

Flow Cytometry Versus Histamine Release Analysis of In Vitro Basophil Degranulation in Allergy to Hymenoptera Venom

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Background: Flow cytometry (FCM) has been proposed for specific allergy in vitro testing. We investigated its biological significance for allergy to Hymenoptera venoms and compared it with the routinely performed basophil histamine release test (HRT).

Methods: Blood samples from 26 allergic and 8 nonallergic donors were incubated with venom at serial concentrations. Basophils were analyzed with anti-CD45-PE-Cyanin 5, Anti-IgE-FITC, and Anti-CD63-Phycoerythrin. HRT was measured by radioimmunoassay.

Results: FCM was as convenient as HRT for measuring basophil reactivity in at least 87% of allergic and 75% of nonallergic subjects. CD63 outer expression was specifically induced in 91% of releaser subjects (86% on HRT) and in 1 of 10 tests in nonallergic donors, or one of six tests (16% on HRT) in allergic patients tested with an irrelevant allergen. Both methods were concordant in 85.7% of the tests. The three discordant patients had low-grade reactions and borderline biological responses on FCM (n = 2) or HRT (n = 1).

Conclusions: The dynamic, physiologic significance of CD63, the dose–response curve, and dependency on ethylene-diaminetetra acetic acid suggested that both tests reflect the same mechanism. *Cytometry Part B (Clin. Cytometry) 52B:13–19, 2003.* © 2003 Wiley-Liss, Inc.

Key terms: basophils; Hymenoptera venom; honeybee; common wasp; *Vespula* spp. (yellow jacket); *Polistes* spp. (paper wasp); allergy; histamine release; CD63; flow cytometry

Allergic reactions to honeybee or wasp venom can be life threatening. Clinical data and skin tests usually lead to the diagnosis (1). However, skin tests may have insufficient specificity and can be dangerous. The presence of specific types of immunoglobulin E (IgE) in serum also indicates a sensitization but is not pathognomonic of the clinical risk (2).

Immediate hypersensitivity is characterized by the allergen-induced mast cell activation and immediate release of highly active biological compounds stored in vesicles (i.e., histamine) or rapidly produced (i.e., leukotrienes) (3,4). The amount of compounds that is released correlates with the severity of the reaction (5) and may be downregulated under desensitization (6). Thus a reliable and safe in vitro analysis of the capacity of basophils to release these compounds under specific challenges is very helpful in confirming the diagnostic and treatment evaluations.

Because mast cells are not available for in vitro analysis, their peripheral homologous counterparts (7), the peripheral blood polynuclear basophils, are the best candidates, although they are rare (0.33–1.35% of leukocytes, or

0.020–0.102 × 10⁹ basophils/L, in our experience). Like mast cells, basophil activation, through membrane IgE cross-linking, can be analyzed by their morphologic changes (4,8) or, more quantitatively, by measuring the release of active compounds. The histamine release test (HRT) (9) is the most frequently used method in routine diagnosis, especially for allergy to venom (10).

Flow cytometry (FCM), a recent, sensitive, and automated technique, allows quantitative analysis of single-cell immunologic analysis, and methods have been developed for basophil identification and activation analysis (11–13). Membrane IgE is the most restricted marker for basophils (14–16). CD63 is normally expressed on the inside of the vesicle membrane (8). Under membrane IgE cross-linking,

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Received 4 October 2001; Accepted 8 November 2002

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/cyto.b.10012

Table 1
Detailed In Vitro Analysis of Basophil Activation by Hymenoptera Venom: FCM Versus HRT in Nonallergic and Allergic Patients*

Allergy	Honeybee	<i>Vespula</i> ^a	<i>Polistes</i> ^b	Positive FCM	Positive HRT
None	4	5	1	0 (0%)	ND
A related allergen	3	1	2	1/6	1/6
The relevant allergen	7	15	0	21 (95%)	19 (86.4%)

*Patients were tested with related or relevant allergens. In two donors and four patients, basophils could not be activated by any method. FCM, flow cytometry; HRT, histamine release test; ND, not determined.

^aCommon wasp.

^bPaper wasp.

the fusion of the vesicle to the plasma membrane allows the expression of CD63 on the external membrane (17) that can be detected directly by FCM. Thus, FCM offers an attractive alternative to HRT for measuring allergen-specific immediate basophil activation (15–21) but remains to be validated for each type of allergen, other than dust mites (22), grass pollens (23,24), and food allergens (25) that are not required in the routine diagnosis, because the conventional tests are sufficient. A biological test is particularly necessary for allergens such as drugs or venom. A few antibiotics and muscle relaxants have been tested recently (26).

We compared an FCM test by using a triple-labeling (CD45, IgE, and CD63) method with the routinely performed HRT to assess the allergen-specific releasability of basophils in patients who had experienced reactions after Hymenoptera stings.

MATERIAL AND METHODS

Twenty-six patients (58% female and 42% male; age, (mean $\pm$ standard deviation): 39.5 ± 18.1 , age range: 6–67 years) were tested with the HRT and FCM techniques. All patients had experienced at least one severe reaction (Müller grades I–IV: general urticaria, Quincke edema, and hypotension) after stings from a honeybee ($n = 8$) or common wasp ($n = 26$; Table 1). The diagnosis of allergy was confirmed with a positive prick test to Hymenoptera venom (Stallergenes-Pasteur, Fresnes, France). All patients were tested with the relevant allergen, and five were tested (six tests) with a related allergen to which they were not allergic (no clinical history and negative prick test). Specific IgE (CAP-RAST, Pharmacia-Upjohn Diagnostics Ltd., Stockholm, Sweden) were most frequently positive (>0.35 kU/l) for the relevant allergen.

Eight healthy donors (age range: 25–58 years; four female and three male) who had no reported history of allergic reaction to bee or wasp stings were tested by the FCM method against the same allergens.

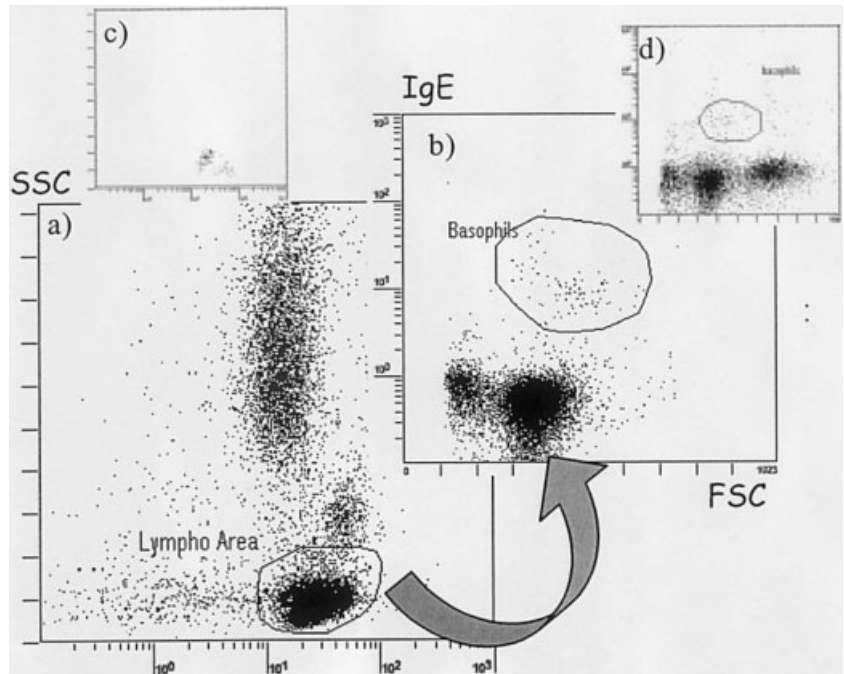
The capacity of basophils to be activated (termed *releasability* by Sanz et al. [22,27]) was evaluated through membrane IgE cross-linking by using a goat anti-human IgE antiserum (Nordic Immunology Ltd., Oslo, Norway) on the HRT or an anti-IgE receptor antibody (Buhlmann, DPC, Paris, France) on the FCM test. Venom from honeybee (*Apis mellifera*), common wasp (*Vespula* spp.), and paper wasp (*Polistes* spp.) were purchased from Staller-

gen SA (Fresnes, France), dialyzed against sterile saline (Spectra MWCO 1000, Spectrum Medical Industries, Houston, TX), and used at serial dilutions (0.5, 0.1, 0.05, and 0.01 μ g/ml at final concentration).

HRT was performed by incubating total heparinized blood, diluted 4:1, in a volume of Tris buffer (pH 7.2) supplemented with serum albumin (10 g/l) and CaCl_2 and MgCl_2 (2 mmol) for 40 min at 37°C with the buffer alone (Ho), the goat anti-human IgE antiserum, or the allergen (Hx). After centrifugation (10 min at 300g), the released histamine was measured in the supernatant by radioimmunoassay (Immunotech, Marseille, France). The results are given as the percentage of the total content of histamine obtained by complete cytolysis of the sample with distilled water (H100) and calculated as follows: $\text{Hx} = (\text{Hx} - \text{Ho})/(\text{H100} - \text{Ho}) \times 100$. The test was considered positive when more than 20% of the histamine was released.

The FCM test was performed on total heparinized blood, diluted 1:2 in sterile endotoxin-free phosphate buffer (0.1 M, pH 7.2), and incubated for 40 min at 37°C, with the buffer alone (Fo), the anti-IgE receptor antibody, or the allergen at the same serial concentrations (Fx). Samples were then labeled with an anti-CD45 phycoerythrin-Cy5 (clone T29/33, Dako, Copenhagen, Denmark), an anti-CD63 phycoerythrin (clone CLB-gran1/2, Caltag SA, South San Francisco, CA), and an anti-IgE fluorescein isothiocyanate (FITC; affinity-purified goat anti-human IgE ϵ chain, Biosource, Camarillo, CA) for 15 min at room temperature. Erythrocyte lysis and fixation were automated (Immunoprep, Q-Prep, Beckman Coulter SA, Hialeah, FL). Samples were analyzed on an EPICS XL, and data were acquired with EPICS System II software (Beckman Coulter SA). Basophils were selected electronically on an IgE/forward scatter dot plot restricted to a large lymphocyte area defined on a CD45/side scatter dot plot (Fig. 1). Immunodetection with specific antibodies were compared with isotypic controls and between activated and nonactivated samples. A minimum of 150 IgE positive events was considered (150–450, corresponding to 35–40,000 lymphocyte events). Results (F) are given as the percentage of identified basophils (Fx) that expressed CD63 above the nonstimulated values (Fo), calculated as follows: $F = \text{Fx} - \text{Fo}$. The positive threshold was defined as 5% above the nonstimulated values.

Fig. 1. **a:** A large lymphocyte gate was defined on a CD45/SSC dot plot. **b:** Basophils were selected on an IgE/FSC dot plot restricted to the lymphocyte area. **c:** The CD45/SSC dot plot gated on IgE⁺ cells shows basophils distributed throughout the lymphocyte area. **d:** The ungated IgE/FSC dot plot shows a less homogeneous IgE⁺ dot plot due to nonspecific staining of polynuclear neutrophils. FSC, forward scatter; IgE, immunoglobulin E; SSC, side scatter.



Results are expressed as mean $\pm$ one standard deviation. Statistical significance was tested with Yates' χ^2 test and Student's paired *t*-test.

RESULTS

Basophil Activation Analyzed by FCM

On FCM, basophils could be selected electronically by using simultaneous CD45/side scatter and anti-IgE/forward scatter plots, even under stimulation (as shown in Fig. 2a). The basophil population represented only 0.09–8.0% of the peripheral blood leukocytes and were located in lymphocyte bit map area defined by CD45/side scatter criteria. The membrane CD63 was not significantly expressed on resting basophils ($1.8 \pm 1.5\%$; Fig. 1c) despite the use of anti-IgE-FITC.

Wide Range of Basophil Releasability

Basophils from two (25%) healthy donors and four (13%) allergic patients could not be activated (non-releaser) by either FCM or HRT with membrane IgE cross-linking or the specific allergen.

In releaser patients, the anti-IgE receptor induced CD63 outer expression in $38.5 \pm 19.7\%$ of basophils, with a wide discrepancy (range: 7.9–75.7%; Fig. 2b). Similarly, on HRT, anti-IgE induced the release of $50.4 \pm 14.5\%$ of the total capacity of histamine (range: 15–86%).

Allergen-Induced Basophil Activation Analyzed by FCM

Specific allergens (0.5–0.01 $\mu\text{g}/\text{mL}$) induced CD63 expression in more than 5% of basophils in 20 (77%) patients (91% of the 22 “releasable” patients; Table 2). The CD63 response curve depended on the allergen concentration and often produced a bell-shape curve (Fig. 3) culminating

at $34.7 \pm 24.8\%$ of IgE⁺ basophils (range: 5.4–75.1%). The peak was obtained at an allergen concentration as low as 0.05 $\mu\text{g}/\text{mL}$ in five (22.7%) patients. The basophil activations induced by the allergen and the anti-IgE receptor

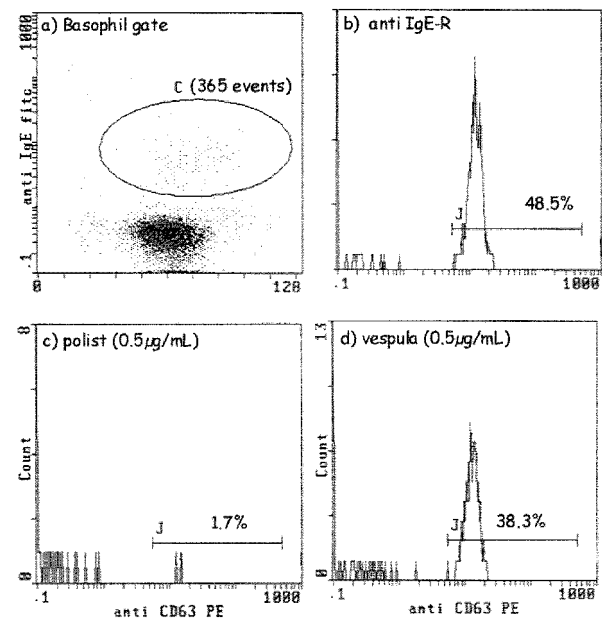


Fig. 2. One representative dot plot of Basophil activation measured by Flow Cytometry. Basophils were electronically selected on CD45/IgE (a). The basophil activation was measured on outer expression of CD63. The anti-IgE Receptor was used as a positive control (b). An extensive CD63 expression was observed following basophil incubation with the relevant allergen (d) and not a related allergen (c).

Table 2
In Vitro Analysis of Basophil Activation in 22 Releasers of 26 Patients Allergic to the Relevant Allergens*

	Anti-IgE receptor	Allergens ($\mu\text{g/ml}$)			
		0.5	0.1	0.05	0.01
% CD63 ⁺ basophils on FCM					
Mean $\pm$ S.D.	34.5 $\pm$ 22.1	30.5 $\pm$ 25.3	19.4 $\pm$ 18.6	16.5 $\pm$ 17.5	8.1 $\pm$ 11.5
Range	3–75	1–75	1–66	1–66.5	1–44.3
% Histamine released on HRT					
Mean $\pm$ S.D.	42.5 $\pm$ 24.1	47.8 $\pm$ 31.3	36.5 $\pm$ 28	27.3 $\pm$ 24.8	6.1 $\pm$ 7.9
Range	1–86	1–100	1–89	1–72	1–33

*Allergic patients were exposed to the venom of the honeybee, the *Vespula* wasp, or the *Polistes* wasp (0.01–0.5 $\mu\text{g/ml}$). The basophil activation was measured as the percentage of basophils that expressed CD63 on FCM or the percentage of total histamine released on the HRT. Histamine releasability was tested by cross-linking of membrane IgE with the anti-IgE receptor on FCM or anti-IgE on HRT. In four patients, basophils could not be activated by any method (IgE: 0.54–2.3 kU/l). The response depended on the allergen dose. FCM, flow cytometry; HRT, histamine release test; IgE, immunoglobulin E; S.D., standard deviation.

were completely inhibited by ethylene-diaminetetra-acetic acid (results not shown).

Specificity of Allergen-Induced Basophil Activation Analyzed by FCM

Specific allergens (0.5–0.01 $\mu\text{g/ml}$) induced outer expression of CD63 on fewer than 5% of basophils in 1 of 10 tests (90%) in healthy donors (Table 3). One donor (subject 6) had no reaction to Hymenoptera venom but appeared to have a serum-specific IgE (spIgE; 1.9 kU/L) reaction and a very high releasability by FCM (45.7%). Similarly, in one allergic patient (subject 15), the nonrelevant allergen induced an outer expression of CD63 (spIgE, 1.15 kU/L; FCM releasability, 70.3%) in one of six (17%) tests (Table 4).

Allergen-Induced Basophil Activation Analyzed by HRT

Specific allergen-induced histamine release was found in 19 (73%) patients (86.4% of the 22 releaser patients;

Table 2) and in one of six (16%) tests with a nonrelevant allergen (Table 4).

The fraction of released histamine depended on the allergen concentration (in a wide range: 0.5–0.01 $\mu\text{g/ml}$), often in a bell-shape curve, with a response peak at 0.1 $\mu\text{g/ml}$ ($n = 4$) or even 0.05 $\mu\text{g/ml}$ ($n = 4$). The largest fraction of induced histamine release was $46.7 \pm 30.8\%$ (range: 20–100%).

FCM and HRT Basophil Activation Tests Were Highly Concordant

Overall, 24 of 28 (85.7%) tests (with relevant or related allergens) were concordant on the FCM and HRT (Table 5). Patient 11, who was clearly allergic (clinical grade III reaction after a wasp sting, positive cutaneous tests, and presence of spIgE for *Vespula*), did not react on either basophil tests.

Discordances Between FCM and HRT

Four tests were discordant: patients 26 and 29 were positive for *Vespula* on FCM (5.4% and 7.5%, respectively) and spIgE (0.86 and 1.62 kU/L, respectively) and negative on HRT (0%). Both had positive cutaneous tests and low-grade clinical reactions (grade I). Patient 12 had a low-grade clinical reaction, suggesting an allergy to the common wasp (positive prick test, but spIgE < 0.35 kU/L, negative on FCM and HRT; Table 2). Patient 15 clearly was allergic to the common wasp (grade III clinical reaction, positive prick test, spIgE = 3.36 kU/L, FCM: 51.9%, HRT: 75%; Table 2) and had spIgE for the honeybee and positive FCM results but negative HRT results (negative prick test, spIgE = 1.15 kU/L, FCM: 8.9%, HRT: 2%; Table 4).

DISCUSSION

Our results confirmed that the quantification of basophil activation by FCM is reliable for the diagnosis of allergy to Hymenoptera venom. One advantage of the FCM method was that we could identify the type of cell that was activated with exposure to the allergen.

Because basophils are smaller than other polynuclear cells (i.e., neutrophil and eosinophil) and their granules have low light diffraction properties, they are not clearly distinguishable from lymphocytes by the physical criteria

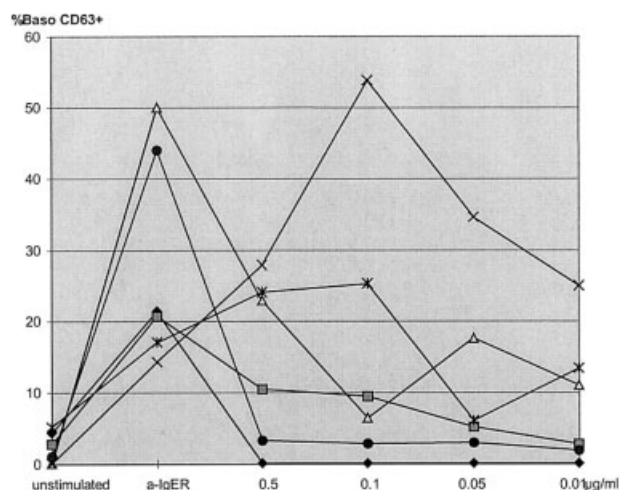


FIG. 3. Representative examples of dose-related responses of in vitro activation of basophils in several patients allergic to wasp venom. Anti-immunoglobulin E receptor (a-IgER) was used as a positive control and compared with the nonstimulated sample. The allergen was added at serial dilutions from 0.5 to 0.01 $\mu\text{g/ml}$, with final concentrations as indicated. Responses from three patients produced a bell-shape curve ($\times$, $*$, Δ); one had a mild response ($\square$) and two were negative ($\bullet$, $\blacklozenge$).

Table 3
In Vitro Analysis of Basophil Activation in Eight Subjects Not Allergic to the Tested Antigens*

Subject	Venom	splgE	% CD63 ⁺ basophils on FCM				
			Rest	Anti-IgE-R	0.5	0.1	0.05
1	Honeybee	ND	0.1	7	1.9	0.1	0.1
2	<i>Vespula</i>	ND	4.4	17	0.1	0.1	0.1
2	Honeybee	ND	"	"	0.1	0.1	0.1
3	<i>Vespula</i>	ND	1.4	15	2.8	2.6	3.9
4	<i>Vespula</i>	ND	2.2	15.2	0.1	0.1	0.1
5	<i>Vespula</i>	ND	0.1	19.9	0.1	0.1	0.1
5	Honeybee	ND	"	"	0.1	0.1	0.1
6	<i>Vespula</i>	1.9	0.7	45.7	6.2	6.8	4.1
6	<i>Polistes</i>	<0.35	"	"	1.5	0.3	0.1
6	Honeybee	<0.35	"	"	1.0	0.1	1.0
7	<i>Vespula</i>	<0.35	2.1	1.0	2.3	0.9	0.1
8	<i>Vespula</i>	ND	1.7	2.0	0.1	0.1	0.1

*Nonallergic subjects were exposed to venom from the honeybee and the *Polistes* and *Vespula* wasps (0.01–0.5 µg/ml). Sensitization was measured with splgE in (kU/l). Basophil activation was measured as the percentage of basophils that expressed CD63⁺ under stimulation on FCM. One donor (subject 6) had specific IgE and a borderline level of CD63⁺ basophils for one allergen on FCM without any clinical history of venom allergy. Anti-IgE-R, anti-immunoglobulin E receptor; FCM, flow cytometry; ND, not determined; splgE, serum-specific immunoglobulin E.

(size and heterogeneity) used with FCM. The use of CD45 helped to distinguish the lymphocytes and basophils from autofluorescent cells or contaminating red blood cells (15).

Basophils express multiple membrane markers (11,14), but none is really specific. The IgE receptor is expressed more strongly on basophils than on eosinophils or monocytes (28,29), especially in allergic patients (30,31) and is specific for IgE. Thus, like others (15–17), we believe that membrane IgE is the best marker available to identify basophils on FCM. One major technical problem was due to the rarity of basophils in some samples. We believe that at least 150 basophils should be analyzed to reliably detect more than 5% of activated basophils. Samples with low basophil counts cannot be considered useful unless most are activated, but that is rarely the case, specially with the allergen.

The basophil activation induced membrane expression of several activation markers or adhesion molecules involved in basophil migration toward the site of the allergy

(11,32–34). We chose CD63 because of its physiologic relevance (8,17), as confirmed by its very early kinetic, bell-shape dose curve and its calcium dependency as observed also on HRT (25,35).

Using total blood is more convenient for routine testing and it is routinely used for HRT. However, FCM require red blood cell lysing that is a crucial step and must be gentle to avoid changing basophils, which are very fragile, especially under activation.

We chose a 40-min challenge to closely resemble HRT conditions, but a shorter incubation time probably would be sufficient because the plateau of basophil degranulation is reached within 20 min (17,35). Interleukin-3 has been proposed to enhance the basophil response, but its benefits are questionable in such a short period (19,36,37).

We tested basophils with the same allergen as that commonly used for skin tests, except that we dialyzed it to remove any contaminating histamine that might interfere with basophil activation (38).

Table 4
In Vitro Analysis of Basophil Activation With Related Allergens in Five Subjects Allergic to Another Venom*

Subject	Venom	IgE	% CD63 ⁺ basophils on FCM					% Histamine released on HRT			
			Rest	Anti-IgE-R	0.5	0.1	0.05	Anti-IgE	0.5	0.1	0.05
11	<i>Polistes</i>	<0.35	1.2	14.6	0.1	0.1		7	2	1	1
12	<i>Vespula</i>	<0.35	1.1	42.9	2.2	1.8	1.9	41	29	7	4
13	Honeybee	1.01	0	14	0.4	0.5		15	1	0	1
13	<i>Polistes</i>	<0.35	"	14	3.5	1.6		"	0	0	0
14	Honeybee	<0.35	2.7	38	0.1	0.1	1.4	86	17	3	2
15	Honeybee	1.15	1.1	70.3	8.9	6.9	5.5	60	1	2	0

*Subjects were exposed to venom from the honeybee and the *Polistes* and *Vespula* wasps (0.01–0.5 µg/ml). Sensitization was measured with splgE (kU/l). Basophil activation was measured as the percentage of basophils that expressed CD63 on FCM or the percentage of total histamine released on the HRT. Histamine releasability was tested by cross-linking of membrane IgE with the use of anti-IgE-R on FCM or anti-IgE on HRT. Anti-IgE-R: anti-immunoglobulin E receptor; FCM, flow cytometry; HRT, histamine release test; splgE, serum-specific immunoglobulin E.

Table 5
*In Vitro Tests of Allergy to Hymenoptera Venom: FCM Versus HRT Results in 26 Tests From Subjects Allergic to the Relevant Allergen**

	FCM–	FCM+	Concordance
HRT–	1 + 4 ^a (subject 11)	2 + 1 ^a (subjects 26, 29, 15 ^a)	3 (13.6%)
HRT+	0 + 1 ^a (subject 12 ^a)	19	19 (86.4%)
Concordance	1 (4.5%)	21 (95.5%)	

*In four patients, basophils could not be activated by any method. Over all, both methods were concordant in 24 of 28 (85.7%) tests ($\chi^2 < 0.0008$). FCM, flow cytometry; HRT, histamine release test.

^aSix more tests were performed with related allergens.

The anti-IgE antibody we used for immunolabeling did not stimulate basophils as shown on nonstimulated samples, even when labeling was performed at room temperature and for only 15 min. Moreover, we simultaneously used an anti-CD45 that was reported to reduce basophil activation by anti-IgE (39). However, ethylene-diaminetetra-acetic acid can be added to the immunolabeling buffer to avoid nonspecific stimulation. The labeling could be performed on ice but for a longer incubation time.

Basophil releasability has been tested with formyl-methionine-leucine-phenylalanine, phorbol myristate acetate, calcium ionophore (ionomycin) (4,33,40) and cross-linking adhesion molecules (11,17,28). We chose membrane IgE cross-linking because it is the most physiologic way of activating basophils. However, because we used anti-IgE-FITC for the immunodetection of basophils, we chose an anti-IgE receptor as the positive control. Moreover, anti-IgE receptor should be less dependent on the serum level of IgE, especially when using total blood containing soluble IgE. Unsurprisingly, our results appeared to be close to the HRT positive control (anti-IgE).

Among patients, we observed a great variability of basophil releasability, and a few patients did not respond at all to either method without reducing the cellular pool of histamine (results not shown). Knoll et al. (17) found that this response could be due to a defect in signal transduction. However, labeling may compete with high levels of serum IgE or possibly the soluble form of the IgE receptor. The clinical significance of "releasability" remains to be elucidated. It was not detected with formyl-methionine-leucine-phenylalanine, thereby arguing against the use of such a nonphysiologic positive control.

We observed a wide discrepancy in the allergen-specific responses. The clinical significance remains to be elucidated, especially with regard to the prognostic value and the evaluation of desensitization procedures. Interestingly, we observed that specific response could be higher than the nonspecific (anti-IgE receptor) response, suggesting a possible alternative pathway (i.e., immunoglobulin G).

The FCM test was positive in one nonallergic donor who had a very high releasability, suggesting a high susceptibility (atopy?). Indeed, the membrane IgE density has been described to be increased in atopic patients (30). Nevertheless, an allergy to *Vespula* could not be excluded absolutely, despite the current lack of clinical event.

FCM proved to be a sensitive and reliable method for in vitro quantification of basophil-specific activation. Sensitivity and specificity were at least as good as histamine release. The FCM method has some technical advantages: the sample can be analyzed immediately after the test procedure, whereas for HRT the supernatant must be stored for the histamine radioimmunoassay; instead of measuring the quantity of histamine release as with HRT, the real proportion of activated basophils can be quantified, which excludes non specific histamine release from other sources such as platelets; and a smaller volume of blood sample is required, making it particularly suitable for infants.

ACKNOWLEDGMENTS

We thank Françoise Duplat for skillful technical assistance, Dr. Jean-Philippe Dubouis (C. H. S. Lons-Saulnier), Dr. Racle Martine (C. H. Annecy), Dr. Roch Sanchez, and Dr. Bruno Girodet (St Luc, and St Joseph Hospital, Lyon) for the clinical diagnosis.

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