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Monitoring human basophil activation via CD63 monoclonal antibody 435

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On activation of human basophilic granulocytes with anti-IgE or with the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine, the expression of the CD63 antigen on the cell surface, detected by monoclonal antibody (MAb) 435, increased up to 100-fold. The kinetics of CD63 up regulation and histamine release were identical, and a strong correlation was found between percentage of MAb 435-binding basophils and extent of histamine release.

Immunoelectronmicroscopy demonstrated that the epitope for MAb 435 in resting basophils is located on the basophilic granule membrane. After basophil activation, MAb 435 bound to the exterior of the plasma membrane. Experiments with various doses of anti-IgE demonstrated that the binding of MAb 435 to basophilic granulocytes follows an all-or-nothing-like response per cell. Basophils either do not bind the MAb at all, or they bind a maximal amount of the MAb. We also measured the up regulation of the CD11/CD18 leukocyte adhesion complex. Here, too, we noted an increase in cell-surface exposure of all subunits after activation. This increase was not as strong as increase found with MAb 435. Thus, MAb 435 is an interesting new tool for investigating the activation of human basophils, in addition to the measurement of mediator release. This MAb may be useful for the detection of basophil activation in vivo. (J ALLERGY CLIN IMMUNOL 1991;88:328-38.)

Key words: Basophils, degranulation, activation, histamine release, CD63, CD18, CD11a,b,c, up regulation

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Activation of basophilic granulocytes induces fusion of cytoplasmic granules with the plasma membrane and the successive release of inflammatory mediators, such as histamine. This fusion phenomenon also changes the surface structure of the plasma membrane.

Activation of basophilic granulocytes can be quantified via measurement of mediators (stored or de novo synthesized) released into the extracellular medium.¹⁻³ Alternatively, the measurement of intracellular messengers, such as changes in cytosolic-free Ca^{++} concentration,⁴ may yield information about the activation state of the basophil. Translocation or activity increase of protein kinase C⁵ has also been used to document basophil activation. However, these methods can only be used to assay activation of basophils in vitro.

In this article, we introduce the measurement of basophil activation by monitoring changes on the basophil surface after activation. This was performed via binding of the MAb 435. This MAb recognizes a glycoprotein with a relative molecular mass of 53,000 (GP 53), and has been clustered as CD63.⁶

Apart from basophilic granulocytes, it has been demonstrated that the CD63 Ag is present in other leukocytes.⁶ In platelets, the CD63 Ag is well characterized and described as an Ag present in intracellular granules.⁷

Recently, the Ag for CD63 has been cloned and analyzed.⁸ Homology has been described between the CD63 Ag and a melanoma-associated Ag. Still, no function is known for the CD63 Ag. Perhaps the highly glycosylated luminal domain of the CD63 molecule protects the granule membrane from enzymatic degradation.⁸

MATERIAL AND METHODS

Material

Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), human albumin (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands), sheep serum containing IgG Abs against human IgE (CLB, No. SH25P01), MAb against CD11a (CLB-LFA-1/2), CD11b (CLB-B2.12), CD11c (Becton Dickinson, Mountain View, Calif., Leu-M-5), and against CD18 (CLB-LFA-1/1), FITC-labeled GAMG Abs (CLB, No. GM17-01-F07), and alkaline-phosphatase-labeled GAMG Abs (Promega Biotec, Madison, Wis.) were obtained from the manufacturers. The concentration of anti-IgE Abs in the sheep serum was calculated by comparing affinity-purified sheep antihuman IgE Abs with serum dilutions for HR from human basophils.

MAb 435 is a MAb of the IgG1 subclass and was raised against a cytochrome b_{558} -enriched fraction from human neutrophils.⁹ Ascites was produced in pristane-primed

Abbreviations used

MAb:	Monoclonal antibody
FMLP:	Formyl-methionyl-leucyl-phenylalanine
PBS:	Phosphate-buffered saline
GAMG:	Goat antimouse immunoglobulin
EM:	Electron microscopy
IEM:	Immunoelectron microscopy
MFI:	Mean fluorescence intensity
Ag:	Antigen
Ab:	Antibody
BSA:	Bovine serum albumin
HR:	Histamine release
FITC:	Fluorescein isothiocyanate
TNP:	Trinitrophenyl

BALB/c mice. MAb 435 was clustered in the Fourth Workshop on Leukocyte Antigens as CD63 and referred to as CLB-gran/12. Purified Ab is now commercially available (CLB-CD63). Immunoglobulins were purified from ascites with protein A-Sepharose as indicated by the supplier (Pharmacia Fine Chemicals). Negative-control Ab was of the IgG1 subclass and directed against TNP.

Five and 10 nanometers of gold-labeled GAMG⁵ and GAMG,¹⁰ respectively (1:40), were obtained from Janssen Pharmaceutica (Beerse, Belgium); Lowicryl K4M was obtained from Bio-Rad, Veenendaal, The Netherlands; and LX112 and Araldite 502 was obtained from Agar Aids, Stansted, England.

Earle's balanced salt solution was obtained from Flow Laboratories (Ayrshire, Scotland). BSA was purchased from Organon Teknica BV (Oss, The Netherlands). Fetal calf serum (Hyclone Laboratories, Logan, Utah) was heat inactivated before use. Phorbol myristate acetate (Sigma Chemical Co., St. Louis, Mo.) and FMLP (Sigma Chemical Co.) were dissolved in dimethylsulfoxide and were stored at -20°C . These agents were diluted at least 300-fold in the cell incubations. The final concentration of dimethylsulfoxide ($\leq 0.3\%$, vol/vol) had no effect on cell viability or HR.

Purification of basophils

Buffy coats from 500 ml of human blood were obtained after informed consent from healthy donors without an allergic history. The basophils were purified by successive isopycnic centrifugation and elutriator centrifugation, as previously described by De Boer and Roos,¹⁰ with minor modifications. During the elutriation procedure, the third fraction was collected between 2580 and 1000 rpm. This fraction was additionally purified over a discontinuous Percoll gradient of 1.068 and 1.075 g/cm^3 .

The cells were counted electronically (counter model ZF, Coulter Electronics, Dunstable, U.K.). The basophil content was determined by differential staining with alcian blue¹¹ and by cytocentrifuge preparations stained with May-Grünwald/Giemsa. The purity of the basophils was $70\% \pm 9\%$

($n = 21$). Platelets were not present, as determined with MAb Y2 against platelet glycoprotein IIIa (Dakopatts, Copenhagen, Denmark) and by EM.

HR

Purified basophils were washed once with Earle's balanced salt solution supplemented with 5% (vol/vol) heat-inactivated fetal calf serum. Incubations were performed in 300 μ l of EF buffer containing 10^5 cells.

Before reagents were added, the cells were prewarmed for 10 minutes at 37° C. After the incubation, the cells were pelleted for 10 seconds at 12000 g , and 250 μ l of the supernatant was collected. The supernatants were mixed with 200 μ l of perchloric acid (12%) and 750 μ l of PBS and were stored at 4° C. For measurement of the total amount of histamine, 10^5 cells were lysed in perchloric acid. Histamine was measured by fluorometric analysis, as described by Siraganian.¹² HR was calculated as percentage of the total amount of histamine in the cells. The spontaneous release was almost always 5%. Occasionally, spontaneous release reached >5%; the results of these experiments were discarded.

EM

Degranulation of basophils. Purified basophils were incubated for 30 minutes at 37° C with 100 ng of anti-IgE per milliliter or with 1 μ mol/L of FMLP. Thereafter, the cells were immediately fixed with 2.5% (wt/vol) glutaraldehyde in 0.1 mol/L of cacodylate buffer (pH 7.2). As a control, untreated basophils were also fixed. The cells were postfixed in 1% (wt/vol) osmium tetroxide in the same buffer and embedded in a mixture of LX112 and Araldite 502. Thin sections were stained with uranyl acetate and lead hydroxide. In each experiment, 200 basophils were observed. To avoid counting the same cell several times in serial sections, a ribbon of sections was taken on each grid, and between the ribbons a thick section was discarded. From each grid only one section was selected for counting cells.

Postembedding IEM. Basophils were fixed in a mixture of 4% (wt/vol) paraformaldehyde and 0.5% (wt/vol) glutaraldehyde in 0.1 mol/L of phosphate buffer, pH 7.2, and embedded at low temperature in Lowicryl K4M. Thin sections were incubated with MAb 435 (1:50) for 2 hours, followed by incubation with GAMG¹⁰ for 1 hour, and were stained with uranyl acetate and lead citrate.

Preembedding IEM. Basophils were incubated at 37° C with anti-IgE for 0, 4, or 30 minutes and immediately fixed with 4% (wt/vol) paraformaldehyde and 0.5% (wt/vol) glutaraldehyde for 15 minutes. After cells were washed, they were incubated with MAb 435 (1:100) for 1 hour, followed by incubation with GAMG⁵ for 1 hour. The cells were then fixed with 2.5% (wt/vol) glutaraldehyde, postfixed with 1% (wt/vol) osmium tetroxide, and embedded in a mixture of LX112 and Araldite 502. Thin sections were stained with uranyl acetate and lead hydroxide.

Immunofluorescence. Cells were washed once and resuspended in PBS with 1% (vol/vol) BSA in a concentration of 10^7 per milliliter. In kinetic experiments, additional

changes in the plasma membrane after sampling were inhibited by immediate fixation with 0.5% (wt/vol) paraformaldehyde. Twenty microliters of this suspension was incubated with 20 μ l of the MABs in the appropriate dilutions. Incubations were performed at 4° C during 30 minutes. The cells were then washed once with PBS supplemented with 1% (vol/vol) BSA, and fluoresceinated Ab against mouse immunoglobulins was added, followed by an incubation for 30 minutes at 4° C. Thereafter, the cells were washed once with PBS with 1% (vol/vol) BSA and subsequently analyzed by flow cytometry in a FACSAN flow cytometer (Becton Dickinson). For each sample, 10,000 cells were analyzed. Cell populations were gated, excluding contaminating monocytes, neutrophils, and eosinophils, at 90° and with forward light scatter. The percentage of positive cells was expressed as the percentage of cells that demonstrated more binding of a certain MAb than of the control IgG1 Ab against TNP.

Sorting. Basophils were stimulated with 1 μ mol/L of FMLP for 20 minutes in EF buffer. Thereafter, the cells were washed twice in PBS with 1% (vol/vol) BSA. The basophils were then incubated with MAb 435 for 30 minutes at 4° C, washed in PBS with 1% (vol/vol) BSA, and subsequently incubated with FITC-conjugated GAMG Abs. After another 30-minute incubation at 4° C, the cells were washed with PBS with 1% (vol/vol) BSA and stored on ice.

Cells were sorted by FACSTAR+ (Becton Dickinson, Palo Alto, Calif.). During and after sorting, the cells were kept on ice. Cells from the MAb 435-negative and MAb 435-positive fraction were counted, and cytocentrifuge preparations were stained with May-Grünwald-Giemsa. Each fraction contained at least 320,000 cells. In each fraction, the mean content of histamine per basophil was determined. The sorting procedure did not result in degranulation of basophils as was checked by sorting of W6/32-positive basophils or basophils that were incubated with a negative control MAb. In the sorted fractions, $\geq 90\%$ of the amount of histamine present in the cells before sorting was found.

RESULTS

Binding of MAb 435 to human basophils

When the binding of MAb 435 to unstimulated basophils was investigated (Fig. 1), a low percentage of the cells appeared to bind this Ab ($5.7\% \pm 4.2\%$; $n = 6$). After stimulation with anti-IgE, a subpopulation of cells strongly bound MAb 435 (Fig. 1). The increase in fluorescence intensity of these cells was twentyfold to 100-fold compared to resting cells. Remarkably, after activation with suboptimal concentrations of anti-IgE, the MFI of the cell population as a whole did not increase, but a fraction of the basophils demonstrated an increased fluorescence intensity. This implies that the expression of the epitope for MAb 435 in activated basophils is an all-or-nothing response.

When basophils were stimulated by FMLP or phor-

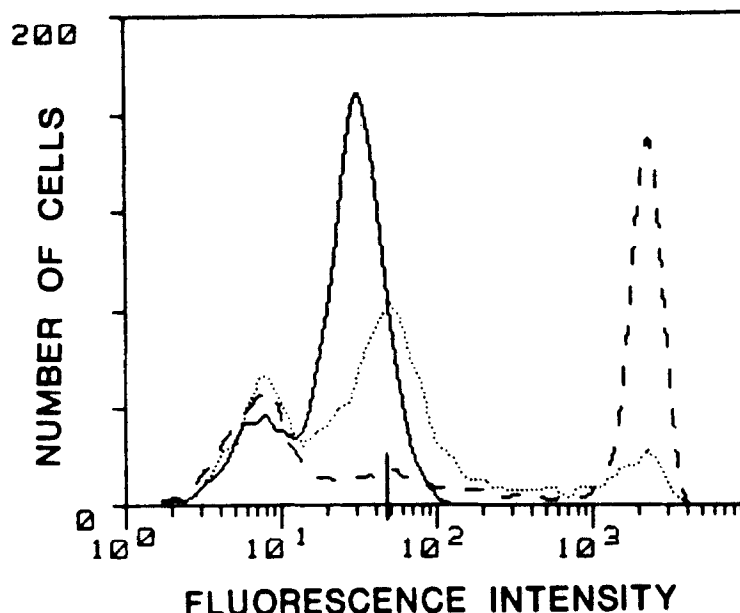


FIG. 1. Binding of MAb 435 to resting and anti-IgE-stimulated basophils. Stimulation was performed for 30 minutes at 37° C. After stimulation, the basophils were incubated with MAb 435 and FITC-labeled GAMG Ab. Binding was analyzed by flow cytometry (—), resting basophils (....), basophils stimulated with 10 ng of anti-IgE per milliliter (resulting in 14% HR) (---), and basophils stimulated with 100 ng of anti-IgE per milliliter (resulting in 70% HR) (-.-). Fluorescence intensity is in arbitrary units. Ninety-nine percent of the basophils demonstrated a background fluorescence with an irrelevant Ab <50 arbitrary units, indicated by the vertical bar. Cells with a fluorescence between 2 and 20 arbitrary units are contaminating lymphocytes. Representative experiment of six performed.

bol myristate acetate, the increase in MAb 435 binding was comparable, both in extent and in its all-or-nothing response to the increase observed after anti-IgE stimulation (data not presented).

In the absence of extracellular Ca^{++} , degranulation of basophilic granulocytes after the activation by anti-IgE or FMLP is inhibited.^{13,14} Under these conditions, the increase in MAb 435 binding was also strongly inhibited ($n = 2$; data not presented).

Stimulation of human basophils by anti-IgE results in a donor-specific HR.¹⁵ In the experiment illustrated in Fig. 1, incubation with 100 ng of anti-IgE per milliliter resulted in 70% HR and in 65% MAb 435-positive cells. To examine further the relationship between HR and MAb 435 binding, we studied the increase in percentage of MAb 435-positive basophils after the activation with FMLP and anti-IgE and compared this increase to the extent of HR. As is illustrated in Fig. 2, A and B, a strong correlation exists between these two parameters. The correlation between MAb 435 positivity and HR after anti-IgE stimulation was 0.95, whereas after FMLP, the correlation was 0.92.

Time course

The HR from basophils after FMLP stimulation is almost instantaneous, in contrast to the anti-IgE-in-

duced HR.¹⁴ We compared the time course of HR with that of the binding of MAb 435. Within 20 seconds of FMLP addition, the percentage of MAb 435-positive cells was maximal (Fig. 3, A), in agreement with the kinetics of the FMLP-induced HR. HR after anti-IgE stimulation was half-maximal within 6 minutes, whereas maximal HR was obtained after 11 minutes. The binding of MAb 435 closely followed these kinetics also (Fig. 3, B). MAb 435-positive basophils demonstrated only maximal binding (as measured by fluorescence intensity) after 11 minutes. Between 3 and 11 minutes after addition of anti-IgE, the responding basophils demonstrated intermediate fluorescence intensity per cell (*inset*, Fig. 3, B).

Comparison of up regulation of CD11, CD18, and CD63 Ags

After activation, human basophils demonstrate increased adherence to endothelial cells.¹⁶ This increased adherence is blocked by a MAb directed against the β -subunit of the leukocyte adhesion complex (CD18). We examined the up regulation of the α -subunits (CD11a, CD11b, and CD11c) and the β -subunit of the leukocyte adhesion complex after activation of human basophils with FMLP and compared these with the up regulation of CD63 (Table I). On

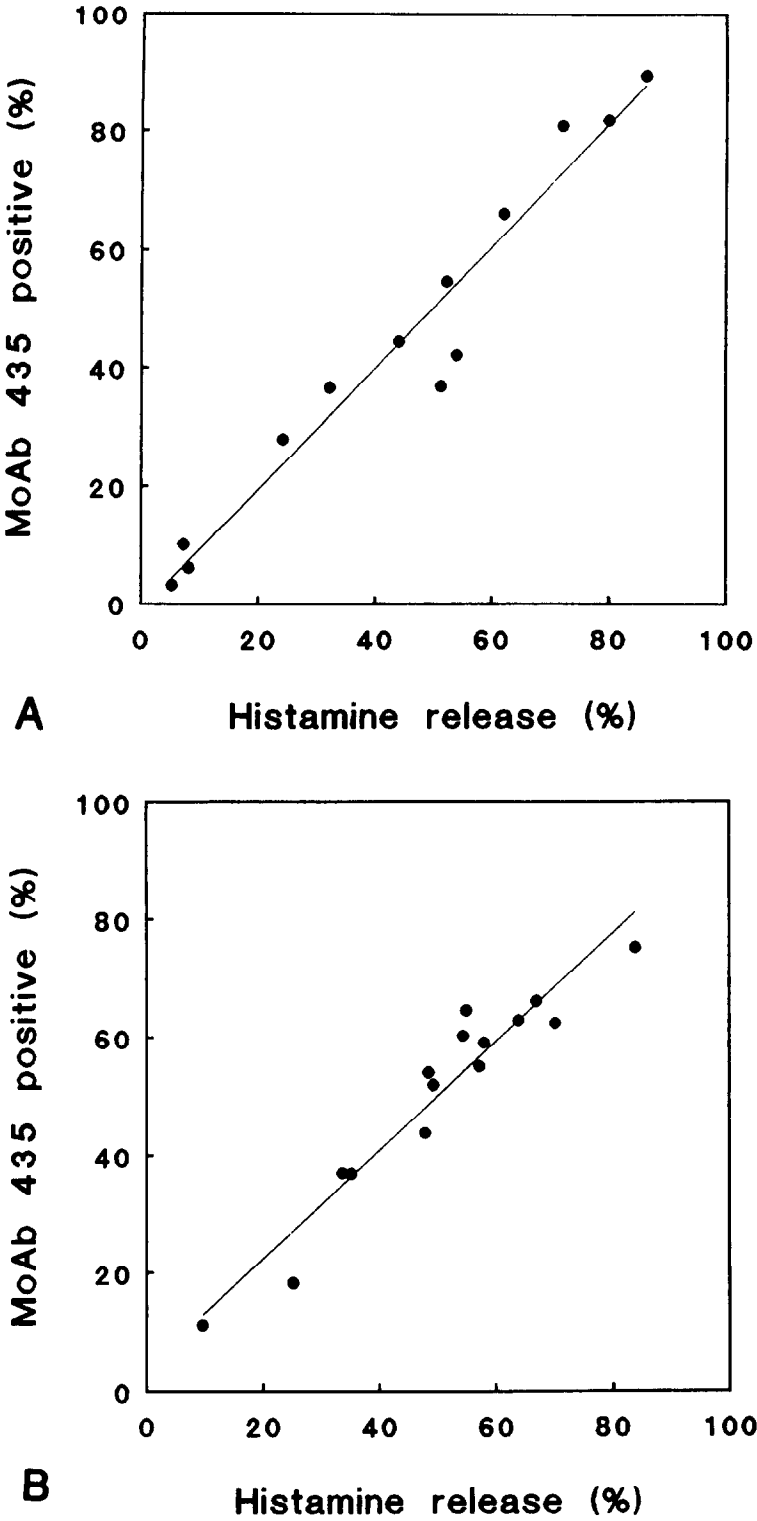


FIG. 2. Correlation of MAb 435 expression with HR. **A**, Basophils were stimulated with 100 ng of anti-IgE per milliliter. **B**, Basophils stimulated with 1 μ mol/L of FMLP during 20 minutes at 37° C in EF buffer. Histamine was measured in the cell-free supernatant and was expressed as percentage of histamine present at $t = 0$. MAb 435 binding was performed, as illustrated in Fig. 1. MAb 435 positivity was determined as the number of basophils that demonstrated high binding of MAb 435. The fold increase of MFI per MAb 435-positive basophils compared to the MFI of the unstimulated basophils was, after anti-IgE addition, 81.3 ± 7.9 , and after FMLP addition, 76.3 ± 8.8 (fold increase in MFI $\pm$ SEM). Results obtained with basophils from 13 donors (**A**) and 15 donors (**B**). The regression for the graphs was 0.95 and 0.92, respectively.

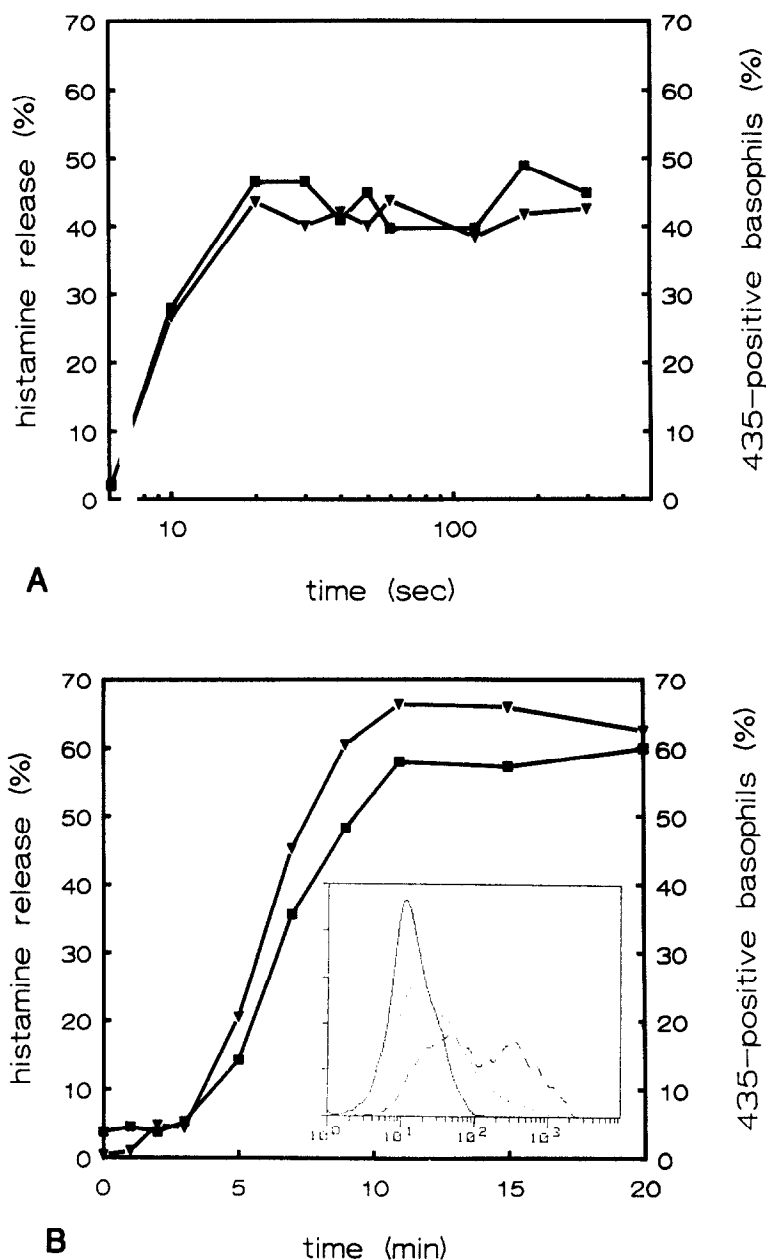


FIG. 3. Time course of HR and MAb 435 binding induced by anti-IgE or FMLP. **A**, Basophilic granulocytes were stimulated with 1 $\mu\text{mol/L}$ of FMLP. **B**, Basophilic granulocytes stimulated with 100 ng of anti-IgE per milliliter. After the indicated intervals, HR (■—■) and MAb 435 binding (▼—▼) were analyzed in the same samples. Histamine was measured in the cell-free supernatant and was expressed as percentage of histamine present in the cells at $t = 0$. MAb 435 binding was performed as illustrated in Fig. 1 on basophils immediately fixed with 0.5% paraformaldehyde. MAb 435 positivity was determined as the number of basophils that demonstrated more binding of MAb 435 compared to the resting basophils. **Inset, B**, MAb 435 binding to resting basophils (—), to basophils after a 5-minute stimulation with anti-IgE (.....), or to basophils after a 7-minute stimulation with anti-IgE (---). Representative experiment of seven performed.

resting basophils, CD11a and CD11b were clearly present, whereas CD11c was only detectable in very low amounts. The β -subunit (CD18) was present on almost all cells. After activation, a marked increase in binding of all subunits of the leukocyte adhesion

complex was observed. The percentage of CD11a-positive cells increased from 35% to 92% and that of CD11c from 7% to 48%. The increase in binding of the leukocyte adhesion complex to the basophils was unimodal, in contrast to the bimodal increase (nega-

TABLE I. Presence of the leukocyte adherence Ags on human basophils before and after FMLP activation

Ag	Resting		FMLP activated	
	% Positive cells	MFI	% Positive cells	MFI
TNP	0.2	8.7	0.4	7.9
CD11a	34.8	44.6	92.4	100.1
CD11b	77.6	64.4	77.6	126.5
CD11c	7.3	20.1	47.9	25.8
CD18	89.2	87.8	97.6	117.3
CD63	9.2	19.3	23.7	209.4

For experimental details, see MATERIAL AND METHODS.

Because of donor-specific responses on stimulation with FMLP, one representative experiment is presented. Similar findings were observed in 11 additional experiments.

TABLE II. Correlation between HR and degranulation of basophils after stimulation with FMLP or anti-IgE

Challenge	HR (%)	No. of basophils examined*		
		Not degranulated	Partly degranulated	Fully degranulated
None	4	187	11	2
FMLP	77	51	28	121
Anti-IgE	12	168	22	10

*Basophils examined by EM were classified as not degranulated, that is, containing numerous basophilic granules and demonstrating no sign of exocytosis; partly degranulated, that is, containing numerous basophilic granules but demonstrating at least one clear site of exocytosis; fully degranulated, that is containing few or no remaining basophilic granules.

tive and strongly positive cells) observed with CD63 (Fig. 1). Although the percentage of CD11b- and CD18-positive cells was hardly affected by cell activation, an increase in the amount of fluorescence per cell was noted. The CD11b-negative cells were contaminating lymphocytes. Thus, most basophils carried CD11b and increased its expression on activation.

Bochner et al.¹⁶ have demonstrated that, at low concentrations of extracellular Ca^{++} , degranulation after anti-IgE stimulation was inhibited, whereas CD11/CD18-mediated adherence of human basophils to endothelial cells was still observed. The increase in adherence could be caused by an increase of the CD11/CD18 complex on the basophil membrane. Therefore, we compared the increase in CD63 expression to the increase in CD11a expression with 1 $\mu\text{mol/L}$ of FMLP at low concentrations of extracellular Ca^{++} , as well as with 10 nmol/L of FMLP at optimal concentrations of extracellular Ca^{++} . In this way, we found a diminished increase in CD63-positive basophils after suboptimal activation compared to optimal conditions, whereas CD11a increase was normal ($n = 3$). For instance, with 10 nmol/L

of FMLP, CD63-positive basophils increased from 1.4% to 6%, whereas 34% of the basophils were positive after stimulation with 1 $\mu\text{mol/L}$ of FMLP. CD11a-positive cells increased from 23% to 74% with 10 nmol/L of FMLP, whereas at optimal FMLP concentrations, 75% CD11a-positive cells were found.

EM

The apparent all-or-nothing response per basophil for the binding of MAb 435 led us to examine the expression of its epitope by EM in connection with the degranulation process. This was performed with the method previously described by Hastie et al.¹⁷ In each experiment, the HR was also measured. In Fig. 4, a partly degranulated basophil is illustrated, characterized by segments of the surface that are irregular; the amount of granules is still high, but membrane-surrounded exocytotic cavities communicating with the exterior are evident. The results obtained by observing 200 basophils, together with the extent of HR, are presented in Table II. These results indicate that the extent of HR closely correlated with the number of degranulated cells.

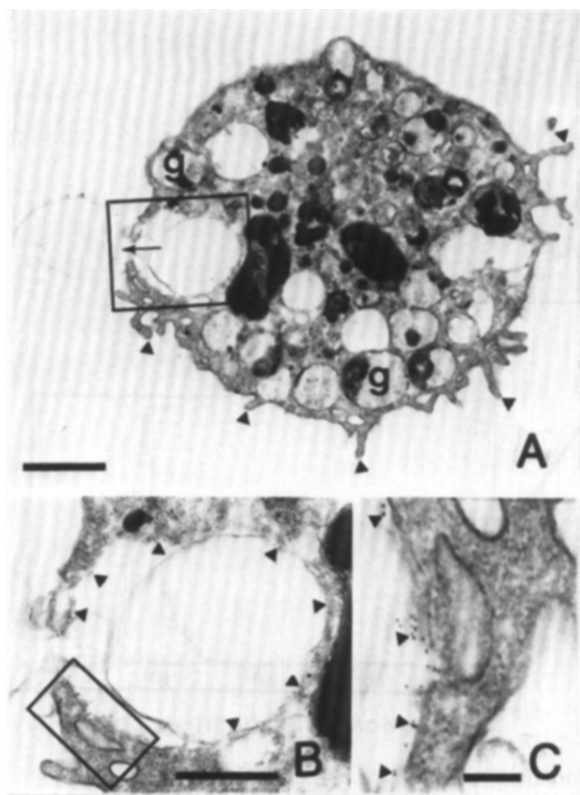


FIG. 4. IEM of a partly degranulated basophil (preembedding IEM). Basophils were stimulated with anti-IgE for 4 minutes, fixed, and incubated with MAb 435 and 5 nm gold-labeled GAMG Ab. HR was 20%. **A**, Low magnification illustrating a section of the whole cell. Part of the cell surface reveals irregular protrusions (arrowheads). The cytoplasm contains typical basophilic granules (*g*). A large membrane-surrounded cavity is evident, which communicates with the exterior (arrow). The cell surface is unlabeled. **B**, Higher magnification of marked area in **A** illustrating that the membrane of the exocytotic cavity is strongly labeled (arrowheads). **C**, Higher magnification of marked area in **B** to better illustrate the gold labeling (arrowheads); bars: **A**, 1 μm ; **B**, 0.5 μm ; **C**, 0.1 μm .

Localization of the MAb 435 epitope

Fluorescence microscopy on air-dried resting basophils revealed that the epitope for MAb 435 was present intracellularly. Further information about the exact localization was obtained by IEM. Localization of the Ag reacting with MAb 435 in resting basophils was investigated by postembedding IEM. After incubation of thin sections of basophils with MAb 435 and gold conjugate, the labeling was found exclusively on the basophilic granules and localized mainly on the granule membrane (Fig. 5). No labeling was found on the cell surface.

To study the localization of the MAb 435 epitopes on the cell membrane, preembedding IEM was used. Basophils were stimulated with anti-IgE for 0, 4, and

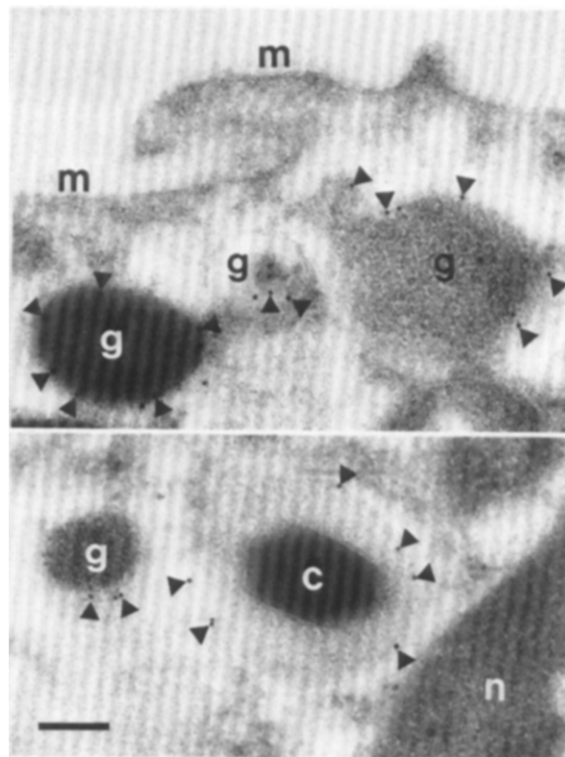


FIG. 5. Intracellular localization of CD63 (postembedding IEM). Resting basophils were embedded in Lowicryl K4M. Thin sections were incubated with MAb 435 and 10 nm gold-labeled GAMG. Labeling (arrowheads) is localized on the membrane of the basophilic granules (*g*). One granule contains a cross-section of a Charcot-Leyden crystal (*C*). The cell membrane (*m*) and the nucleus (*n*) are unlabeled; bar, 0.2 μm .

30 minutes, respectively, fixed, and then incubated with MAb 435 and GAMG.⁵ At time zero (i.e., without stimulation), the cells appeared the same as described above (see also Table II) for resting basophils; after 4 minutes and, most prominently, after 30 minutes of stimulation, the number of activated basophils was higher. In all three experiments, the resting cells, containing all basophilic granules and revealing a smooth cell surface, were almost unlabeled. The partly degranulated basophils were strongly labeled on the membrane of exocytotic cavities (Fig. 4) and/or segments of the cell surface with an irregular appearance. Fully degranulated basophils revealed an irregular cell surface that was highly labeled.

Sorting of MAb 435-positive basophils

The all-or-nothing pattern of the MAb 435 binding per cell, combined with the correlation between HR and MAb 435 binding, might indicate an all-or-nothing response for HR as well. To test this hypothesis, we activated basophils with 1 $\mu\text{mol/L}$ of FMLP and

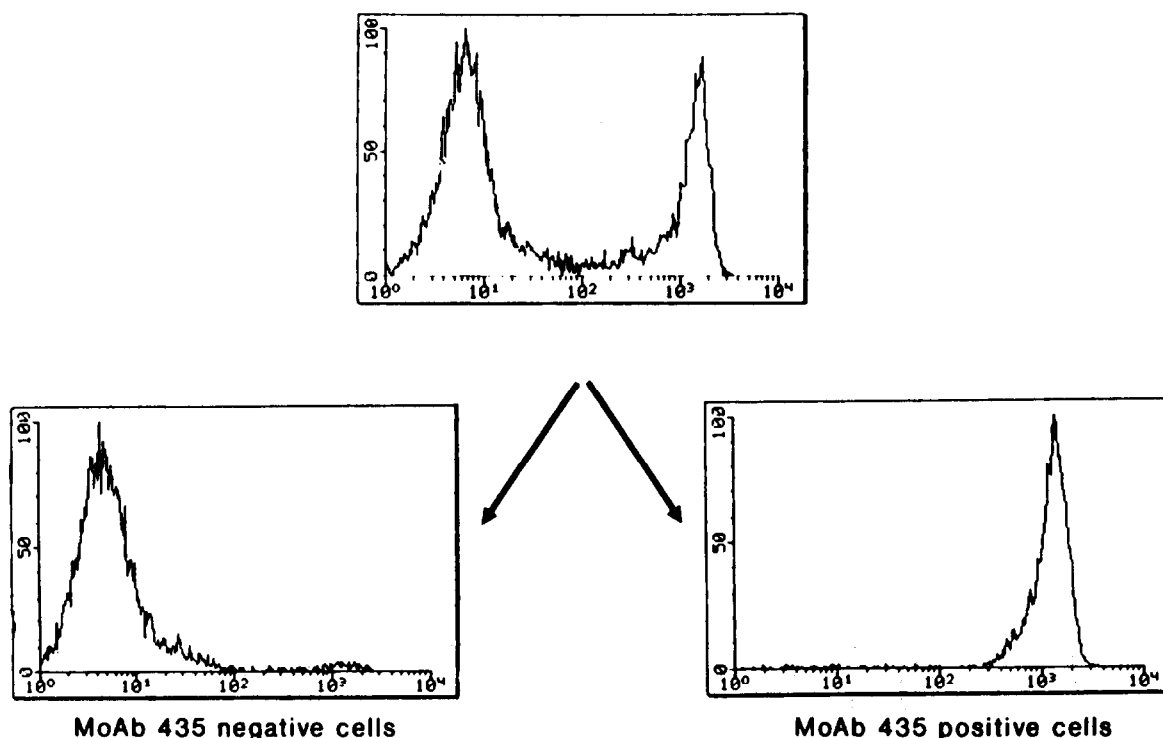


FIG. 6. Sorting of activated basophils. After activation with 1 $\mu\text{mol/L}$ of FMLP, basophils were incubated with MAb 435, followed by FITC-labeled GAMG. The *horizontal axis* in the figures illustrates the relative amount of fluorescence per cell in arbitrary units, and the *vertical axis* illustrates the number of cells with a specific amount of fluorescence. In the **top panel**, the binding pattern of MAb 435 to the basophil suspension before sorting is illustrated. In the **lower two panels**, the binding pattern of MAb 435 to the sorted cell suspensions are illustrated; **left panel**: MAb 435-negative cells (18% basophils, 0.62 pg of histamine per basophil); **right panel**: MAb 435-positive cells (>95% basophils, 0.14 pg of histamine per basophil). The percentage of basophils in the MAb 435-negative and positive fraction was determined by May-Grünwald/Giemsa staining. The mean amount of histamine per basophil was corrected for the percentage of basophils.

subsequently incubated the cells with MAb 435, followed by fluoresceinated Ab against mouse immunoglobulins. This suspension was then sorted by a fluorescence-activated cell sorter into a cell population that had bound MAb 435 and a population that had not bound MAb 435. The fluorescence of the basophil suspension before and after sorting is illustrated in Fig. 6. After stimulation, the cells were kept on ice to prevent the synthesis of histamine. When the 435-positive fraction was analyzed by microscopic analysis of cytocentrifuge preparations stained with May-Grünwald/Giemsa, a heterogeneous pattern of completely and partly degranulated basophils was obvious. In the 435-negative fraction, 18% basophils, all intact, were present. The amount of histamine per basophil was lower in the 435-positive cell population (0.14 pg of histamine per basophil), compared to the 435-negative cell population (0.62 pg of histamine per basophil) (Fig. 6). The amount of histamine in the 435-negative basophils was below the average of 1.3 pg of histamine per basophil in nonstimulated

suspensions. We therefore repeated this experiment twice with basophils from two other donors (Table III). Although the amount of histamine released and the percentage of MAb 435-positive basophils differed between the donors, the MAb 435-positive cells still contained 25% and 19% of the histamine present in the MAb 435-negative cells.

DISCUSSION

We have demonstrated that degranulation of human basophils leads to increased expression of CD63 Ag on the plasma membrane. This protein is recognized by MAb 435, and thus, it is now possible to measure basophil activation through binding of a MAb. The increase in CD63 expression after stimulation ranged up to 100-fold, but this increase varied per donor. This phenomenon is probably due to donor-specific amounts of CD63 Ag present in the basophils (like the amount of histamine in the basophils).

Human basophilic infiltration has been demonstrated *in vivo*,¹⁸ and most probably this process will

TABLE III. Sorting of 435 positive basophils

Donor	HR (%)	435-positive (%)	After sorting	
			Histamine per cell (pg)	
			435-negative	435-positive
51	40	35	0.62	0.14
53	37	36	1.7	0.42
3	14	15	1.5	0.28

Basophilic granulocytes obtained from three different donors were stimulated for 30 minutes with 1 μ mol/L of FMLP. After stimulation, the HR was measured. MAb 435 binding and sorting was performed as described in Fig. 6.

be preceded by activation and adherence of basophils. The increased adherence of activated human basophils to endothelial cells is largely blocked by MAbs against the β -chain of the leukocyte adhesion complex (CD18).¹⁶ We confirmed previous observations¹⁰ that human basophils carry all four subunits of the leukocyte adhesion complex. Moreover, we now demonstrate that activation of these cells results in an increased expression of these proteins on the basophil surface. IEM revealed that the CD63 Ag is located at the granule membranes. The correlation between the extent (Fig. 2) and kinetics (Fig. 3) of HR and MAb 435 binding suggests that the histamine-containing granule membrane is recognized by MAb 435. Granules different from granules that contain histamine, of the size and in the number as granules labeled with MAb 435 in the IEM studies, have, to our knowledge, never been described. Thus, in all probability, CD63 is located on the membranes of the histamine-containing granules.

From the difference in up regulation of CD63 and CD11a under suboptimal conditions (low extracellular Ca^{++} or low FMLP concentrations), it follows that these are differently regulated processes. Most probably, CD63 and CD11a have a different intracellular localization.

The kinetics of HR and MAb 435 binding after stimulation with FMLP are different from kinetics observed with anti-IgE. Possibly, the fusion of the MAb 435-positive (histamine-containing) granules with the plasma membrane is slower after anti-IgE than after FMLP stimulation. Also, the fusion process itself might be different between the two stimuli. The similar time course of intracellular Ca^{++} mobilization after stimulation by FMLP or anti-IgE (Knol EF, Mul FPJ, Hooibrink, et al. Manuscript submitted) rules out large differences between the kinetics of receptor-ligand binding (in case of FMLP stimulation) and the cross-linking induced by anti-IgE.

An interesting phenomenon of the MAb 435 binding during the activation of basophils is the all-or-nothing response per cell. Such a phenomenon has

previously been described by Pruzansky et al.¹⁹ who used alcian blue staining to quantitate the extent of basophil activation. This method is, however, limited in that negative basophils are at least 80% degranulated; therefore, the number of completely degranulated basophils is not quantitated. Another controversy in this study is that some incompletely stained basophils (partly degranulated) were observed. This amount ranged up to 10%, but these cells were not separately classified.

EM studies of Dvorak et al.,²⁰ not addressing the all-or-nothing phenomenon, nevertheless demonstrated that, by the time HR was complete, only fully degranulated and nondegranulated basophils were present.

We have also measured basophil activation by EM methods, as described by Hastie et al.¹⁷ Their study demonstrated that four of 10 activated cell preparations contained partly degranulated basophils. However, three of these four preparations were stimulated by Ag during 5 minutes or less, whereas the IgE-mediated release reaction takes about 10 minutes (Fig. 3). For instance, in Fig. 4, we illustrate a degranulating basophil 4 minutes after anti-IgE stimulation. As is obvious, the degranulation was still incomplete. The inset in Fig. 3, B, also illustrates an intermediate binding of MAb 435 after 5 and 7 minutes of stimulation with anti-IgE. Also, Hastie et al.¹⁷ determined very low numbers of basophils because of the loss of basophils in the activated cell suspensions. With the same method, we now found that degranulation of human basophils does not result in strictly divided populations of fully and nondegranulated basophils, but some partly degranulated basophils can also be observed. Our results are in favor for an all-or-nothing response for most of the basophils, but some basophils remain partly degranulated.

The heterogeneity of the histamine content in the MAb 435-positive basophils may be explained by the width of the fluorescence intensity of the MAb 435-positive basophils (Fig. 6). This intensity ranges from about 300 to 3000 relative units of fluorescence. This

heterogeneity might be due either to variation in the number of granules per basophil or in the amount of histamine per granule.

We postulate that MAb 435 recognizes an Ag on the membrane of the histamine-containing granules. The results presented in Fig. 5 suggest that the Ag is present on the inside of the granule membrane. Recently, the CD63 Ag has been cloned⁸ and demonstrated to contain four putative transmembrane regions. Probably, the Ag is anchored in the granule membrane, and fusion of the granules with the plasma membrane causes the exposure of the CD63 Ag to the outside of the basophil. When basophils are stimulated, a limited number of cells are triggered for HR and increased MAb 435 binding. These cells extrude most, but not always all, of their granule contents.

Measurement of activation via binding of MABs creates a new opening in basophil research in that now in vivo basophil activation can be measured. This is in contrast to the current methods, which are limited to assays of in vitro activation.

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