UNIVERZA V LJUBLJANI
BIOTEHNIŠKA FAKULTETA

Nina ČELESNIK SMODIŠ

UGOTAVLJANJE SENZIBILIZACIJE IN SPREMLJANJE IMUNOTERAPIJE Z REKOMBINANTNIMI ALERGENI IZ STRUPOV KOŽEKRILCEV

DOKTORSKA DISERTACIJA

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RECOMBINANT ALLERGENS FOR MONITORING *HYMENOPTERA* VENOM SENSITIZATION AND IMMUNOTHERAPY

DOCTORAL DISSERTATION

Ljubljana, 2014

Doktorska disertacija je zaključek Univerzitetnega podiplomskega študija Biomedicine s področja genetike na Biotehniški fakulteti Univerze v Ljubljani. Raziskovalno delo je bilo opravljeno na Univerzitetni kliniki za pljučne bolezni in alergijo Golnik, v Laboratoriju za klinično imunologijo in molekularno genetiko.

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AI Specifična imunoterapija (SIT) s strupi kožekrilcev je edino učinkovito zdravljenje za preprečitev sistemskih preobčutljivostnih reakcij, povzročenih s piki žuželk iz redu kožekrilcev. Za uvedbo SIT je ključna potrditev senzibilizacije s primarnim povzročiteljem. Večina bolnikov je zaščitena že z uvedbo prvega vzdrževalnega odmerka, vendar natančni mehanizmi imunske tolerance niso povsem razjasnjeni. Primarnega povzročitelja smo potrjevali s kožnimi testi in določanjem sIgE za nativne strupe kožekrilcev. V primeru nejasnih, dvojno pozitivnih ali negativnih rezultatov za strup čebele in ose, pa s poglavitnimi vrstno-specifičnimi in navzkrižno reaktivnimi rekombinantnimi alergeni na nivoju IgE reaktivnosti in biološke aktivnosti ali testom aktivacije bazofilcev (BAT). Diagnostično uporabnost rekombinantnih alergenov smo primerjali z nativnimi. Prav tako smo preučevali vlogo receptorja FcεRI in z njim povezane vloge bazofilcev pri spodbuditvi kratkotrajne zaščite SIT s strupi kožekrilcev in ugotavljali specifičnost teh sprememb. Ugotovili smo majhno diagnostično občutljivost komercialno dostopnega alergena rApi m 1 iz *E. coli* (57 %) za ugotavljanje preobčutljivosti za strup čebele in veliko diagnostično občutljivost rVes v 5 in rVes v 1 iz Sf9 (92 %) za ugotavljanje preobčutljivosti za strup ose. Z uporabo rekombinantnih alergenov v testu BAT, rApi m 1 in rVes v 5 iz *E. coli* ter rApi m 2 iz Sf3, smo ugotovili primarno senzibilizacijo pri 93 % dvojno pozitivnih bolnikov po piku neznanega kožekrilca. Pri večini je bil vzrok za dvojno pozitivnost v navzkrižno reaktivni hialuronidazi (rApi m 2) z visoko biološko oz. alergogeno aktivnostjo. S testom BAT smo potrdili povzročitelja pri 81 % bolnikov z negativnimi sIgE in kožnimi testi. V primeru dvojno pozitivnega rezultata za strup čebele in ose je bil klinično pomemben tisti strup, ki je pri testu BAT spodbudil večji celični odziv. Pred prvim vzdrževalnim odmerkom SIT smo ugotovili statistično značilno zmanjšano občutljivost bazofilcev za protitelesa anti-FcεRI in za SIT-specifičen alergen. Enako smo ugotovili tudi za SIT-nespecifične alergene, ki niso bili vključeni v terapijo. Desenzibilizacija bazofilcev je bila povezana z zmanjšanim izražanjem receptorja FcεRI. Povzamemo lahko, da je komercialno dostopen alergen rApi m 1 omejeno primeren za ugotavljanje preobčutljivosti za strup čebele, medtem ko sta rVes v 5 in rVes v 1 primerna za ugotavljanje preobčutljivosti za strup ose. Uporaba rekombinantnih alergenov v testu BAT omogoča ugotovitev primarne senzibilizacije dvojno pozitivnih bolnikov, pri katerih ne gre zanemariti pomembne vloge hialuronidaze kot navzkrižno reaktivnega proteina. Uporaba testa BAT pri obravnavi dvojno negativnih bolnikov pogosto omogoča uvedbo SIT. Učinek kratkotrajne SIT s strupi kožekrilcev je za alergen nespecifičen in privede do desenzibilizacije bazofilcev po IgE/FcεRI poti, kar je možen mehanizem kratkotrajne zaščite.

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AB Venom immunotherapy (VIT) is the only effective treatment for the prevention of severe systemic allergic reactions induced by *Hymenoptera* stings. It has been proven to be effective as soon as the maintenance dose is achieved, but the mechanisms responsible for the early protection have not yet been explained in detail. The decision to commence VIT requires confirmation of allergic sensitization to the culprit insect. Confirmation of *Hymenoptera* venom allergy started with skin tests along with quantification of sIgE to native whole venom extracts. Diagnostic utility of recombinant species-specific and cross-reactive major allergens in form of IgE reactivity and biological activity or of basophil activation test (BAT) was evaluated in patients with inconclusive, double positive or negative results. Furthermore, diagnostic utility of recombinant major allergens was compared with native allergens. In addition, our aim was to evaluate the role of high-affinity IgE receptor (FcεRI), the related basophil function and its allergen specificity in the induction of short-term VIT protection. We found low diagnostic sensitivity of commercially available rApi m 1 (*E. coli*) for diagnosis of honeybee (57%) and a high diagnostic sensitivity (92%) of rVes v 5 and rVes v 1 (Sf9) for diagnosis of *Vespula* allergy. With the use of *E. coli*-expressed rApi m 1 and rVes v 5 and Sf3-expressed rApi m 2 in BAT we were able to identify honeybee and/or *Vespula* allergy in 93% of double positive patients with an unknown culprit insect. In majority of them the cause for double positivity seemed to be in protein-based sensitization to hyaluronidase (rApi m 2), with a very high allergenic activity. BAT allowed prompt diagnosis in 81% of patients with negative venom-specific IgE and skin tests. In the case of double positive BAT, the culprit insect correlated with the venom that induced a significantly higher basophil response. We demonstrated a marked reduction of basophil threshold sensitivity to anti-FcεRI and VIT-specific venom before the first maintenance dose. Moreover, a significant and comparable decrease was also evident for non-VIT allergens. This suppression was also related to the changes at the level of FcεRI expression. In conclusion, commercially available rApi m 1 has limited clinical usefulness for detection of honeybee allergy, whereas rVes v 5 and rVes v 1 should be helpful for the serological dissection of *Vespula* allergy. Recombinant-based BAT allowed identification of the disease-causing insect in majority of double positive patients. Our results also suggest the importance of hyaluronidase in case of protein based cross-reactivity. The routine use of BAT should facilitate prescription of VIT in complex cases with negative results. Short-term VIT induces desensitization of FcεRI-mediated basophil response, which seems to be allergen-nonspecific. This suppression could be highly relevant for the development of early protective mechanisms.

KAZALO VSEBINE

KAZALO ZNANSTVENIH DEL

OBJAVLJENA ZNANSTVENA DELA

OSTALO POVEZOVALNO ZNANSTVENO DELO

KAZALO PREGLEDNIC

KAZALO SLIK

KAZALO PRILOG

KRATICE IN OKRAJŠAVE

Api m 1	fosfolipaza A2
Api m 2	čebelja hialuronidaza
BAT	test aktivacije bazofilcev (ang.: Basophil activation test)
cAMP	ciklični adenozin monofosfat
CCD	navzkrižno reaktivne ogljikohidratne determinante (ang.: Cross-reactive carbohydrate determinants)
CD	določena molekula izražena na površini celice (ang.: Cluster of differention)
EAACI	Evropska akademija za alergologijo in klinično imunologijo (ang.: European Academy of Allergy and Clinical Immunology)
FcεRI	receptor velike afinitete za protitelesa IgE
FEIA	fluorescenčni encimskoimunski test (ang.: Fluorescence enzyme immuno assay)
IFN-y	interferon γ
IgA	imunoglobulin razreda A
IgE	imunoglobulin razreda E
IgG	imunoglobulin razreda G
IgG_1	imunoglobulin razreda G, podrazred G1
IgG_4	imunoglobulin razreda G, podrazred G4
IL	interlevkin
LAMP-3	lizosomski membranski protein 3
LLR	velika lokalna reakcija (ang.: Large local reaction)
n	nativni
OSR	oljna repica (ang.: Oilseed rape)
r	rekombinantni
Sf	celična linija iz vrste insektov *Spodoptera frugiperda*
sIgE	specifična protitelesa imunoglobulinov razreda E
SIT	specifična imunoterapija
TGF-β	dejavnik tumorske rasti β
Tr1	regulatorni limfociti T tipa 1
Treg	regulatorni limfociti T $CD4^+25^+$
Ves v 1	fosfolipaza A1
Ves v 2	osja hialuronidaza
Ves v 5	antigen 5

1 PREDSTAVITEV PROBLEMATIKE IN HIPOTEZE

Piki žuželk iz redu kožekrilcev (*Hymenoptera*) pri večini ljudi povzročijo začasen lokalni vnetni odziv z bolečino, srbežem, rdečino ter oteklino. Pri približno 5 odstotkih populacije pa lahko povzročijo sistemske preobčutljivostne reakcije (Golden in sod., 1989; Charpin in sod., 1992; Bilo in sod., 2005; Sturm in sod., 2014).

Znotraj našega geografskega okolja so najpogostejši piki čebel (*Apis mellifera*), os (*Vespula* spp.) in sršenov (*Vespa crabro*) (Müller, 1990).

Preobčutljivost za strup kožekrilcev predstavlja resen zdravstveni problem in je eden pogostejših vzrokov za težjo in potencialno usodno sistemsko anafilaktično reakcijo s prizadetostjo dihal in obtočil, ki se zgodi nemudoma po stiku z alergenom (Sampson in sod., 2006; Bilo in Bonifazi, 2009; Bilo, 2011).

Približno 75 % bolnikov z anamnezo težje sistemske preobčutljivostne reakcije, le-to ponovno doživijo ob ponovnem piku kožekrilca (Bonifazi in sod., 2005). Pri teh bolnikih je potrditev senzibilizacije s primarnim povzročiteljem ključna za uvedbo zdravljenja s specifično imunoterapijo (SIT) s strupi kožekrilcev, ki preprečuje nadaljne sistemske preobčutljivostne reakcije. Vendar veliko bolnikov identitete kožekrilca ne prepozna. V diagnostičnem postopku pa pogosto naletimo na dvojno pozitivne lahko pa tudi na dvojno negativne rezultate za strup čebele in ose, kar predstavlja problem pri izbiri ustreznega alergena za SIT. Zato je diagnostične metode na področju preobčutljivosti za strupe kožekrilcev potrebno izboljšati ter ugotoviti primarno senzbilizacijo pri vseh bolnikih z velikim tveganjem za potencialno življenje ogrožajočo preobčutljivostno reakcijo.

SIT je edino učinkovito zdravljenje za preprečitev sistemskih preobčutljivostnih reakcij, povzročenih s piki kožekrilcev. Večina bolnikov je zaščitena že z uvedbo prvega vzdrževalnega odmerka, vendar natančni mehanizmi imunske tolerance niso povsem razjasnjeni. Prav tako ne poznamo *in vitro* metode s katero bi lahko spremljali potek in uspešnost zdravljenja, saj se dolgotrajna toleranca za alergen ne vzpostavi pri približno 15 – 25 % bolnikov, zdravljenih s strupom čebele in 5 % bolnikov, zdravljenih s strupom ose (Golden, 2005).

1.1 PREOBČUTLJIVOSTNA REAKCIJA ZA STRUPE KOŽEKRILCEV

Alergija za strupe kožekrilcev spada med takojšnje preobčutljivostne reakcije tipa I posredovane s protitelesi imunoglobulinov razreda E (IgE). Navzkrižna povezava

alergena in specifičnih protiteles IgE (sIgE), povezanih z receptorji velike afinitete za protitelesa IgE (FcεRI) na površini mastocitov in bazofilcev, sproži signalizacijsko kaskado kar se v nekaj minutah odrazi z degranulacijo in s sproščanjem mediatorjev preobčutljivostnega odziva: vazoaktivnih aminov (npr. histamina), lipidnih mediatorjev (prostaglandinov, trombocit-aktivirajočega faktorja, levkotrienov), kemokinov ter drugih citokinov (IL-4, IL-5 in IL-13) (Larche in sod., 2006; Knol, 2006). Posledica učinka mediatorjev, ki se sprostijo iz efektorskih celic, je razvoj kliničnih simptomov preobčutljivostne reakcije.

Preobčutljivostne reakcije tipa I so posredovane z IgE, kljub temu več kot tretjina oseb s prisotnimi sIgE ne razvije simptomov in/ali bolezni ob stiku s specifičnim alergenom (Bilo in sod., 2005; Bilo in Bonifazi, 2011; Sturm in sod., 2014). Aktivacija efektorskih celic, kot so mastociti in/ali bazofilci, je torej ključna za klinični razvoj bolezni.

Preobčutljivostna reakcija po piku kožekrilca lahko poteka v obliki lokalne ali sistemske reakcije. Za veliko lokalno preobčutljivostno reakcijo (ang.: Large local reaction, LLR) je značilna oteklina premera ≥ 10 cm, ki nastane na mestu pika in lahko vztraja več dni. Klinično sliko sistemske preobčutljivostne reakcije pa se najpogosteje ovrednoti s klasifikacijo po Muellerju, s stopnjevanimi razredi od I do IV (Pregl. 1). Glede na resnost kliničnega stanja, sistemske preobčutljivostne reakcije razdelimo v lažjo obliko, s prizadetostjo kože, podkožja in sluznic, ter težjo obliko, za katero je značilna prizadetost dihal in obtočil (Bilo in sod., 2005; Bilo in Bonifazi, 2011).

Preglednica 1: Razporeditev sistemskih preobčutljivostnih reakcij za strupe kožekrilcev po Muellerju (Mueller, 1966; Bilo in sod., 2005: 1342)

Table 1: Classification of systemic reactions to insect stings by Mueller (Mueller, 1966; Bilo et al., 2005: 1342)

Stopnja reakcije	Klinična slika
I	generalizirana koprivnica, srbenje, oslabelost, strah
II	poleg simptomov st. I še vsaj 2 od navedenih: angioedem, stiskanje v prsih, slabost, bruhanje, diareja, bolečina v trebuhu, omotica
III	poleg simptomov st. II še vsaj 2 od navedenih: dušenje, piskanje, stridor, motnje govora, hripavost, oteženo požiranje, zmedenost
IV	poleg simptomov st. III še vsaj 2 od navedenih: hipotenzija, kolaps, izguba zavesti, inkontinenca urina in/ali blata, cianoza

1.2 SESTAVA STRUPA KOŽEKRILCEV

Strup kožekrilcev sestoji iz biogenih aminov, bazičnih peptidov in proteinov z veliko molekulsko maso, večinoma encimov, ki predstavljajo glavne alergogene komponente strupa (Pregl. 2). Poglavitna alergena čebeljega strupa sta fosfolipaza A2 (Api m 1) ter hialuronidaza (Api m 2) (Müller, 2002; Bilo in sod., 2005; Bilo in Bonifazi, 2011; Bilo in sod., 2012). Poglavitni alergeni osjega strupa so antigen 5 (Ves v 5), fosfolipaza A1 (Ves v 1) in hialuronidaza (Ves v 2) (Müller, 2002; Bilo in sod., 2005; Bilo in Bonifazi, 2011).

Preglednica 2: Alergeni iz strupov kožekrilcev (Bilo in sod., 2012: 1155; Hoffman, 2006; Blank in sod., 2010, 2013)

Table 2: Allergens of *Hymenoptera* venoms (Bilo et al., 2012: 1155; Hoffman, 2006; Blank et al., 2010, 2013)

Kožekrilec	Alergen	Biokemijsko ime
čebela	Api m 1	*fosfolipaza A2
osa	Ves v 1	*fosfolipaza A1
čebela / osa	Api m 2 / Ves v 2	*hialuronidaza
osa	Ves v 5	*antigen 5
čebela	Api m 3	kisla fosfataza
čebela	Api m 4	melitin
čebela / osa	Api m 5 / Ves v 3	dipeptidil peptidaza IV
čebela	Api m 6	s cisteinom bogati inhibitor tripsina
čebela	Api m 7	serinska proteaza CUB
čebela	Api m 8	karboksilesteraza
čebela	Api m 9	serinska karboksilesteraza
čebela	Api m 10	ikarapin
čebela	Api m 11	poglavitni protein »royal jelly«
čebela / osa	Api m 12 / Ves v 6	vitelogenin

* poglavitni alergeni

* major allergens

1.2.1 Rekombinantni alergeni iz strupov kožekrilcev

Rekombinantne alergene iz strupov kožekrilcev se proizvaja v bakterijskih (nastanejo neglikozilirani proteini) ali evkariontskih (nastanejo glikozilirani proteini) sistemih za izražanje genov.

Proizvodnja neglikoziliranih alergenov, za katero se najpogosteje uporablja *Escherichia coli* (*E. coli*), je relativno preprosta in cenovno ugodna. Poleg tega ne vsebujejo potencialno navzkrižno reaktivnih in/ali klinično nepomembnih ogljikohidratnih epitopov. Vendar izguba glikozilacije lahko vpliva na zvijanje proteinov ter s tem na njihovo terciarno strukturo, ki je osnova za epitopsko funkcionalnost alergenov. Pri

neglikoziliranih rekombinantnih alergenih je zaradi odsotnosti ogljikohidratnih epitopov lahko zmanjšana njihova občutljivost za ugotavljanje preobčutljivosti.

Pri proizvodnji glikoziliranih rekombinantnih alergenov se struktura proteinov in njihova epitopska funkcionalnost ohrani. Glikozilirani rekombinantni alergeni imajo tako primerljive lastnosti vezave protiteles IgE kot nativni alergeni (Soldatova in sod., 1998). Za njihovo pripravo se pogosto uporablja kvasovke ali z bakulovirusom okužene celične linije insektov. Takšna proizvodnja je dražja in mnogo bolj kompleksna. Po drugi strani vezava IgE na ogljikohidratne epitope, ki jih vsebujejo tako nativni kot tudi glikozilirani rekombinantni alergeni, lahko povzroči lažno pozitivne rezultate kar zmanjšuje njihovo specifičnost (Müller in sod., 2009; Mittermann in sod., 2010).

V izogib omenjenih pomanjkljivostim, se je rekombinantne alergene iz strupov kožekrilcev pred kratkim začelo proizvajati v celičnih linijah insektov (*Spodoptera frugiperda* – Sf), ki ne vsebujejo encima α-1,3-fukoziltransferaze (Seismann in sod., 2010). Posledično se tvorijo proteini brez α-1,3-fukoze, ključne strukture N-glikozirajočega vezavnega mesta. Ti rekombinanti imajo primerljivo strukturo in epitopsko funkcionalnost kot nativni alergeni, a so brez navzkrižno reaktivnih in/ali klinično nepomembnih ogljikohidratnih epitopov.

1.3 DIAGNOSTIKA PREOBČUTLJIVOSTI ZA STRUPE KOŽEKRILCEV

Glede na priporočila smernic Evropske akademije za alergologijo in klinično imunologijo (ang.: European Academy of Allergy and Clinical Immunology, EAACI) je osnova za diagnosticiranje preobčutljivosti za strupe kožekrilcev natančna klinična anamneza. V nadaljevanju diagnostičnega postopka povzročitelja potrjujemo s kožnimi testi in določanjem specifičnih protiteles IgE proti nativnim izvlečkom celokupnega strupa kožekrilcev v krvi (Bilo in sod., 2005; Bonifazi in sod., 2005; Bilo in Bonifazi, 2009). Vendar identiteta primarnega povzročitelja preobčutljivostne reakcije pogosto ostane neznana. V primeru nejasnih, dvojno pozitivnih ali negativnih rezultatih za strup čebele in ose, je potrebno poseči po dodatnih diagnostičnih metodah. Na področju diagnosticiranja preobčutljivosti za strupe kožekrilcev sta na voljo tehnologiji celičnega *in vitro* testa aktivacije bazofilcev ter uporaba rekombinantnih alergenov iz strupov kožekrilcev.

1.3.1 Kožni testi

Za diagnosticiranje preobčutljivosti za strupe kožekrilcev se izvajajo kožni vbodni in intradermalni testi. Za kožne vbodne teste na volarno stran podlahti vnašamo običajno

koncentracije strupov od 0,01 do 100 µg/ml, za intradermalne pa koncentracije od 0,001 do 1 µg/ml. Občutljivost kožnih vbodnih testov je tudi pri najvišji koncentraciji 100 µg/ml strupa (60 – 70 %), manjša od občutljivosti intradermalnih testov (82 – 93 %) (Sturm in sod., 2004, 2011a; Bilo in sod., 2005; Ebo in sod., 2007b; Peternelj in sod., 2009). Zato je pri bolnikih z negativnimi kožnimi vbodnimi testi priporočljivo izvesti še intradermalne teste (Bilo in sod., 2005).

1.3.2 Določanje specifičnih protiteles IgE

Pri diagnosticiranju preobčutljivosti za strupe kožekrilcev se za določevanje sIgE zelo pogosto uporablja sistem ImmunoCAP-FEIA (Thermo Fisher Scientific, Uppsala, Švedska). S tem sistemom v serumu ali plazmi bolnikov kvantitativno merimo koncentracijo sIgE za alergen na principu fluorescenčno encimskoimunskega testiranja (ang.: Fluorescence enzyme immuno assay, FEIA). Meritveno območje za sIgE obsega vrednosti od 0 do 100 kU/l. Referenčna vrednost za sIgE je < 0,35 kU/l.

Občutljivost sistema ImmunoCAP-FEIA za določevanje sIgE proti nativnim strupom kožekrilcev je nekoliko manjša (76 – 91 %) od občutljivosti intradermalnih kožnih testov (do 93 %) (Sturm in sod., 2004, 2011a; Bilo in sod., 2005; Peternelj in sod., 2009; Vos in sod., 2012, Ebo in sod., 2013). Specifičnost je zaradi (pre)visokega deleža (do 60 %) dvojno pozitivnih rezultatov za strup čebele in ose prav tako omejena, saj je večina 90 – 95 % preobčutljiva le za posamezen strup kožekrilca (Jappe in sod., 2006; Mittermann in sod., 2010; Eberlein in sod., 2012; Müller in sod., 2012). Pogost problem predstavljajo lažno dvojno pozitivni rezultati, ki nastanejo zaradi prisotnosti navzkrižno reaktivnih protiteles IgE, usmerjenih proti homolognim strukturam obeh strupov (Hemmer in sod., 2001; Müller in sod., 2009; Mittermann in sod., 2010).

V prvi fazi *in vitro* diagnostičnega postopka določamo koncentracijo sIgE proti nativnim izvlečkom celokupnega strupa kožekrilcev v krvi (Bilo in sod., 2005; Bonifazi in sod., 2005; Bilo in Bonifazi, 2009). Razvoj rekombinantne tehnologije pa je omogočil proizvodnjo posameznih komponent iz strupov kožekrilcev (Pregl. 2). Možno je določevanje koncentracije sIgE proti posameznim alergenom iz strupov kožekrilcev, ki so vrstno-specifični in ne vsebujejo navzkrižno reaktivnih struktur.

Prvi komercialno dostopen rekombinantni alergen Api m 1 je prišel na tržišče konec leta 2009, namenjen za široko diagnostično uporabo v sistemu ImmunoCAP-FEIA. Poglavitni nativni (n) alergen čebeljega strupa (nApi m 1) je pri diagnosticiranju preobčutljivosti za strup čebele nadomestil nov rekombinantni (r) neglikoziliran alergen (rApi m 1) proizveden v *E. coli*. Nekoliko kasneje, leta 2010 in 2011, sta komercialno

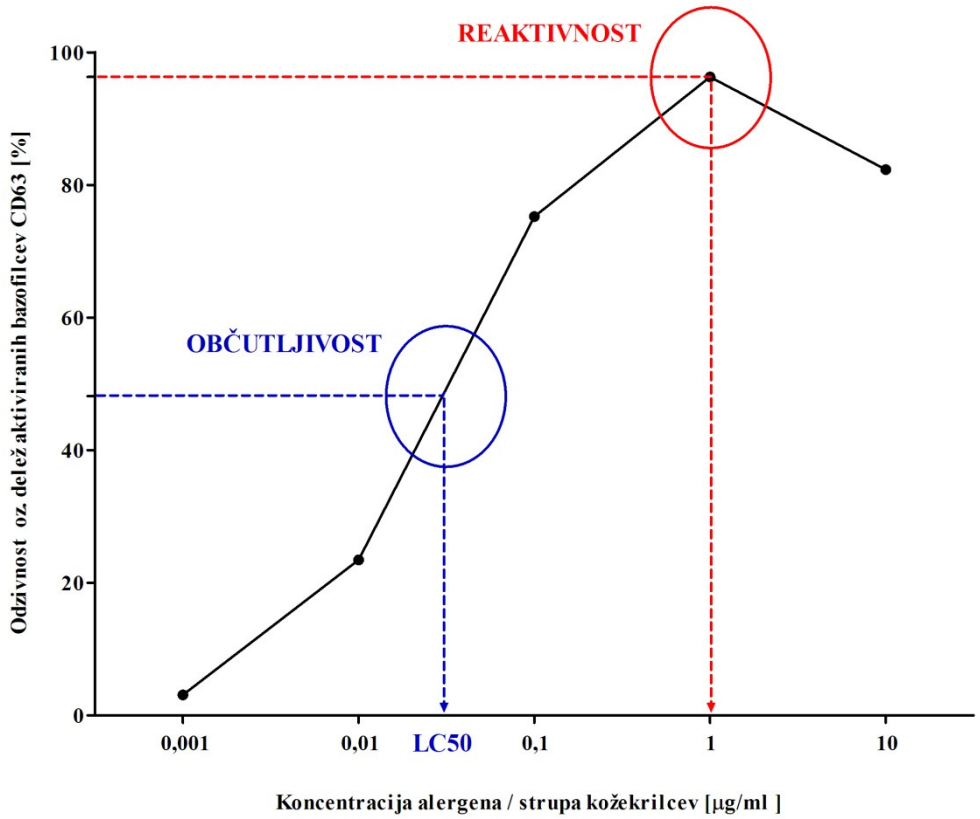
dostopna postala še poglavitna alergena osjega strupa, rVes v 5 ter rVes v 1, proizvedena v celičnih linijah insektov Sf9.

Dodana vrednost novih komercialno dostopnih poglavitnih rekombinantnih alergenov za strup čebele in ose v primerjavi z nativnimi je v tem, da so vrstno-specifični in ne vsebujejo navzkrižno reaktivnih ogljikohidratnih epitopov. Vendar je diagnostično občutljivost novih rekombinantnih alergenov potrebno testirati na veliki skupini dobro opredeljenih bolnikov ter ugotoviti njihovo ustreznost za široko diagnostično uporabo. S tem namenom smo diagnostično občutljivost rApi m 1 za ugotavljanje preobčutljivosti za strup čebele preverili na veliki skupini 184 preiskovancev z jasno definirano preobčutljivostjo za strup čebele in diagnostično občutljivost rVes v 5 in rVes v 1 za ugotavljanje preobčutljivosti za strup ose na 200 preiskovancih z jasno definirano preobčutljivostjo za strup ose, ter ju primerjali z diagnostično občutljivostjo nativnega alergena za strup posameznega kožekrilca.

1.3.3 Test aktivacije bazofilcev

S celičnim *in vitro* testom aktivacije bazofilcev (ang.: Basophil activation test, BAT) s pomočjo pretočnega citometra v periferni krvi merimo izražanje označevalcev, ki se pojavijo na površini bazofilcev po aktivaciji z alergenom. Navzkrižna povezava receptorjev FcεRI s protitelesi IgE in alergenom na površini bazofilcev povzroči aktivacijo in spremenjeno izražanje številnih površinskih proteinov, kot so CD45, CD63, CD69 in CD203c (Hamilton in Adkinson, 2004). Izražanje 53 kDa velikega glikoproteina (lizosomskega membranskega proteina 3, LAMP-3) – površinskega označevalca CD63, se je izkazalo kot najbolj napovedno pri procesu anafilaktične degranulacije (Ebo in sod., 2008; MacGlashan, 2010). Pri neaktiviranih bazofilcih je CD63 izražen znotrajcelično na membrani granul, ki vsebujejo histamin. S celično aktivacijo se granule z eksocitozo zlijejo s plazemsko membrano, kar omogoči sledenje njegovega izražanja na zunajcelični membrani bazofilcev (Knol in sod., 1991).

Pri testu BAT vzorec krvi *in vitro* stimuliramo z različnimi koncentracijami alergena v logaritemskem merilu, npr. z od 0,01 do 1 μg/ml strupa (Peternelj in sod., 2008a). Pogosto uporabljamo tribarvno tehniko anti-CD123/anti-HLA-DR/anti-CD63, kjer so bazofilci označeni kot $CD123^+/HLA\text{-}DR^-$ celice. Odzivnost oz. delež aktiviranih bazofilcev ($CD123^+/HLA\text{-}DR^-/CD63^+$) lahko izrazimo v obliki sigmoidne krivulje, ki se med preiskovanci močno razlikujejo. Spremljamo lahko dve neodvisni spremenljivki, celično reaktivnost in celično občutljivost (MacGlashan, 1993) (Slika 1).



Slika 1: Sigmoidna krivulja odzivnosti bazofilcev CD63 po stimulaciji s strupom kožekrilca
Odzivnost oz. delež aktiviranih bazofilcev CD63 [%] (os y) in različne koncentracije alergena v logaritemskem merilu, od 0,001 do 10 µg/ml strupa kožekrilcev (os x). Celična reaktivnost merjena pri konc. 1 µg/ml strupa označena z rdečo in celična občutljivost kot LC50 označena z modro.

Figure 1: CD63 basophil dose-response curve after venom stimulation
CD63 basophil activation [%] (y axis) and various allergen concentrations in logaritmic scale, from 0.001 till 10 µg/ml of *Hymenoptera* venom (x axis). Cellular reactivity at 1 µg/ml of venom stimulation marked with red and cellular sensitivity as LC50 marked with blue.

Celična reaktivnost odraža maksimalen odziv celic, običajno pri stimulaciji z višjimi koncentracijami alergena in predstavlja vrh sigmoidne krivulje (MacGlashan, 1993; Ebo in sod., 2008). Pri preobčutljivosti za kožekrilce, reaktivnost bazofilcev merimo pri koncentraciji 1 µg/ml strupa (Slika 1) in je pomembna za diagnostično potrditev IgE senzibilizacije z referenčno vrednostjo ≥ 15 % aktiviranih bazofilcev (Košnik in sod., 2005; Peternelj in sod., 2008b, 2009; Korošec in sod., 2009; Žitnik in sod., 2012).

Celična občutljivost, kot polovična vrednost maksimalnega odziva, predstavlja mejno vrednost aktivacije celic pri stimulaciji s submaksimalnimi koncentracijami alergena (Slika 1). Občutljivost bazofilcev lahko izražamo z različnimi spremenljivkami: kot razmerje odzivnosti merjene z dvema koncentracijama alergena (0,1/1) (Košnik in sod., 2005; Žitnik in sod., 2012), s primerjavo odziva pri različnih submaksimalnih

koncentracijah alergena (Lalek in sod., 2010; Zidarn in sod., 2012), kot koncentracijo alergena, ki sproži 50 % maksimalnega odziva celic (LC50 ali C50) (Mikkelsen in sod., 2010; Eberlein in sod., 2012) ter najpogosteje kot CD-sens (Johansson in sod., 2005; Nopp in sod., 2006, 2009). Pri izračunu za CD-sens recipročno vrednost koncentracije alergena, ki je sprožil 50 % maksimalnega odziva celic, množimo s 100. Tako višja vrednost CD-sens predstavlja višjo občutljivost celic.

V najnovejših raziskavah občutljivost bazofilcev pridobiva vedno večji pomen. Nakazuje se, da je pri preobčutljivosti za kožekrilce pomembna za ugotavljanje primarne senzibilizacije (Eberlein in sod. 2012), pri napovedovanju stranskih učinkov specifične imunoterapije (Košnik in sod., 2005; Žitnik in sod., 2012), ter tudi pri spremljanju uspešnega odziva na zdravljenje in razvoja dolgotrajne tolerance (Peternelj in sod., 2008a, Žitnik in sod., 2012; Eržen in sod., 2012).

Pri diagnosticiranju preobčutljivosti za strupe kožekrilcev se je BAT izkazal kot visoko občutljiva (85 – 100 %) in specifična metoda (83 – 100 %) (Erdmann in sod., 2004; Sturm in sod., 2004; Eberlein-König in sod., 2006; Peternelj in sod., 2008b, 2009; Košnik in Korošec, 2009).

1.3.4 Bolniki z dvojno pozitivnimi rezultati

Dvojno pozitivnost, tako za čebelji kot tudi za osji strup, s standardnimi rutinskimi diagnostičnimi testi zasledimo pri 30 – 60 % bolnikov preobčutljivih za strupe kožekrilcev (Müller in sod., 2009, 2012; Mittermann in sod., 2010; Sturm in sod., 2011a; Eberlein in sod., 2012). Do pojava dvojne pozitivnosti pride zaradi senzibilizacije z obema strupoma ali zaradi navzkrižne reaktivnosti (King in sod., 1996; Hemmer in sod., 2001, 2004; Bilo in sod., 2005; Bonifazi in sod., 2005; Eržen in sod., 2009; Bilo in Bonifazi, 2011).

Navzkrižna reaktivnost med strupoma je prisotna zaradi vezave specifičnih protiteles IgE na homologne strukture proteinov in/ali na klinično nepomembne navzkrižno reaktivne ogljikohidratne determinante (ang.: Cross-reactive carbohydrate determinants, CCD) obeh strupov (Van der Veen in sod., 1997; Van Ree R in Aalberse, 1999; Hemmer in sod., 2001; Van Ree R, 2002; Altmann, 2007; Eržen in sod., 2009; Mertens in sod., 2010). Najpogostejši navzkrižno reaktiven protein je hialuronidaza, katero vsebujeta strupa iz obeh družin kožekrilcev. Do navzkrižne reaktivnosti med hialuronidazama (Api m 2 in Ves v 2) lahko privede njuna 50-odstotna homologija peptidnih epitopov ali CCD (Hoffman in Wood, 1984; Wypych in sod., 1989; Bonifazi in sod., 2005; Jin in sod., 2010). Poleg hialuronidaze, homologne navzkrižno reaktivne

strukture vsebujeta tudi proteina dipeptidil peptidaza IV (Api m 5 in Ves v 3) (Blank in sod., 2010) in vitelogenin (Api m 12 in Ves v 6) (Blank in sod., 2013) (Pregl. 2).

Delež dvojno pozitivnih diagnostičnih testov je (pre)visok, saj je večina bolnikov preobčutljiva za strup le enega kožekrilca, ki ga pogosto ne znajo identificirati. Ko identiteta kožekrilca ostaja neznana, je potrebna nadaljnja diagnostična obravnava, da ugotovimo ali je vzrok za dvojno pozitivnost v primarni dvojni senzibilizaciji ali navzkrižni reaktivnosti. Ugotovitev primarne senzibiliziranosti bolnikov je ključnega pomena za izbiro ustreznega alergena za zdravljenje s SIT (Müller in sod., 2009; Mittermann in sod., 2010; Hofmann in sod., 2011). V pomoč je možen dodaten pristop z inhibicijskimi testi, testom aktivacije bazofilcev ter določevanje specifičnih protiteles IgE za rekombinantne alergene.

Z inhibicijskimi testi lahko ugotovimo primarno senzibilizacijo pri približno 15 % dvojno pozitivnih bolnikov (Straumann in sod., 2000; Košnik in Korošec, 2009). Za izvedbo inhibicijskih testov je potrebna relativno visoka koncentracija sIgE v krvi, metoda ima pogosto težavno interpretacijo rezultatov in je zato manj primerna za vsakodnevno rutinsko diagnostiko (Müller in sod., 2009; Hofmann in sod., 2011). Test aktivacije bazofilcev je z merjenjem celične reaktivnosti omogočil določitev primarne senzibilizacije pri 17 – 33 % dvojno pozitivnih bolnikov (Sturm in sod., 2004; Peternelj in sod., 2008b, 2009; Mertens in sod., 2010). Njegova izvedba je možna tudi pri bolnikih z nizkimi koncentracijami sIgE (Košnik in Korošec, 2009). Test aktivacije bazofilcev je zmanjšal delež dvojno pozitivnih rezultatov ter s tem izboljšal specifičnost, kljub temu jih pri testiranju z nativnimi alergeni še vedno zasledimo pri okoli treh četrtinah do dveh tretjinah dvojno pozitivnih bolnikov (Sturm in sod., 2004; Eberlein-König in sod., 2006; Peternelj in sod., 2008b, 2009; Košnik in Korošec, 2009; Mertens in sod., 2010). Rekombinantni alergeni iz strupov kožekrilcev ne vsebujejo klinično nepomembnih CCD, ki povzročajo lažno dvojno pozitivne rezultate (Müller in sod., 2009; Mittermann in sod., 2010). Vendar je zelo pomembna kakovost oziroma epitopska funkcionalnost alergena. V »in-house« sistemu se je kombinacija poglavitnih vrstno-specifičnih in navzkrižno reaktivnih rekombinantnih alergenov iz strupa čebele in ose (rApi m 1, rVes v 5, rApi m 2) izkazala z visoko napovedno vrednostjo primarne senzibilizacije (Mittermann in sod., 2010). Medtem ko je bila napovedna vrednost komercialno dostopnih rekombinantnih alergenov rApi m 1 ter rVes v 5 s sistemom ImmunoCAP-FEIA, znatno nižja (Eberlein in sod., 2012).

Za dopolnitev omenjenih pomanjkljivosti smo združili oba pristopa z uporabo poglavitnih rekombinantnih alergenov v testu aktivacije bazofilcev pri bolnikih s sistemsko preobčutljivostno reakcijo po piku neznanega kožekrilca in dvojno pozitivnimi sIgE in testom aktivacije bazofilcev z nativnimi alergeni. Občutljivost in biološko aktivnost rekombinantnih alergenov smo želeli potrditi na nivoju aktivacije

bazofilcev, ki so poleg mastocitov ključni za nastanek simptomov preobčutljivostne reakcije. Preizkusili smo diagnostično uporabnost glikoziliranih ter neglikoziliranih, vrstno-specifičnih ter navzkrižno reaktivnih rekombinantnih alergenov čebeljega in osjega strupa pridobljenih z različnimi celičnimi kulturami ter jih primerjali na nivoju celične in IgE reaktivnosti. Naš namen je bil ugotoviti ali je uporaba poglavitnih rekombinantnih alergenov v testu aktivacije bazofilcev primerna za določitev primarne senzibilizacije z nativnimi alergeni dvojno pozitivnih bolnikov.

1.3.5 Bolniki z negativnimi rezultati

Pri vseh bolnikih s prepričljivo klinično anamnezo sistemske preobčutljivostne reakcije po piku kožekrilca, ne zaznamo za strup specifičnih protiteles IgE (< 0,35 kU/l) ali pozitivnega kožnega testa (Day in sod., 1994; Hoffman, 2003). Za strup specifičnih protiteles IgE in pozitivnega kožnega vbodnega testa niso zaznali pri 4 % od 1219 bolnikov s težjo sistemsko reakcijo po piku kožekrilca (Korošec in sod., 2009). Golden in sod. (2001) jih v svoji raziskavi niso zaznali celo pri 18 % bolnikov in ocenjujejo, da se pri tretjini preobčutljivih bolnikov, senzibilizacija za strup ne odrazi s pozitivnim intradermalnim kožnim testom. Ti bolniki lahko naknadno ponovno doživijo življenje ogrožajočo težjo sistemsko reakcijo (Golden in sod., 2001; Hoffman, 2003). V več kot 30 odstotkih primerov anafilaktične reakcije s smrtnim izidom po piku kožekrilca, so bila za strup specifična protitelesa IgE zelo nizka ali celo nezaznavna (Hoffman, 2003).

Za diagnosticiranje bolnikov, ki se ne odzovejo na standardna rutinska testiranja, se za potrditev senzibilizacije vedno bolj uveljavlja celični *in vitro* test BAT. V primerjavi z določanjem sIgE in kožnimi vbodnimi testi je imel večjo občutljivost (90 % proti 76 % in 64 %) ter primerljivo ali večjo pozitivno napovedno vrednost (84 % proti 77 % in 22 %) (Peternelj in sod., 2009). Merjenje reaktivnosti bazofilcev omogoča identifikacijo primarnega kožekrilca pri približno dveh tretjinah bolnikov z negativnimi sIgE in kožnimi testi (Ebo in sod., 2007a; Korošec in sod., 2009).

Pri bolnikih s prepričljivo anamnezo sistemske preobčutljivostne reakcije za strup kožekrilca in negativnimi sIgE in kožnimi testi so predhodne raziskave pokazale veliko diagnostično občutljivost testa aktivacije bazofilcev pri potrjevanju senzibilizacije. Zato smo celični *in vitro* test BAT v kompleksnih primerih z dvojno negativnimi rezultati standardnih rutinskih diagnostičnih testov za strup čebele in ose, začeli uporabljati v vsakodnevni rutinski diagnostiki. V preiskovanem obdobju 2 let in pol smo pri bolnikih z anamnezo težje sistemske preobčutljivostne reakcije diagnostično uporabnost testa BAT, želeli primerjati z drugimi možnimi diagnostičnimi pristopi ter določiti njegovo napovedno vrednost v primeru dvojno pozitivnega rezultata za strup čebele in ose.

1.4 MEHANIZEM SPECIFIČNE IMUNOTERAPIJE

Edino učinkovito zdravljenje za preprečitev sistemskih preobčutljivostnih reakcij, povzročenih s piki kožekrilcev, je specifična imunoterapija – SIT s strupi kožekrilcev. Indicirana je pri bolnikih, ki so doživeli težjo sistemsko preobčutljivostno reakcijo s prizadetostjo dihal in obtočil in pri bolnikih z lažjo sistemsko preobčutljivostno reakcijo ter pridruženimi dejavniki tveganja.

Čeprav je za vzpostavitev dolgotrajne zaščite potrebno vsaj 3 – 5 let zdravljenja s SIT, je večina bolnikov zaščitena že z uvedbo prvega vzdrževalnega odmerka (Forck, 1986; Goldberg in Confino-Cohen, 2010). Standardizirani vzdrževalni odmerek 100 μg strupa preprečuje nadaljnje sistemske preobčutljivostne reakcije za strupe kožekrilcev pri 75 – 95 % bolnikov (Bonifazi in sod., 2005; Golden, 2005). Goldberg in Confino-Cohen (2010) sta vzpostavitev kratkotrajne zaščite potrjevala z izvedbo provokacijskega testa s pikom čebele pri 79 bolnikih z zaključeno uvodno fazo SIT s strupom čebele. V 1. tednu po prejetju prvega vzdrževalnega odmerka 100 μg čebeljega strupa, 89 % bolnikov po provokacijskem testu s pikom čebele ni imelo težav, 5 % je imelo manjšo lokalno reakcijo, pri 6 % bolnikov pa se je razvila sistemska preobčutljivostna reakcija. Slednji so po zvišanju vzdrževalnega odmerka na 200 do 250 μg, brez težav prestali ponoven provokacijski test.

SIT je izredno učinkovito zdravljenje, ki zmanjša tveganje za nadaljnje sistemske reakcije, prepreči morbidnost in smrtnost ter izboljša kvaliteto življenja bolnikov (Bilo in Bonifazi, 2011). Kljub izredni učinkovitosti SIT, mehanizmi imunske tolerance na celičnem in molekularnem nivoju niso povsem razjasnjeni. Prav tako trenutno ni na voljo *in vitro* metode s katero bi lahko spremljali potek in uspešnost zdravljenja ter vzpostavitev tolerance za alergen.

Med predlaganimi mehanizmi dolgotrajne zaščite je porast specifičnih blokirajočih protiteles predvsem IgG_4 in tudi IgG_1, IgA ter hkratno znižanje vsebnosti specifičnih IgE (Till in sod., 2004; Akdis M in Akdis CA, 2007). Možen mehanizem na ravni limfocitov T je povišana sinteza specifičnih regulatornih limfocitov T (Treg), le-ti naj bi z izločanjem IL-10 in TGF-β spodbudili mehanizme tolerance za alergen (Kerstan in sod., 2011). Tretji predlagan mehanizem je na nivoju spremenjene odzivnosti efektorskih celic preobčutljivostnega odziva, in sicer bazofilcev, mastocitov in/ali eozinofilcev (Goldberg in Confino-Cohen, 2010; Till in sod., 2004; Akdis M in Akdis CA, 2007). Vendar ni jasno ali so mehanizmi dolgotrajne zaščite primerljivi z mehanizmi kratkotrajne zaščite ali se od njih popolnoma razlikujejo.

Med zgodnjimi učinki SIT so zasledili porast znotrajceličnega cAMP, povečano razgradnjo triptofana, povišano sintezo specifičnih limfocitov T ter IL-10 in večjo

aktivnost receptorja za histamin (Bussmann in sod., 2010; Novak in sod., 2012; Akdis in sod., 1998; Jutel in Akdis, 2011; Bellinghausen in sod. 1997; Pierkes in sod., 1999; Bauer in sod., 2007).

Mehanizmi kratkotrajne zaščite se razvijejo že med uvodno fazo SIT ob postopnem zviševanju subkutano injiciranega strupa kožekrilca. Pri hitrih oziroma zelo hitrih shemah SIT s strupi kožekrilcev (ang.: rush in ultra-rush venom immunotherapy) kumulativni odmerek 100 μg strupa dosežemo že v nekaj urah oziroma v nekaj dneh od pričetka terapije. Tako kratek časovni okvir verjetno ne zadošča, da bi spodbudil spremembe na nivoju populacij limfocitov T ali pri vrednostih humoralnega imunskega odziva, kot so protitelesa IgE in IgG_4. Zgodnji učinki SIT se najverjetneje navezujejo na spremenjene odzivnosti efektorskih celic, kot so bazofilci in mastociti. Med uvodno fazo SIT so v raziskavah namreč zasledili močno zmanjšano sproščanje mediatorjev alergijskega odziva iz efektorskih celic, histamina, sulfidolevkotrienov ter citokinov (Jutel in sod., 1996; Pierkes in sod., 1999; Košnik in sod., 2003; Bauer in sod., 2007).

Ključno vlogo pri posredovanju signala in sprožitvi kaskade takojšnjih preobčutljivostnih reakcij tipa I ima receptor velike afinitete FcεRI za protitelesa IgE. V obliki tetramera se receptor FcεRI nahaja na površini bazofilcev in mastocitov. Kot trimer je vezan na površino eozinofilcev in antigen-predstavitvenih celic. Vezava α-podenote receptorja na konstantno domeno Cε3 protiteles IgE, sproži prenos signala preko fosforilacije tirozinskih aminokislinskih ostankov na β-podenoti in γ-podenoti receptorja (Kraft in Kinet, 2007). Navzkrižna povezava receptorjev FcεRI z IgE in alergenom tako sproži signalizacijsko kaskado, kar se v nekaj minutah odrazi z degranulacijo in s sproščanjem mediatorjev alergijskega odziva iz efektorskih celic (Knol, 2006). MacGlashan je pred kratkim prikazal, da se ob kratkotrajni *in vitro* desenzibilizaciji bazofilcev s submaksimalno stimulacijo, ki povzroči celično neodzivnost za alergen, zmanjša izražanje receptorja FcεRI na njihovi površini (2012).

Čeprav so pri hitrih in zelo hitrih shemah SIT bolniki večinoma zaščiteni že po 24 do 48 urah, vzrok njihove kratkotrajne zaščite ni znan. Možno je, da do spremembe pride na celičnem nivoju pri posredovanju signala preobčutljivostne reakcije, kjer ima ključno vlogo receptor velike afinitete FcεRI na površini bazofilcev in mastocitov. Želeli smo preučiti vlogo bazofilcev in receptorja velike afinitete FcεRI na njihovi površini, pri spodbuditvi kratkotrajne zaščite SIT s strupi kožekrilcev. Za razjasnitev mehanizmov imunske tolerance na celičnem in molekularnem nivoju smo na skupini 60 odraslih in 48 otrok z različnimi shemami SIT spremljali odzivnost bazofilcev na stimulacijo s protitelesi anti-FcεRI in sledili nivoju izražanja gena za α-podenoto receptorja FcεRI (*FCER1A*) in receptorja FcεRI na površini bazofilcev pred pričetkom terapije, med uvodno fazo in tik pred uvedbo prvega vzdrževalnega odmerka SIT s strupi kožekrilcev.

Prav tako smo želeli preveriti ali so te zgodnje celične spremembe za alergen specifične ali nespecifične. Zato smo na skupini primarno dvojno senzibiliziranih bolnikov za strup čebele in ose pred pričetkom terapije in tik pred uvedbo prvega vzdrževalnega odmerka SIT s strupom enega kožekrilca, poleg odzivnosti na anti-FcεRI, spremljali tudi odzivnost bazofilcev na stimulacijo s specifičnim strupom kožekrilca s katerim smo izvajali SIT, kot tudi z nespecifičnim strupom kožekrilca, ki ni bil vključen v terapijo. Nadalje smo pri nekaterih bolnikih sledili tudi odzivnosti bazofilcev na poglavitni rekombinantni alergen rVes v 5 ali rApi m 1, s katerim SIT nismo izvajali ali na inhalacijski alergen v primeru dodatne ko-senzibilizacije. Bazofilce bolnikov preobčutljivih za strup čebele ali ose smo tudi izolirali ter jih *de novo* senzibilizirali s specifičnimi protitelesi IgE za alergen pršice in spremljali odzivnost bazofilcev na stimulacijo z alergenom pršice v enakih časovnih točkah.

Glede na zastavljeno problematiko ter izsledke predhodnih raziskav, smo predpostavili sledeče hipoteze:

- Občutljivost komercialno dostopnih poglavitnih rekombinantnih alergenov za strup čebele in ose v primerjavi z nativnimi je manjša, če vključimo reprezentativno število dobro opredeljenih bolnikov s preobčutljivostjo za posamezen strup kožekrilca.

- Rekombinantni alergeni strupov iz kožekrilcev so primerni za uporabo v biološkem testu aktivacije bazofilcev – BAT. Določeni glikozilirani rekombinantni alergeni so v bioloških testih bolj odzivni in primernejši za uporabo od neglikoziliranih. Predpostavljamo, da bomo z uporabo rekombinantnih alergenov v testu BAT ugotovili primarno senzibilizacijo dvojno pozitivnih bolnikov s sistemsko preobčutljivostno reakcijo po piku neznanega kožekrilca.

- Predvidevamo, da nam bo uporaba celičnega *in vitro* testa BAT v rutinski diagnostiki preobčutljivosti za strupe kožekrilcev omogočila potrditev povzročitelja pri bolnikih s prepričljivo anamnezo težje sistemske reakcije in negativnimi specifičnimi protitelesi IgE ter kožnimi testi. Menimo, da bo njegova občutljivost in specifičnost ter napovedna vrednost v primeru dvojno pozitivnega rezultata večja, v primerjavi z drugimi diagnostičnimi metodami.

- Mehanizem kratkotrajne zaščite SIT je povezan z ekspresijo gena za α-podenoto FcεRI (*FCER1A*) in z izražanjem receptorja FcεRI na površini bazofilcev. Po uvedbi prvega vzdrževalnega odmerka SIT je zmanjšana ekspresija gena *FCER1A* ter izražanje receptorja FcεRI na površini bazofilcev, kar se kaže v zmanjšani odzivnosti bazofilcev ob stimulaciji s protitelesi anti-FcεRI. Občutljivost bazofilcev je zmanjšana tako ob stimulaciji s specifičnim alergenom kot tudi ob stimulaciji z nespecifičnim alergenom, ki ni vključen v imunoterapijo.

2 ZNANSTVENA DELA

2.1 OBJAVLJENA ZNANSTVENA DELA

2.1.1 Majhna diagnostična občutljivost komercialno dostopnega alergena rApi m 1 za ugotavljanje preobčutljivosti za strup čebele

Low sensitivity of commercially available rApi m 1 for diagnosis of honeybee venom allergy

Peter Korošec, Rudolf Valenta, Irene Mittermann, **Nina Čelesnik Smodiš**, Renato Eržen, Mihaela Zidarn, Mitja Košnik

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Prvi komercialno dostopen neglikoziliran rekombinantni alergen fosfolipaze A2 (rApi m 1) iz *E. coli* je pri diagnosticiranju preobčutljivosti za strup čebele nadomestil nativnega (nApi m 1). Njuno diagnostično občutljivost smo primerjali na veliki skupini 184 preiskovancev z jasno definirano klinično sliko po piku čebele. Vključili smo 20 bolnikov z veliko lokalno reakcijo (LLR), 15 s sistemsko preobčutljivostno reakcijo stopnje I, 32 stopnje II, 74 stopnje III in 34 stopnje IV po Muellerju. Določili smo vrednosti celokupnih ter specifičnih IgE za nativni strup čebele in ose ter za rApi m 1, nApi m 1 in oljno repico (CCD). Vsi bolniki so imeli pozitivne sIgE za nativni strup čebele in negativne sIgE za nativni strup ose. Srednje vrednosti celokupnih in vseh specifičnih IgE so bile med stopnjami preobčutljivostnih reakcij med seboj zelo primerljive. Pozitivne sIgE za rApi m 1 in nApi m 1 je imelo 63 % oz. 95 % preiskovancev z LLR, 64 % oz. 73 % stopnje I, 57 % oz. 94 % stopnje II, 51 % oz. 87 % stopnje III in 63 % oz. 97 % stopnje IV po Muellerju. Kar 91 % bolnikov je imelo pozitivne sIgE za nApi m 1 in le 57 % za rApi m 1. Razlika je še toliko bolj očitna na skupinah s težjo obliko sistemske preobčutljivostne reakcije po piku čebele (stopnje III in IV po Muellerju). Od 16 pozitivnih bolnikov za CCD, je bilo 10 pozitivnih za oba alergena in le 6 samo za rApi m 1. Ugotovili smo majhno diagnostično občutljivost komercialno dostopnega alergena rApi m 1 iz *E. coli*. Ti rezultati nakazujejo na omejeno diagnostično uporabnost rApi m 1 za ugotavljanje preobčutljivosti za strup čebele.

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Low sensitivity of commercially available rApi m 1 for diagnosis of honeybee venom allergy

To the Editor:

Several studies have shown that the major recombinant allergen in honeybee venom,[1] phospholipase A_2 allergen (Api m 1), might be useful for *in vitro* and *in vivo* diagnosis of honeybee venom allergy.[2-5] Correctly refolded[2] rApi m 1 has demonstrated *in vivo* diagnostic sensitivity up to 95%.[3] In *in vitro* determinations of specific IgE (sIgE) against rApi m 1, sensitivity between 78%[3] and 97%[4] and even up to 100%[5] has been reported. Analytic specificity of recombinant allergens was evaluated by using IgE immunoblotting, ELISA, or both,[3,5] and one report used a commercially available liquid-phase detection system (ADVIA Centaur; Siemens Medical Solution Diagnostics, Deerfield, Ill).[4]

Venom recombinants are produced in the bacterial expression systems as nonglycosylated proteins and in eukaryotic systems as glycosylated allergens. Glycosylated allergens expressed in eukaryotic cells, such as baculovirus-infected insect cells, have an IgE-binding capacity comparable with that of natural allergens.[6] However, IgE reactivity to carbohydrate epitopes can lead to false-positive results, which decrease the diagnostic specificity of glycosylated recombinants.[4,5] In late 2009, the first nonglycosylated recombinant venom allergen component was available for the widely used ImmunoCAP solid-phase assay (CAP-FEIA; Phadia, Uppsala, Sweden). This allergen was a novel *Escherichia coli*–expressed nonglycosylated Api m 1, which was a substitute for the previously used nApi m 1. Because this recombinant is potentially very useful and commercially available, we wanted to evaluate its diagnostic utility and compare it with the nApi m 1 allergen in a routine clinical laboratory setting by analyzing a large group of patients with well-defined honeybee venom allergy before adopting its use in our clinical practices.

In total, 184 subjects (mean age, 43 years; age range, 16-75 years; 101 women) with established honeybee venom allergy (20 with large local reactions and 15 with Mueller grade I, 32 with grade II, 74 with grade III, and 34 with grade IV reactions) were recruited during a 5-year period. In all subjects honeybee and wasp (*Vespula* species) venom–specific IgE and total IgE levels were prospectively measured with CAP-FEIA. Tests for CAP-FEIA recombinant *E coli*–expressed nonglycosylated Api m 1 (i208) and nApi m 1 (k203) were performed in early winter 2010 from stored (at −40°C) samples. CAP-FEIA to oilseed rape (OSR; canola, *Brassica napus*; f316) was used as an indicator of cross-reacting carbohydrate determinants (CCDs).[7] Fifteen samples were missed for nApi m 1 (we included 169 subjects), 9 samples were missed for rApi m 1 (we included 175 subjects), and 10 samples were missed for CCD measurements (we included 174 subjects). Thus we tested 168 sera for all allergens. In 2010, the production of nApi m 1 (k203) was discontinued.

In all study subjects we demonstrated a positive specific IgE response to honeybee venom (2.1 kU/L; range, 0.41->100 kU/L) and a negative specific IgE response (<0.35 kU/L) to wasp venom (*Vespula* species, Fig 1). Next, we examined the subjects for the presence of specific IgE antibodies to rApi m 1 and nApi m 1, as detected by using the CAP-FEIA system (Fig 1). Surprisingly, we found that more subjects had positive specific IgE responses for nApi m 1 (153/169 [91%]) than for rApi m 1 (100/175 [57%]; $P < .0001$, Fisher exact test). These marked differences were even more prominent in subjects who had experienced severe anaphylactic reactions. After a honeybee sting, rApi m 1 recognized only 51% and 63% of subjects in the Mueller grade III and IV subgroups, respectively. On the other hand, nApi m 1 recognized 87% of subjects in the Mueller grade III subgroup and almost all subjects (97%) in the Mueller grade IV group ($P < .0001$, Fisher exact test). The median concentration of rApi m 1 in 100 subjects with positive responses was 4.4 kU/L (range, 0.36->100 kU/L), which was comparable with the median concentration of nApi m 1 (4.8 kU/L; range, 0.36->100 kU/L) in 153 subjects with positive responses. The major finding was that 53 subjects who had clearly positive responses for nApi m 1 (3.4 kU/L; range, 0.37->100 kU/L) had negative IgE reactivity for rApi m 1. Nevertheless, nApi m 1 had still missed 9% of subjects with established honeybee venom allergy.

Most likely, carbohydrate epitopes of the native allergen did not influence these results because only 9% (16/174) of our subjects showed IgE reactivity to CCDs (Fig 2). The proportion of IgE positivity for CCDs found in this study was comparable with results found in other studies of venom-monosensitized subjects (ie, 14.3% OSR positive responses in honeybee venom–sensitized patients and 12.5% in wasp venom–sensitized patients).[7] On the other hand, in venom double-positive sera a much higher proportion of CCD positivity was reported.[7] There were no significant differences between CCD positivity according to the severity of sting reactions and no matching between IgE reactivity to OSR and reactivity to rApi m 1 and nApi m 1. Namely, 10 of 16 CCD-positive subjects had positive responses for both rApi m 1 and nApi m 1, and only 6 CCD-positive subjects had positive responses just for nApi m 1. It is important to note that OSR was used as a measure for carbohydrate-specific IgE because OSR pollen, but not Gramineae pollen, contains MMF glycans, which are characteristic for honeybee Api m 1.[7] The discrepancy between nApi m 1 and rApi m 1 reactivity might be influenced also by the other bee venom epitopes, such as Api m 2, Api m 3, Api m 4, or Api m 10.[5] Nevertheless, the notion that Api m 1 represents a major allergen has been shown in numerous studies,[1-5] including this one with native CAP-FEIA Api m 1 allergen.

Recently, an initial report about the diagnostic value of CAP-FEIA i208 rApi m 1 was published.[8] Unfortunately, the strength of this communication was limited by the low number of subjects studied. Only 34 subjects with a history of honeybee venom allergy (only 18 of whom were honeybee monosensitized) limited the clinical relevance and prevented a comprehensive and

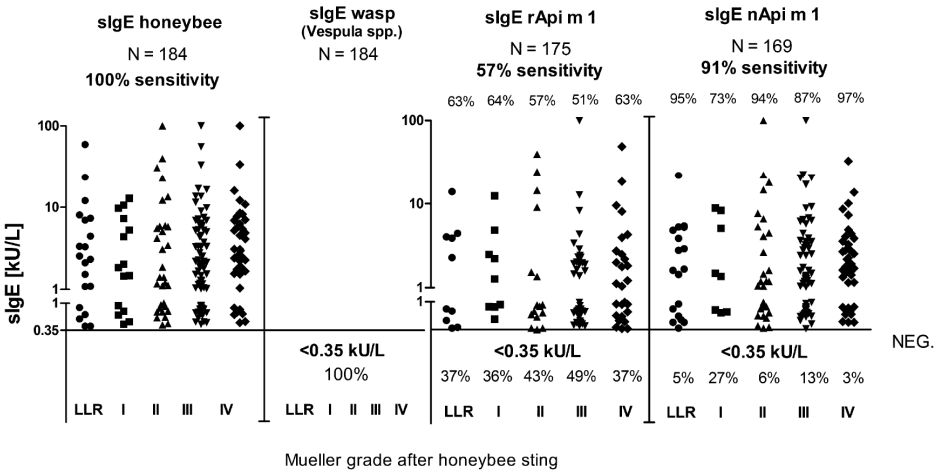

FIG 1. Honeybee venom, wasp venom, rApi m 1, and nApi m 1 CAP-FEIA sIgE measurements in patients with honeybee venom allergy. *LLR,* Large local reaction.

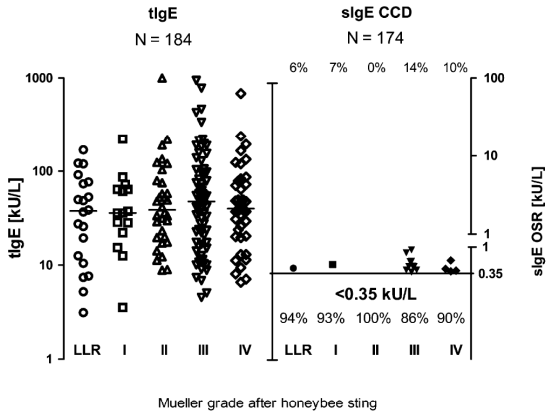

FIG 2. Total IgE *(tIgE)* and sIgE reactivity for CCDs measured with OSR CAP-FEIA in patients with honeybee venom allergy. The *horizontal solid bars* indicate the median value for each subgroup. *LLR,* Large local reaction.

statistically sound analysis. The diagnostic sensitivity of rApi m 1 reported in this communication was 79%.[7]

Previous reports also suggest that total serum IgE is a potential risk factor of the severity of systemic venom-induced anaphylactic reactions.[9,10] The median concentration of total IgE was 37.8 kU/L (range, 3.1-169 kU/L), 36 kU/L (range, 3.6-223 kU/L), 38.9 kU/L (range, 8.9-1000 kU/L), 47.7 kU/L (range, 4.6-947 kU/L), and 40 kU/L (range, 6.6-685 kU/L) in the large local reaction and Mueller grade I, II, III, or IV subgroups, respectively (Fig 2). Similar honeybee venom–specific IgE rank was highly comparable between subjects with different severity grades (Fig 1). Therefore an influence of total and specific IgE on the severity of systemic reactions after a previous honeybee field sting could not be shown.

In conclusion, our results suggest that the current CAP-FEIA rApi m 1 has limited clinical usefulness for the detection of honeybee venom allergy because of its low diagnostic sensitivity. Thus improved diagnostic tests are needed.

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IgE to Gly m 5 and Gly m 6 is associated with severe allergic reactions to soybean in Japanese children

To the Editor:

Soybean is 1 of 8 foods believed to cause a majority of food-induced allergic reactions in children.[1,2] However, the prevalence of soybean allergy in Japan might be higher than in Europe and the United States, with soybean reported as the fifth most common food allergen causing anaphylaxis.[3] Soybeans contain about 40% protein, the majority of which is composed of the 2 storage proteins β-conglycinin and glycinin, which have been recently designated Gly m 5 and Gly m 6.[4] Four other proteins are officially accepted as allergens, and at least an additional 12 have been reported as IgE-reactive proteins.[5] Data regarding soybean allergens associated with clinical symptoms in children are limited. In this study we have examined the IgE reactivity pattern to 5 soybean and 3 cross-reactive allergens in a group of children with and without soybean allergy. Furthermore, we have investigated the clinical usefulness of analyzing specific IgE antibodies to Gly m 5 and Gly m 6.

There were 74 subjects (range, 0.6-16.3 years), of whom 33 were given diagnoses of soybean allergy (symptomatic group) based on challenge outcome (n = 29) or clinical history after intake (n = 4; 3 experienced apparent skin symptoms and 1 experienced anaphylaxis). The symptomatic group was further divided into subjects with severe symptoms (n = 14) and mild symptoms (n = 19). Severe symptoms were defined as a combination of skin, respiratory, or gastrointestinal symptoms, whereas mild symptoms were defined as isolated skin symptoms, oral symptoms, or both (Table I). The remaining 41 subjects were sensitized to soybean without any symptoms from soybean (nonsymptomatic group). Tolerance in the nonsymptomatic group was either confirmed by means of food challenge (n = 22) or a history of daily ingestion of soybean products (n = 19). Food challenges were conducted in accordance with the Japanese guidelines.[6]

IgE reactivity to 8 different allergens was tested in an in-house, qualitative multiplexed immunoassay, essentially as reported elsewhere.[7] The 8 allergens included in the setup were Gly m 5, Gly m 6, rGly m 4, soybean Kunitz trypsin inhibitor (Sigma-Aldrich, St Louis, Mo), soybean agglutinin (Vector Laboratories, Peterborough, United Kingdom), Cross-reactive carbohydrate determinants (CCDs) purified from digested bromelain (essentially MUXF3), profilin from timothy pollen (rPhl p 12), and lipid transfer protein from peach fruit (rPru p 3). Native Gly m 5 and Gly m 6 were essentially purified according to the method of Thanh and Shibasaki.[8] All recombinant allergens, as well as the CCD reagent, were produced at Phadia AB (Uppsala, Sweden).

IgE antibody levels to soybean, Gly m 5, and Gly m 6 were analyzed in serum by using ImmunoCAP (Phadia AB), all of which were commercially available. The lower limit of quantitation of the tests was 0.10 kU_A/L. The Fisher exact test was used to determine differences regarding the prevalence of IgE reactivity analyzed by using the multiplex assay (categorical data). The Spearman rank correlation test was used in the analysis of associations between IgE concentrations. The relationship between IgE concentrations and clinical status outcome was analyzed by using logistic regression analysis. Odds ratios were estimated by using regression models, and 95% CIs were generated according to the Wald test.

Among the children in the symptomatic group with mild symptoms, all had skin symptoms, and 3 had oral symptoms (Table I). Respiratory symptoms, mostly coughing and wheezing, were the most frequent symptoms (n = 12) in the severe group. The multiplex immunoassay showed that among the children in the symptomatic group, 67% had IgE reactivity to Gly m 5 (49% in the nonsymptomatic group), 58% to Gly m 6 (39% in the nonsymptomatic group), 21% to Gly m 4 (20% in the nonsymptomatic group), and 6% to soybean agglutinin and soybean trypsin inhibitor (7% and 10%, respectively, in the nonsymptomatic group). The number of subjects with IgE reactivity to lipid transfer protein, profilin, and CCDs varied between 12% and 15% (7% to 17% in the nonsymptomatic group). No significant difference in the frequency of IgE reactivity between the symptomatic and nonsymptomatic groups was observed for any of the allergens included in the study. However, a tendency toward a higher frequency of IgE reactivity in the symptomatic group was noted for both Gly m 5 and Gly m 6 ($P = .16$ for both). Therefore quantitative analysis of IgE to Gly m 5 and Gly m 6 was performed to investigate the true prevalence.

Analysis with ImmunoCAP demonstrated that all children had IgE levels to soybean, Gly m 5, and Gly m 6 of greater than 0.1 kU_A/L, except one in the nonsymptomatic group. The IgE levels to both Gly m 5 and Gly m 6 correlated with the IgE levels to soybean ($r_S = 0.89$ and $r_S = 0.86$, respectively). The IgE levels to soybean and Gly m 5 were significantly higher in the symptomatic group than in the nonsymptomatic group ($P < .01$). With respect to the specific IgE levels in the 2 groups, the risk of being allergic to soy increased significantly with increasing levels of IgE. For IgE to soybean, the odds increased 1.51-fold (95% CI, 1.10-2.08), and for IgE to Gly m 5, the odds increased 1.48-fold (95% CI, 1.08-2.02) per logarithmic unit increase, respectively. Significant differences were noticed between the severe and nonsymptomatic groups in IgE levels to soybean, Gly m 5, and Gly m 6 (Fig 1). The IgE responses to soybean, Gly m 5, and Gly m 6 were not statistically different between the children with mild symptoms and the nonsymptomatic children. Significant differences in the IgE levels to soybean were detected between the mild and severe symptom groups but not in the IgE levels to Gly m 5 and Gly m 6.

Knowledge about specific soybean allergens associated with clinical symptoms is restricted to a few publications. Many studies demonstrating IgE reactivity to soybean proteins in sera from soybean-sensitized subjects have been published, but the patient material has generally been small and often with an unclear diagnosis. In this study we have examined IgE reactivity to 5 soybean and 3 cross-reactive allergens in sera from 74 Japanese children. To the best of our knowledge, this group, consisting of symptomatic and nonsymptomatic subjects, is the largest defined clinical sample tested with the aim of identifying important soybean allergens.

2.1.2 Velika diagnostična občutljivost komercialno dostopnih alergenov rVes v 5 in rVes v 1 za ugotavljanje preobčutljivosti za strup ose

High sensitivity of CAP-FEIA rVes v 5 and rVes v 1 for diagnosis of Vespula venom allergy

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V predhodni raziskavi smo pokazali omejeno diagnostično uporabnost komercialno dostopnega alergena rApi m 1 iz *E. coli* za ugotavljanje preobčutljivosti za strup čebele. Zato smo želeli ugotoviti diagnostično uporabnost novih komercialno dostopnih alergenov rVes v 5 in rVes v 1 iz Sf9 za ugotavljanje preobčutljivosti za strup ose, pred diagnostično uporabo v rutinski klinični praksi. Vključili smo skupno 200 preiskovancev z jasno definirano klinično sliko po piku ose, 19 z veliko lokalno reakcijo (LLR), 17 s sistemsko preobčutljivostno reakcijo stopnje I, 42 stopnje II, 68 stopnje III in 54 stopnje IV po Muellerju. Najprej smo določili vrednosti sIgE za nativni strup ose in čebele ter za rVes v 5. Pri bolnikih, ki so bili negativni za rVes v 5 smo določevali še rVes v 1. Z indirektno encimskoimunsko metodo smo bolnike, ki so bili negativni za komercialno dostopna alergena, testirali še z alergeni iz celičnih kultur, rVes v 2, rVes v 5 in nPhl p 4. Vsi bolniki so imeli pozitivne sIgE za nativni strup ose in negativne sIgE za nativni strup čebele. Pozitivne sIgE za rVes v 5 je imelo 79 % preiskovancev z LLR, 100 % stopnje I, 95 % stopnje II, 94 % stopnje III in 89 % stopnje IV po Muellerju. Od preostalih 31 rVes v 5 negativnih bolnikov, je imelo 15 pozitivne sIgE za rVes v 1. Skupno je bilo kar 92 % bolnikov pozitivnih s komercialno dostopnima alergenoma rVes v 5 ali rVes v 1. Od 16 negativnih bolnikov, sta bila 2 z encimskoimunsko metodo pozitivna za rVes v 2, 2 za rVes v 5 ter 4 za nPhl p 4. Ugotovili smo veliko diagnostično občutljivost komercialno dostopnih rekombinantnih alergenov rVes v 5 in rVes v 1 iz Sf9 za ugotavljanje preobčutljivosti za strup ose. V prvi fazi *in vitro* diagnostičnega postopka ostaja določevanje sIgE proti nativnemu strupu ose, ki vsebuje vse alergene. Uporaba rVes v 5 in rVes v 1 bi za ugotavljanje preobčutljivosti za strup ose lahko koristila v primeru dvojno pozitivnih oz. nejasnih rezultatov rutinskih diagnostičnih testov.

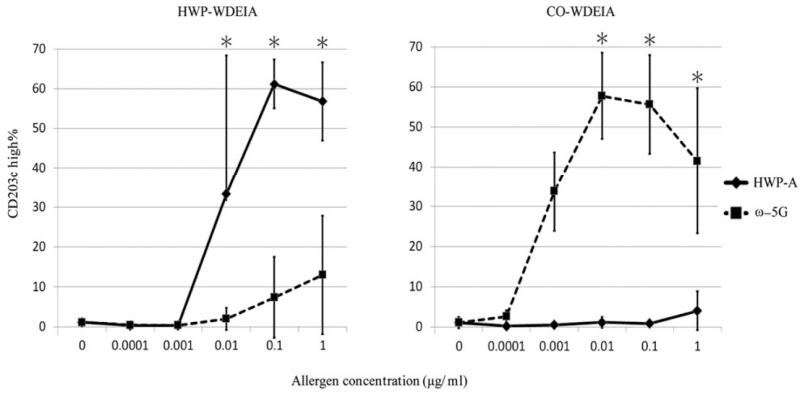

FIG 1. Expression of CD203c on basophils induced by HWP-A and ω-5 gliadin *(ω-5G)*. Mean levels of CD203c expression in 5 patients with HWP-WDEIA and 5 patients with CO-WDEIA are presented. Data are expressed as means ± SEMs. *$P < .01$ when comparing HMP-A and ω-5 gliadin, as determined by using the Student *t* test.

and proline, IgE produced against HWPs probably cross-reacts with natural wheat proteins. In fact, preincubation of sera with HWP-A clearly revealed a decreased binding of IgE to natural wheat proteins.

In conclusion, measurement of basophil CD203c expression induced by various preparations of wheat proteins is highly useful in predicting causative allergens in patients with WDEIA. Furthermore, the basophil activation test based on the expression of CD203c might help determine causative allergens for a wide variety of food allergies.

This study was approved by the Ethics Committee of the Shimane University Faculty of Medicine (approval nos. 469 and 703). All participants provided written informed consent.

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High sensitivity of CAP-FEIA rVes v 5 and rVes v 1 for diagnosis of *Vespula* venom allergy

To the Editor:

Ves v 5 (antigen 5) is a 23-kDa protein from *Vespula* venom, and it is recognized as the most potent allergen in venoms of the *Vespidae* family.[1] There is a high sequence similarity of Ves v 5 within species of the same genus, such as yellow jacket, that is, *Vespula* (>95%); however, when it is compared with other genera such as *Dolichovespula* or *Polistes*, the sequence identity is much lower (about 60%).[2] Another potential *Vespula* allergen is a 37-kDa phospholipase A1, known as Ves v 1.[1] Neither Ves v 5 nor Ves v 1 is found in honeybee venom. Ves v 5 and Ves v 1 recombinant allergen components, both expressed in insect cells, became available in 2010 and 2011, respectively, for analyses on the ImmunoCAP solid-phase IgE assay (CAP-FEIA; Phadia, Uppsala, Sweden).

Very recently we demonstrated that the current CAP-FEIA recombinant major honeybee venom allergen rApi m 1 (i208) has a limited clinical usefulness for the detection of honeybee venom allergy because of its low diagnostic sensitivity, which

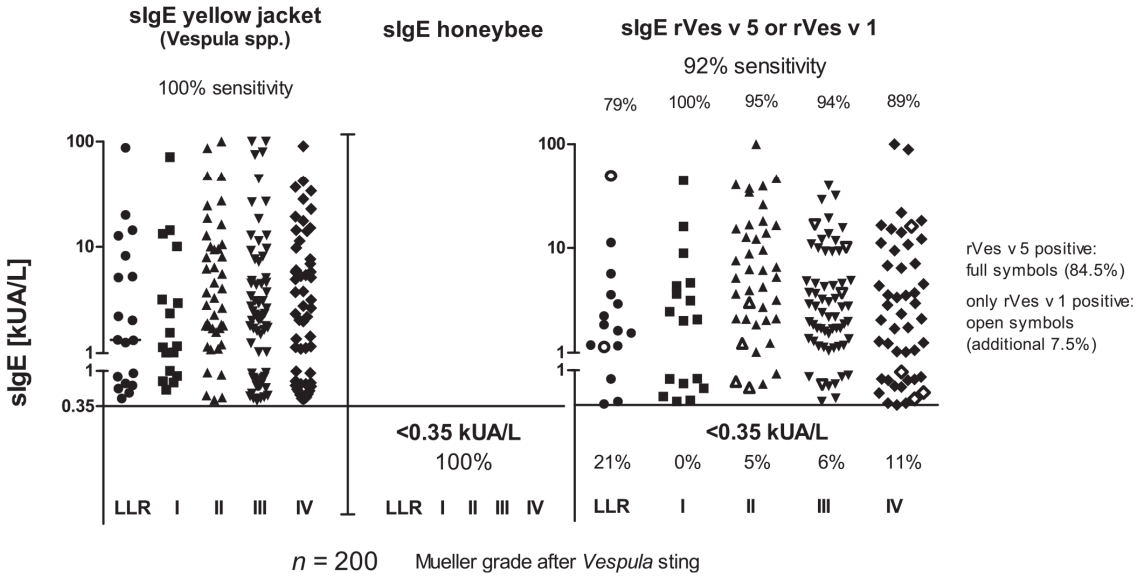

FIG 1. Wasp and honeybee venom, recombinant vesp v 5 and vesp v 1 CAP-FEIA specific IgE measurement in wasp venom–allergic subjects.

was about 60%.[3] Similarly, low sensitivity for CAP-FEIA rApi m 1 was recently also demonstrated by another group.[4] For that reason we wanted to evaluate the diagnostic sensitivity of novel recombinant Ves v 5 (rVes v 5) and rVes v 1 in a routine clinical laboratory setting by analyzing a group of *Vespula* venom allergic patients.

In total, 200 subjects (mean age, 42 years; range, 16-81 years; 99 women) with established *Vespula* venom allergy (19 with large local reactions and 17, 42, 68, and 54 with Mueller grade I, II, III, and IV reactions, respectively) were recruited during a 4-year period. We included only those subjects in which the culprit insect was the yellow jacket. In all subjects, honeybee and yellow jacket (*Vespula* species) venom-specific IgE levels were measured with CAP-FEIA. Tests for CAP-FEIA rVes v 5 (i209) and rVes v 1 (i211) were performed in 2011 from serum samples stored at −40°C. We first tested the samples for rVes v 5, and if they were negative (<0.35 kUA/L), we tested them with rVes v 1. In addition, samples that were negative for rVes v 5 and rVes v 1 in the CAP were also tested for IgE reactivity to rVes v 2b (*Vespula hyaluronidase*), rVes v 5, and glycosylated nPhl p 4 by IgE dot-blotting as described previously.[5] rVes v 2b was produced by baculovirus-infected insect cells.[6]

In all study subjects, we demonstrated a positive specific IgE response (2.24 kUA/L [range, 0.41 to >100]) to yellow jacket venom and a negative specific IgE response (<0.35 kUA/L) to honeybee venom (Fig 1). The median concentration of specific IgE antibodies to yellow jacket venom was 1.34 (range, 0.44-87.4) in large local reactions and 1.17 (range, 0.57-71.2), 3.5 (range, 0.42 to >100), 2.16 (range, 0.41 to >100), and 2.51 kUA/L (range, 0.43-90.3) in Mueller grade I, II, III, or IV subgroup, respectively. Next, we examined the subjects for the presence of specific IgE antibodies to rVes v 5 or rVes v 1. We found that 84.5% of the subjects (169 of 200) had positive specific IgE for rVes v 5. Of 31 negative subjects, 15 subjects were positive for rVes v 1 (additional 7.5%). Thus, altogether 184 of 200 subjects (92%) were positive for either rVes v 5 or rVes v 1. The median concentration of rVes v 5 or rVes v 1 in all positive subjects was 2.78 kUA/L (range, 0.36 to >100) and 1.63 (range, 0.36-49.4), 2.08 (range, 0.39-44.7), 5.7 (range, 0.59 to >100), 2.58 (range, 0.39-39.6), and 2.24 kUA/L (range, 0.36 to >100) with sensitivities of 79% (15 of 19), 100% (all 17), 95% (40 of 42), 94% (64 of 68), and 89% (48 of 54) in large local reactions and Mueller grade I, II, III, or IV subgroup, respectively. Consequently, the diagnostic sensitivity of Ves recombinants was comparably high in all subjects who had experienced systemic reactions following a yellow jacket sting and also in those who experienced very severe reactions of Mueller grades III and IV. It was slightly lower (79%) only in those patients who experienced local reactions. Of the 16 sera that were negative to rVes v 5 and rVes v 1 in the CAP, 2 sera were positive to dot-blotted rVes v 2b (additional 1%). Both subjects experienced severe reactions of Mueller grade III or IV. In the dot blot, an additional 2 sera were positive to rVes v 5 and an additional 4 sera contained IgE antibodies specific for carbohydrates on Phl p 4.

These results are comparable with the previous rVes v 5 diagnostic evaluations by commercially available liquid phase detection system (ADVIA Centaur; Siemens Medical Solution Diagnostics, Deerfield, Ill).[7] In this report, specific IgE to rVes v 5 were demonstrated in 87% of 100 *Vespula* allergic subjects. An even higher ratio of identification (100%) was observed in our previous report by using in-house IgE immunoblotting and/or ELISA rVes v 5, but on limited number on *Vespula* allergic 20 patients.[5]

In this study, we demonstrated that additional use of Ves v 1 significantly enhances the diagnostic sensitivity (for almost 8%)

of diagnostic tests based on recombinant yellow jacket venom allergens. Nevertheless, rVes v 5 and rVes v 1 had missed 8% of subjects with established allergy. Testing for rVes v 2b added only a minor contribution to diagnostic sensitivity. Primarily, 3 allergens were recognized as responsible for *Vespula* venom allergy, beyond Ves v 5 and Ves v 1, also Ves v 2, which occurs in isoforms.[1] Recently, a novel 100-kDa glycosylated protein with homology to dipeptidyl peptidases with allergenic potential, namely, Ves v 3, was reported as a yellow jacket allergen.[7] rVes v 3 showed IgE reactivity in approximately 50% of Vespula allergic subjects (in overall 54 tested)[8] and might be useful to diagnose the few patients who are not identified with rVes v 5, 1, and 2.

Clinically, we cannot afford to miss a patient who is sensitive to insect venom; thus, the whole venom (that contains all the venom allergens as a single test) needs to be the first line of laboratory evaluation. However, the identification of the disease-causing insect venom in venom allergy is often difficult. In such cases, commercially available CAP-FEIA tests based on recombinant rVes v 5 and rVes v 1 allergens should be helpful for the serological dissection of *Vespula* venom allergy.

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Specific allergen concentration of WHO and FDA reference preparations measured using a multiple allergen standard

To the Editor:

Allergen measurements require well-defined allergen standards. Allergists rely on these measurements for dosing patients on immunotherapy with the aim of achieving maintenance doses of 5 to 20 μg of specific allergen that have been associated with clinical efficacy.[1] Allergists need to know that allergen measurements made by manufacturers are consistent and can reliably be used in clinical practice. Allergen measurements are widely used in the indoor air quality industry to assess exposure in homes, the workplace, schools, and commercial buildings. They are routinely used for assessing health risks associated with allergen exposure, for assessing the efficacy of allergen avoidance procedures, and for developing new allergen control products.[2]

Measurements of allergens by ELISA rely on standards of known allergen concentration, but few national or international allergen standards exist. The World Health Organization/International Union of Immunological Societies (WHO/IUIS) Allergen Standardization Committee initiated a program to develop purified allergen standards for calibration of *in vitro* measurements. This initiative was funded by the European Union to develop certified reference materials for allergenic products ("Development of Certified Reference Materials for Allergenic Products and Validation of Methods for their Quantification," acronym CREATE). The aims of CREATE were to develop international reference materials with verifiable allergen content.[3,4]

Our goal was to apply the principles of allergen standardization developed in CREATE to other purified allergens. We developed a single "universal" allergen standard (UAS) for use in ELISA and in a fluorescent multiplex array for indoor allergens.[5] Purified proteins are essential in multiplex systems to reduce nonspecific interactions. Eight purified allergens (Der p 1, Der f 1, Der p 2, Fel d 1, Can f 1, Rat n 1, Mus m 1, and Bla g 2) were combined in the UAS. The protein concentration of the purified allergens was determined by amino-acid analysis, in keeping with CREATE. A detailed validation of the UAS and comparison with previous ELISA standards will be published elsewhere.[6] Here, we report the concentration of specific allergens in WHO/IUIS and US Food and Drug Administration (FDA) reference preparations using the single multiallergen standard.

Specific allergen concentrations of WHO/IUIS and FDA reference preparations were determined by using ELISA (Table I). The WHO/IUIS *Dermatophagoides pteronyssinus* standard 82/518 has been widely used as a standard for measurements of allergen exposure with a reported concentration of 12.5 μg Der p 1 per ampoule.[7] A value of 7.2 μg Der p 1 per ampoule was obtained by using the UAS, which is similar to estimates of 5 μg Der p 1 per ampoule reported previously.[8,9] The WHO/IUIS dog hair standard has an assigned potency of 100,000 IUs per ampoule and contained 20.4 μg Can f 1 per ampoule as determined by using the UAS.

2.1.3 Klinična uporabnost testa aktivacije bazofilcev za diagnosticiranje preobčutljivosti za strupe kožekrilcev s poudarkom na posameznikih z negativnimi specifičnimi protitelesi IgE

Clinical routine utility of basophil activation test for diagnosis of Hymenoptera-allergic patients with emphasis on individuals with negative venom-specific IgE antibodies

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Pri bolnikih s prepričljivo anamnezo sistemske preobčutljivostne reakcije za strup kožekrilca in negativnimi specifičnimi protitelesi IgE (sIgE), so predhodne raziskave pokazale veliko diagnostično uporabnost testa aktivacije bazofilcev (BAT) pri potrjevanju senzibilizacije. Naš namen je bil ugotoviti diagnostično uporabnost testa BAT v rutinski klinični praksi pri obravnavi bolnikov z negativnimi rezultati rutinskih diagnostičnih testov za strup čebele in ose. V študijo smo prospektivno vključili 21 bolnikov z anafilaktično reakcijo (mediana stopnje III po Muellerju) po piku kožekrilca in negativnimi sIgE. S testom BAT smo diagnosticirali 81 % bolnikov (17 od 21), z intradermalnimi kožnimi testi pa 57 % (12 od 21). Trije bolniki s preobčutljivostjo za strup ose so imeli pozitivne sIgE za rVes v 5. Štirje bolniki (19 %) pa so ostali negativni z vsemi testi. V primeru dvojno pozitivnega rezultata za strup čebele in ose je bil klinično pomemben tisti strup, ki je pri testu BAT spodbudil večji celični odziv. Test BAT nam je omogočil potrditev povzročitelja pri večini bolnikov z anamnezo težje sistemske preobčutljivostne reakcije po piku kožekrilca in negativnimi sIgE in kožnimi testi za strupe kožekrilcev. Rutinska uporaba tega celičnega *in vitro* testa, v kompleksnih primerih z negativnimi rezultati rutinskih diagnostičnih testov, pogosto omogoča uvedbo specifične imunoterapije z ustreznim strupom kožekrilca.

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Clinical Routine Utility of Basophil Activation Testing for Diagnosis of Hymenoptera-Allergic Patients with Emphasis on Individuals with Negative Venom-Specific IgE Antibodies

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Key Words
Hymenoptera venom allergy · Diagnostic tests · Basophil activation test

Abstract
Background: Previous reports suggest the usefulness of basophil activation testing (BAT) in Hymenoptera-allergic patients with negative venom-specific IgE antibodies. We sought to evaluate the diagnostic utility of this testing in a routine clinical laboratory setting. ***Materials and Methods:*** Twenty-one patients with anaphylactic reactions to Hymenoptera sting (median grade III) and negative venom-specific IgE were routinely and prospectively tested with BAT. ***Results:*** We were able to diagnose 81% (17 of 21) of patients with BAT and 57% (12 of 21) with intradermal skin testing. Three wasp venom-allergic patients showed IgE positivity to rVes v 5. Four patients (19%) were negative for all tests. In the case of double-positive BAT, the culprit insect correlated with the venom that induced a significantly higher basophil response. ***Conclusions:*** BAT allows the identification of severe Hymenoptera-allergic patients with negative specific IgE and skin tests. The routine use of this cellular test should facilitate prescription of venom immunotherapy in complex cases with inconclusive diagnostic results.

Introduction

Previous reports have clearly described patients with a history of systemic reactions after Hymenoptera sting, but with negative venom-specific IgE and skin test results [1–3]. Moreover, Golden et al. [1] showed that those patients could subsequently experience another severe reaction to sting because they demonstrated that after the sting challenge, the frequency of systemic reaction is comparable between patients with a positive (21%) and a negative (22%) skin test result. Furthermore, in cases of fatal sting anaphylaxis, venom-specific IgEs are very low or even undetectable in more than 30% of patients [3].

It was previously demonstrated that in patients with a positive history of anaphylactic sting reaction and negative venom-specific IgE and skin testing, the CD63 basophil activation test (BAT) could be a very useful tool that can improve overall sensitivity of the diagnostic evaluation [3, 4]. For this reason, a few years ago, we started using the BAT in routine diagnostic testing of patients with negative venom-specific IgE antibodies, preferably in those with a severe clinical reaction. In this report, we analyze the clinical utility of this cellular testing in our practice during the last 2.5 years, with special emphasis on comparison with other possible testing approaches for

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double-negative patients and with the diagnostic and culprit consideration in the case of double-positive honeybee and wasp venom basophil response. We also analyzed whether the result of basophil testing in these complex cases could make it possible to accurately decide on and apply venom immunotherapy (VIT).

Materials and Methods

Study Patients

Twenty-one patients were recruited at the University Clinic of Respiratory and Allergic Diseases in Golnik from December 2009 to June 2012 (table 1). They all had a convincing history of at least one severe anaphylactic reaction to Hymenoptera sting (median Mueller grade III) and negative venom-specific IgE antibodies (table 1, 2). All investigations, except IgE reactivity to recombinant venom allergens, were prospectively performed in a routine clinical and laboratory setting.

Specific IgE, Total IgE and Tryptase Measurement

The serum concentration of specific IgE to wasp, honeybee, optional European hornet venom, rApi m 1, rVes v 5, and rVes v 1, as well as of total IgE and tryptase was measured with ImmunoCAP-FEIA (Phadia, Uppsala, Sweden). In all patients, the measurements of specific IgE to wasp and honeybee venom were repeated within 1–2 months.

Intradermal Skin Testing

An intradermal skin test was done with up to 1 μg/ml of wasp and honeybee venom allergen as previously described [3].

Basophil Activation Test

A BAT was performed as previously described [3, 5–8]. Briefly, whole blood was incubated with 0.1 or 1 μg/ml of honeybee or wasp venom (Hal Allergie, Leiden, The Netherlands) or with 0.55 μg/ml of anti-FcεRI monoclonal antibody (mAb; Bühlmann Laboratories, Schönenbuch, Switzerland), or with 2 μM fMLP (Sigma, USA) as a control at 37°C for 15 min. Degranulation was stopped by chilling on ice, after which anti-CD63, anti-CD123 mAb and anti-HLA-DR mAbs (BD Biosciences, USA) were added and incubated for 20 min. Finally, whole-blood probes were lysed, washed, fixed and analyzed within 2 h on a FACSCalibur flow cytometer (BD Biosciences). The threshold value for diagnostically positive results was defined as 15% of CD63-positive basophils. This threshold was previously described in detail [3, 6, 8].

Table 1. Demographic data and the results of serum-specific IgE, total IgE and tryptase measurement

Patient No.	Sex	Age years	sIgE bee/wasp	Recombinant sIgE			Tryptase μg/l	Total IgE
				Api m1	Ves v 5	Ves v 1		
1	F	63	<0.35/<0.35	<0.35	<0.35	<0.35	5.1	7.4
2	M	54	<0.35/<0.35	<0.35	<0.35	<0.35	3.0	16.6
3	F	33	<0.35/<0.35	<0.35	<0.35	<0.35	3.6	22.3
4	M	20	<0.35/<0.35	<0.35	<0.35	<0.35	4.1	741
5	M	55	<0.35/<0.35	<0.35	**0.47**	<0.35	4.6	11.4
6	F	74	<0.35/<0.35	<0.35	<0.35	<0.35	4.8	12
7	F	19	<0.35/<0.35	<0.35	<0.35	<0.35	6.7	45.7
8	F	40	<0.35/<0.35	<0.35	<0.35	<0.35	4.5	2.9
9	M	55	<0.35/<0.35	<0.35	<0.35	<0.35	7.4	19.1
10	F	52	<0.35/<0.35	<0.35	<0.35	<0.35	3.3	256
11	F	45	<0.35/<0.35	<0.35	<0.35	<0.35	6.6	8.1
12	F	40	<0.35/<0.35	<0.35	<0.35	<0.35	2.5	62.1
13	M	65	<0.35/<0.35	<0.35	<0.35	<0.35	5.0	13.5
14	M	50	<0.35/<0.35	<0.35	**0.43**	<0.35	7.5	39.5
15	M	43	<0.35/<0.35	<0.35	<0.35	<0.35	6.7	19.4
16	F	44	<0.35/<0.35	<0.35	<0.35	<0.35	3.1	266
17	F	49	<0.35/<0.35	<0.35	<0.35	<0.35	9.2	13.1
18	M	26	<0.35/<0.35	<0.35	<0.35	<0.35	5.4	16.3
19	M	16	<0.35/<0.35	<0.35	**0.83**	<0.35	1.4	251
20	F	33	<0.35/<0.35	<0.35	<0.35	<0.35	3.0	5.56
21	M	27	<0.35/<0.35	<0.35	<0.35	<0.35	41.9	3.25

Patients No. 11, 19 and 21 also showed negative specific IgE to hornet venom. In all patients, measurement of specific IgE to wasp and honeybee venom was repeated within 1–2 months. Data given in bold indicate positive sIgE results.

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Results

Intradermal Skin Test

Twelve patients showed positive and 9 patients negative results for the intradermal skin test; 2 patients were positive for bee venom, 6 for wasp venom and 4 for both venoms (table 2).

Basophil Activation Test

The median percentages of basophil response to stimulation buffer (negative control), anti-FcεRI mAb and fMLP-positive control were 3% (range 2–4), 97% (28–100) and 60% (19–86), respectively. The median response to stimulation with allergens was 4% (range 2–72) and 17% (5–87) at 0.1 and 1 μg of honeybee venom, and 6% (range 1–68) and 46% (3–95) at 0.1 and 1 μg of wasp venom, respectively.

The cutoff value for positive results of allergen stimulation was set at 15% of CD63-positive basophils. Thus, 17 patients showed positive and 4 patients negative BAT results; 4 patients were positive for bee venom, 3 for wasp venom, and 10 patients for both venoms (table 2).

IgE Reactivity to Recombinant Venom Allergens

Three patients showed positive IgE reactivity to rVes v 5 and 18 patients were negative for rVes v 5. None of the patients demonstrated IgE reactivity for rVes v 1 and rApi m 1 (table 1).

Sensitivity in Patients with Negative sIgE

The diagnostic sensitivity of the intradermal skin test and BAT in patients with negative sIgE was 57% (12 of 21) and 81% (17 of 21), consequently. All 12 patients that tested positive for the intradermal test also showed posi-

Table 2. Clinical data and the results of basophil activation and intradermal skin tests

Patient No.	BAT, %		Intradermal skin tests		Culprit insect/time interval[1]	Clinical reaction	Planned VIT
	bee 0.1/1	wasp 0.1/1	bee 0.1/1	wasp 0.1/1			
1	4/**23**	**16/57**	neg./neg.	neg./neg.	?/3 years, 7 years	II	wasp
2	4/9	**25/71**	neg./neg.	**pos.**/n.d.	wasp, hornet/18 years, 37 years	III	wasp
3	**27/65**	11/**49**	neg./**pos.**	neg./**pos.**	bee/15 years	III	bee
4	2/**17**	4/6	neg./**pos.**	neg./neg.	?/3 months	IV	bee
5	4/7	**23/86**	neg./neg.	neg./**pos.**	wasp/4 years	III	wasp
6	2/**16**	2/5	neg./neg.	neg./neg.	?/2 months, 6 years	III	bee
7	3/5	5/6	neg./neg.	neg./neg.	bee/4 months	III	no
8	9/**19**	**16/30**	**pos.**/n.d.	**pos.**/n.d.	?/2 months, 5 years	IV	bee and wasp
9	4/**33**	5/**77**	neg./neg.	neg./**pos.**	wasp/2 months	IV	wasp
10	4/7	6/10	neg./neg.	neg./neg.	bee, wasp/2 months, 4 years	III	no
11[2]	7/**17**	2/4	neg./neg.	neg./neg.	hornet/12 years	IV	no
12	**19/25**	**15/46**	neg./neg.	**pos.**/n.d.	wasp/10 years	II	wasp
13	**72/87**	4/**36**	**pos.**/n.d.	**pos.**/n.d.	bee/1 months, 1 per 2–3 years, 20 years	III	bee
14	6/**16**	6/**53**	neg./neg.	neg./neg.	wasp/2 years	IV	wasp
15	8/9	5/9	neg./neg.	neg./neg.	wasp/4 months	IV	no
16[3]	6/**19**	1/3	neg./**pos.**	neg./neg.	wasp/2 months	IV	no
17	4/13	5/9	neg./neg.	neg./neg.	wasp/4 months	IV	no
18	4/**16**	**24/84**	neg./neg.	**pos.**/n.d.	wasp/5 months	II	no
19	4/12	**26/65**	neg./**pos.**	neg./**pos.**	hornet/1.5 years	IV	wasp
20	5/**22**	**68/95**	neg./neg.	neg./**pos.**	wasp/1 years	IV	wasp
21	3/**25**	10/**70**	neg./neg.	neg./neg.	hornet/8 years	III	wasp

Data given in bold indicate positive BAT or intradermal skin test results. n.d. = Not defined.
[1] Time interval between last sting reaction and the start of diagnostic evaluation.
[2] In patient No. 11, VIT was not planned due to conflicting diagnostic and culprit insect data.
[3] In patient No. 16, VIT was not planned yet due to further testing for conformation of culprit venom.

tive results for BAT. All 4 patients that tested negative for BAT also showed negative results for the intradermal test. The sensitivity of specific IgE to rVes v 5 was 14% (3 of 21), and 23% (3 of 13) for wasp BAT-positive and/or intradermal-positive subjects.

Sensitivity in Patients with Negative sIgE and Intradermal Skin Test

Out of 9 patients that tested negative for the sIgE and intradermal skin test, 5 of them had a positive BAT response. Thus, the diagnostic sensitivity of the BAT in this subgroup of patients was 56%.

Specificity

The specificity was analyzed according to the culprit data, which were apparent for 10 intradermal and 13 BAT-positive patients (table 2). In 6 subjects with a single positive intradermal skin test, the culprit data were matching, and in 1 subject, they were not. Three subjects showed double-positive intradermal test results, but a single-positive culprit insect. In 3 subjects with a single-positive BAT, the culprit data were matching, and in 2 subjects, they were not. Eight subjects showed a double-positive BAT, but a single-positive culprit insect. However, in all those double-positive patients, the basophil response to culprit insect venom was markedly higher than the response to the clinically irrelevant venom: median 20% (range 5–72) and 74% (range 46–95) at 0.1 and 1 μg of culprit venom, and 5% (range 3–19) and 25% (range 16–49) at 0.1 and 1 μg of irrelevant venom, respectively ($p \leq 0.01$; fig. 1). Consequently, the diagnostic specificity of the intradermal test was 60% (6 of 10), and of BAT, with regard to higher response, 85% (11 of 13).

Adherence to VIT

The VIT was planned in 14 subjects; in 4 patients, it was indicated with bee venom, in 9 patients with wasp venom, and in 1 patient with both venoms. It was also planned in 4 patients (in 3 with wasp venom and in 1 with bee venom) that showed only positive BAT results. It was not indicated in 4 subjects negative for all tests, in 2 subjects with conflicting culprit and testing data (No. 11 and 16; table 2), and in 1 subject with a grade II reaction without risk factors.

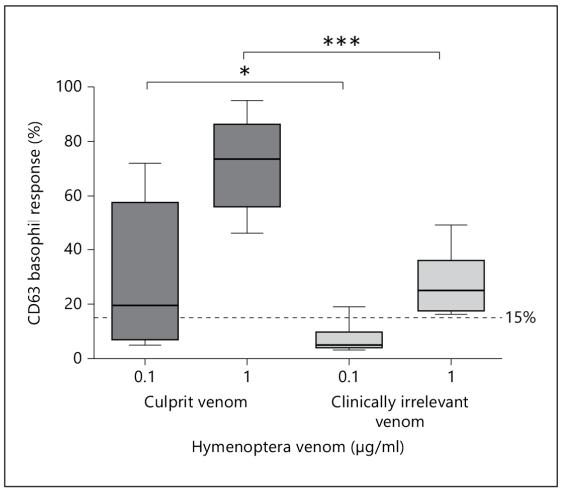

Fig. 1. Basophil response to culprit and clinically irrelevant venom in double-positive patients (n = 8). * $p < 0.05$; *** $p < 0.001$.

Discussion

In this study, we showed that the routine use of BAT allows the prompt identification of severe Hymenoptera venom-allergic patients with inconclusive specific IgE and skin test results. Thus, clinical utilization of BAT in practice should facilitate the prescription of VIT in complex cases.

Because VIT decisions rely on confirmation of allergic sensitization, it is clear that the management of those patients requires very sensitive diagnostic evaluation. According to current guidelines, the standard tests include skin testing and analysis of the serum for specific IgE to whole venom extract [9–11]. Nevertheless, a significant number of patients, and also those that may subsequently experience another severe or even fatal reaction to a sting, demonstrate negative sIgE and skin tests [1–3]. For such patients, the need for additional diagnostic approaches is obvious. In the field, a Hymenoptera venom allergy BAT was largely tested for the last 10 years either as a diagnostic or prognostic tool, or for monitoring VIT [3–8, 12–15]. However, those studies were carried out in an advance research setting on selected patients, and thus, there is a lack of data about the practical utility of BAT in a routine clinical setting and how those results could actually affect immunotherapy decision making. For this reason, in our study, all testing in all patients, except sIgE to recombinant venom allergens, was initiated prospectively by a practitioner allergist as a standard diagnostic evaluation of severe complex cases at our clinic.

Similar to a previous study [3], we clearly demonstrated that in patients with negative venom-specific IgE,

the diagnostic sensitivity of BAT is about 80% and the sensitivity of the intradermal skin test is about 50%. In other words, with BAT, we can successfully identify more than half of severe Hymenoptera-allergic patients with both negative sIgE and intradermal skin tests. Recently, it was suggested that more than half of wasp-allergic patients with negative sIgE to wasp venom might have positive IgE reactivity to rVes v 5 [15]. We also recently tested 25 patients with an anaphylactic reaction after wasp sting, but with negative wasp venom-specific IgE, and found that 32% of them showed positive rVes v 5 results [16]. However, all 25 of these wasp venom-allergic subjects showed clearly positive wasp-venom basophil responses. Furthermore, in the current report, only 14–23% of patients showed positive IgE reactivity to rVes v 5 and none of the patients were positive for other recombinants. Therefore, the addition of rVes v 5 or other recombinant IgE testing might be useful for diagnosing only a few patients.

Diagnosis and selection of the disease-causing insect venom is very important because VIT is the only effective prophylaxis for life-threatening reactions in patients allergic to Hymenoptera venom. Furthermore, in Hymenoptera-allergic patients, double IgE positivity to bee and wasp venom is often evident, and thus, the identification of the culprit venom could be difficult [17, 18]. Moreover, in the case of Hymenoptera-allergic patients with negative venom-specific IgE antibodies, both BAT and/or intradermal tests can show double-positive results [3], and this double positivity can hamper the identification of the relevant venom for immunotherapy, especially if the patient has an anaphylactic reaction to only one insect. Importantly, when we analyzed BAT double-positive patients in whom a single insect causing an allergic reaction has been identified, we showed a markedly higher basophil response to the culprit venom than to the clinically irrelevant venom. Similar observations were recently demonstrated by Eberlein et al. [19] which showed a much stronger basophil reaction to the clinically relevant insect venom than to the irrelevant one in double IgE-positive patients. On the other hand, in cases with double-positive intradermal tests, there was no correlation with a single culprit insect, and similar observations were also demonstrated by Eberlein et al. [19]. These results clearly suggest the clinical relevance of basophil response, not only for conformation of sensitization or for monitoring VIT [5, 6], but also as a tool for dissecting bee and wasp allergy as a basis for accurately prescribing the culprit venom for immunotherapy.

In summary, we demonstrated that in complex cases with inconclusive diagnostic results, BAT is more clinically sensitive and relevant than any other type of testing. Therefore, Hymenoptera-venom BAT should become more broadly available in clinical practice and should be routinely performed in patients that have a history of a reaction suggestive of severe insect sting anaphylaxis, but in whom testing for specific IgE antibodies and skin tests yield negative results.

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2.1.4 Kratkotrajna imunoterapija s strupi kožekrilcev povzroči z FcεRI posredovano desenzibilizacijo bazofilcev

Short-term venom immunotherapy induces desensitization of FcεRI-mediated basophil response

Nina Čelesnik Smodiš, Tina Vesel, Matija Rijavec, Mira Šilar, Renato Eržen, Mitja Košnik, Simona Eva Kloft Žitnik, Tadej Avčin, Peter Korošec

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Natančni mehanizmi vzpostavitve imunske tolerance pri kratkotrajni imunoterapiji s strupi kožekrilcev niso povsem razjasnjeni. Naš namen je bil preučiti vlogo receptorja velike afinitete FcεRI in z njim povezane vloge bazofilcev pri spodbuditvi kratkotrajne zaščite specifične imunoterapije (SIT) s strupi kožekrilcev. V študijo smo vključili 60 odraslih in 48 otrok. Pred pričetkom terapije in tik pred uvedbo prvega vzdrževalnega odmerka 100 μg strupa zelo hitre sheme SIT (5. dan) smo spremljali občutljivost bazofilcev (CD-sens) na stimulacijo s protitelesi anti-FcεRI, ekspresijo gena za α-podenoto receptorja FcεRI (*FCER1A*) in izražanje receptorja FcεRI na površini bazofilcev. Enakim parametrom smo sledili pred pričetkom terapije, med uvodno fazo in tik pred uvedbo prvega vzdrževalnega odmerka 70 in 100 μg strupa tudi pri hitri shemi SIT (1 – 2. ter 5. teden). Pri vseh vključenih preiskovancih smo pred uvedbo prvega vzdrževalnega odmerka ugotovili močno zmanjšano občutljivost bazofilcev pri stimulaciji s protitelesi anti-FcεRI tako pri zelo hitri kot tudi pri hitri shemi SIT. Z različnimi dinamikami med shemami SIT je bila pri 34 – 100 % vključenih bolnikov zmanjšana tudi ekspresija gena in izražanje receptorja FcεRI na celični površini. Ugotovili smo, da kratkotrajna SIT privede do močne desenzibilizacije bazofilcev po IgE/FcεRI poti, kar je možen mehanizem vzpostavitve imunske tolerance pri kratkotrajni SIT s strupi kožekrilcev.

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ORIGINAL ARTICLE ANAPHYLAXIS

Short-term venom immunotherapy induces desensitization of FcεRI-mediated basophil response

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basophils; high-affinity IgE receptor/FcεRI; *Hymenoptera* allergy; short-term venom immunotherapy.

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Abstract

Background: The precise immunological mechanisms for the early clinical protection of venom immunotherapy (VIT) have not yet been explained. Our aim was to evaluate whether high-affinity IgE receptor (FcεRI) and the related basophil function have a role in the induction of short-term VIT protection.
Methods: We included 60 adults and 48 children. Basophil threshold sensitivity (CD-sens) to anti-FcεRI stimulation, and FcεRI gene and cell-surface expression were assessed at the beginning and just before the first maintenance dose (MD) of 100 μg of ultra-rush VIT (day 5) and at the beginning, during buildup, and just before the first MD of 70 μg and of 100 μg of semi-rush VIT (weeks 1–2 and 5).
Results: We demonstrated a significant reduction in CD-sens to anti-FcεRI stimulation before the first MD in both ultra-rush and semi-rush VIT in all included subjects. FcεRI gene and/or cell-surface expression was decreased in 34–100% of subjects, with different dynamics between VIT protocols.
Conclusion: We found a marked desensitization of FcεRI-activated basophils after short-term VIT. This suppression, which could be highly relevant for the development of early protective mechanisms, might be also related to the changes at the level of FcεRI expression.

The clinical efficacy of venom immunotherapy (VIT) has been documented and well established. Although long-lasting allergen tolerance requires at least 3–5 years of treatment, its early protection has been confirmed already after the maintenance dose (MD) had been achieved (1,2). The early protective mechanisms that lead to unresponsiveness to the sensitizing allergen seem to develop during the buildup phase of VIT (1–3). Rush and ultra-rush venom immunotherapy protocols have proven to be as safe and efficient as conventional immunotherapy (1,2,4–6).

Despite its effectiveness, the precise immunological mechanisms for the immediate protection of VIT have not yet been explained. The proposed early markers for protective mechanisms during specific immunotherapy include increased tryptophan degradation, the rise in IL-10 levels, and IL-10-producing cell types, and up-regulation of histamine receptor 2 (3,7–12). Studies have shown reduced histamine, sulfidoleukotriene, and cytokine release during the early course of VIT, implying that their early suppression is one of the first protective mechanisms (11,13).

The high-affinity IgE receptor (FcεRI) is a key molecule on the surface of mast cells and basophils that participates in signal transduction of IgE-mediated response, and the initial FcεRI-mediated signal strength plays a major role in regulating effector cells' deactivation (14). In recent studies, the clinical efficacy of anti-IgE treatment was demonstrated within 7 days of the initial treatment, as soon as reduction in basophil FcεRI expression had been initiated (15,16). This indicates a critical role of basophils and FcεRI in the early process of clinical desensitization. Moreover, it was recently demonstrated that the subthreshold desensitization of basophils leads to the loss of FcεRI expression (17).

Because some previous data suggest an important role of FcεRI in modulating allergic response, we hypothesized that

Abbreviations
VIT, venom immunotherapy; FcεRI, high-affinity IgE receptor; MD, maintenance dose.

FcεRI changes could also be related to the early alternations induced by VIT. For this reason, we evaluated basophil threshold sensitivity to anti-FcεRI stimulation and FcεRI gene and cell-surface expression at the beginning and just before the first MD in ultra-rush and/or semi-rush VIT.

Methods

Study population

Sixty adults (mean age 46 years; range 19–73; 34 men) with a history of systemic allergic reaction after *Hymenoptera* sting [Mueller grades IV (55%), III (37%), and II (8%)], 18 children (11 years; 4–17; 13 boys) after honeybee sting [IV (6%), III (33%), II (44%), and I (17%)], and 30 children (11 years; 5–17; 25 boys) after *Vespula* sting [IV (17%), III (53%), II (27%), and I (3%)] were included in this prospective study. In 106 subjects, the sensitization was confirmed by venom-specific IgE and/or skin tests. Only two subjects had negative standard test results, but a positive basophil activation test (18). Thirty-four adults were enrolled in ultra-rush VIT with *Vespula* and 26 with honeybee venom. Eighteen children were enrolled in ultra-rush VIT with honeybee, and 30 children were enrolled in semi-rush VIT with *Vespula* venom. One child was missed before MD of 70 μg and 4 before MD of 100 μg. The control group consisted of five healthy, nonallergic individuals (31 years; 24–41; two men).

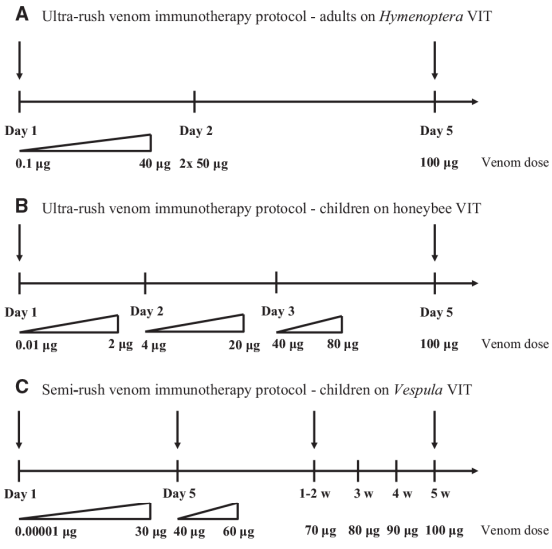

Figure 1 (A–C) Blood sampling (↓) in A 60 adults and B 18 children was performed just before ultra-rush VIT and the first MD of 100 μg and in C 30 children just before semi-rush VIT, during buildup, before the first MD of 70 μg and of 100 μg. One child was missed before MD of 70 μg and 4 before MD of 100 μg. MD: maintenance dose

All patients gave written informed consent for treatment with specific immunotherapy and participation in the study. The study was approved by the Slovenian National Medical Ethics Committee.

Study protocol

Blood samples of patients on ultra-rush VIT were taken before the beginning of treatment and at the end of the buildup phase, before the first MD of 100 μg administration. Blood samples of patients on semi-rush VIT were taken before the beginning of treatment, during buildup, before the first MD of 70 μg, and before the first MD of 100 μg. Blood samplings were performed just before the injection of allergen. The ultra-rush and semi-rush protocols are shown in Fig. 1. The control group was monitored on days 1 and 5.

Basophil threshold sensitivity to anti-FcεRI stimulation and CD-sens calculation

A basophil activation assay was performed on the heparinized whole blood incubated with serial dilutions of anti-FcεRI mAbs (Buhlmann Laboratories, Basel, Switzerland) from 550 to 0.55 ng/ml, at 37°C for 15 min. Degranulation was stopped by chilling on ice, after which anti-CD63, anti-CD123, and anti-HLA-DR mAb (BD Biosciences, Franklin Lakes, NJ, USA) were added and incubated for 20 min. Finally, whole-blood probes were lysed, washed, fixed, and analyzed within 2 h on a FACSCalibur flow cytometer (BD Biosciences).

Basophil sensitivity was determined as the anti-FcεRI mAbs concentration giving a 50% of maximum CD63% up-regulation. CD sensitivity (CD-sens) was calculated as the inverse value of this threshold anti-FcεRI mAb concentration multiplied by 100, as previously demonstrated (19–21). The higher value for CD-sens represents higher basophil sensitivity.

FcεRI gene expression profiles

Total RNA was isolated from whole-blood samples using the PAXgene Blood miRNA Kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Basophils were obtained from heparinized whole blood, enriched and purified by Ficoll density centrifugation, followed by magnetic cell sorting (MACS Basophil Isolation Kit II, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Total RNA was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified by Qubit® fluorometer (Invitrogen Corporation, Carlsbad, CA, USA). Following reverse transcription, cDNA was quantified by real-time PCR (ABI PRISM 7500 Real-Time PCR System) at standard conditions using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Expression levels of the α-subunit of high-affinity IgE receptor (*FCER1A*) (Hs00175232_m1) were normalized against 18S rRNA endogenous control (Applied Biosystems). All measurements were taken in triplicate for each sample and time point, and the relative expression was analyzed using the $\Delta\Delta C_t$ method.

FcεRI cell-surface expression

The number of FcεRI receptors per basophil (CD123 + / HLA-DR - cells) was analyzed using a FITC-conjugated antibody to FcεRI (eBioscience, San Diego, CA, USA) and standard curve of Calibration Beads (Dako Cytomation, Glostrup, Denmark) as previously described (20).

Absolute basophil cell count

For the absolute basophil count (CD123 + /HLA-DR - cells), we added AccuCount Fluorescent microbeads (Spherotec Inc., Libertyville, IL, USA) to fixed samples before analysis. The absolute number of basophils per µl of whole blood was calculated using the following equation: (number of events for basophil region/number of events for microbead region) × (number of microbeds used in test/volume of the whole-blood sample initially used).

Statistical analyses

Data distribution was evaluated using the D'Agostino–Pearson normality test. Depending on the distribution of the data, we used either a Wilcoxon's matched pairs test or a paired *t*-test. Data were expressed as median (IQR) or mean (95% CI). Probability values (P) of < 0.05 were accepted as significant. Analyses were performed using GraphPad Prism 5.

Results

Basophil activation

The results of %-CD63 basophil response were analyzed as dose-response curves (Fig. 2A–B) and CD-sens (Fig. 3A–B) after stimulation with serial dilutions of anti-FcεRI mAbs in 22 adults on ultra-rush VIT, in 30 children on semi-rush VIT, and in 5 healthy controls.

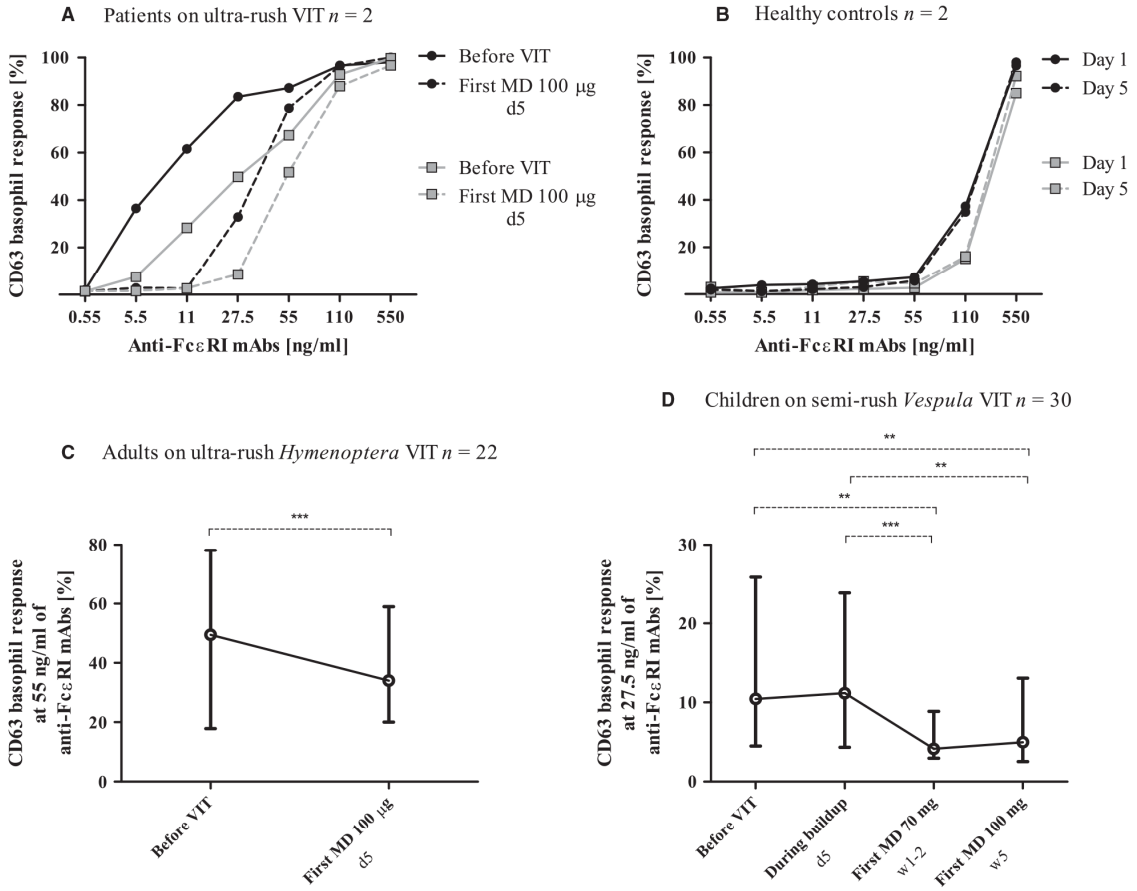

Figure 2 (A–D) CD63 basophil response in A two patients before ultra-rush VIT and the first MD of 100 µg and in B two healthy controls on days 1 and 5. CD63 basophil response at 55 ng/ml of anti-FcεRI mAbs in C 22 adults before ultra-rush VIT and the first MD of 100 µg and at 27.5 ng/ml in D 30 children before semi-rush VIT, during buildup, before the first MD of 70 and of 100 µg. Data are presented as dose-response curves or median values with IQR after stimulation with serial dilutions of anti-FcεRI mAbs. $^{**}P < 0.01$ $^{***}P < 0.001$

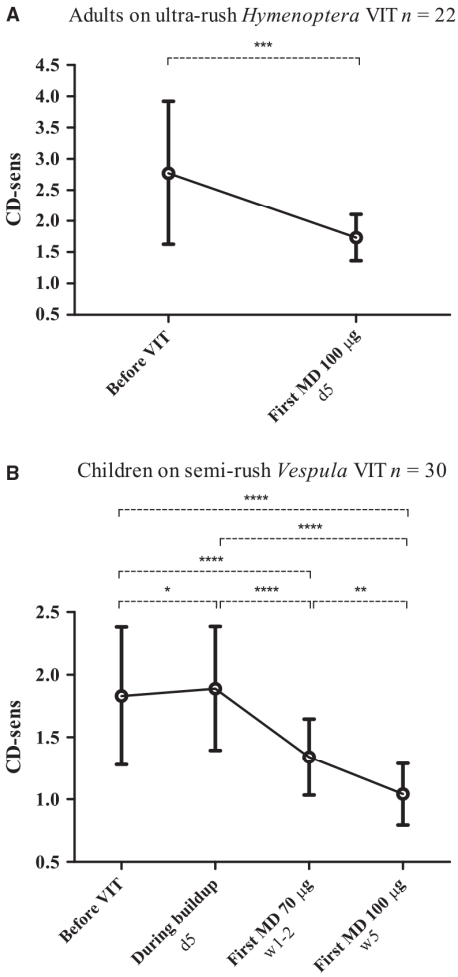

Figure 3 (A, B) Basophil threshold sensitivity (CD-sens) to anti-FcεRI stimulation in A 22 adults before ultra-rush VIT and the first MD of 100 µg and in B 30 children before semi-rush VIT, during buildup, before the first MD of 70 µg and of 100 µg. Data are presented as mean values with 95% CI of mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

Stimulation with submaximal concentration of anti-FcεRI

The CD63 basophil response following stimulation with submaximal concentrations from 110 to 5.5 ng/ml decreased significantly in both groups of patients. In adults, the decreases before the first MD of 100 µg were the most significant when tested at submaximal concentrations of 55 ng/ml (median 34.1%; IQR 20.1–59.0), 27.5 ng/ml (3.9%; 2.3–7.3), and 11 ng/ml (2.6%; 2.1–3.8), when compared to before ultra-rush VIT (for 55 ng/ml: 49.5%, 17.9–78.1, $P < 0.001$, Fig. 2C; for 27.5 ng/ml: 8.1%, 4.1–27.7, $P < 0.001$; and for 11 ng/ml: 4.7%, 2.9–24.7, $P < 0.001$).

In 30 children, the decreases before the first MD of 70 and 100 µg were the most significant at submaximal concentrations of 55 ng/ml (34.1%; 10.0–67.6; $P < 0.05$% and 26.9%; 8.7–46.9; $P < 0.001$, respectively) and 27.5 ng/ml (4.1%; 3.0–8.9; $P < 0.01$% and 5.0%; 2.5–13.1; $P < 0.01$, respectively) when compared to before semi-rush VIT (for 55 ng/ml: 39.4%; 13.0–68.6 and for 27.5 ng/ml: 10.5%; 4.5–25.9), whereas they slightly increased during buildup (for 55 ng/ml: 49.0%; 13.9–71.8; and for 27.5 ng/ml: 11.2%; 4.3–23.9; Fig. 2D).

The CD63 basophil response at maximal concentration of 550 ng/ml was comparable between all time points for both study groups (adults: median 96.9% and 96.7%, children: median between 90.4% and 91.6%). Similar response was found for minimal concentration of 0.55 ng/ml (adults: median 1.9% and 1.5%, children: median between 3.4% and 2.2%).

The group of healthy controls had similar responses on days 1 and 5 at each of the concentration tested (the differences were less than 5%; $P > 0.05$).

Basophil threshold sensitivity to anti-FcεRI stimulation (CD-sens)

Second, CD-sens, a measure of basophil threshold sensitivity to anti-FcεRI mAb stimulation, was estimated for both study populations and healthy controls. CD-sens in adults decreased significantly before the first MD of 100 µg (mean 1.7; 95% CI 1.4–2.1) in comparison with before ultra-rush VIT (2.8; 1.6–3.9; $P < 0.001$, Fig. 3A). Similar decrease was found in children before the first MD of 70 and 100 µg (1.3 and 1.0; 1.0–1.6 and 0.8–1.3; $P < 0.0001$, respectively) vs. before semi-rush VIT (1.8; 1.3–2.4), while CD-sens slightly increased during buildup (1.9; 1.4–2.4; $P < 0.05$, Fig. 3B). The decreases in CD-sens before the first MD were evident in all included subjects (100%).

In healthy controls, no discrepancy in CD-sens between the time points was noticeable (day 5: 0.7; 0.2–1.1 vs. day 1: 0.7; 0.2–1.2).

FcεRI gene expression profiles

FcεRI gene expression was analyzed on whole-blood samples of 60 adults and 18 children on ultra-rush VIT, in 30 children on semi-rush VIT, and in 5 healthy controls.

We found a significantly reduced expression of FcεRI gene before the first MD of 100 µg of ultra-rush VIT vs. before treatment, both in adults and in children ($P < 0.001$, Fig. 4A–B). The decrease was evident in 63% of adults and in 100% of children. However, in semi-rush VIT, FcεRI gene expression levels, when compared to before VIT, were significantly reduced only during buildup ($P < 0.01$, in 67% of subjects), and no differences were found before the first MD of 70 or 100 µg (to 34% of subjects; Fig. 4C). In control group, FcεRI expression remained at the same level on day 5 as it was on day 1 ($P > 0.05$).

To assess whether the decline in FcεRI gene expression is cellular related, human basophils were isolated in six adults before ultra-rush VIT and before the first MD of 100 µg. There were a decrease in four patients and a slight increase in two (Fig. 4D).

FcεRI cell-surface expression

FcεRI cell-surface expression was analyzed in 30 children on semi-rush VIT. We found a significant decrease before the first MD of 70 μg (mean 120 × 10^3 mol per cell; 95% CI 103–137 × 10^3; $P < 0.05$, in 59% of subjects), but no changes during buildup (136 × 10^3; 115–156 × 10^3) and before the first MD of 100 μg (135 × 10^3; 119–150 × 10^3), when compared to before VIT (127 × 10^3; 111–144 × 10^3, Fig. 5).

Absolute basophil cell count

Absolute basophil count was performed in 19 adults on ultra-rush VIT and in 30 children on semi-rush VIT. The blood basophil numbers before the first MD of 100 μg in ultra-rush protocol (12 basophils per μl; IQR 7–18) were highly comparable to prior to treatment (14 basophils per μl; 10–20, Fig. 6A). Absolute basophil count in semi-rush VIT; at the level of the first MD of 70 μg and 100 μg (19 and 15 basophils per μl; 11–30 and 10–25; respectively) was also comparable to before treatment (21 basophils per μl; 12–32); however, it decreased during buildup phase (11 basophils per μl; 8–18; $P < 0.001$, Fig. 6B).

Discussion

Venom immunotherapy unambiguously remains the treatment of choice for the prevention of severe systemic allergic reactions induced by *Hymenoptera* stings (22–24). It has been proven to be effective as soon as the MD is achieved (1,2), but the mechanisms responsible for the early protection have not yet been explained in detail (25). This study showed that short-term VIT induced a marked desensitization of FcεRI-mediated basophil activation, which seems to be associated with down-regulation of FcεRI expression.

Basophil desensitization was evident before the first MD, within 5 days of ultra-rush or a few weeks of semi-rush VIT, but not during buildup phase. These changes are comparable with previous observations that demonstrated decreased IgE receptor-induced histamine and sulfidoleukotriene release. Pierkes et al. (11) proposed that this effect is partially due to the induction of IL-10 and IFN-γ production of T cells, and Novak et al. (12) suggested that desensitization could be rapidly induced by means of up-regulation of histamine receptor 2. Our results show that clinically induced basophil desensitization could also be associated with the reduction in FcεRI expression, and this observation may provide an additional explanation for the early effect of VIT. This correlates with a

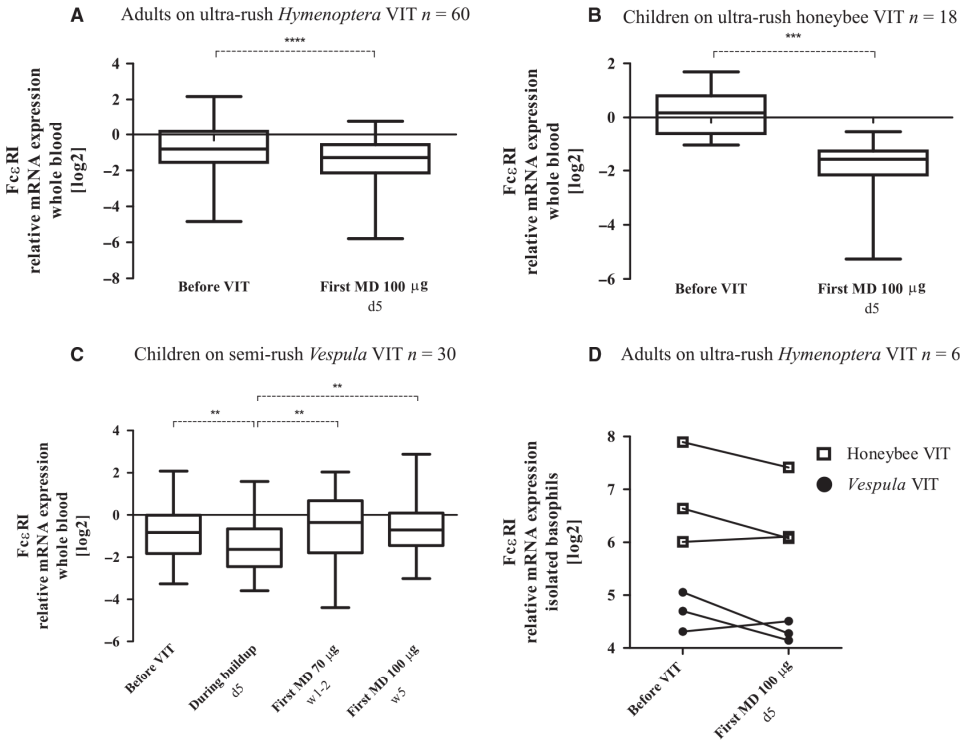

Figure 4 (A–D) FcεRI relative mRNA expression profiles of whole-blood samples in A 60 adults and B 18 children before ultra-rush VIT and the first MD of 100 μg and in C 30 children before semi-rush VIT, during buildup, before the first MD of 70 μg and of 100 μg and of isolated basophils in D 6 adults before ultra-rush VIT and the first MD of 100 μg. In A–C, the box plot indicates the median (horizontal line), interquartile range (box), and minimal and maximal values (whiskers), and in D, data are presented as before–after plots. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$

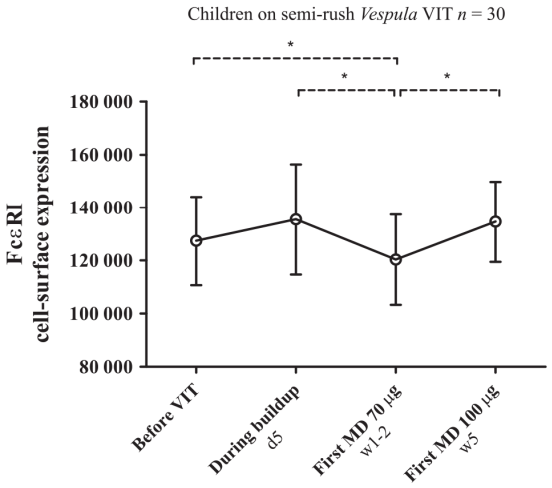

Figure 5 FcεRI cell-surface expression in 30 children before semi-rush VIT, during buildup, before the first MD of 70 µg and of 100 µg. Data are presented as mean values with 95% CI of mean. $*P < 0.05$.

recent study of MacGlashan, which demonstrated that under conditions of *in vitro* subthreshold stimulation, basophils become desensitized due to the loss of FcεRI expression (17). In this study, desensitization leads to the loss of Syk as well; however, priming with IL-3 completely blunted the loss of Syk, but showed no effect on FcεRI.

While the FcεRI-mediated basophil desensitization was similar, both in ultra-rush and in semi-rush VIT protocol, there was a discrepancy concerning the FcεRI expression. Namely, the decrease in whole-blood FcεRI gene expression was significant only in ultra-rush protocols. In a limited number of patients, this was also confirmed at the level of isolated basophils. In semi-rush protocol, whole-blood FcεRI gene expression did not show any significant changes, although we demonstrated a significant decrease in FcεRI cell-surface expression. The observed discrepancy in gene expression might be influenced by the number of basophils. However, the blood basophil numbers at the MD level in ultra-rush and semi-rush protocol were comparable to the state before starting VIT and were decreased only during buildup phase. Similar decreases in blood basophil count during buildup phase (12, 26) and returning to baseline values at the time of the first MD, after 1 week of immunotherapy, were also demonstrated previously (26). Early basophil desensitization seems to be a dynamic process that in different points might include different changes in the sensitivity of FcεRI signaling elements (14, 17, 27). Nevertheless, the differences between ultra- and semi-rush protocol might be also influenced by the cellular kinetic of basophils, which is relatively rapid with the turnover of several days (28). Interestingly, a similar kinetics as in ultra-rush VIT protocol was also demonstrated in anti-IgE treatment model, in which a significant FcεRI expression and basophil phenotype alternations were evident within 7 days of the initial treatment (15,16).

Although we did not assess the rate of clinical protection by sting challenge, all patients completed the buildup phase and reached the MD of 100 µg of venom. In fact, none of the previous (3,10–13) or the current study of early VIT-related markers objectively assessed the rate of early clinical protection as shown previously (1,2). We recently demonstrated in our 8-year follow-up study that after VIT a significant and approximately fourfold decrease in allergen-specific basophil response was demonstrated in all tolerant subjects, which suggests that basophil changes could be crucial for successful induction of tolerance after completing a successful course of VIT (29). Similar allergen-specific basophil changes were also observed after 2–4 years of children honeybee VIT (30). Unfortunately, these studies involved only anti-FcεRI response to maximum stimulation (at 550 ng/ml), and thus, we were not able to assess whether FcεRI-mediated subthreshold basophil desensitization also appears in later course of VIT. In contrast, in current study, we did not assess the allergen specificity of FcεRI-mediated desensitization, that is, if beyond VIT allergen, these changes are also relevant for possible non-VIT allergens. Obviously, both those aspects should be clarified in further studies.

In summary, FcεRI-mediated basophil desensitization may be an important factor for inducing the early protective state

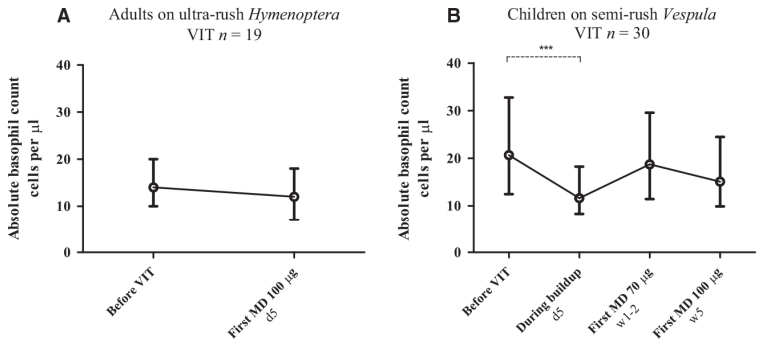

Figure 6 (A, B) Absolute basophil count (CD123 + /HLA-DR - cells) in A 19 adults before ultra-rush VIT and the first MD of 100 µg and in B 30 children before semi-rush VIT, during buildup, before the first MD of 70 µg and of 100 µg. Data are presented as median values with IQR. $***P < 0.001$

that must be achieved before the application of the first maintenance dose of VIT. Furthermore, a detailed understanding of these mechanisms would allow the development of novel interventions for promoting or monitor the silencing of basophil FcεRI pathway.

Authors' Contributions

P.K., N.Č. involved in the conception and design of the manuscript; N.Č., T.V., M.R., M.Š., R.E., M.K., S.E.K.Ž., and T.A. involved in the analysis and interpretation of the data; P.K. and N.Č. involved in drafting the manuscript for important intellectual content.

Conflicts of interests

All authors declare that there is no conflict of interest.

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2.1.5 Kratkotrajna imunoterapija s strupi kožekrilcev povzroči z FcεRI posredovano desenzibilizacijo bazofilcev, ki je za strup nespecifična

Down-regulation of FcεRI-mediated CD63 basophil response during short-term VIT determined venom-nonspecific desensitization

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Pri kratkotrajni specifični imunoterapiji (SIT) s strupi kožekrilcev smo ugotovili desenzibilizacijo bazofilcev po IgE/FcεRI poti. Nadalje smo želeli ugotoviti ali je FcεRI desenzibilizacija bazofilcev za alergen specifična ali nespecifična. Vključili smo 11 primarno dvojno senzibiliziranih bolnikov za čebelji in osji strup hkrati ter pred pričetkom terapije in tik pred uvedbo prvega vzdrževalnega odmerka SIT s strupom enega kožekrilca, spremljali občutljivost bazofilcev (CD-sens) na stimulacijo s protitelesi anti-FcεRI ter na čebelji in osji strup. Pri nekaterih bolnikih smo sledili tudi odzivnosti bazofilcev na poglavitni rekombinantni alergen rApi m 1 ali rVes v 5 ali na inhalacijski alergen (pelodi trav) v primeru dodatne ko-senzibilizacije. V enakih časovnih točkah, smo pri dodatnih 7 bolnikih, izvedli še pasivno senzibilizacijo bazofilcev. Bazofilcem smo odstranili za čebelji ali osji strup specifična protitelesa IgE (sIgE) ter jih senzibilizirali s specifičnimi protitelesi IgE za pršico. Pri vseh 18 vključenih preiskovancih smo pred uvedbo prvega vzdrževalnega odmerka ugotovili močno zmanjšano občutljivost bazofilcev pri stimulaciji s protitelesi anti-FcεRI in z za SIT-specifičnim strupom kožekrilca. Nadalje smo pri 10 od 11 primarno dvojno senzibiliziranih bolnikih pred uvedbo prvega vzdrževalnega odmerka ugotovili primerljivo statistično značilno zmanjšano občutljivost bazofilcev pri stimulaciji z za SIT-nespecifičnim strupom kožekrilca. Primerljiv nespecifičen učinek smo pokazali pri stimulaciji s poglavitnim vrstno-specifičnim rekombinantnim alergenom strupa, ki ni bil vključen v terapijo. Pri poli-senzibiliziranem bolniku smo ugotovili zmanjšano občutljivost bazofilcev za pelode trav. Pri 7 bolnikih s pasivno senzibiliziranimi bazofilci za alergen pršice smo ugotovili statistično značilno zmanjšano občutljivost bazofilcev za *de novo* senzibilirajoči alergen pršice. Kratkotrajna SIT s strupi kožekrilcev privede do desenzibilizacije bazofilcev za specifičen strup kožekrilca, s katerim izvajamo SIT kot tudi za nespecifičen strup kožekrilca, ki ni vključen v SIT. Za razliko od specifičnega učinka dolgotrajne SIT, je učinek kratkotrajne SIT s strupi kožekrilcev verjetno za strup nespecifičen.

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Down-Regulation of FcεRI-Mediated CD63 Basophil Response during Short-Term VIT Determined Venom-Nonspecific Desensitization

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Abstract

Background: We recently showed a desensitization of FcεRI-mediated basophil response after short-term VIT. Our aim was to evaluate the allergen specificity of this desensitization.

Methods: In 11 *Hymenoptera*-venom double positive subjects, basophil threshold sensitivity (CD-sens) to anti-FcεRI, honeybee, and *Vespula* venom was assessed at the beginning and just before the first maintenance dose (MD) of single ultra-rush VIT. In some patients we also monitored CD-sens to rApi m 1 and/or rVes v 5 or other co-sensitizations (i.e., grass pollen). In additional 7 patients, basophils were stripped and sensitized with house dust mite (HDM) IgEs at the same time points.

Results: We demonstrated a marked reduction of CD-sens to anti-FcεRI and VIT-specific venom before the first MD in all 18 subjects included. Furthermore, in 10 out of 11 double positive subjects, a significant and comparable decrease before the first MD was also evident for non-VIT venom; this nonspecific decrease was further supported by the opposite recombinant species-specific major allergen. In one subject with additional grass pollen allergy, a decrease of CD-sens to grass allergen was also demonstrated. Similarly, in 7 cases of patients with passively HDM-sensitized basophils, a significant reduction of CD-sens was also evident to *de novo* sensitized HDM allergen.

Conclusions: Short-term VIT induced basophil desensitization to VIT-specific as well as to VIT-nonspecific venom. As opposed to long-term VIT, which induces venom-specific changes, the effect of short-term VIT seems to be venom-nonspecific.

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Introduction

Venom immunotherapy (VIT) is unambiguously the treatment of choice for prevention of severe systemic allergic reactions induced by *Hymenoptera* stings [1–3] and the early protective mechanisms that lead to unresponsiveness to the sensitizing allergen seem to develop during the course of short-term VIT, as soon as the maintenance dose (MD) is achieved [4–6]. Despite its effectiveness, the precise immunological mechanisms for the immediate protection of VIT have not yet been explained. Recently we showed that short-term VIT induced a marked desensitization of FcεRI-mediated basophil activation and that this desensitization was evident in both adults and children before the first MD, within 5 days of ultra-rush or a few weeks of semi-rush VIT, but not during the buildup phase [7]. These changes were comparable with other reports that demonstrated decreased IgE receptor-induced histamine, sulfidoleukotriene, and cytokine release during short-term VIT [8,10,11]. Unfortunately, these studies did not clarify the allergen specificity of this desensitization (i.e., if these changes are also relevant for possible non-VIT co-sensitizing allergens beyond VIT allergen), as shown previously by means of decreased peripheral leukocyte sensitivity to mediator release during short-term immunotherapy to the allergen injected and other unrelated sensitizing allergen not included in the therapy [12–15]. Similar nonspecific effect was found during short-term *in vitro* subthreshold basophil desensitization [16,17].

For this reason, we carried out a complex follow-up study to evaluate basophil threshold sensitivity to anti-FcεRI, VIT, and non-VIT venom in double positive adult subjects at the beginning and just before the first MD of single ultra-rush VIT. In some patients we also monitored basophil sensitivity to opposite, non-VIT venom major allergens such as rVes v 5 or rApi m 1 or other co-sensitizing aeroallergens. To further assess whether these changes were cellular-based, we set up a controlled experimental design of a passive IgE sensitization of stripped honeybee (HBV) or *Vespula* venom (VV) basophils. Thus, at the beginning and just before the first MD, the patients' basophils were isolated and sensitized with house dust mite (HDM) serum IgE antibodies and followed up for basophil threshold sensitivity to HDM allergen.

Finally, all patients were monitored for whole blood FcεRI gene and basophil cell-surface expression.

Materials and Methods

Study population

Eleven subjects (mean age 41 years; range 23–55; 10 men) with double positive sIgE and basophil activation test (BAT) to HBV and VV were included in this prospective study (Table 1). Double positivity to HBV and VV was confirmed in nine (1–3, 6–11) with clinical history and recombinant Api m 1 and Ves v 5 or v 1 by sIgE and/or BAT. Subjects nos. 4 and 5 were positive only to rVes v 5, but also had a clear history of anaphylaxis after honeybee sting (Mueller grade IV and I, respectively) and double positive sIgE, skin, and/or BAT to both venoms [18]. The clinical relevance of an additional grass pollen allergy in subject no. 11 was confirmed by sIgE, skin test, BAT, and recombinant major allergens Phl p 1, 5b. For the passive IgE sensitization experiment, seven subjects (39 years; 23–56; 4 male) with single positive clinical history, venom-specific IgE and/or BAT and species-specific recombinants to HBV or VV were included in the study (Table 2). They all had negative sIgE, skin test, and BAT to HDM allergen.

In the double positive group, the venom for initial ultra-rush VIT was selected according to greater severity of induced anaphylaxis (5 Mueller grade IV, 5 III, and 1 II), and therefore eight subjects were enrolled in initial ultra-rush VIT with HBV and three with VV (Table 1). From the single positive group, three subjects with Mueller grades IV, III and II were enrolled in HBV and one with grade IV and three with grade III in VV ultra-rush VIT (Table 2). The ultra-rush *Hymenoptera* VIT protocol was performed as previously described in detail [7]. Blood samples were taken before the beginning of treatment and at the end of the buildup phase, before the first MD administration at day 5. All subjects gave written informed consent and the study was approved by the Slovenian National Medical Ethics Committee.

Basophil threshold sensitivity and CD-sens calculation

A basophil activation assay was performed on the heparinized whole blood incubated with basophil stimulation buffer with IL-3 (Bühlmann, Switzerland) containing serial dilutions of anti-FcεRI mAbs (550–0.55 ng/ml; Bühlmann), HBV (1–0.001 μg/ml) and VV (1–0.001 μg/ml; Hal Allergie, Netherlands) or rApi m 1 (8–0.08 μg/ml) and/or rVes v 5 (1–0.001 μg/ml) expressed in *E. coli* [19] or grass pollen (10–0.1 μg/mL; AQUAGEN SQ, grass pollen mix L299, ALK Abello, Spain), at 37 °C for 15 minutes. Degranulation was stopped by chilling on ice, after which anti-CD63, anti-CD123, and anti-HLA-DR mAb (BD Biosciences, USA) were added and incubated for 20 minutes. Finally, whole blood probes were lysed, washed, fixed, and analyzed within 2 hours on a FACSCalibur flow cytometer (BD Biosciences) [7,20–22].

Basophil sensitivity was determined as the allergen concentration giving a 50% of maximum CD63% up-regulation. CD sensitivity (CD-sens) was calculated as the inverse value of this threshold allergen concentration multiplied by 100, as previously demonstrated [7,23–25]. The higher value for CD-sens represents higher basophil sensitivity.

Passive IgE sensitization

Peripheral blood mononuclear cells (PBMCs) from heparinized blood of HBV or VV single positive donors were first separated by Ficoll-Paque (GE Healthcare Bio-Sciences AB, Sweden) density gradient and washed twice with RPMI 1640 (GIBCO, USA) and again with RPMI 1640 with 0.5% BSA (Sigma, USA; RPMI-BSA). PBMCs were then treated with lactic acid buffer (pH 3.9) containing 13.4 mM lactic acid, 140 mM NaCl, and 5 mM KCl (Sigma) for 3.5 minutes, and with 15 μl PBS-TrisBase (Immuno-Concepts, USA) and washed again with RPMI-BSA. The cells were re-suspended in a solution containing sera from subjects allergic to HDM allergen, 32 μl 0.1 M EDTA (Sigma), and 8 μl heparin 250 IU/ml (Krka, Slovenia), and placed in a CO_2 incubator for 90 min at 37 °C. Afterwards the cells were washed,

Table 1. Clinical data, sIgE and BAT in double positive subjects.

Patient no.	Age (years)	Sex	Mueller grade		Initial VIT	sIgE (kUA/L)		Diagnostic BAT (%)		sIgE (kUA/L)		
			HB	V		HBV	VV	HBV	VV	rApi m1	rVes v5/v1	OSR
						(i1)	(i3)	1/0.1 μg/mL	1/0.1 μg/mL	(i208)	(i209/i211)	(f316)
1	49	M	IV	LLR	HBV	13.0	1.23	97/41	56/3	1.95	1.46	1.63
2	26	M	III	II	HBV	5.24	2.23	94/73	67/3	0.94	0.66	<0.35
3	54	M	III	LLR	HBV	14.0	2.41	70/46	65/5	2.65	0.84	0.67
4	47	M	IV	I	HBV	1.71	1.12	95/77	72/7	<0.35	0.37	<0.35
5	36	M	I	IV	VV	2.26	8.14	56/5	85/56	<0.35	2.27	2.35
6	31	M	III	II	HBV	4.41	0.81	96/84	94/61	0.86	1.07	<0.35
7	55	M	nk	IV	VV	4.59	0.70	73/42	73/19	1.04	<0.35	<0.35
8	31	M	I	IV	VV	9.94	25.0	72/4	71/6	0.35	4.45	11.2
9	55	F	II	nk	HBV	3.50	0.36	94/43	76/2	1.21	0.38	<0.35
10	23	M	III	LLR	HBV	9.74	5.00	82/74	66/18	3.25	1.97	3.70
11*	40	M	III	LLR	HBV	10.7	3.06	94/93	96/28	0.59	0.49	3.86

M: male, F: female, HB(V): Honeybee (venom), V(V): *Vespula* (venom).
LLR: large local reaction, nk: the degree of reaction after the sting is not known.
Diagnostic BAT: the threshold value for diagnostically positive results was defined as 15% of CD63-positive basophils [20–22].
*Patient with additional grass pollen sensitization: skin prick test (mixed grasses; HAL Allergy) pos; sIgE Timothy (g6): 31.1 kUA/L; rPhl p 1,5b (g213): 9.15 kUA/L.
All sIgE were measured with ImmunoCAP-FEIA (Phadia, Thermo Fisher Scientific, Uppsala, Sweden).
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Table 2. Clinical data, sIgE and BAT in subjects for passive IgE sensitization.

Patient no.	Age (years)	Sex	Mueller grade	VIT	sIgE (kUA/L)		Diagnostic BAT (%)		sIgE (kUA/L)		
					HBV	VV	HBV	VV	rApi m1	rVes v5/v1	OSR
					(i1)	(i3)	1/0.1 μg/mL	1/0.1 μg/mL	(i208)	(i209/i211)	(f316)
12	40	F	II (HB)	HBV	0.36	<0.35	73/78	10/6	<0.35	<0.35	<0.35
13	55	F	III (V)	VV	<0.35	12.3	6/3	72/64	<0.35	69.20	<0.35
14	35	M	IV (HB)	HBV	11.6	1.47	96/86	5/2	0.49	<0.35	3.14
15	56	M	IV (V)	VV	<0.35	1.17	3/1	76/4	<0.35	0.36	<0.35
16	23	F	III (V)	VV	<0.35	21.9	14/3	51/4	<0.35	1.81	<0.35
17	32	M	III (HB)	HBV	1.33	<0.35	89/29	36/2	4.01	<0.35	0.49
18	33	M	III (V)	VV	<0.35	4.21	14/10	77/8	<0.35	1.14	<0.35

M: male, F: female, HB(V): Honeybee (venom), V(V): *Vespula* (venom).
Diagnostic BAT: the threshold value for diagnostically positive results was defined as 15% of CD63-positive basophils [20–22].
All sIgE were measured with ImmunoCAP-FEIA (Phadia, Thermo Fisher Scientific, Uppsala, Sweden).
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re-suspended in RPMI-BSA, and immediately used in BAT, where they were stimulated with serial dilutions of *D. pteronyssinus* (333.3–1.665 ng/ml; Bühlmann). The BAT protocol was completely the same as for heparinized whole blood samples [7,20–22].

FcεRI gene expression profiles

Whole blood FceRI gene expression levels were determined as previously described [7]. Briefly, total RNA was isolated from whole blood using the PAXgene Blood miRNA Kit (PreAnalytiX GmbH, Switzerland) and quantified by Qubit fluorometer (Invitrogen Corporation, USA). Following reverse transcription, cDNA was quantified by real-time PCR (ABI PRISM 7500 Real-Time PCR System) at standard conditions using TaqMan Universal PCR Master Mix (Applied Biosystems, USA). Expression levels of the α-subunit of high-affinity IgE receptor (*FCER1A*) (Hs00175232_m1) were normalized against ribosomal 18s RNA Endogenous Control (Applied Biosystems). All measurements were performed in triplicate for each sample and time point and relative expression were analyzed using the ΔΔCt method.

FceRI cell-surface expression

The number of FceRI receptors per basophil (CD123+ HLA-DR– cells) was analyzed using a FITC-conjugated antibody to FceRI (eBioscience, USA) and standard curve of Calibration Beads (Dako Cytomation, Denmark) as previously described [7,24].

Absolute basophil cell count

For the absolute basophil count (CD123+ HLA-DR– cells) we added AccuCount Fluorescent microbeads (Spherotec Inc., USA) to fixed samples before analysis. The absolute number of basophils per μl of whole blood was calculated using the following equation: (number of events for basophil region/number of events for microbeads region) × (number of microbeds used in test/volume of the whole blood sample initially used) as previously described [7].

Statistical analyses

Depending on the distribution of the data, we used either a Wilcoxon matched pairs test or a paired *t*-test. Data were expressed as median (IQR) or mean (95% CI). Probability values (*P*) of less than 0.05 were accepted as significant. Analyses were performed using GraphPad Prism 5.

Results

Basophil threshold sensitivity (CD-sens) to VIT and non-VIT venom

A marked reduction of CD-sens to anti-FcεRI and VIT-specific venom was demonstrated before the first MD in comparison to before VIT in all subjects included (before VIT: median 6.4, IQR 2.5–9.3 and 884, 389–2034 vs. before MD: 3.9, 2.2–5.6 and 775, 312–1143, respectively; $P<.001$; Figure 1A, B). Furthermore, a significant and comparable decrease was also evident in non-VIT venom in 10 out of 11 double positive subjects (385, 219–719 vs. 213, 178–676, $P<.01$; Figure 1C).

Basophil response to recombinant species-specific major allergens

To determine that the observed basophil desensitization to non-VIT venom is not related to cross-reactive epitopes, we included the opposite recombinant species-specific major allergens. Patients nos. 6 and 10 were followed at the beginning and before the first MD of HBV VIT. CD-sens to anti-FcεRI and HBV markedly decreased before the first MD when compared to before HBV VIT (before VIT: 2.3; 1538 and 41.1; 524 vs. before MD: 2.1; 1042 and 1.4; 322, separately; Figure 2A, B). Moreover, a comparable decrease was evident in response to non-VIT rVes v 5 allergen (1136 and 621 vs. 877 and 535, separately; Figure 2C). Patient no. 7 was monitored during VV VIT. Similarly, the decrease in CD-sens before the first MD versus the state before VV VIT was evident to anti-FcεRI (before VIT: 4.0 vs. before MD: 2.6), rVes v 5 (144 vs. 122) and non-VIT rApi m 1 (152 vs. 132), shown in Figure 2D–F.

Basophil response in patient co-sensitized to grass pollen allergen

To further clarify the allergen specificity of basophil desensitization, patient no. 11 suffering from allergic rhinitis was followed before treatment and the first MD of HBV VIT. An evident reduction in CD-sens was demonstrated before the first MD in comparison to before HBV VIT to anti-FcεRI (before VIT: 15.6 vs. before MD: 3.9) and all co-sensitizing allergens i.e., HBV (6667

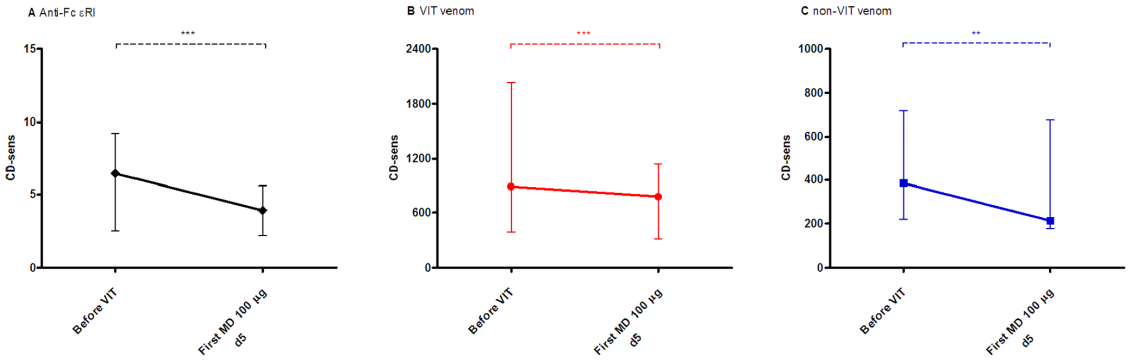

Figure 1. A–C. Basophil threshold sensitivity (CD-sens). Basophil threshold sensitivity (CD-sens) in 11 double and 7 single positive subjects in **A** to anti-FcεRI and in **B** to VIT venom and in 11 double positive subjects in **C** to non-VIT venom stimulation before treatment and the first maintenance dose of single ultra-rush VIT. Data are presented as median values with interquartile range. ***P*<.01 ****P*<.001.
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vs. 3448), VV (719 vs. 676) and also to grass pollen (20.5 vs. 12.1), demonstrated in Figure 3A–D.

Passive IgE sensitization

To further assess whether these basophil changes were cellular-based, we conducted a controlled experiment of passive IgE sensitization of 7 HBV or VV stripped basophils with sera of HDM allergic subjects before treatment and the first MD of VIT. All individual CD63 basophil dose-response curves to anti-FcεRI, VIT-specific venom and to *de novo* sensitized HDM allergen were decreased at the level of the first MD when compared to before VIT (patients nos. 12–18; Figure 4A–F). CD-sens of HBV (patients

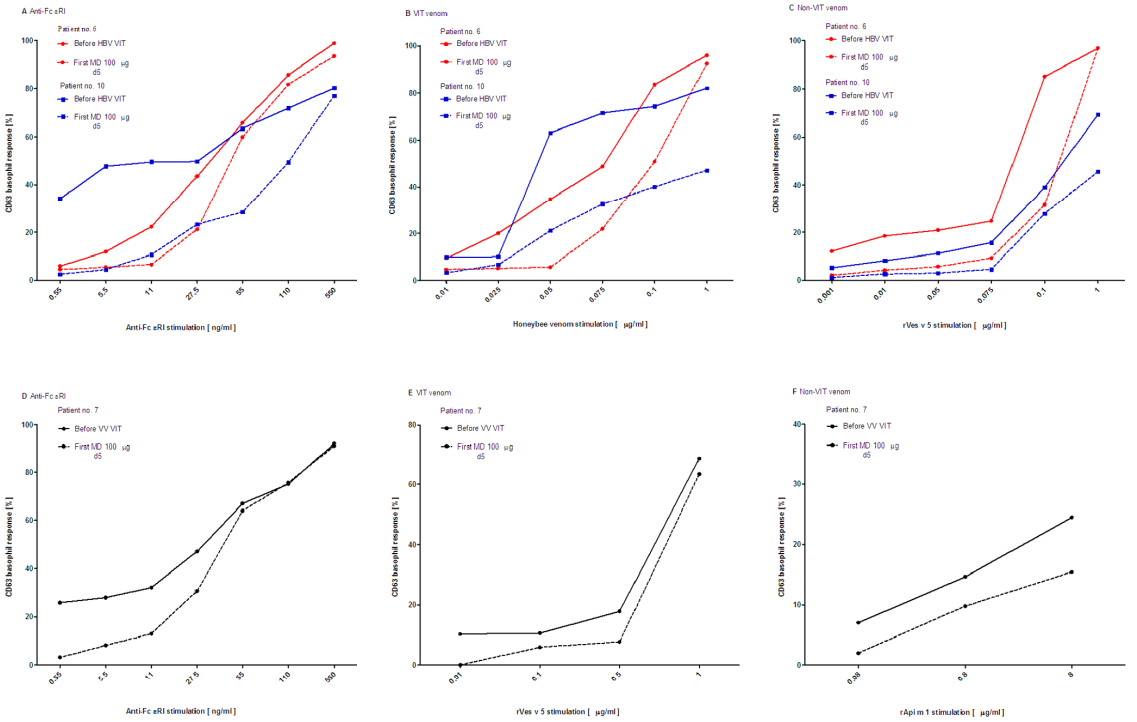

Figure 2. A–F. Basophil response to recombinant species-specific major allergens. CD63 basophil dose-response curves in double positive patients nos. 6 and 10 in **A** to anti-FcεRI in **B** to honeybee and in **C** to rVes v 5 stimulation before treatment and the first maintenance dose of honeybee ultra-rush VIT and in double positive patient no. 7 in **D** to anti-FcεRI in **E** to rVes v 5 and in **F** to rApi m 1 stimulation before treatment and the first maintenance dose of *Vespula* ultra-rush VIT.
doi:10.1371/journal.pone.0094762.g002

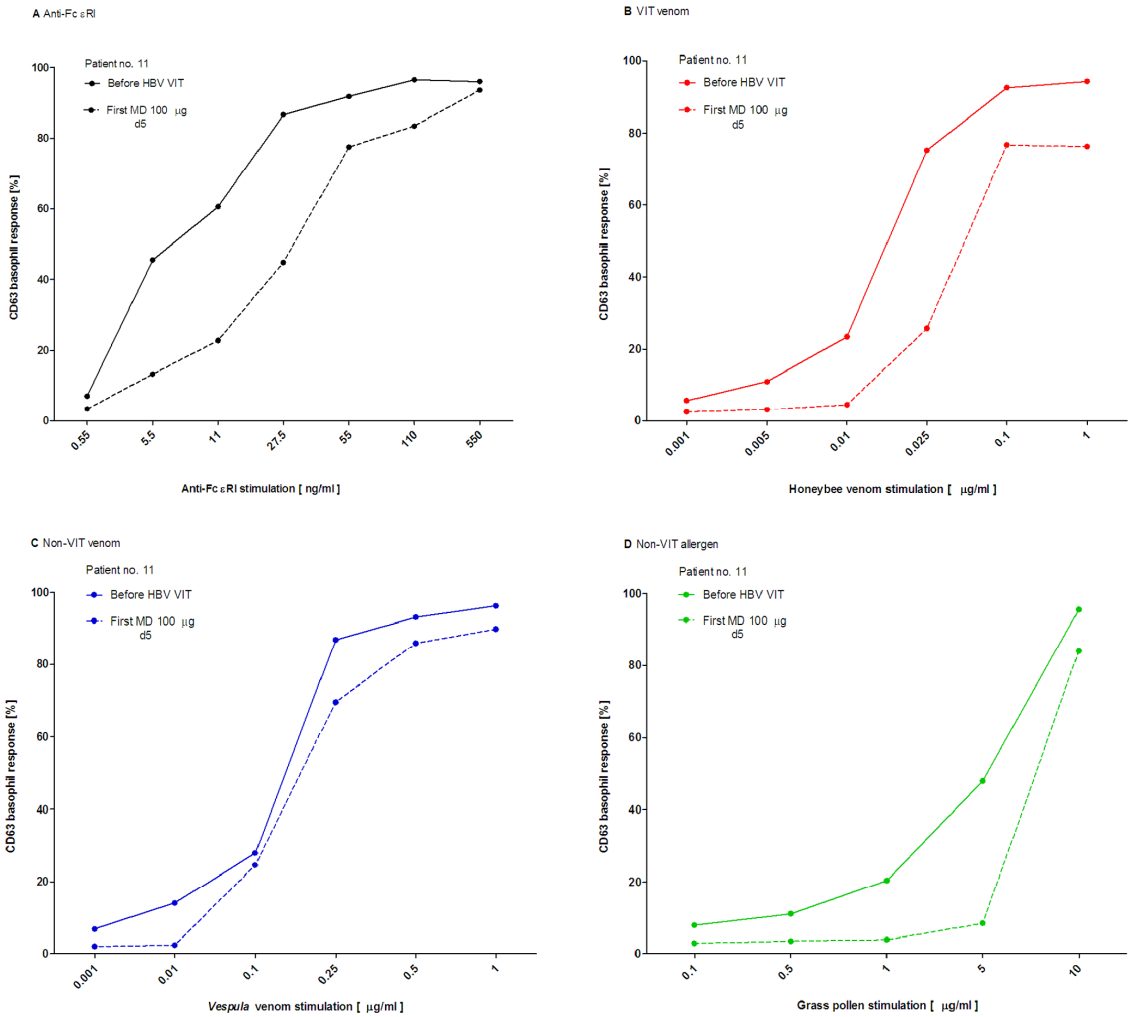

Figure 3. A–D. Basophil response in patient co-sensitized to grass pollen allergen. CD63 basophil dose-response curve in poly-sensitized patient no. 11 in **A** to anti-FcεRI in **B** to honeybee in **C** to *Vespula* venom and in **D** to grass pollen stimulation before treatment and the first maintenance dose of honeybee ultra-rush VIT.
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nos. 12, 14 and 17) and VV allergic subjects (patient nos. 13, 15, 16 and 18) before the first MD was significantly decreased when compared to before VIT to anti-FcεRI (before VIT: median 6.5, IQR 1.6–9.2 vs. before MD: 3.9, 1.4–5.9; $P<.05$; Figure 5A) and to VIT-specific venom (403, 296–7143 vs. 340, 244–3571; $P<.05$; Figure 5B). Moreover, after passive IgE sensitization of those stripped basophils with HDM-specific IgEs, a similar reduction was found to *de novo* sensitized HDM allergen. CD-sens to HDM stimulation at the level of the first MD versus before VIT was significantly decreased (before VIT nos. 12–18: 5.6, 1.3–42 vs. 3.9, 1.1–26; $P<.05$; Figure 5C).

FcεRI gene and cell-surface expression

We found a reduced expression of FcεRI gene before the first MD of VIT versus before treatment in 11 of 18 subjects included (61%) (before VIT mean −0.040, 95% CI −0.654 to 0.574 vs. before MD: −0.174, −0.868 to 0.520); however, the differences were not significant. A similar reduction was also evident in basophil FcεRI cell-surface expression (84×10^3 mol per cell, $64-104\times10^3$ vs. 80×10^3, $61-100\times10^3$ in 8 of 13 subjects included (62%); $P>.05$).

Absolute basophil cell count

The blood basophil numbers before the first MD were highly comparable to prior to treatment (before VIT mean 23 basophils per µl; 95% CI 18–28 vs. before MD: 24; 17–31).

Discussion

We recently showed that short-term VIT induced a marked desensitization of FcεRI-mediated basophil response [7] and

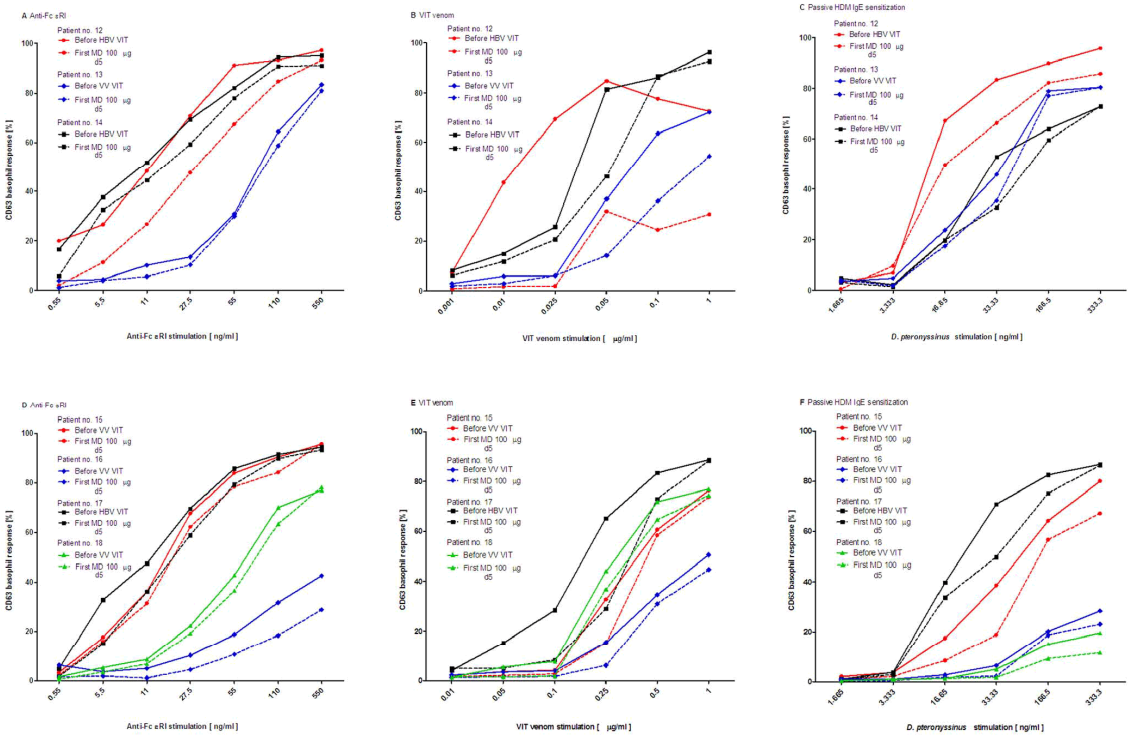

Figure 4. A–F. Passive IgE sensitization (dose-response curves). CD63 basophil dose-response curves in patients nos. 12–18 in **A** and **D** to anti-FcεRI in **B** and **E** to VIT venom and in **C** and **F** after passive IgE sensitization of stripped basophils also to house dust mite stimulation, all before treatment and the first maintenance dose of ultra-rush VIT.
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hereinafter we seek to determine the allergen specificity of this desensitization. In the current study, before the first MD within 5 days of ultra-rush VIT, we demonstrated the basophil desensitization to anti-FcεRI and to VIT-specific as well as to VIT-nonspecific venom. In limited number of patients this nonspecific basophil desensitization was further supported by opposite venom recombinant species-specific major allergen and also in case of other co-sensitizations (i.e., grass pollen). Furthermore, the venom-nonspecific base of these changes was demonstrated in the controlled experimental design of stripped patients' basophils passively sensitized with HDM IgEs and stimulated with HDM.

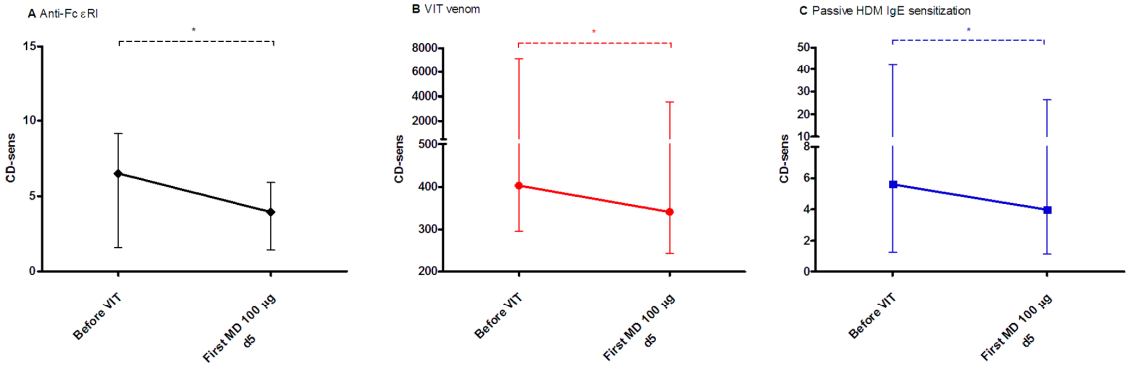

Figure 5. A–C. Passive IgE sensitization (CD-sens). CD-sens in in patients nos. 12–18 in **A** to anti-FcεRI in **B** to VIT venom and in **C** after passive IgE sensitization of stripped basophils also to house dust mite stimulation before treatment and the first maintenance dose of ultra-rush VIT. Data are presented as median values with interquartile range. *$P<.05$.
doi:10.1371/journal.pone.0094762.g005

It has been shown that VIT is effective as soon as the MD is achieved [4,5], and in the case of an ultra-rush protocol this is in a timeframe of a few days; however, for induction of long-lasting protection (i.e., tolerance), the treatment should last at least 3 to 5 years [1–3,26]. The precise mechanisms of action for both the quickly established protection and the induction of long-term tolerance have not yet been explained, despite different approaches that followed the action from blocking IgGs up to Treg cells [6,8,11,26–28]. Recent reports have focused on the basophils. Induction of tolerance in adults was demonstrated to be significantly associated with an approximately fourfold decrease in basophil response to VIT-venom submaximal stimulation [21]. Similar results were shown in children after 6 months and 2 to 4 years of VIT [20]. The mechanisms responsible for basophil suppression after long-term or VIT withdrawal is unknown: first, it seems that these changes are not related to the blocking role of sIgGs [21,29], as shown in the pollen immunotherapy model [30,31] and, second, those studies did not show any alternations in basophil response to non-VIT co-sensitizing allergens [20,21].

Unlike the long-term action, knowledge about the early protective changes after short-term VIT became more evident with two recent reports and also current data, clearly showing a prompt desensitization of FcεRI-mediated basophil activation either by up-regulation of histamine receptor 2 or by down-regulation of FcεRI expression [7,9]. These FcεRI-pathway-mediated alternations obviously suggest that early events might be cellular-based and thus not only relevant for VIT-venom response, as demonstrated previously [8,11,32]. For this reason, we followed double positive subjects during initial single short-term VIT and found basophil desensitization to both VIT and non-VIT venom. This nonspecific desensitization was also confirmed by non-VIT venom major recombinant allergens (rVes v 5 and rApi m 1) or co-sensitizing aeroallergens and thus is not very likely to be related to cross-reactive epitopes. Furthermore, to better show the cellular-based desensitization, patients' basophils were stripped for IgEs, sensitized with HDM-specific IgEs, and stimulated with HDM allergen. Similarly, we found a significant reduction of CD-sens to *de novo* sensitized HDM allergen. Furthermore this experiment on washed basophils indicates that different humoral factors from sera or plasma-like different antibodies or cytokines-may not be critical for early basophil desensitization [6,8,27,28,30,31]. Beside decreased mediator release, a depletion of circulating effectors cells has been proposed as a potential early protective mechanism [8,11]. Pierkes at al. observed reduced basophil numbers at the maintenance level, 1 week after the beginning of treatment [8], while most studies depicted reductions only during buildup [7,9,10,33] with return to pretreatment baseline values at the maintenance level, within 1–2 weeks or 6 months after the beginning of treatment [7,10,33], which is in concordance with our findings. The discrepancies observed could be an issue of different samplings and timeframes between immunotherapy protocols applied (rush or semi-rush vs. ultra-rush), thus also affecting the dynamic cellular turnover of basophils [34].

What was important in the analysis of early cellular response was (for all of the stimuli we used) the basophil threshold sensitivity approach with the construction of complete dose response curves and not only with limited concentrations, as in our previous study when monitoring HBV immunotherapy in children at the same time points [20]. In the previous study, the basophils were stimulated with a maximum concentration of anti-FcεRI mAbs (550 ng/ml) and with 1 and 0.1 μg/ml of venom and thus only insignificant basophil changes before the first MD were evident. Comparable insignificant findings would be found in the current report if only those limited concentrations were analyzed.

The foundation of specific immunotherapy is based on the allergen specificity of this treatment. Our challenging results suggest that, in contrast to long-term VIT, which induces venom-specific changes, the effect of short-term VIT may be venom-nonspecific. Similar nonspecific effect of short-term desensitization was observed as decreased peripheral leukocyte histamine release, specifically to desensitizing allergen as well as nonspecifically to other co-sensitizing allergens [12–14,16,17]. Currently, we do not know the clinical relevance of these early basophil changes and whether they are also clinically relevant for co-sensitizing allergens. Nevertheless, the anti-IgE treatment model demonstrated that a reduction of symptoms occurs when the basophils become comparably desensitized, as shown in our model [35,36]. In addition, this study was not designed to assess whether FcεRI-mediated basophil desensitization appears in the later course of VIT because we primarily focused on the point before obtaining the first MD of 100 μg of venom, which is a hallmark for the long maintenance period.

In summary, this study showed that early VIT-induced suppression of cellular FcεRI pathway results in comparable basophil desensitization to both VIT-specific and VIT-nonspecific venom. Furthermore this nonspecific desensitization was evident in case of other co-sensitizations (i.e., grass pollen) and with HDM passive IgE sensitization. Thus, further preferably larger multi-centre studies are warranted a) to examine the clinical relevance of this early cellular desensitization, not only concerning VIT allergen, but also other co-sensitizing allergens; b) to address the later course of these changes; and c) to examine in detail the exact mechanism of this cellular shift because this would allow the development of novel interventions for promoting or monitor the silencing of basophil FcεRI pathway.

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Author Contributions

Conceived and designed the experiments: PK NČS. Performed the experiments: NČS MŠ MR. Analyzed the data: NČS MŠ MR RE MK. Contributed reagents/materials/analysis tools: NČS MŠ MR RE MK. Wrote the manuscript: NČS PK.

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2.2 OSTALO POVEZOVALNO ZNANSTVENO DELO

2.2.1 Uporaba poglavitnih rekombinantnih vrstno-specifičnih alergenov v testu aktivacije bazofilcev za diagnosticiranje s strupi kožekrilcev dvojno pozitivnih bolnikov

Diagnosis of double positive *Hymenoptera* venom allergic patients with recombinant species-specific major allergens used in basophil activation test

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v pripravi

V primeru dvojne pozitivnosti, tako za čebelji kot tudi za osji strup, je potrebno z nadaljnjo diagnostično obravnavo ločiti med primarno dvojno senzibilizacijo in navzkrižno reaktivnostjo. Pri reševanju problematike dvojne pozitivnosti je bil naš namen ugotoviti diagnostično uporabnost poglavitnih rekombinantnih alergenov v testu aktivacije bazofilcev (BAT). Vključili smo 14 bolnikov s sistemsko preobčutljivostno reakcijo po piku neznanega kožekrilca in dvojno pozitivnimi sIgE in testom BAT z nativnim alergenom čebeljega in osjega strupa. Ugotavljali smo biološko aktivnost in/ali IgE reaktivnost za rApi m 1, rApi m 2, rVes v 5, rVes v 2, rVes v 1 in navzkrižno reaktivne ogljikohidratne determinante (CCD). Za navzkrižno reaktiven protein hialuronidaze (rApi m 2) smo testirali alergene z različnimi stopnjami glikozilacije (brez, nizko in visoko glikozilirane), proizvedene v različnih sistemih za izražanje genov (*E. coli*, kvasovke ter celične linije insektov High Five in Sf3). Z uporabo poglavitnih rekombinantnih alergenov v testu BAT smo pri 4 bolnikih ugotovili senzibilizacijo za strup ose, pri 5 za strup ose in za hialuronidazo, pri 1 le za hialuronidazo in pri 3 bolnikih za strup ose in čebele ter hialuronidazo hkrati. Pri 1 bolniku smo prikazali biološko aktivnost le za CCD. V testu BAT smo pri vseh 9 pozitivnih bolnikih za Sf3 izraženo hialuronidazo, ki ne vsebuje navzkrižno reaktivnega α-1,3-fukoziliranega N-vezavnega mesta, ugotovili visoko biološko oz. alergogeno aktivnost. Uporaba poglavitnih rekombinantnih alergenov, ki ne vsebujejo CCD, v testu BAT nam je omogočila ugotovitev primarne senzibilizacije pri veliki večini dvojno pozitivnih bolnikov. Naši rezultati nakazujejo na pomembno vlogo hialuronidaze, kot navzkrižno reaktivnega proteina.

Diagnosis of double positive *Hymenoptera* venom allergic patients with recombinant species-specific major allergens used in basophil activation test

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Keywords: *Hymenoptera* allergy, double positivity, recombinant allergens, hyaluronidase

Abbreviations
HBV: honeybee venom
VV: *Vespula* venom
CCDs: cross-reactive carbohydrate determinants
VIT: venom immunotherapy
BAT: basophil activation test

Abstract

Introduction

Double positivity to honeybee and *Vespula* venom necessitates supplementary testing to distinguish genuine double sensitization from cross-reactivity.

Objective

We sought to test the usefulness of venom recombinant-based basophil activation test (BAT).

Materials and Methods

Fourteen *Hymenoptera* venom allergic patients with an unknown culprit insect and double-positive sIgE and BAT to *Vespula* and honeybee whole venom extracts were included in the study. We evaluated the BAT and/or IgE reactivity to rApi m 1, rApi m 2, rVes v 5, rVes v 2, rVes v 1 and CCDs. For Api m 2 (honeybee hyaluronidase) we also tested different recombinants, expressed in *E. coli*, *Pichia* and insect cells (High Five - glycosylated and Sf3 - without alpha-1,3-core fucosylation).

Results

With recombinant-based BAT we were able to identify 4 patients with *Vespula*, 5 patients with *Vespula* and cross-reactive hyaluronidase, 1 patient with only hyaluronidase and 3 patients with *Vespula*, honeybee and hyaluronidase allergy. In one patient only the reactivity to CCDs was demonstrated. In BAT with Sf3-expressed hyaluronidase a very high allergenicity was showed in all 9 positive patients.

Conclusions

Recombinant-based BAT allows the identification of honeybee and/or *Vespula* allergy in majority of double-positive patients. Furthermore, our results also suggest the importance of hyaluronidase in case of protein based cross-reactivity.

1 Introduction

The identification of the disease-causing insect in *Hymenoptera* venom allergy is often difficult, as about 30 to 60% of the patients have IgE antibodies that react with both honeybee (HBV) and *Vespula* venom (VV) (1-5). Apart from genuine double sensitisation, double positivity is related to the presence and recognition of protein e.g. hyaluronidase or carbohydrate cross-reactive epitopes (CCDs) (4, 6-12). CCDs frequently lack allergenic activity and hence may give rise to false positive diagnostic results (7, 11, 13-18). This possesses the diagnostic and clinical problem of identifying the relevant venom for venom immunotherapy (VIT), especially if the patient has an anaphylactic reaction to only one, possibly an unknown insect.

Two novel approaches were recently clinically evaluated to diagnose HBV and/or VV allergy in double-positive patients, the recombinant rApi m 1, rVes v 5 and rVes v 1 ImmunoCAP-FEIA IgE testing (5, 19-24) and the basophil activation test (BAT) with venom extracts and cellular sensitivity analysis (4). Both approaches improved our diagnostic procedures, although they still have some major limitations. Namely, the recombinant IgE testing showed the limited usefulness of commercially available rApi m 1 for detecting HBV allergy due to its low sensitivity (57% to 79%) and/or false negative results in 10-30% of patients (5, 19-21, 23, 25). On the other hand, the sensitivity of comparable rVes v 5 and rVes v 1 IgE testing for detecting VV allergy was very good (overall sensitivity of 92-100%) (5, 22-24). In BAT, single positive results are evident only in about one quarter to one third of double-positive subjects (17, 26-28), and thus it was recently suggested that in case of double-positive BAT the clinically relevant information about the culprit insect might be associated with the venom which induced higher basophil allergenic activity (4, 29).

To overcome some of these limitations we combined both approaches by introducing *Hymenoptera* venom recombinant-based BAT in a complex group of patients with anaphylactic reaction to an unknown culprit insect and double-positive sIgE and BAT results with whole venom extracts. Those patients were then tested with recombined-based BAT with species-specific major HBV and VV allergens and cross-reactive hyaluronidase. The results were then compared with recombinant IgE reactivity, and/or response to recombinants from different expression systems in particular for hyaluronidase allergen.

2 Methods

2.1 Study population

Fourteen *Hymenoptera* venom-allergic patients (mean age 45 years; range 21-66; 10 men) with a history of anaphylactic reaction (2 Mueller grade IV (14%), 9 II (64%) and 3 I (21%)) to an unknown culprit insect were included in this study. All 14 patients showed double-positive specific IgE antibodies (sIgE) and BAT with honeybee and *Vespula* whole venom extracts. Clinical data, sIgE and BAT with whole venom extracts measurements are shown in Table 1.

All patients gave written informed consent for participation and the study was approved by the Slovenian National Medical Ethics Committee.

Table 1 Clinical data, sIgE and BAT with whole venom extracts and sIgE to CCDs

Patient no.	Sex	Culprit insect	Mueller grade	HBV (i1) sIgE [kUA/L]	VV (i3) sIgE [kUA/L]	BAT [%] HBV extract 1/0.1 µg/mL	BAT [%] VV extract 1/0.1 µg/mL	OSR/MUXF3 (f316/o214) sIgE [kUA/L]
1	M	NK	II	2.15	5.55	74/4	94/10	<0.35
2	F	NK	II	2.08	1.33	91/55	97/96	<0.35
3	M	NK	I	8.65	12.5	25/16	20/2	1.04/0.42
4	M	NK	IV	1.89	29.4	93/10	99/96	0.41/<0.35
5	M	NK	I	11.5	5.19	96/95	94/43	<0.35
6	M	NK	II	2.79	4.03	35/6	59/10	0.41/<0.35
7	M	NK	II	0.43	0.87	30/2	97/80	<0.35
8	F	NK	II	3.61	1.32	46/16	34/18	<0.35
9	M	NK	II	4.83	8.56	93/18	95/43	<0.35
10	M	NK	II	5.49	1.42	99/97	98/87	0.74/0.36
11	M	NK	I	2.13	2.48	63/6	88/3	<0.35
12	F	NK	II	0.58	1.61	28/2	94/90	<0.35
13	F	NK	IV	1.75	0.96	95/57	62/11	<0.35
14	M	NK	II	1.26	16.5	28/7	72/76	1.05/0.91

NK: not known
HBV: Honeybee venom
VV: *Vespula* venom

2.2 *Hymenoptera* venoms and allergens

Natural (n) honeybee and *Vespula* whole venom extracts were purchased at Hal Allergie (Leiden, Netherland) and bromelain from Sigma (St Louis, Mo, USA). nPhl p 4 was purified from timothy grass pollen as previously demonstrated (2, 30). Recombinant (r) rApi m 1, rApi m 2 and rVes v 5 were expressed in nonglycosylated form in *E. coli*. rApi m 2 and rVes v 2 were produced by baculovirus-infected Sf3 insect cells lacking alpha-1,3-core fucosylation. rApi m 2 was also produced as a glycosylated protein by baculovirus-infected High Five (HF) insect cells and by *Pichia pastoris*. The production of proteins, their purity and concentration determination was performed as previously described (2).

2.3 Basophil activation test (BAT)

A basophil activation assay was performed on the heparinized whole blood incubated with 0.1 and 1 µg/mL of HBV or VV extracts or with serial dilutions of rApi m 1 *E. coli* (0.07-70 ng/mL), rVes v 5 *E. coli* (0.001-10 ng/mL), rApi m 2 Sf3 (0.0002-200 ng/mL), rApi m 2 *E. coli* (0.2-200 ng/mL), rApi m 2 HF/*Pichia* (0.001-100 ng/mL), nPhl p 4 (3×10^{-7}-10^{-1} ng/mL) and bromelain (0.01-1000 ng/mL) or 0.55 µg/ml of anti-FcεRI mAb (Buhlmann Laboratories, Switzerland), or 2 µM fMLP (Sigma, USA) as a control, at 37 °C for 15 minutes. Degranulation was stopped by chilling on ice, after which anti-CD63, anti-CD123, and anti-HLA-DR mAb (BD Biosciences, Franklin Lakes, NJ, USA) were added and incubated for 20 minutes. Finally, whole blood probes were lysed, washed, fixed, and analyzed within 2 hours on a FACSCalibur flow cytometer (BD Biosciences).

The threshold for basophil activation was determined as the allergen concentration giving ≥ 15% of CD63% up-regulation (C15) (18, 29, 31-32).

2.4 Determination of sIgE antibodies

The serum concentration of sIgE against HBV (i1), VV (i3), CCDs (OSR - f316 and MUXF3 - o214), nApi m 1 (k203), rApi m 1 (i208), rVes v 5 (i209) and rVes v 1 (i211) was measured with ImmunoCAP-FEIA (Phadia, Thermo Fisher Scientific, Uppsala, Sweden).

2.5 Hyaluronidase IgE reactivities / IgE dot-blotting

Comparable amounts of rApi m 2 expressed in Sf3, *E. coli*, HF, *Pichia* and rVes v 2a expressed in Sf3 and HSA as a control were pre-bound to nitrocellulose membranes (Whatman Protran, Sigma, USA) and incubated with patients` sera (dilution 1:10) at 4 °C overnight. Bound human IgE antibodies were detected with ^{125}I-labeled antihuman IgE antibodies and visualized by autoradiography (2, 33-34).

2.6 Statistical analyses

Data distribution was evaluated using the D'Agostino–Pearson normality test. Since the majority of data were not normally distributed, we expressed them as median and range. Methods were tested for agreement with the kappa test. Analyses were performed using GraphPad Prism 5.

3 Results

3.1 Double positivity with whole venom extracts and CCD sensitization

Specific IgE and CD63 basophil response following stimulation with 1 and 0.1 μg/mL of venom were double-positive for whole HBV and VV extract in all 14 patients. CCDs sensitization was evident in 5 patients (36%). Three patients were double-positive for OSR and MUXF3 and 2 exclusively for OSR. For details, see Table 1.

3.2 Biological activity of differently expressed recombinant *Hymenoptera* venoms and allergens

We aimed to test the biological activity in form of CD63 basophil response of differently expressed species-specific recombinant *Hymenoptera* venoms and allergens. For HBV hyaluronidase - Api m 2 we included several protein forms with a diverse degree of glycosylation depending on the type of cells and the conditions used for expression. Nonglycosylated *E. coli*-expressed rApi m 2 appeared not to be correctly biologically active as 5% of CD63 basophil response was obtained with 2 and 200 ng/mL and a maximal response of 30% with 20 ng/mL of allergen stimulation (Figure 1B). Furthermore we tested glycosylated variants of rApi m 2 expressed in *Pichia* and insects cells, lines HF and Sf3, the later was without alpha-1,3-core fucosylation (Figure 1A, C-D). We obtained sigmoid-shaped dose-response curves with a high-level of allergenicity with insect cell-expressed rApi m 2, C15 0.1 and 0.2 ng/mL, respectively. Sf3 insect cell-expressed rApi m 2 was chosen for further analyses in recombinant-based BAT.

Nonglycosylated *E. coli*-expressed major allergens of HBV and VV, phospholipase A2 - rApi m 1 and antigen 5 - rVes v 5, showed comparable CD63 basophil dose-response curves when tested, C15 0.7 and 0.1 ng/mL, respectively (Figure 1E, F).

In quest for CCDs reactivity we used natural plant glycoproteins nPhl p 4 and bromelain. The highly glycosylated grass pollen allergen nPhl p 4 showed a considerably higher-level of allergenicity as carbohydrate-containing bromelain, C15 3×10^{-6} and 100 ng/mL, respectively (Figure 1G, H).

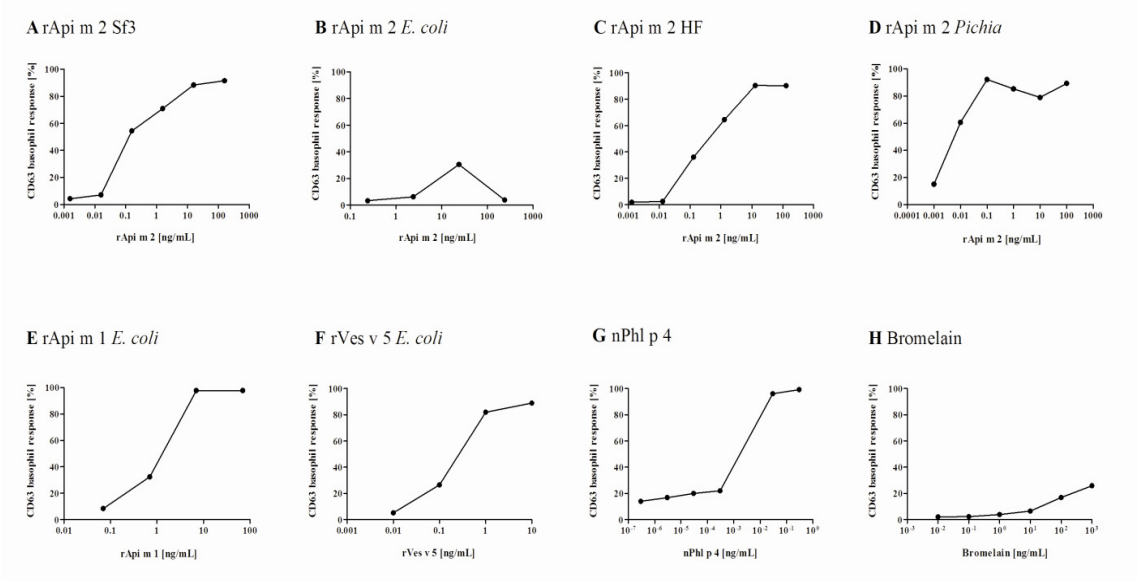

Figure 1 CD63 basophil dose response curves of recombinant *Hymenoptera* venoms and allergens
Biological activity in form of CD63 basophil dose response curves of honeybee venom hyaluronidase – rApi m 2, expressed in **A** in Sf3 insect cells, in **B** in *E. coli*, in **C** in High Five insect cells and in **D** in *Pichia pastoris*; and in **E** of honeybee venom phospholipase A2 – rApi m 1, expressed in *E. coli*; in **F** of *Vespula* venom antigen 5 – rVes v 5; in **G** of natural Timothy grass pollen Phl p 4; and in **H** of bromelain.

3.3 Recombinant-based basophil activation

To distinguish genuine from cross-sensitization in double-positive *Hymenoptera* venom allergic patients we used a combination of nongycosylated *E. coli*-expressed recombinant species-specific major allergens rApi m 1 and rVes v 5 and Sf3-expressed hyaluronidase rApi m 2 (without alpha-1,3-core fucosylation). The results of recombinant-based BAT are presented in Figure 2.

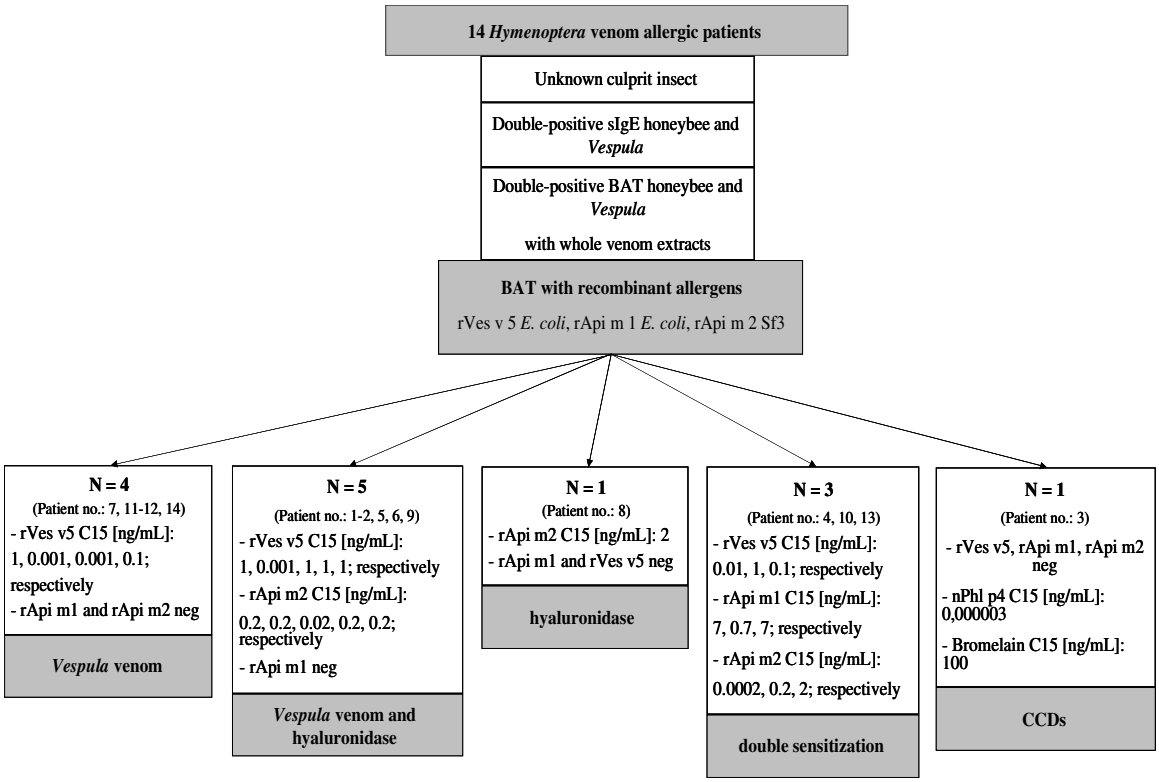

Figure 2 Flowchart with recombinant-based BAT results

Flowchart of the inclusion of the study subjects with results of recombinant-based CD63 basophil activation using species-specific major allergens: nonglycosylated *E. coli*-expressed rVes v 5 and rApi m 1 and cross-reactive hyaluronidase - rApi m 2, expressed in Sf3 insect cells, without alpha-1,3-core fucosylation. The threshold for basophil activation was determined as the allergen concentration giving ≥ 15% of CD63% up-regulation (C15).

With recombinant-based BAT we were able to identify 4 patients with *Vespula* sensitization, as they responded to stimulation with major *Vespula* allergen, C15 1, 0.001, 0.001 and 0.1 ng/mL, separately and remained negative when tested with HBV allergens. Five patients were positive when tested with *Vespula*, C15 1, 0.001, 1, 1 and 1 ng/mL and cross-reactive hyaluronidase, C15 0.2, 0.2, 0.02, 0.2 and 0.2 ng/mL, respectively and negative with major HBV allergen. One patient reacted only with hyaluronidase, C15 2 ng/mL and three patients were displaying a genuine double sensitization to both species-specific major allergens rVes v 5, C15 0.01, 1 and 0.1 ng/mL and rApi m 1, C15 7, 0.7 and 7 ng/mL and to hyaluronidase, C15 0.0002, 0.2 and 2 ng/mL, separately.

In one patient, who did not react with any of the species-specific major allergens, nor hyaluronidase, additional CCDs reactivity with nPhl p 4 and bromelain was demonstrated, C15 3×10^{-6} and 100 ng/mL, respectively.

3.4 Recombinant-based IgE reactivity

Patients with basophil response to major VV and/or HBV allergens were tested with natural and/or recombinant major allergens with ImmunoCAP-FEIA for IgE reactivity.

From the 12 patients with positive BAT to rVes v 5, 9 (75%) showed IgE reactivity to rVes v 5 and 5 (42%) of them also to rVes v 1 (Table 2A). From the 3 patients with positive BAT to rApi m 1, only 1 (33%) showed IgE reactivity to rApi m 1. However they all reacted to nApi m 1 (100%) (Table 2B).

Table 2A, B sIgE in rVes v 5 **A** and rApi m 1 **B** positive patients with BAT

A

Patient no.	rVes v 5 (i209) sIgE [kUA/L]	rVes v 1 (i211) sIgE [kUA/L]
1	2.34	0.87
2	1.38	<0.35
4	2.82	19.1
5	2.74	0.56
6	3.57	0.99
7	<0.35	<0.35
9	11.6	<0.35
10	<0.35	<0.35
11	1.24	<0.35
12	1.71	<0.35
13	<0.35	<0.35
14	15.7	0.55

B

Patient no.	rApi m 1 (i208) sIgE [kUA/L]	nApi m 1 (k203) sIgE [kUA/L]
4	<0.35	1.66
10	0.40	2.00
13	<0.35	0.48

3.5 Hyaluronidase sensitization

Nine out of 14 patients (64%) showed protein-based BAT positivity with HBV hyaluronidase - rApi m 2. Due to high basophil allergenic activity to Sf3-expressed HBV hyaluronidase (C15 0.0002-2 ng/mL) we additionally tested all patients for IgE reactivity to differently glycosylated hyaluronidases from HBV and for potential cross-reactivity with homologous hyaluronidase from VV - Ves v 2 (Table 3).

Table 3 Hyaluronidase CD63 basophil response and IgE reactivities

Patient no.	BAT	Dot blots				
	rApi m 2 Sf3 C15 [ng/ml]	rApi m 2 Sf3	rApi m 2 *E. coli*	rApi m 2 HF	rApi m 2 *Pichia*	rVes v 2a Sf3
1	0.2	+	+	+	+	+
2	0.2	+	+	+	+	-
3	neg	-	-	-	-	-
4	0.0002	+	+	+	+	+
5	0.02	+	+	+	+	+
6	0.2	+	+	+	+	+
7	neg	-	-	+	-	-
8	2	+	-	-	+	-
9	0.2	+	+	+	+	-
10	0.2	+	+	+	+	-
11	neg	+	+	+	+	-
12	neg	-	-	+	-	+
13	2	+	-	+	np	np
14	neg	-	-	+	-	-

BAT: C15: concentration of the allergen witch elicits ≥ 15% CD63 basophil response
IgE reactivities: -: negative; +: positive; np: not performed.

IgE reactivities of Sf3 and *Pichia*-expressed rApi m 2 showed an excellent agreement with basophil response (κ .84 and .82, respectively) and *E. coli*-expressed a moderate agreement (κ .57), whereas HF-expressed showed only a slight agreement (κ .10). In other words all 9 patients with positive BAT also had IgE reactivity to Sf3, 8 to *Pichia* (1 was missed) and 7 to *E. coli*-expressed rApi m 2. One patient which was negative with BAT, showed IgE reactivity to all three rApi m 2 variants. Highly glycosylated rApi m 2 from HF insect cells had the highest IgE reactivity, as it was positive in 12 out of 14 patients (86%).

Furthermore 5 out of 13 patients (38%) showed protein cross-reactivity with rVes v 2a hyaluronidase. Four of those patients also showed basophil and IgE reactivity to all HBV hyaluronidases. In the one remaining patient basophil response was negative and IgE reactivity was positive only with HF-expressed rApi m 2 (Table 3). All of Ves v 2 positive patients were sensitized with other major VV allergens, rVes v 5 and rVes v 1.

4 Discussion

Double positivity to honeybee and *Vespula* venom occurs in about one half of patients and thus necessitates supplementary testing to distinguish between genuine double sensitization and cross-reactivity. In quest for primary sensitization a panel of recombinant species-specific major *Hymenoptera* venom allergens without cross-reacting N-glycans was used in basophil activation test. With recombinant-based BAT, we were able to define the honeybee and/or *Vespula* allergy in 93% of complex patients with an unknown culprit insect and double-positive sIgE and BAT results with whole venom extracts. Furthermore, our results also demonstrated the importance of BAT hyaluronidase positivity in these patients.

Venom recombinant allergen-based IgE testing has been extensively studied for the last few years (1-2, 5, 19-24). Initial studies demonstrated that nonglycosylated species-specific major allergens rApi m 1 and rVes v 5 used in discontinued ADVIA Centaur system or in-house IgE immunoblot/ELISA systems have unambiguously discriminated between HBV and VV allergy (1-2). On the other hand its use in routinely accessible ImmunoCAP-FEIA system raised some concern regarding their diagnostic sensitivity. While IgE reactivities to CAP rVes v 5 and 1 have been shown to detect virtually all VV allergies (5, 22-24), HBV major allergen rApi m 1 showed limited usefulness due to its low sensitivity (5, 19-21, 23). For that reason we questioned our selves if recombinant venom allergens could be even more useful in BAT which enables measurements of allergenic activity and is not limited to measurements of only IgE reactivity. With that cellular-based recombinant approach we were able to identify the sensitization pattern in 13 out of 14 double-positive patients, while with recombinant-based CAP IgE reactivity testing alone we would miss 5 rVes v 5 and/or rApi m 1 sensitized patients. A recent study elucidating double positivity by Eberlein et al. demonstrated that the venom which induced higher basophil allergenic activity in BAT was in accordance to the culprit insect in up to 90% of patients, whereas recombinant IgE sensitization testing was accordant only in about 10%. It seems that venom recombinant IgE testing reflected the double sensitization pattern independent of CCDs; meanwhile BAT added more clinically relevant information about the culprit insect (4). The clinical relevance of higher venom basophil allergenic activity is currently not entirely determined due to ethnical limitations on diagnostic sting challenges. Nevertheless studies which monitored basophil response during VIT and performed sting challenges suggested good clinical relevance of decreased basophil-related allergenic activity (32, 35). Moreover BAT has proved to be an indispensable diagnostic and prognostic tool when elucidating *Hymenoptera* venom allergic patients with double-negative sIgE and skin test results (29, 31, 36).

A vast majority of double-positive patients exhibited basophil and/or IgE reactivity to cross-reactive honeybee venom hyaluronidase, Api m 2 and nearly one half of them showed peptide-specific cross-reactivity with its 50% homologous hyaluronidase from *Vespula* venom, Ves v 2. Although the highest rApi m 2 reactivity of 86% seen with highly glycosylated High Five insect cells is likely partially induced by CCDs, an overall protein-based reactivity in CCDs-depleted allergens e.g. Sf3 and *E. coli*-expressed was detected in 71% and 57%, respectively. Even though IgE reactivities of Sf3 and *E. coli*-expressed HBV hyaluronidases were in substantial agreement, there was a considerable discrepancy regarding their biological activity. Namely, *E. coli*'s rApi m 2 appeared of good IgE reactivity (2), whereas its basophil response was inadequate. In contrast, insect cell-expressed allergen demonstrated accurate sigmoid-shaped dose-response curves with a high-level of allergenicity. Thus *E. coli*-expressed hyaluronidase seems to be lacking a functional epitope structure to elicit a proper degranulation pattern of effector cells. Similarly, superior biologic activity of hyaluronidase expressed in *Spodoptera frugiperda* insect cells as compared

with *E. coli* was observed previously (37). Biologic activity of insect cell-expressed allergen was comparable with natural enzyme, whereas *E. coli*'s reached only 20% to 30% of its natural activity (37). Therefore testing of novel recombinant allergens for its biological activity prior to adopting its use is essential.

The high level of rApi m 2 sensitization in about two thirds of double-positive patients was not surprising, since hyaluronidase is considered as one of the major allergens in HBV as well as VV (9, 12) and as the most important cross-reactive allergen (10, 38-39). On the other hand the role of hyaluronidase has been recently reassessed and recognized as being modest (40). Moreover the majority of its reactivity has been assigned to CCDs and not to the peptide itself. However the study focused exclusively on VV allergy and its hyaluronidase IgE reactivity that was estimated to be protein-based in 35% of double-positive sera which is similar to 38% Ves v 2 reactivity found in our results. Nevertheless in addition to IgE reactivities assessments of differently expressed allergen, our study evaluated the allergenic activity in form of effector cells activation. A very high allergenicity in basophil response to HBV hyaluronidase, without CCDs interference, was demonstrated in a substantial number of our selective double-positive patients, thus indicating on the importance of protein based cross-reactivity (21, 41). Apart from genuine double sensitization it is not completely understood whether systemic reactions to both insects might also arise from protein-based cross-reactivity. A careful evaluation of such patients is needed for a better understanding of potential significance of hyaluronidase cross-reactions.

With induction of recombinant species-specific major allergens devoid of CCDs in a highly sensitive basophil activation test we demonstrated an improvement of its specificity and were able to identify the primary sensitization in vast majority of double-positive *Hymenoptera* venom allergic patients. Recombinant-based BAT significantly improves current diagnostic procedures when elucidating double positivity in complex patients and could be helpful in selection of appropriate VIT.

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3 RAZPRAVA IN SKLEPI

3.1 RAZPRAVA

3.1.1 Diagnostična uporabnost rekombinantnih alergenov

3.1.1.1 Diagnostična uporabnost komercialno dostopnega alergena rApi m 1 za ugotavljanje preobčutljivosti za strup čebele

Fosfolipaza A2 (Api m 1) predstavlja poglavitni alergen čebeljega strupa (Sobotka in sod., 1976). Rekombinantno izražen alergen so uporabili v različnih sistemih *in vivo* ter *in vitro* za diagnosticiranje preobčutljivosti za strup čebele. Diagnostična občutljivost *in vivo* je bila 95 % (Müller in sod., 1997), medtem ko je z različnimi sistemi *in vitro* znašala od 78 do 100 % (Müller in sod., 1997, 2009; Mittermann in sod., 2010). Rezultati raziskav z »in-house« encimskoimunskimi metodami (dot blot in/ali ELISA) in sistemom ADVIA Centaur (Siemens Medical Solution Diagnostics, Deerfield) so torej pokazali, da je določanje specifičnih protiteles IgE proti rekombinantno izraženemu alergenu diagnostično uporabno za ugotavljanje preobčutljivosti za strup čebele (Müller in sod., 1995, 1997, 2009; Mittermann in sod., 2010).

Prvi komercialno dostopen rekombinantno izražen alergen (rApi m 1) iz *E. coli*, namenjen za široko diagnostično uporabo na sistemu ImmunoCAP-FEIA, je pri diagnosticiranju preobčutljivosti za strup čebele nadomestil nativnega (nApi m 1). Njegova prednost v primerjavi z nativnim je prav gotovo v odsotnosti navzkrižno reaktivnih in/ali klinično nepomembnih ogljikohidratnih determinant. Vendar izguba glikozilacije lahko vpliva na terciarno strukturo, ki je osnova za epitopsko funkcionalnost alergena. Zato smo diagnostično občutljivost nativnega in rekombinantnega alergena Api m 1 za ugotavljanje preobčutljivosti za strup čebele, primerjali na veliki skupini 184 preiskovancev z različnimi stopnjami preobčutljivostnih reakcij po piku čebele.

Celotna skupina preiskovancev je imela jasno definirano klinično sliko po piku čebele ter pozitivne sIgE za nativni strup čebele in negativne sIgE za nativni strup ose. Ugotovili smo, da je kar 91 % (153 od 169) preiskovancev imelo pozitivne sIgE za nApi m 1 in le 57 % (100 od 175) preiskovancev za rApi m 1 (Korošec in sod., 2011). Razlika je bila še večja, če smo njuno diagnostično občutljivost medsebojno primerjali na skupinah s težjo obliko sistemske preobčutljivostne reakcije po piku čebele, stopnje III in IV po Muellerju. Senzibilizacijo za strup čebele je rApi m 1 potrdil pri le 51 % z anamnezo težje sistemske preobčutljivostne reakcije stopnje III in 63 % stopnje IV, medtem ko je nApi m 1 potrdil senzibilizacijo za strup čebele pri 87 % stopnje III in kar

97 % stopnje IV. Kar 53 preiskovancev je bilo pozitivnih z nativnim in negativnih z rekombinantnim Api m 1. Kljub temu nApi m 1 v primerjavi z nativnim izvlečkom celokupnega strupa čebele, še vedno ni zaznal 9 % preiskovancev z jasno definirano preobčutljivostjo za strup čebele.

Ogljikohidratne determinante nativnega Api m 1 najverjetneje niso vplivale na te rezultate, saj je le 9 % mono-senzibiliziranih preiskovancev imelo pozitivne sIgE za CCD, kar je primerljivo s 13 % v predhodnih raziskavah (Hemmer in sod., 2001). Prav tako ni bilo razlik v pozitivnosti za CCD med stopnjami preobčutljivostnih reakcij ali ujemanja med IgE reaktivnostjo za CCD in IgE reaktivnostjo za rApi m 1 in nApi m 1. Deset od 16 pozitivnih za CCD, je bilo namreč pozitivno za oba alergena in le 6 samo za nApi m 1. Za določevanje sIgE proti CCD smo uporabili alergen oljne repice (ang.: Oilseed rape, OSR), ki vsebuje glikane MMF značilne za čebeljo fosfolipazo A2 – Api m 1 (Hemmer in sod., 2001). Na ugotovljeno neskladnost med nativnim in rekombinantnim Api m 1 so morda vplivali tudi drugi alergeni čebeljega strupa, kot so Api m 2, Api m 3, Api m 4, Api m 10, itd. (Pregl. 2). Vendar so številne raziskave, vključno s komercialno dostopnim nativnim alergenom, potrdile Api m 1 za poglavitni alergen čebeljega strupa z diagnostično občutljivostjo okoli 90 %. (Sobotka in sod., 1976, Müller in sod., 1995, 1997, 2009; Mittermann in sod., 2010).

V nedavno objavljeni raziskavi so Hofmann in sod. (2011) ravno tako preizkušali diagnostično uporabnost komercialno dostopnega alergena rApi m 1 na sistemu ImmunoCAP-FEIA. Poročali so o 79 % diagnostični občutljivosti za ugotavljanje preobčutljivosti za strup čebele testirani na 34 preiskovancih, od katerih je bilo le 18 mono-senzibiliziranih za strup čebele. Raziskava je torej vključevala majhno število preiskovancev, ki so bili večinoma hkrati senzibilizirani tako za čebelji kot tudi za osji strup, kar močno zmanjšuje statistično moč in klinično relevantnost te študije.

Izsledki nekaterih predhodnih raziskav so pokazali, da vrednosti celokupnih IgE morda predstavljajo dejavnik tveganja za razvoj resnejše sistemske preobčutljivostne reakcije po piku kožekrilca (Sturm in sod., 2007; Blum in sod., 2011). Pri bolnikih z najtežjo sistemsko preobčutljivostno reakcijo so opazili statistično značilne manjše vrednosti celokupnih in specifičnih IgE, v primerjavi z nižjimi stopnjami sistemskih preobčutljivostnih reakcij (Blum in sod., 2011). Nasprotno, mi nismo ugotovili nobene medsebojne odvisnosti med koncentracijami celokupnih ali specifičnih IgE in resnostjo sistemske preobčutljivostne reakcije po piku čebele. Vrednosti celokupnih in specifičnih IgE so bile med veliko lokalno reakcijo ter stopnjami sistemskih preobčutljivostnih reakcij zelo primerljive.

Ugotovili smo majhno diagnostično občutljivost komercialno dostopnega alergena rApi m 1 iz *E. coli* na sistemu ImmunoCAP-FEIA (Korošec in sod., 2011). Ti rezultati nakazujejo na pomanjkljivo diagnostično uporabnost rApi m 1 za ugotavljanje preobčutljivosti za strup čebele. Komercialno dostopen alergen rApi m 1 je v trenutni obliki omejeno primeren za diagnostično uporabo ter ga je potrebno izboljšati.

3.1.1.2 Diagnostična uporabnost komercialno dostopnih alergenov rVes v 5 in rVes v 1 za ugotavljanje preobčutljivosti za strup ose

Antigen 5 (Ves v 5) predstavlja poglavitni alergen strupov iz družine *Vespidae* (Hoffman, 1993). Več kot 95 % podobnost v zaporedju Ves v 5 je znotraj vrst roda *Vespula* (npr. *V. germanica* in *V. vulgaris*) in okoli 60 % podobnost z drugimi rodovi kot sta *Dolichovespula* in *Polistes* (King in sod., 1996). Poglavitna alergena osjega strupa sta tudi fosfolipaza A1 (Ves v 1) in hialuronidaza (Ves v 2) (Hoffman, 1993). Čebelji strup ne vsebuje niti antigena 5 niti fosfolipaze A1.

Ves v 5 in Ves v 1 proizvedena v celičnih linijah insektov Sf9, namenjena za široko diagnostično uporabo na sistemu ImmunoCAP-FEIA, sta postala prva komercialno dostopna rekombinantno izražena alergena osjega strupa. V raziskavi smo pokazali omejeno diagnostično uporabnost komercialno dostopnega alergena rApi m 1 iz *E. coli* za ugotavljanje preobčutljivosti za strup čebele (Korošec in sod., 2011). Podobno majhno diagnostično občutljivost alergena rApi m 1 kot v naši raziskavi, so potrdili tudi Sturm in sod. (2011b). Zato smo želeli ugotoviti diagnostično uporabnost novih komercialno dostopnih alergenov rVes v 5 in rVes v 1 za ugotavljanje preobčutljivosti za strup ose, pred diagnostično uporabo v rutinski klinični praksi.

Diagnostično občutljivost rekombinantnega Ves v 5 in Ves v 1 za ugotavljanje preobčutljivosti za strup ose, smo preizkušali na veliki skupini 200 preiskovancev z različnimi stopnjami preobčutljivostnih reakcij po piku ose. Celotna skupina preiskovancev je imela jasno definirano klinično sliko po piku ose ter pozitivne sIgE za nativni strup ose in negativne sIgE za nativni strup čebele. Ugotovili smo, da ima 84,5 % (169 od 200) preiskovancev pozitivne sIgE za rVes v 5. Od preostalih 31 preiskovancev, ki so imeli negativne sIgE za rVes v 5, je imelo 15 preiskovancev (dodatnih 7,5 %) pozitivne sIgE za rVes v 1. Torej je bilo skupno kar 92 % (184 od 200) preiskovancev pozitivnih bodisi za rVes v 5 ali rVes v 1 (Korošec in sod., 2012). Diagnostična občutljivost rekombinantnih osjih alergenov za ugotavljanje preobčutljivosti za strup ose je bila primerljivo visoka pri vseh preiskovancih s sistemsko preobčutljivostno reakcijo po piku ose, tudi pri osebah s težjo sistemsko preobčutljivostno reakcijo stopnje III in IV po Muellerju. Diagnostična občutljivost je

bila rahlo manjša (79 %) le pri preiskovancih, ki so po piku ose doživeli veliko lokalno reakcijo.

Od 16 preiskovancev, ki so bili negativni s komercialno dostopnima alergenoma rVes v 5 ali rVes v 1, sta bila 2 preiskovanca (dodaten 1 %) pozitivna z osjo hialuronidazo (rVes v 2) z indirektno encimskoimunsko metodo (dot blot). Oba sta doživela težjo sistemsko preobčutljivostno reakcijo razreda III in IV po piku ose. Z indirektno encimskoimunsko metodo smo ugotovili IgE reaktivnost za rVes v 5 še pri 2 preiskovancih ter za nativni alergen Phl p 4 (pelodi trav), ki je bogat z ogljikohidrati, še pri 4.

Naši rezultati so primerljivi z rezultati raziskav pridobljenih z že opuščenim sistemom ADVIA Centaur. Müller in sod. (2009) so prikazali 87 % diagnostično občutljivost rVes v 5 za ugotavljanje preobčutljivosti za strup ose, testirano na 100 preiskovancih s preobčutljivostjo za strup ose. Še večjo diagnostično občutljivost (100 %) rekombinantnega Ves v 5 so ugotovili z »in-house« encimskoimunskimi metodami (dot blot in/ali ELISA) (Mittermann in sod., 2010). Vendar je ta raziskava vključevala le 20 preiskovancev. Primerljivo diagnostično občutljivost (90 %) komercialno dostopnega alergena rVes v 5 kot v naši raziskavi, so potrdili tudi Hofmann in sod. (2011). V trenutni raziskavi smo pokazali, da dodatna vključitev rVes v 1 v testiranje, občutno poveča diagnostično občutljivost rekombinantnih alergenov strupa ose (za skoraj 8 %). Vendar sta kljub temu rVes v 5 in rVes v 1 v primerjavi z nativnim izvlečkom celokupnega strupa ose, še vedno zgrešila 8 % oseb z jasno definirano preobčutljivostjo za strup ose. Dodatno testiranje z rVes v 2 je le malenkostno prispevalo k izboljšanju celokupne diagnostične občutljivosti rekombinantnih osjih alergenov. Po mnenju Jina in sod. (2010) naj bi večina reaktivnosti rVes v 2 izvirala iz CCD in ne iz proteina samega. Pred kratkim so Blank in sod. (2010) opisali nov 100 kDa velik glikoziliran protein osjega strupa Ves v 3, ki je homologen s čebeljo dipeptidil peptidazo (Api m 5). V tej raziskavi je bilo približno 50 % od 54 vključenih preiskovancev pozitivnih z rVes v 3 zato bi z njim veljalo testirati preostale naše preiskovance, ki so bili negativni z vsemi tremi poglavitnimi alergeni osjega strupa.

Ugotovili smo veliko diagnostično občutljivost komercialno dostopnih alergenov rVes v 5 in rVes v 1 iz Sf9 za ugotavljanje preobčutljivosti za strup ose (Korošec in sod., 2012). V rutinski klinični praksi je zelo pomembno, da potrdimo senzibilizacijo pri vseh bolnikih s sistemsko preobčutljivostno reakcijo po piku kožekrilca. Vendar s trenutno dostopnimi rekombinantnimi alergeni, del bolnikov žal še vedno zgrešimo. Zato v prvi fazi *in vitro* diagnostičnega postopka ostaja določevanje sIgE proti nativnemu izvlečku celokupnega strupa ose, ki vsebuje vse alergene strupa. Uporaba novih komercialno dostopnih rekombinantnih alergenov Ves v 5 in Ves v 1 bi lahko

koristila v primeru dvojno pozitivnih oz. nejasnih ali nasprotujočih si rezultatov rutinskih diagnostičnih testov.

3.1.1.3 Diagnostična uporabnost rekombinantnih alergenov v testu aktivacije bazofilcev za ugotavljanje primarne senzibilizacije dvojno pozitivnih bolnikov po piku neznanega kožekrilca

Eden izmed osnovnih problemov pri rutinski *in vitro* diagnostiki preobčutljivosti za strupe kožekrilcev je (pre)visok delež (do 60 %) dvojno pozitivnih sIgE za nativni strup čebele in ose. Specifičnost *in vitro* testiranja z nativnimi izvlečki celokupnega strupa kožekrilcev je torej omejena (Sturm in sod., 2004; Košnik in Korošec, 2009). Večino lažno dvojno pozitivnih rezultatov povzročajo navzkrižno reaktivna IgE usmerjena proti klinično nepomembnim CCD (Hemmer in sod., 2001). Sistemi za določevanje sIgE pogosto uporabljajo visoke koncentracije alergena. Posledično detektiramo tudi nizko afinitetna protitelesa IgE (Aalberse in sod., 2001), ki so klinično manj pomembna (Sturm in sod., 2011a). V primeru ko identiteta kožekrilca ni znana, rezultati pa dvojno pozitivni, je potrebna nadaljnja diagnostična obravnava. Med najpogosteje uporabljene dodatne *in vitro* diagnostične pristope, zagotovo sodita test aktivacije bazofilcev in uporaba rekombinantnih alergenov iz strupov kožekrilcev.

Specifičnost testa aktivacije bazofilcev je bila boljša v primerjavi z določanjem sIgE (87 % proti 67 %) (Sturm in sod., 2004). V nasprotju s sistemi za določevanje sIgE, pri testu BAT detektiramo le visoko afinitetna protitelesa IgE, ki so potrebna za aktivacijo bazofilcev (Sturm in sod., 2011a). Pri testu BAT bazofilci torej niso aktivirani s klinično nepomembnimi sIgE proti ogljikohidratnim epitopom (Košnik in Korošec, 2009), kar je verjetno vzrok za občutno nižji delež dvojno pozitivnih rezultatov testa BAT (17 %) v primerjavi z določanjem sIgE s sistemom ImmunoCAP-FEIA (62 %) (Sturm in sod., 2011a).

Novejše raziskave so se reševanja problematike dvojne pozitivnosti lotile z določevanjem sIgE proti rekombinantnim alergenom iz strupov kožekrilcev, ki ne vsebujejo CCD. Začetne raziskave na že opuščenem sistemu ADVIA Centaur in različnih »in-house« metodah (dot blot in/ali ELISA) so pokazale veliko diagnostično uporabnost poglavitnih rekombinantnih neglikoziliranih vrstno-specifičnih alergenov čebeljega in osjega strupa (rApi m 1 in rVes v 5) pri ugotavljanju primarne senzibilizacije (Müller in sod., 2009; Mittermann in sod., 2010).

Po drugi strani se diagnostična uporaba komercialno dostopnih rekombinantnih alergenov zaradi majhne diagnostične občutljivosti ne zdi primerna. Medtem ko se kombinacija alergenov rVes v 5 in rVes v 1 pri ugotavljanju preobčutljivosti za strup

ose kaže za zelo uspešno (diagnostična občutljivost med 92 in 100 %) (Korošec in sod., 2012; Müller in sod., 2012; Sturm in sod., 2012; Ebo in sod., 2013), se poglavitni alergen čebeljega strupa rApi m 1 pri ugotavljanju preobčutljivosti za strup čebele ni najbolje izkazal. Zaskrbljujoča je majhna diagnostična občutljivost (57 – 79 %) alergena in/ali negativni rezultati pri 10 – 30 % bolnikov (Hofmann in sod., 2011; Korošec in sod., 2011; Sturm in sod., 2011b, 2012; Jakob in sod., 2012; Müller in sod., 2012). Vzrok so iskali v morebitni senzibilizaciji z drugimi alergeni strupa (Hofmann in sod., 2011; Korošec in sod., 2011; Sturm in sod., 2011b; Müller in sod., 2012) ter v izbiri preiskovancev (Hofmann in sod., 2011; Korošec in sod., 2011; Sturm in sod., 2011b) in celo geografskem območju njihovega prebivališča (Sturm in sod., 2011b, 2012). Sturm in sod. (2012) so namreč zasledili razlike v senzibilizaciji z rApi m 1 med severom (79 %) in jugom Evrope (57 %). Razlog za majhno diagnostično občutljivost je verjetno tudi slaba kakovost, oziroma nezadostna epitopska funkcionalnost alergena. Številne raziskave, vključno s komercialno dostopnim nativnim alergenom, so namreč potrdile Api m 1 za poglavitni alergen čebeljega strupa z diagnostično občutljivostjo okoli 90 % (Sobotka in sod., 1976, Müller in sod., 1995, 1997, 2009; Mittermann in sod., 2010; Korošec in sod., 2011).

Zato smo želeli ugotoviti diagnostično uporabnost poglavitnih rekombinantnih alergenov iz strupov kožekrilcev v testu aktivacije bazofilcev, ki nam poleg IgE reaktivnosti, omogoča merjenje biološke oz. alergogene aktivnosti rekombinantnih alergenov. V testu BAT smo uporabili skupino poglavitnih vrstno-specifičnih in navzkrižno reaktivnih rekombinantnih alergenov iz strupov kožekrilcev, ki ne vsebujejo CCD. Kombinacija rApi m 1 in rVes v 5 iz *E. coli* ter rApi m 2 iz Sf3, nam je s celičnim *in vitro* pristopom omogočila ugotovitev primarne senzibilizacije pri 93 % bolnikov s sistemsko preobčutljivostno reakcijo po piku neznanega kožekrilca in dvojno pozitivnimi sIgE in testom BAT z nativnim strupom čebele in ose. Če bi določevali le IgE reaktivnost za komercialno dostopna alergena rVes v 5 in/ali rApi m 1, bi zgrešili pomembne senzibilizacije pri kar tretjini bolnikov.

Velika večina dvojno pozitivnih preiskovancev je imela pozitiven BAT in/ali sIgE za navzkrižno reaktiven protein čebelje hialuronidaze - rApi m 2 in skoraj polovica od njih je bila pozitivna tudi z osjo hialuronidazo - rVes v 2. Ker sta bila oba alergena izražena v celičnih linijah insektov (Sf3) in nista vsebovala CCD, je do navzkrižne reaktivnosti med obema hialuronidazama (rApi m 2 in rVes v 2) najverjetneje privedla njuna 50-odstotna homologija peptidnih epitopov. Čeprav je velika, 86 % IgE reaktivnost z visoko glikozilirano rApi m 2 iz celične linije High Five verjetno delno povzročena z CCD, je visok delež 71 % in 57 % dvojno pozitivnih bolnikov reagiralo tudi s peptidnimi epitopi rApi m 2 iz celične linije insektov (Sf3) in *E. coli*, ki CCD nista vsebovali. Kljub temu, da sta bili IgE reaktivnosti rApi m 2 iz Sf3 in *E. coli* v odlični skladnosti, sta bili njuni biološki aktivnosti povsem različni. rApi m 2 iz Sf3 je imela

pravilno obliko sigmoidne krivulje aktiviranih bazofilcev z visoko biološko oz. alergogeno aktivnostjo, nasprotno pa je bila biološka aktivnost rApi m 2 iz *E. coli* povsem neprimerna. Zdi se, da ima neglikozilirana rApi m 2 izražena v *E. coli* neustrezno terciarno strukturo in funkcionalnost epitopov, da bi ustrezno aktivirala efektorske celice. Podobno se je rApi m 2 iz *E. coli* s slabšo biološko aktivnostjo že pokazala v raziskavi Soldatove in sod. (1998). V Sf3 izražena rApi m 2 je imela primerljive encimske lastnosti kot nativen encim, medtem ko je rApi m 2 iz *E. coli* ohranila le 20 – 30 % encimske funkcionalnosti.

Kar dve tretjini dvojno pozitivnih bolnikov z ugotovljeno senzibilizacijo za rApi m 2 nas ni presenetilo, saj hialuronidaza velja za najpomembnejši navzkrižno reaktiven protein (Hoffman in Wood, 1984; Wypych in sod., 1989; Bonifazi in sod., 2005) in poglavitni alergen čebeljega in osjega strupa (Bilo in sod., 2005; Bilo in Bonifazi, 2011). Vseeno nekateri njene pomembne vloge niso potrdili. Ravno nasprotno, pripisali so ji vlogo manj pomembnega alergena za katerega naj bi veljalo, da večina njegove reaktivnosti izvira iz ogljikohidratnih determinant in ne iz peptida samega (Jin in sod., 2010). Vendar so se v omenjeni raziskavi osredotočili le na bolnike s preobčutljivostjo samo za osji strup. Ugotovili so, da je imelo 35 % dvojno pozitivnih preiskovancev sIgE za peptidne epitope rVes v 2, kar je podoben odstotek (38 %) kot v naši raziskavi. Poleg IgE reaktivnosti različno glikoziliranih hialuronidaz, smo v naši raziskavi preverjali tudi njihovo biološko aktivnost na nivoju aktivacije bazofilcev. Pri veliki večini dvojno pozitivnih bolnikov smo na celičnem nivoju ugotovili zelo visoko biološko aktivnost hialuronidaze, brez CCD, kar potrjuje pomembno vlogo hialuronidaze in njene reaktivnosti na ravni proteina. Enako so potrdili tudi Seismann in sod. (2010) ter Sturm in sod. (2011b). Vzrok za sistemske preobčutljivostne reakcije tako po piku čebele kot tudi ose je v primarni dvojni senzibilizaciji z obema strupoma. Vseeno se na tem mestu poraja vprašanje ali bi morda za primarno dvojno senzibilizacijo lahko bila odgovorna tudi sIgE usmerjena proti homolognim peptidnim epitopom hialuronidaze. Vsekakor bo za boljše razumevanje le-tega potrebno natančno ovrednotenje klinične pomembnosti te senzibilizacije.

Z uporabo poglavitnih vrstno-specifičnih in navzkrižno reaktivnih rekombinantnih alergenov brez CCD v visoko občutljivem testu aktivacije bazofilcev, smo še izboljšali njihovo specifičnost in uspeli ugotoviti primarno senzibilizacijo pri veliki večini dvojno pozitivnih bolnikov. Test aktivacije bazofilcev z uporabo rekombinantnih alergenov iz strupov kožekrilcev je pomembna izboljšava trenutnega *in vitro* diagnostičnega pristopa pri obravnavi kompleksnih dvojno pozitivnih bolnikov in je lahko v veliko pomoč pri izbiri ustreznega alergena za zdravljenje s SIT.

3.1.2 Diagnostična uporabnost testa aktivacije bazofilcev pri bolnikih z negativnimi sIgE in kožnimi testi

Pri bolnikih z anamnezo težje sistemske preobčutljivostne reakcije po piku kožekrilca in negativnimi kožnimi testi in sIgE za strupe kožekrilcev je nujen dodaten diagnostični pristop za potrditev senzibilizacije, ki omogoča uvedbo SIT. Ena izmed najobetavnejših *in vitro* diagnostičnih metod na tem področju je zagotovo test aktivacije bazofilcev z merjenjem izražanja označevalca CD63 na celični površini (Ebo in sod., 2007a; Korošec in sod., 2009).

V zadnjih 10 letih se je test BAT na področju preobčutljivosti za strupe kožekrilcev izkazal kot nepogrešljivo diagnostično in prognostično orodje ter kot ustrezna metoda za spremljanje SIT s strupi kožekrilcev (Sturm in sod., 2004, 2012; Eberlein-König in sod., 2006; Ebo in sod., 2007a, 2007b; Peternelj in sod., 2008a, 2008b, 2009; Korošec in sod., 2009; Žitnik in sod., 2012; Eržen in sod., 2012). Vendar so bile vse te študije zasnovane v raziskovalne namene ter kot takšne vključevale le izbrane populacije preiskovancev. Posledično primanjkuje podatkov o diagnostični uporabnosti testa BAT v rutinski klinični praksi ter o vplivu njegovih rezultatov na predpisovanje zdravljenja s SIT s strupi kožekrilcev. Zato so bile vse meritve v trenutni raziskavi izvedene prospektivno, kot del rutinske diagnostične obravnave kompleksnih bolnikov s preobčutljivostjo za strupe kožekrilcev in negativnimi standardnimi rutinskimi diagnostičnimi testi.

Podobno kot v predhodni raziskavi (Korošec in sod., 2009) smo pri obravnavi bolnikov z negativnimi sIgE dokazali 80 % diagnostično občutljivost testa BAT in približno 50 % diagnostično občutljivost intradermalnih kožnih testov (Korošec in sod., 2013). Uporaba testa BAT nam je torej omogočila potrditev senzibilizacije pri več kot polovici bolnikov s težjo preobčutljivostno reakcijo po piku kožekrilca in negativnimi tako sIgE kot tudi intradermalnimi kožnimi testi. V nedavni raziskavi so Sturm in sod. (2012) ugotovili, da ima več kot polovica bolnikov s preobčutljivostno reakcijo po piku ose z negativnimi sIgE za nativni strup, pozitivne sIgE za poglavitni rekombinantni alergen osjega strupa, rVes v 5. Vos in sod. (2012) so nakazali, da bi vzrok lahko bil v primanjkljaju tega poglavitnega alergena v nativnem pripravku osjega strupa. Podobno so testirali 25 bolnikov z anamnezo sistemske preobčutljivostne reakcije po piku ose in negativnimi sIgE za nativni strup in ugotovili, da je imelo 8 bolnikov (32 %) res pozitivne sIgE za rVes v 5 in 1 bolnik (4 %) za rVes v 1. Vendar je vseh 25 bolnikov (100 %) imelo pozitiven BAT s strupom ose (Korošec in sod., 2012). Tudi v trenutni raziskavi je le 14 % preiskovancev imelo pozitivne sIgE za rVes v 5 in za noben drug rekombinantni alergen osjega strupa. Tako bi določevanje sIgE za rekombinantne alergene osjega strupa lahko bilo uporabno le pri majhnem številu obravnavanih bolnikov.

V primeru negativnih rezultatov rutinskih diagnostičnih testov ali dvojno pozitivnih rezultatov za strup čebele in ose, je pogosto identifikacija primarnega povzročitelja preobčutljivostne reakcije lahko težavna. Tudi pri bolnikih z negativnimi sIgE in kožnimi vbodnimi testi so s testom BAT in/ali intradermalnimi kožnimi testi rezultati lahko dvojno pozitivni (Korošec in sod., 2009). V naši raziskavi je 48 % preiskovancev imelo dvojno pozitivni BAT za strup čebele in ose. Od tega jih je 80 % doživelo preobčutljivostno reakcijo po piku le enega izmed kožekrilcev. Vendar so vsi imeli statistično značilno večjo odzivnost bazofilcev pri stimulaciji s klinično pomembnim strupom kožekrilca. Če povzamemo, v primeru dvojno pozitivnega rezultata ter anamnezo preobčutljivostne reakcije po piku le enega izmed kožekrilcev, je bil klinično pomemben tisti strup, ki je pri testu BAT spodbudil večji celični odziv. Enako so ugotovili tudi Eberlein in sod. (2012), ki so v svoji raziskavi preučevali problematiko dvojne pozitivnosti. Večina bolnikov z dvojno pozitivnimi sIgE in testom BAT ter anamnezo preobčutljivostne reakcije po piku le enega izmed kožekrilcev, je imela statistično značilno večjo odzivnost oz. občutljivost bazofilcev pri stimulaciji s klinično pomembnim strupom kožekrilca. Test BAT je z merjenjem celične občutljivosti omogočil določitev primarne senzibilizacije pri kar 90 % dvojno pozitivnih bolnikov. Po drugi strani je bila napovedna vrednost primarne senzibilizacije komercialno dostopnih alergenov, rApi m 1 in rVes v 5, le okoli 10 %. Povzeli so, da IgE reaktivnost omogoča vpogled v vzorec senzibilizacije, ki ni odvisen od CCD, medtem ko celična občutljivost, ki ji sledimo s testom aktivacije bazofilcev, odraža klinično pomembnost senzibilizacije. Nasprotno, v primeru dvojno pozitivnega rezultata intradermalnih kožnih testov nismo ne mi in ne Eberlein in sod. (2012) ugotovili nobene korelacije s primarnim povzročiteljem preobčutljivostne reakcije. Ti rezultati nakazujejo na klinično pomembnost občutljivosti bazofilcev, ne le za potrditev senzibilizacije ali spremljanje zdravljenja s SIT (Peternelj in sod., 2008a; Žitnik in sod., 2012), temveč tudi pri ugotavljanju primarne senzibilizacije dvojno pozitivnih bolnikov.

Test BAT nam je omogočil potrditev povzročitelja pri bolnikih z anamnezo težje sistemske preobčutljivostne reakcije po piku kožekrilca in negativnimi sIgE in kožnimi testi za strupe kožekrilcev (Korošec in sod., 2013). V kompleksnih primerih z negativnimi rezultati rutinskih diagnostičnih testov je bila njegova diagnostična občutljivost in specifičnost ter klinična pomembnost večja, v primerjavi z drugimi diagnostičnimi metodami. Rutinska uporaba tega celičnega *in vitro* testa, v kompleksnih primerih z negativnimi rezultati rutinskih diagnostičnih testov, pogosto omogoča uvedbo specifične imunoterapije z ustreznim strupom kožekrilca.

3.1.3 Vloga receptorja FcεRI pri vzpostavitvi kratkotrajne zaščite specifične imunoterapije s strupi kožekrilcev

SIT s strupi kožekrilcev je edino učinkovito zdravljenje za preprečitev sistemskih preobčutljivostnih reakcij, povzročenih s piki kožekrilcev (Bonifazi in sod., 2005; Bilo in Bonifazi, 2011; Bilo, 2011). Večina bolnikov je zaščitena že z uvedbo prvega vzdrževalnega odmerka, vendar natančni imunološki mehanizmi vzpostavitve kratkotrajne zaščite ali dolgotrajne tolerance za alergen niso povsem razjasnjeni. Prav tako trenutno ne poznamo *in vitro* metode s katero bi lahko spremljali potek in uspešnost zdravljenja ter vzpostavitev tolerance za alergen in s katero bi še pravočasno prepoznali nezaščitene bolnike.

V raziskavah so med samim potekom ali po uspešno zaključeni SIT navajali porast za alergen-specifičnih blokirajočih protiteles IgG_4, limfocitov Treg (Tr1) in IL-10 ter znižanje serumskih vrednosti za alergen-specifičnih protiteles IgE in občutljivosti kožnih testov (Wetterwald in sod., 1985; Jutel in sod., 1996; Akdis in sod., 1998; Pierkes in sod., 1999). Prav tako so sledili potencialnim biomarkerjem, osteopontinu in genskim ekspresijskim profilom (Konno in sod., 2005; Niedoszytko in sod., 2010). Za vrednosti humoralnega imunskega odziva, protitelesa IgG in IgE, niso uspeli dokazati nobene napovedne vrednosti z vzpostavitvijo imunske tolerance (Golden in sod., 1992; Lerch in sod., 1998). Medtem ko se za povečano vsebnost limfocitov Treg, s posledično povečanim sproščanjem protivnetnih citokinov IL-10 ter TGF-β dozdeva, da naj bi se s svojimi učinki pomembno vpletala v zaščitne mehanizme SIT. Čeprav ni jasno ali prispevajo tudi k vzpostavitvi dolgotrajne imunske tolerance (Bussmann in sod., 2010). Za osteopontin so zasledili statistično značilne večje vsebnosti pri preiskovancih po uspešno zaključeni SIT v primerjavi z nezdravljenimi preiskovanci (Konno in sod., 2005). Niedoszytko in sod. (2010) so ugotovili pomembne razlike v vzorcu ekspresije genov med bolniki, ki so po končani SIT vzpostavili toleranco za alergen ter med bolniki po neuspešno zaključenem zdravljenju.

Eržen in sod. (2012) so pri bolnikih po uspešno zaključeni SIT zabeležili spremembe v odzivnosti bazofilcev. Pri vseh osebah z negativnim provokacijskim testom s pikom kožekrilca, z vzpostavljeno imunsko toleranco po zaključeni SIT, so ugotovili štirikrat manjšo za alergen-specifično odzivnost bazofilcev ob stimulaciji s submaksimalno koncentracijo alergena. Pri osebi s pozitivnim provokacijskim testom, pri kateri se imunska toleranca po zaključeni SIT ni vzpostavila, se odzivnost bazofilcev ni bistveno spremenila oziroma je bila le-ta celo nekoliko večja. Rezultati te raziskave nakazujejo, da bi zmanjšana občutljivost bazofilcev lahko bila ključna za vzpostavitev imunske tolerance po končanem zdravljenju s specifično imunoterapijo. Primerljivo zmanjšanje specifične občutljivosti bazofilcev, so ugotovili tudi pri 85 % otrok po 6. mesecih in po 2. – 4. letih terapije s strupom čebele (Žitnik in sod., 2012). Natančen mehanizem

supresije bazofilcev po dolgotrajni SIT ni znan; ali gre za humoralni, znotrajcelični ali je celo kombinacija obeh, vendar te celične spremembe najverjetneje niso povezane z blokirajočo vlogo za strup-specifičnih protiteles IgG (Varga in sod., 2009; Eržen in sod., 2012), kot je značilno za SIT z inhalacijskimi alergeni (Nopp in sod., 2009; Lalek in sod., 2010). Prav tako navedene raziskave niso pokazale nobenih sprememb v odzivnosti bazofilcev za nespecifične ko-senzibilirajoče alergene.

Za razliko od dolgotrajnih zaščitnih mehanizmov, zgodnji zaščitni mehanizmi najverjetneje spodbujajo celične spremembe in niso vezani le na specifičen alergen s katerim se izvaja terapija, kot je bilo sprva mišljeno (Jutel in sod., 1996; Pierkes in sod., 1999; Mikkelsen in sod., 2010).

Rezultati naše raziskave so pokazali, da je kratkotrajna SIT s strupi kožekrilcev povzročila močno desenzibilizacijo bazofilcev po IgE/FcεRI poti. Bazofilci so bili desenzibilizirani pri vseh bolnikih tik pred uvedbo prvega vzdrževalnega odmerka, znotraj 5. dni zelo hitre ter v nekaj tednih hitre sheme, a ne med uvodno fazo SIT (Čelesnik Smodiš in sod., 2012). Te spremembe so primerljive z izsledki predhodnih raziskav, kjer so med kratkotrajno SIT dokazali zmanjšano sproščanje mediatorjev alergijskega odziva iz efektorskih celic, histamina in sulfidolevkotrienov. Pierkes in sod. (1999) so ugotovili, da je do zmanjšanega sproščanja mediatorjev najverjetneje privedlo povečano sproščanje IL-10 in IFN-γ s strani limfocitov T, medtem ko Novak in sod. (2012) menijo, da je za to odgovorno povečano izražanje receptorja za histamin (H2R). Naši rezultati nakazujejo, da bi klinično inducirana desenzibilizacija bazofilcev lahko bila povezana tudi z zmanjšanim izražanjem receptorja velike afinitete FcεRI, kar dodatno pojasnjuje zgodnje zaščitne mehanizme SIT. To sovpada z ugotovitvami pred kratkim objavljene raziskave MacGlashana (2012), ki je pokazal, da se med pogoji submaksimalne stimulacije *in vitro*, bazofilci desenzibilizirajo zaradi izgube izražanja receptorja FcεRI. *In vitro* desenzibilizacija bazofilcev je v tej raziskavi vodila tudi do izgube kinaze Syk, kar je z dodatkom IL-3 izzvenelo, medtem ko na izražanje FcεRI ni imelo nikakršnega vpliva. Zmanjšano izražanje receptorja FcεRI na površini bazofilcev se je pokazalo kot klinično pomembno pri zdravljenju z anti-IgE terapijo (Eckman in sod., 2010; Savage in sod., 2012). Klinična učinkovitost anti-IgE terapije je sovpadala z zmanjšanim izražanjem receptorja FcεRI na površini bazofilcev, medtem ko je njegovo izražanje na površini mastocitov ostalo nespremenjeno (Eckman in sod., 2010; Savage in sod., 2012). Klinična učinkovitost terapije se je torej pokazala takoj, ko je bilo zmanjšano izražanje receptorja FcεRI na površini bazofilcev.

Pri vseh vključenih preiskovancih smo ugotovili primerljivo, močno zmanjšano občutljivost bazofilcev pri stimulaciji s protitelesi anti-FcεRI, tako pri zelo hitri kot tudi pri hitri shemi SIT. Razlike med protokoloma SIT smo opazili v ekspresiji gena za α-podenoto receptorja FcεRI (*FCER1A*). Statistično značilno zmanjšano ekspresijo gena

FCER1A na celotni krvi smo zasledili le pri bolnikih na zelo hitri shemi SIT. Zmanjšano ekspresijo gena *FCER1A* smo pri manjši skupini preiskovancev potrdili tudi na izoliranih bazofilcih. Nasprotno, pri bolnikih na hitri shemi SIT v ekspresiji gena na celotni krvi tik pred uvedbo prvega vzdrževalnega odmerka nismo ugotovili nobenih sprememb, je bilo pa v tej točki zmanjšano izražanje receptorja FcεRI na površini bazofilcev.

Na opazovano neskladnost v ekspresiji gena na celotni krvi med protokoloma SIT, bi lahko vplivalo tudi število bazofilcev v krvi. Zmanjšano število cirkulirajočih efektorskih celic v periferni krvi naj bi poleg zmanjšanega sproščanja njihovih mediatorjev prav tako imelo zaščitno vlogo (Jutel in sod., 1996; Pierkes in sod., 1999). Vendar je število bazofilcev v krvi pri zelo hitri in hitri shemi SIT ostalo nespremenjeno. Primerljivo število bazofilcev so imeli bolniki pred pričetkom terapije in tik pred uvedbo prvega vzdrževalnega odmerka, medtem ko smo zmanjšano število bazofilcev v krvi zasledili med uvodno fazo SIT. Podobno zmanjšano število bazofilcev v krvi med uvodno fazo SIT (Plewako in sod., 2006; Novak in sod., 2012; Nullens in sod., 2013) z vrnitvijo na bazalno raven ob uvedbi vzdrževalnega odmerka, po 1. tednu ali 6. mesecih terapije, so ugotovili tudi Plewako in sod. (2006) in Nullens s sod. (2013). Najverjetneje je zgodnja desenzibilizacija bazofilcev dinamičen proces, ki v različnih časovnih točkah vključuje različne spremembe v občutljivosti molekul signalizacijske poti FcεRI (MacGlashan in Miura, 2004; Gibbs in sod., 2006; MacGlashan, 2012). Na opažene razlike med protokoloma SIT bi lahko vplivala tudi zelo hitra celična dinamika bazofilcev, saj se preobrat v celičnem ciklu odvije že v približno 3. dneh (Karasuyama in sod., 2011). Primerljivo celično dinamiko z zmanjšano odzivnostjo bazofilcev in izražanjem receptorja FcεRI, kot pri zelo hitri shemi SIT, so opazili tudi že po 7. dneh anti-IgE terapije (Eckman in sod., 2010; Savage in sod., 2012).

Da bi preverili ali so te zgodnje celične spremembe za alergen specifične ali nespecifične, smo sledili primarno dvojno senzibiliziranim bolnikom za strup čebele in ose med uvodno fazo kratkotrajne SIT s strupom le enega kožekrilca. Ugotovili smo močno desenzibilizacijo bazofilcev tik pred uvedbo prvega vzdrževalnega odmerka tako s specifičnim strupom kožekrilca, s katerim smo izvajali SIT, kot tudi z za SIT-nespecifičnim strupom kožekrilca, ki ni bil vključen v terapijo (Čelesnik Smodiš in sod., 2014). Nespecifičen vpliv kratkotrajne SIT smo potrdili tudi z rekombinantnim vrstno-specifičnim poglavitnim alergenom (rVes v 5 ali rApi m 1) ali s ko-senzibilizirajočim inhalacijskim alergenom, katera prav tako nista bila vključena v terapijo. Na ta način smo potrdili, da nespecifičen vpliv najverjetneje ni vezan na navzkrižno reaktivne ogljikohidratne determinante. Nadalje smo za dodatno potrditev celične desenzibilizacije, bazofilcem bolnikov odstranili za čebelji ali osji strup specifična protitelesa IgE, jih pasivno senzibilizirali z za pršico sIgE in stimulirali z alergenom

pršice. Tik pred uvedbo prvega vzdrževalnega odmerka smo ugotovili močno celično desenzibilizacijo za *de novo* senzibilizirajoči alergen pršice. S poskusom pasivne senzibilizacije na spranih bazofilcih smo hkrati lahko dokazali, da zgodnje spremembe v celičnem odzivu med kratkotrajno SIT s strupi kožekrilcev niso vezane na različne humoralne dejavnike iz seruma ali plazme – različna protitelesa ali citokine (Akdis in sod., 1998; Pierkes in sod., 1999; Nopp in sod., 2009; Lalek in sod., 2010; Bussmann in sod., 2010; Jutel in Akdis, 2011).

Da smo lahko zasledili zgodnje spremembe v celičnem odzivu, je bila zelo pomembna izvedba poskusov stimulacije bazofilcev *in vitro* s serijo redčitev posameznih alergenov, ki so omogočili izris posameznih sigmoidnih krivulj deleža aktiviranih bazofilcev CD63 ter izračun celične občutljivosti (Slika 1). Ko so namreč sledili celičnemu odzivu med uvodno fazo SIT s strupom čebele pri otrocih v enakih časovnih točkah, so bili poskusi izvedeni le z maksimalno koncentracijo protiteles anti-FcεRI (550 ng/ml) in omejenima koncentracijama strupov (1 in 0,1 μg/ml). Posledično niso zasledili zgodnjih celičnih sprememb pred uvedbo prvega vzdrževalnega odmerka (Žitnik in sod., 2012). Do podobnih zaključkov bi vodila tudi trenutna raziskava, če bi analizirali le-te omejene koncentracije alergenov.

Osnova zdravljenja s specifično imunoterapijo s strupi kožekrilcev temelji na spodbuditvi specifičnih sprememb v odzivnosti za alergen. Naši rezultati nakazujejo, da v nasprotju z učinki dolgotrajne SIT, kratkotrajna SIT s strupi kožekrilcev spodbudi za alergen nespecifične spremembe. Nespecifičen vpliv kratkotrajne SIT so opazili tudi že v nekaterih drugih raziskavah. Pokazali so, da do zmanjšanega sproščanja mediatorjev iz efektorskih celic med kratkotrajno SIT, privede tako stimulacija celic z za imunoterapijo-specifičnim alergenom, kot tudi z nespecifičnim alergenom s katerim imunoterapije sicer ne izvajamo (May in Aduna, 1971; May in sod., 1972; May in Williams, 1973). Podoben nespecifičen učinek se je pokazal tudi med poskusi kratkotrajne desenzibilizacije bazofilcev *in vitro* (Sobotka in sod., 1979; MacGlashan in Lichtenstein, 1981). Vendar trenutno še vedno ne poznamo klinične pomembnosti teh zgodnjih celičnih sprememb in prav tako ne vemo ali bi lahko bile klinično pomembne tudi za druge ko-senzibilizirajoče alergene. Vendar vemo, da primerljiva desenzibilizacija bazofilcev pri zdravljenju s terapijo z anti-IgE vodi do izboljšanja kliničnih simptomov preobčutljivosti (Eckman in sod., 2010; Savage in sod., 2012). V okviru doktorske disertacije nismo ocenjevali ali FcεRI desenzibilizacija bazofilcev nastopi tudi v kasnejšem poteku SIT. Osredotočeni smo bili le na uvodno fazo SIT, ki je ključna za vzpostavitev zaščite za kasnejšo vzdrževalno fazo.

Ugotovili smo, da kratkotrajna SIT s strupi kožekrilcev privede do desenzibilizacije bazofilcev po IgE/FcεRI poti (Čelesnik Smodiš in sod., 2012, 2014). Naši rezultati so tudi pokazali, da kratkotrajna SIT s strupi kožekrilcev privede do desenzibilizacije

bazofilcev za specifičen strup kožekrilca, s katerim izvajamo SIT kot tudi za nespecifičen strup kožekrilca, s katerim SIT ne izvajamo. Ta nespecifičen vpliv smo potrdili tudi v primeru dodatne ko-senzibilizacije z inhalacijskim alergenom in v primeru pasivne senzibilizacije bazofilcev (Čelesnik Smodiš in sod., 2014).

Nadaljnje raziskave, ki bodo vključevale večje raziskovalne centre so potrebne, da ugotovijo klinično pomembnost teh zgodnjih celičnih specifičnih in nespecifičnih sprememb. Prav tako je potrebno raziskati pojavnost zgodnjih celičnih sprememb v kasnejših fazah SIT ter natančno ovrednotiti mehanizem FcεRI desenzibilizacije bazofilcev. FcεRI desenzibilizacija bazofilcev je morda zelo pomembna za vzpostavitev zgodnje klinične zaščite pred piki kožekrilcev za katero je ključno, da se doseže pred aplikacijo prvega standardiziranega vzdrževalnega odmerka SIT. V tem primeru bi sledenje izražanju gena in receptorja FcεRI na površini bazofilcev ter z njim povezane odzivnosti, koristilo za napovedovanje uspešnega odziva na zdravljenje s SIT. Natančno razumevanje tega zaščitnega mehanizma bi lahko pripomoglo k razvoju novih terapij za spodbuditev utišanja in/ali sledenje izražanja signalizacijske poti FcεRI bazofilcev.

3.2 SKLEPI

V sklopu doktorske disertacije smo sklenili sledeče ugotovitve:

- Komercialno dostopen alergen rApi m 1 ima majhno diagnostično občutljivost in je posledično omejeno primeren za ugotavljanje preobčutljivosti za strup čebele.

- Komercialno dostopna alergena rVes v 5 in rVes v 1 imata veliko diagnostično občutljivost za ugotavljanje preobčutljivosti za strup ose.

- Občutljivost in diagnostična uporabnost komercialno dostopnih poglavitnih vrstno-specifičnih rekombinantnih alergenov je večja za strup ose kot za strup čebele.

- Rekombinantni alergeni iz celičnih kultur so primerni za uporabo v biološkem testu aktivacije bazofilcev. Določeni glikozilirani rekombinantni alergeni imajo ustreznejšo biološko aktivnost in večjo diagnostično uporabnost od neglikoziliranih.

- Uporaba rekombinantnih alergenov iz strupov kožekrilcev v testu aktivacije bazofilcev izboljša njihovo specifičnost in omogoča ugotavljanje primarne senzibilizacije dvojno pozitivnih bolnikov po piku neznanega kožekrilca.

- Hialuronidaza je pomemben navzkrižno reaktiven protein čebeljega in osjega strupa z visoko biološko oz. alergogeno aktivnostjo, ki ni povezana z ogljikohidratnimi epitopi.

- Odzivnost bazofilcev ima visoko diagnostično napovedno vrednost pri bolnikih z negativnimi sIgE in kožnimi testi. V primerjavi z intradermalnimi kožnimi testi ima boljšo diagnostično občutljivost in specifičnost.

- Uporaba celičnega *in vitro* testa aktivacije bazofilcev pri obravnavi kompleksnih bolnikov z negativnimi rezultati rutinskega diagnostičnega testiranja pri večini bolnikov omogoča uvedbo zdravljenja s specifično imunoterapijo.

- Kratkotrajna SIT s strupi kožekrilcev povzroči FcεRI desenzibilizacijo bazofilcev, ki je povezana z manjšo ekspresijo gena in/ali izražanjem receptorja FcεRI na površini bazofilcev.

- Kratkotrajna SIT s strupi kožekrilcev privede do desenzibilizacije bazofilcev za specifičen alergen, s katerim izvajamo SIT kot tudi za nespecifičen alergen, ki ni vključen v SIT. Zdi se, da je učinek kratkotrajne SIT s strupi kožekrilcev za alergen nespecifičen.

- Kratkotrajna SIT s strupi kožekrilcev povzroči desenzibilizacijo bazofilcev po IgE/FcεRI poti, kar je možen kratkotrajni zaščitni mehanizem SIT.

- Izražanje receptorja FcεRI in njegove odzivnosti bi lahko bila uporabna *in vitro* metoda s katero bi spremljali potek in uspešnost zdravljenja s SIT.

4 POVZETEK (SUMMARY)

4.1 POVZETEK

Piki žuželk iz redu kožekrilcev lahko povzročijo sistemske preobčutljivostne reakcije, ki so pogost vzrok za težjo in potencialno usodno sistemsko anafilaktično reakcijo. Bolniki z anamnezo težje sistemske preobčutljivostne reakcije, v veliki večini le-to ponovno doživijo. Zato je pri teh bolnikih potrditev senzibilizacije s primarnim povzročiteljem ključna za uvedbo zdravljenja s SIT s strupi kožekrilcev. Vendar veliko bolnikov identitete kožekrilca ne prepozna. V diagnostičnem postopku pa pogosto naletimo na dvojno pozitivne lahko pa tudi na dvojno negativne rezultate za strup čebele in ose, kar predstavlja problem pri izbiri ustreznega alergena za SIT. SIT s strupi kožekrilcev je edino učinkovito zdravljenje, ki v 75 – 95 % zaščiti pred nadaljnjimi sistemskimi preobčutljivostnimi reakcijami. Kljub njeni izredni učinkovitosti, natančen imunološki mehanizem še vedno ni povsem razjasnjen. Prav tako ne poznamo *in vitro* metode s katero bi lahko spremljali potek in uspešnost zdravljenja.

Pogost problem dvojne pozitivnosti z nativnimi alergeni predstavljajo lažno pozitivni rezultati, ki nastanejo zaradi prisotnosti navzkrižno reaktivnih in/ali klinično nepomembnih ogljikohidratnih determinant. Zato smo želeli ugotoviti diagnostično uporabnost novih komercialno dostopnih poglavitnih vrstno-specifičnih rekombinantnih alergenov čebeljega in osjega strupa, ki CCD ne vsebujejo. Diagnostično občutljivost rApi m 1, za ugotavljanje preobčutljivosti za strup čebele, smo preizkušali na veliki skupini 184 mono-senzibiliziranih preiskovancev s preobčutljivostjo za strup čebele in diagnostično občutljivost rVes v 5 in rVes v 1, za ugotavljanje preobčutljivosti za strup ose, na 200 mono-senzibiliziranih preiskovancih s preobčutljivostjo za strup ose. V primerjavi z nApi m 1 alergenom (91 %) se je rApi m 1 izkazal s slabšo diagnostično občutljivostjo (57 %) za ugotavljanje preobčutljivosti za strup čebele, razlika pa je bila še večja pri skupinah s težjo sistemsko preobčutljivostno reakcijo (51 % proti 87 % in 63 % proti 97 %). Nasprotno, sta se rVes v 5 in rVes v 1 izkazala z veliko diagnostično občutljivostjo za ugotavljanje preobčutljivosti za strup ose (92 %). Komercialno dostopen alergen rApi m 1 iz *E. coli* ima majhno diagnostično občutljivost in je posledično omejeno primeren za ugotavljanje preobčutljivosti za strup čebele, medtem ko imata komercialno dostopna alergena rVes v 5 in rVes v 1 iz Sf9 veliko diagnostično občutljivost ter bi v primeru dvojno pozitivnih oz. nejasnih rezultatov rutinskega testiranja bila diagnostično uporabna za ugotavljanje preobčutljivosti za strup ose.

Ko v primeru dvojne pozitivnosti identiteta kožekrilca ostaja neznana, je potrebno z nadaljnjo diagnostično obravnavo ločiti med primarno dvojno senzibilizacijo in navzkrižno reaktivnostjo. Diagnostično uporabnost poglavitnih vrstno-specifičnih in

navzkrižno reaktivnih rekombinantnih alergenov čebeljega in osjega strupa, proizvedenih v različnih sistemih za izražanje genov, smo želeli preizkusiti v biološkem sistemu na nivoju aktivacije bazofilcev pri skupini dvojno pozitivnih preiskovancev s sistemsko preobčutljivostno reakcijo po piku neznanega kožekrilca. Pri 14 bolnikih, z dvojno pozitivnimi sIgE in testom BAT z nativnim alergenom čebeljega in osjega strupa, smo ugotavljali biološko aktivnost in/ali IgE reaktivnost za rApi m 1, rApi m 2, rVes v 5, rVes v 2, rVes v 1 in za CCD. Z uporabo poglavitnih rekombinantnih alergenov v testu BAT smo pri 4 bolnikih ugotovili senzibilizacijo za strup ose, pri 5 za strup ose in za hialuronidazo, pri 1 le za hialuronidazo in pri 3 bolnikih za strup ose in čebele ter hialuronidazo hkrati. Pri 1 bolniku smo prikazali biološko aktivnost le z navzkrižno reaktivnimi ogljikohidratnimi determinantami. Pri vseh 9 pozitivnih bolnikih smo v testu BAT za Sf3 izraženo hialuronidazo, ki ne vsebuje navzkrižno reaktivnega α-1,3-fukoziliranega N-vezavnega mesta, ugotovili visoko biološko oz. alergogeno aktivnost, medtem ko je bila biološka aktivnost neglikozilirane hialuronidaze iz *E. coli* povsem neprimerna. Uporaba poglavitnih rekombinantnih alergenov, ki ne vsebujejo CCD, v testu BAT nam je omogočila ugotovitev primarne senzibilizacije pri veliki večini dvojno pozitivnih bolnikov. Naši rezultati nakazujejo na pomembno vlogo hialuronidaze, kot navzkrižno reaktivnega proteina.

Pri vseh bolnikih s prepričljivo klinično anamnezo sistemske preobčutljivostne reakcije po piku kožekrilca, ne zaznamo za strup specifičnih protiteles IgE ali pozitivnega kožnega vbodnega testa. Potrditev senzibilizacije za strupe kožekrilcev pa je predpogoj za uvedbo SIT, ki prepreči nadaljnje sistemske reakcije pri teh bolnikih. Želeli smo ugotoviti rutinsko uporabnost celičnega *in vitro* testa BAT v primerjavi z drugimi možnimi diagnostičnimi pristopi za potrjevanje povzročitelja pri bolnikih s težjo sistemsko preobčutljivostno reakcijo in negativnimi rezultati rutinskih testov. Prav tako smo želeli določiti njegovo napovedno vrednost v primeru dvojno pozitivnega rezultata za strup čebele in ose. V študijo smo prospektivno vključili 21 bolnikov s težjo sistemsko preobčutljivostno reakcijo po piku kožekrilca in negativnimi sIgE za strup čebele in ose. S testom BAT smo potrdili senzibilizacijo pri 81 % bolnikov, z intradermalnimi kožnimi testi pa pri 57 %. V primeru dvojno pozitivnega rezultata za strup čebele in ose je bil klinično pomemben tisti strup, ki je pri testu BAT spodbudil večji celični odziv. Rutinska uporaba celičnega *in vitro* testa BAT nam je pri večini bolnikov s težjo sistemsko preobčutljivostno reakcijo po piku kožekrilca ter negativnimi sIgE in kožnimi testi omogočila potrditev senzibilizacije. Test BAT ima v primerjavi z intradermalnimi kožnimi testi boljšo diagnostično občutljivost in specifičnost. Tako nam njegova uporaba v rutinski diagnostiki pri obravnavi kompleksnih bolnikov z negativnimi rezultati pogosto omogoča uvedbo zdravljenja s SIT z ustreznim strupom kožekrilca.

Čeprav je za vzpostavitev dolgotrajne zaščite s SIT s strupi kožekrilcev potrebno vsaj 3 – 5 let zdravljenja, je večina preobčutljivih bolnikov zaščitena že z uvedbo prvega vzdrževalnega odmerka. Vzrok njihove kratkotrajne zaščite ni povsem razjasnjen. Naš namen je bil preučiti vlogo receptorja velike afinitete FcεRI in z njim povezane vloge bazofilcev pri spodbuditvi kratkotrajne zaščite SIT s strupi kožekrilcev. Nadalje smo želeli ugotoviti ali so morda te zgodnje celične spremembe za alergen specifične ali nespecifične. Najprej smo vključili večje število preiskovancev, 60 odraslih in 48 otrok, pri katerih smo pred terapijo, med uvodno fazo in tik pred uvedbo prvega vzdrževalnega odmerka pri zelo hitri ali hitri shemi SIT spremljali občutljivost bazofilcev na stimulacijo s protitelesi anti-FcεRI, ekspresijo gena za α-podenoto receptorja FcεRI (*FCER1A*) in izražanje receptorja FcεRI na površini bazofilcev. V nadaljevanju pa smo spremljali 11 primarno dvojno senzibiliziranih bolnikov v enakih časovnih točkah pri zelo hitri shemi SIT s strupom enega kožekrilca. Poleg navedenega smo sledili tudi odzivnosti bazofilcev za čebelji in osji strup ter za rApi m 1 in/ali rVes v 5 ali za inhalacijski alergen v primeru dodatne ko-senzibilizacije. Pri dodatnih 7 bolnikih smo izvedli še pasivno senzibilizacijo bazofilcev, kjer smo izoliranim bazofilcem odstranili za čebelji ali osji strup specifična protitelesa IgE ter jih *de novo* senzibilizirali s specifičnimi protitelesi IgE za alergen pršice v enakih časovnih točkah.

Pri vseh vključenih preiskovancih smo tik pred uvedbo prvega vzdrževalnega odmerka ugotovili močno zmanjšano občutljivost bazofilcev pri stimulaciji s protitelesi anti-FcεRI in z za SIT-specifičnim strupom kožekrilca tako pri zelo hitri kot tudi pri hitri shemi SIT. Nadalje smo ugotovili primerljivo statistično značilno zmanjšano občutljivost bazofilcev pri stimulaciji z za SIT-nespecifičnim strupom kožekrilca. Primerljiv nespecifičen učinek smo pokazali pri stimulaciji s poglavitnim vrstno-specifičnim rekombinantnim alergenom strupa, ki ni bil vključen v terapijo. Pri poli-senzibiliziranem bolniku smo pred prvim vzdrževalnim odmerkom strupa čebele, ugotovili zmanjšano občutljivost bazofilcev za pelode trav. Pri 7 bolnikih s pasivno senzibiliziranimi bazofilci za alergen pršice, smo ugotovili statistično značilno zmanjšano občutljivost bazofilcev za *de novo* senzibilirajoči alergen pršice. Z različnimi dinamikami med shemami SIT je bila pri 34 – 100 % vključenih bolnikov zmanjšana tudi ekspresija gena in izražanje receptorja FcεRI na celični površini. Ugotovili smo, da kratkotrajna SIT privede do močne desenzibilizacije bazofilcev po IgE/FcεRI poti, kar je možen mehanizem vzpostavitve imunske tolerance pri kratkotrajni SIT s strupi kožekrilcev. Kratkotrajna SIT s strupi kožekrilcev privede do desenzibilizacije bazofilcev za specifičen alergen, s katerim izvajamo SIT kot tudi za nespecifičen alergen, ki ni vključen v SIT. Za razliko od specifičnega učinka dolgotrajne SIT, je učinek kratkotrajne SIT s strupi kožekrilcev verjetno za alergen nespecifičen. Izražanje receptorja FcεRI in njegove odzivnosti bi lahko bila uporabna *in vitro* metoda s katero bi lahko spremljali potek in uspešnost zdravljenja s SIT.

4.2 SUMMARY

Hymenoptera stings can induce allergic systemic and occasionally fatal reactions. In case of severe *Hymenoptera* venom allergy, a re-sting may cause life-threatening reactions. In such patients, correct diagnosis is an absolute prerequisite for effective management, i.e. venom-specific immunotherapy. However the identification of the offending insect is not always straightforward, as majority of patients are unable to identify the stinging *Hymenoptera* and standard diagnostic tests often yield inconclusive, double positive or even negative results. Venom immunotherapy (VIT) is the only effective treatment, which reduces the risk of a subsequent systemic reaction in 75–95% of treated patients. Despite its effectiveness, the precise immunological mechanisms have not yet been fully explained. Moreover there is no *in vitro* test for monitoring early induction of protective mechanisms and tolerance during VIT.

The accurate diagnosis of venom allergy and in particular of the culprit insect can be hampered by several factors. For instance serologic diagnosis of insect venom allergy is often difficult because of the presence and recognition of cross-reactive carbohydrate determinants (CCDs) in insect venom extracts that lack allergenic activity and hence may give rise to false positive results. Thus commercially available recombinant species-specific major allergens devoid of CCDs were evaluated for its diagnostic utility. Recombinant Api m 1 was tested in 184 subjects with established honeybee venom allergy and rVes v 5 and rVes v 1 in 200 subjects with established *Vespula* venom allergy. Diagnostic sensitivity was significantly higher for nApi m 1 compared to rApi m 1 allergen (91% vs. 57%). These marked differences were even more prominent in subjects who had experienced severe anaphylactic reactions (51% vs. 87% and 63% vs. 97%, respectively). On the other hand we found high diagnostic sensitivity of rVes v 5 and rVes v 1 (92%) for diagnosis of *Vespula* venom allergy. In conclusion, our results suggest that the current commercially available rApi m 1 has limited clinical utility for the detection of honeybee venom allergy due to its low diagnostic sensitivity. Meanwhile commercially available tests based on rVes v 5 and rVes v 1 in difficult cases should be helpful for the serological dissection of *Vespula* venom allergy.

Double positivity to honeybee and *Vespula* venom necessitates supplementary testing to distinguish genuine double sensitization from cross-reactivity. We sought to test the diagnostic utility of differently expressed recombinant species-specific and cross-reactive major honeybee and *Vespula* venom allergens used in basophil activation test (BAT) on a complex group of patients with an anaphylactic reaction to an unknown culprit insect. Fourteen *Hymenoptera* venom allergic patients with double-positive sIgE and BAT results with native whole venom extracts were evaluated for biological activity and/or IgE reactivity to rApi m 1, rApi m 2, rVes v 5, rVes v 2, rVes v 1 and CCDs. With recombinant-based BAT we were able to identify 4 patients with *Vespula*,

5 patients with *Vespula* and cross-reactive hyaluronidase, 1 patient with only hyaluronidase and 3 patients with *Vespula*, honeybee and hyaluronidase sensitization. In one patient only the reactivity to CCDs was demonstrated. In BAT with Sf3-expressed hyaluronidase, without alpha-1,3-core fucosylation, a very high allergenic activity was showed in all 9 positive patients, while *E. colis*' appeared of poor and inadequate biological activity. Recombinant-based BAT allows the identification of honeybee and/or *Vespula* allergy in majority of double-positive patients. Furthermore, our results also suggest the importance of hyaluronidase in case of protein based cross-reactivity.

A considerable number of patients with a history of systemic reactions after *Hymenoptera* sting demonstrate negative venom-specific IgE and skin test results. Furthermore, those patients could subsequently experience another severe or even a fatal reaction to a sting. As VIT decisions rely on confirmation of allergic sensitization, it is clear that the management of these patients requires further diagnostic evaluation. Previous reports suggest the usefulness of BAT in *Hymenoptera* venom allergic patients with negative standard diagnostic test results. We sought to evaluate the diagnostic utility of this cellular testing in a routine clinical laboratory setting, with special emphasis on comparison with other possible testing approaches for double-negative patients and with the diagnostic and culprit consideration in the case of double-positive honeybee and *Vespula* venom basophil response. Twenty-one patients with a severe anaphylactic reaction to *Hymenoptera* sting and negative venom-specific IgE were routinely and prospectively tested with BAT. We were able to diagnose 81% of patients with BAT and 57% with intradermal skin testing. In the case of double-positive BAT, the culprit insect correlated with the venom that induced a significantly higher basophil response. In summary, BAT allows the prompt identification of severe *Hymenoptera* venom allergic patients with negative specific IgE and skin tests. We demonstrated that in such patients BAT is more clinically sensitive and relevant than any other type of testing. The routine use of this cellular test should facilitate prescription of venom immunotherapy in complex cases with inconclusive diagnostic results.

Although long-lasting allergen tolerance requires at least 3 to 5 years of treatment, VITs' early protection has been confirmed already after the maintenance dose (MD) had been achieved. The precise early immunological mechanisms of VIT that seem to develop during the build-up phase have not yet been fully explained. Our aim was to evaluate the role of high-affinity IgE receptor (FcεRI) and the related basophil function in the induction of short-term VIT protection and to evaluate its allergen specificity. We initially included 60 adults and 48 children. Basophil threshold sensitivity (CD-sens) to anti-FcεRI stimulation, and FcεRI gene and cell-surface expression was assessed at the beginning, during build-up and just before the first MD of ultra-rush or semi-rush VIT. Furthermore 11 *Hymenoptera*-venom genuinely double sensitized subjects were followed for the same parameters as well as for basophil threshold sensitivity to

honeybee, and *Vespula* venom at the same VIT time points during single ultra-rush VIT. In some patients we also monitored CD-sens to rApi m 1 and/or rVes v 5 or other co-sensitizations (i.e., grass pollen). In additional 7 patients, basophils were isolated, stripped and sensitized with house dust mite (HDM) IgEs at the same time points.

We demonstrated a marked reduction of CD-sens to anti-FcεRI and VIT-specific venom before the first MD in all subjects included. Furthermore a significant and comparable decrease before the first MD was also evident for non-VIT venom; this nonspecific decrease was further supported by the opposite recombinant species-specific major allergen. In one subject with additional grass pollen allergy, a decrease of CD-sens to grass allergen was also demonstrated. Similarly, in 7 cases of patients with passively HDM-sensitized basophils, a significant reduction of CD-sens was also evident to *de novo* sensitized HDM allergen. FcεRI gene and/or cell-surface expression was decreased in 34% up to 100% of subjects, with different dynamic between VIT protocols. We found a marked desensitization of FcεRI-activated basophils after short-term VIT. This suppression, which could be highly relevant for the development of early protective mechanisms, might be also related to the changes at the level of FcεRI expression. Short-term VIT induced basophil desensitization to VIT-specific as well as to VIT-nonspecific venom. As opposed to long-term VIT, which induces venom-specific changes, the effect of short-term VIT seems to be venom-nonspecific. Furthermore, a detailed understanding of this cellular shift would allow the development of novel interventions for promoting or monitor the silencing of basophil FcεRI pathway.

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