

Basophil Sensitivity in Patients Not Responding to Venom Immunotherapy

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Key Words

Venom immunotherapy • Treatment failure • Basophil sensitivity • CD63

Abstract

Background: Treatment failure of venom immunotherapy (VIT) is not rare and the risk and pathogenic factors for those failures are so far poorly understood. For that reason we evaluated allergen-specific basophil sensitivity in patients who did not tolerate field re-stings after completed VIT treatment. **Methods:** Basophil responsiveness was evaluated by flow cytometry analyses of basophil CD63 surface expression induced by different concentrations of bee or wasp venom (1, 0.1 and 0.01 $\mu\text{g/ml}$) in 14 treated patients who had experienced systemic allergic reactions (Muller grades II–III) and 17 treated patients who had no reactions after the field re-stings. We also included a group of 28 Hymenoptera venom-allergic patients who had not received VIT. **Results:** In 14 patients who still reacted to bee or wasp sting, basophil response at a venom concentration of 0.1 $\mu\text{g/ml}$ was significantly higher than in patients who tolerated field re-stings ($p = 0.03$; t test). Basophil response was also slightly higher at a concentration of 1 $\mu\text{g/ml}$, but not to statistical significance ($p = 0.12$; t test). There was no difference in the response to direct cross-linking of the IgE and in venom-specific IgE and IgG4 serum concentrations between those 2 groups ($p > 0.8$; Fisher's exact test, t test). Patients who tolerated field re-stings have also significantly lower basophil response in comparison to patients who had not received VIT,

both at 0.1 and 1 $\mu\text{g/ml}$ of venom concentrations ($p < 0.001$; t test). **Conclusions:** The results suggest that basophil venom-specific sensitivity is associated with the efficiency of VIT.

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Venom immunotherapy (VIT) is an efficient and the only viable option for prophylaxis in patients allergic to Hymenoptera venom [1]. However, not all patients are protected against re-stings after completed VIT. The percentage of those patients varies between different studies, which seems to be related to patient selection criteria, different treatment protocols and observation periods [2–9]. Haugaard et al. [8] reported no systemic reaction after 28 sting challenges in 25 adults who had received VIT for a mean period of 42.8 months. In another study, systemic reactions after a sting challenge occurred only in 2% of patients who had mostly received 4–6 years of VIT [9]. However, a higher incidence of systemic reaction was observed by van Halteren et al. [4], with an overall relapse rate of 8% after a sting challenge in patients with VIT given for a median duration of 40 months and by Lerch and Müller [2], where 7.5% of the patients exhibited re-sting reactions. In the study by Lang et al. [7], field stings up to 13 years after completion of at least 3 years of VIT resulted in an overall relapse rate of 14.8%, and Golden et al. [6] reported systemic reaction in 14% of patients after discontinuation of VIT of at least 5 years.

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The pathogenic factors for VIT treatment failures are so far poorly understood and there is still no in vitro test to predict the success of VIT treatment. At the moment, a potentially harmful sting challenge is the only method available to evaluate the efficacy of VIT [10]. However, it remains unclear whether sting challenge can reliably predict a field sting reaction [2, 11]. Furthermore, the lack of a reaction to a single sting challenge has been shown to be of limited clinical significance, because a subsequent sting can still cause a systemic reaction [11].

Flow cytometry quantification of basophil activation by measurement of CD63 expression [12] is a very sensitive and specific cellular in vitro method for identification of Hymenoptera venom allergy [13–18]. As basophils play an important role in the pathogenesis of allergic anaphylactic reactions [19], we evaluated allergen-specific basophil response in patients who did not tolerate field stings after VIT treatment. Therefore, the aim of our study was to evaluate, by measuring CD63 surface expression as a marker of basophil activation, whether there is any correlation between basophil in vitro sensitivity to allergen-specific stimulation and the efficacy of VIT.

Materials and Methods

Study Subjects

Thirty-one patients (median age 56 years; age range 32–76; 16 women) who underwent 2–7 years of VIT within the past 4–15 years, with a history of at least 1 Hymenoptera sting after completion of VIT, were included in the study. We also included a group of 28 patients who did not receive VIT (median age 40 years; age range 20–72; 11 women) with a history of at least 1 systemic anaphylactic reaction of Muller grades II–IV. Measurement of specific immunoglobulin E (IgE) and IgG4 in serum was performed by CAP-FEIA (Pharmacia, Uppsala, Sweden). All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the study. The study was approved by the local ethics committee.

Basophil Activation Assay

A basophil activation test was performed by BD FastImmune (BD Biosciences, San Jose, Calif., USA) in patients after completion of VIT and known history of field re-sting responses, as described previously [18]. Briefly, whole blood (with heparin anticoagulant) was preincubated with basophil stimulation buffer with IL-3 containing a final concentration of 0.01, 0.1 or 1 µg/ml of honey bee or wasp venom (Hal Allergie, Düsseldorf, Germany), 0.55 µg/ml of anti-FcεRI mAb (Buehlmann Laboratories, Basel, Switzerland) or 2 µM fMLP (Sigma, St. Louis, Mo., USA) at 37°C for 15 min. Chilling on ice stopped degranulation and thereafter FITC-conjugated anti-CD63 mAb, PE-conjugated anti-CD123 mAb and PerCP-conjugated anti-HLA-DR mAb (BD Biosciences) were added and incubated for 20 min on ice. Finally, whole blood probes were lysed, washed, fixed and analyzed within 2 h

Table 1. Demographic data of 2 groups of patients after completed VIT

a Patients who experienced systemic allergic reactions (Muller grades II–III) after field re-stings

Pa-tient	Age	Sex	Culprit insect	Muller grade before VIT	Years on VIT	Number of field stings after VIT
1	66	M	wasp, hornet	IV	5.5	several (wasp)
2	52	F	wasp	IV	3	several ¹
3	54	F	wasp	IV	3	several
4	45	F	wasp, bee	III	5	1 (bee)
5	50	F	wasp	IV	5	5 at once
6	63	F	wasp	III	5	several
7	40	M	bee	III	2	3
8	51	F	bee	IV	6	1
9	69	F	bee	IV	2.5	1
10	34	M	bee	IV	5	1
11	49	F	wasp	IV	4	1
12	55	F	wasp	IV	7	1
13	57	F	wasp	III	3	7 at once, next time 1
14	74	F	bee	III	5	3 ²

b Patients without reactions after field re-stings

Pa-tient	Age	Sex	Culprit insect	Muller grade before VIT	Years on VIT	Number of field stings after VIT
1	68	F	bee	III	4.5	3
2	73	M	wasp, hornet	IV	5	several (wasp)
3	44	F	bee	IV	4	1
4	60	M	wasp, bee	III	5	1 (wasp)
5	53	F	wasp	III	4.5	several
6	60	F	bee	III	2	2 at once, next time 1
7	72	M	bee	IV	3	2
8	46	M	wasp	III	5	several
9	61	F	wasp	III	5	several
10	53	M	bee	IV	3	several
11	76	M	wasp	III	4	several
12	57	M	bee	III	3	3 or 4
13	60	M	bee	IV	3	several
14	47	M	wasp	IV	4	2
15	67	M	wasp	III	5	several
16	32	M	hornet	IV	6	2 at once (wasp)
17	52	M	bee	III	6	around 20

¹ This patient had no allergic reactions after the first few field stings.

² This patient had no allergic reactions after the first 2 field stings.

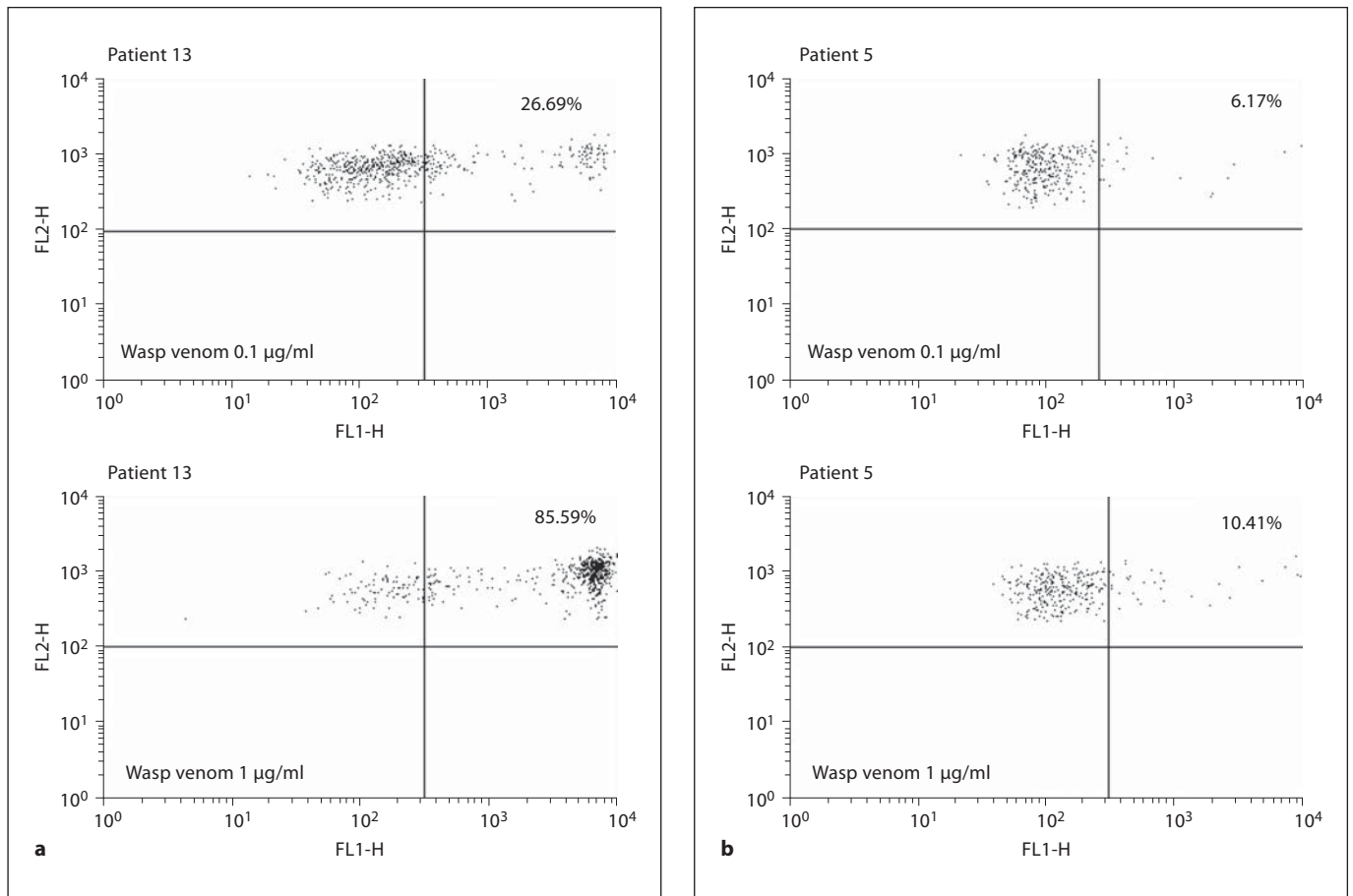


Fig. 1. Flow-cytometric analysis of basophil in vitro response to 0.1 or 1 $\mu\text{g/ml}$ of wasp venom in patients after completed VIT. Patient 13 (**a**) had experienced systemic allergic reactions of Muller grade III and patient 5 (**b**) had no reactions after the field re-sting. FL1 represents staining with anti-CD63 mAb and FL2 staining with anti-CD123 mAb.

on a FACSCalibur flow cytometer (BD Biosciences). Data were acquired with a threshold on FL2 set to eliminate most of CD123[−] cells, and at least 600 CD123⁺ cells per sample were acquired. Basophils were identified as low side scatter, CD123⁺ and HLA-DR[−] cells and the percentage of degranulated basophils was measured on FL1 (CD63).

Statistical Analyses

The distribution of data was analyzed by skewness and kurtosis, and the ratios to their standard errors. In those situations in which data showed severe nonnormality, a nonparametric test was used (Mann-Whitney test); otherwise, we performed unpaired *t* tests. Fisher's exact test was used to compare different categories of patients. Probability values of $p < 0.05$ were accepted as significant. Analyses were performed with SPSS (version 10.0) and GraphPad Prism (version 4.0). Data are presented as means with SEM in parentheses.

Results

Fourteen patients had experienced systemic allergic reactions of Muller grades II–III (table 1a) and 17 patients had no allergic reactions after the field re-stings with the respective Hymenoptera species (table 1b). The duration of VIT was comparable in both groups of patients (mean 4.4 and 4.2 years; table 1a and b). The interval between field re-sting and basophil assay measurements was comparable in both groups (mean 3.1 and 3.7 years). In 14 patients who still reacted systematically, basophil response at venom concentration of 0.1 $\mu\text{g/ml}$ was significantly higher [28.9% (7.2); $p = 0.034$; *t* test] than in patients who tolerated field re-stings [13.5% (2); table 2a and b; fig. 1a, 1b, 2]. Basophil response was also slightly higher at concentrations of 1 $\mu\text{g/ml}$ [60.6% (7.9) vs. 43.1%

Table 2. Basophil in vitro venom response determined by the percentage of surface CD63-expressing CD123+ HLA-DR+ cells and specific IgE results after completed VIT

a Patients who had experienced systemic allergic reactions (Muller grades II–III) after field re-stings

Patient	Venom (1 µg/ml) ¹	Venom (0.1 µg/ml) ¹	Venom (0.01 µg/ml) ¹	Specific IgE kU/l	VIT	Reaction after VIT
1	16.05	5.84	5.26	0.38	wasp	yes (II) ²
2	87.24	28.87	6.69	<0.35	wasp	yes (II)
3	19.22	10.85	13.57	<0.35	wasp	yes (III)
4	58.39	15.01	5.53	1.96	wasp, bee	yes (II) ³
5	79.78	26.06	6.24	0.64	wasp	yes (II)
6	94.39	64.96	7.73	<0.35	wasp	yes (II)
7	80.36	13.24	5.49	3.71	bee	yes (III)
8	34.48	9.06	3.03	1.93	bee	yes (II)
9	47.01	5.14	3.12	0.46	bee	yes (II)
10	93.79	94.37	69.55	0.88	bee	yes (II–III)
11	86.11	66.38	4.08	5.07	wasp	yes (III)
12	20.26	17.6	12.23	<0.35	wasp	yes (II)
13	85.59	26.69	5.13	<0.35	wasp	yes (III)
14	45.29	21.15	12.68	<0.35	bee	yes (III)

Figures in parentheses are Muller grades.

¹ In vitro stimulation was performed with the same venom as VIT.

² This patient was stung by wasps and not yet by a hornet after VIT. He had systemic allergic reactions after stings from both insects before VIT.

³ This patient was only stung by a bee after VIT and not yet by a wasp, therefore in vitro stimulation was performed by bee venom.

b Patients without reactions after field re-stings

Patient	Venom (1 µg/ml) ¹	Venom (0.1 µg/ml) ¹	Venom (0.01 µg/ml) ¹	Specific IgE kU/l	VIT	Reaction after VIT
1	94.54	31.71	15.15	5.96	bee	no
2	68.78	6.33	4.99	<0.35	wasp	no ²
3	42.29	13.06	7.52	<0.35	bee	no
4	7.01	6.21	6.75	<0.35	wasp, bee	no ³
5	10.41	6.17	5.18	0.67	wasp	no
6	5.26	7.46	2.55	1.28	bee	no
7	11.24	8.88	5.35	<0.35	bee	no
8	64.47	27.35	10.49	4.91	wasp	no
9	62.34	12.41	8.25	<0.35	wasp	no
10	11.06	9.66	9.51	0.68	bee	no
11	72.48	9.71	8.5	0.36	wasp	no
12	11.54	10.24	7.44	<0.35	bee	no
13	23.67	6.53	4.72	0.35	bee	no
14	58.27	25.1	8.87	<0.35	wasp	no
15	88.24	19.2	7.09	0.52	wasp	no
16	21.4	4.9	4.65	0.8	wasp	no ⁴
17	79.35	25.07	7.98	1.08	bee	no

¹ In vitro stimulation was performed with the same venom as VIT.

² This patient was stung by wasps and not yet by a hornet after VIT. He had systemic allergic reactions after stings from both insects before VIT.

³ This patient was only stung by a wasp after VIT and not yet by a bee, therefore in vitro stimulation was performed by wasp venom.

⁴ This patient was only stung by 2 wasps after VIT and not by a hornet, though the culprit insect was a hornet.

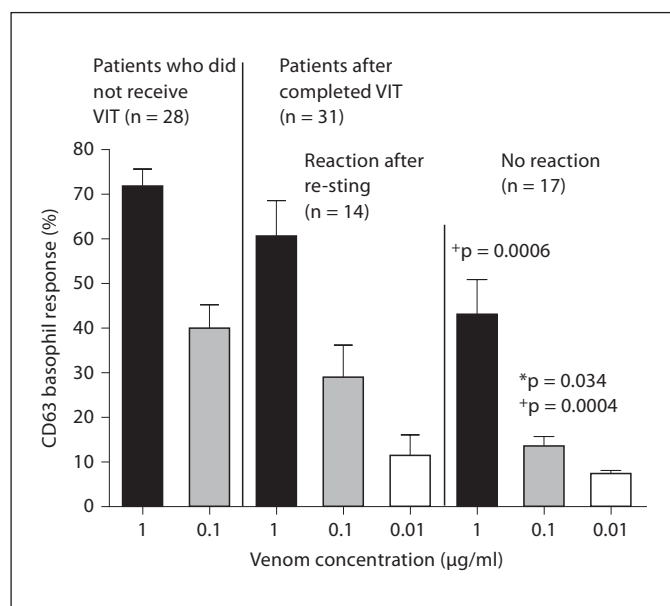


Fig. 2. Basophil in vitro responsiveness at 1, 0.1 and 0.01 µg/ml of venom in patients who did not receive VIT, and after completed VIT and with a history of field re-sting responses. * Significantly different from patients who still reacted to bee or wasp sting. + Significantly different from patients who had not received VIT.

(7.7)] and 0.01 µg/ml of venom [11.5% (4.6) vs. 7.4% (0.7)], but not of statistical significance ($p = 0.12$, $p = 0.76$; t test, Mann-Whitney test; table 2a and b; fig. 1a, 1b, 2). Six of 17 patients who tolerated field re-stings had negative CD63 response (cut-off of 15%) [15, 18] for all tested venom concentrations in comparison to the group who still reacted systematically, where all 14 patients showed positive CD63 response ($p = 0.021$; Fisher's exact test; table 2a and b). There was no difference in venom-specific IgE and IgG4 serum concentrations between those 2 groups of patients [6 of 14 and 8 of 17 patients had negative venom sIgE (<0.35 kU/l); $p = 1$; Fisher's exact test; IgG4; 1,544 µg/l (483), 1,409 µg/l (334); $p = 0.81$; t test; fig. 3a and b; table 2a, 2b]. Patients who tolerated field re-stings after completed VIT had significantly lower basophil response in comparison to Hymenoptera venom-allergic patients who had not received VIT [39.9% (5.2) at 0.1 µg/ml; 71.8% at 1 µg/ml; $p < 0.001$; t test], which was not the case for patients who still reacted systematically ($p > 0.16$; fig. 2). There was no significant difference in the response to stimulation buffer alone [4.9% (0.4); 4.6% (0.4); 3.4% (0.3)], to anti-FcεRI mAb [81.7% (3.8); 81.1% (5); 77% (3.8)] or to stimulation with fMLP [51.4% (5.1); 61.1% (4.5); 45.8%

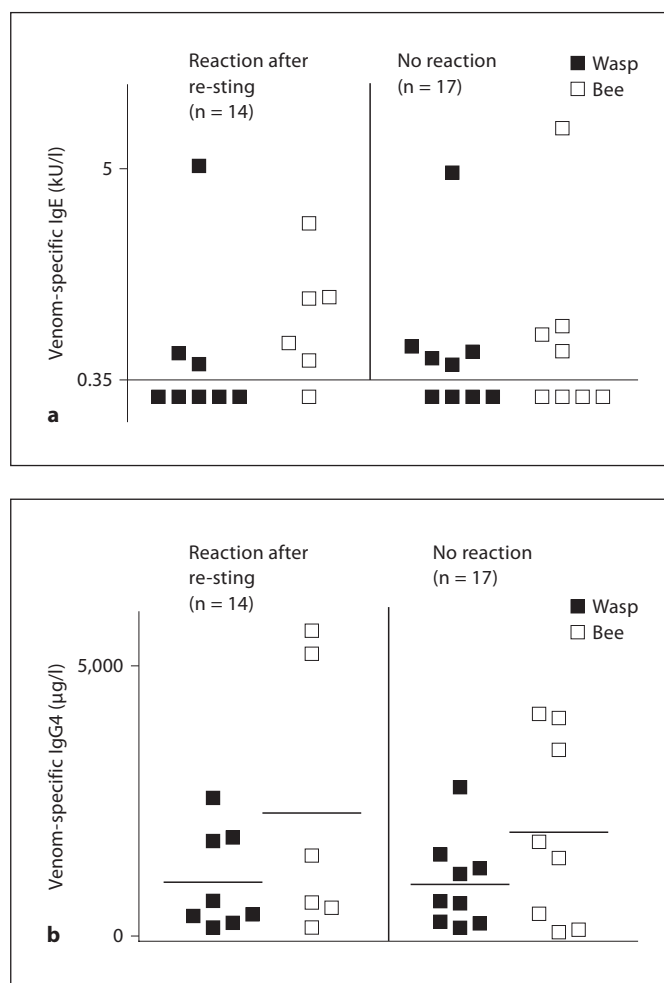


Fig. 3. Venom-specific IgE (a) and IgG4 (b) serum concentrations in patients after completed VIT and with a history of field re-sting responses. The horizontal solid bars indicate the mean value for each group.

(3.5)] between groups with completed VIT (tolerating field re-stings or still reacting systematically) and patients who had not received VIT (data not shown).

Discussion

Our study was the first to evaluate the basophil responsiveness in patients with completed VIT and a subsequent history of field re-sting reactions. We demonstrated significantly different basophil in vitro venom responsiveness between patients who still reacted and patients who tolerated field re-stings. Moreover, we also

demonstrated that a parallel group of patients who had not received VIT had a comparable basophil venom responsiveness in comparison to reactors and a significantly higher responsiveness in comparison to nonreactors after completed VIT.

In patients who still reacted systematically we found markedly increased basophil responsiveness at submaximal venom concentrations (0.1 µg/ml) in comparison to protected patients; however, both groups still showed comparable positive basophil venom response to stimulation with higher (maximal) venom concentration (1 µg/ml). Those allergen concentrations were carefully selected and tested [18, 20]. Similar to our findings, Ebo et al. [17] also demonstrated a significantly decreased CD63 expression for submaximal venom stimulation, but just in patients after 6 months and/or 3 years of VIT treatment. Both observations suggest the importance of basophil responsiveness for successful VIT treatment, which could be manifested by changes in basophil sensitivity to allergen-specific in vitro stimulation, but not by basophil in vitro venom maximal response (at top of dose response) [15]. MacGlashan [21] showed that basophil sensitivity is an independent intrinsic property connected to the intracellular signaling elements, which does not correlate with maximal response. Evaluation of basophil sensitivity is also a relevant tool for identification of patients at higher risk for adverse reaction during VIT [18].

Efficacy of VIT was assessed by the history of field reactions after the re-sting of the respective Hymenoptera species. To avoid, as much as possible, a subjective influence on the interpretation of the re-sting data, we selected only patients with a clear history of significant field reactions of at least Muller grade II. Nevertheless, systemic reactions [7] and even fatalities [22] have been reported in subjects following previously tolerated field re-stings. This was also the case in 2 of our 14 patients with treatment failure, which also experienced systemic reactions after previously tolerated field re-stings from the respective insect. It has also been assumed that VIT can be stopped if the venom skin test or specific IgE result becomes negative, although this occurs in a minority of patients within the first 6 years of treatment [4, 23]. We could not confirm this assumption for specific IgE, as we found no correlation between the venom-specific IgE serum concentrations and the outcome of the field sting, which agrees with previous reports [2, 24].

In conclusion, the results suggest that higher basophil venom-specific sensitivity is associated with the VIT treatment failure and that the dose response-dependent basophil activation test might be a helpful tool for identification of patients at higher risk for systemic allergic reactions after completed VIT.

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