



Isolation and characterization of allergen-binding cells from normal and allergic donors

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Abstract

Background: Flow cytometry of the immune system so far has been limited to the analysis of subpopulations according to lineage markers. The cells involved in a particular immune response could not be assayed due to their low frequency. Here we show the potential of antigen-specific high gradient magnetic cell sorting to enrich cells for visualisation in multiparameter cytometry, functional studies and immortalization. **Objectives:** The aim of this study was the development of an efficient technology for staining and isolation of antigen-binding cells from human peripheral blood. In particular, allergen-specific cells from normal and allergic donors should be analysed and compared to develop a cellular diagnosis of allergy. **Study design:** The rare antigen-specific cells were sorted by high-gradient magnetic cell sorting with MACS. Haptenized phospholipase A₂ (PLA₂), the major allergen of bee venom, or haptenized ParoI, the major allergenic component of *Parietaria officinalis*, were used as antigens. The cells from normal and allergic donors, binding to the allergen were characterized phenotypically by immuno-fluorescence. Allergen-specific B-cells were immortalized by EBV transformation. **Results and conclusion:** Allergen-specific cells can be enriched from blood of both allergic and normal donors to purities of up to 75%, by high gradient magnetic cell sorting. The specificity of labelling with allergen was confirmed by establishing allergen-specific EBV-transformed B-cell lines from the sorted cells. Clear differences exist in the cellular composition of allergen-binding cells from normal compared to allergic donors. In normal donors the allergen-binding cells are B-cells expressing CD19 and CD21. In allergic donors, in addition to allergen-binding B-cells, occurring in about equal absolute numbers as in normal donors, basophilic granulocytes are labeled by allergen. These cells express CD38, CD9 and CD25 on their surface, and stain for IgE.

Keywords: Allergen binding cell; *Parietaria officinalis*; Phospholipase A₂; Magnetic cell sorting; EBV-lines

Abbreviations: PBMC, peripheral blood mononuclear cells; PLA₂, phospholipase A₂; ParoI, *Parietaria officinalis* major allergen; IL, interleukin; EBV, Epstein-Barr virus; MACS, magnetic cell sorter.

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1. Introduction

Allergy or hypersensitivity of the immune system in its various forms (types I–IV) affects more than 20% of the human population. In immediate type hypersensitivity (type I), the allergic immune reaction is mediated by allergen-specific antibodies, mostly of the IgE class [1].

The antibodies can be directed against a variety of antigens, e.g., molecules from pollen, fungi, food, house dust mite, hymenoptera venoms or animal danders. Recently, some advance has been made in understanding the generation of allergen-specific IgE on the cellular and molecular level [2,3]. B-cells expressing allergen-specific IgM or IgG can be instructed to switch towards the expression of allergen-specific IgE by the cytokines, interleukin 4 and interleukin 13, expressed by Th-lymphocytes and other cells [4]. However, the prime event shifting an immune response to a domination by IL-4 and thus IgE, is still not known, as is the basis of allergic memory. The direct analytical and preparative assessment of cells expressing allergen-specific antibodies would greatly facilitate the further molecular and cellular analysis of the human allergic immune response and, in addition, provide a valuable diagnostic tool. Here we describe the use of allergens for specific immunofluorescent and magnetic labeling and the isolation of allergen-binding cells from human peripheral blood.

The antigens we have used are phospholipase A₂, the major allergen of bee venom [5], and *ParoI* the major allergen of *Parietaria officinalis* [6]. The allergens have been haptenized with digoxigenin for indirect staining of the cells with fluorochromated anti-dig antibodies and anti-dig superparamagnetic microbeads. Labeled cells are enriched by high-gradient magnetic cell sorting with MiniMACS. The sorted cells are directly submitted to immunocytometric multiparameter, single cell analysis. Drastic differences between cells from normal and allergic individuals are detected. Antigen-specific cytometric diagnosis can directly reveal the specific serological and cellular state of a given immune reaction. From the sorted allergen-specific B-cells, we also have obtained specific EBV-lines, expressing human monoclonal, allergen-specific antibodies.

2. Materials and methods

2.1. Antigens and preparation of conjugates

Phospholipase A₂ from bee venom was purchased from Sigma, Freiburg, Germany (P 9279). *ParoI* was purified as described elsewhere [7]. Both allergens were conjugated to digoxigenin (dig), using the DIG Antibody Labelling Kit from Boehringer Mannheim, Germany.

For the conjugation, 0.5–1.0 mg/ml of the protein were incubated with digoxigenin-succinimid ester at a final concentration of 50 µg/ml for 1 h at room temperature. Unbound digoxigenin was removed by separation over a PD10-column (Pharmacia Freiburg, Germany). Preparations were made at different molar ratios of protein to dig and tested out by titration on PBMC for their ability to stain specifically a distinct population of the PBMC but not monocytes or macrophages.

2.2. Cells

Blood was obtained from normal donors (blood bank of the University of Cologne), or from allergic patients with known history of severe allergy either against bee venom or *Parietaria*. Nearly all of the allergic patients had elevated levels of allergen-specific IgE serum antibodies. Patients allergic against bee venom all showed a positive reaction in skin tests performed to confirm the diagnosis.

2.3. Magnetic and fluorescent labeling of cells with antigen

The blood was diluted with two volumes of RPMI and aliquots of 30 ml were layered onto 15 ml of Ficoll-hypaque (Pharmacia, Freiburg, Germany). After centrifugation for 20 min at RT with 800 × g, cells of the interphase were collected and washed three times with cold phosphate-buffered saline (PBS). Cells were resuspended in a small volume of PBS/0.5% BSA/10 mM EDTA, and incubated for 10 min on ice with 5 mg/ml of the murine monoclonal antibody A20/44 (IgG₁) to block unspecific binding. Allergen-digoxigenin conjugates were added at 10 µg/ml and the cells were incubated for another 10 min. After washing with PBS/BSA/EDTA, the cells were then labeled for 10 min at RT with superparamagnetic microbeads coupled to anti-digoxigenin antibodies

(Miltenyi Biotec, Bergisch-Gladbach, Germany), resulting in a 1/5 dilution of microbeads to the cell suspension. Finally, the cells were stained with 1/50 volume of PE-coupled anti-digoxigenin antibodies, by incubation for 10 min on ice, and finally washed again. Prior to magnetic separation the cells were deaggregated by passage through a 40 μ m nylon-mesh (Partec, Münster, Germany) [8].

2.4. Antigen-specific magnetic cell sorting

For magnetic isolation of allergen-binding cells we used the MiniMACS-system, described in detail elsewhere [9] (Miltenyi Biotec, Bergisch-Gladbach, Germany). The columns were prewashed with 4 ml of degassed PBS/BSA. Half of the cell samples were loaded onto the column, the column was washed, and the other half of the cells was applied. Then the columns were washed with 5 ml PBS/BSA. To elute the bound cells, the columns were removed from the magnet and the cells washed off the matrix with 1 ml of PBS/BSA. The separation was controlled by flow cytometry, using a FACScan and FACScan Research or Cellquest software (Becton Dickinson, San Jose, CA). For cytometric analysis, 10 μ g/ml of propidium-iodide (PI) was added in order to identify dead cells, not only according to forward versus side scatter, but also according to relative F2 vs. F3 fluorescence [10].

2.5. Immunofluorescence

The following antibodies were used for immunophenotyping: α CD3, α CD5, α CD10, α CD14, α CD16, α CD19 [12–16] as fluorescein-isothiocyanate (FITC)- or phycoerythrin (PE)-conjugates, all obtained from Becton Dickinson. α CD9, α CD21, α CD23, α CD38 [17–20] all obtained from Immunotech, Dardilly, France as FITC-conjugates. The isotype-specific antibodies were all purchased as Biotin (Bio)-, or horseradish-peroxidase (HRPO)-conjugates α IgG₁-Bio, α IgG₄-Bio, α IgE-Bio, α IgG-HRPO, α IgM-HRPO, α IgE-HRPO, α κ -HRPO, α λ -HRPO were obtained from SBA, Birmingham, Alabama, USA; α Digoxigenin-beads from Miltenyi Biotec, Bergisch Gladbach, Germany; α Digoxigenin-PE was a generous gift from A. Thiel. Streptavidin-Cyochrome was purchased from Pharmingen, San Diego, USA.

2.6. Generation of EBV-lines

Feeder cells were generated from autologous PBMC by depletion of B-lymphocytes with α CD19-superparamagnetic beads by high gradient magnetic cell sorting with MACS using the A2 column (Miltenyi Biotec, Bergisch Gladbach, Germany) with a 0.45- μ m needle. The negative cells were incubated for 30 min at 37°C with 50 μ g/ml mitomycin C (Medac, Hamburg, Germany). To remove excess mitomycin before adding the feeder cells to the sorted cells, the prospective feeder cells were washed three times in 50 ml of RPMI, supplemented with 5% FCS. After the last washing step, T-lymphocytes were suppressed by adding 1 μ g/ml cyclosporin A (Sandoz, Basel, Switzerland), and cultured in the medium described below. EBV-lines were generated from the antigen-positive fractions of the magnetic sorts, by incubating 100–1000 cells per well in 96-well flat bottom plates (Costar, MA, USA) with 1/4 volume of supernatant of the EBV-secreting marmoset cell line B95-8 [21]. These cells were layered onto 2×10^5 autologous feeder cells per well, generated from PBMC as described above, cultured in RPMI with 10% FCS, 50 U/ml penicillin, 50 μ g/ml streptomycin, and 2 mM L-glutamine. After 2 weeks, aggregates presumably representing the progeny of single cells were picked under the microscope and diluted over a series of wells. For most of the lines generated this way, monoclonality was later confirmed by restriction analysis of JH-rearrangements.

2.7. Western slot blots

Different amounts of unconjugated PLA₂, or bovine serum albumin (BSA) as negative control, were blotted onto nitrocellulose-filters (Hybond C extra, Amersham, Braunschweig, Germany), using the micro-sample-filtration manifold (Schleicher-Schuell, Dassel, Germany). After washing with PBS, the filters were blocked for 1 h with Trizma-Base, NaCl, pH 7.6, containing 3% Tween 20 (TBS) (Serva, Heidelberg, Germany), and 5% of dry milk powder. The culture supernatants of the EBV-lines generated were deaggregated and sterilized by filtration, diluted with one volume of TBS/Tween/dry milk powder and applied to the filters. After incubation for 1 h, filters were washed 3 times for 5 min in TBS/Tween20. Then the

filters were incubated with HRPO-conjugates of antibodies, specific for the various human isotypes and diluted in TBS/Tween/dry milk, for 40 min at RT. After washing as described before, filter-bound HRPO was quantitated using the ECL-System (Amersham, Braunschweig, Germany). Autoradiographs were obtained on X-OMAT-AR-films (Eastman-Kodak, New York, USA).

2.8. DARIA (double antibody radioimmunoassay)

The DARIA was essentially carried out as described elsewhere [22]. For the determination of *ParoI*-specific human IgE or IgG, 50 μ l of super-

natant from EBV-lines were mixed with 50 μ l of an appropriate dilution of an IgE or IgG myeloma protein. This solution was incubated for 4 h at room temperature with 100 μ l of borate-buffered saline (BBS, pH 8.0) containing 1 ng of radiolabeled *ParoI* (10–20 000 counts/min). Antigen-antibody complexes were precipitated by addition of an appropriate amount of goat anti-human IgE or IgG for 16 h at 4°C. Precipitates were collected on Whatman GF/A filters. After washing the filters in 10 ml of BBS, the bound radioactivity was determined in a gamma counter. Amounts of antibodies were expressed as: $((a - b)/(B_{\max} - b)\%)$,

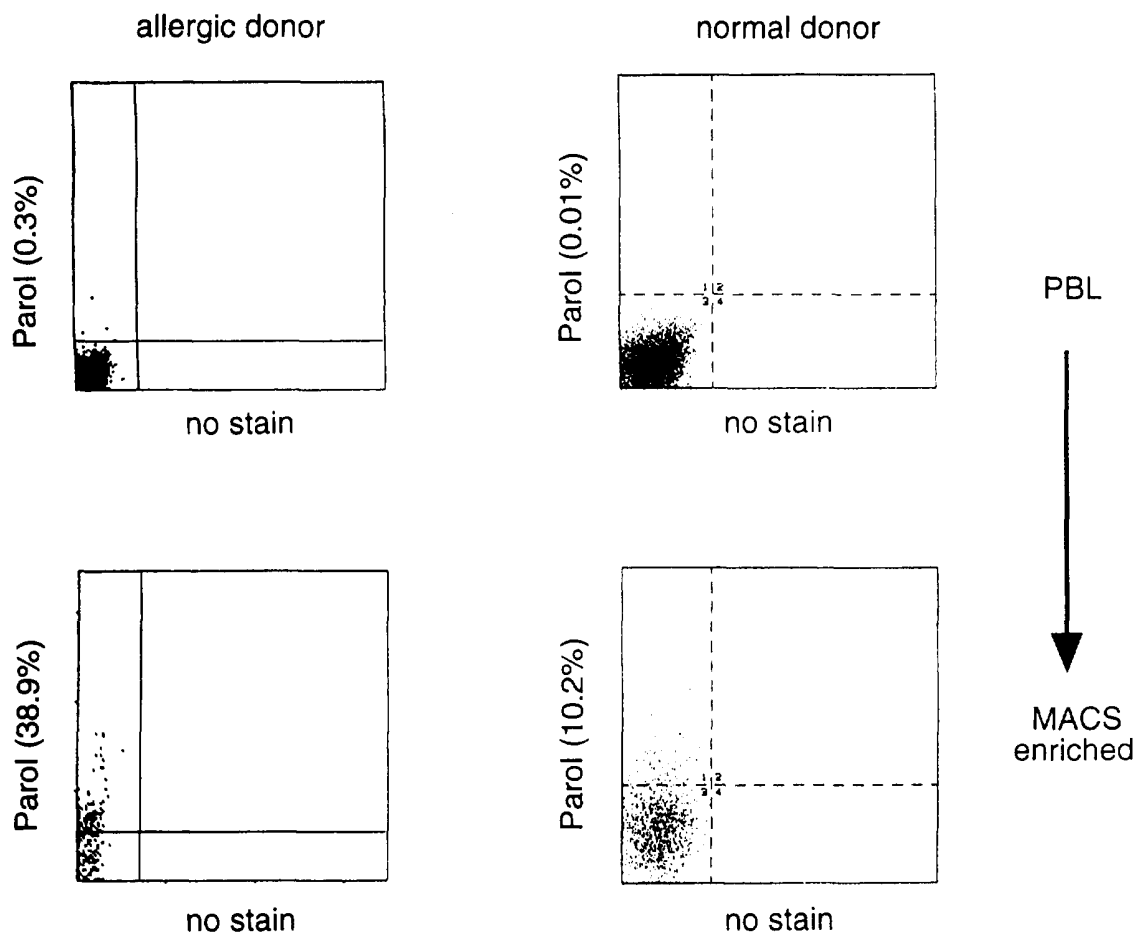


Fig. 1. Enrichment of *ParoI*-binding cells from PBL of a normal and an allergic donor with the MiniMACS. The specific cells were tagged with digoxigenized *ParoI* and labeled with anti-dig-microbeads and anti-dig-PE.

where a = counts/min bound by the tested supernatant, b = background counts/min (counts/min bound by a pool of sera without IgE against *ParoI*) and $B_{\max}$ = counts/min bound by the positive reference serum pool (a pool of sera with high levels of *ParoI*-specific IgE). Supernatants binding less than $1.3 \times$ background counts/min were considered negative.

2.9. May-Gruenwald/Giemsa staining

After MACS-separation the fractions enriched for allergen-binding cells were resuspended in 300 μ l of RPMI1640 supplemented with 30% FCS and collected by centrifugation for 10 min at $400 \times g$

at 4°C. The pellets were resuspended in 100 μ l RPMI/30% FCS. Cytospins were prepared in a Cytospin centrifuge (Shandon-Elliott), spinning the cells onto the slides at 1000 rev./min for 3 min. The cytospin preparations were air-dried overnight at room temperature. The slides were then incubated for 5 min in May-Gruenwald solution (Merck, Darmstadt, Germany), followed by incubation in a 1:1 dilution of May-Gruenwald: Weisebuffer (Merck Darmstadt, Germany) for another 5 min. The final staining step was carried out by immersing the slides in a 1:10 dilution of Giemsa solution (Merck Darmstadt, Germany) in water for 15 min. The slides were then washed in

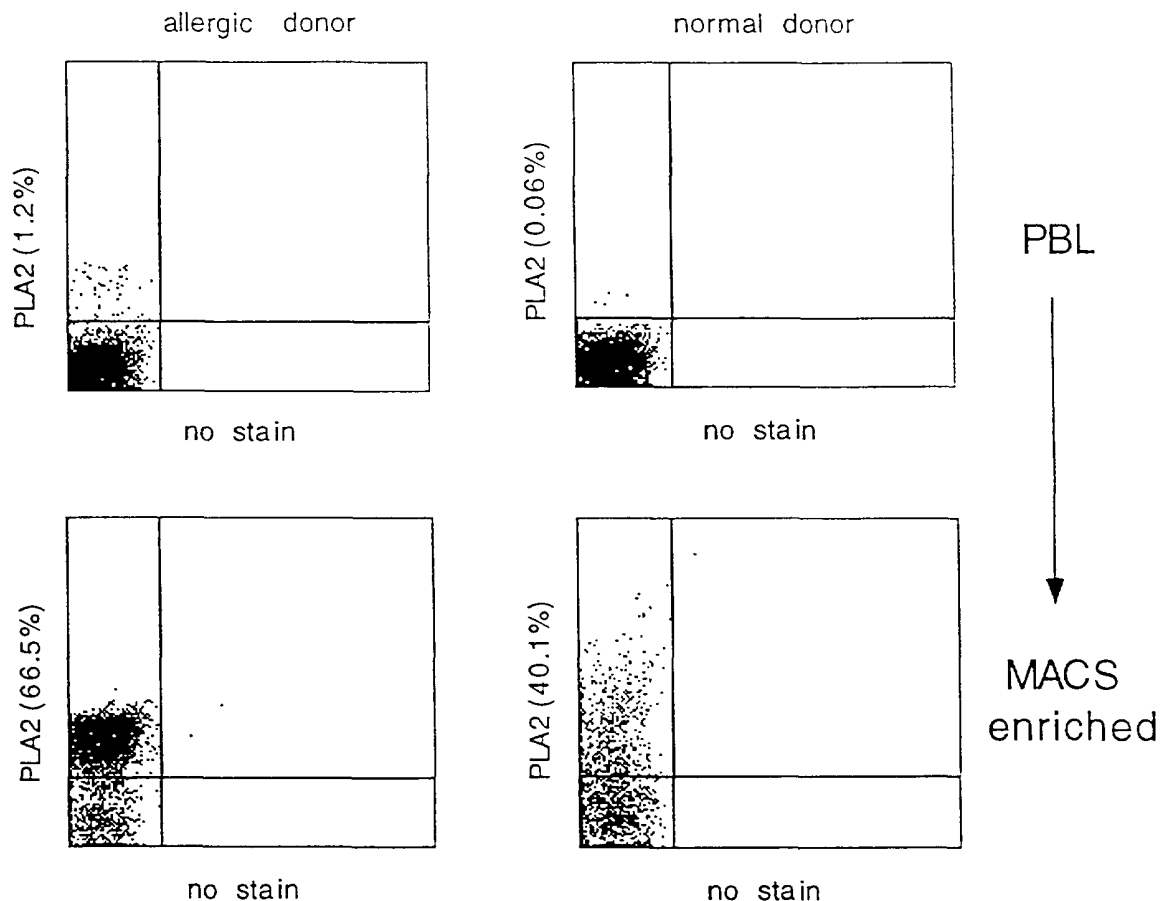


Fig. 2. Enrichment of PLA₂-binding cells from PBL of a normal and an allergic donor with the MiniMACS. The specific cells were tagged with digoxigenized PLA₂ and labeled with anti-dig-microbeads and anti-dig-PE.

distilled water and air dried for 30 min. Finally, the preparations were embedded in Eukitt (Kindler Freiburg, Germany), covered with a cover glass and analyzed in a Zeiss Axiolab microscope.

3. Results

3.1. Enrichment of allergen-binding cells

For the isolation of allergen-binding cells from peripheral blood (50 ml from allergic donors or 500 ml from normal donors) the blood was depleted of erythrocytes, neutrophils and

eosinophils on a Ficoll-gradient. The mononuclear cells obtained that way were stained with digoxigenin-conjugates of either PLA₂ or ParoI, anti-dig-MACS microbeads and anti-dig-antibodies conjugated to phycoerythrin. As determined by flow-cytometry, the frequency of allergen-binding cells was below 0.1% in the blood of normal donors (15 sortings), whereas samples from allergic donors contained between 0.4 and 2.3% of allergen-binding cells (10 sortings) before MACS-separation. The percentages of allergen-binding cells before MACS were calculated by acquiring 100 000 events with the FACScan. The

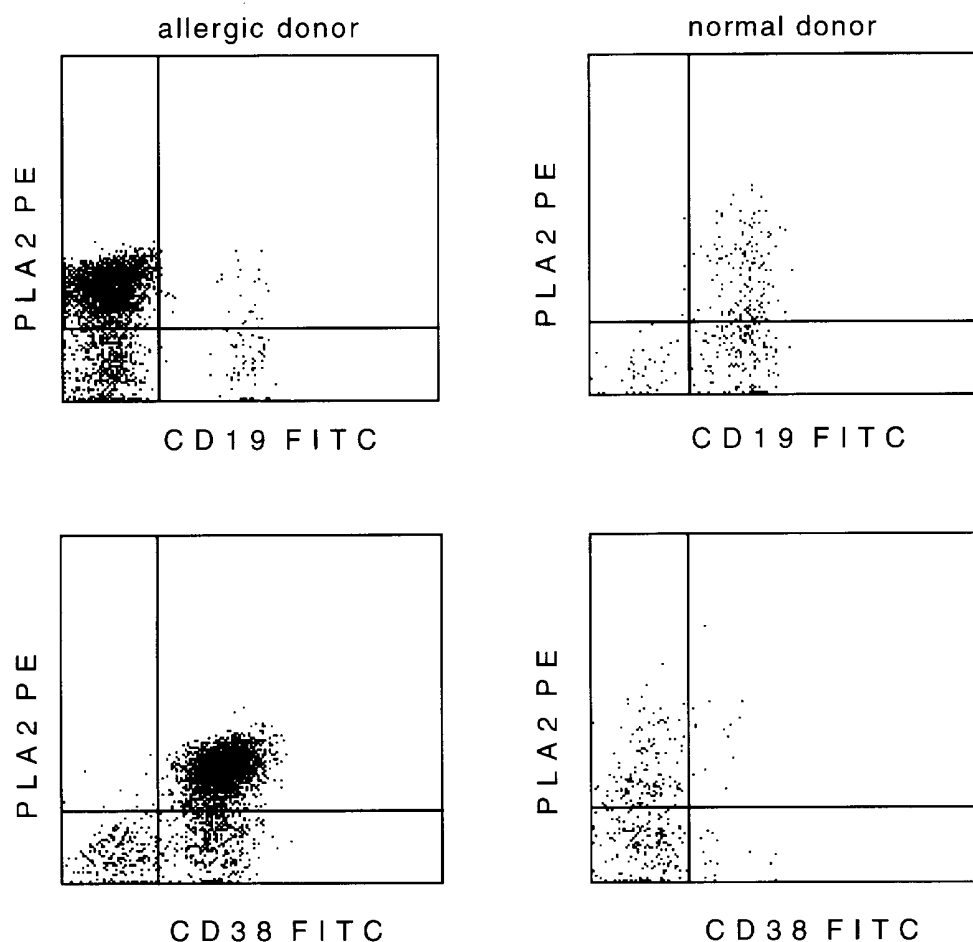


Fig. 3. Counterstaining of the MACS-enriched PLA₂-binding cells from an allergic and a normal donor with antibodies directed against a B-cell marker (CD19) or against a marker for basophilic granulocytes and plasmacells (CD38).

allergen-binding cells were enriched by high gradient magnetic cell sorting with the MiniMACS to frequencies of up to 75% (allergic donors) and 2–45% (non-allergic donors) (Figs. 1,2). From some allergic donors (50 ml of blood) up to 8×10^5 allergen-binding cells, mostly basophilic granulocytes and 3–11% B-cells, were enriched, whereas from the blood of normal donors at the most 6×10^4 allergen-binding cells per 50 ml of blood were enriched, nearly all of them B-lymphocytes. The exact determination of frequencies is difficult due to the fact that the allergen-binding cells, especially those from normal donors, display considerable heterogeneity of labeling and thus some of the cells of the positive population may overlap with the negative population.

3.2. Phenotype of the allergen-binding cells

The indirect staining of the digoxigenized allergen with anti-dig-PE was used to monitor the magnetic cell sortings, it also allowed the phenotypical analysis of the sorted allergen-binding cells by multiparameter flowcytometry. The cells were counterstained with fluorescein-, and Cychrome-conjugated antibodies against various surface-markers, with the remaining PE-negative cells serving as an internal control. The allergen-binding cells from both, normal and allergic donors, did not express CD3, CD14 or CD16, i.e., markers for T-cells, monocytes or NK-cells (data not shown). The frequency of labeled cells was very low, and cell types known for their ability to bind fluorochromed conjugates unspecifically,

like monocytes, were not enriched by MACS. The specificity of labeling was proven further by the generation of allergen-specific B-cell lines from the sorted cells, as described later. A drastic difference was observed when comparing allergen-binding cells from blood of allergic and non-allergic donors, with regard to expression of CD19, a B-cell marker, and CD38, a marker of plasma cells and basophilic granulocytes. The absolute numbers of allergen-binding CD19⁺ B-cells are about the same. However, about 90% of the allergen-binding cells enriched from the blood of normal donors, but only 3–12% from allergic donors are CD19⁺ B-cells (Fig. 3). Almost all of these cells also express CD21, the receptor for EBV, making them a suitable target for transformation and thus for the generation of allergen-specific B-cell lines. These results are summarized in Table 1.

The majority of the enriched allergen-binding cells from blood of allergic donors are cells staining for CD38, a marker expressed mostly by plasma cells and basophilic granulocytes. Besides CD38, these cells express CD25 on their surface and stain brightly for CD9, a marker of pre B-cells and basophilic granulocytes. These cytometric analyses, together with the May-Gruenwald/Giemsa staining, clearly identify these cells as basophilic granulocytes. They make up to 72–95% of the enriched, allergen-binding cells (Fig. 3, Table 1), while in normal blood donors only a few of these cells are observed. The specific antigen-receptor of basophilic granulocytes most likely is

Table 1

Percentages of PLA₂-binding cells from blood of normal and allergic donors before and after enrichment with MACS

	Normal donor				Allergic donor			
	1	2	3	4	ZI	ET	BO	GA
Before MACS	0.02	0.1	0.03	0.08	1.89	1.75	0.68	0.36
After MACS	12.2	18.2	4.7	45.8	92.2	44.8	60.0	23.8
CD 38	3.0	9.1	12.1	9.6	93.2	72.0	90.9	82.0
CD 19	95.1	82	78.5	97.7	2.8	11.8	7.5	11.3
19/21	90.0	81.2	71.5	95.3	2.6	12.1	6.2	10.7

For phenotypical analysis the PLA₂-specific cells were counterstained with anti-CD38-FITC or anti-CD19-FITC. The results of a three-colour analysis, staining the cells with PLA₂-PE, anti-CD19-Cychrome and anti-CD21-FITC are also shown.

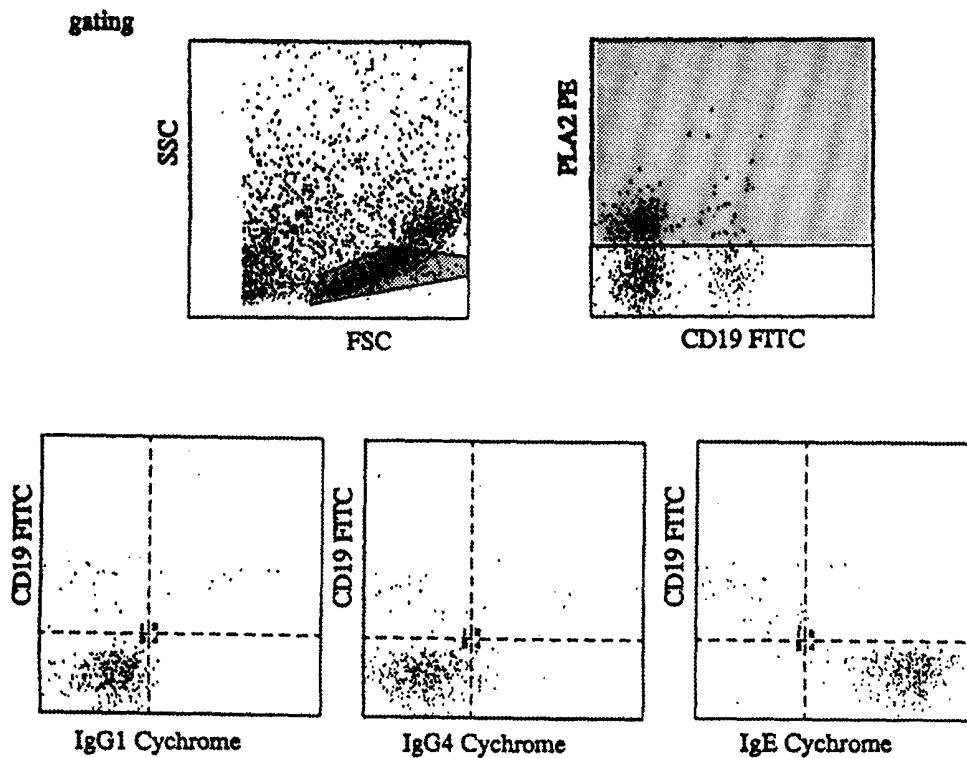


Fig. 4. Phenotypic analysis of PLA₂-binding cells from an allergic donor. Positive cells are labeled with PLA₂-dig and stained with anti-dig-PE. Counterstainings are performed with anti-CD19-FITC and Cychrome-labeled anti-isotype antibodies. The first gate was set excluding dead cells and monocytes. The second gate was set on the PLA₂-binding, PE-stained cells. Gated cells were analyzed for surface immunoglobulin.

Fc-receptor-bound immunoglobulin. Accordingly, nearly all of the allergen-binding CD38⁺ cells stain for surface IgE, some also for IgG₄, but none for IgG₁ (Fig. 4). From some sorts of cells from non-allergic donors, the enriched allergen-binding cells were submitted to EBV-transformation. We established three EBV-lines producing IgG, and two lines producing IgM specific for PLA₂, as determined in a Western Blot (Figs. 5 and 6). Several lines producing *ParoI*-specific antibodies could be established from non-allergic donors. Their specificity could be determined in a *ParoI*-specific double antibody radio immunoassay (DARIA) (Table 2). Out of 25 EBV-lines tested, six produced *ParoI*-specific IgE, and four EBV-lines produced different IgG-subclasses.

4. Discussion

Resolution of flow cytometric analysis so far has been largely restricted to discrimination of discrete cell lineages. The most important parameter of immune reactions, antigen specificity, has not been available for analysis, although the reacting, specific cells probably behave different from the cells of other specificity in the same subpopulation and it is the specific reaction that is the real aim of cellular analysis. For clinical diagnosis, antigen-specific cytometry would add valuable information on the cells and molecules involved in the pathology of the immune response, like allergy, arthritis, infections or autoimmune-reactions. While T-cells recognize antigen in a rather com-

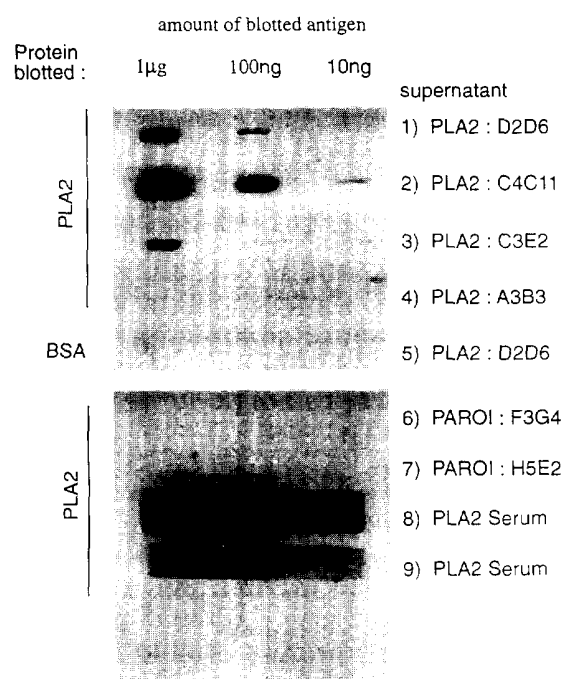


Fig. 5. Western slot blot analysis of the supernatants of EBV-clones. Different amounts of PLA₂ or BSA (as negative control) were blotted. The filters were incubated with the supernatants of the clones generated from a PLA₂-specific sorting (lanes 1–5), or from a sorting with *ParoI* (lanes 6 and 7). Lanes 8 and 9 were incubated with serum from patients with severe allergy against bee venom. The filters were developed with an antibody specific for human IgG.

plex form, as peptides associated with MHC molecules, B-cells directly recognize the native antigen through specific antibodies. Thus, in principle, the labeling of B-cells with antigen should be feasible. In practice, however, the direct analysis of B-cells stained with a particular antigen is hardly impossible due to their low frequency among cells in, e.g., peripheral blood.

Here we describe the enrichment of the rare, antigen-binding cells by high gradient magnetic cell sorting with MACS to purities of up to 75%, which enables the phenotypic, genotypic and functional analysis of these cells. The MACS technology has already proven its power in the sorting of other types of rare cells like CD34⁺ cells, fetal cells from maternal blood and B-cells expressing

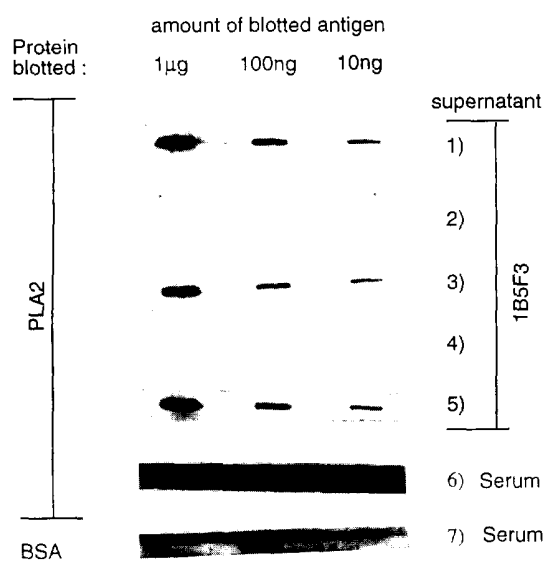


Fig. 6. Western slot blot analysis of the supernatant of a EBV-clone. Different amounts of PLA₂ or BSA (as negative control) were blotted. The filters were incubated with the supernatant of a clone generated from a PLA₂-specific sorting (lanes 1–5). Lanes 6 and 7 were incubated with the serum from a patient with severe allergy against bee venom. The filters were developed with antibodies of different specificities: lanes 1,6,7, anti-κ/anti-λ; lane 2, anti-λ; lane 3, anti-κ; lane 4, anti-IgG; lane 5, anti-IgM.

Table 2
Result of the DARIA analysis using supernatants of EBV-lines from a *ParoI*-specific sorting

EBV-line supernatant	<i>ParoI</i> -specific IgE
<i>ParoI</i>	
E2C3	58 ^a
G4B8	35
F2F6	49
E2D3	32
G2E10	32
F2G7	34
H3G3	Negative
G4D7	Negative
F3D5	Negative
H3E5	Negative
F3E4	Negative
PLA ₂	
A3G3	Negative

^aThe values are given as percentages of the values obtained using a 1/10 dilution of a standard serum with high level of *ParoI*-specific immunoglobulin.

rare isotypes on their surface [8–11]. In this study, we used the antigens PLA₂ and *ParoI*, haptenized with digoxigenin, to label B-lymphocytes and antibody-armed effector cells specifically. These antigens are prominent allergens and available in purified form. Blood samples from patients allergic against one or the other allergen were available. A crucial point in the evaluation of the usefulness of a given technology is the proof of specificity of labeling. In the present experiments we have shown that the B-cells are labeled specifically by generating allergen specific EBV-lines from the sorted cells. The frequency of specific EBV-lines generated corresponds roughly to the frequency of allergen-binding CD19⁺ B-cells in the MACS sorted population. We tested a panel of cytokines and polyclonal activators including IL2, IL4, IL6, SAC, PHA and others, to prevent breakdown of secretion which had been a problem for EBV-lines previously (data not shown). When cultured in the presence of 100 U/ml IL-2 [23], these cells stably secrete the specific antibodies for more than 4 months. A relatively high number of EBV-clones specific for *ParoI* are typed as IgE, although the cells have been sorted from the blood of a presumably non-allergic donor. However, the specificity of this isotype-classification typing will have to be confirmed. In the case of the PLA₂-specific EBV-clones, the antibodies produced are either IgM or IgG, which had been expected, since those cells were derived from the blood of a non-allergic donor. The EBV-lines not only serve as specificity control, but obviously also provide allergen-specific human monoclonal antibodies, which can be used as reference reagents and for studies of repertoire and B-cell biology. The B-cells, too, will be valuable tools to unravel the role of specific B-lymphocytes in antigen presentation. In the blood of allergic individuals, in addition to B-lymphocytes, the allergen-labelled basophilic granulocytes and perhaps also plasma cells, both which have been reported to express CD38, are detectable. The fact, that allergen-binding CD38-positive cells are only present in allergic patients is in itself suggesting that the labelling of these cells by allergen is specific and occurs via FcER-bound allergen-specific IgE, arming the basophilic granulocytes. Whether or not plasmablasts had been

labeled by allergen is not clear now. Among the CD38⁺ cells, some very few had the histological appearance of plasmablasts. However they were by far outnumbered by the granulocytes. Until now, we have not typed or enumerated them further. Donohoe et al. stained IgE-positive cells from normal and allergic donors, and describe these cells as highly differentiated B-cells, but it is not clear whether those are cells secreting the immunoglobulins or not [24]. A new technique will be helpful to answer this question allowing to stain and sort live cells according to the secreted products [25]. Apart from B-lymphocytes, basophilic granulocytes and perhaps plasmablasts, no other cell types stained for the allergen. This is in contrast to the results published by Oshiba et al. who enriched about 12% T-cells together with antigen-specific B-cells, by using tetanus toxoid as antigen, coupled to magnetic beads [26]. It seems, however, hard to understand how a T-cell could specifically, via TcR, bind to an antigen that is not processed and presented in MHC context. The aim of the present study was to adopt MACS technology to the enrichment of antigen-specific leukocytes to facilitate antigen-specific cytometry of these rare cells. In a reproducible fashion we succeeded to enrich cells binding to a particular antigen, from below background to frequencies of up to 75%. In some of the enriched populations of the present study, the allergen-binding B-cells have been phenotyped as CD19⁻ and CD21⁺. In the blood of allergic and non-allergic individuals, equal absolute numbers of allergen-binding B-cells were present, most of them expressing IgM, some IgG₁ and only very few IgG₄ or IgE.

In blood of allergic, but not in blood of non-allergic individuals, we found a relatively high number of CD38-expressing antigen-binding cells. These cells could also be stained with CD9 and weakly with CD25, and according to staining with May-Gruenwald and Giemsa appear to be basophilic granulocytes. The specific staining and enrichment of basophilic granulocytes with a particular antigen requires that the number of FcR-bound immunoglobulins exceeds 10 000 molecules per cell, i.e., a considerable proportion of the FcR-bound immunoglobulin has one particular specificity in these individuals. Most of that im-

munoglobulin is IgE. Some cells might also have specific Fc-receptor-bound IgG₄, but none IgG₁.

The clear staining of basophilic granulocytes offers a new way of diagnostic monitoring of allergy. The cellular analysis of the reacting effector cells has clear advantages over the commonly used provocation tests, since it does not have the side effects and risks for the patients. It provides detailed information about the cells involved in that particular immune reaction.

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