

Review article

Flow-assisted allergy diagnosis: current applications and future perspectives

Physicians predominantly rely upon quantification of serum-specific immunoglobulin E (IgE) and/or skin test to confirm clinically suspected IgE-mediated allergy. However, for various reasons, identification of the offending allergen(s) and potentially cross-reactive structures is not always straightforward. Flow-assisted allergy diagnosis relies upon quantification of alterations in the expression of particular basophilic activation markers. Actually, upon challenge with a specific allergen, basophils not only secrete quantifiable bioactive mediators but also upregulate the expression of different markers which can be detected efficiently by flow cytometry using specific monoclonal antibodies. Currently, the technique has been applied in the investigation of IgE-mediated allergy caused by classical inhalant allergens, food, *Hevea* latex, hymenoptera venoms and drugs. It is also appreciated; the technique proves valuable in the diagnosis of non-IgE-mediated (anaphylactoid) reactions such drug hypersensitivity and the detection of autoantibodies in certain forms of chronic urticaria. This review will not address immunologic features, characteristics and general pitfalls of flow-assisted analysis of *in vitro*-activated basophils as summarized elsewhere. After a recapitulation of the principles and some specific technical issues of flow-assisted analysis of *in vitro*-activated basophils, we principally focus on the current clinical and research applications of the basophil activation tests. Personal experience of both research groups is provided, where appropriate. Finally, a viewpoint on how the field might evolve in the following years is provided.

**D. G. Ebo¹, J. Sainte-Laudy²,
C. H. Bridts¹, C. H. Mertens¹,
M. M. Hagendorens¹,
A. J. Schuerwegh¹, L. S. De Clerck¹,
W. J. Stevens¹**

¹Department of Immunology, Allergology, Rheumatology, University Antwerp (UA), Antwerpen, Belgium; ²Laboratoire d'Immunologie, CHU Dupuytren Limoges, France

Key words: allergy; CD203c; CD63; flow cytometry; review.

Prof. Dr. W. J. Stevens
Immunologie – Allergologie – Reumatologie
Universiteit Antwerpen
Campus Drie Eiken
Universiteitsplein 1
2610 Antwerpen (Wilrijk)
Belgium

Accepted for publication 29 November 2005

Allergy diagnosis generally relies upon an evocative clinical history and appropriate confirmatory tests such as quantification of serum-specific immunoglobulin E (IgE) antibodies, skin tests and eventually controlled challenge tests. However, for several reasons, correct identification of the causative allergen(s), is not always straightforward. This review will not address immunologic features, characteristics and general pitfalls of flow-assisted analysis of *in vitro*-activated basophils as summarized elsewhere (1–4).

Functional *in vitro* tests have generally focused on basophil mediator release assays such as histamine and leukotriene release tests (5, 6). However, as addressed in these reviews, different practical and technical shortcomings have restricted their validation and clinical application.

The discovery of CD63 as a basophil activation antigen constituted the start of the development of a flow cytometric technique to analyse and quantify allergen-specific *in vitro* activation of peripheral blood basophils. Based upon these observations, Sainte-Laudy et al. (7, 8) and subsequently Sabbah et al. (9, 10) developed a flow-

assisted basophil activation test (BAT) relying upon anti-IgE to characterize basophils and anti-CD63 to assess activation of these cells. Since, clinical and research applications of the BAT have increased rapidly.

Flow-assisted analysis of *in vitro*-activated basophils: principles

Flow cytometric analysis of *in vitro*-activated peripheral blood basophils rests upon quantification of phenotypic alterations, i.e. changes in expression of basophilic activation markers after challenge with a specific allergen. Electron microscopic studies have shown that basophil activation may follow two main pathways: (i) the anaphylactic degranulation (AND), which is characterized by quick morphological changes, exocytosis of the intracellular granules and release of pre-formed mediators and (ii) the piecemeal degranulation (PMD) characterized by slow morphological changes without exocytosis of the granules (11). Figure 1 summarizes the AND-type degranulation. Upon stimulation with specific allergens

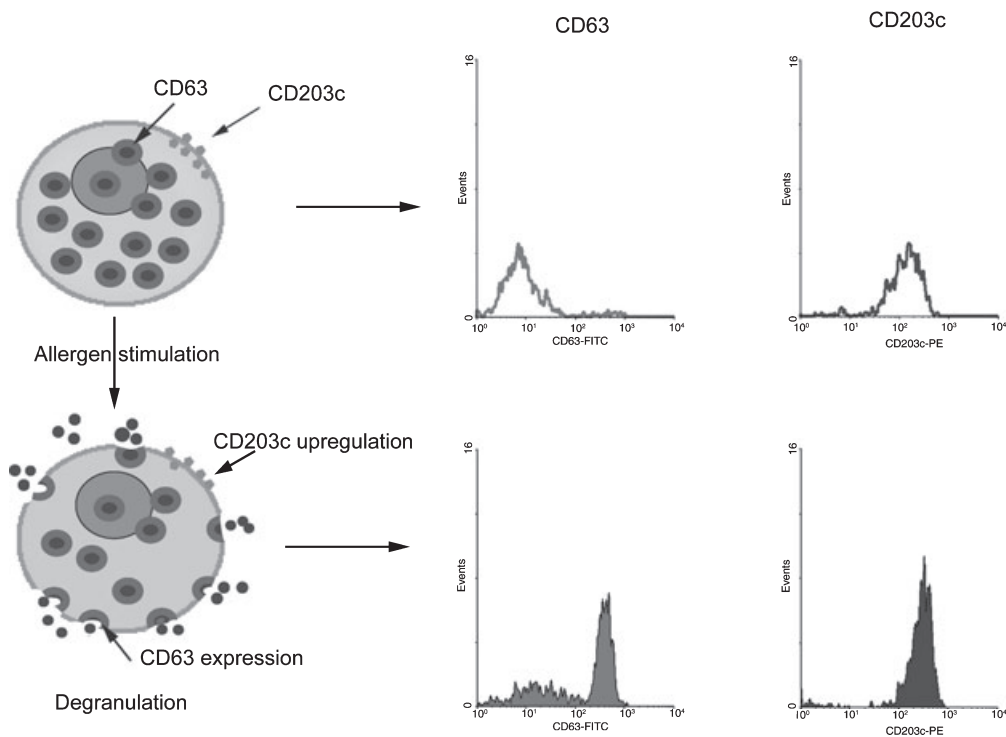


Figure 1. In resting basophils CD63 is mainly anchored to the intracellular granule and is barely detectable on the surface membrane (top panel, open histogram). On activated basophils (e.g. by allergen, anti-IgE and anti-FcεRI), as a result of the fusion between the granule and cell membrane, CD63 is expressed with high density (bottom panel, closed histogram). Although CD203c is constitutively expressed on basophils (top panel, open histogram), it can still be up-regulated in response to allergen-specific, anti-IgE and anti-FcεRI stimulation (bottom panel, closed histogram).

Table 1. CD63 and CD203c: general characteristics

	CD63	CD203c
Synonyms	Gp53 or lysosome-associated membrane protein (LAMP)-3	Neural cell surface differentiation antigen E-NPP3 (also: PD-Iβ, B10, gp130 ^{RB13-6})
(Super)family	Transmembrane-4 super family (tetraspanins)	Pyrophosphatase/phosphodiesterases (E-NPPs)
Resting basophils	Barely detectable	Constitutively present (+)
IgE-activated basophils	Upregulation starts within 10 min Expressed with a high density (at least 1 log scale)	Upregulation starts within 5 min and peaks within 10–15 min Upregulation less prominent when compared with CD63

that cross-link IgE bound to its high-affinity receptor (FcεRI), basophils not only secrete and generate a variety of quantifiable bioactive mediators, but also upregulate the expression of different activation markers [for review, see Refs (12, 13)]. These changes can be detected on a single-cell basis by multicolour flow cytometry using specific monoclonal antibodies. At present, the most commonly applied antigens in flow cytometric studies for basophilic activation are CD63 and CD203c.

The major characteristics of both activation markers are summarized in Table 1. CD63 [gp53 or lysosome-associated membrane protein (LAMP)-3] is a member of the transmembrane-4 superfamily and is expressed by different cell types, e.g. basophils, tissue mast cells, macrophages and platelets (14–16). In resting basophils, CD63 is anchored at the intracytoplasmic granules and is barely detectable on the outer surface membrane of

basophils and mast cells, both in healthy subjects and allergic patients. In contrast, as a result of the fusion of the granule with the outer cell membrane, CD63 is expressed with a high density on IgE-activated basophils (2, 17–28). The exact function of basophilic CD63 remains to be disentangled, but anti-CD63 antibodies have recently been demonstrated to inhibit adhesion of mast cells to the extracellular matrix proteins, fibronectin and vitronectin, and to inhibit IgE-dependent activation of adherent mast cells (29).

CD203c (neural cell surface differentiation antigen E-NPP3) (also: PD-Iβ, B10, gp130^{RB13-6}) belongs to the so-called ectonucleotide pyrophosphatase/phosphodiesterases (E-NPPs) multigene family that also comprises E-NPP1 (PC-1, PDNP-1) and E-NPP2 (PD-Iα, PDNP2, autotoxin) [for review, see (30)]. These type II transmembrane metalloenzymes have broad substrate specificity

and catalyse the cleavage of phosphosulphate and phosphodiester bonds of numerous molecules comprising deoxynucleotides and nucleotide sugars. In peripheral blood, CD203c is exclusively and constitutively expressed on the surface of basophils (31, 32). CD203c is upregulated very quickly (<5 min) in response to specific allergen, anti-IgE and anti-FcεRI stimulation (2, 27, 28, 33–38). Upregulation and response to specific activators and inhibitors of CD63 and CD203c antigens appear to follow different kinetics and seem to be directed through alternative signal transduction pathways (2, 28, 39, 40). In contrast to CD63, CD203c is exclusively expressed on the outer basophil membrane, and upon activation of the cell expression of CD203c is less prominent when compared with CD63. The expression of CD63 seems to be a pathway closer to the AND degranulation than the expression of other activation markers such as CD203c (28).

Whether the novel identified basophil identification antigen, CRTH2(DP2) (chemoattractant receptor-homologous molecule expressed on T-helper 2 cells) (41–43), and the recently described activation antigens CD13, CD164 (behaving as CD203c) and CD107a (paralleling CD63 expression) (28) can be applied in flow-assisted allergy diagnosis and/or improve the technique, remains to be established.

Flow-assisted analysis of *in vitro*-activated basophils: technical aspects and validation

Complete description of the different techniques applied for *in vitro* analysis and quantification of flow-assisted allergy diagnosis is beyond the scope of this review. This section will rather focus and compare the major common technical features and issues of the different assays.

Whole blood vs isolated basophils

Flow-assisted analysis and quantification of *in vitro*-activated basophils can be applied either upon whole blood or upon basophils isolated by buffy coat centrifugation or sedimentation over dextran. The use of whole blood has the advantage of being very practical with simple manipulation (less centrifugation steps) mirroring physiological conditions. However, interference with serum components (e.g. IgG-blocking antibodies, anti-IgE and anti-receptor antibodies, circulating drugs) cannot completely be ruled out. Alternatively, cell separation is generally accompanied by loss of basophils and *in vitro* activation of cells can occur.

Pre-activation with IL-3

There is no general consensus on the need of pre-activation with interleukin (IL)-3. Nevertheless, it has

been demonstrated that short pre-activation (up to 10 min) of the basophils with IL-3 (2 ng/ml) does not upregulate CD63 expression by itself but primes the cells with increased mediator release or CD63 expression for a given stimulus concentration [e.g. *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), specific allergen, C5a]. In addition, priming with IL-3 has been observed to increase assay sensitivity (19). This increase of assay sensitivity might be particularly relevant when investigating allergens causing relative little specific stimulation, as is often the case for drugs. For some allergens, priming with IL-3, by shifting dose-responses to the left, might allow to widen the sometimes narrow gap between specific allergen stimulation and unspecific activation or cytotoxicity as IL-3-primed basophils may react to allergen concentrations 10 to 100 times less than those needed for non-IL-3 pre-treated cells (44). Tested in the same technical conditions, CD63 expression and histamine release gave similar results for grass pollen-induced basophil activation (45).

For venom-induced basophil activation, the picture is slightly different as IL-3 priming increase both the amount of histamine released and the sensitivity (46). The mechanism of basophil IL-3 priming remains unknown. Early events of the biochemical cascade such as Syk and Shc phosphorylation and calcium flux are not affected whereas a point of upregulation was found at the level of Raf-1-Mek-Erk (47).

Interleukin-3 priming has not been used for the CD203c (2), but it would be interesting to compare the efficacy of the two activation markers used in the same technical conditions.

Stimulation with allergen, including positive and negative control

Allergen selection. Obviously, proper selection of allergen is a critical issue. Currently, flow cytometric analysis of *in vitro*-activated basophils generally relies upon the use of natural allergen extracts that have been proven quite satisfactory (see section: Flow-assisted Analysis of *In Vitro*-activated Basophils: Clinical Applications). It should, however, be kept in mind that allergens extracted from natural sources may be heterogeneous with varying composition, presence of non-specific stimulants and/or inhibitory components such as preservatives, endotoxins and lectins. To some extent, these problems might be circumvented by the use of recombinant allergens which are becoming available for an increasing number of important allergens.

Commercially available skin-test extracts are rarely suitable for basophil activation experiments as they generally contain toxic and/or inhibitory preservatives. To some extent, this problem might be circumvented by dialysing the extract. For drugs, the injectable form is recommended.

Dose-response experiments. Basophil responses can be highly heterogeneous between different patients. However, allergen-induced upregulation of CD63 and CD203c is generally not restricted to a single stimulation concentration but implies different stimulation concentrations spanning several log scales (26, 35, 37, 48–50). This offers the opportunity to restrict basophil activation experiments to an 'optimal' concentration that discriminates between patients and controls. Unfortunately, this might not apply to all allergens. Drugs, such as neuromuscular blocking agents, might show a considerably smaller range of basophil-activating concentrations (51) (D.G. Ebo, pers. obs.).

At present, allergen stimulation concentrations are sometimes deduced from former histamine and/or leukotriene release experiments. This might not always be appropriate, as upregulation of CD63 and/or CD203c appear not simply to reflect the excreting function of the basophil, i.e. release of mediators, but merely represents a distinct point in the signalling pathway or a different functional endpoint (37, 39, 52). In a comparison between histamine release and upregulation of activation markers, several studies demonstrated bell-shaped dose-response curves for histamine release and rather sigmoid dose-response curves for expression of CD63 (52) and/or CD203c (4, 37), indicating ongoing upregulation of activation markers for a challenge concentration where mediator release has already decreased. In addition, Kahlert et al. (37) demonstrated CD203c expression to precede and to be more sensitive than histamine release. In fact, depending on the allergen tested, the sensitivity was 5–100-fold higher with the flow cytometric assay of activation markers.

Another important reason to perform dose-finding experiments is to establish the allergen stimulation concentrations that evoke maximal and sub-maximal cell stimulation, respectively. Own results seem to suggest that the BAT can monitor successful venom immunotherapy (VIT), provided the cells are challenged sub-maximally (see below: section Hymenoptera Venom).

It is also of paramount importance to identify stimulation concentrations that are cytotoxic or result in unspecific stimulation of the cells, as this might result in false-negative and false-positive results respectively. Finally, the optimal allergen concentration may vary from one protocol to another (isolated basophils vs whole blood, priming with IL-3, etc.).

Positive and negative control stimulation. It has to be emphasized that for correct interpretation in each assay, relevant positive and negative control stimulation has to be included. In most published papers, the positive control is a polyclonal or monoclonal anti-IgE antibody. An attractive alternative positive stimulant is monoclonal anti-FcεRI (53). As activation with fMLP does not mimic an IgE-mediated activation of the basophil, it cannot be considered as an appropriate positive control.

Approximately 5–10% of the individuals tested failed to upregulate CD63 and CD203c with regard to IgE-mediated basophilic activation. Although these non-responders represent a substantial number of the patients tested and are capable of influencing sensitivity and specificity of the test, they are quite often not mentioned in the results of the published studies. Obviously, in non-responders, basophil activation assays are lost as a diagnostic tool. Recent publications indicate a central role for the tyrosine kinases lyn and syk in FcεRI-mediated basophil degranulation (54, 55). Syk was reported to be absent, and levels of lyn to be reduced in non-releasing basophils but mRNA of both proteins were detectable at normal levels. Moreover, maximum histamine release correlated with expression of both kinases. Whether, pre-activation of the basophils with IL-3 might convert the non-releaser status in a releaser status (2, 56), or a passive sensitization procedure might help circumvent the problem remains to be established. Given the rather disappointing results of passive sensitization protocols with (stripped) donor basophils, the development of novel tools such as mast cell lines or stable 'humanized' rat basophil leukaemia cells lines seem of particular interest (57).

False-negative results might also be explained by other causes such as recent exposure to the allergen with transient refractoriness of the cells and/or temporarily reduced levels of allergen-specific circulating and membrane-bound IgE. In contrast, false-negative results can also be observed when analysis is delayed. Therefore, ideally, testing should be carried out between 6 weeks and 12 months after the acute event. As already addressed elsewhere, intake/administration of drugs, particularly glucocorticosteroids, immunomodulators, immunosuppressive drugs and anti-IgE might interfere with the tests (3). Alternatively, false-negative results can also have technical causes such as poor storage of blood sample (recommendation: maximum 24 h at 4°C), inappropriate stimulation of the cells with poorly defined allergens that contain inhibitory or cytotoxic substances, application of cytotoxic allergen stimulation concentrations.

Anyhow, in the event of negative findings, one should always carefully check for all these possible confounders and it might be worthwhile to redo the test, as it happens in some patients that non-responder status is temporary. Negative control stimulation, to assess spontaneous expression of activation markers, generally implies incubation of the cells with stimulation buffer.

Characterization and activation markers

At present, the most commonly used technique to analyse and quantify *in vitro* activated basophils relies upon characterization of the cells by anti-IgE and assessment of their activation status by anti-CD63, commercially available from Bühlmann (washed leucocytes) (Bühlmann Laboratories AG, Basel, Switzerland) and Orpegen (Orpegen Pharma GmbH, Heidelberg, Germany) and

BD (whole blood) (Pharmingen, Biosciences, Erembodegen-Aalst, Belgium). At the time of this writing, a Medline search for 'CD63 and allergy and diagnosis' reveals over 50 citations. For an extensive technical description of the anti-IgE/anti-CD63 technique as performed in the Department of Immunology Antwerp, the reader is referred elsewhere (26). It has been argued the anti-IgE/anti-CD63 technique to suffer from different drawbacks. First, the need for an additional anti-IgE antibody to identify basophils. IgE-antibody that could potentially activate the cells and generate false-positive results. Secondly, surface-bound IgE being selectively expressed not only by basophils but also by monocytes and dendritic cells. Thirdly, CD63 expression in peripheral blood not only to be basophil lineage-specific but also expressed by platelets. Several of these issues have been commented elsewhere (3).

In the CD63-based assay, recently, an alternative labelling with anti-CD123 and anti-human leucocyte antigen (HLA) DR antibodies that characterize the basophils as CD123+/HLA DR- cells has been described (58–61).

As in peripheral blood where CD203c expression is restricted to basophils, the test offers the advantage not to require anti-IgE or anti-CD123/HLA DR to identify the cells and allows to perform a 'single colour basophil activation assay' [for review, see Ref. (2)]. Acquisition of the basophils and analysis of their activation status can theoretically be performed with a single labelling procedure. However, the CD203c protocol is not always straightforward. First, acquisition of resting basophils upon CD203c is sometimes hampered due to low spontaneous expression of the marker. Secondly, upregulation of CD203c by activated basophils is generally less prominent than upregulation of CD63 (CD63 stains with greater fluorescence intensity, see Fig. 1). Thirdly, in patients with elevated spontaneous expression of CD203c, upregulation can completely be absent and lead to false-negative results.

In the absence of comprehensive clinical studies, it remains to be established whether the novel identified basophil identification antigen CRTH2(DP2) (chemo-attractant receptor-homologous molecule expressed on T-helper 2 cells) (41–43), and the recently described activation antigens CD13 and CD164 (behaving as CD203c) and CD107a (paralleling CD63 expression) (28) can be applied in flow-assisted allergy diagnosis and/or will improve the technique. A CRTH2-CD3-CD203c method is currently available from Beckman Coulter (Beckman Coulter, Mijdrecht, The Netherlands).

Flow-assisted analysis of *in vitro*-activated basophils: clinical applications

Analysis of *in vitro*-activated patient basophils

Currently, flow cytometric analysis of *in vitro*-activated patient basophils using CD63 and CD203c proved

reliable for the diagnosis of different IgE-mediated allergies including classical inhalant allergens (pollen, house dust mite), primary and secondary food allergies, natural rubber latex allergy, hymenoptera venom allergy, and drug allergies (Table 2). In addition, the technique allowed component-resolved diagnosis of pollinosis (35) and hymenoptera venom allergy (34). Moreover, flow cytometric analysis of *in vitro*-activated basophils was repeatedly demonstrated to constitute a valuable instrument to assess and to compare the (residual) allergenicity of natural allergen extracts, chemically modified and recombinant allergens (4, 35, 37, 38, 62–64). Finally, most recently, flow-assisted analysis of *in vitro*-activated basophils has been applied to assess passive IgE-sensitization by blood transfusion (65).

The efficacy of flow cytometry particularly in terms of sensitivity is highly dependent on the allergen type investigated. For proteins (pollen, house dust mite, latex, venoms, foods) the picture is globally comparable and the efficacy might increase with the use of recombinants. For drugs, however, the much lower observed sensitivity of the test probably reflects a weaker allergenicity of these molecules. In fact, in order to elicit the IgE receptor aggregation pathway, allergens must be, at least divalent (66). Results obtained with the RBL-2H3 cell line (basophilic leukaemia cell line) have shown that high-order oligomers (trivalent, tetravalent, etc.) may induce a stronger signal leading to an increase of the IgE receptor clustering (67). Other factors are also involved as the lifetime of the clustering which must exceed 100 s (68) and the number of receptors clustered (69). The earlier steps may be also reversible (70). All these factors and the nature of the microenvironment may lead to selective basophil responses.

Inhalant allergens. Several studies have evaluated application of flow cytometry in the diagnosis of pollinosis (19–21, 35, 48). Overall, flow cytometric quantification of CD63 and/or CD203c on peripheral blood basophils exposed to natural and/or recombinant pollen allergens demonstrated a highly reliable approach to diagnose pollen allergy. In the study by Hauswirth et al. (35) a significant correlation between CD63 and CD203c expression ($R = 0.76$) was observed.

Two of the above-mentioned studies also investigated flow cytometric analysis of *in vitro*-activated basophils in the diagnosis of house dust mite allergy (19, 20). The overall sensitivity and specificity of flow-assisted diagnosis in classical inhalant allergy equalled or exceeded 90%.

Latex. Application of the technique in the diagnosis of latex allergy was first described by Ebo et al. (23). It was concluded that flow cytometric analysis of *in vitro*-activated basophils is not only highly sensitive and specific for the diagnosis of IgE-mediated allergy, but it was also demonstrated that the technique might be helpful to discriminate between clinical relevant and

Table 2. Flow cytometric analysis of *in vitro* allergen-activated basophils: sensitivity and specificity data

Stimulus	Reference test	Sensitivity	Specificity	Numbers	Reference
House dust mite (HDM)	H + IgE and/or ST	56–78	91–100	20	(19)
<i>Dactylis glomerata</i>	H + IgE and/or ST	73–100	100		
Cypress pollen	H + ST + PT	91	100	75	(48)
HDM and <i>Lolium perenne</i>	H + IgE + ST	93	98	128	(20)
Latex	H + IgE + ST	93	92	102	(23)
Latex	H + ST	93	100	73	(53)
Latex	H + IgE and/or ST	80	97	79	(74)
Wasp venom	H	92	80	70	(49)
Wasp venom	H	85	83	87	(58)
Bee venom	H	91	90		
β -lactam	H + ST	50	93	88	(25)
β -lactam	H $\pm$ ST $\pm$ IgE $\pm$ PT	49	91	110	(84)
Metamizol	H $\pm$ PT	42	100	55	(85)
Aspirin and NSAID	H $\pm$ PT	15–55	74–100	90	(86)
Neuromuscular blocking agent	H	64	93	50	(51)
Neuromuscular blocking agent	H $\pm$ ST	54	100	60	(24)
Neuromuscular blocking agent	H	79	100	24	(27)
Carrot–celery–hazelnut	H (OAS)	85–90	80–90		(81)
Apple	H (OAS)	90	100	59	(26)
		88*	75*		
Apple (Mal d 1)	H (OAS)	75	64	54	(50)
Celery (Api g 1)		65	86		
Carrot (Dau c 1)		75	82		
Serum of patients with CIU	H + ASST	20	70	65	(102)

Numbers: total number of patients and controls.

H, history; ST, skin test; PT, provocation test; ASST, autologous serum skin test.

In the study by Cozon et al. (19) sensitivity and specificity vary according to different IL-3 pre-activation protocols.

*In an additional comparison between birch pollen-allergic patients with and without oral allergy syndrome (OAS).

irrelevant latex IgE results resulting from sensitization to cross-reactive carbohydrate determinants (CCD). Cross-reactive carbohydrate determinants that have been demonstrated, on several occasions, to exhibit pronounced effects on the specificity of specific IgE quantification and to mimic allergy (71–73).

Since several independent groups have confirmed these data with sensitivities and specificities generally exceeding 90% (53, 74). In a study by Boumiza et al. (36) sensitivity for the CD63-based assay was only 50% when compared with 75% for the CD203c technique. However, this study has been commented and the conclusion of the authors that application of CD203c is a marked improvement over CD63 has, at least, to be considered as premature (75, 76).

Food. Flow-assisted diagnosis of IgE-mediated food allergy was first reported by Moneret-Vautrin et al. (77). From this study in patients with heterogeneous primary food allergies, the authors concluded that the flow cytometric assay gives comparable results to the leukotriene (LTC₄) release test and was more efficient than the histamine release test. The technique also proved highly efficient in the diagnosis of IgE-mediated allergy from the fish parasite *Anisakis simplex* (60).

In individual patients, the technique confirmed diagnosis of primary food allergies such as anaphylaxis from

sesame (*Sesame indicum*) seed and oil (78), the food additive guar gum (*Cyamopsis tetragoloba*) (79), an oral allergy syndrome from papaya (*Carica papaya*) (80).

Finally, the technique has also been assessed in the diagnosis of secondary food allergies, i.e. food allergies that result from cross-reactivity to pollen allergens. According to history and healthy controls, the flow cytometric assay demonstrated a comparable sensitivity and specificity with skin tests and specific IgE for several birch pollen-associated food allergies such as carrot, celery and hazelnut (81). Almost similar results were obtained in a study on birch pollen-associated apple allergy (26). The latter paper, however, also demonstrated that validation of a diagnostic test is inappropriate when it failed to identify conditions that could have affected the outcome of the test. Actually, in a separate comparison between birch pollen-allergic patients with and without apple allergy, it was shown that cross-reactivity considerably reduced specificity of IgE quantification and (albeit to a lesser test) the flow cytometric technique.

Whether application of recombinant allergens will further improve the technique remains to be established. In a first experiment, with the Bet v 1 homologues Mal d 1, Api g 1 and Dau c 1 [the major allergens of birch (*Betula verrucosa*), apple (*Malus domestica*), celery (*Apium graveolens*) and carrot (*Daucus carota*) respectively] sensitivity and specificity of the test varied between

65% and 75%, and 68 and 100% respectively (50). (For a comparison with natural extracts, see Table 2.)

Hymenoptera venom. Four studies have investigated flow cytometric analysis of *in vitro*-activated basophils in the diagnosis of anaphylaxis from wasp and bee venom. The first group to publish on the reliability of flow-assisted diagnosis of venom allergy reported an absolute sensitivity and specificity of the test with a good correlation between this method and mediator release tests (46). Almost similar observations on CD63 expression and histamine release were found by Lambert et al. (82). Unfortunately, robust interpretation from the data might to some extent be hampered by issues such as absence of clear dose-finding experiments and joint reporting of the results obtained for different hymenoptera venoms (vespula, polistes, hornets, bees, etc.).

In more recent series by Sturm et al. (58) and Erdmann et al. (49), the sensitivity and specificity of flow cytometric analysis of *in vitro* venom-activated basophils was comparable with or superior to more established diagnostic tests such as venom skin tests and specific IgE, with a sensitivity and specificity of 85–90%. Moreover, patients could be identified even after a long interval to the relevant sting reaction and despite a low IgE result.

From the Erdmann paper it appears that flow cytometric analysis of *in vitro* activated basophils failed to monitor successful venom immunotherapy and to predict the outcome of a controlled sting challenge (49). However, comparison between maximal and sub-maximal stimulation conditions demonstrated a significant decrease of basophilic CD63 expression during maintenance venom immunotherapy. Actually, after prolonged maintenance therapy of 3 years, for sub-maximal stimulation conditions, up to 60% of the patients had a negatization of their basophil activation test (D.G. Ebo, unpubl. obs.). However, 'refractoriness' in our whole-blood assay might not really represent a basophil property, but rather mirror less IgE that is cross-linked on the cell membrane because of decreased specific IgE and/or induction of blocking antibodies.

On several occasions, the technique has also been demonstrated to take the sting out of difficult cases with inconclusive IgE and skin test results (58, 61, 83) and to provide a tool for prediction of side effects from venom immunotherapy (61). A CD203c-based flow assay confirmed the diagnosis of hymenoptera venom allergy in 20 of 22 patients and demonstrated expression of this activation marker to be consistent with expression of CD63 (33).

Drugs and related compounds. Diagnosis of drug allergy is difficult, as a broad spectrum of different drugs (or metabolites) can elicit immune and non-immune-mediated pathologies with distinct and sometimes unclear pathomechanism. The causative structure (epitope) is frequently unknown, the result of an *in vitro* or *in vivo* test

might not be predictive for the clinical outcome, and the reference test for diagnosis, the challenge test, is complicated and sometimes dangerous endeavour. Problems are frequently compounded by the fact that different drugs are administered simultaneously. Consequently, the availability of a safe, quick and reliable assay allowing simultaneous testing of different drugs would be more than welcome. Ideally, such a test should provide the physician with a tool that, apart from identification of the responsible compound(s), might also allow assessment of cross-reactivity and tailor safe alternatives.

Neuromuscular blocking agents. Evidence has accumulated that flow cytometry can contribute to the diagnosis of anaphylactic and anaphylactoid reactions from neuromuscular blocking agents that bear quaternary ammonium (NH_4^+) ions. In a series including 21 patients with definite anaphylaxis from these drugs, the sensitivity and specificity of a CD63-based basophil activation assay was 64% and 93%, respectively (51). More recently, comparable sensitivity and specificity figures were obtained in a study by Monneret et al. (24) and Sudheer et al. (27). In an own comparison between 14 patients suffering from anaphylaxis with profound hypotension and bronchospasm after administration of rocuronium and a positive skin test for rocuronium and eight individuals who tolerated administration of rocuronium and demonstrated a negative skin test for rocuronium, sensitivity and specificity of the test was 91.7% and 100% respectively. But two patients had to be excluded because of non-responsiveness to drug and positive control stimulation with anti-IgE. In line with the series of Refs (24, 27, 51), flow cytometry also proved helpful to identify cross-reactivity between rocuronium and other muscle relaxants (particularly with the other aminosteroid vecuronium) and to tailor a potentially safe alternative (D.G. Ebo, unpubl. obs.).

Beta-lactam antibiotics. In a comparative analysis between quantification of specific IgE and basophilic CD63 expression, in 58 patients suffering from skin-test proven β -lactam allergy and 30 healthy control individuals, sensitivity and specificity of the assays approximated 38% and 87% for IgE and 50% and 94% for the basophil activation test respectively (25). As demonstrated in Table 1 almost similar results were found in the study by Torres et al. (84).

Aspirin and non-steroidal anti-inflammatory drugs. Two studies from the same group have evaluated flow cytometric analysis of *in vitro* activated basophils in the diagnosis of 'hypersensitivity' from aspirin, metamizol and other non-steroidal anti-inflammatory drugs (NSAIDs) (85, 86). From the first study, sensitivity and specificity of the test for metamizol was 42% and 100% respectively. In the second study, the sensitivity for the different NSAID varied from 15% for metamizol and 55% for naproxen, whereas specificity generally exceeded 90%. Additional comprehensive studies are eagerly awaited to confirm these results. Particularly, because aspirin-induced respiratory as well as cutaneous reactions

are typical pseudo-allergic manifestations that result from inhibition of cyclooxygenase-1 (COX-1) with subsequent depletion of prostaglandin E2 and unrestrained synthesis of cysteinyl leukotrienes and mediator release from mast cells and eosinophils (87, 88). Erdmann et al. (89) did not find the BAT to be reliable for the diagnosis of hypersensitivity from NSAID.

Miscellaneous. In isolated cases, the technique helped to identify the plasma expander hydroxyl ethyl starch (90), chemophor (the emulsifier of the intravenous cyclosporine formulation) (91), bovine serum albumin present in a semen culture medium for artificial insemination (92), omeprazole (93), different low-molecular-weight heparins (94, 95), the antiseptics povidone (96) and chlorhexidine (97), viscotoxins of mistletoe (*Viscum album*) (98), the dye patent blue (59) and the enzyme hyaluronidase (99) as the causative compound in patients with sometimes life-threatening anaphylactoid reactions. Moreover, in some of these patients the technique contributed to establish the individual therapeutic alternative and/or allowed identification of potentially cross-reactive structures.

Analysis of *in vitro*-activated donor basophils

Application of flow-assisted analysis and quantification of *in vitro*-activated basophils can also be applied on cells drawn from non-allergic and/or allergic donors. In a first application, generally referred as a passive sensitization procedure, basophils (stripped) of non-allergic donors, prior to allergen challenge, are incubated with serum of the patient (77, 100). Passive sensitization protocols to detect allergen-specific IgE in the patients' serum, however, are laborious and difficult to standardize and to reproduce as it is highly dependent on the basophil releasability of the individual donor.

A second application comprises detection of mediator-releasing antibodies in a typical form of chronic urticaria, i.e. auto-immune urticaria. The auto-immune subclass of chronic idiopathic urticaria has been characterized by the presence of cell-activating IgG autoantibodies against the IgE molecule or α -chain of the high-affinity Fc ϵ RI on basophils and mast cells. Currently, these antibodies are usually detected by autologous serum skin tests and confirmed by histamine release studies [for review, see Ref. (101)]. As already addressed above, a particular application of the flow cytometer is the autoimmune basophil activation test. In these settings, basophils of allergic or non-allergic individuals are stimulated with patients' serum in order to detect the presence of basophil-activating autoantibodies. At present, this application has been reported in three studies. First, the paper by Wedi et al. (102) who, with the use of an atopic donor, demonstrated increased CD63 expression in 70%, 45% and 35% of the patients with a positive and negative autologous serum test and controls respectively. Secondly, the report by Gyimesi et al. (103) who

demonstrated a strong correlation between the ability of patients' sera to raise a positive autologous serum skin test and CD63 expression by the basophils of atopic donors. Recently, these data were confirmed in a third study by De Swerd et al. (104), who found positive basophil activation in 68% of chronic idiopathic urticaria patients with a positive autologous serum skin test and 41% of the chronic idiopathic urticaria patients with a negative autologous serum skin test.

Conclusion, future perspectives and speculative viewpoints

Over the last two decades, the development of sophisticated flow cytometric instrumentation has expanded the application of flow cytometry in several fields of medicine such as haematology, oncology, immunology. Since the mid-1990s, it is known that flow-assisted analysis of *in vitro*-activated basophils applying CD63 or CD203c can constitute an efficient and safe instrument and provides the physician with a novel diagnostic approach that allows quick and simultaneous multiple allergy testing. The technique has been clinically validated for several classical IgE-mediated allergies including indoor and outdoor inhalant allergies, primary and secondary food allergies, natural rubber latex allergy, hymenoptera venom allergy and some drug allergies. However, additional comprehensive studies are required to further validate the technique and allow its entrance in routine diagnostic application. Currently, it seems justified already to speculate on some particular issues.

First, as flow cytometric analysis of *in vitro*-activated basophils more closely resembles the *in vivo* pathway leading to symptoms, it is presumed that the technique could further be adopted to discriminate between clinically relevant and irrelevant IgE results, to evaluate the (residual) allergenicity of natural or recombinant allergens and to select and monitor specific (component-resolved) immunotherapy. Furthermore, it is anticipated that the technique might also be applied to demonstrate occurrence of natural tolerance, e.g. out-grow of food allergies in children. It remains to be elaborated whether for some of these purposes, e.g. follow-up of immunotherapy, sub-maximal stimulation of the cells might be more appropriate than maximal stimulation, or vice versa.

In vitro follow-up of provocation tests might be another application of flow-assisted analysis of basophils. Actually, the *ex vivo* measurement of spontaneous CD63 expression may parallel recent *in vivo* basophil activation during challenge tests.

Although the technique does not allow differentiating between IgE-dependent and IgE-independent basophil activation, even if the CD63 group of activation markers are closely linked to AND, this should not be interpreted as a shortcoming. It is anticipated that it might constitute a unique tool in the diagnosis of IgE-independent anaphylactoid reactions as well as for the diagnosis of

IgE-mediated anaphylaxis when a specific IgE assay is unavailable. Particularly, for drug and occupational allergy diagnosis, flow cytometric analysis of *in vitro*-activated basophils might become an important acquisition and scale back the need for potentially harmful provocations.

Whether the introduction of novel activation markers will broaden and/or improve the applications of the BAT

needs to be established. It is tempting to speculate that the technique might benefit from combined application of different activation markers.

Finally, it is anticipated that the development of stable 'humanized' basophil cell lines might further broaden the application of flow-assisted allergy diagnosis and render the technique more accessible by rendering superfluous the need for fresh basophils (57).

References

1. Sanz ML, Maselli JP, Gamboa PM, Oehling A, Dieguez I, de Weck AL. Flow cytometric basophil activation test: a review. *J Investig Allergol Clin Immunol* 2002;**12**:143–154.
2. Bühring HJ, Streble A, Valent P. The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Int Arch Allergy Immunol* 2004;**133**:317–329.
3. Ebo DG, Hagendorens MM, Bridts CH, Schuerwegh AJ, De Clerck LS, Stevens WJ. In vitro allergy diagnosis: should we follow the flow? *Clin Exp Allergy* 2004;**34**:332–339.
4. Valent P, Hauswirth AW, Natter S, Sperr WR, Bühring HJ, Valenta R. Assays for measuring in vitro basophil activation induced by recombinant allergens. *Methods* 2004;**32**:265–270.
5. Crockard AD, Ennis M. Basophil histamine release tests in the diagnosis of allergy and asthma. *Clin Exp Allergy* 2001;**31**:345–350.
6. Demoly P, Lebel B, Arnoux B. Allergen-induced mediator release tests. *Allergy* 2003;**58**:553–558.
7. Sainte-Laudy J, Vallon C, Guerin JC. [Analysis of membrane expression of the CD63 human basophil activation marker. Applications to allergologic diagnosis]. *Allerg Immunol (Paris)* 1994;**26**:211–214.
8. Sainte-Laudy J, Vallon C, Guerin JC. Diagnosis of latex allergy: comparison of histamine release and flow cytometric analysis of basophil activation. *Inflamm Res* 1996;**45**(Suppl. 1):S35–S36.
9. Sabbah A, Drouet M, Sainte-Laudry J, Lauret MG, Loiry M. Contribution of flow cytometry to allergologic diagnosis (in French). *Allerg Immunol (Paris)* 1997;**29**:15–21.
10. Sabbah A, Plassais R, Grenapin S, Drouet M, Lauret MG, Loiry M et al. Testing basophil activation by flow cytometry in the diagnosis of allergy to hymenopteran venom (in French). *Allerg Immunol (Paris)* 1998;**30**:44–48.
11. Dvorak AM. Cell biology of the basophil. *Int Rev Cytol* 1998;**180**:87–236.
12. Bochner BS. Systemic activation of basophils and eosinophils: markers and consequences. *J Allergy Clin Immunol* 2000;**106**(Suppl. 5):S292–S302.
13. Ghannadan M, Hauswirth AW, Scherthaner GH, Müller MR, Klepetko W, Schatzl G et al. Detection of novel CD antigens on the surface of human mast cells and basophils. *Int Arch Allergy Immunol* 2002;**127**:299–307.
14. Nieuwenhuis HK, van Oosterhout JJ, Rozemuller E, van Iwaarden F, Sixma JJ. Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. *Blood* 1987;**70**:838–845.
15. Metzelaar MJ, Wijngaard PL, Peters PJ, Sixma JJ, Nieuwenhuis HK, Clevers HC. CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. *J Biol Chem* 1991;**266**:3239–3245.
16. Grutzkau A, Smorodchenko A, Lippert U, Kirchhof L, Artuc M, Henz BM. LAMP-1 and LAMP-2, but not LAMP-3, are reliable markers for activation-induced secretion of human mast cells. *Cytometry* 2004;**61A**:62–68.
17. Knol EF, Mul FP, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. *J Allergy Clin Immunol* 1991;**88**(3 Pt 1):328–338.
18. Fureder W, Agis H, Sperr WR, Lechner K, Valent P. The surface membrane antigen phenotype of human blood basophils. *Allergy* 1994;**49**:861–865.
19. Cozon G, Ferrandiz J, Peyramond D, Brunet J. Detection of activated basophils using flow cytometry for diagnosis in atopic patients. *Allergol Immunopathol (Madr)* 1999;**27**:182–187.
20. Sanz ML, Sanchez G, Gamboa PM, Vila L, Uasuf C, Chazot M et al. Allergen-induced basophil activation: CD63 cell expression detected by flow cytometry in patients allergic to *Dermatophagoides pteronyssinus* and *Lolium perenne*. *Clin Exp Allergy* 2001;**31**:1007–1013.
21. Saporta M, Kamei S, Persi L, Bousquet J, Arnoux B. Basophil activation during pollen season in patients monosensitized to grass pollens. *Allergy* 2001;**56**:442–445.
22. Yoshimura C, Yamaguchi M, Iikura M, Izumi S, Kudo K, Nagase H et al. Activation markers of human basophils: CD69 expression is strongly and preferentially induced by IL-3. *J Allergy Clin Immunol* 2002;**109**:817–823.
23. Ebo DG, Lechkar B, Schuerwegh AJ, Bridts CH, De Clerck LS, Stevens WJ. Validation of a two-color flow cytometric assay detecting in vitro basophil activation for the diagnosis of IgE-mediated natural rubber latex allergy. *Allergy* 2002;**57**:706–712.
24. Monneret G, Benoit Y, Debard AL, Gutowski MC, Topenot I, Bienvenu J. Monitoring of basophil activation using CD63 and CCR3 in allergy to muscle relaxant drugs. *Clin Immunol* 2002;**102**:192–199.
25. Sanz ML, Gamboa PM, Antepara I, Uasuf C, Vila L, Garcia-Aviles C et al. Flow cytometric basophil activation test by detection of CD63 expression in patients with immediate-type reactions to betalactam antibiotics. *Clin Exp Allergy* 2002;**32**:277–286.
26. Ebo DG, Hagendorens MM, Bridts CH, Schuerwegh AJ, De Clerck LS, Stevens WJ. Flow cytometric analysis of in vitro activated basophils, specific IgE and skin tests in the diagnosis of pollen-associated food allergy. *Cytometry B Clin Cytom* 2005;**64**:28–33.
27. Sudheer PS, Hall JE, Read GF, Rowbottom AW, Williams PE. Flow cytometric investigation of peri-anesthetic anaphylaxis using CD63 and CD203c. *Anaesthesia* 2005;**60**:251–256.

28. Hennersdorf F, Florian S, Jakob A, Baumgartner K, Sonneck K, Nordheim A et al. Identification of CD13, CD107a, and CD164 as novel basophil-activation markers and dissection of two response patterns in time kinetics of ige-dependent upregulation. *Cell Res* 2005;**15**:325–335.
29. Kraft S, Fleming T, Billingsley JM, Lin SY, Jouvin MH, Storz P et al. Anti-CD63 antibodies suppress IgE-dependent allergic reactions in vitro and in vivo. *J Exp Med* 2005;**201**:385–396.
30. Goding JW, Grobben B, Slegers H. Physiological and pathophysiological functions of the ecto-nucleotide pyrophosphatase/phosphodiesterase family. *Biochim Biophys Acta* 2003;**1638**:1–19.
31. Bühring HJ, Simmons PJ, Pudney M, Muller R, Jarrossay D, van Agthoven A et al. The monoclonal antibody 97A6 defines a novel surface antigen expressed on human basophils and their multipotent and unipotent progenitors. *Blood* 1999;**94**:2343–2356.
32. Bühring HJ, Seiffert M, Giesert C, Marxer A, Kanz L, Valent P et al. The basophil activation marker defined by antibody 97A6 is identical to the ecto-nucleotide pyrophosphatase/phosphodiesterase 3. *Blood* 2001;**97**:3303–3305.
33. Platz IJ, Binder M, Marxer A, Lischka G, Valent P, Bühring HJ. Hymenoptera-venom-induced upregulation of the basophil activation marker ecto-nucleotide pyrophosphatase/phosphodiesterase 3 in sensitized individuals. *Int Arch Allergy Immunol* 2001;**126**:335–342.
34. Binder M, Fierlbeck G, King T, Valent P, Bühring HJ. Individual hymenoptera venom compounds induce upregulation of the basophil activation marker ecto-nucleotide pyrophosphatase/phosphodiesterase 3 (CD203c) in sensitized patients. *Int Arch Allergy Immunol* 2002;**129**:160–168.
35. Hauswirth AW, Natter S, Ghannadan M, Majlesi Y, Scherthner GH, Sperr WR et al. Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. *J Allergy Clin Immunol* 2002;**110**:102–109.
36. Boumiza R, Monneret G, Forissier MF, Savoye J, Gutowski MC, Powell WS et al. Marked improvement of the basophil activation test by detecting CD203c instead of CD63. *Clin Exp Allergy* 2003;**33**:259–265.
37. Kahlert H, Cromwell O, Fiebig H. Measurement of basophil-activating capacity of grass pollen allergens, allergoids and hypoallergenic recombinant derivatives by flow cytometry using anti-CD203c. *Clin Exp Allergy* 2003;**33**:1266–1272.
38. Reininger R, Swoboda I, Bohle B, Hauswirth AW, Valent P, Rumpold H et al. Characterization of recombinant cat albumin. *Clin Exp Allergy* 2003;**33**:1695–1702.
39. Majlesi Y, Samorapoompichit P, Hauswirth AW, Scherthner GH, Ghannadan M, Baghestanian M et al. Cerivastatin and atorvastatin inhibit IL-3-dependent differentiation and IgE-mediated histamine release in human basophils and downmodulate expression of the basophil-activation antigen CD203c/E-NPP3. *J Leukoc Biol* 2003;**73**:107–117.
40. Monneret G, Boumiza R, Gravel S, Cossette C, Bienvenu J, Rokach J et al. Effects of prostaglandin D(2) and 5-lipoxygenase products on the expression of CD203c and CD11b by basophils. *J Pharmacol Exp Ther* 2005;**312**:627–634.
41. Nagata K, Hirai H, Tanaka K, Ogawa K, Aso T, Sugamura K et al. CRTH2, an orphan receptor of T-helper-2-cells, is expressed on basophils and eosinophils and responds to mast cell-derived factor(s). *FEBS Lett* 1999;**459**:195–199.
42. Cosmi L, Annunziato F, Galli MIG, Maggi RME, Nagata K, Romagnani S. CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur J Immunol* 2000;**30**:2972–2979.
43. Boumiza R, Debard AL, Monneret G. The basophil activation test by flow cytometry: recent developments in clinical studies, standardization and emerging perspectives. *Clin Mol Allergy* 2005;**3**:9.
44. de Weck AL, Stadler BM, Dahinden CA. New perspectives in the modulation of allergic inflammation. *Int Arch Allergy Appl Immunol* 1989;**90**(Suppl. 1):17–21.
45. Sainte-Laudy J, Sabbah A, Vallon C, Guerin JC. Analysis of anti-IgE and allergen induced human basophil activation by flow cytometry. Comparison with histamine release. *Inflamm Res* 1998;**47**:401–408.
46. Sainte-Laudy J, Sabbah A, Drouet M, Lauret MG, Loiry M. Diagnosis of venom allergy by flow cytometry. Correlation with clinical history, skin tests, specific IgE, histamine and leukotriene C4 release. *Clin Exp Allergy* 2000;**30**:1166–1171.
47. Vilarino N, Miura K, MacGlashan DW, Jr. Acute IL-3 priming up-regulates the stimulus-induced Raf-1-Mek-Erk cascade independently of IL-3-induced activation of Erk. *J Immunol* 2005;**175**:3006–3014.
48. Paris-Kohler A, Demoly P, Persi L, Lebel B, Bousquet J, Arnoux B. In vitro diagnosis of cypress pollen allergy by using cytofluorimetric analysis of basophils (Basotest). *J Allergy Clin Immunol* 2000;**105**(2 Pt 1):339–345.
49. Erdmann SM, Sachs B, Kwiecien R, Moll-Slodowy S, Sauer I, Merk HF. The basophil activation test in wasp venom allergy: sensitivity, specificity and monitoring specific immunotherapy. *Allergy* 2004;**59**:1102–1109.
50. Erdmann SM, Sachs B, Schmidt A, Merk HF, Scheiner O, Moll-Slodowy S et al. In vitro analysis of birch-pollen-associated food allergy by use of recombinant allergens in the basophil activation test. *Int Arch Allergy Immunol* 2005;**136**:230–238.
51. Abuaf N, Rajoely B, Ghazouani E, Levy DA, Pecquet C, Chabane H et al. Validation of a flow cytometric assay detecting in vitro basophil activation for the diagnosis of muscle relaxant allergy. *J Allergy Clin Immunol* 1999;**104**(2 Pt 1):411–418.
52. MacGlashan DW, Jr. Graded changes in the response of individual human basophils to stimulation: distributional behavior of events temporally coincident with degranulation. *J Leukoc Biol* 1995;**58**:177–188.
53. Sanz ML, Gamboa PM, Garcia-Aviles C, Vila L, Dieguez I, Antepara I et al. Flow-cytometric cellular allergen stimulation test in latex allergy. *Int Arch Allergy Immunol* 2003;**130**:33–39.
54. Kepley CL, Youssef L, Andrews RP, Wilson BS, Oliver JM. Syk deficiency in nonreleaser basophils. *J Allergy Clin Immunol* 1999;**104**(2 Pt 1):279–284.
55. Lavens-Phillips SE, MacGlashan DW, Jr. The tyrosine kinases p53/56lyn and p72syk are differentially expressed at the protein level but not at the messenger RNA level in nonreleasing human basophils. *Am J Respir Cell Mol Biol* 2000;**23**:566–571.

56. Kepley CL, Youssef L, Andrews RP, Wilson BS, Oliver JM. Multiple defects in Fc epsilon RI signaling in Syk-deficient nonreleaser basophils and IL-3-induced recovery of Syk expression and secretion. *J Immunol* 2000;**165**:5913–5920.
57. Vogel L, Luttkopf D, Hatahet L, Hausteiner D, Vieths S. Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 2005;**60**:1021–1028.
58. Sturm GJ, Bohm E, Trummer M, Weiglhofer I, Heinemann A, Aberer W. The CD63 basophil activation test in Hymenoptera venom allergy: a prospective study. *Allergy* 2004;**59**:1110–1117.
59. Ebo DG, Wets RD, Spiessens TK, Bridts CH, Stevens WJ. Flow-assisted diagnosis of anaphylaxis to patent blue. *Allergy* 2005;**60**:703–704.
60. Gonzalez-Munoz M, Luque R, Nauwelaers F, Moneo I. Detection of *Anisakis simplex*-induced basophil activation by flow cytometry. *Cytometry B Clin Cytom* 2005;**68**:31–36.
61. Kosnik M, Silar M, Bajrovic N, Music E, Korosec P. High sensitivity of basophils predicts side-effects in venom immunotherapy. *Allergy* 2005;**60**:1401–1406.
62. Gronlund H, Bergman T, Sandstrom K, Alvelius G, Reininger R, Verdino P et al. Formation of disulfide bonds and homodimers of the major cat allergen Fel d 1 equivalent to the natural allergen by expression in *Escherichia coli*. *J Biol Chem* 2003;**278**:40144–40151.
63. Drew AC, Eusebius NP, Kenins L, de Silva HD, Suphioglu C, Rolland JM et al. Hypoallergenic variants of the major latex allergen Hev b 6.01 retaining human T lymphocyte reactivity. *J Immunol* 2004;**173**:5872–5879.
64. Ball T, Edstrom W, Mauch L, Schmitt J, Leistler B, Fiebig H et al. Gain of structure and IgE epitopes by eukaryotic expression of the major Timothy grass pollen allergen, Phl p 1. *FEBS J* 2005;**272**:217–227.
65. Johansson SG, Nopp A, Hage M, Olofsson N, Lundahl J, Wehlin L et al. Passive IgE-sensitization by blood transfusion. *Allergy* 2005;**60**:1192–1199.
66. Levine B. The nature of the antigen-antibody complexes which initiate anaphylactic reactions. II. The effect of molecular size on the abilities of homologous multivalent benzylpenicilloyl haptens to evoke PCA and passive Arthus reactions in guinea pig. *J Immunol* 1965;**94**:121–131.
67. Kane P, Erickson J, Fewtrell C, Baird B, Holowka D. Cross-linking of IgE-receptor complexes at the cell surface: synthesis and characterization of a long bivalent hapten that is capable of triggering mast cells and rat basophilic leukemia cells. *Mol Immunol* 1986;**23**:783–790.
68. Schweitzer-Stenner R, Pecht I. Parameters determining the stimulatory capacity of the type I Fc epsilon-receptor. *Immunol Lett* 1999;**68**:59–69.
69. DeLisi C, Siraganian RP. Receptor cross-linking and histamine release. I. The quantitative dependence of basophil degranulation on the number of receptor doublets. *J Immunol* 1979;**122**:2286–2292.
70. Riviera J, Gonzalez-Espinosa C, Kovarova M, Parravicini V. The architecture of IgE-dependent mast-cell signalling. *ACI Int* 2002;**14**:25–36.
71. Mari A, Iacovacci P, Afferni C, Barletta B, Tinghino R, Di Felice G et al. Specific IgE to cross-reactive carbohydrate determinants strongly affect the in vitro diagnosis of allergic diseases. *J Allergy Clin Immunol* 1999;**103**:1005–1011.
72. Mari A. IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the in vivo and in vitro reactivity. *Int Arch Allergy Immunol* 2002;**129**:286–295.
73. Ebo DG, Hagendorens MM, Bridts CH, De Clerck LS, Stevens WJ. Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. *Clin Exp Allergy* 2004;**34**:137–144.
74. Hemery ML, Arnoux B, Dhivert-Donnadieu H, Rongier M, Barbotte E, Verdier R et al. Confirmation of the diagnosis of natural rubber latex allergy by the Basotest method. *Int Arch Allergy Immunol* 2005;**136**:53–57.
75. Ebo DG, Lechkar B, Schuerwegh AJ, Bridts CH, De Clerck LS, Stevens WJ. Comments regarding 'Marked improvement of the basophil activation test by detecting CD203c instead of CD63' by Boumiza et al. *Clin Exp Allergy* 2003;**33**:849–843.
76. de Weck AL, Sanz ML. For allergy diagnostic flow cytometry, detection of CD203c instead of CD63 is not at all an improvement in other hands. *Clin Exp Allergy* 2003;**33**:849–852.
77. Moneret-Vautrin DA, Sainte-Laudy J, Kanny G, Fremont S. Human basophil activation measured by CD63 expression and LTC4 release in IgE-mediated food allergy. *Ann Allergy Asthma Immunol* 1999;**82**:33–40.
78. Stevens WJ, Ebo DG, De Clerck LS, Bridts CH. Anaphylaxis to sesame (*Sesamum indicum*) seed and sesame oil. *J Allergy Clin Immunol* 2002;**109**:250.
79. Bridts CH, Ebo DG, De Clerck LS, Stevens WJ. Anaphylaxis due to the ingestion of guar gum. *J Allergy Clin Immunol* 2002;**109**:221.
80. De Clerck LS, Ebo DG, Bridts CH, Stevens WJ. Oral allergy syndrome due to the consumption of *Carica papaya*. *J Allergy Clin Immunol* 2003;**111**:103.
81. Erdmann SM, Heussen N, Moll-Slodowy S, Merk HF, Sachs B. CD63 expression on basophils as a tool for the diagnosis of pollen-associated food allergy: sensitivity and specificity. *Clin Exp Allergy* 2003;**33**:607–614.
82. Lambert C, Guilloux L, Dzviga C, Gourgaud-Massias C, Genin C. Flow cytometry versus histamine release analysis of in vitro basophil degranulation in allergy to Hymenoptera venom. *Cytometry B Clin Cytom* 2003;**52**:13–19.
83. Eberlein-Konig B, Rakoski J, Behrendt H, Ring J. Use of CD63 expression as marker of in vitro basophil activation in identifying the culprit in insect venom allergy. *J Investig Allergol Clin Immunol* 2004;**14**:10–16.
84. Torres MJ, Padiar A, Mayorga C, Fernandez T, Sanchez-Sabate E, Cornejo-Garcia JA et al. The diagnostic interpretation of basophil activation test in immediate allergic reactions to betalactams. *Clin Exp Allergy* 2004;**34**:1768–1775.
85. Gamboa PM, Sanz ML, Caballero MR, Antepara I, Urrutia I, Jauregui I et al. Use of CD63 expression as a marker of in vitro basophil activation and leukotriene determination in metamizol allergic patients. *Allergy* 2003;**58**:312–317.
86. Gamboa P, Sanz ML, Caballero MR, Urrutia I, Antepara I, Esparza R et al. The flow-cytometric determination of basophil activation induced by aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) is useful for in vitro diagnosis of the NSAID hypersensitivity syndrome. *Clin Exp Allergy* 2004;**34**:1448–1457.
87. Szczeklik A, Stevenson DD. Aspirin-induced asthma: advances in pathogenesis, diagnosis, and management. *J Allergy Clin Immunol* 2003;**111**:913–921.
88. Mastalerz L, Setkowicz M, Sanak M, Szczeklik A. Hypersensitivity to aspirin: common eicosanoid alterations in urticaria and asthma. *J Allergy Clin Immunol* 2004;**113**:771–775.

89. Erdmann SM, Ventocilla S, Moll-Slodowy S, Sauer I, Merk HF. Basophil activation tests in the diagnosis of drug reactions (in German). *Hautarzt* 2005;**56**:38–43.
90. Ebo DG, Schuerwegh A, Stevens WJ. Anaphylaxis to starch. *Allergy* 2000;**55**:1098–1099.
91. Ebo DG, Piel GC, Conraads V, Stevens WJ. IgE-mediated anaphylaxis after first intravenous infusion of cyclosporine. *Ann Allergy Asthma Immunol* 2001;**87**:243–245.
92. Orta M, Ordoqui E, Aranzabal A, Fernandez C, Bartolome B, Sanz ML. Anaphylactic reaction after artificial insemination. *Ann Allergy Asthma Immunol* 2003;**90**:446–451.
93. Gamboa PM, Sanz ML, Urrutia I, Jauregui I, Antepara I, Dieguez I et al. CD63 expression by flow cytometry in the in vitro diagnosis of allergy to omeprazole. *Allergy* 2003;**58**:538–539.
94. Caballero MR, Fernandez-Benitez M. Allergy to heparin: a new in vitro diagnostic technique. *Allergol Immunopathol (Madr)* 2003;**31**:324–328.
95. Ebo DG, Haine SE, Hagendorens MM, Bridts CH, Conraads VM, Vorlat A et al. Hypersensitivity to nadroparin calcium: case report and review of the literature. *Clin Drug Invest* 2004;**24**:421–426.
96. Le Pabic F, Sainte-Laudy J, Blanchard N, Moneret-Vautrin DA. First case of anaphylaxis to iodinated povidone. *Allergy* 2003;**58**:826–827.
97. Ebo DG, Bridts CH, Stevens WJ. Anaphylaxis to an urethral lubricant: chlorhexidine as the “hidden” allergen. *Acta Clin Belg* 2004;**59**:358–360.
98. Bauer C, Oppel T, Rueff F, Przybilla B. Anaphylaxis to viscotoxins of mistletoe (*Viscum album*) extracts. *Ann Allergy Asthma Immunol* 2005;**94**:86–89.
99. Ebo DG, Goossens S, Opsomer F, Bridts CH, Stevens WJ. Flow-assisted diagnosis of anaphylaxis to hyaluronidase. *Allergy* 2005;**60**:1333–1334.
100. de Leon MP, Drew AC, Glaspole IN, Suphioglu C, Rolland JM, O’hehir RE. Functional analysis of cross-reactive immunoglobulin E antibodies: peanut-specific immunoglobulin E sensitizes basophils to tree nut allergens. *Clin Exp Allergy* 2005;**35**:1056–1064.
101. Grattan CE. Autoimmune urticaria. *Immunol Allergy Clin North Am* 2004;**24**:163–181.
102. Wedi B, Novacovic V, Koerner M, Kapp A. Chronic urticaria serum induces histamine release, leukotriene production, and basophil CD63 surface expression—inhibitory effects of anti-inflammatory drugs. *J Allergy Clin Immunol* 2000;**105**:552–560.
103. Gyimesi E, Sipka S, Danko K, Kiss E, Hidvegi B, Gal M et al. Basophil CD63 expression assay on highly sensitized atopic donor leucocytes—a useful method in chronic autoimmune urticaria. *Br J Dermatol* 2004;**151**:388–396.
104. De Swertdt A, Van Den KC, Kasran A, Cadot P, Neyens K, Coorevits L et al. Detection of basophil-activating IgG autoantibodies in chronic idiopathic urticaria by induction of CD63. *J Allergy Clin Immunol* 2005;**116**:662–667.