MSI Direct MALDI-MSI	Small molecules/peptide range: 100-30,00 d Protein range: 6,000-70,000 d
Saline	30 min	2	Direct MALDI-MSI Direct MALDI-MSI	Small molecules/peptide range: 100-3,000 d Protein range: 6,000-70,000 d
Api m 2	30 min	2	Direct MALDI-MSI Direct MALDI-MSI	Small molecules/peptide range: 100-3,000 d Protein range: 6,000-70,000 d