

Assays for measuring in vitro basophil activation induced by recombinant allergens

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Accepted 21 August 2003

Abstract

The diagnosis of type I allergy is essentially based on clinical data, skin tests, and measurements of allergen-specific IgE. However, the determination of specific IgE per se does not permit a definitive conclusion concerning the response of effector cells to the respective allergen(s) and consecutive clinical symptoms in all patients. In an attempt to overcome this problem, a number of basophil-activation assays have been developed during the last few years. Today, allergen-induced activation of blood basophils can be employed as a specific and reliable measure of IgE-dependent responses in sensitized individuals. Using recombinant allergens and basophil-specific markers, these novel assays appear to serve as simple and useful tests in component-resolved diagnosis of type I allergies. In the current article, the biochemical, functional, and technical background of these basophil tests is discussed.

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Keywords: Allergy; IgE; IgE-receptor; Histamine; CD63; CD203c

1. Introduction

Immunoglobulin E (IgE)-dependent type I allergies represent a substantial medical problem in industrialized countries worldwide [1,2]. Thus, about 20–30% of the urban population is suffering from symptoms of IgE-dependent reactions to environmental allergens [1,2]. The symptoms that are caused by such IgE-dependent reactions include asthma, rhinitis, conjunctivitis, and atopic dermatitis [1,2]. In many patients, these symptoms require continuous treatment and medical care, and sometimes, the emerging reactions are grave or even life-threatening [2].

Recent progress has led to the identification, biochemical characterization, and cloning of various allergens [3,4]. Allergens are glycoproteins that expose a number of IgE-binding sites [3–5]. Sequence homologies of allergens in various species and cross-sensitization in

allergic patients have been described [3,4]. During the past few years, a number of allergens have been cloned and provided in recombinant form [3–7]. The availability of recombinant allergens has also led to the development of specific (component resolved) diagnostic tests [8]. However, still many questions remain to be answered. Likewise, in individual patients, striking differences are sometimes noted when biologic skin tests (prick-test) are compared with serology test results [9–11]. This notion points to an important role of effector cells and their activation during an allergic response. It has also been shown that depending on their structure and glycosylation status, allergens may differ significantly from each other with regard to the activation of IgE-bearing effector cells [12,13].

Therefore, more recent studies have focused on allergen-induced activation of IgE-bearing cells, i.e., peripheral blood basophils, in allergic individuals. These cells store a number of vasoactive mediators (histamine, and others) in their granules and expose high affinity IgE binding sites (= *FcεRI*) [14–17]. When IgE receptors on basophils are cross-linked by an allergen, the cells

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release their mediators into the extracellular space thereby contributing to allergic symptoms in sensitized individuals [18–20]. Using natural or recombinant allergens, this reaction can be studied *in vitro* and can be employed to measure the allergen-dependent activation of basophils [8,21,22].

During the past few years, three important assays, that are based on IgE-dependent basophil activation and can be employed for allergen-diagnostics, have been introduced and applied utilizing recombinant allergens: the histamine release test, the CD63-based ‘baso-test,’ and the CD203c-activation assay.

2. Histamine release test

2.1. Background of assay

Utilizing defined allergens, the histamine release test provides direct information concerning the releasability of IgE-bearing basophils. In fact, when IgE-receptors on basophils are cross-linked by an allergen (through IgE) or anti-IgE antibody, the cells undergo degranulation [23–25]. Allergen-induced (IgE-mediated) histamine release is a dose-dependent reaction with a bell-shaped peak seen in typical dose–response curves [23–25]. The capacity of basophils to release histamine in response to an IgE-dependent stimulus (=releasability) is dependent on several factors including the nature of disease (in severe atopic diseases, a high spontaneous release is often seen), previous drug intake, presence of cytokines and hormones, or previous challenge with activating agents (desensitization) [25]. In addition, approximately 5% of the individuals are non-responders with regard to IgE-mediated histamine release [23–25]. In these donors, a defect in IgE-receptor-signaling pathways (Syk-deficiency in basophils) has recently been proposed [26]. Histamine release is optimal at 37 °C and occurs in the presence, but not in the absence, of extracellular calcium [23–25].

2.2. Sampling of blood and preparation of basophils

The assay is usually performed on peripheral blood basophils (mast cells also express IgE-receptor, but are tissue-fixed cells). In principle, whole blood, dextran-isolated granulocytes, or Ficoll-isolated mononuclear cell fractions (which also contain substantial amounts of basophils) can be used as primary source of cells. If basophils are going to be cultured for several hours before the test is performed, we recommend to use Ficoll-isolated mononuclear cell fractions rather than dextran-isolates or whole blood. With regard to blood sampling it is recommended that the donors have not taken drugs or food shortly (several hours) before blood donation. As anticoagulant, heparin is recommended.

2.3. Selection and application of test-allergens

The type of allergen is selected based on clinical data and prior skin test- or serology test results. The test can be prepared using crude allergen extracts or recombinant allergens [5–8]. Today, the general recommendation is to use recombinant allergens whenever possible [8]. In each assay, an anti-IgE antibody is run in parallel as a positive control (to show that cross-linking of IgE receptor is sufficient to cause histamine release). With regard to anti-IgE, the optimal concentration to cause histamine release ranges between 0.1 and 3 µg/ml [16–20,24,25]. However, in case of allergens, the effective range may not be known, and may vary among allergens, and also among allergic individuals. In this regard it is also important to be aware that the dose–response curves produced in IgE-receptor cross-linking experiments are mostly monophasic with a bell-shaped peak that may cover only a (relatively) small concentration-range [18–20,24,25]. Therefore, the allergens (also recombinant) must always be applied in serial dilution starting at 1.0 µg/ml. Some of the allergens may be very effective and show histamine release between 0.01 and 0.0001 µg/ml or even in a lower test-range. Fig. 1 shows a dose-dependent release of histamine from basophils (of an allergic patient) in response to a recombinant allergen (Bet v 1).

2.4. Release reaction and measurement of histamine

Usually the histamine release solution contains Pipes, and always contains Ca^{2+} . The release reaction is optimal at 37 °C and occurs within 10–15 min [18–20,24,25]. It is recommended that basophils are incubated with

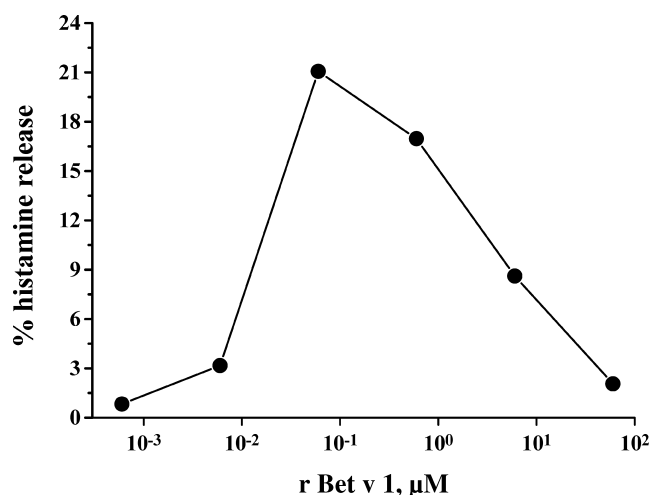


Fig. 1. Dose-dependent histamine release from basophils induced by recombinant (r) Bet v 1 in an allergic donor. Basophils were incubated with various concentrations of r Bet v 1 as indicated. Histamine release is expressed as a percentage of total histamine. Note bell shape of a dose–response curve.

allergens for 20–30 min at 37 °C. Thereafter, cells should immediately be subjected to centrifugation at 4 °C. After centrifugation, the cell-free supernatants are recovered by aspiration. In addition, a cell lysate-sample is stored. Probes should be stored at or below –20 °C. The histamine concentration can be measured by various techniques such as RIA, ELISA, or fluorometry [18–20,24–28]. In our hands, the RIA works well [27,28]. In each experiment, the spontaneous release of histamine should be measured and should be less than 5% of total (extracellular + cellular) histamine. In some atopic individuals, especially those who react against multiple (auto)allergens, the spontaneous histamine release may be rather high, so that the test cannot be performed. In fact, in donors with a spontaneous histamine release of >5%, the results cannot be judged as being informative (diagnostic). In case of such result, it is recommended that the test is repeated some days later in the same patient, and that all potentially interfering factors (previous intake of food or drugs, sampling, storage times) are excluded.

2.5. Calculations and interpretation of test-results

Histamine release (releasability) should always be given as a percentage of total histamine. As mentioned above the result should only count if the spontaneous release of histamine is less than 5%. For diagnostic purposes, a percentage of histamine release of >10% (or >5% after subtraction of spontaneous release) can be regarded as positive result provided that the reaction is clearly dose-dependent. Based on the magnitude of reaction, donors can be categorized as non responders (<5%), low responders (5–19%), intermediate (20–50%), and good responders (>50%). Such categorization is always subjective and based on arbitrary figures, and therefore may not be very useful in clinical terms. Also, the clinical effectiveness (harmfulness) of the allergen may not only depend on the maximum amount of mediator released but also on the allergen and minimal concentrations of allergen that are sufficient to cause histamine release. In addition, it should be pointed out that histamine is not the only critical mediator produced by basophils, and release of such mediators may be differentially regulated in these cells.

3. The CD63 basophil test

CD63 is a tetraspan antigen expressed on various myeloid cells including blood basophils and blood monocytes [29]. Resting basophils express only low amounts of the antigen [14,30–32]. However, when exposed to an allergen or anti-IgE antibody, basophils express substantial amounts of CD63 on their surface [30–35]. In recent years, the flow cytometric determina-

tion of CD63 and its allergen-induced upregulation on basophils in sensitized individuals has been introduced as a novel assay [31–35]. Thus, utilizing CD63, basophil responses can be measured and quantitated in allergic individuals.

3.1. Sampling of blood

The test should be performed in individuals who do not have a severe infection or an active chronic inflammation. In allergic individuals, it is important to be aware that these patients often take drugs that interfere with basophil activation. If at all possible, these drugs should not be taken at or shortly before the time of blood sampling. After donation, blood samples should be worked up within a few hours. As anticoagulant, heparin (but not other anticoagulants) should be used.

3.2. Test

In principle, whole blood or enriched cells (isolated granulocytes) can be used to perform the CD63 basophil test. The use of whole blood may be preferable in routine and screening examinations. Blood (cell) samples are incubated with (recombinant) allergens and control substance at 37 °C. We recommend to use at least two or three different concentrations of allergen (see histamine release), and to incubate basophils with the allergen(s) for 15–20 min. In fact, allergen-induced upregulation of CD63 is a rapid process with a plateau reached after 5–10 min [32–36]. After incubation, cells are washed and subjected to two-color flow cytometry using CD63 antibody and a second “basophil-specific” reagent. As basophil-specific reagent, a CD203c antibody or anti-IgE antibody can be employed [32–36]. Other alternatives are CD123 and CD17 [37]. In our experience, the CD203c antibody is preferable since it is a basophil-specific reagent [36]. Fig. 2 shows a flow cytometric evaluation of the increase in CD63 and CD203c on

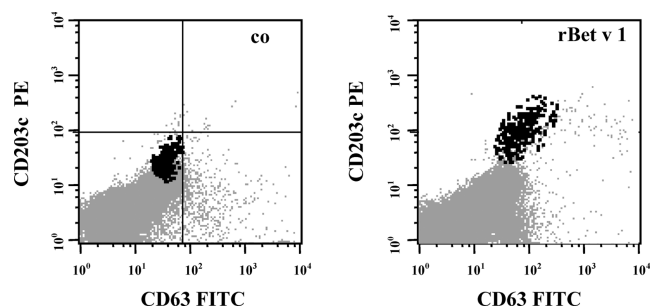


Fig. 2. Allergen-induced upregulation of CD63 and CD203c on basophils in a sensitized donor. Basophils were incubated with control medium (left panel) or rBet v 1, 1 µg/ml (right panel) for 10 min. Then, cells were analyzed for expression of CD63 and CD203c. As visible, both antigens were found to be upregulated after exposure of basophils to recombinant allergen.

basophils in response to a recombinant allergen (Bet v 1) in a sensitized individual. In case of anti-IgE antibody, one must be aware that also (activated) monocytes express IgE-receptors in atopic individuals [38,39]. Similarly, in case of CD123 or CD17, expression on monocytes and other blood leukocytes must be taken into account [29].

3.3. Interpretation of test results

The increase in surface expression of CD63 is either given as change in percentage of positive basophils or as change in mean fluorescence intensity (MFI) [33–36]. We recommend to refer to the MFI-change in all tests, if possible. With regard to the percentage count, the cut off level (negative/positive-gate) should be determined with great caution. It has been proposed that a percentage count of more than 15% ‘positive’ basophils after allergen-challenge would indicate an allergic state. However, depending on the antibody used, normal unstimulated basophils may express CD63 at a much higher percentage level [31]. With regard to the MFI, the increase in CD63 is given as a relative value (stimulation index = SI) resulting from a comparison between stimulated and unstimulated cells ($MFI_{stim} : MFI_{control}$). An SI exceeding 2.0 can be regarded as a safe indication of basophil activation induced by the test substance. The dose-dependency can be regarded as the second convincing indication for a specific reaction. In some cases, the allergen-induced increase in CD63 may be visible but is not diagnostic ($SI < 2.0$). In such cases, we recommend to repeat the test using a fresh sample of blood after some days. Like the CD203c-test, the CD63-test is a highly sensitive assay and therefore may show positive reactions even in patients who do not have definitive allergies, but only a transient increase in specific IgE after antigen-challenge. Also, the test result should always be interpreted in light of the overall clinical situation of the patient (drug intake, season, immunotherapy, recent allergen-challenge, co-existing medical problems, and disorders).

4. The CD203c basophil-activation assay

The enzyme ecto-nucleotide pyrophosphatase (phosphodiesterase) 3 = ENPP-3 (CD203c) is expressed on the surface of human basophils, but is not expressed on other peripheral blood cells [40,41]. Thus, ENPP-3/CD203c is a basophil-specific determinant. In response to IgE-receptor cross-linking on basophils, the levels of CD203c rapidly increase [36,40]. Likewise, in sensitized individuals, exposure of basophils to an allergen or anti-IgE antibody is followed by a dose- and time-dependent increase in surface expression of CD203c [36,40,42,43]. Fig. 3 shows the dose-dependent increase in CD203c in

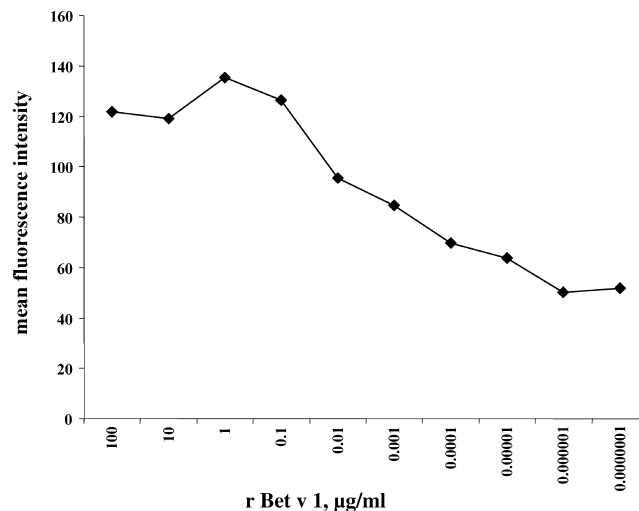


Fig. 3. Dose-dependent effect of r Bet v 1 on expression of CD203c on basophils obtained from a sensitized patient.

response to a recombinant allergen in a Bet v 1 allergic donor. Both natural and recombinant allergens can be used to promote expression of ENPP-3/CD203c [36,40,42,43]. However, today, it is recommended to use recombinant allergens whenever possible. The increase in CD203c can readily be measured in whole blood samples using conventional multicolor flow cytometry without any preanalytical step or second basophil-labeling reagent. Based on these notions, a novel basophil-activation assay employing CD203c has recently been proposed [36,43].

4.1. Sampling of blood

The test should only be performed in individuals who do not have a severe infection or an active chronic inflammation. This may be a problem in severe atopic individuals who are often suffering from chronic inflammation. In allergic individuals, it is important to be aware that these patients often take drugs that interfere with basophil activation. If at all possible, these drugs should not be taken at or shortly before the time of blood sampling. After donation, blood samples should be worked up within a few hours. As anticoagulant, heparin (not other anticoagulants) should be used.

4.2. Test

In principle, whole blood or enriched cells (isolated granulocytes) can be used to perform the CD203c test. The use of whole blood is preferable in routine and screening tests. Blood (cell) samples are incubated with recombinant allergens and control substances at 37 °C. We recommend to use at least two or three different concentrations of allergen (see histamine release) and to incubate basophils with the allergen(s) for 15–20 min.

In fact, allergen-induced upregulation of CD203c is a rapid process with a plateau reached after 5–15 min [36,40,42,43]. After incubation, cells are washed and subjected to flow cytometry using CD203c antibody. Alternatively, the CD203c antibody can be directly applied together with allergen, which keeps the test time to a minimum (30–60 min in total). So far, only a few CD203c antibodies have been generated. Of these, the CD203c antibody 97A6 is the most suitable reagent and is available in PE-labeled form [40]. Expression of CD203c can be measured by conventional flow cytometry in single color and as a single step process. All in all, when compared with other basophil tests, the CD203c baso-assay appears to be a very simple and rapid test.

4.3. Interpretation of test results

Baseline levels of CD203c expressed on basophils may differ significantly among individuals. In fact, a number of different factors may influence the expression of this antigen. One important factor are T cell-derived and basophil-activating cytokines. Likewise, IL-3 promotes the expression of CD203c (unpublished observation). Therefore, higher levels of CD203c may be found during the course of an inflammatory process. We therefore recommend to exclude those patients who are suffering from severe inflammation. The increase in surface expression of CD203c is given as a relative value (stimulation index = SI) comparing stimulated with unstimulated cells ($MFI_{stim}/MFI_{control}$) [36,40,42,43]. An SI exceeding 2.0 can be regarded as a safe indication of basophil activation induced by the test substance [36,40,42,43]. The dose-dependency can be regarded as the second convincing indication for a specific reaction. In some cases, the allergen-induced increase in CD203c may be visible but is not diagnostic ($SI < 2.0$). In such cases, we recommend to repeat the test using a fresh sample of blood after a certain time.

The CD203c test is probably the most sensitive IgE-specific basophil-activation assay developed so far. Therefore, this assay may show positive reactions even in patients who do not have definitive allergies. Likewise, a positive reaction may be documented after a transient slight (even invisible) increase in specific IgE after antigen challenge (for example, a hymenoptera sting) [42]. Therefore, the test result should always be interpreted in light of the overall (clinical) situation of the patient (drug intake, season, immunotherapy, recent exposure to allergens, and co-existing diseases).

5. Concluding remarks

A number of useful assays measuring basophil-activation initiated through specific IgE and the IgE receptor have been developed during the past few years.

When employing recombinant allergens and novel basophil markers, these assays appear to be simple tests and most helpful in the diagnosis of IgE-dependent allergies. Flow cytometry-based assays such as the CD63- or CD203c test are particularly rapid and can provide information about basophil responsiveness to an allergen within 60 min.

Acknowledgments

This study was supported by: Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich–FWF—Grant Nos. F018-01 and F-018-09

References

- [1] A.B. Kay, *Allergy and Allergic Diseases*, Blackwell Science, Oxford, UK, 1997.
- [2] R. Dattwyler, A.P. Kaplan, K.F. Austen, in: *Allergy*, first ed., Churchill Livingstone, New York, 1985, pp. 599–607.
- [3] R. Valenta, D. Kraft, *Curr. Opin. Immunol.* 7 (1995) 751–756.
- [4] R. Valenta, S. Vrtala, S. Laffer, S. Spitzauer, D. Kraft, *Allergy* 53 (1998) 552–561.
- [5] R. Valenta, D. Kraft, *Immunol. Rev.* 179 (2001) 119–127.
- [6] R. Valenta, W.R. Sperr, F. Ferreira, P. Valent, C. Sillaber, M. Tejkl, M. Duchenne, C. Ebner, D. Kraft, O. Scheiner, J. *Allergy Clin. Immunol.* 91 (1993) 88–97.
- [7] T. Ball, S. Vrtala, W.R. Sperr, P. Valent, M. Susani, D. Kraft, R. Valenta, *J. Biol. Chem.* 269 (1994) 28323–28328.
- [8] R. Valenta, J. Lidholm, V. Niederberger, B. Hayek, D. Kraft, H. Grönlund, *Clin. Exp. Allergy* 29 (1999) 896–904.
- [9] J. Bousquet, B. Lebel, H. Dhivert, Y. Bataille, B. Martinot, F.B. Michel, *Clin. Allergy* 17 (1987) 529–536.
- [10] L. Rasaneh, P. Kuusisto, M. Penttilä, M. Nieminen, J. Savolainen, M. Lehto, *Allergy* 49 (1994) 342–347.
- [11] V. Niederberger, P. Stübner, S. Spitzauer, D. Kraft, R. Valenta, K. Ehrenberger, F. Horak, *J. Invest. Dermatol.* 117 (2001) 848–851.
- [12] R. van Ree, R.C. Aalberse, *J. Allergy Clin. Immunol.* 103 (1999) 1000–1001.
- [13] C. Afferni, P. Iacovacci, B. Barletta, G. Di Felice, R. Tinghino, A. Mari, C. Pini, *Clin. Exp. Allergy* 29 (1999) 1087–1094.
- [14] P. Valent, P. Bettelheim, *Crit. Rev. Hematol. Oncol.* 10 (1990) 327–352.
- [15] A.M. Dvorak, *Int. Rev. Cytol.* 180 (1998) 87–236.
- [16] F.H. Falcone, H. Haas, B.F. Gibbs, *Blood* 96 (2000) 4028–4038.
- [17] T. Ishizaka, R. De Bernardo, H. Tomioka, L.M. Lichtenstein, K. Ishizaka, *J. Immunol.* 108 (1972) 1000–1008.
- [18] K. Ishizaka, T. Ishizaka, *Immunol. Rev.* 41 (1978) 109–148.
- [19] L.M. Lichtenstein, *J. Immunol.* 197 (1971) 1122–1130.
- [20] L.M. Lichtenstein, G. Marone, L.L. Thomas, F.J. Malveaux, *J. Invest. Dermatol.* 71 (1978) 65–69.
- [21] S. Vrtala, W.R. Sperr, I. Reimitzer, R. Ree, S. Laffer, W.D. Müller, P. Valent, K. Lechner, H. Rumpold, D. Kraft, R. Valenta, *J. Immunol.* 151 (1993) 4773–4781.
- [22] S. Vrtala, M. Susani, W.R. Sperr, P. Valent, S. Laffer, C. Dolecek, D. Kraft, R. Valenta, *J. Allergy Clin. Immunol.* 97 (1996) 781–787.
- [23] A.M. Dvorak, S.J. Galli, E.S. Schulman, L.M. Lichtenstein, H.F. Dvorak, *Fed. Proc.* 42 (1983) 2510–2515.
- [24] T. Ishizaka, K. Tomioka, K. Ishizaka, *J. Immunol.* 106 (1971) 705–710.

- [25] L.M. Lichtenstein, D.W. MacGlashan, J. Allergy Clin. Immunol. 77 (1986) 291–294.
- [26] C.L. Kepley, L. Youssef, R.P. Andrews, B.S. Wilson, J.M. Oliver, J. Immunol. 165 (2000) 5913–5920.
- [27] P. Valent, J. Besemer, M. Muhm, O. Majdic, K. Lechner, P. Bettelheim, Proc. Natl. Acad. Sci. USA 86 (1989) 5542–5546.
- [28] W.R. Sperr, H. Agis, H. Semper, R. Valenta, M. Susani, W. Sperr, M. Willheim, O. Scheiner, E. Liehl, K. Lechner, P. Valent, Int. Arch. Allergy Immunol. 114 (1997) 68–73.
- [29] P.W. Modderman, in: W. Knapp et al. (Eds.), Leukocyte Typing IV, Oxford University Press, 1989, p. 1042.
- [30] B.S. Bochner, J. Allergy Clin. Immunol. 106 (2000) 292–302.
- [31] P. Valent, G.-H. Scherthaner, W.R. Sperr, G. Fritsch, H. Agis, M. Willheim, H.-J. Bühring, A. Orfao, L. Escribano, Immunol. Rev. 179 (2001) 74–81.
- [32] E.F. Knol, F.P.J. Mul, H. Jansen, J. Calafat, D. Roos, J. Allergy Clin. Immunol. 88 (1991) 328–338.
- [33] P. Gane, C. Pequet, H. Crespeau, P. Lambin, F. Leynadier, P. Rouger, Cytometry 19 (1995) 361–365.
- [34] J. Sainte-Laudy, A. Sabbah, C. Vallon, J.C. Guerin, Inflamm. Res. 47 (1998) 401–408.
- [35] A. Pâris-Köhler, P. Demoly, L. Persi, B. Lebel, J. Bousquet, B. Arnoux, J. Allergy Clin. Immunol. 105 (2000) 339–345.
- [36] A.W. Hauswirth, S. Natter, M. Ghannadan, G.-H. Scherthaner, W.R. Sperr, H.-J. Bühring, R. Valenta, P. Valent, J. Allergy Clin. Immunol. 110 (2002) 102–109.
- [37] M. Willheim, H. Agis, W.R. Sperr, M. Koller, H. Kiener, G. Fritsch, W. Füreder, A. Spittler, W. Graninger, O. Scheiner, P. Valent, J. Immunol. Methods 182 (1995) 115–129.
- [38] B. Sihra, O. Kon, J. Grant, A. Kay, J. Allergy Clin. Immunol. 99 (1997) 699–706.
- [39] D. Maurer, E. Fiebiger, B. Reininger, B. Wolff-Winiski, M.H. Jouvin, O. Kilgus, J.P. Kinet, G. Stingl, J. Exp. Med. 179 (1994) 745–750.
- [40] H.-J. Bühring, P.J. Simmons, M. Pudney, R. Muller, D. Jarrossay, A. van Agthoven, M. Willheim, W. Brugger, P. Valent, L. Kanz, Blood 94 (1999) 2343–2356.
- [41] H.-J. Bühring, M. Seiffert, C. Giesert, A. Marxer, L. Kanz, P. Valent, K. Sano, Blood 97 (2001) 3303–3305.
- [42] I. Platz, M. Binder, A. Marxer, G. Lischka, P. Valent, H.-J. Bühring, Int. Arch. Allergy Immunol. 126 (2001) 335–342.
- [43] M. Binder, G. Fierlbeck, T.P. King, P. Valent, H.-J. Bühring, Int. Arch. Allergy Immunol. 129 (2002) 160–168.