The release of basogranulin in response to IgE-dependent and IgE-independent stimuli: Validity of basogranulin measurement as an indicator of basophil activation

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Background: Basogranulin, the novel basophil granule protein recognized by the monoclonal antibody BB1, can be released by stimulation with anti-IgE antibody or calcium ionophore. However, the kinetics and regulation of its secretion are unknown.
Objective: We quantified basogranulin and histamine release in response to a range of stimuli to assess whether basogranulin secretion is a reliable marker of basophil activation.
Methods: Isolated peripheral blood basophils were stimulated with anti-IgE antibody, calcium ionophore, *N*-formyl-Met-Leu-Phe, and complement C5a. The released basogranulin and histamine were quantified by dot blotting with BB1 and a fluorometric method, respectively. Basogranulin localization was confirmed by flow cytometry.
Results: Both basogranulin and histamine displayed a bell-shaped response curve when basophils were challenged with anti-IgE. Half-maximal release occurred within 30 seconds. Basogranulin levels were maximal by 15 minutes, whereas those for histamine continued increasing to 30 minutes. Wortmannin, a PI3-K inhibitor, suppressed the release of both mediators. Basophils from donors with the "nonreleaser" phenotype secreted neither mediator in response to anti-IgE. Non-IgE-dependent stimuli released both mediators in parallel in a concentration-dependent manner. The correlation between the relative amounts of each mediator released was highly significant ($r = .901$, $P < .0001$, n = 87). Flow cytometry revealed that some of the secreted basogranulin adhered to the cell surface.
Conclusions: Basogranulin is secreted along with histamine in response to both FcεR I-related and unrelated stimuli. It is therefore a valid marker of basophil activation and could provide the basis for an immunoassay that distinguishes between basophil and mast cell activation. (J Allergy Clin Immunol 2003;112:102-8.)

Key words: *Basophil, basogranulin, histamine, IgE, A23187, f-Met-Leu-Phe, C5a, wortmannin, nonreleaser, BB1*

Mechanisms of allergy

Basophils and mast cells release various pro-inflammatory mediators such as histamine, leukotrienes, and cytokines on stimulation either through the cross-linking of IgE on high-affinity IgE receptors (FcεR I) or through non-IgE-dependent secretagogues such as activated complement factor 5 (C5a).[1-3] Both cell types are critically involved in allergy and inflammatory disease[2,4,5] but have different spatial and temporal roles. Basophils circulate in the peripheral blood, whereas mast cells mature and reside in tissue. However, at sites of allergic inflammation, basophils might be recruited to the affected tissues, where they might contribute to the inflammatory process.[6-8] Despite such differences in distribution and kinetics, the similarities in their staining properties with basic dyes and in the products they secrete create difficulties in distinguishing their respective roles, especially at sites of local inflammation. Recently, the basophil-specific antibodies 2D7[9] and BB1[10,11] have been developed and have provided novel opportunities for precise studies about the different roles of these two cell types in disease.[12-18]

The antigen for BB1 is a highly basic protein (pI = 9.6) that forms large macromolecular complexes (~5 × 10^6 Da).[11] Immunoelectron microscopy reveals that it is stored within cytosolic granules that are enfolded with the known granule membrane marker CD63.[11] In view of its distinct differences from known proteins, we have proposed the name "basogranulin."[11] Its biologic activity and pathophysiologic role remain unknown, but it was observed to be secreted by basophils in response to stimulation by both anti-IgE antibody and calcium ionophore.[10] Basogranulin, therefore, has the potential to serve as a specific marker of basophil activation. Its detection in biologic fluids might allow better discrimination between the effects of basophils and mast cells than current methods involving the measurement of histamine, tryptase, or prostaglandin D_2.

Abbreviations used

FcεR I:	Fc epsilon receptor I
fMLP:	*N*-formyl-Met-Leu-Phe
PAG:	PIPES-buffered saline containing albumin and glucose
PAG-CM:	PIPES-buffered saline containing albumin, glucose, calcium, and magnesium
PAG-EDTA:	PIPES-buffered saline containing albumin, glucose, and EDTA
PI3-K:	phosphatidyl inositol 3-kinase

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As an essential first step in the validation of basogranulin as a marker of basophil activation and also in the understanding of any role it might play in inflammation, we have examined the control and kinetics of its release by basophils. We report here on the effect of IgE-dependent and IgE-independent stimuli at different concentrations, as well as time course studies and the effects of selective inhibitors of signaling pathways. Comparison is made with the release of histamine to show that basogranulin release is a reliable qualitative and quantitative measure of basophil activation.

MATERIALS AND METHODS

Materials

Ficoll-Paque Plus was purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks, United Kingdom). MACS basophil isolation kit and LS separation columns were obtained from Miltenyi Biotec (Bisley, Surrey, United Kingdom). Calcium ionophore A23187, complement C5a, and fMLP were purchased from Sigma (Poole, United Kingdom). Wortmannin was purchased from Calbiochem (Nottingham, United Kingdom). Sheep anti-human IgE antisera (IgG concentration = 14.4 mg/mL) was obtained from Serotec (Oxford, United Kingdom). BB1 (basophil-specific monoclonal antibody, IgG2a isotype) was produced and biotinylated as previously described.[11]

Basophil purification

Basophils were purified from human peripheral blood obtained after informed consent from healthy donors (Approval No. 176/01, Southampton Joint Ethics Sub-Committee). Drawn whole blood, anticoagulated with K3EDTA, was diluted 2-fold with PIPES-buffered saline (110 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L PIPES, pH 7.3) and layered on Ficoll-Paque Plus (d = 1.077 g/mL). After centrifugation at 400*g* for 30 minutes, the interface fraction was collected and washed with PIPES-buffered saline containing 30 μg/mL human serum albumin (Sigma; Fraction V), 1 mg/mL glucose, and 1 mmol/L EDTA (PAG-EDTA). The cells were resuspended in ice-cold PAG-EDTA, and basophils were stained with Alcian blue (Sigma)[19] and counted in a Neubauer hemocytometer. Further purification of basophils was performed with the MACS basophil isolation kit (a negative-selection immunomagnetic protocol) according to the manufacturer's instructions. Isolated basophils were then resuspended in 1 mL of PAG-EDTA, and the number and purity of basophils were determined. Median purity of isolated basophils used in this study was 86%.

Cell stimulation

Basophils were resuspended in PAG containing 1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ (PAG-CM). Resuspended cells (1.44×10^5 cells/240 μL/tube) were incubated with 30 μL of wortmannin or buffer at 37°C for 10 minutes. Cells were then stimulated by the addition of 30 μL anti-IgE antibody (final concentration: 0.0001%-1% of original solution), A23187 (0.01-1 μmol/L), fMLP (0.01-1 μmol/L), or C5a (0.001-0.1 μmol/L) for 15 minutes. Control cells were resuspended in PAG-CM alone and either kept on ice or incubated at 37°C for the "on-ice" and "spontaneous" controls, respectively. The reaction was stopped by transferring tubes to ice-cold water for 2 minutes. After centrifugation at 450*g* for 5 minutes at 4°C, the supernatant was collected and stored at –70°C. For time-course studies, cells were stimulated with 0.1% anti-IgE antibody, and the reaction was terminated at 0.5, 1, 5, 15, and 30 minutes. A sample of unstimulated cells from each preparation was set aside for the determination of the total amounts of basogranulin and histamine in the whole cell lysate.

Dot blot analysis for basogranulin

The relative amount of basogranulin in the supernatant was determined by a modification of the dot blot technique described previously.[10] Aliquots (100 μL) of cell supernatants, or cell lysates prepared by ultrasonication of the basophil preparation, were applied to the wells of a dot blot apparatus (Bio-Rad, Hemel Hempstead, United Kingdom) that contained a polyvinylidene fluoride membrane. The blot was removed; blocked with PBS containing 0.1% Tween 20, 5% ECL blocking agent (Amersham Pharmacia Biotech), 0.3% H_2O_2, and 0.1% NaN_3; and probed with 0.5 μg/mL biotinylated BB1 antibody. The signal was developed with ExtrAvidin-horseradish peroxidase conjugate (Sigma) followed by Super-Signal West Pico chemiluminescent substrate (Pierce, Cheshire, United Kingdom). Spots were visualized, and the intensity of signals was quantified with the GeneGnome cooled CCD imaging system and supporting software (SynGene, Cambridge, United Kingdom). The percentage of basogranulin released into the supernatant was estimated by linear regression analysis using serially diluted cell lysate as the standard.

Histamine analysis

Histamine was measured by a glass fiber–based fluorometric assay.[20] Samples of cell supernatant or lysate were applied to glass fiber–coated microtiter plates and incubated for 1 hour at 37°C. The plates were then rinsed with deionized water, dried, and the histamine concentration determined by the *o*-phthaldialdehyde method[20] using an automated system (Reference Laboratory, Copenhagen, Denmark). The percentage release of histamine into supernatants was estimated in the same way as for basogranulin.

Flow cytometry

Basophils (>85% purity) were stimulated with 1 μmol/L A23187 or buffer alone and analyzed for surface basogranulin by fixation with 2% paraformaldehyde for 10 minutes on ice or, for intracellular basogranulin, by treatment with a permeabilizing solution (Becton Dickinson, Mountain View, Calif) for 10 minutes. Cells were then washed twice with PBS containing 2% goat serum, 0.2% Tween 20, and 0.1% NaN_3; divided into aliquots; and stained with either BB1 (10 μg/mL) or isotype control (IgG_{2a}; 10 μg/mL) for 30 minutes on ice followed by FITC-conjugated goat anti-mouse immunoglobulin (Becton Dickinson). After further washes, the cells were analyzed by means of a FACScan flow cytometer (Becton Dickinson). Basophils were gated by forward scatter and side scatter to eliminate debris or minor contaminating cells.

Statistics

All data are expressed as the mean ± SE for the percentage release of the total cell content. Although the number of replicates varied between treatments because of variations in the yield of purified basophils in individual experiments, each study was repeated at least 3 times with cells from different donors. Significant differences were analyzed by ANOVA followed by either the Bonferroni or the Dunnet post hoc test. Correlation between basogranulin and histamine release was analyzed by means of Pearson's correlation test. *P* values less than .05 were considered significant.

RESULTS

Anti-IgE antibody–induced basogranulin and histamine release

Treatment of basophils with anti-IgE antibody at dilutions of 0.0001% to 1% induced basogranulin release in a bell-shaped concentration-response manner with an opti-

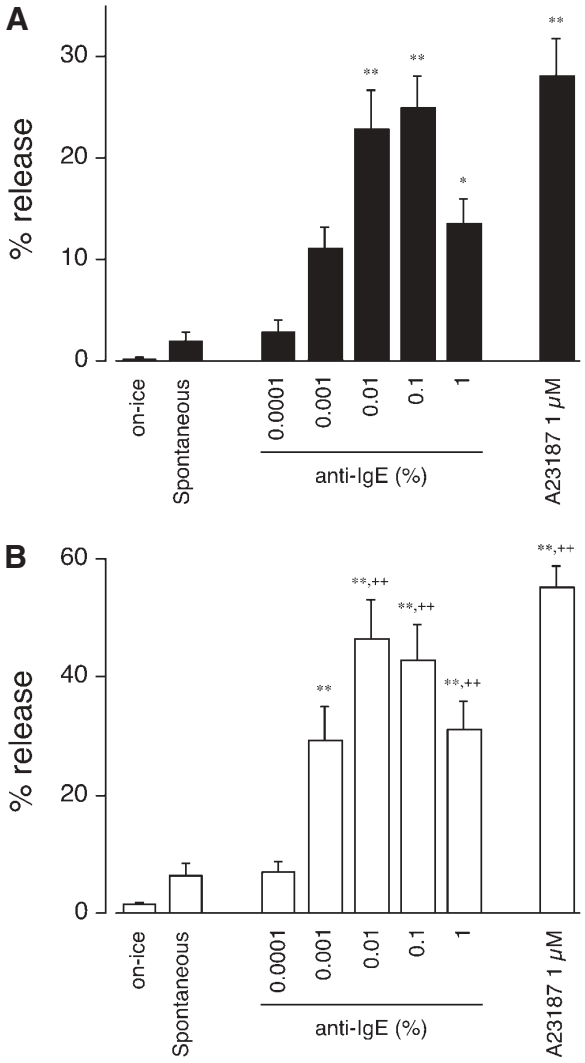

FIG 1. Basogranulin **(A)** and histamine **(B)** release from basophils induced by anti-IgE antibody or A23187. Data are expressed as the mean ± SE for percentage release of total cell content (n = 4-5). **P* < .05 and ***P* < .01 compared with spontaneous release. ++*P* < .01 compared with basogranulin.

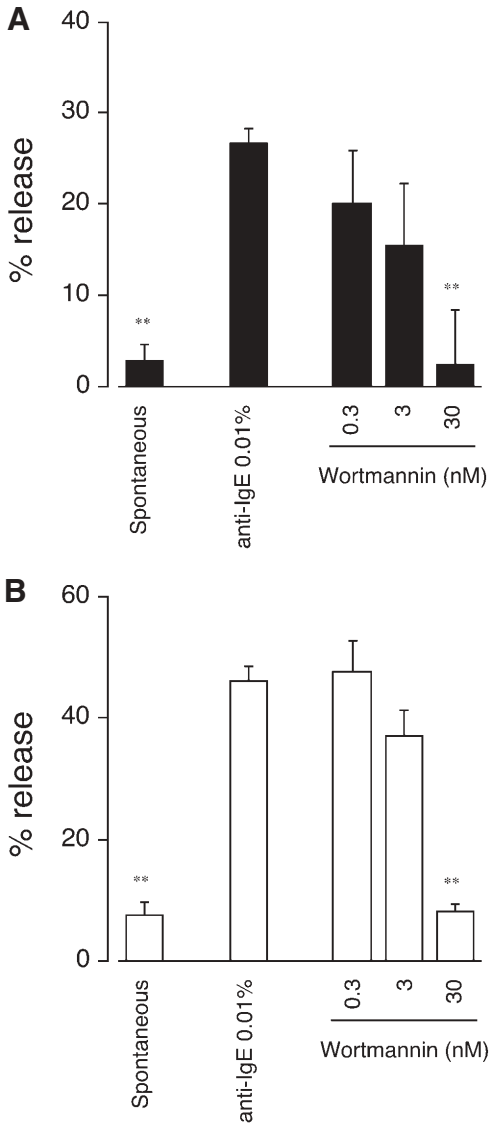

FIG 2. Effect of wortmannin (0.3-30 nmol/L) on basogranulin **(A)** and histamine **(B)** release induced by 0.01% anti-IgE antibody. Data are expressed as the mean ± SE for percentage release of total cell content (n = 3-4). ***P* < .01 compared with anti-IgE–stimulated group.

mum anti-IgE concentration of 0.1%, which corresponded to release of 25.0% ± 3.0% of the total cellular basogranulin (Fig 1, *A*). The pattern of release of histamine was similar, although there was a trend for the bell-shaped response to be shifted to the left, with an optimum concentration of 0.01%. Histamine release as a proportion of the total histamine content of the cells was significantly greater than that for basogranulin expressed in the same way ($P < .01$) at corresponding concentrations of stimuli: 0.01% to 1% anti-IgE antibody and 1 μmol/L A23187 (Fig 1, *B*). Maximal release for histamine (46.3% ± 6.8%) was substantially greater than that of basogranulin (25.0% ± 3.0%). Corresponding differences were found in levels of spontaneous release of basogranulin (0.1% ± 0.3% at 4°C and 1.9% ± 0.9% at 37°C) and histamine (1.4% ± 0.5% and 6.3% ± 2.1%, respectively). Incubation of cells for 10 minutes with 0.3 to 30 nmol/L wortmannin, an inhibitor of PI3-K before stimulation with 0.01% anti-IgE antibody suppressed release of both basogranulin and histamine in a concentration-dependent manner (Fig 2, *A, B*).

Basogranulin release was detected within 0.5 minute of the addition of anti-IgE and reached maximal values 15 minutes after stimulation with 0.1% anti-IgE antibody. It then descended slightly at 30 minutes. Histamine release paralleled that of basogranulin from 0.5 to 15 minutes, but then continued to increase to 30 minutes (Fig 3). At 30 minutes, a significant difference between the release of the 2 mediators was observed ($P < .01$).

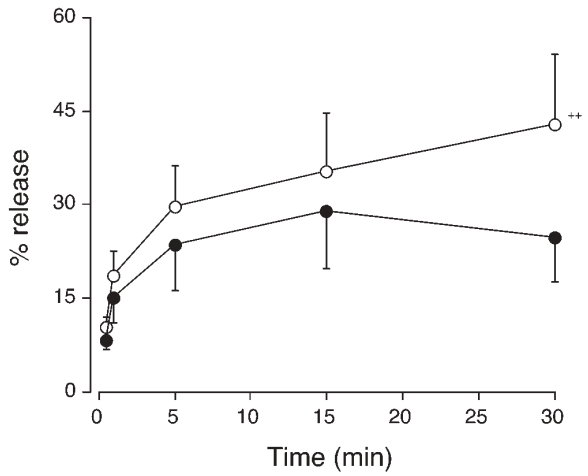

FIG 3. Time course of the release of basogranulin *(closed circles)* and histamine *(open circles)* induced by 0.1% anti-IgE antibody. Data are expressed as the mean ± SE (n = 5). $^{++}P < .01$ compared with basogranulin release at the same time point.

In agreement with previous reports,[21] we found that the basophils from some donors did not release histamine in response to anti-IgE antibody (maximum increase over spontaneous levels less than 10%). Neither did these donors' basophils release basogranulin in response to the same stimulus (Fig 4, *A, B*). Stimulation of these cells with 1 μmol/L A23187, however, did induce secretion of both basophil products.

FcεR I–unrelated basogranulin and histamine release

The calcium ionophore A23187 (0.01-1 μmol/L) induced the secretion of both basogranulin and histamine in a concentration-dependent manner (21.7% ± 5.9% and 48.2% ± 7.2% at 1 μmol/L, respectively) (Fig 5, *A, B*). The bacterial peptide fMLP (0.01-1 μmol/L) and anaphylatoxin C5a (0.001-0.1 μmol/L) also induced the release of both mediators in a concentration-dependent manner (24.5% ± 7.4% and 46.1% ± 8.6% for basogranulin and histamine, respectively, at 1 μmol/L fMLP, and 26.1% ± 5.0% and 48.7% ± 8.3%, respectively, at 1 μmol/L C5a).

Basogranulin and histamine release induced by all stimuli and the levels of spontaneous release were significantly correlated ($r = .901$, $P < .0001$, n = 87), and the percentage release of basogranulin was almost always less than that of histamine (Fig 6).

Flow cytometric analysis of surface and intracellular basogranulin

Because the values obtained for basogranulin secretion were consistently lower than those for histamine, we examined the fate of secreted basogranulin by flow cytometry. Analysis was performed with gating for the basophil population to distinguish responses from those caused by debris and minor cell contamination (gated events: 77%-92% of total). As shown in a representative histogram (Fig 7), some cell surface basogranulin was observed before stimulation (29.2% of cells for gated

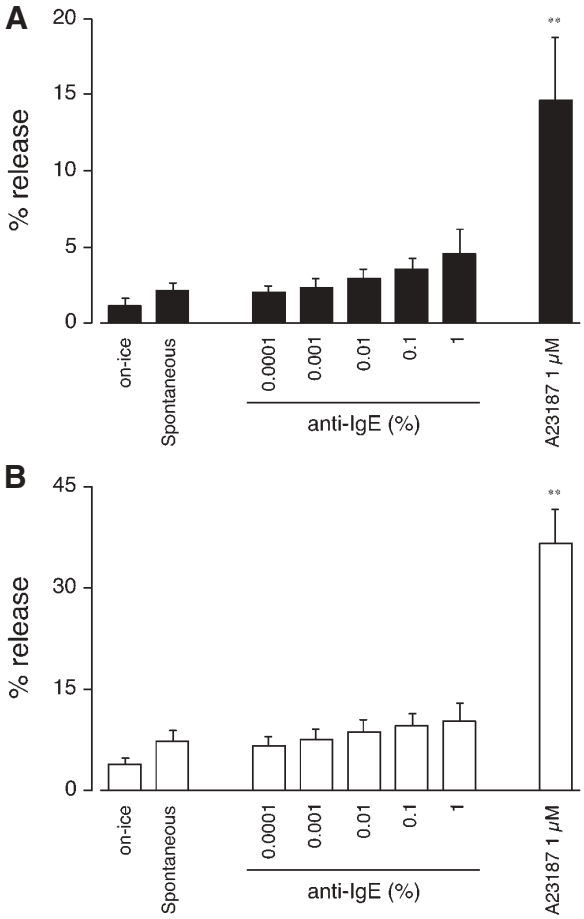

FIG 4. Basogranulin **(A)** and histamine **(B)** release from "nonreleaser" basophils induced by anti-IgE antibody or A23187. Data are expressed as the mean ± SE ($n = 3$-5). $^{**}P < .01$ compared with spontaneous release.

population, Fig 7, *A*) but increased markedly after stimulation with 1 μmol/L A23187 (44.9%, Fig 7, *B*). In contrast, intracellular basogranulin was observed in 87.8% of cells before stimulation (Fig 7, *C*), decreasing to 62.1% afterwards (Fig 7, *D*).

DISCUSSION

In this study we have shown that basogranulin is released by basophils in parallel with histamine in response to the same stimuli, after similar dose-response curves, and with similar kinetics. Basogranulin release, therefore, seems to be under the control of the same receptors and signaling pathways as those for histamine release. Stimulation of the FcεR I with anti-IgE antiserum (Fig 1), of the fMLP receptor with fMLP, and of the C5a receptor (CD88) with anaphylatoxin C5a (Fig 5) all provoked basogranulin release in parallel with histamine, as did the calcium ionophore A23187, which stimulates increases in cytosolic Ca^{2+} levels directly.[22] These studies indicate that basogranulin represents a valuable marker for basophil activation over a range of conditions.

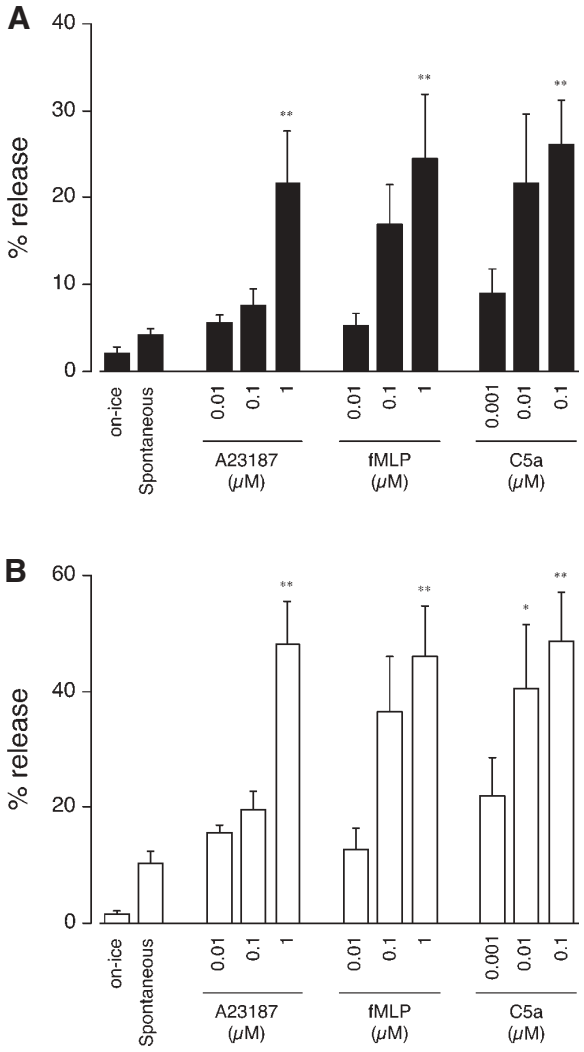

FIG 5. Basogranulin **(A)** and histamine **(B)** release induced by calcium ionophore A23187, peptide fMLP, and C5a. Data are expressed as the mean ± SE (n = 4-7). **P* < .05 and ***P* < .01 compared with spontaneous release.

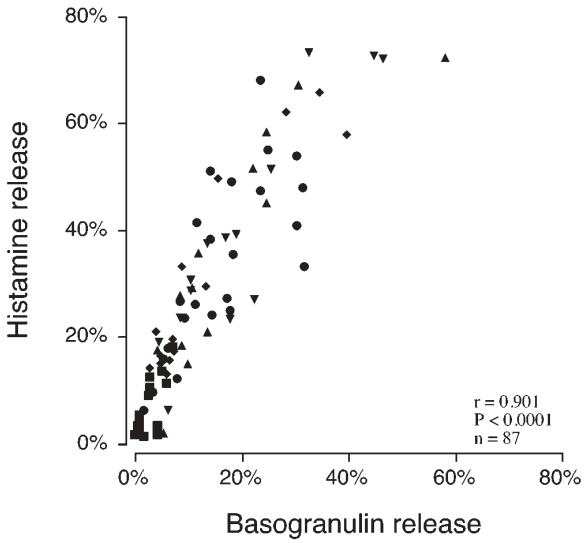

FIG 6. Correlation between basogranulin and histamine release. Percentage release is shown for nonstimulated controls (■) and cells stimulated with anti-IgE antibody (0.0001% to 1%) (●), A23187 (0.01-1 μmol/L) (◆), fMLP (0.01-1 μmol/L) (▲), or C5a (0.001-0.1 μmol/L) (▼). Analysis was by Pearson's test.

PI3-K is one of the important kinases activated by receptor cross-linking and is involved in regulating histamine release.[23-25] Wortmannin, which has inhibitory actions on PI3-K, abolished not only histamine but also basogranulin release in a concentration-dependent manner (Fig 2). Therefore, the dominant signal pathway for basogranulin release seems to pass through PI3-K as does the signal for histamine. The tyrosine kinase Syk has also been identified as a key step in the signal transduction pathway initiated by FcεR I cross-linking,[26] and its absence in basophils from 10% to 20% of donors has been suggested to be the basis for the so-called nonreleaser phenotype.[21] We also encountered this phenotype in our study and found that such basophils had a low releasability of basogranulin and of histamine in response to anti-IgE (Fig 4). Because these cells are capable of releasing both mediators in response to calcium ionophore, it would seem to indicate a defect in signal transduction rather than in the secretory apparatus per se. Basogranulin and histamine thus seem to be released together under the same control mechanisms, an idea that is in accord with the subcellular localization of basogranulin in the secretory granules[11] where histamine has also been shown to be stored.[27] The biologic role of basogranulin must be understood in the context of an extracellular milieu containing histamine, acidic proteoglycans, and the other constituents of the basophil secretory granule.

The major difference observed between basogranulin and histamine release was quantitative. Although statistical analysis revealed the release of these two mediators to be highly correlated (r = .901, P < .0001, n = 87), the release of basogranulin expressed as a percentage of total cell content was almost invariably lower than that of histamine (Fig 1, *B*, and Fig 6), which is not what one would expect for mediators stored and released together. One possible explanation might be denaturation or proteolytic degradation of basogranulin, because BB1 only recognizes the native form of the protein.[10,11] The advent of purified preparations of basogranulin will allow this possibility to be investigated, but it seems unlikely for two reasons. First, for the relative amount of basogranulin to be consistently less than that of histamine at even the earliest time points, the degradation/denaturation would have to be very rapid, yet basogranulin in the supernatants seemed to be quite stable during subsequent handling (for dot blot assay, etc). Second, basogranulin in the whole cell lysate might be expected to be exposed to the same degrading/denaturing factors as are present in the supernatant, so it is not clear why they would act preferentially in the supernatant. Another possibility is that basogranulin might adsorb on the cell surface after secretion

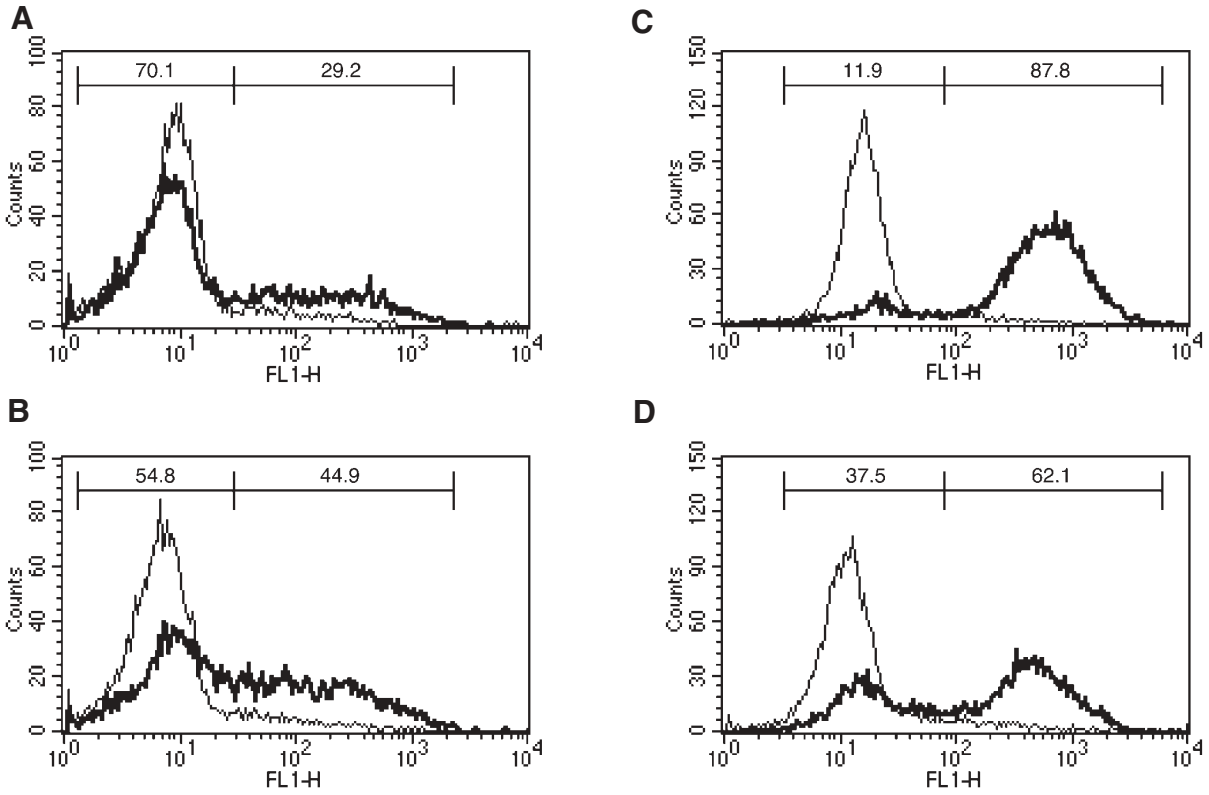

FIG 7. Flow cytometric analysis of surface and intracellular basogranulin in purified basophils before **(A** and **C)** and after stimulation with A23187 **(B** and **D)**. The cells were either fixed to determine surface expression **(A** and **B)** or permeabilized to stain for intracellular basogranulin **(C** and **D)** using BB1 *(thick line)* or isotype control antibody *(thin line)*. The percentages of total gated events are shown.

because of its highly positive charge. We have previously reported that some basogranulin can be detected on the basophil surface by flow cytometry.[10] Comparison of cell surface and intracellular basogranulin with flow cytometry before and after stimulation revealed an increase in the surface-bound basogranulin after activation (Fig 7). These findings suggest that basogranulin is not dispersed as freely as histamine after secretion and provides the most likely explanation for the apparently lower degree of basogranulin release.

These studies indicate that basogranulin release is as reliable a qualitative and quantitative measure of basophil activation as is histamine release. Basophil activation tests serve as useful clinical indices for allergic disease,[28-32] and because histamine is relatively abundant in basophils (2 pg/cell) and is easily measured, it has served as the standard indicator for basophil activation for many years, whether in plasma,[33] serum, or other biologic fluids,[34] or ex vivo stimulation tests.[35,36] However, although this method is useful for isolated peripheral blood leukocytes, when more complex biologic fluids are tested, discrimination of the source of histamine, whether from basophils or mast cells, is not possible. Prostaglandin D_2 and tryptase, being secreted predominantly from mast cells, provide evidence for mast cell activation,[37,38] and low levels of these mediators in biologic fluids with high levels of histamine might be suggestive of basophil activation. However, the value of such indirect approaches to implicate basophil activation has been undermined by recent studies indicating that helper T-cell subsets can produce prostaglandin D_2 in response to antigen stimulation[39] and that some basophil populations can secrete substantial quantities of tryptase.[40] Because basogranulin is specific for basophils,[10] measurement of basogranulin in complex biologic fluids should provide direct evidence for a role for basophils in health and disease.

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