

IMMUNOBLOT STUDIES IN ALLERGIC PATIENTS TO HYMENOPTERA VENOM BEFORE AND DURING IMMUNOTHERAPY*

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SUMMARY:

Background: Hymenoptera venom immunotherapy (VIT) is immunologically effective in patients with systemic allergic reactions after hymenoptera stings.

Objective: To evaluate the effect of VIT on specific IgE and IgG4 immunoblotting bands in VIT-treated patients.

Material and Methods: Specific IgE and IgG4 immunoblotting bands for hymenoptera venom were performed with ALA-BLOT in sera of 17 patients (8 allergic to honeybee venom, 8 to wasp and 1 to polistes venom) before and during successful VIT (1 and 3 years). Before immunotherapy, all patients had experienced moderate/severe systemic reactions to a hymenoptera sting, with positive skin tests and venom-specific IgE. During immunotherapy all patients suffered field stings, without any systemic reaction.

Results: Before VIT we detected several immunoglobulin-binding bands in different regions, with different individual patterns. After VIT, we observed in some patients (5/8 for honeybee venom, 6/8 for wasp and 1/1 for polistes) complete disappearance of some IgE-binding bands, mainly the 15 kDa region (honeybee) and 23 and 44 kDa regions (wasp and polistes). All patients showed decreased intensity of IgE-binding bands, most pronounced in regions 16, 44 and 52 kDa (honeybee); 44 and 35 kDa bands (wasp) and 23 kDa (polistes). Some patients showed *de novo* appearance of IgG4-binding bands (4/8 for honeybee and 8/8 for wasp venom), mainly in 52 kDa (honeybee) and in 23 and 44 kDa regions (wasp). All patients showed increased intensity of IgG4 bands that were already present before VIT, more pronounced in 52 and 44 kDa (honeybee) and in 44 and 35 kDa regions (wasp).

Conclusions: During successful VIT there are changes in intensity and number of IgE and IgG4 binding bands, which could reflect the immunological improvement induced by VIT. These changes are more pronounced/frequent in wasp VIT, a fact that could explain the best results usually seen in these patients.

Key-words: Allergy - Hymenoptera - Immunotherapy - IgE - IgG4 - Immunoblotting.

INTRODUCTION

Since the introduction of pure insect venom specific immunotherapy (VIT) in 1974, its efficacy has been shown by a number of studies. Nevertheless, the immunologic mechanisms underlying this treatment are still unclear (1)

The indications for VIT are a clinical history of systemic reactions with respiratory or cardiovascular symptoms in adults or children plus positive skin tests or serum specific IgE; or mild to moderate reactions with urticaria and angioedema in highly exposed adults with repeated reactions plus positive diagnostic tests (1, 2).

The monitoring of the immune response during VIT can be done by skin tests or serial assays of venom specific IgE and IgG/IgG4 antibodies (3-5), which

could eventually provide data to help the allergologist's decision to stop or continue VIT, since it can be considered that, in presence of the above mentioned immunological changes, VIT interruption can be considered after a period of 3 to 5 years (6).

The fact that nonallergic beekeepers exhibit specific IgG4 antibodies in their sera (7) and that specific IgG, especially IgG4 increases during immunotherapy led to the supposition that these antibodies could protect against immediate allergic reactions. Nevertheless, it seems that there are no single levels of IgG or IgG4 that could have predictive value regarding the occurrence of systemic reactions following a sting challenge (8).

The purpose of this study was to analyse immunoblotting changes in patients under VIT, comparing wasp, polistes and honeybee VIT in regard to the evolution of the number and intensity of positive IgE and IgG4-binding bands.

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MATERIAL AND METHODS

Patients

All patients had a history of severe clinical reactions after hymenoptera sting and positive skin tests and increased serum specific IgE to hymenoptera venoms.

We studied 8 patients allergic to honeybee venom (7 males and 1 female, mean age: 42 ± 9 years), 8 patients allergic to wasp venom (4 males and 4 females, mean age: 38 ± 13 years) and 1 female patient allergic to *Polistes* venom (60 years old) before and during VIT (3 years). During VIT all patients were re-stung in the field, at least once, without any systemic reaction.

Serum of highly sensitized honeybee and wasp venom allergic patients served as positive controls, and serum of 15 nonatopic patients without history of insect sting, as negative controls.

Western blot analysis

Immunoblotting was performed with the AlaSTAT AlaBLOT specific IgE and IgG4 test system (DPC, Bad Nauheim, Germany) to insect venom allergens according to the manufacturer's instructions.

Briefly, venom extracts were electrophoresed by SDS-PAGE, electrotransferred to nitrocellulose membrane strips and then incubated with 50 μ L of diluted patient sera for 120 min at room temperature. Anti-IgE or anti-IgG4 monoclonal antibodies labelled with the reporter enzyme alkaline phosphatase were immobilized at sites where patient IgE or IgG4 was bound to venom extracts on the membrane strip. By adding a chromogenic substrate for the reporter enzyme, venom bands recognized by a patient's specific IgE or IgG4 were visualized. The colour development was stopped after 15 min. The bands obtained were quantified by densitometry after scanning using the Biosoft Software (Biosoft, Oxford, UK). Signals in controls or patients were regarded as being positive if their intensity (i.e. relative area under the curve) was in excess of the intensity plus 2 standard deviations of the blots using negative control sera.

To identify individual bands, molecular masses were assessed by measuring the distance between the edge of the dye front and the centre of the band of interest. To calculate the molecular mass in kilodaltons (kDa), which allows identification of the respective allergen, a lot-specific formula provided by the manufacturer was used.

RESULTS

■ **Honeybee venom allergic patients- IgE and IgG4-binding bands:** Several IgE binding bands were detected in the regions of 10, 13, 15, 16, 44 and 52 kDa (Table 1). The 10 kDa region may represent the tetramer of melittin (9) and the regions of 15 kDa may represent the molecular size of phospholipase A2 in different glycosylated forms (9). 16 kDa and 44 kDa bands represent the major allergens Api m1 (phospholipase A2) and Api m2 (hyaluronidase), respectively (10). IgE binding to 13 and 52 kDa bands was already identified by other authors (7, 9)

The most frequent IgE-binding bands were situated in the 16 kDa and 44 kDa regions. All patients showed positivity in the 16 kDa region and, in the 44 kDa, seven of the 8 patients (87,5%) also showed positive results. In the 13 and 15 kDa regions there were six patients (75%) with positive binding bands and in the 10 and 52 kDa regions only two patients (25%) showed positive IgE-binding bands before VIT.

During VIT we could see complete disappearance of some IgE-binding bands: two patients in the 13 kDa region; 5 patients in the 15 kDa regions, 1 patient in the 44 kDa and 52 kDa regions. In the 10 and 16 kDa regions we didn't see a complete disappearance of any bands; however, there were significant reductions in the IgE binding intensity (mean decrease of 44 and 73%, respectively). In the other bands we have observed mean decreases of IgE-binding intensity of 57% (13 kDa), 67% (15 kDa), 71% (44 kDa) and 39% (52 kDa), after three years of VIT (table 1).

Regarding IgG4-binding bands we found very similar results to IgE-binding bands: same regions (10, 13, 15, 16, 44 and 52 kDa) and approximately the same frequency of positive bands (table 2).

All patients showed positivity in the 16 kDa region and, in the 44 kDa and 15 kDa regions, seven of the 8 patients (87,5%) also showed positive bands. In the 13 kDa region there were six patients (75%) with positive binding bands, in the 52 kDa region only two patients (25%) and in the 10 kDa region only one patient showed positive IgG4-binding bands before VIT.

During VIT we observed de novo appearance of IgG4-binding bands in the 13 kDa region (1 patient), 44 kDa region (1 patient) and 52 kDa region (3 patients). This positivity, which was not present before VIT, appeared first at 12 months and was further increased at 36 months.

In the three regions where most patients had positive

IgG4-binding bands before VIT (15, 16 and 44 kDa) there was an increase in IgG4 binding intensity in these regions after three years of VIT (mean increase of 49% in 15 kDa region; 35% in 16 kDa and 66% and for 44 kDa: %). In the 10 kDa region, only one patient showed evidence of IgG4 binding, even after three years of VIT. In the 13 kDa region there was a mean increase of 50% and in the 52 kDa region there was a mean increase of 160% in IgG4-binding intensity after three years of VIT

■ **Wasp/Polistes venom allergic patients- IgE and IgG4-binding bands:** Several IgE-binding bands were detected in the regions of 22, 23, 35, 44 and 52 kDa (Table 3). 23 kDa bands may correspond to antigen 5 of wasp or polistes venom (Ves v5 and Pol a5), 35 kDa may correspond to phospholipase A1 of wasp or polistes (Ves v1 and Pol a1). 44 kDa bands may correspond to Ves v2 (hyaluronidase) (10, 11) Fig. 1.

Before VIT, we identified in all wasp venom allergic patients (Table 3) IgE binding to 35 kDa region. Seven

patients (87.5%) showed IgE binding bands to 23 and 44 kDa regions. In the 22 kDa and in the 52 kDa regions, only two patients had positive bands.

In the course of wasp VIT we could see complete disappearance of some IgE-binding bands: 4 patients in the 23 kDa, 1 patient in the 35 kDa and 4 patients in the 44 kDa region. In the 22 and 52 kDa regions we didn't see a complete disappearance of any bands; however, there were significant reductions in the IgE binding intensity (mean decrease of 44 and 59%, respectively). In the other bands we have observed mean decreases of IgE-binding intensity of 71% (23 kDa), 55% (35 kDa), 75% (44 kDa), after three years of VIT (table 3).

Regarding specific IgG4 to wasp venom (Table 4), it was only in the 35 kDa region that we detected IgG4 binding in all patients before VIT, with a mean increase of 117% during VIT. Three patients in the 23 and two patients in the 44 kDa regions, showed de novo appearance of positive bands in these regions, identi-

	10 KDa			13 KDa			15 KDa			16 KDa			44 KDa			52 KDa		
	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y
1	0	0	0	0	0	0	9	6	4	25	17	8	6	0	0	0	0	0
2	0	0	0	13	0	0	15	0	0	25	11	6	17	6	3	21	0	0
3	0	0	0	15	0	0	14	4	0	22	10	7	16	8	6	0	0	0
4	0	0	0	0	0	0	0	0	0	38	25	18	19	16	9	0	0	0
5	24	12	12	28	13	15	16	0	0	101	72	22	107	98	30	0	0	0
6	8	8	6	20	6	5	10	0	0	90	70	10	98	38	20	0	0	0
7	0	0	0	3	4	3	10	8	0	12	7	9	50	22	7	0	0	0
8	0	0	0	3	2	2	0	0	0	12	9	6	0	0	0	54	40	23

Table 1: Honeybee venom IgE binding regions (binding intensity at different time points 0, 1 year and 3 years of VIT).

	10 KDa			13 KDa			15 KDa			16 KDa			44 KDa			52 KDa		
	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y
1	0	0	0	31	36	36	33	54	54	70	101	101	0	15	15	0	0	0
2	0	0	0	0	10	12	41	59	80	99	133	141	10	13	40	0	8	11
3	0	0	0	12	15	17	101	150	162	137	179	184	20	29	39	0	0	0
4	0	0	0	9	31	32	153	165	179	162	171	184	34	45	48	6	13	17
5	43	49	57	42	58	62	115	132	148	150	154	160	111	131	123	0	18	27
6	0	0	0	9	13	18	15	28	37	24	36	50	16	35	48	0	9	15
7	0	0	0	30	38	50	33	45	70	60	72	90	12	24	35	0	0	0
8	0	0	0	0	0	0	0	0	0	9	11	45	7	9	32	9	15	37

Table 2: Honeybee venom IgG4 binding regions (binding intensity at different time points 0, 1 year and 3 years of VIT).

fiable at one year after the start of VIT. However, in both regions, one patient did not show any IgG4 binding even after 3 years of VIT. The mean increase in band intensity was 79% for 23 kDa and 128% for 44 kDa region. In the 22 and 52 kDa regions, no patient showed IgG4 binding before VIT; however, during VIT, three and seven patients, respectively, showed IgG4 binding bands in these two regions.

In the patient allergic to *Polistes* venom, we could only detect IgE binding to the 23 and 35 kDa regions, (Fig. 2) and IgG4 binding to 23 kDa region, with an increase in IgG4 binding and a decrease in IgE binding intensity during VIT, including the disappearance of the 23 kDa band at three years of VIT (tables 3 and 4).

As it can be seen in Tables I to IV, there are individual patterns in these variations and there are no apparent correlations between variations of intensity in immunoglobulin binding bands or between intensity of IgG4 or IgE binding bands, before or during VIT.

	22 KDa			23 KDa			35 KDa			44 KDa			52 KDa		
	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y
1	0	0	0	14	0	0	21	12	8	7	0	0	0	0	0
2	0	0	0	5	4	2	24	19	12	0	0	0	0	0	0
3	0	0	0	0	0	0	8	3	2	5	0	0	0	0	0
4	0	0	0	20	14	7	23	17	5	12	7	3	0	0	0
5	24	12	12	24	16	0	42	31	22	13	6	3	0	0	0
6	8	8	6	27	11	0	25	19	12	17	0	0	20	10	7
7	0	0	0	15	9	8	79	28	20	9	0	0	14	11	6
8	0	0	0	55	18	12	6	2	0	18	7	4	0	0	0
Pol	0	0	0	9	3	0	35	32	25	0	0	0	0	0	0

Table 3: Wasp and *Polistes* venom IgE binding regions (binding intensity at different time points 0, 1 year and 3 years of VIT).

*Pol – *Polistes* venom allergic patient.

	22 KDa			23 KDa			35 KDa			44 KDa			52 KDa		
	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y	0	1Y	3Y
1	0	0	0	109	131	140	41	46	78	103	119	129	0	0	15
2	0	17	30	28	31	47	31	42	55	15	19	37	0	0	21
3	0	6	15	27	15	28	16	29	74	19	62	150	0	0	27
4	0	0	0	0	0	0	36	90	145	9	11	24	0	17	50
5	0	9	25	25	38	47	18	42	71	0	0	35	0	0	0
6	0	0	0	0	10	17	7	13	24	12	28	39	0	0	19
7	0	0	0	0	16	17	3	12	23	0	0	0	0	6	23
8	0	0	0	0	138	132	8	30	35	0	69	97	0	54	86
Pol	0	0	0	23	170	175	50	32	64	0	0	0	0	0	0

Table 4: Wasp and *Polistes* venom IgG4 binding regions (binding intensity at different time points 0, 1 year and 3 years of VIT).

*Pol – *Polistes* venom allergic patient.

DISCUSSION

It has been shown by numerous studies that VIT provides effective protection for 90-95% of patients with a history of sting anaphylaxis who are subsequently resting after therapy (12). Besides clinical data obtained from accidental field stings or from in-hospital sting challenges, the immunologic parameters used to evaluate the response to VIT are serum measurements of specific IgE and IgG or IgG4, the latter constituting the predominant antibody response during VIT (13). Despite the fact that most patients do have a significant increase in specific IgG4 titer after reaching maintenance doses, several investigators have been unable to show a relationship between increased IgG4 and clinical protection (9,14). The same considerations apply to specific IgE: usually specific IgE values and skin tests gradually decrease during VIT, being below initial values after one year of VIT. However, these tests do not have a significant predictive value, the exception being if both skin tests and specific IgE become negative, which indicates clinical protection (1). Other authors have put forward the hypothesis that it is the ratio of IgE/IgG4 to single

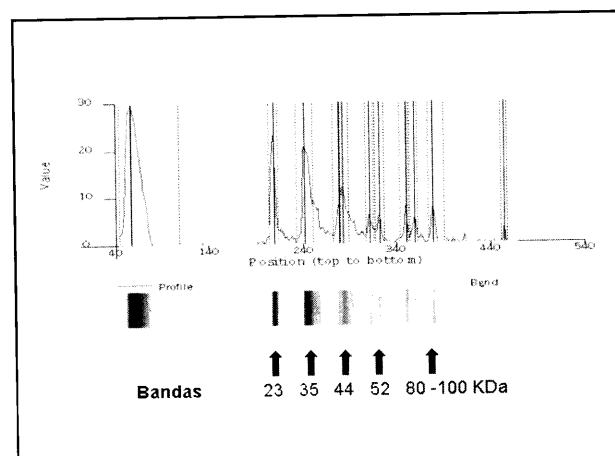


Figure 1: sIgE immunoblotting for wasp venom.

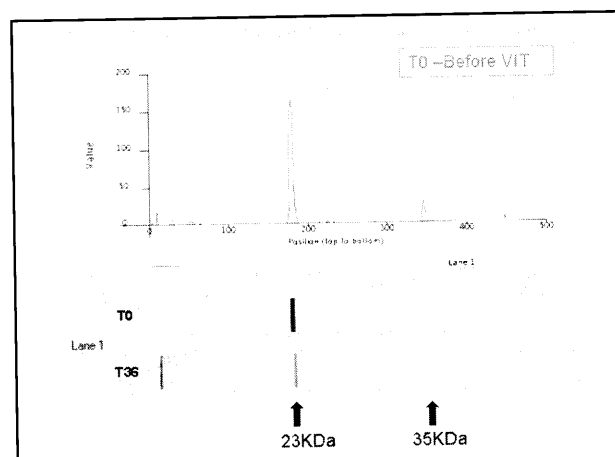


Figure 2: sIgE immunoblotting for *Polistes* venom.

allergens that better reflects protection (9). In their study analysing reactors and non reactors to sting challenge after VIT, the authors propose that protection is indicated by the presence of significant amounts of specific IgG4 to all single allergenic components to which the patient has specific IgE, whereas absent or insufficient IgG4 to only one allergen to which IgE is present is associated with allergic symptoms (9). If this hypothesis is true, immunoblotting techniques are absolutely necessary in order to detect single band variations in specific IgE and IgG4 titers. However, the assessment of VIT efficacy by sting challenges is not without criticism, since some non-reactors to a sting challenge can present systemic reactions to subsequent stings (15). This fact together with the ethical problems raised by this type of challenges stresses the need for a more practical method of assessing VIT efficacy, mainly if it can show, at least, close correlations with the outcomes of a sting challenge.

In our study all patients suffered field stings during the course of VIT and there were no systemic reactions in any patient. Despite being an uncontrolled challenge, we believe these negative field stings can be regarded as evidence in favour of VIT efficacy in patients who previously had systemic reactions. In fact, our data showed the existence of variations in the intensity of specific IgE and IgG4 bands following VIT, which could reflect the immunological protection of VIT in patients with hymenoptera allergy.

In honeybee venom allergic patients only two patients (4 and 8) did not present disappearance of any IgE-binding band. In the remaining 6 patients we could observe disappearance of a total of 9 IgE-binding bands. Regarding IgG4-binding bands we could see appearance of 5 new bands in 4 patients at the end of three years of VIT. If we analyse the ratio IgE/IgG4 of single allergenic bands we can see only two examples of persistence of IgE-binding bands without appearance of any IgG4-binding bands (10 kDa in patient 6 and 13 kDa in patient 8). However, the intensity of any of these two IgE-binding bands was extremely weak and therefore, probably not responsible for any clinical symptoms after challenge.

In wasp/polistes venom allergic patients there were also only two patients (2 and 4) who did not present disappearance of any IgE-binding bands and, in total, we could register the disappearance of 10 IgE-binding bands in 7 patients. However, the main difference to honeybee venom allergic patients was seen in IgG4-binding bands: all wasp-allergic patients showed *de novo* appearance of at least one IgG4-binding band not present before VIT, in a total of 15 new IgG4-binding

bands. Furthermore the mean increase in intensity in the total IgG4-binding bands was approximately 400% in wasp versus 55% in honeybee venom. Regarding IgE-binding bands the mean decrease in intensity was 68% in wasp versus 71% in honeybee venom.

These changes also reflect in the ratios of single allergen IgE/IgG4-binding intensity, which have shown a consistent tendency towards decreased values over the three years of VIT, more pronounced in wasp VIT where we observed reductions of IgE/IgG4 ratios of more than ten times in almost all bands.

Indeed, it is quite interesting to see that the immunologic changes that we describe are much more pronounced in the wasp than in honeybee VIT patients, a fact that could help to explain the better clinical results observed in wasp VIT by several authors (16).

Since our study has only included patients that we regarded as having been successfully treated with VIT it is really impossible to state which magnitude of change in IgE and/or IgG4-binding intensity will be the most helpful to discriminate between successful and non-successful VIT or to help in the decision of stopping VIT. However, we believe that the data from this study emphasizes the importance of looking at individual patterns and at changes in the intensity of IgE/IgG4 binding to single allergen bands. We also believe that best VIT results can be expected when there is a simultaneous decrease in intensity of a certain IgE-binding band (preferably to zero) together with an increase in the intensity of the respective IgG4-binding band.

CONCLUSIONS

During successful VIT there are evident decreases in the intensity and number of specific IgE-binding bands and a complementary increase in the number and intensity of IgG4-binding bands. These changes are more pronounced or more frequent in wasp than in honeybee VIT, a fact that could explain the better results usually seen in wasp-venom allergic patients. The significant decrease in the ratio of specific IgE/IgG4 to single allergens can reflect the immunological improvement induced by VIT in a more accurate way than simple serum specific IgE or IgG4 measurements to venom extracts. More studies are needed in order to determine if these immunoblot findings can be correlated with relevant clinical data, namely with the outcomes of sting challenges in successfully and non-successfully treated patients, in order to determine if this could really be a better and safer tool for the assessment of the efficacy of VIT.

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