

Follow-Up of Venom Immunotherapy on Flow Cytometry and Definition of a Protective Index

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Key Words

Venom allergy · Intracutaneous tests · Basophil activation · CD63 · Flow cytometry · CCR3 · Immunotherapy

Abstract

Background: A major problem of venom-specific immunotherapy (VIT) is the absence of reliable parameters for deciding treatment discontinuation. **Aim of the Study:** Intracutaneous tests (ICTs), the basophil activation test (BAT), specific IgEs (sIgEs) and blocking factor (BF) activity were measured during VIT. We made an evaluation by means of a protective index (PI) including ICT, BAT and BF values. **Material and Methods:** A population of 45 patients who had experienced a systemic reaction after an insect sting were tested before VIT (T0), at 1 week (T1w), at 10 weeks (T10w) and at 21 weeks (T21w), and, for a subgroup of 17 patients, at 3–5 years (T3–5y). Basophil activation (expressed in % CD63 and in the area under the curve) and BF activity were measured by flow cytometry using the CCR3/CD63 protocol. **Results:** The first 21 weeks of follow-up showed no significant variation in the ICT, sIgE and BAT measurements, except for BAT, by eliminating weak negative anti-IgE responses. In these conditions, the decrease in basophil activation was significant at T10w ($p = 0.009$) and T21w ($p = 0.009$). Increased BF activity

was also significant at T10w ($p = 0.008$) and T21w ($p = 0.002$). The PI threshold calculated from the mean $\pm$ 3 standard errors (SE) was $64.8 (14.7 \pm 16.7, n = 25)$ at T0. PI increase was significant at T3–5y ($3,430 \pm 6,282; p < 0.001$). **Conclusion:** VIT induced a significant decrease in ICT values and basophil activation, along with an increase in serum BF activity, significant after 10 weeks of VIT. Evaluated in a larger population, the PI could represent a new tool for the clinico-biological follow-up of VIT efficacy.

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Introduction

Blood basophils have been considered for a long period of time as accessory cells due to their low concentration in human blood ($30\text{--}50/\text{mm}^3$). In contrast, nowadays, the role of basophils as key cells in allergic inflammation is widely accepted [1]. Moreover, the frequent use of new diagnostic tests based on the analysis of basophil activation on flow cytometry confirms that basophils represent a reliable image of a patient's immunoallergological status [2–4].

In the field of venom allergy diagnosis, basophil activation measured by flow cytometry has been the aim of a lot

of papers dealing both with venom allergy diagnosis and venom immunotherapy (VIT) follow-up [2, 5–8].

The efficacy of Hymenoptera venom-specific immunotherapy (VIT) is widely accepted, but the mechanisms involved are still unclear. Among the different putative mechanisms, the best-documented ones are the increase in specific IgA (sIgA) or sIgG and particularly sIgG4, the upregulation of CD8 suppressor cells, the decrease in cellular reactivity and the repolarization of T cells toward Th1 type and the involvement of Treg cells [9–12].

The evaluation of the degree of protection is a key question and the different parameters tested so far, like intracutaneous tests (ICTs), sIgE, sIgG and sIgG4 are not sufficiently reliable by themselves for predicting VIT outcome.

In vivo, ICT negativity after VIT has been proposed [13] but has not been confirmed by other authors [14]. However, the association of negative ICTs and negative sIgEs was positively correlated to a diminution of the risk of relapse [13].

In vitro, VIT follow-up by sIgG or sIgG4 has no predictive value [14, 15]; these sIgG persist after the end of VIT [16] with a significant decrease after 1 year of maintenance [17]. The use of the sIgE/sIgG ratio has also been proposed [18], but sIgE level evolution is highly patient dependent [19].

VIT follow-up based on the measurement of venom-induced basophil reactivity has been the aim of numerous papers for a long time. In 1974, Lichtenstein et al. [20] showed that basophil reactivity measured by histamine release decreased during VIT with a high sIgG increase.

Decreased basophil reactivity measured for suboptimal venom concentrations (100 ng/ml) after 6 months of VIT was proposed by Kucera et al. [6], this effect being emphasized after 3 years. The authors showed that basophil reactivity was negative in 60% of the cases, in contrast to sIgEs which were negative in only 17.9%. Using a venom concentration of 1,000 ng/ml, no difference was observed between reactors and nonreactors.

Decreased basophil activation measured by histamine release has been associated with decreased ICT values [21], recently confirmed by flow cytometry using CD63 [22] or CD203c [23] activation markers.

The so-called blocking factors (BF) or blocking antibodies, described a long time ago by Maunsell [24], are autologous antibodies produced by patients treated by specific immunotherapy that have been used recently for the follow-up of VIT [25].

On the basis of a paper published previously by our group [26], we present here an investigation using ICTs,

sIgE measurements and the basophil activation test (BAT) of 45 patients allergic to wasp, hornet and/or bee venom, who had experienced a systemic reaction after being stung; the decision was made to use VIT and they were then tested. We also proposed the calculation of a protection index (PI), calculated by an algorithm comprising ICTs, basophil activation expressed in the area under the curve (AUC) and BF activity, in order to measure the degree of protection during and after VIT.

Material and Methods

Patients

A population of 45 patients, aged 20–76 years (median 51 years), who had experienced a severe systemic reaction (grade 2–4 according to the Müller scale) after being stung by a bee (n = 7), wasp (n = 28) or hornet (n = 10) or for whom a second VIT was decided after a first ineffective VIT (n = 12), were tested before VIT (T0), at 1 week (T1w), T10w and T21w. At 3–5 years (T3–5y), a subgroup of 15 patients was tested (bee: n = 2, hornet: n = 3 and wasp: n = 10).

This protocol was accepted by the ethics committee of the Li-moges Hospital and all patients gave their informed consent.

Intracutaneous Tests

ICTs were performed using venom extracts (Stallergènes, France) at 5 concentrations, ranging from 100 ng/ml (10^{-3} of the 100 µg/ml stock solution) to 10 pg/ml, with a dilution ratio of 10. Results were expressed according to the following scale: 0 = negative at 100 ng/ml, 1 = positive at 100 ng/ml, 2 = positive at 10 ng/ml, 3 = positive at 1 ng/ml, 4 = positive at 0.1 ng/ml and 5 = positive at 0.01 ng/ml.

Venom Immunotherapy

VIT protocol was based on a progressive increase in the doses over 1.5 days, from 10 pg/ml to 100 µg/ml, with an interval of 30 min between injections, and followed with boosters at 1, 3, 6, 10, 15 and 21 days and then every 6 weeks. As hornet venom was no longer available in France, patients who had been stung by hornets were also treated with *Vespula* venom extract.

Specific IgE

sIgE values were determined by the UniCap method (Thermo Fisher Scientific) and results were expressed according to the manufacturer's recommendation, i.e. from 0.1 kUA/l to 100 kUA/l. sIgE values were measured at T0, T21w and at T3–5y.

Flow Cytometric Analysis of Basophil Activation

The flow cytometric analysis of basophil activation was performed according to the Flow2 CAST protocol (Bühlmann, Switzerland).

Allergen-Dependent Basophil Activation

This method has already been described [26]. In brief, whole blood taken before venom injection was mixed either with 4 dilutions of the tested venom (250, 83, 28 and 9 ng/ml of the same venom used for ICTs), with positive controls [ready-to-use anti-

IgE receptor (anti-RiGE), Bühlmann] and anti-IgE (Beckmann Coulter monoclonal anti-D2, 0.5 µg/ml) or with stimulation buffer alone (2 negative controls). An aliquot of ready-to-use labeling-antibodies mixture (anti-CCR3 PE and anti-CD63 FITC) was added and samples were incubated for 15 min at 37°C in a water bath. Erythrocytes were lysed (ready-to-use lysing buffer, 10 min at room temperature in the dark), tubes were centrifuged (1,800 rpm for 5 min) and the cell pellet was resuspended in 300 µl of PBS buffer. Basophils (CCR3+++ cells) were gated and the CD63 activation-marker density on the basophil membrane was measured by flow cytometry. Results were expressed in the AUC after having chosen the best-fit curve (logarithmic, exponential and polynomial of degree 2, Excel software) related to the 4 venom dilutions tested. The AUC is defined as the integral of the best-fit curve: basophil reactivity (% CD63) = $F(x)$ µg/ml venom, where x stands for the allergen concentration, and calculated from 250 ng/ml to 9,4 ng/ml (final concentrations within the test tube).

BF Activity Measurement

An aliquot of whole-blood taken on EDTA was washed with PBS buffer and resuspended in activation buffer. The tested serum was diluted 1:4 and 1:12 in a negative serum pool (serum negative for venom-specific IgEs taken from patients with no history of venom allergy and kept at -20°C). A single target venom concentration (83 ng/ml in RPMI 1640 buffer) was mixed v/v either with serum dilutions and or with the negative serum pool (100% control) and incubated for 30 min at room temperature, in parallel with 2 negative controls (RPMI 1640 alone). An aliquot of these dilutions was then mixed v/v with the washed cell pellet and incubated for 15 min at 37°C in a water bath.

The other steps have been described above. Results were expressed in % CD63 and in a percentage calculated versus the 100% control.

Calculation of a PI

We set up an algorithm comprising ICTs, BAT expressed in AUC and BF activity calculated for the 1:12 serum dilution leading to a PI, calculated according to the following formula:

$$(\text{BF activity } \%) / [(AUC/2) \times \log_{2.5} \text{ ICT}] \times 10^6.$$

In order to obtain an index equally weighted for these 3 parameters with a variation range of around 100, the ICT values were expressed in powers of 2.5, i.e. (2.5^x), the value of 'x' being 0, 1, 2, 3, 4 and 5, respectively, according to the ICT scale.

Statistical Analysis

Of the 45 tests, 4 showed a high spontaneous activation (>15%) and were excluded, and 1 was not analyzed due to a technical problem. Of the 40 remaining tests, at T0, 4 showed an anti-RiGE response of <15%, 9 showed an anti-IgE response of <15% and 2 showed both an anti-R and anti-IgE response of <15%.

We defined 8 groups of tests: group 1 = all tests, group 2 = antecedent of ineffective previous VIT, group 3 = weak or negative response to anti-IgE or anti-RiGE at T0, group 4 = hornet-stung patients, group 5 = weak or negative anti-IgE response or anti-RiGE at T0 excluded, group 6 = weak or negative anti-IgE response or anti-RiGE at T0 and antecedent ineffective previous VIT excluded, group 7 = all tests without antecedent of ineffective previous VIT and group 8 = ineffective previous VIT alone.

All statistical results were calculated by the XLSTAT-Pro software using the Mann-Whitney U test.

Results

Evolution of the Different Parameters during the First 21 Weeks of VIT

Intracutaneous Testing

Of the 28 ICTs performed, the number of positive tests observed, respectively, for the 6 venom concentrations (0–5) was 0, 10, 8, 7, 1 and 2 at T0 and 4, 7, 8, 7, 2 and 0 at T21w. The difference was not significant.

Specific IgE

The mean sIgE levels (n = 24) measured at T0 (12.7 ± 26.9 kUA/l) and at T21w (19.2 ± 27.4 kUA/l) were not significantly different.

Basophil Activation

Only patients who were tested at least at T0 and T21w were included in the analysis.

Group 1. Compared to T0, at all time points, mean AUC values were not significantly different (n = 29; fig. 1a).

Group 2. At all time points, differences in mean AUC values were not significant (n = 7; fig. 1a).

Group 3. The anti-IgE control was negative (<15% CD63) in 11 cases, whereas the anti-RiGE was negative in only 3 cases, 3/3 showing a negative response to the anti-IgE. Of these 11 tests, 8 showed a negative (<15% CD63) venom-induced basophil response for all the venom dilutions tested.

Compared to T0 ($3,328 \pm 4,412$), a significant increase in mean AUC (fig. 1a) was observed at T10w ($10,255 \pm 5,723$, p = 0.003) and T21w ($11,178 \pm 7,306$, p = 0.004). The anti-IgE responses were, respectively, for T0, T1w, T10w and T21w, $4.5 \pm 6.1\%$, $18 \pm 11\%$, $43 \pm 22\%$ (p < 0.001) and $46 \pm 32\%$ (p < 0.01). Only 2/11 anti-IgE responses were <15% at T21w.

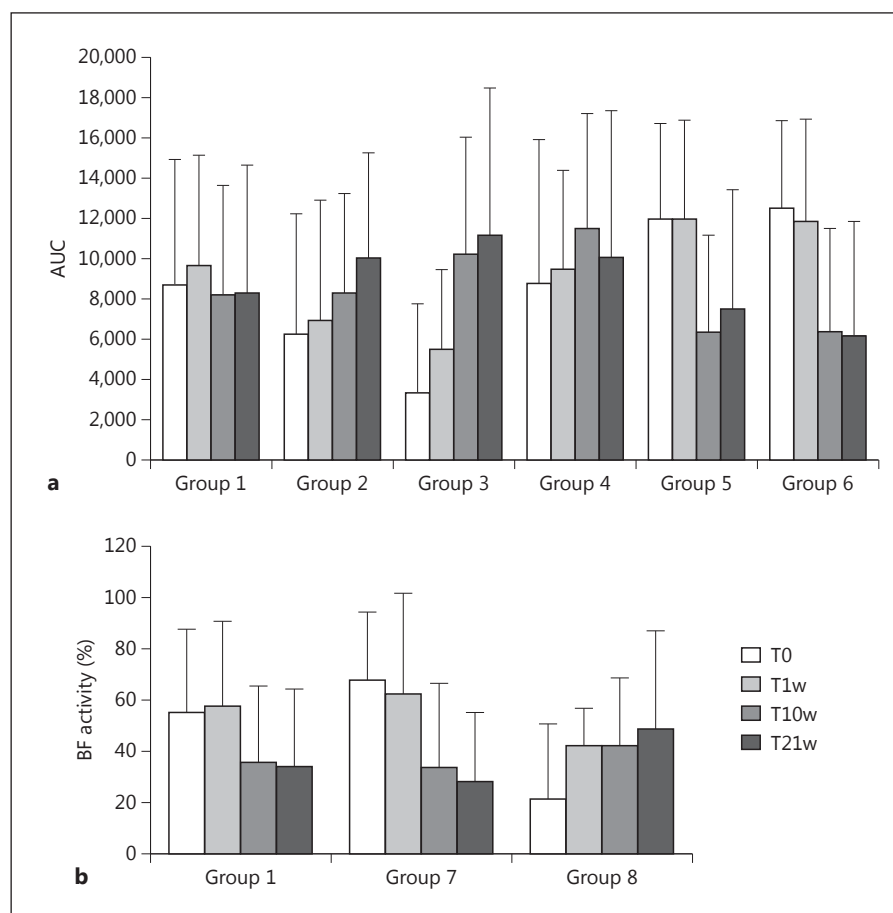
The highest venom concentration (250 ng/ml) induced a weak but positive activation (between 5 and 15% CD63) in 5/11 cases and a negative response (<5%) in 3/11 cases; in these 3 cases, the anti-RiGE induced a weak (2/3) or normal (1/3) response.

Group 4. At all time points, differences in AUC values were not significant (n = 9; fig. 1a).

Group 5. Compared to T0 ($12,051 \pm 4,658$), the mean AUC decrease was significant at T10w and T21w ($6,391 \pm 4,765$, p = 0.009 and $7,529 \pm 5,883$, p = 0.009), respectively (n = 18, fig. 1a).

Group 6. Compared to T0 ($12,514 \pm 4,332$), the mean AUC decrease observed at T10w and T21w, respectively, was significant ($6,380 \pm 5,115$, p = 0.008 and $6,206 \pm 5,619$, p = 0.036; fig. 1a).

Fig. 1. a Evolution of basophil activation expressed in AUC related to group 1 (n.s.), group 2 (n.s.), group 3 ($p = 0.003$ at T10w and $p = 0.003$ at T21w), group 4 (n.s.), group 5 ($p = 0.009$ at T10w and $p = 0.009$ at T21w) and group 6 ($p = 0.008$ at T10w and $p = 0.036$ at T21w) and measured at T0, T1w, T10w and T21w. **b** Evolution of BF activity measured for 1:12 serum dilution expressed in % CD63 versus the 100% negative serum control for groups 1, 7 and 8. The BF activity increase was significant for group 7 at T10w ($p = 0.008$) and T21w ($p = 0.002$). Results were expressed as mean $\pm$ SD.



Blocking Factors

BF activity was calculated for the 1:4 and 1:12 serum dilution and for groups 1, 7 and 8 ($n = 7$).

At all time points, the variation in serum blocking activity was not significant for the 1:4 dilution for 2 of the groups tested, but was significant when compared to T0 ($67.7 \pm 26.3\%$) for group 7 and for the 1:12 dilution at T10w (30.7 ± 32.6 , $p = 0.008$) and at T21w ($27.8 \pm 26.8\%$, $p = 0.002$), respectively (fig. 1b).

Protective Index

PI values were calculated for group 5. At T0, mean PI was 14.7 ± 16.7 leading to a threshold, set at mean ± 3 SD, of 64.8. Compared to T0, mean PI related to T21w was not significantly different (68.9 ± 99.4). 0/25 PI values at T0 and 4/18 at T21w were ≥ 64.8 .

VIT Follow-Up of Subgroup of Group 2 Patients

The evolution of the different parameters related to the 15 patients tested at T3–5y are described in tables 1 and 2.

Intracutaneous Testing

Compared to T0, ICT results observed at T3–5y were significantly different ($p = 0.009$).

Specific IgEs

Mean sIgE levels measured at T0 and T3–5y were, respectively, 5.1 ± 6.7 kUA/l and 3.3 ± 3.8 kUA/l. Mean sIgE levels observed were not significantly different from mean sIgE levels observed at T0.

Basophil Activation Test

Compared to T0 (AUC $9,832 \pm 11,300$), VIT induced a significant decrease in basophil reactivity at T3–5y (AUC $5,168 \pm 5,175$; $p = 0.047$).

Blocking Factors

Compared to T0 ($48.2 \pm 31.1\%$), BF activity observed at T3–5y was significantly different for the 1:12 serum dilution ($7.8 \pm 5.4\%$; $p = 0.013$).

Table 1. Main data related to the 15 patients tested at T0, T21w and at T3–5y

Patient	Previous VIT	Culprit insect	T0				T21w					T3–5y					Re- stung	Clini- cal reac- tion	
			AUC	ICT	sIgE	BF, %	PI	AUC	ICT	sIgE	BF, %	IP	AUC	ICT	sIgE	BF, %			IP
1		wasp	8,074	3	4	94.6	0.9	19,264	2	22	6.1	15.7	10,500	1	5.8	12.1	67	no	
2		wasp	4,413	2	1.5	64.6	25.8	8,766	2	3.1	78.7	7.8	2,700	2	0.7	15.3	101.2	no	
3		wasp	301	2	2.7			8,445	2	24	13.2	33.1	10,300	1	4.8	3.4	75	no	
4		wasp	401	1	0.7			5,650	1	9.4			100	0	0.8	6.5	18,700	yes	no
5		wasp	8,252	2	1.2	56.1	17.2	1,770	3				100	0	0.3	4.2	7,664	no	
6		hornet	4,554	1	0.9			20,209	1	3.1	51.1	19.4	11,900	0	3.5	5	160	yes	no
7		bee	19,000	3	11.6	98.10	0.13	2,400	3	14.9	4.6	51	780	1	2	8	943.6	yes	no
8		wasp	18,600	1	2.3	24.2	32.6	4,700	1	2.3			100	0	0.9	16	16,800	no	
9	yes	wasp	10,707	3	3.7	0.9	11.9	11,793	4	5.7	87.8	0.5	4,200	3	2.5	0.1	29.7	no	
10		bee	15,000	5	24	72	0.4	2,000	2	10.3			1,095	0	1.9	0.6	726.2	yes (twice)	no
11		hornet	12,432	1	0.3	55.8	28.4	100	1	0.2			100	0	0.1	18	6,560	yes	no
12		wasp	11,600	1	3	59.6	27.9	10,500	1	6.9	23	58.7	8,600	2	5.2	6.3	35.1	no	
13		wasp	16,217	3	15.5	6.7	7.4	8,167	0	29			12,900	0	15.2	9.2	140.8	no	
14	yes	hornet	19,000	3	4.4		6.8	18,100	3	20.2	64.4	2.5	12,100	2	4.4	8	24.5	no	
15		wasp	16,031	2	0.7			3,159	1	0.9	7.1	235.3	2,050	0	0.8	4.7	929.8	yes (twice)	no

Protective Index

Compared to T0, mean PI value calculated at T3–5y was significantly different (22 ± 19.8 and $3,430 \pm 6,262$; $p < 0.001$)

Applying the cut-off calculated above (64.8), no PI above the cut-off was observed at T0, 1/9 was observed at T21w and 12/15 were observed at T3–5y.

Based on these results, we suggest the following decision be applied at T3–5y:

- ICTs positive for a venom concentration ≤ 1 ng/ml or observation of a systemic reaction after a Hymenoptera sting: continue VIT.
- ICTs positive for a venom concentration ≥ 10 ng/ml: calculate the PI (if the anti-IgE-positive or anti-RIgE-positive controls are $>15\%$ CD63).

Patients Restung after VIT Discontinuation

Within the group of 15 patients tested at T0 and at T3–5y, 6 had been restung (2 of them twice) without any local/regional or systemic reaction. The 2 patients treated after a previous unsuccessful VIT were not restung.

The 2 patients who had previously been treated unsuccessfully by VIT and tested at T3–5y had a low PI at T3–5y (29.7 and 24.5) associated with high skin and basophil reactivity (table 1). As they were not restung, it was impossible to correlate the calculated PI values with the clinical data.

Table 2. Evolution of the different parameters studied related to the subgroup of 22 patients tested at T0, T21w and T3–5y

Parameter	Time point	Mean	SD	Statistical significance/T0
ICT	T0	2.2	1.15	
	T21w	1.8	1.08	n.s.
	T3–5y	0.8	1.01	0.009
sIgE	T0	5.09	6.7	
	T21w	10.9	9.5	n.s.
	T3–5y	3.3	3.8	n.s.
BAT (AUC)	T0	10,972	6,462	
	T21w	8,334	6,579	n.s.
	T3–5y	5,168	5,175	0.047
BF	T0	48.2	31.1	
	T21w	37.3	33.4	n.s.
	T3–5y	7.8	5.4	0.013
PI	T0	22	19.8	
	T21w	47.1	73.5	n.s.
	T3–5y	3,430	6,262	<0.001

n.s. = Not significant.

Discussion

BAT results are usually denoted as a percentage of cells expressing a high density of the chosen activation membrane marker (here, CD63). As the shape of the activation

curve and its maximum may vary from one patient to another, results related to one given allergen concentration may not be relevant to basophil reactivity. In contrast, results expressed as sensitivity (allergen concentration related to 30 or 50% maximum response) [4, 27] or, here, in AUC, bypass this difficulty. AUC, taking into account all the allergen dilutions tested and the shape of the activation curve, is applicable even for low responses and is, in our experience and that of other authors [28], more relevant to basophil reactivity and less patient-dependent than basophil reactivity related to one given allergen concentration. The venom concentrations used here (250 µg/ml to 9 ng/ml) were in line with those used by other authors [5, 29].

The follow-up of VIT from T0 to T21w showed that ICT and sIgE measurements were not significantly different at T21w compared to T0, in contrast to BF activity, already significantly higher at T10w. BAT variation was not significant for the whole population (group 1) but became significant after the exclusion of the low anti-IgE or anti-RIgE responders (group 5).

These results confirmed, as already described [26], that a negative/poor anti-IgE response may signify that a negative/poor allergen-induced response may falsely underestimate a patient's immunological status.

We have no explanation or hypothesis concerning the increase in anti-IgE-induced basophil reactivity observed during VIT follow-up (only 2/11 anti-IgE responses <15% at T21w, and the related venom-induced responses were also <15%), other than a possible upregulation of IgE receptors on the basophil membrane, induced by VIT or stimulation of the kinase-mediated pathways which are involved in nonreleasers [29]. The hypothesis of drug interference is not relevant as the samples were taken after strict drug avoidance.

Patients stung by a hornet and treated with wasp venom (group 4) showed, in contrast to group 5, no variation of the mean AUC at T21w. The number of patients included in the follow-up at 3–5y was too low for drawing conclusions. The use of wasp venom for VIT of patients allergic to hornet venom may induce different kinetics of BF synthesis from those induced in patients allergic to wasp venom and treated with wasp venom. These patients should, accordingly, be tested at later time points, after T21w.

The production by the immune system of specific BF is an early phenomenon as BF activity increased significantly after 10 weeks VIT. Moreover, increased BF activity was concomitant with a significant decrease in BAT, at least after having excluded the low anti-IgE and anti-RIgE responses.

The follow-up of the subgroup of patients showed that at T3–5y, ICT measurements decreased significantly, as did basophil activity, whereas BF activity increased significantly and sIgE variation was not significant. These results led us to propose the calculation of a PI by means of an algorithm, incorporating the 3 parameters which, in vivo and in vitro, reflected the variation of the immunological status induced by VIT.

Mean PI values increased significantly at T3–5y and were not significant at T21w.

Concerning VIT-induced basophil reactivity inhibition, an interesting putative mechanism could be the upregulation of histamine receptor H2 (H2R) which induces a potent suppression of mediator release including cytokines, and is observed as early as in the first 6 h of the build-up phase of rush VIT [30].

The kinetics of basophil reactivity suppression have also been extensively studied [24, 31]. Jutel et al. [9] observed that basophil response significantly decreased after the build-up phase of ultra-rush VIT, suggesting an early immunological tolerance status. They also showed that serum BF appeared from the start of the maintenance phase.

A rise of surface CD63 expression has been observed during VIT, indicating in vivo basophil activation with the probable release of preformed mediators (including IL4) which may also induce an upregulation of membrane markers [32].

Mikkelsen et al. [33] studied *Vespula* venom-induced basophil reactivity during the VIT build-up phase of 7–11 weeks, and an increase in basophil reactivity was observed at week 3 with a return to initial baseline at week 7. No significant variation in blood basophil concentration during the duration of the study was observed.

Which factors may influence basophil reactivity? The density of low/high-affinity receptors for IgEs or the density of bound/ratio-specific/total IgEs? IgE receptor density on the basophil membrane is known to be upregulated by total IgE concentration [34]. Other factors such as the Fc affinity for its receptor and Fab affinity for the specific allergen may also exert a major influence, but we lack data in these fields.

An interesting study [25] based on the use of the test devised by Maunsell [24] (study of BF by ICTs) has shown that during venom rush VIT, skin reactivity and sIgEs decreased significantly at 5 years whereas sIgG4 increased with a tendency to decrease after 3 years VIT. The authors showed that a natural blocking activity was observed in 28.6% of the tested patients but that this activity increased during VIT. The majority of the treated patients retained

skin reactivity at 5 years for 100 ng/ml venom (7/15 in our study).

The inhibitory effect of the BF may be mediated by antibodies (sIgG, sIgG4 or sIgA) and several authors have demonstrated that IgG or IgG4 contained in sera taken from patients under VIT showed an inhibitory activity on the basophil response [35].

Interestingly, an ex vivo study of basophil membrane markers [32, 36] showed that several markers such as CD63 increased during rush VIT but had also significantly decreased 1 week after the end of rush VIT.

Suppression of other functions by VIT has been pointed out as being due to the secretion of IL4 and IL13 [37] and the side effects observed during VIT proved to be correlated with higher allergen basophil-induced IL4 and IL3 secretion.

VIT induces a progressive reduction of sIgEs (not significant here) over the usual 3- to 5-year interval and increase in sIgG, sIgG4 or sIgA values have been regarded as being responsible for a decrease in cutaneous mast cell and circulating basophil reactivity [14].

The two main mechanisms involved in BF activity are allergen immune-capture and the coaggregation of high-affinity IgG and IgA receptors with high-affinity IgE receptors being expressed on basophil membrane.

Low-affinity receptors for IgG (FcγIIRA and FcγIIRB) are represented on the human basophil membrane. Stimulation of ITIM motifs results in an inhibition of IgE-dependent activation [38]. Coaggregation of IgE and IgG or IgA receptors results in an inhibition of human basophil activation involving SHIP-1 [39, 40]. In this study, an absence of variation of BF activity was observed for the 7 patients for whom a previous VIT had been ineffective (fig. 1b), but the increase in BF activity in group 6 was significant and favored the involvement of these BF in the VIT immunological mechanism.

Different attempts to include both in vivo and in vitro parameters within the calculation of an index for the follow-up of VIT have not been successful so far. The main parameters to have been studied are the ratio of sIgEs/total IgEs, the negativity of both ICT and sIgE values and the rise of Th1 cytokines.

Haye and Dosen [31] showed that 22% of patients treated with wasp venom experienced reactions after 5 years and ICTs became negative in 65% of the cases. Golden et al. [41] showed that half of the patients who had systemic reactions to a sting after stopping VIT had a history of a systemic reaction occurring during VIT (to an injection or a sting).

In summary, the 21-week follow-up of patients who had experienced a systemic reaction after a wasp, bee or hornet sting showed that, in contrast to ICT values and sIgEs, a significant decrease in basophil reactivity was observed after 10 weeks VIT paralleled by an increase in serum blocking activity. The results observed in the different subgroups underline the importance of the anti-IgE control, with a poor or negative basophil response being associated with a risk of false negativity of the allergen-induced basophil activation.

The effect of VIT was evaluated by a 'protective index' which included measuring the allergen-induced reactivity of the 2 main cells involved in the anaphylactic reaction, i.e. skin mast cells (by ICTs), circulating basophils (by BAT), and also the activity of circulating BF. Our hypothesis is that these antibodies may play a major role in VIT efficacy. Compared to T0, and in spite of a significant variation of the biological parameters (basophil reactivity and BF activity), the mean PI measured at T21w was not significantly different. However, the follow-up of a subgroup of patients continuing to receive VIT showed that a significant PI increase was observed at T3–5y due to a significant variation of all 3 parameters included in the PI calculation. This clinico-biological parameter may represent a new tool for the follow-up of VIT efficacy, but it will need to be evaluated in larger populations and in patients who experience a post-VIT reaction, as all of the patients in this study who were restung were clinically anergic after 3–5 years of VIT.

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