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Diclofenac induces basophil degranulation without increasing CD63 expression in sensitive patients

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Summary

Diclofenac (Dc) induces an IgE-independent basophil (Ba) degranulation in susceptible individuals. CD63 Ba expression is utilized as an *in vitro* test for diagnosis of drug hypersensitivity. We tested the ability of Dc to induce CD63 Ba expression by flow cytometry (BAT) and Ba degranulation using light microscopy (HBDT) in patients sensitive to Dc. We studied 14 patients with diclofenac hypersensitivity, also two patients sensitive to *Dermatophagoides pteronyssinus* (Dp), and 12 normal controls. HBDT was performed by mononuclear cells toluidine blue staining. BAT determined CD63 expression in antiCD63/anti-IgE/anti-CD45-labelled whole blood. In each case, the percentage of activated Ba post-stimulation with 1 and 10 μg/ml Dc was determined. Positive controls included N-formyl-methionyl-leucyl-phenylalanine (fMLP) peptide-induced activation. IgE-mediated Ba activation was induced with a Dp allergenic extract. With Dc 1 μg/ml, mean HBDT in Dc-susceptible individuals was 33·62 ± 18·35% and 8·49 ± 4·79% in controls ($P = 0·0001$). Mean BAT was 2·04 ± 1·68% and 1·93 ± 1·40% in controls ($P = 0·8$). Ba preincubation with Dc did not affect fMLP-induced CD63 expression, neither in Dc-sensitive individuals ($P = 0·8$) ($n = 4$) nor in subjects without Dc hypersensitivity ($P = 0·25$) ($n = 4$). Ba from the two patients sensitive both to Dc and Dp responded to Dp but not to Dc by BAT: Dc, 1·99 ± 0·78%; Dp: 60·87 ± 9·28%; but showed degranulation by HBDT: Dc, 30·53 ± 1·02%, Dp: 48·78 ± 22·17%. Dc induces Ba degranulation in sensitive patients in a way that does not induce CD63 expression and is different from IgE-mediated and fMLP-mediated degranulation. Our results suggest that CD63 expression is not a reliable diagnostic method for diclofenac allergy.

Keywords: allergy, basophil, CD63, exocytosis, NSAIDs

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Introduction

Severe adverse drug reactions account for 5% of hospital admission [1], affects 6·7% of already hospitalized patients and contributes to death in 0·32% patients [2]. Hypersensitivity comprises approximately 10% of all adverse drug reactions.

Drugs induce hypersensitivity through basophils (Ba) and mast cells activation by at least two methods. One is driven by an IgE-dependent immune mechanism and the second is due to a non-immune process, leading to degranulation without IgE intervention [3–5]. The first is known as 'allergic' and the latter as 'pseudoallergic' or 'anaphylactoid'. Clinical features of both adverse drug reactions are similar whatever mechanism of Ba activation is triggered.

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently used drugs that may promote an anaphylactoid response [6,7]. Nowadays, the gold standard for identification of NSAID-sensitive patients is based on placebo-controlled provocation challenge with increasing doses of the drug [5–8]. *In vitro* diagnosis of pseudoallergic adverse drug reactions is still a challenge, as available tests have low positive and negative predictive values or are difficult to reproduce [6,7].

In vitro demonstration of human Ba activation has been proposed as a surrogate marker of *in vivo* hypersensitivity. It can be determined by light microscopy (human Ba degranulation test: HBDT) based on the disappearance of methachromatically stained Ba cytoplasmic granules after exposure to a specific allergen or drug [9]. The reliability of

this method relies on obtaining good enrichment of these cells, constituting less than 1% of peripheral blood leucocytes [5,9]. Ba activation can also be quantified by measuring histamine [10] and/or cysteinyl–leukotriene release [11].

Since the description of CD63 antigen it has been possible to monitor human Ba activation by flow cytometry. CD63 is detected mainly on membranes of the cytoplasmic granules and is expressed on the Ba surface after degranulation induced by IgE-mediated stimulation or after challenge with the N-formyl-methionyl-leucyl-phenylalanine (fMLP) peptide [12]. Flow cytometry is a valuable tool for identifying and analysing a large number of cells from a mixed cell population, even when they are represented in low numbers in the original mix. Identification of Ba is based both on expression of the common leucocyte antigen CD45 [13] and on the presence of IgE on the cell surface, a surrogate marker of the high-affinity receptor for IgE (FcεRI). In this defined population, cell activation can be assessed before and after allergen or drug challenge by the increased expression of CD63 antigen on the plasma membrane [14].

Over the past 10 years a number of studies on Ba activation tests (BAT) have been published which focused on their use as a diagnostic tool in IgE-mediated hypersensitivity reactions to various allergens and drugs [15]. BAT is based on flow cytometry quantification of allergen-induced CD63 expression. Increase in CD63 expression in true allergic responses have been correlated positively with positive skin prick tests [16], IgE-specific detection [17–19] and histamine and leukotriene C4 (LTC4) release [20,21]. However, the usefulness of BAT in the diagnosis of non-IgE-mediated reactions, as in the case of NSAIDs sensitivity, remains to be clarified [3,17].

In the present work we tested the ability of diclofenac (Dc) to induce CD63 Ba expression by BAT and Ba degranulation using HBDT in patients sensitive to this NSAID and compared these findings with those obtained with *Dermatophagoides pteronyssinus* (Dp) in dust mite allergic patients and in a control group without adverse reactions to this drug.

Materials and methods

Drugs

Dc for intravenous (i.v.) use at final concentration of 1 and 10 μg/ml (Baliarda, Buenos Aires, Argentina), according to the plasmatic C_{max} of a therapeutic dose [22]. Preliminary dose–response assays showed that these concentrations did not trigger non-specific Ba activation. fMLP was obtained from Sigma and used at a final concentration of 10^{-6} M.

Allergenic extract

Aqueous sterile filtered standardized extract of Dp with a 10-μg/ml Der p1 concentration at a final dilution of 1 : 200. (Alergo Pharma, Buenos Aires, Argentina).

Table 1. Antecedents and symptoms of studied patients.

Patient	Antecedents	Symptoms
1. FS	Polyps, rhinitis, asthma	Asthma exacerbation
2. EM	None	Urticaria, angioedema
3. MM	Polyps, rhinitis, asthma	Asthma exacerbation
4. BM	Polyps, rhinitis, asthma	Asthma exacerbation
5. DC	Rhinitis	Urticaria, angioedema
6. CL	None	Anaphylactic reaction
7. MP	Asthma	Urticaria, angioedema
8. RS	Asthma	Anaphylactic reaction
9. MF	Rhinitis, asthma	Angioedema
10. HN	Polyps, rhinitis, asthma	Asthma exacerbation
11. SR	Polyps, rhinitis, asthma	Angioedema, rhinitis, asthma exacerbation
12. GL	Rhinitis	Angioedema
13. HB	Rhinitis	Angioedema
14. FS	None	Angioedema

Patients

Fourteen patients with typical clinical features of an immediate adverse reaction after Dc exposure (Table 1), including two patients also Dp-sensitive, and a control group of 12 subjects without a history of an adverse reaction to NSAIDs, including three with Dp allergy, were studied. Patients were studied between 3 and 6 months after the last episode of NSAID sensitivity. The diagnosis of allergy to dust mite was based on a positive skin prick test.

All included subjects were informed about the study and agreed to participate. The study was approved by the Ethics Committee of the Unidad de Alergia, Asma e Inmunología Clínica.

Ba stimulation assays

Blood specimens were collected from patients using heparin-containing tubes and stored at 4°C for a maximum of 4 h. Peripheral blood mononuclear cells (PBMC) for HBDT or whole blood cells for BAT were incubated for 15 min at 37°C with: (a) Dc, final concentration 1 μg/ml; (b) Dc, final concentration 10 μg/ml; (c) Dp, final dilution 1 : 200; (d) fMLP (Sigma), final concentration 10^{-6} M used as a positive control; and (e) phosphate-buffered saline (PBS) solution as a negative control. The reaction was stopped by placing the tubes in an ice bath.

HBDT

PBMC were isolated by differential centrifugation on Ficoll-Hypaque (Pharmacia) from 12 ml of venous blood diluted 1 : 2 with PBS solution, washed twice with PBS and suspended in PBS. Ba stimulation assay was performed on aliquots of PBMC. Cells were stained with toluidine blue and Ba with metachromatic-stained granules were counted in a Neubauer chamber. At least 50 Ba were counted from the

negative control tube in a predefined area of the chamber. The number of Ba in the same area of the chamber was then scored and the percentage of Ba degranulation was calculated according to the following formula: 1 – stimulated number of Ba/control number of Ba × 100. The threshold for positivity was determined by control, mean + 2 standard deviations (s.d.) (1 μg/ml: 8·49%, s.d. 4·79; 10 μg/ml: 12·24%, s.d. 8·73).

BAT

Ba stimulation assay was performed on aliquots of 90 μl of whole blood cells. After stimulation, as described above, cells were washed with PBS and incubated for 30 min at 4°C with biotinylated anti-human IgE (Vector, California, USA), washed and incubated for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-human CD63 (Caltag, California, USA), phycoerythrin (PE)-conjugated streptavidin (Vector) and phycoerythrin-cyanin-5 (PECy5)-conjugated anti-human CD45 (Cytognos, Salamanca, Spain). Erythrocytes were removed, adding 2 ml of lysing solution (Quicklysis; Cytognos). Cells were left for 10 min at room temperature, washed with PBS, resuspended in FACS-Flow (Becton Dickinson, California, USA) and analysed within 1 h with a fluorescence-activated cell sorter (FACScan; Becton Dickinson) using CellQuest software. Acquisition was performed on at least 200 Ba for each sample. Initial gating (R1) of cells was performed using forward scatter (linear scale) and side scatter (linear scale) around the lymphocytes. The second gate (R2) was set around $CD45^{dim}$ IgE^{bright} cells on a dot-plot of R1 displaying anti-IgE *versus* anti-CD45 fluorescence (logarithmic amplification). A logic gate (R1 and R2) was defined to evaluate the surface CD63 expression. The threshold for positivity was determined by control, mean + 2 s.d. (1 μg/ml: 1·93%, s.d. 1·40; 10 μg/ml: 1·90%, s.d. 2·06). The results were expressed as a percentage of Ba $CD63^+$.

Ba recovery after the assay

The number of Ba IgE^+ $CD45^+$ present for every 50 000 $CD45^+$ cells in R1 was determined for each sample after PBS or drug incubation.

Dc effect on fMLP and Dp response

Ba were preincubated for 15 min at 37°C with Dc (1 μg/ml final concentration) and followed subsequently by a further 15 min incubation with fMLP 10^{-6} M or Dp (dilution 1 : 200); HBDT and BAT were then performed.

Statistical analysis

Results were expressed as mean values ± s.d. Groups were compared with a two-tailed Student's *t*-test using GraphPad PRISM program. A *P*-value less than 0·05 was considered significant.

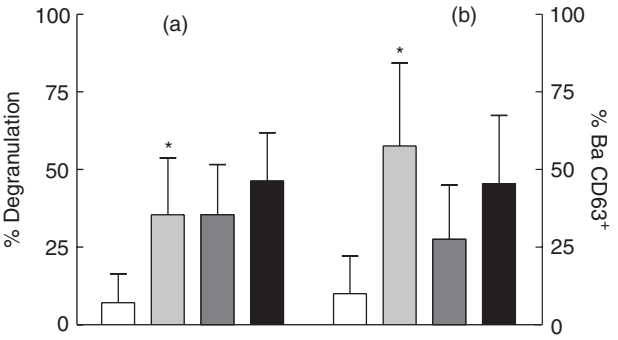

Fig. 1. Results of basophil (Ba) activation by human Ba degranulation test (HBDT) (a) and Ba activation test (BAT) (b) after challenge with a *Dermatophagoides pteronyssinus* (Dp) allergen extract and the N-formyl-methionyl-leucyl-phenylalanine (fMLP). Whole blood from Dp-sensitive patients (grey bars, $n = 5$) and controls (white bars, $n = 3$) were stimulated with Dp diluted 1/200 and incubated 15 min at 37°C. The response to fMLP (10^{-6} M) of Ba from all allergic subjects (black bars, $n = 17$) and controls (striped bars, $n = 9$) included in this work was also compared. Values represent mean ± s.d. *$P = 0{\cdot}05$ compared to Dp non-sensitive controls.

Results

HBDT and BAT comparably detect Dp- and fMLP-induced Ba activation

In a first step, experiments were performed on both allergic and healthy individuals in order to compare Ba activation using HBDT and BAT. We evaluated Dp response on five Dp-sensitive patients and three controls. Figure 1 shows mean Ba degranulation by HBDT and $BaCD63^+$ by BAT after Dp stimulation. There were significant differences between control and patient responses (HBDT: $P = 0{\cdot}03$; BAT: $P = 0{\cdot}03$) but not between methods (controls: $P = 0{\cdot}82$; patients: $P = 0{\cdot}33$). We also compared the results using fMLP as stimulus on Ba from all the individuals included in this study. The response was highly variable among subjects. No significant difference was found between HBDT and BAT (controls: $P = 0{\cdot}35$; patients: $P = 0{\cdot}91$). Allergic individuals showed a higher but no significant response to fMLP compared with controls.

Dc induces HBDT Ba degranulation but does not increase BAT CD63 expression in Dc sensitive patients

With 1 μg/ml of Dc, 12 of the 14 patients studied showed a positive HBDT but only one patient had a very weak positive BAT. With 10 μg/ml of Dc, eight of 12 patients studied showed a positive HBDT and only one of 13 patients studied had a weak positive BAT. Mean percentages of HBDT Ba degranulation in 14 Dc-sensitive patients and

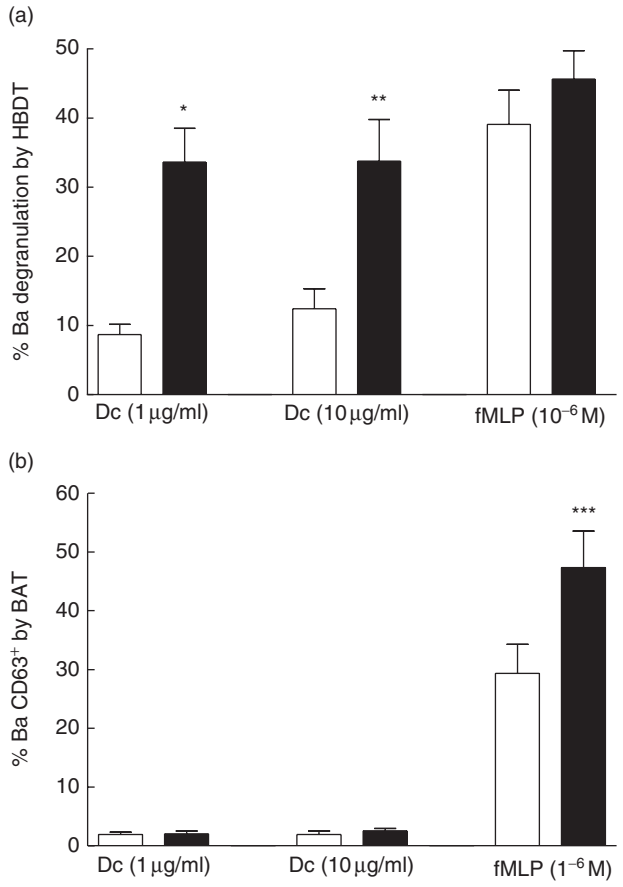

Fig. 2. Human basophil (Ba) degranulation test (HBDT) (a) and Ba activation test (BAT) (b) results (mean ± s.d.) of Ba from 14 Dc-sensitive patients (black bars) and 12 control donors (white bars) stimulated with 1 or 10 μg/ml of diclofenac (Dc) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) as positive control. *$P = 0{\cdot}0001$; **$P = 0{\cdot}0049$; ***$P = 0{\cdot}037$.

12 non-sensitive controls, stimulated with Dc 1 μg/ml, were 33·62 ± 18·35% and 8·49 ± 4·79% ($P = 0{\cdot}0001$) and 33·79 ± 20·75% and 12·24 ± 8·73% ($P = 0{\cdot}0049$) with Dc 10 μg/ml (Fig. 2a). However, a mean increase in CD63 expression by BAT in the same patients did not show significant variations with any Dc concentrations. Mean BAT was 2·04 ± 1·68% with Dc 1 μg/ml in Dc-susceptible individuals and 1·93 ± 1·40% in controls ($P = 0{\cdot}8$), and 2·54 ± 1·52% in Dc-sensitive subjects and 1·90 ± 2·06% in non-Dc-susceptible subjects ($P = 0{\cdot}4$) with Dc 10 μg/ml (Fig. 2b). Moreover, stimulation of Ba from Dc-sensitive patients with fMLP induced statistically significant ($P = 0{\cdot}03$) increases in CD63 expression (47·33 ± 23·25%) in comparison with controls (29·37 ± 16·96%). We evaluated some patients and controls more than once in order to determine internal reproducibility. Individual results are shown in Table 2.

Dc did not affect Dc sensitive Ba recovery

No significant difference was found between the number of Ba recovered after Dc incubation compared with the number of Ba incubated with only PBS, either in patients or in controls (Table 3). In contrast, the number of Ba from Dc-sensitive patients but not from controls decreased after fMLP stimulation ($P = 0{\cdot}02$).

Ba from individuals sensitive to Dc and Dp exhibit responses to Dp similar to Ba from individuals sensitive to Dp but insentitive to Dc

We compared Ba activation induced by Dp in patients sensitive to both Dc and Dp with Ba from Dc-insensitive Dp-sensitive controls. Ba from two patients sensitive to both Dc and Dp responded to Dp by BAT 60·87 ± 9·28% and by HBDT (48·78 ± 22·17%) in a manner not significantly different from Dp response of Ba from subjects allergic to dust mite, but non-susceptible to Dc ($n = 3$): BAT: 55·64 ± 35·95% ($P = 0{\cdot}84$); HBDT: 26·48 ± 11·40% ($P = 0{\cdot}36$).

In contrast, Dc response of Ba from the two patients sensitive to both stimuli was 1·99 ± 0·78% by BAT and 30·53 ± 1·02 by HBDT. Figure 3 shows a flow cytometry analysis of Ba from a whole blood sample from a Dc- and Dp-sensitive patient after challenge.

Dc did not inhibit CD63 expression induced by fMLP or Dp

We observed that CD63 expression induced by fMLP was not affected by Ba preincubation with Dc, neither in Dc-sensitive individuals (fMLP = 60·7 ± 10·49%; Dc + fMLP = 56·80 ± 22·30%, $P = 0{\cdot}8$, $n = 4$), nor in subjects without Dc hypersensitivity (fMLP = 39·58 ± 20·19%; Dc + fMLP = 45·88 ± 21·22%, $P = 0{\cdot}25$, $n = 4$). Our findings also showed that CD63 expression induced by Dp extract was not inhibited by Ba preincubation with Dc, neither in subjects sensitive only to Dp (Dp = 55·9 ± 36·56%; Dc + Dp = 69·67 ± 31·03%, $P = 0{\cdot}29$, $n = 3$) nor in patients sensitive to both stimuli (Dp = 60·85 ± 10·11%; Dc + Dp = 67·05 ± 2·75%, $P = 0{\cdot}45$, $n = 2$).

Discussion

It has been shown that Ba activation driven by an IgE-dependent mechanism can be monitored by an increase in Ba CD63 expression [3,18,19,23,24] and by Ba degranulation observed with HBDT [9]. Our results confirm these findings.

NSAIDs sensitivity is expressed clinically in three different ways, as an urticarial reaction, anaphylaxis or as an exacerbation of asthma and rhinosinusitis. Multiple efforts to demonstrate a specific IgE to NSAIDs have been unsuccessful. The suggested mechanism of this pseudoallergic reaction includes the blockade of the cyclo-oxygenase enzyme (COX) with resultant overproduction of leukotrienes (LT), especially LT3, 4 and 5; a rapid depletion of prostaglandin E2

Table 2. Examples of human basophil (Ba) degranulation test (HBDT) and Ba activation test (BAT) internal reproducibility.

	Dc (1 μg/ml)	Dc (10 μg/ml)	Dp (1/200)	fMLP (10^{-6} M)
HBDT (% Ba degranulation)				
EY (control)	13·04	19·56	23·91	36·96
	6·25	10·42	12·50	47·92
			16·67	66·67
NG (control)	6·25	10·42		56·25
	15·38	23·08		50·00
	11·11	18·52		33·33
	3·85	n.d.		34·60
GR (control)	9·37	6·25		25·00
	n.d.	0·00		4·26
DF (Dp-sensitive)			17·40	21·74
			51·61	64·52
			35·72	45·24
FS (Dc-sensitive)	31·49	61·12		55·56
	60·00	48·00		50·00
BAT (% Ba CD63+)				
EY (control)	0·22	0·65	2·96	8·34
	2·44	4·92	4·82	11·79
NG (control)	0·60	0·78		44·00
	2·57	2·86		42·04
	2·08	3·33		52·16
	1·94	3·20		42·16
GR (control)	0·20	0·00		25·46
	n.d.	2·30		57·80
DF (Dp-sensitive)			88·00	50·00
			51·64	45·48
			86·80	32·00
FS (Dc-sensitive)	1·44	1·14		47·00
	2·58	4·06		67·10

Dc: diclofenac; Dp: *Dermatophagoides pteronyssinus*; fMLP: N-formyl-methionyl-leucyl-phenylalanine; n.d.: not determined.

(PGE_2) and Ba stored mediators release. This classic anaphylactoid response appears to be related only to blocking the COX 1 isoform of the enzyme, as patients with aspirin-induced asthma have been shown to tolerate selective COX 2 inhibitors [5]. The cross-reactivity among NSAIDs to induce symptoms varies from one clinical form to another, but there is very little cross-reactivity with the newer NSAIDs that inhibit only COX 2 [5–7].

Our results show that Dc activates Ba in sensitive individuals in a way that do not induce CD63 expression but promotes Ba degranulation as assessed by disappearance of metachromatic-stained cytoplasmic granules by light microscopy, and that this mechanism is different from the IgE and fMLP-mediated Ba activation. These results could not be explained by Dc-induced cytolysis of sensitive Ba by flow cytometry.

Gamboa *et al*. have shown that Ba from 43·3% of NSAID-sensitive patients expressed CD63 after stimulation with Dc [25]. These results were obtained using higher drug concentrations, longer periods of drug exposure, a two-colour label of Ba and an anti-IgE as positive control. We favour a PBS control as a negative cut-off, considering that 8–20% of normal individuals fail to degranulate in response to cross-linking of the IgE high-affinity receptor [26]. Also, we preferred fMLP as a positive control because it induces Ba activation by an IgE-independent mechanism, as has been described for many drugs such as NSAIDs [27]. We used a tricolour staining in our tests. It has been recommended to include anti-CD45 to avoid debris and other IgE-reactive leucocytes when cells of interest are near the lymphocyte region [14,15].

Table 3. Number of basophils (Ba) after acquiring 50·000 CD45+ cells of R1.

	Controls ($n = 12$)	Patients ($n = 14$)
PBS	1253 ± 585	800 ± 399
Dc 1 μg/ml	1551 ± 1078	829 ± 465
Dc 10 μg/ml	1477 ± 853	840 ± 455
fMLP (10^{-6} M)	1302 ± 705	640 ± 439*

Values are given as mean ± s.d. *$P = 0·05$ compared with negative controls (phosphate-buffered saline: PBS). Dc: diclofenac; fMLP: N-formyl-methionyl-leucyl-phenylalanine.

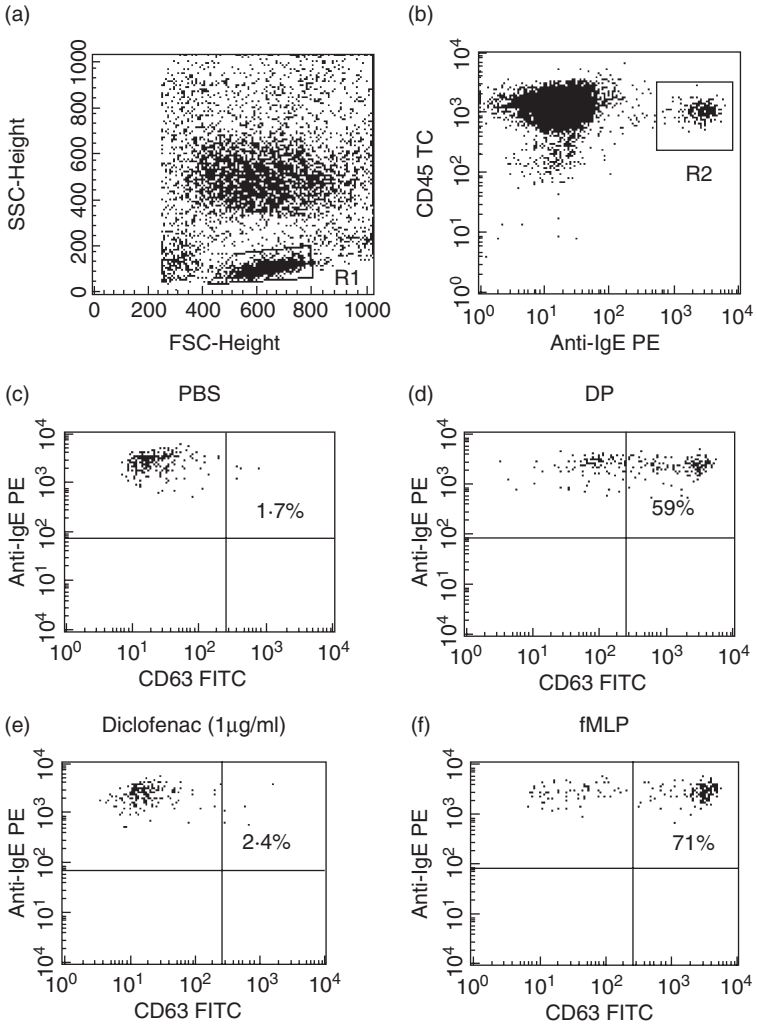

Fig. 3. Flow cytometry analysis of Ba from a whole blood sample from a Dc- and Dp-sensitive patient after challenge. Ba were identified on the basis of CD45 expression and the presence of IgE on their surface. Percentages of Ba $CD63^+$ are indicated. (a) First gate R1. (b) Second gate R2. (c–f) CD63 expression on Ba included in the logic gate (R1 AND R2); (c) negative control (PBS); (d) stimulated with 10 μg/ml Dp allergen extract at a final dilution of 1/200; (e) stimulated with 1 μg/ml of Dc; (f) positive control (fMLP 10^{-6} M).

Our observations suggest the existence of two different degranulation mechanisms, as described by Dvorak *et al.* They have defined two general patterns of mediator release from human Ba: anaphylactic degranulation (AND) with a massive and rapid extrusion of granules to the extracellular milieu, and piecemeal degranulation (PMD), which includes the release of focal and complete granule contents in the absence of granule–granule or granule–plasma membrane fusions. Dvorak *et al.* have described PMD in tissue Ba in contact allergy skin lesions. This secretory process was identified by light microscopy as Ba-retained granule containers in the cytoplasm of viable cells that are devoid of metachromatically stained content. Electron microscopy revealed that Ba in PMD exhibit empty or partially empty granule chambers that do not fuse with each other or with the plasma membrane [28].

The PMD phenotype has also been identified in eosinophils and mast cells during inflammatory reactions [29]. The AND process has been described in Ba and mast cells during IgE-dependent immediate hypersensitivity responses [29–31]. fMLP has been found to induce a unique sequence of morphological events in *ex vivo* human Ba that included PMD in the early degranulation phase followed by AND (PMD occurs in seconds and AND peaked by 1 min) [31].

Our results suggest that Dc-sensitive Ba could undergo the PMD process in response to Dc stimulation, losing their metachromatic granule contents observed by HBDT but without granule–plasma membrane fusion, which does not increase CD63 expression as registered by BAT. Moreover, the recovery of Ba was similar after PBS or Dc exposure in both controls and patients, demonstrating that the lack of metachromatic staining was not associated with cellular lysis.

Overall, our results, even in a small population, suggest that CD63 expression is not a reliable method for diclofenac allergy diagnosis.

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